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Errata for the thesis: Respiratory Complications of Organophosphorus Pesticide Poisoning by Dr Elspeth Hulse, University of Edinburgh (2016)

1. P 86. Section 2.2.9.2 line 5 should read "The samples were sectioned to 4µm using a microtome and approximately 2-4cm^2 of the tissue specimens (which had to contain evidence of bronchi and bronchioles for scoring purposes) were placed on slides and dried for 15 min at 37°C, then 60°C for 25 min."
2. P 112. Section 3.3.2.1 line 7 should read "There were no significant differences (Kruskal-Wallis) between groups at time -10 min or 6 hours, with the mean (SD) PaO_2/F_IO_2 ratio of No NMBA, Pre-OP NMBA and Post-OP NMBA at 6 hours being 421(±97), 370(±175) and 346(±168) mmHg respectively (Figure 3.6).
3. P152. Table 5.1 Treatment groups for the pulmonary aspiration study. Group 3 (OA) dose should read "0.08g/Kg IV over 30 min".
4. P 230. Line 26 (last line) should read "The BALF from Solv+GJ treated pigs grew fewer bacterial colonies and, perhaps as a consequence, contained lower CRP concentrations at 48 hours compared with BALF from OP+GJ treated pigs."

Respiratory Complications of Organophosphorus Pesticide Poisoning

Dr Elspeth J Hulse

Thesis presented at the University of Edinburgh for the degree of

Doctor of Philosophy

Doctor of Philosophy – The University of Edinburgh – 2016

Declaration

The findings contained within this thesis are the result of my work as an individual and as a member of a research team. Large animal research is optimised by a multidisciplinary approach that requires variously skilled people collectively able to perform studies safely and efficiently. My work was embedded within such a team and I made a considerable contribution by (i) processing data from studies undertaken before I joined the research team (study 1 and 3); (ii) being a member of the team (co-designing) during Professor Eddleston's study (study 2); and (iii) designing, conducting and leading (practically and intellectually) the team within my own studies (minipig aspiration, human and *ex vivo*;Table 1).

This work has not been submitted for any other degree or diploma and to the best of my knowledge contains no material published or written by any other person, except where stated in the text. NB. Sections of chapter 1 were published extensively in December 2014 in the American Journal of Respiratory and Critical Care Medicine (AJRCCM) as part of a clinically relevant text concerning the diagnosis, management and complications of OP pesticide ingestion and OP nerve agent exposure (Hulse et al., 2014b). I designed the concept of this paper and wrote the first draft of the chapter. Similarly, chapter 2 contains elements of the methodology paper I wrote to detail the creation of a direct and indirect lung injury within the same animal at the same time (Hulse et al., 2014a). The idea for this paper was my own and I wrote the first draft.

Study title in thesis	Date	Study description	Personnel involved and their role in the study
Study 1	2008	Indirect lung injury caused by orogastric OP in a minipig model	Michael Eddleston (ME) (designed and conducted), Professor John Simpson (co-designed and conducted), Andrew Conway Morris (ACM) (conducted and performed data analysis), Professor John Harris, Richard Edward Clutton (REC) (co-designed and conducted) and vets. Tracey Davey (TD) (electron microscopy). Sionagh Smith (SS) (histopathology), Elspeth Hulse (EH)/ACM (data analysis).
Study 2 + dose ranging study	2012	Indirect lung injury caused by orogastric OP & dose ranging study in a minipig model	ME (designed and conducted), EH (co-designed, conducted and performed data analysis), REC (conducted) & vets, Kosala Dissanyake (surgery), Adrian Thompson (AT) (laboratory processing and sampling), TD (electron microscopy).

Study 3	2010	Pilot aspiration study in a minipig model	ME (designed and conducted), David Dorward (co-designed and conducted), AT, REC and vets (co-designed and conducted). EH (performed data analysis).
Minipig OP aspiration study (chapter 5)	2012-2013	Pulmonary aspiration of OP in a minipig model	EH (designed, conducted and performed data analysis), ME (co-designed and conducted), Gordon Drummond (co-designed), REC (co-designed and conducted) & vet team, Kev Dhaliwal (advised), Professor Tim Walsh (advised), AT (laboratory processing and sampling), Dave Binnie (vet team), Kosala Dissanyake (surgery), Helena Carruthers (vet team), SS and William Wallace (WW) (histopathology), shared universities research facility (SuRF) (histopathology/TLR staining), TD (electron microscopy), Dr James Gibbons (microbiology), Scottish Agricultural College (blood white cells and oestrodiol), Mr Martin Connell (image analysis advice).
Human OP aspiration study (chapter 6)	2013-2014	Pilot feasibility study into pesticide ingestion and aspiration in Sri Lanka	EH (designed, conducted and performed data analysis), ME (co-designed), Professor Indika Gawarammana (co-designed), Dr Vasanthi Pinto (Sri Lankan ICU/Anaesthetics Research liaison and sample taking), Dr Kamal Naser (Sri Lankan Toxicology ICU liaison), local ward doctors at the University of Peradeniya hospital (sample taking), South Asian Clinical Research Toxicology Collaboration (SACTRC) research assistants (sample taking), Mr Iriyagama (microbiology assistance), Shonna Johnston (flow cytometry analysis assistance), SuRF unit (TLR staining).
Ex vivo lung perfusion study (chapter 7)	2013	EVLP study into OP aspiration and direct and indirect lung injury	EH (designed, conducted and performed data analysis), Professor Danny McAuley (co-designed), Dr Alistair Proudfoot (advised), Dr U Hamid (Co-designed and conducted), Dr Mara Rocchi and Stephano Guido (TLR expression), SS (histopathology), TD (electron microscopy).

Table 1: Study personnel involved with the study design, conduct and subsequent data analysis. ME: Professor Michael Eddleston, REC: Professor Eddie Clutton, EH: Elspeth Hulse, GD: Dr Gordon Drummond, AT: Mr Adrian Thompson (laboratory), SS: Dr Sionagh Smith and WW: Dr William Wallace histopathology, TD: Mrs Tracey Davey electron microscopy Newcastle University, SuRF: Shared Universities Research Facility. EVLP – *ex vivo* lung perfusion.

Acknowledgements

I thank my supervisor Michael Eddleston for his patience, calm leadership style and for giving me room to investigate ideas and conduct experiments with relative freedom. I also thank him for giving me faith in my own independent thinking and creativity.

The military, academic anaesthetists and my colleagues in the Royal Navy Medical Services have supported me completely during this PhD. They agreed to time out of anaesthetic training, and funded two trips to Sri Lanka to further the human research into OP pesticide poisoning. For this I am immensely thankful.

A massive thank you to the pig Intensive Care management team: Adrian Thompson, Professor Eddie Clutton, Professor Michael Eddleston, Fran Reed, Bryony Few, Helena Carruthers, Roy Davies, Dave Binnie and the other veterinarians involved. These 48 hour studies ran back-to-back and required dedication, careful planning and large amounts of Haribo (Bonn, Germany) and coffee!

My thanks must also go to the laboratory specialists who taught, advised and helped me with the large amount of immunoassays and bronchoalveolar lavage fluid measurements of cells and inflammatory markers. These individuals included: Mr Adrian Thompson, Dr Forbes Howie, Professor Adriano Rossi, Dr David Dorward, Bastiaan Vliegenthart, Shonna Johnston (flow cytometry), Dr Mara Rochii, and Mike Millar (immunohistochemistry). A special thank you to the histopathologists -Dr William Wallace, Dr Sionagh Smith, Tracey Davey (electron microscopy specialist) and Martin Connell (CT analysis) who helped with the visual analysis of my work.

The Sri Lankan feasibility study would never have happened without the help of: Professor Indika Gawarammana, Dr Vasanthi Pinto, Dr Kamal Naser, Dilani Pinnaduwa, Mr Iriyagama (who taught me basic microbiology), Nilanthi Dissanyake and all the research assistants, anaesthetists and ward doctors who made the human studies possible. To the late Nimal who helped look after and protect Isla (my eldest daughter) and all of us when we were living in Sri Lanka. It was a tragedy that he was run over by two buses outside our house in September 2014 and we pray that he is now at peace in heaven.

The final and most important thanks should go to my long suffering husband Matt and children born at either end of the PhD; Isla (4 years old) and Sana (1 year old). He, along with my mother (Angela Isbister) and mother in law (Pam Hulse), has provided buckets of childcare which has enabled me to study and work effectively with the knowledge that my children were being cared for safely and with much love and patience. And thank you to my Saviour and inspiration the Lord Jesus Christ, without whom I would never have entered the academic world of research or indeed might not be here at all, Thank you.

Abstract

Of the 800,000 suicides recorded globally every year, over a third are due to pesticide ingestion, the majority of which occur in rural Asia with organophosphorus (OP) compounds.
These anticholinesterase pesticides cause an acute cholinergic syndrome characterised by decreased consciousness, excessive airway secretions and respiratory failure. A combination of these clinical features is the most common cause of death.

Up to 30% of OP pesticide poisoned patients are admitted to the Intensive Care Unit (ICU) for tracheal intubation and lung ventilation, but up to half die. It is not understood why the case fatality for intubated poisoned patients is so high, but one hypothesis is that the patients, when unconscious, aspirate their stomach contents (including the OP and the solvent present in its agricultural formulation) causing a toxic lung injury which contributes to the observed high mortality.

In this PhD, I aimed to characterise the lung injury caused by OP pesticide self-poisoning through both indirect (ingestion) and direct (aspiration) means. To achieve this, I analysed data from previous toxicological minipig work and designed and conducted a specific minipig pulmonary aspiration study which was complemented by an experimental OP poisoning *ex vivo* lung perfusion model and human data from pesticide poisoned patients in Sri Lanka.

I first investigated the pulmonary pathophysiology resulting from orogastric administration of OP pesticide without aspiration. Analysis of my group's Gottingen minipig *in vivo* work demonstrated that orogastric placement of agricultural OP (dimethoate EC40) produced lung injury via exposure to blood-borne pesticide. Pathological lung changes consisted of alveolar and interstitial oedema, pulmonary haemorrhage and modest neutrophilia with increased concentrations of protein, IL-6 and IL-8 when compared with controls, but with low concentrations of TNF-α and IL-10 in bronchoalveolar lavage fluid (BALF). In a second study, OP poisoned minipigs had increased concentrations of BALF protein, neutrophils, IL-8 and CRP six hours after orogastric poisoning when compared with their baseline values. Electron microscopy images of both studies demonstrated damage to the alveolar capillary membrane secondary to systemic OP poisoning.

Prior to conducting the main pulmonary aspiration study in minipigs, there was considerable refinement of the processes involved through use of: (i) pilot aspiration and dose ranging studies; (ii) the development of a specific pulmonary histopathological scoring system; and (iii) employment of modern human anaesthetic equipment and intensive care patient management protocols. After this period of model development, an *in vivo* 48 hour study using Gottingen minipigs (n=26) was conducted to investigate the pulmonary pathophysiology in animals given either sham bronchoscopy (sham control) or 0.5 mL/kg of: saline (saline control), porcine gastric juice [GJ], OP (dimethoate EC40) + GJ [OP+GJ], or solvent (cyclohexanone) + GJ [Solv+GJ] into the right lung under bronchoscopic guidance.

The results showed that in a minipig model OP and GJ placed into one lung created a direct (right) and indirect (left) lung injury significantly different to controls, and in some respects worse than GJ alone 48 hours after poisoning. The direct lung injury caused by OP+GJ was characterised by significantly worse pathology ($p=0.0003$) in terms of: pulmonary neutrophilia, alveolar haemorrhage, necrosis, oedema and fibrin deposition, when compared with sham controls at 48 hours. Lungs injured directly with OP+GJ also had significantly higher concentrations of BALF neutrophils ($p\leq0.01$), protein ($p\leq0.05$), IL-6 ($p\leq0.01$), IL-8 ($p\leq0.01$) and CRP ($p\leq0.05$) at 24 hours, and BALF protein ($p\leq 0.01$), and CRP ($p\leq 0.05$) when compared with sham controls at 48 hours. The BALF from OP+GJ minipigs at 48 hours also had higher numbers of aerobic bacteria than other groups, suggesting the development of pneumonia could be a source of additional lung injury.

Lung damage might also have resulted from a reduction in the surfactant component responsible for the lowering of alveolar surface tension. Direct lung injury with OP+GJ caused a proportional reduction of beneficial pulmonary surfactant phosphatidylcholine (PC) species 16:0/16:0 [29(±4) % vs. 38(±4) %] when compared with sham controls at 48 hours. Unlike the other groups, OP+GJ (direct and indirectly-injured) lungs had type 2 alveolar cell ultrastructural morphological differences in the lamellar bodies that stored the surfactant. The lamellar bodies were more numerous and more dense in the OP+GJ lungs compared with other groups and could signify a failure of surfactant release or some other pathology pertinent to OP aspiration lung injury.

Computed tomography analysis showed that direct lung injury with OP+GJ caused significantly more lung tissue to be poorly or non-aerated [77 (±13) % ; $p\leq0.0001$ when compared with sham]

as opposed to 62 (±27) % in GJ, 53(±13)% in sham and 47(±0.2)% in saline control animals by 47.5 hours and was mainly due to pulmonary haemorrhage and oedema fluid.

The key differences between aspiration of OP+GJ versus GJ alone was that the majority of inflammatory markers (e.g. BALF protein, IL-6 and CRP) appeared to increase from 24-48 hours in OP+GJ treated animals, but decreased in GJ pigs, possibly signifying resolution. Treatment with GJ alone produced less severe histopathological damage, bacterial BALF numbers and percentage of poorly and non-aerated lung tissue. Importantly, there was less evidence of indirect lung injury within the GJ pigs when compared with animals treated with OP+GJ.

Solvent placed into the lung seemed to offer some form of protection from the effects of GJ aspiration. This was dramatically demonstrated by the histopathology scores, proportional percentage of beneficial phosphatidylcholine (PC) species 16:0/16:0 and the percentage of poorly and non-aerated lung tissue all approaching control animal levels by 48 hours in minipigs that had Solv+GJ placed in the directly-injured (right) lung. Further evidence of benefit was provided by statistically significant reductions ($p \leq 0.05$) in BALF concentrations of IL-8, IL-6 and CRP in minipigs which had aspirated Solv+GJ when compared with OP+GJ and/or GJ minipig groups at 24 hours.

The pathophysiology of aspirated OP+GJ was also investigated in a pilot ovine *ex vivo* lung perfusion (EVLP) model (n=4). Lungs directly-injured with OP+GJ had higher concentrations of total protein (4300 mg/L vs. 350 mg/L) with a proportional reduction of beneficial pulmonary surfactant phosphatidylcholine species 16:0/16:0 (27% vs.34%) when compared with control lungs. Analysis of toll-like receptor (TLR) lung tissue expression in the OP+GJ directly and indirectly-injured lungs indicated that inflammatory mechanisms might also involve upregulation of TLR 3 and 5, unlike other lung injuries e.g. those induced with lipopolysaccharide, which typically upregulates TLR 2 and 4.

To compare OP-induced lung injury in humans and the minipigs, a small feasibility study was conducted in the ICUs of the University of Peradeniya hospital, Sri Lanka. Unfortunately, ethics review and recruitment proved more difficult than expected and we failed to recruit to target. We did however find raised BALF concentrations of IL-6, IL-8 and CRP and low concentrations of

TNF, IL-1β, IL-10 in intubated OP poisoned patients at 24 hours when compared with controls. We also found that two plasma micro-RNA biomarkers thought to be involved in inflammation and lung injury, MiR-21 and MiR-146a, had significantly reduced expression in OP-poisoned patients with aspiration compared to non-intubated control patients from the UK ($p=0.008$ and $p=0.0083$ respectively).

The work from this thesis has allowed the characterisation of both indirect and direct lung injuries caused by OP pesticide ingestion and aspiration. The minipig model showed that at 48 hours the lung injury created by aspiration of OP+GJ appeared more severe than GJ alone, but the addition of the solvent cyclohexanone seemed protective and even beneficial in the context of GJ aspiration. The cytokine expression profiles from both the human and minipig work, combined with the preliminary TLR lung tissue analysis from the EVLP model, suggest that OP+GJ aspiration is unlike normal GJ aspiration and classic ARDS. Increased concentrations of aerobic bacteria in the minipig OP+GJ lungs at 48 hours and evidence of suppression of plasma miR-21 and miR-146a in OP poisoned patients could be linked, and may involve cholinergic immune system modulation. These molecular mechanisms need to be investigated further in both *in vitro* and *in vivo* models.

These discoveries indicate the complex nature of the pulmonary injury that occurs after OP pesticide poisoning, and suggests that damage is not caused by gastric contents alone. Preliminary findings indicate that aspiration of OP+GJ could create favourable conditions for the development of aspiration or ventilator-associated pneumonia but this would need confirmation in larger clinical studies. The potential roles of micro RNA as a biomarker of OP poisoning and lung injury, and solvent as a therapy for aspiration should be explored in further pre-clinical studies.

Lay Summary

Background

Up to 200,000 people die every year by drinking pesticides through self-harm, mostly in rural Asia. The pesticides are commonly organophosphorus (OP) compounds which cause drowsiness, increased airway secretions and muscle weakness which can stop effective breathing and lead to death.

Up to a third of patients poisoned with OP pesticide are admitted to the Intensive Care Unit for assisted breathing, but up to half of these patients die. It is not understood why so many die, but one reason could be that at the time of poisoning the patients may 'breathe in' (termed aspiration) some of the acidic juice in their stomach (including the pesticide, or solvents mixed with the pesticide) causing lung injury, increasing the risk of death.

In this PhD, I aimed to investigate the lung injury caused by both drinking and 'breathing in' OP pesticide.

Methods

I looked at data from previous animal studies and designed and conducted a study in pigs that were deeply anaesthetised to model patients in intensive care. In the latter study, animals received mixtures of OP pesticide and stomach juice, stomach juice alone, or pesticide solvent and stomach juice into their right lungs at the beginning of the experiment. Some animals had nothing placed in their lungs (control animals).

The design of the study allowed me to document the injury caused by the direct placement of OP pesticide and stomach juice into the right lung, but also the (indirect) injury caused to the left lung at the same time by chemicals absorbed into the blood stream.

This work was complemented by other experimental work on animal lungs and by looking at OP pesticide poisoned patients in Sri Lanka where poisoning has been historically common.

Results

The animal study showed that after 2 days the lung injury caused by direct placement of OP pesticide and stomach juice appeared more severe than that caused in the control animals and often more pronounced than a lung injury caused by stomach juice alone. The animals who had OP and stomach juice in their lung also grew more bacteria from lung washings after 2 days when compared with the other groups. OP pesticide and stomach juice placed in the right lung also caused injury to the left lung meaning that some toxins were absorbed into the bloodstream and affected the other lung. Solvent and stomach juice placed in a lung caused much less lung damage than OP pesticide and stomach juice or stomach juice alone. This suggests that the solvent seems to protect or prevent gross injury after the lung was exposed to stomach juice.

Conclusion

The results from all the animal and human work suggest that aspiration of OP pesticide and stomach juice into the lungs creates a measureable and distinctive lung injury. This lung injury appears different to other more common forms of lung injury and suggests that damage is not caused by stomach juice alone.

Publications from this thesis

Clutton RE, Reed FR, Eddleston ME, Hulse EJ (2013). “Prolonged anaesthesia in minipigs.” *Ellegaard Gottingen Minipigs international* newsletter 39:11-15

Hulse, E. J., et al. (2013). "Translational toxicological research: investigating and preventing acute lung injury in organophosphorus insecticide poisoning." *Journal of the Royal Army Medical Corps* 160(2): 191-192.

Hulse, E., et al. (2014). "A model describing the use of a bronchial blocking device and a sheathed bronchoscope for pulmonary aspiration studies in the Göttingen minipig*." Laboratory Animals* 48(2): 164-169.

Hulse, E. J., et al. (2014). "Respiratory complications of organophosphorus nerve agent and insecticide poisoning. Implications for respiratory and critical care." *American Journal of Respiratory and Critical Care Medicine* 190(12): 1342-1354.

Hulse EJ, Eddleston M. “Poisoning: pesticides and Agricultural Chemicals*” Oxford Textbook of Critical Care Medicine,* Editors A Webb, M Shapiro, M Singer, P Suter. Oxford University Press, Oxford, in press.

Presentations from this thesis

Nov 2015	Newcastle, Edinburgh, Cambridge and Sheffield Universities (NECS) Respiratory Research Collaboration Meeting, Cambridge, UK
Oct 2015	Veterinary Comparative Respiratory Society (VCRS), Royal College of Surgeons, Edinburgh
Dec 2014	Colt Foundation Military Medicine research day, Royal Society of Medicine, London. National finalist.
Nov 2014	Trainee competition, Tri-service anaesthetic society, Royal College of Anaesthetists, London.
Sep 2014	Medical Faculty, University of Rajarata, Anuradhapura, Sri Lanka
Sep 2014	UK Military Research Strategy Setting Exercise, RCDM, Birmingham.
May 2014	Society of Naval Anaesthetists, Plymouth. Winner best trainee presentation.
Dec 2013	3 minute thesis presentation, University of Edinburgh, winner joint 1st place for the CMVM school.
Nov 2013	Royal College of Surgeons, Edinburgh
Dec 2012	Colt Foundation Military Medicine research day, Royal Society of Medicine, London. National finalist.

Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
AI	active ingredient
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BuChe	butylcholinesterase
CNS	central nervous system
CO	cardiac output
CRP	C reactive protein
CT	computed tomography
CVP	central venous pressure
CXR	chest x-ray
DMSO	dimethyl sulphoxide
EC40	(dimethoate) emulsifiable concentrate 40%
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
$ETCO_2$	end tidal carbon dioxide measurement
EVLP	*ex vivo* lung perfusion
FCS	fetal calf serum
F_IO_2	fraction of inspired oxygen
GC	gastric contents (own minipigs stomach contents on the day of study)
GCS	Glasgow coma score
GJ	gastric juice (pre-prepared standardised stomach contents from abattoir pigs)
HMSC	human smooth muscle cells
ICU	intensive care unit
IL	interleukin
IMS	intermediate syndrome
LB	lamellar body
LD_{50}	lethal dose of substance required to kill 50% population

LiDCO	lithium dye dilution measurement of cardiac output
LOA	limits of agreement (95%)
Log pow	logarithm of partition coefficient between octanol and water
M 1,2,3,5	muscarinic receptor sub type
miRNA	micro ribonucleic acid
MAP	mean arterial pressure
mRNA	messenger ribonucleic acid
NMBA	neuromuscular blocking agent
NMJ	neuromuscular junction
NTE	neuropathy target esterase
OD	optical density
OP	organophosphorus
PAM	pralidoxime -2-pyridine aldoxime methyl chloride
PEEP	positive end expiratory pressure
PMHx	past medical history
Pplat	plateau airway pressure
RAGE	receptor for advanced glycated end products
RR	respiratory rate
SD	standard deviation
SPD	surfactant protein D
TLR	toll like receptor
TNF-α	tumour necrosis factor alpha
VAP	ventilator associated pneumonia
V_{DL}	volume of distribution within the body
VILI	ventilator induced lung injury
V_T	tidal volume
WHO	world health organisation

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Chapter 1: Introduction

Introduction

Poisoning with pesticides after deliberate or accidental exposure is a major global problem. In 1990, the World Health Organization (WHO) estimated that 20,000 people died every year from accidental pesticide poisoning, while over 200,000 died from self-poisoning (WHO, 1990). A comprehensive systematic review (Gunnell et al., 2007) and large Indian study (Patel et al., 2012) suggest that pesticide self-poisoning kills around 350,000 people every year. Organophosphorus (OP) pesticides are the most important, being responsible for around 2/3 of deaths and several million non-lethal poisoning cases annually (Gunnell et al., 2007, Eddleston, 2000).

More harmful, but less common, are weaponised OP compounds, the effects of which were graphically demonstrated with the use of Sarin gas in Damascus, Syria, in 2013 (Sellestrom et al., 2013). Although the methods of absorption differ between weaponised (topical, inhalational) and self-harm (ingestion, topical) OP poisonings, the chemical structure, systemic effects, and clinical management are comparable (Figure 1.1B) (Sidell et al., 2008, True and Dreisbach, 2001). Studies discussing the effects of OP nerve agent poisoning are referred to in this thesis, but only if they add to the understanding of the pulmonary pathophysiology.

Respiratory complications following OP pesticide poisoning are common and are probably the leading cause of death following ingestion. These complications are discussed in this chapter, but the mechanisms are still not clearly understood.

Around a third of patients who self-harm through ingestion of OP pesticides undergo tracheal intubation and mechanical lung ventilation, but up to half of these die (Banday et al., 2015). One theory for the high mortality observed within intubated OP pesticide-poisoned patients is that prior to receiving medical care patients aspirate their stomach contents into their lungs, causing injury (Adnet and Baud, 1996). The pathophysiology of such a lung injury is not known, nor is its potential to increase mortality amongst OP poisoned patients.

Therefore, the aim of these PhD related studies (section 1.12) was to explore the pulmonary pathophysiological mechanisms and lung injury created through ingested and aspirated OP pesticide and how this might contribute to the increased mortality seen in intubated OP pesticide poisoned patients.

1.1 Chemistry

ACh is the neurotransmitter for all preganglionic autonomic nerve fibres, all post ganglionic parasympathetic fibres, a few post-ganglionic sympathetic fibres and at neuromuscular junctions. ACh is also a neurotransmitter in the brainstem and is involved in cognition, arousal and sleep patterns (Sarter and Bruno, 1999). ACh is mainly produced within neurons but can be synthesised and secreted as a paracrine or autocrine hormone in the respiratory tract and is thought to regulate mucociliary clearance, macrophage function and modulate sensory nerve fibre activity (Proskocil et al., 2004, Kummer et al., 2008). This non-neuronal function of ACh is gaining much interest in the field of medicine as its manipulation may help treat respiratory diseases and certain cancers (Wessler and Kirkpatrick, 2012).

ACh takes effect through stimulation of either muscarinic or nicotinic receptors both centrally and peripherally. There are 5 muscarinic (M1-5) G-protein coupled receptors and several pentameric nicotinic ligand-gated cation receptors present in the respiratory system (Kummer et al., 2008). Muscarinic, and possibly nicotinic (Dehkordi et al., 2006), stimulation can control airway tone and secretions with the actions of the M3 receptor predominating in the airways (Wess et al., 2007).

The enzyme acetylcholinesterase (AChE) is present in the clefts of the post-synaptic neuromuscular junction, red cell membranes and respiratory epithelium and under normal circumstances hydrolyses acetylcholine into acetic acid and choline (Figure 1.1C) (Kummer et al., 2008).

OP pesticides exert their main toxic effect by binding to the AChE enzyme through phosphorylation (formation of P-O bond) at the serine residue, rendering the enzyme inactive (Figure 1.1D).

The majority of OP pesticides are either dimethyl or diethyl forms (Figure 1.1A); inhibition of AChE produces a diethylated or dimethylated phosphate moiety that does not vary according to the individual OP involved (Eyer, 2003).

Inhibited AChE may reactivate spontaneously, reactivate more quickly with the aid of an oxime drug (e.g. pralidoxime), or become irreversibly bound to the OP, a process known as ‘aging’ which can occur from min to hours (Buckley et al., 2011).

Both parathion and dimethoate are ‘thion’ pro-poisons that require activation by cytochrome P450s in the gut or liver from phosphorothioates (P=S group) to active ‘oxons’ that contain the P=O group. Profenofos exists as an oxon and does not require activation (Table 1.1).

Most OP pesticides are lipophilic and are absorbed quickly from the gut. Their lipophilicity also means they will be stored preferentially in fatty tissues within the body and can cause persistent, delayed or recurrent OP pesticide poisoning as they are released from these sites (Eddleston et al., 2006, Peter et al., 2008). If the OP is highly water soluble (e.g. dimethoate) the volume of distribution (V_{DL}) will be smaller and the compound retained mainly within the vascular compartment enabling recurrent inhibition of the AChE within the blood (Table 1.2).

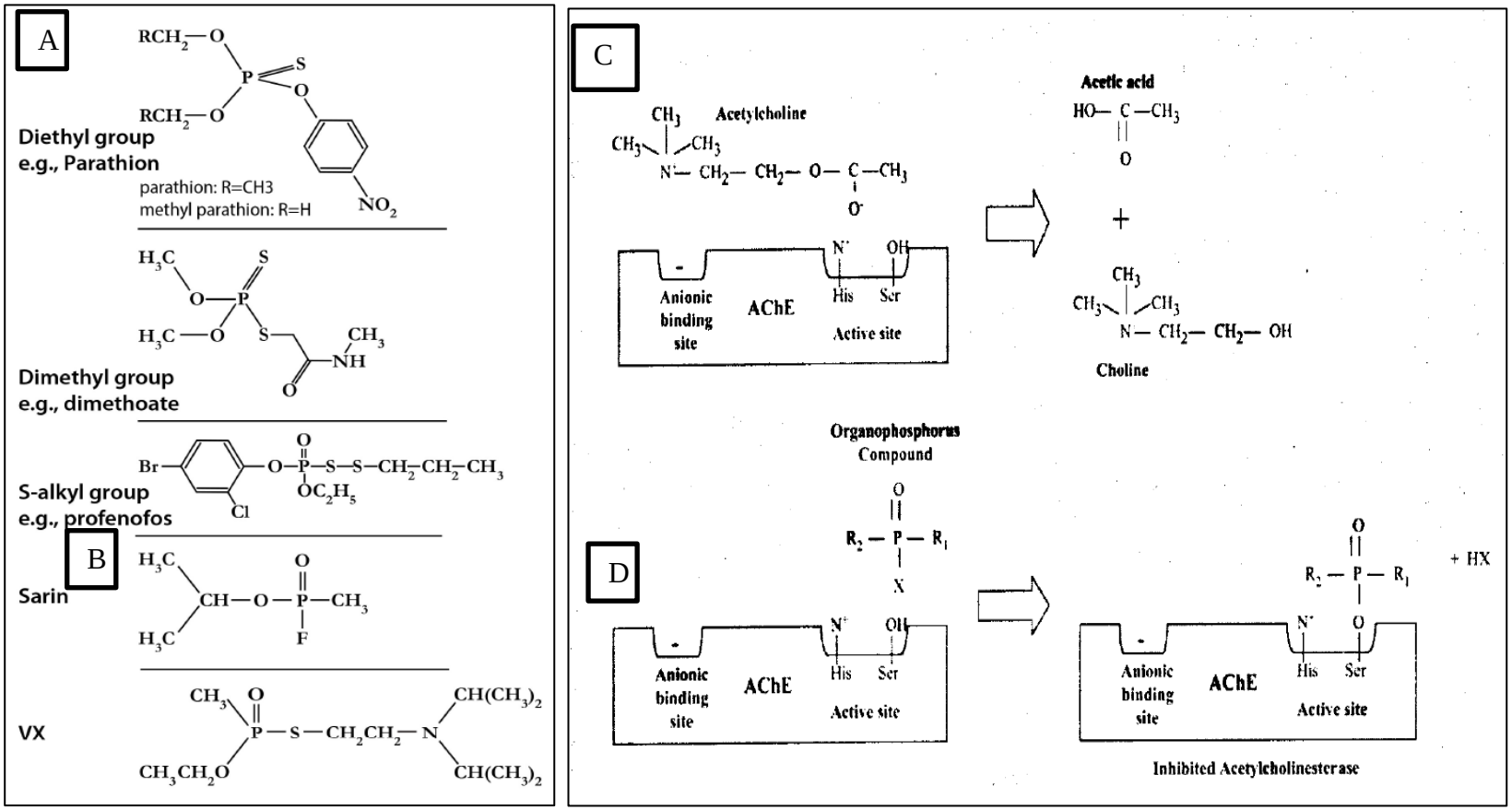


Figure 1.1: Structures of OP pesticides and nerve agents, and AChE action. (A) Structures of the diethyl, dimethyl, and S-alkyl OP pesticides parathion, dimethoate, and profenofos, respectively. (B) Structures of the OP nerve agents sarin and VX. (C) The normal metabolic action of AChE is to catalyse the breakdown of the neurotransmitter acetylcholine (ACh) creating choline and acetic acid. (D) The action of organophosphorus pesticides (compounds) on AChE through phosphorylation of the serine residue and the creation of the HX 'leaving group'. Over time an R group from the OP can leave and 'age' the AChE and the OP becomes irreversibly bound. AChE = acetylcholinesterase enzyme, ACh = acetylcholine. Pictures A and B reproduced from (Hulse et al., 2014b) with permission. Pictures C and D reproduced from Erdman *et al* with permission (Erdman, 2004).

	Chlorpyrifos	Dimethoate	Profenofos
WHO and EPA toxicity class	II Moderately toxic	II Moderately toxic	II Moderately toxic
Rat oral LD_{50} (mg/kg)	¥229-350	¥310	ŧ400-§714
Alkyl groups	Diethyl	Dimethyl	S-alkyl
Fat solubility (log P_{OW})	¥4.7-5.0	¥0.7	ŧ4.68
Thion or oxon	Thion	Thion	Oxon
Formulation g/L solvents	¥Up to 500 with Xylene	¥Up to 500 with Xylene alone or with cyclohexanone	§Up to 720 with Xylene, ethylbenzene, 1,2,4 - trimethylbenzene

Table 1.1: Table describing the chemical characteristics of three types of OP pesticide. Data used from ¥World Health Organisation (WHO) data sheets, §material safety data sheets, **ŧNational Institute of Health (US) websites and** (Eddleston et al., 2005). Log P_{OW} = the logarithm of the partition coefficient between octanol and water which is a measure of lipophilicity; <1 signifies a water soluble chemical, whereas > 4 indicates a highly fat soluble chemical.

The metabolism of OPs is usually through a variety of different mechanisms including cytochrome p450 oxidation by the liver and hydrolysis by plasma esterases and paronoxases. The inactive metabolites are excreted in the urine. Genetic polymorphisms can affect the rate of metabolism of OP pesticides (Erdman, 2004).

1.2 Animal models

The literature portrays marked species variability in OP pharmacokinetics/dynamics and response to treatments raising questions about the validity of these models in the study of human poisoning. For example, Table 1.2 details the species difference in pharmacokinetics of the diethyl OP pesticides: parathion and diazinon. Although oral absorption of the OP pesticides is rapid (due to their lipophilic nature), there is a high rate of first pass metabolism by the liver leading to low bioavailability, most notably in the dog (1-29%), which has half the bioavailability of the rabbit (68%).

Species	Oral Dose Mg/kg	Bioavailability percentage	V_{DL} (L/kg)	Elimination ½ life (h)	Clearance (L/h/kg)
Rabbit (parathion)	3	68	7.58 (±6.45)	2.54 (±1.67)	6.59 (±3.36)
Dog (parathion)	10	1-29	n/a	n/a	n/a
Rat (diazinon)	80	35.5	22.93 (±4.82)	2.86 (±0.58)	4.6 (±1.05)

Table 1.2: Pharmacokinetics of parent compounds in the blood of different species following oral administration of the organophosphorus pesticides parathion and diazinon. Data adapted from table by Timchalk *et al* (Timchalk, 2010). V_{DL}= volume of distribution, h = hours, n/a = not available, bioavailability = the amount of drug available after absorption and first pass metabolism in the liver. Mean and (SD) shown.

Rodents (mouse and guinea pig) have seven times the carboxylesterase activity of rabbits or humans potentially allowing them to detoxify OP pesticides more efficiently (KalisteKorhonen et al., 1996). Other work has confirmed the near absence of plasma carboxylesterase in humans, primates and pigs (Li et al., 2005, Dorandeu et al., 2007). Thus whilst animal toxicological studies are vital in helping us understand the pharmacology of OP pesticide poisoning, the pharmacokinetics and dynamics across species varies widely, and so extrapolations to human poisoning should be performed carefully.

Primate and porcine (Dorandeu et al., 2007) models are currently considered the most clinically relevant animal models and therefore data from these species are presented preferentially in this thesis (Table 1.3).

Physiological variable	Human	Healthy pig
Lung morphology	3 lobes in right lung, 2 in left	4 lobes in right lung [cranial, middle accessory and caudal], 2 in left. Has similar pulmonary pleura and vascular circulation to humans (Rogers et al., 2008)
Submucosal glands	Present in trachea and bronchial airways density $1mm^{-2}$	Present in trachea and bronchial airways at density $1mm^{-2}$ (Ballard and Inglis, 2004) but decreased presence of sub mucosal glands in smaller airways (Judge et al., 2014)
Pulmonary electrolyte and water transfer	ENaC, CFTR and chloride channels present in humans and pigs.	Although pigs have similar electrolyte transfer mechanisms to humans (Liu et al., 2007) they have Increased permeability to water (Crews et al., 2001).
Pulmonary elastic properties	Humans have more lung elastance and less air space collapse at expiration. Human lungs have a collagen/elastin ratio of 3.1:1(McLaughlin.Tr, 1966).	Pigs have increased air space collapse at expiration (De Robertis et al., 2001) and have half the lung elastance (Protti et al., 2011) compared to humans. Pig lungs have more elastin and less collagen than human lungs giving a collagen/elastin ratio 2.6:1 (McLaughlin.Tr, 1966).
Ease of bronchoscopy	Allows video bronchoscope with 2.1mm working channel for biopsy	Allows video bronchoscope with 2.1mm working channel for biopsy.
Bronchoalveolar lavage volume returns	Variable but should be > 30% return (Meyer et al., 2012)	75-80%*
BALF cellular numbers*	$12.9\pm2 \times 10^4$/mL	$163\pm73 \times 10^4$/mL (mean ± SD)

BALF cellular profiles*:		
Alveolar macrophages (%)	85.3±.6	85±6 (mean±SD)
PMN (%)	1.6±0.7	7±5 (0.01-9% neutrophils)*
Lymphocytes (%)	11.81±1.1	8±3
Eosinophils (%)	0.19±0.06	0
Pulmonary intravascular macrophages (Schneberger et al., 2012)	Induced in disease states and poorly understood	Innate (constitutive)
Blood volume	80mL/kg in adults	60.5 mL/kg (in large white pigs)(Anderson et al., 1969)
Blood profiles #		
WBC x10^9/L	4-11	9.46±2.66 (mean ±SD)
Neutrophils x 10^9/L	1.7 – 7.5	2.56±1.3
Lymphocytes x 10^9/L	1.5 -4.5	6.59±1.84
Eosinophils x 10^9/L	0.0-0.5	0.19±0.09

Table 1.3: Differences between human and porcine pulmonary and haematological physiological variables. CFTR; cystic fibrosis transmembrane conductance regulator, ENaC; epithelial sodium channels, PMN; polymorphonuclear granulocyte (neutrophil).* data adapted from paper by (Judge et al., 2014). # data from 6 month old female Ellegaard Gottingen minipigs - 2008 Ellegaard laboratory data. Human blood profile values from http://www.cddft.nhs.uk/GP_Pathology_Handbook/Haem-Trans/Info/FBC_ref_ranges.htm

It is important to appreciate that the OP pesticide used in toxicological animal studies is often the active ingredient (e.g. dimethoate), as opposed to the commercial formulation, which contains solvents and surfactants (e.g. dimethoate EC40 also contains cyclohexanone, xylene and a surfactant). The commercial formulation is usually the product used in human poisoning through ingestion, and has been found to be more toxic than the active ingredient alone when administered

by gavage in minipig studies (Eddleston et al., 2012). Where the commercial formulation has not been used in animal studies, this has been highlighted for the reader.

1.3 Pharmacology

Organophosphorus pesticides not only inhibit acetylcholinesterase (AChE) but also esterase enzymes and butyrylcholinesterase (BuChE)(Lotti, 2001). AChE breaks down acetylcholine centrally and peripherally at cholinergic synapses (neuromuscular junction), red cell membranes and partly in respiratory epithelium, reducing activity (Nachmansohn and Feld, 1947, Kummer et al., 2008). Inhibition results in excess acetylcholine and cholinergic overstimulation within the peripheral, central, and autonomic (parasympathetic and sympathetic) nervous systems and tissues (Pope, 1999).

Red cell AChE activity is commonly used as a marker of clinical severity of OP toxicity but its function remains uncertain (Brewer, 1974). In recent years the membrane bound AChE has been found to be co-located with the Y_T blood group antigens (Rao et al., 1993) (the absence of which causes paroxysmal nocturnal haemoglobinuria) and is genetically different from the AChE form found elsewhere in the body (NCBI, 2016).

In one study 30% AChE activity was associated with normal muscle function, but with 10% AChE activity, patients showed deranged muscle function and required high doses of atropine for control of other symptoms (Eddleston et al., 2008). But, the measurement of red cell AChE inhibition does not always correlate with clinical severity; mild cholinergic symptoms have been observed in patients (n=95) with S-alkyl OP pesticide poisoning who had severe AChE inhibition (Eddleston et al., 2009).

Butylcholinesterase which is present in plasma, can hydrolyse acetylcholine as well as scavenge and inactivate organophosphorus compounds (Satoh, 2010). Controversy exists as to the importance of BuChE, but in AChE knock-out mice (AChE-/-) exposure to a BuChE inhibitor, or the nerve agent VX, produces a similar toxidrome to OP poisoning, implying that BuChE, and possibly other mechanisms, may be important in the metabolism of acetylcholine and functioning of

autonomic and neuromuscular signalling in the long term absence of AChE (Hartmann et al., 2007, Duysen et al., 2001).

Whilst many other enzymes (e.g. hydrolases and cytochrome p450 enzymes) are inhibited by OP compounds, the clinical significance of their inhibition is currently unclear (Casida and Quistad, 2004, Eyer et al., 2003).

1.4 Acute clinical presentation

The acute cholinergic crisis creates a toxidrome of muscarinic (miosis, hypersalivation, emesis, nausea, bronchorrhea, bronchospasm, alveolar oedema, bradycardia, hypotension) and nicotinic (sweating, muscle weakness, fasciculations, paralysis, with occasional hypertension and tachycardia) features (Lotti, 2001). The combination of pin-point pupils, sweating, and fasciculations is pathognomic of OP poisoning. Although it is unclear whether the central features of unconsciousness, seizures and respiratory depression are predominantly muscarinic or nicotinic in origin, they contribute to respiratory failure and complications (Figure 1.2) (Tsao et al., 1990, Houze et al., 2008, Bakry et al., 1988).

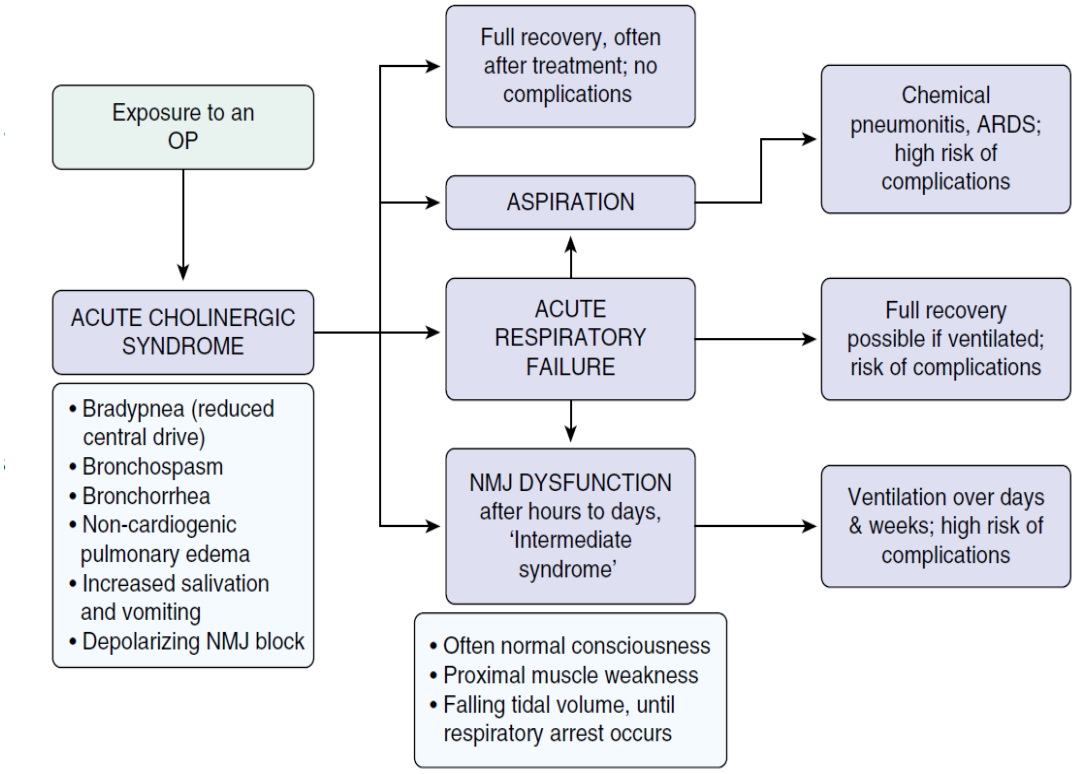


Figure 1.2: Respiratory system toxicity secondary to organophosphate pesticide poisoning. Figure taken from (Hulse et al., 2014b) with permission from American Thoracic Society© (US publication).

1.5 Deaths from OP pesticide poisoning

Most deaths occur acutely through an OP-induced respiratory arrest. This centrally driven apnoea produces hypoxia which is worsened by peripheral acute cholinergic effects and seizures. In others, death occurs later from distributive shock, neuromuscular junction (NMJ) dysfunction, recurrent cholinergic toxicity, or from complications of reduced consciousness and respiratory failure.

OP pesticide ingestion can cause rapid poisoning with reports of a death within five min from mevinphos ingestion (Lokan and James, 1983) and a requirement for intubation and ventilation within 15-30 min of poisoning secondary to parathion ingestion (Eyer et al., 2003). Many people poisoned by highly toxic pesticides die in the community before presentation to hospital, often due to a lack of adequate pre-hospital management, transport and intubation and ventilation facilities

(Banday et al., 2015). However, patients poisoned by the relatively less toxic WHO Class II thion pesticides (e.g. chlorpyrifos) do not generally require intubation and ventilation until 2 to 4 hours post exposure. Recurrent cholinergic toxicity and respiratory failure may also occur much later for highly fat soluble thion OP pesticides such as fenthion (Eddleston et al., 2006). These variations can be explained by the chemistry of these compounds discussed above (Table 1.1).

If patients survive to hospital presentation, up to a third of cases require intubation and ventilation, depending on the OP involved (Table 1.4) (Banday et al., 2015, Muley et al., 2014). This causes intense resource problems in rural Asian district hospitals (where most patients present), as there are often inadequate numbers of trained doctors, ventilators, and intensive care beds (Senarathna, 2006). The overall in-patient case fatality of OP pesticide poisoning is usually between 10 and 20%, but may reach over 50% in patients who require intubation (Table 1.4).

Study author (year)	Location and facility type: ICU/poison centre/ general hospital	Type of OP insecticide poisoning documented	No. OP poisonings (n)	Overall case fatality	No. requiring intubation and ventilation	Intubated patient case fatality	Mean No. days ventilation or length of ICU stay (range)	Respiratory complications, points of interest
ΨNalin (1973) (Nalin, 1973)	General hospital admission Guyana	Malathion	264	20% (53/264)	27% (72/264)	n/a	n/a	'Occasional' complication of aspiration pneumonia
Du toit (1981) (Dutoit et al., 1981)	Teaching hospital, South Africa	Oxydemeton methyl (68%)	157	3% (5/157)	11% (17/157)	12% (5/41)	6.6 (±4.2) days ventilation	Early tracheostomy was encouraged when the nursing standard was sub-optimal
Kamat (1983-1986) (Kamat et al., 1989)	Respiratory ICU, Bombay, India	Fenitrothion (39), fenthion (38), and carbamate (33)	227	45% (102/227)	50% (113/227)	71% (80/113)	3 days ventilation (max 23 days)	Autopsy data (n=85) showed that of those that died within 24 hours, 75% (27/36) had pulmonary interstitial oedema and 25% had parenchymal haemorrhage. Of those who died after 24 hours, 69% (34/49) had lobar or segmental consolidation and 30% (15/49) had

								interstitial oedema.
Bardin (1987) (Bardin et al., 1987)	ICU, South Africa	OPs	61	16% (10/61) respiratory infection and septicaemia in 7 cases	CPAP or intubation needed in 56% (34/61) cases	n/a	All patient median stay 8.0 (2-30) days	28% (17/61) had abnormal CXR showing possible aspiration and pulmonary oedema with 56% (34/61) having a PaO_2 <10kPa on air on admission. Respiratory infection in 59% (36/61) patients.
Karalliedde (1988) (Karalliedde and Senanayake, 1988)	Government teaching hospital, Sri Lanka	Dimethoate, methamidophos, malathion, monocrotophos, fenthion	92	18% (17/92)	7% (6/92)	67% (4/6)	Up to 28 days ventilation.	22% unconscious on admission, not all intubated. 14% (13/92) respiratory depression in conscious patients 48-96 hours after poisoning
Kamat (1989) (Kamat et al., 1989)	Respiratory ICU, Bombay, India	OPs	81	28% (23/81)	52% (42/81)	52% (22/42)	4.4 days ICU stay	Pulmonary infection in 36% (29/81). Improved survival rates due to aggressive atropinisation and better airway management

Tsao (1990) (Tsao et al., 1990)	General hospital, Taipei, Taiwan	80 cases OP poisoning, 13 carbamate poisoning, 14 had both or an uncertain agent	107	21% (22/107) mainly from respiratory failure. Seven in first 24 hours, 11 during 24-96 hours	40% (43/107)	51% (22/43)	12-473 hours ventilation (19.7 days)	26% (28/107) presumed aspiration pneumonia
Bardin (1990) (Bardin and Vaneeden, 1990)	ICU, South Africa	OPs	44	7% (3/44)	32% (14/44)	21% (3/14)	7.3 (±6.5) days	Mortality due to respiratory infection
ΨMekonnen (1991) (Mekonnen, 1991)	General hospital, Ethiopia	OPs	50	20% (10/50)	66% (33/50)	n/a	n/a	24% (12/50) aspiration pneumonia
Kasilo (1991) (Kasilo et al., 1991)	6 general hospitals, Zimbabwe	Dimethoate, parathion, malathion, and chlorpyrifos	606	8% (48/606)	n/a	n/a	Mean period of hospitalisation 8 (1-29) days	
De Bleeker (1993)(De Bleecker et al., 1993)	ICU, Belgium	Ethyl-parathion, methyl-parathion, Dimethoate	19	16% (3/19)	n/a	n/a	n/a	37% (7/19) developed bronchopneumonia
Goswamy (1994)(Goswamy et al., 1994)	ICU, Bombay, India	Diazinon, Malathion, and sumithion	13	15% (2/13)	n/a	n/a	2.3 days ventilation	23% (3/13) type 1 respiratory failure

Zilker (1996) (Zilker T, 1996)	ICU, Germany	Parathion	55	42% (23/55)	96% (53/55)	43% (23/53)	18 survivors extubated after 3.5 days (±0.7), 12 survivors after 23 days (±5.1)	Main causes of death were cardiac and hypotension 65% (15/23), pulmonary 22% (5/23).
Goel (1998) (Goel et al., 1998)	Teaching hospital, India	Dimethoate, phenitrothion , phorate, chlorpyrifos	103	9% (9/103)	35% (36/101) secondary to respiratory failure (30 in the first 24 hours)	22% (8/36)	n/a	Signs of respiratory insufficiency in 43% patients. Of those that were intubated 47% (17/36) developed nosocomial pneumonia and 3 had pneumothoraces
Lee (2001) (Lee and Tai, 2001)	Medical ICU, Singapore	Malathion (100%)	23	13% (3/23)	74% (17/23)	n/a	7.1 (+/-6.7) days of ventilation	Intubations were because of bronchial secretions (83%), decreased GCS (78%), pneumonia (78%) and paralysis (57%)
Sungur (2001) (Sungur and Guven, 2001)	Medical ICU, Turkey	Dichlorvos (51%), ethyl-parathion (11%), fenthion (9%)	47	28% (13/47)	21% (10/47)	50% (5/10)	4.1 (+/-3.2) days of ventilation.	Aspiration pneumonia in 21% (10/47) patients. IMS in 19% (9/47) patients.

Karki (2001) (Karki et al., 2001)	General hospital, Nepal	Methyl-parathion (62%), dichlorvos (13.5%)	37	8% (3/37)	8% (3/37)	n/a	Mean duration of treatment 5.5 (2-20)	16% (6/37) developed aspiration pneumonia, 8% (3/37) developed ARDS
Verhulst (2002) (Verhulst et al., 2002)	Paediatric ICU, South Africa	Most unknown, but included chlorpyrifos, diazinon, and parathion (mainly enteral (60%) with some transcutaneous (15%) poisoning)	54	7% (4/54)	35% (19/54)	21% (4/19)	2 (1-39) median days of ventilation in survivors.	Two intubated patients developed pulmonary oedema, one ARDS.
Krupesh (2002) (Krupesh, 2002)	ICU, Bangalore, India	OPs	39	15% (6/39)	74% (29/39)	20% (6/29)	6.17 days of ventilation	All those who died developed haemorrhagic pancreatitis which progressed to ARDS.
Godhwani (2004) (Godhwani and Tulsiani, 2004)	ICU, Rajasthan, India	OPs (29%), carbamates (29%), unknown but probably OPs (37%)	82	12% (10/82)	65% (53/82)	21% (11/53)	5.79 days	6% (5/82) developed aspiration pneumonia. Ventilated patients required frequent suctioning to

								maintain a patent airway.
Munidasa (2004) (Munidasa et al., 2004)	ICU, Sri Lanka	OPs	71	41% (29/71)	100% (71/71)	41% (29/71)	Median 4 (1-27) days of ventilation	Systolic blood pressure<100mm Hg and those patients needing FiO_2>0.4 to keep saturations above 92% in the first 24 hours are predictors of poor outcome.
Hussain (2005) (Hussain and Sultan, 2005)	Surgical ICU, Karachi, Pakistan	OPs	52	8% (4/52)	100% (52/52)	8% (4/52)	7.33 (2-12) days of ventilation.	ARDS in 10% (5/52) from aspirating before intubation
Roberts (2005) (Roberts et al., 2005)	Tertiary hospital, Australia	Chlopyrifos, diazinon, fenthion	41	2% (1/41)	22% (9/41)	11% (1/9)	16 (0.5-51) median days of ventilation. 0.5-19.5 median days spent in hospital	Pneumonia in 3 patients
Rao (2005)(Rao et al., 2005)	General hospital, Warangal, India	Monocrotophos, chlorpyrifos, quinalphos	653	26% (171/653)	32% (211/653)	n/a	n/a	9% (60/653) died within the first 3 hours of hospitalisation
Eddleston (2006)(Eddleston et al., 2006)	Three Sri-Lankan general hospitals	Chlorpyrifos, dimethoate, fenthion, quialphos	376	12% (46/376)	24% (90/376) 2/3rd intubated in first 24	51% (46/90)	Intubated for a median of 219 hours (IQR 154-276 hours)	9 patients pneumonia, 4 unexpected respiratory arrest

					hours, 1/3rd after 24 hours			
Noshad (2007) (Noshad et al., 2007)	General hospital, Tabriz, Iran	OPs	80	9% (7/80)	13% (10/80)	50% (5/10)	4.3-5.2 days of ventilation	Patients died secondary to pneumonia, ARDS and cardiopulmonary arrest. Cases of aspiration pneumonia were excluded from this study.
Tsai (2007) (Tsai et al., 2007)	Teaching hospital, Taiwan	OPs	75	8% (6/75)	28% (21/75)	n/a	Mean ICU stay 3.5 (+/- 6.1) days	5 patients had aspiration pneumonia
Lin (2008) (Lin et al., 2008)	Poisons control centre, Taiwan	Mevinphos (18%), chlorpyrifos (17%), methamidophos (8%), dimethoate (5%)	4799	13% (610/4799)	n/a	n/a	n/a	Dimethyl OPs caused majority of deaths
Peter (2008)(Peter et al., 2008)	Medical ICU Vellore, India	Monocrotophos (17%), quinalphos (17%),	35	9% (3/35)	86% (30/35)	n/a	8.6 (+/-6.6) days of ventilation for those without signs of encephalopathy, 14.5 (±4.3) days for those with delayed	71% (25/35) developed IMS 46% (16/35) developed pneumonia. 57%

		phorate (11%)					encephalopathy (mean + SD)	(20/35) had a tracheostomy sited.
Wang (2010) (Wang et al., 2010a)	General hospital, Taiwan	109 OP poisoning, 46 carbamate poisoning	155	20% (31/155)	n/a	Patients with acute respiratory failure and pneumonia mortality was 47%	ICU stay 6.2 (+/-7.4) days	22% (34/155) developed early pneumonia. Charcoal stained sputum was a significant predictor.
Shaikh (2011) (Shaikh, 2011)	Medical unit and ICU, Hyderabad, Pakistan	OPs	100	18% (18/100)	40% (40/100)	n/a	Duration of hospital stay 4.5 (+/-2.5) days (range 3-22 days)	Respiratory failure 42%, sepsis 5%, seizures 9%, pulmonary oedema 19%, ARDS 10%,
Imran (2013) (Imran et al., 2013)	Medical ICU, Pakistan	OPs	145	19% (27/145)	32% (46/145)	n/a	2.7 (1-8.1) days, mean hospital stay 6.8 (1-18) days	21% (31/145) cases IMS all required ventilation. Two of the IMS patients could not be weaned from ventilation and died.
Indira (2013) (Indira et al., 2013)	Medical wards and ICU, India	All cholinergic insecticide poisoning (OPs and carbamates)	176	28% (50/176)	36% (64/176)	78% (50/64) secondary to respiratory failure. 29 pts in the cholinergic	91.2 (48-168) hours ventilation in the 20 autopsied patients before they died. Those with IMS were ventilated for between 1-7 days.	36% (64/176) developed respiratory failure and were all intubated. Autopsy data suggested that

						phase, 20 during intermediate syndrome, 1 x aspiration pneumonia		cause of death was specifically 40% (8/20) pneumonia, 35% (7/20) ventilator failure. IMS 18% (31/176) appearing at a mean 45 hours and lasting 1-7 days.
Hrabetz (2013) (Hrabetz et al., 2013)	ICU, Germany	Di-methyl OPs (19), Di-ethyl OPs (8), generic OPs (6)	33	15% (5/33)	97% (32/33)	na	Median duration ventilation 168 (0-1058) hours.	Aspiration pneumonia in 82% (27/33). Gastric lavage was conducted in 61% (20/33). Cardiopulmonary resuscitation was conducted in 18% (6/33), cerebral seizures 15% (5/33)
Ahmed (2014) (Ahmed et al., 2014)	ICU, Udar Pratesh, India	OPs	86	19% (16/86)	All intubated 100%	19% (16/86)	4.83 (+/-3.41) days of ventilation	8 patients were brought dead to the emergency department making mortality rate 28%. 10% (9/86) developed VAP, one patient

								pulmonary oedema.
Muley (2014) (Muley et al., 2014)	Main hospital, Gujurat, India	OPs	76	11% (8/76)	34% (26/76)	31% (8/26)	Hospital stay 8.5 ± 7.49 days	Admission GCS 12+/-3 Intermediate syndrome in 16% (12/76)
Banday (2015) (Banday et al., 2015)	Department of Medicine, rural hospital, Karnataka, India	OPs: dimethoate (25%), chlorpyrifos (11%)	133	32% (42/133)	40% (53/133)	79% (42/53)	6.85 ± 4.32 days of ventilation	34% (45/133) had altered GCS on admission. 29% (39/133) had multi organ failure Episodic convulsions in (14%) patients, severe bradycardia (25%), hypotension (11%)

Table 1.4: Literature review of case series reporting intubation, ventilation, and mortality after OP pesticide poisoning. **Ψ** Studies where intubation was inferred due to severity of described symptoms i.e. Coma. IMS; intermediate syndrome, ARDS; acute respiratory distress syndrome, VAP; ventilator associated pneumonia. Data reproduced with permission from the American Thoracic Scoiety© (Hulse et al., 2014b).

1.6 Respiratory complications of the acute cholinergic syndrome

Acute cholinergic syndrome	Local airway effects Alveolar fluid and bronchorrhea ARDS Central nervous system effects Neuromuscular junction effects
Complications of the acute cholinergic syndrome	Aspiration pneumonitis and pneumonia, Complications of ventilation Immunomodulation
Intermediate effects of OP poisoning	Neuromuscular junction dysfunction and intermediate syndrome Delayed or recurrent cholinergic toxicity Overlapping acute and intermediate poisoning effects
Delayed effects of OP poisoning	OP-induced delayed polyneuropathy Delayed pulmonary sequelae

Table 1.5: Respiratory complications of OP poisoning

Respiratory complications of OP poisoning occur during, and as a consequence of, the acute cholinergic crisis, delayed NMJ dysfunction, and recurrent cholinergic toxicity (Table 1.5). In a large Sri Lankan case series of proven OP pesticide poisonings, there were two commonly observed patterns of respiratory failure: (i) respiratory failure in unconscious patients during the acute cholinergic crisis requiring intubation within 2 hours (58% of intubated patients) and (ii) respiratory failure in conscious patients without cholinergic signs often more than 24 hours after poisoning (32%) (Eddleston et al., 2006) (Figure 1.3). Patients who were intubated after 24 hours required a significantly longer period of ventilation than those intubated within 24 hours: median (interquartile range) 219 (154-276) hours. The remaining 10% patients were intubated between 2-24 hours, and showed equal distribution between the two observed patterns of respiratory failure.

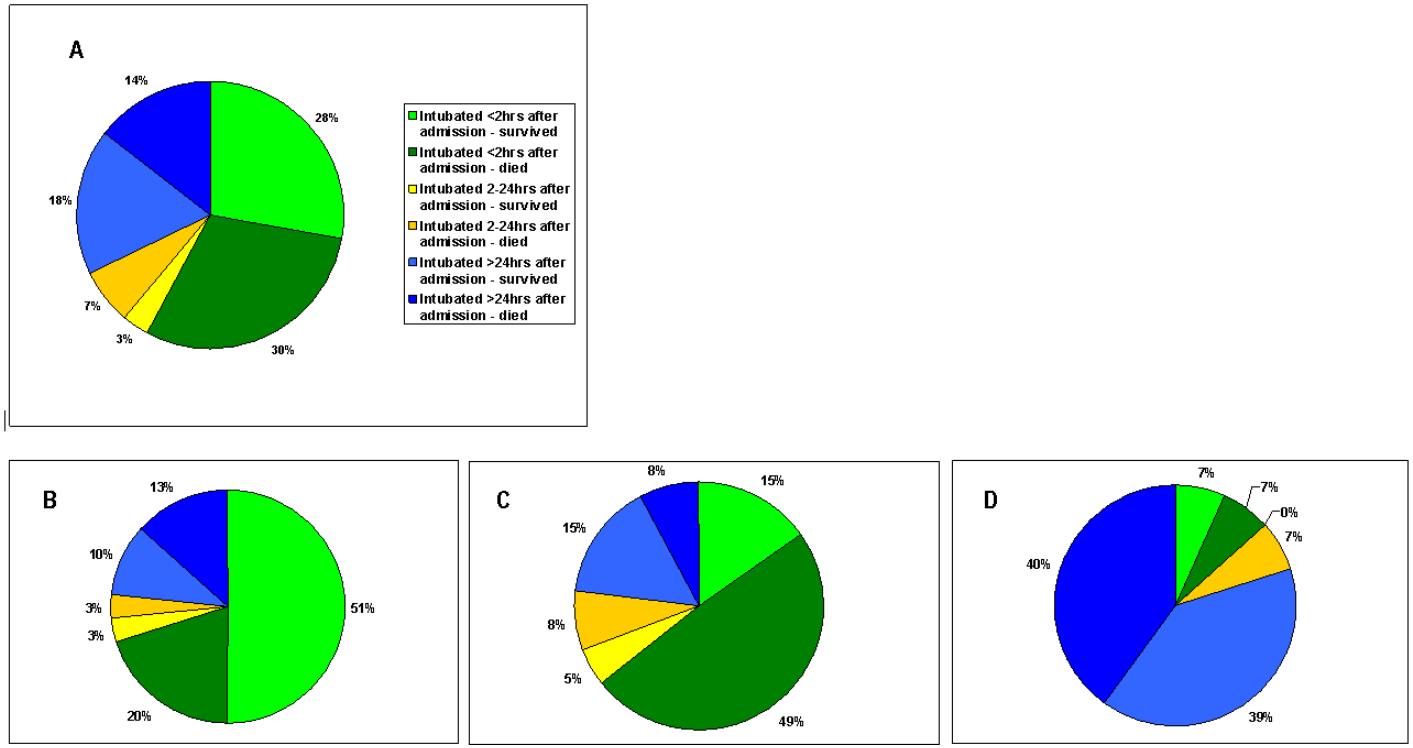


Figure 1.3: Timing of respiratory failure and outcome according to OP pesticide ingested. Timing of respiratory failure according to outcome for A) all intubated patients (n=90), and patients poisoned by B) chlorpyrifos, C) dimethoate and D) fenthion. Green colouring is for intubations within 2 hours of admission, blue for intubations after 24 hours, and yellow for intubation at intermediate times. Dark colouring indicates patients who died after intubation. Data reproduced from (Hulse et al., 2014b) with permission from the American Thoracic Society ©.

The distinction between early respiratory failure in unconscious patients and later respiratory failure in conscious patients combined with isolated neuromuscular junction (NMJ) dysfunction, [first reported by Wadia (Wadia et al., 1974)] was also observed in a small patient cohort using electrophysiology. Patients intubated within 24 hours (n=7) were found to have a median Glasgow coma score (GCS) of 10/15 (range 4-13) and normal peripheral nerve/muscle electrophysiology at intubation (phrenic nerve/diaphragm function was not tested). In comparison, those intubated after 24 hours (n=5) had a median GCS of 15/15 (range 12-15) with obvious abnormal peripheral nerve/muscle electrophysiology indicating disruption of neuromuscular junction transmission (Jayawardane et al., 2012).

1.6.1 Acute cholinergic syndrome

During the acute cholinergic crisis, respiratory failure can occur from: local pulmonary muscarinic effects e.g. bronchoconstriction, bronchorrhoea and alveolar oedema; central depression of the respiratory centre; and a flaccid paralysis though depolarising block of the muscles of respiration (Nachmansohn and Feld, 1947, De Candole et al., 1953, Namba, 1971, Wadia et al., 1974). Studies across several animal species indicate that the relative importance of the different mechanisms varies by species (De Candole et al., 1953). The exact balance of the three mechanisms in causing death in humans is uncertain; however, central respiratory failure predominates in non-human primates (see below), suggesting that this is likely to be the dominant mechanism in humans.

1.6.1.1 Local airway effects

Animal studies show that OP compounds cause bronchospasm, most likely due to local effects (Rezk et al., 2007, Segura et al., 1999, Gundavarapu et al., 2014). Bronchial smooth muscle predominantly contracts in response to muscarinic M3 receptor stimulation, with some involvement of nicotinic and M2 receptors [the latter demonstrates negative feedback control (Wess et al., 2007)]. The non-specific muscarinic antagonist drug atropine is highly effective at reversing both bronchorrhea and bronchoconstriction.

1.6.1.2 Alveolar fluid and bronchorrhea

Alveolar fluid has been observed in many cases of OP pesticide poisoning (Table 1.4). In the largest published autopsy case series of 85 patients with OP pesticide poisoning (who were treated with appropriate doses of atropine: 12-24mg every hour, up to 1g/24 hours), 75% of patients dying within 24 hours (n=36) showed pulmonary interstitial oedema while 25% showed parenchymal haemorrhage (Kamat et al., 1989).

Bronchorrhoea results from neuronal and non-neuronal cholinergic stimulation of the mucus glands, cilia, and cells producing periciliary fluid (Kummer et al., 2008, Takemura et al., 2013). Although atropine turns off excess fluid production, it does not increase the removal of alveolar fluid via the interstitial space to the lymphatic system (Takemura et al., 2013). Fluid removal therefore limits the rate of improvement in pulmonary oxygenation after atropine therapy. Sympathetic nervous stimulation may assist fluid removal from the alveoli through β receptor activation (Matthay et al., 2002). However, the effect of salbutamol in OP poisoned guinea-pigs was short-lived suggesting the involvement of other mechanisms (Segura et al., 1999). A series of animal and clinical studies is required to improve the understanding of the central and/or local pathophysiology of OP-induced alveolar oedema following poisoning (Carey et al., 2013), as its resolution could correlate with patient survival, in the context of acute lung injury (Ware and Matthay, 2001).

1.6.1.3 Disruption of the alveolar capillary membrane

Animal studies show disruption of the pulmonary endothelial-epithelial barrier by blood-borne OP compounds. Intravenous VX given to open chest anaesthetised dogs caused alveolar oedema secondary to an increase in pulmonary capillary permeability (Lainee et al., 1991) and similarly in *ex vivo* rabbit lung perfusion studies using intravenous parathion (AI) dissolved in propylene glycol (Delaunois et al., 1992). The authors believed that cholinergic stimulation of M1 and M3 receptors on C nerve fibres caused the increased permeability of endothelial cells and subsequent pulmonary oedema, with stimulation of the M2 receptor having an inhibitory effect (Delaunois et al., 1994). Although M2 receptors have been confirmed on C-nerve fibres in rat skin, muscarinic receptor subtypes have yet to be confirmed on C-nerve fibres within the human lung (Haberberger and Bodenbenner, 2000).

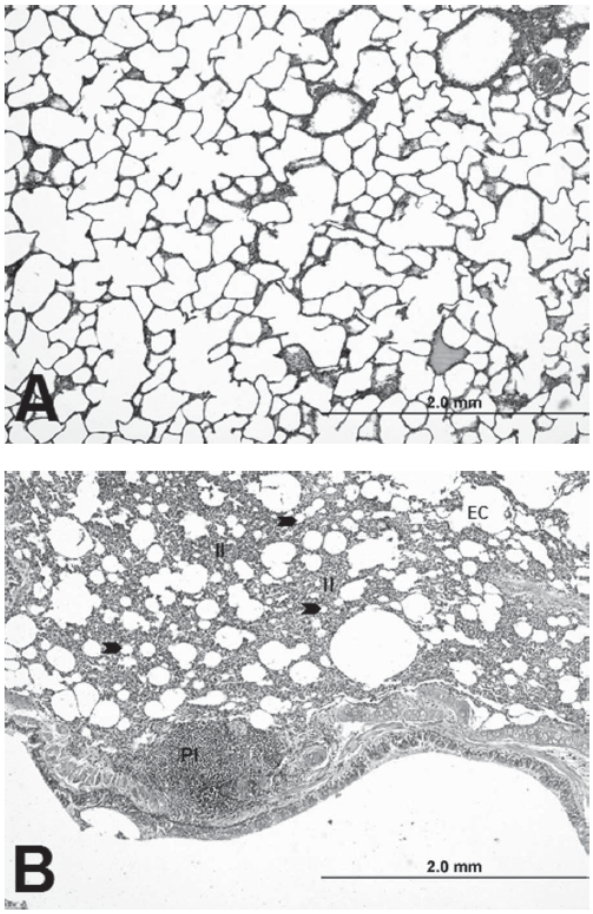


Figure 1.4: Pulmonary histopathological changes following orogastric administration of fenthion in sedated rabbits. The OP pesticide was administered using an orogastric tube. After 6 hours the rabbits were euthanised under anaesthetic. Control lung (A) has normal alveolar architecture with (B) showing affected lung following fenthion ingestion. There is alveolar and interstitial oedema, peribronchial inflammation (PI), emphysematous changes (EC), with alveolar destruction (black arrows). Image taken from Yavuz *et al* with permission (Yavuz et al., 2008), www.tandfonline.com.

In vivo rabbit studies using orogastric fenthion [51% commercial formulation] showed that after 6 hours of administration, serious lung tissue damage was observed including: alveolar and interstitial oedema and haemorrhage, inflammation, emphysematous changes and alveolar destruction (Figure 1.4) (Yavuz et al., 2008). Similar histopathological findings were found in chronically poisoned rats given either chlorpyrifos (AI) mixed with 0.5% dimethyl sulphoxide (DMSO) by gavage

every day for 28 days (Uzun et al., 2010) or dimethoate (AI) mixed with their drinking water for 30 days (Amara et al., 2012).

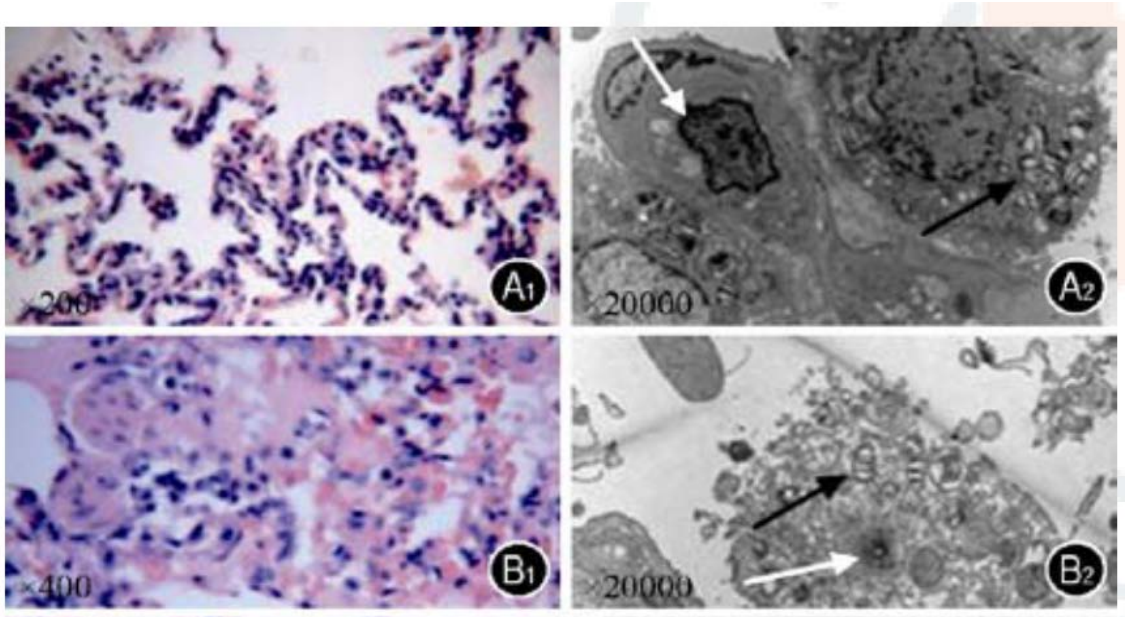


Figure 1.5: Pulmonary histopathological changes following orogastric administration of dichlorvos in anaesthetisted pigs. The OP pesticide was administered using an orogastric tube, and the pigs euthanised after 6 hours whilst under anaesthetic. Control lung (A1) has normal alveolar architecture (A2) showing normal type 2 epithelial cells with lamellar bodies (black arrow) and normal cell nuclei (white arrow). The OP pesticide poisoned pig lung shows (B1) alveolar and interstitial oedema with evidence of inflammatory cells and (B2) alveolar fluid and cellular debris, altered type 2 epithelial cell morphology with vacuole like lamellar bodies, absent or swollen mitochondrial cristae (black arrow) with nuclear lysis (white arrow). Images taken from He *et al* (He et al., 2012a) with permission. Images A1 and B1 haematoxylin and eosin stained lung tissue x200-400 magnification, images A2 and B2 are electron microscopy images of the same lung tissue at x20,000 magnification.

In vivo pig studies using orogastric dichlorvos [80% commercial formulation] showed a similar pattern lung injury (Figure 1.5). Additional features included a large amount of cellular debris and oedema in the alveolar spaces, with abnormal type 2 alveolar epithelial cell morphology, destruction of mitochondria and erosion of the alveolar capillary membrane (He et al., 2012b). Pigs that had ingested dichlorvos demonstrated reduced PaO_2/F_IO_2 ratios, and significantly increased extravascular lung water over time (6 hours), when compared with controls. The post mortem wet to dry lung weight ratio was also significantly increased in the OP pesticide group when compared

with controls (6.32±2 vs. 2.83±0.7) signifying marked pulmonary oedema. Human studies are lacking.

1.6.2 Acute respiratory distress syndrome (ARDS)

Acute respiratory distress syndrome (ARDS) is the 'umbrella' term used to describe acute lung inflammation with non-cardiogenic pulmonary oedema and respiratory failure. It is caused by various stimuli (e.g. sepsis, pulmonary aspiration, trauma) and its pathogenesis involves damage of the alveolar-capillary membrane leading to activation of the inflammatory and coagulation pathways (Figure 1.6) (Ware and Matthay, 2000). Neutrophils are important to the development of ARDS and elevated levels in the bronchoalveolar lavage fluid (BALF) correlate with patient survival (Grommes and Soehnlein, 2011).

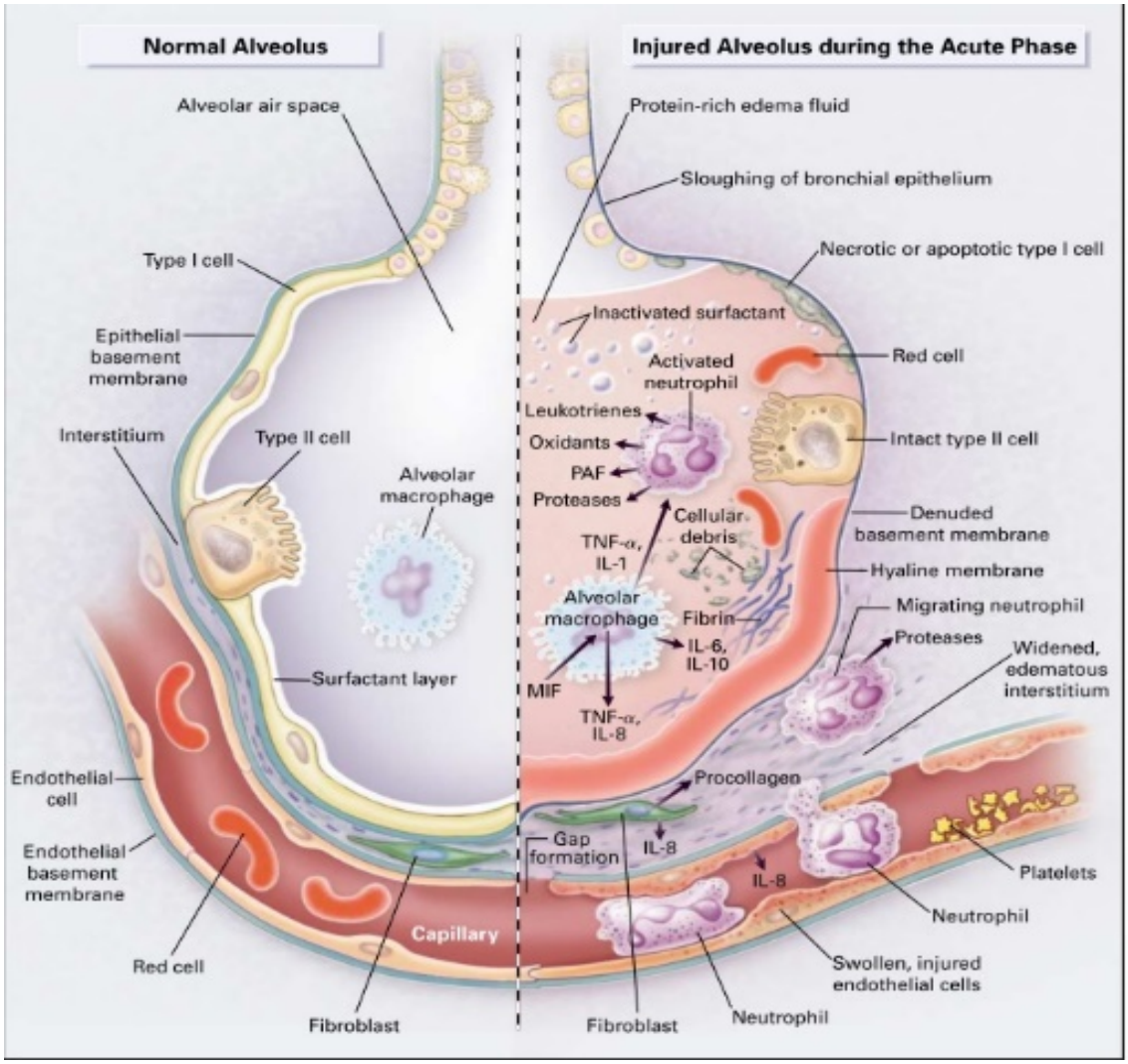


Figure 1.6: A normal (left) and injured (right) alveolus in the acute phase of acute respiratory distress syndrome (ARDS). In the acute phase of ARDS, there is sloughing of both the bronchial and alveolar epithelial cells, with the formation of protein-rich hyaline membranes on the basement membrane. Alveolar macrophages may secrete cytokines, interleukin-1, 6, 8, and 10, and tumour necrosis factor a (IL-1, 6, 8, 6, 10, and TNF-**α), which can act locally to** stimulate chemotaxis and activate neutrophils. Neutrophils (attracted by IL-8) adhere to the injured capillary endothelium and move through the interstitium into the alveolar air space, which is filled with protein-rich oedema fluid. They can then release free radicals including oxidants and platelet activating factor (PAF). The influx of protein containing oedema fluid into the alveolus can also lead to the inactivation of protective lung surfactant. Image from (Ware and Matthay, 2000) used with permission, Copyright Massachusetts Medical Society.

1.6.2.1 OP poisoning and ARDS features

ARDS has been reported in several cases of OP pesticide poisoning (Table 1.4) (Akgur SA, 2001). It may result directly from pulmonary complications of poisoning, such as aspiration or inhalation, or indirectly via haematogenous exposure to OP pesticide. As ARDS may be undiagnosed in over 50% of non-poisoning cases (Ferguson et al., 2005), it is possible that it is also frequently undiagnosed in OP poisoned patients.

Guinea pigs exposed by inhalation to VX and soman display ARDS as shown by a dose-dependent increase in alveolar fluid, substantial increases in bronchoalveolar lavage protein and neutrophils, with vascular engorgement and inflammation (Wright et al., 2006, Perkins et al., 2013). Primates exposed to inhalational sarin or soman also develop a pulmonary neutrophilia, which is statistically significant at day 4 in soman poisoning when compared with control animals (Anzueto et al., 1990). It is unclear whether OP pesticide induces a pulmonary neutrophilia as oral fenthion EC only caused an increase in alveolar macrophages, with oral dichlorvos EC producing an 'inflammatory cell aggregation' in the lung tissue, neither specifying the presence of neutrophils (Yavuz et al., 2008, He et al., 2012a).

Intraperitoneal dichlorvos (AI) in rats caused destruction of alveolar type 2 cells (loss of lamellar-like bodies) and surfactant production, with mild oedema in alveolar type 1 cells (Wang et al., 2010b).

The other histopathological changes within the alveolar capillary membrane, and increases in lung parenchymal oedema in response to oral OP pesticide exposure have already been discussed above (figure 1.4 and 1.5).

The pulmonary histopathology resulting from ingested OP pesticide could be caused by excess levels of free radical compounds in the lung (Pena-Llopis, 2005). Rats who were chronically poisoned displayed increased lung levels of oxidation products and antioxidant enzymes after a month's exposure to oral OP pesticide (Uzun et al., 2010, Amara et al., 2012). Mitochondria are a site of cellular oxygen consumption, and one proposed mechanism for OP-induced free radical

formation is through mitochondrial dysfunction, which can lead to oxidative damage and eventual cell death (Karami-Mohajeri and Abdollahi, 2013).

Animals exposed to OP compounds develop inflammatory changes in the lung. Rats poisoned with inhalational Sarin had increased concentrations of inflammatory cytokines [IL-1β, IL-2 and TNF-α] within the lung tissue and BALF at 24 hours post exposure (Gundavarapu et al., 2014). However, the differences between the mean BALF cytokine levels in the control and sarin exposed rats were essentially small (between 5-20 pcg/mL) and below that usually observed in ARDS.

OP pesticide studies investigating pulmonary inflammation after poisoning are lacking. In an occupational asthma study, guinea pigs exposed to subcutaneous 1mg/kg parathion (AI mixed with peanut oil), showed significantly increased expression of mRNA TNF-α and IL-1β in BALF macrophages, compared with vehicle controls, 24 hours after exposure (Proskocil et al., 2013). Yet the experimental dose of OP pesticide was extremely low [dermal LD_{50} for guinea pigs is 45 mg/kg] and did not produce a pulmonary neutrophilia. These results are unlikely to reflect the effects of exposure to large doses of OP compounds that occur with self-poisoning.

Toll-like receptors (TLRs) are pattern recognition receptors present on lung and infiltrating cells and are involved in the innate immune system. The receptors are stimulated by environmental toxins and pathogens when normal host defences fail and mediate the production of cytokines and chemokines required for an adaptive immune response. Murine knock out models have implicated TLR 2 and 4 in the development of non-infectious acute lung injury, but it is not known which TLRs are involved with the lung injury created through OP ingestion and aspiration (Lafferty et al., 2010).

In conclusion, the precise nature of pulmonary injury and immune-pathophysiology resulting from OP pesticide poisoning has not been elucidated, and may therefore differ from classic ARDS.

1.6.3 Central nervous system effects

Clinical experience of rapid respiratory arrest with OP nerve agents and potent pesticides (see above) suggests that the predominant early mechanism in humans is likely to be central. The absence of clinical studies of OP-induced acute clinical respiratory arrest has prompted the use of animal models. Central respiratory depression can be objectively distinguished from NMJ

dysfunction by recording phrenic nerve activity and the diaphragm's response to phrenic nerve stimulation (De Candole et al., 1953).

In non-human primates, lethal sarin and soman vapour doses caused apnoea and hypoxia (PaO_2 <50mmHg) within 5 min of administration (Anzueto et al., 1990). Diaphragm NMJ function (as measured by pressure generation through bi-lateral phrenic nerve stimulation) was still intact and 70-80% of normal in sarin poisoned and normal in soman poisoned animals at the start of the apnoea. This indicated that apnoea was as a result of absent phrenic nerve signalling (central effect) at the time of respiratory arrest. Diaphragmatic NMJ function did deteriorate over the following hours, returning to > 70% of baseline by day four. Phrenic nerve studies have been conducted in OP poisoned patients (Singh et al., 2000) to investigate the intermediate syndrome within 48 hours of poisoning, but not for acute cholinergic respiratory failure. Clinically orientated phrenic nerve neurophysiological and diaphragmatic electromyographic studies are required during the respiratory failure caused by acute cholinergic syndrome in order to determine whether central or NMJ dysfunction predominates in human poisoning.

The mechanism of OP-induced central respiratory depression is unknown. The proposed respiratory centre pacemaker (timing system) - the Pre-Boetzinger complex (Pre Bot-C) - in the venterolateral medulla has glutamatergic and muscarinic control. Excess acetylcholine may alter the function of the Pre Bot-C or other linked hindbrain areas to depress respiratory activity e.g. switch off the respiratory timing mechanism (Gaspari and Paydarfar, 2011, Burchfiel and Duffy, 1982). Pre-treatment with centrally acting, but not peripherally acting, anticholinergic drugs protected against respiratory failure in rat models of OP poisoning, suggesting central muscarinic control (Bird et al., 2003). The relative contribution of afferent and efferent pathways and central respiratory networks for either central apnoea or lung dysfunction are not well understood (Carey et al., 2013).

OP nerve agents, and to a lesser extent pesticides, can paradoxically cause seizures (excitatory activity) secondary to large increases in brain acetylcholine (Lallement et al., 1998). This can worsen cerebral hypoxia and compromised respiratory efforts (Sellestrom et al., 2013, Banday et al., 2015).

Diazepam prevented central respiratory depression in OP pesticide poisoned rats, perhaps by reducing seizure like activity, allowing normal respiratory centre signalling (Dickson et al., 2003).

OPs can cause brain damage though CNS inflammation. Animal models have shown that glial cell activation occurs after exposure to nerve agents, with release of chemokines and cytokines in areas of the brain responsible for respiratory control (Banks and Lein, 2012).

1.6.4 Neuromuscular junction effects

During an acute cholinergic crisis, excess acetylcholine at NMJs leads to muscle fasciculations and in time, a flaccid paralysis due to a depolarising neuromuscular blockade. In his original description of NMJ dysfunction, Wadia noted fasciculations and paralytic signs in 27% and 26% of patients respectively, following diazinon pesticide poisoning (Wadia et al., 1974). A case series noted that fasciculations and paralysis at the start of poisoning were associated with an increased occurrence of intermediate syndrome (see below) (John et al., 2003).

1.7 Delayed respiratory complications of the acute cholinergic syndrome

An overall view of the respiratory complications of the acute cholinergic syndrome is shown in Figure 1.7.

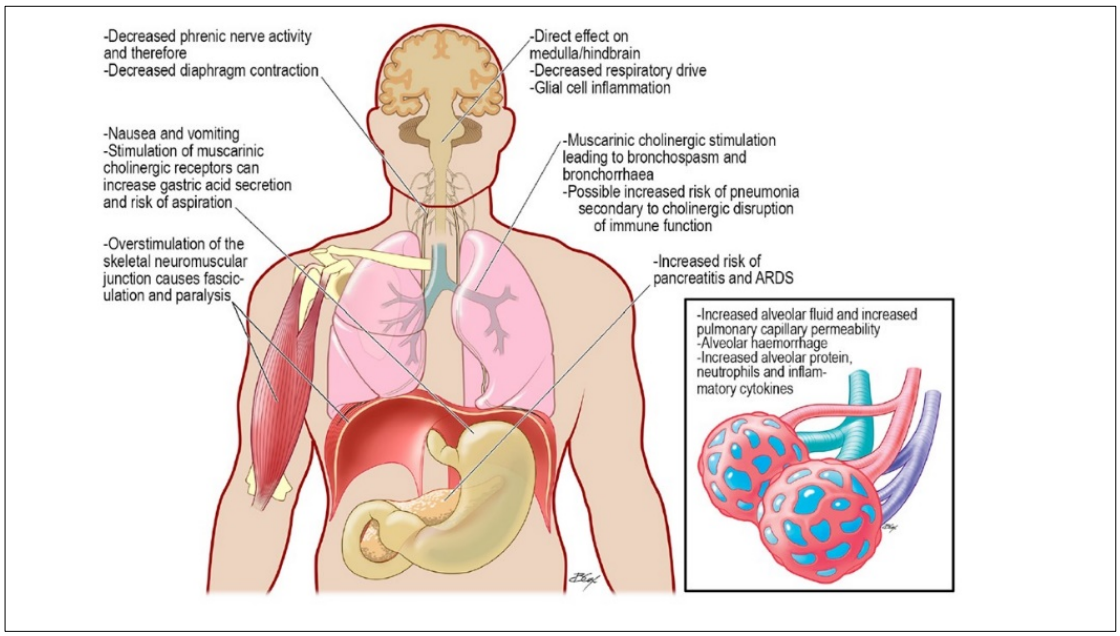


Figure 1.7: Pulmonary complications of organophosphorus poisoning. Exposure to OP compounds causes the acute cholinergic syndrome characterised by: reduction in central respiratory drive, bronchospasm, depolarising neuromuscular junction (NMJ) block and hypoxia due to bronchorrhoea and alveolar oedema. This may resolve or develop into acute respiratory failure that requires urgent treatment. Reduced consciousness and loss of airway control in the cholinergic syndrome increases the risk of aspiration, resulting in chemical pneumonitis that will worsen oxygenation and may progress to ARDS-like pathology. Overstimulation of the NMJ causes chronic peripheral dysfunction that may occur simultaneously with the acute cholinergic syndrome or after it has resolved with normal cerebral function (then termed the 'intermediate syndrome'). This NMJ dysfunction often requires days to weeks of mechanical ventilation with associated risk of ventilator-associated pneumonia and barotrauma. Image from (Hulse et al., 2014b) used with permission from the American Thoracic Society©.

1.7.1 Aspiration pneumonitis and pneumonia

Published clinical case series suggest that as many as 30% of OP pesticide poisoned patients aspirate their stomach contents causing either pneumonitis (chemical injury secondary to stomach acid) and/or eventual aspiration pneumonia (aspiration of bacterial matter)(Table 1.4). In an autopsy case series of 85 patients with OP pesticide poisoning, over two-thirds of the 49 patients who died after 24 hours showed segmental or lobar consolidation, possibly due to aspiration injury (Kamat et

al., 1989). This finding is not confined to resource-poor ICUs, as aspiration pneumonia was reported in 82% (27/33) of OP pesticide poisoned patients admitted to a German ICU (Hrabetz et al., 2013).

OPs cause vomiting, increased secretions, loss of consciousness, and loss of airway protection, with or without seizures. When these features occur before hospital presentation, aspiration is common. Patients can also aspirate after gastric lavage or forced emesis (Eddleston et al., 2007). Aspiration in the context of self-poisoning increases duration of hospital stay, morbidity, case fatality (Isbister et al., 2004), and cost to the health service.

Animal studies show that gastric acid secretion is increased through cholinergic stimulation of M3 and M5 receptors as well as inhibition of somatostatin release from D cells (Schubert and Peura, 2008, Aihara et al., 2005). The presence of low pH (<2.5), high aspirate volume (>0.3 mL/kg), food particles, and bacteria with pro-inflammatory cells increases the risk of severe lung injury after aspiration due to any cause (Marik, 2001, Bregeon et al., 2008). Gastric contents aspiration is estimated to cause 15-20% of ICU ARDS cases (Meade et al., 2008, Erickson et al., 2009) with case fatality as high as 40-50% (Matthay and Rosen, 1996). It is not known what proportion of reported 'aspiration pneumonias' after OP pesticide poisoning are due to pre-hospital aspiration, unsafe gastric lavage, and/or ventilator-associated pneumonia (VAP).

1.7.2 Complications of lung ventilation

Lung ventilation for acute cholinergic poisoning or NMJ dysfunction increases the risk of complications such as VAP and ventilator-induced lung injury (VILI) (Slutsky and Ranieri, 2013). VAPs are hospital acquired pneumonias that occur after 48 hours of tracheal intubation. In western hospitals, VAPs affect between 9-27% of intubated patients (Klompas, 2007), with an attributable mortality rate of 13% (Melsen et al., 2013). There is a paucity of data for Asian hospitals but 2-55% of intubated ICU patients develop a VAP, with a case fatality of 14-73% (Chawla, 2008). Although the incidence of these complications in OP poisoned patients is currently unknown, adherence to modern VAP prevention strategies (Hunter, 2012, Walsh et al., 2013, Price et al., 2014) and best ICU practice may reduce morbidity in these patients.

1.7.3 Immunomodulation

Lung inflammation is modified by the cholinergic nervous system – at least in murine models, with cholinergic activation producing a broadly anti-inflammatory pulmonary effect (Tracey, 2007). Importantly, these observations have been made using 'sterile' models of inflammation (e.g. lipopolysaccharide- or acid-induced inflammation) rather than aspiration. The effect appears to be mediated by the macrophage alpha-7 nicotinic acetylcholine receptor (α7nAChR) whose stimulation reduces the damage caused during the pulmonary response to sterile irritants (Wang et al., 2003). No similar primate or porcine studies have been published.

In the face of infection this effect could be harmful: α7nAChR stimulation in rodent pneumonia is associated with decreased neutrophil accumulation and a slower clearance of bacteria (Giebelen et al., 2009). Stimulation of α7nAChR on neutrophils *in vitro* leads to impaired superoxide generation and bacterial killing (Xu et al., 2008). Rats chronically exposed to subcutaneous VX and sarin or nicotine showed a decrease in metabolic and phagocytic neutrophil activity, combined with a reduction in plasma TNF-α, IL-1β and IL-6. The authors conclude this was largely due to cholinergic stimulation of α7nAChR within the monocyte phagocytic system (Zabrodskii et al., 2013).

The human lung is extensively innervated by the parasympathetic nervous system (Gwilt et al., 2007). If cholinergic immunosuppression also occurs in humans, OP poisoning should cause such effects. No clinical studies have yet been performed in OP poisoned patients; although, a study of Polish factory workers producing OP pesticides showed an increased incidence of upper respiratory infections (70% vs. 30%) and reduced *in vitro* neutrophil function compared to controls (Hermanowicz and Kossman, 1984). Clinical research is required to determine how cholinergic effects on inflammation might affect outcome in OP poisoned patients.

1.8 Intermediate effects of OP compound poisoning

1.8.1 NMJ dysfunction and the intermediate syndrome

The cholinergic crisis is often followed by paralysis of proximal muscles, particularly those involved in respiration. This paralysis may occur after resolution of cholinergic signs and is termed 'Type II

paralysis' (Wadia et al., 1974) or 'intermediate syndrome' (IMS) (Senanayake and Karalliedde, 1987).

In the original case series (Wadia et al., 1974) NMJ paralysis occurred in 18% (36/200) of diazinon OP pesticide poisoned patients – the same as that found in a recent second large prospective observational cohort study (31/176, 18%) (Indira et al., 2013). Respiratory failure frequently lasts for more than 7 days. In a case series of patients with laboratory proven OP pesticide poisoning who were intubated more than 24 hours post-exposure, the median time to final extubation was 219 (interquartile range 154-276) hours (Eddleston et al., 2006). This prolonged ventilation increases the risk of serious complications.

As originally defined (Senanayake and Karalliedde, 1987), the intermediate syndrome occurs 24 to 96 hours after OP pesticide exposure and consists of weakness or paralysis of the respiratory muscles, proximal limb muscles, neck flexors, and motor cranial nerves in the absence of cholinergic symptoms. Its incidence varies according to the OP involved and the severity of poisoning. Dimethyl OP pesticides such as methyl-parathion and fenthion are commonly implicated, but IMS does occur with diethyl OP pesticides such as parathion (De Bleecker et al., 1993). Amongst a case series of patients poisoned by the WHO Class II pesticides fenthion, chlorpyrifos and dimethoate, only fenthion poisoned patients were commonly intubated for the first time after 24 hours (Figure 1.3D).

A recent clinical study of 78 OP pesticide (mostly chlorpyrifos) poisoned patients showed ten patients with progressive changes in their hand muscle response to repetitive nerve stimulation (ulnar and median nerves) correlated with the severity of intermediate syndrome (Jayawardane et al., 2008). Five patients developed respiratory failure, with four of them showing severe and characteristic decrement effects prior to respiratory arrest. Thirty patients developed a *forme fruste* manifestation of the intermediate syndrome with less weakness and without progression to respiratory failure. In these, repetitive nerve stimulation showed modest change but no severe decrement (decreases in the magnitude of evoked responses) (Figure 1.8). However, as the amplitude can differ substantially between patients, the quality of data could have been improved

by describing each patient's response to nerve stimulation as a percentage of the initial amplitude. This information was not available in the paper.

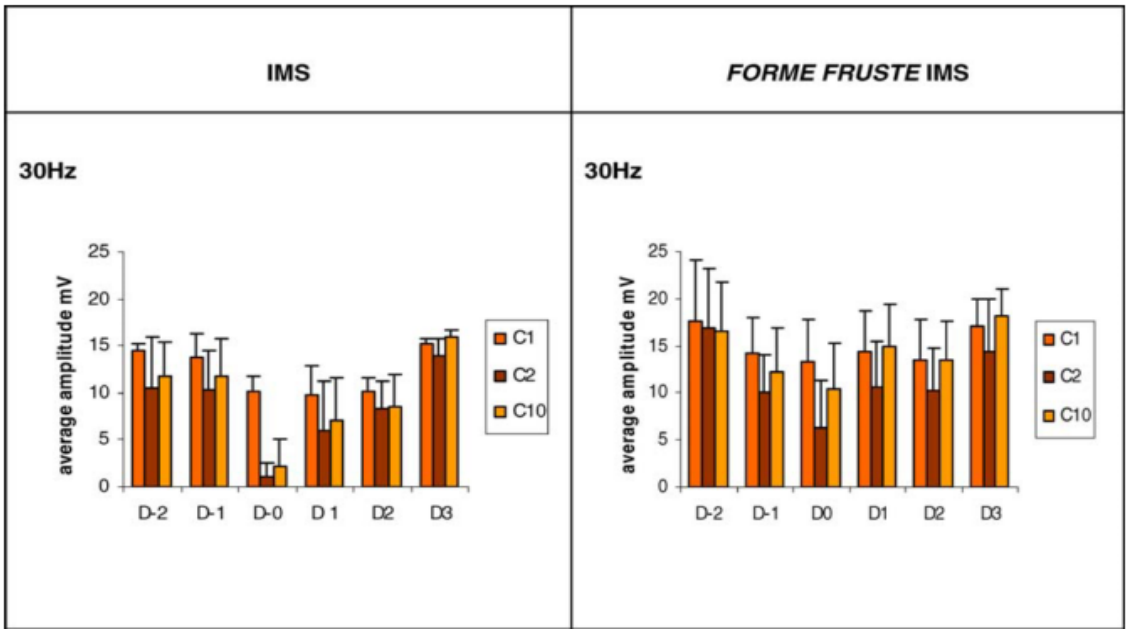


Figure 1.8: Comparison of average amplitudes C1, C2 and C10 between IMS and *forme fruste* IMS patient groups. The average amplitude of C1 (first), C2 (second), and C10 (tenth) compound muscle action potentials in the clinical IMS and forme fruste IMS at 30Hz frequency. Changes are plotted for each day relative to day 0 (D0). D0 in the clinical IMS group is the day they were diagnosed as clinical IMS. The D0 in *forme fruste* group is the day they were detected to have maximal muscle weakness. The forme fruste group has higher amplitudes at D0, D1 and D2 than IMS group indicating modest, but not severe decrement. Image is from (Jayawardane et al., 2008) used with permission.

This study revealed that the IMS has a spectrum of NMJ dysfunction and that characteristic changes in repetitive nerve stimulation can identify a subgroup of patients at high risk of developing respiratory failure (Jayawardane et al., 2008).

Neuromuscular junctional dysfunction is proposed to occur due to overstimulation of nicotinic receptors resulting in downregulation (De Bleecker et al., 1993, Senanayake and Karalliedde, 1987). Such dysfunction is typically unresponsive to both atropine and oximes (Wadia et al., 1974, Khan et al., 2001). Recent work suggests that NMJ dysfunction after poisoning with agricultural

dimethoate formulations is due to a combination of metabolites of the active ingredient (omethoate) and solvent (cyclohexanol) (Eddleston et al., 2012).

1.8.2 Critical illness polyneuropathy/myopathy (CIP/CIM)

The pathophysiology of CIP/CIM is unclear, but occurs in 50-70% of ICU patients ventilated for ≥7 days. The condition is characterised by limb and respiratory muscle weakness and can prolong weaning from mechanical ventilation (Hermans et al., 2008). The incidence of critical care polyneuropathy with OP poisoning is unknown and may be difficult to distinguish from IMS. No cases have been reported in the literature.

Some clinicians propose that IMS results from skeletal myopathy (John et al., 2003). Muscle necrosis at the motor end plate has been observed in animals after exposure to both OP pesticide and nerve agents (Sidell and Hurst, 1997) and at the diaphragm after human parathion poisoning (De Reuck and Willems, 1975). Raised creatinine kinase levels, indicating muscle injury, have been observed in OP pesticide poisoned patients (John et al., 2003), but the role of extended bed rest or seizure activity was not excluded. The current evidence does not suggest that muscle necrosis is important in the development of respiratory failure attributable to OP poisoning.

1.8.3 Delayed or recurrent cholinergic toxicity

Due to their chemical properties, highly fat soluble OP pesticides such as dichlofenthion (Davies et al., 1975) and fenthion (Eddleston et al., 2005) may cause delayed onset cholinergic poisoning. This usually occurs more than 24-48 hours post exposure although recurrent cholinergic features may occur days to weeks after poisoning, perhaps as fat stores are metabolised and OP released. In a case series of five dichlofenthion-poisoned patients, one apparently stable patient developed sudden fatal cholinergic respiratory toxicity 60 hours post exposure. A second patient required resuscitation after 40 hours, while a third patient required atropine for 47 days to prevent recurrent cholinergic toxicity (Davies et al., 1975). Such recurrent effects are likely to explain the delayed encephalopathy and coma reported 4-7 days post admission for a small case series of patients poisoned by fat soluble OP pesticides (Peter et al., 2008).

1.8.4 Overlap of acute cholinergic toxicity and NMJ dysfunction/intermediate syndrome

The intermediate syndrome usually occurs after resolution of the acute cholinergic crisis (Wadia et al., 1974). However, cholinergic symptoms and NMJ dysfunction sufficient to require long term ventilation (i.e. IMS) are not mutually exclusive and may overlap (Wadia et al., 1974, John et al., 2003, Eddleston et al., 2006). In a large case series of Sri Lankan patients requiring intubation, some dimethoate-poisoned patients regained consciousness as the acute cholinergic syndrome settled, yet were paralysed and required ventilation for days to weeks. In these patients, there was clear overlap between cholinergic features and intermediate syndrome-like NMJ dysfunction (Eddleston et al., 2006).

1.9 Late respiratory complications of OP poisoning

1.9.1 OP-induced delayed polyneuropathy

OP pesticide poisoning may be complicated weeks later by an OP-induced delayed polyneuropathy (OPIDP) which results from inhibition of neuropathy target esterase (NTE) in axons (Glynn, 2006, Richardson et al., 2013). NTE catalyses the deacylation of phosphatidylcholine - the major phospholipid of eukaryotic cell membranes - to soluble products. Its inhibition causes paralysis with swelling and degeneration of distal long axons in the legs and spinal cord. OPIDP has been known to cause respiratory failure through phrenic nerve involvement (Rivett and Potgieter, 1987).

1.9.2 Delayed pulmonary sequelae

Monitoring of patients exposed to non-blistering nerve agent attacks during the Iran-Iraq war in the 1980s revealed long term pulmonary sequelae. In 201 survivors (>80% life-long non-smokers), 11% had abnormal spirometric variables with 58% patients having an abnormal chest CT, most commonly showing air trapping and emphysematous changes (Ghanei et al., 2010). It is unclear whether similar chronic lung damage occurs after acute ingestion and aspiration of OP pesticides.

Chronic OP pesticide exposure has also been linked with the development of lung cancer (diazinon) and paediatric asthma-like airway hyper-reactivity in two recent human cohort studies (Jones et al., 2015, Raanan et al., 2015).

1.10 Importance of solvents, surfactants and contaminants

OP poisoning may be worsened by the ingestion of co-formulated compounds such as solvents e.g. cyclohexanone, xylene or petroleum fractions (Eddleston et al., 2012, Casida and Sanderson, 1961, Nalin, 1973). Porcine studies indicate that cardiovascular toxicity, reduced consciousness, and NMJ dysfunction following exposure to agricultural dimethoate formulations are due to both the dimethoate (AI) and the solvent (Eddleston et al., 2012). Aspiration of both solvents and surfactants, as well as gastric contents and OP, may exacerbate pulmonary damage (Nogue et al., 2003).

Poor storage, or sun (UV) exposure of OP pesticides can create contaminants, toxic metabolites or by-products (Chukwudebe et al., 1989) which can potentiate the cholinergic toxicity of the OP. This was observed in a large cohort of OP pesticide workers using malathion who were accidentally exposed to the toxic by product iso-malathion (Baker et al., 1978).

Trialkyl-phosphorothiotaes are toxic metabolites created after OP pesticides are exposed to sunlight and cause lung injury and death in rats within 4 days of exposure. The lung injury consisted of a swollen alveolar capillary membrane, destruction of type 1 alveolar epithelial cell, increased alveolar macrophages and leucocyte infiltration of the pulmonary interstitium (Dinsdale et al., 1982). This lung injury bears some resemblance to that created by systemic OP poisoning and may therefore be involved in the overall pathogenesis of OP pesticide-induced lung injury.

1.11 Management of OP pesticide poisoning

The management of severe OP toxicity requires urgent resuscitation with oxygen and judicious fluid management, plus the intravenous administration of doubling doses of atropine to patients with cholinergic features: pinpoint pupils, excess sweating, bronchorrhea, bradycardia, hypotension, and dyspnoea (Abedin et al., 2012, Eddleston et al., 2004). If oxygen is unavailable, atropine can be given in its absence during resuscitation (Konickx et al., 2014). The role of oximes to reactivate inhibited AChE is controversial: a recent Cochrane review suggested that there is insufficient evidence to determine whether oximes are beneficial or harmful in OP pesticide poisoning (Buckley et al., 2011).

1.11.1 Airway management of OP pesticide poisoning

Airway control is vital to reduce the risk of complications, indicating the need for early tracheal intubation and mechanical ventilation. The possibility of recurrent cholinergic toxicity and NMJ dysfunction necessitates the regular and careful monitoring of poisoned patients to identify those who require intubation, extubation or tracheostomy for prolonged ventilation (Durbin, 2010). In a western ICU, 36% (12/33) of OP (mainly parathion or oxydemeton-methyl) poisoned patients required re-intubation and 6% (2/33) required tracheostomy for prolonged ventilation (Hrabetz et al., 2013).

1.12 Reason for thesis

Approximately one third of patients who self-harm through ingestion of OP pesticide are intubated and ventilated to manage the effects of acute cholinergic toxicity, but up to 50% die (Table 1.4). One theory for the high mortality observed within intubated OP pesticide-poisoned patients is that prior to medical intervention, patients aspirate their stomach contents (including the OP and its solvent) into their lungs causing injury. The pathophysiology of such an aspiration lung injury is unknown, neither is its potential to increase mortality amongst OP poisoned patients.

This thesis explores the existing knowledge base of pulmonary injury secondary to systemic OP pesticide poisoning, and then focuses on characterising the specific lung injury created through aspiration of OP pesticide and gastric juice by using an *in vivo* Gottingen minipig model, ovine ex vivo lung perfusion model and a pilot study in pesticide-poisoned patients.

1.12.1 Aims

1. To characterise the lung injury created through OP ingestion using data from previous *in vivo* minipig studies
2. To characterise some of the pulmonary pathophysiological mechanisms and lung injury created through aspiration of OP pesticide and gastric juice using an *in vivo* minipig model
3. To assess the type and severity of lung injury created by aspiration of gastric juice alone, gastric juice with OP (dimethoate EC40) or gastric juice with solvent (cyclohexanone) and how they differ.

4. To further explore the pathophysiology of OP pesticide poisoning and aspiration by using an *ex vivo* lung perfusion model
5. To conduct a pilot feasibility study in OP poisoned patients from Sri Lanka.

1.12.2 Hypothesis

Unconscious patients who both ingest and aspirate OP pesticide may develop a toxic lung injury which is capable of contributing to the increased mortality observed in intubated OP poisoned patients.

1.12.3 Research questions

Question 1: What pulmonary pathophysiology is caused by orogastric administration of OP pesticide in the Gottingen minipig?

Before investigating the pathophysiology of aspirated OP pesticide and gastric contents, it was necessary to first examine the lung injury created through orogastric placement of OP pesticide alone. Due to a lack of information in the literature concerning this subject, the results of two previous studies undertaken by my research group were analysed (chapter 3).

The existing minipig model was updated by incorporating modern human ICU care practices into a model of pulmonary aspiration (chapter 4). This involved providing protective ventilation (tidal volumes of 6-8 ml/kg) and using ventilator associated pneumonia (VAP) prevention strategies. This improved model was used in the minipig pulmonary aspiration study and allowed question 2 (chapter 5) to be answered:

Question 2:

a. *What pulmonary pathophysiology is caused by the aspiration of (i) gastric juice (GJ) alone, (ii) OP (dimethoate EC40) with GJ and (iii) solvent (cyclohexanone) with GJ?*

b. *Which combination produces the worst lung injury?*

The next challenge was to investigate whether a similar toxic lung injury occurred in intubated human OP pesticide poisoned patients who had aspirated (question 3; chapter 6). It was particularly important to compare the pathophysiology of the minipig with that observed in human OP poisoning to determine whether the minipig model might be considered appropriate for future treatment studies.

Question 3:

Is the lung injury observed in the minipig pulmonary OP aspiration model similar to that observed in human OP poisoning and aspiration?

Whilst in Sri Lanka, plasma was collected to measure the presence (or absence) of micro RNA (miRNA) biomarkers of toxic lung injury. If there were meaningful measurements, these biomarkers could help predict those OP pesticide poisoned patients who have aspirated – those who may need access to vital limited resources such as ambulance transport, intubation and ventilation and enhanced respiratory critical care (question 4).

Question 4:

Are there plasma micro RNA biomarkers that indicate OP poisoning and aspiration in humans?

To further understand the pathophysiology of this toxic lung injury, an ovine *ex vivo* lung perfusion model was developed, and used for a small pilot study (chapter 7). It was hoped that an *ex vivo* lung perfusion (EVLP) model could provide an adequate, high throughput, low cost alternative to the high cost and intensive staffing requirements of the *in vivo* minipig model.

Some of the pilot data (e.g. pulmonary toll like receptor (TLR) expression) generated a further research question which was briefly explored using previously collected minipig and human samples.

Question 5

a. *Can an ovine EVLP model be successfully developed to allow observation of OP pesticide pulmonary aspiration lung injuries?*
b. *Which TLRs are preferentially expressed in ovine lung secondary to pulmonary aspiration of OP (dimethoate EC40) with gastric juice or solvent (cyclohexanone) with gastric juice in an EVLP model?*
c. *Are TLR 3 and TLR 5 also expressed in porcine lung and human BALF cells secondary to pulmonary aspiration of OP pesticide and gastric contents?*

Chapter 2: Materials and methods

2.1 Materials

2.1.1 General chemicals and compounds

Chemicals	Company
Acetylthiocholine iodide	Cat no. A5751, Sigma, Dorset, UK
Acheson's silver dag	Agar Scientific, Essex, UK
Analytical grade Methanol (Sri Lanka)	Product code M/4000/17, Fisher Scientific, MA USA
Bovine serum albumin (BSA)	Cat no. BSAV-0050-01, BioFx laboratories, inc MD USA). After April 2014 I used BSA cat no. BP9700-100, Fisher Scientific, MA, USA.
Calibration gas (QuickCal)	No. 755581, GE Healthcare, Finland
Cold sterilisation fluid	Medistel (tristel), Cambridgeshire, UK
Cyclohexanone	Product no. W390909 99.8%, (98.14g/mol) [mw 98.15 g/mol, density 0.94 g/mL), Sigma-Aldrich, Dorset, UK
Dulbecco's Modified Eagle Medium (EVLP)	Cat no. 31053-044, Invitrogen, Paisley, UK
Dimethoate emulsifiable concentrate 40% (EC40)	(400 g/1000 mL) batch 37M 60217 06/0206, CHEMINOVA, Dorset, UK
DPX mounting medium	Cat no. 03702689, Raymond A Lamb, laboratory supplies
DTNB (5,5-dithio-bis-(2-nitrobenzoic acid))	Cat no. D8130, Sigma-Aldrich, Dorset, UK
Ethanol (100%-50%) made with RNAse free water or MiliQ water as per methods	Sigma-Aldrich, Dorset, UK
Ethopropazine hydrochloride	Cat no. E5406, Sigma-Aldrich, Dorset, UK
Foetal Calf Serum	Cat no. 10270-106, Gibco, Life Technologies, Paisley, UK
Formaldehyde 4% buffered pH7 ±0.2 (Formalin 10%)	VWR, cat numbers: 361388Q (now discontinued) and 9713.9025, Cellstor 20l 10% neutral buffered formalin, Cell path, Powys, UK (BAF-0010-20A)
Immersion oil (Immersol 518 N)	Zeiss, Cambridgeshire, UK
Methyl-D Choline (intravenous use surfactant tracer)	Made in Southampton, UK
Molecular grade Chloroform	Product no. 496189, Sigma-Aldrich, Dorset, UK
Normal goat serum; heat inactivated in a water bath at 56°C for 30 min.	Product No. G0-605, Biosera , Sussex, UK
Oleic acid	Sigma-Aldrich, Dorset, UK

Osmium Tetroxide (Transmission electron microscopy (TEM) fixation)	Pioloform, Agar Scientific, Essex, UK
Precellys 24 homogeniser	Bertin technologies, France
RNAse free water	Cat No. 1017979, Qiagen, Manchester, UK
Sulphuric acid	Prod no.102765G BDH AnalaR, Leicestershire, UK
TEM stains: 2% aqueous Uranyl Acetate and Lead Citrate	Leica UK Ltd, Milton Keynes, UK
Tween 20	Cat no. P2287, Sigma, Dorset, UK

Table 2.1: General chemicals and compounds

2.1.2 Anaesthetic drugs/equipment

Drug/Equipment	Model/ Manufacturer
Air filled blanket to reduce minipig heat loss on the surgical table	Bair hugger™, 3M, Bracknell, UK
Antibacterial mouth gel	1% Corsadyl w/w (chlorhexidine gluconate) dental gel, GSK, Middlesex, UK
Arterial line (catheter)	Cat no. 01159.09, Vygon, UK
Arterial monitoring sets (tubing and sensors)	Cat no. MX9622, logical® medex, Edwards Life Sciences and Smiths Medical, Kent, UK
Antibiotics/antifungals for gastric juice treatment	Metronidazole 500 mg bottle Gentamicin 2 x 80 mg/2 mL vials Vancomycin 1g /vial Amphotericin B 50 mg vial (30 mg used) powdered drugs reconstituted as per instructions
BAL 70 mL specimen traps	Cat no. MST-4000, Pennine Healthcare, UK,
BAL alligator forceps	Cat no. 100507 Disposable alligator cup biopsy forceps, Conmed Linvatec, Wilts, UK
Bronchoscopes	VETVU VFS-2A veterinary fiberscope, Krusse, UK Video bronchoscope (BRS-5000; Vision Sciences, Orangeburg, NY, USA)
Bronchoscope sheaths	Endosheath, Vision Sciences, NY, USA
Cardiac output and invasive blood pressure monitoring system	Lithium dye dilution cardiac output (LiDCO) and pulse contour analysis of cardiac output (Pulse CO) London, UK.
Central venous line (multilumen)	Cat no. CA-22-703, Teleflex, PA, USA,

Epidural catheters (16swg)	Cat no. 100/382/816, Portex, Smiths Medical, Kent, UK
Fentanyl citrate	500 mcg/10 mL vials
Flexible yankauer suckers (oral secretion removal)	Cat no. 8888501007, size 18F, 25cm long, Argyle, UK
Heat Moisture Exchange filters for ventilator circuits	Cat no. 1644, Intersurgical, clearguard, Berkshire, UK
Heparin sodium	5000IU/mL, 5 mL vials, Wockhardt, Wrexham, UK
ICU bay monitors	Datex ohmeda S/5, MCAIOV respiratory module, GE healthcare, Amersham, UK
Intravenous Hartmann's solution	Cat No. VM 10347/4009 XVD710 POM-V, 100mL bags, AQUPHARM, York, UK
Intravenous (0.9%) saline	Cat No. Vm 10434/4079 POM-V, 1000 mL bags, Vetivex 1 Dechra, Northwich, UK
Ketamine hydrochloride	Cat no. L090/4 E122054PBE, 100 mg/mL, Vetalar V, Pharmacia & Upjohn, Sandwich, UK
Lithium chloride	Cat No. PL17048/0001, 0.15 mmol/mL, LiDCO, London, UK
Midazolam	Cat.no PL00031/01/0126, 10 mg/2 mL, Hypnovel, Roche, Welwyn Garden City, UK
Mini-bronchoalveolar lavage catheter	Combicath, Plastimed, France
Norepinepherine	Cat No. PL04515/0240, 1 mg/mL, Hospira, Maidenhead, UK
Patient transfer monitors	Phillips, Surrey, UK and Propaq, Welch Allyn, Buckinghamshire, UK
Pentobarbital	Euthatal; Merial, Harlow, Essex, UK
Positive end expiratory (PEEP) valve, non-disposable	Cat no. 8407475,Drager, Germany
Propofol 1%	1 g/100 mL bottles, Fresenius Kabi, Cheshire, UK
Suction catheters	Cat no. OSC-1114/60, Prestrol oppo suction catheters 14FG, Pennine healthcare, UK
Suction machine (for 6 hourly removal of oral/tracheal secretions and BAL) and tubing	Suction machine: SAM, MG electric, Colchester, UK Tubing: Cat no. CT-40323m, 6 mm FF, Pennine healthcare, Derby, UK
Three lumen octopus intravenous connector for TIVA	Cat no. 0841.03, Vygon, UK

Torque controlled blocker (TCB) Univent endotracheal tube (ETT)	Cat no. 1202933, sizes 7.5 and 8.0 mm internal diameter (13 or 13.5 mm outer diameter), Fuji Systems, Tokyo, Japan
Urometers	Cat no.00-1227, Flexicare, Glamorgan, UK
Ventilators	Servo 300A, Maquet, Sweden Oxylog® 1000, Drager, Lubeck, Germany

Table 2.2: Anaesthetic drugs and equipment

2.1.3 Solutions, buffers and kits

All solutions were prepared with Milli-Q® water unless stated otherwise.

Solutions and Buffers	Recipe
BD Cytometric Bead Array (CBA) Human Inflammatory Cytokines kit	Cat no. 551811, New Jersey, USA Includes: - Human IL-1β, IL-6, IL-8, IL-10, TNF-α and IL-12p70 capture beads - Human inflammatory cytokine PE Detection Reagent - Human inflammatory cytokine standards - Cytometer set up beads - PE positive control detector - FITC positive control detector - Wash buffer - Assay diluent - Serum enhancement buffer
Cell growth medium for BAL cells	500 mL Dulbecco's Modified Eagle Medium (DMEM) cat no. BE12-614F, Lonza, Slough, UK 50 mL Foetal calf serum cat no. 50260-500, Biosera, Sussex, UK 5 mL Penicillin streptomycin, cat no. DE17-603E, Lonza 5 mL L-glutamine, cat no. BE17-605E, Lonza
DTNB (5,5-dithio-bis-(2-nitrobenzoic acid)) mix	39.6 mg dissolved in 10 mL phosphate buffer (AChE/Hb mix) 10mM
ELISA (sandwich immunoassay) porcine kits IL-8, IL-6, CRP and SP-D (human)	IL-6 (cat.no DY686), CXCL8/IL-8 (cat no. DY535), CRP (cat no.DY2648) and human SP-D (cat no. DY1920) RnD systems, Abington, UK

Ethopropazine mix	10.5 mg dissolved in 5 mL HCl, shake and heat.
Human basic flow cytomix kit + CRP beads	Cat no. BMS 8420FF, Ebioscience, CA, USA
KwikDiff ™solutions 1,2 and 3	Cat No. 9990700, Thermo electron corporation. Use neat as required for staining bronchoalveolar lavage derived cells. Solution 1 – methyl alcohol Solution 2 – Xanthene dye and buffer Solution 3 – Thiazine dye and buffer
Phosphate buffer (Cobas Fara centrifugal analyser)	0.1M pH 7.4 Solution 1:NA_2HPO_4 14.2g/L, Solution 2: KH_2 PO_4 13.6g/L To 800 mL of solution 1 and solution 2 to give pH 7.4.
Phosphate buffered saline (PBS)	PBS: 5 x phosphate buffered saline tablets were dissolved in 1000 mL MilliQ water [1 tablet dissolved in 200 mL water gives 10mM phosphate buffer, 2.7mM KCL, 137mM NaCl], cat no. P4417-100 Sigma Aldrich, MO, USA
Plasma diluent for ELISAs	Foetal calf serum added to PBS to make 20-60% solutions.
Porcine RAGE ELISA kit (competitive immunoassay)	Cat no. PR0356, NeoBiolab, MA, USA
Porcine surfactant associated protein type D ELISA kit (competitive immunoassay)	Cat no. PS 0177, NeoBiolab, MA, USA
Porcine vWF ELISA kit (sandwich immunoassay)	Cat no. PV 0008, NeoBiolab, MA, USA
Reagent 1 (AChE assay)	10 mL phosphate buffer 445 µL DTNB 46.5 µL ethopropazine
Reagent 2 (AChE assay)	Acetylthiocholine solution: 36.4 g dissolved in 10 mL dH2O, 12.6mM
Reagent diluent (to dilute non plasma samples for ELISA and block ELISA plates)	1% BSA in PBS
Scanning electron microscopy (SEM)/ Transmission electron microscopy (TEM) buffer for tissue samples	2% gluteraldehyde buffered with sodium cacodylate, Agar Scientific, Essex, UK
Scott's tap water substitute (histology blueing agent)	Cat no. 380290001, Leica, UK

Stop solution for ELISA	2N H_2SO_4 made from dissolving 5.4 mL pure sulphuric acid in 100 mL dH2O
Substrate solution for ELISA	1:1 mixture of colour reagent A (H_2O_2) and colour reagent B (Tertramethylbenzidine) (R&D systems cat No. DY999) or TMB/ES001 Millipore, MA, USA
Transformation solution (Hb assay)	200 mg K_3 (Fe $(CN)_6$) 50 mg KCN 1000 mg $NaHCO_3$ Dissolve the above in 1L dH2O, 0.5 mL Triton x-100 then added. Store in a light proof bottle.
Wash buffer (ELISA)	0.05% Tween 20 in PBS, pH 7.2-7.4

Table 2.3: Solutions, buffers and kits

2.1.4 Antibodies and primers

Antibodies/Primers/enzymes/kits	Cat number and Company
Agilent R2100 bioanalyser	Agilent Technologies, UK
Applied biosystems 7500 (RT-PCR)	Life Technologies, Paisley , UK
B actin	Primers and probes designed in Moredun Science park and manufactured by Life technologies, Paisley, UK
Micro RNA 21 5p miScript Primer Assay (human)	Cat no. MS00009079, Qiagen, UK 5'UAGCUUAUCAGACUGAUGUUGA
Micro RNA 39 3p miScript Primer Assay (human)	Cat no. MS00019789 , Qiagen, UK 5'UCACCGGGUGUAAAUCAGCUUG
Micro RNA 122 5p miScript Primer Assay (human)	Cat no. MS00003416, Qiagen, UK 5'UGGAGUGUGACAAUGGUGUUUG
Micro RNA 146a 5p miScript Primer Assay (human)	Cat no. MS00003535, Qiagen, UK 5'UGAGAACUGAAUUCCAUGGGUU
Micro RNA 1287 5p miScript Primer Assay (human)	Cat no. MS00014497, Qiagen, UK 5'UGCUGGAUCAGUGGUUCGAGUC
MiRNeasy plasma spike-in control lyophilized *C. elegans* miR-39 miRNA mimic	Cat no. 219610, Qiagen, Manchester, UK
miScript PCR Starter Kit: miScript Nucleics mix, miScript Reverse Transcriptase mix, HiSpec buffer, QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer, RNAse free water	Cat no. 218193, Qiagen, Manchester, UK

Novacastra Bond Polymer Refine Detection Kit	Peroxide block Ph6 buffer Bond primary antibody diluent Polymer reagent DAB chromogen Hematoxylin counterstain, Leica biosystems, Milton Keynes, UK
QIA shredder column	Cat no. 79654, Qiagen, UK
QIAzol lysis reagent	Cat no. 79306, Qiagen, UK
Quantifast RT-PCR Plus Kit	Cat no. 204482, Qiagen, UK
RNeasy MinElute Cleanup Kit: RNeasy MinElute Spin columns, collection tubes, RNase free reagents and buffers (RWT/RPE)	Cat no. 74204, Qiagen, Manchester, UK
TLR 1-10 primer/probes	Eurofin MWG operon, Germany Probes were 5'-FAM (flurophore)/3'-BHQ1 (quencher) conjugated
TLR 3 primary rabbit antibody	Cat no. NB100-56571, Novus biologicals, CO, USA
TLR 5 primary rabbit antibody	Cat no. NBP2-24827, Novus biologicals, CO, USA

Table 2.4: Antibodies and primers and associated extraction kits

2.1.5 Miscellaneous equipment

Equipment	Manufacturer/ type model
BD Accuri™C6 flow cytometer	BD, UK
Blood gas analyser	EPOC® – portable blood gas electrolyte and critical care analyser and single use cartridges, Woodley equipment, Ltd, UK
Capnograph for physiological dead space measurement	Datex-Engstorm Normocap 200, Finland
Capnograph calibration gas	cat no. 755581, Quick Cal™, GE Healthcare, Finland
Cardiac by-pass machine, reservoir and oxygenators	Jostra Maquet HL30, Sweden D905 E0S, Dideco oxygenator, Sorin group, Japan Quadrox oxygenator, Maquet, Sweden
40µm cell strainer	Falcon no. 352340, BD, UK
Centrifuges	R-8C laboratory centrifuge, REMI, Mumbai, India Sigma, SciQuip 2-6, Shrewsbury, UK
Cobas Fara centrifugal analyser	Roche Diagnostics, Welwyn Garden City, UK

Computerised Tomography scanner	16 slice GE lightspeed CT scanner, GE Healthcare, Buckinghamshire, UK
Cover slips	Menzel-Glaser, 18 x 18mm square, part no. MNJ-300-010W, Thermo Scientific, MA, USA
Cytocentrifuge	Cytospin 2, Shandon, Techgen, Zellik, Belgium
Cytocentrifuge filter card	No. 11922355, Shandon, white, Thermo Scientific, MA, USA
Dry gas meter	Harvard apparatus, MA, USA
ELISA plate heater/humidifier	Grant-bio PHMT, Cambridge, UK
ELISA plate- non tissue culture treated	Cat no. 35 1172, Falcon, Leicestershire, UK
ELISA plate reader	Molecular devices OptiMax tunable microplate reader, Sunnyvale CA, USA
ELISA plate washer	LP41 Plate washer, Adil instruments, Biorad, France
Haemocytometer	Improved Neubauer BS.748, Hawksley, UK
Incubator/shaker for 96 well plates	IEMS™ Thermoelectron corporation
Microscopes	• 1900's mirror directed microscope (monocular lens) in Sri Lanka • Laborlux K, Leitz • Amoeba celestron USB (for sri lanka) • Olympus BH2 plus reticule • Olympus slide scanner VS120 • Olympus Research Microscope AX70 Provis (NY, USA) • LSM 710 inverted confocal microscope (Zeiss)
Microscope slides	Menzel-Glaser, frosted end, BS7011, Thermo Scientific, MA, USA
Nano-Drop ND-1000 spectrometer	NanoDrop Technologies Nikon, Nanodrop Thermo Scientific, USA
Pipettes (electronic)	Cat no. 118171A Eppendorf, Hamburg, Germany
Precellys® 24 tissue homogenizer	Cat. No. EQ03119.200.RD000.0, Bertin Technologies, MA, USA
QImaging LightCycler® 480 384-well qPCR plates and plastic covers	Roche Applied Science, Welwyn Garden City, UK
Rotaflow pump, custom tubing packs and membrane oxygenators	Maquet, Sweden & Dideco D905 EOS , Sorin Group, Gloucestershire, UK
Scanning electron microscope	Stereoscan 240 scanning electron microscope, housed within EM Research Services, Newcastle University. Digital images collected with Orion 6.60.6 software.

Thermal cycler	Peltier thermal cycler PTC-200 GRI
Transmission electron microscope	The grids were examined using a Philips CM 100 Compustage (FEI) Transmission electron microscope and digital images were collected using an AMT CCD camera (Deben)
Water bath	Grant, Sub, Grant instruments, Cambridge, UK

Table 2.5: Miscellaneous equipment used in the research

2.1.6 Software

Data acquisition tool	Software used
AX70 Provis microscope pictures	Axiovision Rel 4.8
BD Accuri™C6 flow cytometer	FCAP Array software v.3
Data capture from the ICU machines	Edinburgh data logger v1.0.55.0 Vetronic Services Limited (copyright)
Image processing	Microsoft Word 2010 PowerPoint tools Analyze®
LightCycler® 480	LightCycler® 480 release 1.5 O SP3
Nano-Drop ND-1000 spectrometer	ND-1000 v3.3.0 (NanoDrop Technologies)
OPTImax tunable microplate reader	SoftMax Pro 4.8
Statistical analysis	Graph Pad Prism version 6.04 for Windows 8, Graph Pad Software, La Jolla California USA, www.graphpad.com Permutation tester 1.0 Stat Boss (2008) for non-parametric data.
Stereoscan 240 scanning electron microscope	Orion 6.60.6 software.

Table 2.6: Software used to process data

2.2 Methods

Methods that have been used in more than one study/chapter are described below, but largely pertain to the main Gottingen minipig pulmonary aspiration study.

The studies in the thesis appear in the order below (Table 2.7).

Study title in thesis	Date	Study description
Study 1	2008	Indirect lung injury caused by orogastric OP in a minipig model, historic data analysis.
Study 2 + dose ranging study	2012	Indirect lung injury caused by orogastric OP and dose ranging study in a minipig model
Study 3	2010	Pilot aspiration study in a minipig model, historic data analysis.
Main minipig OP aspiration study (chapter 5)	2012-2013	Pulmonary aspiration of OP in a minipig model (n=26)
Human OP aspiration study (chapter 6)	2013-2014	Pilot feasibility study into pesticide ingestion and aspiration in Sri Lanka
Ex vivo lung perfusion study (chapter 7)	2013	EVLP study into OP aspiration and direct and indirect lung injury

Table 2.7: Order of studies within thesis. EVLP = *ex vivo* lung perfusion.

2.2.1 Historic data analysis

The data collection method for individual studies conducted between 2008-2011 ranged from hand-written anaesthetic and research notes, to fully automated electronic recordings (Excel files, Microsoft) using software developed specifically for recording physiological data in the minipig laboratory (Edinburgh data logger v1.0.55.0 Vetronic Services Limited©).

Recorded respiratory data included, but was not limited to; arterial oxygenation (PaO_2), F_IO_2, oxygenation of the minipig, arterial blood gas measurement and pulmonary mechanics. This was occasionally supplemented with histopathology reporting and electron microscopy images of lung tissue. The bronchoalveolar fluid and plasma inflammatory markers were analysed using similar

methods described below. The results of these measurements were made available to me in an Excel spreadsheet.

Data were extracted, analysed and entered into a statistical software package to create graphs, and if numbers permitted, statistical analysis (Graph Pad Prism version 6.04 for Windows 8). Methodologies were derived from the same notes, and confirmed with the investigators where possible.

Whilst analysing the frequency distribution of thesis data it became apparent that the data were non-parametric (see below-statistics), and so non-parametric statistical tests were used where appropriate. Statistical omnibus testing used Kruskal-Wallis, followed by either Dunn's post-test or permutation testing dependent on the number of results.

2.2.2 Animal handling

The animal studies were approved by the Institutional Ethical Review Committee (Moredun Research Institute) and was licensed under the Animal (Scientific Procedures) Act 1986.

At the end of the experiment, or when specified physiological limits were crossed e.g. MAP of less than 45 mmHg unresponsive to fluid resuscitation and vasopressors, the minipigs were euthanised with a lethal dose (150 mg/kg) pentobarbital injection (Euthatal; Merial, Essex) under anaesthesia. Death was confirmed by the absence of vital signs and cardiac electrical activity.

2.2.2.1 Replacement, refinement and reduction

In order to adhere to current national (UK) guidelines encouraging the replacement, refinement or reduction of animals used during research we used a single animal unit to observe two different types of lung injury (indirect and direct) during the minipig pulmonary aspiration study (chapter 5) (Kilkenny et al., 2010). This technique demonstrated study refinement, and reduced the numbers of animals required.

This thesis tried to follow the ARRIVE reporting guidelines (Kilkenny et al., 2010) where possible.

2.2.2.2 Maintenance and husbandry

Following shipment from Ellegaard Denmark, Göttingen female minipigs were kept for at least one week in laboratory housing and cared for according to Ellegaard Göttingen minipig (Bollen et al., 2000, Svendsen, 2006) and Home Office (UK) guidelines. The mean ±SD (range) body mass of the 26 animals in the aspiration study on the day of study was 27.9 ±1.9 (23.5 – 31.5) kg. The animals' age ranged from 9 to14 months. The animals were typically housed in groups of 4 where ad libitum water was available. Specialised high-fibre low-energy feed (Miniporc 801586 SMP) was provided (400 - 600 g per day). Pens were 2 m^2 and the bedding was wheat-straw. Light intensity and duration was 100 - 200 lux for 12 hours. Humidity was held at 50 -70 % and ambient temperature at 20 - 22°C. There were daily health checks at feeding time with a veterinary inspection at least once a week. Food was not withheld at the normal feeding times and they had constant access to straw (which they may have ingested) before being studied.

2.2.2.3 Anaesthesia for minipigs

Intramuscular (IM) midazolam 0.5 mg/kg and ketamine 5 mg/kg were given before anaesthesia. After 15 min or when profound sedation was present, the pig was lifted to the surgery table and positioned in sternal recumbency. Induction of anaesthesia used isoflurane from a calibrated vaporizer (2.5 – 4%) combined with oxygen (4L/min) through a Bain breathing system and a Hall pattern mask. The marginal ear vein was cannulated using a 20 or 22 SWG cannula (Vygon, UK). When the jaw was relaxed, a bandage strip was passed under the maxilla and the head lifted to a 45˚ angle. Using a size 4 Miller laryngoscope blade and a Portex stylet, the *rima glottidis* was exposed. The dorsal epiglottic surface and the laryngeal mucosa were sprayed using a 2% lidocaine solution delivered by a laryngo-tracheal mucosal atomisation device (LMA MADgic; Teleflex, UK). The facemask was then re-positioned for 1 min to allow ventilation with the isoflurane/oxygen mixture. Propofol (2 mg/kg), fentanyl (2.5 µg/kg) and rocuronium (1 mg/kg) were injected intravenously in sequence, over 20, 10 and 2 sec respectively. The head was lifted as before and the trachea intubated with a size 7.5 or 8.0 mm internal diameter (13 or 13.5 mm outer diameter) TCB Univent tube (Fuji Systems, Tokyo, Japan). Providing no resistance was apparent, the tube was advanced some 5 – 10 cm and the bronchial cuff inflated with 10 - 20 mL air (maximum capacity

50 mL). Correct tube position was confirmed by capnography and chest wall movements synchronous with the gas delivery from a preset volume-controlled, pressure-limited mechanical ventilator (Seimens Servo 300A; Maquet, UK). Thereafter, anaesthesia was maintained using a total intravenous technique namely propofol 298.7 ± 44mg/hr and fentanyl 145 ± 45 mcg/hr (mean and SD) for all pigs, based on 16 readings per pig over 48 hours. Using the mean minipig weight as a denominator this equates to a mean (range) of propofol 10.6 (7.1-14.6) mg/kg/hr and fentanyl 5.2 (1.8-12.5) mcg/kg/hr. The doses were based on a porcine study that used TIVA for laparotomy (Martin-Cancho et al., 2004), but was adjusted for clinical signs as detailed below. It was observed that less anaesthetic was required for OP poisoned pigs secondary to the central nervous system depression.

2.2.2.4 Standard ICU care and monitoring

The lungs were ventilated using a mixture of oxygen and medical air (F_IO_2 0.5) with a tidal volume of 6 – 8 mL/kg delivered at 15 - 25 breaths per min. Peak inspiratory pressure (Ppeak) was limited to < 25 cm H_2O, positive end expiratory pressure (PEEP) was set at 5 cm H_2O and end-tidal CO_2 maintained (mean and SD) at 5.5 ± 0.8 kPa. If oxygenation fell (PaO_2 ≤80mmHg/10.5 kPa) then the oxygen was increased to F_IO_2 1.0 and titrated down to achieve PaO_2 ≥10.5kPa. In this thesis, reference is also made to the plateau airway pressure (Pplat) which is the positive pressure applied to small airways and measured during an inspiratory pause by the ventilator, as opposed to the peak inspiratory pressure (Ppeak) during inspiration.

The depth of anaesthesia was monitored using cranial nerve reflexes (palpebral corneal reflexes, ocular position, jaw tone and tongue curl), autonomic nervous signs and the bispectral index (BIS; Aspect Medical Systems, Covidien, Ireland). Once the animal was anaesthetised and placed on its back, a jugular vein and a carotid artery were surgically exposed in the neck and cannulated with a multilumen central venous catheter (Teleflex, PA, USA) and arterial catheter (Vygon, UK) respectively. Intravenous anaesthetic delivery was then switched from the marginal auricular to the multilumen venous catheter.

All animals received 10mL/kg of intravenous fluid (Hartmann's or Saline) for 30 min at the beginning of the study before commencing maintenance fluids (5 mL/kg/hr). Low central venous pressure (CVP) combined with a mean arterial blood pressure (MAP) ≤ 55mmHg and/or urine output < 0.5 mL/kg/hr was addressed by increasing the maintenance fluids from 5 to 20 mL/kg/hr for 30 min with IV Hartmann's solution or 0.9% sodium chloride for infusion. A norepinepherine infusion (4mg in 50 mL 5% dextrose - 80μg/mL) was an added intravenous therapy in minipigs that were not responsive to fluid therapy.

Attempts to maintain normothermia i.e. a nasopharyngeal temperature of 37- 38°C, involved using a forced air warming device (Bair hugger®, 3M, Brussels, Belgium) and temperate ambient temperatures (22-24°C). Blood potassium was kept ≥ 3.5 mmol/L and glucose ≥ 5 mmol/L by intravenous infusions of potassium chloride (13 mmol/L) or 20 mL 40% glucose, respectively.

Cardiorespiratory data including the end tidal CO_2, CVP, MAP, cardiac output (LiDCO®/Pulse CO), respiratory compliance (chest wall and pulmonary), peak and plateau airway pressures were captured from the Datex Ohmeda S/5 monitor and MCAiOV respiratory module (GE healthcare, Amersham, UK) using data logger software (v1.0.55.0 Vetronic Services Limited©). Computerised readings that showed negative values for cardiorespiratory data e.g. lung compliance, or MAP were regarded as artefactual and excluded from the final data analysis.

PaO_2/F_IO_2 ratio range	ARDS definition
≤300 mmHg and greater than 200 mmHg	Mild ARDS
≤200 mmHg and greater than 100 mmHg	Moderate ARDS
≤100 mmHg	Severe ARDS

Table 2.8 ARDS definitions according to the Berlin criteria (Ranieri et al., 2012)

Respiratory physiological variables like PaO_2/F_IO_2 ratios are reduced in ARDS and are used in its diagnosis (Table 2.8). These criteria are predictive of mortality and duration of mechanical ventilation during ARDS (Ranieri et al., 2012). Increased plateau airway pressures with reduced pulmonary compliance can occur in lung injury and are observed in OP pesticide poisoning (Rezk et al., 2007, Segura et al., 1999). Cardiovascular depression is also a known complication of severe OP pesticide poisoning (Davies et al., 2008). Therefore it was important to monitor these variables.

To reduce the risk of ventilator associated pneumonias, every six hours the minipigs were turned (right lateral to left lateral position), had oral secretions removed by suction and antibacterial mouth gel applied to the buccal mucosa (chapter 4).

The minipigs were also positioned in dorsal recumbency (on their backs) with their heads and torso position maximised by elevating the surgical table as much as possible. The tables achieved a maximum of 25° and 15° respectively which was less than the 30- 45° required for preventing human VAP (Hunter, 2012). In four pigs, 12 measurements were taken over the 48 hour study and the mean surgical table elevation was 16°.

2.2.3 Standardised gastric juice

Studies of aspiration in animals have used laboratory prepared mixtures of HCl ± gastric enzymes at a controlled pH. This does not reflect 'real life' pulmonary aspiration because the gastric contents usually contains food particulate matter (Raghavendran et al., 2005, Knight et al., 1993), and can contain microbes and pro-inflammatory cytokines (in the case of human ICU patients)(Bregeon et al., 2008), all of which can increase the severity of lung injury.

I decided to use a more 'real life' gastric juice mixture for the main pulmonary aspiration and EVLP study. This was achieved by collecting the gastric contents from 6 large white pigs immediately following abattoir slaughter (Grampian Country Park Halls Ltd, Broxburn, UK). The pooled gastric contents were filtered through 4 x layers of surgical gauze to remove large particulate matter. The pH of the mix was 3.6 and microscopy and culture (performed by the Veterinary Pathology Unit, Royal (Dick) School of Veterinary Studies, Edinburgh) confirmed the presence of moderate, highly mixed bacterial growth. The predominant organisms were *Lactobacillus sp*. and *Bacillus sp*. with occasional enteric bacteria, yeasts and moulds.

In order to prevent pneumonia caused through aspiration of these microbes and thereby confusing the pathophysiology of future aspiration studies, the gastric juice was treated to kill all the microorganisms present. Calculations by myself in discussion with Dr I Laurenson (Consultant microbiologist, Royal Infirmary of Edinburgh) were made to ensure that at least 30 mg/L of each antibiotic, with the addition on an antifungal agent, was used to treat the gastric juice.

Two litres of the filtered gastric juice, acidified to pH 2.0 (Meers et al., 2011a, Wynne and Modell, 1977) using hydrochloric acid, was treated with metronidazole 500 mg, vancomycin 1 g, gentamicin 140 mg, and amphotericin B 30 mg and left for 30 min at room temperature before storage at -80°C (Bregeon et al., 2008). The mix was thawed, readjusted to pH2.0 with HCl, aliquoted into 20 mL samples and re-frozen at -80°C.

An aerobic bacterial count and bacterial identification was performed on a selection of the thawed gastric juice used within the study (chapter 5).

Pulmonary aspiration studies have often used 1- 2 mL/kg of 'aspirate medium', but volumes as large as 2mL/kg combined with a lower pH (≤pH2) can have detrimental effects and increased mortality in rat aspiration studies (James et al., 1984). I hypothesised that 0.5 mL/kg of GJ at pH 2.0 would be an adequate volume to create a single lung injury in the minipig whilst reducing the chance of spill over into the contralateral lung.

2.2.4 Creation of the unilateral lung injury

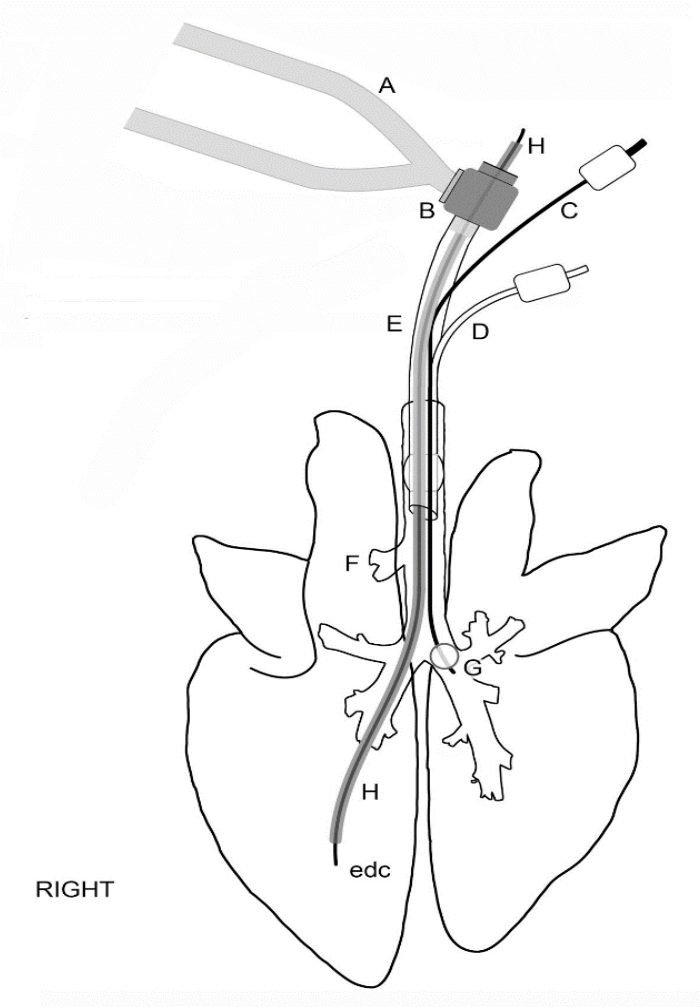


Figure 2.1 Diagram illustrating the instrumentation of minipigs during the pulmonary aspiration study (chapter 5). A: Ventilator tubing from Seimens Servo 300A ventilator; B: bronchoscope dual-axis swivel adapter; C: bronchial blocker inflation tube; D: endotracheal tube cuff inflator; E: torque controlled bronchial blocker (TCB; Univent endotracheal tube); F: right accessory bronchus; G: bronchial blocker cuff in left main bronchus; H: bronchoscope (BRS-5000; Vision Sciences, UK); edc: epidural catheter. Drawing by Professor RE Clutton based on sketches by myself (Hulse et al., 2014a) reproduced with permission.

Before instillation, a sterile endosheath was placed on the bronchoscope (BRS-5000; Vision Sciences, USA) incorporating a 2.1 mm diameter working channel. A sterile 16 SWG epidural catheter (Portex Epidural Catheter; Smiths Medical, UK; edc; Figure 2.1) was then placed

approximately three-quarters of the length down the working channel. The catheter connector was fitted to the end of the epidural catheter without a filter. The lungs were ventilated with 100% O_2 for 1 min and rocuronium 1.0 mg/kg was injected IV and allowed 45 - 90 sec to achieve diaphragmatic paralysis. The bronchoscope was then passed through a dual-axis swivel adapter (Portex; B; Figure 2.1) into the TCB Univent tube until the carina was visible. The bronchial blocker (G; Figure 2.1) was advanced approximately 2- 4 cm into the left bronchus under bronchoscopic vision using the torque-controlled guide to facilitate positioning. The cuff was then inflated with 2- 6 mL air and the bronchial blocker bung removed to allow gas release from the left lung. The bronchoscope was then advanced through the right main stem bronchus into the right lower lobe until it was wedged. At this stage the epidural catheter was pushed down the remainder of the working channel into the right lower lung lobe until further advance became impossible. The syringe containing 0.5 mL/kg mixtures of GJ, saline, dimethoate EC40 +GJ or cyclohexanone +GJ was then connected to the epidural catheter and injected.

Further rocuronium was given if bronchospinal reflexes occurred during bronchoscopy. If SpO_2 fell below < 80% then the bronchoscope was withdrawn and the lungs ventilated with 100% O_2 until values had recovered to 95% or greater.

On completion of installation, bronchoscopy was continued to ensure that the epidural catheter was withdrawn completely into the end of the TCB Univent tube, the bronchial blocker cuff was deflated and that the bronchial blocker was fully retracted. The bronchoscope was then removed and the bronchial blocker bung closed.

2.2.5 Bronchial lung sampling and processing

All sampling and bronchoalveolar lavage fluid (BALF) processing was performed by myself. The bronchial sampling was performed at 24.5 hour and 48 hours. The sample was taken at 24.5 hour rather than 24 hour to allow the CT scan to be taken at 24 hour with the bronchoalveolar lavage performed afterwards. For ease of reading the 24.5 hour sample will hereon in be referred to as the 24 hour sample.

2.2.5.1 Bronchoalveolar lavage fluid sampling and bronchial wall biopsy

Bronchoalveolar lavages (BALs) were conducted at 24 and 48 hours in all pigs for both right and left lungs, apart from the positive control pig receiving oleic acid (right lung only).

After one min of ventilation with 100% oxygen and administration of rocuronium 1mg/kg IV, the bronchoscope (BRS-5000; Vision Sciences, USA) was advanced into the right lower lung until the bronchoscope wedged at approximately the bifurcation of the 3rd-4th generation airway.

The first 6 minipigs received 60 mL BAL aliquots. These were reduced to 50 mL aliquots for subsequent minipigs because of concerns that the volume might be excessive and traumatic, aggravating the treatment induced lung injury (discussed further in chapter 5). The aliquot of room temperature sterile 0.9% saline was instilled through the working channel of the bronchoscope endosheath. Lavage was delivered in 3 aliquots, each being left *in situ* for 5-10 secs before being aspirated into a closed suction specimen trap (Pennine Healthcare, UK) between each aliquot.

On completion of BAL, alligator forceps were passed down the working channel and a bronchial wall biopsy sample was taken. Samples were placed in 10% buffered formalin and underwent overnight paraffin fixation. The right lung was lavaged first and then biopsied, followed by the same procedure in the left lung.

2.2.5.2 BALF processing

The BALF was placed on ice before being transferred to a 50 mL falcon tube where the return volume was noted. The BALF was centrifuged at 3500 rpm for 10 min (Sigma 2-6, Shrewsbury, UK) and the supernatant aliquoted for further testing, before storage at -80°C.

The volume of cell pellet was estimated (using known volumes in similar falcon tubes, range 0.1-2 mL), and placed on ice until a cell count and cytospin could be performed. The study protocol was changed for the last 20 minipigs who had 5 mL of cell growth medium added to their BAL cell pellets in order to improve cellular viability prior to cell counting.

2.2.5.2.1 BALF haemocytometer cell count

Standard procedures were followed for the BALF cell count (Meyer et al., 2012). The cell pellet and cell growth medium were re-centrifuged and the medium removed by aspiration. The cell pellet was then re-suspended in an appropriate volume of saline to create a suitable concentration for an immediate haemocytometer cell count. The 50μL of cell suspension was then mixed with 50μL of trypan blue and left for 2-3 min at room temperature to determine the percentage of live and dead cells.

Trypan blue suspension (10μL) was placed in the haemocytometer and cells counted (dead cells showed up bright blue) using light microscopy at x40 magnification. The number of live and dead cells were counted in 80 small squares and the following formula applied:

(DxN)/(K) x 1000 (to convert cells/mm^3 to cells/mL) x total volume of cellular suspension (mL) = total number of cells in the BALF

Where D = dilution factor (to take into account trypan blue staining), N = average number of cells per small square, K= the volume of each small square (0.00025mm^3).

Cellular viability was calculated by dividing the number of live or dead cells by the total number of cells then multiplied by 100.

2.2.5.2.2 Cytospin

The cell pellet and saline mixture was placed through a 40 μm cell strainer to remove any clumps of mucus. An aliquot (200 μL) of the cell suspension was mixed with 200 μL saline to create a 1 in 2 mix, and then 200 μL of this solution was added to 200 μL saline to create a 1 in 3 mix. Aliquots (200 μL) of each of these mixtures were placed in separate cytospin cassettes preloaded with filter paper and glass slides. The cassettes were then placed in a Shandon centrifuge 4 (Techgen, Zellik, Belgium) for 3 min at 300 rpm. When finished, the slides were removed from the cassette and air-dried.

2.2.5.2.3 Kwik Diff staining and mounting of BALF cells

Each air-dried slide was dipped in Kwik Diff fixing solution #1, 5 times (one second per dip), Kwik Diff solution #2 (xanthene dye and buffer), 5 times (one second per dip), then Kwik Diff solution #3 (thiazine dye and buffer), 5 times (one second per dip). Time was allowed between immersions in the different solutions to allow the excess to drip off before blotting the edges on absorbent paper.

Once dry, DPX mounting medium was applied to the cytospin and a coverslip placed over the top.

2.2.5.2.4 Light microscopy and counting of BALF cells

BALF cells were counted by myself using light microscopy at x40 magnification. Blinding of this process was achieved by blocking the identity of the slides and randomizing the order of examinations.

In each cytospin slide, approximately 500 BALF white cells [525 ± 38 (mean ± SD) for minipig pulmonary aspiration study – chapter 5] were counted in at least 3-4 different microscopic fields of view, in a clockwise direction in accordance with standard procedures (Baughman, 2007). Alveolar macrophages, neutrophils, monocytes, lymphocytes or eosinophils were identified and counted. The presence and extent of RBC contamination was noted by counting a further 100 cells (including RBCs) and calculating the percentage of red cells ((number of red cells / number of BALF white cells + red cells) x 100) for each cytospin slide.

2.2.6 Pulmonary surfactant compositional analysis

Lung surfactant is a lipoprotein secreted by type 2 alveolar epithelial cells and is a detergent-like compound that keeps the lung expanded during respiration. Phospholipids, in particular phosphatidylcholine (PC) species, are the major lipid component of surfactant. Variability in the molecular composition of PC species has been described in several lung diseases, including neonatal and adult ARDS, but the cause of variation in composition is poorly understood (Dushianthan et al., 2012).

Dipalmitoyl phosphatidylcholine (DPCC) [PC 16:0/16:0] is the most abundant PC species in human and porcine lung surfactant and is responsible for the reduction of alveolar surface tension (Lang et al., 2005). Without this, the lung would find it difficult to overcome the forces required to efficiently open distal airways required for gas exchange and lung oxygenation. The proportion of saturated PC 16:0/16:0 of the total PC species is significantly reduced in ARDS secondary to pneumonia and aspiration, often combined with an increase in the unsaturated PC species PC 16:0/18:1, PC 16:0/18:2, PC18.0/18:2 and PC16:0/20:4 found in lavage fluid (Schmidt et al., 2007, Dushianthan et al., 2014). This proportional reduction 16:0/16:0 PC species is thought to be due to reduced synthesis, increased breakdown or by dilution through lung oedema (Dushianthan et al., 2014).

A model to investigate surfactant PC composition, synthesis and metabolism has recently been established using a non-radioactive stable isotope – methyl D_9 choline chloride (choline chloride (trimethyl-D9)) (Dushianthan et al., 2014). Choline is found in the lipids that make up all cell membranes and is also an integral constituent of surfactant. All of the choline in pulmonary PC is derived exclusively from plasma. Methyl D_9 choline chloride is incorporated into the patient's synthesized surfactant, and so BAL collection allows the observation of the rate and PC composition of new surfactant production.

Newly synthesized PC fractions in ARDS patients have lower PC 16:0/16:0 composition at earlier time points after the *methyl*- D_9-choline infusion, reaching endogenous levels by 48 hours after the infusion. The enrichment of *methyl*-D_9-choline into total PC is over 70% greater in ARDS patients compared with controls at 24 and 48 hours (Dushianthan et al., 2014).

It was decided that the analysis of PC species composition and synthesis (using methyl D_9 choline) in BALF samples from both the minipig aspiration study and *ex vivo* lung perfusion study would help to understand the pathophysiology of aspiration of OP+GJ or Solv+GJ when compared with GJ alone.

BALF samples from a selection of 10 minipigs involved in the pulmonary aspiration study and 10 ex vivo lung perfusion (EVLP) BALF samples were sent to Dr Vikki Goss at the University of

Southampton who kindly performed phospholipid extraction and mass spectrometry analysis using the following methods (Dushianthan et al., 2014).

2.2.6.1 Phospholipid extraction

Total lipid extraction was performed using a modified Bligh and Dyer (Bligh and Dyer, 1959) method automated on a Tecan robotic system. Samples were made up to a volume of 800 µL with 0.9% NaCl and dimyristol-PC (PC14:0/14:0) added as internal standard. One mL of dichloromethane and 2 mL of methanol were added to the samples, followed by an additional 1 mL of dichloromethane and 1 mL of distilled water. Greater resolution was attained by centrifuging the mixture at 400 g at 20°C for 20 min. The lower lipid layer was removed and dried under a nitrogen concentrator at 40°C. Once dried, 1 mL of chloroform was added to the mixture and re-dried to be analysed by mass spectrometry.

2.2.6.2 Mass spectrometry analysis

The phospholipid fraction was suspended in a mixture of methanol-butanol-water-concentrated NH4OH (6:2:1.6:0.4 v/v), and was injected by loop injection using an Aquity UPLC system (Waters Corporation, UK) at a rate of 0.05 µL/mL into the electrospray ionisation interface of a Xevo TQ mass spectrometer (Waters Corporation, UK) (ESI-MS/MS). The newly produced PC species was calculated from MS/MS fragmentations of precursor ion m/z +184 and m/z + 193 respectively. Pre-made Excel spread sheets were used to quantify ion peaks after corrections for ^{13}C-isotopic effects.

2.2.7 Biochemical assays

Biological samples had the minimum amount of freeze thaw cycles to avoid sample degradation. BAL protein content, blood AChE/Hb and alanine transaminase (ALT) content was conducted by myself, Mr A Thompson and Dr F Howie using the Cobas Fara centrifugal analyser (Roche Diagnostics Ltd, Welwyn Garden City, UK). I carried out the majority of ELISAs with some assistance for CRP measurements from Mr A Thompson.

Some researchers prefer to report the non-cellular contents of BALF by calculating the epithelial lining fluid (ELF) concentrations of these components. This is done by correcting for the

concentration of urea in both plasma and BALF, which under normal conditions should be in equilibrium and of equal concentration (between plasma and ELF)(Rennard et al., 1986). However, as has been shown in this thesis, OP poisoning (directly and indirectly) causes heterogeneous lung injuries with varying damage to the alveolar capillary membrane. Consequently, it is unknown whether the urea concentration between plasma and ELF would be truly equal in the sampled lung areas. Therefore, I have chosen to report the BALF non-cellular contents as per mL of BAL fluid for all experiments in this thesis (Baughman, 2007, Haslam and Baughman, 1999).

2.2.7.1 Broncho alveolar lavage fluid protein content

ARDS is caused by pulmonary inflammation and the breakdown of the alveolar capillary membrane and can be demonstrated through an increase in BALF total protein and albumin (Ware and Matthay, 2000). It was thought that aspiration of OP+GJ would cause a similar injury and so we measured BALF total protein and albumin in the minipig aspiration, human and EVLP studies.

2.2.7.1.1 Albumin measurement

Porcine, ovine and human BALF albumin measurements were determined using a commercial mouse microalbumin kit (Olympus Diagnostics Ltd, Watford, UK) adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd, Welwyn Garden City, UK). The immunoturbidimetric assay was standardised against purified mouse albumin standards (Sigma Chemical Co. Poole, UK) with samples diluted in PBS as appropriate. Where diluted samples were returned with concentration measuring less than 3 mg/L they were repeated neat and that result was used for data analysis. Intra batch standard accuracy for the minipig and EVLP ovine samples was coefficient of variance (CV) ≤14%.

2.2.7.1.2 Total protein measurement

Protein measurements were carried out utilising the dye-binding assay of Bradford (Bradford, 1976) adapted for use on the Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK).

The Bradford reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 mL 95 % ethanol. One hundred mL 85 % (w/v) phosphoric acid was added to this solution and the

mixture stirred for 30 min. The solution was then diluted with distilled H_2O to a final volume of 1000 mL, filtered through Whatman grade 1 filter paper and stored at room temperature in a closed bottle. Bradford reagent (256 μL) was added to each cuvette, which was incubated for 100 sec at 37°C prior to sample addition, with an initial absorbance reading (595 nm) taken at 95 sec. Following the addition of 25 μL of sample plus 50 μL distilled H_2O (diluent) to the cuvettes, a further incubation took place for 180 sec at 37°C. A final absorbance was then read at 595 nm.

A standard curve was constructed using bovine serum albumin (BSA) as standard, and distilled water as the diluent, covering the range 0 to 100 mg/L. The difference between the final and initial absorbances was calculated, and a standard curve plotted. The protein concentrations of samples were interpolated from the standard curve. Samples were diluted with distilled water to fall in the middle portion of the standard curve. A quality control (QC) was run with every rotor to assess the reproducibility of the results. BAL sample dilutions were between 10 and 150 fold. Intra batch standard accuracy for the minipig and *ex vivo* samples was CV ≤11%.

2.2.7.2 ELISA analysis of porcine IL-6, IL-8, CRP and human SP-D

Interleukin 8 (IL-8) is a pro-inflammatory cytokine released by many different cells and tissues and in the context of gastric aspiration, IL-8 seems to be the main cytokine causing neutrophil recruitment and activation with subsequent lung injury (Folkesson et al., 1995, Inci et al., 2008, Meers et al., 2011a). Interleukin 6 (IL-6) is secreted by endothelium, and other tissues and has both pro and anti-inflammatory roles. Statistically significant increases of plasma IL-6 and IL-8 have been observed in human patients with ARDS (Ware et al., 2013), in which approximately 20% of ARDS ICU admissions might have been due to aspiration (Briel et al., 2010). C reactive protein (CRP) is an acute phase protein produced in the liver and to a lesser extent locally in the lung. Its main function is to find and remove microorganisms and apoptotic cells through activation of the complement system (Agassandian et al., 2014). Levels of CRP are raised in ARDS (higher in survivors) (Bajwa et al., 2009) and systemic OP poisoning (Lee et al., 2013). It was therefore considered important to measure these inflammatory markers throughout the studies to help

understand the pathophysiology of aspiration of OP+GJ. The use of surfactant protein D (SP-D) is discussed below.

ELISA sandwich development kits for porcine IL-6 (cat.no DY686), CXCL8/IL-8 (cat no. DY535), CRP (cat no.DY2648) and human SP-D (cat no. DY1920) were purchased from R&D systems, Abingdon, UK. They contained standards, capture and detection antibodies and streptavidin-HRP. Buffers and solutions were made as above. The R&D ELISA kits were developed for measurement of cell supernatants and not plasma. The company suggested using FCS mixtures with PBS as a diluent, and so for measurement of plasma IL-6 the diluent used was 60%FCS and PBS and not the usual reagent diluent. The validation of the ELISA for plasma IL-6 is discussed below.

2.2.7.2.1 ELISA blanks, sample dilutions and repeats

Reagent blanks (containing no sample or buffer but exposed to all other solutions) were used in ELISAs measuring porcine IL-6, IL-8, CRP BALF and CRP plasma. Their optical densities (OD) were subtracted from the sample OD results. These blanks were also considered the zero standards for calculation of the standard curve. Blanks containing FCS 60% in PBS were used for porcine plasma IL-6. CRP plasma standards only required dilution in PBS (with no FCS) due to the extremely high dilutions which were in excess of 1 in 50,000.

Detection ranges for these ELISAs were from their lowest to highest standards:

IL-6 – 125-8000pcg/mL

IL-8 – 125-8000pcg/mL

CRP – 78-5000pcg/mL

SP-D – 78-5000pcg/mL

Samples that were less or more than the ELISA detection range were repeated at the appropriate dilution. OD values that remained under the detection range after repeat neat testing were accepted and used in the remaining calculations performed using Graph Pad Prism 6.04 (see calculation of R&D ELISA results) in order to produce graphs and assess the data.

All BALF samples for CRP measurement were tested as 1 in 60 dilutions. Twelve high outliers were retested at a greater dilution (1 in 200), but two remained out of range. Those high samples along

with 18 low (<78pcg/mL) sample readings could not be retested due inadequate reserves of reagents and/or standards.

2.2.7.2.2 Basic R&D ELISA protocol

The capture antibody was diluted in PBS to a working concentration as per instructions and then 100 µL placed on a 96 well ELISA plate overnight at room temperature, or at 4°C for 3 nights (over weekends). The plate was washed 3 x 300 µL using wash buffer and then blocked with 300 µL reagent diluent for 1 hour. One hundred µL of samples or standards were added to appropriate wells with duplicates and left at room temperature for 2 hours. The plate was then washed 3 x 300 µL, and the detection antibody was diluted in reagent diluent to a working concentration and 100µL added to all wells and left for 2 hours at room temperature. After a repeat plate wash (3 x 300 µL), 100 µL streptavidin-HRP (1 in 200 dilution with reagent diluent) was added for 20 min and then the plate re-washed. One hundred µL of substrate solution was added to all wells for 20 min, then 50 µL of stop solution was also added to all wells. A plate reader allowed the OD to be measured at 450nm, then readings from 540nm were subtracted.

2.2.7.2.3 Calculation of R&D ELISA results

To calculate the results, the blank OD values were averaged and subtracted from the OD values of samples and standards, negative values were zeroed. Data were then entered into Graph Pad using the blank values as the zero standards. The x axis was $\log_{10}$ of concentration of standards and then using a sigmoidal 4 parameter logistic (PL) curve the unknowns were interpolated and $\log^X$ transformed to give calculated concentrations. The mean values of the duplicate wells were calculated and entered into a table for graph production (Graph Pad Prism 6.04, USA).

2.2.7.2.4 Validation of R&D ELISA kits for porcine plasma (IL-6)

The R&D duo sets were not validated by the company for the complex matrices of plasma and serum. Consequently, the company recommended using PBS supplemented with FCS for the standard and sample diluent instead of reagent diluent.

In order to find and validate an appropriate diluent for the standards, spike/recovery tests were initially performed using mixtures at either end of the spectrum suggested- PBS with 20% and 60% FCS. Other dilutions were not tested as 60% FCS and PBS were found to be adequate diluents.

2.2.7.2.4.1 Method to determine whether 20% FCS+ PBS or 60% FCS +PBS was suitable as a standard diluent for measurement of plasma IL-6 using an R&D ELISA kit

1. Plasma from two minipigs involved in the aspiration study taken at 44 hours (chapter 5) [one sham bronchoscopy pig (N1) and one OP+GJ pig (N2)] was thawed and vortexed.
2. Excessive lipid in the plasma sample resulting from the propofol anaesthetic was removed. In order to achieve this 1 mL of each plasma sample was aliquoted into 1.5 mL Eppendorf tubes and centrifuged at 8000 rpm for 2 min. The visible lipid layer at the surface, was removed by placing the end of a 0.5 mL Eppendorf tip within the lipid layer. The lipid adhered to the plastic and most was removed by withdrawing the tip.
3. A spiking stock solution of 80 ng/mL was made using the R&D recombinant porcine IL-6 provided in the kit.
4. The standards were prepared by diluting the IL-6 standard in either 20% or 60% FCS + PBS. A seven point standard curve using 2- fold serial dilutions was created with a high standard of 8000 pcg/mL.
5. To check for standard curve performance, a third standard curve using the recommended reagent diluent (1% BSA in PBS) was created.
6. Three tubes were labelled: neat, spiked and control
7. One mL of each plasma sample (N1 and N2) was placed in a tube and labelled ‘neat’.
8. Plasma samples (980 μL) from both N1 and N2 were placed in different tubes and spiked with 20 μL of the spiking stock solution. This was labelled ‘spiked’.
9. As a control, 980 μL of the diluent (20% or 60% FCS + PBS) used for the standard curve was also spiked with 20 μL of the spiking stock solution. This was labelled ‘control’.
10. The 3 samples were vortexed briefly.
11. The 3 samples and standards then underwent basic R&D ELISA protocol and concentration calculations.
12. In order to calculate the percentage recovery the following calculation was used:

Percentage recovery = (observed – neat)/expected x 100

Observed = spiked sample IL-6 concentration

Neat = un-spiked sample IL-6 concentration

Expected = control spiked sample IL-6 concentration

13. The spiked control recovery percentage was calculated. If between 80-120% of the expected concentration, the recovery from within the spiked sample was also calculated. If this was also between 80-120%, then the diluent was deemed to be acceptable.

The results of the IL-6 plasma spike tests in determining an appropriate diluent for ELISA are recorded in Appendix B.

2.2.7.3 ELISA analysis of porcine RAGE, VWF and SP-D

In order to examine the integrity of the alveolar capillary membrane after aspiration injury, substances known to be secreted or released specifically by damaged type 1 and 2 alveolar epithelial and endothelial cells were measured. Raised levels of the soluble receptor for advanced glycation end-products (RAGE) are associated with damage to the type 1 alveolar epithelial cells and are raised in acute lung injury (Ware et al., 2013). RAGE ligands can trigger the pro-inflammatory nuclear factor κB (NF-κB) pathway and subsequent cytokine release leading to tissue injury (Chavakis et al., 2004). Surfactant protein D is produced by type 2 alveolar epithelial cells and is important for the lungs host defence response, protecting against infection and inflammation (Nayak et al., 2012). Increased plasma with reciprocal reduced BALF SP-D levels are found in a range of lung diseases and ALI (Eisner et al., 2003) (Cheng et al., 2003). It is thought this is due to leakage from the injured alveoli into the blood stream. Von Willebrand factor (vWF), an antigen released mainly by the endothelium, is important for blood clotting and platelet adhesion. Raised plasma vWF (indicating endothelial activation or injury) is associated with increased multi-organ failure and mortality in patients with ARDS (Ware et al., 2004).

2.2.7.3.1 Measurement of receptor for advanced glycated end products (RAGE) in porcine plasma

Two quantitative competitive immunoassay kits (Neo biolab, MA, USA) were used to measure plasma RAGE [detection range 0.1-10 ng/mL]. All kit components and samples were brought to room temperature before use and solutions made as per instructions.

One hundred μL of sample or standard was added to the appropriate number of wells in a pre-prepared 96 well microtitre plate coated with RAGE specific antibody. Due to cost limitations, only the 24 and 48 hour plasma samples from the 26 pigs could be tested. The 48 hour porcine plasma samples were tested first using a 'best guess' (1 in 4) dilution based on the literature. Subsequent testing used neat plasma for the 24 hour samples. Fifty μL of RAGE-HRP conjugate was added to all wells except PBS blanks, and mixed using a multichannel pipette. Tests were conducted in the same location on subsequent days. The plate was covered and placed in a humidified heating block at 37°C for 1 hour. The well contents were then discarded and underwent 5 x 300 μL washes (plate washer LP41, Adil instruments, CA, USA) using the pre-prepared wash solution. Fifty μL of substrate A and the 50 μL of substrate B were added to all wells. The plate was then covered and incubated at room temperature for 15 min. Fifty μL of stop solution was then added to each well. The OD was read at 450 nm using a p6 well microtiter plate reader (molecular devices OptiMax tuneable microplate reader, Sunnyvale CA, USA).The mean OD of the blank wells was subtracted from the rest of the OD values (samples and standards) and negative values were zeroed. The mean OD for sample or standard duplicate wells was calculated. If the mean OD for a sample was above that of the zero standard it was counted as zero concentration.

The data were processed by using the OD of other non-zero standards divided by the zero standard, then multiplied by 100 to create x- axis values. The y axis was $\log_{10}$ of concentration of standards. A line was created using linear regression and the unknown concentrations interpolated. The concentrations then underwent $Y = 10^{Y}$. Replicate values were averaged for data analysis.

2.2.7.3.2 Measurement of von Willebrand factor (vWF) in porcine plasma

Two quantitative sandwich immunoassay kits (Neo biolab, MA, USA) were used to measure plasma vWF [detection range 0.1-50 ng/mL]. All kit components and samples were brought to room temperature before use and solutions made as per instructions.

Fifty µL of neat plasma sample or standard was added to the appropriate number of wells in a pre-prepared microtitre plate coated with vWF specific antibody and incubated at room temp for 1 hour. The well contents were then discarded and underwent a wash in a plate washer (plate washer LP41, Adil instruments, CA, USA) 5 x 300 µL using the pre-prepared wash solution. Fifty µL of conjugate was added to all wells and incubated for 1 hour at 37°C in a humid chamber. The plate was re-washed, then 50 µL substrate A and 50 µL substrate B from the kit, was added. The plate was then covered and incubated at room temperature for 15 min. Fifty µL of stop solution was then added to each well. OD values were immediately read at 450 nm. The mean OD of the blank wells was subtracted from the rest of the OD values (samples and standards) and negative values were zeroed. The mean OD for sample or standard duplicate wells was calculated. Appropriate standard curves were achieved as per instructions. The concentrations were logged and then a line produced through linear regression, the unknown concentrations were interpolated from the graph. The concentrations then underwent log transformation (10^x).

2.2.7.3.3 Measurement of surfactant associated protein type D (SP-D) in porcine BALF

Two quantitative competitive immunoassay kits (Neo biolab, MA, USA) were used to measure SP-D [detection range 1-250 ng/mL]. All kit components and samples were at room temperature before use and solutions made according to instructions. In order to maximise the use of the test kits a preliminary experiment (results not shown) was performed to determine whether different dilutions of plasma or BALF would perform best i.e. values were on the steep part of the standard curve. The results indicated that BALF, not plasma, should be used at a 1 in 10 dilution to best meet this requirement.

One hundred µL of sample or standard plus 10 µL of balance solution was added to the appropriate number of wells in a pre-prepared microtiter plate coated with SP-D specific antibody. Fifty µL of

SP-D-HRP conjugate was added to all wells except PBS blanks, and mixed using the multichannel pipette. The plate was covered and placed in a humidified heating block at 37°C for 1 hour. The well contents were then discarded and underwent a wash in a plate washer (plate washer LP41, Adil instruments, CA, USA) 5 x 300 µl using the pre-prepared wash solution. Fifty µL of substrate A and 50 µL of substrate B were added to all wells. The plate was then covered and incubated at room temperature for 15 min. Fifty µL of stop solution was then added to each well.

The remaining sample processing was as that described for RAGE (above).

2.2.7.4 Measurement of red blood cell acetylcholinesterase (AChE) activity

AChE was measured in diluted whole blood and then dilution factor was accounted for by multiplying by 21 (0.2 mL venous blood in 4 mL 0.9% saline). Porcine and human AChE measurements were determined using a modified Ellman method described by by Worek *et al*, and adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd, Welwyn Garden City, UK)(Worek et al., 1999). Twenty µL sample and 40 µL sample wash (water) followed by 210 µL reagent 1 (see above in solutions and buffers) was added to a cuvette, spun and warmed to 37°C. Background absorbance was measured at 436 nm. Ten µL reagent 2 (a plasma butylcholinesterase inhibitor) was added, to produce a total volume 280 µL, the absorbance of which was measured over 5 min at 436 nm. Standards were human red blood cells suspended in water at 500x, 1000x and 2000x dilutions. Within-run precision of standards (replicates) CV was <6%, intra-batch precision (2012-2014 standards) was CV ≤20%.

2.2.7.4.1 Measurement of haemoglobin (Hb) in AChE blood samples

Haemoglobin (Hb) was measured in diluted whole blood and then dilution factor was accounted for by multiplying by 21 (0.2 mL blood in 4 mL 0.9% saline). Porcine and human Hb measurements were determined using a modified Ellman method by Worek *et al*, adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd, Welwyn Garden City, UK)(Worek et al., 1999).

Twenty µL sample was added to 260 µL transformation solution. Absorbance was measured at 546 nm. Controls and standards were of known concentrations of human Hb. Within-run precision (replicates) was <6%, intra-batch precision (2012-2014 standards) was CV <9.5%.

2.2.7.4.2 Data processing of AChE and Hb measurements

The whole blood AChE concentrations were divided by the Hb concentration to adjust for the presence of red cells carrying the AChE. These data were then normalised for each pig by dividing each result by the -30 min time point AChE/Hb result and multiplying by 100 to give a percentage.

2.2.8 Computed Tomography (CT)

Whilst a digital image is made up of 2-dimensional pixels, CT images are composed of 3-dimensional voxel. This allows in-depth analysis of an image and generates vast quantities of data. It allows accurate measurement of poorly or non-aerated (wet) areas of lung beyond that visible to the human eye, and has been used successfully in the evaluation of animal lung aspiration injury models (Heuer et al., 2012).

Helical CTs were conducted by certified radiographers from Burgess Diagnostics (UK) on a mobile (lorry-loaded) 16 slice GE Lightspeed CT scanner (GE Healthcare, Buckinghamshire, UK). The CT transfer procedures for the studies are discussed in detail in chapter 4. All CT analysis was performed by myself with technical advice from Mr Martin Connell.

2.2.8.1 Pilot aspiration study (study 3)

Helical CT scans were taken for five pigs at various time points between -1 hour (pre-poisoning) and at post mortem approximately 48 hours after poisoning (results detailed in chapter 4). Animals were scanned in dorsal recumbency, in expiration and with no positive end expiratory pressure (PEEP). Each scan lasted approximately 11 sec. Lung ventilation was resumed after the CT scan was completed (see chapter 4).

2.2.8.2 Pulmonary aspiration study

A new CT scan protocol was developed to ensure a greater and more uniformly aerated lung CT scan than the pigs described above with zero positive end expiratory pressure. CT lung scans were taken for 12 pigs at -30 min, 4, 8, 24, 32 and 47.5 hours. The minipigs were positioned in dorsal recumbency, and their lungs ventilated (PPV) until the point of scanning whereupon ventilation was suspended for 10-20 sec for the duration of the scan. During this period airway pressure was held

at 5cm H_2O using a PEEP valve (Drager, Germany) positioned in the expiratory limb of the ventilator circuit. For both studies, the soft reconstruction (type of CT image 'view') of the CT scan lung images were analysed using Analyze 10.0® on a Macintosh PC.

2.2.8.3 Creation of 2D lung maps

The results of initial trials with the automatic lung mapping tool (available in the software package Analyze 10.0 ®) failed to produce an accurate reflection of minipig lung anatomy in the pilot and main pulmonary aspiration studies. This was because density similarities between soft tissue, blood, fluid and consolidated lung prevented the true outlining of the lung anatomy that was required to perform whole lung density analysis.

Therefore, the 2D lung images had to be traced by hand. Lungs would typically lie between slices 20-470, and every 10th lung slice between these parameters (i.e. 20th, 30th, 40th slice etc.) was traced around using the 'spline edit' function. When tracing around the lung slice was complete, the slice would be filled in, creating a 2D lung map in the transverse plane (right lung in red, left lung in green; Figure 2.2A). Each slice was spaced at 1.25mm, with sample slices spaced at 12.5mm apart (every 10th slice) along the length of the lung (cranio-caudal axis) which was approximately 500mm.

Where it was difficult to differentiate soft tissue from consolidated or fluid-filled lung in the image, the intensity of the image was changed to show different Hounsfield unit voxels on the greyscale to allow visualisation of the lung. Where possible, the major blood vessels and bronchi were excluded from the 2D lung slice, but if these could not be excluded (due to anatomy or blurring of borders/tissues) they were incorporated into the 2D map. This would also include surrounding lung/pleural fluid.

2.2.8.4 Creation of 3D lung object maps

The above procedure produced 40 x 2D lung maps which were propagated in the cranio-caudal axis using a smoothing function and created a 3D reconstruction of each lung (Figure 2.2B). If the 3D lung image had obvious missing lung segments, or extra segments deviating from normal porcine lung anatomy, the 2D lung maps were re-traced and a new image propagated.

If the lung volumes at different time points were significantly different (≥100,000 mm^3 difference) from the baseline (-30 min) volume in both lungs (appendix A), then the image was reassessed, and in the absence of an obvious cause, the 2D and 3D lung maps were re-drawn.

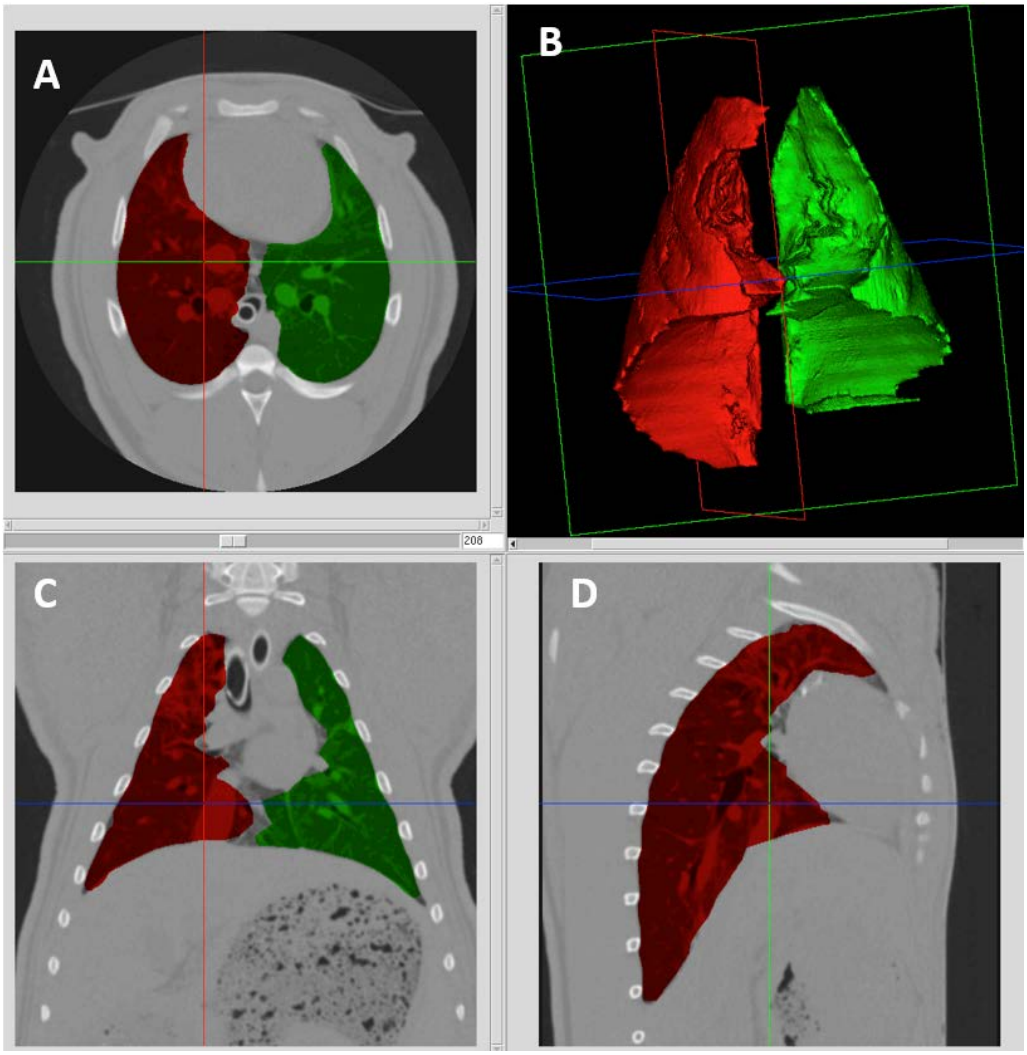


Figure 2.2: Images depicting the creation of the 2D and 3D lung maps in minipigs. Images show the transverse plane 2D lung map (A), the 3D reconstruction of the lungs (B) and the coronal (C) and sagittal view (D) of the lungs (green – left lung, red – right lung). The above images were created from a saline control pig in the pilot aspiration study.

2.2.8.5 Measurement of the density of the 3D lung object maps

To compare the lung injuries from the different treatment groups, the percentage of voxels within different areas of lung aeration -particularly poorly aerated or non-aerated lung tissue (-499 to +250 Hounsfield units)- were measured (Heuer et al., 2012). The percentage [number of voxels between -499 and +250HU/ number of voxels between -999 and +250 HU x 100] and not the actual number of voxels was used as an initial measure because of variation within minipig lung volume and individual CT scan voxel size (see appendix A). Further in depth analysis of the CT data is discussed in chapter 5.

Using the 'region of interest' function in the programme Analyze 10.0 ®, the 3D object map was superimposed onto the original CT to count and identify the density value (Hounsfield unit) of each voxel, between -999HU and +250HU within this 3D object map. Using these data, histograms were made indicating the density spread of each lung showing the number of voxels vs. density in Hounsfield units (Figure 2.3). These data were further explored by using the programme to create 3D representations of the parts of the lung with any particular density bracket i.e. to only show the denser lung areas (Figure 2.3).

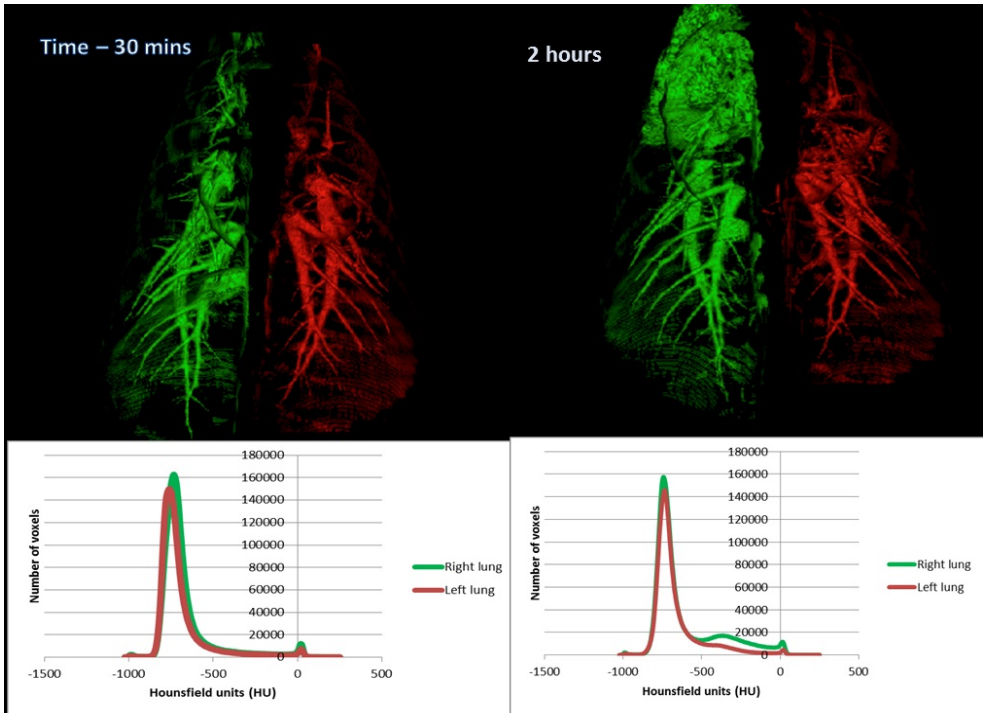


Figure 2.3: 3D CT lung images showing areas of the lung between -250 and +250 HU considered to represent fluid and/or consolidation before (-30 min, left) and after (2 hours, right) aspiration of OP+GJ into the right upper lung lobe. The graphs below the images show the number of voxels (y axis) and their density (HU) spread (x axis). After 2 hours the right lung (green line) now has a peak in the 'wet area' denoted between -250 and +250 HU.

2.2.9 Histology

This section details the practical procedures used to fix and stain both bronchial and lung tissue for the experiments within this thesis.

The histopathology scoring system was devised over a number of years by myself and others and this process is extensively described below. The histopathology scoring system was critical in designing the model and powering of the main aspiration study.

2.2.9.1 Bronchial biopsy

Bronchial biopsies for the main aspiration study were taken by myself from both right and left lungs and placed in 10% neutral buffered formalin. The samples were clipped and placed through graded alcohols (0-100%), xylene and embedded in paraffin-wax at 60°C overnight using a Leica ASP

tissue processor. A selection of samples were sectioned to 4 μm by a microtome, placed on slides and dried at 37°C overnight. These processes were carried out by the Shared Universities Research Facility (SuRF) at the University of Edinburgh (Queen's Medical Research Institute, Edinburgh).

2.2.9.2 Lung sample processing and sectioning

Lung sample collection and fixation is covered in individual chapters.

Post mortem bi-lateral, upper and lower lung samples (1cm wide transverse cross sections 'slabs') were fixed in 10% buffered formalin and sent to the University of Edinburgh veterinary histopathology laboratory where sections were further cut, placed through graded alcohols (70-100%), xylene and embedded in paraffin-wax at 60°C. The samples were sectioned to 4 μm using a microtome and approximately 5mm^2 of the tissue specimens (which had to contain evidence of bronchi and bronchioles for scoring purposes) were placed on slides and dried for 15 min at 37°C, then 60°C for 25 min. If there was obvious pathology within a part of the lung 'slab' then two slides (sample sections) were made for that area (RUL, LUL, RLL or LLL) of lung. The number of slides made was determined by the technician in the veterinary histopathology laboratory.

2.2.9.3 Haematoxylin and Eosin staining

The lung sample sections were dewaxed with xylene, then rehydrated with absolute alcohol before being rinsed in tap water, stained with Harris' haematoxylin and re-washed in tap water.

The sections were then washed in sequential order: 1% acid alcohol; tap water; Scott's tap water substitute; tap water. The sections were stained with 1% aqueous eosin for 30 sec, then dehydrated in absolute alcohol and placed in xylene and mounted using DPX medium and covered with coverslips.

2.2.9.4 Process of histopathology scoring

For the main aspiration study slides were scored 'blindly' by myself (slides had no group allocation identifier) in random order using the specifically designed histopathological scoring system 1.1 (see below) with light microscopy. Some areas of lung had duplicate slides created as deemed necessary (e.g. areas of obvious injury) by the veterinary histopathology department. In this instance, areas of

lung were given a mean score for statistical analysis. Images were captured using AX70 Provis (Olympus, USA) microscope and software.

The score was generated by assessing the whole tissue sample on the slide (approximately 5mm^2), with the worst score attainable e.g. if there were three bronchioles but only one bronchiole had the presence of neutrophils, the score would be based solely on the bronchiole that had neutrophils, and not as a mean score of the three bronchioles.

2.2.9.5 Development of a histopathological scoring system

Ingestion of OP may create a haemorrhagic and oedematous lung (He et al., 2012a, Kamat et al., 1989). Aspiration of GJ creates varying degrees of neutrophilia within the airways, interstitium and alveoli (Meers et al., 2011a).

The literature detailed some basic pulmonary histopathological scoring systems for OP ingestion or gastric aspiration, but none were specific to either (i) aspiration of combinations of OP and GJ, or (ii) aspiration injuries in the minipig (Yavuz et al., 2008, Downing et al., 2008, Meers et al., 2011a, Bregeon et al., 2008, Meers et al., 2011b, Inci et al., 2008, Amara et al., 2012).

Consequently, there was a requirement for the development of a repeatable, accurate and validated pulmonary histopathology scoring system for OP poisoning through ingestion and aspiration in minipigs.

This was made possible by using the pulmonary histopathology samples from both study 1 (orogastric placement of OP) and study 3 (orogastric placement and aspiration of OP) with the expertise of: Dr Sionagh Smith (SS) (Histopathologist, Royal (Dick) School of Veterinary Studies (R(D)SVS), University of Edinburgh), Dr William Wallace (WW) (Consultant respiratory histopathologist, Royal Infirmary of Edinburgh), Professor RE Clutton (Professor of Anaesthesia at R(D)SVS, University of Edinburgh) and Dr Gordon Drummond (previous Senior Statistical Editor for the Journal of Physiology and Honorary Clinical Senior Lecturer at the Department of Anaesthesia, Royal Infirmary of Edinburgh).

The flow chart depicts the processes leading to the development of the final histopathological scoring system (1.1) used for the main pulmonary aspiration study (Figure 2.4).

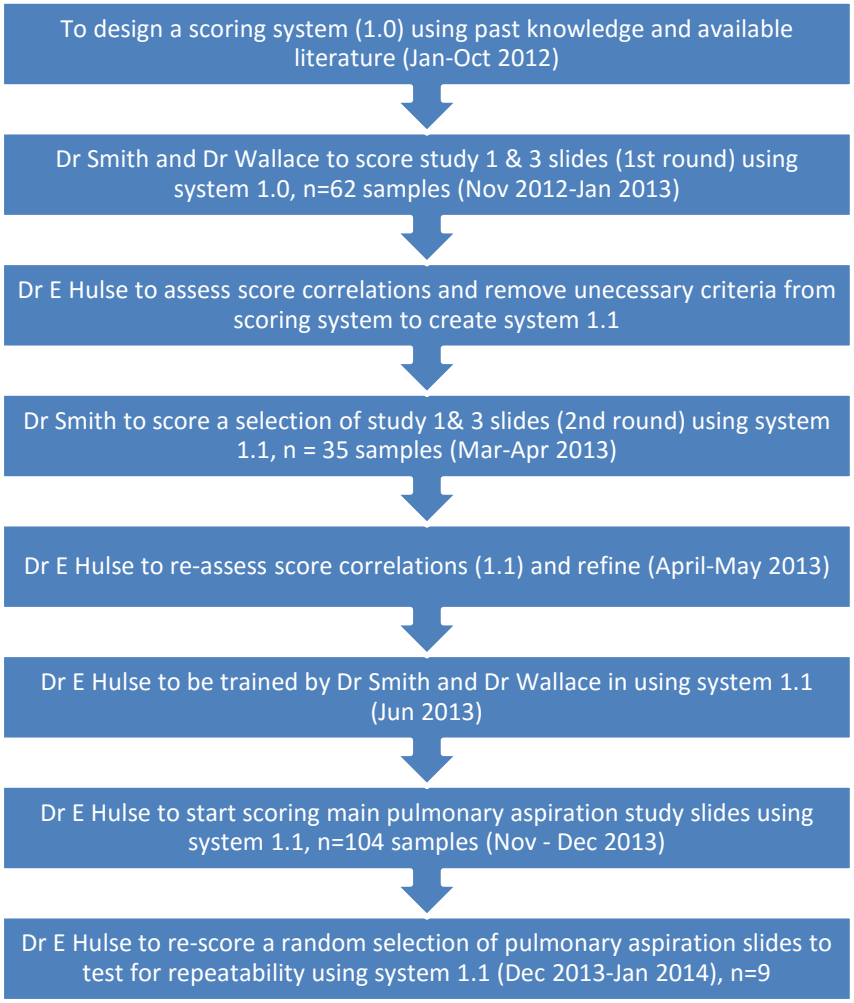


Figure 2.4: The stages involved in the development of the system used to score pulmonary injury caused by orogastric placement and aspiration of organophosphorus pesticide.

2.2.9.5.1 Histopathological scoring system 1.0

The initial histopathology scoring system 1.0 looked at eight different aspects of lung injury, with each feature able to score between zero and three points (maximum 24 points) (Table 2.9).

Structure	Lesion	Extent	Score
1. Bronchial lumens	Neutrophils	None <10 per airway 11-50 per airway >50 per airway	0 1 2 3
2. Bronchiolar lumens	Neutrophils	None <10 per airway 11-50 per airway >50 per airway	0 1 2 3
3. Bronchial/bronchiolar	Epithelial necrosis /degeneration	None Mild Moderate Severe	0 1 2 3
4. Perivascular	Inflammation / fibrin	None Mild Moderate Severe	0 1 2 3
5. Alveoli / interstitium	Oedema	None <25% 25-50% >50%	0 1 2 3
6. Alveoli	Inflammatory cells (neutrophils)	None - few Mild increase Moderate Marked	0 1 2 3
7. Interstitial	Inflammatory cells (neutrophils)	None - few Mild increase Moderate Marked	0 1 2 3
8. Anywhere	Haemorrhage / necrosis / fibrin	None Up to 5% of section 5-50% of section >50% of section	0 1 2 3
Other (descriptive notes only): e.g. thrombosis, emphysema, fibrosis, vasculitis		Max score (24)	

Table 2.9: Histopathological scoring system 1.0

The eight minipig lungs from study 1 that had undergone bronchoalveolar lavage were selected for histopathological scoring. These included two IV oleic acid (positive control), two saline control (by gavage) and 4 OP-poisoned (by gavage) minipigs (Table 3.1). All five minipigs in study 3 had samples selected for histopathological analysis. Bilateral cranial, caudal and occasionally middle lung sections (from the right lung) were taken from both right and left lungs, producing 4-5 samples

per pig, a total of 62 samples for scoring. Slides with approximately 5mm^2 tissue samples (which had to include some bronchioles) were prepared from formalin fixed tissue samples.

Each examiner SS and WW marked the same set of slides (one slide per area of lung, n=62) in a random fashion using 1.0 scoring system and both were blinded to the groups from which the slides belonged. The scores were not based on fields of view analysed, but on the worst score attainable within the whole tissue sample (5mm^2). The results from SS and WW were entered into dot plot where the correlation was calculated for non-parametric data using Spearman's rank coefficient (Graph Pad Prism 6.04). If the scoring of SS and WW was similar, there was an increase in the observed monotonic relationship nearing the value of 1. The scores awarded by SS vs. WW were also entered into a Bland-Altman analysis to identify the bias (mean difference) and limits of agreement (LOA) (Bland and Altman, 1986).

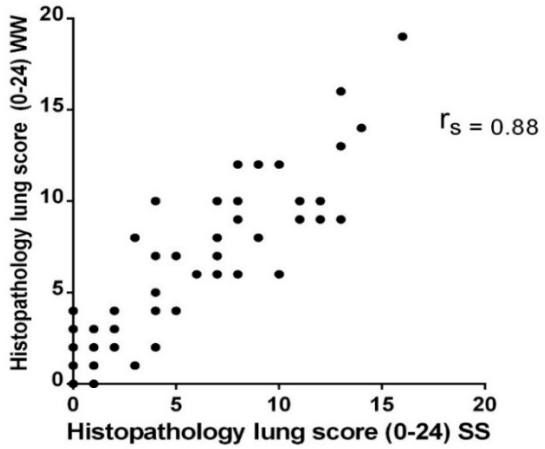


Figure 2.5: Scatter dot plot showing individual pulmonary histopathology scores for all lung samples from study 1 and 3 using system 1.0, WW vs. SS, Spearman's rank coefficient r_s =0.88.

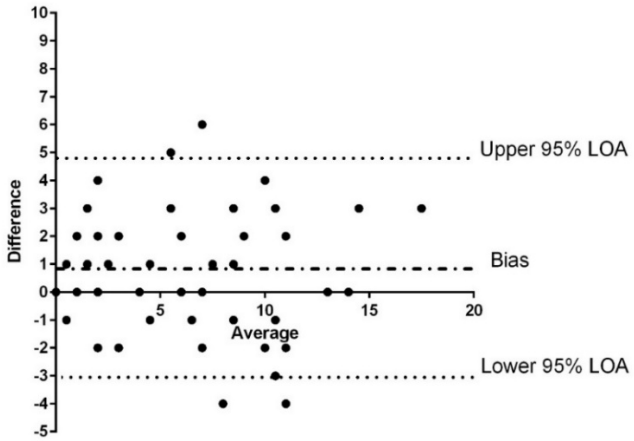


Figure 2.6: Bland-Altman analysis of scoring system 1.0 WW vs. SS, difference (WW-SS) vs. average. Bias (0.9) and 95% limits of agreement (LOA -3 to 4.9) are shown.

These histopathological scores demonstrated the scoring system's (1.0) ability to measure acute lung injury in a range of pathologies (e.g. oleic acid, aspiration of GJ mixtures and orogastric placement of OP) (Figure 3.5; Figure 4.7). The scores from SS and WW also showed a strong correlation with a Spearman's rank coefficient of 0.88 (Figure 2.5). However, Bland-Altman analysis revealed that the data had a relatively wide confidence interval showing 95% limits of agreement (LOA) between -3 and 4.9 score points (Figure 2.6). The positive bias (0.9) was due to WW having a tendency to over score in comparison with SS, though this was only statistically significant for the saline control in study 1 (p=0.024).

2.2.9.5.2 Histopathological scoring system 1.1

After discussion with the statistician (Dr Drummond) and histopathologists (SS and WW), we removed the bronchial epithelial necrosis and perivascular inflammation components (due to low scoring across pathologies). This measure resulted in improved correlation (0.9) and LOA (-3.6 to 3.6) (Figure 2.7, Figure 2.8) and created the histopathological scoring system 1.1.

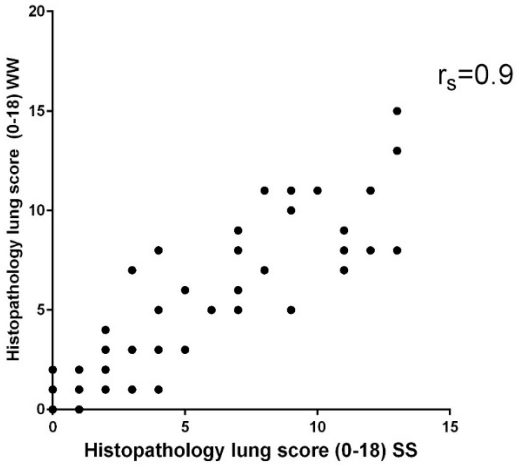


Figure 2.7: Scatter dot plot showing individual pulmonary histopathology scores for all lung samples from study 1 and 3 using the amended scoring system 1.1, WW vs. SS, Spearman's rank coefficient r_s =0.9.

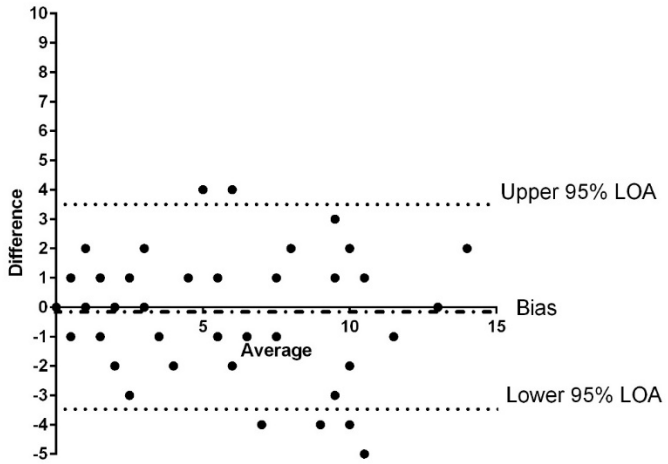


Figure 2.8: Bland-Altman analysis of amended scoring system 1.1, WW vs. SS, difference (WW-SS) vs. average. Bias (-0.01) and 95% limits of agreement (LOA -3.6 to 3.6) are shown. These data are from studies 1 and 3.

To exclude scoring differences between upper and lower lung samples, and to further improve score agreement, correlation and Bland-Altman analysis was conducted for both upper and lower lung samples using system 1.1 and samples from studies 1 and 3.

Analysis of the lung scores revealed slightly wider LOA within the lower lung (caudal) samples especially with greater lung damage. The mean (SD) bias of cranial and medial lung samples was 0.16 (1.63), 95% limits of agreement -3 to 3.3, compared with caudal bias -0.19 (2.0), 95% limits of agreement -4.1 to 3.7 (Figure 2.9).

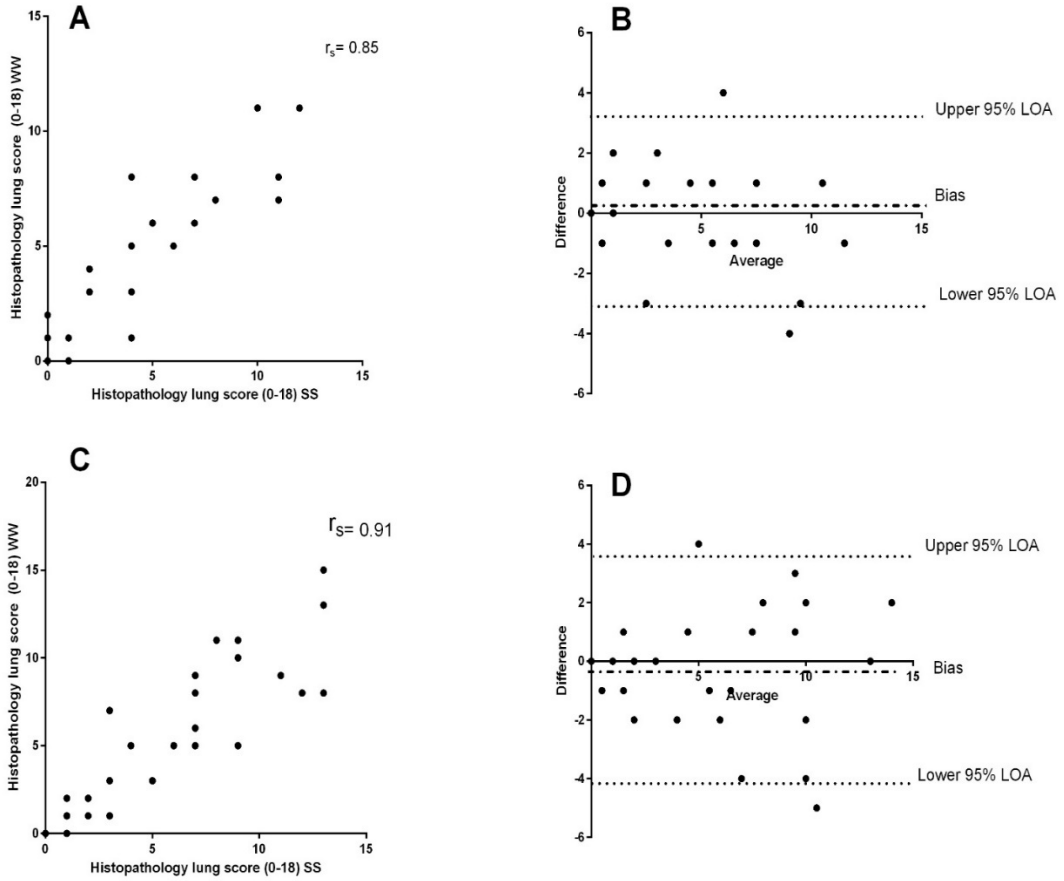


Figure 2.9: Scatter dot plots and Bland-Altman analysis (Difference (WW-SS) vs. mean) showing the correlation and spread of cranial and middle (A and B) vs. caudal (C and D) histopathology scores by SS and WW using the amended pulmonary histopathology scoring system 1.1. The Spearman's rank correlation coefficient R_s, bias and 95% LOA are marked on each graph.

Despite these minor differences in LOA, it was decided to include both upper and lower lung scores to the overall scoring system. This was not least because the pulmonary aspiration mixtures were to be placed in the right lower lung for the main aspiration study.

2.2.9.5.3 Histopathology scoring system 1.1 is repeatable within assessors

SS 'blindly' re-scored a random selection of samples (n=35) from studies 1 and 3 using the 1.1 system approximately 8 months after the first scoring session (Figure 2.10). The correlation (R_S=0.95) and LOA (-3.86 to 2.49) were tighter than those of comparisons with WW when using system 1.1 (Figure 2.7). It was concluded that the histopathology scoring system 1.1 was repeatable and could be used for the main pulmonary aspiration study.

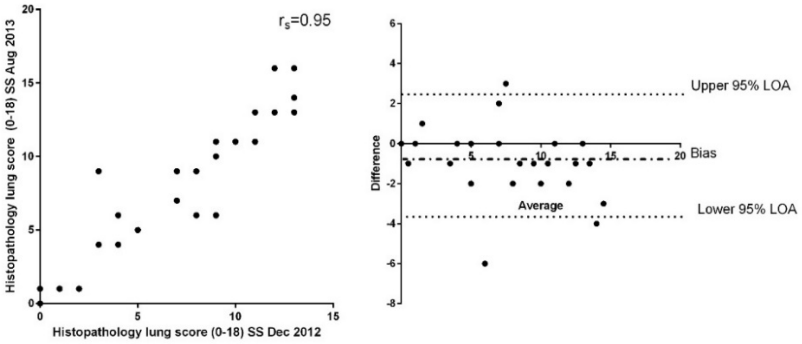


Figure 2.10: Repeatability of histopathology scoring within an assessor (SS) using a scatter dot plot and Bland-Altman analysis. Bland-Altman analysis of SS awarded scores December 2012 vs. SS August 2013 scoring using system 1.1(difference (SS 2012-SS 2013) vs. average), Bias (-0.69) and 95% limits of agreement (LOA -3.86 to 2.49) are shown. Spearman's rank correlation coefficient R_S is 0.95.

In conclusion, we created a specific, repeatable histopathological scoring system with acceptable correlation and inter-observer agreement for the study of lung injury caused by orogastric administration or aspiration of OP ±GJ. Yet, it must be noted that 4-5 lung tissue samples were taken from each individual minipig (representing right and left upper lung and right and left lower lung), but were treated as individual samples. This means that the samples may have had less variance than they would if they were truly from separate animals and we are therefore guilty of pseudoreplication (Drummond and Vowler, 2012). However, as noted by the authors Drummond *et al*, sometimes pseudoreplication is unavoidable due to the high costs of large animal work and the requirement to obtain the maximum amount of information from each animal.

2.2.10 Tissue imaging

Two samples of right and left basal lung tissue (approximately 1 mm^3) were prepared after fixation with 10% neutral buffered formalin. These were placed into 2% buffered (sodium cacodylate) gluteraldehyde and sent to Mrs Tracey Davey at the Newcastle University, Electron Microscopy (EM) Research Services unit for further processing and microscopy.

2.2.10.1 Scanning electron microscopy (SEM)

The samples, upon delivery to the Newcastle University EM unit, were rinsed in several changes of buffer. Dehydration was carried out using the following protocol; 25% ethanol (30 min), 50% ethanol (30 min), 75% ethanol (30 min), 100% ethanol (two hours) with a final dehydration with carbon dioxide in a Baltec critical point dryer. Samples were mounted either on an aluminium stub with Acheson's silver dag and then dried overnight, or onto sticky carbon discs.
Samples were then coated with gold, standard 15nm, using a Polaron SEM coating unit and examined using a Stereoscan 240 scanning electron microscope (Cambridge instruments, UK). Digital images were collected with Orion 6.60.6 software.

2.2.10.2 Transmission electron microscopy (TEM)

On arrival at the Newcastle University EM unit, the samples underwent a secondary fix with 1% osmium tetroxide.

Samples were dehydrated using the following protocol; 25% acetone (30 min), 50% acetone (30 min), 75% acetone (30 min), 100% acetone (two hours). Samples were impregnated with 25% resin in acetone, 50% resin in acetone, 75% resin in acetone (60 min each), then 100% resin for a minimum of 3 changes over 24 hours.

Samples were embedded in 100% resin at 60 °C for 24 hours and then sectioned (0.5 μm) and stained with 1% toluidine blue in 1% borax. Ultrathin sections (70 nm) were then cut using a diamond knife on a RMC MT-XL ultramicrotome or a Leica EM UC7 ultramicrotome. The sections were stretched with chloroform to eliminate compression and mounted on Pioloform-filmed copper grids.

Finally, samples were stained with 2% aqueous uranyl acetate and lead citrate (Leica, UK) and the grids examined using a CM 100 Compustage (FEI) transmission electron microscope (Phillips, Netherlands). Digital images were collected using an AMT CCD camera (Deben, UK).

2.2.11 Statistics

2.2.11.1 Frequency distribution

Frequency distribution of the physiological and non-physiological data exhibited non-parametric traits. A representative example of the physiological data (Figure 2.11), as well as the CT graphs above (Figure 2.3), show the negatively skewed data sets and their non-parametric nature.

Frequency Table	
Class	*Count*
93.07178-148.07177	5
148.07178-203.07177	6
203.07178-258.07177	4
258.07178-313.07177	6
313.07178-368.07177	10
368.07178-423.07177	11
423.07178-478.07177	12
478.07178-533.07177	14
533.07178-588.07177	7
588.07178-643.07177	3

Your Histogram	
Lowest Score	93.07178
Highest Score	614.6939
Total Number of Scores	78
Number of Distinct Scores	78
Lowest Class Value	93.07178
Highest Class Value	643.07177
Number of Classes	10
Class Range	55

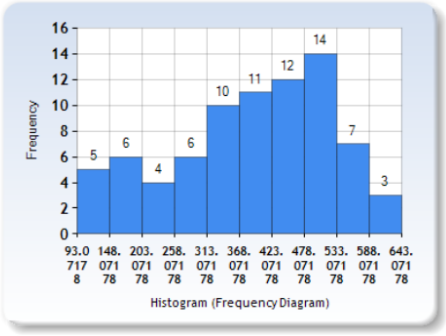


Figure 2.11 Frequency distribution of PaO_2/FiO_2 data in five OP poisoned minipigs from the pulmonary aspiration study (chapter 5). A left skewed graph can be seen indicating a likely non-parametric distribution of data. Graph created by using www.socscistatistics.com

2.2.11.2 Omnibus and post hoc tests

Statistical omnibus testing was performed using either Friedman (repeated measures) or Kruskal-Wallis tests. If these were found to be significant, then further post hoc analysis using Dunn's post-test or permutation testing (Ludbrook and Dudley, 1998) were used.

Data are expressed as mean ±standard deviation (SD) unless otherwise stated, and values of $p \leq 0.05$ were considered significant and denoted as follows; $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****.

Power calculations for the main pulmonary aspiration study are detailed in chapter 5.

Chapter 3: Indirect lung injury caused by orogastric administration of organophosphorus pesticide

3.1 Introduction

Chapter one highlighted the paucity of animal data describing lung injury caused by OP pesticide ingestion.

In order to better understand the pulmonary pathophysiology of ingested OP, and to help guide the design of future OP aspiration studies, I reviewed all the research team's previous OP poisoned minipig studies from 2008 – 2012 and extracted relevant respiratory information.

3.1.1 General respiratory system findings from early studies

From its inception in 2008, the pesticide poisoning minipig model was developed from a basic anaesthesia research model into a high fidelity intensive care unit (ICU) for minipigs with 24 hour veterinary/ medical supervision. Animals were all poisoned by gavage (unless otherwise stated) which involved a plastic tube being placed from the oropharynx to the stomach and left *in situ* to be used for the installation of OP mixtures.

The studies from 2008-2011 were designed to establish the minipig toxicology model and to learn more about the pathology of the lung, heart and neuromuscular junction (NMJ) secondary to OP pesticide poisoning.

Pulmonary histopathology for those poisoned with high dose OP by gavage (e.g. 2.5 mL/kg dimethoate EC40) generally showed evidence of acute interstitial pneumonia (inflammation of the gas exchange areas of the lung including interstitial neutrophilia) with septal oedema, lymphangectasia and alveolar haemorrhage. In contrast chlorpyrifos EC48 (2.2-2.4 mL/kg) seemed to cause less damage with a predominant alveolar histocytosis (increased number of lung macrophages) and septal oedema.

Immediate *post mortem* analysis of minipigs receiving high dose (2.5 mL/kg) (n=3), but not low dose dimethoate EC40 (<2.5mL/kg) or saline by gavage (n=5), showed neuromuscular junction (NMJ) dysfunction after 12 hours. This was demonstrated by the failure of indirect (via phrenic nerve stimulation) but not direct diaphragmatic stimulation to elicit a contraction. The failed indirect stimulation may have resulted from excess acetylcholine at the NMJ (depolarising block) as a result of AChE inhibition. It has also been seen (to a lesser extent) in primates exposed to inhalational OP nerve agents (Anzueto et al., 1990). Impulses from the central respiratory centre that fail to be transmitted to the diaphragm (via the phrenic nerve) will result in apnoea and hypoxia if mechanical ventilation is not provided during this period of neuromuscular block/dysfunction.

The early studies lasted between 3-20 hours and the minipig lungs were usually ventilated using a Manley minute volume divider ventilator. This was incapable of providing protective ventilation (< 6 - 8 mL/kg tidal volumes) and could have contributed to pulmonary injury through ventilator induced lung injury (VILI) during the studies. The fraction of inspired oxygen (F_IO_2) was also high (>0.95) and could have worsened any VILI present by causing oxidative damage to the respiratory system. Oxygen toxicity can increase right-left intrapulmonary shunting, absorption atelectasis, cellular damage to the tracheobronchial tree, free radical formation, increased susceptibility to pulmonary infection, respiratory failure and increased mortality (Capellier and Panwar, 2011). Evidence suggests that pulmonary changes secondary to hyperoxia can occur as early as 17 hours in healthy humans, and so injury secondary to this mechanism cannot be ruled out in some of the early model development animal studies (Davis et al., 1983).

Despite these confounding factors, a few studies warranted further inspection and are described in more detail in the following chapter. These included a 12 hour long study conducted in 2008 with 8 minipigs (study 1), and another 6 hour long study (partly designed and run by myself) conducted in 2012 with 15 minipigs (study 2). These animals (8 pigs in study 1, 10/15 pigs in study 2) underwent sequential bronchoalveolar lavage and *post mortem* histology (including electron microscopy) which produced important information about the architecture and passage of cellular and non-cellular components across the alveolar capillary membrane in control and OP poisoned minipigs.

In ARDS there is an alveolar capillary leak which allows protein to move from the blood vessel to the alveolar space. If there is concurrent inflammation present in the lung then cytokines such as IL-8, IL-1β, TNF-α also rise in the alveolar space. Consequently, some of these markers were measured from the lavage fluid collected in study 1 and 2 (Ware and Matthay, 2000). As ARDS is a predominantly neutrophil driven process the number and type of white cells present in the lavage fluid were also documented where possible.

3.1.2 Aim of the chapter

a. To present evidence of lung injury caused through orogastric placement of OP by extracting data from previous minipig studies
b. To identify any underlying common mechanism of lung injury caused by ingestion of OP pesticide

3.2 Methods

Unless otherwise stated, animals were handled, anaesthetised, monitored and sampled in a similar manner to that described in methods (chapter 2). However, animals were anaesthetised using the volatile gas isoflurane in these studies.

The data collection method for these studies ranged from hand written anaesthetic and research notes, to fully automated electronic recordings (Excel files) using software developed specifically for recording physiological data (Edinburgh data logger v1.0.55.0 Vetronic Services Limited©).

Recorded respiratory data included, but were not limited to; arterial oxygenation (PaO_2), F_IO_2, arterial blood gas component measurement and pulmonary mechanics. This was supplemented with lung histopathology and electron microscopy.

The data were entered into a statistical software package to create graphs, and if numbers permitted, statistical analysis (Graph Pad Prism version 6.04 for Windows 8). The methods used in these studies were obtained from the notes, and confirmed with the investigators where possible.

3.2.1 Study 1- methods

This study involved collaboration with a respiratory research team led by Professor John Simpson, University of Edinburgh. Members of this team performed the bronchoalveolar lavage sampling and subsequent measurement of inflammatory markers and protein concentrations in the BALF.

3.2.1.1 Study groups

Study 1 lasted 12 hours and was conducted in 8 male minipigs, weight 21.4 (± 2.1) kg, who received either 2.5 mL/kg high-dose dimethoate EC40 (n=4) or saline (n=2) by gavage with two oleic acid (positive) controls (Table 3.1). The OP pesticide pigs also received IV oxime treatments to explore whether pralidoxime reactivated dimethoate inhibited AChE and to simulate real life poisoning. The positive control with oleic acid was included to ascertain that the immunoassays were working correctly. Minipigs were ventilated with 100% (FiO_2 >0.95) oxygen. Unfortunately, PaO_2/F_IO_2 ratios were not calculated for all groups because blood gas data were incompletely recorded.

Pig group	Dose	Treatments (2 hours post poisoning) IV
Dimethoate EC40 high-dose (n=4) [OP pigs]	2.5 mL/kg	Pralidoxime loading dose 20 mg/kg, followed by an infusion 15.5 mg/kg/hr
Saline control (n=2)	2.5 mL/kg	none
Oleic acid control (n=2)	1st – IV 0.25 mL/kg, 2nd 0.17 mL/kg	none

Table 3.1: Minipig groups and treatments received for study 1, IV = intravenously.

3.2.1.2 Bronchoalveolar lavage fluid sampling and analysis

All minipigs received a single-sided bronchoalveolar lavage (BAL) at baseline (time -30 min), and at 4 and 12 hours using a 60 mL sterile saline aliquot introduced through the working channel of a bronchoscope (VETVU VFS-2A Veterinary fiberscope, Krusse, UK). Bronchial biopsies were also taken at 12 hours. The samples and BALF were processed for cellular and non-cellular content in a manner similar to that described in chapter 2 (methods).

3.2.1.3 Histopathology and imaging

Post mortem, the lungs were removed from the thorax and filled via the trachea with 10% formalin until a fluid pressure of approximately 25 cm H_20 was achieved. Bilateral cranial, caudal and occasionally middle lung sections (from the right lung which has 4 rather than 2 lobes) were cut from all (right and left) lungs, producing 4-5 samples per pig. The lung histopathology was scored using system 1.0 (see methods chapter 2).

Small lung samples (≤1mm^3) were sent to Mrs Tracey Davey for electron microscopy at the Newcastle University (see methods).

3.2.2 Study 2-methods

3.2.2.1 Study groups

Study 2 involved 15 female minipigs, weight 29.1 (± 2.3) Kg, who all received high-dose dimethoate EC40 (2.5 mL/kg) by gavage. The objective of the study was to observe the effect of OP poisoning on the NMJ with, or without the non-depolarising neuromuscular blocking agent (NMBA), rocuronium.

This study provided the opportunity to introduce and develop new ICU techniques that would be used for the main pulmonary aspiration study. The study also allowed further investigation of the indirect lung injury caused by ingestion of high-dose dimethoate and to conduct dosing studies (after completion of study-2) for the main aspiration study (chapter 4).

The pigs were randomly allocated to groups receiving rocuronium before (pre-OP NMBA), and after OP poisoning (post-OP NMBA), and to a control group receiving no rocuronium (No NMBA) (Table 3.2).

Pig group	Dose
Dimethoate + rocuronium before poisoning (n=5) [Pre-OP NMBA]	2.5 mL/kg dimethoate EC40 (gavage)+ IV NMBA [3mg/kg loading dose rocuronium and 1 mg/kg/hr infusion] before poisoning
Dimethoate + rocuronium 2 hours after poisoning (n=5) [post-OP NMBA]	2.5 mL/kg dimethoate EC40 (gavage) + IV NMBA [3mg/kg loading dose rocuronium and 1 mg/kg/hr infusion] 2 hours after poisoning
Dimethoate only (n=5) [No NMBA]	2.5 mL/kg dimethoate EC40 (gavage)

Table 3.2: Minipig study groups and dose of rocuronium received in study 2, IV: intravenously, NMBA = neuromuscular blocking agent.

3.2.2.2 Ventilation of minipigs

The minipigs were handled and instrumented as described previously (chapter 2), but were protectively ventilated (tidal volumes 6-8 mL/kg tidal volume) with a lower F_IO_2 0.5 to avoid VILI and hyperoxic lung damage respectively.

3.2.2.3 Bronchoalveolar lavage fluid sampling and analysis

Ten of these minipigs underwent bronchoscopy and BALF analysis: Pre-OP NMBA (n=3), Post-OP NMBA (n=4) and no NMBA (n=3). The minipigs had a single sided BAL at baseline (time -30 min) and at 6 hours using a 60 mL sterile saline aliquot through the working channel of a bronchoscope (VETVU

VFS-2A veterinary fiberscope, Krusse, UK). The BALF was aspirated using a 60 mL syringe after a 5 second dwell time, and the return volume was documented.

The BALF was centrifuged at 3500 rpm for 10 min at 4°C and the supernatant aliquoted and frozen at -80°C for further analysis. The cell pellet was re-suspended in an appropriate volume of room temperature saline and a BALF cell count was performed using a haemocytometer. A differential white cell count mean (range), 518 (500-551) cells, was achieved by preparing a cytospin slide using a Shandon cytocentrifuge 2 (Techgen, Zellik, Belgium) and Diffquick staining (Dade Behring, Newark, NJ).

Levels of protein in BALF samples were measured using a Bradford assay on the Cobas Fara centrifugal analyser (Roche Diagnostics, Welwyn Garden City, UK), with C-reactive protein (CRP) and IL-8 measured using commercial ELISAs as per chapter 2 (methods).

3.2.3 Statistical analysis

3.2.3.1 Study 1

The results from pigs treated with oleic acid were not used as part of an omnibus test. Statistical analysis was performed by using unpaired permutation tests comparing the OP and saline control pigs BALF protein, inflammatory markers and histopathology results.

3.2.3.2 Study 2

Statistical analysis involved Friedman analysis for repeated measures with Dunn's post-test between groups (PaO_2/F_IO_2 ratios). Kruskal-Wallis test was used for omnibus group analysis of data at 0 or 6 hours. Paired permutation testing was used for analysis of data within a group (0 hours vs. 6 hours) for all OP poisoned minipigs e.g. cytokine data.

3.3 Results

3.3.1 Study 1- Results

The oleic acid treated pigs survived for only 2 hours due to the severity of poisoning. The OP and saline pigs survived until the end of the experiment at 12 hours.

3.3.1.1 Bronchoalveolar lavage quality

The BALF return volumes were acceptable with a mean (range) 79 (42-100) %, meaning that the samples were a reliable reflection of the cellular and non-cellular content of the alveolar space (Meyer

et al., 2012, Judge et al., 2014). In 3 of 4 OP pigs, bloody bronchoalveolar lavage fluid was collected between 4-12 hours. This was not observed in the other minipigs undergoing bronchoscopy. It remains unknown whether this was due to trauma from the BAL, or part of the pathological process of indirect OP-induced lung injury.

3.3.1.2 Bronchoalveolar lavage protein and cellular contents

OP poisoned minipigs had greater protein concentrations in the BALF at both 4 and 12 hours than saline controls. Although not significant when compared with one another, the concentration of protein at 12 hours was 787(±301) mcg/mL in the OP poisoned vs. 294 (±113) mcg/mL in saline control minipigs (Figure 3.1A). The OP-treated pigs had less white cells, 2.9 (±1.8) x 10^5/mL, than the saline controls, 6.2 (±2.8) x 10^5/mL, at 12 hours, the difference of which was not significant (Figure 3.1B). Unfortunately, there were no cytospin slides available for the minipigs, and so no conclusions regarding the BAL white cell type and dominance within the BALF e.g. alveolar macrophage vs. neutrophil numbers, could be made.

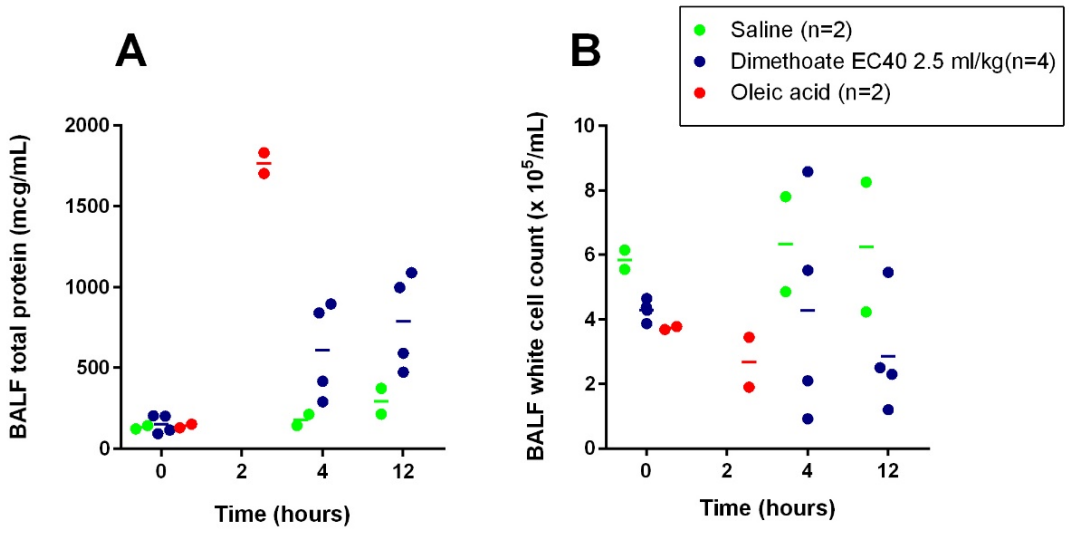


Figure 3.1: Dot plots of BALF protein and white cell numbers in minipigs treated with 2.5 mL/kg dimethoate EC40, 2.5 mL/kg saline by gavage (negative control), or intravenous oleic acid (positive control). Graphs show BALF total protein content (A), and white cell numbers (B) at -30 min (time 0), 2, 4 and 12 hours. There was no statistical difference between OP poisoned and saline controls at 12 hours using permutation testing. Mean values shown by the horizontal coloured lines.

3.3.1.3 Raised inflammatory markers within the BALF of OP poisoned minipigs

In study 1, the oleic acid control animals only survived until 2 hours but did show increases in BALF IL-6 and IL-8 demonstrating that the inflammatory marker ELISA assays were working correctly (Figure 3.2 A, B).

BALF IL-8 concentrations were raised in OP-treated pigs: mean (SD) 1227 (±1439) pcg/mL when compared with saline controls 416 (±531) pcg/mL. This was reflected in the plasma concentrations with OP-treated pigs having 116.1 (±77.9) pcg/mL vs. 36.1 (±8.5) pcg/mL in saline controls, 12 hours after poisoning (Figure 3.2 A, C).

BALF IL-6 concentrations were also raised in OP-treated pigs: mean (SD) 737 (±988) pcg/mL) when compared with saline controls 67 (±56) pcg/mL. Yet, this was not echoed in the plasma concentrations with OP pigs: 14.3(±28.7) vs.43.2 (±61.1) pcg/mL in the saline controls, 12 hours after poisoning (Figure 3.2 B, D).

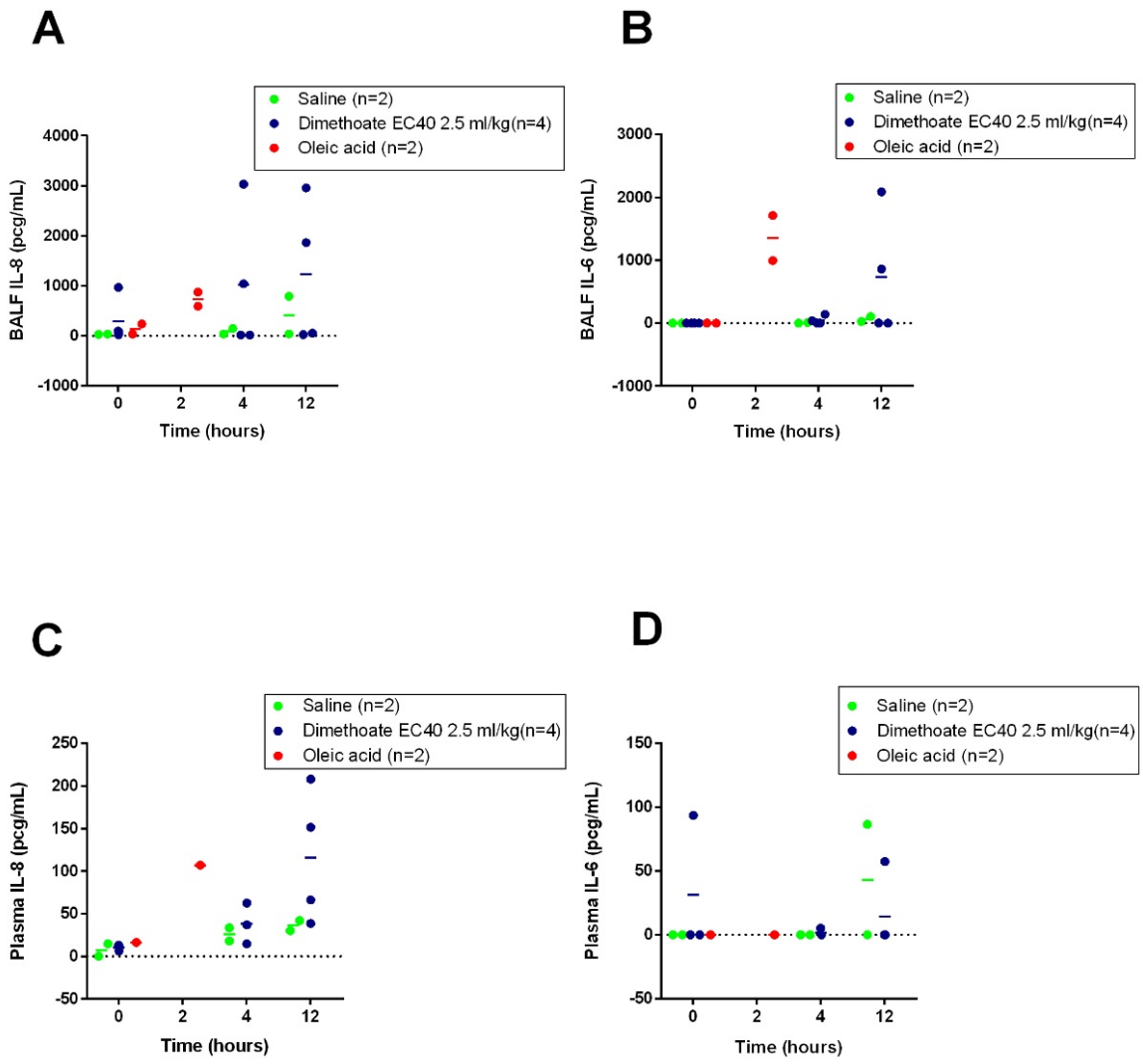


Figure 3.2: Dot plots of BALF and plasma concentrations of IL-6 and IL-8 of minipigs that had received 2.5 mL/kg dimethoate EC40, 2.5 mL/kg saline by gavage, or intravenous oleic acid. Graphs show BALF IL-8 (A), BALF IL-6 (B), plasma IL-8 (C) and plasma IL-6 (D) at -30 min (time 0), 2, 4 and 12 hours. Mean values are shown by the horizontal coloured bars. Permutation testing between OP and saline pigs at 12 hours was not significant. BALF: bronchoalveolar lavage fluid.

BALF concentrations of TNF-α were <10 pcg/mL in all pigs apart from one OP pig at 4 hours (86pcg/mL). Plasma concentrations of TNF-α were also generally low with OP-treated pigs having mean (SD) concentrations of 67.5 (±53) pcg/mL vs. 81.9(±72) pcg/mL in saline pigs (Figure 3.3). IL-10 levels un-recordable in both the BALF and plasma.

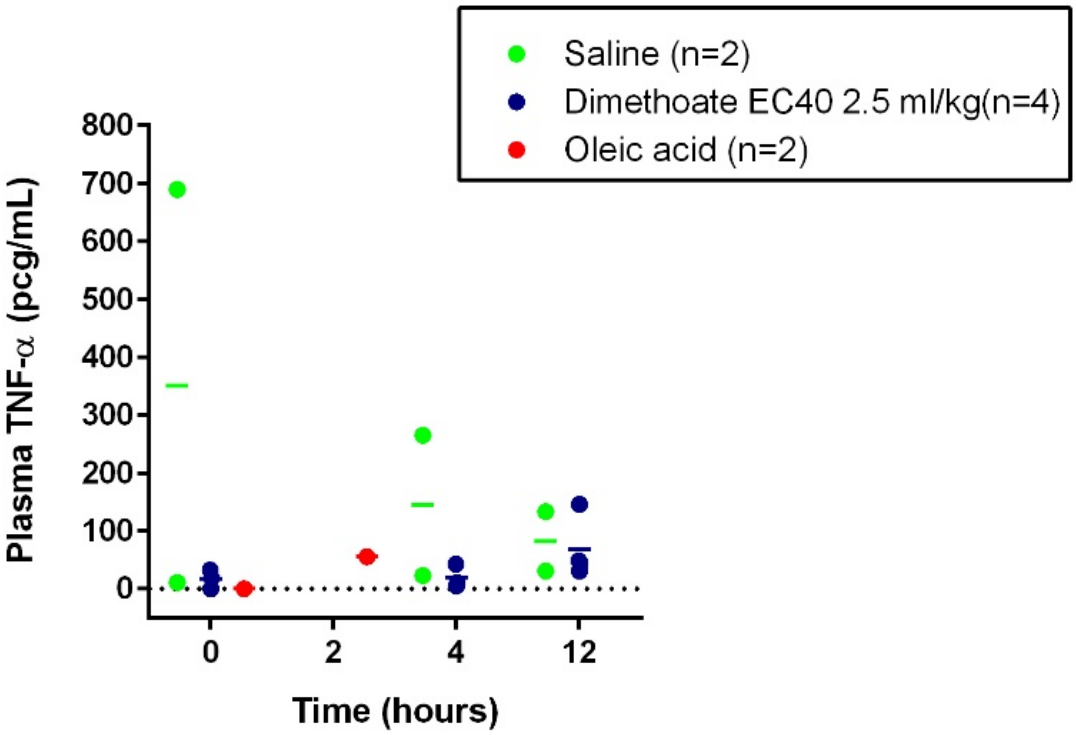


Figure 3.3: Dot plot of plasma TNF-**α in** minipigs that had received 2.5 mL/kg dimethoate EC40, 2.5 mL/kg saline by gavage, or intravenous oleic acid at -30 min (time 0), 2, 4 and 12 hours. Mean values are shown by the horizontal coloured bars. There were no significant differences.

The elevated cytokines levels in the BALF compared with plasma suggests that orogastric placement of OP caused a local pulmonary, rather than a systemic, inflammatory reaction (Bhargava and Wendt, 2012).

3.3.1.4 Analysis of the alveolar capillary membrane in an OP poisoned pig

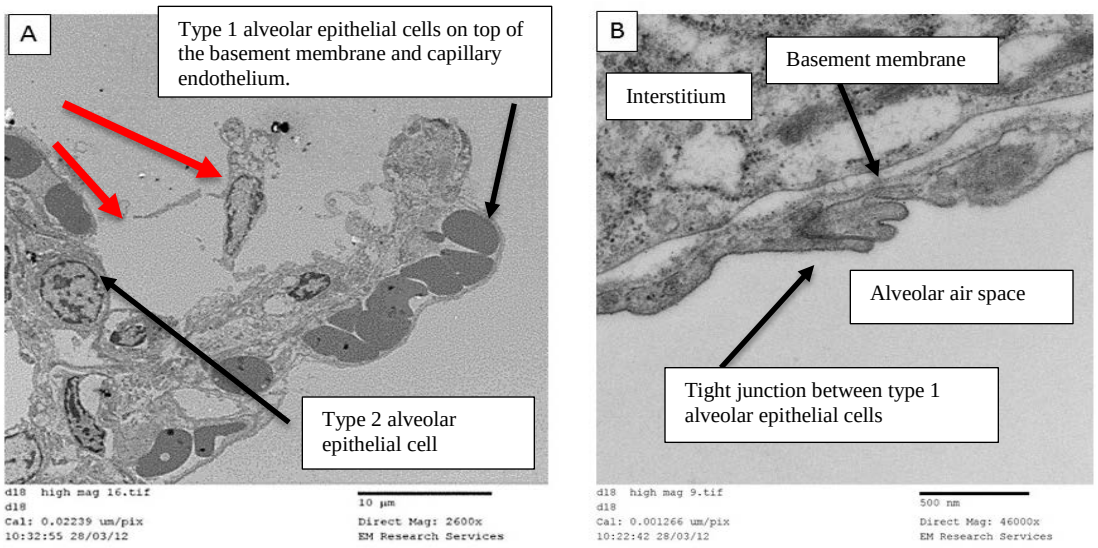


Figure 3.4: Transmission electron microscopy image (x2600- x46000) of the alveolar capillary membrane of a minipig exposed to high dose (2.5 mL/kg) dimethoate EC40. Red arrows indicate defects in the alveolar capillary membrane breaking away into the alveolar air space (A), which may be artefactual or pathological. An intact 'tight junction' between type 1 alveolar epithelial cell is also shown (B) from the same pig. Images taken by Tracey Davey, EM unit, Newcastle University.

A review of the first set of EM images from one saline and one OP poisoned pig showed intact alveolar capillary membranes with no breaks in the epithelial or endothelial tight junctions between cells (Figure 3.4B). However, examination of a second set of EM images revealed evidence of a break in the alveolar capillary membrane in the same OP poisoned pig (Figure 3.4 A). This could have been artefactual and would need to be confirmed in more samples.

3.3.1.5 Orogastric placement of OP creates a significant histopathological lung injury when compared with saline controls

Histopathological examination of tissue from the saline control pigs (n=2) showed evidence of mild alveolar haemorrhage, lymphangiectasia, interstitial neutrophilia, with fibrin deposition in one sample.

Lung histopathology from OP poisoned minipigs (n=4) had evidence of histocytosis, lymphangectasia with alveolar (±interstitial) haemorrhage, oedema and neutrophilia. There was also a new descriptive finding of fibrin deposition within the alveoli. The involvement of fibrin suggests imminent proliferation (repair of tissue) and the beginnings of fibrosis in the lung – the second/third stages of acute respiratory

distress syndrome (Bhargava and Wendt, 2012). However, fibrin was also observed in a sample from a saline control animal, meaning that mechanical lung ventilation and/or the sampling processes may have led to some degree of damage.

Pigs with OP poisoning by gavage had more histopathological damage than controls (p=0.023). As the score was predominantly a measure of neutrophils and oedema in the airways, this suggests that OP poisoning in this model caused a measureable indirect lung injury when compared with control lungs (Figure 3.5).

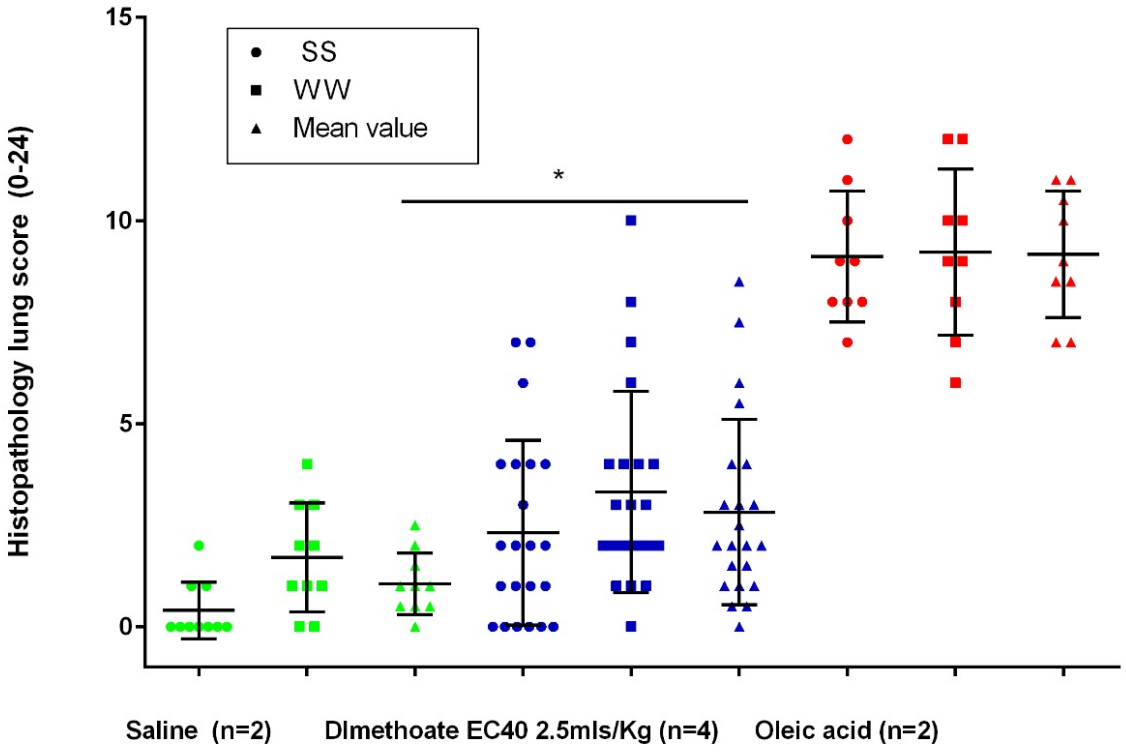


Figure 3.5: Dot plot showing histopathology scoring for each lung sample from minipigs that had received orogastric saline (2.5 mL/kg), dimethoate EC40 (2.5 mL/kg) or intravenous oleic acid. Lung sections were scored using the histopathology 1.0 scoring system (maximum score of 24 points for each sample). Scores from both evaluators (SS and WW) and mean scores are shown for comparison. Permutation testing between saline (mean score) and dimethoate EC40 2.5 mL/kg (mean score) minipig lungs was significant (p=0.023). Mean (SD) shown.

The mean histopathological scores for the saline pigs were of particular importance as they were the control group for powering the subsequent main aspiration study (see chapter 5; 5.2.9.1 for further details).

3.3.2 Study 2- Results

All the pigs in study 2 survived without complications until the study ended at 6 hours.

3.3.2.1 Orogastric administration of OP significantly reduces PaO_2/FiO_2 ratios over time

The PaO_2/F_IO_2 ratio decreased significantly over time in all animals (15/15). The mean (SD) at treatment -10 min was 459 (±109) mmHg vs. 358 (±129) mmHg at 6 hours (p= 0.0023). Values in some minipigs were consistent with mild acute respiratory distress syndrome (see methods for ARDS description) (Figure 3.6). Friedman analysis of all 3 groups revealed significant differences over time (p=0.0084), with intergroup analysis (using Dunn's post-test) showing that No NMBA minipigs had significantly higher PaO_2/F_IO_2 ratios than Pre-OP NMBA, but not Post-OP NMBA pigs over the study's 6 hour duration (p≤0.01). There were no significant differences (Kruskal-Wallis) between groups at time – 10 min or 6 hours, with the mean (SD) PaO_2/F_IO_2 ratio of No NMBA, Pre-OP NMBA and Post-OP NMBA at 6 hours being 421 (± 97), 307 (±129) and 346 (±168) mmHg respectively (Figure 3.6).

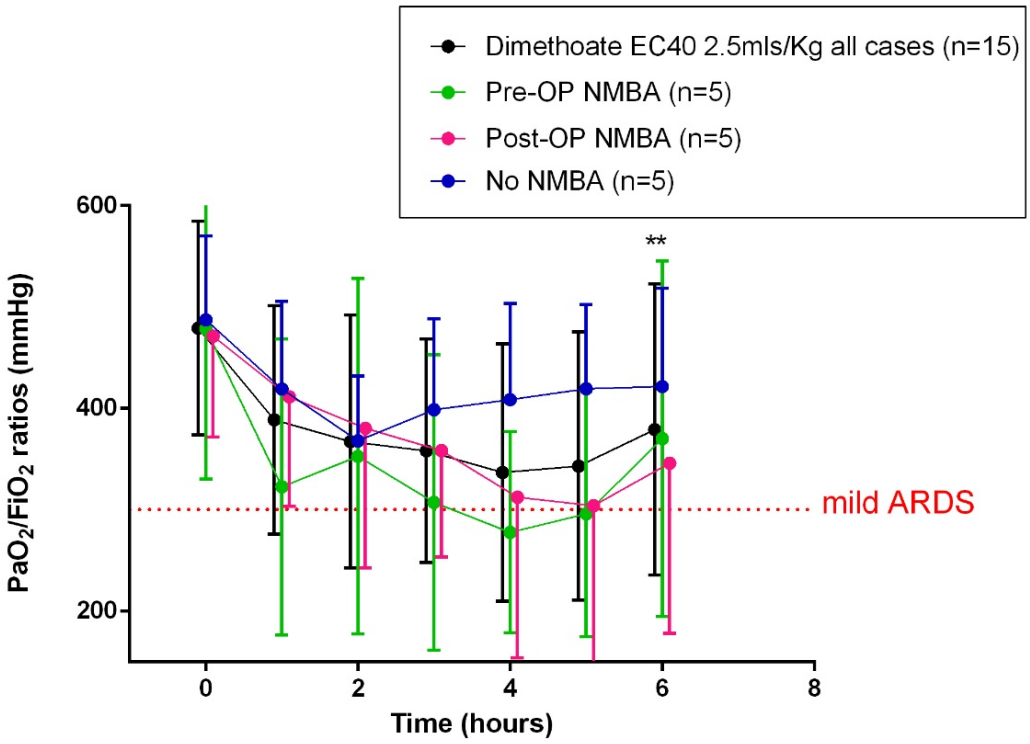


Figure 3.6: PaO_2/F_IO_2 ratios of 15 minipigs in study 2 who all received orogastric high dose OP (2.5 ml/kg dimethoate EC40) and were monitored over 6 hours. Friedman analysis between the 3 groups over time was significant (p=0.0084), with Dunn's post-test showing a significant difference between No NMBA and Pre-NMBA **minipigs (p≤0.01). Permutation testing of** PaO_2/F_IO_2 ratios at time – 10 min vs. time 6 hours in all minipigs was significant p = 0.0023 (as shown above on graph - asteriks). The red dotted line denotes mild ARDS. ARDS – acute

respiratory distress syndrome, NMBA: neuromuscular blocking agent. Mean and SD are shown on the graph.

3.3.2.2 Non-significant increases in white cells and neutrophils in the BALF of OP poisoned minipigs

The BALF return volumes (percentage of total volume instilled -mean (range)) of 71 (57-83)% were acceptable (Meyer et al., 2012). There was a non-significant increase in BALF cells from all groups over 6 hours (Table 3.3)

Groups	BALF cell numbers x 10^7	
	0 hours	6 hours
No NMBA (n=3)	2.1(1.3)	2.8 (2.9)
Pre-OP NMBA (n=3)	1.3 (0.6)	1.4 (0.7)
Post-OP NMBA (n=4)	1.5 (0.5)	2.8 (3.5)
All OP pigs (n=10)	1.6 (0.8)	2.4 (2.5)

Table 3.3: Total white cell numbers in the BALF (x 10^7) of minipigs that received orogastric 2.5 mL/kg dimethoate EC40 and either No NMBA (n=3), Pre-OP NMBA (n=3) or Post-OP NMBA (n=4). No significant differences between the groups, or any minipigs between 0 and 6 hours were seen. NMBA – neuromuscular blocking agent. Mean (SD) shown as above.

Cytospin analysis showed that the predominant BALF cell type was the alveolar macrophage. However, there was also a non-significant increase in neutrophils within the BALF of all OP poisoned minipigs (n=10) at 6 hours (Table 3.4).

BALF white cell type (%)	Time 0 hours	Time 6 hours
Alveolar macrophage	76 (8)	74 (7)
Lymphocytes	11 (6)	8 (5)
Neutrophils	6 (4)	13 (4)

Table 3.4: Percentage of cell types within the BALF of (n=10) minipigs that had orogastric administration of 2.5 mL/kg dimethoate EC40 and received no NMBA (n=3), pre-OP NMBA (n=3), post-OP NMBA (n=4) and had a bronchoalveolar lavage at 0 and 6 hours. Comparisons of

alveolar macrophages, lymphocytes and neutrophils at 0 and 6 hours in all pigs using paired permutation testing was insignificant. Data from 2 minipigs at 6 hours and one pig at time 0 were missing as the cytospin quality did not allow counting of more than 500 cells. Numbers of eosinophils and basophils were negligible at both time points. BALF – bronchoalveolar lavage fluid. Mean and (SD) shown.

3.3.2.3 Raised inflammatory markers within the BALF of OP poisoned minipigs

BALF concentrations of protein, IL-8 (p=0.002) and CRP (p=0.0273) were modestly raised in OP poisoned minipigs 6 hours after poisoning, possibly as a result of indirect lung injury and inflammation (Table 3.5; Figure 3.7). However, there were no negative (saline) or positive (oleic acid) controls with which to draw comparisons.

BALF non-cellular components	Time 0 hours	Time 6 hours
Total protein (mcg/mL)	146 (68)	223 (138)
CRP (pcg/mL)	1609 (1445)	3365 (2377)*
IL-8 (pcg/mL)	30.4 (27.1)	1837 (2927)**

Table 3.5: BALF non-cellular contents showing measurements of total protein, CRP and IL-8 at 0 and 6 hours in OP poisoned minipigs (n=10) who had orogastric administration of 2.5 mL/kg dimethoate EC40 and received No NMBA (n=3), Pre-OP NMBA (n=3), Post-OP NMBA(n=4). Paired permutation testing of (n=10) pigs that underwent bronchoscopy at 0 vs. 6 hours showed that there were significant increases in BALF CRP (p=0.0273) and BALF IL-8 (p=0.002), but not BALF total protein (p=0.176). Mean (SD) shown.

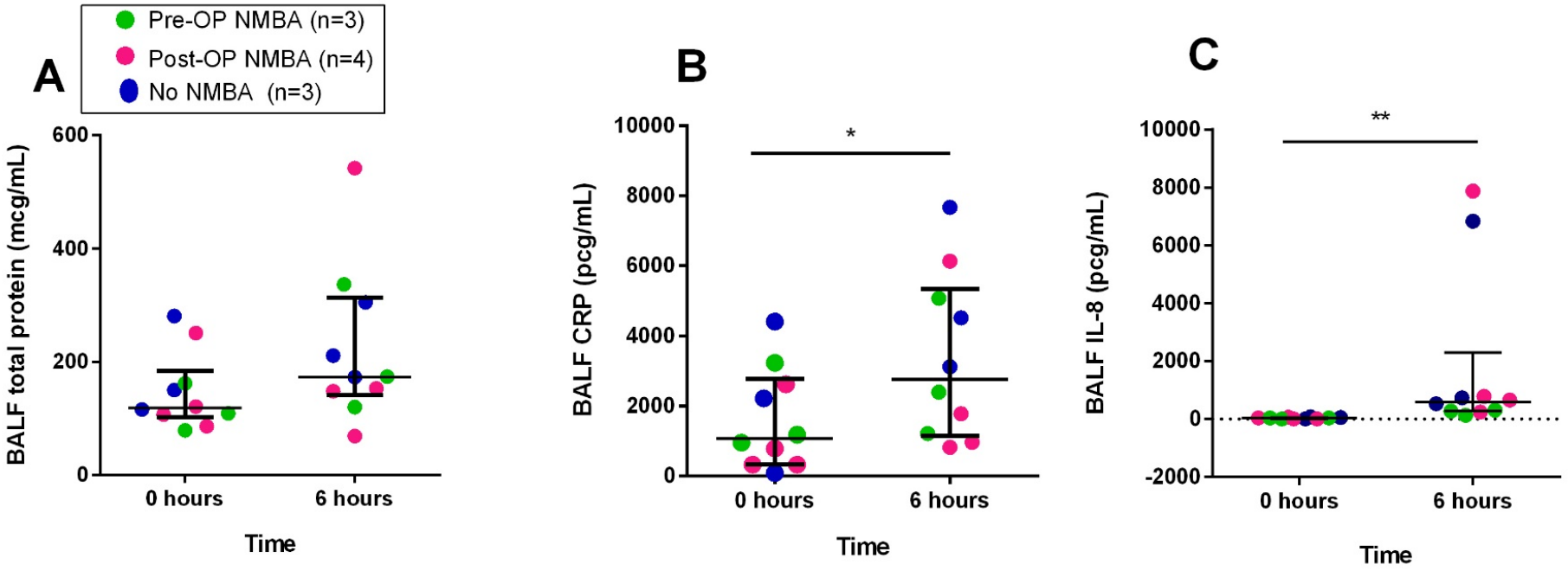


Figure 3.7: Dot plots of BALF total protein (A), C-reactive protein (CRP) (B) and interleukin-8 (IL-8) (C) at time 0 and 6 hours in minipigs that had been given 2.5 mL/kg dimethoate EC40 by gavage and received no NMBA (n=3), pre-OP NMBA (n=3), post-OP NMBA(n=4). Paired permutation testing in OP poisoned minipigs that had received bronchoscopy at 0 vs. 6 hours (n=10) showed there were significant increases in BALF CRP (p=0.0273) and BALF IL-8 (p=0.002), but not BALF total protein (p=0.176). Median and IQ ranges are shown.

3.4 Discussion

Evidence from previous work combined with the results of study 1 and 2 suggest that orogastric placement of large volumes (2.5 mL/kg) of dimethoate EC40 causes a measureable indirect lung injury typified by inflammation and oedema with possible damage to the alveolar capillary membrane. These findings are consistent with both human post mortem findings [lung oedema and haemorrhage] (Kamat et al., 1989) and in animals that have had orogastric administration of OP pesticide [alveolar and interstitial oedema, haemorrhage, emphysema, peribronchial inflammation, and destruction of alveoli] (Yavuz et al., 2008, He et al., 2012a).

Evidence for alveolar capillary membrane damage

In study 1 a non-significant increase in BALF protein concentration occurred over 12 hours in OP poisoned pigs when compared with saline controls, and a similar smaller rise in BALF protein within OP poisoned minipigs in study 2, six hours after poisoning. This suggests an increased production or movement of protein from the vasculature into the alveolar space after OP poisoning in pigs.

In study 1 there was inconclusive evidence from the EM images as to whether the alveolar capillary membrane was disrupted secondary to pathological or artefactual processes (Figure 3.4). Raised protein levels in the alveolar space in the presence of an intact membrane might be explained by a local pulmonary production of proteins and/or inflammatory cytokines, combined with possible active transport of proteins (Kim and Malik, 2003).

The presence of bloody lavages collected from three OP poisoned pigs in study 1 could be related to the pathological process, with the breakdown of the alveolar capillary membrane allowing erythrocytes passage from the vasculature into the alveoli. Bloody lavage was not noted in study 2 pigs, perhaps as the study was of a shorter duration. This would imply that alveolar capillary damage, particularly to the endothelium, may occur between 6 and 12 hours.

Evidence for lung inflammation

White cell numbers within the BALF collected in study 1 from OP-treated and saline control pigs did not change over time. In contrast, samples from OP-treated study 2 animals showed modest, though insignificant, increases in white cell numbers and neutrophil percentages.

The histopathology scores (largely neutrophil and oedema based) awarded in study 1 showed significant differences between groups with OP-treated pigs displaying greater lung injury than saline control pigs ($p=0.023$). This statistic was achieved through removal of the score co-variance from each assessor (WW and SS) by applying a statistical test to their mean (WW +SS/ 2) individual scores. However, it must be noted that these results were based on replicate samples from the same animals. These pseudoreplicates may not carry as much variance as actual individual animal samples (discussed further in chapter 4) and may therefore have less statistical significance.

Despite this, it seems probable that systemic OP poisoning causes a mild pulmonary inflammatory reaction involving the recruitment of neutrophils to the lung in minipigs.

Lung injury in the context of ARDS results from injury to the alveolar capillary membrane with subsequent triggering of inflammatory and coagulation pathways. This leads to increases in IL-6, IL-8, TNF-α and IL-1β in the BALF and plasma, combined with a pulmonary neutrophilia (Bhargava and Wendt, 2012). CRP can be produced locally in the lung and liver in response to many chemical stimuli including IL-6. Its function is to stimulate the complement system to mop up necrotic tissue and raised levels highlight systemic inflammation (Agassandian et al., 2014).

OP-treated pigs in study 1 had increased BALF concentrations of IL-6 and IL-8 with a modest reciprocal increase in plasma IL-8 when compared with saline controls animals over 12 hours. In study 2, OP-treated pigs had a measureable lung injury with reduced PaO_2/F_IO_2 ratios and increased BALF concentrations of CRP and IL-8 when comparing the time 0 and 6 hour lavages. IL-8 is a powerful neutrophil chemoattractant, and its increased presence in the BALF and plasma from OP pigs in study 1 and 2 suggest that neutrophils are involved in the lung injury process (Baggiolini and Clarklewis, 1992).

Orogastric administration of high dose OP in study 1 led to relatively low concentrations of BALF and plasma TNF-α and the anti-inflammatory IL-10. This differs from other causes of ARDS e.g. sepsis and trauma, which usually include raised concentrations of TNF-α and lL-10 (Suter et al., 1992). Therefore, high dose OP ingestion may create a lung injury that differs to ARDS with its own individual cytokine profile. This possibility requires further investigation in greater numbers in both animal and human studies.

Inflammatory pulmonary cytokines have not been explored in great detail for either poisoning with OP nerve agents or pesticides (see chapter 1). Therefore, this information contributes to the understanding of the indirect lung injury produced through OP pesticide ingestion.

Study limitations

In study 1, the lungs were not protectively ventilated, with use of high oxygen concentrations (F_IO_2<0.95 – previously discussed). Therefore, VILI may have contributed to the increased concentrations of protein, IL-8 and neutrophils found in the BALF (Slutsky and Ranieri, 2013). Yet, there were still increases in the BALF protein, IL-6 and IL-8 of the OP pigs compared with saline controls, indicating the possibility of another cause of lung injury within the OP group (e.g. the indirect effects of the OP).

Sequential bronchoalveolar lavage could also have caused increases in inflammatory cytokines and neutrophils within the BALF of instrumented minipigs in both studies (Vonessen et al., 1991, Terashima et al., 2001). Study 2 had no saline controls receiving bronchoscopy and lavage at – 10 min and 6 hours, and so the degree of lung injury caused by the BAL process in OP pigs could not be compared. However, the effect of recurrent lavage (and potentially VILLI) in study 1 saline control minipigs was associated with a mild increase in plasma and BALF IL-8 over 12 hours (Figure 3.2 A, C).

It is possible that the muted response observed in study 2 represents an inadequate period (six hours) for the full reaction to occur, with neutrophil pulmonary sequestration tending to peak at around 24 hours (Reutershan et al., 2005).

In combining three different study groups for data analysis, study 2 may have obscured some significant group differences. However, the only observed significant difference between groups was the PaO_2/F_IO_2 ratios over time (No NMBA vs. Pre-OP NMBA), which was not significant between groups at 6 hours (Figure 3.6). The lack of NMBA may have assisted lung ventilation (through persistent diaphragmatic activity and subsequent splinting of dependant lung areas) leading to better PaO_2/F_IO_2 ratios than the Pre-NMBA group.

3.5 Conclusion

Orogastric administration of OP created a measureable, statistically significant lung injury (as shown by histopathology) when compared with saline controls.

A consistent finding in these studies, despite confounding factors e.g. hyperoxia and VILI, and low animal numbers, was that orogastric administration of OP caused acute and indirect lung injury. This systemic injury was characterised by reduced PaO_2/F_IO_2 ratios, histopathological changes (interstitial and alveolar oedema, haemorrhage and neutrophilia), with the presence of raised concentrations of BALF protein, IL-6, IL-8 and CRP. The underlying mechanism seems to involve the pulmonary recruitment of neutrophils, perhaps in response to secreted IL-8 by the damaged alveolar epithelium and endothelium, and/or resident macrophages and monocytes (Baggiolini and Clarklewis, 1992). Systemic OP poisoning caused a predominantly local pulmonary as opposed to a systemic inflammatory reaction as concentrations of IL-6 and IL-8 were high in the BALF, but not in the plasma. This toxic systemic OP lung injury is unlike 'classic' ARDS as it displays low concentrations of BALF and plasma TNF- α with negligible IL-10 (Bhargava and Wendt, 2012).

Possible damage to the alveolar capillary membrane has been demonstrated in both study 1 and 2 OP poisoned pigs and could be responsible for the increased quantities of protein, inflammatory cytokines and oedema present in the lung parenchyma. Some authorities have suggested that increased endothelial permeability secondary to OP poisoning is due to stimulation of muscarinic receptors on C-fibers within the lung (Delaunois et al., 1994). However, the process of pulmonary inflammation secondary to OP poisoning is poorly understood.

In conclusion, orogastric placement of large volumes of OP can cause an indirect lung injury that has similarities, but some differences to mild-moderate ARDS. It has yet to be determined whether, or how, this systemic lung injury contributes to the increased mortality seen within intubated OP poisoned patients. The implications are that human OP poisoned patients should receive protective ventilation on the ICU combined with airway aspiration avoidance to prevent the aggravation of any existing indirect lung injury.

Chapter 4: Development of a pulmonary aspiration model in the Gottingen minipig

4.1 Introduction

Pigs have been used to investigate the effects of gastric contents, toxins, bacterial and viral inoculates, trauma and positive pressure ventilation on lung function (Bode et al., 2010, Forster et al., 2010, Gargani et al., 2007, Heuer et al., 2012, Inci et al., 2008, Matute-Bello et al., 2008, Meers et al., 2011a, Protti et al., 2011, Spieth et al., 2011, Fodor et al., 2014). Minipigs have been used extensively in toxicology research (Bode et al., 2010) and in our team's previous work (chapter 3). Pigs have clear advantages over smaller rodent models of lung injury, including ease of bronchoscopy, bronchoalveolar lavage (BAL) and serial lung biopsies and have many similarities to the human lung (Table 1.3) (Judge et al., 2014). Therefore, it seemed rational to use the Gottingen minipig for the main pulmonary aspiration model.

To study aspiration in pigs and obtain useful results, our team first had to conduct a pilot aspiration study (study 3) in 2010 using five minipigs.

Study 3 aimed to determine whether it was possible to: (i) create a direct and/or indirect pulmonary injury within the same animal; (ii) use computed tomography (CT) scanner to repeatedly image the lungs within an anaesthetised animal; (iii) refine the practice of conducting long clinical studies within an ICU setting; and (iv) create histopathological specimens to form the basis of a scoring system that would allow comparison of injury between lungs and models, and assist in the statistical powering of future aspiration studies. The development of the histopathology scoring system is discussed in chapter 2, with the powering of the main aspiration study in chapter 5.

Integral to this process was the development of a CT imaging analysis technique that allowed the quantitative measurement of direct and indirect lung injury through aspiration of OP+GJ. This work was conducted by myself and Mr Martin Connell (Clinical Scientist at the Clinical Research Imaging Centre, University of Edinburgh) using the software programme Analyze 10.0®. [NB. This has been described in the methods (chapter 2) as the methodology was used in more than one study.]

The lessons learned from study 3 combined with expert advice, and a methodology literature review, culminated in new techniques and protocols (e.g. protective ventilation (6-8 mL/kg) and

reduced F_IO_2) that were implemented where possible in study 2 (chapter 3) and a subsequent dose-ranging study within the same minipigs.

Study 3 used 1/5th (0.63-0.71 mL/kg dimethoate EC40) of a toxic human oral dose (Eddleston et al., 2012) to poison three minipigs, first by orogastric administration, then by pulmonary aspiration (of stomach contents – discussed below). However, only one of the three OP-poisoned minipigs in study 3 survived 48 hours, which was the intended study duration for the main pulmonary aspiration study. It was concluded that although there may have been other contributing factors (i.e. differing gastric aspiration contents), this dose was excessive for an aspiration study intended to last 48 hours.

Consequently, dose ranging studies were conducted in a further 8 minipigs which had already undergone OP poisoning with 2.5 mL/kg dimethoate EC40 by gavage in study 2. These were randomised to receive either 1/12.5th, 1/25th or 1/50th of a toxic dose (2.5 mL/kg) of dimethoate EC40 placed in a lung. These experiments allowed the cardiorespiratory effects of varying degrees of OP poisoning to be recorded and enabled a suitable choice of OP dose for the main pulmonary aspiration study.

4.1.1 Aims

- To conduct a pilot aspiration study to test the model design/techniques and explore the effects of unilateral aspiration on bilateral pulmonary function
- To provide histopathological data to assist powering of the main aspiration study
- To identify an appropriate OP dose for the main pulmonary aspiration study
- To discuss the lessons learned from these processes and how they were used to improve the main pulmonary aspiration study design.

4.2 Methods

4.2.1 Study 3 (pilot aspiration study)

The 48-hour pilot study was designed and conducted by Professor Michael Eddleston and the research team (including Dr David Dorward) in 2010 at the Moredun Research Institute, Pentlands Science Park Ltd. The study was conducted before I joined the group - I processed the data from the pilot work and used it to develop the main pulmonary aspiration model and scoring systems for histopathology and CT analysis.

4.2.1.1 Animals, anaesthesia, observations and monitoring

Five male minipigs aged 8-9 months, body mass 20.6 (± 0.96) kg, were used in the study and housed with food and water available in a similar manner to that described in chapter 2. Anaesthesia consisted of a pre-anaesthetic medication with intramuscular ketamine/midazolam with maintenance of anaesthesia achieved using the volatile agent isoflurane mixed in an oxygen/air mixture (50:50). The pigs' lungs were protectively ventilated (tidal volumes <8 mL/kg) using a Servo 300 ICU ventilator (Maquet, Sweden) and cardiorespiratory parameters were monitored as described in chapter 2.

Regular arterial and venous blood samples were taken to measure serial arterial lactate and venous AChE levels (chapter 2). Pulmonary CT scans of all the minipigs were also taken at regular intervals to allow lung volume and density analysis.

4.2.1.2 Poisoning of the minipigs

The pilot study design followed a clinically relevant model (poisoning by orogastric administration then aspiration) rather than a mechanistic one (aspiration only). Three minipigs were poisoned by orogastric administration of 0.63-0.71 mL/kg dimethoate EC40 at time 0. Thirty min later their stomach contents were aspirated using the orogastric tube and were placed into a using the working channel of a bronchoscope (VETVU VFS-2A veterinary fiberscope, Krusse, UK). Two other minipigs received a similar volume of saline by orogastric administration, with their stomach contents placed into the right lung 30 min later. This was in order to produce and observe both direct, and indirect pulmonary injuries.

The volume of stomach contents administered to the lungs of the first pig (OP+GC) was 50 mL. It was noted to contain particulate matter. After administration, the pig suddenly became very sick with poor oxygenation suggestive of severe lung injury. The animal was subsequently euthanised whilst under anaesthesia. For the remaining four pigs, a smaller volume of stomach fluid (10 mL) was used and resulted in less severe lung injury (Table 4.1).

Pig number and group	Dose given by gavage	Volume of gastric contents (GC) administered to lung
1. OP+GC	Dimethoate EC40 0.65 mL/kg (13 mL)	50mL
2. Saline+GC	Saline 0.63 mL/kg (15mL)	10mL
3. OP+GC	Dimethoate EC40 0.63 mL/kg (13mL)	10mL
4. OP+GC	Dimethoate EC40 0.71 mL/kg (15 mL)	10 mL
5. Saline + GC	Saline 0.68 mL/kg (15mL)	10mL

Table 4.1: Study 3 minipig interventions in the OP pesticide and gastric contents (GC) group and saline and GC control group. GC: gastric contents of the animals own stomach after poisoning by gavage.

4.2.1.3 CT transfer procedure and data processing

The mobile CT scanner (16 slice GE lightspeed CT scanner, GE Healthcare, Buckinghamshire, UK) was located in a specialised vehicle parked outside the laboratory. In order to be scanned, the pigs were transferred from the laboratory to the scanner, a process which involved the disconnection of the pig from the anaesthetic breathing system within the laboratory and using a self-inflating ventilation bag (Ambu-bag®) to deliver 21% oxygen (room air) via the endotracheal tube during transfer and scanning.

The transfer was performed by a veterinary anaesthetist with this procedure on their personal license. The animal was lifted by two people to the scanner with heart rate and pulse oximeter (oxygen saturations) monitoring attached. Once the scan was complete, the pig was carried back to the laboratory and placed on the original breathing system. During the CT transfer and scan, 'top-ups' of 0.5-1.0 mg/kg IV propofol anaesthetic were administered. The CT imaging was analysed as described in chapter 2.

4.2.1.4 Histopathological processing of minipig lungs

The minipig lungs were removed from the thoracic cavity at the end of the experiment and filled with 10% formalin via the trachea at a standard pressure (25 cm H_2O). Smaller sections of lung were then cut from the apical, middle and lower lung lobes and placed in formalin for further histopathological processing as detailed in chapter 2 (methods).

4.2.2 Dose ranging study

Dose ranging studies were conducted in eight minipigs that had already been poisoned with OP (2.5 mL/kg dimethoate EC40) by gavage six hours previously in study 2. These included three No NMBA, three Post-OP NMBA and two Pre-OP NMBA pigs. The minipigs were then randomised to receive either 1/12.5th, 1/25th or 1/50th of a toxic dose (2.5 mL/kg) of dimethoate EC40 made up to 0.5 mL/kg with 0.9% saline. The mixture was placed one bronchus via the working channel of a bronchoscope (VETVU VFS-2A veterinary fiberscope, Krusse, UK) at time 0 and physiological observations were recorded for 45 min. Because all the minipigs had already received a lethal dose of OP pesticide they were not expected to survive 45 min after bronchial installation.

The PaO_2/F_IO_2 ratios were chosen to measure lung oxygenation, while airway pressure and compliance were used to demonstrate mechanical lung dysfunction secondary to aspiration i.e. increased peak airway pressures combined with reduced compliance. Total respiratory system compliance [= tidal volume (expiratory)/ (Pplateau – PEEP)] was measured by the Datex Ohmeda S/5 M-CAiOV module. Alveolar dead space (the volume of lung being ventilated but not perfused) was estimated using the formula: [(arterial $PaCO_2$-end tidal CO_2) /arterial $PaCO_2$] x tidal volume. It was expected that this would increase in the presence of lung injury (Nuckton et al., 2002).

The anaesthesia and monitoring from study 2 were similar to that described in chapters 2 and 3.

4.2.2.1 Electron microscopy images of direct and indirectly-injured lung

Small lung samples (1-2 mm^3 of both direct and indirect lung injury) from minipigs that were involved in both study 2 and dose ranging studies (n=6) were sent to Mrs Tracey Davey for tissue processing and electron microscopy at Newcastle University (see methods chapter 2).

4.3 Results

4.3.1 Study 3 (pilot aspiration study)

Study duration was 2, 35 and 48 hours for the three OP+GC pigs and 46 and 48 hours for the two saline+GC control pigs. The volume of stomach contents administered to the lungs in the first pig (OP+GC) was 50 mL. The condition of this pig deteriorated acutely after treatment with poor oxygenation suggestive of severe lung injury. The animal was subsequently euthanised. The volume of stomach fluid was reduced for the subsequent pigs which reduced the severity of injury.

4.3.1.1 AChE inhibition in OP+GC pigs

OP poisoning was confirmed by the fall in normalised activity of AChE relative to haemoglobin concentration over time (Figure 4.1). Most (OP+GC) minipigs had complete AChE inhibition 4 hours after orogastric OP administration.

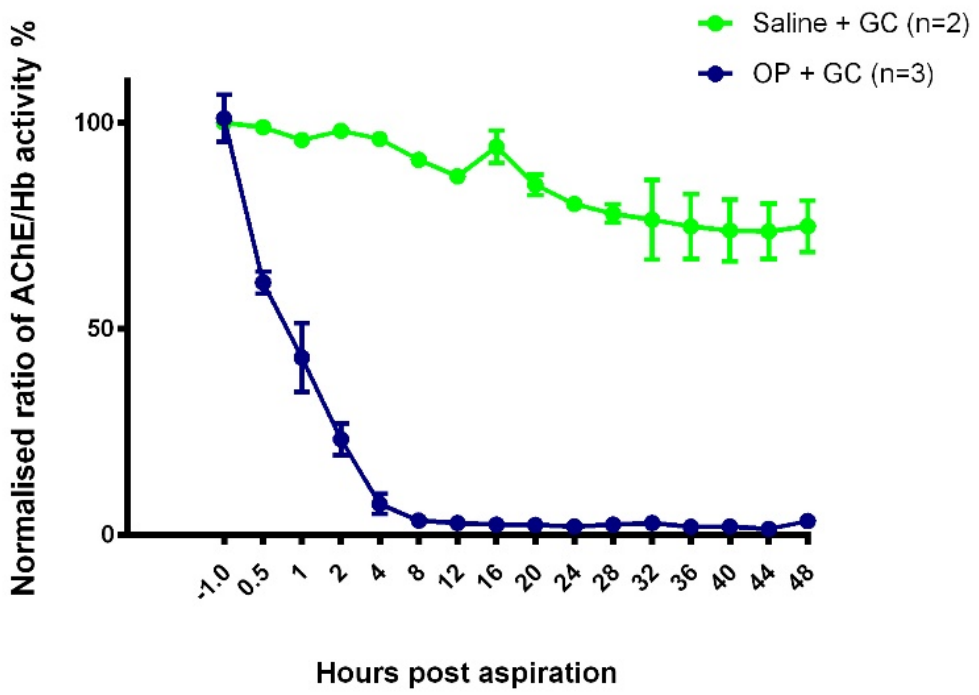


Figure 4.1: Normalised ratio of AChE/Hb activity over time. Plots show the mean and SD bars. AChE: acetylcholinesterase, Hb: haemoglobin.

4.3.1.2 Physiological observations

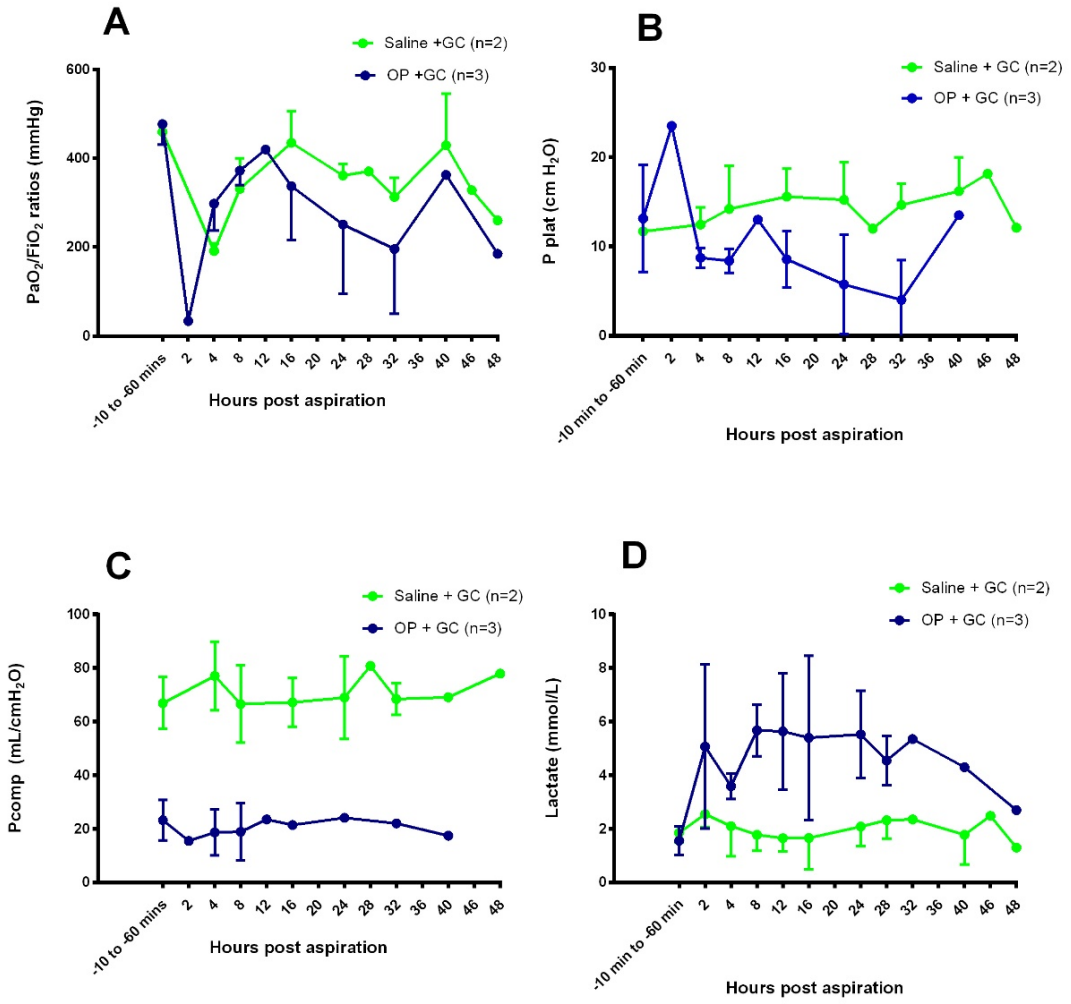


Figure 4.2: Study 3 physiological data: (A) PaO_2/F_IO_2 ratio (mmHg) as a measure of oxygenation, (B) plateau airway pressure (Pplat, cmH_2O), (C) pulmonary compliance (Pcomp, mL/cm H_2O) and (D) arterial blood lactate concentration (mmol/L). Data were collected initially from between -10 and -60 min before the start and up to 48 hours after the experiment. Note one OP+GC pig died at two and the other at 35 hours. Mean and SD shown.

Both groups had results consistent with lung injury with OP+GC having the lowest PaO_2/F_IO_2 ratios over time (Figure 4.2A). The mean PaO_2/F_IO_2 values were also lower in the OP+GC group than the saline+GC group (293 mmHg vs. 348 mmHg) (Table 4.2). Plateau airway pressures (Pplat - Figure 4.2B) peaked at two hours after OP+GC aspiration injury and then remained greater in the saline+GC. Of note, the saline+GC pigs received larger tidal volumes than the OP+GC pigs and this could explain the increased Pplat in saline+GC pigs (Table 4.2). However

the Pplat did not exceed 25cmH_2O in any pigs indicating an apparent successful protective ventilation strategy in both groups.

Measured variable	OP+GC (n=3) Mean(±SD)	Saline+GC (n=2) Mean (±SD)
PaO_2/F_IO_2 ratio [mmHg]	293(131)	348(83)
Plateau airway pressure [cmH_2O]	11(5.8)	14.2(2.1)
Compliance [mL/ cmH_2O]	20.5(3.0)	71.4(5.5)
Tidal volume [mL]	188(63)	921(76)
Arterial lactate [mmol/L]	4.5(2.0)	2(0.6)
Mean arterial pressure [mmHg]	63.4(6.7)	68(9.4)
Norepinephrine - 40mcg/mL concentration [mL/hr]	10.6(12)	2.4(2.3)
Norepinephrine [mcg/kg/hr]*	20.9	4.6

Table 4.2: Study 3 (pilot aspiration study) measured physiological variables, biochemical and norepinephrine use in minipigs over the duration of the study.* Data based on mean values of animal group -weight and mL/hr norepinephrine infusion. Data are presented as mean (measured variables over study duration) and (SD) shown.

The respiratory system compliance was greater within the saline+GC group when compared with the OP+GC group throughout the experiment, possibly indicating better lung function (Figure 4.2C). However, equipment measurement errors may have been responsible for the observed differences in tidal volume and compliance (see discussion).

The mean blood lactate concentration over the study duration was greater in the OP+GC group than the saline+GC group (4.5 mmol/L vs. 2 mmol/L)indicating greater lactate production (secondary to tissue hypoxia or catecholamine driven glycolysis) or decreased removal from the blood (predominantly hepatic) (Figure 4.2D; Table 4.2).

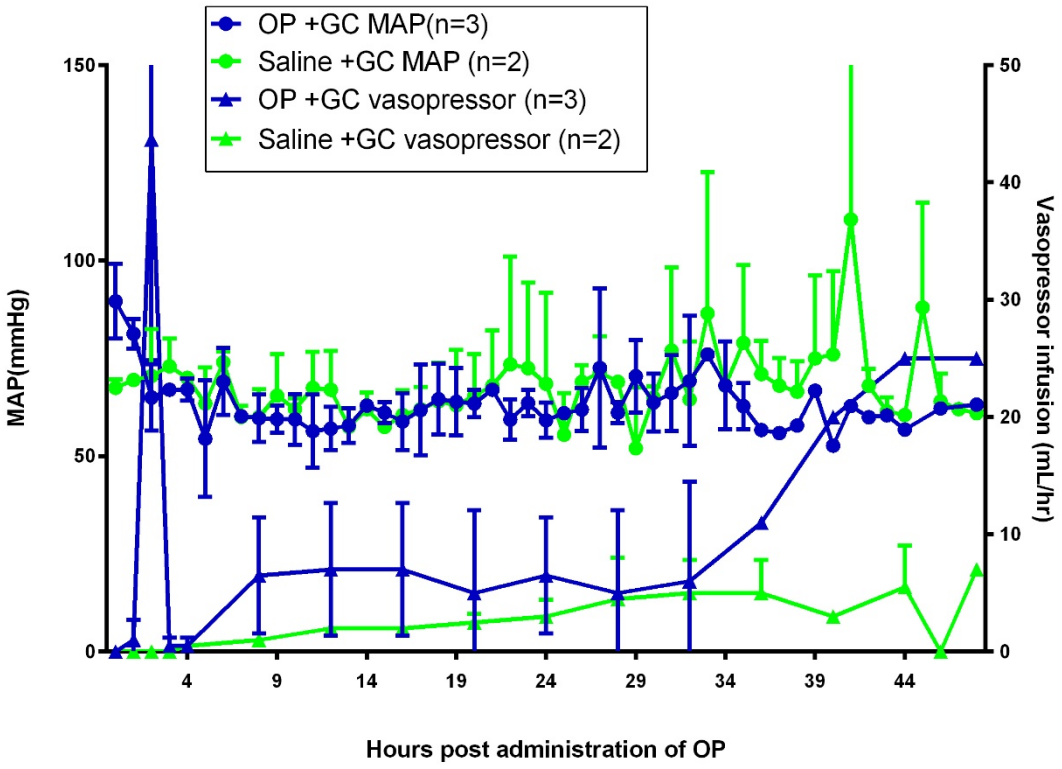


Figure 4.3: Graph depicting the mean arterial pressure (MAP) of pigs [left y-axis] and the corresponding infusion rate of norepinephrine (40mcg/mL) mL/hr [right y-axis]. Note that the OP+GC pigs survived two, 35 and 48 hours. Mean and SD shown.

To determine whether hypotension (and thereby potential tissue hypoxia) or the vasopressor infusion caused the increased lactate concentration in the OP+GC pigs we measured the MAP and norepinephrine use in the pigs. The mean MAP for the duration of the study was similar in both groups (63 mmHg OP+GC vs. 68mmHg saline+GC) with an increased use of norepinephrine in the OP+GC group at the beginning and towards the end of the experiment in one pig (Table 4.2). This suggested a degree of hypotension within the OP+GC group at either end of the experiment. After 32 hours OP+GC lactate concentrations started to fall in the remaining OP pig which also saw a rise in the norepinephrine infusion rate (Figure 4.2D; Figure 4.3). This suggests that the high lactate levels in this pig were not linked to norepinephrine use. However mean use of norepinephrine in each group was 10.6(±12) mL/hr in OP+GC pigs vs 2.4(±2.3) mL/hr in saline+GC pigs and therefore may still have contributed to an increase in the lactate concentration of the OP+GC group overall.

4.3.1.3 Computed tomography analysis

Lung density analysis of CT data from a representative OP+GC pig created a visual time sequence of aspiration which showed an increase in lung density over time in the direct (right – green) and to a lesser extent in the indirectly (left – red) injured lungs (Figure 4.4).

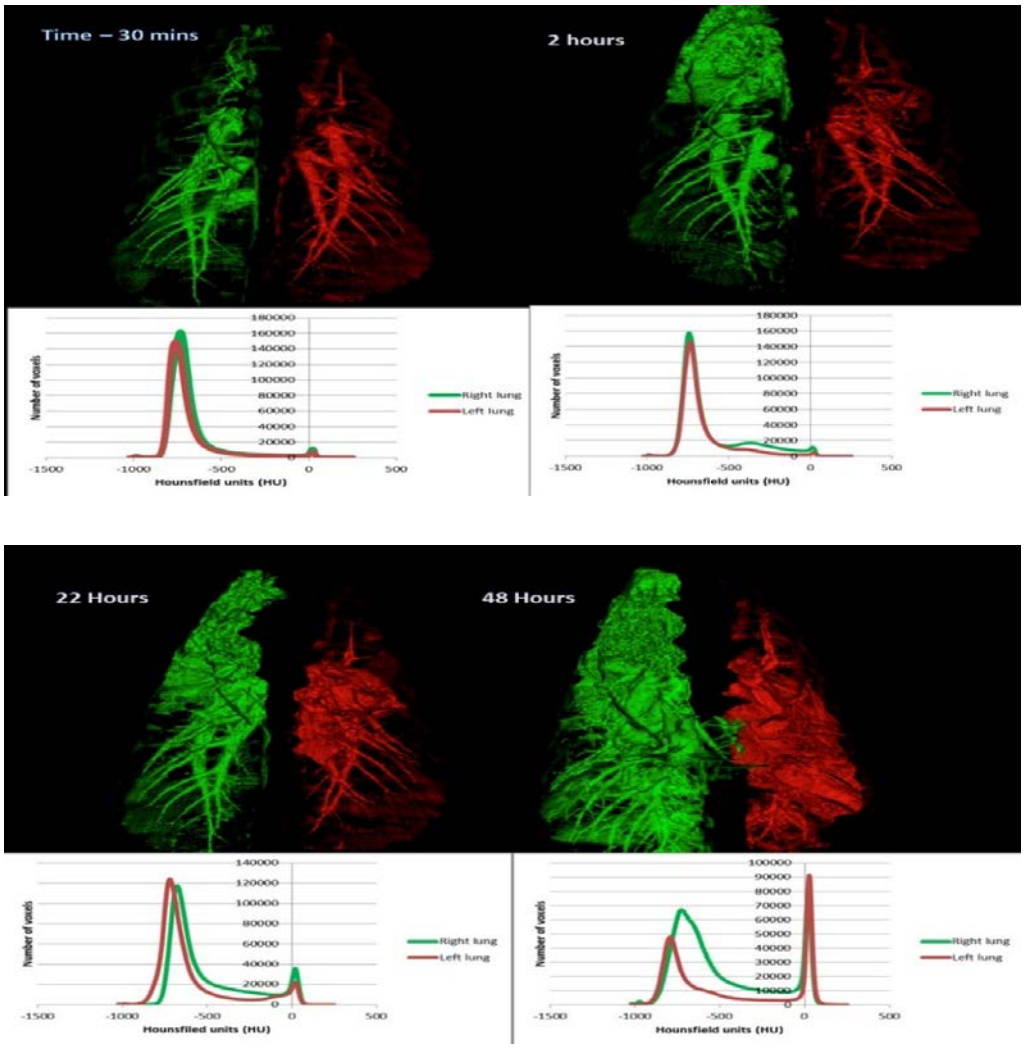


Figure 4.4: Timeline (-30 min to 48 hours) showing the computed tomography (CT) representation of the changes in the density of voxels in a minipig poisoned by orogastric administration of OP with subsequent aspiration of gastric contents and placement into the right lung to simulate human OP pesticide poisoning. The pictures represent areas of the right (green) and left (red) lungs that have poorly or non-aerated lung tissue (-250 to +250HU) which includes fluid, haemorrhage and/or consolidation. The injury is initially created in the right apical lung at two hours and then extends to the lower right and left lungs by 48 hours. Note y axis variation as each 3D lung map has a variable number of voxels dependent on the voxel and lung size within each CT lung scan.

Lung damage can be compared within groups by analysing CT scans at similar time points in different minipigs and calculating percentages of lung tissue (voxel percentage) displaying over distended, normally aerated, poorly and non-aerated lung tissue (Heuer et al., 2012).

As part of the CT data analysis the first CT scan was checked to confirm into which lung (right or left) the aspiration mixture (OP+GC or saline+GC) had been placed (Table 4.3).

Order of pigs	Group	Side of lung aspiration
1	OP+GC	Right lung
2	Saline+GC	Left lung
3	OP+GC	Left lung
4	OP+GC	Right lung
5	Saline + GC	Right lung

Table 4.3: Laterality of aspiration lung injury in study 3. OP: organophosphorus pesticide, GC: gastric contents.

Data from the CT scans taken between 24-27 hours showed that placement of either OP+GC or saline+GC in the lung created a direct and indirect lung injury (example shown in Figure 4.4). The percentage difference of poorly aerated lung tissue in the directly-injured vs. indirectly-injured lung was greater in the OP+GC group than the saline+GC group: OP+GC mean (SD) direct 38 (±21) % vs. indirect 19 (±11) % with saline+GC mean (SD) direct 27 (±13) % vs. indirect 26 (±22) % (Table 4.4; Figure 4.5). The similar extent of injury in both the saline+GC right and left lungs may have occurred through accidental spillage of GC into both lungs e.g. during CT transfer.

Group	Direct lung injury % normal lung aeration (-900 to -500HU)	Direct lung injury % poorly aerated lung tissue (-500 to -100HU)	Indirect lung injury % poorly aerated lung tissue (-500 to -100HU)
Saline+GC	56 (±5)	27 (±13)	26 (±22)
OP+GC	44 (±31)	38 (±21)	19 (±11|)

Table 4.4: CT findings at 24-27 hours detailing the percentage of poorly and normally aerated lung tissue in direct lung injury with either Saline+GC or OP+GC. Mean (SD) shown.

Although both groups had similar percentage aeration in the indirectly-injured lungs, in the directly-injured lungs OP+GC had more poorly aerated lung tissue mean (SD) 38(±21)% vs. 27(±13)% and less normal aerated lung tissue 44(±31)% vs. 56(±5)% than the saline+GC pigs suggesting a greater degree of lung injury (Table 4.4).

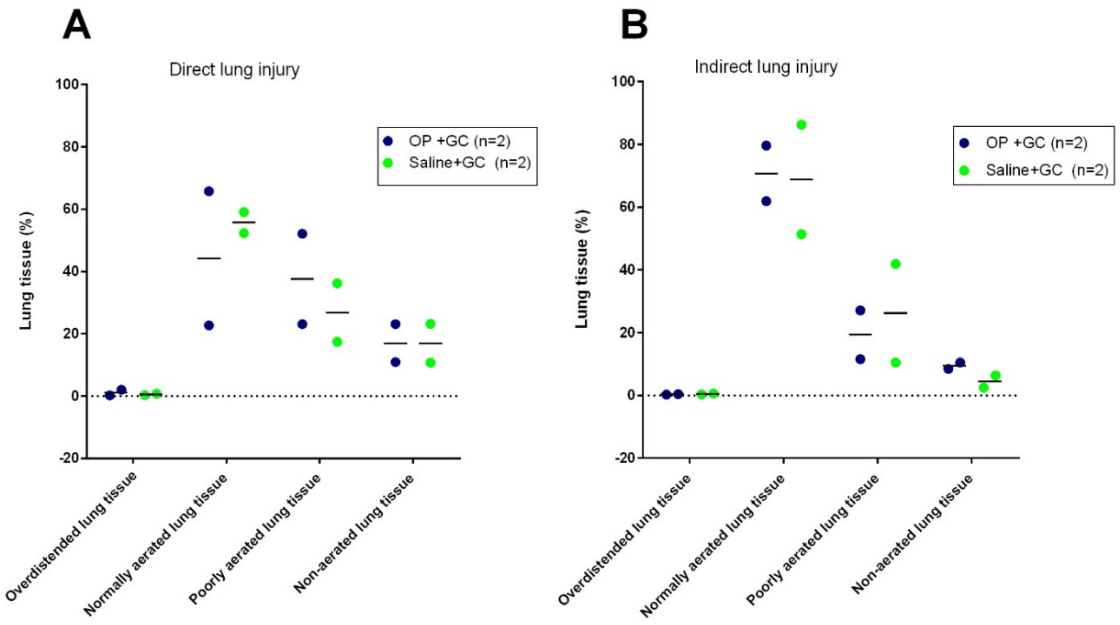


Figure 4.5: Dot plot showing the variation in aeration of lung tissue (%) as determined by analysis of CT images taken from minipig lungs given either OP+GC (n=2) or saline+GC (n=2) into the directly-injured lung. (A) shows the direct and (B) the indirect lung injuries caused 24-27 hours after poisoning. The OP+GC directly-injured pig lungs had less normally aerated lung tissue and increased poorly aerated lung tissue when compared with Saline+GC pigs. The OP+GC pigs also had an increase in the percentage of poorly aerated lung tissue in the directly-injured lung when compared with the indirectly-injured lung, unlike the Saline+GC pigs.The bar denotes the mean. Overdistended lung: -1000 to -900HU, normally aerated lung: -900 to -500HU, Poorly aerated lung: -500 to -100 HU, Non-aerated lung: -100 to +250HU.

4.3.1.4 Histopathological analysis

Bilateral lung samples from cranial, caudal and middle areas (some right lungs only) were examined in all study 3 pigs giving four to five samples per pig (OP+GC; n= 13, saline+GC; n= 8). Both SS and WW scored all these samples using the histopathological scoring system 1.0 (Figure 4.6).

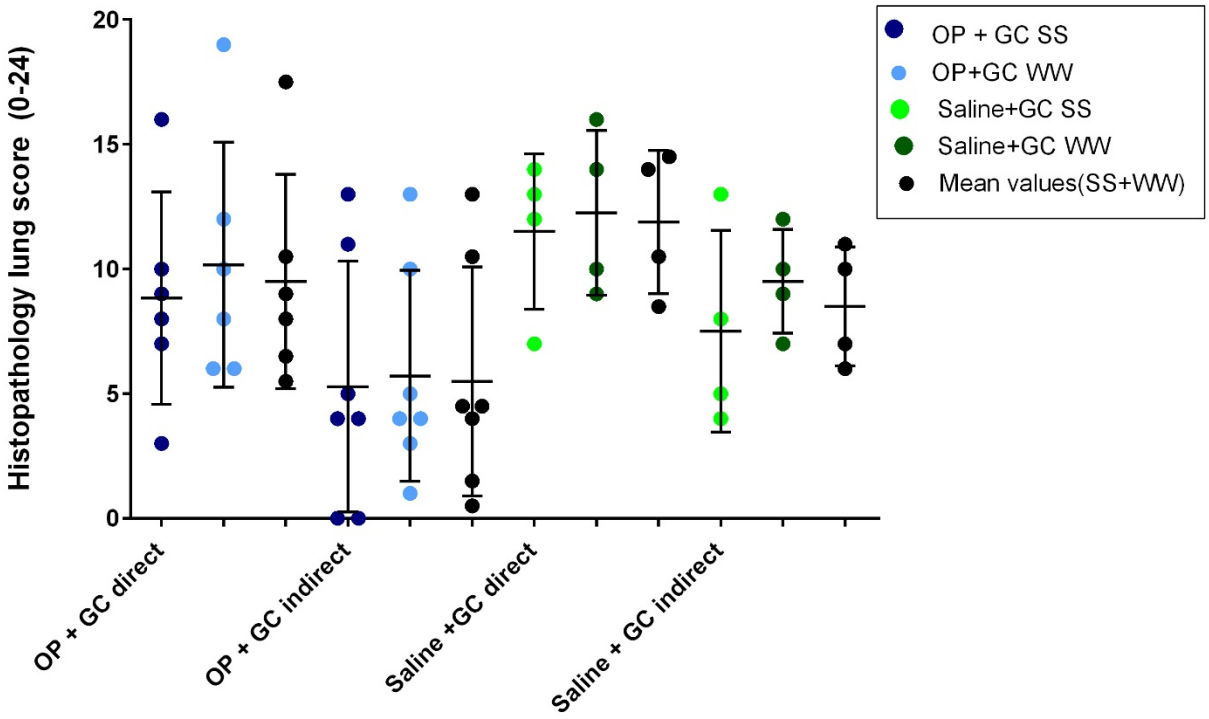


Figure 4.6: Dot plot showing the histopathology scoring for study 3 in direct and indirectly-injured minipig lungs using the 1.0 system (maximum score: 24 points per sample), mean and SD shown. Scores from both SS and WW and mean scores (black) are shown for comparison. Kruskal-Wallis analysis and permutation testing using score means between groups showed differences were non-significant. Mean and SD shown. GC: gastric contents.

Direct lung injuries from either OP+GC, or saline+GC scored higher than their indirect injuries, though this was not statistically significant (Figure 4.6). However, saline+GC pigs had non-significantly greater scores than OP+GC pigs for both direct (11.9 (±2.9) vs. 9.5(±4.3)) and indirect (8.5(±2.4) vs. 5.5(±4.6)) lung injuries, respectively.

4.3.2 Dose range study results

Most (6 out of 8) of the minipigs survived for the 45 min following the second OP aspiration poisoning. Two of the three pigs that had the highest dose (1/12.5th of a lethal OP dose) died by 15 min, leaving one pig to complete the full 45 min study.

4.3.2.1 Aspiration of OP creates a dose response effect on airway physiology

The mean PaO_2/F_IO_2 ratios of all pigs at the start of the dose ranging experiments exceeded 300mmHg. By the end of the experiment the 1/12.5th pig and 1/25th group had developed moderate ARDS and 1/50th group mild ARDS (Table 4.5).

Time	1/12.5th OP dose PaO_2/F_IO_2 ratio mmHg mean (SD)	1/25th OP dose PaO_2/F_IO_2 ratio mmHg mean (SD)	1/50th OP dose PaO_2/F_IO_2 ratio mmHg mean (SD)
-30 min before dose ranging studies (end of study 2)	333(84)	383(96)	320(105)
45 min after lung installation of OP	126	181(45)	241(51)
Mean ratio over 45 min	177(104)	240(96)	261(40)

Table 4.5: PaO_2/F_IO_2 ratios in poisoned minipigs (dimethoate EC40 2.5 mL/kg by gavage) that also then received either 1/12.5th, 1/25th or 1/50th of a lethal OP dose (2.5 mL/Kg dimethoate EC40) mixed with 0.9% saline to 0.5 mL/kg and placed in the right lung. The -30 min PaO_2/F_IO_2 ratios were taken at the end of study 2 and before the dose ranging studies. Mean and (SD) shown.

There was a convincing dose-response effect to placement of intrapulmonary OP and saline mixtures at time 0, with the most detrimental effects occurring in the 1/12.5th OP dose group. When compared with the least injurious 1/50th dose group, the 1/12.5th group produced the greatest alveolar dead space (39(±11) mL vs. 23(±17) mL) and peak airway pressures (17(±5) cmH_20 vs. 14(±5) cmH_20) with the lowest airway compliance (7.5(±2.6) mL/cmH_20 vs. 13.5(±2.0) mL/cmH_20) and PaO_2/F_IO_2 ratios (131(±79) mmHg vs. 241(±30) mmHg) 15 min after poisoning (Figure 4.7).

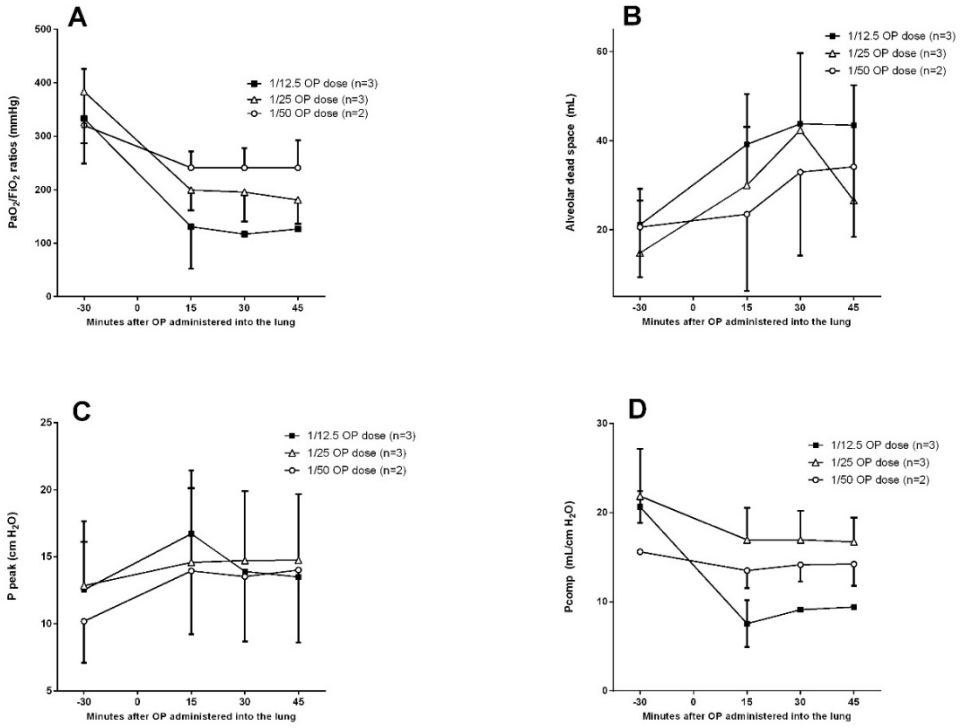


Figure 4.7: Pulmonary physiological responses to the dose ranging OP aspiration studies showing:(A) the PaO_2/F_IO_2 ratio, (B) the alveolar dead space [($PaCO_2$-end tidal CO_2)/$PaCO_2$ x tidal volume], (C) the peak pulmonary pressure measured within the airway (Ppeak in cm H_2O), (D) the static pulmonary compliance (Pcomp in mL/cmH_2O). The time -30 min figures are the last readings from the previous study 2. This was followed by a 30 min pause before starting the dosing study. Mean and SD shown.

4.3.2.2 Orogastric OP placement combined with additional indirect or direct lung injury with OP causes damage to the alveolar capillary membrane

EM images taken from minipigs exposed first to orogastric 2.5 mL/kg dimethoate EC40 and then a saline mixture (0.5 mL/kg) of 1/25th a normal toxic dimethoate EC40 dose into a lung showed evidence of both direct and indirect lung injury. There was evidence of swelling and damage to the alveolar capillary membrane which was floating in the alveolar space in both the direct and indirectly-injured lungs, with the additional presence of erythrocytes and fibrin deposition in the alveolar spaces of the directly-injured lung (Figure 4.8 A, E and F). Fibrin deposition is a complication of the acute phase of ARDS (Ware and Matthay, 2000). Direct lung injury, including damage to the alveolar capillary membrane, was also seen after placing smaller dimethoate EC40 (1/50th lethal dose) doses into the right lung (Figure 4.8 B).

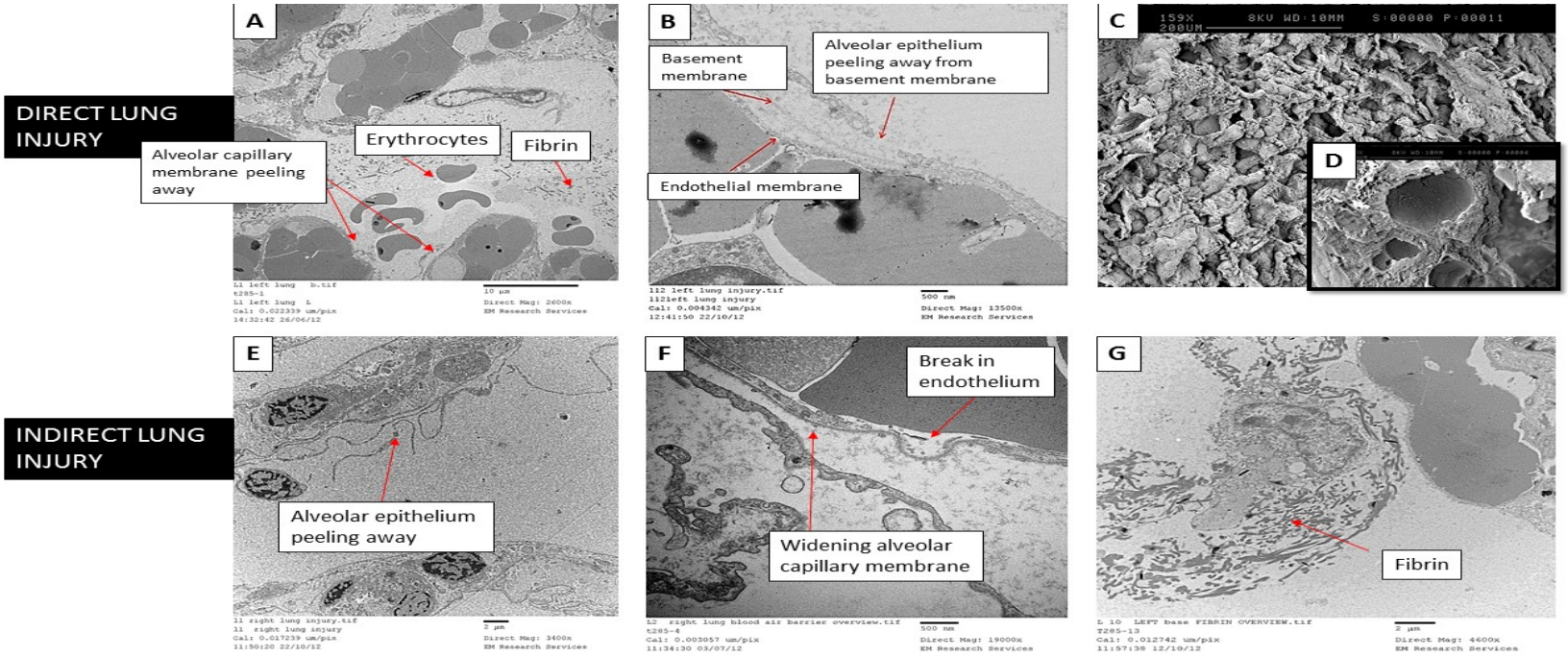


Figure 4.8: Effects of direct and indirect organophosphorus (OP) on minipig lungs. Comparison of lung architecture in anaesthetised minipigs 7 hours after orogastric placement of 2.5 mL/kg dimethoate EC40 and subsequent installation of dimethoate saline solutions [1/12.5th (C and D), 1/25th (A) or 1/50th (B) of lethal 2.5 mL/kg dose of dimethoate EC40 made up to 0.5 mL/kg with saline for pulmonary administration] into a lung (direct injury; A, B, C and D) with the contralateral lung showing further systemic injury (indirect lung injury; E, F, and G). Indirectly-injured lungs were are seen in Images E and F where animals had a 1/25th and image G a 1/12.5th of a lethal 2.5 mL/kg dose of dimethoate EC40 made up to 0.5 mL/kg with saline for pulmonary administration. Images C and D show scanning electron microscopy images (original magnification: x159–635) of lungs that had had 1/12.5th normal toxic dose placed in the lung. Direct injury shows destruction of the alveolar capillary framework, with fibrin mesh and clot formation within the alveoli. The alveoli display ragged edges and increased thickening of the alveolar capillary membrane (D) in contrast to normal lung architecture (see chapter 5 EM pictures of controls). Transmission electron microscopy images (original magnification: x2600-19,000) of directly-injured (A and B) and indirectly-injured (E, F and G) lungs. Both indirect and direct injury cause alveolar capillary membrane swelling which has led to the alveolar epithelium peeling away into the alveolar space with fibrin deposition in and around the alveolar capillary membrane. Free erythrocytes are observed in image A from a possible break in the endothelium. All images were edited in PowerPoint, Microsoft 2010.

4.4 Discussion

During the pilot study, the cardiorespiratory effects of OP+GC vs. saline+GC aspiration were examined in the minipig aspiration model. The technical aspects of the model design were also assessed and are discussed below.

The pilot study generated histopathology scores which helped to produce a specific, repeatable pulmonary histopathological scoring system for OP poisoning and aspiration and provided data to power the main aspiration study. An appropriate dose of OP pesticide (dimethoate EC40) for pulmonary installation in the main aspiration study was also identified.

Study 3 – pilot aspiration study

In study 3, direct and indirect pulmonary aspiration injury was created using both OP+GC and saline+GC in pigs and was measured using physiological and histopathological outcomes, and clinical imaging.

Evidence of acute lung injury (PaO_2/F_IO_2 <300mmHg) was present in both the OP+GC and saline+GC pigs at various time points. Although the PaO_2/F_IO_2 ratios were generally lower in the OP+GC group, the mean values were not significantly different, indicating comparable injuries.

Plateau airway pressures (P plat) remained <25cmH_20 in both groups indicating that protective ventilation was maintained throughout the experiment. However Pplat was unexpectedly greater in the saline+GC than in the OP+GC group. OP poisoning in humans would usually increase Pplat due to bronchospasm, bronchorrhoea and increases in alveolar fluid. The data (Table 4.2) indicated that the saline+GC pigs received very large tidal volumes compared with OP+GC pigs and so increases in Pplat may have been due to increased distension of the lung in the saline+GC pigs. Alternatively, the gastric juice component may have been more harmful (e.g. contained more particulate matter) in the saline+GC group, creating greater lung dysfunction and inflation pressures (Meers et al., 2011a).

Compliance in the OP+GC pigs was one third of that in the saline+GC pigs. This may have resulted from bronchospasm and oedematous changes secondary to the cholinergic effects of OP poisoning. However, this does not explain the low compliance before the experiment began

in the OP+GC pigs. The discrepancies in group tidal volumes could account for the increased compliance seen in the saline+GC pigs, but would mean they had been receiving a V_T equalling 45 mL/kg which would have caused significant barotrauma. A more likely explanation is that the saline+GC pigs may have had an adult, rather than paediatric, spirometry sensor in the ventilation circuit, which when processed by the data collection monitor (Datex Ohmeda S/5 M-CAiOV module) in paediatric mode could have calculated large inaccurate V_T measurements (e.g. 900 mL instead of 240 mL-data based on an experiment performed by Professor E Clutton). This is probably the reason for abnormal compliance measurements.

Differing respiratory variables may also originate in the model itself. Porcine respiratory mechanics are different to humans. For example, air space collapse occurs more easily in pig lungs than humans at the end of expiration, demonstrating different elastic lung mechanics (De Robertis et al., 2001). OP-induced bronchospasm and bronchorrhoea/alveolar oedema in response to cholinergic overstimulation may also be different in pigs compared with humans, although the extent of this is unknown.

Normal venous lactate values in a Gottingen minipig range between 1 and 2.4 mmol/L (Maleck et al., 2002), and 1.2 (±1.2) mmol/L (median and IQR) in commercial pigs (Hofmaier et al., 2013). In the current study the mean blood lactate concentration for the duration of the study was greater in the OP+GC, 4.47 (±1.96) mmol/L, than the saline+GC group, 2 (±0.63) mmol/L), indicating increased production (secondary to tissue hypoxia or catecholamine driven glycolysis) or decreased (hepatic) removal from the blood. The MAP was similar between groups but norepinephrine use in the OP+GC group (21 mcg/kg/hr) was greater than in the saline+GC group (5 mcg/kg/hr). Raised lactate concentrations with elevated vasopressor requirements may have occurred in response to OP-induced hypotension with subsequent tissue hypoxia (Davies et al., 2008). Yet, elevated lactate levels secondary to vasoconstriction of peripheral vessels and subsequent tissue hypoxia and/or catecholamine driven glycolysis can also occur with norepinephrine infusions themselves (Lundholm, 1950).

However, the OP+GC pig that possessed increased norepinephrine requirements (the only OP+GC pig that survived until 48 hours) displayed a simultaneous lowering of its arterial lactate concentration (Figure 4.2; Figure 4.3), perhaps through reduced tissue hypoxia or increased removal from the blood.

Visual examination of the CT scans suggested that a direct and indirect lung injury was created after pulmonary installation of either the OP+GC or saline+GC mixtures (Figure 4.4). However, it was recognised that some of the pulmonary mixture may have splashed between both lungs, especially during movement of the animals to the CT scanner. Proof that this might have occurred was shown in the quantitative CT analysis which revealed that the saline+GC group had similar percentages of poorly aerated lung tissue in both the direct and indirectly-injured lungs (27% vs 26%). However, this was not the case with the OP+GC group (38% vs 19%), suggesting that this was not a universal problem.

Direct lung injury through aspiration of GC mixtures was greater in the OP+GC group than the saline+GC group (38% vs. 27% poorly aerated lung tissue) potentially indicating additional lung injury caused by aspirated OP. Paradoxically, aspirated saline+GC created an equal (if not worse) histopathological lung injury compared with OP+GC, which suggests the pulmonary injury came from the aspiration of the gastric contents, and not the OP. However, 2/3 pigs receiving OP+GC did not survive until 48 hours, at which point, their histopathology score might have been greater.

Other confounding factors included: (i) different lung installation volumes - the first pig had 50mL, (rather than 10mL) resulting in euthanasia of the animal at 2 hours (ii) the GC was not standardised with respect to pH (James et al., 1984), the presence of particulates (Knight et al., 1993) or microbial gastric matter (Bregeon et al., 2008), all of which affect the severity of aspirational lung injury.

Pulmonary aspiration dose ranging study

The minipigs showed a dose-response effect to the pulmonary aspiration of mixtures containing 0.9% saline and dimethoate EC40. The 1/12.5th dose resulted in only one of three pigs surviving past 15 min after OP aspiration, suggesting that the dose may have been excessive. Whereas, the 1/50th dose caused only mild ARDS despite previous high-dose OP poisoning, suggesting this dose was too low.

Therefore the 1/25th dose (0.1ml/kg dimethoate EC40) was chosen as the pigs receiving this all survived to 45 min with development of a moderate ARDS.

EM images from study 2, albeit after a second OP poisoning (variable) dose, showed direct and indirect lung injury with swelling and breakage of the alveolar capillary membrane with fibrin

deposition (Figure 4.8 E, F and G). The presence of fibrin in the airways could indicate the development of an ARDS-like injury secondary to systemic OP poisoning (Bastarache, 2009).

The greatest limitation of this study was that the minipigs had already been poisoned with high-dose orogastric OP (2.5 mL/Kg dimethoate EC40) and subjected to bronchoscopy and lavage from the earlier study (study 2) creating a background lung injury. This makes it difficult to apply causation of lung injury to any one factor (e.g. Indirect or direct OP toxicity, saline bronchoalveolar lavage, or dose ranging study). However, the aim of the dose ranging study – to find an appropriate dose for the main aspiration study – was achieved.

Aspiration model development

Study 3 and the dose ranging study highlighted that changes to the model were required for the main aspiration study. This led to further reading of the literature, and the engagement of specialists in: intensive/respiratory care and transfer medicine, specialised airway equipment and the maintenance of general medical equipment. Important areas that required development are described below (Table 4.6;Table 4.7).

Key development areas for the main aspiration study
The creation of a more reliable unilateral lung injury without contamination of the contralateral lung (Figure 4.9)
The decision to use a pure pulmonary aspiration model vs. systemic poisoning and aspiration
A method for standardising the GJ mix for aspiration (Table 4.7)
Provision of a higher quality (human standard) ICU care (Table 4.7)
Provision of an improved transfer system for the animals to and from the CT scanner (Figure 4.10).

Table 4.6: Key development areas for the main aspiration study

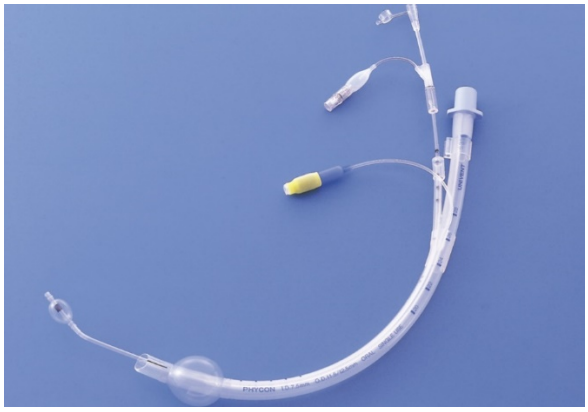


Figure 4.9: A torque-controlled blocker (TCB) Univent endotracheal tube (Fujisystems, Japan). This specialised endotracheal tube (ETT) ventilates the trachea as a normal ETT, but has a second lumen within the ETT that houses an extendable bronchial blocker. The bronchial blocker can be advanced into the lung that is to be protected (left lung in the aspiration studies) followed by the inflation of a balloon within that bronchus. This afforded some protection of the left lung during administration of the OP and GJ mixture into the right lung via a bronchoscopy.

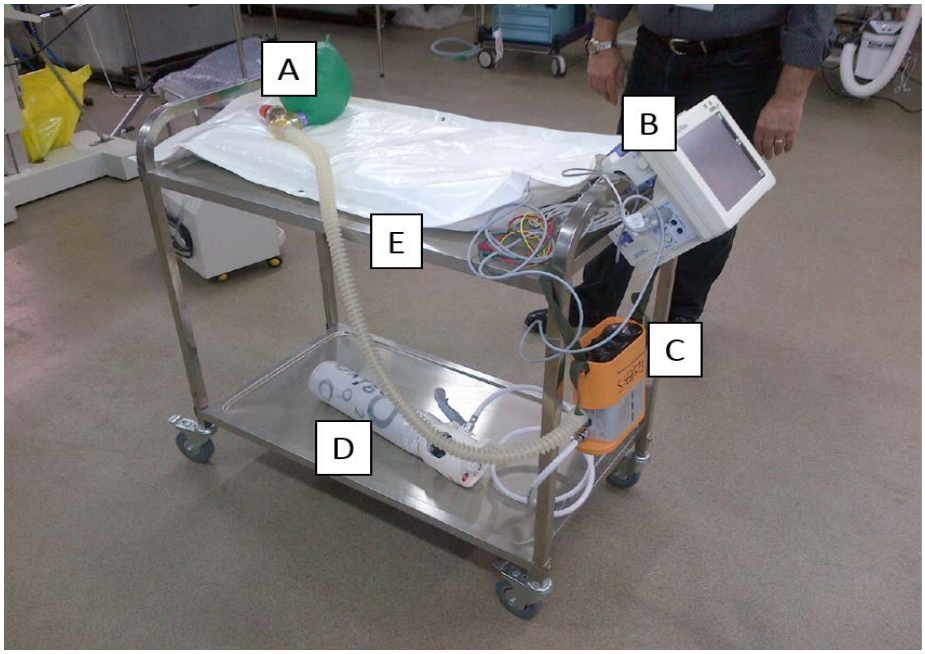


Figure 4.10: The CT transfer trolley for the Gottingen minipig was specifically designed to allow simple transfer from the ICU bay to the CT scanner located in the vehicle parked outside. Improvements included an oxylog® 1000 portable ventilator (C), combined with a calibrated positive end expiratory pressure (PEEP) valve (A) that would allow the lungs to be scanned at a constant airway pressure in every animal. A monitor (B) that displayed the animal's blood oxygen saturations (saturations probe used on tongue), electrocardiograph and invasively measured blood pressure which gave an audible alarm when values fell below acceptable levels. The use of lightweight aluminium oxygen

cylinders (D) allowed safe delivery of oxygen concentrations up to 100% whilst the trolley and specialised carry mat (E) allowed the minipig to be transferred safely and comfortably to the CT scanner with minimal movement of the lung contents. These measures also protected the researchers from significant ergonomic stress when moving the equipment and/or animal. The intravenous pumps for the anaesthetic agents are not shown in this diagram, but allowed the constant delivery of anaesthetics to the animal throughout the transfer and scanning process.

The use of a pure aspiration study, as opposed to OP poisoning by orogastric administration followed by aspiration, would remove the variable gastric contents composition e.g. differing volumes, pH, particulate composition. It would also change the nature of the control animals e.g. intrapulmonary placement of saline alone, vs. Saline+GC. This was important as the main aspiration study was powered using control minipig lung histopathology changes secondary to orogastric saline, and not to aspiration of saline+GC.

Lung isolation during installation of the OP ± GC, and using a standardised gastric juice mix were introduced to improve the studies accuracy (in placing the mixture in one lung only) and repeatability. The calibration and servicing of key ICU equipment and standardisation of monitor settings were also seen as vital to reduce measurement error. Other improvements made to the model are described below (Table 4.7).

Process	Old technique	Proposed new technique	Reason for improvement
Anaesthetic	Volatile agent e.g. isoflurane	Total intravenously controlled anaesthetic (TIVA)(Martin-Cancho et al., 2004)	• To allow a smooth anaesthetic akin to normal ICU practice • To allow uncomplicated CT transfers with IV anaesthetic in place • Avoid the need for other methods of volatile gas scavenging. • Isoflurane has an inhibitory effect on AChE (Dorandeu et al., 2007) and anti-inflammatory effect on the lung (Faller et al., 2012). Other volatiles also have an anti-inflammatory role (Müller-Edenborn et al., 2014).
Machine servicing and calibration	As required basis	All anaesthetic apparatus e.g. SERVO ventilator, Datex monitor and respiratory module, portable monitors, valves etc. to be checked, serviced and calibrated if required. Standardised paediatric settings for all monitors when collecting data for the minipigs.	• To improve accuracy of data collected • All animals to be placed on paediatric settings to avoid discrepancies of tidal volume and compliance measurements.
CT transfer (Meers et al., 2011a)	Lift pig manually to scanner and ventilate using an Ambu bag for ventilation (room air 21% oxygen)	To use a transfer trolley with monitoring, ventilation (up to 100% O_2) and TIVA (Figure 4.10) Use of a positive end expiratory pressure (PEEP) non-disposable valve (Drager, Germany) within the portable respiration circuit.	• To allow the safe transfer of the minipig with monitoring, anaesthetic and a method of delivering more than 21% oxygen. • A stable transfer will reduce likelihood of aspirated contents moving from the directly-injured (right) to indirectly-injured (left) lungs. • A trolley can improve ergonomic conditions for staff lifting (30kg) animals • The PEEP valve allows the CT lung scan of each animal to occur at a standardised airway pressure of 5cm H_20.
Unilateral (direct) lung injury	Placement of a bronchoscope (VETVU VFS-2A veterinary fiberscope, Krusse, UK) in the right lung	Placement of a video bronchoscope in right lower lung and temporary use of	• Using a video bronchoscope (BRS-5000, Vision Sciences, USA) with sterile sheath (endosheath, Vision Sciences) and working channel (2.1mm) will allow the research team to: observe placement of the bronchoscope and epidural catheter, reduce the

	and administering the minipig stomach contents down the working channel.	a TCB uninvent bronchial blocker in the left lung. Placement of an epidural catheter down the working channel of the sterile sheath and advancing into lower right lung Use of larger (30kg) minipigs to allow the easier placement of the bronchoscope and working channel for lavage and biopsy.	introduction of bacteria into the lower respiratory tract and allow regular sterile BAL and lung biopsies. • Torque controlled blocker (TCB) and Univent ETT allows use of a normal endotracheal tube in the beginning, and bronchoscope placement of the bronchial blocker in the indirectly-injured (left) lung during the placement of the aspiration mix at the beginning of the experiments. • The epidural catheter placed in the working channel will allow the administration of the OP + GJ mix at the lowest areas of the right lower lung, hopefully preventing wash back into the left lung on eventual bronchial blocker removal and subsequent turning and movement for CT scans.
Poisoning methodology and standardised gastric juice	Poisoning with dimethoate EC40 by orogastric placement first, and then by installation of stomach contents placed into the right lung. Using the minipigs own gastric juice for aspiration injury	Pure pulmonary aspiration model – the pig would receive no dimethoate EC40 by gavage, but only a standardised measured mixture of porcine gastric juice and dimethoate EC40/solvent into the right lung. Standardised porcine gastric juice would be collected form an abattoir, filtered, made to pH2 with HCl and treated with antibiotics/antifungal drugs and frozen at -80°C.	• The ability to observe a measured direct and indirect lung injuries without overt systemic poisoning confusing the clinical picture. • Standardised porcine gastric juice would mean all minipigs received GJ with the same pH, amount of particulate matter and microorganisms allowing for better comparison of intervention groups.
VAP prevention and human ICU standards	No VAP prevention strategy, veterinary ICU standards.	• Regular (6 hourly) sterile suctioning of the airway • 'head up' positioning with regular turning • Regular oral chlorhexidine gluconate gel (6 hourly) • Wearing of surgical gloves for all oral/airway procedures	• To reduce the rate of VAP by positioning the pig 'head up', regular suctioning combined with good hand and oral cleaning with 1% chlorhexidine gluconate (antibacterial) gel (Hunter, 2012, Price et al., 2014). • Prevention of atelectasis and pressure sores by regular turning of pigs • To prevent would infection over 48 hour periods with sterile dressings.

		• Surgical dressings for suprapubic, central venous and arterial catheters. • Routine treatment and normalisation of plasma glucose and electrolytes with judicious fluid management. • Ventilation with the ARDSnet protocol 6-8 mL/Kg, 5cm H_20 PEEP, plateau pressure <30cm H_2O(Brower et al., 2000, Spieth et al., 2011)	• To prevent ventilator induced lung injury (VILI), atelectotrauma and barotrauma by using an ARDS protective ventilation strategy.
Urinary catheterisation and central vascular access	Suprapubic catheterisation via a mini-laparotomy in male pigs Surgical cut down in the neck to allow placement of central arterial and venous cannulae.	Use of female minipigs to allow insertion of urinary catheters via the urethra. To attempt ultrasound placement of central venous cannulae using a sheathed needle.	Being able to place a urinary catheter, or central vascular access without the requirement for invasive surgery would feasibly reduce the overall systemic inflammatory insult. [NB urethral catheterisation was attempted by the veterinary team in 2 pigs using 6 and 8 FG foley catheters in the main aspiration study and abandoned due to its difficulty. Central venous access was attempted in an anaesthetised minipig using an ultrasound scanner but the depth of the vascular vessels in the neck and the thickness of the minipig skin and tissues meant that this was not possible for the main aspiration study]. Consideration was given to ultrasound placement of vascular cannulae in the minipig groin where the vessels are more superficial, but it was felt that these may easily become infected during a 48 hour study.
Animal sexing	Use of male pigs	Use of female pigs to assist in urethral catheter placement created a necessity to check for the homogeneity of females by measuring oestrodiol levels in all pigs at the beginning of the study.	Oestradiol is known to have an anti-inflammatory effect on blood vessels (Chakrabarti et al., 2008) and increased levels can reduce mortality from influenza A in mice through reduction in pro-inflammatory markers in the lung (Robinson et al., 2011).

Lung histopathology	Pouring 10% formalin into excised minipig lungs (via trachea) at a pressure of 25 cmH_2O immediately post mortem	To conduct a tracheostomy post mortem and fill the lungs with 10 % neutral buffered formalin under a standardised pressure with the lungs still in the thoracic cavity. After 2-3 hours the lungs would be removed and allowed to stand in 10% formalin for 2-3 days before cutting samples for histopathological analysis.	To optimise conditions for histopathology and immunohistochemistry of lung tissue secondary to acute lung injury, intratracheal placement of 10% neutral buffered formalin in-situ prior to lung removal was thought the best process (Braber et al., 2010).
Control animals	Saline + GJ placed in lung as control treatments	Use of sham bronchoscopy controls, saline controls and a positive control	It was decided to have both sham (n=5) bronchoscopy (bronchoscope placed in the lung with no mixture given), and saline (n=5) control (saline placed down the bronchoscope into the lung at the beginning of the experiment) minipigs to observe if there were any differences between the animals, and if the differences were slight they could be combined to create a greater number of controls. It is known that BAL (insertion of saline via a bronchoscope working channel and removing it through suction) can cause lung injury itself (Vonessen et al., 1991), but it was not known what lung injury would be caused through the installation and absorption of a small volume of saline into a minipigs lungs. After consultation with Dr K Dhaliwal (Senior clinical lecturer, Respiratory medicine, University of Edinburgh) and Professor M Eddleston it was decided to add a single positive control of intravenous oleic acid that would allow the future aspiration study inflammatory marker analysis to be compared with a known lung injury. This process would also confirm the correct functioning of the inflammatory marker ELISAs.

Table 4.7 Table describing the proposed Gottingen minipig aspiration model improvements.

Chapter 5: An investigation of the pathophysiology of pulmonary aspiration of gastric juice (GJ), organophosphorus pesticide with GJ and solvent with GJ in a Gottingen minipig model

5.1 Introduction

Human clinical experience shows that self-poisoning combined with decreased consciousness can lead to aspiration of gastric contents (Adnet and Baud, 1996, Montassier and Le Conte, 2012) with detrimental effects on the length of hospital stay (Liisanantti et al., 2003) and mortality (Isbister et al., 2004). OP pesticide ingestion causes decreased consciousness alongside excessive muscarinic symptoms of bronchorrhoea, increased salivation, nausea and vomiting which makes pulmonary aspiration of the gastric contents (including the OP and solvents themselves) more likely.

Although the literature describes pulmonary aspiration as being common in OP poisoning (chapter 1), whether or how it contributes to the very high mortality (up to 50%) within intubated OP pesticide poisoned patients is unknown. From our research team's previous porcine work, it is clear that solvents also have a role in increasing the severity of systemic OP poisoning (Eddleston et al., 2012).

Therefore our hypothesis was that aspiration of OP pesticide with gastric juice (GJ) could cause an acute lung injury, which in humans is capable of prolonging intensive care ventilation, hospital stay, and may also increase mortality.

This hypothesis generated two initial research questions:

1. What severity of lung injury is created through aspiration of GJ alone, OP pesticide with GJ, or solvent with GJ when compared with controls?
2. How does the pulmonary pathophysiological response to aspiration of these mixtures differ?

The study designed to answer these questions used groups of minipigs to create and observe specific pulmonary aspiration injuries following installation of: gastric juice (GJ) alone, OP pesticide (dimethoate EC40, which includes the solvent cyclohexanone) with GJ (OP+GJ), and solvent (cyclohexanone) with GJ (Solv+GJ) compared with positive and negative controls (Table 5.1).

To optimise the use of minipigs (replacement, refinement and reduction of animals in research (NC3Rs)) the study design allowed simultaneous observation of both direct and indirect lung injuries within the same animal by creating a unilateral pulmonary aspiration lung injury. This was tested and developed through the pilot aspiration study (study 3; chapter 4) alongside other model refinements (chapter 4).

A range of test were employed to quantify the lung injury and observe the pathophysiology caused through aspiration of these mixtures:

- Measurement of cardiorespiratory physiological variables, e.g. physiological dead space
- Pulmonary CT voxel density analysis
- Pulmonary histopathological scores
- Cell counts and pro-inflammatory cytokine concentrations in BALF and blood
- Microscopy, culture and aerobic bacterial count of the GJ aspirate, tracheal (baseline) and BALF 48 hour samples
- Pulmonary surfactant phosphatidylcholine composition

The rationale for using these tests is explained throughout the chapters.

5.1.1 Aims of the chapter

- To investigate and describe the pulmonary pathophysiology of aspiration of porcine GJ alone, OP (dimethoate EC40) with GJ, or solvent (cyclohexanone) with GJ
- To quantify and observe the severity of direct and indirect lung injury created by OP with GJ, Solv with GJ and GJ alone, when compared with controls

5.2 Methods

The study took place between November 2013 and May 2014 at the Moredun Research Institute, Edinburgh, UK with a core research team of 11-13 people including human and veterinary anaesthetists, research, laboratory and radiography staff.

Two 48 hour studies were conducted back-to-back in week long intervals from 0530 Monday to 1200 Friday using a staff rota system. This meant at least two staff were on site at all times, with more staff made available to assist during CT/BAL sampling points in the mornings. I would be present every day to assist in the daily management of the study and also partook in the night shift rota.

On completion of the week long studies (Friday 1200) a great deal of sample handling still remained (e.g. histopathology sampling, BALF/blood processing). To achieve this I stayed at the Moredun site later on Friday afternoons and returned the following Monday to process the histopathology samples and tidy the laboratory.

Methods particular to this pulmonary aspiration study are detailed below; the remainder of the methods can be found in Chapter 2.

5.2.1 Groups and randomisation

Twenty six Gottingen minipigs were randomly allocated to one of six treatment groups for the study (Table 5.1). There were two control groups; sham bronchoscopy and saline controls. This was to differentiate the effect of the administration of fluid (saline) into the lung at time 0 and to determine whether it created a different lung injury from sham bronchoscopy. If the results were similar between groups (i.e. no statistical difference) then combining the results of both controls groups would be an option for further intergroup statistical analysis. The dimethoate EC40 and complementary cyclohexanone doses for aspiration were calculated during the dose ranging studies (chapter 4). The oleic acid dose was based on a porcine study from 1991 showing that 0.08 mL/kg oleic acid infused IV over 30 min created a stable measureable acute lung injury in similar sized Yorkshire pigs for up to 4 hours (Sumping et al., 1991). During study 1, two previous Gottingen

minipigs had been poisoned with either 0.17 or 0.25 mL/kg oleic acid IV and were euthanised after only two hours due to cardiovascular instability. From the available evidence, I hypothesised that a dose of 0.08 mL/kg had a greater chance of producing a lung injury that could be observed for 48 hours.

Group	No. minipigs	Treatments	Dose	Purpose
Group 1 (sham)	5	Control: Sham, had bronchoscopic instrumentation into the right lower lung with no administration of mixtures.	n/a	To observe the effect of one lung initial instrumentation
Group 2 (saline)	5	Control: saline placed in the right lower lung via bronchoscope	0.5 mL/kg (e.g.30kg pig – 15 mL)	To observe the effect of saline administration into one lung
Group 3 (OA)	1	Positive control: IV oleic acid	0.08 mL/kg IV over 30 min	Positive control causing ARDS in order to ascertain normal immunoassay function
Group 4 (GJ)	5	Porcine GJ placed in the right lower lung via bronchoscope	0.5 mL/kg	To observe a normal pulmonary aspiration injury with GJ
Group 5 (OP+GJ)	5	Porcine GJ with OP dimethoate EC 40 placed in the right lower lung via bronchoscope	1/25 of an oral lethal dose = 3 mL dimethoate EC40 [containing 1.2g dimethoate and 1.2 g cyclohexanone] + 12 mL GJ for 30 kg pig. Total volume 0.5mL/Kg	To observe a pulmonary aspiration injury with GJ with OP-dimethoate EC40
Group 6 (Solv+GJ)	5	Porcine gastric juice with solvent cyclohexanone placed in the right lower lung via bronchoscope	1/25 of oral lethal dose= 1.26 mL (1.2g) cyclohexanone + 13.7 mL GJ for 30 kg pig. Total volume 0.5mL/Kg	To observe a pulmonary aspiration injury with GJ with cyclohexanone

Table 5.1 Treatment groups for the pulmonary aspiration study

The acquisition costs of the mobile CT scanner were substantial, therefore only the first 12 minipigs in the study underwent block randomisation to produce a representative sample of the treatment groups. The following animals obtained pulmonary CTs: 2 x group 1, 2 x group 2, 2 x group 4, 3 x group 5, and 3 x group 6. The remaining 14 minipigs were randomised to the six treatment groups and did not receive CT scans. Randomisation was done by entering the minipig number into a computer [www.random.org] program.

The creation of a standardised porcine GJ and unilateral lung injury are discussed in methods chapter 2.

All but the first two female minipigs had plasma oestradiol measured to assure hormonal homogeneity within the study groups.

5.2.2 Study protocol

Two minipigs were anaesthetised simultaneously for the 48 hour aspiration studies. Ethics, animal husbandry, standard ICU monitoring and anaesthesia are described in methods, Chapter 2.

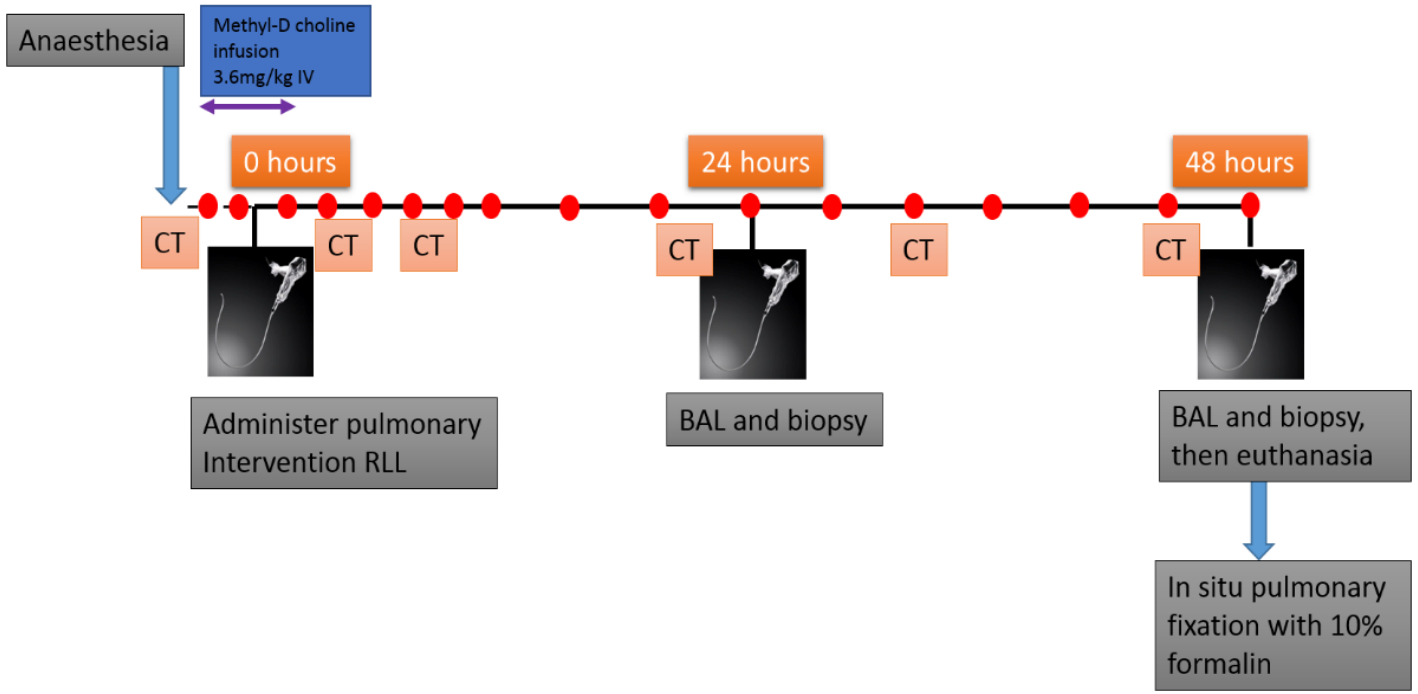


Figure 5.1 Minipig pulmonary aspiration study protocol. Each minipig was anaesthetised approximately 2 hours before time 0 to allow time for surgical placement of arterial, central venous and urinary catheters. The pulmonary surfactant analysis tracer isotope (methyl-D_9 choline chloride 3.6mg/Kg) was infused intravenously from -30 min for 3 hours (purple line). The red dots denote sampling points for arterial and venous blood ± urine and physiological deadspace measurement. Computed tomography (CT) of the minipig lungs occurred at -30 min, 4, 8, 24, 32 and 47.5 hours. The intervention mixture e.g. 0.5 mL/kg gastric juice, was placed in the right lower lung (RLL) at time 0. Bronchoscopy, bronchoalveolar lavage (BAL) and pulmonary biopsy for both the direct (right) and indirect (left) lungs happened at 24 and 48 hours. After the 48 hour BAL and biopsy sample the minipig was euthanised, a tracheostomy created and the lungs were filled with 10% neutral buffered formalin at 25 cm H_2O over 2-3 hours.

The study timeline (Figure 5.1) describes the key points for administration of the pulmonary aspiration mixes, CT scans, BAL, biopsy and sampling. The rationale behind the pulmonary surfactant (Phosphatidylcholine) analysis and infusion of methyl-D_9 choline chloride is discussed in methods section.

5.2.3 Physiological dead space

Physiological dead space was calculated using the Enghoff-modified Bohr equation (Enghoff, 1938, Nunn and Holmdahl, 1990):

$V_D/V_T = (PaCO_2 - P\bar{E}CO_2)/PaCO_2$

Where V_D is the dead space volume, V_T the tidal volume (breath volume), $PaCO_2$ is the partial pressure of CO_2 in arterial blood, and $P\bar{E}CO_2$ is the partial pressure CO_2 in mixed (dehumidified) expired gas.

The animals tidal volume (V_T) was calculated by measuring the volume of expired gas using a dry gas meter (Harvard Apparatus, MA, USA) over 5 min and then dividing that volume by the number of breaths taken within that time (Figure 5.2D). $PaCO_2$ was calculated from an arterial blood sample taken midway through this 5 min period using a blood analyser (EPOC®, Woodley, UK). At the end of 5 min the peak airway pressure (Ppeak) and $P\bar{E}CO_2$ were recorded from the ICU ventilator (Servo 300A, Maquet) and capnograph (Normocap 200, Datex-engstorm, Finland; Figure 5.2E) respectively.

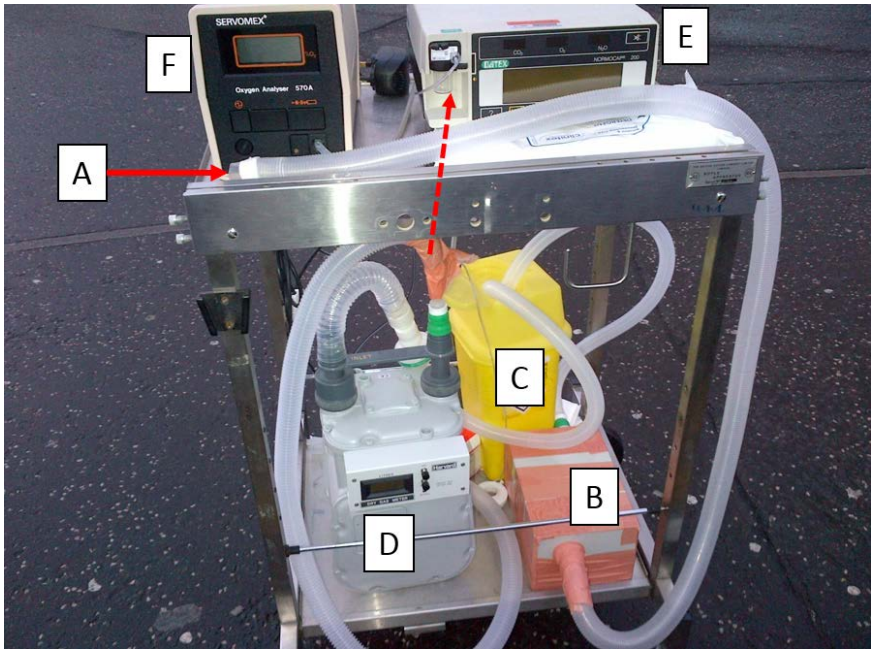


Figure 5.2: Physiological dead space measurement equipment. Exhaled air from the ICU ventilator (Servo 300A, Maquet) was collected via a tube (A) and passed through a gas mixing box containing baffles (B) and a dehumidifying box (C) containing ice and water. A heat and moisture exchange (HME) filter (Intersurgical, UK) was also placed within the gas circuit to prevent any residual water within the gas mixture from reaching the dry gas meter (D) (Harvard apparatus, MA, USA). Waste gas from the dry gas meter (D) was syphoned off (red dotted line) to a capnograph (E) (Normocap 200, Datex-Engstorm, Finland) to measure mixed end tidal CO_2 (P_ECO_2). The oxygen analyser (F) was not used in our experiments. The capnograph was calibrated at the beginning of each week of experiments using a known gas mixture (Quick cal™, GE Healthcare, Finland).

Mechanical dead space - the volume of tubing and equipment that lies in between the mouth and the ventilator circuit e.g. length of endotracheal tube outside of the mouth, HME filter, spirometry module insert - was subtracted from V_D before further adjustments for mechanical ventilation and compressible volumes (Singleton et al., 1972). For an average minipig ventilated with an 8.0 mm diameter ETT, mechanical dead space was 72.5 mL.

The tidal volume was then corrected (V_Tc) for removal of compressed volume (Vv) by application of a correction factor S:

$$V_D/V_Tc = S \times (V_D/V_T) + (1-S)$$

Where S is the correction factor = V_T/V_Tc, and

$$VTc = Pb \times (Vv+Vt)/(Pb+Ppeak) - Vv, \text{ where } Vv = Vcp \times (Pb/Ppeak)$$

Where Pb is the barometric atmospheric pressure, with Ppeak being the peak airway pressure in the endotracheal tube, Vv is the compressible volume of the ventilator (measured at approximately 1006 mL), Vcp is the volume compressed by the ventilator and the tidal volume (V_T) was calculated as above (Forbat and Her, 1980).

The data are presented as percentage change (%) from the initial (t -30 min) V_D/V_{TC} over time.

5.2.4 Blood sampling and processing

Arterial (0.3 mL) and venous blood (11 mL) were collected at regular intervals as per the study protocol. From these, arterial blood (0.1mL) was placed in an arterial blood gas and electrolyte analyser (EPOC®, UK) to determine: PaO_2, $PaCO_2$, pH, lactate, Glucose, Na+, K+, urea and creatinine. Venous blood (0.2 mL) was added to 4 mL of 4°C 0.9% saline, mixed and aliquoted for AChE measurement. The remaining venous blood was added to EDTA tubes, mixed and centrifuged at 3900 rpm for 7 min. The resultant plasma and red cell pellet was aliquoted for further testing and stored at -80°C.

An extra EDTA tube was filled with whole blood at t= -30 min, 24 and 47.5 hours to allow measurements of oestradiol, haemoglobin and white cell numbers within each animal. These assays were processed by the laboratory staff at the Scottish Agricultural College (SAC) Veterinary Services, Bush Park, Penicuik, Scotland.

5.2.5 Bronchoalveolar lavage sampling

Bronchoscopy at the start of the experiment (time 0) may have caused a degree of lung injury; pro-inflammatory mediator release (Terashima et al., 2001) and neutrophil recruitment (Vonessen et al., 1991). Lung injury secondary to bronchoscopy is made worse by large volumes of lavage fluid and can make the awake, conscious human patient febrile, and suffer headache and transient hypoxia (Terashima et al., 2001, Dhillon et al., 1986, Meyer, 2004). The BAL methodology in study 1 used 60mL saline flushes and biopsies at t-30min, 2-4 hour and 12 hours. In humans (70kg) it is normal practice to use BAL volumes between 100-300 mL saline (Meyer et al., 2012), but the ideal BAL volume sufficient to analyse BALF without causing injury in a minipig, is unknown. The first six minipigs had 60 mL BAL volumes which was reduced to 50 mL BAL for the remaining 20 minipigs. The decision to reduce the volume of BAL was

taken was after a discussion between myself and the veterinary staff in order to reduce possible lung damage caused through an excessively large lavage (further discussion in results section).

5.2.6 Microbiological sampling and analysis

Aerobic bacterial counts from the porcine GJ (aspiration mixtures) were taken at the beginning of the experiment to confirm the absence of live microorganisms and thus its ability to cause bacterial contamination of the airways.

Tracheal aspirates were also taken at the beginning of the experiment in preference to time 0 BAL (Vonessen et al., 1991) to avoid lung injury. Tracheal aspirates allowed measurement of the existing airway microbiota, and helped to confirm the absence of a pre-existing pneumonia in the animal. At the end of the experiment, BALF samples were cultured to determine whether animals had developed a bacterial pneumonia by 48 hours.

Dr James Gibbons MVB PhD and staff at the Microbiology Veterinary Pathology Unit, University of Edinburgh provided advice and performed the aerobic cultures of tracheal secretions, GJ and BALF samples.

The first two minipigs did not provide tracheal aspirates and 48 hour BALF cultures as these animals were involved in experimental refinement.

5.2.6.1 Tracheal aspirate sampling

Tracheal aspirates from 24 anaesthetised minipigs were taken at t -30 min by placing a 14G suction catheter (Prestrol, Pennine Healthcare) through the endotracheal tube into the bronchial tree and applying gentle suction (approximately 16-20 kPa using the suction machine). The catheter was then removed and secretions placed in a sterile plastic container by flushing the catheter with 10 mL of sterile 0.9% saline.

5.2.6.2 Porcine gastric juice sampling

Aliquots of thawed porcine GJ (20 mL) were mixed together with gentle agitation. From these aliquots, the pulmonary aspiration mixtures with OP and solvent were prepared in sterile syringes. A sample of the excess GJ (2-3 mL) was sent to the microbiology laboratory for culture and analysis.

5.2.6.3 Bronchoalveolar lavage fluid sampling

After the 48 hour BAL from the right lung, a second BAL was performed in the right lung using 50 mL room temperature 0.9% saline and was aspirated into a sterile pot for microbiological analysis. Sampling occurred before the right lung biopsy to avoid blood contamination.

5.2.6.4 Aerobic bacterial culture

Neat BALF or GJ (20 μL) and 20 μL of a 1/100 dilution of the sample (diluted in sterile 0.9% saline) were inoculated in duplicate onto horse blood agar and MacConkey agar plates. The inoculum was spread across the plates and incubated aerobically at 37°C for 18- 24 hours. The number of colonies on each plate was counted after 24 hours and 48 hours incubation (to allow for the detection of slow-growing organisms).

The total aerobic colony count (TAC) forming unit per millilitre (cfu/mL) was calculated as:
(no. of cfu) x 50 (to convert 20 μL to mL) x dilution factor.
For example if 70 colonies present on plate from neat sample then the calculation would be:
70 x 50 x 1(no dilution) = 3500 = 3.5 x 10^3 cfu/mL fluid.

The TAC was calculated as the average number of colonies (using all plates) after 48 hours incubation from plates which contained between 30 – 300 colonies. If there were more than 300 colonies, then a further sample dilution (100x) and culture was conducted. Where fewer than 30 colonies were present when using a neat sample, the number of colonies present were counted and TAC calculated using the above formula.

5.2.7 Decontamination, sterilisation and reuse of equipment

To minimise the risk of cross-contamination, used airway equipment e.g. TCB Univent endotracheal tubes, laryngoscope blades, facemasks, laryngo-tracheal mucosal atomisation devices (LMA MADgic; Teleflex, UK), and on one occasion two x bronchoscope endosheaths (due to a failed delivery), were decontaminated and sterilised before re-use.

Decontamination of the equipment involved washing with soap and water and was followed by sterilisation through a 30 min immersion in a 20:1 mixture of water: proprietary sterilant for

medical endoscopy and surgical equipment (Medistel; Tristel, UK). After this, the equipment was thoroughly rinsed and flushed with water after immersion and before re-use.

5.2.8 Lung fixation

Immediately after euthanasia, the TCB Univent ETT was removed from the trachea and replaced by a surgically sited tracheostomy using a size 6.0mm ETT. The ETT was sutured using 1.0 silk to the surrounding skin and trachea to prevent dislodgement.

The minipig was taken to the *post mortem* room and the 6.0mm ETT attached to a container (via a plastic tube) held at a height to deliver 10% neutral buffered formalin (VWR/Cellpath, UK) at approximately 25 cm H_2O over 2-3 hours.

After this period, the ETT was disconnected from the tubing and the lungs were carefully excised from the thorax and placed in a large basin containing 10% neutral buffered formalin with the ETT open to air. The basin was sealed and the lungs allowed to 'fix' for 48-60 hours.

Whole sections of right and left upper and lower lung samples 1-2cm thick 'slabs' (4 per pig) were cut and placed in 10% buffered formalin for further processing by the histopathology section of the Veterinary Pathology Unit, Royal (Dick) School of Veterinary studies, University of Edinburgh. Small samples (1-2 mm^3) from both right and left lower lungs were also sent to the Newcastle University for EM analysis. Further lung fixation methodology is discussed in Chapter 2.

5.2.9 Statistical analysis specific to the pulmonary aspiration study

Statistical analysis used in this study is described in Chapter 2 (methods).

For BALF analysis, positive control (oleic acid) data are shown in the bar graphs, but were not used for statistical analysis.

5.2.9.1 Power calculations for the minipig pulmonary aspiration study

Calculating the power of the minipig pulmonary aspiration study was performed by myself under the guidance of Ms Nicola Williams, senior statistician at Oxford University in October 2012.

Our research group has previously used minipig groups of n=5 for OP toxicity studies (power 90%, alpha error 5%). This group size was based on data detailing the area under the curve (AUC) of AChE inhibition over time between control and OP-poisoned pigs.

To power the main aspiration study, pulmonary histopathology scores from previous OP toxicity studies (orogastric administration and aspiration; studies 1 and 3) were used to calculate the power and sample size. The pulmonary histopathological score was thought to be a more appropriate measurement of aspiration lung injury as opposed to the AChE inhibition between treatment and control groups.

5.2.9.1.1 Use of pulmonary histopathological data for powering

Histopathological data (scores) from two minipigs that had received orogastric saline (no aspiration) and survived for 12 hours in study 1 were used to represent the control group (n=10 samples). Histopathological data from three directly-injured lungs secondary to aspiration of OP pesticide and gastric contents from study 3 were used to represent the treatment group (n=6 samples) for comparison. The control group animals from the pilot aspiration study 3 were not used because they had saline and their stomach contents (including GJ and particulates) placed into a lung. This produced a significant lung injury itself and the histopathology scores would therefore not provide accurate powering data for the main aspiration study.

The histopathology scores were awarded by SS using the histopathology scoring system 1.0. The overall mean score and SD was calculated for each control and treatment groups.

Assuming a minimum of n= 5 in each group, and an alpha error of 5%, the power of the study was calculated using the free statistical power calculator from dssreserach.com [https://www.dssresearch.com/KnowledgeCenter/toolkitcalculators/statisticalpowercalculators.aspx].

The control group had an overall mean histopathological score (0.4) and SD (0.28), with a treatment group mean (8.83) and SD (2.75).

Assuming a minimum of five animals in each group and a two tailed test with an alpha error of 5% = *power 100%.*

Using SS scores with the later refined validated histopathological scoring system 1.1 (which gave similar control scores and a mean (8.0), SD (1.8) for the intervention pigs) the power would increase because of the tighter SD's. This would also be reflected in WW scores using system 1.1 producing a mean (8.17) SD (2.02) for intervention and mean (0.7) and SD (0.42) for the control minipigs.

The main aspiration study was not sufficiently powered to compare active treatment groups e.g. OP+GJ vs. GJ vs. Solv+GJ.

5.2.9.1.2 Comments on using pulmonary histopathological data for powering

The above calculations were based on a small sample of animals and from different studies (studies 1 and 3) at different time points which may have affected the validity of these calculations.

Study 1 animals had survived for 12 hours without protective lung ventilation, whereas the OP pigs from study 3 had survived two, 35 and 48 hours with protective lung ventilation. A longer time with ventilation could feasibly have caused more lung damage through prolonged OP toxicity, with non-protective ventilation causing ventilator induced lung injury.

Study 1 saline control pigs also experienced bronchoalveolar lavage (BAL) at 0, 4 and 12 hours which itself is known to cause lung injury (Vonessen et al., 1991). These pigs may therefore not be a true representation of 'control' animals. However, as the BAL technique was to be used in the main aspiration studies (including the control animals), the comparison of histopathological data from study 1 and 3 minipigs was not entirely without merit.

These calculations were made assuming a normal distribution of data and needed to be adjusted. If non-parametric data are suspected, approximately 15% more animals should be added to each group using the asymptotic relative efficiency (ARE) principle (Randles and Wolfe, 1979). However, using the above data, n=2 in each group created a power = 99.1%, so using n=5 in each group should have mitigated against this.

5.2.9.2 Computerised tomography statistical analysis

Friedman's test was used to compare the mean percentages of poorly and non-aerated lung (between -499 and +250 Hounsfield units (HU)) for each pig to assess for significant changes

between the five groups [sham, saline, OP+GJ, GJ, Solv+GJ] over time in the right, left and whole (right and left) lungs. This region of interest was chosen to include both normal lung which was poorly aerated (-500 to -100HU) possibly secondary to the effects of OP e.g. bronchospasm and collapse, and lung filled with fluid e.g. blood, oedema, consolidation (-100HU to +250HU) (Drummond, 1998, Heuer et al., 2012).

If this was found to be significant, the more dense part of the lung HU profile [a list of the percentage of voxels within each 5HU sections between -499 and +250 HU: 149 measurements] of each lung, at each time point, was compared between groups using Friedman analysis and post hoc testing with Dunn's test which is described in Table 5.5.

5.2.9.2.1 Intra-rater reliability testing

To determine the extent of intra-observer reliability (repeatability within an assessor), a random 10% of the CT scans (6/60) for both right and left lungs were re-analysed to compare the number of voxels for each 5HU section within the whole lung (-1025 to +256HU: 256 measurements). This created 3072 (256 5HU x 2 (right and left lungs) x 6 scans) measurements for comparison and results are shown as Bland-Altman plots and Spearman's rank coefficients.

5.3 Results

All the minipigs survived until the 48 hour sampling point.

5.3.1 Use of control groups for statistical analysis

Plateau airway pressures ($p \leq 0.001$), physiological dead space ($p \leq 0.05$), and CT lung density analysis (all apart from directly-injured lung at 47.5 hours) for directly and indirectly-injured lungs ($p \leq 0.0001$) showed statistically different measurements between sham bronchoscopy and saline control groups. These results combined with an increase in non-cellular BALF contents at 48 hours in the directly-injured lungs of saline when compared with sham control groups indicated that the saline control group was often experiencing a greater degree of lung injury when compared with the sham control group.

Although this study was not powered to show differences between sham and saline control lungs, these findings prompted the decision to keep the control group data separate, not combined, for future statistical analysis of aspiration study data.

5.3.2 Plasma AChE activity is reduced in OP+GJ minipigs

To confirm OP poisoning the normalised plasma AChE activity was measured in all pigs. Despite the low dose (1/25th of a lethal human and minipig dose of dimethoate EC40) of OP used in the aspiration study, the AChE activity was significantly depressed in the OP+GJ group when compared with the other groups at 47.5 hours (Figure 5.3).

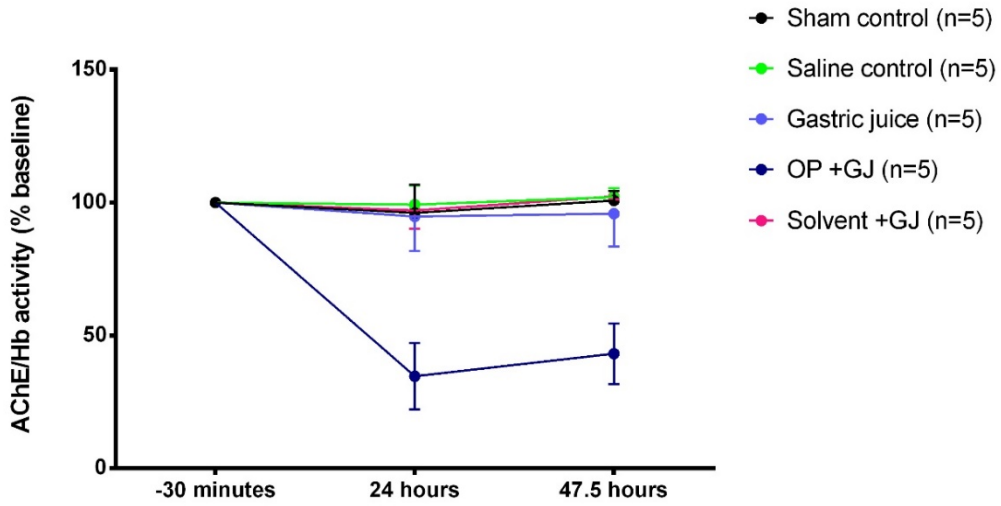


Figure 5.3: Effects of pulmonary treatments on plasma AChE activity (relative to Hb concentration and normalised to percentage baseline). Friedman analysis of AChE activity showed significant differences **(p≤0.05*)** between groups with OP+GJ being significantly reduced when compared with: sham, saline, **GJ (p≤0.01**)** and Solv+GJ **(p≤0.05*) groups at 47.5 hours using permutation testing. Mean and SD shown.**

5.3.3 Hormonal homogeneity within the female minipig study group

Oestradiol can have an anti-inflammatory effect and has reduced lung inflammation in a mouse influenza model (Robinson et al., 2011). It was therefore important to establish the plasma oestradiol concentrations of the female minipigs in the study.

Oestradiol levels were measured in 24 minipigs at the beginning of the experiment (t = - 30 min). All but five had oestrodiol levels reported as < 5 pmol/L. Statistical analysis using the Kruskal-Wallis test showed no statistical difference between groups (p>0.5). [NB. Results were calculated after excluding the data from the oleic acid treated pig and entering results reported as <5 pmol/L as 2.5 pmol/L].

5.3.4 Physiological observations

5.3.4.1 The minipig pulmonary aspiration model produced cardiovascular stability and adequate tissue perfusion

5.3.4.1.1 Cardiovascular observations

High dose dimethoate ingestion is known to cause hypotensive shock in humans (Davies et al., 2008) and minipigs (Eddleston et al., 2012) through direct cardiotoxicity, vasodilatation and

distributive shock (low systemic vascular resistance (SVR)). However, the minipig aspiration model was required to produce cardiostability in order to conduct a 48 hour single organ failure (lung) study.

Intravenous norepinephrine was only used in two pigs to increase mean arterial pressure (MAP); one OP+GJ pig at a very low dose (80-800 mcg/hr) for 2 hours at the beginning of the experiment, and one pig receiving IV oleic acid, which required a constant norepinephrine infusion in doses up to 0.12 mg/kg/hr (data not shown in graphs/tables).

Overall, OP+GJ pigs had the lowest MAP (70 ± 4.9 mmHg) and SVR (1589 ±307 Dynes/cm^5) when compared with the other groups throughout the duration of the experiment. This was probably due to OP-induced vasodilatation (without direct cardiotoxicity) which consequently produced a larger stroke volume (volume ejected from the heart) and cardiac output (CO) within the OP+GJ pigs (Table 5.2).

Group	Mean ± (SD) MAP – [% baseline]	Mean ± (SD) –MAP [mmHg]	Mean ± (SD) CO – [% baseline]	Mean ± (SD) - CO [L/ min]	Mean ± (SD) SVR – [% baseline]	Mean ± (SD) SVR – [Dyne/sec/cm^5]
Sham	88 (4.7)	75 (4.4)	110 (25)	3.2 (0.7)	95 (26.7)	2019 (565)
Saline	82 (6.0)	84 (6.1)	106 (14)	2.9 (0.4)	91 (17.4)	2393 (447)
GJ	92 (5.2)	77 (4.7)	114 (27)	3.4 (0.8)	97 (23.9)	2007 (478)
Solv+GJ	83 (6.2)	76 (5.8)	116 (33)	3.6 (1.0)	85 (12.9)	2006 (380)
OP+GJ	89 (5.8)	70 (4.9)	121 (34)	3.7 (1.0)	73 (15.4)	1589 (307)

Table 5.2 Cardiovascular physiological data for minipigs in the aspiration study. Values show actual and normalised (% change from baseline) data for the duration of the experiment (0 to 48 hours). Graphs for MAP % change from baseline data are seen in Figure 5.4.

5.3.4.1.2 Arterial lactate and pH

The arterial lactate concentration is often used as marker of tissue hypoperfusion secondary to circulatory shock (Weil and Afifi, 1970). Lactate was raised in the OP+GJ group, which was not statistically significant when compared to the other groups (Figure 5.4C). The corresponding arterial pH was reduced in the OP+GJ group when compared with sham ($p \leq 0.001$) and saline ($p \leq 0.0001$) controls and Solv+GJ ($p \leq 0.0001$) treated pigs. However, this

is of little clinical significance as the pH values for all animals were largely within the normal range for pigs [pH 7.4-7.53] (Figure 5.4 D)(Hannon et al., 1990).

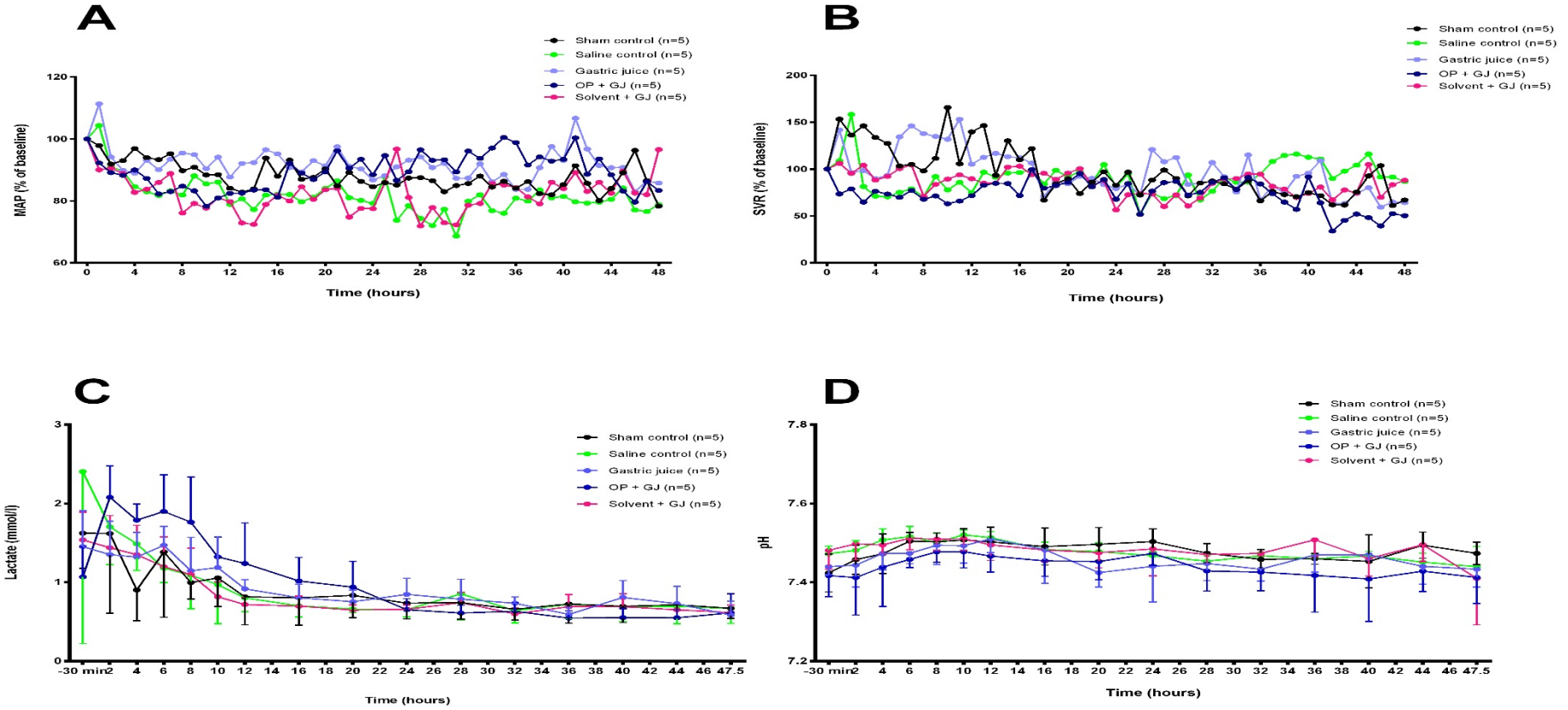


Figure 5.4 Physiological data from minpigs receiving pulmonary treatments. Graphs show the (A) mean arterial pressure (MAP - % change from baseline), (B) systemic vascular resistance (SVR - % change from baseline), (C) arterial plasma lactate concentration (mmol/L) and (D) arterial pH of all minipigs during the pulmonary aspiration study. Graphs A and B show the mean values only, with C and D showing the mean and SD. Friedman analysis of arterial lactate concentration (C) showed no significance between groups, but arterial pH (D) was significant **(p≤ 0.0001) with Dunn**'s post-test showing OP+GJ group was **significantly reduced when compared with sham (p≤0.001) and saline (p≤0.0001) controls, and Solv+GJ groups (p≤0.0001) over ti**me.

5.3.4.2 Reduced PaO_2/F_IO_2 ratios and higher plateau airway pressures are found in minipigs that have aspirated mixtures of OP+GJ, Solv+GJ or GJ alone.

To determine the severity of acute lung injury caused through the aspiration of GJ, OP+GJ, or Solv+GJ several parameters were measured: PaO_2/F_IO_2 ratios, plateau airway ventilation pressures and physiological dead space (Chapter 2 – methods).

5.3.4.2.1 PaO_2/F_IO_2 ratios

PaO_2/F_IO_2 ratios are often used in human clinical practice to guide treatment as reduced ratios show some correlation with ARDS mortality rates (all causes) and duration of mechanical ventilation, especially when using standardised ventilation protocols (Ranieri et al., 2012, Chen and Ware, 2015). In this experiment, PaO_2/F_IO_2 ratios were significantly reduced in pigs that had aspirated OP+GJ ($p \leq 0.05$), Solv+GJ ($p \leq 0.01$) and most notably in the GJ ($p \leq 0.0001$) alone group, in comparison with the sham control over time.

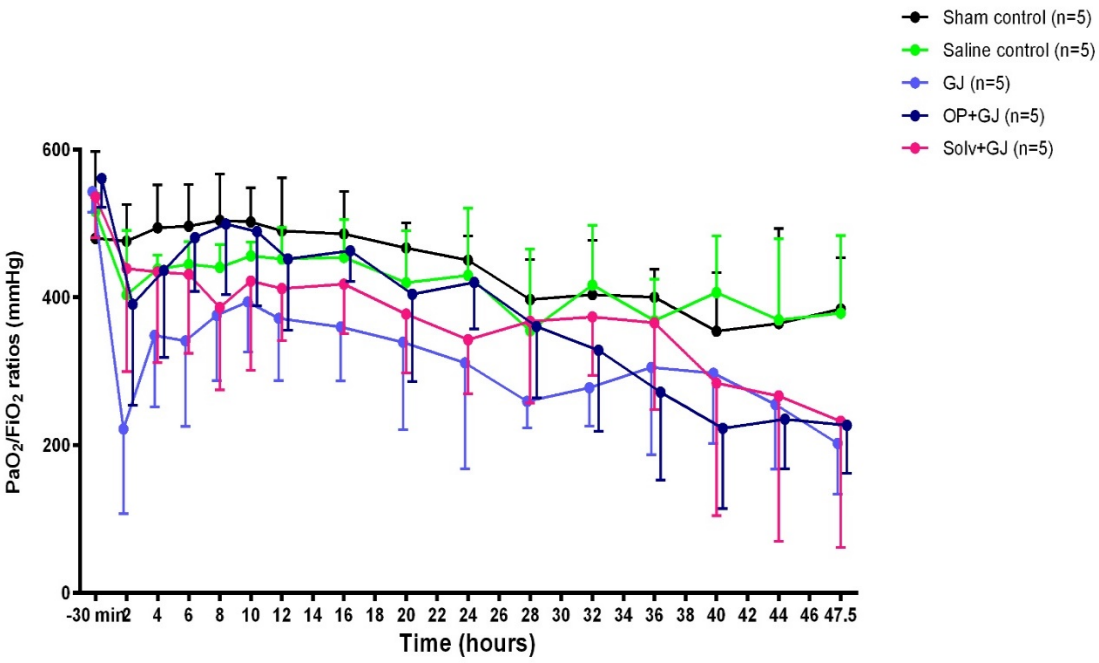


Figure 5.5 PaO_2/F_IO_2 ratios in minipigs receiving pulmonary treatments. Friedman analysis showed a significant difference between groups ($p < 0.0001$) and Dunn's post **test showed treatment groups (OP+GJ ($p \leq 0.05$), Solv+GJ ($p \leq 0.01$), GJ ($p \leq 0.0001$)) had** significantly lower ratios than the sham control group over time. Data from a saline control minipig between 24-47.5 hours have been omitted due to an undetected pneumothorax (discussed below) between 32-48 hours. The graph shows mean and SD.

At the end of the experiment, all (apart from those in the control groups) had developed a mild-moderate ARDS with PaO_2/F_IO_2 ratios of 227 (±65) mmHg, 203 (±69) mmHg and 232 (±171) mmHg for OP+GJ, GJ and Solv+GJ treated pigs at 47.5 hours respectively (Figure 5.5). The PaO_2/F_IO_2 ratios for sham bronchoscopy were 385 (±70) mmHg, with saline controls 317 (±166) mmHg.

5.3.4.2.2 Plateau airway pressures

Ventilation of oedematous and even normal lung can create high airway pressures that cause barotrauma in the lung. This has structural, physiological, biological, and systemic side effects and is reviewed elsewhere (Slutsky and Ranieri, 2013). Protective mechanical ventilation aims to avoid this in humans by ventilating at plateau airway pressures (Pplat) ≤30 cm H_20.

All groups showed some increases in Pplat values, with OP+GJ, Solv+GJ and GJ groups showing significant ($p \leq 0.0001$) increase compared with sham control over time (Figure 5.6). This may have been a result of increased resistance (bronchospasm), oedema and lung injury within these aspiration groups (Segura et al., 1999, Mendelson, 1946, Wynne and Modell, 1977).

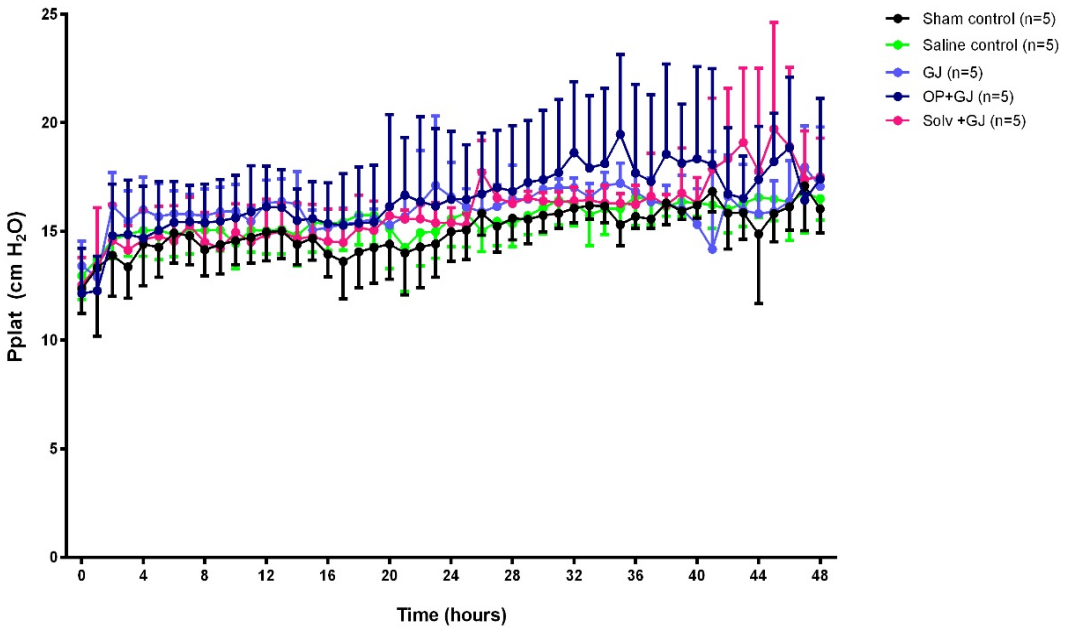


Figure 5.6: Plateau airway pressure (Pplat) for minipigs receiving pulmonary treatments. Friedman analysis showed a significant difference between groups (P<0.0001) and Dunn's post test showed Pplat for the treatment groups (OP+GJ, Solv+GJ, GJ) were **significantly (p≤0.0001)** greater than the sham control group over time. There was no statistical difference between GJ and OP+GJ, or GJ and Solv+GJ, but Solv+GJ was **significantly lower than OP+GJ (p≤ 0.01)**. The saline control group omits the data from 24-48 hours for the minipig that suffered a pneumothorax. The graph illustrates means with SD.

Plateau airway pressures were significantly lower with Solv+GJ (p ≤0.01) compared with OP+GJ, but not GJ treatments, suggesting that something other than the cyclohexanone component of dimethoate EC40 i.e. dimethoate active ingredient (AI), was responsible for the increased airway pressures. This might have been due to the OP-induced cholinergic effects on the airways (Segura et al., 1999).

5.3.4.2.3 Physiological dead space

Pulmonary or physiological dead space is that part of the tidal volume (breath) that does not participate in gas exchange within the lung. In mechanically ventilated patients it consists of anatomical (e.g. bronchi and ventilator equipment) and alveolar dead space. An increase in physiological dead space is a feature of early phase ARDS, and has been associated with an increased risk of death (Nuckton et al., 2002, Raurich et al., 2010, Chen and Ware, 2015). Physiological dead space is approximately 150 mL for healthy humans (V_D/V_T < 0.3) (Fowler,

1948), more if anaesthetised. The data are represented as the percentage change in corrected physiological dead space (V_D/V_{TC}) over time, with the expectation that the value would increase over time, for those animals with more severe lung injury.

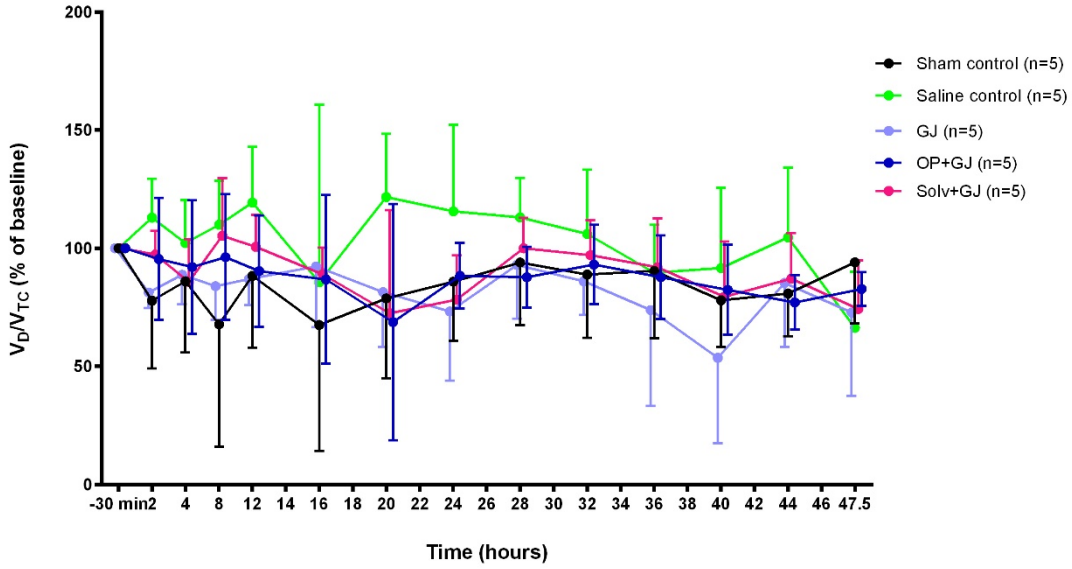


Figure 5.7: Physiological dead space (V_D/V_{TC} -% of baseline) for minipigs receiving pulmonary treatments. Friedman analysis showed a significant difference between **groups (P<0.0036) and Dunn's post test showed that sham (p≤0.05) and GJ (p≤0.01)** groups had significant decrease in the percentage change of V_D/V_{TC} compared to the saline control group over time. The saline control group omits the data from 24-48 hours for the minipig that had a pneumothorax. The graph illustrates means with SD; some x values have been staggered to show error range of values between groups.

All groups experienced a decrease in the percentage change of V_D/V_{TC} illustrating improved ventilation and reduction in physiological dead space over time (Figure 5.7). The animals within the saline group performed least well (higher percentage of dead space from baseline), and caused the observed statistical differences between groups. This can be explained because the saline group started (t -30 min) with the lowest actual V_D/V_{TC} and had subsequent higher readings after anaesthesia (as would normally be expected) leading to increased baseline values (%).

5.3.5 Bronchoalveolar lavage fluid analysis

Bronchoalveolar lavage allows the analysis of the number and types of cells that predominate in the lung during aspiration of various compounds. It also allows the measurement of many small molecules e.g. albumin and inflammatory markers. These combined, give important clues in understanding how the pathophysiology of aspiration of OP+GJ or Solv+GJ differs from the aspiration of GJ alone.

5.3.5.1 Quality of the BAL

The BALF procedure was modified after the first six minipigs by reducing the BAL aliquot from 60 mL to 50 mL for the remaining 20 minipigs. This was in order to decrease the perceived harm (by myself and the veterinary team) that such a large aliquot could have on the animal, potentially increasing the lung injury.

For all pigs, BAL return volume was acceptable at 56 ± 16% with no significant difference for percentage volume return (%), or actual volume return (mL) between groups (Meyer et al., 2012). Permutation testing comparing the number of cells and total protein content contained in the 60mL (n=8 tests) vs. 50 mL (n=12 tests) BALs within the same groups e.g. sham bronchoscopy group, showed no significant difference between samples. These results suggest that there was no significant effect of 50 mL vs. 60 mL BAL on cellular or non-cellular contents in this study, and therefore the lower BALF volume did not decrease any observed lung injury. The BALF cellular viability was examined (Meyer, 2004) in 20 pigs and found to be 85.4 ± 10.6% (mean ±SD).

5.3.5.1.1 The presence of red blood cells (RBC) in the bronchoalveolar lavage fluid

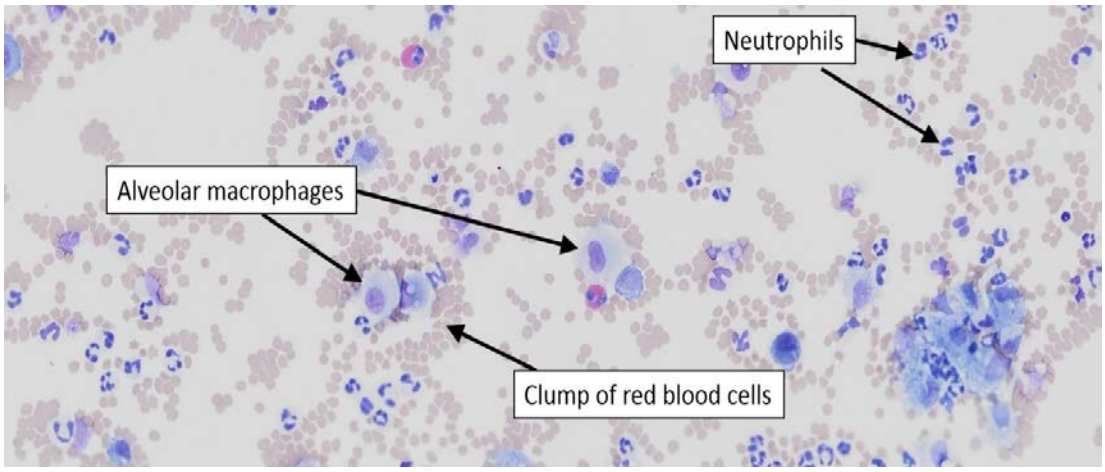


Figure 5.8 BALF cellular contents from a representative OP+GJ directly-injured (right) lung at 48 hours. This cytospin image shows the presence of numerous neutrophils with the occasional alveolar macrophage. Many red blood cells can also be seen. Cells have been stained with Diffquik® (photo by E Hulse using Olympus slide scanner VS120 at 20x).

The presence of red blood cells (% of 100 counted cells) within BALF was determined for all minipigs and was 20.4 ± 28.5%. There were no statistically significant differences between groups, but directly-injured lungs from OP+GJ pigs had the greatest RBC count (51± 39% of 100 cells) at 48 hours (Figure 5.8 and 5.9A). The greatest RBC count identified in the indirectly-injured (left) lungs at 48 hours was obtained from the Solv+GJ lungs, 36 ±50% (Figure 5.9B). It is not known whether the OP+GJ direct lung injury high RBC content was due to the pathophysiology, contamination from lavage and biopsy processes, or a combination of these. BALF samples from the indirectly-injured sham, and Solv +GJ, pig lungs at 48 hours had 98% and 97% RBC contamination, respectively. These samples were removed from the total number of cells analysis, but were included for a BALF white cell count (WCC) differential count. The high RBC contamination in these samples may have resulted from the lung biopsy and lavage in the directly-injured right lung (prior to indirect left lung sampling) causing a bloody residual lavage wash to be vented into the left lung through positive pressure ventilation.

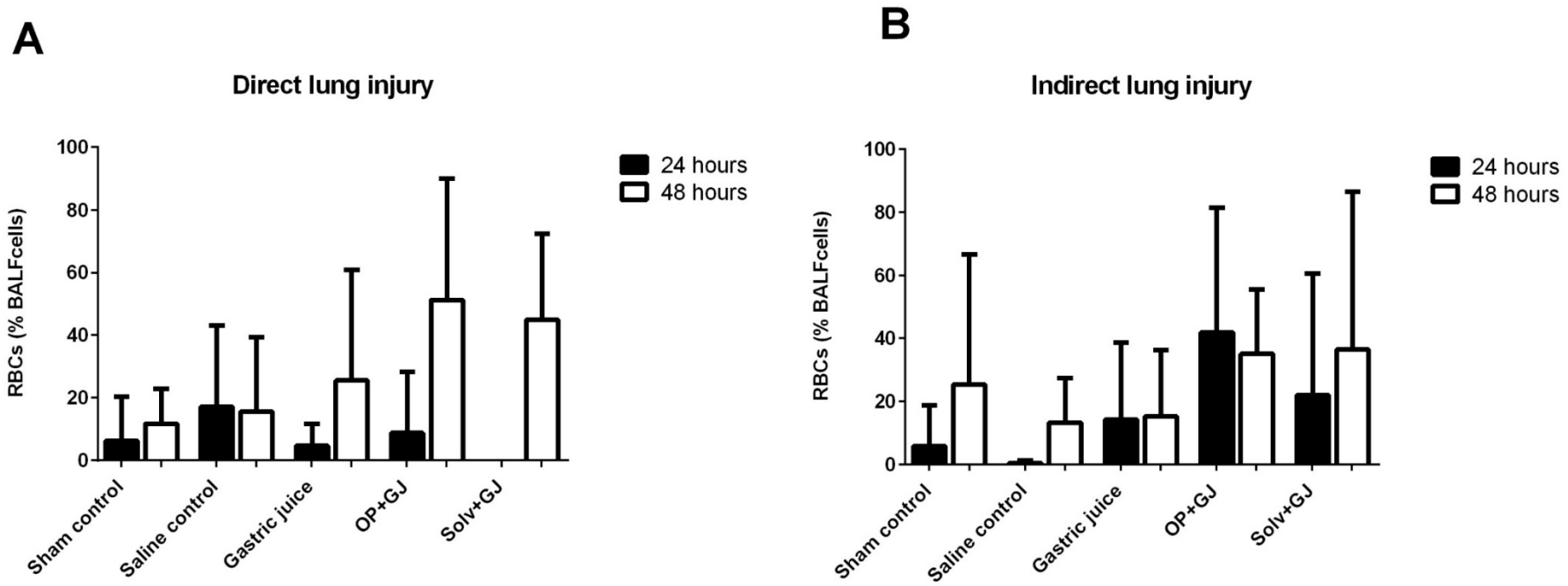


Figure 5.9: The presence of red blood cells in the BALF from minipigs receiving pulmonary treatments. Red blood cells (RBC) within the BALF of samples at 24 and 48 hours in the (A) directly-injured (right) and (B) indirectly-injured (left) lungs. The graph represents the percentage of BALF cells that are red blood cells in a count of 100 cells. No statistical significance was found between the groups. Mean and SD shown.

5.3.5.2 Bronchoalveolar lavage fluid cell numbers are increased after pulmonary installation of GJ, OP+GJ or Solv+GJ when compared with controls

The GJ pigs had the greatest number of BALF cell numbers (red and white cells) at 24 hours in both the directly and indirectly-injured lungs, and significantly more than the right sham ($p=0.02$) or saline ($p=0.02$) lungs (Table 5.3). By 48 hours, OP+GJ and GJ pigs had the greatest number of cells in the directly-injured (right) lungs although not significantly more than the other groups (Figure 5.10A). Although not significant, the greatest number of cells in the left lung at 48 hours belonged to the Solv+GJ and OP+GJ groups.

5.3.5.3 OP+GJ placement in the directly-injured lung causes bilateral pulmonary neutrophilia at 24 hours

OP+GJ, GJ and Solv+GJ treated pigs had a significantly greater percentage of neutrophils ($p \leq 0.01$) in the directly-injured lung at 24 hours than sham control pigs, but this difference disappeared at 48 hours (Figure 5.11 A). This was due to the substantial increase in control BALF neutrophil numbers in both lungs between 24-48 hours, probably as a result of bilateral BAL and biopsy at 24 hours.

In the indirectly-injured (left) lungs, OP+GJ had the greatest number of neutrophils at 24 and 48 hours, although the differences were not statistically significant (Figure 5.11 B; Table 5.3). GJ, and Solv+GJ pigs, but not OP+GJ treated pigs, had statistically lower percentages ($p=0.016$) of neutrophils in the left rather than right lungs at 24 hours. Increased neutrophils present in the OP+GJ indirectly-injured lung could represent a mechanism of lung injury secondary to systemic OP poisoning. It indicates that something - possibly IL-8 released from damaged pulmonary tissue e.g. endothelium, macrophages - caused neutrophils to migrate from the circulation into the indirectly-injured (left) lung as part of an inflammatory process (Baggiolini and Clarklewis, 1992).

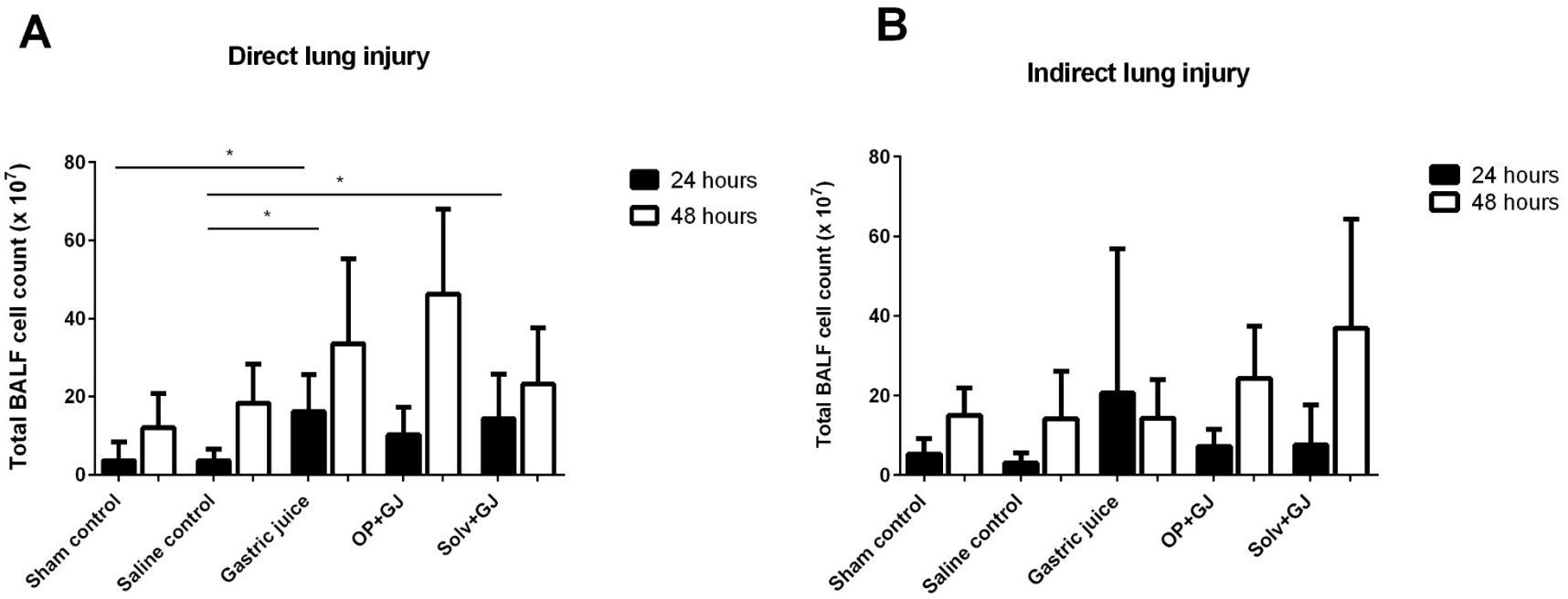


Figure 5.10: Total BALF cell counts from the (A) directly-injured (right) and (B) indirectly-injured (left) lungs at 24 and 48 hours. Kruskal-Wallis analysis of right or left lungs showed that only the difference between right lung groups at 24 hours (p=0.025) was significant. There was a significant difference between right and left lungs at 24 hours (p=0.03), but not 48 hours. Data from one sham and one Solv+GJ left lung (48 hours) BAL has been omitted from the graph and data analysis due to gross blood contamination. Post hoc analysis of directly-injured (right) lung 24 hour groups using permutation tests are shown above. The graphs show mean and SD.

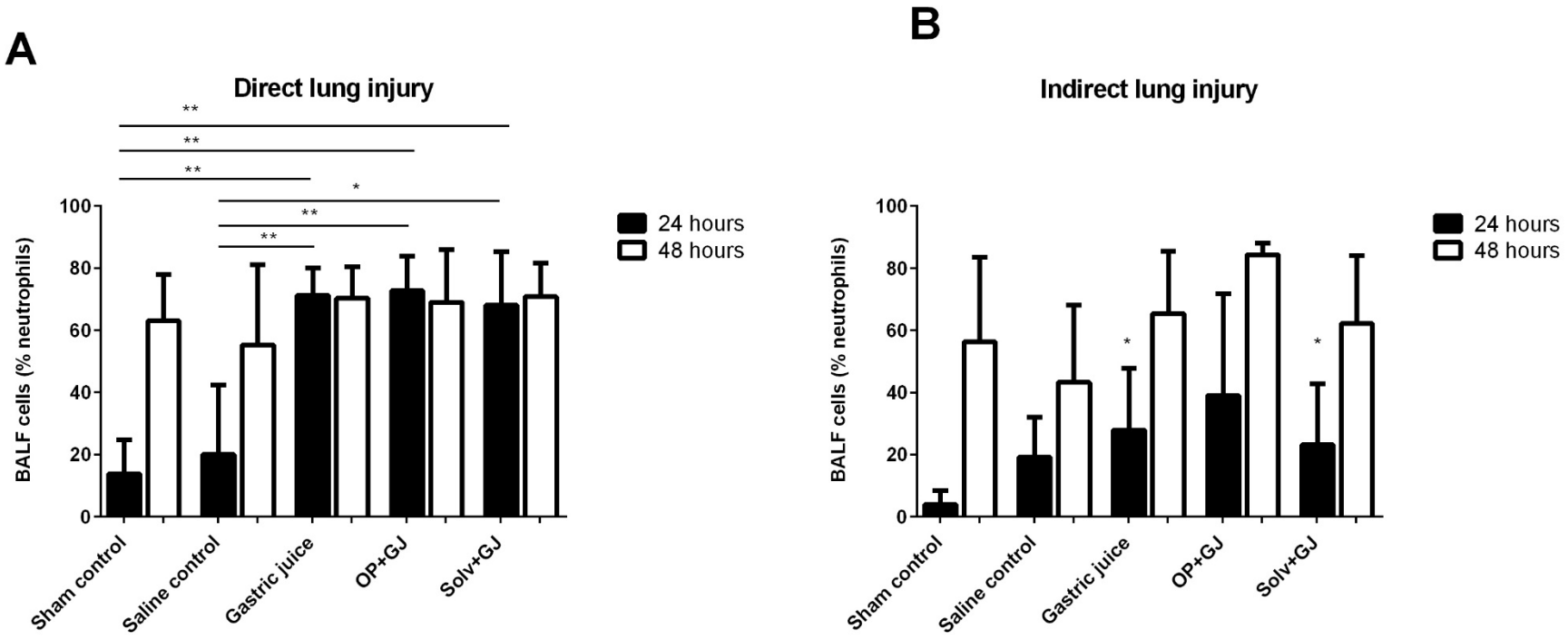


Figure 5.11 Percentage of BALF white cells that are neutrophils from the (A) directly-injured (right) and (B) indirectly-injured (left) lungs at 24 and 48 hours. Kruskal-Wallis analysis showed that only directly-injured lungs at 24 hours were significantly different (p= 0.002) between groups. There was a significant difference between right and left lungs at 24 hours (p= 0.0002) with post hoc permutation testing showing a significant difference between the GJ (p=0.016) and Solv + GJ (p=0.016) pig lungs, but not at 48 hours (shown by the asteriks and no bar). Data from one 48 hour indirectly-injured (left) lung from the Solv+GJ group were omitted due to a lost cytospin slide. Post hoc analysis using permutation tests are shown above. Graphs show means and SD.

Groups	Cell numbers x 10^7	Neutrophils (% of BALF WCs)	Total protein (mg/L)	Albumin (mg/L)	SP-D (ng/mL)	IL-6 (pcg/mL)	IL-8 (ng/mL)	CRP (ng/mL)
Sham 24 hrs (D)	3.6 (4.7)	13.8(10.9)	195.8 (182.8)	53.6 (64.8)	177 (54.3)	101(132.8)	0.34 (0.31)	20.1 (42.1)
Sham 24 hrs (I)	5.3 (3.9)	4.0 (4.4)	156 (142.4)	61.4 (62.8)	n/a	n/a	n/a	n/a
Sham 48 hrs (D)	12 (8.8)	63 (15.0)	813 (232.4)	187.2 (81.7)	68.6 (15.3)	743.7(377.1)	12.52 (7.8)	7.5 (10.1)
Sham 48 hrs (I)	15 (6.9)	56.3 (27.3)	1031 (818.4)	208.8 (209.4)	n/a	n/a	n/a	n/a
Saline 24 hrs (D)	3.6 (2.9)	20.1 (22.4)	226.6 (176.4)	85.4 (70.1)	111.1 (22.2)	206.1 (284.2)	0.57 (0.6)	1.6 (1.0)
Saline 24 hrs (I)	3.1 (2.5)	19.2 (12.9)	137.0 (101)	60 (49.3)	n/a	n/a	n/a	n/a
Saline 48 hrs (D)	18.3 (10)	55.3 (25.8)	1178 (671.8)	311 (202.8)	70 (29.3)	1419(1255)	29.04 (23.9)	29.8 (59)
Saline 48 hrs (I)	14.2 (11.9)	43.3 (24.9)	1076 (1080)	218 (219.3)	n/a	n/a	n/a	n/a
GJ 24 hrs (D)	16.2 (9.4)*, #	71.2 (8.9)**, ##	2967 (1619)**, ##	734.4 (368.1)*, #	79.9 (56.2)	4189 (2323)**, ##	38.8 (38.9)**, ##	130.1 (158)##
GJ 24 hrs (I)	20.6 (36.2)	27.9 (19.9)	464 (334.2)	153.6 (130)	n/a	531.9 (603.4)	5.0 (3.9)**	16.7 (16.0)
GJ 48 hrs (D)	33.5 (21.8)	70.3 (10.0)	1916 (630.5)**	555.2 (237.7)*	51.3 (13.5)	1820 (923.7)	39.2 (29.7)	61 (53.1)*

GJ 48 hrs (I)	14.3 (9.7)	65.3 (20.2)	1475 (1544)	280.8 (328.4)	n/a	1629 (2255)	26.0 (31.8)	62.3 (59.5)
OP+GJ 24 hrs (D)	10.2 (7.0)	72.7 (11.2)**, ##	1751 (902.3)*, #	549.6 (297.8)*, #	102.5 (21.6)	2796 (1228)**, ##	8.9 (3.4)**, ##	337.2 (323.6)*, ##
OP+GJ 24 hrs (I)	7.23 (4.2)	39 (32.8)	1842 (3692)	355.2 (625.6)	n/a	1615 (2822)	4.6 (7.0)	215.7 (404.8)
OP+GJ 48 hrs (D)	46.3 (21.7)	69.0 (17.0)	3032 (1036)**, #	776.8 (211.8)**, #	48.7 (13.7)	2993 (1884)	16.2 (17.4)	580.9 (584.3)*, #, $
OP+GJ 48 hrs (I)	24.3 (13.1)	84.3 (3.7)	2194 (1259)	499.2 (236.7)	n/a	1877 (1573)	6.7 (4.7)	462.9 (706.9)**, $
Solv+GJ 24 hrs (D)	14.4 (11.4)#	68.1 (17.1)**, #	1059 (494.7)*. #	360.8 (165.3)**, ##	91.5 (63.4)	1228 (828)**, ##, $	3.8 (1.5)**, ##, $$, @	34.3 (27.3)##, @@
Solv+GJ 24 hrs (I)	7.5 (10)	23.1 (19.7)	343.8 (193.6)	112 (74)	n/a	243.9 (414.9)	1.5 (0.8)*	6.54 (5.8)
Solv+GJ 48 hrs (D)	23.2 (14.4)	70.8 (10.7)	2296 (436.1)**, #	632 (127.5)**, #	56.2 (17.4)	1889 (1026)	19.22 (17.2)	75.2 (118.8)@
Solv+GJ 48 hrs (I)	36.9 (27.4)	62.1 (22.0)	1757 (1099)	407.2 (299.4)	n/a	2031 (1931)	23 (20.2)	339.2 (440.6)*

Table 5.3: BALF data for all measured cellular and non-cellular contents in minipigs receiving pulmonary treatments. Mean (±SD) shown. Statistically significant differences between groups: * group vs. sham control, # group vs. saline control, $ group vs. GJ, @ group vs. OP+GJ at similar time points. BALF: bronchoalveolar lavage fluid, WC: white cell, D:direct lung (right) injury, I:indirect lung (left) injury, n/a: not available.

5.3.5.4 A rise in pulmonary neutrophils is mirrored by a non-significant reduction in plasma neutrophils

The concentration of neutrophils in plasma was measured to determine whether the rise in BALF neutrophils was mirrored by a decrease in the plasma concentration. There was a decrease in the plasma neutrophil concentration over time in most groups, which was not statistically significant (Figure 5.12).

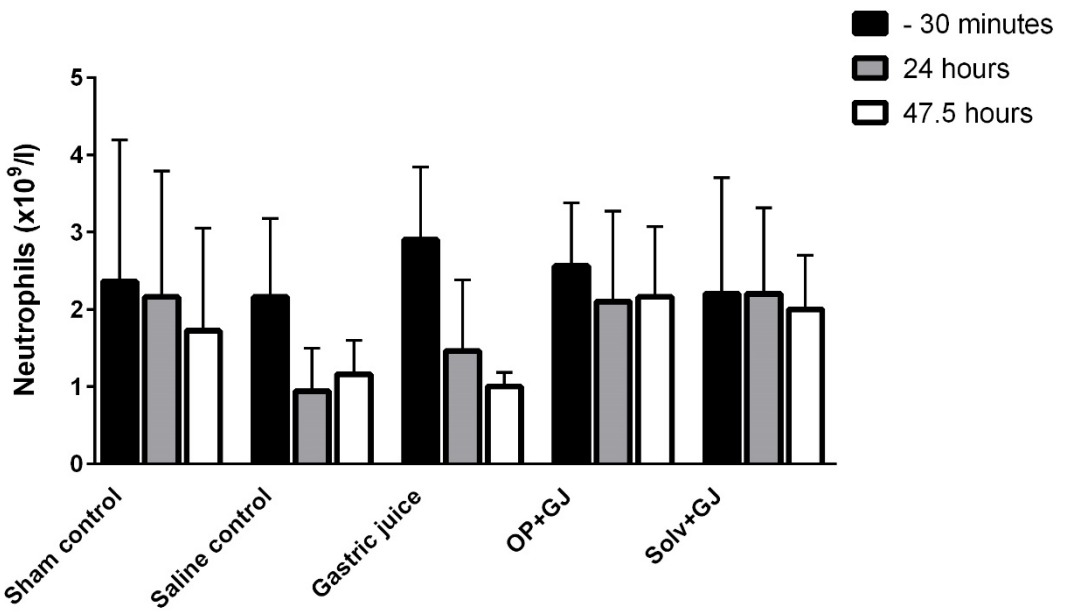


Figure 5.12: Concentration of neutrophils in the plasma in all groups at -30 min, 24 and 47.5 hours. One plasma sample result from Solv+GJ 47.5 hours was lost by the testing laboratory. Graph shows mean ± SD.

5.3.5.5 Increased total protein from minipig lungs that have had pulmonary installation of GJ, OP+GJ or Solv+GJ

Pulmonary inflammation with the breakdown of the alveolar capillary membrane signals the development of ARDS and can be demonstrated through an increase in BALF total protein and albumin (Ware and Matthay, 2000). Therefore, non-cellular BALF proteins were measured in the minipig model.

Total BALF protein and albumin concentrations were statistically greater in those pigs that had had pulmonary installation of GJ, OP+GJ or Solv+GJ compared with sham controls at both 24

and 48 hours for the directly-injured (right) but not the indirectly-injured (left) lungs (Figure 5.13, Figure 5.14).

GJ treated pigs had the greatest mean concentration of protein (3000 mg/L) and albumin (730 mg/L) at 24 hours in the directly-injured (right) lungs. By 48 hours, OP+GJ had the greatest mean concentration of protein (3000 mg/L) and albumin (780 mg/L), with GJ reducing to approximate OP+GJ 24 hour levels in the directly-injured lungs (Table 5.3). Although these differences were not significant, it suggests that GJ had a peak injury effect at 24 hours, with the OP+GJ lung injury peaking at 48 hours, or may in fact still have been developing.

OP+GJ treated pigs had the greatest concentration of protein and albumin in the indirectly-injured (left) lungs at both 24 and 48 hours. Also, GJ and Solv+GJ, but not OP+GJ treated pigs, had a statistically lower concentration of protein and albumin in the indirectly-injured lungs at 24 hours. This is further evidence that systemic OP pesticide poisoning created a measureable indirect lung injury.

Although not statistically significant, BALF from both lungs of Solv+GJ treated pigs had consistently less protein than OP+GJ at 24 and 48 hours suggesting that the OP-induced pulmonary damage was not caused by the cyclohexanone component of the dimethoate EC40. Solv+GJ treated pigs also had lower protein and albumin in both lungs when compared with GJ at 24 hours indicating that cyclohexanone may have had some protective qualities in the context of GJ aspiration in the first 24 hours. This effect was lost at 48 hours possibly due to repeated BAL and biopsy, or it being metabolised and/or utilised. Perhaps the solvent cyclohexanone was acting as a protective barrier on the intact alveolar epithelium, preventing movement of protein across the membrane, or reducing breaks in the alveolar capillary membrane?

It must be noted that protein from BAL contamination with blood (see RBC presence in BALF above), could have contributed to the elevated BALF total protein and albumin (Morrison et al., 1986) present in all groups, especially OP+GJ.

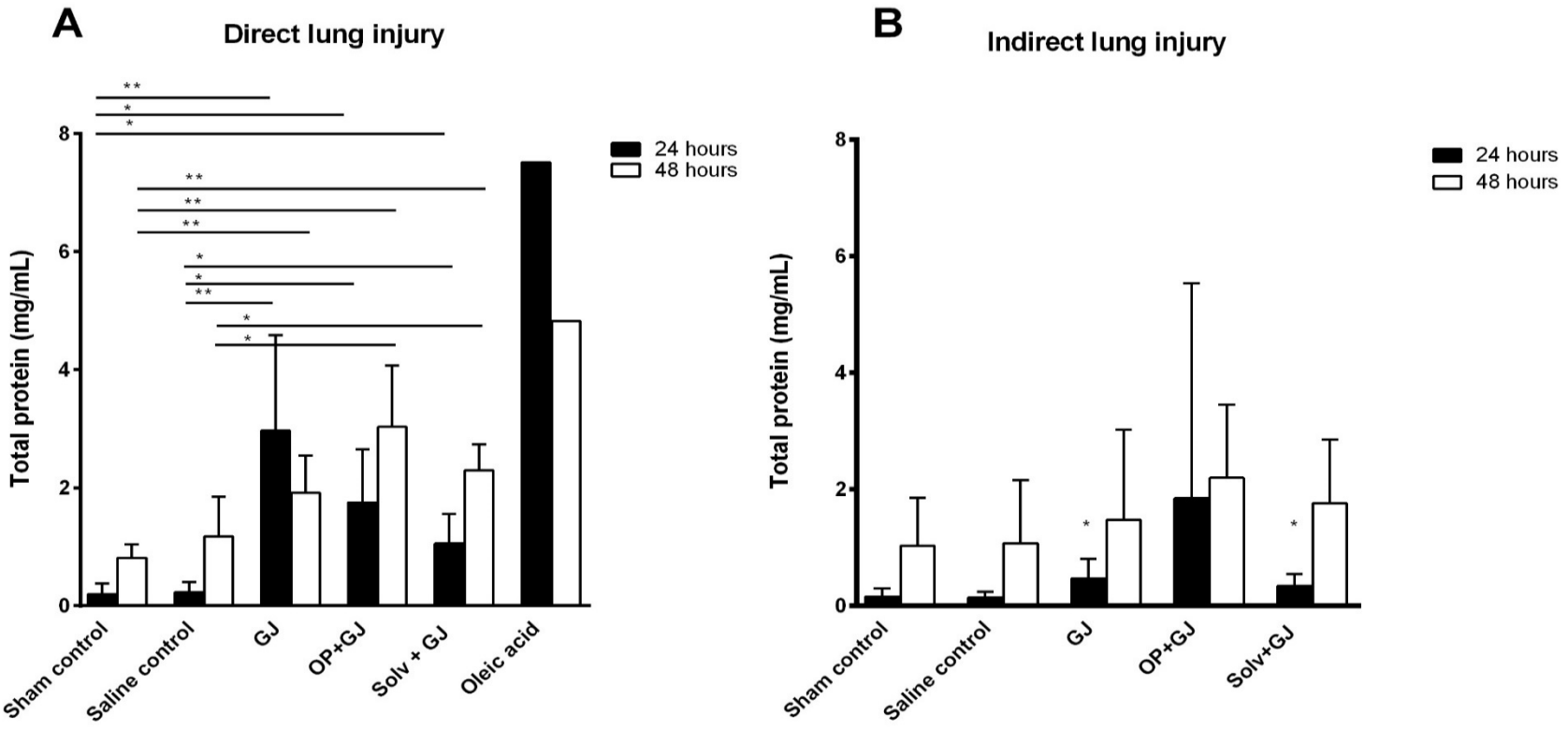


Figure 5.13 Protein concentrations in the BALF of minipigs receiving pulmonary treatments. BALF total protein in (A) directly-injured (right) and (B) indirectly-injured (left) lungs at 24 and 48 hours. Kruskal-Wallis analysis of right lungs at 24 (p=0.0022) or 48 (p=0.0023) hours was significantly different between groups, but not for left lungs. Right and left lungs at 24 (p=0.0004) and 48 (p=0.0223) hours were significantly different with post hoc tests (asteriks no bars) showing GJ and Solv+GJ lungs were significantly different at 24 hours (p=0.0238), but not at 48 hours. Post hoc analysis using permutation tests are detailed above. Mean and SD shown.

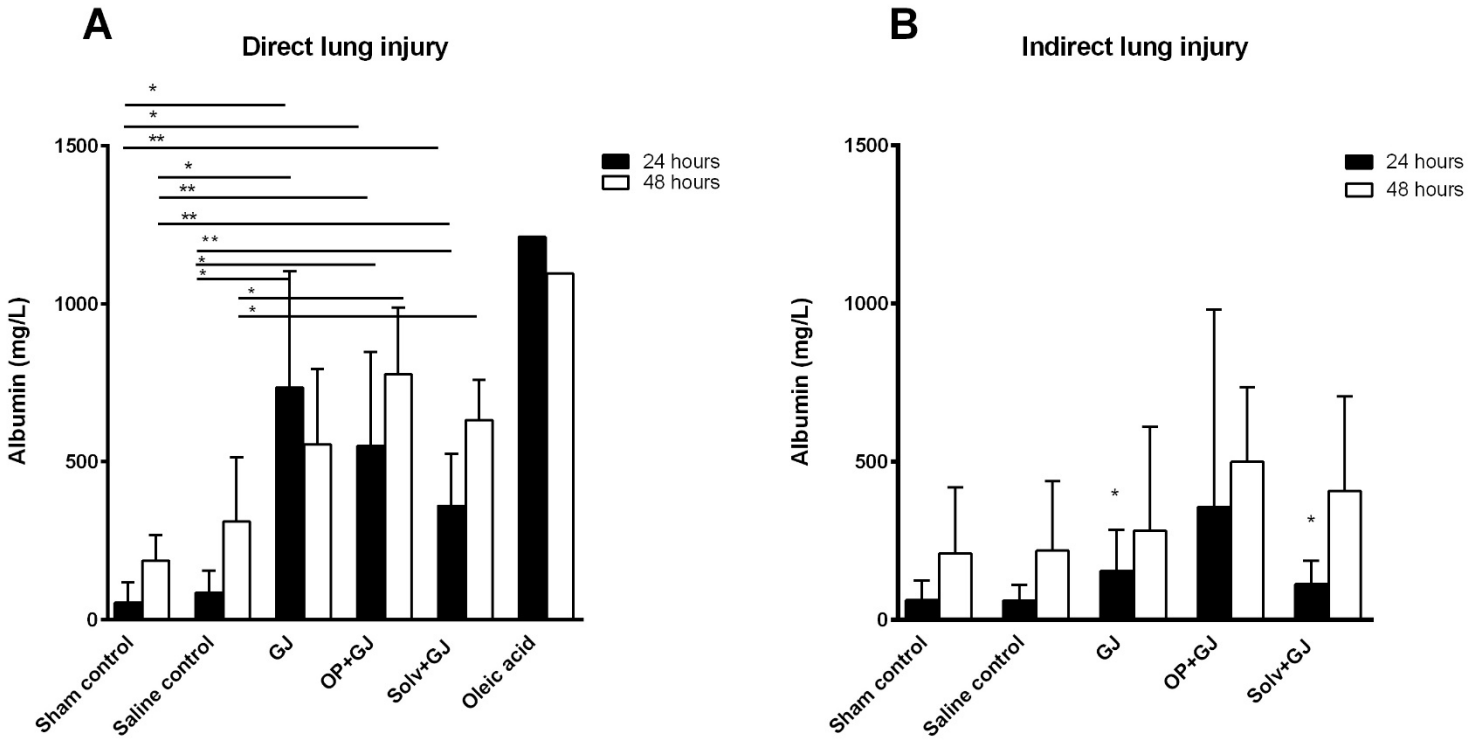


Figure 5.14: Albumin concentrations in the BALF of minipigs receiving pulmonary treatments. Albumin in BALF from (A) directly-injured (right) and (B) indirectly-injured (left) lungs at 24 and 48 hours. Kruskal-Wallis analysis of right lungs of all groups at 24 (p=0.0032) or 48 (p=0.0041) hours was significant between groups, but not for left lungs. Right and left lungs at 24 (p=0.0018) and 48 (p= 0.0085) hours had a significant difference, post hoc tests (asteriks no bars) showed GJ (p=0.0238) and Solv+GJ (p=0.0159) lungs showed a difference at 24 hours, but not at 48 hours. Post hoc analysis using permutation tests detailed as above. Mean and SD shown.

5.3.5.6 Specific markers for damage to the alveolar capillary membrane failed to show significant differences between groups

5.3.5.6.1 Receptor for advanced glycation end products (RAGE)

The receptor for advanced glycation end-products (RAGE) is multi-ligand cell surface protein predominantly found in the basal membrane of the type 1 alveolar epithelial cells and not in the lung micro-vasculature (Uchida et al., 2006). RAGE ligands can trigger the pro-inflammatory nuclear factor κB (NF-κB) pathway and subsequent cytokine release leading to tissue injury (Chavakis et al., 2004).

Significant increases in the soluble form of RAGE (produced by the membrane bound RAGE (Raucci et al., 2008)) have been observed in BALF from rats in response to 10mg/kg intra-tracheal hydrochloric acid and in BALF and plasma from humans with lung injury compared with controls (Uchida et al., 2006, Ware et al., 2013). Other types of lung injury e.g. hyperoxia and/or hypoxia have also shown increased plasma RAGE in animal models (Gopal et al., 2015). ARDS patients show dynamic reductions in plasma RAGE (n=24) with the application of ventilatory manoeuvres designed to improve oxygenation in humans (Jabaudon et al., 2015). Thus it was hypothesised that plasma RAGE might be a good measure of type 1 alveolar epithelial injury from aspiration of GJ mixtures.

Two ELISA experiments were conducted on consecutive days to measure both the 24 and 48 hour plasma RAGE in all minipigs. PBS blanks from the 24 hour sample ELISA plate had to be used to correct both plates (24 and 48 hour plates) due to conjugate being accidentally placed into the 48 hour sample blanks. Although these ELISA experiments were conducted in the same location on subsequent days, this action could have caused slight variations in the results of the 48 hour plasma RAGE samples.

Despite this, we found the lowest concentrations of plasma RAGE in the OP+GJ and Solv+GJ treated pigs when compared with the other groups at similar time points, but with no statistical difference (Figure 5.15).

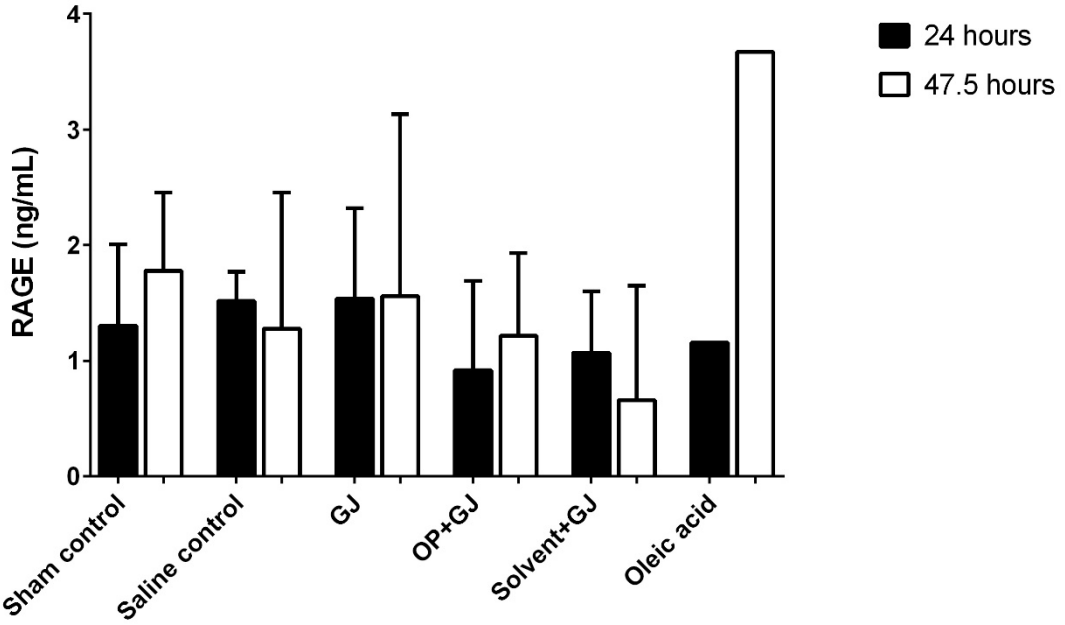


Figure 5.15: Concentrations of plasma receptor for advanced glycation end products (RAGE) in all minipigs at 24 and 47.5 hours. Kruskal-Wallis analysis of data was insignificant for 24 and 47.5 hours. Mean and SD shown.

5.3.5.6.2 Surfactant protein D (SP-D)

Surfactant protein D is produced by type 2 alveolar epithelial cells and is important for the lung's host defence response, protecting against infection and inflammation (Nayak et al., 2012). Increased plasma with reciprocal reduced BALF SP-D concentrations (possibly due to leakage from the lung into the capillaries) is found in a range of lung diseases and lung injuries (Eisner et al., 2003, Cheng et al., 2003). Lower concentrations of SP-D in BALF have been observed in non-survivors of ARDS possibly contributing to dysfunction in surfactant homeostasis and increasing the chances of pulmonary infection and inflammation (Greene et al., 1999, Cheng et al., 2003).

Lungs directly-injured with OP+GJ had the lowest concentration of BALF SP-D at 48 hours (49±14 ng/mL) compared with GJ (51±14 ng/mL) or Solv+GJ (56±17 ng/mL) although the differences were not statistically significant (Figure 5.16;Table 5.3).

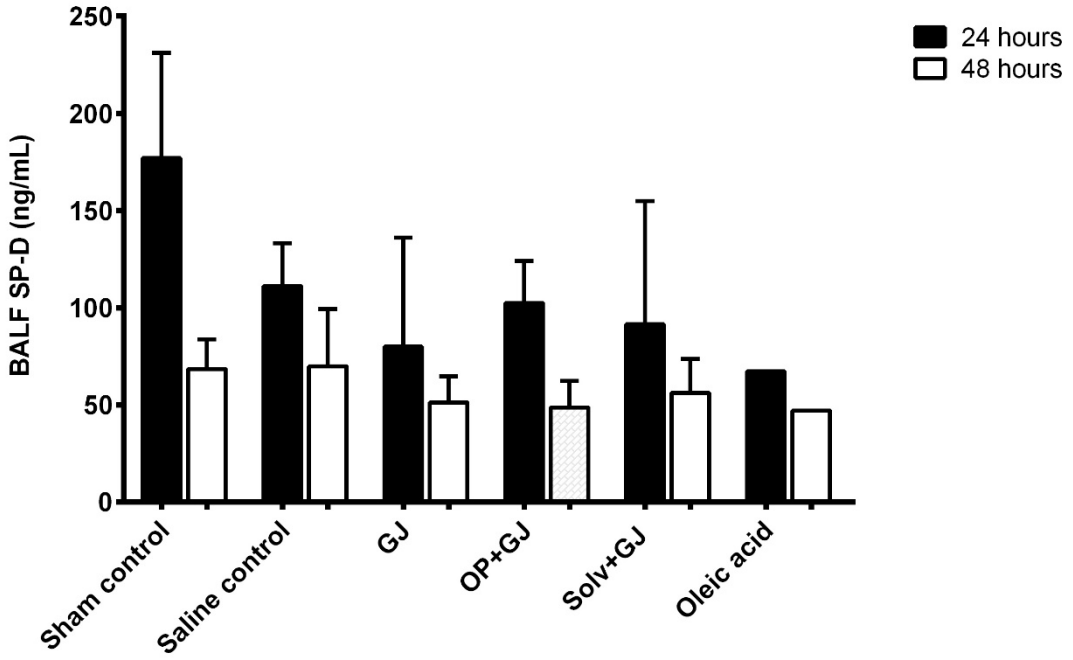


Figure 5.16: Concentrations of surfactant protein D (SP-D) in BALF from all minipigs at 24 and 48 hours in directly-injured (right) lungs. Kruskal-Wallis analysis found no significant difference between the groups. Mean and SD shown.

5.3.5.6.3 Von Willebrand factor (vWF)

Von Willebrand factor (vWF) is an antigen released mainly by the vascular endothelium and is required for blood clotting and platelet adhesion. Increased plasma vWF concentrations (indicating endothelial activation or injury) are associated with increased multi organ failure and mortality in patients with ARDS (Ware et al., 2004). Tests conducted on 48 hour plasma samples displayed undetectable concentrations of vWf in all but one OP+GJ treated pig and so results are not shown.

5.3.5.7 Pulmonary installation of GJ, OP+GJ or Solv+GJ causes significant increases in BALF IL-8, IL-6 and CRP at 24 hours

In the context of gastric aspiration, the pro-inflammatory IL-8 appears to be the main cytokine causing neutrophil recruitment and activation with subsequent lung injury (Folkesson et al., 1995, Inci et al., 2008, Meers et al., 2011a).

Interleukin 6 (IL-6) is secreted by endothelium, and other tissues and has both pro and anti-inflammatory roles. It assists in the transition from innate to adaptive immune functions including neutrophil apoptosis and B and T cell differentiation (Scheller et al., 2011).

Statistically significant increases of plasma concentrations of IL-6 and IL-8 have been observed in human patients with ARDS (Ware et al., 2013). Pulmonary aspiration of gastric contents is responsible for 20% of ARDS ICU admissions (Briel et al., 2010). It was therefore important to measure these cytokines in this aspiration study.

C reactive protein (CRP) is an acute phase protein produced in the liver and to a lesser extent locally in the lung that is elevated during systemic inflammation in response to cytokines e.g. IL-6, IL-1β and TNF-α. Its main function is to find and remove microorganisms and apoptotic cells through activation of the complement system (Agassandian et al., 2014). CRP levels are raised in ARDS (being greater in survivors) (Bajwa et al., 2009) and systemic OP poisoning (Lee et al., 2013).

5.3.5.7.1 BALF and plasma IL-8 and IL-6 measurement

Concentrations of BALF IL-8 and IL-6 in the directly-injured (right) lung were significantly raised in OP+GJ, GJ and Solv+GJ treated groups when compared with either sham or saline controls at 24 hours (Figure 5.17, Figure 5.18, Table 5.3).

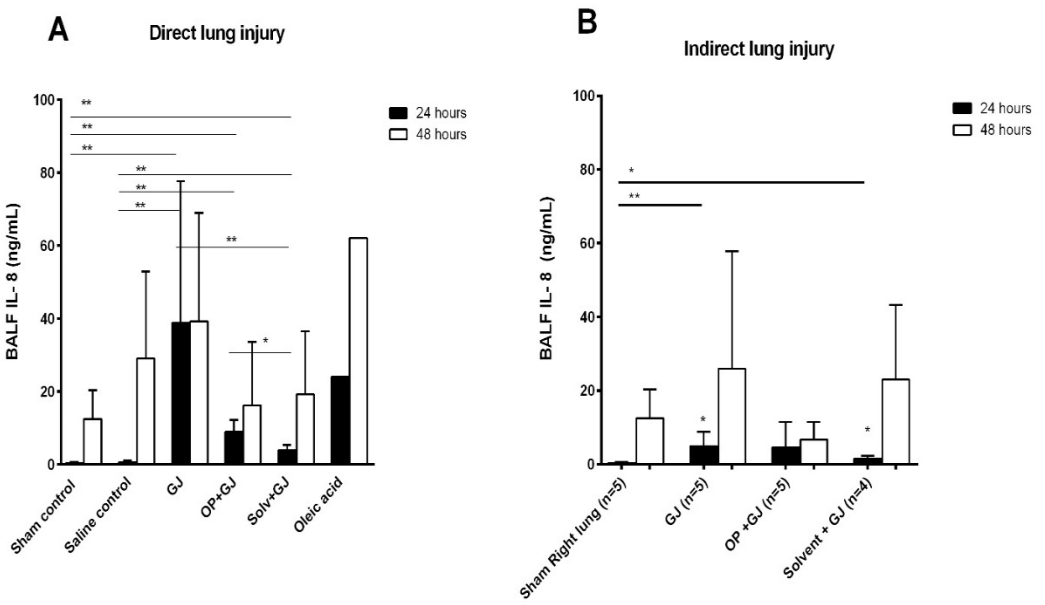


Figure 5.17: BALF interleukin 8 (IL-8) in (A) directly (right) and (B) indirectly injured (left) lungs at 24 and 48 hours. Kruskal-Wallis analysis showed that directly-injured (p=0.0004) and indirectly-injured (p=0.0376) lungs at 24 hrs, but not 48 hours, had significant differences between groups. Comparisons bewteen right and left lungs at 24 (p=0.0067) but not 48 hours had a significant difference. Post hoc tests showed that IL-8 was significantly lower in the indirectly-injured lungs of GJ (p=0.0238) and solv+GJ (p=0.0238) pigs when compared with directly-injured (right) lungs (asterisks no bar). Post hoc analysis using permutation tests is detailed as above. Mean and SD shown on the graph.

In the directly-injured lung, the GJ group had the greatest concentrations of BALF IL-8 at both 24 (39±39 ng/mL) and 48 hours (39±30 ng/mL). In contrast, Solv+GJ treated pigs had the lowest concentration of BALF IL-8 in the directly-injured lungs (4±2 ng/mL), and significantly less so than GJ or OP+GJ treated pigs (9±3 ng/mL), at 24 hours (Figure 5.17 A; Table 5.3). This suggests that the solvent cyclohexanone may have a protective effect in the lung, whether mixed within dimethoate EC40 or on its own with GJ, thereby causing reduced BALF IL-8 concentrations.

The IL- 8 concentrations within the indirectly-injured (left) lungs of both GJ (5±4 ng/mL) and Solv+GJ (2±1 ng/mL) groups were significantly raised when compared with representative sham control lungs (0.3±0.3 ng/mL), but were significantly lower than their contralateral directly-injured lungs (unlike OP+GJ lungs (5±7 ng/mL)) at 24 hours (Figure 5.17 B).

Plasma IL-8, but not BALF IL-8, was un-recordable suggesting secretion of IL-8 was produced predominantly by local lung parenchyma or infiltrating cells e.g. alveolar macrophages, rather than from a systemic inflammatory reaction. It is possible that the pulmonary inflammatory reaction/damage caused by aspiration was not great enough to produce raised plasma IL-8 concentrations.

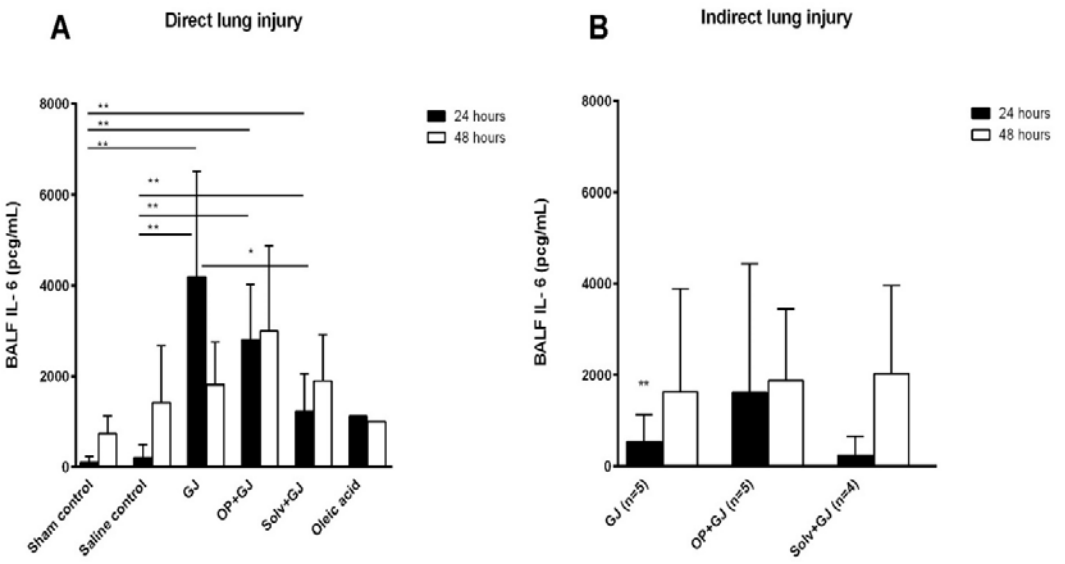


Figure 5.18: BALF interleukin 6 (IL-6) in (A) directly-injured (right) and (B) indirectly-injured (left) lungs at 24 and 48 hours. Kruskal-Wallis analysis between right and left lungs showed that only the right lungs at 24 hrs (p=0.0006) were significantly different between groups. Right and left lungs at 24 (p=0.0062) but not 48 hours had a significant difference. Post hoc tests showed that IL-6 was significantly lower in the indirectly-injured lung of GJ (p=0.0079) pigs than the directly-injured (right) lungs (asterisk no bar). Post hoc analysis using permutation tests is detailed as above. Mean and SD shown on the graph.

In the directly-injured (right) lungs, GJ treated pigs had the greatest mean concentration of IL-6 at 24 hours (4200 pcg/mL) which reduced to control animal levels at 48 hours (1820 pcg/mL). In contrast, OP+GJ treated pigs had the highest concentration of IL-6 at 48 hours (3000 pcg/mL) suggesting an ongoing inflammatory process (Figure 5.18 A).

Solv+GJ treated pigs had significantly less BALF IL-6 in the directly-injured (right) lung (1200 pcg/mL) when compared with GJ treated pigs at 24 hours, suggesting a protective effect of the solvent cyclohexanone. Only GJ treated pigs had a statistically lower concentration of IL-6 in the indirectly-injured (left) lungs (530 pcg/mL) compared with the right lung at 24 hours. This

signified that an indirect lung injury was unlikely in this group (Figure 5.18 B). Whereas OP+GJ pigs had higher concentrations (1615 pcg/mL) of IL-6 at 24 hours signifying indirect lung injury. By 48 hours all groups had raised IL-6 in the indirectly-injured lung, possibly due to the effects of recurrent lavage and biopsy (Table 5.3).

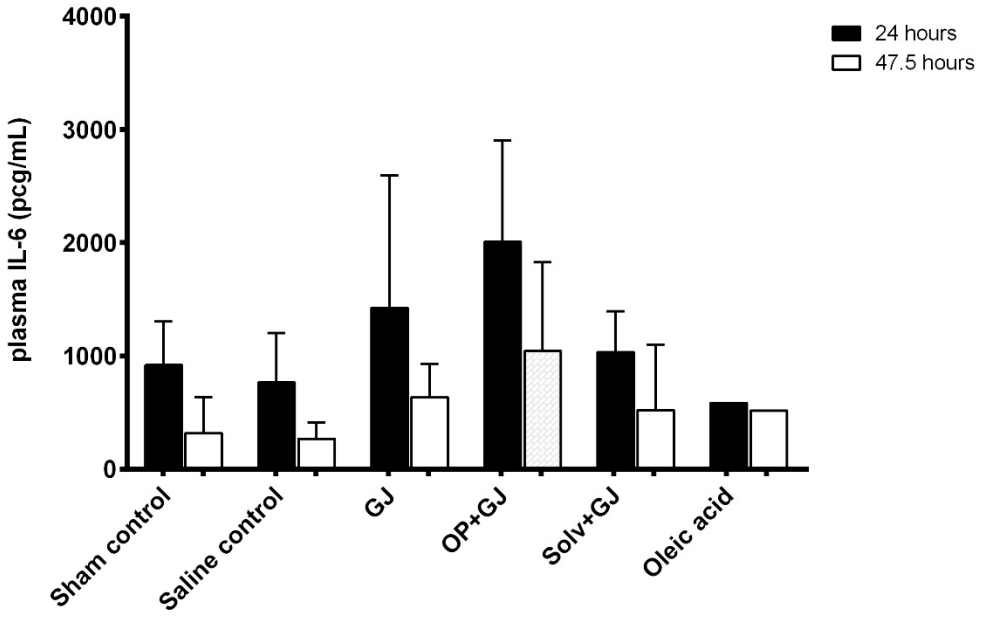


Figure 5.19: Concentrations of plasma interleukin 6 (IL-6) in minipigs at 24 and 47.5 hours. Kruskal-Wallis analysis of group data was insignificant for 24 and 47.5 hours. Mean and SD shown.

Plasma concentrations of IL-6 were greatest in OP+GJ treated pigs at 24 and 47.5 hours, but not statistically significant (Figure 5.19).

5.3.5.7.2 CRP

Mean CRP concentrations in BALF were significantly raised in OP+GJ (340 ng/mL), GJ (130 ng/mL) and Solv+GJ (34 ng/mL) directly-injured lungs when compared with saline controls (1.6 ng/mL) (p=0.0079) at 24 hours. Only OP+GJ directly-injured lungs (p=0.0159) also had significantly raised CRP levels compared with sham (20 ng/mL) control at 24 hours (Figure 5.20 A). Notably, the OP+GJ treated pigs had significantly greater CRP concentrations in the directly-injured lungs at 48 hours (580 ng/mL) than any other group (including GJ and Solv+GJ groups), implying marked inflammation.

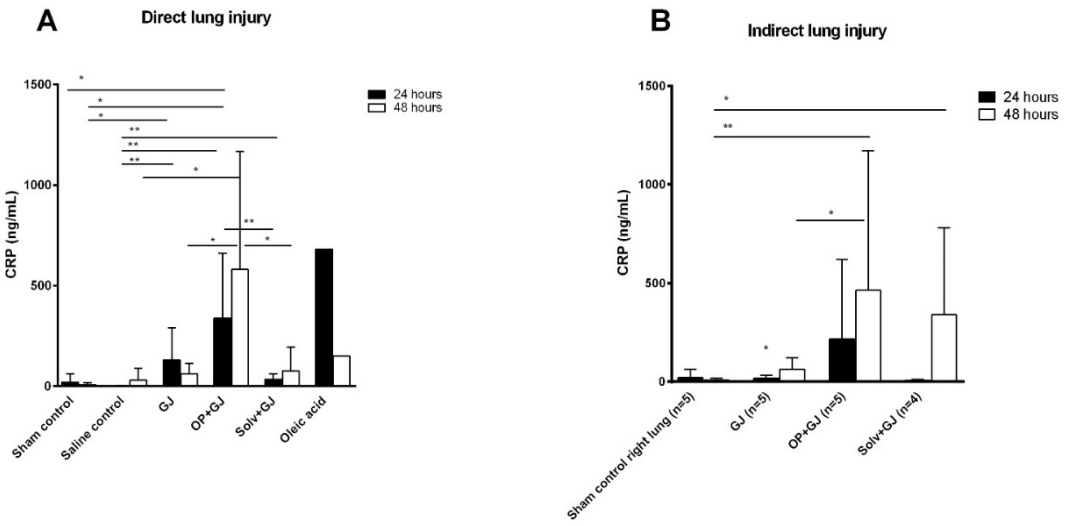


Figure 5.20: Concentrations of BALF C-reactive protein (CRP) in (A) directly-injured (right) and (B) indirectly-injured (left) lungs at 24 and 48 hours. Kruskal-Wallis analysis showed that the differences between groups in the right (p=0.002) lungs at 24 hrs, and right (p=0.0124) and left (p=0.0105) lungs at 48 hours were significant. Right and left lungs at 24 (p=0.0028) but not 48 hours had a significant difference. Post hoc tests showed that CRP was significantly lower in the indirectly-injured (left) (p=0.0159) than the directly-injured lungs of GJ pigs (asterisk no bar). Post hoc analysis using permutation tests is detailed as above. Mean and SD shown on the graph.

Solv+GJ treated pigs had significantly less BALF CRP when compared with OP+GJ treated pigs at 24 hours (34 ng/mL vs. 340 ng/mL; p=0.0079) and 48 hours (75 ng/mL vs. 580 ng/mL; p=0.0317) in directly-injured lungs (Figure 5.20 A). This echoes previous findings and suggests that cyclohexanone is not responsible for the damage observed in dimethoate EC40 aspiration (as represented here by the OP+GJ injury), and that the solvent may have an anti-inflammatory effect on the lung. Although the results show that Solv+GJ directly-injured lungs had less CRP compared with GJ treated pigs at 24 hours, this finding was non-significant (unlike IL-6 and IL-8) with similar concentrations in both groups by 48 hours. This could mean that cyclohexanone and GJ mixtures cause a similar amount of lung inflammation as GJ alone, or that the beneficial effects of topical (lung) cyclohexanone, in the context of GJ aspiration, had decreased by 48 hours.

OP+GJ induced the greatest concentrations of CRP at both 24 and 48 hours in the indirectly-injured lung, with GJ treated pigs having significantly less than OP+GJ (p=0.0476) at 48 hours. GJ treated pigs also had a significant reduction (p=0.0159) of CRP in the indirectly-injured lungs

when compared with the directly-injured lungs at 24 hours (Figure 5.20 B). This emphasises the ability of intrapulmonary OP+GJ to cause an indirect (systemic) lung injury in contrast to the placement of GJ alone.

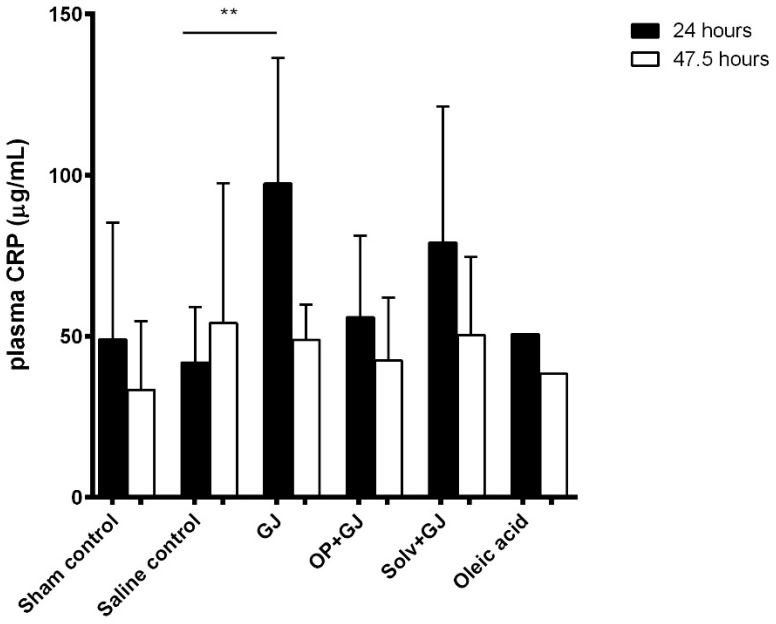


Figure 5.21: Plasma concentration of C-reactive protein (CRP) in minipigs receiving pulmonary treatments. Kruskal-Wallis showed significant difference between groups at 24 hours (p=0.0489) but not 47.5 hours. Post hoc permutation testing showed saline control and GJ treated pigs had a significant difference (p=0.0079) at 24 hours. Mean and SD are shown on the graph.

The only significant finding involving plasma CRP concentrations was that GJ treated pigs had greater concentrations (p=0.0079) compared with saline controls at 24 hours. There were no observed differences between aspiration groups (Figure 5.21). The high BALF and low plasma concentrations of IL-6, IL-8 and CRP suggest that the response to pulmonary installation of GJ mixtures was more localised to the lung, rather than producing a systemic reaction.

5.3.6 Microbiological results from porcine GJ, tracheal aspirates and bronchoalveolar lavage fluid

5.3.6.1 Porcine gastric juice used in the aspiration study had no significant bacterial growth

Fourteen gastric juice aliquots collected in the study were sent for microscopy and culture. Eleven of these samples had colony counts ≤6 despite using neat porcine GJ. In these cases, the colonies present were counted, and the total aerobic count (TAC - cfu/mL) was calculated using the formula above (methods- chapter 5). The TAC of the porcine GJ was 243 (±141) cfu/mL. The individual GJ sample TAC (cfu/mL) can be seen below (Figure 5.22).

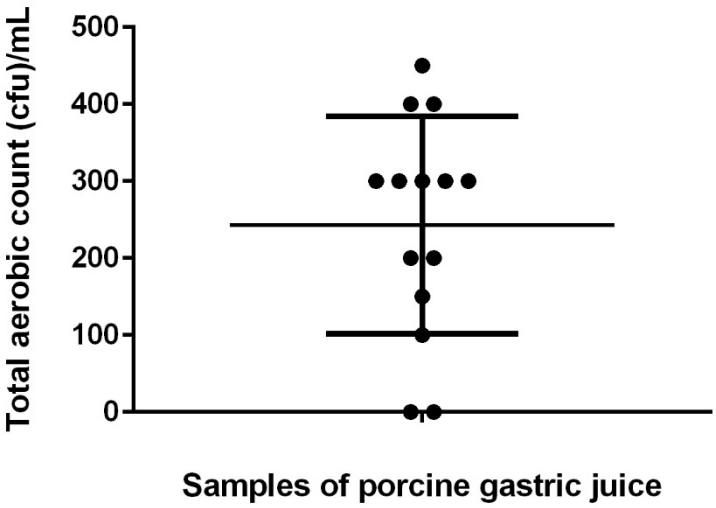


Figure 5.22: Dot plot showing bacterial growth from porcine gastric juice aliquots (n=14) used in the aspiration study. Fourteen GJ samples were sent for aerobic bacterial culture and total aerobic count (TAC) reported as colony forming units (cfu)/mL. Mean and SD shown.

Using the formula for the TAC (cfu/mL = colony number x 50 x dilution factor), it was possible to calculate that the porcine GJ samples had a range of 0-9 colonies per plate. Samples with few colonies usually contain colonies secondary to bacterial contamination e.g. from air, plate, sample container. It is generally accepted that plates with less than 20 colonies per plate can be disregarded due to inaccuracies which occur through plate contamination (Breed and Dotterrer, 1916). Thus the GJ used in the main aspiration study was likely to be bacteria free, and not the source of any observed bacterial growth within the minipig lungs.

Moreover, the GJ sample mainly contained the *Bacillus* species which were morphologically unlike the bacteria seen in the BALF specimens (see below), and therefore unlikely to have caused the high TAC in the aspiration study groups (OP+GJ and GJ groups) at 48 hours.

5.3.6.2 BALF aerobic bacterial numbers raised in GJ and OP+GJ pigs at 48 hours

BALF samples from OP+GJ (p=0.0002), GJ (p=0.0065) and Solv+GJ (p=0.0127) treated pigs had significantly higher numbers of aerobic bacteria at 48 hours when compared with pooled -30 min tracheal samples (Figure 5.23).

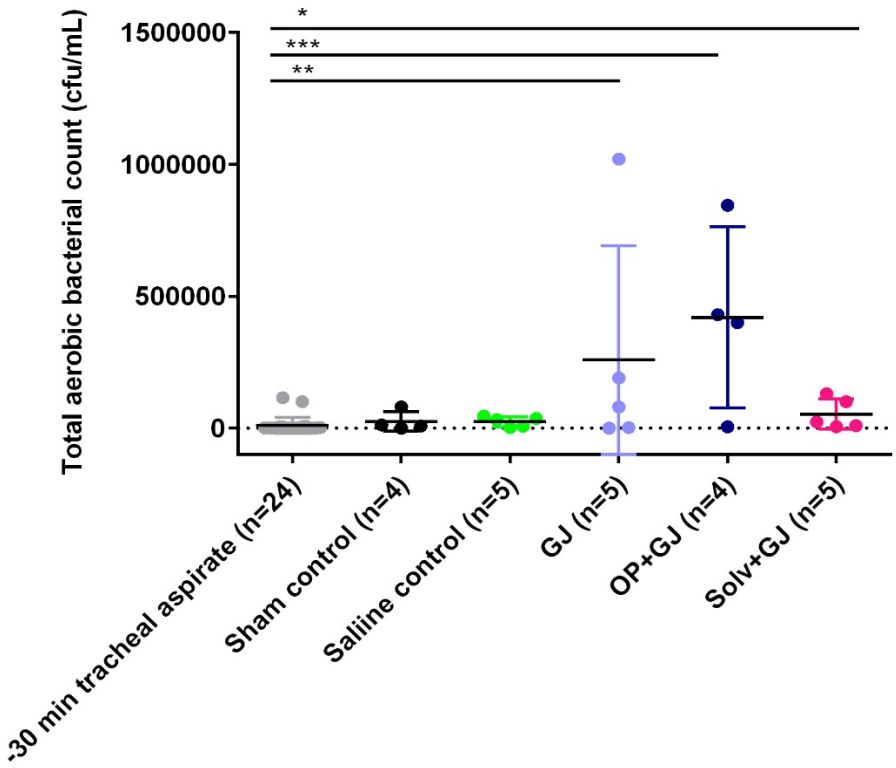


Figure 5.23: Number of aerobic bacteria (cfu/mL) in -30 min tracheal aspirates and 48 hour BALF samples. Kruskal-Wallis showed significant difference between all groups (p=0.0011), which became non-significant when testing only 48 hours BALF samples. Post hoc permutation testing results with Mean and SD are shown on the graph.

Although not statistically significant, OP+GJ and GJ treated pigs had higher numbers of aerobic bacteria at 48 hours in the BALF when compared with the controls and Solv+GJ treated pigs.

The bacteria species found in the 48 hour BALF samples were predominantly *Escherichia coli (E.coli)*, *DNAase negative staphylococcus* and *Klebsiella pneumoniae* (*K.pneumonia)* (Figure

5.24). The organisms present in the greatest numbers in both the GJ and OP+GJ treated pigs were *E. coli* (n=2), *K. pneumonia (*n=2*)*, *Pseudomonas luteola* (n=1) and *Chryseobacterium indologenes (*n=1*)*. *K. pneumonia* and *Pseudomonas luteola* are commonly associated with ventilator associated pneumonias in humans (Park, 2005).

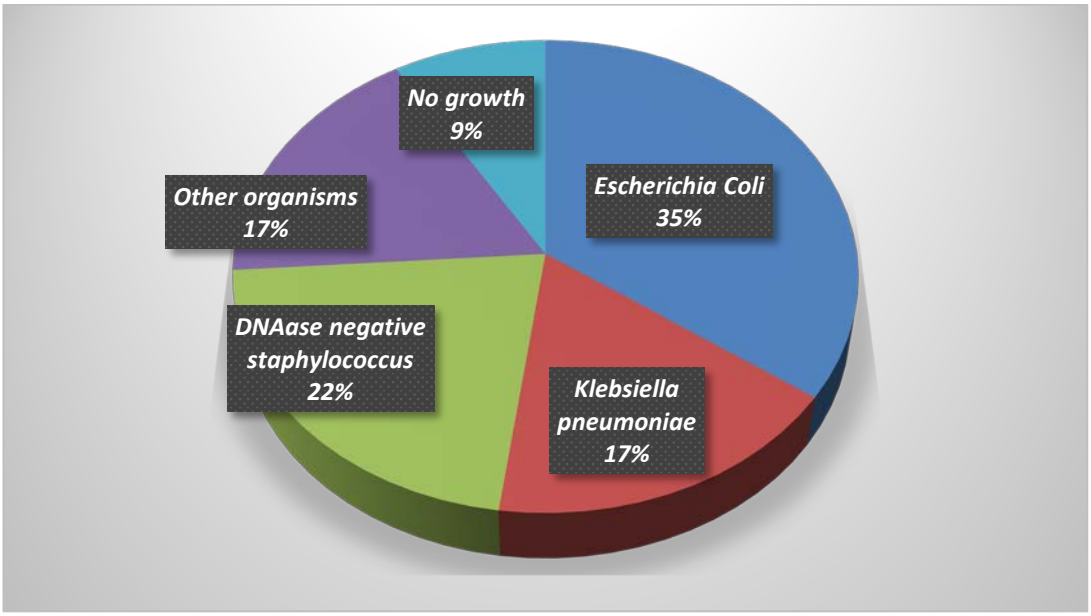


Figure 5.24: Percentage of types of aerobic bacteria grown in 23 minipig 48 hour BALF samples. The pig that received oleic acid grew *E. Coli* (<1000cfu/mL) and is omitted from this graph.

5.3.7 Direct lung injury secondary to OP+GJ pulmonary installation creates a higher percentage of poorly and non-aerated lung tissue than control, GJ, and Solv+GJ minipig lungs

Serial pulmonary CT scans were conducted in 12 minipigs over the 48 hour study period. This allowed measurement, through image analysis, of the direct and indirect lung injuries caused through aspiration of various compounds (see above methods).

Figure 5.25 displays serial pulmonary CT scans from a minipig that had OP+GJ placed in the right lower lung. A right sided opacity is visible at 4 hours (B); it develops over 24 hours (D), and eventually shows bilateral lung injuries with pleural effusions at 32 hours (E).

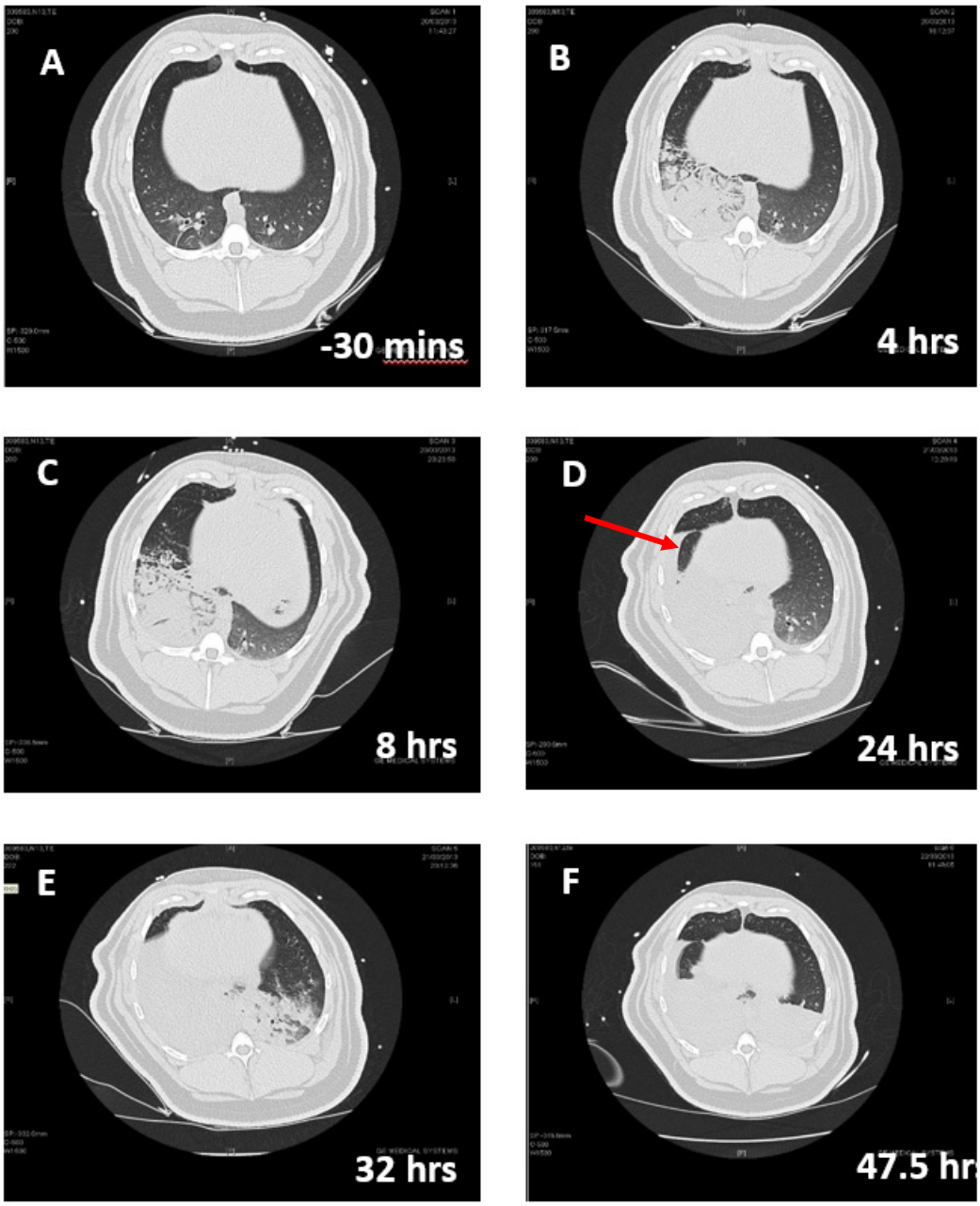


Figure 5.25: Serial pulmonary CT images before (A) and after (B-F) instillation of a mixture of OP+GJ into the right lung of a minipig at time 0. At time 4 hours (B) there is an obvious right sided lower area of consolidation which enlarges over time to engulf most of the right lung at 24 hours (D) with a large pleural effusion seen in the fissure (red arrow). By 32 hours the contralateral left lung is also involved showing dorsal consolidation (E) which worsens at 47.5 hours (F). The right lung recovers slightly at 47.5 hours with more aeration sternally (anteriorly) (F).

After the number and density of voxels within the CT lung scans had been analysed (OP+GJ pig; Figure 5.25), images were created showing only those voxels displaying non-aerated lung

tissue (-250 to +250 HU). These images are displayed below with a similarly processed set of control lungs (Figure 5.26). As the animals were placed supine (dorsal recumbency) for the duration of the experiment, all pigs developed some dependant lung area (basal and dorsal) consolidation. This was observed throughout the experiment and can be seen in the sham control pig (Figure 5.26) (Niehues et al., 2012). The OP+GJ treated pig showed an obvious consolidation of the dorsal/caudal right lung at 4 hours which enlarged over time. By 47.5 hours the OP+GJ treated pig also had greater left sided lung consolidation than the sham control suggesting greater indirect damage to the lungs.

Figure 5.27 shows in more detail the HU analysis of the lungs of an OP+GJ treated pig for both the directly-injured (right -green) and indirectly-injured (left -red) lungs. In the course of time, the proportion of voxels denoting well ventilated and aerated lung (-1000 to -500HU) reduced, whilst those in the poorly and non-aerated lung area (-500 to +250HU) increased. This was more prominent in the directly-injured (right - green) lung indicating the presence of oedema,haemorrhage, consolidation and poor aeration.

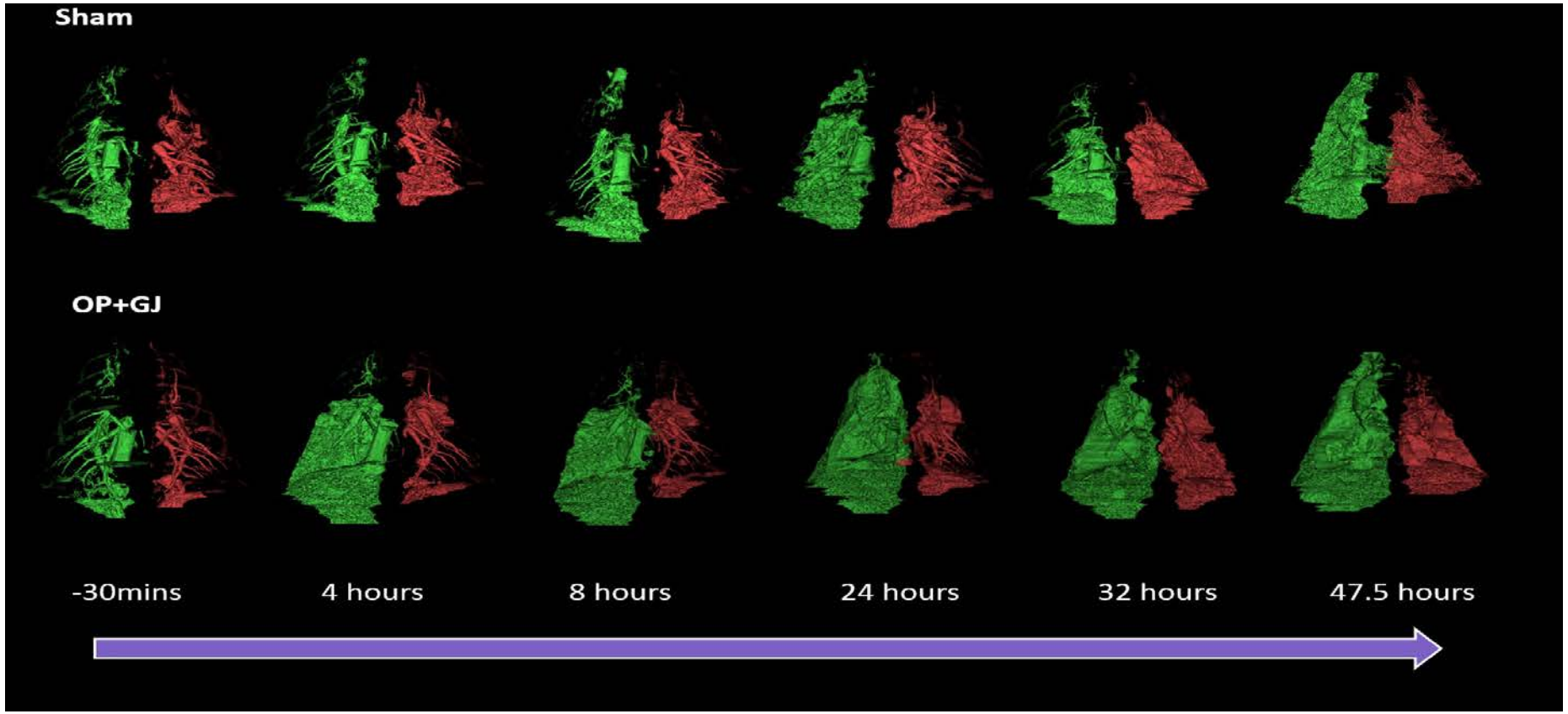
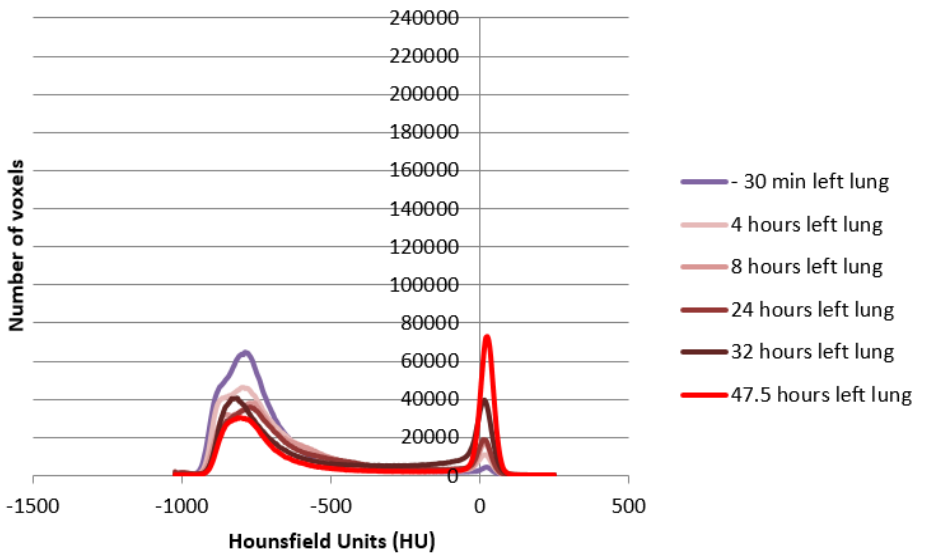


Figure 5.26: Pulmonary CT images showing only non-aerated voxels with a density between -250 and +250HU before and after instillation of a mixture of OP+GJ into the right lung of a minipig (bottom) compared with a sham control minipig (top) over 48 hours. At time 4 hours in the OP+GJ pig there is a right sided (green) dorsal/caudal area of consolidation which enlarges over time to engulf most of the right lung at 24 hours. By 32 hours the contralateral left lung shows signs of increased density in the dorsal area which slightly worsens at 47.5 hours. The sham pig shows obvious blood vessels and caudal consolidation (due to anaesthetic) at -30 min, and from 24 – 47.5 hours develops some dorsal and basal consolidation common to all study animals probably secondary to the pigs positioning for the experiment. The green denotes right lung and red, left lung.

A



B

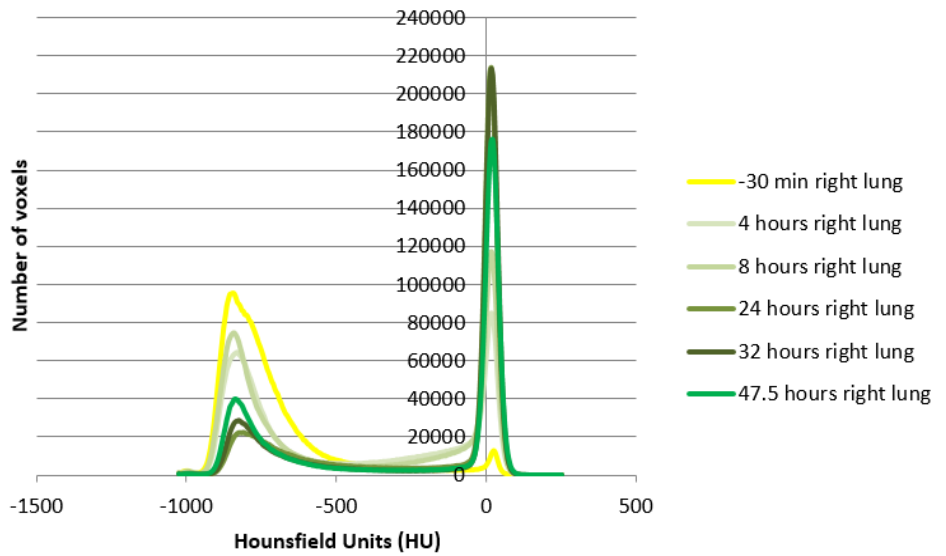


Figure 5.27: Frequency distribution of the number of voxels (y axis) against voxel density (x axis) for representative OP+GJ treated pig lungs over time (-30 min to 47.5 hours). The indirectly-injured (left) lung (A) is represented in red, directly-injured (right) lung (B), green. The directly-injured (right) lung has a peak amount of voxels in the dense area (-250 to +250HU) at 32 hours which then slightly resolves at 47.5 hours, which mirrors the CT images in Figure 5.25. The indirectly-injured (left) lung (A) has less overall volume (2 x lobes) than the right lung (4 x lobes), hence the smaller peaks at beginning and end of experiment, but the spread of voxels shows that a good proportion of voxels denoting well ventilated, aerated lung (-1000 to -500HU) still remain at 47.5 hours, unlike that in the directly-injured (right) lung.

5.3.7.1 CT lung density analysis is repeatable

Further experiments were conducted to establish whether the measurement of lung densities using the above methods was repeatable.

Measurements of the CT scans were repeated some weeks later after the initial measurement on the following randomly selected CT lung scans: pigs N14- 8 hours, N13- 24 hours, N12 -30 min, N7- 47.5 hours, N6- 24 hours and N8- 32 hours (N denoting the number of pig, and the time the scan was taken during the experiment). The original and repeat CT scan data generated 3072 'number of voxel' measurements for comparison (see methods). These were entered into a statistical package (Graph Pad 6.1) and analysed using Bland-Altman analysis and correlation graphs. There were 6 extreme outliers (re-scores) which were removed. The bias was -145 (±1063) voxels indicating a non-significant tendency to award greater scores on the second occasion, especially for measurements with high voxel numbers (Figure 5.28A). Despite this, the Spearman's rank coefficient was >0.99 (p≤0.0001) (Figure 5.28B) indicating a strong intra-rater reliability for this CT measurement tool.

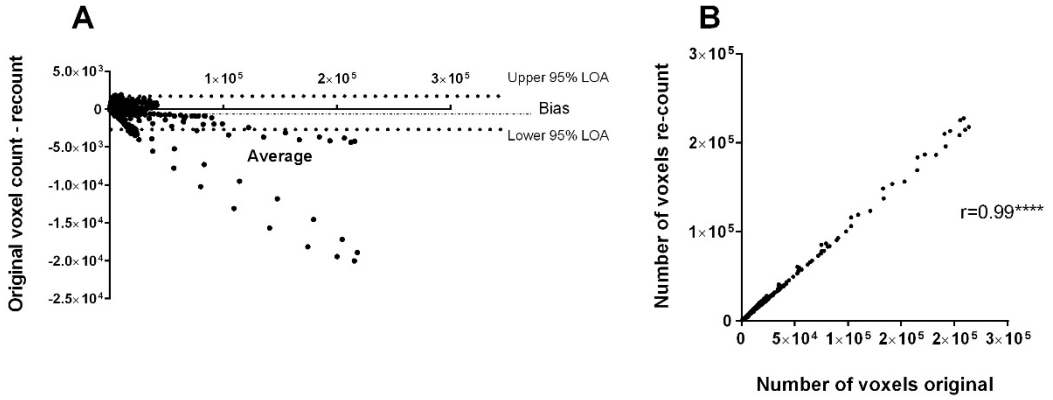


Figure 5.28: Repeatability of CT lung density analysis. Diagram (A) Bland-Altman analysis showing the difference between the original and re-scored number of voxels in each 5HU section for each lung plotted against the average number of voxels for that section. The bias was -145 (±1063), with the 95% limits of agreement (LOA) 1939 and -2228. There was a slight tendency to score greater during the re-score, especially for the sections with high numbers of voxels. A dot plot of the original vs. the recount number of voxels (B) shows a strong linear correlation, with the Spearman's rank coefficient being 0.99, (p <0.0001) with the 6 outliers mentioned above removed. These results show that this method of CT analysis had good intra-rater reliability.

5.3.7.2 Pulmonary installation of OP+GJ creates greater quantities of poorly or non-aerated lung tissue when compared with other groups at 47.5 hours

There was a clear change in the quantity of poorly and non-aerated lung tissue over time in all minipigs that underwent CT lung analysis (Figure 5.29). The change in mean percentages of poorly and non-aerated lung tissue was analysed globally with the Freidman omnibus test for all time points (-30 min, 8, 24, 32 and 47.5 hours) and revealed significant differences between groups for the right lung (p=0.019), left lung (p=0.0087) and lungs combined (p=0.0123).

Groups	-30 min	24 hours	47.5 hours
Sham (n=2)	15.3 (10.2)	34 (7.1)	53.1 (13.4)
Saline (n=2)	19.6 (4.2)	27.8 (14.9)	47 (0.2)
GJ (n=2)	19.6 (9.3)	46.9 (4.8)	61.6 (26.9)
OP+GJ (n=3)	16.2 (5.2)	65.7 (5.2)	76.7 (12.7)
Solv+GJ (n=3)	15.5 (3.4)	65.7 (7.9)	50.8 (24.2)

Table 5.4: Percentage (mean ±SD) of poorly and non-aerated lung tissue (%) [-499 to +250 HU] in the directly-injured (right) lung at time -30 min, 24 and 47.5 hours.

OP+GJ pigs had the greatest proportion of poorly and non-aerated lung tissue in directly-injured lungs at 47.5 hours, with saline controls having the least (Figure 5.29 A; Table 5.4).

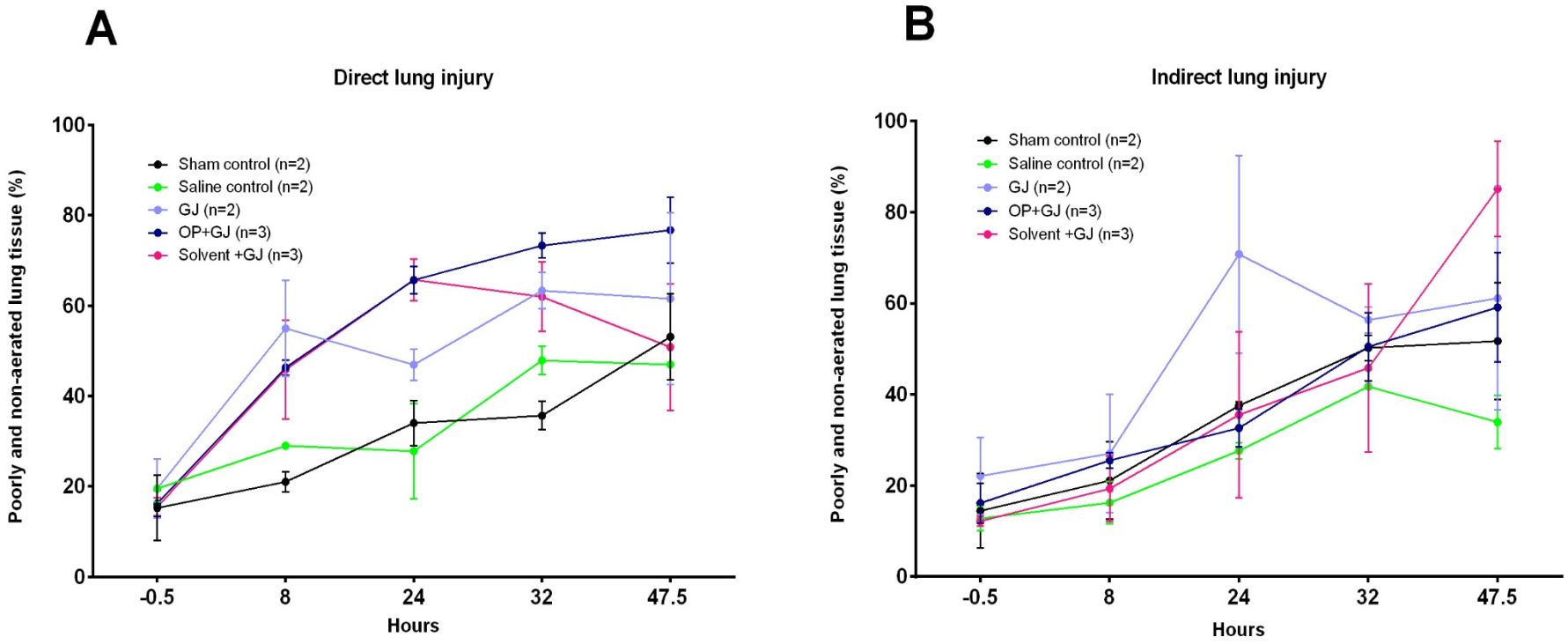


Figure 5.29: Graphs showing the mean percentage of poorly and non-aerated lung tissue [-499 to +250 HU] from (A) directly-injured (right) and (B) indirectly-injured (left) lungs at -30 min, 8, 24, 32 and 47.5 hours. Friedman analysis showed the right (p=0.0199), left (p=.0087) and lungs combined (p=.0123) changes were significant over time. Post hoc analysis was carried out by using further Friedman and Dunn's post-test analysis at each time point on detailed HU profiles [150 x 5HU sections] of lung within -499 to +250 HU bracket for each pig (results shown in Table 5.5). Data from one saline control pig left lung at 32 hours have been omitted because it suffered a large pneumothorax which was drained before the 47.5 hour CT scan. Comparison of percentages of poorly and non-aerated lung tissue of right and left lungs (all time points) using permutation tests showed a significant difference (p=0.0256) between OP+GJ treated lungs only. Graphs show mean and standard error of the mean (SEM) deviation.

In order to compare individual groups at each time point, post hoc analysis was conducted by repeating Freidman and Dunn's post-tests on detailed HU profiles of the poorly and non-aerated (-499 to +250HU) sections of lung at individual time points (Table 5.5). This confirmed the presence of clear differences of poorly aerated lung tissue between groups at each time point, with OP+GJ directly-injured lungs possessing the greatest percentage of poorly or non-aerated lung tissue at 47.5 hours.

Pulmonary installation of both GJ and OP+GJ caused a significant increase ($P \leq 0.0001$) in the percentage of poorly and non-aerated lung tissue in the directly-injured (right) lung at 24 and 47.5 hours when compared with sham controls (Table 5.4; Table 5.5).

The Solv+GJ treated pigs initially had an increase of poorly and non-aerated lung tissue similar to the OP+GJ and GJ treated pigs, but at 24 hours (2/3 pigs) this started to decrease. By 47.5 hours the Solv+GJ treated pigs had significantly less poorly and non-aerated lung tissue (51%) than either GJ (62%) ($p \leq 0.0001$) or OP+GJ (77%) ($p \leq 0.001$) treated animals and were not statistically different to sham (53%) or saline controls (47%) (Table 5.5). This suggests that the solvent cyclohexanone may have had a protective effect against GJ induced lung injury and that an increase in the poorly and non-aerated lung tissue seen in the OP+GJ treated pigs was not caused by the cyclohexanone component of dimethoate EC40 alone (Figure 5.29 A).

The indirectly-injured (left) lungs all developed an increase in the percentage of poorly and non-aerated lung tissue over time with no significant differences between aspiration groups and saline controls by 47.5 hours (Figure 5.29 B). There were however statistically significant differences between sham control vs. saline or GJ ($p \leq 0.0001$), and Solv+GJ and OP+GJ ($p \leq 0.05$) (Table 5.5). The increase in poorly and non-aerated lung tissue in the GJ indirectly-injured lung could have resulted from one of the two pigs having a partial left lung collapse (observed on the CT scan with less lung volume than baseline) secondary to mucous plugging or increased secretions.

Comparisons between right and left lungs within each group showed that only OP+GJ directly-injured lungs were significantly less aerated ($p = 0.0256$) than the indirectly-injured lung, emphasising the extent of the direct lung injury.

Groups	30 min (I)	30 min (D)	8 hour (I)	8 hour (D)	24 hour (I)	24 hour (D)	32 hour (I)	32 hour (D)	47.5 hour (I)	47.5 hour (D)
Sham control vs. Saline	****	****	****	****	****	****	****	****	****	ns
Sham control vs. GJ	****	****	ns	****	ns	****	ns	*	****	****
Sham control vs. Solv+GJ	****	ns	****	**	****	***	ns	****	*	ns
Sham control vs. OP+GJ	ns	****	*	****	****	****	****	ns	*	****
Saline vs. GJ	****	ns	****	ns	****	ns	****	***	ns	****
Saline vs. Solv+GJ	ns	****	****	****	ns	*	***	ns	ns	ns
Saline vs. OP+GJ	****	****	****	***	ns	ns	****	****	ns	****
GJ vs. Solv+GJ	****	****	****	****	****	ns	ns	ns	ns	****
GJ vs. OP+GJ	**	****	ns	ns	****	ns	****	***	ns	ns
Solv+GJ vs. OP+GJ	****	****	****	ns	ns	**	****	****	ns	***

Table 5.5: Statistical differences in the proportion of poorly and non-aerated lung tissue (%) for all groups and lungs at all time points. Post hoc analysis was conducted by using Friedman and Dunn's post-test analysis at each time point on detailed HU profiles [150 x 5HU sections] of lung within -499 to +250 HU bracket for each pig. Data from one saline control pig left lung at 32 hours have been omitted because it suffered a large pneumothorax which was drained **before the 47.5 hour CT scan. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001. N**s: not significant, HU: Hounsfield Unit, CT: computerised tomography, D: direct (right) lung injury, I :indirect (left) lung injury.

5.3.8 Pulmonary installation of OP+GJ creates greater histopathological lung scores when compared with sham control, GJ and Solv+GJ pig lungs

The 18-point histopathology scoring system 1.1 (chapter 4), which was largely based on the presence of neutrophils and oedema within the lung parenchyma, was used to quantify lung injury after pulmonary installation of either OP+GJ, GJ or Solv+GJ. Both histopathological scoring, and transmission and scanning electron microscopy images confirmed that OP+GJ placed into a lung created significant airway and interstitial neutrophil infiltration, haemorrhage, oedema, necrosis with extensive fibrin deposition (Figure 5.30 G,H; Figure 5.31).

Both GJ ($p \leq 0.05$) and OP+GJ ($p \leq 0.001$) lungs combined had greater scores when compared with sham or saline controls (Figure 5.32 and Table 5.6). Analysis of individual lungs showed OP+GJ scores were significantly greater than the sham right ($p=0.005$) and left ($p=0.037$), and saline left ($p=0.0053$) lungs. GJ scores were only greater ($p=0.0135$) than the right sham control lung (Table 5.6). This suggests that OP+GJ treated animals caused a direct and indirect measureable lung injury when compared with sham controls. There was no statistical difference between GJ and OP+GJ for lungs combined or individually, although the graphical trend indicates that OP+GJ lungs had higher scores (Figure 5.32).

OP+GJ combined lungs had significantly higher scores than Solv+GJ treated lungs ($p=0.031$), particularly when compared with the indirectly-injured lung ($p=0.0187$). This again supports the idea that other ingredients (e.g. surfactants) in dimethoate EC40, rather than the solvent cyclohexanone, caused pulmonary damage. Although the reduced score of Solv+GJ treated lungs - compared to GJ treated lungs - was not significant, Solv+GJ lungs were also not statistically different from total, right or left lung sham or saline controls. This implies that the solvent cyclohexanone may have beneficial properties in reducing lung injury secondary to pulmonary installation of GJ or OP+GJ.

5.3.8.1 Histopathology scoring system 1.1 is repeatable for the main pulmonary aspiration study

In order to re-affirm that the histopathological scoring system 1.1 was repeatable within an assessor, a 10% selection of the lung slides from the main pulmonary aspiration study were blindly re-scored 6 months after the initial scoring. Bland-Altman analysis of the data displayed

a bias, mean (SD), of 0.8 (±1.7), LOA between -2.5 and 4.1, with a Spearman's rank coefficient 0.93 (Figure 5.33). The results were of similar magnitude to the histopathologist's (SS) own repeatability scores and confirmed that the scoring system was repeatable.

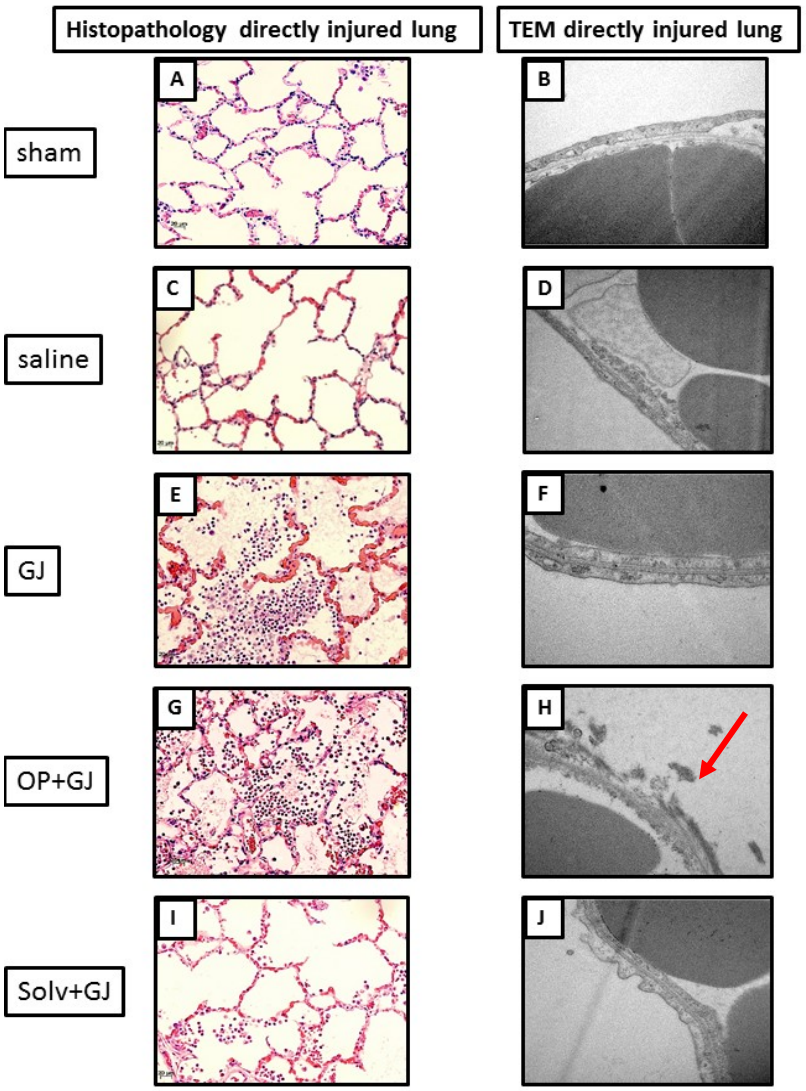


Figure 5.30: Direct effects of pulmonary installation of mixtures of GJ, OP+GJ and Solv+GJ on minipig lung. Comparison of lung architecture in representative minipigs 48 hours after sham bronchoscopy (A and B), administration of saline (C and D), GJ (E and F), OP+GJ (G and H) and Solv+GJ (I and J) into the directly-injured (right) lung. Light microscopy images (original magnification: x20) with haematoxylin and eosin. Direct injury with GJ (E) and with OP (G) caused alveolar and interstitial oedema, neutrophil infiltration, haemorrhage, fibrin deposition, vascular congestion, and necrosis. The

haemorrhage and necrosis was more pronounced in the OP+GJ lungs (see Figure 5.31) but aspirated Solv+GJ caused less of these lung injury features (I). Images edited in PowerPoint. Transmission electron microscopy (TEM) images of the same lungs detailing their alveolar capillary membranes (B, D, F, H J) (original magnification: x25, 000). Direct injury with GJ, OP+GJ and Solv+GJ (F, H and J) seemed to cause alveolar capillary membrane swelling. Aspirated OP+GJ (H) also led to peeling of the alveolar epithelium into the alveolar space and fibrin deposition (red arrow) in and around the alveolar capillary membrane.

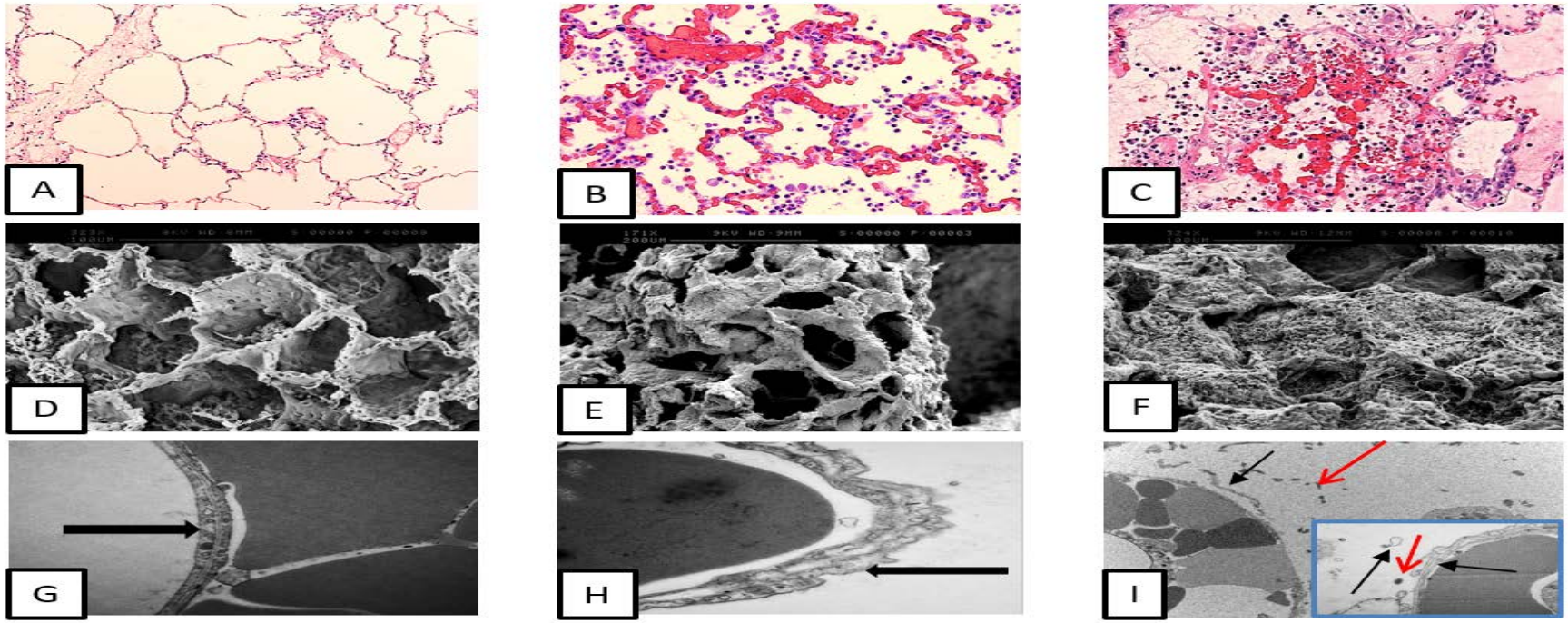


Figure 5.31 Effects of indirect and direct lung injury secondary to pulmonary installation of OP on example minipig lungs. Comparison of lung architecture in anesthetised minipigs 48 hours after sham bronchoscopy (control pig; A, D, and G), OP+GJ into the contralateral lung (indirect injury; B, E, and H), and OP+GJ into the right lung (direct injury; C, F, I). (A–C) Light microscopy images (original magnification: x10–20) with haematoxylin and eosin. Compared with indirect injury, direct lung injury caused greater alveolar and interstitial oedema, neutrophil infiltration, haemorrhage, fibrin deposition, vascular congestion, and necrosis. Images edited in PowerPoint. (D–F) Scanning electron microscopy images (original magnification: x171–324) of the similarly affected lungs. Direct lung injury showed extensive destruction of the alveolar capillary framework, with increased fibrin and clot formation. (G–I) Transmission electron microscopy images (original magnification: x4600-25,000) of similarly affected lungs. Both indirect and direct lung injury caused alveolar capillary membrane swelling. The black arrow signifies the alveolar capillary membrane in control (G), indirect (H) and directly-injured (I) lungs. After direct injury, the alveolar epithelium appears to be peeling into the alveolar space (I) and fibrin deposition (red arrow) in and around the alveolar capillary membrane. Figure reproduced from (Hulse et al., 2014b) with permission from the American Thoracic Society©.

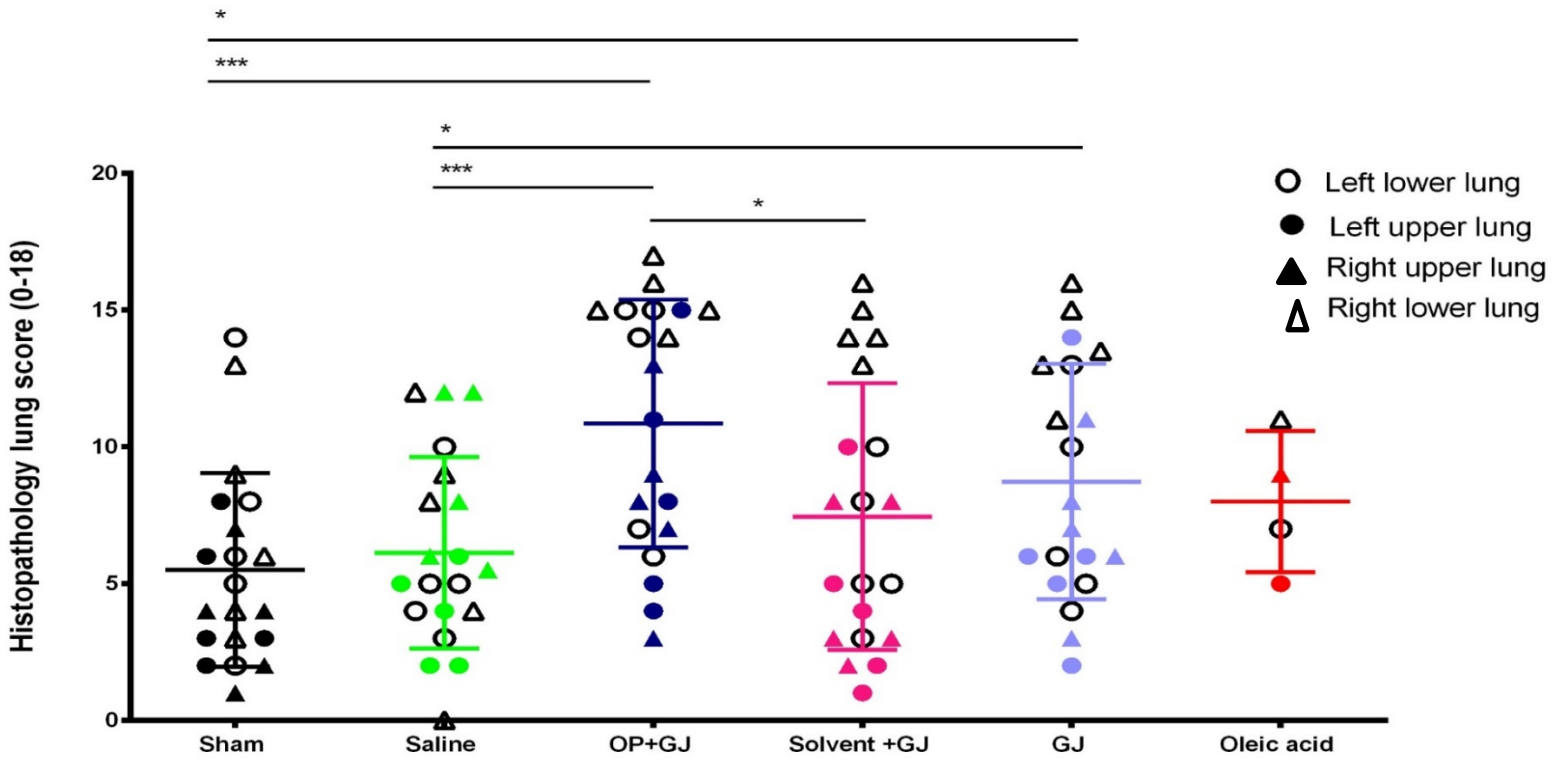


Figure 5.32: Dot plot showing the spread of histopathology scores awarded to upper and lower (cranial and caudal) parts of the lung in all groups. After placement of 0.5mL/kg saline, GJ, OP+GJ or Solv+GJ into the right lower (caudal) lung, the lung damage was not necessarily evenly spread throughout the lung. Therefore samples were taken from both cranial (upper) lung and injured caudal (lower) lung sections, from both directly and indirectly-injured lungs in the same animal. Triangles are directly-injured (right) lungs, circles are indirectly-injured (left) lungs. Empty triangles or circles denote lower, as opposed to upper lung samples. The greatest scores generally belonged to the right caudal (lower) lung samples (empty triangles). Kruskal-Wallis analysis of group data was significant (p=0.0016) with results of permutation tests as detailed above. Mean and SD shown.

Group A	Group B	Combined or individual lung	P value	Significance
Sham	OP+GJ	Combined lungs	0.0003	***
Sham	Solv+GJ	Combined lungs	0.16	ns
Sham	GJ	Combined lungs	0.0156	*
Saline	OP+GJ	Combined lungs	0.001	***
Saline	Solv+GJ	Combined lungs	0.33	ns
Saline	GJ	Combined lungs	0.045	*
OP+GJ	Solv+GJ	Combined lungs	0.031	*
OP+GJ	GJ	Combined lungs	0.139	ns
GJ	Solv+GJ	Combined lungs	0.39	ns
Sham	OP+GJ	Directly-injured lung	0.005	**
Sham	Solv+GJ	Directly-injured lung	0.06	ns
Sham	GJ	Directly-injured lung	0.0135	*
Saline	OP+GJ	Directly-injured lung	0.054	ns
Saline	Solv+GJ	Directly-injured lung	0.38	ns
Saline	GJ	Directly-injured lung	0.164	ns
OP+GJ	Solv+GJ	Directly-injured lung	0.38	ns
OP+GJ	GJ	Directly-injured lung	0.51	ns
GJ	Solv+GJ	Directly-injured lung	0.7	ns
Sham	OP+GJ	Indirectly-injured lung	0.037	*
Sham	Solv+GJ	Indirectly-injured lung	0.84	ns
Sham	GJ	Indirectly-injured lung	0.455	ns
Saline	OP+GJ	Indirectly-injured lung	0.0053	**
Saline	Solv+GJ	Indirectly-injured lung	0.63	ns
Saline	GJ	Indirectly-injured lung	0.11	ns
OP+GJ	Solv+GJ	Indirectly-injured lung	0.0187	*
OP+GJ	GJ	Indirectly-injured lung	0.154	ns
GJ	Solv+GJ	Indirectly-injured lung	0.299	ns

Table 5.6: Histopathology lung score statistical analysis. Table showing outcome of permutation tests of either combined (n=20), directly-injured (right, n=10) or indirectly-injured (left, n=10) lung histopathology scores between group A and group B. Results **shown as p value, ≤0.05*, p≤0.01**, p≤0.001*****. Tests between directly and indirectly-injured lungs of the same animals showed no significant difference. Ns: non-significant.

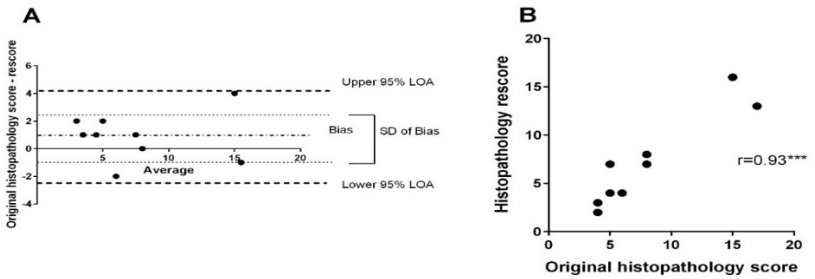


Figure 5.33: Repeatability of histopathology scoring within an assessor for the main aspiration study using a scatter dot plot and Bland-Altman analysis. Bland-Altman analysis shows the difference between the original histopathology score and rescore (A) using system 1.1. The mean (SD) bias was 0.8 (1.7), with the 95% limits of agreement (LOA) -2.5 and 4.1. This shows there was a slight tendency to score lower during the rescore. A dot plot of the original histopathology scores vs. the rescore (B) shows a linear positive correlation, with the Spearman's rank coefficiant being 0.93, ($p < 0.0003$) ***. These results show that the histopathology scoring using system 1.1 was repeatable.

5.3.9 Pulmonary surfactant profiles

Pulmonary surfactant profiles consisting of various phosphatidylcholine (PC) species are altered during lung injury and can provide insight into the pathophysiology of pulmonary aspiration of GJ, or mixtures thereof. Lung injury causes reduction in the saturated PC 16:0/16:0 species responsible for reduction of alveolar surface tension with elevation of the unsaturated PC species (e.g. PC 16:0/18:1) (Schmidt et al., 2007, Dushianthan et al., 2014).

Due to a failed mail delivery of the choline isotope for the day of study, two pigs did not receive an infusion of *methyl*-D_9 choline at the beginning of the experiment, and so these animals were removed from further surfactant analysis. Furthermore, staff changes at the University of Southampton limited the number of PC composition experiments that could be completed for this thesis. Therefore not all pigs were tested, and those that were tested, had the directly-injured (right) lung and 48 hour samples preferentially examined.

5.3.9.1 OP+GJ directly-injured lungs have altered pulmonary surfactant profiles compared with sham control lungs

At 24 and 48 hours after pulmonary installation of OP+GJ, directly-injured lungs had less of the beneficial PC species 16:0/16:0, and more of the unsaturated PC species 18:0/20:4 when compared with other lungs. This indicated the development of a pulmonary surfactant profile similar to that observed in ARDS patients (Figure 5.34) (Dushianthan et al., 2014).

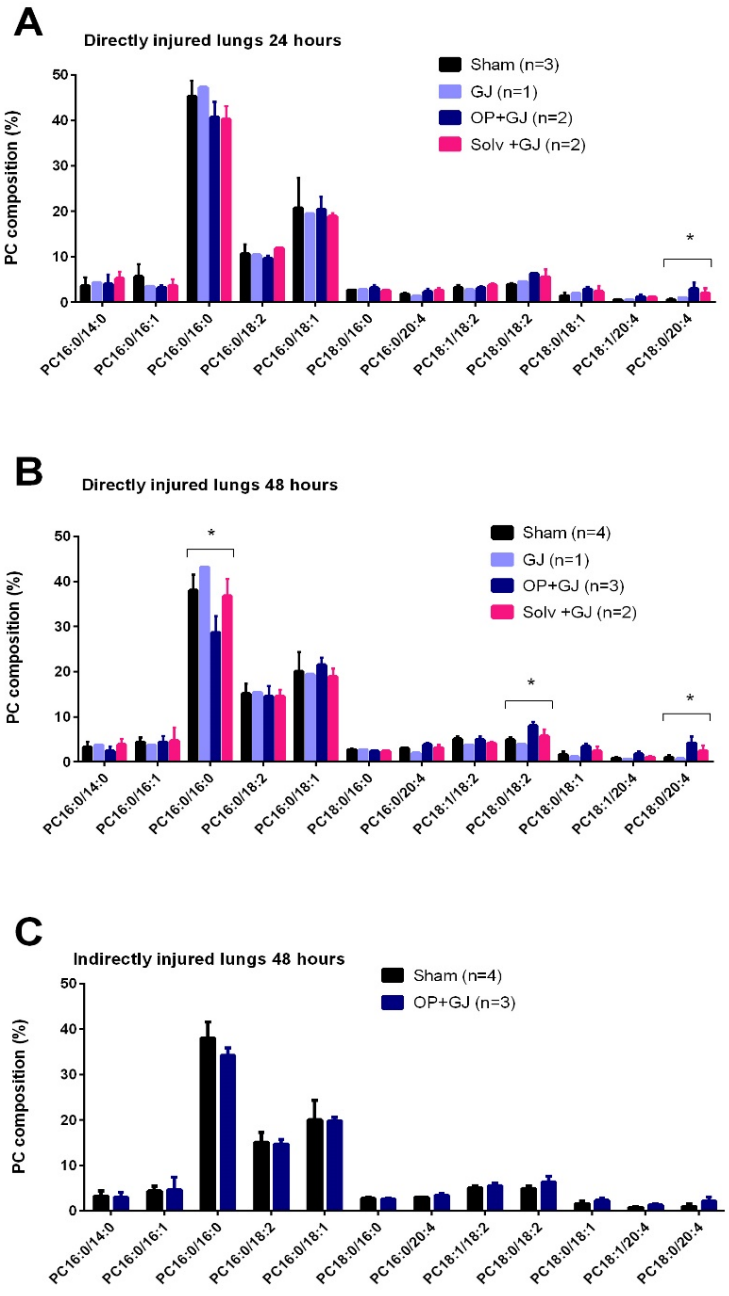


Figure 5.34: Surfactant abnormalities observed within minipigs receiving pulmonary treatments. Graphs showing the BALF pulmonary surfactant PC species composition (%) of the directly-injured (right) lung at 24 (A), 48 hours (B) and indirectly-injured (left) lung at 48 hours (C). Figure C uses the same sham control data from the right lung BALF (B) for comparison. OP+GJ treated lungs cause a reduction in the PC species 16:0/16:0 responsible for reducing alveolar surface tension in the lung, with increases in unsaturated PC species (e.g. 16:0/18:1, 18:0/18:2). Kruskal-Wallis testing was conducted on PC species that showed obvious group differences [16:0/16:0, 16:0/18:1, 18:0/18:2, and 18:0/20:4] in sham, OP+GJ and Solv+GJ pig lungs. Further intergroup testing using Dunn's post-test was not significant. Mean and SD shown.

By 48 hours, there were significant differences ($p<0.05$) when comparing directly-injured lungs from all groups (sham control, OP+GJ and Solv+GJ) in the percentage of total PC species that was 16:0/16:0, 18:0/18:2 and 18:0/20:4 (Figure 5.34B). Yet, there were no significant changes between individual groups, probably due to low numbers. Although not significant, the OP+GJ directly-injured lungs showed reduced percentage of PC 16:0/16:0 and elevated unsaturated PC species 18:0/18:2, 18:0/20:4 when compared with other groups at 48 hours (Figure 5.34 B). At 48 hours the OP+GJ directly-injured lungs had 29% and GJ 43% with Solv+GJ (37%) having near sham control levels (38%) of the beneficial PC species 16:0/16:0 (Table 5.7). The indirectly-injured (left) OP+GJ lung at 48 hours also had less PC 16:0/16:0 than sham control with slight increases in some unsaturated species [18:0/18:2, 18:0/20:4] indicating a potential indirect (systemic) lung injury secondary to OP+GJ aspiration (Table 5.7; Figure 5.34 C).

Group	Percentage (%) of total PC species that was beneficial (16:0/16:0)
Sham (direct) n=4	38(±3.5)
GJ (direct) n=1	43.1
Solv+GJ (direct) n=2	36.7(±3.7)
OP+GJ (direct) n=3	28.7 (±3.6)
OP+GJ (indirect) n=3	34(±1.7)

Table 5.7: Percentage of total PC species containing the beneficial phosphatidylcholine species 16:0/16:0 in directly and indirectly-injured lungs at 48 hours. Mean and (SD) shown.

The injury pattern observed i.e. low percentage of beneficial PC [16:0/16:0] and greater percentage of unsaturated PC species suggests that either: (i) OP+GJ pigs had a significant type 2 alveolar cellular injury that reduced total PC quality or production; (ii) the PC species was being diluted with proteinaceous fluid; (iii) there was a change in the ratio of saturated: unsaturated PCs that were produced and/or metabolised within the context of OP+GJ lung injury, or; (iv) a combination of all of the above. TEM image analysis of alveolar type 2 epithelial cells (responsible for surfactant production) showed ultrastructural lamellar body abnormalities within the OP+GJ directly-injured lungs when compared with other groups (Figure 5.35). The lamellar bodies appeared greater in number and denser. As the function of the lamellar body is

to release, retrieve and recycle pulmonary surfactant (Schaller-Bals et al., 2000, Andreeva et al., 2007), this could be a site of injury secondary to pulmonary installation of OP+GJ.

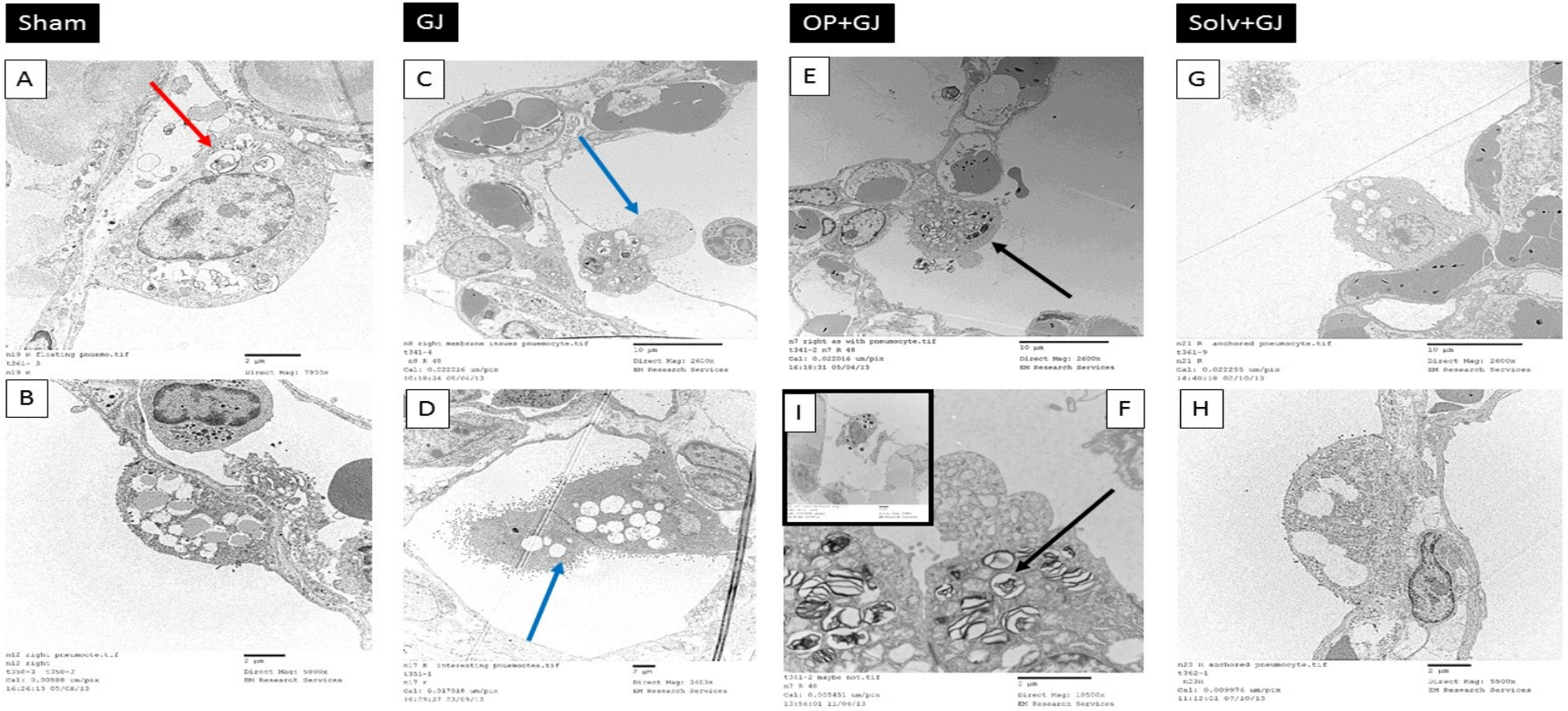


Figure 5.35: Transmission electron microscopy images showing type 2 alveolar epithelial cells (AT2 cells) from the directly-injured (right) lungs in all groups at 48 hours. There are two representative examples of each group: sham bronchoscopy (A, B) showing AT2 cells with healthy lamellar bodies (red arrow), gastric juice (C, D) showing the release of surfactant into the alveolar space (blue arrows), OP+GJ (E, F) showing lamellar bodies densely packed (black arrows) with the inset (I) showing an indirectly-injured lung which also has similar lamellar body changes within the AT2 cell. Solv +GJ lungs are shown (G, H) with no obvious lamellar body damage. Images taken between x2600-10, 500 magnification and edited in PowerPoint. Images were selected from minipig lungs in which lamellar bodies were seen.

5.3.10 Lung injury model analysis

Study refinements

Measurement of the effectiveness of study refinements was possible for some aspects e.g. the reduction of BAL volume from 60 mL to 50 mL, which had no measureable effect on the concentration of cellular and non-cellular components (see above results).

The effect of other study refinements may not have been fully realised e.g. using DMEM for increasing the cellular viability of the BALF cell pellet (n=20 samples) may have been overshadowed by simultaneously introducing trypan blue. Prior to this, it is possible more of the BALF cells from the pellet may have expired (despite being placed on ice (4°C) and with production of cytospin slides and cell counts taking place ≤2-3 hours after sampling).

BALF bacterial culture

Ideally, the microbiological analysis should have included both anaerobic and aerobic bacterial culture for a complete assessment of airway flora. However, Dr J Gibbons (microbiologist) found no difference in the type of bacteria grown in four BALF samples that were cultured anaerobically. This suggested that the type of bacteria growing in the BALF samples were facultive organisms, and could have been grown under either aerobic or anaerobic conditions.

On three occasions the aerobic bacteria grown from the 48 hour BALF were the same in both animals being studied simultaneously. This could have been caused by cross contamination of bacteria between animals. The animals each had their own sterile airway equipment, were on closed respiratory circuits and the ICU room had a positive pressure so as to force waste contaminated air out of the room. Therefore, likely sources of cross contamination must have come from people or equipment in contact with both pigs e.g. poor staff hand hygiene, and/or use of the same antibacterial mouth gel (same tube) for both animals.

Incidents

One minipig in the saline control group developed a left sided pneumothorax, probably after the 24 hour bronchial biopsy. This became apparent with worsening PaO_2/F_IO_2 ratios in the presence of cardiovascular stability. During the 47.5 hour CT scan, the lesion was noted and

was successfully drained via needle thoracocentesis (by Professor Clutton and myself) under anaesthesia. The animal was subsequently euthanised (as per protocol) on return to the laboratory.

For this reason, some of the data collected for this animal during the 24-48 hour sampling period were excluded from further analysis which included: PaO_2/F_IO_2 ratios, plateau airway pressures and physiological dead space. The 32 hour CT scan for this animal was also excluded as it showed an increased volume of air in the left lung secondary to the pneumothorax. Statistical analysis of the physiological variables did not change with its removal from the dataset.

Protocol violations

There were three unintended protocol violations. In one pig, the (left) bronchial blocker balloon was not inflated during the installation of the OP+GJ mixture into the right lung and so some of the mixture could have spilled over into the left lung. Despite this, the percentages of poorly and non-aerated lung tissue in this animal were virtually the same as another similarly treated pig at the same time point (8 hours), indicating that the accident had little effect.

Methyl-D_3 choline was undelivered, and so not infused, in two pigs at the beginning of the experiment. Data from these pigs were removed from the statistical analysis of surfactant composition.

Methyl choline has been used as a bronchoconstrictor in other studies, but at the doses used in this study (which were below normal human dietary requirements), it is unlikely to have affected the respiratory airways in the two pigs (one receiving OP+GJ, the other GJ) that did not receive it.

The mean plateau airway pressure (over 48 hours) from the OP+GJ treated pig that did not receive methyl D_3 choline vs. pigs within the same group showed no statistical difference 15.6 (1.46) vs. 16.7 (1.7) cm H_20, p= 0.6 using permutation testing. It was concluded that the lack of the infusion in these pigs probably had little clinical effect e.g. the infusion did not cause excess bronchoconstriction requiring increased airway pressures to deliver 6-8 ml/kg V_T. Therefore, all data from these animals were used for analysis, except surfactant composition.

5.4 Discussion

This study was designed to investigate the pathophysiology and severity of lung injuries caused by pulmonary installation of mixtures of porcine GJ, OP (dimethoate EC40) with GJ and solvent (cyclohexanone) with GJ in the Gottingen minipig.

Pulmonary installation of OP+GJ created a statistically more severe lung injury when compared with sham control animals. The model also succeeded in revealing how pulmonary installation of OP+GJ created a subtly different, and in some cases a more severe, direct and indirect lung injury when compared with normal GJ aspiration. The OP+GJ directly-injured lung had greater: BALF cell numbers, BALF concentrations of total protein, albumin, IL-6 and CRP; numbers of aerobic bacteria; greater histopathology scores; and increased percentage of poorly and non-aerated lung tissue, when compared with lungs from the GJ treated pigs at 48 hours. Although these are interesting findings, the study was only powered to observe differences between OP+GJ and controls, not within active aspiration groups (e.g. OP+GJ vs. GJ or Solv+GJ treated animals).

GJ treated pigs showed limited evidence of indirect lung injury. This was shown by significantly increased: BALF IL-8 at 24 hours, and poorly and non-aerated lung tissue (left) when compared with sham controls at 47.5 hours. Despite this, the evidence suggests that OP+GJ treated pigs displayed a more convincing picture of indirect lung injury which is discussed below.

The solvent cyclohexanone is synergistically toxic in systemic OP poisoning (Eddleston et al., 2012). However, it may actually be of paradoxical therapeutic benefit in the context of OP+GJ or GJ aspiration.

Pulmonary aspiration model in the Gottingen minipig

The model successfully created a single organ, mild-moderate lung injury without compromising the cardiovascular system of the minipig. Despite the OP pigs having lower MAPs and SVRs there were no differences in cardiac output (table 5.2), with normal arterial pH and lactate values found in all groups. This allowed interpretation of the results in the context of lung injury alone, not because of cardiovascular shock and/or organ hypoperfusion.

Arguably, the lung injury encountered in the current study was too minor given there were no significant changes in the physiological dead space or cardiovascular values of the minipigs (Fraisse et al., 2007). Yet, significant differences were present in the other measured variables e.g. the neutrophilia, haemorrhage and oedema present in the lung histopathology of treatment groups.

All the female pigs in the study were hormonally homogeneous with the OP+GJ treated pigs showing a marked reduction in AChE activity, confirming OP pesticide poisoning.

Lung inflammation

GJ, OP+GJ, and Solv+GJ placed in the right lung caused mild to moderate ARDS as shown by reduced PaO_2/F_IO_2 ratios and increased Pplat which appeared to worsen over time. These treatment groups developed a marked pulmonary influx of proteinaceous fluid, neutrophils and inflammatory markers when compared with sham controls. BALF from OP+GJ directly-injured lungs had significantly more neutrophils, total protein, albumin, IL-6, IL-8 and CRP at 24 hours and total protein, albumin and CRP at 48 hours, than either sham or saline controls.

BALF analysis showed that GJ directly-injured (right) lungs had the greatest concentrations of protein, albumin, IL-8 and IL-6 at 24 hours in comparison with other groups. By 48 hours, BALF protein, albumin and IL-6 concentrations were reduced in GJ treated pigs, and had been surpassed by OP+GJ treated pigs. Whilst there was no statistical difference between OP+GJ and GJ at 48 hours, the data suggest an ongoing inflammatory process in the OP+GJ pig lungs in contrast to resolution of inflammation in the GJ treated lungs.

Gastric acid aspiration inflicts a two-hit injury consisting of a chemical burn that occurs seconds to hours after aspiration, followed by an inflammatory process (including migration of neutrophils) peaking at 4-6 hours (Marik, 2001, Engelhardt and Webster, 1999). Regeneration and recovery of the lung (particularly of sensitive alveolar epithelial type 2 cells) can take between 3-7 days in normal acid aspiration (Engelhardt and Webster, 1999). In the current study, bronchoalveolar lavage and plasma measurements of inflammatory cytokines only occurred at 24 and 48 hours and so may have missed an earlier peak of pulmonary inflammation. However, from the available evidence (including CT analysis), the GJ treated lungs had a peak injurious effect at around 24 hours and therefore must have included injury

from not only acid, but particulate injury as well. In the OP+GJ injured animals the peak injurious effect was at 48 hours (and possibly beyond) indicating the presence of another cause of injury in addition to the acid and particulate lung injury e.g. the toxic effect of OP and its emulsifiable contents.

Plasma levels of IL-6 and IL-8 between groups were not significantly different, indicating that this inflammatory process was not systemic, but more localised to the lung.

Concentrations of CRP in the directly-injured (right) lung BALF -but not plasma- were significantly greater in 48 hour samples from OP+GJ treated pigs compared with all other groups, signifying an ongoing localised inflammatory process. CRP binds to necrotic lung tissue and/or microorganisms and activates the complement system for immune complex removal (Agassandian et al., 2014). The complement system is vital for the adaptive immune response and can itself cause leucocytosis, phagocytosis and an increase in vascular permeability (Kirschfink and Mollnes, 2003). Therefore, the large increase in BALF CRP concentrations within OP+GJ treated pigs could be ascribed to (i) increased necrotic lung tissue (as observed in histopathology scores) or (ii) increased concentration of bacteria within the lung (Figure 5.23) or (iii) a combination of the above. CRP can be produced locally in the lung but its role in lung pathology is not well understood (Agassandian et al., 2014). Further research using *in vitro* pulmonary epithelial cell experiments might identify these pathways.

Lung oedema and haemorrhage

Orogastric administration of OP pesticide causes pulmonary oedema and haemorrhage in animals (Yavuz et al., 2008) and humans (Kamat et al., 1989). It is thought to be due to an increase in the cholinergically dependant endothelial permeability [as demonstrated in isolated and perfused rabbit lung with paroxon in the perfusate] (Delaunois et al., 1995) and alveolar destruction [as shown in studies of commercial fenthion with solvents given to rabbits via orogastric tube] (Yavuz et al., 2008). Inhaled OP also produces alveolar thickening and destruction with increased capillary congestion and extravasated RBCs (Atis et al., 2002). It was unknown whether intrapulmonary OP+GJ would cause similar lung injuries.

CT analysis showed that the OP+GJ directly-injured (right) lungs were markedly oedematous having the greatest mean percentage of poorly and non-aerated lung tissue amongst all groups

between 32 and 47.5 hours. The plots in Figure 5.29 suggest that the GJ induced lung injuries plateaued, and may even have been regressing (indicating resolution of tissue injury) between 32-47.5 hours after the initial insult. In contrast, plots for the OP+GJ treated pigs demonstrated ongoing injury at 47.5 hours with 77±13% lung tissue being poorly and non-aerated. Yet, the effects of GJ and OP+GJ treatments on the proportion of poorly and non-aerated lung tissue evident on CT analysis were not significantly different at 47.5 hours after administration. Therefore, the observed differences between GJ and OP+GJ pulmonary treatments should be confirmed in longer 72 hour repeat pulmonary aspiration studies, with increased group size.

There were significant baseline [-30 min (right and left lungs) CT scans] differences in the percentage of poorly and non-aerated lung tissue between groups (Table 5.5). Only the following group comparisons had no statistical difference at baseline: sham vs. Solv+GJ and saline vs. GJ in the right lung, with sham vs. OP+GJ and saline vs. Solv +GJ in the left lung. These baseline differences could have influenced the observed changes in lung density encountered later in the experiment. The majority of the significant -30 min findings are due to the large percentage of poorly and non-aerated tissue in the saline group lungs at the beginning of the experiment, possibly due to under ventilation of these animals at the beginning of the study.

Despite these limitations, the data overall highlights (i) the worsening aeration of OP+GJ directly-injured lungs, and (ii) the apparent resolution of lung injury within the Solv+GJ directly-injured lungs, in which two thirds (2/3 animals) approached control animal levels of poorly and non-aerated lung tissue at 47.5 hours.

Lungs directly-injured with OP+GJ were not only more poorly aerated; gross and histopathological examination indicated that they contained more blood than lungs from other groups. The presence of blood was confirmed by histopathology, TEM and SEM images as well as BALF RBC counts (Figure 5.8; Figure 5.31). Blood in the OP+GJ treated lungs could have arisen from the alveoli (microvasculature), parenchyma or bronchi. As the control animals had similar trauma to the bronchi and parenchyma caused by bilateral bronchoscopy and biopsy, the source of blood in the OP+GJ treated animals was unlikely to be from these procedures.

The TEM images often demonstrated breaks in the alveolar capillary membrane of OP+GJ treated lungs – possibly from a toxic or direct chemical effect – and could be the source of RBC leak into the alveolar spaces.

Measureable lung and alveolar cell injury

Using the histopathology scoring system 1.1 (largely based on the presence of airway, alveolar and interstitial neutrophils, haemorrhage, necrosis and oedema) OP+GJ treated lungs (individually or combined) were awarded the greatest mean score compared with controls, GJ or Solv+GJ treated lungs. Lungs directly-injured with OP+GJ had statistically greater lung injury scores than that found in sham, saline and Solv+GJ pig lungs. This indicated that OP+GJ treated lungs had greater lung injury when compared with all other groups, although not statistically different from GJ injured lungs.

Specific alveolar cell injury markers (RAGE, SP-D and vWF) failed to show any significant differences between groups. The positive control oleic acid had an increase in plasma RAGE concentration at 47.5 hours, and a reduction in BALF SP-D at 48 hours illustrating that the ELISAs were functioning appropriately. The conclusion was that the study was underpowered to demonstrate significant differences using these markers of lung injury.

There was an unexpected reduction in plasma RAGE in OP+GJ treated animals (particularly at 24 hours) and Solv+GJ treated pigs (particularly at 47.5 hours). One explanation is that the OP, and/or solvent, caused direct toxic damage to the type 1 alveolar epithelial cells. Bleomycin appears to cause a similar pattern of lung injury - reducing the amount of soluble RAGE released into the circulation (Hanford et al., 2003).

Surfactant protein-D is produced by type 2 alveolar epithelial cells and is responsible for clearing foreign material (e.g. bacteria and viruses) and promoting resolution of inflammation in the lung (Nayak et al., 2012). Despite all groups in the current study having a reduction in BALF SP-D over time, OP+GJ treated pigs had the lowest concentration of SP-D at 48 hours. This reduction is in keeping with other ARDS findings and can be explained by: (i) decreased production by type 2 alveolar epithelial cells (i.e. toxic injury as described above) (ii) increased utilisation by the lung e.g. phagocytosis of necrotic tissue (iii) loss of SP-D into the circulation via alveolar capillary leak and (iv) dilution with the influx of proteinaceous fluid into the alveolar space (Nayak

et al., 2012). Future studies may consider measuring plasma SP-D to elucidate the role of alveolar capillary leak.

Pulmonary surfactant is produced by the type 2 alveolar epithelial cells and stored, released and recycled by lamellar bodies within these cells. Although we were unable to measure the quantity of surfactant, we were able to examine the compositional changes between groups. At 48 hours the percentage of total PC species that was beneficial (PC 16:0/16:0) was less in the OP+GJ directly-injured (29%) and indirectly-injured lungs (34%) when compared with sham controls (38%). OP+GJ treated lungs also had more of the unsaturated PC species. This pattern has also been observed in ARDS patients, some of whom have aspirated (Schmidt et al., 2007). The authors speculated that there was an alteration in the metabolism of surfactant in these patients. Unfortunately, it was not possible to investigate surfactant metabolism in the current study because the collaborating authority -Dr Goss- left the mass spectrometry department at the University of Southampton.

The TEM images of the type 2 alveolar epithelial cells in OP+GJ treated pigs showed ultra-structural differences from other study groups. In particular, the lamellar bodies (LB) looked larger and more dense. Orogastric administration of OP (dichlorvos EC80) has previously been observed to cause changes in type 2 pneumocyte morphology in a porcine model. Nuclear autolysis with increased chromatin deposition, swollen mitochondrial cristae and LB changes including vacuole like lamellar bodies have been observed (He et al., 2012a). The vacuolization within the LB bodies was also seen in rats given intraperitoneal AI dichlorvos (Wang et al., 2010b). Other direct toxic lung injuries can cause changes in the surfactant profile. In a rodent model, silica increases the quantity of unsaturated (unfavorable PC species) surfactant, with decreases in saturated (favorable) PC species combined with increases in lamellar body number and size (Miller and Hook, 1990).

Lamellar bodies are responsible for the release, storage and recycling of surfactant, and possibly SP-D (Andreeva et al., 2007). Therefore, it is possible that OP+GJ aspiration alters the normal lamellar body and surfactant homeostasis, leading to decreased release/retrieval of SP-D and beneficial surfactant PC 16:0/16:0 species.

Further quantitative analysis of the surfactant type produced combined with ultrastructural type 2 alveolar epithelial cell measurements (number and size of lamellar bodies) within each group is required to elucidate whether the amount, or quality (composition) of surfactant exerts a detrimental effect on the lung and its ability to ventilate.

Difference between GJ and OP+GJ aspiration direct lung injuries

At 24 hours, the BALF cells and total protein in the directly-injured lungs and IL-8 in the indirectly-injured lungs were more significantly increased in GJ treated pig lungs than those damaged by OP+GJ when compared with controls. The PaO_2/F_IO_2 ratios were also more significantly reduced in the GJ treated ($p \leq 0.0001$) pigs than those receiving OP+GJ ($p \leq 0.05$) when compared with sham controls over time. Yet, the plots and data from measured respiratory parameters and inflammatory markers show that OP+GJ was often greater than GJ direct lung injury by 48 hours.

There were only two statistically significant differences between the OP+GJ and GJ groups in the directly-injured lung. These were: (i) BALF CRP (which was greater in OP+GJ treated than GJ treated lungs at 48 hours ($p \leq 0.05$)); and (ii) the 32 hour CT scan, which showed a greater percentage of poorly and non-aerated lung tissue in the OP+GJ directly-injured lung ($p \leq 0.001$). In the indirectly-injured lung OP+GJ had significantly more CRP in the BALF at 48 hours than GJ affected lungs ($p \leq 0.05$). Also, GJ indirectly-injured lungs had significantly more poorly and non-aerated lung tissue than OP+GJ left lungs ($p \leq 0.0001$) at both 24 and 32 hours, but not 48 hours. This was due partly to one of the two GJ pigs having a partial lung collapse due to increased secretions or mucous plugging as evidenced by the CT scan.

The OP+GJ and GJ directly-injured lungs may also have had different injuries to the type 2 alveolar epithelial cells. Although OP+GJ and GJ groups had similarly low BALF SP-D at 24 and 48 hours, a single GJ treated pig showed a normal surfactant profile, with TEM images (n=2) also showing normal type 2 alveolar epithelial cells that were secreting surfactant e.g. functioning normally (Figure 5.34), unlike the directly-injured OP+GJ lungs.

As the model was designed to give the same aspiration volume, OP+GJ treated pigs only had approximately 12-13 mL of porcine GJ rather than the 15 mL used in the GJ group. This may have limited the lung injury observed in the OP+GJ directly injured lung and explain why there were fewer statistically significant differences between the groups.

Lung fluid bacterial numbers

The microbiological analysis of BALF showed that the greatest aerobic bacterial numbers (cfu/mL) were obtained from the OP+GJ and GJ treated lungs, and were significantly raised compared with pooled -30 min tracheal samples, but not sham controls at 48 hours. This was probably due to small group numbers.

VAP is diagnosed in humans with signs of purulent secretions, pyrexia, blood leucocytosis (>12 x 10^9) and a BALF bacterial count > 10^4 (cfu/mL) (Hunter, 2012). Although no pig had a leucocyte count consistent with a diagnosis of VAP, the diagnostic bacterial count > 10^4 (cfu/mL) would have included several tracheal and BALF samples consistent with a VAP diagnosis. These included -30 min tracheal samples (n=2) and the following 48 hour BALF samples: sham control (n=1), saline control (n=3), GJ (n=3), OP+GJ (n=3) and Solv+GJ (n=3). Elevated bacterial counts in the tracheal and BALF samples might be because pigs are more prone to respiratory disease and pneumonias (Brockmeier et al., 2002), despite these Gottingen minipigs (Ellegaard, Denmark) being reared in a barrier facility which is heavily monitored for microbiological activity within the animals.

Neither the tracheal nor the BALF samples grew bacteria known to be associated with porcine respiratory disease complex (Brockmeier et al., 2002), but predominantly grew *Escherichia coli (E.coli)*, and *Klebsiella pneumonia* which are well known in human ICUs as sources of ventilator associated pneumonia (Park, 2005). It is unknown whether these bacteria grew because of contamination from the (ex-human ICU) ventilator, respiratory equipment or the research team themselves, although all measures were taken to reduce these possibilities (see methods).

The presence of GJ and/or dimethoate EC40 may have rendered the pulmonary environment more suitable to grow those bacteria in greater numbers, allowing the development of ventilator or

aspiration related pneumonia. CRP concentrations were greater in the OP+GJ treated pigs, and CRP is known to increase in the presence of bacteria (Agassandian et al., 2014). Humans exposed to occupational OPs are more prone to respiratory tract infections, so it may be that patients who ingest and aspirate OP+GJ are more prone to VAP/ aspiration pneumonia than normal ICU patients (Hermanowicz and Kossman, 1984). This may be the reason for the increased mortality rate seen in intubated OP poisoned patients. As VAPs by definition have to occur after 48 hours of mechanical ventilation, longer pulmonary aspiration studies in a greater number of minipigs are required to find statistical difference in BALF bacterial numbers between OP+GJ, GJ and Solv+GJ treated groups.

Indirect lung injuries

At 24 hours, the BALF of GJ and Solv+GJ indirectly-injured (left) lungs had significantly reduced neutrophil percentages and concentrations of albumin, total protein, and IL-8 (with GJ also showing significantly reduced IL-6 and CRP) compared with the directly-injured (right) lungs. In contrast, OP+GJ indirectly-injured lungs showed no significant difference with the directly-injured (right) lungs for any measured cellular or non-cellular BALF component at 24 hours. By 48 hours the differences in inflammatory markers between groups became less clear as the impact of recurrent BAL and biopsy probably caused their own individual lung injuries (Vonessen et al., 1991). Yet, the OP+GJ indirectly-injured lung still had statistically greater CRP levels than the GJ indirectly-injured lung at 48 hours.

There was no significant difference between the histopathology scores of directly or indirectly-injured lungs in animals of the same groups, but only the OP+GJ indirectly-injured lung scores were significantly greater than both controls and the Solv+GJ indirectly-injured lungs (Table 5.6).

There was a significant difference between the direct and indirectly-injured lungs in OP+GJ treated pigs during CT analysis. This may have been due to the severity of the directly-injured lung compared with the modest indirect lung injury.

Lungs injured by GJ alone had some evidence of indirect lung injury shown by significantly increased BALF IL-8 ($p \leq 0.01$) when compared with sham control, and significantly increased poorly

and non-aerated lung tissue at 47.5 hours when compared with sham control ($p \leq 0.0001$) and at 8, 24, and 32 hours when compared with saline controls ($p \leq 0.0001$). However the CT scans were recorded from only two GJ treated pigs, one of which had radiological evidence of partial (indirect lung) lung collapse, possibly due to increased secretions or mucous plugging.

These findings suggest that OP+GJ placed in one lung creates a simultaneous indirect systemic injury in the contralateral lung that is most apparent in the first 24 hours and probably results from the OP (dimethoate) rather than the GJ or solvent (cyclohexanone) components.

Cyclohexanone reduces signs of lung injury secondary to aspiration of GJ or OP+GJ

Commercial material safety data sheets (MSDS) detail chemical safety information. MSDS sheets state that the inhaled solvent cyclohexanone can cause respiratory tract oedema and chemical pneumonitis. Cyclohexanone is also synergistically toxic in the context of dimethoate EC40 ingestion (Eddleston et al., 2012). Unexpectedly, Solv+GJ placed in the lungs of Gottingen minipigs caused less damage than GJ or OP+GJ treated pigs, and seemed to reduce some of the adverse side effects of GJ, or OP+GJ. Importantly, in the current study, the dose of cyclohexanone within the Solv+GJ and OP+GJ groups was the same (e.g 1.26 mL for a 30kg pig).

In the first 24 hours, Solv+GJ treated pigs showed an increase in the proportion of poorly and non-aerated lung tissue similar to that seen in GJ and OP+GJ directly-injured lungs. However, after this time, it dramatically reduced in two out of the three Solv+GJ treated animals to control animal levels by 47.5 hours. This indicated statistically significant lung injury resolution. The beneficial effects of solvent present in lungs exposed to GJ were also confirmed through histopathology scoring: Solv+GJ treated pigs had no statistical difference to sham or saline control lungs. The Solv+GJ treated pigs lungs (combined) also had significantly less scores than OP+GJ treated lungs (combined).

Solv+GJ treated pigs also had significantly lower plateau airway pressures in comparison with OP+GJ treated pigs, despite similar reductions in PaO_2/F_IO_2 ratios. The absence of the OP component in Solv+GJ treated pigs probably meant less bronchoconstriction (due to no AChE

inhibition), and therefore lower airway pressures. The Solv+GJ treated pigs still experienced an aspiration injury, hence the low PaO_2/F_IO_2 ratios, but other evidence (described below) illustrated that these pigs had a less severe injury than GJ or OP+GJ treated animals. Logically therefore, one might conclude that PaO_2/F_IO_2 ratios were not a good measure of lung injury within this aspiration model.

At 24 hours, Solv+GJ treated pigs had less BALF total protein, albumin, IL-6, IL-8 and CRP in both directly and indirectly-injured lungs compared with OP+GJ or GJ treated pigs. This was statistically significant in the directly-injured lung when measuring BALF IL-8 (OP+GJ and GJ), IL-6 (GJ) and CRP (OP+GJ).

The only significant difference at 48 hours was that Solv+GJ treated animals had lower BALF CRP concentrations when compared with BALF from OP+GJ treated animals in the directly-injured lung. Perhaps group differences for measured inflammatory markers in the directly-injured lung were not as pronounced as the 24 hour results because the possible beneficial/protective effect of cyclohexanone had worn off - being metabolised or removed from the lung. If this had been the case, a second dose of cyclohexanone applied at 24 hours, would have produced similar, or greater differences than were observed. BALF CRP from Solv+GJ and OP+GJ treated animals in the indirectly-injured lungs at 48 hours were similar, perhaps due to a lack of cyclohexanone in the Solv+GJ indirectly-injured lung.

These findings suggest that cyclohexanone could possibly exert a topical (local) anti-inflammatory effect on the alveolar epithelium, reducing release of inflammatory cytokines, and development of interstitial oedema and/or haemorrhage. Solv+GJ treated animals also had a surfactant PC composition and ultra-structural lamellar body appearance in keeping with control animals. Therefore, the beneficial effects of cyclohexanone may arise from its ability to restore normal function to the alveolar epithelial type 2 cells, especially its ability to produce and secrete pulmonary surfactant and associated proteins.

The BALF from Solv+GJ treated pigs grew fewer bacterial colonies and, perhaps as a consequence, contained lower CRP concentrations at 48 hours compared with BALF from GJ or OP+GJ treated

pigs. It is possible that cyclohexanone also has an antibacterial effect within the lung, which reduced the number of bacterial colonies as well as the extent of inflammation. The growth of bacteria can be reduced in the presence of organic solvents, but this is dependent on the solvent's ability to penetrate the bacterial cell wall, and/or interfere with intracellular functioning (Rajagopal, 1996).

It is also possible that reduced significant differences in BALF inflammatory markers between OP+GJ and GJ groups (especially at 24 hours) were because the cyclohexanone component in the dimethoate EC40 preparation, may have been reducing inflammation. This could be confirmed by performing another aspiration experiment using dimethoate active ingredient (AI) alone. This was initially considered, but funds were unavailable.

5.5 Conclusion

This OP pesticide aspiration experiment revealed the direct and indirect toxic effects of OP+GJ on the pulmonary epithelium through single lung installation in a 48 hour Gottingen minipig model.

Pulmonary placement of OP+GJ created a direct lung injury consisting of neutrophil infiltration, marked (alveolar and interstitial) oedema, haemorrhage, with alveolar destruction and fibrin deposition. Inflammation of the lung parenchyma in the directly-injured lung was confirmed by significantly increased concentrations of BALF protein, albumin, IL-6, IL-8 and CRP at 24 hours, and BALF protein, albumin and CRP at 48 hours when compared with control groups.

Aspiration of GJ alone produced similar effects to that of OP+GJ, which on balance, were less severe. The increased damage caused by OP+GJ was demonstrated by the ongoing rise in concentration of BALF inflammatory markers, the increased proportion of poorly and non-aerated lung tissue and the greater histopathology scores awarded to samples taken at 48 hours after poisoning. Additionally, OP+GJ treated pigs had unfavourable surfactant profile changes (similar to that observed with ARDS), more severe than that seen in the GJ treated pig.

Mixing cyclohexanone with GJ did not create a greater lung injury than GJ alone. Instead, it seemed to offer some protective benefit to lungs injured directly with either GJ, or OP+GJ. Some of these benefits involved the lowering of the numbers of aerobic bacteria and inflammatory markers (e.g. IL-6, IL-8) present within the BALF 24-48 hours after pulmonary installation of Solv+GJ. How the solvent exerted these beneficial effects needs to be explored in further *in vitro* and animal studies.

In conclusion, OP+GJ aspiration creates a measureable direct and indirect lung injury which is statistically greater than sham controls 48 hours after poisoning. The lung injury caused by GJ alone appeared less severe than OP+GJ treated animals, but would need to be confirmed in appropriately powered studies with larger numbers of animals.

Chapter 6: Pesticide self-poisoning and lung injury in ventilated patients on Sri Lankan ICUs

6.1 Introduction

The diagnosis of human pulmonary aspiration syndromes [aspiration pneumonitis: acidic chemical injury, or aspiration pneumonia: aspirated oropharyngeal bacteria causing pneumonia] is notoriously difficult and often goes unnoticed (Marik, 2001). Due to the central and peripheral cholinergic effects of OP pesticide toxicity (e.g. unconsciousness, vomiting and increased salivation) it is unsurprising that pulmonary aspiration has been described in 5% to 80% poisoning cases (Godhwani and Tulsiani, 2004, Hrabetz et al., 2013). Post mortem findings describe 40-70% of OP poisoned patients having lung consolidation (Indira et al., 2013, Kamat et al., 1989), which could have resulted from aspiration pneumonitis, aspiration pneumonia, combined with the direct and indirect effects of ingestion of OP. There are no known animal studies that have previously described lung injury resulting from OP poisoning and aspiration syndromes.

The minipig pulmonary aspiration study (chapter 5) found that aspirated OP pesticide combined with gastric juice created an acute direct lung injury that developed over 48 hours and was characterised by a pulmonary neutrophilia, oedema, haemorrhage and necrosis, with a statistically significant increase in the concentrations of BALF protein and inflammatory markers [IL-6, IL-8 and CRP] when compared with controls over 24-48 hours. There was also a reduction in the beneficial pulmonary surfactant phosphatidylcholine (PC) species 16:0/16:0 with increased numbers of aerobic bacteria in minipigs that had aspirated OP+GJ when compared with controls. Molecular mechanisms have yet to be confirmed, but this aspiration lung injury seemed to have many features similar to the acute phase of ARDS (Bhargava and Wendt, 2012), but with differences to normal GJ aspiration lung injury (e.g. the ability of aspirated OP+GJ to create a marked indirect lung injury with ongoing injurious processes in both lungs at 48 hours).

The next objective was to explore whether the lung injury observed in the minipig aspiration model was present in cases of human OP pesticide poisoning with evidence of aspiration.

Using the clinical networks set up in Sri Lanka by Professor M Eddleston, I attempted to conduct a pilot feasibility study to observe the lung injury caused by OP pesticide poisoning and aspiration. This process allowed me to assess whether it was possible to conduct a larger observational study

in the future, to determine whether the pig model reflected human OP poisoning and aspiration and also gave me experience in the management of OP poisoning. Sri Lanka was chosen as a location as it once had one of highest rates of pesticide self-poisoning in the world. Although this improved in 1990-2000s due to government legislation (restricting the use of the most toxic pesticides), poisoning remains very common (de Silva et al., 2012).

Comparisons were made between the minipig model and human pesticide poisoning by collecting physiological data from pesticide poisoned patients and by measuring an extended panel of BALF and plasma inflammatory markers, including some of those measured in the minipig study, at 24 and 48 hours after OP poisoning. The inflammatory panel was extended to include markers that are known to be raised in the BALF and plasma of ARDS patients e.g. TNF-α and IL-1β allowing a more comprehensive comparison of OP-induced lung injury vs. ARDS in humans. This was complemented, where possible, with analysis of the BALF cellular contents and bacterial culture.

Ventilator associated pneumonias (VAPs) are hospital-acquired infections that occur more than 48 hours after intubation. The criteria used to diagnose VAP, and the bacteria causing VAP, are similar to those encountered in aspiration pneumonia and are therefore sometimes difficult to separate (Raghavendran et al., 2011). In the US, the incidence of VAPs for intubated patients is 9% (Rello et al., 2002, Klompas et al., 2011). Although accurate estimates are difficult to generate (due to differing diagnostic criteria), in a recent meta-analysis of patient data from randomised prevention studies, Melsen and colleagues concluded that VAPs cause 13% of all ICU deaths (Melsen et al., 2013). There is a paucity of reliable data for Asian hospitals; however, published reports suggest an incidence of 2-55% with a case fatality of 14-73% for general ICU ventilated patients (Chawla, 2008, Xie et al., 2011).

In order to estimate the incidence of pneumonia within Sri Lankan OP poisoned patients, an audit was conducted to (i) establish the current incidence of VAP and (ii) document the VAP prevention strategies (e.g. sitting the patient up > 30°) currently used in the Sri Lankan ICUs in which I was collecting patient data.

To optimise the use of samples collected in Sri Lanka, some potential biomarkers to indicate the presence of organ dysfunction (lung and liver) secondary to severe OP poisoning with or without aspiration of stomach contents were measured. Reduced blood acetylcholinesterase activity (% of normal) is classically used by physicians to diagnose and assess the severity of OP poisoning, but there are draw backs. The sample needs to be cooled immediately to prevent inhibitor-enzyme complex reactivation at room temperature. The test has marked inter- and intra-individual variability (pre-exposure levels are recommended) and hence, % AChE inhibition can have variable clinical presentations (i.e. severe poisoning symptoms may have low % AChE inhibition and vice versa) which complicates the interpretation of the results and the implications for clinical care (Marsillach et al., 2013, Rajapakse et al., 2014). A point of care test (Test-mate) exists for both AChE and BuChE but has had varying reported reliability (Rajapakse et al., 2011). Therefore, a thermostable (requiring no sample or test refrigeration) point of care test to be used as an adjunct to AChE testing may prove of future benefit in quantifying the severity of OP poisoning, e.g. in terms of liver or lung involvement.

MicroRNAs (miRNA) are small non-coding RNA molecules that regulate transcription and post transcription of large numbers of genes by binding to seed sequences (base pairing) of mRNA, often resulting in down regulation of gene expression at the target mRNA/protein level. There is considerable ongoing research evaluating these miRNAs as biomarkers for different diseases. MiRNA are also thermostable when used as a dried serum blot held at room temperature and so offer additional benefits as a point of care test without the requirement for cold storage (Patnaik et al., 2010).

Pulmonary miR-21 and miR-146a are increased in animal models of high volume ventilator induced lung injury, and are thought to be (amongst others) critical in modulating inflammation in acute lung injury (Vaporidi et al., 2012, Sessa and Hata, 2013). These miRNA could possibly serve as adjunct biomarkers of toxic lung injury to help predict those OP pesticide poisoned patients who may have aspirated and require early intubation and lung ventilation, with enhanced respiratory critical care.

MiR-122, the most abundant micro RNA in the liver, is important for lipid metabolism, local tumour and inflammation suppression, and is being investigated as a toxic liver injury marker (raised plasma

expression) for acetaminophen poisoning (Wen and Friedman, 2012, Antoine et al., 2013). Severe OP poisoning not only causes unconsciousness and pulmonary aspiration, but can also cause liver dysfunction (Gomes et al., 1999, Kerem et al., 2007, Cecchi et al., 2012). Thus OP-induced liver injury might increase plasma miR-122 and act as a surrogate biomarker for severe OP poisoning and aspiration. A thermostable, easy to measure biomarker (plasma or whole blood) could be used in conjunction with AChE/BuChE in rural locations to indicate whether an OP poisoned patient is severely poisoned and/or has aspirated. This could help hospitals allocate appropriate ambulance services and direct the patient to medical facilities with available ventilators, of which there is often a shortage (Gunnell et al., 2007).

6.1.1 Aims

The aims of this pilot feasibility study were:

(i) to compare and contrast the human clinical respiratory pathophysiology with that found in the minipig pulmonary aspiration study

(ii) to conduct a VAP audit of ICU patients in Sri Lankan ICUs

(iii) to evaluate a limited number of plasma miRNA biomarkers for OP pesticide poisoning and pulmonary aspiration.

6.2 Methods

The idea and design for the study was my own, but developed through discussion with Professor M Eddleston, Dr G Drummond and Professor I Gawarammana (SACTRC & University of Peradeniya, Sri Lanka).

The study was granted ethical approval by the Institutional Ethical Review Committee (IERC), Faculty of Medicine, University of Peradeniya, Sri Lanka. Data were collected during two study periods; June – August 2013, and July-September 2014 at the University of Peradeniya hospital, Peradeniya, Sri Lanka.

An amendment to extend the age of inclusion from 75 to 90 years, and to take initial admission plasma samples from poisoned patients was granted in July 2014.

Only the methods particular to the human study are described below with the remainder contained in chapter 2 (methods).

6.2.1 Patient identification and consent

In this pilot feasibility study, three groups of poisoned patients and one group of surgical control patients were selected. The patient numbers were based on the power calculations (see below).

The poisoned patients were those suspected of OP poisoning, with (OP+ASP, n=5) and without (OP+No ASP, n=5) evidence of aspiration, and non-OP pesticide poisoning with signs of aspiration (NON OP+ASP, n=5). The poisoned patients included those admitted to the 3 bedded toxicology intensive care unit (ToxICU), or 10 bedded main intensive care unit (MICU), University of Peradeniya, Sri Lanka, for intubation and ventilation. Patients in the control group (n=5) were those admitted for elective surgery, intubated and ventilated, with procedures lasting 3-5 hours.

Suitable control patients were identified by Dr Vasanthi Pinto (Head of the ICU in Peradeniya Teaching Hospital) on the daily surgical theatre lists. Suitable pesticide poisoned patients were identified by local anaesthetists, SACTRC researchers and/or by myself on daily ward rounds of the MICU and ToxICU using the study inclusion/exclusion criteria.

Consent was sought from the relatives of those patients who had pesticide poisoning and were intubated and ventilated. The relatives were given a patient information sheet and consent form in their own language (Tamil or Sinhala) and were allowed to seek advice and ask questions with the local SACTRC researchers and/or myself (with an interpreter).

Consent from the control patients was sought from the patient themselves by the SACTRC researchers, local Sri Lankan anaesthetists and/or myself (with an interpreter) after informing them of the study, providing an information sheet and answering any questions they had.

Patients or relatives acting on behalf of poisoned patients were able to withdraw from the study at any point if they wished.

6.2.2 Power calculation

There were insufficient human data to perform power calculations for this pilot study and so the numbers in each group (n=5) were based on the predicted available poisoned patient cohort in Peradeniya and the power calculations from the main minipig aspiration study using lung histopathology scores.

6.2.3 Inclusion and exclusion criteria

6.2.3.1 Inclusion criteria

- Male or female
- Age 18-75 years (received an extension to age 90 in July 2014)
- Pesticide poisoning or elective surgery case
- Admission to MICU/ToxICU
- Intubated and ventilated within first 48 hours of poisoning, or as part of the surgical procedure (controls)
- Informed consent from patient, Next Of Kin or family
- Clinical need for a urinary catheter

6.2.3.2 Exclusion criteria

- Failure to obtain informed consent from patient/family/NOK
- Age <18 or >75 (>90) years
- Patients who were pregnant
- History of untreated TB or ongoing pneumonia
- Known chronic pulmonary disease i.e. COPD, emphysema, chronic uncontrolled asthma
- Broncho-pulmonary dysplasia
- Cystic Fibrosis
- Cardiogenic pulmonary oedema
- Extreme cardiovascular instability
- Intra cranial hypertension
- Persistent pulmonary air leak
- Advanced malignancy
- Pulmonary haemorrhages
- Major trauma
- Bleeding diathesis: INR >1.5, platelets < 100 x 10^9/L
- Taking blood thinning agents e.g. heparin, oral clopidogrel or warfarin
- Previous bronchoscopy or bronchoalveolar lavage on ICU
- Mixed pesticide overdoses

6.2.4 Confirmation of aspiration in pesticide poisoned patients

Confirmation of aspiration in pesticide poisoned patients was conducted by examining the patient notes for evidence of aspiration e.g. low pulse oximetry readings (SpO_2 <90%) and low PaO_2 (<10kPa) on arterial blood gas (ABG) measurements on admission. If aspiration could not be confirmed, further information was gathered from the nurses, doctors, SACTRC researchers and/or the relatives with an interpreter.

If a chest x-ray (CXR) was taken on admission this was also examined for evidence of aspiration. Typically these would show opacities in the right lung. However, most patients did not have admission ABGs or CXRs and aspiration was diagnosed on the basis of history, examination and low pulse oximeter readings on admission.

6.2.5 Sampling time points

For the poisoned patients, physiological data, ABG values, urine, and a mini-bronchoalveolar lavage (see below) were collected at 24 and 48 hours after poisoning. Venous blood was sampled on admission (after July 2014 amendment) and at 24 and 48 hours if possible. For the control patients, a single set of samples and physiological data was taken after the elective surgery had finished (mean time- 4 hours), just before waking the patient.

Not all patients were sampled at both 24 and 48 hours due to the patient location, early patient extubation and/or other factors out-with the investigator's control.

6.2.6 Patient and physiological data

The following information was collected for each patient on admission from the available patient notes: name; age; sex; ID number; poisoning history; medical history e.g. alcoholism, with ABG, CXR and medical examination results e.g. lung sounds.

The following information was gathered at study sampling points (if possible): SpO_2, ventilator settings, peak airway pressure, ABG (PaO_2, $PaCO_2$, pH, with lactate and Glucose concentrations), evidence of VAP prevention e.g. patient bed elevation, patient temperature, non-invasive blood pressure (NIBP), full blood count information.

Physiological dead space measurement (chapter 5) was attempted in several cases but abandoned due to futility because of the anaesthetic internal circuit design (in several different machines). For example, the presence of CO_2 absorbing granules in the anaesthetic circuit would mean measured CO_2 (required for dead space measurement) would be reduced producing inaccurate dead space measurements.

6.2.7 Mini bronchoalveolar lavage sampling

Mini-bronchoalveolar lavage (miniBAL) was performed by the ICU doctors under my direction. The patient's lungs were ventilated on 100% oxygen for 2-3 min, before the doctor took the BAL sample using a sterile technique.

The miniBAL lavage samples were obtained using a Combicath® (Figure 6.1). Use of the Combicath® is a relatively common procedure in mechanically ventilated patients in the diagnosis of ventilator associated pneumonia, and has also been used in the measurement of drugs and antioxidants within the lung (Boselli et al., 2005, Fujitani and Victor, 2006, Duflo et al., 2002).

The Combicath® is a fine sterile plastic catheter that is inserted into the endotracheal tube. It was passed blindly into the lung and used as described in Figure 6.1. Advancement of the inner catheter dislodged a small polyethylene absorbable plug at the end of the catheter which allowed complete sterile sampling of the lower lung (Figure 6.1B). Twenty mL of room temperature sterile saline was injected down the catheter using a 50mL syringe with a small volume (<2 ml) being aspirated back into the syringe for analysis (Figure 6.1A). On two occasions a second 20 mL bolus was required to retrieve a sample. This practice was in keeping with the Combicath's normal use.

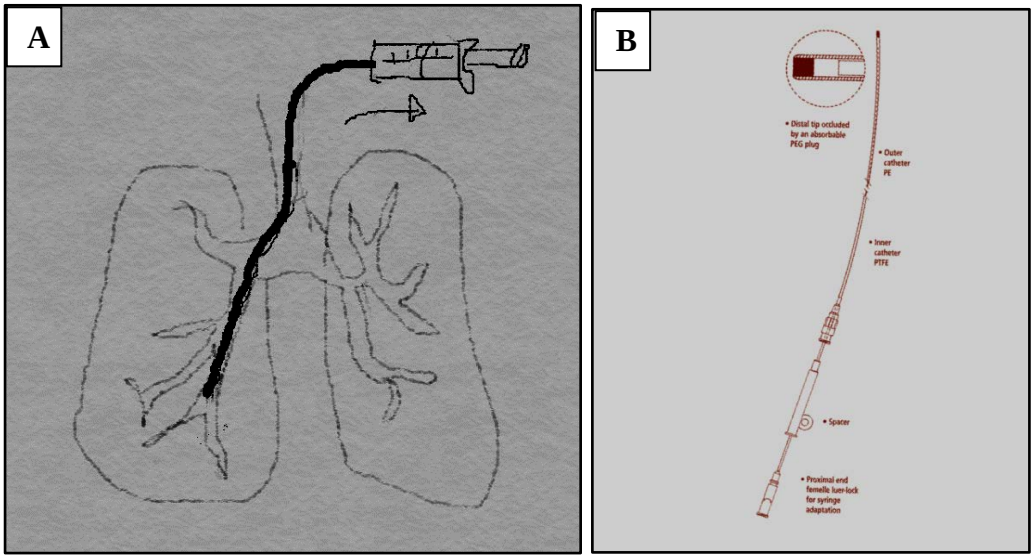


Figure 6.1: A Combicath® used for mini-bronchoalveolar lavage (miniBAL) in intubated ICU patients. The catheter (B) is passed blindly into the lower lung until resistance is felt, and pulled back 2-3cm. The spacer is removed allowing the inner catheter to be pushed (telescoped) down the outer catheter (displacing the polyethylene plug) until the proximal end connects with the outer catheter. A 50mL syringe containing 20-40mL sterile 0.9% saline is attached to the Luer lock of the inner catheter, and the contents is injected into the lower lung and then immediately aspirated into the 50mL syringe retrieving 1-3mL (A). Image (A) drawn by E Hulse using Fresh Paint, Microsoft 2015, image B from Keomed.com.

6.2.7.1 Mini Bronchoalveolar lavage fluid processing

The lavage sample was placed on ice and centrifuged at 2500 rpm for 20 min (R-8C laboratory centrifuge, REMI, Mumbai, India). The supernatant was aliquoted and stored in containers at -20°C (in the hospital ward) and then at -80°C (in the SACTRC laboratory freezer before shipment to the UK in dry ice). The cell pellet volume was estimated and a volume of sterile saline was added for slide preparation. The Combicath was designed to measure bacterial numbers and not the number of cells in a BALF sample (>500 cells required for white cell differentiation), but it did allow two slides per patient to be prepared in order to examine the predominant cell types.

6.2.7.2 Creation of a slide to examine bronchoalveolar lavage cells

Twenty µl of the BALF cell pellet mix was pipetted onto a glass slide and smeared with the side of the pipette along the length of the slide. As the humidity in Sri Lanka was above 80% the slide had

to be dried on an improvised hot plate rather than in room air alone. Once dried, the cells on the slide were fixed by immersion in 100% methanol for 1 sec, five times. Once air dried again, they were stained with Kwik Diff ™ stain as per the minipig protocol, and dried. Some slides were fixed in methanol and then kept for toll like receptor (TLR) staining back in the UK (chapter 7). In the early development of this technique, some of the early slides became unusable because of cellular destruction. This resulted in the availability of only eight slides (from all groups) that could be used for either white cell identification (below) or TLR analysis (chapter 7).

6.2.7.3 Bacterial culture of bronchoalveolar lavage fluid

The culture of neat BALF was attempted in a few cases (n=7) using the local microbiological laboratory facilities (Figure 6.2). The samples taken from pesticide poisoned patients were mostly obtained at 48 hours (n=4) with two being taken at 24 hours (n=2). One control sample was taken at 4 hours. A few samples were not cultured either due to the lack of sample, or lack of access to the laboratory. Mr Iriyagama (head microbiological technician) or myself used a sterile wire loop to spread the BALF sample (20-50 μL) on chocolate, MacConkey and blood agar plates and incubated them at 37°C for 24-48 hours in an anaerobic chamber. If there were bacteria present after this time Mr Iriyagama would perform further identification tests e.g. gram staining. Quantification of any bacteria present was not possible due to laboratory staff restraints.

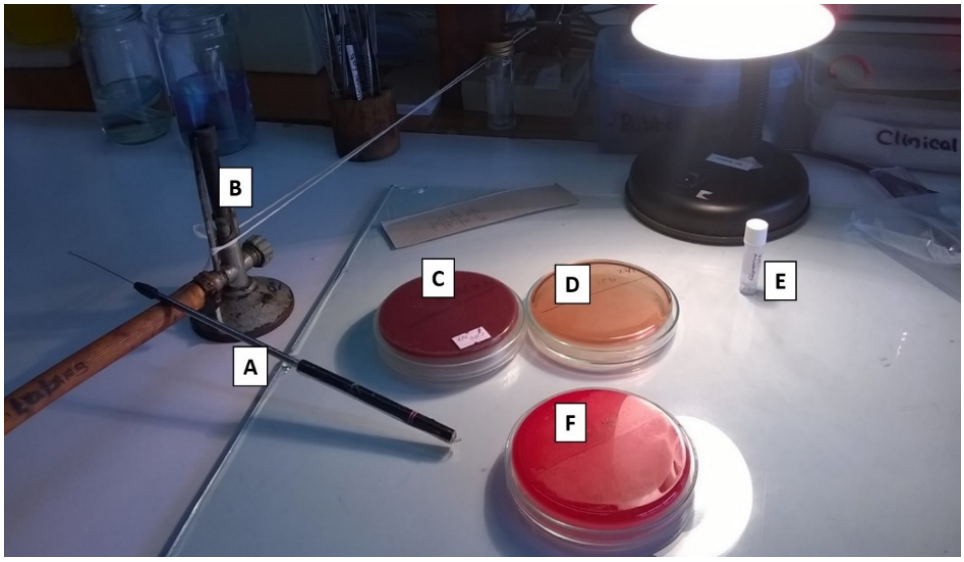


Figure 6.2: Microbiological equipment used for culturing bacteria from human BALF. The wire loop (A) was heated in the Bunsen burner (B) for 30 sec and cooled. The loop was then used to remove a sample of BALF from the collection tube (E) and spread in a zig zag pattern over chocolate agar (C), MacConkey agar (D) and blood agar (F). The agar plates were then sealed and placed in an anaerobic chamber at 37°C for 24-48 hours.

6.2.8 Blood sampling and processing

Ten mL of venous blood was taken from either the antecubital fossa or femoral vein. Blood (0.2 mL) was added to 4 mL ice cooled sterile 0.9% saline and aliquoted into two vials for measurement of AChE activity in the UK. These samples were immediately placed on ice (4°C) and frozen at the earliest opportunity.

The remainder of the venous blood was aliquoted into two EDTA blood collection tubes. The EDTA tubes were centrifuged at 2500 rpm for 20 min (R-8C laboratory centrifuge, REMI, Mumbai, India). The plasma and red cell pellet were then aliquoted and frozen (-20°C then -80°C).

In some cases, as part of normal ICU care, one of the two blood filled EDTA tubes was sent for measurement of a neutrophil count in the local laboratory. This results were made available on completion.

Arterial blood samples were analysed either on a local ICU blood gas analyser or a hand held blood gas analyser (EPOC, Woodley, UK).

6.2.9 Micro RNA analysis of human plasma

Extraction of micro RNA was conducted on plasma samples stored in EDTA using RNeasy MinElute Cleanup Kit and reverse transcription using miScript PCR starter kit (Qiagen, Manchester, UK).

6.2.9.1 Extraction of micro RNA from EDTA plasma

Plasma samples were defrosted and agitated by hand. Fifty µL of sample was added to 150 µL RNase free water and mixed with a pipette in a 1.5 mL collection tube.

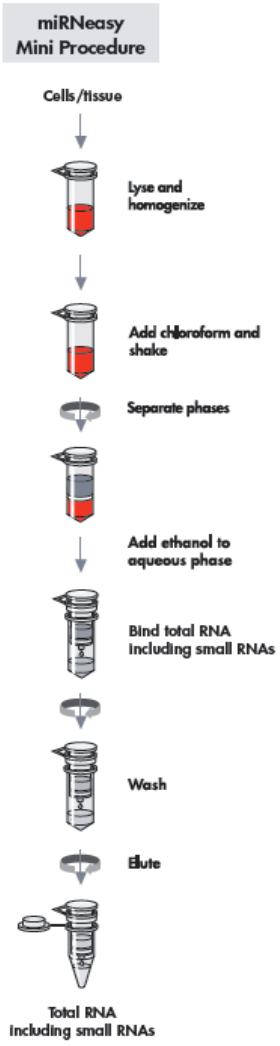


Figure 6.3: RNA extraction procedure using miRNeasy (Qiagen, UK)

One mL of Qiazol lysis reagent (to lyse fatty tissues and cells, inhibit RNases, and remove cellular DNA and protein through organic extraction) was added, mixed and left to homogenize for 5 min at room temperature.

MiRNeasy miR-39 spike-in- control (3.5 μL) and 200 μL of chloroform (to dissolve RNA) were added to all samples. After this they were shaken vigorously for 15 sec and left to incubate for 2-3 min at room temp and centrifuged for 15 min at 12,000 g at 4°C. On completion, 600 μL of the clear upper aqueous layer was transferred to a new collection tube where 900 μL 100% ethanol was added (to precipitate RNA) and mixed by pipette.

Seven hundred μL of the 1.5 mL mix was transferred to an RNeasy MinElute spin column in a 2 mL collection tube and centrifuged 8000 g for 15 sec at room temperature. The fluid at the bottom of the spin column 'flow through' was discarded and the process repeated with the remaining sample to collect the RNA in the spin column.

Seven hundred μL buffer RWT was added to the spin columns, centrifuging at 8000 g for 15 sec, discarding the flow. This process was repeated with 500 μL Buffer RPE.

Five hundred μL 80% ethanol (RNA wash) was then added to the spin columns, and centrifuged at 8000 g for 2 min. On completion, the flow through and collection tube were discarded and the spin column placed in a new tube (with the lids cut off) and centrifuged at 8000 g for 5 min to dry the membrane. The flow through was discarded.

The spin column was placed in a new 1.5 mL collection tube and 14 μL RNase free water was added to the spin column membrane to dissolve the extracted RNA, followed by a final centrifuge at 8000 g for 1 min. The spin columns were discarded and tubes sealed for further use. The process is summarised above (Figure 6.3).

6.2.9.2 Reverse Transcription and creation of complementary DNA (cDNA)

A 1.5 mL tube on ice was used to create a mastermix for all samples containing the following volumes per reaction: 4 μL miScript HiSpec buffer, 2 μL miScript Nucleics mix, 7 μL RNase free

water, 2 μL miScript Reverse Transcriptase (RT) mix. This mix contained all components required for first-strand cDNA synthesis.

Fifteen μL of mastermix was pipetted into wells on a 96 well plate and to this 5 μL of the extracted micro RNA sample was added. The plate was sealed and centrifuged at 1500 rpm for 2 min and then placed in a thermal cycler with the programme: Incubation at 37°C for 60 min, then 95°C for 5 min to inactivate miScript RT mix.

On completion, the 20 μL RT mix was aspirated from the well plate and added to 1.5 mL storage tubes containing 200 μL RNase free water (1 in 11 dilution). These were stored in a freezer -80°C prior to PCR.

6.2.9.3 Real time polymerase chain reaction (PCR)

A PCR mastermix was created in a 1.5 mL tube at room temperature containing the following volumes per reaction: 5 μL QuantiTect SYBR green PCR master mix, 1 μL miScript universal primer, 1 μL miScript primer assay (miR39- internal control /miR-21/miR-122/miR-146a), 2 μL RNase free water.

One μL cDNA samples were placed in a 384 well plate, where 9 μL of PCR master mix was added to each sample and gently mixed. All samples were made in duplicate.

The plate was sealed and centrifuged at 1500 rpm for 2 min and placed in a lightcycler (Roche, Welwyn Garden City, UK) for an initial 15 min at 95°C. It then went through the following 3-step cycle for 45 cycles: denaturation 15 sec 94°C, annealing 30 sec 55°C and extension for 30 sec at 70°C. Data were analysed using LightCycler® 480 release 1.5 O SP3 software to generate Ct (cycle threshold) values. [NB. A positive reaction is detected by the accumulation of a fluorescent signal. The Ct value is the number of cycles required for the fluorescent signal to cross the threshold of a background signal. Consequently, samples with much target nucleic acid will have lower Ct values (≤29 cycles) as they reach threshold more quickly. Samples with little or no target nucleic acid will have values ≥35 cycles.]

Each sample was assayed in duplicate and the average Ct value calculated. Average Ct values were compared within groups and relative quantification was calculated using the miR-39 (internal control gene) to determine the fold differences in the expression of the target gene (Ct target gene – Ct miR-39) creating a ΔCt value (Kishore et al., 2014). A further normalisation was applied by removing the average signal produced by the control group (ΔCt target gene – mean ΔCt control group target gene) producing ΔΔCt values. The relative gene expression was then calculated using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001). Statistical tests were conducted on these calculated data and not the raw data.

6.2.9.4 Additional human control plasma samples for miRNA testing

Because of the low numbers of Sri Lankan surgical control patients recruited, and the fact that they all had colorectal cancer with potential oncogenic levels of expression of miR-21 (see below study limitations), it was decided to re-measure the miRNA in additional human controls.

Healthy human (UK) non-intubated controls (n=8) were sourced from the Partaking in Alcohol Related to serum RNA Total Increase (PARRTI) study (this was studying miRNA changes pre and post alcohol ingestion). This study had ethical permission for samples to be used as controls in miRNA testing of other groups of toxicological patients. Samples were taken from healthy young adults (medical students) before the intake of alcohol. EDTA plasma samples were analysed for levels of miR-39, miR-21, miR-146a and miR-122. As the plasma samples were from healthy non-intubated controls the expression for all the miRNA in this group was expected to be lower than that observed in the pesticide poisoned patients. The testing of these extra control samples was conducted by Mr Bastiaan Vliegenthart a fellow toxicology PhD student with whom I collaborated.

6.2.9.5 Statistical analysis of micro RNA results

The function of this experiment was to assess potential biomarkers and not to observe the change in miRNA expression over time between groups. Therefore all plasma samples within a group (irrespective of the time of sampling) were analysed using an omnibus Kruskal-Wallis test between control, OP (OP+ASP and OP+No ASP) and NON-OP + ASP groups.

6.2.10 Measurement of inflammatory cytokines in the plasma and BALF using Cytometric Bead Array (CBA) analysis

The cytokines IL-8 and IL-6 were measured in the minipig aspiration model at 24 and 48 hours, but IL1β, TNF-α, IL-12p70 and the anti-inflammatory cytokine IL-10 can also be raised during ARDS development (Ware and Matthay, 2000). To further understand the lung injury caused by OP ingestion and aspiration a CBA Human Inflammatory cytokine kit (BD, New Jersey, USA) was used to quantitatively measure BALF and plasma interleukins 8 (IL-8), 1β(IL-1β), 6 (IL-6), 10 (IL-10), tumour necrosis factor (TNF) and 12p70 (IL-12p70). It should be noted that TNF-α -as quoted from the literature- and the measured TNF in the CBA kit are the same due to a change in scientific terminology.

CRP, a general marker of inflammation and the presence of apoptotic cells, was also raised in the minipig model and therefore measured using a basic FlowCytomix kit (eBioscience, CA, USA).

6.2.10.1 Human Inflammatory Cytokine measurement

The cytokine standards were prepared as per instructions in eight serial dilutions with assay diluent providing a detection range from 20-5000 pcg/mL.

The amount of each capture bead (e.g. IL-1β-6,-8,-10, TNF, -12p70) required for the experiment was calculated using the formula [number of tests x 25 μL/6]. Each of the six bead aliquots was added together in a tube and vortexed. [NB. when testing plasma samples the mixed capture beads were centrifuged at 200 g for 5 min, the supernatant discarded and replaced with a similar volume of serum enhancement buffer as per instructions. These were vortexed and incubated at room temperature for 30 min. Serum enhancement buffer blocks human anti-mouse antibodies found in some people's serum that can lead to false positives.]

The human BALF samples were tested neat (undiluted), 1 in 10 and 1 in 20 dilution, with the plasma samples tested neat (undiluted) and 1 in 4 dilutions using assay diluent.

Using a curved bottom 96 well plate, 25 μL of the mixed capture beads, 50 μL of standards or sample then 25 μL Human Inflammatory Cytokine PE Detection Reagent were pipetted into the

wells. The plate was covered and shaken for 5 min at 1150 rpm on a plate shaker (IEMS™, Thermoelectron Corporation).

The plate was then incubated at room temperature for 3 hours, before being centrifuged at 1500 rpm for 5 min (LMC-3000, Grant Bio centrifuge). The supernatant was discarded and 120 μL of wash buffer added to each well and shaken for 2 min at 1125 rpm before analysis on a BD C Sampler™ flow cytometer.

For plasma samples 25 μL of mixed beads and 50 μL standard or sample were shaken for 5 min at 1150 rpm and then incubated for 1.5 hours. The plate was centrifuged at 1500 rpm for 5 min and the supernatant removed. 125 μL wash buffer was added to each well, shaken for 2 min at 1150 rpm, centrifuged and the supernatant removed again. 25 μL human inflammatory cytokine PE detection reagent plus 100μL assay diluent were added to each well, shaken for 5 min at 1150 rpm and then incubated at room temperature for 1.5 hours. After a final centrifuge at 1500 rpm for 5 min and removal of the supernatant, 120 μL of wash buffer was added to each well, shaken for 2 min at 1150 rpm and then analysed on a flow cytometer (BD Accuri™ C6).

The antibody coated beads present in the wells of the plate were designed to bind to the cytokines (present in the sample) under investigation and emit a fluorescence signal unique to that bead/cytokine. This allowed simultaneous measurement of multiple cytokines in the same sample, at the same time using flow cytometry.

Standard curves were created by calculating the median fluorescent intensity (MFI) of the detector antibody for each cytokine in the standards. Using software (FCAP Array software v.3) the samples' MFI values were placed on the standard curve to calculate their cytokine concentration within the sample. The sample results were accepted if there were ≥80 events (beads) detected for each cytokine measurement.

6.2.10.2 Human CRP simplex bead test

The cytokine standards were prepared as per instructions in 7 serial dilutions (plus blanks) with assay diluent providing a detection range from 0.1-70 ng/mL (100-70,000 pcg/mL).

The human BALF samples were tested neat (undiluted), and at a 1 in 250 dilution, with the plasma samples tested at 1 in 250 and 1 in 2500 dilutions using assay diluent.

Using a curved bottom 96 well plate 25 μL of standards or sample, 25 μL of the CRP beads, then 50 μL biotin-conjugate mixture were pipetted into the wells. The plate was covered and protected from light and shaken for 2 hours at 500 rpm on a plate shaker.

The plate was centrifuged at 1500 rpm for 5 min (LMC-3000, Grant Bio centrifuge). The supernatant was discarded and then 100 μL of assay diluent was added to each well, re-centrifuged and the supernatant discarded. This process was repeated once.

One hundred μL assay buffer and 50 μL streptavidin-PE solution were added to all wells, covered, protected from light and shaken for 1 hour at 500 rpm.

The plate was centrifuged at 1500 rpm for 5 min (LMC-3000, Grant Bio centrifuge). The supernatant was discarded and then 100 μL of assay diluent was added to each well re-centrifuged and the supernatant discarded. The process was repeated once.

The forward and side scatter parameters were adjusted so that the bead population was identified. 120 μL assay diluent was added to each well and analysed on a flow cytometer (BD Accuri™ C6).

99% of samples had > 290 beads present. Standard curves were created and results analysed.

6.2.11 Human BALF SP-D measurement

Human BALF SP-D was measured by using an R&D development ELISA kit (cat no. DY1920) purchased form R&D systems, Abingdon, UK. It contained standards, capture and detection antibodies and streptavidin-HRP with a range of 156 – 10,000 pcg/mL. Buffers and solutions were made as in the methods chapter 2. Reagent diluent blanks were used and subtracted from the final absorbance readings. Samples were tested at neat (undiluted), and at a 1 in 25 dilution for one high outlier.

The capture antibody was diluted in PBS to a working concentration as per instructions and then 50 µL placed on an ELISA plate overnight at room temperature. The plate was washed 3 x 300 µL using wash buffer and then blocked with 300 µL reagent diluent for 1 hour. 50 µL of samples or standards were added to appropriate wells with duplicates and left at room temperature for 2 hours. The plate was then washed 3 x 300 µL, and the detection antibody was diluted in reagent diluent to a working concentration and 50 µL added to all wells and left for 2 hours at room temperature. After a repeat plate wash (3 x 300 µL) 100 µL streptavidin-HRP (1 in 200 dilution with reagent diluent) was added for 20 min and then the plate re-washed as before. 100 µL of substrate solution was added to all wells for 20 min, and at the end of that, 50 µL of stop solution was also added to all wells. A plate reader allowed the optical density to be measured at 450 nm, then readings from 540 nm were subtracted. Calculation of ELISA results were as that described in chapter 2.

6.2.12 Ventilator associated pneumonia audit in Sri Lankan ICUs

Permission to conduct the audit was granted by Dr Vasanti Pinto, head of the ICU, University of Peradeniya (10 bed unit), Dr Kamal Naser, on the toxicology ward ICU (3 beds) and Nuwara Eliya general hospital ICU (4 beds). Data were collected from all intubated patients using a basic data collection form (criteria described below). Data were collected between July-September 2013 on randomly selected days based on my location and work commitments (convenience sampling). The VAP diagnostic audit criteria were based on the hospitals in Europe linked for infection control (HELICS) VAP definitions (Walsh et al., 2013).

Criteria used in audit for potential VAP:

- CXR changes suggestive of pneumonia +
- Temperature >38°C or white cell count of > 12 000 mm^3 or < 4000 mm^3 +
- Two chest signs e.g. new onset purulent sputum or change in character and signs of ventilator distress, or worsening gas exchange or ventilation requirements.

This definition was amended to allow use of a single CXR for diagnosis (despite cardiac or pulmonary disease) as serial CXRs were often unavailable. Also, only those with an admission

diagnosis of pulmonary infection, but not signs of systemic infection, were excluded in the VAP numbers.

One organophosphate (OP) poisoned patient was audited twice, once for his first intubation day 1, and then again for his second period of intubation on day 2. These were counted as separate events. VAP was not diagnosed at either time as there was no CXR, although he had clinical symptoms of VAP during the second assessment. Additional clinical ICU information was collected; the report is found at appendix C.

6.3 Results

The pilot study failed to recruit the desired number of patients due to several factors. Effective public health measures in Sri Lanka e.g. restricting the use of the most toxic (WHO class II) pesticides and the 'safe storage of pesticides' programme, meant that the number of OP pesticide poisoned patients that required intubation and ventilation was reduced.

Patient numbers were also reduced because: (i) ethical permission was delayed on both visits, (ii) there was difficulty in recruitment as some relatives did not want the patient to be involved with the study (iii) some ward doctors did not allow patient recruitment and/or refused to take samples due to the perceived lack of medico-legal protection in performing the studies procedures e.g. miniBAL (it was for this reason that there were more plasma samples taken than BAL samples) (iv) some relatives were not present in time to give consent for sampling within the 48 hour window (v) some patients were extubated before the 48 hour sampling point, or before consent was given.

During the first study period (2013) only three surgical controls were recruited (reasons discussed below), but during the subsequent study period (2014) additional ICU non-infective controls were sought (after ethical approval) for 24 and 48 hour post intubation and ventilation sampling. However, some of the ICU consultants would not allow sampling due to the perceived additional harm for the patients; this resulted in no extra control patients being recruited.

Not all selected physiological variables were recorded at the scheduled sampling time points either because (i) there was no staff assistance, (ii) it was collected but not recorded or (iii) the required equipment was missing, or failed to work.

6.3.1 Patient demographics

In total, only 3 of target 5 control and 11 of target 15 pesticide poisoned patients were recruited to the study. In this patient cohort pesticide poisoning was more common in males (9:2, males: females) with a mean age 42(±19) years (range 19-72). The OP+ASP group was younger, mean 26.5 (±9.5) years (range 19-40). Of the 11 pesticide poisoned patients, n=7 also consumed alcohol, with n=7 receiving gastric lavage and n=10 liquid charcoal via a nasogastric tube for treatment of

pesticide poisoning. Eight patients required transfer to the University of Peradeniya hospital from a rural hospital, and patients were intubated within 12 (±8) hours of OP self-poisoning secondary to respiratory failure. The patient characteristics and poisoning details at admission are detailed below (Table 6.1).

Patient No. and Graph code shape	Age (years) mean (SD)	Sex (M:F)	Pesticide type/ volume	Alcohol consumed	Gastric lavage	Charcoal	Rural hospital transfer to Peradeniya (No.hours after diagnosis)	PMHx	Time of intubation post poisoning (hours)	Drugs given on admission	Clinical information aiding initial diagnosis
1 (circle)	67	M	Surgical control					Colorectal cancer			
2 (square)	69	F	Surgical control					Colorectal cancer			
3 (triangle)	56	F	Surgical control					Colorectal cancer			
Group mean (control)	64±(7)	1:2	Surgical control								
4 (circle)	26	M	OP - unknown	Yes	Yes	Yes	Yes (n/a)	Hypothyroidism	8		ABG PaO_2 8.2, decreased sats 75%, OP smell
5 (square)	19	F	OP – Chlorpyrifos EC40 (Indur)	No	n/a	Yes	Yes (n/a)	None	n/a	Co-amoxiclav and metronidazole	CXR – at 6 hours signs of right sided aspiration

6 (triangle)	21	M	OP-diazinon (diazol) 50 mL	Yes	Yes	Yes	Yes (9.5)	None	8	Atropine 10mg + infusion, co-amoxiclav, PAM 3g, adrenaline for bradycardia	GCS 5/15, lowest recorded sats 56%, evidence of aspiration at scene (vomit), respiratory depression, bi lateral crepitations, bradycardia, test-mate AChE U/gHb 19.0
7 (diamond)	40	M	OP-unknown	Yes	No	Yes	No	No	13	1.8mg atropine	Miosis, sweating, bilateral crepitations, lowest sats 88%
Group mean (OP+ASP)	26.5±(9.5)	3:1									
8 (circle)	72	M	OP-unknown	No	No	Yes	Yes (n/a)	Alcoholism	8	Atropine 6mg, cefuroxime,	Frothing at mouth, OP

										metronidazole, PAM 2g	smell. Intubated secondary to respiratory failure.
9 (square)	48	M	OP - unknown	Yes	Yes	Yes	No	Alcoholism, previous glyphosphate ingestion the previous year	5	Atropine 1.2mg	Miosis, respiratory arrest. Test-mate 12 hours post poisoning - 2.3U/gHb.
10 (triangle)	52	M	OP- unknown	Yes	Yes	Yes	Yes (2)	Old Right CVE with left sided body weakness	6.5	Atropine 6mg, diazepam 5mg, omeprazole, cefotaxime, metronidazole	GCS 15/15 and lungs clear on admission, respiratory distress then developed and was intubated. Seizures treated with diazepam. Test-mate -

											1.6 AChE U/gHb.
11 (diamond)	58	M	OP-phenthoate	Yes	No	No	Yes (17)	None	13.5	Atropine 21 mg, midazolam, cefuroxime, metronidazole	Sweating, miosis, bradycardia, developed respiratory difficulty and intubated and transferred to Peradeniya. Test-mate - 4.7 Ug/Hb
12 (hexagon)	43	M	OP-unknown 100 mL	Yes	Yes	Yes	Yes (3.5)	None	31	Atropine 10 mg	Found lying on ground, on arrival in Peradeniya GCS 15/15, sats 99%, RR 29, test-mate -0.3 AChE U/gHb

Group mean (OP+ No ASP)	54.6±(11.2)	5:0							12.8± (10.7)		
13 (circle)	68	M	Carbamate - Carbofuran	n/a	Yes	Yes	No	Asthma	Within 24 hours	Cefuroxime, metronidazole, ranitidine,	Sats 80%, RR 50, CXR showed opacity in right mid-zone.
14 (square)	20	F	Carbamate	n/a	Yes + forced emesis	Yes	Yes (<1.5)	None	1h 25 min	Atropine 1.8 mg + infusion, cefuroxime, metronidazole and dopamine	GCS 3/15 in ambulance, history of vomiting, poor respiratory effort, CXR showed bilateral infiltrates R>L, BP 79/60 test-mate -2.2 AChE U/gHb
Group mean	44±(34)	1:1									

(NON OP+ ASP)											

Table 6.1: Patient characteristics and clinical information of control and pesticide poisoned patients at admission to University Hospital Peradeniya, Sri Lanka. Groups are colour coded: surgical controls (grey), OP+ASP (blue), OP+No ASP (green) and NON-OP+ASP (purple). Test-mate results – normal level is 27±2.9 U/gHb in adult humans, levels below this indicate OP or carbamate poisoning. CXR: chest x-ray, BP: blood pressure, sats: pulse saturations of oxygen (%), RR: respiratory rate, D: day, Dx: diagnosis, GCS: Glasgow coma score, PMHx: past medical history. Mean (SD) shown.

6.3.2 Patient physiological variables at formal sampling points

All (n=11) pesticide poisoned patients in this pilot study were ventilated due to respiratory failure at some point after poisoning, predominantly (4/11) using the synchronised intermittent mandatory ventilation (SIMV) setting. This indicated that the patients were not able to breath adequately with pressure support on their own. All the measured peak airway pressures (Ppeak) were below 25 cmH_2O i.e. protected ventilation was used, but most poisoned patients (n=7) were sitting in bed angled at only 8.3 (±4.3)°, thereby increasing their risk of pulmonary micro aspiration and VAP (Hunter, 2012).

The poisoned patients were largely cardiovascularly stable with only one NON-OP+ASP and one OP+ No ASP patient (patients 10 and 13) receiving vasopressor support at the time of sampling (Table 6.2).

Both OP poisoned and NON OP+ASP patients had evidence of suppression of AChE activity in venous whole blood (normal 586 ± 5U/ mmol (Worek et al., 1999)). This was because the two NON-OP+ASP patients were believed to have consumed carbamates [i.e. Curater] which also reversibly inactivates the enzyme AChE (reduce activity) akin to OPs (Fukuto, 1990). Patient 5 (OP+ASP) had an unexpectedly high level of AChE activity, perhaps due to the reactivation of the enzyme (as she was treated with pralidoxime at the peripheral hospital), or the patient did not in fact ingest an AChE inhibiting pesticide (Figure 6.4).

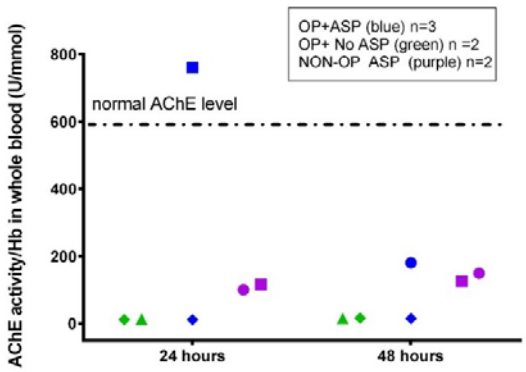


Figure 6.4: Dot plots showing AChE activity in venous whole blood when corrected for haemoglobin concentration (U/mmol). The differences between OP+ASP and NON-OP+ASP patients was not significant using permutation testing. Not all patients were tested at both time points due to early extubations. The normal AChE activity level in humans is 586 ± 5 U/mmol and indicated by the broken line. The plot codes (colour and shape) for individual patients are detailed in Table 6.2.

Patient No. and Graph code shape	Pulse saturations (%)	Ventilator setting	FiO_2	RR	PEEP (cm H2O)	Ppeak (cm H2O)	PaO2 (mmHg)	PaCO2 (mmHg)	Lactate (mmol/L)	pH	PaO_2/FiO_2 ratio (mmHg)	BP (mmHg)	Temp (°F)	Bed elevation (°)	Plasma neutrophils (x 10^9 L)	Complications/ Outcome
1 (circle)	99	SIMV	0.5	12	0	14	203	38		7.33	406			Head down	10.9	None known
2 (square)	99	SIMV	0.9	12	0	16	332	33		7.37	369			0	22.6	Required some oxygen on the ward due to low saturations. Possible pre-existing lung infection
3 (triangle)	100	SIMV	0.56	16	0	19	186	35.3		7.38	332	112/68		0	13.6	None known
4 (circle)	[100]	[SIMV]	[0.4]	[16]	[8]	[19]	[110.2]	[34.8]	[1.73]	[7.4]	[276]		[98.6]	[15]		n/a
5 (square)	100	SpV	0.6	18	0		226.1	27.9	0.6	7.4	377		98.2	10.3	12.2	Extubated D2, discharged D3.
6 (triangle)	100		0.5	14	5							145/94	98.4			Self-extubated D2, discharged from hospital D7.
7 (diamond)	99 [100]		0.7 [0.65]	16 [14]	5 [5]	25 [25]	[79.5]	[34.8]	3.6 [1.55]	[7.44]	[122]		98.4 [98.4]	14.2 [11]	[13.3]	Self-extubated D5, re-intubated D8, extubated D13. Dx IMS

8 (circle)	100 [98]	SIMV [SIMV]	0.4 [0.5]	14 [12]	5 [5]	13 [13]	163.4 [183.4]	24.3 [33.9]		7.5 [7.5]	409 [367]		98 [98]	10.3 [1.0]	12.7 [15.7]	Extubated D5, re-intubated D8 for increased RR40 sats 85%. Dx IMS and pneumonia. Eventually extubated and discharged.
9 (square)	99		0.35	14	5							120/80	98		14.1	Extubated D13, Dx, probable IMS. No other complications.
10 (triangle)	99 [100]		0.6 [0.5]	14 [16]	6 [6]	25 [25]	184.1 [101]	57.2 [68.4]	4.3 [4.4]	7.1 [6.9]	307 [202]	Sys 84 [74/49]	98.4 [98.4]	6.5 [5.0]	2.4 [4.5]	Required vasopressors - nor adrenaline, adrenaline developed multi-organ failure and died D3 in cardiac arrest.
11 (diamond)	100 [100]	Mandatory A/C	0.6 [0.6]	14 [14]	5 [5]	16 [16]	[200.4]	[38.3]	[3.27]	[7.4]	[334]		98.4 [98.2]	20.5 [14]	[7.6]	Extubated D16, probable IMS.

12 (hexagon)	[99]		[0.5]	[17]	[5]							[140/90]	[100]		9.42	Extubated D11, neck flexor weakness. Probable IMS.
13 (circle)	100 [97]	SIMV [SIMV]	0.5 [0.4]	12 [14]	5 [5]	25 [25]	128.7 [122.2]	44.8 [45.1]	2.0 [1.6]	7.2 [7.3]	257 [306]	[85/56]	98 [102]	9.1 [7.8]	14.3 [13.3]	D6 developed liver failure and heart failure and placed on inotropes: dopamine, dobutamine, noradrenaline. Eventually died.
14 (square)	100 [100]	SIMV+PCV [SIMV+PCV]	0.5 [0.5]	14 [14]	5 [5]	20 [20]	199 [195]	28.7 [23.6]	4.9 [1.6]	7.1 [7.3]	398 [390]	129/62 [98/51]	98 [98]	9.0 [9.2]	19.9 [22.1]	D6 grew pseudomonas from endotracheal tube, treated as pneumonia. Extubated D11 probable IMS, afebrile.

Table 6.2: Individual patient physiological data of control (taken at 3h 15 min, 5h 25 min and 4h 15 min respectively) and pesticide poisoned patients at 24 and 48 hours (except patient 6 – 14.5 hours after poisoning) in Sri Lanka. Groups are colour coded: surgical controls (grey), OP+ASP (blue), OP+No ASP (green) and NON-OP+ASP (purple). Data shown outside the brackets are 24 hour data with 48 hour data in the square brackets. SIMV: synchronized intermittent mandatory ventilation, SpV: spontaneous ventilation, PEEP: positive end expiratory pressure, Ppeak: peak airway pressure within the ventilation circuit, A/C: assisted control ventilation, PCV: pressure controlled ventilation, RR: respiratory rate, IMS: intermediate syndrome.

6.3.3 Patient outcomes and respiratory findings

Two patients died after pesticide poisoning whilst in hospital: one from cardiac arrest and multi-organ failure on day 4 after an unknown OP+No ASP poisoning (patient 10) and one from cardiac failure several days after NON-OP+ASP poisoning (patient 13) secondary to carbofuran ingestion. Over half (5/9) of intubated OP poisoned patients developed probable (as no formal NMJ analysis was performed) intermediate syndrome (IMS) (ventilated ≤5 days), one being ventilated for up to 16 days (patient 11; Table 6.2). The OP's causing IMS were mostly unknown except for one case involving phenthoate poisoning. There was possibly one carbamate induced IMS (patient 14) who was ventilated for 11 days. IMS is rare in carbamates, but has been previously described (Paul and Mannathukkaran, 2005).

6.3.3.1 Reduced PaO_2/F_IO_2 ratios in pesticide poisoned patients

We first compared the incidence of acute lung injury as documented by reduced arterial PaO_2/F_IO_2 ratios (≤ 300 mmHg) at 24 and 48 hrs in the poisoned patients. Lung injury (ARDS) was only present in two of four patients from the OP+ASP group at 48 hours, one patient from the NON-OP+ASP group at 24 hours and one patient from the OP+No ASP group at 48 hours with none of three patients from the control group for whom arterial blood samples were available (Figure 6.5). However, to make a meaningful conclusion, this measurement would have to be repeated in a larger patient group, with more similar controls whose lungs had been ventilated for 24-48 hours.

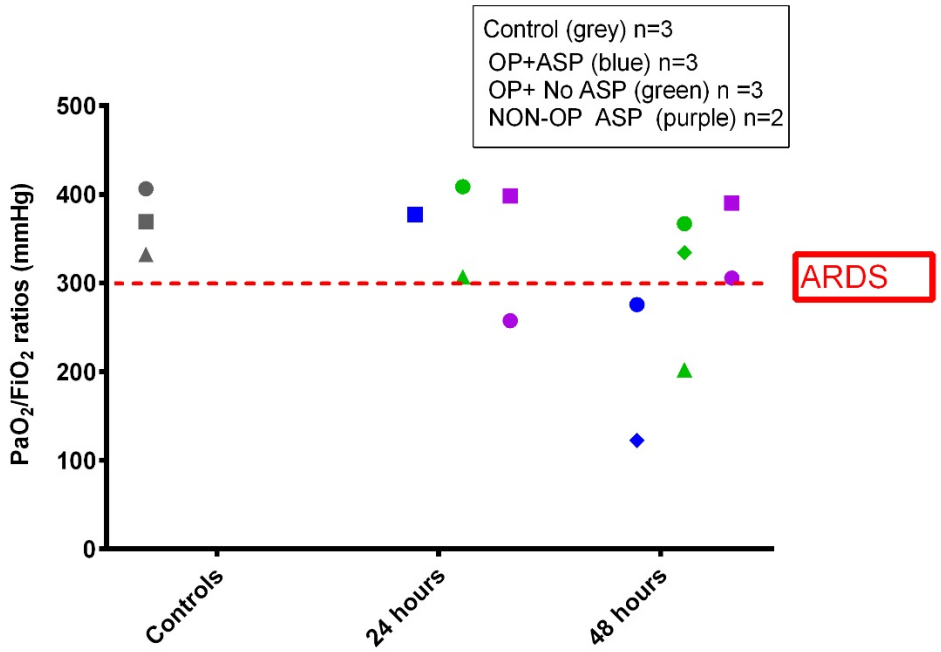


Figure 6.5: Dot plot showing arterial PaO_2/F_IO_2 ratios in control and pesticide poisoned Sri Lankan patients. Red dotted line shows the line below which the diagnosis of acute **respiratory distress (ARDS) is made (≤300 mmHg).**

6.3.3.2 BALF analysis

The BALF return volume from patients was 0.85 (± 0.5) mL which was lower than the quoted 1-2 mL from other studies using the Combicath (Fujitani and Victor, 2006). This may have been due to operator inexperience with the technique. Nevertheless, the volume returned allowed the measurement of inflammatory markers, identification of cell types and bacteria present. There were fewer BALF samples (24 hours= 6 samples, 48 hours=4 samples) than plasma samples from pesticide poisoned patients because the local doctors were less likely to perform the procedure.

6.3.3.2.1 Bronchoalveolar lavage fluid cellular content

The miniBAL sampling technique did not provide a cell pellet large enough to provide the cell numbers required (>500) for statistical analysis. Prepared slides (from representative groups) allowed a limited white cell identification. Neutrophils appeared to be the predominant BALF white cell type within the OP+ASP and NON-OP+ASP groups (Table 6.3; Figure 6.6). This concurred with the minipig model cytospin analysis which showed marked pulmonary neutrophilia for OP+GJ aspiration group. Patient 2 (control group) had an unusually high

percentage of neutrophils on the BALF cell slide (59%), but also had a high plasma neutrophilia, and thus may have had underlying pulmonary and systemic inflammatory processes, perhaps as part of the colorectal malignancy co-morbidity (e.g. metastases) (Table 6.2).

Patient number and group	Time of BALF sample	No. cells counted on slide	Alveolar macrophages (% cells)	Neutrophils (% cells)	Lymphocytes (% cells)
Patient 2 Control	5h 25 min	207	41	59	-
Patient 4 OP+ASP	48 hours	204	21	79	-
Patient 5 OP+ASP	24 hours	209	21	79	-
Patient 8 OP+No ASP	24 hours	209	89	2	8
Patient 13 NON-OP+ASP	24 hours	218	46	52	1

Table 6.3: Percentage white cell types present in the BALF of representative control and pesticide poisoned patients. Limited slides were available for examination because of cellular destruction or use in the TLR analysis (see methods chapter 6).

The human BALF samples were notably less blood-stained that the minipig BALF samples, probably owing to the more gentle sampling technique. A formal bronchoalveolar lavage using a bronchoscope would be required to determine whether OP pesticide poisoning with aspiration actually produces a more bloody lavage [secondary to possible alveolar capillary and endothelial leak or breaks] compared with normal aspiration.

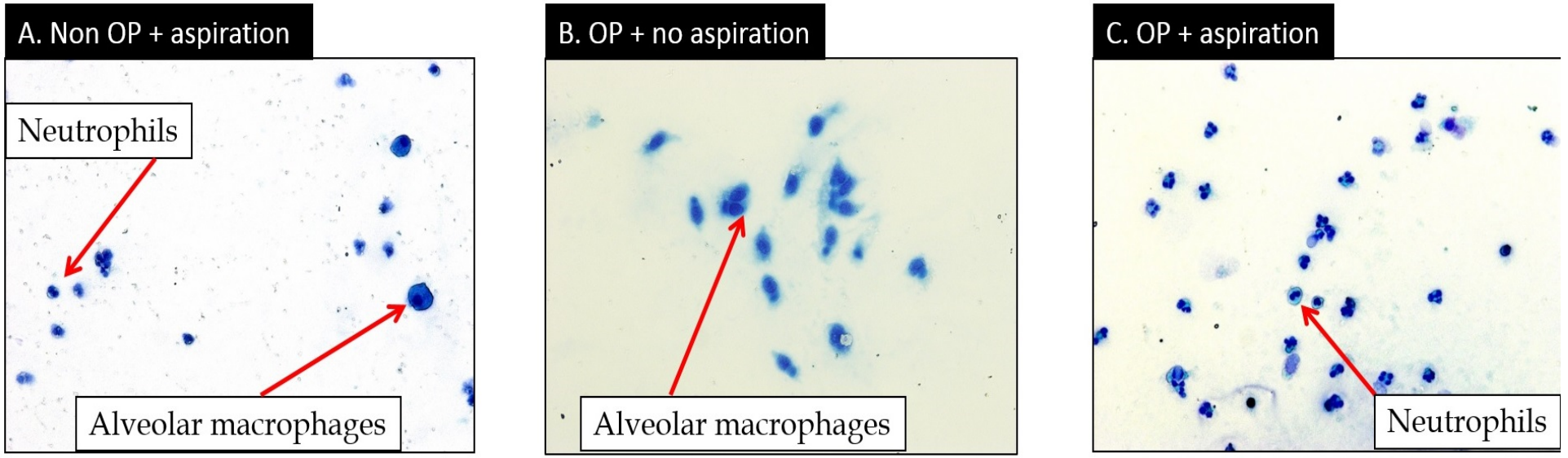


Figure 6.6: Representative images of cells taken from the BALF in NON-OP+ASP (A), OP+No ASP (B) and OP+ASP (C) patients suspected pulmonary aspiration of gastric contents 24 hours after pesticide ingestion. Slides were prepared as per methods above, and stained with Kwik Diff™. The presence of alveolar macrophages and neutrophils are highlighted with red arrows. Light microscopy images at x20 were taken using Axiovision Rel 4.8 software.

6.3.3.3 Bronchoalveolar lavage fluid concentrations of protein and albumin are increased in OP+ASP and NON-OP+ASP groups

Both BALF protein (Figure 6.7A) and albumin (Figure 6.7B) were measured as described in chapter 2 (methods) and both were found to be greater in the OP+ASP and NON-OP+ASP groups compared with controls and OP+No ASP group. The quantity of total protein and corresponding albumin were similar within the aspiration groups indicating that the increase in protein was largely due to an increased alveolar capillary leak e.g. albumin, as opposed to a large influx of inflammatory molecules. The differences were not statistically significant due to low numbers.

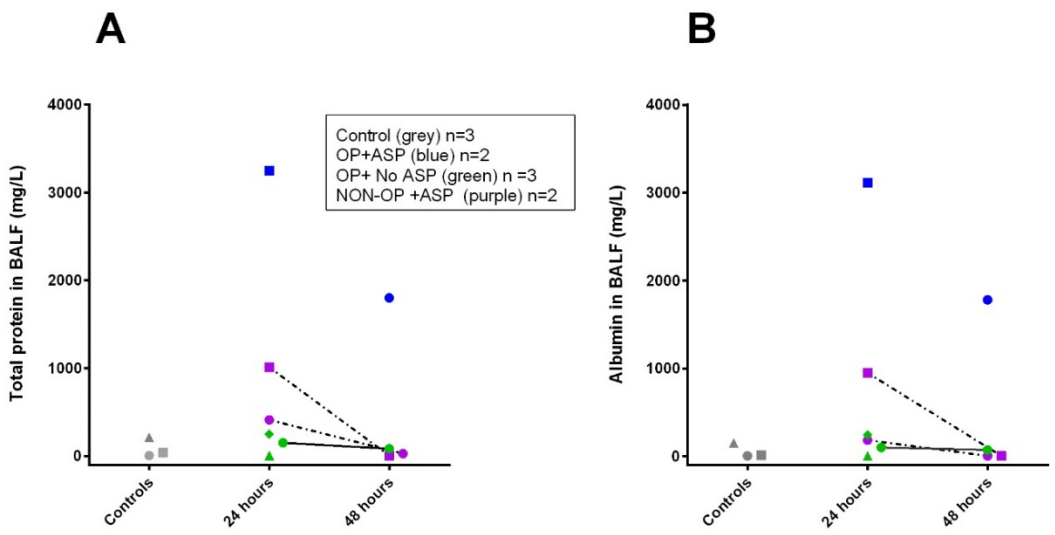


Figure 6.7: Dot plots of total protein (A) and albumin (B) contained in BALF of surgical control and pesticide poisoned patients at 24 and 48 hours. The dotted line connects the 24 and 48 hour sample from the same NON-OP+ASP patients, with the continuous line connecting OP+No ASP aspiration same patient samples. The limit of assay detection was 5 mg/L and so values calculated as <5 mg/L were entered as 5 mg/L.

6.3.3.4 Inflammatory cytokine concentrations were raised in bronchoalveolar lavage fluid from OP+ASP and NON-OP+ASP groups

During the development of aspiration lung injury and ARDS, inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α and the anti-inflammatory IL-10 may be released by alveolar macrophages, neutrophils and local pulmonary endothelium (Raghavendran et al., 2011, Bhargava and Wendt, 2012).

At 24 hours, pesticide poisoned patients had greater concentrations of BALF IL-1β (4/6 patients), IL-6 (5/6) and IL-8 (6/6) than controls, with the OP+ASP or NON-OP+ASP groups showing the greatest concentrations overall (Figure 6.8). BALF IL-8 (p=0.03) and IL-1β (p=0.05) concentrations were also significantly different between controls, OP (OP+ASP and OP+No ASP combined) and NON-OP+ASP patients at 24 hours during omnibus testing. However sub-group analysis (permutation testing) showed no significant differences between groups individually. Increased BALF IL-6 and IL-8 concentrations in the OP+No ASP group supports the argument that orogastric administration of OP caused an indirect lung injury through systemic absorption, like that seen in the minipig model (indirect lung injury in OP+GJ treated pigs), at 24 hours.

By 48 hours, the BALF concentrations of IL-6 and IL-8 in OP+No ASP, and NON-OP+ASP patients returned to control levels. The OP+ASP concentrations of IL-6 and IL-8 remained high in patient 4 at 48 hours, as was found in the minipig study, suggesting a possible ongoing lung injury in this patient. No 48 hour samples were available for patient 5 as they had already been extubated (Table 6.2).

BALF concentrations of TNF, IL-10 and IL-12p70 were not raised in the OP poisoned patients (OP+ASP and OP+ No ASP) at 24 hours with similarly low concentrations found in the control group. Patient 13 from the NON-OP+ASP group had elevated TNF and IL-10 concentrations (Table 6.4).

Measured cytokine	Control (n=3)	OP (ASP+ No ASP) n=4)	NON-OP + ASP (n=2)
TNF (pcg/mL)	0	2.8 (±5)	49 (±70) [patient 14: 0, patient 13: 99]
IL-10 (pcg/mL)	0	1(±1.5)	3.5(±4.9) [patient 14: 0, patient 13 : 6.93]
IL-12p70 (pcg/mL)	0	0.01(±0.02)	0.6(±0.8)

Table 6.4: BALF cytokine concentrations of TNF, IL-10 and IL-12p70 in control and pesticide poisoned patients at 24 hours. The mean concentrations reported are all below the detection range of the assays except in the NON-OP+ASP group for TNF. 48 hour concentrations were lower than those reported for 24 hours (results not shown). Mean (SD) shown.

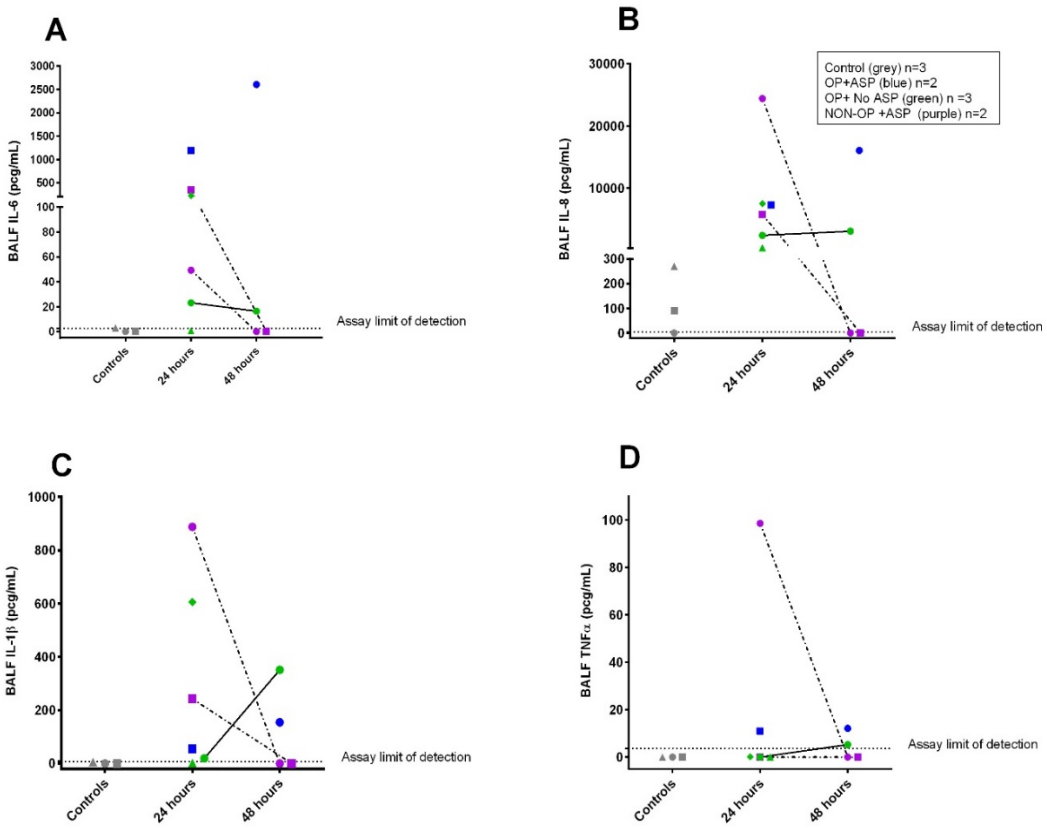


Figure 6.8: Dot plots of BALF concentrations of IL-6 (A), IL-8 (B), IL-**1β** (C) and TNF (D) of control and pesticide poisoned patients at 24 and 48 hours. Kruskal-Wallis analysis of BALF controls vs. all OP (n=4) vs. NON-OP+ASP patients (n=2) revealed significant differences for BALF IL-8 (p=0.03*) and IL-1B (p=0.05 *) at 24 hours with no significant subgroup analysis with permutation testing. The dotted line connects the 24 and 48 hour sample from the same NON-OP+ASP patient, with the continuous line connecting OP+No ASP patients. The assay limits of detection were 2.5pcg/mL (IL-6), 3.6 pcg/mL (IL-8), 7.2 pcg/mL (IL-1B), 3.3 pcg/mL (IL-10), 3.7 pcg /mL (TNF) indicated by the dotted lines above.

6.3.3.5 Plasma inflammatory cytokine concentrations are similar between control and pesticide poiosned patients

There were more plasma than BALF samples collected in the pesticide poisoned patients because the local doctors were more agreeable to sampling blood than BALF.

Measurement of the same cytokine panel in plasma revealed that concentrations of IL-6, IL-8 and TNF in pesticide poisoned patients were not statistically different from controls (Figure 6.9 A, B and D). However, there were greater concentrations of these cytokines in OP+No ASP group. The information regarding poisoning severity (type of OP and volume consumed) was not available and so it is not known whether OP+No ASP patients had a more lethal dose of OP

(and therefore more systemic toxicity) than the OP+ASP patients. The concentration of BALF IL-8 in the aspiration groups was greater than the plasma, indicating a predominantly local pulmonary, rather than systemic inflammatory effect. IL-8 is a powerful neutrophil chemoattractant and the increased percentage of neutrophils in OP +ASP and NON-OP+ASP cytospin slides may have been caused through this mechanism (Baggiolini and Clarklewis, 1992) (Figure 6.6).

Plasma concentrations of IL-1β (<4pcg/mL) and IL 12p70 (<5pcg/mL) were very low in all groups and at all time points. Plasma concentrations of the anti-inflammatory cytokine IL-10 were greater in controls than all the poisoning groups (Figure 6.9 C).

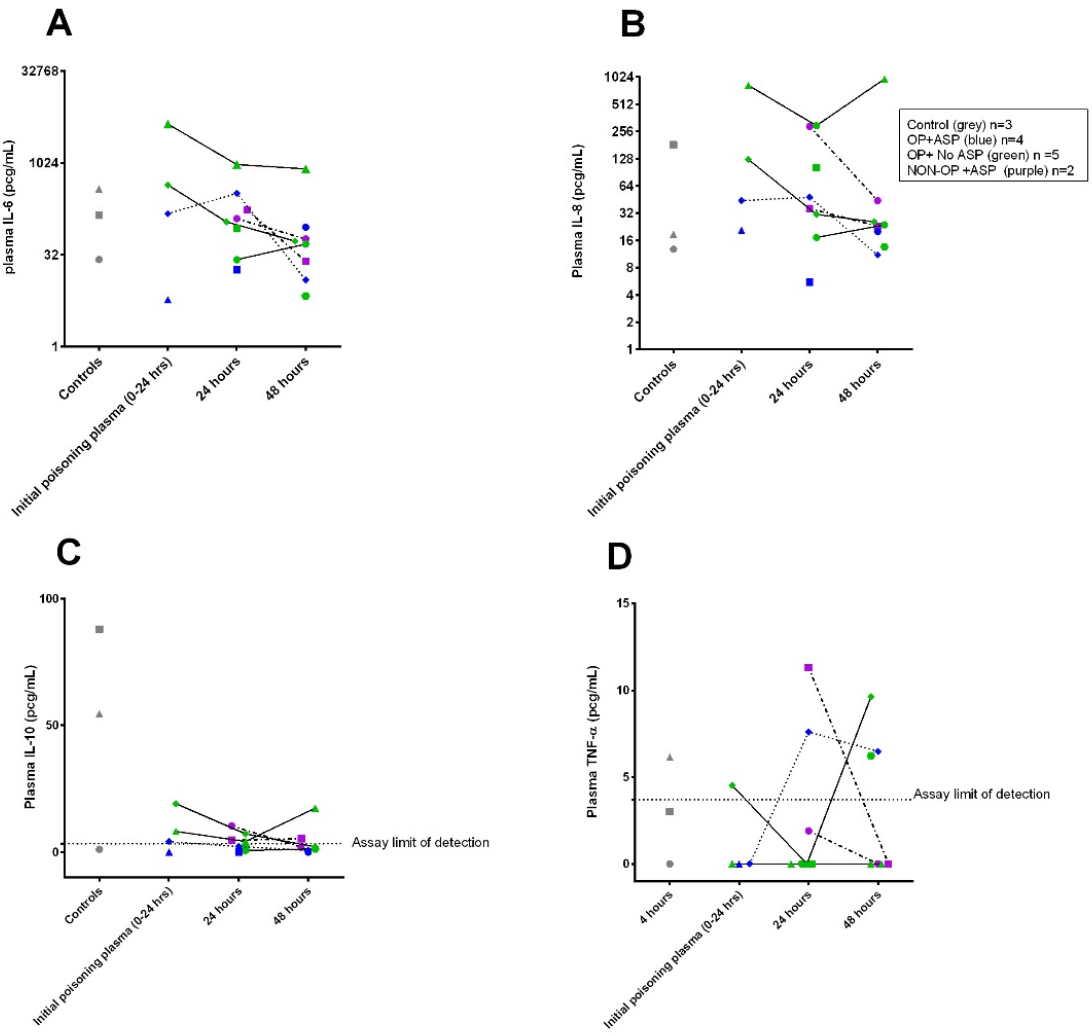


Figure 6.9: Dot plots of plasma concentrations of IL-6 (A), IL-8 (B), IL-10 (C) and TNF (D) in controls and pesticide poisoned patients. The dotted lines connect the 24 and 48 hour samples from either the same NON-OP+ASP or OP+ASP patients, with the continuous line connecting OP+ No ASP patient samples. There was very little or no IL-**1β or IL**-12p70 in the plasma (results not shown). Kruskal-Wallis testing did not find significant differences between groups. The assay limits of detection were 2.5pcg/ml (IL-6), 3.6 pcg/ml (IL-8),3.3 pcg/ml (IL-10), 3.7 pcg /ml (TNF) indicated by the dotted lines above. [NB.Figures 1.9 A and B are semi-logarithmic].

6.3.3.6 OP poisoned patients with and without aspiration have elevated concentrations of C-reactive protein in bronchoalveolar lavage fluid and plasma

All BALF samples from pesticide poisoned patients contained high concentrations of CRP when compared with controls. NON-OP+ASP patients had greater CRP concentrations (48.8(±42) pcg/mL) than OP poisoned patients combined (28.8(±23.3) pcg/mL) with low

concentrations in control samples (1(±1) pcg/mL) at 24 hours indicating pulmonary inflammation and/or the presence of apoptotic cells within the lung (Figure 6.10A) (Agassandian et al., 2014). Omnibus testing showed significant differences between controls, OP (OP+ASP and OP+No ASP combined) and NON-OP +ASP patients at 24 hours ($p=0.03$), but not with sub-group analysis (permutation testing).

Plasma CRP was raised in 1/3 controls, 2/4 OP+ASP, 4/6 OP+ No ASP and 1/2 NON-OP +ASP poisoned patient between admission and 24 hours after pesticide ingestion, but by 48 hours the CRP concentrations in all the samples were within the normal range (<10 mcg/mL) (Figure 6.10B). This suggests there was an initial systemic inflammatory reaction in pesticide poisoned patients, which resolved by 48 hours. Raised plasma CRP has been previously observed in OP poisoned patients (Lee et al., 2013), but the figures in our study were not statistically significant due to small study size.

The greatest BALF CRP concentration at 48 hours was in an OP+ASP patient whose corresponding plasma CRP was normal. This echoed findings in the minipig model and seems to indicate an ongoing, predominantly local (pulmonary), rather than systemic, inflammation. This needs to be confirmed in more human patients.

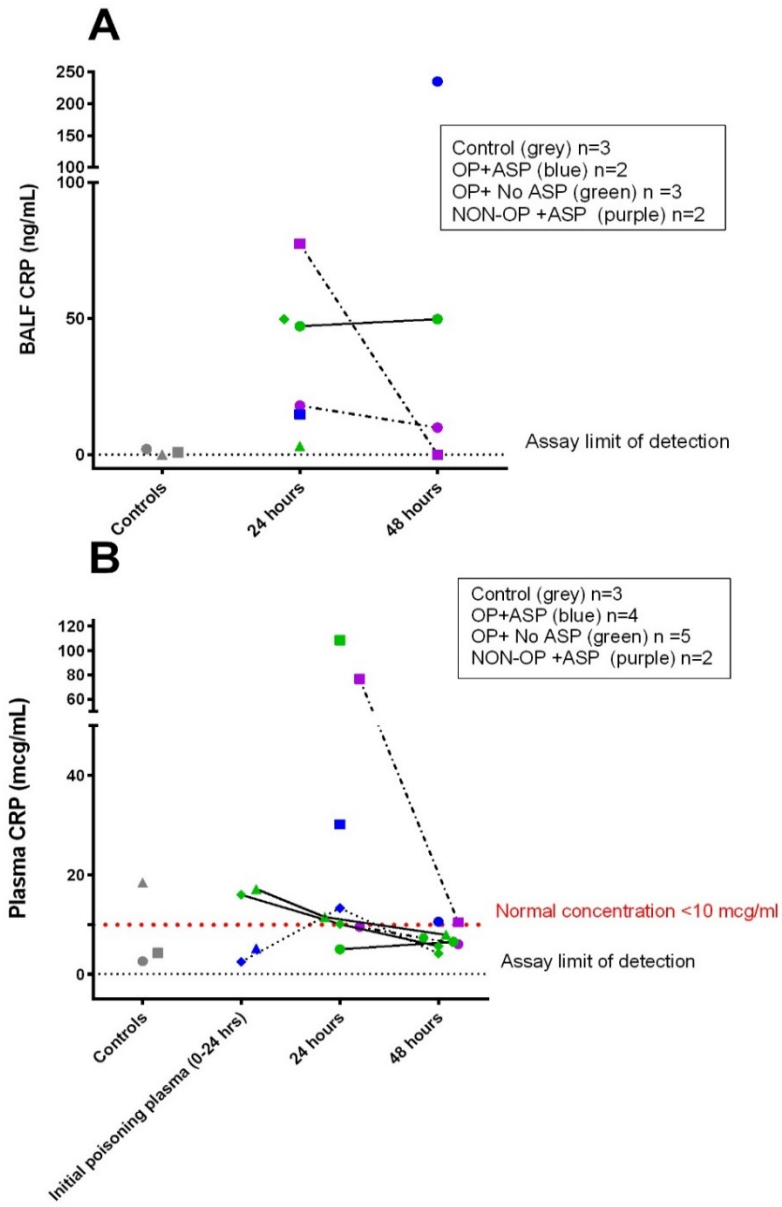


Figure 6.10: Dot plots of BALF CRP (A) and plasma CRP (B) concentrations from control and pesticide poisoned patients. Kruskal-Wallis analysis of BALF controls vs. all OP patients (n=4) and NON-OP+ASP patients (n=2) at 24 hours found significant differences (p = 0.03*), but with no significant subgroup analysis with permutation testing. The plasma CRP concentration differences between groups were not significant. The dotted lines connect the 24 and 48 hour sample from either the same NON-OP+ASP or OP+ASP patient, with the continuous line connecting OP+ No ASP patients. The assay limit of detection was 0.1 mcg/mL indicated by the dotted line. The red dotted line indicates the level at or below the normal plasma CRP concentration. CRP: C reactive protein, BALF: bronchoalveolar fluid.

6.3.3.7 Bronchoalveolar lavage fluid from OP poisoned patients has increased concentrations of surfactant protein D (SP-D)

Surfactant protein-D, secreted and produced by type 2 alveolar epithelial cells, is important for the lung host defence response and the resolution of inflammation (Nayak et al., 2012).

BALF samples from 1/2 OP+ASP and 2/3 OP+No ASP poisoned patients contained greater concentrations of SPD in the combined OP+ASP and OP+No ASP groups (2873(±3831) pcg/mL), than the control samples (404(±205) pcg/mL)at 24 hours. The NON-OP+ASP group had low concentrations 48.8(±42) pcg/mL at 24 hours (Figure 6.11). The greatest SP-D concentrations at both 24 and 48 hours were in OP+ASP patients. However these concentrations were still far below those found within the minipig model which was 102.5 ng/mL for OP+GJ treated pigs (direct lung injury) at 24 hours.

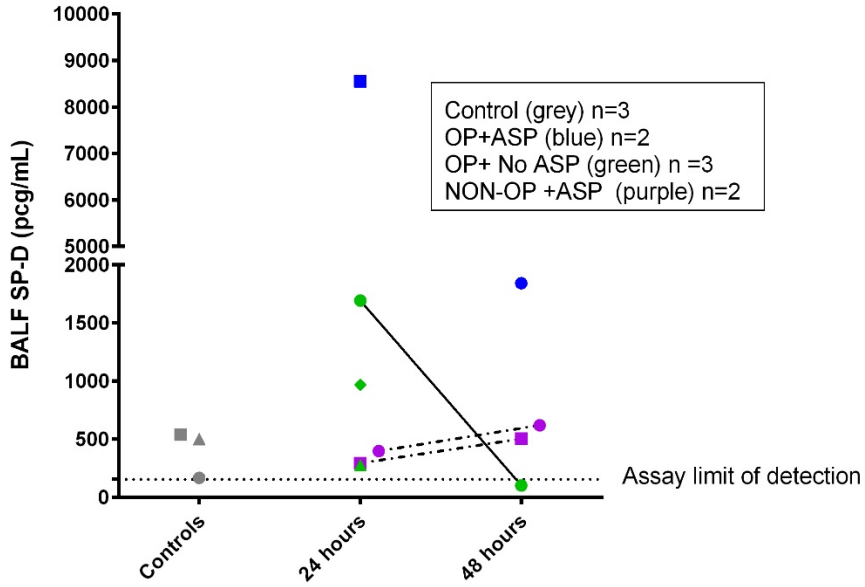


Figure 6.11: Dot plot of BALF SP-D concentrations from control and pesticide poisoned patients. There were no statistical (test) differences between groups. The broken dotted lines connect the 24 and 48 hour sample from the same NON-OP+ASP patient, with the continuous line connecting OP+No ASP patient samples. The assay limit of detection was 156 pcg/mL indicated by the dotted line. BALF: Bronchoalveolar fluid, SP-D: surfactant protein D.

6.3.3.8 Bacterial growth within bronchoalveolar lavage fluid was low between 24-48 hours, but patients remain at risk of ventilator associated pneumonia

Only one BALF sample (from a total of 6 poisoned and one control patient) from an OP+No ASP patient (patient 8) grew bacteria (*Pseudomonas* spp. and coliforms) 48 hours after poisoning. This sample may have been compromised as it was stored at 25-30°C (room temperature) for 36 hours before formal culture due to the laboratory being shut out of hours on the weekend. Sputum samples left for 24 hours at room temperature will promote growth of *Pseudomonas aeruginosa* and inhibit growth of *Haemophilus influenza* (Nelson et al., 2010).

From the time of admission, patient 8 was receiving intravenous cefuroxime and metronidazole for presumed aspiration pneumonia. Despite this, the patient developed probable VAP at day 6-7 after poisoning showing signs of respiratory compromise with the production of yellow sputum. No microbiological specimen was sent, but the patient was placed on the additional antibiotic co-amoxiclav and was eventually extubated after a prolonged period of ventilation on the ToxICU. The reason for the prolonged ventilation was probably due to IMS (see chapter 1).

Only one other NON-OP+ASP patient had a positive endotracheal secretion culture, with *Pseudomonas* spp detected on day 7 after poisoning.

Generally, pesticide poisoned patients did not have abnormal body temperatures, apart from one NON-OP+ASP patient at 48 hours. Unlike our study, others have found that OP poisoned patients usually develop a hypothermia on admission followed by an increase in core temperature which is partly iatrogenic secondary to high doses of atropine (atropine therapy was documented in 8/11 of our poisoned patients)(Moffatt et al., 2010). As no patients received regular paracetamol, this suggests that most patients in this study did not have an active systemic/pulmonary infection or cholinergic toxicity at 48 hours after poisoning. However, 7/11 poisoned patients were placed on antibiotics on admission as prophylaxis for aspiration pneumonia. Antibiotics often consisted of - a broad spectrum cephalosporin for gram positive and negative bacteria and anaerobic bacterial cover with metronidazole. Many of the pesticide poisoned patients also had above normal plasma neutrophil concentrations (Table 6.2) and so sub-clinical inflammatory processes, possibly

aspiration pneumonitis (non-bacterial) or pneumonia (bacterial), cannot be ruled out despite most poisoned patients (9/11) being haemodynamically stable .

6.3.4 The expression of MiRNAs -21,-146a and -122 are significantly reduced in OP pesticide and aspiration lung injury compared with non-intubated healthy control patients in the UK

Plasma expression of miRNAs miR-21, miR-146a and miR-122 were measured in all patients at all time points to uncover any differences between groups, and in so-doing, exploring their suitability as adjunct biomarkers to assess the severity of OP poisoning and involvement of other organs e.g. lungs and liver.

Omnibus testing of plasma miR-21, miR-146a and miR-122 did not find any significant differences between all 4 groups (control, OP+ ASP, OP + No ASP and NON-OP+ASP) using the Sri Lankan controls (n=3) (Figure 6.12 A, C and E). However, all three control patients were undergoing surgery for colonic carcinoma. Therefore concurrent inflammatory processes may have been present, and as a result, caused elevation of miR-21(Kanaan et al., 2012). Hepatocellular cancer is also linked with raised plasma miR-21, thus metastatic liver disease in any of the Sri Lankan controls could have contributed to the miR-21 signal (Tomimaru et al., 2012).

In contrast, the results from omnibus testing using controls from the Partaking in Alcohol Related to serum RNA Total Increase (PARRTI) study (n=8), showed significant differences across all patient groups for miR-21 ($p=0.003$), miR-146 ($p=0.0038$) and miR-122 ($p=0.0272$) (Figure 6.12 B, D and F).

MiR-21 ($p=0.008$), miR-146a ($p=0.0083$) and miR-122 ($p=0.0037$) plasma expression was significantly reduced in the OP+ASP group compared to UK study (PARRTI) controls. MiR-21 ($p=0.0001$) and miR-146a ($p=0.0001$) plasma expression was also significantly reduced in OP + No ASP when compared with UK study controls (Table 6.5).

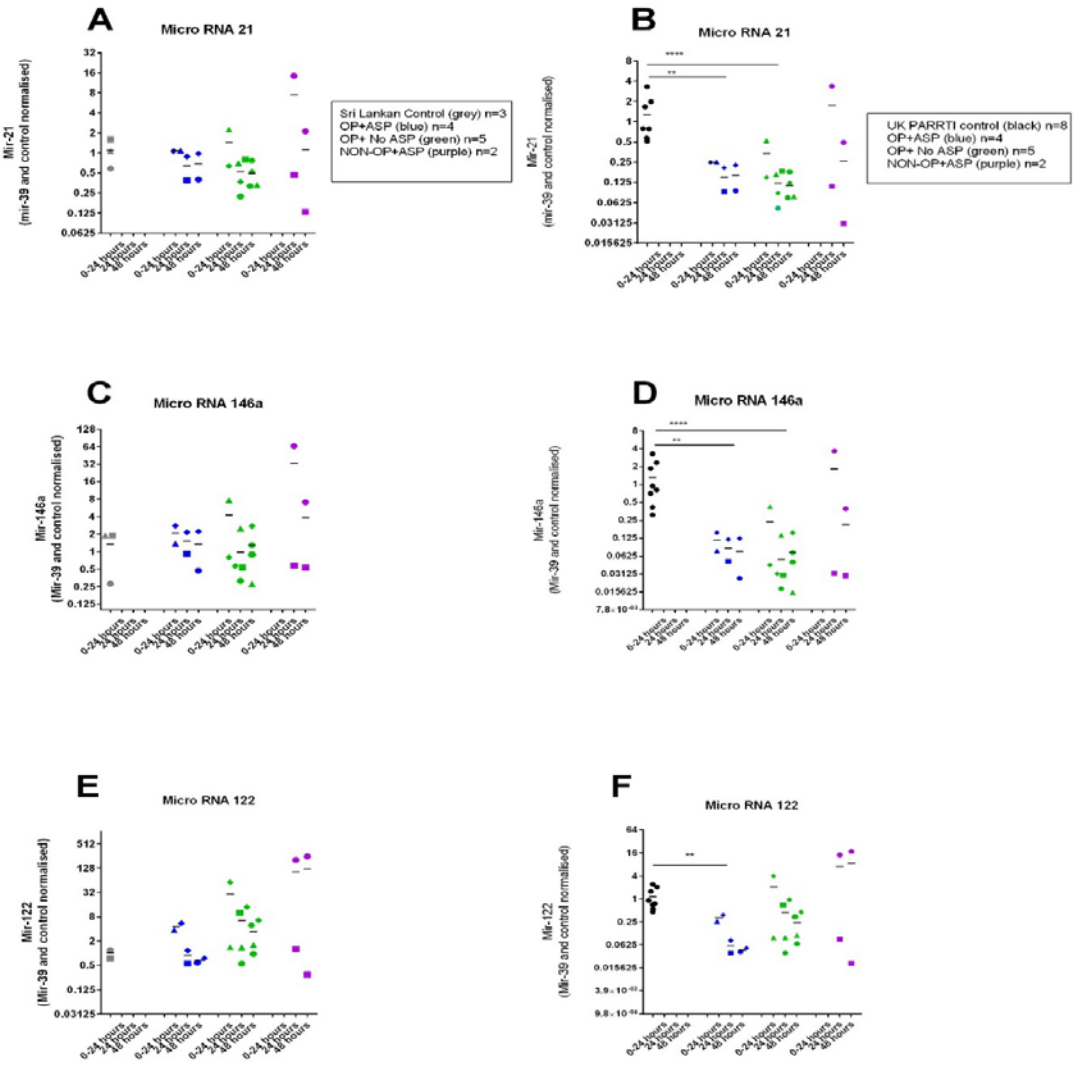


Figure 6.12: Relative quantification of plasma miRNA -21, -122 and -146a in pesticide poisoned and control (Sri-Lankan and UK) patients. Relative quantification used the PCR and the $2^{-\Delta\Delta CT}$ method (normalised using miR-39 and controls) the results of which were used for permutation testing. Sri Lankan plasma controls were taken at a mean time of 4 hours 20 min after completion of non-emergency surgery (figures A, C and E) with UK PARRTI study controls being taken from awake non-intubated healthy human adults from the UK (figures B, D and F). Kruskal-Wallis omnibus testing showed there were no significant differences between Sri Lankan control and all other groups. KW analysis between the UK controls and all other groups showed significant differences for miR-21** **($p \leq 0.01$)**, miR-146a** **($p \leq 0.01$)** and miR-122 ***($p \leq 0.05$)**. The results of further analysis between groups (including all time points) using permutation testing are shown on the graph (above). Group means are illustrated with a straight line. RNA: Ribonucleic acid, PCR: polymerase chain reaction, (PARRTI) study: Partaking in Alcohol Related to serum RNA Total Increase.

Plasma Micro RNA	Group comparisons	P value
MiR-21	UK Control vs. OP + ASP UK Control vs. OP + NO ASP UK Control vs. NON OP+ASP OP + ASP vs. OP + NO ASP OP ASP vs. NON OP+ASP	0.008** 0.0001**** NS NS NS
MiR-146a	UK Control vs. OP + ASP UK Control vs. OP + NO ASP UK Control vs. NON OP+ASP OP + ASP vs. OP + NO ASP OP ASP vs. NON OP+ASP	0.0083** 0.0001**** NS NS NS
MiR-122	UK Control vs. OP + ASP UK Control vs. OP + NO ASP UK Control vs. NON OP+ASP OP + ASP vs. OP + NO ASP OP ASP vs. NON OP+ASP	0.0037** NS NS NS NS

Table 6.5: Statistical comparison plasma expression of miR- 21, miR-146a and miR-122 in UK control and pesticide poisoned patients. Plasma expression of these miR-RNA in Sri Lankan controls had no statistical differences with the pesticide poisoned patients. The table shows results of permutation testing between the groups detailed above. P was considered **significant * p≤0.05, ** p ≤0.01, *** p≤0.001, **** p≤ 0.0001. NS= non**-significant.

6.3.4.1 MiR-122 has a linear relationship with alanine aminotransferase within plasma from pesticide poisoned patients

MiR-122 is a marker of toxic liver injury and its expression in plasma has a direct correlation with the liver enzyme alanine aminotransferase (ALT) plasma concentration, both being raised in acetaminophen overdose (Antoine et al., 2013). To assess whether this pattern was also seen in OP poisoning, we measured plasma ALT and examined its correlation with plasma miR-122. The Spearman's rank coefficient was 0.67 and showed a positive linear relationship, confirming that miR-122 is an acceptable marker of liver injury in the context of pesticide poisoning (Figure 6.13). Although uncommon, raised liver enzymes have been observed in OP poisoning (Banday et al., 2015).

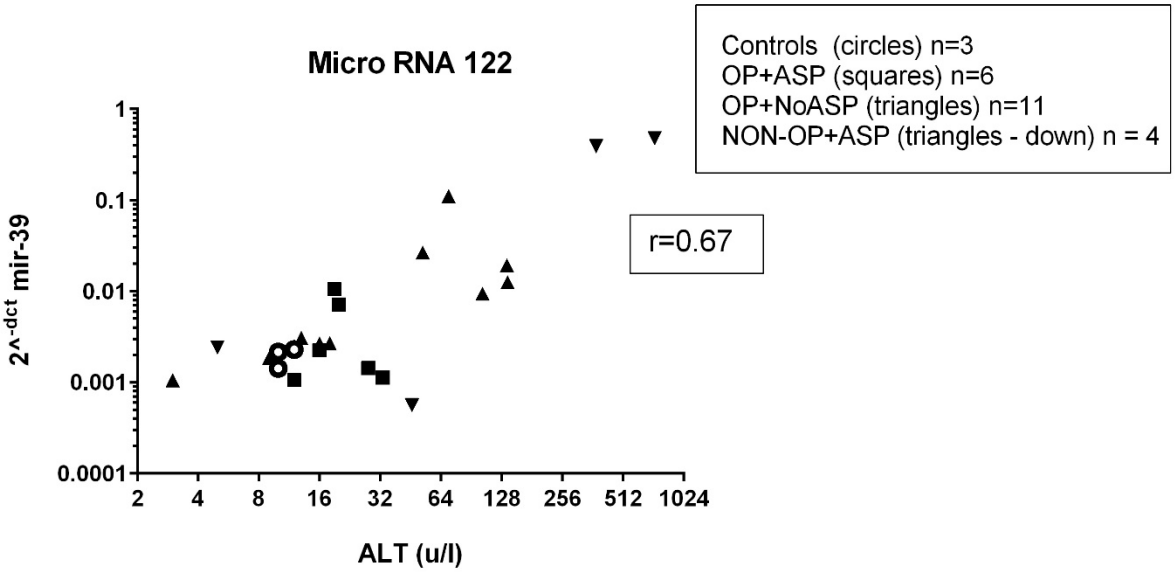


Figure 6.13: A scatter dot plot of the relative quantification of plasma miR -122 using the $2^{-\Delta ct}$ method (normalised using miR-39 only) vs. plasma concentration of alanine transferase (ALT) of all Sri Lankan surgical control and pesticide poisoned patients. The relationship was significant at p=0.0004 (***). The Spearman's Rank coefficient r: 0.67.

6.3.5 Ventilator associated pneumonia audit

There were 55 patients included in the audit; two thirds were male (35/55) and half (26/55) required mechanical lung ventilation for ≥ 3 days (according to the notes). The mean age of the adult patients (n=50) was 49 (±8.2, range 17-77) years old and 8 (range 2-15) years old for children under 16 years old (n=5).

The main reasons for intubation and ventilation were: post-operative complications (18%), OP poisoning (16%) and infections (15%).

Five patients (5/26 – 19%) fulfilled the criteria for VAP (none from OP poisoning) with 4/5 having a qualitative endotracheal or sputum culture as per HELIC's guidelines. There were at least two other possible cases of VAP which fell short of a definitive diagnosis due to lack of a CXR in one, and lack of documented chest signs in the other (OP poisoned patient). There was an additional possible VAP in a post-OP poisoning patient who fulfilled the criteria, but had only been ventilated for 2 days at the assessment point.

Nearly all (53/55) intubated patients were receiving combinations of antibiotics, antivirals or antifungals, with only 15% (7/55) receiving single agent therapy. The most common agents used were the antibiotics metronidazole (26%) and cefuroxime (14%). The most common bacteria cultured from endotracheal tube (ETT) aspirates or sputum samples from all intubated patients (19/55) was *Acinetobacter* (40%) and *Pseudomonas* spp. (32%). Those patients who fulfilled diagnostic criteria for a VAP had cultures positive for *Acinetobacter* (n=3) with one negative sample (n=1).

Thirteen percent (7/55) of patients had no observed filter (heat and moisture exchanger (HME) or bacterial/viral) in the breathing circuit. This could have made patients vulnerable to pneumonia from organisms present within the machinery of the ventilator. There were no observed routine changes of ETT's or protocols for tracheostomy placement in patients ventilated for 5-7 days. These practices (e.g. bacterial filters, routine ETT changes and early tracheostomy placement (Durbin, 2010)) can decrease pulmonary complications (including VAP) and mortality.

The bed angulation of those eligible to sit up (54/55) was 12 (±5) degrees, range (0-29). Bed angulation was limited in some cases due to faulty ICU beds.

6.4 Discussion

This small pilot feasibility study was limited by the difficulties faced with recruitment of patients due to: (i) the short time frame, (ii) reduced incidence of OP pesticide poisoning, (iii) late ethics approval, and (iv) an unwillingness of some ICU doctors to assist in the study (see limitation section below for further discussion).

This current human pilot study demonstrated that OP pesticide poisoning and aspiration caused a direct and indirect lung injury typified by increases in alveolar protein, IL-6, IL-8, CRP and neutrophils. Results were similar to that observed in the minipig study and suggest that the minipig model may be an acceptable platform for the further research and testing of novel treatments for OP pesticide poisoning and aspiration lung injuries. However, larger clinical studies are required to obtain enough data for a meaningful comparison between animal model and human patients.

This experience taught me the processes involved within a clinical trial and some of the pitfalls and difficulties involved. The pilot study produced valuable information that will hopefully facilitate future funding for a larger observational study in Asia.

Direct lung injury caused through ingestion and aspiration of OP pesticide + gastric contents

The OP+ASP patients had a worse lung injury when compared with control and NON-OP+ASP groups at 48 hours as indicated by the lower PaO_2/F_IO_2 ratios.

BALF samples were only available from two individual OP +ASP patients [one sample from 24 hours, the other patient sampled at 48 hours only]. However, both showed evidence of lung injury. Their BALF had an increased proportion of neutrophils on representative slides; the greatest concentrations of BALF protein, albumin, IL-6 and SP-D at both 24 and 48 hours; and the greatest concentrations of BALF IL-8 and CRP at 48 hours, when compared with all other groups. In OP+ASP patients (n=4), plasma concentrations of IL-6 and IL-8 at both time points were not significantly raised, with only a mild increase in plasma CRP at 24 hours compared to controls.

The limited evidence from our human and animal work suggests that IL-8 may be released by the damaged lung/endothelium in association with aspiration of OP with gastric contents. This could

cause increased migration of neutrophils, fluid and protein into the airspaces (Baggiolini and Clarklewis, 1992, Folkesson et al., 1995). Simultaneously, increased IL-6 may have been produced by the damaged endothelium, and in response, concentrations of pulmonary CRP were raised (Scheller et al., 2011, Agassandian et al., 2014).

In minipigs, the OP+GJ treated group had one of the lowest BALF SP-D concentrations. In contrast, the OP+ASP patients had the greatest BALF SP-D concentrations when compared with other groups. This might have been because (i) the type 2 alveolar epithelial cells were not damaged by the aspiration of OP and gastric contents in humans and were able to produce more protective SP-D (ii) less SP-D was lost into the circulation due to a reduced alveolar capillary membrane leak (iii) less dilution of SP-D through a smaller BALF aliquot or less oedema fluid. BALF SP-D is known to be raised in human ARDS survivors and reduced in those who die from the syndrome (Greene et al., 1999). This could explain the survival of the two OP+ASP patients who had raised concentrations of BALF SP-D.

Overall, the findings suggest that OP+ASP patients had a greater inflammatory lung injury than patients from other groups. Inflammation was mainly confined to the lung (as shown by the low concentration of plasma inflammatory markers) and may still have been developing beyond 48 hours.

These findings are mainly in keeping with the minipig model, but would need to be confirmed in larger numbers of pesticide poisoned humans.

How OP aspiration lung injury might differ from 'normal' gastric contents aspiration lung injury

Cytokine profiles related to human aspiration lung injuries are largely unknown. However, animal studies indicate lung injury secondary to aspiration of gastric particles and acid can cause increased concentrations of: BALF IL-8 (also shown in the minipig and our Sri-Lankan study),TNF-α and the anti-inflammatory IL-10 (Raghavendran et al., 2011). Perhaps low concentrations of BALF IL-10 and TNF are seen in OP+ASP patients because (i) the sampling points missed the peak period of

cytokine release (ii) the cytokines are not involved in this specific lung injury process, and/or (iii) the OP pesticide modulates the inflammatory response by decreasing the anti-inflammatory IL-10 produced both locally in the lung (neutrophils and macrophages) and systemically. The latter could potentially augment lung injury or create a more favourable environment for the development of pneumonia (Queiroz et al., 1999, Siewe et al., 2006). Cholinergic control of the immune system has an important anti-inflammatory effect. Disorder in this system, secondary to OP pesticide poisoning, could feasibly result in greater and different types of lung injury compared with aspiration of gastric contents alone (Tracey, 2007).

The specific inflammatory pathways could be elucidated by standardised *in vitro* experiments exposing alveolar macrophages and endothelial cells to OP+GJ and GJ alone and characterising the released cytokine profiles. These effects should also be investigated in *in vivo* models, because of the systemic effects OP poisoning might have on the lung.

Indirect lung injury caused through ingestion of OP pesticide

The OP+No ASP patients had increased BALF concentrations of IL-1β, IL-8 and CRP at both 24 and 48 hours, and IL-6 at 24 hours. This indicates pulmonary inflammation and the creation of an indirect lung injury after OP pesticide ingestion. These patients had less protein and a decreased number of neutrophils in the BALF suggesting a more intact alveolar capillary membrane than that seen in the OP+ASP patients. Plasma concentrations of inflammatory markers in OP+No ASP patients were largely similar to controls, except in one patient (patient 17) who had concentration increases 10x IL-6 and 5x IL-8 than that of the highest control patient, suggestive of mild systemic inflammation. It must be considered, that some OP+No ASP patients may in fact have had some degree of micro aspiration which was not able to be confirmed. This may have contributed to the observed results.

However, overall these indirect lung injury results echo the minipig study findings from both chapter 3 (previous minipig OP poisoning by gavage) and chapter 5 (minipig pulmonary aspiration study).

Direct lung injury caused through ingestion and aspiration of non-OP pesticide + gastric contents

When the OP poisoned patients (OP+Asp and OP+No ASP) were combined and compared with controls and NON-OP+ASP patients, there were significant differences between groups at 24 hours for BALF concentrations of IL-1β, IL-8 and CRP.

Subgroup analysis using permutation testing showed no significant differences between two individual groups. The group with the greatest concentrations of BALF IL-1β, IL-8 and CRP at 24 hours was the NON-OP+ASP patients. This could be explained by: (i) a lower pH in the gastric contents of NON-OP+ASP patients, (ii) increased aspirated volumes or increased presence of gastric particulate matter in the NON-OP+ASP patients, (iii) it simply created a worse lung injury compared with other groups (Raghavendran et al., 2011). As the compound identity was not formally analysed, the NON-OP+ASP patients may have in fact ingested an OP (due to evidence of AChE inhibition). However, poisoning literature finds that histories are fairly reliable and so the patients probably did ingest what they were reported to have ingested (Camilleri, 2015). At 48 hours, the high concentrations of cytokines and protein in the BALF had reduced to near normal levels indicating some form of injury resolution, unlike the single OP+ASP patient sampled at 48 hours.

Aspiration pneumonia and VAP in pesticide poisoned patients

Due to the stomach's low pH caused by HCl, the aspiration of gastric contents usually produces a sterile chemical aspiration pneumonitis. If the gastric contents (including swallowed oropharyngeal secretions) contain bacterial matter, aspiration pneumonia may then develop. Live bacteria in gastric contents is more likely when the gastric pH has been raised (Gray and Shiner, 1967), for example with H_2 blockers (ranitidine) in the treatment of gastric ulcer symptoms. Ventilator associated pneumonias (VAPs) are hospital-acquired infections that occur more than 48 hours after intubation. The criteria used to diagnose VAP, and bacteria causing VAP, are similar to that in aspiration pneumonia (Raghavendran et al., 2011).

In our pesticide poisoned patient cohort, the 48 hour BALF sample from one OP+No ASP patient (patient 8) grew bacteria (*Pseudomonas* and coliforms). The same patient 1 week later (alongside one NON-OP+ASP patient) developed probable ventilator associated pneumonias.

Most pesticide poisoned patients who were admitted, intubated and ventilated received prophylactic intravenous antibiotics (usually a broad spectrum cephalosporin and anaerobic protection by metronidazole) for a number of days to prevent aspiration pneumonia. This may have prevented the development of either aspiration pneumonia or VAP. However, best practice is to treat aspiration pneumonitis conservatively unless clinical signs suggesting pneumonia are present 48 hours later, otherwise this can select resistant organisms and create a 'difficult to treat' pneumonia (Marik, 2001).

Current UK hospital guidelines recommend that airway colonisation and aspiration must be prevented to reduce the risk of VAP development and increased time on the ventilator. Simple techniques like oral decontamination with chlorhexidine 1-2%, sitting the patient upright at 30-45 degrees, and early extubation on the ICU can be effective in preventing VAP (Hunter, 2012).

The audit conducted in 2013, indicated that 19% (5/26) of intubated patients ventilated for more than 48 hours developed probable VAP. This was likely to have been an underestimation because there was no consistent ICU staff vigilance for VAP in requesting CXRs, blood tests and microbiological sampling of endotracheal secretions or sputum.

VAP prevention in the Sri Lankan ICUs was hampered by: (i) a lack of antibacterial mouth gel (bicarbonate of soda with lime juice was used), (ii) occasional lack of viral/bacterial filters within the respiratory circuit [13% had no filters – data from audit], (iii) Infrequent changes of endotracheal or tracheostomy tubes (allowing colonisation of airways), and (iv) occasional re-use of endotracheal suction catheters. Data from the audit also showed that the mean bed angulation of those eligible to sit up (54/55) was 12°, and 8° in our aspiration study, well below that recommended to avoid VAP and/or aspiration pneumonia (Raghavendran et al., 2011, Hunter, 2012) .

Nearly all intubated ICU patients, of which 16% were OP poisoned patients, were on some form of antibiotic, antifungal or antiviral agent. Such a 'blanket' antimicrobial approach is likely to strengthen antibiotic resistance and growth of resistant organisms in those ICUs.

Although none were diagnosed in the audit, the above elements combined could potentially increase the incidence of VAP within intubated pesticide poisoned patients in Sri Lanka.

Micro RNA biomarkers

MiR-21 and miR-146a

MiRNA profiling of ARDS is in its infancy, but animal models of high volume ventilation lung injury and oleic acid induced lung injury have found increased levels of expression of miR-21 and miR-146a in pulmonary tissue, and may be important in ARDS development and pathogenesis (Vaporidi et al., 2012, Lee et al., 2014).

MiR-21, regarded as an important inflammatory control trigger, is found in many tissues and cell types and is involved in cell growth and movement (Sheedy, 2015). Some anti-inflammatory therapies aimed at blocking the effects of miR-21 have had modest success in controlling inflammatory conditions like lupus and psoriasis (in mice). Yet, miR-21 is complex and it behaves differently under various circumstances with both pro and anti-inflammatory roles in different cell types (Guinea-Viniegra et al., 2014, Garchow et al., 2011, Sheedy, 2015). In its anti-inflammatory capacity, miR-21 has been shown to upregulate the production of IL-10 (O'Neill et al., 2011) which was negligible in the OP+ASP plasma and BALF- perhaps due to reduced miR-21 expression. Whether the decrease in plasma expression of miR-21 in OP poisoned patients is causing an overall pro- or anti-inflammatory state in the lung remains to be investigated.

Zeng *et al* showed that lipopolysaccharide (LPS) induced acute lung injury in rats led to pulmonary upregulation of pro- inflammatory cytokine expression and increased concentrations of BALF IL-1β, TNF-α, IL-6 and miR-146a (lung tissue only) (Zeng et al., 2013). They demonstrated miR146a overexpression (alveolar macrophages exposed to LPS) was also capable of negatively regulating expression of the same inflammatory cytokines [IL-1β, TNF-α, IL-6] in the macrophages. MiR-146

has also been found to have a negative regulatory effect on inflammatory gene expression in lung smooth muscle cells, fibroblasts and epithelial cells (Comer et al., 2014).

It was therefore unexpected that circulating plasma miR-21and miR-146a had a significantly reduced expression in OP poisoned patients with and without aspiration, compared with non-intubated UK study controls. It is possible the systemic and local toxic OP effect suppressed the production (or release) of miR-146a and miR-21 in the plasma through the disruption of the cholinergic anti-inflammatory pathway. This would explain the cytokine profile within the OP poisoned patients BALF and why it differs from other lung injures e.g. LPS induced lung injury has increased TNF-α and IL-10 in the BALF, whereas OP+ASP patients did not (Sheedy, 2015, O'Neill et al., 2011).

These differences may be important if confirmed in future larger studies investigating both plasma and lung expression of miRNAs, reflecting possible pathophysiological differences with other lung aspiration injuries. In addition, further studies are required to determine whether these miRNAs have potential as biomarkers of OP poisoning severity and OP aspiration.

MiR-122

Hepatocellular carcinoma, alcoholic liver disease and poisoning with acetaminophen in mice and humans cause a decrease in the liver cell expression of miR-122, with a corresponding increase in circulating (plasma) miR-122 (Szabo and Bala, 2013, Antoine et al., 2013). The raised plasma levels are likely due to the release of miR-122 from damaged hepatocytes.

Alcohol dependence is a major risk factor for self-harm and acute pesticide self-poisoning (Van Der Hoek and Konradsen, 2005) and was suspected to have been consumed with the pesticide in 4/5 of the OP+ No ASP and 3/4 of the OP+ ASP patients. Therefore, miR-122 could have been raised in some of the poisoned patients, especially those with chronic alcohol abuse. However, there was a significant reduction in expression of circulating miR-122 in the OP+ASP compared with UK controls. There was no significant reduction seen with Sri Lankan controls which could have been due to liver disease resultant from possible metastatic hepatocellular carcinoma (discussed below).

There was a strong linear correlation between the liver enzyme ALT plasma concentration and miR-122 plasma expression (Figure 6.13) indicating that miR-122 was an adequate measure of liver injury in the context of OP and non-OP pesticide poisoning. The OP+ASP patients had normal concentrations of ALT (< 50u/l), while the majority of OP+No ASP patients had modestly raised ALT and miR-122. The greatest plasma miR-122 expression and ALT activity came from a NON-OP+ASP patient. The patient had consumed the carbamate carbofuran without alcohol, developed hepatic failure, and died a few days later.

Without knowing whether a patient had a history of alcohol abuse, it is difficult to surmise whether raised plasma miR-122 levels were secondary to alcoholic liver disease and/or the toxic effect of the pesticide. However, it is still unknown why OP+ASP patients had a reduced plasma miR-122 expression when compared with UK controls and would need to be investigated further.

Control patients

It is possible that the Sri Lankan control patients with colorectal cancer had had liver micro metastases (not visible on ultrasound), resulting in above normal plasma miR-122 and miR-21 expression, potentially masking differences between study groups (Szabo and Bala, 2013, Kanaan et al., 2012). Against this theory, is that the same patients had normal concentrations of the liver enzyme alanine transaminase, suggesting grossly normal hepatic function.

To remove this potentially confounding factor, plasma samples from non-intubated healthy (UK) control patients (n=8) from a different toxicological study (PARRTI study) were tested for expression of miR-122, miR-146a and miR-21. There were inherent flaws in using these controls: they were likely from a different ethnic group, they were not intubated and exposed to ventilator-induced lung stresses, and they were not exposed to miniBALs which may have increased lung injury markers.

Study limitations

Identification of the pesticide

Accurate identification of the pesticide or poison ingested was not possible as the admitting physician was reliant on the first responders either bringing in the bottle that the patient had been drinking from (which anecdotally often had incorrect labelling or no label), and/or giving an accurate medical history of the poisoning episode.

In this small study there were no funds available to formally identify the pesticide consumed, but we were able to test blood from some patients for their AChE activity. This allowed identification of those who had ingested an OP or carbamate pesticide by observing their reduced AChE activity.

One patient (patient 5) thought to have ingested an OP pesticide [chlorpyrifos] with evidence of aspiration had normal AChE levels at her only sampling point at 24 hours, shortly after which she was extubated (Figure 6.4). This may be explained by the ingestion of very little of the OP, or it was not in fact an OP pesticide e.g. it may have been a carbamate with reversible inactivation of AChE, or a completely different non-AChE inhibiting pesticide. However, the by-stander history stipulated the type of OP and commercial formulation – chlorpyrifos/Indur EC40 which makes the history more credible (Camilleri, 2015).

There were no local AChE control samples taken as the Sri Lankan research handheld AChE device was not functioning at the time. Subsequent samples in poisoned patients were brought to the UK for testing.

Reduced numbers of patients

The number of fatal pesticide poisonings has declined in Sri Lanka over recent years (de Silva et al., 2012). The reasons are multifactorial and include: the banning of the most toxic pesticides; better transport and infrastructure to deliver patients to hospital; better medical treatment and education of Sri Lankans as to the dangers of pesticide poisoning; and a switch to using less toxic medicinal drugs for overdose (de Silva et al., 2012).

The reduction in serious OP poisoned patients was evident during both my 3-month research trips to Peradeniya, Sri Lanka. Moreover, the clinical audit I performed, designed to capture those patients intubated and ventilated for ≥ 48 hours over a 2.5 month period, in both Peradeniya and Nuwara Eliya hospitals (2013), showed only 16% (9/55) were due to OP pesticide poisoning.

During my second visit, some patients had been recruited to another study: two OP+No ASP patients were co-enrolled in an interventional study attempting to treat neuromuscular junction dysfunction by Professor M Eddleston. This involved the patients receiving a low dose infusion of the neuromuscular blocking drug – rocuronium in order to partially block the neuromuscular junction. NMJ blockers have been shown to reduce mortality in severe ARDS (Papazian et al., 2010), but it is unknown whether they have any beneficial effect on the pulmonary function in OP+No ASP patients, most of whom did not have PaO_2/F_IO_2 ratios consistent ARDS during our sampling points.

Study controls

When planning the study, we did not know what ICU control patients (e.g. intubated secondary to trauma or sepsis) would be available in Sri Lanka. I hypothesised that the best available control group would be those undergoing intubation and ventilation for non-emergency surgery in the same location/hospital, using the same equipment as the poisoned patients. The Sri Lankan anaesthetic consultants selected general surgical patients for sampling (e.g. colorectal cancer removal) as the surgery (and intubation and ventilation) would last > 3 hours.

There were two main disadvantages with this choice of surgical controls:

(i) The surgical controls were only ventilated for an average of 4 hours, compared with the 24 and 48 hours in the poisoned patients. This meant that ventilator induced lung injury, observed in longer periods of ventilation, may not have occurred in the control group (Slutsky and Ranieri, 2013).

(ii) The control surgical patients all had colorectal cancer, and may therefore have had increased circulating IL-6, IL-8, IL-10 and CRP concentrations (O'Hara et al., 1998, Malicki et al., 2009, Kersten et al., 2013). Use of these controls may have obscured real increases in the plasma concentrations of these inflammatory markers in the pesticide poisoned patients.

The presence of oncogenic miRNA in the Sri Lankan controls is discussed above.

On the second trip, a request was made to the ethics committee for intubated and ventilated ICU patients with no infective disease to act as a new control group. However, whilst the local ethics committee approved of this, the medical staff were unwilling to allow samples to be taken.

Reduced number and quality of bronchoalveolar lavage fluid samples

I was not allowed to perform the sample collection myself as I did not have Sri Lankan Medical Council registration. Therefore the BALF sampling procedure had to be safe and simple enough for the local doctors to perform in intubated ICU patients.

To achieve this, I proposed a mini bronchoalveolar lavage (mini BAL) technique using a telescopic catheter instead of a formal bronchoscopy and lavage. Whilst generally more acceptable, and despite obtaining local ethics approval from the research committee for this method, medical staff at the University of Peradeniya hospital were not always supportive in trying to obtain the human samples required. Thus control and poisoned patient BALF sampling procedures were limited.

The mini BAL provided a much smaller lavage sample volume and cell pellet than a normal BAL using a bronchoscope (3 x 50 mL). Some argue that mini BAL is not as accurate as a normal BAL in differentiating between ARDS and at-risk ARDS patients for observations of alveolar inflammation (neutrophil numbers and protein) though they did not use a Combicath, and used much larger lavage volumes (Perkins et al., 2006). Without access to a cytospin or quality microscope, the production of slides containing BALF cells was inferior when compared with UK laboratory standards.

6.5 Conclusion

The evidence, although based on very low patient and sample numbers, suggests that human OP pesticide poisoning, through ingestion with or without aspiration, can cause measureable direct and indirect lung injuries. The inflammatory profile in OP poisoned patients with aspiration was consistent with that seen in the minipig study. This supports the idea that OP pesticide produces a lung injury characterised by increased concentrations of BALF protein, IL-6, IL-8 and CRP with a pulmonary neutrophilia within 48 hours. These findings suggest that the minipig model may be a suitable platform for the testing of pulmonary therapies for OP poisoning in the future.

OP pesticide poisoning and aspiration may also differ from classic ARDS and 'normal' gastric contents aspiration. This was demonstrated through reduced BALF and plasma concentrations of TNF and IL-10, and warrants further investigation.

OP poisoning and aspiration may render patients more prone to aspiration or ventilator associated pneumonias. However, a larger observational study within a VAP aware ICU environment would be required. The lack of pesticide identification in poisoned patients meant that the beneficial effects of solvent aspiration (as observed in the minipig study) were unable to be confirmed in the human OP+ASP group.

Despite significant changes in plasma expression of miR-21, miR-146a and miR-122 between UK controls and OP+ASP patients, this study needs to be repeated in larger numbers of pesticide poisoned patients with local Sri Lankan controls. The miRNA data combined with inflammatory marker findings may yet assist further understanding of the molecular pathways involved with OP poisoning and aspiration.

Chapter 7: The development of an *ex vivo* lung perfusion model to further explore the pathophysiology of pulmonary aspiration of OP and gastric juice

7.1 Introduction

A combination of OP with GJ instilled into the lung created a direct injury in the minipig consisting of a significantly greater pulmonary neutrophilia and histopathological changes e.g. oedema, haemorrhage, and necrosis, in the lung parenchyma when compared with controls (chapter 5). There were also significantly increased concentrations of protein and inflammatory markers [IL-6, IL-8 and CRP] within the BALF between 24-48 hours when compared to controls. The OP+GJ treated pigs had evidence of an indirect lung injury which distinguished it from an aspiration injury with GJ alone.

Human OP poisoning with and without aspiration also displayed direct and indirect measureable lung injuries (chapter 6). The inflammatory profile in OP poisoned patients with aspiration was similar to that seen in the minipig study with increases in BALF concentrations of protein, IL-6, IL-8 and CRP when compared with controls. These same OP poisoned patients also had low concentrations of BALF TNF and IL-10 at 24 and 48 hours after poisoning. Low concentrations of BALF TNF were also observed in OP poisoned minipigs (poisoned with OPs by gavage) in chapter 3 and suggests that OP pesticide poisoning (± aspiration) produces a lung injury that differs to classic ARDS or gastric contents aspiration (Agouridakis et al., 2002, Suter et al., 1992, Bouros et al., 2004).

Whilst further experiments involving bronchoscopy, lavage and biopsy of directly and indirectly-injured lungs might assist detailed investigation into the pulmonary pathophysiology encountered in aspiration syndromes associated with OP poisoning, the overall cost of animals and technical staffing prompted an alternative approach: the development of an *ex vivo* lung perfusion (EVLP) model. The EVLP model could be designed to provide an efficient, low cost alternative to the *in vivo* minipig model. An EVLP model would also allow the observation of OP pesticide aspiration and lung injury at a more lethal concentration than the *in vivo* minipig model might allow.

This chapter details the initial development of an ovine EVLP model and the data obtained from a pilot study. Some of this pilot data indicated that pulmonary toll like receptor (TLR) expression in OP+GJ treated lungs was different to normal ARDS and so this was superficially explored by re-

examining previously collected minipig (chapter 5) and human (chapter 6) samples. Unfortunately, there were no funds remaining to examine the cytokine expression profile (as in previous studies) in the BALF of the lungs used in the ovine EVLP model pilot study.

The chapter concludes with a discussion detailing (i) how the EVLP model might be improved and (ii) how the pilot data might guide further research towards the immune-pathological mechanisms involved with pulmonary aspiration of OP+GJ.

7.1.1 Aim of chapter

- To describe the creation a pilot EVLP model of OP pesticide aspiration
- To describe the investigation of the pulmonary pathophysiology of aspiration of OP+GJ using the pilot EVLP model
- To discuss potential EVLP model improvements and how the pilot data might guide future studies to better understand the immuno-pathological mechanisms involved with the pulmonary aspiration of OP+GJ

7.2 Methods

The experiments were conducted during the week beginning 22nd April 2013. They were designed and managed by myself and assisted by my co-investigator Dr U Hamid, cardiothoracic registrar, Queens University Hospital Belfast (QUHB) and Professor D MacAulay (QUHB). Dr Alistair Proudfoot (Imperial College London) provided advice on the methodology.

Data are reported as mean (±SD). This includes data calculated from one pair of EVLP ovine lungs from -30 min until the end of the experiment, unless otherwise stated.

7.2.1 Collection of sheep lungs and autologous blood

Four sheep (12 months old) killed using a schedule one method at the Moredun Research Institute provided the lungs and autologous blood for these series of experiments.

Each sheep was stunned and killed with insertion of a pneumatically delivered intracranial bolt. This was followed rapidly by surgical exposure of the top of the trachea which was clamped to avoid damage caused through aspiration. The sheep's blood was then collected from the left carotid (after incision) in a sterile bucket with 20, 000 units of heparin. The autologous whole blood was placed on ice and taken to the cardio by-pass machine in a laboratory close by and added to the circuit with 1.0-1.5L Hartmann's solution.

The heart lung bloc was carefully excised by Dr U Hamid using the technique described below via a thoracotomy (Table 7.1) [NB: instructional videos were filmed during this process and are available upon request].

Procedures to remove heart lung bloc for *ex vivo* lung perfusion
1. Open the pericardium
2. Free lower bases of the right lung by dividing inferior (caudal) pulmonary ligament
3. Divide inferior (caudal) vena cava
4. Cut along the mediastinal pleura in front of the phrenic nerve
5. Divide the ascending aorta
6. Divide the fascia posterior (dorsal) to the heart to remove the lung-heart en bloc.
7. Divide descending aorta close to right ventricle.
8. Divide and trim left atrial appendage to allow free drainage of the perfusate into the CPB system.
9. Insert a by-pass cannulae and suture into the pulmonary artery (PA) using a purse string suture.
10. Pass a PA catheter (Tru-cath, Omega critical care, UK) through the by-pass cannulae and secure in place to allow measurement of the PA pressures.

Table 7.1: Procedure to remove and dissect the heart lung bloc from a sheep. Descriptions in brackets are used to confirm location of tissue using veterinary, as opposed to human, descriptors. PB=cardiopulmonary bypass, PA=pulmonary artery.

Once the heart-lung bloc was removed, it was intended that the vasculature be flushed using a low potassium colloid (Perfadex, Sweden) often used in human EVLP transplant surgery. Perfadex is used to optimise tissues by providing some nutrients and removing clots and inflammatory proteins present in the blood. However, the fluid was not delivered for the study and cooled 0.9% saline (4°C) was used for the first two heart-lung blocs. Thereafter, cooled Dulbecco's modified eagle medium (DMEM) was used as a Perfadex substitute. DMEM is a cell culture medium containing amino acids, vitamins and glucose to aid tissue preservation. The third set of lungs was flushed with cooled DMEM (850 mL) with the fourth set flushed with DMEM (110 mL) and cold (4°C) saline (390 mL) because of insufficient DMEM.

A 50mL syringe housing was connected to the by-pass cannula (in pulmonary artery) to act as a funnel. The heart-lung bloc was flushed to remove old blood by pouring either the cooled (4°C) saline (1L) or DMEM solutions into the syringe housing draining through the heart-lung bloc [pulmonary artery – pulmonary veins – left atrial stump]. Leaks found in the bloc at this stage were

sealed with 4.0 vicryl sutures. The heart-lung bloc was then placed in a clean clinical waste bag and surrounded by ice until placement on the CPB machine.

7.2.2 Priming of the by-pass circuit and centrifugal pump

The Maquet HL-30 cardiac by-pass machine and Rota flow® centrifugal pump were used for these EVLP experiments and set up as per Figure 7.1. The components of the EVLP by-pass circuit included a roller pump, reservoir, centrifugal pump, water bath and membrane gas exchanger connected with sterilised plastic tubing.

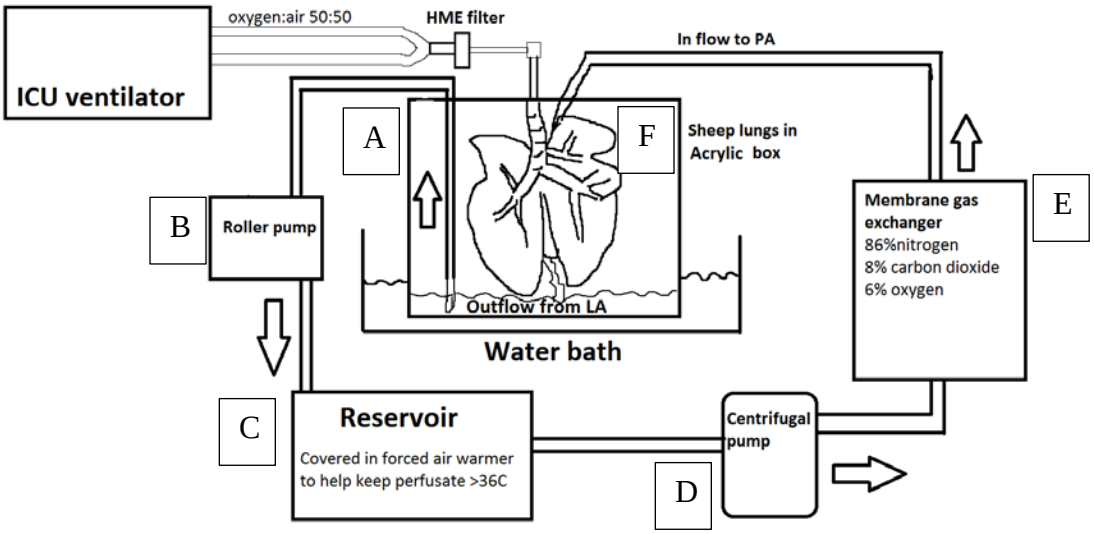


Figure 7.1: Ex vivo lung perfusion (EVLP) circuit. Oxygenated perfusate is pumped from the left atrial (LA) outflow collection at the bottom of the acrylic box (A) where it is passed through a roller pump (B) into the top of the reservoir (C) to allow for removal of clot and air bubbles. Perfusate is forced through a membrane gas exchanger (E) via a centrifugal pump (D) that deoxygenates and adds CO_2 to the perfusate. This venous-like perfusate mix is returned via a cardiac by-pass catheter to the pulmonary artery (PA) (F). The roller pump was added during the second and subsequent experiments to aid return of perfusate to the reservoir.

In order to prime this system with Hartmann's solution, the inflow and outflow tubing ends were joined together so as to complete the circuit. With the tubing in the roller pump disengaged and a

clamp on the bottom of the reservoir, the Hartmann's fluid bag was connected via separate tubing to the top of the reservoir and allowed to fill.

A second clamp was placed on the tubing on the membrane side of the centrifugal pump. Holding the rota flow centrifugal pump upside down to fill, the first clamp was released allowing the solution to flow from the reservoir to the centrifugal pump. When the air had been removed from the centrifugal pump using this process, it was placed in its housing as per Maquet instructions. The centrifugal pump was started on a low flow and then the second clamp removed to allow de-airing of the system (air collection in the reservoir). A 50 mL syringe was used to inject the autologous heparinised blood into the top of the reservoir.

A forced air warmer (Bair hugger™) was wrapped around the reservoir and tubing placed in the water bath whilst circulating to begin the gradual heating of the perfusate to >36.0°C.

7.2.3 Placement of lungs on EVLP circuit

A size 30 (7.5mm) Univent ETT was placed in the trachea and sutured in place using a 1.0 silk suture. Left over silk suture was used to attach the lungs and ETT to a metal bar that straddled the entrance to the acrylic box so that it was suspended above the collecting perfusate.

A catheter mount and HME filter were attached to the ETT and then placed on the ICU ventilator (switched off).

The by-pass cannula was attached to the in-flow tubing (Figure 7.1) and perfusion was commenced at a low flow to allow de-airing of the heart-lung bloc. The outflow pipe was then placed in the perfusate falling from the left atrium into the bottom of the acrylic box. The outflow tubing was placed in a roller pump which was started once there was sufficient volume in the box to pump back to the reservoir. The top of the acrylic box was covered with low-density polyethylene sheeting (Clingfilm, UK) to prevent evaporation during the experiment.

7.2.4 EVLP circuit warm up period

Once the EVLP circuit had been primed and the lungs placed within circuit, an attempt was made to heat the perfusate to >37.0°C. However, the absence of a heat exchanger made this difficult to achieve. During the warm-up period the lungs were ventilated and the perfusate flow rates were slowly increased prior to pulmonary installation of GJ mixtures (Table 7.2).

Cypel *et al* used whole sets of lungs from 35kg pigs with 40% of cardiac output (approximately 1.5 L/min) perfusate flow to avoid pulmonary hypertension and hydrostatic oedema formation (Cypel et al., 2008). Sheep have similar cardiac outputs to pigs (Hales, 1973) and because the mean sheep weight was 38.5kg, 40% CO was calculated to be 1.5 L -2 L /min. After discussions with Dr U Hamid, whose research involved EVLP in human lungs and supported the use of lower flows (causing less damage) of approximately 0.5 L/min (Hamid et al., 2014), we decided to start perfusate flow at 0.5 L/min, increasing thereafter to 1L /min -a point midway between Dr U Hamid's and Cypel's documented flows (Table 7.2).

Time	10 min	20 min	30 min	40 min	50 min	60 min
Temperature of perfusate °C	25	25	30	32	34-36	>36
Perfusate flow (L/min)	0.5	0.5	0.6	0.8	0.8	1.0
Mean PA pressure (mmHg)	≤20	≤20	≤20	≤20	≤20	≤20
LA pressure (mmHg)	0	0	0	0	0	0
Ventilation	None	None	None	Start		Lung recruitment manoeuvre (described below)
Tidal volume (mL/kg)				6-8	6-8	6-8
Respiratory rate (breaths/min)				7-20	7-20	7-20
Plateau airway pressure (cmH_2O)				≤25	≤25	≤25
FiO_2				0.5	0.5	0.5
Membrane gas exchanger (CO_2 8%, O26%, N2 86%)	None	None	None	Start 0.5 L/min	0.5 L/min	1L/min

Table 7.2: Proposed protocol for *Ex vivo* Lung Perfusion circuit warm up period. PA= pulmonary artery, PV= pulmonary vein, LA=left atrium.

Protective ventilation (tidal volume 6-8 mL /kg, PEEP 5 cmH_2O and plateau pressure <25 cmH_2O) was commenced once the perfusate temperature had reached 32°C. Respiratory rate was adjusted to maintain pulmonary venous $PaCO_2$ between 35-45 mmHg and thus stabilize perfusate pH. A recruitment manoeuvre was employed at the end of the warm up period by using an Ambu bag and room air attached to the ETT. The Ambu bag was squeezed for (x2) 10 sec in an attempt to re-

expand collapsed alveoli in the distal airways, whilst keeping MPAP ≤25mmHg and peak airway pressure to a minimum.

7.2.5 Creation of unilateral aspiration lung injury in an EVLP model

Animal ID	Intervention	Length of experiment from intervention
Sham 1 (S1)	Sham bronchoscopy	3 hours
Sham 2 (S2)	Sham bronchoscopy	3 hours
OP+GJ (S3)	Dimethoate EC40 5 mL [dimethoate 2g + cyclohexanone 2g] + GJ 5 mL	6 hours
Solv+GJ (S4)	Cyclohexanone 5.75 mL [cyclohexanone 5.4g] + GJ 5.75 mL	6 hours

Table 7.3: Sequence of EVLP experiments. Pulmonary mixtures (described in interventions) given to S3 and S4 were 0.25 mL /kg animal weight. The duration of EVLP experiments was determined by lung survival and the ability to ventilate oedematous lungs. S1: sham 1, S2: sham 2, S3: OP+GJ treated lungs, S4: solvent+GJ treated lungs, GJ: porcine gastric juice.

In S1 and S2 a bronchoscopy was performed (t=0) in the right lung without treatment instillation. Direct lung injury was created in S3 and S4 by instilling 0.25 mL /kg (animal weight) treatment mixtures (t=0) (Table 7.3) using an epidural catheter placed in the sterile working channel of the bronchoscope. The indirect lung was protected with a bronchial blocker during mixture administration in a similar manner to the main aspiration minipig study (see methods chapter 2).

7.2.5.1 Porcine gastric juice

The gastric juice used in the current study was prepared in the same way as that described in chapter 5 for the minipig aspiration studies. Further details can be found in the chapter 2 (methods).

7.2.6 Pulmonary surfactant compositional analysis

The minipig studies revealed that OP+GJ aspiration decreases levels of the beneficial phosphatidylcholine (PC) surfactant species 16:0/16:0 with increases in the unsaturated PC species present in BALF. It was therefore necessary to measure the composition of PC species within the EVLP model.

To this end, 50mg (per litre of perfusate) of methyl-D_9 choline was added to the warmed perfusate immediately before each experiment This allowed the rate of metabolism of pulmonary surfactant within the mode to be studied. Unfortunately, Dr Goss moved from the University of Southampton and so only the compositional data, rather than the metabolic data, were processed.

Phospholipid extraction and mass spectrometry analysis on 10 EVLP BALF samples was conducted in the same manner as described in chapter 2 (methods).

7.2.7 Blood sampling and processing during EVLP experiment

Arterial (0.3 mL) and venous (10.5 mL) blood were sampled at regular intervals from the out flow and in flow tubing respectively (Figure 7.1).

Both arterial and venous blood (0.1 mL) were placed in an arterial blood gas and electrolyte analyser (EPOC®, UK) to determine the perfusate: PaO_2, $PaCO_2$, pH, lactate, Glc, K+, and anion gap.

Venous blood (0.2 mL) was added to 4 mL of 4°C 0.9% saline, mixed and aliquoted for AChE measurement. The remaining venous blood was added to EDTA tubes, mixed and centrifuged at 3900rm for 7 min. The resultant plasma and red cell pellet was aliquoted for further testing and stored at -80°C.

7.2.8 Bronchial lung sampling and processing

7.2.8.1 Bronchoalveolar lavage fluid sampling and bronchial wall biopsy

The bronchoscope (BRS-5000; Vision Sciences, USA) was advanced into the right lower lung until the bronchoscope wedged at approximately the 3rd- 4th airway generation bifurcation. BAL was conducted by instilling 50 mL of room temperature sterile 0.9% saline through the sterile working channel of the bronchoscope. The lavage was delivered in 3 aliquots, leaving 5-10 sec and aspirating into a closed suction sampling pot between each aliquot.

On completion of the BAL, alligator forceps were passed down the working channel and a tissue sample taken from the bronchial wall. The sample was placed in 10% buffered formalin and underwent overnight paraffin fixation.

BAL was conducted on the right lung first and then biopsied. This process was repeated in the left lung. Lung sampling occurred at 3 (S1-S4) and 6 hours (S3 and S4 only).

7.2.8.2 Bronchoalveolar lavage fluid and bronchial biopsy processing

BALF processing, cytospin preparation, light microscopy, protein measurement and bronchial biopsy processing were as described in chapter 2 (methods)

7.2.9 Steady state management of EVLP experiment

7.2.9.1 Physiological measurements

Respiratory variables, PA pressures and temperatures were collected throughout the experiment using the Datex Ohmeda (m-caiov module) monitor to capture data.

The centrifugal and roller pumps within the EVLP model produced a pulsatile pressure waveform. Consequently, the mean pulmonary artery pressure (MPAP), as the functional unit for pulmonary vascular resistance (PVR) calculations, was calculated using the formula: MPAP= (PA systolic-diastolic)/3 + diastolic PA pressure (mmHg).

Pulmonary vascular resistance was calculated using the formula: PVR= [MPAP- left atrial pressure (LAP) (mmHg)] x 80/ PA (or EVLP perfusate) flow (L/min) [dynes/sec/cm^5]. As the left atrium was opened, the LAP was assumed to be zero as it was equal to atmospheric pressure.

Oxygen capacity of the perfusate was calculated using; $\Delta P_aO_2/F_IO_2$, where ΔP_aO_2 = left atrium perfusate PaO_2 (oxygenated)– pulmonary artery perfusate PaO_2 (deoxygenated) (mmHg) (Cypel et al., 2008).

7.2.9.2 Electrolyte disturbances

Lactate accumulation within the perfusate is common in EVLP (Sanchez et al., 2012) and can only be removed with the aid of a complex haemofilter or more commonly - regular exchange of the perfusate. In the EVLP study, all the autologous blood was added to the perfusate at the start of the experiment. Consequently, the perfusate quickly became acidic, due to excess lactate, and was managed by altering the respiratory rate (to increase removal of CO_2) and/or by adding boluses of

25 mL of 8.4% sodium bicarbonate to the perfusate to maintain a physiological pH (range 7.35-7.45). Anion gap data are shown in results section. Extra fluid boluses (Hartmann's or 0.9% saline) of 100-200 mL were occasionally required to maintain the reservoir volume.

Hypokalaemia in the perfusate (<3.5 mmol /L) was managed by adding boluses of 13 mmol of potassium chloride into the perfusate.

Hypoglycaemia in the perfusate (<3.5 mmol /L) was managed by adding 20-40 mL boluses of 40% glucose solution into the perfusate.

7.2.10 Histology

7.2.10.1 Lung sample collection, processing and sectioning

At the end of each experiment, the lungs (S2-S4) along with the ETT (sutured in situ) were placed in a plastic box containing 10% buffered formalin. Tubing containing 10% buffered formalin was connected to the proximal end of the ETT and the lungs filled at a pressure of 25cm H_20 over a 1-2 hour period.

Bilateral basal (caudal) lung tissue samples 'slabs' were taken and stored in 10% neutral buffered formalin for at least another 48 hours.

Samples were sent to the Veterinary Histopathology Laboratory at the University of Edinburgh where sections were cut, placed through graded alcohols (70-100%) and xylene, and embedded in paraffin-wax at 60°C. The samples were sectioned to 4μm using a microtome, dried (for 15 min at 37°C, then 60°C for 25 min) and finally stained with haematoxylin and eosin.

Smaller sections of lung tissue (1-2 mm^3) were also cut and placed into 2% buffered gluteraldehyde for analysis at the Newcastle University electron microscopy facility (see chapter 2).

7.2.10.2 Haematoxylin and eosin staining

The slides were dewaxed with xylene, with sections rehydrated with absolute alcohol. The slide was rinsed in tap water and stained with Harris' haematoxylin. The slide was rewashed in tap water.

The slide was then washed in sequential order: 1% acid alcohol, tap water, Scott's tap water substitute, and then tap water again. The slides were stained with 1% aqueous eosin for 30 sec, then dehydrated in absolute alcohol and finally placed in xylene, mounted using DPX medium and covered with coverslips.

Tissue samples were blindly scored by myself (slides had a number, but no group identifier) in a random order using the specially designed histopathological scoring system 1.1 (see chapter 4). Images were captured using AX70 Provis (Olympus, USA) microscope and software.

7.2.11 Toll Like Receptor (TLR) analysis of ovine lung tissue after aspiration injury

Dr Mara Rocchi and Mr Stefano Guido from the Moredun Research Institute, Edinburgh, assisted with this section of the experiment by conducting TLR extraction from the ovine lung tissue and reverse transcription polymerase chain reaction RT-PCR following the MIQE guidelines on RTqPCR analysis (Bustin et al., 2009). Expression of TLR-1 to 10 was measured in lung tissue samples from the EVLP model, alongside the calibrator- ovine peripheral blood mononuclear cells (PBMCs).

7.2.11.1 Ovine lung tissue processing

Approximately 1-2mm^3 sections of right and left lower (caudal) lung tissue were excised and placed in an RNA stabilisation solution (RNAlater™, Qiagen, UK) at the end of each EVLP experiment (prior to fixing with 10% formalin) and stored at temperatures between 4°C and -20°C.

7.2.11.1.1 RNA isolation, measurement and assessment of quality

Lung tissue (30mg) from each sample was homogenised using a Precellys 24 homogeniser in 0.6mL of RLT lysis buffer. Total RNA was isolated using an RNeasy minikit (Qiagen, UK) used according to manufacturer's instructions, and then quantified using the Nanodrop. RNA Isolation was performed in duplicate. DNA contamination was reduced by using a QIA shredder column and DNAse treatment during isolation. Reactions were conducted on ice (4°C) to prevent early complementary DNA (cDNA) production.

RNA quality and integrity was measured using a eukaryotic total RNA Nano LabChip on an Agilent R2100 bioanalyser (spectrophotometer). An RNA sample is considered suitable for RT-PCR if the

RNA integrity number (RIN) is between 5-8 (Becker et al., 2010). A 260nm (specific for nucleic acids)/280 nm (specific for proteins) wavelength absorbance ratio of 1.8, with a maximal (>1.8) 260/230 (background and contaminant absorption) ratio is acceptable (Becker et al., 2010). Due to the lung damage caused by the organophosphorus pesticide (dimethoate EC40) and solvent (cyclohexanone) in the EVLP experiment, some RNA isolation and quality measurements were repeated for the directly-injured (right) lung (see Table 7.5).

RNA extraction was conducted in the same way for the ovine PBMCs (taken from two donor sheep) used to calibrate the RT-PCR.

7.2.11.1.2 Reverse Transcription (RT) quantitative Polymerase Chain Reaction (qPCR)

The process of RT-qPCR allows the cloning of expressed genes through a 2-step process; firstly by reverse transcribing the RNA of interest into its cDNA, and secondly by amplifying the cDNA fragment for subsequent measurement (Bustin and Mueller, 2005).

The selected samples and calibrator were assayed with TLR1-10 primers and dual labelled 5'-FAM (flurophore)/3'-BHQ1 (quencher) conjugated probes (Eurofins, MWG Operon, Germany) (Table 7.4). Beta actin levels were also measured within the samples using its respective primers and probe to act as an internal control gene to normalise the expression values of target TLR1-10 genes (Bustin et al., 2009).

Measured cDNA	Primer sequence (F=forward or R=reverse)	Probe sequence
B- actin	OvBact-F: 5'-GCCTCGACCAAGAGCTGA AG-3' OvBact-R: 5'-GGG CAG CCT TTC GGA TCT-3'	5'-FAM-TGA TCG GCG AGT ATG GGC TCC G-TAMRA-3'
TLR 1	OvTLR1-F GAAGATGCTGAGAGCCTTCAAGAC OvTLR1-R GCTGACTGACACGTCCAAATTAAA	TGCACATTGTTTTCCCCACAGGAAAGAAAT
TLR 2	OvTLR2-F CTGCTGGAGCCCATTGACA Ov-TLR2-R TATTGCAGCTCTCAAATTCAACCA	AGGCCGTTCCCCAGCGCTTCT
TLR 3	Ov-TLR3-F TCGATGGACTGAAACAGACGAAT Ov-TLR3-R AGGAGTCATTACCCATCACACGTA	TCACCACGCTGGACCTTTCCCGTA
TLR 4	Ov-TLR4-F ACTCTTGTCATTGGATACATTTCTTTATG Ov-TLR4-R GTAAGATTTAGCCAAGTGAGGTTCCTT	TGCAGTTTCAACCGTATCACGGCCT
TLR 5	Ov-TLR5-F CCCTGTCCCAGTTCCATCTG Ov-TLR5-R CCACCTCAAGTACTGCCGTTTC	TGAAGCATCAGTCCATCAGAGGGTTCG
TLR 6	Ov-TLR6-F GCAACCCTCCGGGAGATAGT Ov-TLR6-R CAATGTAAACAAAATGACAGCTTCTG	CAACGCCATGATCAAAGACAAAGAATCTCC TA
TLR 7	Ov-TLR7-F TTAACCATACCGAGGTGACTATTCC Ov-TLR7-R AGACCACACTCTGGCCCTTGT	AGATGTGACTTGCATGGGACCAGGAGC
TLR 8	Ov-TLR8-F ACTGAAAAGTGCGTCCCTAGGA Ov-TLR8-R TATGCTGAAGGTTATTACGGGCTAA	CAGTGGGAACCGCCTTGACCTTCTG
TLR 9	Ov-TLR9-F TTCTCTCTACAAACTAGAGAAAGACTGGT Ov-TLR9-R GTCTTGGTGATGTAGTCATAGAGGAAGT	CTGGGCAGGCTCCAAGTGCTCG
TLR 10	Ov-TLR10-F CGAGCTGCTCTTCATGTTAATTTATT Ov-TLR10-R ACTGCAGAACCTCGAAACACTTC	AGGGACATGTGAACTACAGACATTCGCAGA ACT

Table 7.4: Primer and probe sequences of ovine toll like receptors TLR 1-10 (OvTLR) RT-qPCR

RT-qPCR was performed using the one step Quantifast RT-PCR Plus Kit according to the manufacturer's instructions. 25µl volumes were used, consisting of: 12.25µl Quantifast Mmix (includes RT polymerase); 1µl primer at 20µM; 0.25µl probe (either 5'-FAM/3'-BHQ1 conjugated) at 20µM; 0.5ul of ROX reference dye; a volume of sample or calibrator to achieve 20 ng of RNA

per reaction with the remaining volume made up with DNAse free water. The PCR measurements were obtained from an Applied Biosystem 7500 under the following cycle conditions: 50°C for 10 min and 95°C for 2 min followed by 40 cycles at 95°C for 10 sec and 60°C for 32 sec. Data were analysed using ABI 7500 software to generate Ct (cycle threshold) values.

Each sample was assayed in duplicate and the average Ct value calculated. Average Ct values were compared within groups and relative quantification was calculated using the Beta actin (internal control gene) to determine the fold differences in the expression of the target gene creating a ΔCt value. A further normalisation was applied by removing the signal produced by the calibrator (PBMCs) that produced ΔΔCt values. The relative gene expression was then calculated using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001).

7.2.12 Minipig lung biopsy processing and staining for TLR 3 and 5

To explore the expression of pulmonary TLRs after OP pulmonary installation, samples from the minipig aspiration study (chapter 5) were retrospectively examined, using bronchial biopsies which had been collected from both right and left lungs at 24 and 48 hours in all but the oleic acid poisoned pig (right lung only). Samples had been placed in 10% neutral buffered formalin for at least 48 hours. The following processes were carried out by the shared universities research facility (SuRF) at the University of Edinburgh (Queen's Medical Research Institute, Edinburgh). This experiment was exploratory and so power calculations were not conducted. Funds were limited and so only TLR 3 and 5 were examined.

7.2.12.1 Minipig bronchial biopsy processing and slide mounting

Six bronchial biopsy samples taken at 48 hours from directly-injured (right) lungs in the minipig aspiration study were selected for TLR staining from the following groups: GJ (n=2), OP+GJ (n=2) and sham bronchoscopy (n=2). They were processed as detailed in the methods section.

7.2.12.2 Immunostaining minipig bronchial biopsy for TLR 3 and 5

The slides were dewaxed with xylene (10 min) and then rehydrated with graded alcohols (70-100%) over 2 min. All reagents used in the following steps were from a Novacastra bond polymer refine

detection kit, except the primary antibodies. Antigen retrieval was performed in a pH 6 buffer under standard conditions in a pressure cooker.

Slides were loaded onto the Leica Bond Max robot and stained with peroxide for 5 min and then primary porcine TLR 3 or 5 antibody (Novus biologicals) at a 1:200 dilution for 120 min. The slides were then placed in polymer for 15 min, DAB chromagen for 10 min and finally haematoxylin for 5 min.

Slides were removed from the robot, dehydrated with graded alcohols (70-100%) and placed in xylene. DPX and coverslips were applied before examination under light microscopy.

7.2.13 Immunostaining human methanol fixed bronchoalveolar lavage fluid cells

Slides containing methanol fixed BALF cells from human pesticide poisoned patients were produced as described in chapter 6. Only two OP+No ASP (n=2) and one NON-OP+ASP (n=1 at 2 time points) patient BALF cell slides were available for examination (discussed in chapter 6).

On return to the UK, the slides were loaded onto the Leica Bond max robot and the following protocol run for each antibody detecting either; TLR 3 or TLR 5 (Novus biologicals). Peroxide block (10 min), serum (normal goat serum) block for 10 min, primary human TLR 3 and 5 antibody (1:1000 dilution) incubation for 30 min. The slides were placed in HRP secondary (goat anti rabbit peroxidase) for 30 min, then exposed to Tyrimide Cy3 (TLR 3 - red) or Fluorescein (FITC) (TLR5-green) for 10 min and then 4',6-diamidino-2-phenylindole (DAPI-blue) for 10 min. Washes were performed within each step (prior to tyrimide staining) using Leica bond wash.

After DPX and coverslips had been applied, the slides were double wrapped in foil and kept refrigerated at 4°C until viewing on a LS710 confocal microscope (Zeiss).

7.3 Results

The EVLP model allowed ovine lungs to be successfully ventilated and perfused for ≥3 hours. The physiological state of each pair of retrieved lungs improved with each subsequent experiment. This resulted in S3 (OP+GJ) and S4 (Solv+GJ) lungs surviving until 6 hours, compared with the S1 and S2 controls (survival 3 hours). Lung oedema with difficulty in ventilation was the main reason to terminate an experiment.

In contrast to the minipig and human OP poisoning aspiration studies, the EVLP study revealed an absence of neutrophils in both the lung parenchyma and BALF after pulmonary administration of OP+GJ. Possible reasons for this are discussed below but could mean that the pathophysiological changes secondary to aspiration of OP+GJ or Solv+GJ observed in the EVLP model may not accurately reflect those within an *in vivo* model.

However, the pilot data from the EVLP model showed that OP+GJ lungs had increased concentrations of BALF protein in both lungs when compared with control or Solv+GJ lungs at 3 and 6 hours respectively. This was similar to that seen in the minipig and human studies and probably results from damage to the alveolar capillary membrane. Electron microscopy images of the EVLP OP+GJ lungs support this explanation.

The experiments with the EVLP model, and immunostaining of the minipig lung and human BALF cells indicate that the immuno-pathological mechanisms of pulmonary aspiration of OP+GJ may involve the stimulation of TLR 3 and 5 within the lung.

7.3.1 Heart-lung bloc and EVLP preparation

The mean sheep weight (n=4) was 38.5 (± 5.9) kg with the volume of blood collected 638 (± 263) mL.

The interval between the animal's death and successful placement of the heart-lung bloc on by-pass was reduced during the course of the experiments (S1; 2 hours, S4 ;1 hour). The mean time from sheep death to installation of pulmonary treatments (t= 0) was 3.1 (± 0.9) hours.

7.3.2 OP inhibition of red cell AChE in the EVLP perfusate

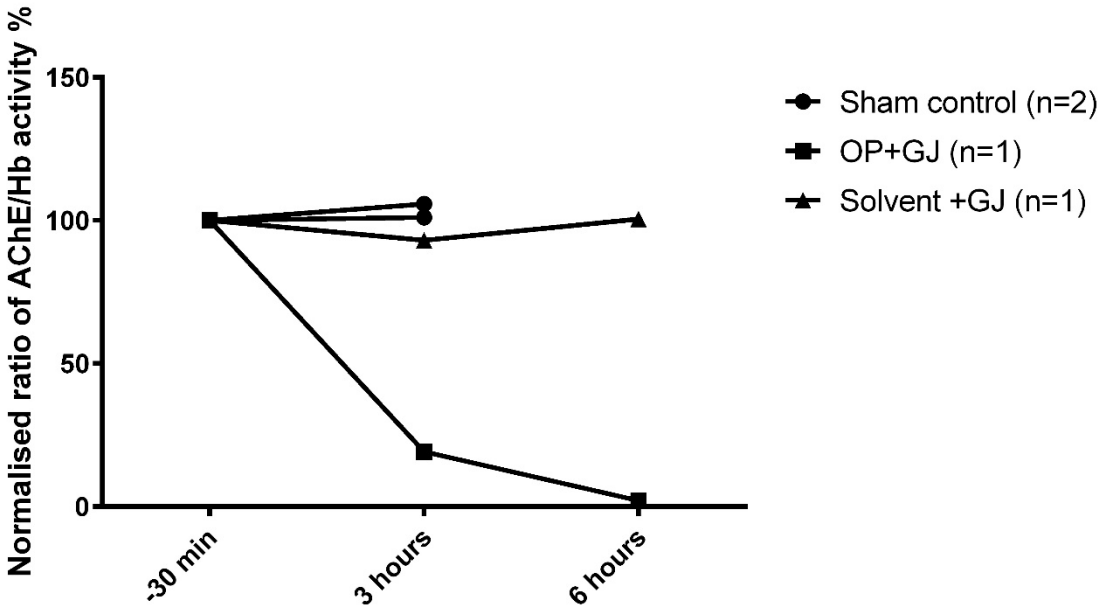


Figure 7.2: Graph showing red cell AChE activity in EVLP perfusate when corrected for haemoglobin concentration (U/mmol).

Figure 7.2 confirms that despite the dilution of the red cells in large volumes of crystalloid, the OP placed in the right lung could inhibit the red cell AChE as it circulated through the EVLP lungs.

7.3.3 Analysis of the respiratory physiological variables of the EVLP model

The lung oxygenation capacity at t -30 min, of each set of lungs, improved over time from S1 (26 mmHg) to S4 (291 mmHg) due to better technique and faster lung retrieval. Although there was a reduction in oxygenation over time for lungs treated with either OP+GJ or Solv+GJ (Figure 7.3A), there were no valid controls (up until 6 hours) for comparison.

Elevations in PVR reduce the pulmonary blood flow and therefore compromise the removal of CO_2 and supply of O_2 to the tissues. Increases in PVR during ARDS can result from: (i) excessive ventilation of lungs (due to the alveolar pressures exceeding the vessel pressures causing subsequent collapse of the vessel) (ii) under-ventilation of lungs (causing hypoxic pulmonary vasoconstriction) and/or with (iii) the presence of microthrombi in the small pulmonary vasculature

(causing mechanical obstruction to blood flow)(Ryan et al., 2014). Repeated collapse and re-opening of the pulmonary vasculature (the latter happening during expiration) can cause further endothelial damage and pulmonary oedema (Schutte et al., 1998). The PVR from 1 hour onwards was reduced in OP+GJ (670±131 dyn.s/cm^5) and Solv+GJ lungs (666±150 dyn.s\cm^5) compared to S1 (1869±1894 dyn.s/cm^5) and S2 control lungs (2001±713 dyn.s/cm^5) (Figure7.3B). Typical EVLP PVR lung values are approximately 500-600 dyn.s/cm^5 (Cypel et al., 2008). Therefore the elevated PVR in the control animals probably reflects the poor quality of the lungs plus damage to vasculature from ischemia-reperfusion injury. The presence of high dose heparin within the perfusate of these experiments means that the raised PVR was unlikely to be caused by microthrombi in the pulmonary vasculature.

All the lungs maintained a Pplat < 25.5 cm H_2O and thereby should have avoided ventilator induced lung injury (Figure 7.3C).

The compliance of a lung is a measure of how well it can expand for any given pressure and is affected by the lung tissue itself and external forces e.g. rib cage -*in vivo*.

Lung compliance = tidal volume (expiratory)/Pplateau – PEEP, was measured by the Datex Ohmeda S/5 M-CAiOV module.

The greatest values of this variable were associated with Solv+GJ treated lungs at 23 (± 1.6) mL/cmH_2O. Values were always < 11.5 mL/cmH_2O in the other three sets of lungs (Figure 7.3D). The normal static lung compliance for a sheep (*in vivo* – anaesthetised) is 2L /kPa (196 mL/cmH_2O)(Collie et al., 1994), and so despite being an *ex vivo* model, all these values represented significant lung dysfunction.

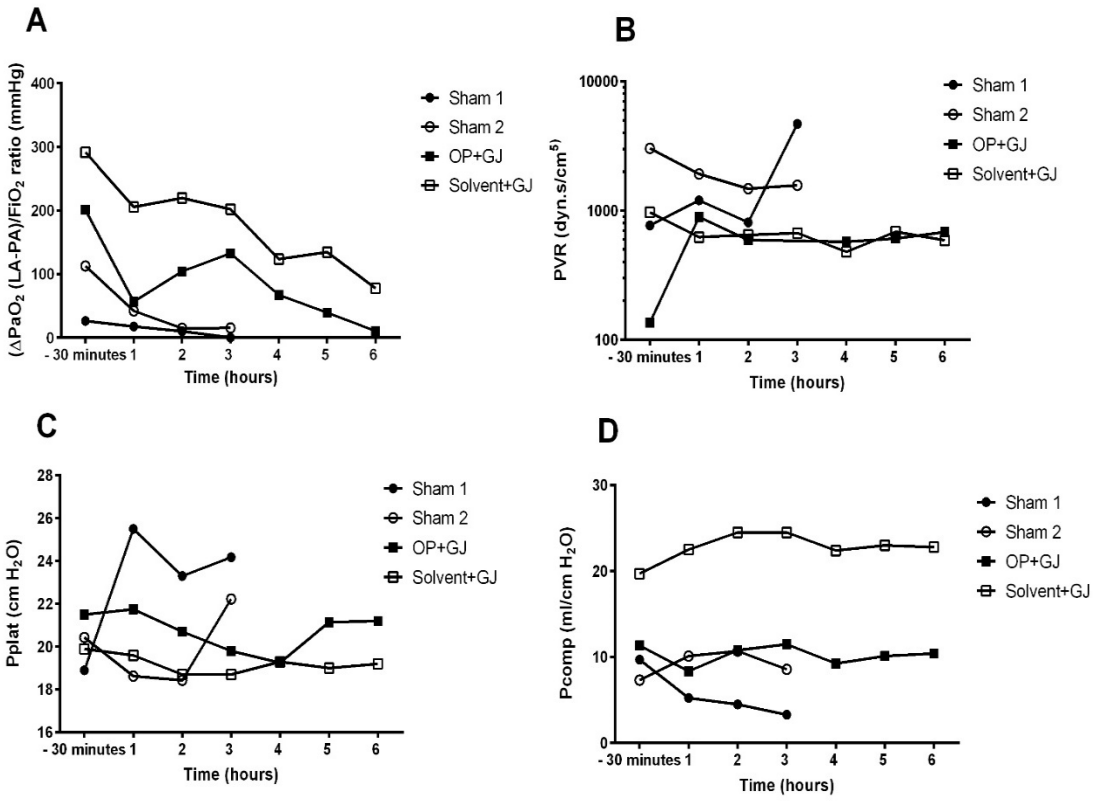


Figure 7.3: Physiological data from the EVLP model. (A) lung oxygenation capacity (**$\Delta PaO_2/F_IO_2$**, mmHg), (B) pulmonary vascular resistance (PVR, dynes/second(dyn.s)/cm_5), (C) plateau airway pressure (Pplat, cmH_2O) and (D) pulmonary compliance (Pcomp, mL /cm H_2O). Data captured 30 min before the start and up to 6 hours after the experiment. OP: organophosphorus pesticide, GJ: gastric juice.

7.3.4 Optimum EVLP flow

A suitable perfusate flow within the EVLP model was necessary to prevent blood vessel collapse which increases PVR (= MPAP x 80/perfusate flow (L/min)) and can lead to endothelial damage. The relationship between the perfusate flow and MPAP within this EVLP model (Figure 7.4) indicates that the optimum perfusate flow rate was approximately 1L/min creating the lowest MPAP's. Below this flow, the pulmonary capillaries may begin to close, necessitating greater pressures to re-open them, increasing PVR.

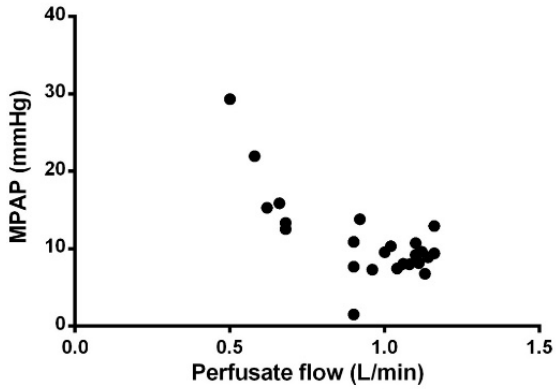


Figure 7.4: A scatter dot plot showing the relationship between perfusate flow (L/min) and the mean pulmonary artery pressure (MPAP, mmHg) within the EVLP model. PVR: [MPAP-LAP (mmHg)] x 80/ pulmonary artery (or EVLP perfusate) flow (L/min) [dynes/sec/cm^5]. LAP was assumed to be at atmospheric pressure as the left atrium was open to air.

The perfusate flow for the controls was 0.86(±0.25) L/min in S1 and 0.65 (±0.04) L/min in S2 with both below the optimum 1L/min flow, which was achieved in the OP+GJ 1.0 (±0.1) L/min, and Solv+GJ 1.1 (±0.08) L/min lungs (Figure 7.5A). Therefore, this may have been a source of lung injury in the control lungs.

7.3.5 Analysis of the metabolic variables of the EVLP model

All experiments (S1-4) maintained a mean perfusate temperature greater than 36°C (Figure 7.5B) assisting normal cell metabolism in the study groups. The blood anion gaps in most of the EVLP perfusates exceeded normal (12-24 mEq/L) blood anion gaps (Jackson et al., 2002), particularly towards the end of each experiment. The perfusate anion gaps for S1 and S2 controls were 27 (±1.6) and 34 (±2.7) mEq/L respectively, with lower values for OP+GJ 25 (±4.4) mEq/L and Solv+GJ 23 (±2.9) mEq/L treated lungs (Figure 7.5C).

A large anion gap indicates the presence of unmeasured ions. In these EVLP experiments it was likely due to lactate, whose concentrations ranged between 9.0-20.0 mmol/L [normal mean for a sheep being 3.5mmol/L](Allison et al., 2008). The greatest values may have been understated: the EPOC (Woodley, UK) device, used for measuring lactate, had a maximum concentration of 20 mmol/L, and so readings could have been greater. S1 had the lowest lactate 11.5 (±1.9), followed

by Solv+GJ 17.4 (±2.2) mmol/L with S2 control and OP+GJ treated lungs having the greatest concentrations (>20 mmol/L)(Figure 7.5D).

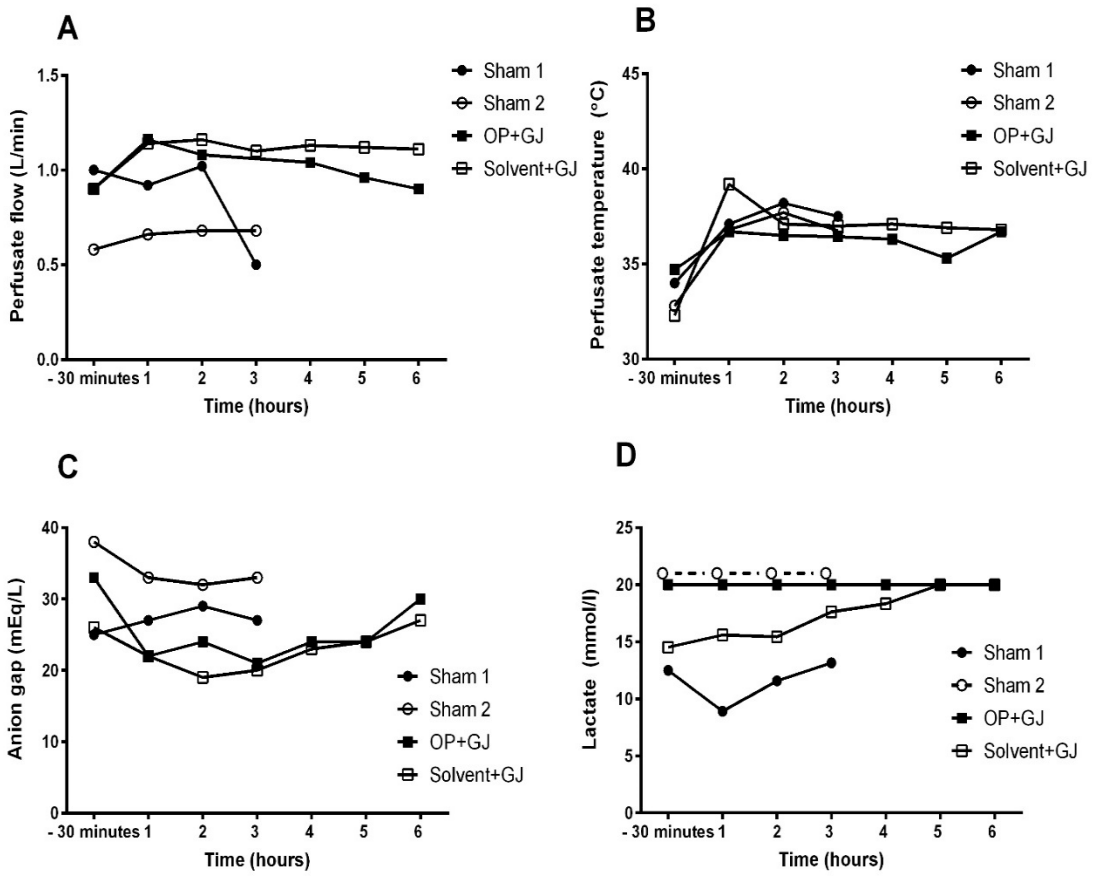


Figure 7.5: EVLP perfusate data. (A) perfusate flow (l/min), (B) perfusate temperature (°C), (C) the perfusate anion gap (mEq/L) [calculated by([Na+] + [K]) – ([Cl^-] + [HCO_3^-]) and (D) lactate concentration (mmol/L). NB the maximum lactate concentration that the EPOC® machine could calculate was 20 mmol/L which was attained by both sham 2 and OP+GJ lungs at the beginning of the experiment.

7.3.6 Bronchoalveolar lavage fluid cellular and non-cellular contents

BAL return volumes (percentage of total volume instilled) with 0.9% sterile saline were acceptable (Meyer et al., 2012) for all lungs (S1-S4, right and left) with mean (SD) 60(±24.9) %.

However, the BALF yielded low numbers of white cells with heavy red blood cell contamination in the OP+GJ and Solv+GJ treated lungs. This meant an accurate cell count and white cell differential (>500 cells required) was not performed on the BALF from these lungs. However, from lavages in which cytospins could be made, the predominant cellular type from all available lavages (S1 (control), OP+GJ and Solv+GJ treated lungs at 3 and 6 hours) was the alveolar macrophage (>90%).

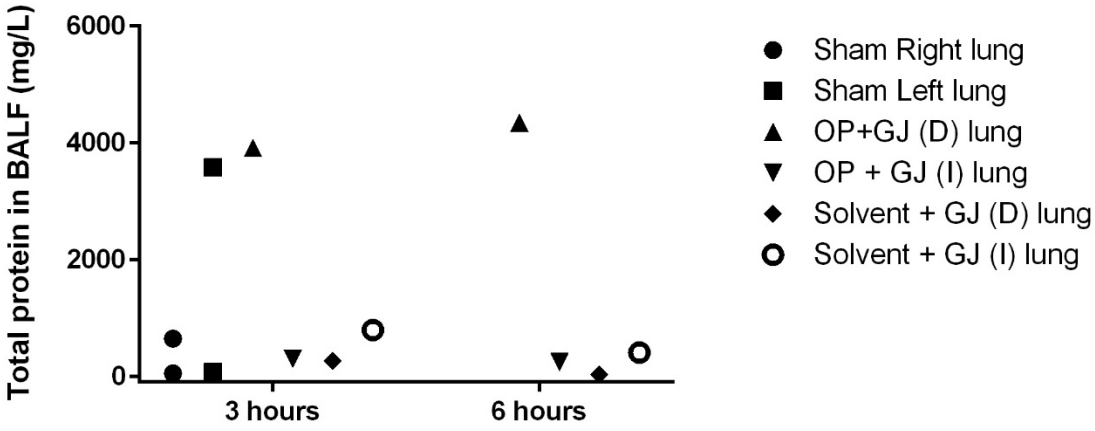


Figure 7.6: Total protein content in the BALF fluid from both directly-injured (D) and indirectly-injured (I) lungs at 3 and 6 hours (mg/L) after pulmonary instillation of mixtures. The high protein concentration in the sham left lung sample was from S2.

The BALF protein concentration was greatest in the OP+GJ directly-injured lungs (3912 mg/L) compared with the mean control 346 (±421) mg/L or Solv + GJ directly-injured lungs (264 mg/L) at 3 hours (Figure 7.6). One of the control (S2) left lungs had a high concentration of protein (3580 mg/L), perhaps secondary to poor EVLP perfusate flow and subsequent endothelial injury. The albumin content of BALF was un-recordable (too low) in all samples indicating that the protein content observed in the BALF probably resulted from either vascular leak (e.g. macroglobulins) or secretion of invading cellular proteins (e.g. enzymes – lactate dehydrogenase) (Dwenger et al., 1986). Blood contamination of the BALF may have been responsible for the elevated BALF protein content (Morrison et al., 1986). However, the Solv+GJ BALF samples were also heavily contaminated with red blood cells and had less protein than the control or directly-injured OP+GJ

lungs at 3 hours. The protein concentration in the OP+GJ indirectly-injured lung was near control values and suggests the absence of an indirect lung injury that was observed in the minipig model.

7.3.7 Pulmonary surfactant phosphatidylcholine (PC) species compositional changes

Pulmonary installation of OP+GJ caused low percentages (of total PC species) of the beneficial saturated PC species 16:0/16:0 in the directly inured lung (27%) and greater percentages in the unsaturated PC species (e.g. 18:0/18:2) by 6 hours. The OP+GJ indirectly-injured lung (46%) and both Solv+GJ treated lungs (direct 39% and indirectly-injured 45%) at 6 hours had similar proportions of PC 16:0/16:0 to the mean control lungs (34%).

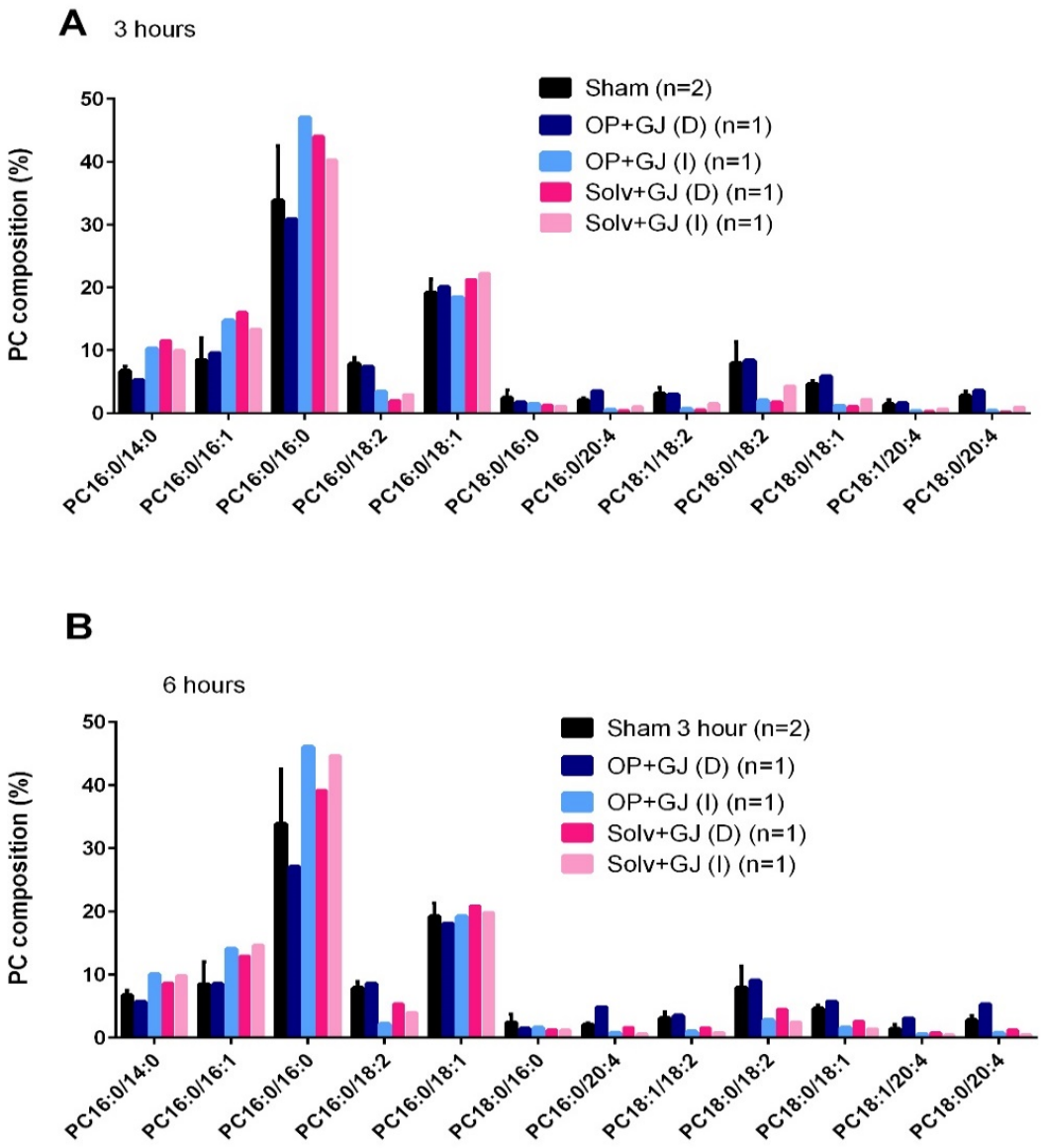


Figure 7.7: Compositional changes in surfactant phosphatidylcholine species occurring after pulmonary installation of treatments. BALF pulmonary surfactant phosphatidylcholine (PC) species composition (% of total PC species) of the directly-injured (right) (D) and indirectly-injured (left) (I) lung at 3 (A) and 6 (B) hours. Figure B used the same sham control data from the right lung BALF (A) at 3 hours for comparison. OP+GJ direct lung injury (D) caused a reduction in the PC species 16:0/16:0 responsible for reducing alveolar surface tension in the lung, with increases in unsaturated PC species (e.g. 18:0/18:2). Mean and SD shown.

7.3.8 Histopathology

The OP+GJ indirectly-injured lungs were similar in appearance to the control lung, with the directly-injured lung displaying capillary engorgement and what appeared to be alveolar cell sloughing and/or fibrin deposition in the alveolar space (Figure 7.8C, black arrow). There were a few alveolar cells present – mostly alveolar macrophages with the occasional neutrophil. The Solv+GJ treated lungs had a similar appearance to the OP+GJ lungs, but the directly-injured lung had less evidence of alveolar sloughing or fibrin deposition (Figure 7.8F). In both OP+GJ and Solv+GJ directly-injured lungs there was a notable absence of neutrophils, similar to the lack of neutrophils observed in the BALF.

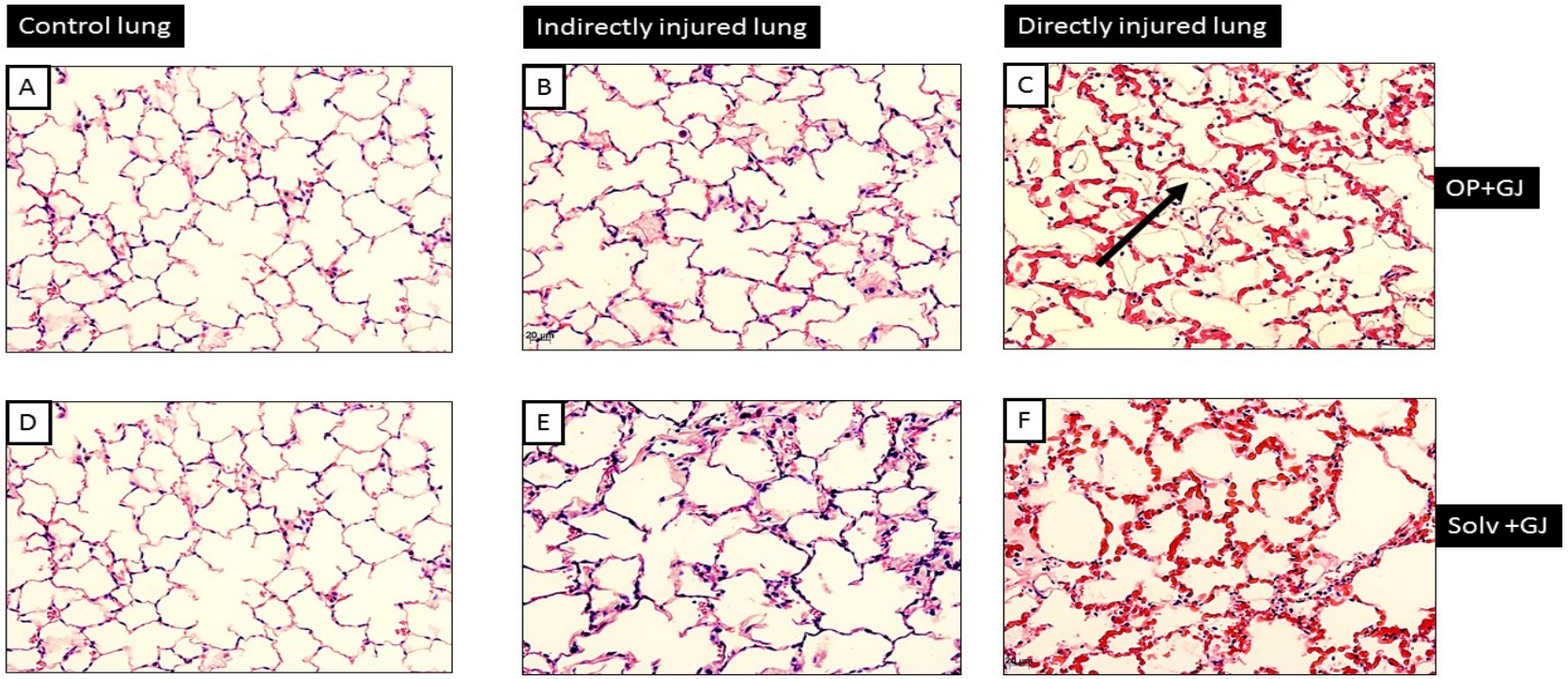


Figure 7.8: Lung injury caused through direct pulmonary installation of either OP (dimethoate EC40)+GJ or solvent (cyclohexanone) + GJ into the directly-injured (right) lung within an EVLP model. Images A and D represent control lung tissue (S2 right lung), B and C (OP+GJ), and E and F (Solv+GJ) show the indirect (left lung) and direct (right lung) lung injuries, respectively. The direct lung injuries for both OP+GJ(C) and Solv+GJ(F) show capillary engorgement in the alveolar capillary membrane with OP+GJ showing more alveolar epithelium sloughing and fibrin deposition (C) within the alveoli (black arrow). Haematoxylin and eosin stained basal lung tissue photographed under light microscopy (AX70 Provis, Olympus, USA) at x20 magnification and edited using Microsoft PowerPoint 2010.

The lack of neutrophils observed in the BALF was reflected in the histopathology scores awarded (Figure 7.9). This was unsurprising as the histopathology scoring system 1.1 was neutrophil dependent, and thus all EVLP lungs had low scores with no obvious differences between treatment groups.

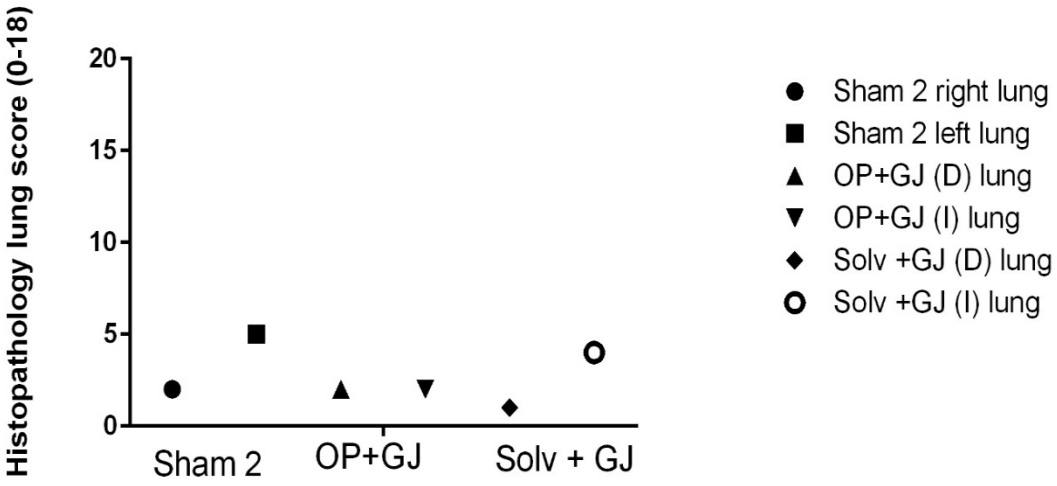


Figure 7.9: EVLP histopathology scores awarded to sham 2, OP+GJ and Solvent + GJ bi basal lung samples using the Histopathology scoring system 1.1.

7.3.9 Electron microscopy of the alveolar capillary membrane

Direct lung injury with OP+GJ was apparent with electron microscopy. This demonstrated greater swelling of the alveolar capillary membrane, capillary engorgement and sloughing of the alveolar cells into the alveolar spaces when compared with S2 control lungs (Figure 7.10). Similar pathological findings to OP+GJ direct lung injury were also seen in the Solv+GJ directly-injured lungs (Figure 7.10 I-L). The directly-injured OP+GJ lung showed additional evidence of lung injury with fibrin and collagen strand formation in the alveoli (Figure 7.10G – inset).

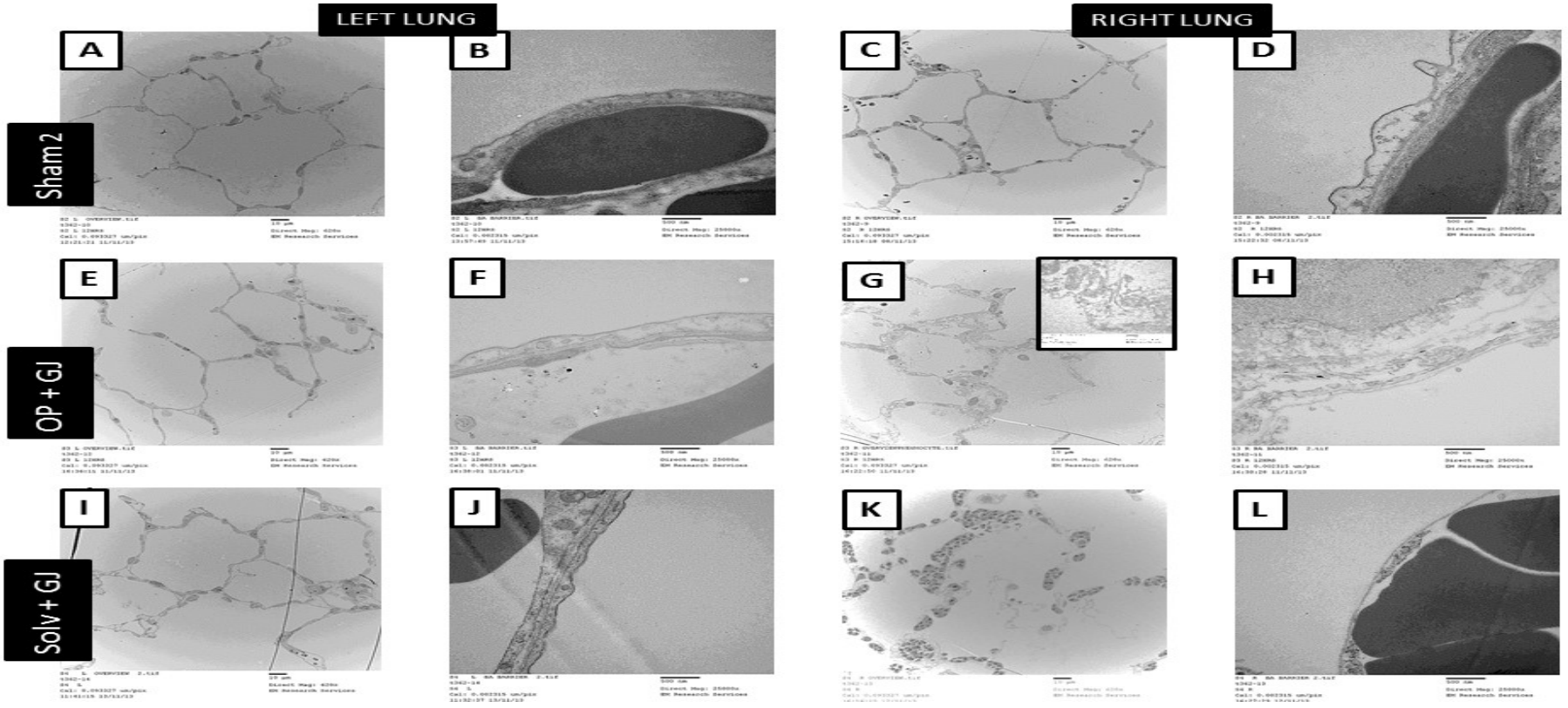


Figure 7.10: Transmission electron microscopic (TEM) images of sham bronchoscopy control ovine lungs (A-D) after 3 hours of EVLP with OP+GJ (E-H) and Solv+GJ (I-L) ovine lungs after EVLP for 6 hours. Images E and I show the indirectly-injured (left) lung and G, K show the directly-injured (right) lung at x620 magnification. B, F and J show the left lung, and D, H and L the right lung alveolar capillary membranes at x25,000 magnification. Direct injury (right lung G, H) with OP + GJ caused alveolar capillary membrane swelling, capillary congestion with subsequent sloughing of the alveolar cells into the alveoli. Fibrin deposition is also present (inset picture - G). Direct injury (right lung K, L) with Solv + GJ caused a similar injury without the apparent fibrin deposition.

7.3.10 TLR analysis of ovine lung tissue

Toll like receptors are pattern recognition receptors present on lung and infiltrating cells, and mainly involved in the innate immune system. They are required to initiate acute inflammation within the lung in response to environmental toxins and pathogens when normal host defences fail (e.g. alveolar macrophages, secretory IgA, surfactant proteins) (Lafferty et al., 2010, O'Neill et al., 2011).

Once triggered, TLRs mediate the production of cytokines and chemokines required for an adaptive immune response and can be both beneficial (maintaining tissue integrity and repair) and detrimental (e.g. lung oedema and fibrosis) for the host. Understanding TLR activation in the context of OP poisoning and aspiration could help explain the pathophysiology observed in both the minipig and human pesticide aspiration studies.

7.3.10.1 Quality of lung tissue for TLR analysis

Table 7.5 details the quantity, quality (260/280nm and 260/230nm absorbance ratios) and integrity of RNA (RIN) that was extracted from the lung tissue samples.

The samples chosen for TLR RT-qPCR had acceptable RIN values (5-8) and 260/280nm wavelength absorbance ratios > 1.8. However, the RNA concentration and 260/230 ratio was reduced in sample #9 (OP+GJ right lung) indicating poor quantity and the presence of contaminants (e.g. protein) in the RNA solution. Unfortunately, this was the best available sample for the OP+GJ directly-injured (right) lung.

Sample type	sample ID#	Lung	time (hours)	RNA conc (ng/μl)	260/280	260/230	RIN
Sham 1	1	R	3	133.20	2.05	1.88	8.30
Sham 1	4	L	3	102.30	2.06	2.15	7.80
Sham 2	5	R	3	135.20	2.04	2.26	8.50
Sham 2	6	R	3	138.50	2.05	2.32	7.80
Sham 2	7	L	3	199.30	2.01	2.28	8.00
OP+GJ	9*	R	6	11.40	2.82	0.24	5.70
OP+GJ	12	L	6	88.00	2.18	2.26	6.90
Solv+GJ	15	L	6	206.70	2.13	2.05	8.70
Solv+GJ	17	R	6	33.80	2.27	2.35	6.00
PMBCs 1	Calibrator			120.60	2.04	2.22	8.6
PMBCs 2	Calibrator			110.30	2.07	2.03	8.4

Table 7.5: Ovine lung tissue and PMBC RNA quality measurements in samples that were selected for RT-qPCR due to their greater absorbance ratios and RIN values. * RNA isolation was repeated twice without improvement in results (best result shown). Sham: sham bronchoscopy, RIN :RNA integrity number (1 = worst performing, 10= best performing).

After calculation of the relative fold increase with the calibrator, sample #4 from sham 1 left lung was found to have a disproportionately large fold increase (199.46) for TLR5, compared with the other four sham samples (#1,5,6 and 7) mean (SD) fold increase 10.6 (±2.5). Repeat testing of sample #4 was attempted but the quality of RNA was too poor and thus the RT-qPCR failed. The BALF protein content of sham 1 left lung was low, suggesting the absence of lung injury and inflammation. Consequently, results from sample #4 TLR 5 expression were excluded from further analysis.

7.3.10.2 TLR expression for lung tissue in EVLP model

Omnibus testing showed that there were significant differences in expression of TLR 1-10 in lung tissue between groups (p=0.0081). Although not significant, the most interesting result was that the OP+GJ indirectly-injured (left) lung had increased expression of TLR 3 (44 fold) and 5 (47 fold) in lung tissue when compared with control mean (SD) fold change: TLR 3; 7.9 (±2), TLR 5; 10.6 (±2.5)

(Figure 7.11). The OP+GJ directly-injured (right) lung had an unexpectedly reduced expression of TLR 3 (24 fold) and TLR 5 (13 fold) in comparison to the indirectly-injured (left) lung. This may have been the result of poor sample quality.

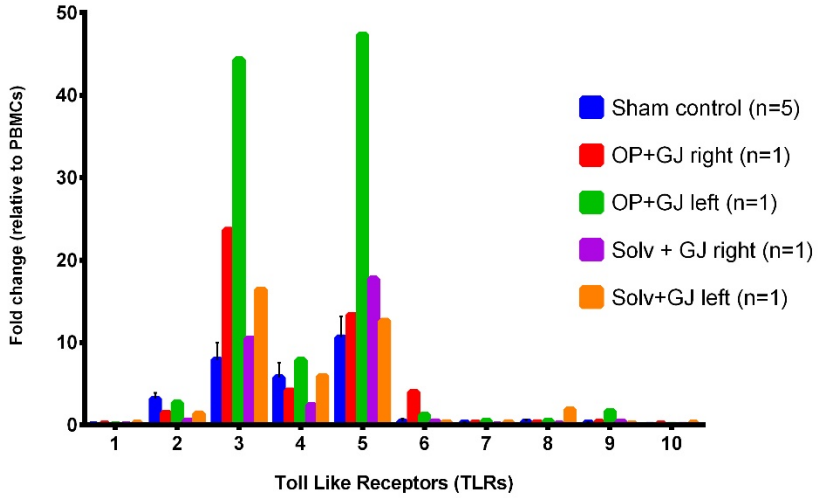


Figure 7.11: Fold change of TLR 1-10 cDNA expression in sham bronchoscopy controls, OP+GJ and Solv+GJ treated lungs using relative quantification and the $2^{-\Delta\Delta CT}$ method (normalised using beta actin and peripheral blood mononucleated cell (PBMC) controls. Sham group (5 samples taken from S1 and S2 EVLP lungs) show mean and SD. Friedman analysis of TLR 1-10 shows significant difference between groups p=0.0081 **.

7.3.11 Immunostaining of minipig bronchial biopsies for toll like receptors 3 and 5

The EVLP study suggested that there was an increase in TLR 3 and 5 expression in lung tissue after pulmonary installation of OP (dimethoate EC40) and GJ when compared with control lungs. To explore this further, bronchial biopsy samples from directly-injured lungs taken at 48 hours from the minipig aspiration study were examined (chapter 5). This experiment was designed to explore the expression of TLR 3 and 5 in lungs that had been exposed to OP+GJ (n=2) when compared with sham bronchoscopy (n=2) controls and GJ exposed (n=2) minipig lungs.

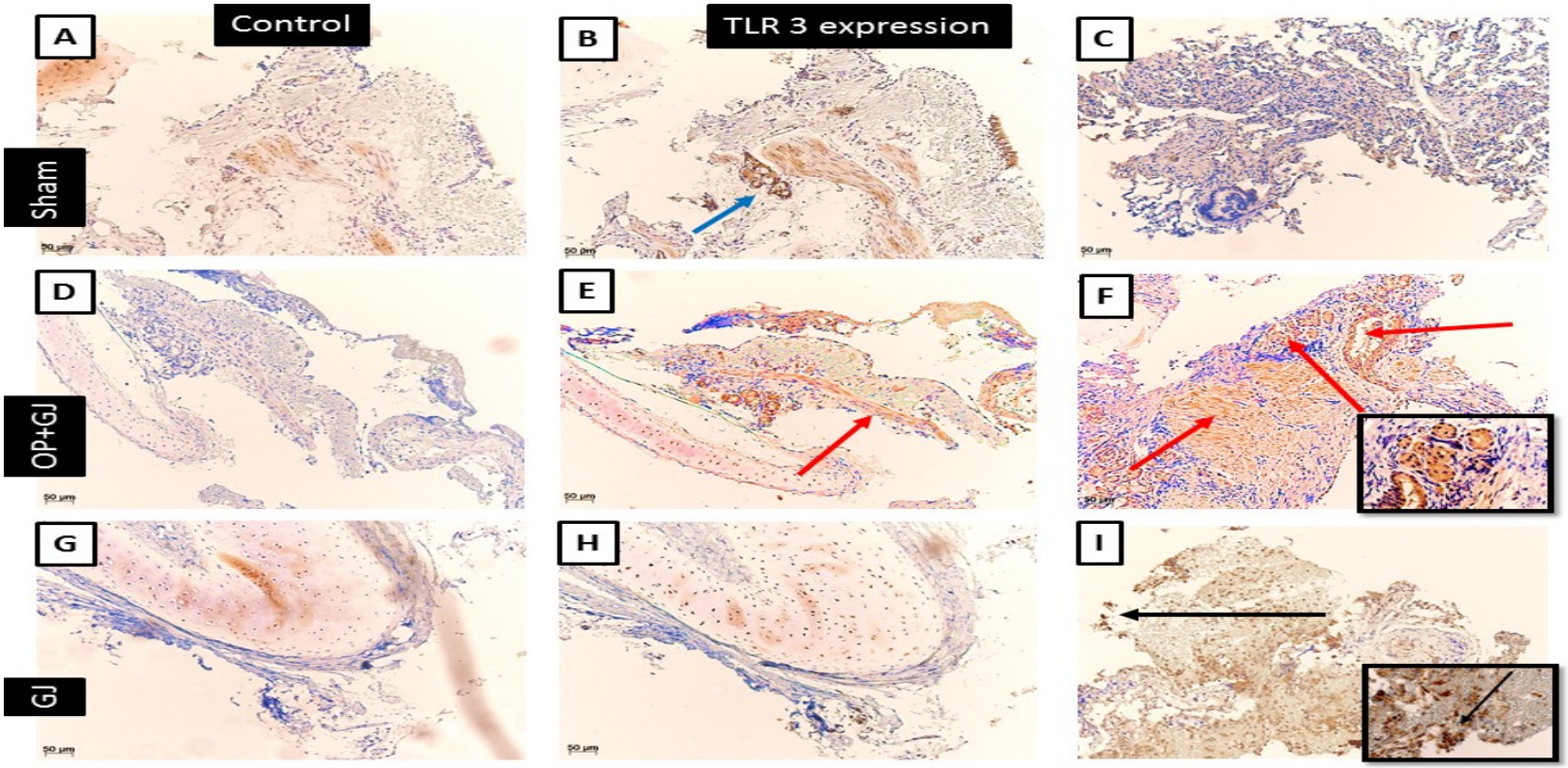


Figure 7.12: Toll like receptor 3 expression in minipig lungs exposed to treatments.TLR3 expression is shown with DAB (brown) staining. Other lung tissue is highlighted with haematoxylin (blue). Bronchial biopsy samples were taken from the right lung at 48 hours after sham bronchoscopy (A, B and C) (n=2) or directly-injured (right) lung installation of either 0.5 mL/kg OP+GJ (n=2) (D, E and F) or GJ alone (n=2) (G, H and I). Control negatives are represented from each group (A, D and G). Sham bronchoscopy minipig lung shows TLR 3 expression on the pseudostratified columnar epithelium, alveolar epithelial surface and strongly in some submucosal ducts (blue arrow). OP+GJ minipig lung tissue has increased expression in submucosal tracts (red arrow) and ducts (inset F) and smooth muscle (red arrows). Gastric juice exposed lung tissue shows TLR 3 expression on pseudostratified columnar epithelium and alveolar epithelium black arrows and inset I). Pictures were taken using Axiovision Rel 4.8 software and an Olympus AX70 Provis microscope with x10 magnification.

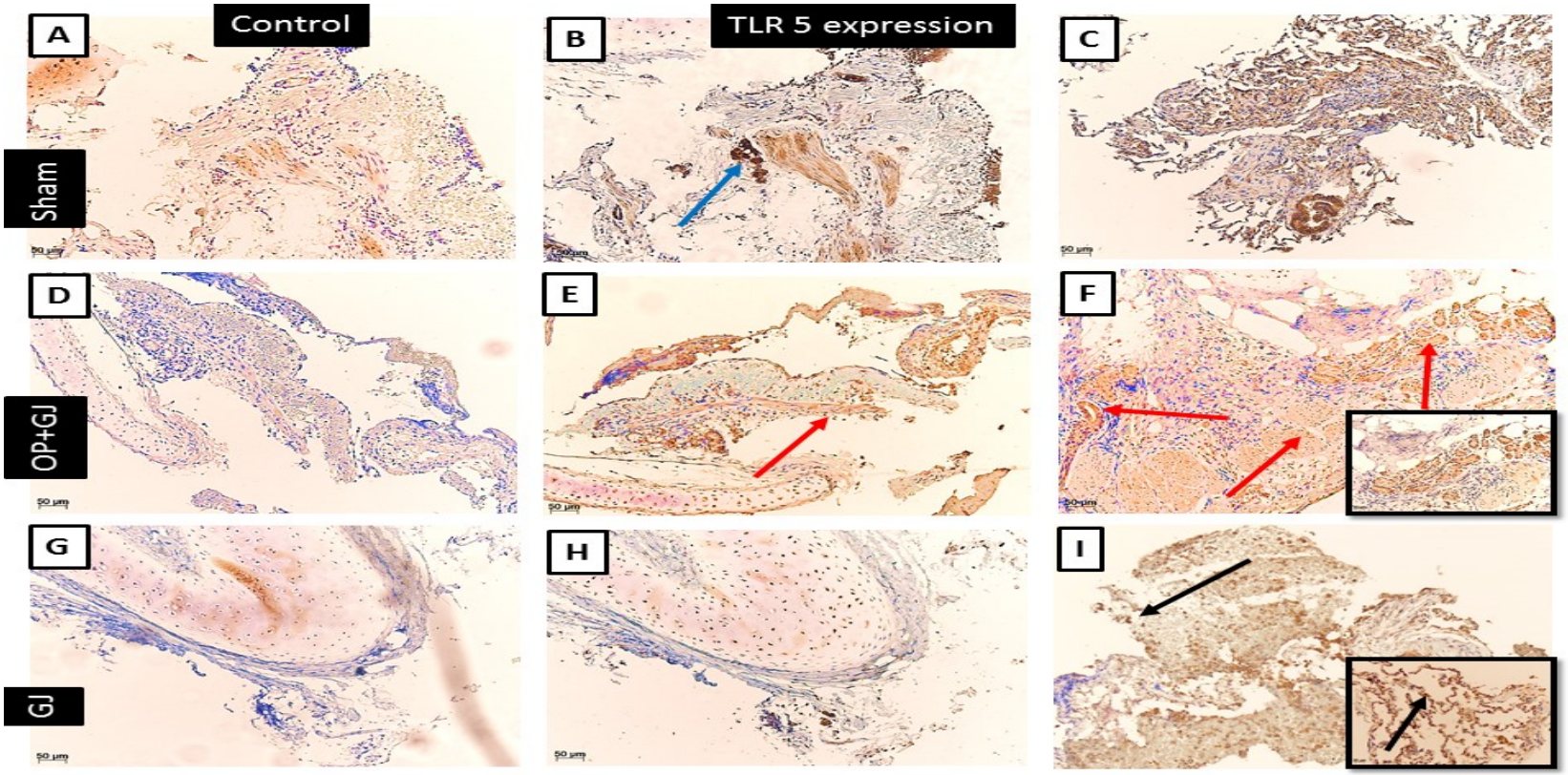


Figure 7.13: Toll like receptor 5 expression in minipig lungs exposed to treatments.TLR5 expression is shown with DAB (brown) staining. Other lung tissue is highlighted with haematoxylin (blue). Bronchial biopsy samples were taken from the right lung at 48 hours after sham bronchoscopy (n=2) (A, B and C) or directly-injured (right) lung installation of either 0.5 mL/kg OP (dimethoate EC40)+GJ (n=2) (D, E and F) or GJ alone (n=2) (G, H and I). Control negatives are represented from each group (A, D and G). Sham bronchoscopy minipig lung shows TLR 5 expression on the pseudostratified columnar epithelium, alveolar epithelial surface and strongly in some submucosal ducts (blue arrow). OP+GJ minipig lung tissue has increased expression in submucosal tracts (red arrow) and ducts (inset F) and smooth muscle (red arrows). Gastric juice exposed lung tissue shows TLR 5 expression on pseudostratified columnar epithelium and alveolar epithelium black arrows and inset I). Pictures were taken using Axiovision Rel 4.8 software and an Olympus AX70 Provis microscope with x10 magnification. Inset pictures in F and I show x20 magnification.

Examination of the lungs of minipigs that underwent sham bronchoscopy revealed TLR 3 staining on the bronchial pseudostratified columnar epithelium, occasional alveolar epithelial surface and some sub-mucosal ducts (blue arrow; Figure 7.12). This pattern was intensified with TLR 5 staining (Figure 7.13).

Minipig lung tissue damaged with OP+GJ also had pseudostratified columnar epithelial involvement, with both TLR 3 (Figure 7.12) and TLR 5 staining (Figure 7.13) -shown by the red arrows. Staining was more obvious in the sub-mucosal duct, tract, and smooth muscle. From the minipig samples tested it is difficult to comment on alveolar epithelial involvement. Minipigs treated with GJ showed some TLR 3 staining on the pseudostratified columnar epithelium and sporadically within the interstitium (Figure 7.12). TLR 5 staining showed a similar pattern to the TLR 3 in GJ treated pigs, but with clearer involvement of the alveolar epithelium (black arrows, Figure 7.13).

7.3.12 Immunostaining for toll like receptors 3 and 5 in human bronchoalveolar lavage fluid cells taken from pesticide poisoned patients

To explore pulmonary TLR 3 and 5 expression in human OP pesticide poisoning, BALF cells from two OP+ No ASP and one NON OP+ASP patients were analysed. Lung tissue samples were not available for testing.

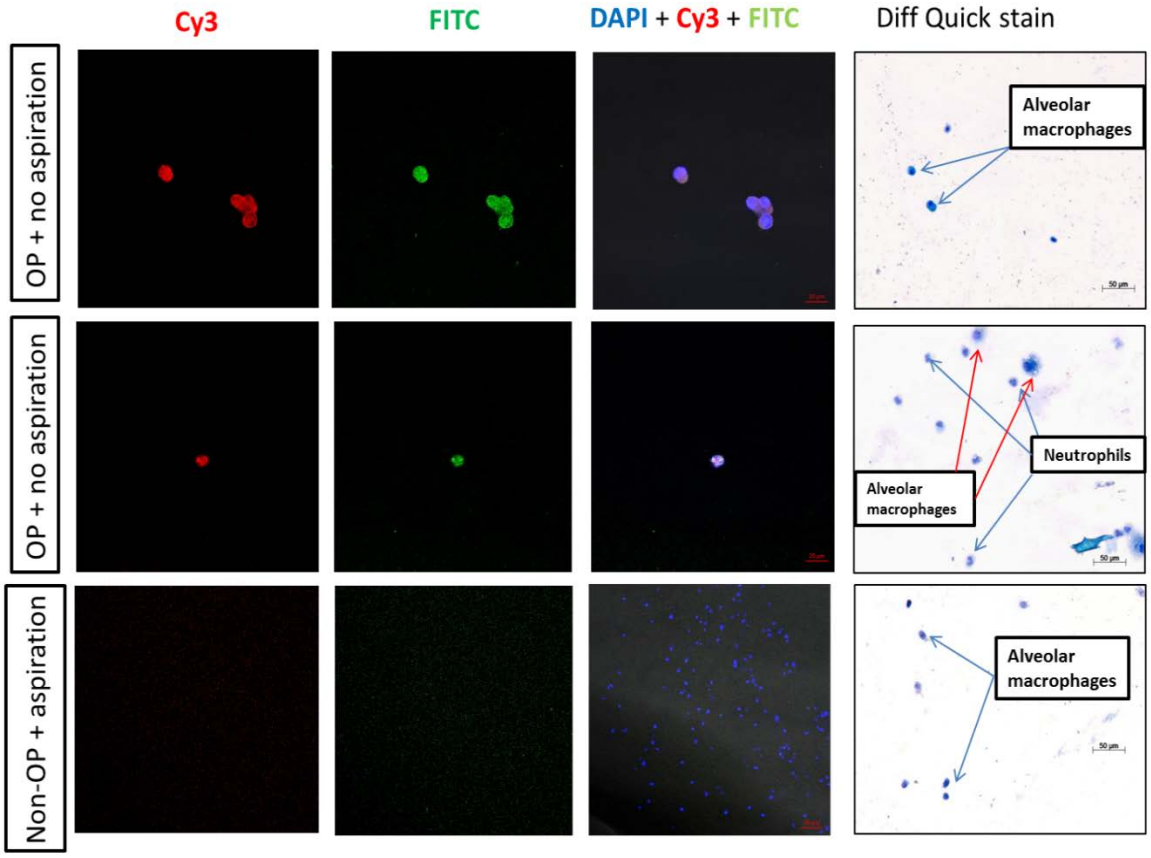


Figure 7.14: TLR 3 and 5 expression on representative BALF cells from one OP poisoned patient with no aspiration (OP +No ASP) and one non-OP pesticide poisoned patient with signs of aspiration (NON-OP+ASP) 24 hours after poisoning. Tyrimide Cy3 (red) shows TLR 3, Fluorescein (FITC) (green) for TLR 5 and 4',6-diamidino-2-phenylindole (DAPI-blue) for nuclear (DNA and RNA) material. Immunofluorescent images were taken at x40 magnification using an LS710 confocal microscope (Zeiss). The Diff Quick stain was performed on the same sample (but not the same slide) to allow identification of the cellular types observed within the BALF. Images of the Diff Quick stained cells were taken using Axiovision Rel 4.8 software and an Olympus AX70 Provis microscope at x20 magnification.

Cellular material from the OP+ No ASP patient showed both alveolar macrophages (top row) and possibly a neutrophil (middle row) with TLR 3 and 5 staining (Figure 7.14). The samples from the NON OP+ASP patient BALF cell Diff Quick stain showed mainly alveolar macrophages with the occasional neutrophil, but with no clear TLR 3 and 5 staining. The immunostaining of BALF cells from this NON OP+ASP patient (bottom row) seemed to show only DAPI stained cellular nuclear fragments, likely due to cellular degradation during the sample preparation of this slide (Figure 7.14).

The sample from the other OP+ No ASP patient had similar (although less marked) staining on presumed alveolar macrophages. White cell labelling for cellular identification (alveolar macrophage vs. neutrophil) was not conducted due to lack of sample and time constraints.

These exploratory findings suggest that alveolar macrophages, and possibly neutrophils, may express both TLR 3 and 5 in response to OP pesticide ingestion. However, there were no controls for comparison and the expression of these receptors in the BALF cells of non-OP pesticide poisoned patients was not clear due to the poor quality of the sample. Further experiments with larger group numbers (including controls) would be required to investigate this hypothesis further.

7.4 Discussion

The aim of this experiment was to develop an EVLP model that could provide an alternative, less expensive and staff intensive method of investigating pulmonary aspiration injuries of OP pesticide. This EVLP model was used for a pilot study to reproduce some of the minipig pulmonary aspiration study findings (chapter 5), and to explore other pulmonary pathophysiological mechanisms.

The EVLP model was moderately successful lasting ≥3 hours for all (n=4) sets of EVLP lungs. Yet, a lack of neutrophils in the perfusate and lung tissue may mean that the study of neutrophil driven lung injuries, such as OP+GJ aspiration, may not be appropriate using this model.

Despite this limitation, the direct lung instillation of OP+GJ in the EVLP model inhibited red cell AChE in the perfusate and increased BALF concentrations of protein (when compared with other groups) combined with EM images showing degraded, swollen alveolar capillary membranes with fibrin deposition- similar to OP+GJ lungs from the minipig study.

The EVLP model, combined with additional studies using minipig lung tissue and human BALF, identified the potential involvement of pulmonary toll like receptors (TLR) 3 and 5 in lung injury secondary to OP+GJ aspiration. This finding would need to be confirmed in further animal and human studies with appropriate controls, but could prove informative and is not currently described in the literature.

Pulmonary installation of OP+GJ produces a lung injury in the EVLP model

Histopathology

Despite the control lungs becoming damaged during the EVLP process, representative histopathology and EM images showed that they were less injured than the OP+GJ and Solv+GJ exposed lungs. OP+GJ and Solv+GJ directly-injured lungs displayed alveolar capillary membrane swelling, vascular engorgement, alveolar epithelial cell sloughing, with the OP+GJ lungs showing additional fibrin and hyaline deposition within the alveoli (Figure 7.8; Figure 7.10). The histopathology scores did not reveal differences between the groups as the system was neutrophil

dependant (discussed below). The heparin dose within the perfusate may also have had an anti-inflammatory effect and reduced fibrin deposition within the lungs (Dixon et al., 2010).

BALF protein

The concentration of BALF protein was greatest in OP+GJ directly-injured lungs at both 3 and 6 hours compared with most controls and Solv+GJ treated lungs (Figure 7.5). This combined with the histopathological changes may indicate a breakdown in the alveolar capillary membrane allowing a direct influx of protein-rich fluid into the alveolar space, consistent with lung injury (Ware and Matthay, 2000). Unexpectedly, the albumin was of such a low concentration that it could not be measured in the BALF. This may have resulted from whole blood dilution in the perfusate. The problem of blood contaminants spuriously increasing BALF protein concentrations (discussed previously) seems unavoidable with OP+GJ aspiration because it produced bloody BALF samples in both minipig and EVLP models.

Indirect lung injury

There was limited evidence that OP+GJ instilled into one lung of the EVLP model produced a concomitant indirect lung injury. The concentration of BALF protein and the surfactant PC species composition in OP+GJ indirectly-injured (left) lungs were similar to those found in the control lungs.

Yet, the OP+GJ indirectly-injured lung had increased expression of TLR 3 and 5, greater than that in the directly-injured lung. However, the low expression (of TLR 3 and 5) in the directly injured lung may have resulted from the poor quality of lung tissue in this experiment, and may in fact have been higher. Nevertheless, these were single data points and were therefore hypothesis generating data rather than conclusive evidence of lung injury.

BALF neutrophil numbers

The low BALF neutrophil numbers combined with the low histopathology scores did not reflect the findings of the *in vivo* minipig study which was typified by a marked pulmonary neutrophilia in the

BALF, alveoli and interstitium. This could have implications for the study of OP+GJ aspiration in an EVLP model, and deserves further investigation.

The observed lack of pulmonary neutrophils in the EVLP model BALF samples could have been due to:

i. Pulmonary interstitial sequestration. This has been encountered in EVLP models using whole blood (Pearse et al., 1989, Pearse and Sylvester, 1992). However, the histopathological examination of lung tissue in the current study did not show convincing pulmonary interstitial neutrophil sequestration.
ii. the marginal pool of neutrophils (contained within the liver, spleen and bone marrow) at death was larger than the circulating pool of neutrophils (Summers et al., 2010).
iii. a lack of a marginal pool of neutrophils in an EVLP model, meaning that neutrophil chemotaxis to the lung could not occur.
iv. poor bronchoalveolar lavage sampling technique of the distal *ex vivo* lung. This seems unlikely as the operator (myself) and method (BAL technique used in the minipig study) were unchanged.
v. a lack of alveolar capillary injury. However, the BALF protein concentration, histology and presence of blood suggests damage to the alveolar capillary membrane.
vi. dilution of the neutrophils in the perfusate and/or destruction within the EVLP circuit. (Pearse and Sylvester, 1992).
vii. the high dose of unfractionated heparin (20,000u) used in the perfusate may have altered neutrophil function and hindered migration to the damaged lung parenchyma (Brown et al., 2003).

Pulmonary installation of OP+GJ could involve stimulation of toll like receptors 3 and 5

Toll like receptors are pattern recognition receptors that react to infectious or non-infectious stimuli in order to mount an inflammatory response as part of the innate immune system. Their stimulation leads to cytokine and chemokine release with movement of white cells and antigen presenting cells to the site of inflammation (Lafferty et al., 2010).

TLRs have been implicated in lung injury. Lung endothelial cells express TLR 4 and are thought to be involved in neutrophil recruitment and capillary sequestration following LPS administration in a lung injury model. Indeed deletion of TLR 2 or 4 in murine models conferred protection from ALI (Lafferty et al., 2010).

EVLP study findings

The EVLP lung tissue from OP+GJ exposed lungs showed relatively low expression for TLR 2 and 4 when compared with controls. However, the OP+GJ indirectly-injured (left) lung exhibited increased expression of TLR3 (44 fold) and TLR 5 (47 fold) when compared with control and Solv+GJ lungs. Unexpectedly, the directly-injured OP+GJ (right) lung showed less TLR 3 and 5 expression when compared with indirectly-injured OP+GJ lung.

The OP+GJ directly-injured (right) lung did have a lower: RIN, 260/230nm absorbance ratio (indicating contaminants) and quantity of RNA in comparison to other samples indicating its poor quality (Table 7.5). These deficiencies could have been caused by OP-induced tissue damage, leading to irregular, and inaccurate RT-qPCR results for TLRs 1-10 (Fleige and Pfaffl, 2006).

The extent to which TLR signalling in the *ex vivo* model was affected by ischaemia-reperfusion injury within the lung is unknown. However Solv+GJ treated lungs underwent the same handling during EVLP and did not show the same elevation in TLR 3 and 5 indicating that these changes were caused by the OP+GJ pulmonary exposure. Moreover, murine models show that TLR 4 up regulation has a pivotal role in the ischaemia-reperfusion injury and oedema in the lung (Zanotti et al., 2009). TLR 4 was not overly expressed in any of the EVLP lungs (Figure 7.10).

Minipig aspiration study findings

To determine whether the observed TLR expression pattern was an effect of OP+GJ aspiration, rather than the result of EVLP lung damage, TLR 3 and 5 expression was also examined in lung tissue from the minipig pulmonary aspiration study. A small sample of minipig *in vivo* bronchial biopsies taken at 48 hours from control (n=2), GJ (n=2) and OP+GJ (n=2) directly-injured (right) lungs were analysed. Funds were unavailable to test other TLRs (a future area of study). Common

areas of staining included the pseudostratified columnar epithelium and alveolar epithelium (Sham and GJ lungs). OP+GJ exposed lung tissue had a particular staining pattern focusing on the submucosal glands, associated tracts and smooth muscle within the bronchial wall.

These areas (glands, ducts and bronchial smooth muscle) are thought to be overstimulated by excess ACh acting on cholinergic receptors during OP poisoning, giving rise to the clinical signs of bronchorrhoea and bronchospasm (Eddleston et al., 2008). Thus the sites of pulmonary cholinergic overstimulation and TLR 3 and 5 increased expression in OP+GJ directly injured lungs might be co-located and somehow related, and should be further investigated.

Human pesticide poisoning study findings

Human BALF cells from an OP poisoned patient with no signs of aspiration showed that alveolar macrophages and neutrophils could express TLR 3 and 5. However, these were absent in the non-OP poisoned patient BALF cells. Unfortunately, white cell labelling and formal identification of the BALF cells was not carried out. Such a finding would require larger numbers of OP poisoned patients for confirmation.

The lack of cellular staining in the lungs of the non-OP poisoned patient could have resulted from the poor quality of the cellular sample. Often BALF samples were taken late at night, placed on the slide and transported by tuk tuk (local Sri Lankan transport) or car to a place where I could dry and methanol fix the slides. This process sometimes took 30-60 min from sampling. This time delay could have degraded the quality of some BALF cellular material. Future studies require a larger patient group with improved sample processing.

The role of TLR 3 in lung injury

TLR 3 is a predominantly intracellular receptor known to be expressed in alveolar macrophages (Kuzemtseva et al., 2014), bronchial epithelial cells, mature dendritic cells, NKT cells and human smooth muscle cells (HSMCs) (Kvarnhammar et al., 2013, Sha et al., 2004). It is triggered by double stranded RNA (DsRNA) (commonly associated with viruses) and the synthetic analogue, Poly I:C

(used for research purposes), leading to production of cytokines e.g. IL-6, IL-8 (Sha et al., 2004, Kvarnhammar et al., 2013).

The minipigs in the experiments described were purchased from their producer in Denmark (Ellegaard), where they were reared in a barrier facility monitored for microbiological activity within the animals. Consequently, they had probably not been exposed to either DsRNA or poly I:C. Similarly, OP pesticide poisoned patients were not knowingly exposed to poly I:C, or suffering from an obvious viral infection, so the TLR3 may have been stimulated by another chemical signal involved with OP+GJ aspiration, including the OP itself?

In response to influenza infection mutant TLR3-/- mice have a decreased inflammatory response, with an increased viral load and greater survival (Le Goffic et al., 2006). In response to respiratory syncytial virus (RSV) TLR3-/- mice also have increased mucous production (Rudd et al., 2006). They are also protected from ALI secondary to decreased neutrophil recruitment, reduced triggering of cellular apoptosis, less lung remodelling and an enhanced surfactant protein expression after a hyperoxic lung injury (Murray et al., 2008).

Thus TLR 3 stimulation in the lung may be harmful. Indeed, increased expression has been found in lung tissue taken from ARDS patients when compared with controls (Murray et al., 2008). Further research should address TLR 3's involvement with OP ingestion and aspiration as it may be a target for future therapies e.g. anti-TLR3 antibodies.

The role of TLR 5 in lung injury

TLR 5 is an extracellular receptor activated by bacteria possessing flagellin e.g. *Pseudomonas* spp. TLR 5 stimulation mobilises NF-kB and promotes TNF-α production (Hayashi et al., 2001). It is present on human; bronchial epithelial cells, mucosal glands, type 2 alveolar epithelial cells, alveolar macrophages, plasma cells and neutrophils (Shikhagaie et al., 2014). Controversy surrounds its presence on HSMCs (Kvarnhammar et al., 2013, Sha et al., 2004, Shikhagaie et al., 2014). *In vitro* experiments using porcine tracheal mucosal have shown that flagellae-stimulated TLR 5, under ACh control, potentiate secretions from sub-mucosal glands (Muramatsu et al., 2013).

The GJ used in the EVLP and minipig aspiration experiments contained dead flagellae (according to the veterinary microbiologist) so these may have stimulated TLR5 expression in the models. However, points against this include: (i) OP+GJ indirectly-injured left lung (i.e. no apparent GJ exposure) had the greatest rates of TLR 5 expression compared with the directly-injured lung, (ii) the Solv+GJ directly-injured lung had relatively low expression of TLR 5 in comparison to OP+GJ lungs during the EVLP experiment.

It was unlikely that pulmonary TLR 5 stimulation in the EVLP model resulted from another source of bacterium, but the possibility cannot be ruled out. However, whilst the OP+GJ treated minipig lung samples had bacteria present at 48 hours post aspiration, the predominant organisms did not possess flagellae, meaning that another molecule or substance (e.g. OP) may have triggered the increase in pulmonary TLR 5 expression in OP pesticide poisoning and aspiration.

EVLP Model analysis

Optimisation of excised lungs

The first two EVLP experiments (control S1 and S2) only lasted 3 hours due to difficulty in ventilating the oedematous lungs. The lungs showed evidence of poor oxygenation and reduced lung compliance, increased PVR and metabolic derangement e.g. increased lactate (Figure 7.4).

Proposed reasons for the observed lung injury in the controls, which developed to some degree in all EVLP lungs, included: method of animal death (catecholamine release causing neurogenic pulmonary oedema); increased warm ischaemia time; and use of whole rather than leuco-depleted blood in the perfusate (Sanchez et al., 2012, Pearse et al., 1989, Avlonitis et al., 2005, Erasmus et al., 2006).

Attempts to improve the initial physiological state of subsequent EVLP lungs (S3 and S4) involved flushing the lungs with DMEM (removing toxins and clots, providing nutrients and cooling lung tissue), speedier lung retrieval (to reduce warm ischaemia time) and early placement on the by-pass machine (Hicks et al., 2006).

Optimisation of perfusate

An EVLP perfusate needs to have sufficient oncotic pressure to allow fluid to move from the lung interstitium to the perfusate in order to avoid lung oedema formation. Those who employ EVLP for organ preservation use a variety of acellular perfusate solutions that often contain albumin (oncotic pressure component) and/or dextrose to protect the endothelium and inhibit coagulation (Van Raemdonck et al., 2015).

The minipig aspiration studies showed that OP+GJ produced a significant pulmonary neutrophilia as part of the observed lung injury. Therefore, it was important that our EVLP model perfusate used autologous whole blood to provide an oncotic component in addition to neutrophils.

Whole blood, which is used in human cardiopulmonary bypass surgery, is known to cause post-operative pulmonary dysfunction with a systemic and pulmonary inflammatory response. A contributing mechanism is thought to be blood exposed to foreign surfaces (e.g. tubing) causing activation of the complement cascade and white cells, with eventual white cell and platelet aggregation in the lung (Wynne and Botti, 2004).

Previous studies have shown that use of ovine whole blood in an EVLP model results in pulmonary oedema, haemorrhage and hypertension (Pearse et al., 1989, Pearse and Sylvester, 1992). These findings were preceded by pulmonary sequestration of leucocytes and platelets. However, this was an early ovine model which used homologous blood and did not describe whether this was cross matched for blood type. The results from these studies may also be due in part to ischaemia – reperfusion injury (Zanotti et al., 2009) and high perfusate flow rates, in the order of 2.5L/min, factors which could have contributed to lung injury in this model.

Porcine EVLP models using autologous leucocyte and platelet depleted blood are associated with less lung damage and apparently allow the retrieval of neutrophils from the BALF (Erasmus et al., 2006, Khalifé-Hocquemiller et al., 2014). Human EVLP organ preservation work can also use acellular perfusate, partly substituted with (10-25%) packed red blood cells (Van Raemdonck et al.,

2015). In this scenario they deliberately have filters and membranes in the circuit to remove harmful leucocytes, inflammatory cytokines and platelets that would cause tissue and endothelial damage.

In conclusion, the use of whole blood in our EVLP model may have contributed to the observed lung injury (oedema and haemorrhage) in all lungs secondary to the presence of leucocytes, platelets and other inflammatory molecules. Although we used all the autologous blood from the animal within the circuit, the oncotic pressure may still have been too low, causing movement of fluid from the perfusate into the lung.

Lung metabolism during EVLP

The lungs are dependent on aerobic, rather than anaerobic metabolism, with a high density of mitochondria in the type 2 alveolar epithelial cells (Fisher et al., 1974). In the current study, there was a concern that the high lactate levels in our EVLP model were contributing to the observed lung oedema and injury.

Lactate production from glucose in lungs under EVLP conditions are known to be high (Fisher et al., 1974, Sanchez et al., 2012), not only due to production from glucose metabolism, but also due to a lack of the normal removal mechanisms i.e. liver, kidney and muscle (Koike et al., 2011). The lung is capable of high lactate clearance as it has a high concentration of lactate dehydrogenase. Furthermore, lactate is readily secreted by the large surface area of the lung into circulation for removal (Sanchez et al., 2012). A rat EVLP model has shown that lactate is not only produced in the lung, but also used as substrate for lung lipid synthesis (Rhoades et al., 1978).

Although high levels of lactate could foreseeably inhibit glycolysis and cause further pulmonary damage, this has apparently not harmed EVLP lungs with acellular perfusate (lactate up to 12mmol/L) that have successfully been used for human transplant (Koike et al., 2011). Above this concentration there may be requirement for further perfusate exchanges or removal, though this is currently unknown.

Optimisation of pulmonary vascular resistance and reduction of pulmonary oedema

Acidic and cold conditions, inappropriate ventilation strategies causing stretching or deflation of the alveolar walls and surrounding capillaries, microthrombi, hypoxic vasoconstriction and negative left atrial pressures can all cause pulmonary vasculature constriction with increases in PVR within EVLP models. This rise in PVR slows the pulmonary blood flow, and can lead to repetitive collapse and reopening of the pulmonary vasculature causing endothelial damage and pulmonary oedema (Cypel et al., 2008, Sanchez et al., 2012).

The PVR in our EVLP experiments was greater (sometimes 3 x) than that found in similar successful EVLP studies (Figure 7.2) (Cypel et al., 2008). This occurred despite a careful 'warm up' period, pH normalisation and protective ventilation in an attempt to reduce PVR. Yet, the perfusate flow rates for the control lungs (S1 and S2) were below the optimum 1L /min (Figure 7.4A) and could therefore have contributed to increased PVR.

The left atrium was not splinted and the height of the reservoir was not altered as described by Cypel *et al* to create a small positive venous afterload pressure (Cypel et al., 2008). Although not all EVLP models have left atrial splinting, its omission could theoretically have caused higher PVR in our model due to microcirculation collapse during ventilation, worsening pulmonary oedema (Sanchez et al., 2012).

Future EVLP experiments

Further experiments exploring OP+GJ aspiration in the EVLP model should consider using leucocyte and platelet depleted autologous blood. This must be combined with acellular perfusate in order to maintain a sufficiently high oncotic pressure to avoid lung oedema formation. Other improvements e.g. mode of animal death – VF arrest rather than exsanguination, reduced lung retrieval times, lung flushing with cooled tissue preservation fluid, and splinting of the left atrium on the by-pass machine should also be considered.

Conclusion

This chapter described the creation and development of an EVLP model capable of maintaining ovine lungs for periods of 3-6 hours.

The EVLP pilot study findings regarding the lung injury created by pulmonary installation of OP+GJ and Solv+GJ had some similarities to the minipig pulmonary aspiration study. Further work was limited by a lack of funds for additional tests combined with flaws in the pilot model design.

Pulmonary installation of OP+GJ into a lung produces a local neutrophilia with evidence of an indirect lung injury as observed in both the minipig aspiration and human pesticide poisoning studies. The apparent lack of neutrophils and evidence of indirect lung injury in our EVLP model may therefore make it unsuitable for further investigation of OP-induced pulmonary aspiration injuries. However, repeating this experiment with an acellular perfusate combined with leucocyte and platelet depleted blood may improve model validity.

Future research investigating the pulmonary TLR expression after OP+GJ aspiration would further the understanding of the immuno-pathophysiological pathways involved in this toxic lung injury.

Chapter 8: Discussion

8.1 Discussion

The overarching hypothesis directing this work was that ingested and aspirated OP pesticide - with its solvent- could create a toxic lung injury capable of contributing to the increased mortality observed in human intubated OP poisoned patients. This thesis examined some of the pulmonary pathophysiological mechanisms and lung injuries created by orogastric and/or pulmonary installation of OP pesticide in the Gottingen minipig and *ex vivo* lung perfusion models. These data were augmented by OP poisoned patient data from Sri Lanka.

The question concerning whether this toxic lung injury is severe enough to increase mortality in intubated OP poisoned patients remains to be answered, but some theories are discussed below.

Pulmonary pathophysiology of ingested OP Pesticide and indirect lung injury

The literature contains few studies detailing the pulmonary effects of ingested, or orogastric administration of OP pesticide. Animal studies describe the breakdown of the alveolar-capillary membrane, with alveolar and interstitial oedema, destruction and intra-parenchymal haemorrhage in response to orogastric placement or ingestion of OP pesticide (He et al., 2012a, Yavuz et al., 2008, Uzun et al., 2010, Amara et al., 2012). Pulmonary interstitial oedema and parenchymal haemorrhage are also features of fatal human OP pesticide self-poisoning through ingestion (Kamat et al., 1989).

The minipigs poisoned with orogastric OP pesticide (indirect lung injury) in study 1 (chapter 3) had increased concentrations of BALF protein, IL-6 and IL-8 when compared with controls, with low plasma concentrations of IL-6 and IL-8 at 12 hours. The histopathology scoring of the OP pigs was significantly greater ($p=0.023$) than the control pigs due to the presence of alveolar and interstitial oedema and neutrophil infiltration. Despite the lack of controls in study 2, the OP poisoned minipigs also showed increased concentrations of BALF protein, neutrophils, IL-8 and CRP six hours after poisoning when compared with their baseline values. Electron microscopy images demonstrated damage to the alveolar capillary membrane secondary to systemic OP poisoning in study 2, and to a lesser extent in study 1.

These findings suggest that there was local pulmonary, rather than systemic, inflammation characterised by protein leakage and neutrophil migration from the endothelium to the alveolar space, possibly resulting from breaks in the alveolar–capillary membrane.

Pulmonary pathophysiology of aspirated OP pesticide and gastric juice

During preparation for the literature review (chapter 1), I found no studies exploring the effects of pulmonary aspiration of mixtures of OP pesticide with gastric contents. Consequently, the originality of the work described in this thesis contributes to the body of knowledge regarding OP poisoning.

The studies described in this thesis demonstrated that OP and gastric juice (GJ) placed into one lung created a direct and indirect lung injury significantly different from controls, and in some respects greater than GJ alone 48 hours after poisoning in a minipig model. The direct lung injury caused by OP+GJ was characterised by significantly greater pathology ($p=0.0003$) as evidenced by pulmonary neutrophilia, alveolar haemorrhage, necrosis, oedema and fibrin deposition when compared with sham controls at 48 hours. Lungs directly-injured with OP+GJ also had significantly greater concentrations of BALF neutrophils ($p\leq0.01$), protein ($p\leq0.05$), IL-6 ($p\leq0.01$), IL-8 ($p\leq0.01$) and CRP ($p\leq0.05$) at 24 hours, and BALF protein ($p\leq 0.01$), and CRP ($p\leq 0.05$) when compared with sham controls at 48 hours. The BALF from OP+GJ treated minipigs at 48 hours also had greater numbers of aerobic bacteria than other groups, suggesting the development of pneumonia could be a source of additional lung injury.

Lung damage might also have occurred due to a reduction in the surfactant component responsible for the lowering of alveolar surface tension. Direct lung injury caused by OP+GJ was associated with a proportional reduction in the beneficial pulmonary surfactant phosphatidylcholine (PC) species 16:0/16:0 [29(±4) % vs. 38(±4) %] when compared with sham controls. Unlike the other groups, lungs directly and indirectly injured with OP+GJ seemed to have type 2 alveolar cell ultrastructural morphological differences in the lamellar bodies that store the surfactant. The lamellar bodies appeared more numerous and dense when compared with other groups and could signify a failure of surfactant release or some other pathology pertinent to OP aspiration lung injury.

Computed tomography analysis showed that direct lung injury with OP+GJ caused significantly more lung tissue to become poorly or non-aerated by 47.5 hours: 77 (±13) %; $p\leq0.0001$ when

compared with sham. The percentage of poorly and non-aerated lung tissue was 62 (±27) % in GJ treated animals, compared with 53(±13) % in sham and 47(±0.2) % in saline control animals by 47.5 hours. Lung volume analysis (appendix A) and histopathology suggest that the loss of aeration in the OP+GJ directly injured lungs was largely due to accumulation of pulmonary haemorrhage and oedema fluid in the alveoli as opposed to collapse of the airways.

Although the main minipig pulmonary aspiration study was underpowered to observe statistical changes between GJ and OP+GJ treated animals, there were some key differences. Most inflammatory markers e.g. BALF concentrations of protein, IL-6 and CRP, increased from 24-48 hours in OP+GJ, but fell in GJ treated pigs- possibly signifying resolution in the lungs of animals exposed to GJ alone. GJ treated pigs also had lower histopathology scores, bacterial BALF numbers and percentage of poorly and non-aerated lung tissue when compared with OP+GJ treated lungs at 48 hours. Importantly, there was also limited evidence of indirect lung injury within the GJ treated pigs when compared with OP+GJ treated pigs.

The EVLP model echoed findings from the minipig model. Six hours after poisoning there was an increased concentration of total protein (4300 mg/L vs. 350 mg/L) with a reduction in the beneficial PC species 16:0/16:0 (27% vs. 34%) in the BALF of ovine lungs directly injured with OP+GJ when compared with control values (taken at 3 hours). *Ex vivo* lungs treated with OP+GJ also produced upregulation of pulmonary toll like receptors 3 and 5 (TLR 3 and 5) in the lung parenchyma six hours after poisoning. This is unlike other acute lung injuries e.g. in response to lipopolysaccharide, which typically stimulates TLR 2 and 4 (Lafferty et al., 2010).
An indirect lung injury could not be reliably demonstrated within the EVLP model. It is likely that an *in vivo* model is required in order to observe OP-induced indirect lung injuries.

The evidence from human OP poisoned patients supported the notion that OP ingestion and aspiration caused a direct and indirect lung injury. This was demonstrated by increased concentrations of BALF protein in both OP+No ASP and OP+ASP patients when compared with controls. Although not significant (because of low patient numbers), this was also accompanied by

similarly raised concentrations of BALF IL-6, IL-8, and CRP in the OP+ASP and OP+ No ASP group when compared with controls.

The human plasma miRNA results also provided information regarding the pathophysiology of OP pesticide and gastric contents aspiration. MiR-21 and Mir-146a (increased expression) are involved with the process of inflammation. In its anti-inflammatory role MiR-21 positively regulates the production of the anti-inflammatory cytokine IL-10 (O'Neill et al., 2011) and can also negatively regulate the production TNF (TNF also induces miR-21)(Sheedy, 2015). Mir-146a negatively regulates the inflammatory process from a variety of cell types within the lung e.g. smooth muscle cells, fibroblasts, epithelial cells and alveolar macrophages (Comer et al., 2014, Zeng et al., 2013). That Sri Lankan OP poisoned patients displayed significantly decreased miR-21 and miR-146a when compared with UK controls with correspondingly negligible BALF and plasma IL-10 concentrations warrants explanation. It implies a dampening or altering of part of the host's anti-inflammatory mechanisms which may be involved in human OP pesticide poisoning. However, although it contradicts the evidence that cholinergic stimulation e.g. OP poisoning, produces a broadly anti-inflammatory response (Tracey, 2007), it confirms the complexity of the immuno-pathological mechanisms involved in OP pesticide poisoning and aspiration.

Ingestion and aspiration of OP pesticide create a unique lung injury

ARDS is characterised by a non-cardiogenic pulmonary oedema with breakdown of the alveolar-capillary membrane, poor oxygenation with activation of the inflammatory and coagulation pathways (Ranieri et al., 2012, Chen and Ware, 2015).The evidence from the minipig and human studies suggests that although ingested and aspirated OP pesticide displays some of the inflammatory hall marks of ARDS e.g. a pulmonary neutrophilia with increased concentrations of BALF IL-6, IL-8 and CRP, there were subtle differences.

TNF-α and IL-1β concentrations peak early in the development of ARDS, leading the process of inflammation and eventual damage of the alveolar capillary membrane (Mukhopadhyay et al., 2006, Agouridakis et al., 2002, Parsons et al., 1992, Bhargava and Wendt, 2012).

IL-10 is an anti-inflammatory cytokine, whose concentration also peaks early in ARDS development. Excessive production, secondary to a genetic polymorphism in humans, actually decreases mortality during ARDS (Gong et al., 2006).

Unlike classic ARDS, BALF concentrations of IL-10 were un-recordable, while TNF-α concentrations were mainly <10pcg/mL (apart from one reading at 86 pcg /ml) in OP poisoned minipigs (n=4) observed over 12 hours (study 1). Plasma concentrations of TNF-α were 67.5 (±53) pcg/mL in OP poisoned minipigs vs. 81.9(±72) pcg/mL in saline treated pigs. Plasma IL-10 was un-measureable in the same OP poisoned minipigs.

Sri Lankan data indicated that OP pesticide poisoned patients had reduced concentrations of BALF and plasma TNF, IL-1β and IL-10 from admission until 48 hours after poisoning when compared with comparable mild ARDS cases from other causes e.g. trauma and sepsis (Table 8.1).

Cytokine	OP poisoned patients BALF (pcg / mL) (n=6)	Mild ARDS BALF (pcg / mL) – mean	OP poisoned patients plasma (pcg / mL) (n=16)	Mild ARDS plasma (pcg / mL) – mean
TNF-**α/TNF**	4.8 (±5.7)	210 (median)-376	1.75 (±3.3)	16 -383 (median)
IL-**1β**	198 (±238)	36, 445	1.1 (±2.1)	8
IL-10	3.2 (±6.0)	111	4.4 (±5.9)	177

Table 8.1: Selective cytokine profiles of OP poisoned patients (OP+ASP and OP+No ASP combined) compared with those with mild ARDS from other causes. BALF (n=6) and plasma (n=16) cytokine levels of TNF, IL-**1β and IL**-10 in all human OP pesticide poisoned patients (OP+ASP and OP+ No ASP combined) from Sri Lanka (samples taken between 0-48 hours after poisoning) compared with patients who had mild ARDS (all causes- samples taken on admission to ICU). Mean (SD) is shown for OP poisoned patients (pcg / mL), mean or medians shown for ARDS data taken from the literature (Agouridakis et al., 2002, Suter et al., 1992, Bouros et al., 2004). The studies quoted measured the cytokines in the following number of patients with mild ARDS: Agouridakis (n=16), Suter (n=7-12), Bouros (n=12).

One explanation for the reduced concentrations of TNF-α, IL-1β and IL-10 in BALF and plasma could be that the direct and indirect lung injuries resulting from OP poisoning were not particularly severe. Radiographic evidence of bilateral infiltrates (required to diagnose ARDS) was often

unavailable in Sri Lankan hospitals as doctors did not perform regular CXR's on the toxicology ICU. However, it is known from human OP poisoning data that at least four patients had PaO_2/F_IO_2 ratios in keeping with mild-moderate ARDS at 48 hours. These patients also had raised concentrations of BALF IL-6, IL-8 and CRP (between 24-48 hours) compared with Sri Lankan controls, indicating pulmonary inflammation.

An alternative explanation for the reduced BALF and plasma TNF-α, IL-10 and IL-1β, combined with raised IL-6, IL-8 and CRP in OP poisoning compared with mild ARDS could be secondary to the disorganisation, or alteration, of the cholinergic anti-inflammatory pathway. Excessive cholinergic stimulation of the nicotinic α7nACh receptor on alveolar macrophages and neutrophils, can reduce the secretion of TNF-α, IL-10 and IL-1β (Zabrodskii et al., 2013, Giebelen et al., 2009, Tracey, 2007). As discussed above, IL-10 production might also have decreased in response to reduced lung miR-21 expression (O'Neill et al., 2011).

Reduced median BALF concentrations of IL-lβ (10pcg/mL), TNF-α (13 pcg/mL) and IL-10 (5pcg/mL) have been observed in patients on day 1 of their ARDS diagnosis (Park et al., 2001). However, the single centre study did not detail the severity of patient ARDS and the BALF markers were measured in low numbers (n=10-28). The patients included in the study mainly had a diagnosis of trauma or sepsis (75%) and may therefore not be a fair comparison for the minipig and human OP poisoning data.

Cytokine expression profiles, TLR receptor stimulation and surfactant profiles may also differ between OP pesticide poisoning, ARDS and normal gastric contents pulmonary aspiration (Table 8.2).

Evidence of lung injury	ARDS (sepsis, trauma CVS shock)	Pulmonary aspiration of gastric contents	OP pesticide poisoning and gastric juice aspiration
Inflammatory mediators in BALF	▲ IL-6, IL-8, and TNF-α, IL-1β, CRP ▲▼ IL-10 (Bhargava and Wendt, 2012)	▲ IL-8 (Folkesson et al., 1995)▲ TNF alpha ++(Goldman et al., 1990, Nader et al., 2007) ▲ IL-10, CRP (Raghavendran et al., 2011)	▲ IL-6, IL-8, CRP, ▼ IL-10, TNF-α, IL-1B
Plasma markers of type 1 alveolar epithelial injury	▲ RAGE(Ware et al., 2013)	▲ RAGE (Jabaudon et al., 2015, Uchida et al., 2006) NB: RAGE was reduced in comparison to controls in minipig aspiration study.	▼ RAGE Although the results were not significant, RAGE concentrations were reduced when compared with controls at 24 hours in the minipig model.
Pulmonary TLR receptors involved	TLR 2 and 4 TLR 3 (influenza) and TLR 5 (bacterial pneumonia)(Lafferty et al., 2010)	TLR 4 (sterile lung injury – acid, in murine model) (Imai et al., 2008)	TLR 3 and 5 (ovine lung tissue exposed to OP+GJ in EVLP model)
Percentage of lung voxels in the CT lung at 47.5 hours that were poorly and non-aerated (as assessed in porcine model chapter 5)	N/A	62(±27)%	77(±13)% (secondary to increased pulmonary oedema and haemorrhage as seen by histopathology)
Percentage of total surfactant PC species containing beneficial PC species (16:0/16:0 minipig studies)	33% 3-5 days after diagnosis of ARDS in humans (Schmidt et al., 2007)	43% direct lung injury (48 hours)	Direct lung injury 29(±3.6)%, indirect lung injury 34(±1.7)% (48 hours)

Table 8.2: Differences between lung injuries produced by mild ARDS, pulmonary aspiration of gastric contents or OP pesticide poisoning and aspiration of gastric juice. Data have been included from previous minipig studies, EVLP model and human OP pesticide poisoned patients from Sri Lanka with a comparative literature search. OP: organophosphorus, TLR: toll like receptor, CT: computed tomography, PC: phosphatidylcholine, EVLP: ex vivo lung perfusion, RAGE: receptor for advanced glycated end-products, IL: interleukin, TNF-**α:** tumour necrosis factor, CRP: C - reactive protein.

Data from the minipig, EVLP and human work combined suggest OP pesticide poisoning and aspiration may create a type of lung injury which differs from normal gastric aspiration or classic ARDS. The mechanisms of this must be further investigated (Table 8.2).

Is solvent contributing to the observed lung injury in OP pesticide poisoning?

Solvent is synergistically toxic when combined with the OP active ingredient, but less so when administered by gavage alone (Eddleston et al., 2012). The solvent found in dimethoate EC40 - cyclohexanone- is a recognised respiratory irritant (Burcham, 2013). It was therefore surprising that the pulmonary installation of cyclohexanone seemed to offer some form of protection from the effects of aspiration of GJ. This was demonstrated by the histopathology scoring, the percentage of beneficial PC species (16:0/16:0) and the percentage of poorly and non-aerated lung tissue all approaching control animal levels by 48 hours in minipigs that had Solv+GJ placed in the directly-injured lung (chapter 5). Further evidence of benefit was provided by statistically significant reductions ($p \leq 0.05$) in BALF concentrations of IL-8, IL-6 and CRP in minipigs that had aspirated Solv+GJ when compared with OP+GJ and/or GJ minipig groups at 24 hours.

However, between 36 and 48 hours the Solv+GJ PaO_2/F_IO_2 ratios began to fall (similar to OP+GJ/GJ levels) whilst the BALF inflammatory markers rose. This suggests that the effect of the cyclohexanone may have 'worn off'. There were insufficient funds to determine if cyclohexanone was synergistically toxic when combined with dimethoate AI in the lungs.

The low numbers of aerobic bacteria present in the Solv+GJ BALF at 48 hours (compared with OP+GJ and GJ groups) indicate that cyclohexanone may also have had an antimicrobial effect.

Thus cyclohexanone has a seemingly anti-inflammatory, antimicrobial action involving the protection of the alveolar capillary membrane and its surfactant components. Whether it acts as a detergent-like compound (similar to surfactant in allowing easy opening of the airways), or as a protective barrier to the effects of GJ on the lung is unknown and should be investigated further as a potential topical therapy for use in the ICU. This could be conducted through use of an established

animal aspiration model by instilling varying doses of topical (nebulised) cyclohexanone at different time points around the start of pulmonary aspiration injury.

Could aspiration of OP pesticide and gastric contents increase case fatality in intubated patients?

The minipig studies demonstrated that pulmonary installation of OP+GJ created a lung injury whose characteristics have not previously been documented. The increased aerobic bacteria, observed in the BALF from OP+GJ injured minipig lungs at 48 hours, could have been the result of cholinergic immunosuppression combined with the unique toxic lung injury detailed above. Animal and *ex vivo* studies show that bacterial clearance and neutrophil function respectively, are reduced in the face of cholinergic stimulation of the α7nACh nicotinic receptor (Giebelen et al., 2009, Xu et al., 2008).
Rats exposed to subcutaneous VX and sarin over 30 days displayed reduced neutrophil phagocytosis and metabolic activity as well as reduced plasma concentrations of TNF-α, IL-1β and IL-6 compared with control animals. The authors surmised that stimulation of the α7nACh receptor was not the only pathological mechanism involved in OP-induced immunosuppression because the effect of nicotine alone on neutrophil phagocytic and metabolic activity was significantly less than the OP nerve agents (Zabrodskii et al., 2013).
Immunosuppression has been documented in workers chronically exposed to OP pesticides. They were found to have deranged blood profiles with a lower proportion (of total white blood cells) of neutrophils and greater proportion of lymphocytes and eosinophils when compared with matched controls (Yaqub et al., 2014). Their neutrophil function (chemotaxis and adhesion) was also impaired, and they experienced greater rates of upper respiratory tract infections (70% pesticide workers vs. 28% controls) and reoccurrence when compared with healthy controls (Hermanowicz and Kossman, 1984). Therefore, aspiration of OP with GJ mixtures could in theory create more favourable conditions for the development of aspiration or ventilator associated pneumonia. In the largest post-mortem autopsy study of OP poisoned patients, 70% (34/49) of those that died 24 hours after poisoning, had evidence of lung consolidation (Kamat et al., 1989).

This may be important because increased rates of pneumonia could account for the increased mortality observed in intubated OP poisoned patients who have aspirated. If this is true, renewed efforts to reduce pneumonia within OP poisoned patients may be beneficial.

8.2 Conclusion

In conclusion, this work has described the pulmonary pathophysiology that results from both ingested and aspirated OP and gastric contents. The lung injury pattern secondary to these injuries suggests that pulmonary aspiration of OP+GJ is potentially more severe than GJ alone, and differs from classic ARDS. The molecular mechanisms and role of OP-induced immunosuppression should be further investigated and combined with a large prospective observational study within OP pesticide poisoned patients to confirm these findings in humans. This may provide a link between OP-induced lung injuries, pneumonia and case fatality of intubated OP poisoned patients.

Appendix A - CT voxel size and lung volume

Pig	Time point (hr)	Voxel depth (mm)	Voxel height (mm)	Voxel width (mm)	Slice thickness (mm)	Volume left lung (mm^3)	Volume right lung (mm^3)	Notes
N3	-30 min	0.5	0.54	0.54	1.25	323479	468861	
N3	8 hour	0.5	0.562	0.562	1.25	360776	358013	
N3	24hr	0.5	0.507	0.507	1.25	409125	456243	
N3	32 hr	0.5	0.521	0.521	1.25	348843	497810	
N3	48hr	0.5	0.505	0.505	1.25	271099	612320	Increased volume because left lung was partly collapsed
N4	-30 min	0.5	0.519	0.519	1.25	324706	367156	
N4	8hr	0.5	0.552	0.552	1.25	215742	448464	
N4	24hr	0.5	0.501	0.501	1.25	362722	416316	
N4	32hr	0.5	0.544	0.544	1.25	418857	447132	
N4	48hr	0.5	0.515	0.515	1.25	299070	556165	Right lung looks satisfactory, left is consolidated so right has more volume
N5	-30 min	0.5	0.513	0.513	1.25	320560 341214	425353 437491	
N5	8hr	0.5	0.501	.0501	1.25	295766	555404	
N5	24 hr	0.5	0.515	0.515	1.25	321424 336049	546089 444693	Right lung bigger because it swells – haemorrhagic oedema with OP+GJ
N5	32 hr	0.5	0.539	0.539	1.25	310131	532138	
N5	48hr	0.5	0.548	0.548	1.25	219970 264987	423452 428621	
N6	-30 min	0.5	0.521	0.521	1.25	373130	481754	

N6	8 hour	0.5	0.527	0.527	1.25	331726	394689	
N6	24hr	0.5	0.552	0.552	1.25	224196	340633	
N6	32 hr	0.5	0.523	0.523	1.25	218542	320657	
N6	48hr	0.5	0.537	0.537	1.25	254523 246, 030	303385 277 892	
N7	-30 min	0.5	0.505	0.521	1.25	234958	357244	
N7	8 hour	0.5	0.478	0.478	1.25	254063	395961	
N7	24hr	0.5	0.52	0.521	1.25	261499	389995	
N7	32 hr	0.5	0.507	0.507	1.25	224189	406118	
N7	48hr	0.5	0.505	0.505	1.25	309220	369670	
N8	-30 min	0.5	0.533	0.533	1.25	222882	319495	
N8	8 hour	0.5	0.517	0.517	1.25	230415	390887	
N8	24hr	0.5	0.507	0.507	1.25	211800 197019	457294 428039	Craggy scan, collapsed left lung
N8	32 hr	0.5	0.521	0.521	1.25	225472	432405	
N8	48hr	0.5	0.542	0.542	1.25	215475	461962 434775	
N9	-30 min	0.5	0.55	0.55	1.25	249721	316686	
N9	8 hour	0.5	0.539	0.539	1.25	320164	363727	
N9	24hr	0.5	0.556	0.556	1.25	244875	331882	
N9	32 hr	0.5	0.539	0.539	1.25	201451	303688	
N9	48hr	0.5	0.533	0.533	1.25	219065	297431	
N10	-30 min	0.5	0.468	0.468	1.25	306877	434100	
N10	8 hour	0.5	0.517	0.517	1.25	407215	530384	Looks satisfactory
N10	24hr	0.5	0.486	0.486	1.25	214589 211114	420263	Looks satisfactory
N10	32 hr	0.5	0.496	0.496	1.25	209555	389232	Looks satisfactory
N10	48hr	0.5	0.505	0.505	1.25	279127	395808	
N11	-30 min	0.5	0.535	0.535	1.25	382485	453252	
N11	8 hour	0.5	0.628	0.628	1.25	329469	416630	
N11	24hr	0.5	0.511	0.511	1.25	307838	538803	Looks satisfactory
N11	32 hr	0.5	0.580	0.580	1.25	na	380970	Large left pneumothorax

								so not performed
N11	48hr	0.5	0.554	0.554	1.25	274100	453869	Post draining of pneumothorax
N12	-30 min	0.5	0.515	0.515	1.25	198594	281088	
N12	8 hour	0.5	0.505	0.505	1.25	204514	315937	
N12	24hr	0.5	0.478	0.478	1.25	213679	279748	
N12	32 hr	0.5	0.507	0.507	1.25	203351	332576	
N12	48hr	0.5	0.560	0.560	1.25	220794	293221	
N13	-30 min	0.5	0.492	0.492	1.25	380877	529975	
N13	8 hour	0.5	0.535	0.535	1.25	356943	686288 624761	Redo right
N13	24hr	0.5	0.576	0.576	1.25	394733	602916 599965	Redo right
N13	32 hr	0.5	0.556	0.556	1.25	414414 414530	627270 542586	Redo right
N13	48hr	0.5	0.617	0.617	1.25	420918 432726	684988 660182	Redo both lungs
N14	-30 min	0.5	0.498	0.498	1.25	279521	397284	
N14	8hr	0.5	0.550	0.550	1.25	300088	372874	
N14	24hr	0.5	0.49	0.498	1.25	218865	391260	
N14	32 hr	0.5	0.542	0.542	1.25	254174	391016	
N14	48hr	0.5	0.60	0.60	1.25	321440	363758	

Table 1: CT scan voxel size and volume of lungs (mm^3). Numbers in red are the volumes of lungs that have been reanalysed. These reanalysed scans were used for statistical voxel data analysis. Numbers highlighted in yellow were CT scans checked for anomalies in right and left lung volumes compared to their -30 min scans. Note: in the pig the right lung has 4 lobes; cranial, middle, accessory, and caudal lobe. The left lung has only 2 lobes; cranial and caudal. Therefore the right lung is larger, which is reflected by the volumes above (König et al., 2007).

Appendix B- Validation of the R&D ELISA IL6 test kit for plasma

The control spike recovery for IL6 using 60% FCS and PBS was 86% (1388/1600pcg/mL) with plasma samples N1 (control) and N2 (OP+GJ) having a recovery rate of 85% and 80% respectively. An accepted recovery rate is between 80-120% and so 60% FCS and PBS was deemed an acceptable diluent for testing the concentration of IL-6 within porcine plasma. Below is an example of the standard curve for IL6 with serial dilutions in the normal reagent diluent (circles) compared with 60% FCS and PBS (squares) (Figure 1).

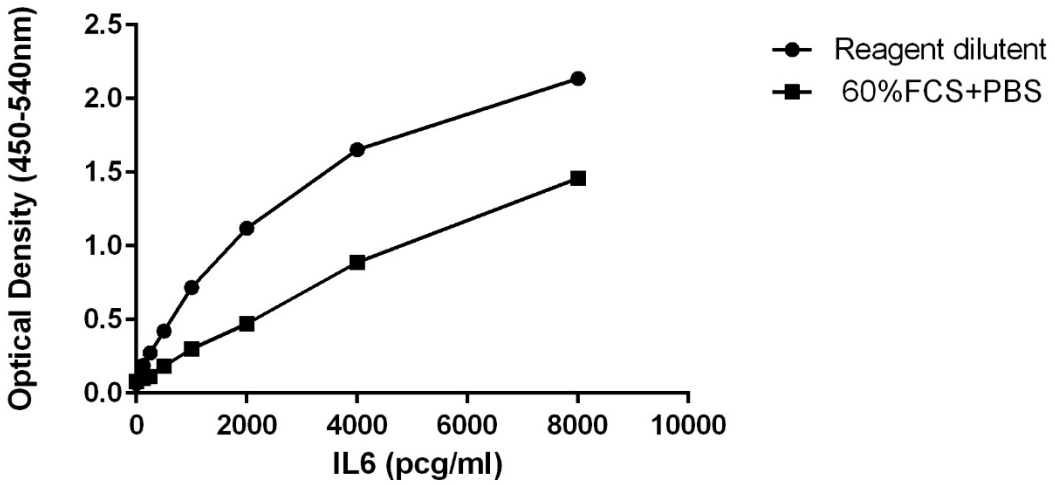


Figure 1: Standard curve of optical density vs. IL6 concentration using standards dissolved in reagent diluent (circles) or 60% fetal calf serum (FCS) and phosphate buffered saline (PBS-squares).

The same experiment was conducted for IL-6 in FCS 20% + PBS but the spike and plasma recoveries lay outside the acceptable range. The experiment was also run for IL-8 and the diluent with best recovery seemed to be 60% FCS and PBS. However, during the experiments with neat plasma it was noted that the levels of plasma IL-8 were extremely low (below lowest standard) in both control and OP+GJ samples, and so measurement of further samples was not completed.

Appendix C: An investigation into normal intensive care unit practices and the occurrence of ventilator associated pneumonia in central Sri Lanka

By Dr Elspeth Hulse, University of Edinburgh, Dr Vasanthi Pinto, University of Peradeniya, Dr Kamal Naser, University of Peradeniya, Dr Nilanthi Dissanayake, University of Peradeniya

Introduction

Ventilator associated pneumonia (VAPs) are hospital-acquired infections that occur more than 48 hours after intubation. In the US, the incidence of VAPs for intubated patients is 9% (Rello et al., 2002, Klompas et al., 2011). Although accurate estimates are difficult to generate, in a recent comprehensive meta-analysis of patient data from randomised prevention studies, Melsen and colleagues concluded that the overall attributable mortality from VAP is 13% (Melsen et al., 2013). There is a paucity of good data for Asian hospitals; however, published reports suggest an incidence of 2-55% with a mortality rate of 14-73% for ventilated patients (Chawla, 2008, Xie et al., 2011). Although diagnosis of VAP can increase a patient's time on ventilators and ICU stay by up to 6 days, it is not always associated with an increase in mortality (Klompas et al., 2011).

Evidence suggests that in order to avoid VAP development, airway colonisation and aspiration must be prevented and the amount of time on the ventilator reduced (Hunter, 2012). Simple techniques like oral decontamination with chlorhexidine 1-2%, sitting the patient upright 30-45 degrees and early extubation on the ICU can thus be very effective at preventing VAP.

This audit was designed to establish the VAP rate in central Sri Lankan ICUs and what current VAP prevention strategies were employed.

Methods

Table 1 A comparison of the American College of Chest Physicians, the CDC National Healthcare Safety Network, and the Hospitals in Europe Linked for Infection Control through Surveillance project (HELICS) clinical definitions of VAP

Definition	Radiological criteria	Clinical criteria	Chest signs
American College of Chest Physicians (Pingleton and colleagues[16])	New or progressive consolidation on chest radiographs	**AND** At least **two** of the following: Fever >38°C **OR** White cell count of >12 000 mm^{-3} or <4000 mm^{-3} **OR** Purulent secretions	
CDC National Healthcare Safety Network (http://www.cdc.gov/HAI/vap/vap.html)	Two or more serial radiographs with at least one of the following: New or progressive and persistent infiltrate OR Consolidation OR Cavitation	**AND** at least one of the following: Fever >38°C **OR** White cell count of >12 000 mm^{-3} or <4000 mm^{-3} **OR** For adults ≥70-yr old, altered mental status with no other recognized cause	**AND two** of the following: New onset of purulent sputum or change in character of sputum or increased respiratory secretions or increased suctioning requirement **OR** New onset or worsening cough or dyspnoea, or tachypnoea **OR** Rales or bronchial breath sounds **OR** Worsening gas exchange (e.g. oxygen desaturations, increased oxygen requirements, or increased ventilator demand)
Hospitals in Europe Linked for Infection Control through Surveillance project (HELICS) (http://helics.univ-lyon1.fr/protocols/icu_protocol.pdf)	A chest X-ray or computed tomography scan suggestive of pneumonia (two or more required for patients with underlying cardiac or pulmonary disease)	**AND** at least one of the following: White cell count of >12 000 mm^{-3} **OR** <4000 mm^{-3} **OR** Temperature >38°C with no other cause	**AND** at least **one** of the following **(two required if microbiology is by qualitative tracheal aspirate culture or if culture is negative)**: New onset of purulent sputum or change in character (colour, odour, consistency, or quantity) **OR** Cough, dyspnoea, or tachpnoea **OR** Auscultatory findings (rales, bronchial breathing, rhonchi, and wheeze) **OR** Worsening gas exchange (e.g. desaturation, increasing FI_{O_2}, or ventilation requirements)

Table 1: VAP internationally accepted diagnostic criteria. Hospitals in Europe Linked for Infection Control (HELICs) criteria is highlighted in yellow.

Permission to conduct the audit was granted by Dr Vasanti Pinto, head of the ICU, University of Peradeniya (10 bed unit), Dr Kamal Naser, on the toxicology ward ICU (3 beds) and Nuwara Eliya general hospital ICU (4 beds). I conducted data collection using a basic data collection form (VAP criteria described below). Data were collected between July-September 2013 on randomly selected days based on my location and work commitments (convenience sampling).

The VAP diagnostic audit criteria was based on the hospitals in Europe linked for infection control (HELICS) VAP definitions (table 1) (Walsh et al., 2013).

Criteria used in audit for potential VAP:

- CXR changes suggestive of pneumonia +

- temp >38°C or white cell count of > 12 000 mm^3 or < 4000 mm^3 +
- Two chest signs e.g. new onset purulent sputum or change in character and signs of ventilator distress, or worsening gas exchange or ventilation requirements.

This definition was amended to allow use of a single CXR for diagnosis (despite cardiac or pulmonary disease) as serial CXRs were often not available. Also, only those with an admission diagnosis pulmonary infection, but not signs systemic infection, were excluded in the VAP numbers.

One organophosphate (OP) poisoned patient was audited twice, once for his first intubation day 1, and then again for his second period of intubation on day 2. These were counted as separate events. VAP was not diagnosed at either time as there was no CXR, although he had clinical symptoms of VAP during the second assessment.

Results

Demographics

- There were 55 patients included in the audit and the male: female ratio was 35/55 (64%):20/55 (36%).
- Mean age and range for adult patients (n=50) was 49 (17-77) years old. Mean age and range for children (>16 years n=5) was 8 (2-15) years old.
- On the day of data collection 9/55 (16%) had only been ventilated for a day, (46/55) 84% for 2 days, 47% (26/55) greater than 2 days. The mean number of days ventilation on day of examination 3.8 days for adults. The children's mean number of days ventilation was not calculated due to 2/5 having excessively long stays > 3-4 months.
- Figure 1 shows the patient diagnosis on admission to the ICU: the majority for post-op complications (18%), OP poisoning (16%) and infections (15%).

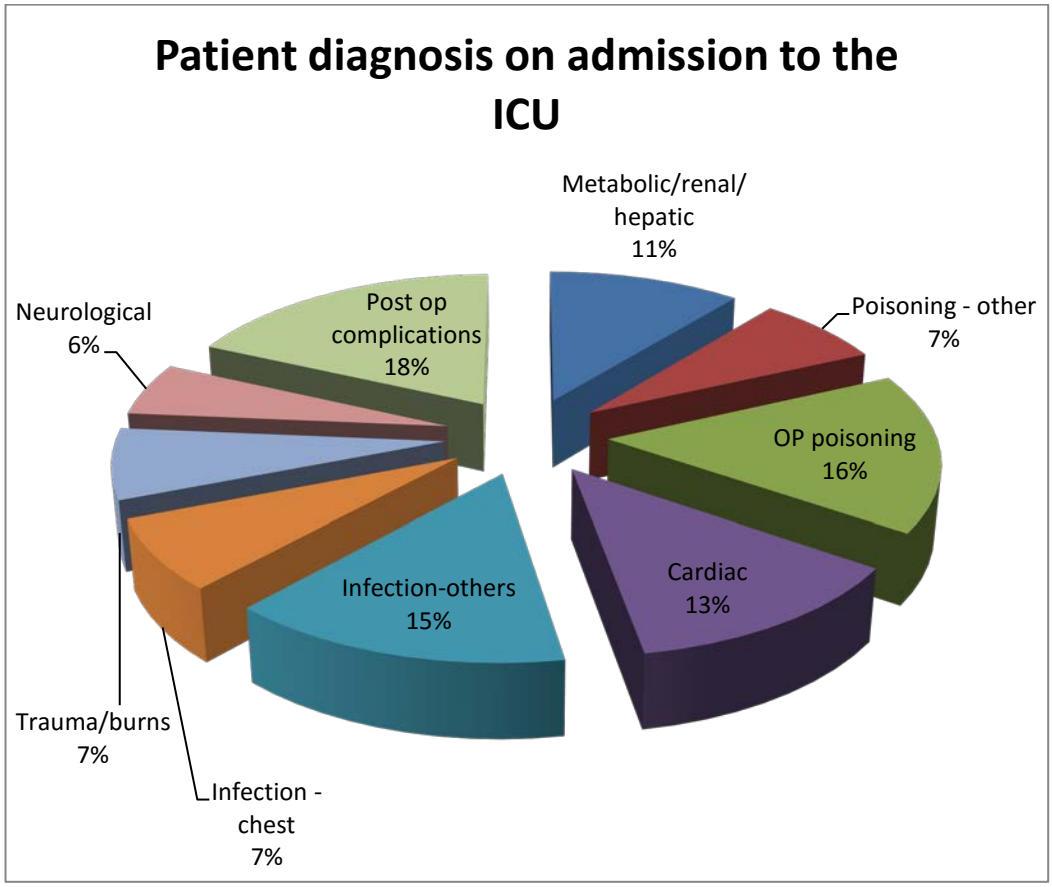


Figure 1: Patient diagnosis on admission to the ICU

- For 10 adult patients, this was their second or third intubation secondary to complications after surgery (6), infection (1), myasthenia gravis (1) and OP poisoning intermediate syndrome (IMS) (2).

VAP diagnosis

- Five patients (5/26 – 19%) fulfilled our criteria for VAP (4/26 in the University of Peradeniya site) alone with 4/5 having a qualitative endotracheal or sputum culture as per HELIC's guidelines.
- There were at least two other possible cases of VAP but fell short of diagnosis due to lack of a CXR in one, and documented chest signs (OP poisoned patient) in the other.

- There was another possible VAP in a post OP poisoning patient who fulfilled the criteria, but had only been ventilated for 2 days.

Antibiotic use

53/55 (96%) intubated patients were on antibiotics/antivirals/antifungals, 15% (7/55) on single agent therapy.

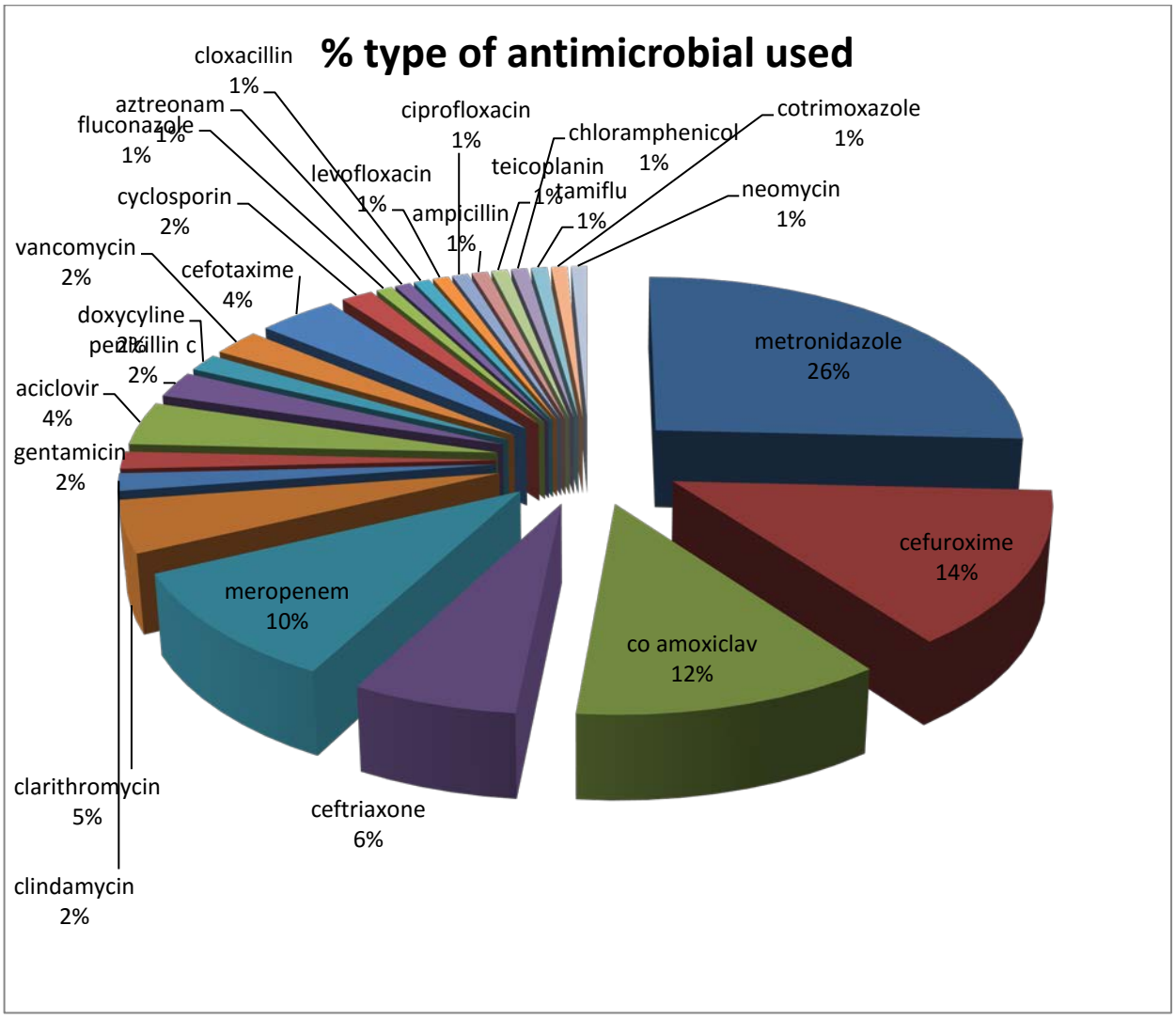


Figure 2: Type of antimicrobial used in ICU (%)

The most commonly used antibiotics were metronidazole and cefuroxime (figure 2). 85% of patients were on two or more agents.

Bacterial culture

The most common bacteria cultured from endotracheal tube (ETT) aspirates and sputum samples (n=19) was *Acinetobacter* and *Pseudomonas* (see figure 3). The cultures were qualitative, not quantitative.

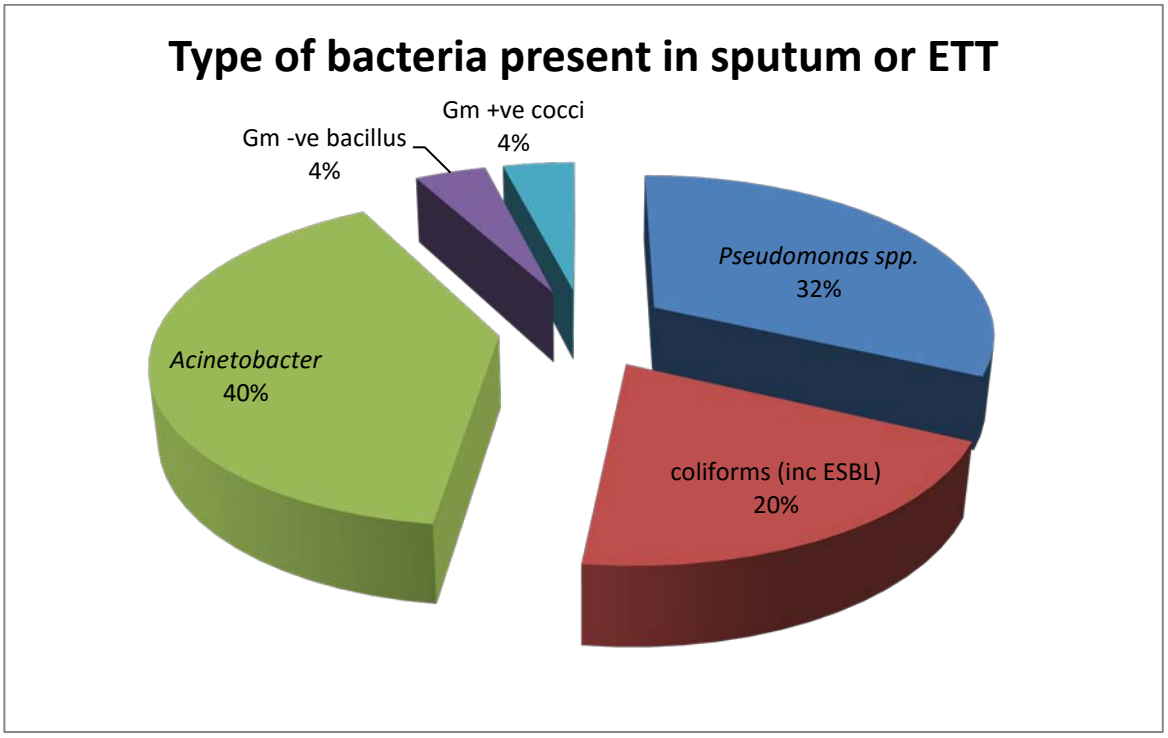


Figure 3: Type of bacteria present on sputum or endotracheal tube (ETT) aspirates on ICU patients (%)

Prevention of airway colonisation

- Chlorhexidine mouth gel was not available for the majority of patients on ICUs and so the mouth was cleaned using a swab covered in various mixtures of saline, bicarbonate of soda and/or lime juice 3-4 times a day.
- 13 % (7/55) had no observed filter (HME or bacterial/viral) in the breathing circuit. The humidifier was not counted as a filter.
- There was no selective decontamination of the digestive tract practised in the ICUs.
- There was no observed routine changes of ETT's after 5-7 days or decision to move to tracheostomy in patients ventilated for >7 days. It was an ad-hoc basis.

Prevention of aspiration

- The mean bed angulation of those eligible to sit up (54/55) was 12°range (0-29).

Other ICU care observations

- 37/55 (67%) received daily nebulised B agonist therapy of the ICU.
- 31/55 (56%) also received daily nebulised anticholinergic (ipravent/apravent) whilst intubated on the ICU.
- Figure 4 illustrates that 91% of patients received DU prevention drugs, the most common of which was ranitidine (82%).
- Tables 2 and 3 describe the ventilation parameters of both adults and children.

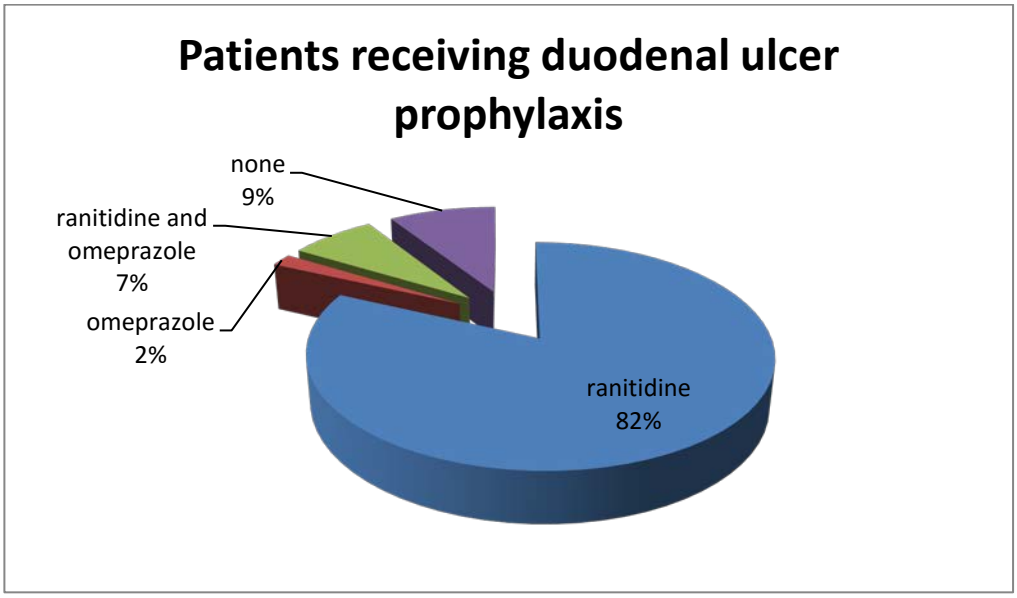


Figure 4: Duodenal ulcer prevention in ICU patients (%)

Mean ventilation variables in adults n= 50
- F_IO2 0.55
- Peak pressure 22 cm H2O
- PEEP 5.4 cm H2O
- Mode of ventilation SIMV + patient control

Table 2: Ventilation variables of ICU patients (n=50)

Mean ventilation variables in children n=5
- F_IO2 0.56
- Peak pressure 16.6cm H2O
- PEEP 4.3 cm H2O
- SIMV + patient control

Table 3: Ventilation variables in children on ICU (n=5)

Only 15% 8/55 patients had some form of thromboprophylaxis treatment (TEDs, clexane) whilst intubated on the ICU.

Discussion

VAP prevention

Although some of the VAPs were probably aspiration pneumonias in origin the VAP rate was high in comparison with western hospitals at 19% (5/26).

A weakness of this audit was that it was based on HELICS criteria, but did not follow it thoroughly when requesting two or more chest signs in the presence of qualitative tracheal cultures or negative cultures. In the hospital in Peradeniya, to my knowledge, all tracheal and sputum samples were qualitative, not quantitative.

However, the true VAP rate might also be hidden due to:
(i) routine antibiotic use on admission to hospital/ICU and intubation
(ii) a lack of routine screening for VAP i.e. regular CXR, BAL or tracheal quantitative culture when sputum colour changes.
Simple measures such as sitting patients up 30-45° and regular chlorhexidine mouth wash have been shown to reduce VAPs (Hunter, 2012). However, some of the ICU beds were broken and could not achieve such a steep incline. Also chlorhexidine mouthwash is expensive, and even the lime juice used for cleansing of the mouth had to be provided by the relatives of the intubated patient.

Some obvious specific issues needed to be controlled in order to prevent VAP. Whilst in Sri Lanka, it was noticed that due to a lack of equipment and funding, the nurses on one ward would re-use suction catheters for individual patients during the day by rinsing them with saline. The provision of an HME/bacterial and viral filter for every ventilator circuit could also help prevent cross contamination.

Although there were low numbers, it seemed that OP poisoned patients may be more prone to VAPs. However, this might be expected secondary to longer periods of ventilation for intermediate syndrome/neuromuscular dysfunction.

Microbiological control

The gold standard for diagnosis of a VAP is quantitative bacterial culture from a lung tissue sample or distal bronchoalveolar lavage sample. The qualitative results available may have just been reflecting local colonisation of the airways in the ICUs. Lower airway microscopy and culture is not routinely done, but is necessary if the specific organism responsible for VAPs is to be appropriately target.

Nearly all ICU patients were on some form of antimicrobial therapy, with most taking more than one agent. The choice of agent seemed extensive with over 25 agents being listed. A more rational approach would be to generate some local hospital guidelines so that every doctor could prescribe the same drugs for the same diagnosis.

A delay of antibiotic prescription until infective signs are present would be more pragmatic, and potentially better for the patient.

Other ICU practices

Ventilation seemed safe with most patients receiving 5cm positive end expiratory pressure (PEEP) and a peak pressure <30 cmH_20 pressure.

Nebulised B agonists and anticholinergics are not routinely used in UK ICUs. B agonists have been postulated to increase the removal of lung water, but the recent Balti-2 trial showed that B agonist use in ARDS patients failed to show benefit and may even be harmful (Gates et al., 2013). These practices could also increase heart rates and promote arrhythmias.

ETT changes should probably occur after 7 days at the very least to prevent colonisation of the airways if tracheostomy is not available.

The true DVT and PE rate for intubated ICU patients at Peradeniya/ Nuwara Eliya hospitals needs to be established before the assumption that no thromboprophylaxis is required and could represent a significant risk for pulmonary embolism development.

There was good overall adherence to DU protection strategies.

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