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Development of a total intravenous anaesthesia regime for use in pigs during live tissue training and biomedical research

Utvikling av et regime for total intravenøs anestesi hos gris brukt til kirurgisk ferdighetstrening og biomedisinsk forskning

Andreas Lervik

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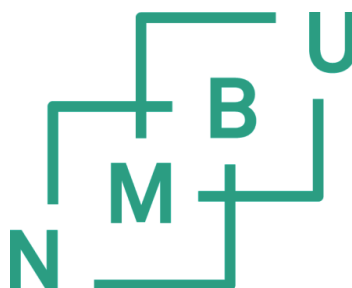
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Philosophiae Doctor (PhD) Thesis

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being supportive and loving. My fantastic children Olivia, Esther and Axel, who always

remind me of the most important things in life, including football, flyfishing, horses and Wi-

Fi.

Abbreviations

BE = Base excess

BIS = Bispectral index

CI = Cardiac index

DAP = Diastolic arterial blood pressure

DO_2 = Delivery of oxygen

ECG= Electrocardiography

EEG = Electroencephalography

EMG = Electromyography

FE´iso = End-tidal isoflurane concentration

HR = Heart rate

LTT = Live tissue training

MAC = Minimum alveolar concentration

MAP = Mean arterial blood pressure

NMB = Neuromuscular blocking drugs

NWR = Nociceptive withdrawal reflex

OE = Oxygen extraction

$PaCO_2$ = Arterial partial pressure of CO_2

PaO_2 = Arterial partial pressure of O_2

PE´CO_2 = Partial pressure of end-tidal CO_2

PR = Pulse rate

Qt = Cardiac output

RR = Respiratory rate

SaO_2 = Arterial oxygen saturation measured in blood

SAP = Systolic arterial blood pressure

SpO_2 = Arterial oxygen saturation measured by pulse oximetry

SV = Stroke volume

SVI = Stroke volume index

TIVA = Total intravenous anaesthesia

TPR = Total peripheral resistance

VO_2 = Oxygen consumption

V_T = Tidal volume

Summary

Large animal models are commonly used in biomedical research or for medical training purposes where cardiovascular instability is encountered. Pigs are frequently used as their cardiorespiratory anatomy and physiology are thought to resemble that of humans closely. General anaesthesia is mostly a legal requirement when using pigs for this purpose. There is however a paucity in the scientific literature when it comes to research on anaesthesia in pigs, with anaesthetic techniques often being extrapolated from those used in other species, including human anaesthesia.

The overall aim of this work was to develop an anaesthetic regime that can be applied when anaesthetising pigs for live tissue training and biomedical research where surgically induced nociception or cardiovascular instability can be encountered.

An experiment was conducted comparing total intravenous anaesthetic protocols with either propofol-ketamine-fentanyl or propofol-ketamine-dexmedetomidine. We concluded that both anaesthetic regimes provided stable cardiovascular conditions in normovolemic pigs. Additionally, solid antinociception was provided, in particular when including dexmedetomidine in the anaesthetic protocol.

This experiment led to new research questions, and further experiments were conducted to examine the cardiovascular function during hypovolemia due to blood loss and the respiratory function during total intravenous anaesthesia with either propofol-ketamine-dexmedetomidine or alfaxolone-ketamine-dexmedetomidine. In addition, the difference in nociceptive response to different modalities of nociceptive stimulation between pigs given fentanyl or dexmedetomidine that was observed during the first experiment was examined further.

The major findings of this experimental work are that total intravenous anaesthesia with either propofol-ketamine-dexmedetomdine or alfaxalone-ketamine dexmedetomdine are suitable anaesthetic regimes in pigs when cardiovascular instability is foreseen during live tissue training or biomedical research, and that these pigs tolerate a substantial blood loss. When using these anaesthetic regimes respiratory supportive measures must be taken. Oxygen supplementation and the ability to provide positive pressure ventilation must be available. Plasma concentrations of both propofol and alfaxalone may increase during moderate to severe blood loss, and dose adjustments can be necessary. Both dexmedetomidine and fentanyl seem to provide solid antinociception in pigs when given as a constant rate infusion. In our study population of juvenile pigs, dexmedetomidine did not negatively influence cardiovascular function to the extent that has been observed in other species.

Sammendrag

Når store forsøksdyr brukes i biomedisinsk forskning eller ved medisinsk ferdighetstrening kan kardiovaskulær instabilitet oppstå. Gris blir ofte brukt som modelldyr, ettersom det er en oppfatning av at artens kardiorespiratoriske anatomi og fysiologi likner menneskets. Generell anestesi av gris til dette formålet er relativt vanlig, men publisert forskning på effektene av ulike anestesiregimer til gris er mangelfull. Ofte ekstrapoleres anestesiteknikker fra andre arter, blant annet fra human anestesiologi.

Hovedformålet med dette vitenskapelig arbeidet var å utvikle et anestesiregime til bruk på gris som anesteseres for medisinsk ferdighetstrening og biomedisinsk forskning der kirurgisk indusert nosisepsjon og kardiovaskulær instabilitet kan oppstå.

I det første eksperimentet ble effektene av total intravenøs anestesi med propofol-ketamin-fentanyl og propofol-ketamin-deksmedetomidin sammenlignet. Hovedfunnet var at begge anestesiregimer gav stabil kardiovaskulær status og solid antinosisepsjon.

Eksperimentet ga opphav til nye forskningsspørsmål. Videre eksperiment ble gjennomført for å undersøke kardiovaskulær funksjon ved hypovolemi og respiratorisk funksjon hos griser anestesert med enten propofol-ketamin-deksmedetomidin eller alfaxalon-ketamin-deksmedetomidin. I tillegg undersøkte vi den forskjellige nosiseptive responsen vi observerte ved ulike modaliteter for nosiseptiv stimulering hos griser som fikk enten fentanyl eller deksmedetomidin i det første eksperimentet.

De viktigste funnene i våre undersøkelser er at total intravenøs anestesi med enten propofol-ketamin-deksmedetomdin eller alfaxalon-ketamin-deksmedetomidin kan brukes til generell anestesi av gris ved medisinsk ferdighetstrening eller biomedisinsk forskning når kardiovaskulær instabilitet forventes, og at disse grisene tolererer et relativt stort blodtap. Når man bruker disse anestesiregimene må respiratorisk støttebehandling være tilgjengelig, og muligheter til å gi tilskudd av oksygen og overtrykksventilering må være til stede. Plasmakonsentrasjoner av både propofol og alfaxalon kan øke ved moderat til kraftig blodtap, og dosejusteringer kan være nødvendig. Både fentanyl og deksmedetomidin gitt som en kontinuerlig infusjon ser ut til å gi solid antinosisepsjon hos gris. I vår studiepopulasjon bestående av juvenile griser så ikke deksmedetomidin ut til å påvirke kardiovaskulær funksjon like negativt hos gris som hos andre arter.

List of papers

Paper I

Lervik A, Raszplewicz J, Ranheim B, Solbak S, Toverud SF, Haga HA. Dexmedetomidine or fentanyl? Cardiovascular stability and analgesia during propofol-ketamine total intravenous anaesthesia in experimental pigs. Vet Anaesth Analg. 2018 May;45(3):295-308. doi: 10.1016/j.vaa.2017.08.012.

Paper II

Lervik A, Toverud SF, Krontveit R, Haga HA. A comparison of respiratory function in pigs anaesthetised by propofol or alfaxalone in combination with dexmedetomidine and ketamine. Acta Vet Scand. 2020 Mar 12;62(1):14. doi: 10.1186/s13028-020-0512-y.

Paper III

Lervik A, Toverud SF, Bohlin J, Haga HA. Macrocirculatory parameters and oxygen debt indices in pigs during propofol or alfaxalone anesthesia when subjected to experimental stepwise hemorrhage. Front. Vet. Sci. | doi: 10.3389/fvets.2021.664112. Accepted 30 Mar. 2021.

Paper IV

Haga HA, Lervik A, Nordgreen J. Inhibition and facilitation of nociceptively evoked muscular activity by fentanyl or dexmedetomidine in isoflurane-anaesthetized pigs. Vet Anaesth Analg. 2021 Mar;48(2):230-238. doi: 10.1016/j.vaa.2020.09.007.

Paper V

Lervik A, Toverud SF, Dulgheriu D, Haga HA. Changes in plasma concentrations of propofol and alfaxalone in a porcine model of haemorrhagic shock. Manuscript.

Introduction

Anaesthesia in large animal models used in medical research, a historical perspective

Large animal models in a broader perspective include all non-rodent mammalian animals used for biomedical research and live tissue training (LTT). In ancient Greece live animals were used for anatomy studies (Hajar 2011), and despite vivisections being performed, moral concerns were not raised, as the *Scala naturae* ranked humans higher than other living creatures. This belief also influenced the later Christian perspective of humans being superior to other animals (Franco 2013). While the use of animal studies decreased during the Roman Empire and in Medieval Europe, Arabic physicians such as Ibn Zuhr and Ibn Al Nafis performed tests on animals in anatomical studies and for the development of surgical methods used in humans (Hajar 2011). In Europe animal testing re-emerged in the Renaissance and during the period of Enlightenment, and experiments related to the science of anaesthesia were also conducted. Andreas Vesalius (1514-1564) used bellows to ventilate the lungs of an asphyxiated dog. Around 1525 Paracelsus (1493-1541) distilled ether and administered the distillate to chickens, thereby anaesthetizing them (Ball & Westhorpe 1996). In 1656 Robert Boyle (1627-1691) injected opium dissolved in alcohol to narcotise a dog (Dorrington & Poole 2013), and the effects of nitrous oxide in several animal species were described by Humphrey Davy (1778-1829) (Davy 1800).

In the late 19th century, the antivivisectionist movement, and the increased demand to avoid suffering in animals had started to influence animal research in Europe. Louis Pasteur (1822–1895) wrote: *"The rabbit should begin to show symptoms on the sixth or seventh day, and die on the ninth or tenth. Usually the rabbit is not allowed to die, but is chloroformed on the last day in order to avoid terminal infections and unnecessary suffering" (Franco 2013)*. In the early 20th century animals were increasingly used for medical research with huge success. Eighty-three of 109 Nobel prizes since 1901 have included studies in non-human vertebrate animals (Franco 2013).

The widespread use of general anaesthesia in animals was delayed well into the 20th century. In particular the discovery of barbiturates in the 1920's and 1930's contributed to increased use of anaesthesia (Grimm & Tranquilli 2015). Despite a reduction in the knowledge gap in veterinary anaesthesia during the last 3-4 decades of the 20th century, worries were still expressed when it came to anaesthesia of experimental animals: "*To most anaesthetists, the field of animal anaesthesia is completely unknown, while on the other hand, most veterinarians have not had the necessary formal training in physiology and pharmacology to assess their anaesthetic agents and techniques realistically. As a result of this there is often an unnecessarily high morbidity and mortality among experimental animals, and inadequate anaesthesia can well render many experimental results suspect" (Holland 1973).* The formation of specialist veterinary anaesthesia colleges in Europe and in North America has however contributed to changing this over the last 30-40 years, with anaesthesia in many laboratory animals now being more sophisticated than ever, utilizing similar drugs and techniques as in human medicine. Paradoxically as the development of anaesthesia has improved animal welfare, it has most likely contributed to the increased use of experimental animals.

Large animal models in medical research and live tissue training; a legal and ethical perspective

The British Cruelty to Animals Act was passed in 1876, and influenced the research using live animals at that time (Finn & Stark 2015). The first Norwegian law to forbid cruelty to animals was written in the Norwegian penal code in 1842, and as one of the first countries in the world Norway passed their Animal Welfare Act in 1935. Today the Directive 2010/63/EU on the protection of animals used for scientific purposes forms the legal standard for how animals may be used in medical research in

Europe. This directive in addition to the Norwegian Animal Welfare Act from 2010 forms the basis for the Norwegian Regulation on Animal Experimentation from 2015.

In §10 of the Regulation on Animal Experimentation the purposes for which animals may be used are listed, including the use of animals for live tissue training in professional or higher education. The §13 of the Norwegian Animal Welfare Act states that approval for these uses cannot be given if animals may be subjected to unnecessary stress or strain, or if the intention can be achieved without the use of animals. In addition, the §14 regulates the use of general or local anaesthesia for experiments that inflict severe injury and cause severe pain to research animals. This will be true for experiments involving surgery and live tissue training. Also, the Annex B of the Regulation on Animal Experiments classifies animal experiments according to severity, where terminal procedures are described as being performed entirely under general anaesthesia, and from which animals should not recover consciousness.

According to the 17th century Cartesian principles animals could be viewed as machines, without consciousness and the capability of feeling pain (Baumans 2004), but this view was challenged by Bentham already in the 19th century. The neurobiological support of Bentham's view on animal suffering is summarized in the Cambridge Declaration on Consciousness in Non-Human Animals from 2012 (Mashour & Alkire 2013). The ethical debate on the use of animals in research is still vivid, and several ethical views on this topic are held. According to the animal rights view sentience gives animals the same protection as humans, and they should never be used in experiments whatever the intention (Carlsson 1986). Peter Singer is not categorical on the use of animals with his modern utilitarian view, and argues that the end could sanctify the means if the cause is good enough (Foex 2007). The use of experimental animals in the modern world is also influenced by a relational view, where some animal species with closer emotional link to humans (apes, dogs, horses) are less

commonly used in experiments. The §20 of the Regulation on Animal Experiments forbids the use of primates, with some exceptions. For apes (*Hominidae*) no such exceptions exist.

Pigs; a good model for trauma in humans? Current knowledge and perspectives.

Pigs (*Sus scrofa domesticus*) were probably domesticated approximately 8-8500 years ago (Caliebe et al. 2017). Today the species includes a number of breeds, with production animals such as the European Land Race breeds, and a variety of breeds purposefully designed for biomedical research with the Göttingen miniature pig and Yucatan pig being the most commonly used (Gutierrez et al. 2015).The number of publications where pigs are used in biomedical research has seen a considerable increase since the early 1980's (Gutierrez et al. 2015), and the species seems to have more or less replaced the use of dogs in cardiovascular and trauma research, as well as in LTT (Wenzel et al. 2000; Swindle et al. 2012), partly due to relational ethical concerns. The pig is today the most commonly used large animal model in preclinical experiments investigating therapies related to trauma, and also for LTT involving medical trauma care providers (Majde 2003).

Compared to many other experimental animals, the anatomy, physiology and metabolism of pigs do of course resemble that of humans more closely in many ways, being a larger, monogastric omnivore animal (Swindle et al. 2012; Pehbock et al. 2015). This fact is however a simplification, and already 20 years ago some important questions related to model design in trauma research were asked at the Military Medicine Workshop on Animal Models in Hemorrhage and Resuscitation Research (Majde 2003). In particular considerations related to animal physiology and experimental procedures including anaesthesia were discussed, with the main conclusion being that all conventional anaesthetics will influence the obtained results in such models. By some authors it has been stated that the cardiovascular response, haematological changes, and ventilator settings used in pigs subjected to trauma resemble human physiology closely (Hildebrand et al. 2013). Haemostasis,

metabolism and control of body temperature on the other hand, being very important in trauma patient, may not be directly comparable between the two species (Hildebrand et al. 2013; Sondeen et al. 2013; Pehbock et al. 2015).

Pigs are often used when performing emergency procedures LTT of civilian and military medical personnel. Efforts are currently made to reduce the use of animals for trauma training purposes, but LTT is still the most common method in many countries, with pigs being the most commonly used species (Gala & Crandall 2019). There is also no clear consensus in the medical literature on whether LTT can be replaced by simulation-based training (Savage et al. 2015; Barnes et al. 2016; Kim et al. 2017). When anaesthetising pigs for LTT, several important aspects should be taken into consideration. The animal must be unconscious, immobile, and proper antinociception should be provided, hence the goals of general anaesthesia should be fulfilled. The anaesthetic protocol should also provide cardiovascular stability during surgery and tolerance to haemorrhage and hypovolemia to avoid premature death of animals used during each training session. This will contribute to the reduction of the total number of animals used, and thereby adhering to the 3 R's principle. When using animals for training human anaesthesiologists, the model should mimic a realistic situation, enhancing the learning outcome.

There are still significant challenges when data from animal studies of resuscitation and trauma research are translated for the benefit for human patients (Reynolds 2012). The reasons for this are probably numerous, including the experimental induction of complex disease states in a previous healthy animal. Other factors that must be considered are the physiological disparities between pigs and humans. Another problem related to this dogmatically taken resemblance of pigs and humans is how anaesthetic drugs are used in in this species. A review of the literature found that the use of neuromuscular blocking drugs (NMB) are fairly commonly used for a multitude of reasons without

describing measures taken to avoid accidental awareness (Bradbury & Clutton 2016). In this authors opinion NMBs are often unnecessary in porcine anaesthesia, and there are good examples of inappropriate use that represent an important animal welfare problem (Monteiro & Smith 2014). There are also other examples in the literature of inappropriate extrapolation of drug effects and doses from human clinical practice to pigs, were low doses of fentanyl have been administered in combination with vecuronium (Rey-Santano et al. 2014; Shen et al. 2021). In other experiments one could question if pigs actually were under general anaesthesia or only sedated, as low doses of propofol combined with low doses of fentanyl were used in combination with pancuronium or a combination of midazolam and fentanyl was used alone (Cavalcante et al. 2011; Bruins et al. 2013). Ketamine has been used as the sole agent during invasive surgical procedures in pigs, until concerns were raised in the mid 1990's about the determination of appropriate anaesthetic depth in the species when using the drug (Boschert et al. 1996). In addition, when reviewing the literature, pigs are commonly anaesthetised for experimental procedures, but there is a paucity in the literature when it comes to studies examining the actual effects of anaesthetic drugs in experimental pigs. This is supported by a literature review by Mikkelsen et al summarizing the use of propofol-remifentanil anaesthesia in pigs undergoing examination of cerebral perfusion. The majority of studies did not specifically discuss the choice of anaesthetic protocols and the potential effect on their study aim (Mikkelsen et al. 2016).

According to the Utstein-style guidelines for laboratory resuscitation research animals must be anaesthetised unless they are unconscious for other reasons such as cerebral ischemia or severe hypothermia. The guidelines also declare that "*if neuromuscular blockers are used, the animals must be insensitive to pain and unconscious*". In addition the physiological effects of the anaesthetic regime used should be considered when planning and reporting outcomes from such research (Idris et al. 1996) No attempt was made to standardise anaesthesia for this type of research, and a later

published survey of the literature found that different anaesthetic regimes have a rather large influence on haemodynamic parameters (Wenzel et al. 2000). In a recent review of pig haemorrhage and trauma models the different anaesthetic regimes used are summarized, with volatile anaesthetics used as a sole agent being by far the most commonly used technique to maintain anaesthesia (Hildebrand et al. 2013). This stands in contrast to the human clinical trauma care situation, where combinations of inhalational and injectable drugs or intravenous drugs alone seems to be more commonly employed (Sikorski et al. 2014; Tobin et al. 2018).

During LTT using a porcine model of trauma at the Norwegian Armed Forces Medical Centre at Sessvollmoen, pigs have been anaesthetized for the last 20 years using a combination of injectable drugs in combination with volatile drugs for maintenance of anaesthesia. In addition, epidural injection of lidocaine has been applied. While animal welfare has been very well maintained at this facility, some challenges have been described by the veterinarians responsible for the use of research animals and the instructors training anaesthesia providers. In particular premature death of some animals and a lack of chronotropic response to blood loss, have been the major concerns. During haemorrhage the chronotropic response observed has been negligible, in contrast to what is normally observed in the clinical situation in humans.

Total intravenous anaesthesia in pigs in trauma research and live tissue training

The induction and maintenance of anaesthesia with intravenous administered drugs is termed total intravenous anaesthesia (TIVA). In veterinary medicine commonly used drug combinations include GABA-agonists such as propofol, drugs with opioid receptor effect and dissociative anaesthetic drugs such as ketamine. In clinical veterinary practice pigs are not frequently subjected to general anaesthesia, mainly due to the costs of performing general anaesthesia and surgery under field conditions in production animals. When used as research subjects however, anaesthesia is often

necessary. Still studies examining the effects of anaesthesia and anaesthetics drugs to the benefit of pigs are less common than in most other species treated by veterinarians.

When reviewing the literature, several anaesthetic and analgesic drugs have been used to deliver TIVA to pigs. The Scottish veterinarian John B (Iain) Glen used mice, rabbits, monkeys and pigs in the development of ICI 35 868, later known as propofol, during the late 1970's. He also examined the effect of a continuous infusion, and the "utilization" rate of propofol in these species (Glen 1980). The pharmacokinetics of propofol has since then been examined in healthy experimental animals including pigs (Cockshott et al. 1992). A study examining the relationship between cardiac output (Qt) and propofol pharmacokinetics, found an 70% increase in plasma concentrations when Qt was reduced by 42% (Kurita et al. 2002). In a second study the researchers investigated the effect of haemorrhage upon propofol plasma concentrations, finding an increase of 38% at the time point of maximum heart rate when approximately 37% of the pigs' blood volume was lost (Kazama et al. 2002). The examined propofol dose was however very low, and a background anaesthesia with isoflurane was used. The same authors examined the influence of haemorrhage on plasma concentrations of propofol and remifentanil infused at 6 mg $kg^{-1} h^{-1}$ and 30 µg $kg^{-1} h^{-1}$ respectively. The concentration of both drugs rose with increasing haemorrhage volume, with the sharpest incline observed with a cumulative blood loss around 40% of the pigs' blood volume (Kurita et al. 2011).

The addition of adjunctive drugs to propofol anaesthesia could reduce the amount of propofol necessary to maintain general anaesthesia. At the same time effects of the adjuncts could be beneficial for animals exposed to surgical nociceptive stimulation or hypovolemia due to haemorrhage or systemic inflammation.

Propofol combined with fentanyl has been used in pigs anaesthetized for cardiovascular research purposes, providing hypnosis and analgesia with a relatively stable cardiovascular status (Kurita et al. 2003; Schoffmann et al. 2009). Propofol has been used in pigs during experimental surgery. In the study by Schoffmann et al. propofol 8 mg kg^{-1} h^{-1} was combined with fentanyl 35 μg kg^{-1} h^{-1} for TIVA during surgical vessel cannulation in piglets. The researchers found stable haemodynamic conditions with a decrease in plasma cortisol concentrations (Schoffmann et al. 2009).

Propofol can also be combined with ketamine for the induction and maintenance of anaesthesia in humans and dogs, resulting in improved cardiovascular function compared to propofol used alone (Smischney et al. 2012; Martinez-Taboada & Leece 2014; Kennedy & Smith 2015), as ketamine seems to offset the decrease in systemic vascular resistance caused by propofol. An investigation comparing isoflurane anaesthesia to a ketamine based protocol in pigs found a higher mean arterial pressure in the ketamine group, but end organ perfusion was not found to be different (Englehart et al. 2008). Additionally ketamine provides antinociception by NMDA-receptor antagonism (Kohrs & Durieux 1998).

Dexmedetomidine is an α-2-receptor agonist with sedative and analgesic properties. Continuous infusion of dexmedetomidine provides antinociception and reduces the minimal alveolar concentration (MAC) of volatile anaesthetics in dogs (Pascoe et al. 2006; Lervik et al. 2012). In pigs, dexmedetomidine induces sedation after intramuscular injection (Nunes et al. 2007). Alpha-2 agonists do however induce unwanted cardiovascular effects such as bradycardia, increased systemic vascular resistance, reduced cardiac output and oxygen delivery in several species even when infused in low doses (Murrell & Hellebrekers 2005; Marcilla et al. 2010; Sano et al. 2010). Interestingly, a study in miniature pigs using a pressure controlled haemorrhagic shock model found that pigs given a combination of ketamine and medetomidine maintained a higher initial blood

pressure compared to propofol-remifentanil treated pigs, and tolerated withdrawal of a larger blood volume (Brezina et al. 2010). Another study in pigs examined the effects of propofol combined with low doses of dexmedetomidine. Hypotension developed while TPR did not change much. Qt dropped as a result of a decrease in HR and SV (Sano et al. 2010). Mikkelsen and colleagues examined the effect of a similar dose of dexmedetomidine in addition to propofol-remifentanil in pigs subjected to hypotension by caval occlusion. They found a decrease in cerebral perfusion in pigs with induced hypotension when dexmedetomidine was used, but not in pigs where normotension was maintained (Mikkelsen et al. 2017).

Alfaxalone is an intravenous anaesthetic agent with a steroid structure, that is commonly used in anaesthetic practice in companion animals. A conception among veterinary anaesthetists is that alfaxalone seems to depress the baroreceptor response less than propofol. In fentanyl premedicated dogs, induction of anaesthesia with alfaxalone decreased the heart rate significantly less than did propofol (Okushima et al. 2015). When the two drugs were compared for anaesthetic maintenance no difference in cardiovascular variables such as mean arterial blood pressure or heart rate could be found (Ambros et al. 2008). In an investigation comparing alphaxalone/alphadolone and propofol administered to maintain anaesthesia in pigs subjected to haemorrhage, a higher heart rate was found at baseline after 2 hours of drug infusion, and after a blood loss of 40 ml kg^{-1} in pigs given alphaxalone/alphadolone (Ruane-O'Hora et al. 2011). Alfaxalone has been used for induction and maintenance of anaesthesia in pigs without causing apnoea after induction, but hypoventilation can result when it is used for maintenance (Keates 2003; Bigby et al. 2017). When used to maintain anaesthesia in cats during ovariohysterectomy, alfaxalone was shown to have less effect on respiration when compared to propofol (Campagna et al. 2015), but when a similar comparison was made in canine clinical patients hypoventilation occurred with both drugs (Suarez et al. 2012).

Aims and objectives

The main aim of the PhD-project was the development of a total intravenous protocol for pigs used in live tissue training and biomedical research that ensured unconsciousness and immobility. At the same time physiological responses to hypovolaemia and spontaneous ventilation should be preserved.

The primary objective was to compare anaesthesia with propofol-ketamine combined with either fentanyl or dexmedetomidine. In particular we wanted to compare the cardiovascular function between pigs administered the two anaesthetic protocols. Additionally, the response to nociceptive stimulation was assessed to ensure comparison of cardiovascular function at similar anaesthetic depth.

Secondary objectives developed after the first experiment:

Firstly, we wanted to investigate the cardiovascular function in pigs anaesthetised with propofol-ketamine-dexmedetomidine during hypovolemia due to blood loss. A lack of chronotropic response to hypovolemia due to blood loss was observed in a pilot study. We thus wanted to compare the cardiovascular function during hypovolemia after blood loss in pigs anaesthetised with propofol-ketamine-dexmedetomidine, when propofol was replaced with alfaxalone. The respiratory function in spontaneously breathing pigs was also compared between the two anaesthetic protocols.

Further, investigations of the different responses elicited by mechanical and electric nociceptive stimulation in pigs administered fentanyl and dexmedetomidine were examined when pigs where anaesthetised by isoflurane. In addition, dexmedetomidine seemed to have surprisingly little influence on heart rate during the first study. We therefor wanted to examine the effect of dexmedetomdine on heart rate during pure isoflurane anaesthesia.

Results - Summary of papers

Pharmacological interventions

For Paper I-III and V Ketamine 15 mg kg^{-1} and midazolam 1 mg kg^{-1} was used for pre-anaesthetic sedation. In Paper IV anaesthetic induction was achieved without prior sedation with isoflurane in oxygen delivered by a face mask. To maintain anaesthesia all drugs were delivered by a continuous infusion through an intravenous catheter placed in an ear vein. In study IV anaesthesia was maintained with inhaled isoflurane in addition to the infused test drugs. The drugs concentrations, doses and infusion rates are summarised in table 1:

Paper	Study group	Drug concentrations	Drug infusion doses
I	Dexmedetomidine	Propofol 20 mg ml^{-1}	8 mg kg^{-1} h^{-1}
		Ketamine 50 mg ml^{-1}	5 mg kg^{-1} h^{-1}
		Dexmedetomidine 4 µg ml^{-1}	2, 4 and 8 µg kg^{-1} h^{-1}
	Fentanyl	Propofol 20 mg ml^{-1}	8 mg kg^{-1} h^{-1}
		Ketamine 50 mg ml^{-1}	5 mg kg^{-1} h^{-1}
		Fentanyl 50 µg ml^{-1}	25, 50 and 100 µg kg^{-1}h^{-1}
II, III, V	Alfaxalone	Alfaxalone 10 mg ml^{-1}	5 mg kg^{-1} h^{-1}
		Ketamine 50 mg ml^{-1}	5 mg kg^{-1} h^{-1}
		Dexmedetomidine 50 µg ml^{-1}	4 µg kg^{-1} h^{-1}
	Propofol	Propofol 20 mg ml^{-1}	8 mg kg^{-1} h^{-1}
		Ketamine 50 mg ml^{-1}	5 mg kg^{-1} h^{-1}
		Dexmedetomidine 50 µg ml^{-1}	4 µg kg^{-1} h^{-1}
IV	Dexmedetomidine	Isoflurane	Mean ± SD FE'Iso (%) 2.0 ± 0.10
		Dexmed. 0.15, 0.625 and 2.5 µg ml^{-1}	0.25, 0.5, 1, 2, 4 and 8 µg kg^{-1} h^{-1}
	Fentanyl	Isoflurane	Mean ± SD FE'Iso (%) 1.9 ± 0.16
		Fentanyl 3.125 , 12.5 and 50 µg ml^{-1}	5, 10, 20, 40, 80 and 160 µg kg^{-1} h^{-1}

Cardiovascular function

In Paper I cardiovascular parameters were compared at similar anaesthetic depth established individually, and the methods used for evaluation of anaesthetic depth are discussed later in the thesis. Trends for cardiovascular parameters over time where graphically displayed.

Similar anaesthetic depth was established at infusion rates of 2 or 4 µg kg^{-1} hr^{-1} of dexmedetomidine and 50 or 100 µg kg^{-1} hr^{-1} of fentanyl. At this time point pigs had significantly higher HR, SAP, DAP, MAP, TPR and DO_2 when anaesthetised with propofol-ketamine-dexmedetomidine compared to when propofol-ketamine-fentanyl was given. CI and arterial lactate concentration were similar with both treatments, while SVI was lower in pigs when given dexmedetomidine.

With both anaesthetic regimes heart rate decreased from baseline over time, with the largest decrease observed with the lowest fentanyl infusion rate. Stroke volume decreased slightly with dexmedetomidine, while a modest increase was seen with fentanyl. MAP and TPR increased over time when dexmedetomidine was administered, with little change observed in the fentanyl group. DO_2 decreased over time with both anaesthetic regimes.

In Paper IV the same test drugs where compared with a different background anaesthetic (isoflurane). No clinically relevant changes in HR or MAP where observed over time with a wide range of infusion rates administered.

In Paper III pigs where subjected to stepwise blood loss to evaluate cardiovascular function, compensation, and the development of oxygen debt with moderate to severe hypovolemia.

In pigs anaesthetised with alfaxalone-ketamine-dexmedetomidine a significantly higher systolic blood pressure was found, but no other statistical differences between groups could be demonstrated. PR increased with increasing blood loss, while arterial blood pressure decreased, as

did CI and SVI. During time periods allowed for compensation, an increase in arterial blood pressure was observed during the early stages of haemorrhage. TPR remained remarkably stable over time in both groups.

Indices of oxygen debt changed as expected over time, with a moderate to severe increase in lactate concentration and a decrease in base excess. No statistical difference could be found when comparing these parameters between groups.

Respiratory function

In Paper II respiratory function was compared between pigs.

Pigs anaesthetised with alfaxalone-ketamine-dexmedetomidine had significantly higher RR and lower $P_E'CO_2$ than pigs anaesthetised with propofol-ketamine-dexmedetomidine, while SpO_2 and V_T was similar between groups. In addition, body weight had a significant effect on SpO_2 and RR in both groups, with a lower SpO_2 and RR found with increasing body weight. Moderate to severe hypoventilation and hypoxemia was present in pigs in both groups, necessitating supportive measures to be taken.

Antinociception

The antinociceptive effects of fentanyl and dexmedetomidine were compared in Paper I and Paper IV using the nociceptive withdrawal reflex (NWR) elicited by electrical stimulation, response to mechanical nociceptive stimulation and cardiovascular response to nociceptive stimulation. The NWR was scored by visually evaluating movement in Paper I and quantified by EMG in Paper IV.

In Paper I the observed NWR was abolished in all pigs with a dexmedetomidine infusion rate of 4 µg kg^{-1} h^{-1}. When fentanyl was administered the NWR was still present in two of eight pigs with the highest infusion rate.

The response to mechanical nociceptive stimulation differed from that observed when electrical stimulation was used in Paper I. The response to claw clamping disappeared in all pigs when the lowest infusion rate of fentanyl was given. In contrast the response to clamping was only abolished in all pigs towards the end of the evaluation period of the highest infusion rate when dexmedetomdine was administered

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In Paper I there was a significant increase in arterial blood pressures in response to nociceptive stimulation in both dexmedetomidine and fentanyl treated pigs at the time point where similar anaesthetic depth was reached, while heart rate did not change. The increase in blood pressure was more pronounced when the pigs were given fentanyl. Upon visual inspection of the descriptive statistics, the increase in MAP was larger at the time points where mechanical nociceptive stimulation was performed in addition to NWR, and the increase was more noticeable when the pigs were given fentanyl.

When isoflurane was used as background anaesthesia in Paper VI, a significant higher odd ratio for not responding to mechanical stimulation was found when fentanyl was given compared to dexmedetomidine, thus confirming the findings from paper I. The NWR was quantified using EMG and expressed as the area under curve (AUC) of the recorded EMG response when plotted against the stimulation dose. The parameter was termed NWR-AUC and was significantly decreased from baseline with the two highest infusion rates of dexmedetomidine given. In pigs given fentanyl the

NWR-AUC increased from baseline with the lowest infusion rate used, before a decline was observed. A significant decrease from baseline was observed with the three highest infusion rates examined. The response to mechanical nociceptive stimulation differed from that observed when electrical stimulation was applied.

When isoflurane was used for background anaesthesia in Paper IV, a significant change in MAP was observed in response to nociceptive stimulation at the highest infusion rate of fentanyl and dexmedetomidine, while the change in pulse rate was non-significant in either group.

Other results

In paper I plasma concentrations of dexmedetomidine and fentanyl was measured at baseline and then every 20 minutes. The plasma concentration of dexmedetomidine at the time point of similar anaesthetic depth was 0.74 ± 0.12 ng ml^{-1}. The fentanyl plasma concentration reached 19.16 ± 4.29 ng ml^{-1} at the similar stage of the experiment. With both drugs an increasing plasma concentration was observed during each infusion period, and it seems that a steady state plasma concentration was not reached.

In pigs subjected to stepwise exsanguination plasma concentrations of propofol and alfaxalone were measured at baseline and after loss of 30% and 50% of the total blood volume (Paper V). The plasma concentrations increased in most pigs after 30% blood loss, and the median increase was above 100% after 50% blood loss. However, the variation in increase was large with both anaesthetic agents at both levels of blood loss. We could not establish a statistically significant difference between the relative increases in plasma concentrations of the two drugs after 50% blood loss.

Another interesting finding in Paper IV was the visually observed shivering and higher spontaneous EMG activity in pigs when fentanyl was given compared to dexmedetomidine. This was notable at the infusion rates up to 40 µg kg^{-1} h^{-1}. Dexmedetomdine depressed spontaneous EMG activity at all infusion rates.

Methodological considerations

Animals

All experiments were conducted according to the Norwegian regulation on animal experimentation, which is based on the Norwegian Animal welfare act and the EU Directive 2010/63/EU. Approval was granted by the Norwegian National Animal Research Authority with FOTS ID 7236 for Paper I, 14277 for Paper II, III and V, and 14629 for paper IV.

Origin of animals

For the first experiment (FOTS ID 7236) 8 mixed breed pigs (75% Norwegian Land Race, 25% Yorkshire and 25% Duroc) were acquired from 2 commercial farms. As far as possible standardisation of experimental animal genotype and phenotype has been looked upon as good laboratory practice when performing studies in research animals, including in pigs, aiming at reducing variation within the study population. As such acquiring animals from two different producers is not ideal and may introduce unwanted bias in an otherwise controlled experimental setting. Data for the last four Papers (FOTS ID 14277 and 14629) were collected from 16 mixed breed pigs (Norwegian Land Race 50% and Duroc 50%) that were acquired in 2 groups of 8 pigs from the Centre for Animal Experimentation at the Norwegian University of Life Sciences.

Body weight

Body weight had a significant effect on SpO_2 and RR in both treatment groups in paper II. We related this finding to a potential larger drug effect in heavier pigs, that probably is related to an allometric dose effect. A potential allometric dose effect has previously been described for medetomidine in pigs (Ranheim et al. 2013) and for propofol in children (Rigby-Jones & Sneyd 2011).

Sex

In Paper I all pigs were castrated, male pigs, while the data collected for the last four papers originated from pigs with different sex, with 10 castrated males and 6 females being included. In Paper III sex had an influence on effect size estimate for the arterial blood pressure measurements when the covariate was included in the mixed model analysis, illustrating that it should be included in the statistical analysis even if the influence of sex in this case was non-significant and small. Alternatively, the study design should include a randomisation that balances groups for sex. Difference in response to trauma and haemorrhage, as well as drug response has been described in between animals and humans of different sex (Pleym et al. 2003; Choudhry et al. 2005).

Taken together the genotype and phenotype of pigs can influence the data and their interpretation when conducting experiment in this species. The translation to other study populations, and more importantly to other species must be made with caution.

Equipotent doses and anaesthetic depth

Anaesthetic and analgesic drugs have different potency, and along with individual variation in the induced anaesthetic effect this makes defining equipotent doses difficult. General anaesthesia is

often characterised as a reversible state of unconsciousness, immobility and amnesia, where in addition, antinociception and dampening of autonomic reflexes is provided (Antognini & Carstens 2002). The ability to define comparable anaesthetic depth is paramount when comparing the effects of anaesthetic and analgesic agents on physiological functions. The characteristics of general anaesthesia mentioned above do not change simultaneously in a unidimensional way along the same axis, hence using one parameter alone is not sufficient. At the same time, creating a composite scoring system for general anaesthesia is challenging. A definitive quantification of anaesthetic depth has proven difficult in several species including pigs. The degree of cerebrocortical depression has been quantified with EEG, and modern EEG algorithms such as the bispectral index (BIS) have been used, also in pigs (Ruíz-López et al. 2020). Response to different modalities of nociceptive stimulation have also been quantified by examining immobility or cardiovascular indicators of nociception in pigs (Haga et al. 2001; Haga et al. 2002; Baars et al. 2013), but no standard for the assessment of anaesthetic depth is available.

In our experiments anaesthetic depth was assessed by examination of the ocular position and reflexes, the motoric response to nociceptive stimulation using electrical and mechanical nociceptive stimuli, the cardiovascular response to nociceptive stimulation, as well as the appearance of burst suppression during EEG monitoring. To define a time point for comparison of pharmacodynamic effects at similar anaesthetic depth in our study, immobility in response to nociceptive stimulation was used. In addition, we did a retrospective evaluation of the cardiovascular response to nociceptive stimulation.

Eye position and ocular reflexes

The evaluation of anaesthetic depth was partly performed by examining eye position and ocular reflexes such as the palpebral reflex and the corneal reflex. This evaluation is often performed

during clinical anaesthesia in animals (Grubb et al. 2020) , and an effect on eye position and movement during ether anaesthesia was also described by Guedel (Guedel 1927). A correlation between eye position and BIS has been described in children, but the individual variation was rather large (Kook et al. 2018). The evidence for the use of eye position and ocular reflexes as a reliable guide to anaesthetic depth in animals is very scarce (Herbert & Murison 2013). Ocular signs will also vary depending on the anaesthetic agents used. In addition, interspecies variation also exists, with equines often having intact palpebral reflexes during a surgical plane of anaesthesia, while it is usually abolished in dogs. Eye position and ocular reflexes must always be used in conjugation with other signs of anaesthetic depth.

Nociceptive stimulation

Electrical nociceptive stimulation was used in paper I with a single stimulus that consisted of a train-of-five of square wave pulses with 1 ms duration and a frequency of 200 Hz. This stimulus was applied over the nerve for 60 seconds with a current intensity of 40 mA and a frequency of 1 Hz. The first time point where absence of motoric response to this electrical stimulus was documented was taken as the time point of similar anaesthetic depth. Electrical current has previously been used to evoke nociception during general anaesthesia in several species, including dogs, pigs and humans (Quasha et al. 1980; Lervik et al. 2012; Spadavecchia et al. 2012). Quantification of the NWR has often been made with EMG, but in addition, visible withdrawal of the stimulated limb has been shown to accompany the reflex response quantified by EMG in pigs (Spadavecchia et al. 2012). In the same publication the authors found that a decreasing NWR amplitude occurred with increasing isoflurane concentrations. The NWR amplitude has also been used to predict movement related to surgery in pigs anaesthetised with ketamine, with movement being abolished when the NWR amplitude approached zero (Baars et al. 2013).

In addition to electrical stimulation, mechanical stimulation was used to evoke motoric response during the experiments in Paper I. Mechanical nociceptive stimulation, often performed as claw or tail clamping, is commonly used during LTT and in clinical veterinary anaesthesia, as well as in animal studies of MAC determination (Antognini & Carstens 2002), with the goal being that the reflex should be abolished.

EEG

EEG was used to quantify the degree of cerebrocortical depression in our studies. Burst suppression may occur in pigs at concentrations below 1 MAC of isoflurane (Rampil et al. 1988), and it is often stated to be associated with a deep level of general anaesthesia in several species, including pigs when intravenous anaesthetics targeting $GABA_A$-receptors are administered (Llonch et al. 2011; Kenny et al. 2014). At the same time movement response to noxious stimuli has been documented in pigs during burst suppression anaesthesia (Haga et al. 2002; Haga et al. 2011). Hence, the presence of burst suppression in pigs during general anaesthesia should be taken as a sign of profound cerebrocortical depression, but spinally generated responses to noxious stimulation can still be observed.

Interestingly a difference in response to the two applied modalities of noxious stimulation was found in Paper I when comparing the two drugs examined. This led us to investigate responses against a different background anaesthesia (isoflurane) in Paper IV, where the findings were similar.

Evaluation of cardiovascular function

Pulse rate, ECG and blood pressure measurement

Instrumentation for cardiovascular evaluation was performed with the pigs in general anaesthesia.

Pulse rate, heart rate, ECG and direct systolic, diastolic and mean arterial blood pressures were

recorded using a multiparameter anaesthetic monitor (GE Carescape B650, GE Healthcare, Finland)

for all studies. All data, including ECG wave forms were automatically downloaded to a computer

and stored for later analysis.

For paper I, III and IV pulse rate was recorded from the invasive blood pressure trace, and for paper

II the pulse rate was recorded from the pulse oximeter trace. ECG was recorded using 3 leads with

adhesive electrodes placed on the palmar and plantar side of the metatarsus or phalanges. For

monitoring during experiments ECG lead II was used.

Direct arterial blood pressure was measured during experiments for paper I, III and IV. An arterial

cannula was percutaneously placed in the cranial tibial artery by a modified Seldinger method (paper

I and III) or with a conventional catheter-over-needle technique in the metatarsal artery (paper IV).

The cannula was connected to non-compliant fluid-filled tubing from a commercially available

pressure transducer. The pressure transducer was connected to the multiparameter monitor and

placed at the level of the right atrium, before zeroing to atmospheric pressure. The same

manufacturer of tubing and transducers, as well as the same length of tubing were used for all

experiments to maintain similar characteristics of the measurement system between individual pigs.

The transducers were not calibrated before each experimental session. The accuracy of disposable

blood pressure transducers has been shown to be very good (Gardner 1996), while to the authors knowledge precision has not been examined.

The signals were at all times observed by one investigator to detect artifacts or arrhythmias. In addition, erroneous measurements were excluded from the analysis after visual inspection during creation of the final database used for statistical analysis. Reasons for exclusion of data could for example be flushing of arterial catheters or movement artifacts, and only readings that were obviously false were rejected.

Cardiac output

Cardiac output was measured using a transpulmonary thermodilution technique in paper I and III. The methodology is based on indicator dilution method, where an indicator is injected rapidly on the venous side of the circulation, and the concentration of the indicator in arterial blood is plotted against time. For the thermodilution technique this includes calculating the cardiac output using the Stewart-Hamilton-equation, where cardiac output is inversely proportional to the area under the curve of temperature change:

$$Qt = \frac{(Tb - Ti) \times Vi \times K}{\int \Delta\, Tb \times dt}$$

T_b =   Temperature blood

T_i =   Temperature injectate

V_i =   Volume of injectate

K =   Correction constant

$\int \Delta\, T_b \times dt$ =   Area under the thermodilution curve

This methodology has traditionally involved placement of a Swan-Ganz-catheter, but for the

transpulmonary technique a bolus of cold saline or 5% glucose solution is injected through a central

vein, and the temperature change is measured in a central artery. To enable rapid injection of 10 ml

of cold saline solution by hand, a catheter introducer sheath placed in the external jugular vein was

used. The saline solution was cooled to below 0°C and kept on ice until injection, ensuring an

injectate temperature below 8°C. A thermistor tip catheter from the producer of the transpulmonary

cardiac output system was placed in the cranial tibial artery with the tip located in the femoral artery

using a modified Seldinger Technique. The thermistor tip catheter was connected to a

multiparameter monitor with a $PiCCO_2$-module installed, that allowed automatic calculation of the

cardiac output. The measurement was always performed in triplicate and recorded manually in a

research protocol. If a deviation of more than 10% from the mean was found, measurements were

repeated until all results were within this range. An average of the three measurements was used in

subsequent analysis and calculations.

Cardiac output measurement using transpulmonary thermodilution has shown good agreement

compared with techniques using thermistor catheters in the pulmonary artery (Sakka et al. 2012),

and a comparable precision has also been shown in pigs when comparing the two methods (Janda et

al. 2006). At the same time variations in agreement was documented in a study comparing the two

methods at different levels of cardiac output (Hüter et al. 2007). All measurements in our study

were performed in triplicates, and the need for repeated measurements due to variations in the 3

measurements was very low.

During low flow states in the experiments for Paper III we experienced problems obtaining

measurements. This was probably caused by recirculation and loss of indicator, leading to

overestimations of cardiac output followed by failing measurements (Nishikawa & Dohi 1993;

Monnet & Teboul 2017; Argueta & Paniagua 2019). The false increase in cardiac output occurring at

lower flow states may theoretically have obscured differences between the two protocols.

Oxygen debt

Blood samples for arterial and mixed venous blood gas analysis were aspirated from catheters

placed in the femoral and pulmonary artery in paper I and III. The samples were analysed within 15-

30 minutes using a bench top blood gas analyser.

To quantify oxygen debt during anaesthesia in paper I, and before and after the induction of

haemorrhagic shock in paper III arterial lactate was analysed. In addition, arterial base excess was

analysed in paper III. The content of oxygen in arterial and mixed venous blood was determined and

the oxygen extraction was calculated in paper I and III.

Oxygen deficit occurs when oxygen delivery is insufficient to meet oxygen demand, and the resulting

accumulated oxygen debt is strongly associated with mortality during haemorrhagic shock (Crowell

& Smith 1964; Dunham et al. 1991; Rixen et al. 2001). For clinical quantification of oxygen debt base

excess and lactate can be used (Rixen & Siegel 2005).

Oxygen extraction increases during states of haemorrhagic shock to compensate for a decrease in

DO_2 to maintain VO_2. When the extraction reaches a critical point anaerobic metabolism will

develop, with an increase in the metabolic acid concentrations (Rivers et al. 2015). The increase in

oxygen extraction will often precede anaerobic metabolism and increased lactate production (Burša & Pleva 2014). This was also visible in our study, where the oxygen extraction reached its near maximum level of around 9 ml dl^{-1} already after the first episode of haemorrhage where 30% of the blood volume was lost, with the increase in lactate following with a progression in blood loss.

Haemorrhagic shock model

In paper III haemorrhagic shock was induced. Total blood volume was estimated to be 65 ml kg^{-1} body weight. A 23 cm, 18G arterial catheter was placed percutaneously in the right femoral artery under ultrasound guidance using a modified Seldinger technique to allow for rapid exsanguination. Thirty percent of the total blood volume was manually withdrawn over 10 minutes from the femoral arterial catheter using a closed collection system with a 3-way stop-cock and a 60 ml syringe, followed by a 20 minutes period to allow for compensation. Thereafter 10% of the total blood volume was withdrawn over 10 minutes, followed by a 10 minutes period to allow for compensation. Thereafter 5% of the total blood volume was withdrawn every 10 minutes, followed by a 10 minutes period to allow for compensation between each period until cardiac arrest occurred. Cardiac arrest was defined as the time point where the $P_{E'}CO2$ dropped below 2.0 kPa.

A variety of models for the induction of haemorrhage has been employed in research related to haemorrhagic shock, including uncontrolled and controlled models. Controlled models can either be guided by blood pressure (Wiggers preparation), volume loss or oxygen debt. Our aim was to compare two anaesthetic regimes and their effect on cardiovascular physiology and oxygen debt in pigs during anaesthesia. A stepwise, volume controlled model allowed us to mimic a situation with reoccurring surgical haemorrhage, as would be encountered during LTT or experimental surgery in pigs. One could criticise our chosen model for several reasons: Surgical trauma was not induced, and the influence of tissue damage on haemodynamic physiology and homeostasis is therefore not

known (Hildebrand et al. 2013). An additional weakness of volume controlled shock models would be the interindividual variation in physiological consequences, that would also include drug metabolism. This is evident when comparing plasma concentrations in Paper V. Also, we did not examine the effect of resuscitation on cardiovascular performance in our pigs. Thus, potential differences between anaesthetic regimes in response to intravenous fluid therapy or other interventions is not known.

Evaluation of respiratory function

The effect of the different anaesthetic drugs on alveolar ventilation was determined by measurement of $P_{E'}CO_2$, tidal volume and respiratory rate in Paper II. $P_{E'}CO_2$ and V_T was measured with a pitot tube with a sample port for gas monitoring (Pedi Lite + Flow Sensor, GE Healthcare, Finland) that was fitted to the end of the endotracheal tube and connected to the monitor using the manufacturers tubing (Spirometry tube, disposable, yellow, GE Healthcare, Finland) and multiparameter anaesthetic monitor with a gas and spirometry module. The capnography trace was continuously inspected by one of the examiners to ensure correct determination of the $P_{E'}CO_2$. According to the manufacturer, the pitot tube allows measurements of tidal volumes from 5 to 300 mL. The respiratory rate was taken from the capnography trace. $P_{E'}CO_2$, inspired tidal volumes and respiratory rates were recorded every 5 seconds for 60 minutes and downloaded to a computer for later analysis.

The effect of the anaesthetic drugs on oxygenation of arterial blood was determined by pulse oximetry. SpO_2 was measured using a pulse oximetry finger sensor (TruSignal finger sensor, GE Healthcare, Finland) placed on the lateral digit of right hind- or front limb. The plethysmography trace was continuously inspected by one of the examiners to ensure proper signal quality. If a flattening of trace or a sudden drop in SpO_2 was observed the probe was repositioned, and the trace

was assessed again. The SpO_2 was recorded every 5 seconds for 60 minutes and downloaded to a computer for later analysis.

Evaluation of the respiratory effects of an anaesthetic regime can involve assessing alveolar minute ventilation and the oxygenation of arterial blood. Alveolar minute ventilation is determined by the V_T, the respiratory rate and the physiological dead space. The $PaCO_2$ is inversely proportional to alveolar minute ventilation and can be determined by arterial blood gas analysis. Arterial blood gas analysis also allows for quantification of the PaO_2, and thus evaluation of pulmonary function. As such, arterial blood gas analysis is considered gold standard when trying to assess respiratory function, but due to study design we decided not to sample arterial blood during the experiment for Paper II.

A major weakness in this study was the use of pulse oximetry, capnography and spirometry as methods for the assessment of oxygenation and alveolar minute ventilation. These methods have their limitations when it comes to both accuracy and precision (Bhavani-Shankar et al. 1992; Carlson & Jahr 1993).

Pulse oximetry

Several studies in human patients compare the performance of pulse oximetry to arterial oxygen saturation (SaO_2) showing a varying accuracy, but also that the accuracy is higher in the range from 80-100% (Jensen et al. 1998; Batchelder & Raley 2007). The precision and accuracy of pulse oximetry in newborn piglets has been examined, using an ear clip sensor placed on the thigh. The investigators found a deviation from measured SaO_2, in particular with values below 60% and during periods where hypoperfusion was suspected (Solevag et al. 2014). The saturations measured in the

current study were all in the range from 80-100%. In addition, none of the pigs experienced hypoperfusion or hypothermia that could have influenced pulse oximeter performance. Pulse oximetry plethysmography curves and capnography waveforms were continuously assessed during data sampling in this study to reduce the risk of inaccurate measurements.

Capnography

$PE'CO_2$ measured by capnography closely resembles $PaCO_2$ in conditions where only minor physiological dead space is present. However, anatomical dead space is constant, and a relatively larger proportion of each breath would be dead space if the tidal volume decreases. This will lead to variations in the measured $PE'CO_2$ that does not correspond to the true $PaCO_2$, and thus not reflect the true alveolar ventilation. The sampled expiratory gas may be contaminated by fresh gas flow from the anaesthetic machine, leading to dilution of the sampled gas. In our study side stream technology was used, and the sample gas was taken from an adapter mounted in the pigs' endotracheal tube without being connected to an anaesthetic breathing system.

Power calculation and statistics

Power, sample size calculations and hypothesis testing

Two conclusions are possible in statistical hypothesis testing: The null hypothesis can either be rejected and the alternative hypothesis accepted, or the null hypothesis is not rejected. When performing hypothesis testing it needs to be decided how certain we want to be prior to rejecting the null hypothesis. The level of probability for falsely rejecting the null hypothesis is the termed the α value, and it follows that if the null hypothesis is rejected, it is done with a probability of being correct given by 1 - α.

When performing statistics, the p-value describes the probability of obtaining the observed result or one more extreme given that the null hypothesis is true. To reject the null hypothesis the $p < \alpha$. To falsely reject the null hypothesis is termed to perform a Type I error. A commonly used α level is 0.05 (Columb & Atkinson 2015). This means that we accept the alternative hypothesis and are 95 % certain that we do not perform a Type I error.

A type II error would be not to reject the null hypothesis when the alternative hypothesis in fact is true. Prior to performing a study, it is good practice to estimate the probability it will result in rejection of the null hypothesis given that the alternative hypothesis is true, i.e. to avoid a Type II error. This probability is denoted $1 - \beta$ and is called the statistical power. The two values α and β are dependent of each other, hence if one is increased the other will decrease. When performing a sample size calculation a power 0.8 is commonly deemed as an acceptable level to aim for in clinical studies (Columb & Atkinson 2015).

Four factors must be included to calculate the sample size needed when performing a study: (1) The minimal clinical relevant difference (2) the estimated variability in the study population, often the standard deviation is used (3) the α-level and (4) the desired power based on the β-level (Columb & Atkinson 2015). Sample size calculations made *a priori* when performing animal experiments aims at preventing to include more animals than necessary, but enough animals to find a clinically relevant difference if present.

Another method that may contribute to the inclusion of an appropriate number of experimental animals in a study is a sequential study design with interim statistical analysis being performed.

When performing sequential studies, a pre-planned approached to interim statistical analyses is important; this will include a clearly defined hypothesis and predetermined criteria for early stopping (Neumann et al. 2017).

In paper II and III an *a priori* power analysis was performed, and the criteria for the interim statistical analysis were determined prior to starting the experiments. Both were based on one of the main outcome parameters used when comparing the effect of haemorrhage between treatment groups, namely arterial lactate concentration. When performing the interim statistical analysis, it became evident that a large number of pigs would have to be included to eventually find a difference in lactate concentration between groups. At the same time, when the final statistical analysis was performed, it was deemed likely that a statistically significant difference could have been established for other parameters examined if more animals had been included. For the final interpretation of the physiological findings' absolute changes over time, visual evaluation of descriptive statistics and the interpretation of 95% confidence intervals were considered equally important to p-values when extrapolating practical consequences of either treatment.

Statistical methods for longitudinal data

Modern technology applied in patient monitoring and for data registration allows researchers to record repeated measurements at a high frequency during anaesthesia, referred to as longitudinal data. A major advantage of such data is that they provide high resolution observations that allows for an accurate description of physiological events during anaesthesia. The frequent sampling of data will however make statistical comparisons more complex. These observations are not independent from each other, thus precluding some traditional statistical techniques. Such longitudinal data can principally be analysed in 3 ways: By using summary statistics, by repeated measures ANOVA or by regression based techniques (Schober & Vetter 2018). Modern regression-

based techniques offer some advantages over the more traditional ANOVA-based approach, such as their ability to correct for within- and between subject categorical factors. In addition non-normally distributed and missing data can be problematic when using ANOVA (Schober & Vetter 2018). A major disadvantage when using modern regression-based analysis, such as mixed effects models is the complexity of the method. When performing such analysis guidance or assistance from professional statisticians will provide security for clinicians.

Synopsis / General discussion

Total intravenous anaesthesia in pigs with either alfaxalone or propofol in combination with ketamine and dexmedetomidine provides excellent cardiovascular stability in healthy, normovolemic pigs. We additionally have demonstrated that pigs anaesthetised with this regime tolerate moderate to severe blood loss, and that antinociception is provided. It is however evident that respiratory support must be provided when administering the doses used in our study, and that dose adjustments are necessary during moderate to severe blood loss.

Cardiovascular findings in normovolemic pigs (paper I and IV)

In paper I pigs anaesthetised with dexmedetomidine in addition to propofol and ketamine displayed higher heart rate than pigs given fentanyl at what we defined as similar anaesthetic depth. The decrease in heart rate from baseline throughout the study period was only modest in the pigs given dexmedetomidine, despite relatively high doses being used. This result was quite surprising, as dexmedetomidine induces prominent bradycardia in several other species (Marcilla et al. 2012; Pascoe 2015; Davy et al. 2017), and a dose dependent reduction of heart rate has also been documented in pigs given lower doses of dexmedetomidine in combination with propofol (Sano et al. 2010), although the breed of pig used was not clearly stated. A potential explanation for the

maintained heart rate in our first study was the concurrent use of ketamine; a drug known to increase heart rate (Traber et al. 1968; Traber et al. 1970). We did however replicate our finding from paper I against a different background anaesthesia in paper IV, making this explanation less likely.

Despite the modest decrease in heart rate in pigs given dexmedetomidine in paper I, a decrease in DO_2 over time was found. This was related to both a lowering of stroke volume and heart rate over time. The DO_2 was however significantly higher than for pigs given fentanyl, mainly due to a higher haemoglobin level in the dexmedetomidine group.

Another finding from a pilot investigation performed in these pigs was the lack of chronotropic response when haemorrhage was induced. This finding could be attributed to the lack of chronotropic response to hypotension observed in humans when propofol is administered (Cullen et al. 1987; Ebert et al. 1992).

Cardiovascular findings in hypovolemic pigs (paper III)

In paper III we demonstrated that a substantial blood loss was possible with both anaesthetic regimes before cardiac arrest occurred in a stepwise exsanguination model. In addition we found that the level of oxygen debt tolerated by the pigs was similar to previous published results (Rixen et al. 2001). We could however not document a significant difference in tolerance to blood loss or oxygen debt at different levels of blood loss between the two anaesthetic agents examined.

Pigs anaesthetised with alfaxalone in combination with ketamine and dexmedetomidine had significantly higher systolic arterial blood pressure than pigs given propofol. A difference could not be established for mean and diastolic arterial blood pressures, but this lack of statistical significance can be related to study power.

After the first experiments the lack of chronotropic response during haemorrhage and hypotension warranted further investigation. We thus wanted to compare this response when alfaxalone was used instead of propofol. Alfaxalone seems to maintain the baroreceptor response better than propofol during anaesthetic induction in dogs (Okushima et al. 2015), and pigs anaesthetised with alphaxlone/alphadolone maintained higher heart rates compared to when propofol was used in pigs subjected to haemorrhage (Ruane-O'Hora et al. 2011). We could not demonstrate a statistical difference in pulse rate between the two treatments during hypotension in paper III. Upon visual inspection of the descriptive statistics a higher mean heart rate in pigs given alfaxalone is evident, with a large variation within both groups. The inclusion of more animals in our experiment could have yielded a different result of the statistical comparison, but both our a priori sample size analysis and the interim statistical analysis were based on the difference in lactate between the two anaesthetic agents. Due to the finding that 458 pigs would have to be included in order to discover a statistically significant difference in lactate between treatments, given that the difference was similar to the difference found after the first 16 pigs, the experiments were halted.

The stepwise haemorrhage model allowed time for compensation between the episodes of blood loss. Both changes in macrocirculatory parameters and indices of oxygen debt indicate that physiological compensation occurred during these time periods. This is particular evident for the parameters where continuous recording was possible. Both a decrease in pulse rate and increase in arterial blood pressures could be observed, in particular when blood loss was less than 55% of the

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total blood volume. In a previous study a 25% probability of death was estimated to occur at an oxygen debt of approximately 75 ml kg^{-1}, that corresponded to an increase in lactate to 5.6 mmol l^{-1}. At the same level of blood lactate in our pigs only 1 of 16 pigs had experienced cardiac arrest. In addition, a reduction in oxygen extraction was observed during the first compensatory period. This was also the only time period where blood gas sampling was performed after and prior to an episode of haemorrhage, thus conclusion can only be made for this phase of the experiment. Taken together anaesthesia with either propofol-ketamine-dexmedetomidine or alfaxalone-ketamine-dexmedetomidine allows for physiological compensation during blood loss up to 50% of the total blood volume, and the development of oxygen debt that is correlated with a high mortality in other studies of haemorrhagic shock in pigs is delayed (Rixen & Siegel 2005).

Respiratory function (paper II)

In pigs given alfaxalone alveolar ventilation was significantly better that in pigs given propofol, with a significantly lower $P_{E'}CO_2$ in pigs given alfaxalone. Tidal volumes were similar between treatments, but pigs anaesthetised with alfaxalone maintained a higher respiratory rate compared to pigs given propofol. A similar difference in $P_{E'}CO_2$ has been published when alfaxalone or propofol was used to maintain anaesthesia in cats, while in a study in dogs such a difference could not be established (Suarez et al. 2012; Campagna et al. 2015). In a pilot study including 12 pigs alfaxalone was used to maintain anaesthesia. Despite some unclarity in their reporting of data, it seems that the investigators found some degree of hypercapnia, despite the pigs having a high respiratory rate. Hypoxemia was avoided, probably due to the supplementation of oxygen (Bigby et al. 2017).

In our statistical analysis we found that body weight had a significant effect on both respiratory rate and SpO_2. One possible explanation for this finding could be that heavier pigs reached higher plasma

concentrations of the infused drugs, and the allometric effect on drug plasma concentration has been discussed elsewhere in the thesis.

In pigs anaesthetised with either alfaxalone-ketamine-dexmedetomidine or propofol-ketamine-dexmedetomidine respiratory function was compromised to an extent that they needed supportive treatment during anaesthesia. The addition of supplemental oxygen therapy or intermittent positive pressure ventilation was necessary in 25% and 37.5% of pigs in the groups respectively. Hence, when using this anaesthetic regime in pigs, means for providing supportive measures must be available.

Antinociceptive effects (paper I and IV)

In paper I we found that depression of the response to electrical nociceptive stimulation occurred in all pigs when 4 µg kg^{-1} h^{-1} of dexmedetomidine was infused, while a response was still elicited in some pigs even at the highest infusion rate of fentanyl. When mechanical nociceptive stimulation was applied, the motor response was only depressed in all pigs at the highest infusion rate of dexmedetomidine, while the motor response disappeared already at the lowest infusion rate of fentanyl. This difference was surprising, but differences in response to different modalities of nociceptive stimulation has previously been described in isoflurane anaesthetised pigs (Spadavecchia et al. 2012), where the NWR was present well past 1 x MAC as defined by mechanical stimulation. Our experiment was not designed to examine possible mechanism for this difference.

In a second experiment different infusion rates of dexmedetomidine and fentanyl were given to pigs against a different background anaesthesia. In this experiment fentanyl depressed the motor response to mechanical nociceptive stimulation more consistent than dexmedetomidine, while the

NWR was more readily depressed by dexmedetomidine. A motor response to claw clamping was observed in some pigs even during the highest infusion rate of dexmedetomidine.

Taken together the finding from both experiments highlights the importance of the chosen stimulation paradigm when evaluating the effect of antinociceptive drugs. In addition, we believe that profound cerebrocortical depression and unconsciousness can be present in pigs despite a motoric response being elicited by nociceptive stimulation. One main goal of general anaesthesia during surgery is however immobility. In the authors experience anaesthesia in pigs for surgical biomedical research and LTT can be performed without movement being observed with infusion rates of propofol-ketamine-dexmedetomidine where mechanical claw clamping elicited a withdrawal response in some animals.

The change in MAP during nociceptive stimulation was larger at the time points where mechanical stimulation was performed in addition to electrical stimulation in paper I. Upon examination of the descriptive statistics it seems evident that the increase in MAP is more prominent during fentanyl infusion. This is even true during the highest infusion rates where a response to mechanical stimulation still was abolished in both groups, meaning that the stimulation time was similar between groups. The difference in change in heart rate response is not as prominent as the change in MAP, but interestingly some animals in both groups respond to nociception with a decrease. Both an increase in MAP and decrease in heart rate in response to nociceptive stimulation has previously been observed in horses and in pigs (Haga et al. 2001; Haga & Dolvik 2005).

Other findings (paper I, IV and V)

The mean plasma concentration of dexmedetomdine at similar anaesthetic depth was 0.74 ng ml^{-1}. This is similar to levels that provide analgesia in other species (Pascoe et al. 2006; Risberg et al.

2014), but a direct comparison has limitations as the methodology differs between studies. In addition, our experiment was not designed to find the lowest plasma concentration where analgesia can be observed in pigs. In our study, this plasma concentration of dexmedetomdine corresponded to an infusion rate of approximately 4 µg kg^{-1} h^{-1}. In combination with propofol and ketamine, this provided profound analgesia including suppression of the cardiovascular response to nociception, however motoric response to clamping of the dewclaw was still present in some pigs. There is scarce information in the literature on the use of low-dose infusions of dexmedetomdine in pigs. A relatively low dose of medetomidine has been used for balanced anaesthesia in pigs undergoing abdominal surgery, but the study was not specifically standardised to evaluate analgesic efficacy (Calzetta et al. 2014). In other studies, higher doses of medetomidine have suppressed the response to mechanical nociceptive stimulation (Nishimura et al. 1993; Sakaguchi et al. 1996; Tendillo et al. 1996; Malavasi et al. 2008). Fentanyl is commonly used in pigs anaesthetised for experimental purposes, but in higher doses than in humans or dogs. Fifty to 200 µg kg^{-1} h^{-1} reduced the minimum alveolar concentration (MAC) of isoflurane by 24.5 to 45.9%, with plasma concentrations ranging from 14 to 59 ng ml^{-1} (Moon et al. 1995). In contrast much lower plasma concentrations are needed for similar MAC reduction in human subjects (McEwan et al. 1993). Most pigs in our study reached the point where no NWR was observed only at the highest infusion rate of fentanyl, with a mean plasma concentration of 19.16 ng ml^{-1}.

Moderate to severe blood loss in pigs anaesthetised with either propofol-ketamine-dexmedetomidine or alfaxalone-ketamine-dexmedetomidine will lead to changes in plasma concentrations of both propofol and alfaxalone, with a substantial increase observed in some individuals after 30 and 50% blood loss. However, we could not detect a statistically significant difference in the change in plasma concentrations between the two drug protocols. When using

either propofol of alfaxalone for anaesthesia during substantial blood loss in pigs, adjustments of the infusion rate are probably necessary.

An additional finding in Paper IV was the effects of fentanyl on the muscular activity in isoflurane anaesthetised pig. Shivering during anaesthesia has been anecdotally reported in pigs and has by some veterinary anaesthetists been attributed to the use of fentanyl. Shivering in anaesthetised pigs after fentanyl administration has been reported in the literature (Ringer et al. 2015), and in a literature review on the use of neuromuscular blocking agents in pigs, several authors attributed their use to the prevention of shivering (Bradbury & Clutton 2016). In Paper IV we found an increased spontaneous EMG activity in pigs given fentanyl at infusion rates from 5 to 20 µg kg^{-1} h^{-1} when compared to baseline, in addition to macroscopical shivering being observed. This was not observed when dexmedetomidine was given. The mechanisms behind this finding are not known and warrant further investigations.

Conclusions

Total intravenous anaesthesia with propofol-ketamine-dexmedetomidine or propofol-ketamine-fentanyl provides stable cardiovascular conditions in healthy, normovolemic pigs. When dexmedetomidine is given as a constant rate infusion to healthy pigs anaesthetised with either propofol-ketamine or isoflurane, the decrease in heart rate is not as prominent as often observed in other species. Pigs anaesthetised with propofol-ketamine-dexmedetomidine or alfaxolone-ketamine-dexmedetomidine tolerate a substantial blood loss before cardiac arrest ensues, and the observed development of oxygen debt is as expected based on previous observations by other authors. Systolic blood pressure is higher in pigs anaesthetised with alfaxolone compared to when propofol is used.

Respiratory function is impaired when healthy pigs are anesthetised with propofol-ketamine-dexmedetomidine or alfaxolone-ketamine-dexmedetomidine, and both hypoxemia and hypoventilation was observed by us. Supportive measures such as oxygen supplementation and positive pressure ventilation must be available when using these anaesthetic regimes in pigs.

Both anaesthesia with propofol-ketamine-dexmedetomidine or propofol-ketamine-fentanyl provide solid antinociception, but based on the cardiovascular response and the observed depression of the NWR, the protocol containing dexmedetomidine seems superior to fentanyl. A difference in response to nociceptive stimulation between pigs given dexmedetomidine or fentanyl was also observed with different modalities of nociceptive stimulation. As such, the modality of nociceptive stimulation must be considered when evaluating the antinociceptive properties of a drug. Additionally, increased muscular activity was documented in pigs when fentanyl was given at lower infusion rates during isoflurane anaesthesia. The reason for this observation was not elucidated.

When administering propofol or alfaxalone intravenously to pigs subjected to moderate to severe blood loss, plasma concentrations of both drugs may increase. Hence, dose adjustments can be necessary.

In conclusion, total intravenous anaesthesia with either propofol-ketamine-dexmedetomidine or alfaxalone-ketamine-dexmedetomidine are suitable anaesthetic protocols when providing general anaesthesia to pigs during live tissue training or biomedical research when cardiovascular instability or surgically induced nociception is foreseen.

Future perspectives

The cardiovascular response to resuscitation was not evaluated in our experiments and should be examined further. Additionally, evaluation of the introduction of these anaesthetic regimes in a controlled, clinical setting, such as during live tissue training, should be performed.

The differences in response between pigs given dexmedetomidine and fentanyl to different modalities of nociceptive stimulation also warrants further investigation. Also, the mechanisms behind the observed increased muscular activity and shivering in pigs given fentanyl should be elucidated.

Additional examinations on the potential influence of this or other anaesthetic regimes on research results should be considered, and further examinations on how anaesthesia influences physiological organ function in healthy pigs or pigs with induced pathology such as sepsis, traumatic brain injury or acute kidney injury warrant attention.

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Papers I-V

RESEARCH PAPER

Dexmedetomidine or fentanyl? Cardiovascular stability and analgesia during propofol-ketamine total intravenous anaesthesia in experimental pigs

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Abstract

Objective To compare cardiovascular function and response to nociception during total intravenous anaesthesia in pigs with propofol, ketamine and either dexmedetomidine or fentanyl administered as a continuous infusion.

Study design Blinded, randomized, balanced, crossover study

Animals Eight immunocastrated male, mixed breed pigs with a mean ± standard deviation body weight of 26.4 ± 1.9 kg for dexmedetomidine and 27.5 ± 3.8 kg for fentanyl treatment.

Methods The animals were anaesthetized twice with either propofol—ketamine—dexmedetomidine (DEX) or fentanyl (FENT). DEX was infused at 2, 4 and 8 $\mu g\ kg^{-1}\ hour^{-1}$ and FENT at 25, 50 and 100 $\mu g\ kg^{-1}\ hour^{-1}$. Each infusion rate was administered for 80 minutes prior to commencing the next. Heart rate (HR), 3-lead electrocardiogram, systolic, mean and diastolic arterial blood pressure (SAP, MAP, DAP) in addition to cardiac output measured by transpulmonary thermodilution was used to monitor cardiovascular function. Mechanical and electrical stimulation (nociceptive withdrawal reflex, NWR) was used to elicit nociceptive responses. Similar anaesthetic depth was determined based on the NWR response. Cardiovascular parameters were compared statistically at this time. Additionally, response to nociceptive stimulation and cardiovascular response over time were compared.

Results DEX-treated pigs had significantly higher HR, SAP, DAP, MAP, systemic vascular resistance, haemoglobin concentration, content of oxygen in arterial and venous blood and oxygen delivery index than FENT-treated pigs at similar anaesthetic depth, whereas stroke volume index was significantly higher in FENT. Motoric response to mechanical nociceptive stimulation was abolished prior to any decrease in NWR response in FENT, whereas the two responses decreased more in unison in DEX. The cardiovascular response to nociception was less pronounced in DEX than in FENT.

Conclusions and clinical relevance Propofol combined with ketamine and either fentanyl or dexmedetomidine provides stable cardiovascular conditions in normovolaemic, healthy pigs. Based on cardiovascular response and depression of NWR, dexmedetomidine apparently provides superior analgesia to fentanyl.

Keywords dexmedetomidine, fentanyl, ketamine, pigs, propofol.

Introduction

Pigs are used frequently for biomedical research or medical training purposes because their cardiorespiratory anatomy and physiology are thought to resemble that of humans closely (Hildebrand et al. 2013).

To decrease the number of animals used in experiments and to ensure animal welfare, cardiovascular stability, unconsciousness and antinociception are equally important features of an anaesthetic regime for these animals. According to a review article on haemorrhage and trauma research in pigs, volatile anaesthetics have most often been used for this purpose (Hildebrand et al. 2013). Inhalant anaesthetic drugs are titrated easily to effect, and a rapid change in alveolar and brain concentrations is possible. However, they cause decreased systemic vascular resistance and induce hypotension in several species including pigs (Lundeen et al. 1983; Tomiyasu et al. 1999). Unconsciousness and immobility are reliably achieved; however, volatile anaesthetic drugs do not provide peripheral or spinal antinociception (Antognini et al. 2005; Kim et al. 2007) and are less potent for inducing immobility in pigs than in other species (Haga et al. 2011).

Anaesthetic protocols based on injectable drugs may have properties more suitable for situations where maintenance of sympathetic tone, systemic vascular resistance and normotension is important (Haskins & Patz 1990; Englehart et al. 2008). Fentanyl is often used as an adjunct in pigs anaesthetized for research purposes where cardiovascular instability is expected (Hildebrand et al. 2013). It provides antinociception, reduces the amount of anaesthetic agent needed and can thereby improve cardiovascular performance, although higher infusion rates are needed in pigs than in several other species (Moon et al. 1995; Kurita et al. 2003). Propofol combined with fentanyl has been used to anaesthetize pigs for cardiovascular research purposes, providing hypnosis and analgesia with a relatively stable cardiovascular status (Kurita et al. 2003; Schoffmann et al. 2009). Propofol can also be combined with ketamine for the induction and maintenance of anaesthesia in humans and dogs, resulting in improved cardiovascular function compared with propofol alone (Smischney et al. 2012; Martinez-Taboada & Leece 2014; Kennedy & Smith 2015), as ketamine seems to offset the decrease in systemic vascular resistance

caused by propofol. Additionally, ketamine provides antinociception by NMDA antagonism (Kohrs & Durieux 1998).

Dexmedetomidine is an α-2-receptor agonist with sedative and analgesic properties. Continuous infusion of dexmedetomidine provides antinociception and reduces the minimal alveolar concentration of volatile anaesthetics in dogs (Pascoe et al. 2006; Lervik et al. 2012). In pigs, dexmedetomidine induces sedation after intramuscular injection (Nunes et al. 2007). However, α-2 agonists induce unwanted cardiovascular effects such as bradycardia, increased systemic vascular resistance, reduced cardiac output and oxygen delivery in several species even when infused in low doses (Murrell & Hellebrekers 2005; Sano et al. 2010). A study in miniature pigs using a pressure-controlled haemorrhagic shock model found that pigs administered a combination of ketamine and medetomidine maintained a higher initial blood pressure and tolerated withdrawal of a larger blood volume than pigs administered propofol and remifentanil and (Brezina et al. 2010). To the best of our knowledge, there are no studies on pigs directly comparing the cardiovascular effects of dexmedetomidine with the more commonly used analgesic drug fentanyl.

The nociceptive withdrawal reflex (NWR) has been used when studying the dose effects of anaesthetic and analgesic drugs in anaesthetized animals (Spadavecchia et al. 2006; Levionnois et al. 2010; Lervik et al. 2012). The NWR has advantages over mechanical dewclaw clamping when applied repetitively because minimal tissue damage and inflammation is evoked. In addition, electrical stimulation intensity, observed limb withdrawal, electromyography activity, and isoflurane concentration correlated strongly in pigs (Spadavecchia et al. 2012), leading us to believe that the NWR can be used when defining anaesthetic depth.

The primary objective of this study was to compare the cardiovascular status at similar anaesthetic depth in pigs anaesthetized with propofol−ketamine in combination with either fentanyl or dexmedetomidine. Secondary objectives were to describe: 1) plasma concentrations at different infusion rates of dexmedetomidine and fentanyl; 2) the response to nociceptive stimulation with the two treatments; and 3) the cardiovascular changes during the course of the experiment.

Materials and methods

Animals

The study was approved by the Norwegian National Animal Research Authority (FOTS ID 7236). Eight immunocastrated, male, mixed breed pigs (Norwegian Land Race 75%, Yorkshire 25% and Duroc 25%) were enrolled and identified based on ear tag number. The pigs were housed in a research animal facility at the Norwegian University of Life Sciences for 7 days prior to and during the wash out period between the experimental sessions. They were fed a combination of a commercial pig diet for slaughter pigs and hay with free access to water. Their health status, including surgical wounds, was monitored twice daily throughout the experimental period.

Study design

A blinded, balanced, cross over study design was used. Treatments were randomized prior to the study in blocks of four. A sequential experimental design was used where a statistical interim comparison for the main outcome variable (cardiac output, $\dot{Q}t$) was performed after eight pigs were included in the study. All investigators involved in the experimental sessions were unaware of the treatment administered to individual animals. There was a minimum 7-day wash-out period between experimental sessions.

Anaesthesia, instrumentation and monitoring

Food, except hay and water, was withheld for 12 hours prior to each experimental session. All pigs were deemed healthy prior to each anaesthesia session based on a clinical examination.

Ketamine (Narketan; Vetoquinol, France) 15 mg kg^{-1} in combination with midazolam (Midazolam; B. Braun, Germany) 1 mg kg^{-1} was administered intramuscularly (IM). After preoxygenation by a face mask, an intravenous (IV) catheter (Venflon Pro; Beckton Dickinson Infusion Therapy, NJ, USA) was placed in the auricular vein, and if needed, propofol (Propofol-Lipuro; B. Braun, Germany) was titrated IV to allow endotracheal intubation. A ventilator (Dräger Evita; Dräger, Germany) was used to deliver mechanical ventilation in a volume-controlled mode. The pigs' lungs were ventilated using 100% oxygen with a respiratory rate (f_R) of 20 breaths minute^{-1}, an inspiratory to expiratory ratio of 1:2 and a positive-end-expiratory pressure of 0 cmH$_2$O, with the tidal volume adjusted to maintain normocapnia [end-tidal carbon dioxide ($P_E'CO_2$) 4.5–6.0 kPa]. Anaesthesia was maintained with propofol at 8 mg kg^{-1} hour^{-1} and ketamine at 5 mg kg^{-1} hour^{-1} IV delivered by a syringe driver (Graseby 3500; Smiths Medical, UK) during the instrumentation period of 90 minutes. All pigs were administered a balanced electrolyte solution (Ringers acetate; Fresenius Kabi, Norway) IV at a rate of 10 ml kg^{-1} hour^{-1} delivered by a volumetric infusion pump (Volumat Agila; Fresenius Kabi, Germany).

For transpulmonary thermodilution measurements and mixed venous blood sampling, a double lumen measuring 60 cm, 6 Fr. balloon pulmonary artery catheter (PAC) (Balloon Wedge Pressure Catheter; Arrow Int. Inc., NC, USA) was placed through an introducer sheath of 10 cm, 6 Fr. (Percutaneous sheath introducer set; Arrow Int. Inc.) after surgical access to one jugular vein. The catheter was advanced while attached to a pressure transducer (TruWave pressure monitoring transducer; Edwards Lifesciences Corp., CA, USA), and placement of the tip in the pulmonary artery was ensured by observation of the characteristic waveform and pressures. In addition, a thermistor tip measuring 20 cm, 5 Fr. thermodilution catheter (PiCCO Catheter; Pulsion Medical Systems SE, Germany) was placed percutaneously by a modified Seldinger technique in the tibial artery. The catheter was connected to a pressure transducer set (TruWave pressure monitoring transducer; Edwards Lifesciences Corp.) fixed at the level of the sternum and zeroed to atmospheric pressure, allowing continuous blood pressure measurement in addition to transpulmonary thermodilution measurement of $\dot{Q}t$. The pigs were monitored using a multiparameter anaesthetic monitor (GE Carescape Monitor B650; GE Healthcare, Finland). Heart rate (HR), f_R, 3-lead electrocardiogram, oxygen saturation, $P_E'CO_2$, fraction of inspired oxygen, systolic, mean and diastolic arterial blood pressure (SAP, MAP, DAP) and body temperature measured from an arterial thermistor were recorded and automatically downloaded to a computer.

At the end of the first anaesthesia, infusions were stopped, central catheters were removed and surgical wounds were sutured and bandaged. The pigs recovered from anaesthesia in the stalls of the research animal facility. A trained veterinary anaesthetist monitored recovery. Meloxicam (Metacam; Boehringer Ingelheim Vetmedica GmbH, Germany) 0.4 mg kg^{-1} was administered IV, and amoxicillin (Clamoxyl Vet; Zoetis, Finland) 20 mg kg^{-1} was administered IM. In case of excitation during

recovery, 5 µg kg^{-1} of dexmedetomidine (Dexdomitor; Orion Corporation, Finland) was administered IV.

At the end of the second anaesthesia, all animals were euthanized by an IV injection of potassium chloride while still anaesthetized.

Infusion of test drugs

A minimum of 90 minutes after endotracheal intubation, baseline (BL) recordings were performed, and an infusion of either dexmedetomidine (Dexdomitor; Orion Corporation, Finland) or fentanyl (Fentanyl; Hameln Pharma Plus GmbH, Germany) was started using a syringe driver (Graseby 3500; Smiths Medical, UK). Dexmedetomidine (0.5 mg mL^{-1}) was diluted in 0.9% NaCl to a concentration of 4 µg mL^{-1} and infused at 2, 4 and 8 µg kg^{-1} hour^{-1}. Fentanyl (50 µg mL^{-1}) was infused at 25, 50 and 100 µg kg^{-1} hour^{-1}. Each infusion rate was started at the lowest rate and administered for 80 minutes prior to commencing the next.

Cardiovascular evaluation

The $\dot{Q}$t was measured at time points BL, 80, 160 and 240 minutes before nociceptive stimulation (see description below) and when increasing the infusion rate to the next level. An additional measurement of $\dot{Q}$t was performed at the first time point when the NWR response reached a score of four as described later. Between NWR determination and measuring $\dot{Q}$t, 2 minutes were allowed at this time point. For the transpulmonary thermodilution measurement, 10 mL of cold saline was injected as a rapid bolus through the side port of the introducer sheath that was fitted with an injectate temperature sensor housing (Pulsion Medical System SE, Germany) after entering patient data in the monitor system. The $\dot{Q}$t measurement was repeated three times at each time point. If a deviation of more than 10% from the mean of these measurements was experienced, additional measurements were performed until three measurements within this range were obtained. In addition to $\dot{Q}$t, HR, SAP, DAP and MAP were recorded from the anaesthetic monitor.

NWR and mechanical nociceptive stimulation

For the NWR, self-adhesive surface electrodes (Ambu Neuroline 700; Ambu, MD, USA) were placed over the fourth metacarpal bone after clipping and cleansing for transcutaneous electrical stimulation of the palmar nerve. The resistance of each electrode pair was kept below 3 kΩ. The electrical stimulus consisted of a train of five 1 ms constant current square-wave pulses delivered at a frequency of 200 Hz with an electrodiagnostic device (Viking Quest; Cephalon, Denmark). This single stimulus was applied over the nerve for 60 seconds with a current intensity of 40 mA and a frequency of 1 Hz. The same investigator always scored the NWR response throughout the experiment on a four-point scale by visual and tactile observation with: 1) clear leg movement visible; 2) less pronounced leg movement visible; 3) weak movement, almost not visible but sensed; and 4) no detectable movement. The NWR determination was performed at time point BL and thereafter every 10 minutes throughout the experimental session.

Mechanical stimulation was applied to the left (lateral and medial interchangeably) dewclaw of the hind limb by a latex-coated forceps with a clamping area 1 × 1 cm. The clamping pressure was monitored with a spring balance attached to one forceps arm, and the force applied at clamping was 100 N. The same investigator recorded the latency to withdrawal of the limb. If no withdrawal reflex was observed during stimulation, the response was recorded as absent. Stimulation was stopped at withdrawal of the limb or if vigorous movement in other limbs or whole body movement was observed. Maximum clamping time for each stimulus was 60 seconds, and latency was set to 60 seconds. Mechanical stimulation was performed at time point 10 and thereafter every 20 minutes throughout the experimental session.

Similar anaesthetic depth was defined as the first time point when the NWR score was 4. In cases where a score of 4 was not reached, data from time point 240 minutes were used for statistical comparison.

Electroencephalography

For recording of a two-channel referential electroencephalography (EEG), needle electrodes (Aiglette; Technomed Europe, Netherlands) were used. Electrodes were placed 1 cm caudal to the lateral angle of the eye and 1 cm medial to the temporal line bilaterally, and these electrodes were referred to an electrode placed in the median plane 2 cm caudal to the recording electrodes. A ground electrode was placed caudal to the atlas wing. The resistance of each electrode pair was maintained below 3 kΩ. The electrodes were connected to an EEG monitor (A-

1000; Aspect Medical Systems, MA, USA). The monitor filters were set as follows: high frequency filter: 50 Hz, 50/60 Hz filter: 50 Hz and low frequency filter: 2.0 Hz. The monitor automatically detected burst suppression and calculated the percentage of epochs in the previous 63 seconds where the EEG signal was considered suppressed. This percentage is called suppression ratio and was updated every 5 seconds.

Blood sampling and analysis

Arterial and mixed venous blood samples were obtained simultaneously from the arterial and PAC at BL and thereafter every 20 minutes before each nociceptive stimulation process throughout the experiment. Samples were drawn into heparinized blood gas syringes (Pico 70; Radiometer, Denmark) and closed by a cap. They were analyzed within 45 minutes using a blood gas analyser (ABL 800 Flex; Radiometer). Simultaneously, heparinized blood samples were withdrawn from the PAC for analysis of dexmedetomidine and fentanyl plasma concentrations. Plasma concentrations for fentanyl were determined using a liquid chromatography–tandem mass spectrometry method developed for the qualitative and quantitative determination of fentanyl in porcine plasma (Berg et al. 2013). Dexmedetomidine was measured using liquid chromatography–tandem mass spectrometry according to an earlier description (Ranheim et al. 2015).

Calculations and statistical analysis

The following was calculated:

$$\text{Haemoglobin (Hb) concentration} = (Hb_{arterial} + Hb_{venous})/2$$

$$\text{Body surface area (BSA) m}^2 = 0.0734 \times BW^{0.656}$$

$$\text{Cardiac index (CI)} = \dot{Q}t \big/ BSA$$

$$\text{Stroke volume index (SVI)} = \dot{Q}t \big/ HR/BSA$$

Systemic vascular resistance (SVR)

$$= 80 \times MAP \big/ \dot{Q}t$$

Content of oxygen in arterial blood
$$(CaO_2) = (1.34 \times Hb \times SaO_2) + (0.025 \times PaO_2)$$

Content of oxygen in mixed venous blood ($C\bar{v}O_2$)

$$= (1.34 \times Hb \times SvO_2) + (0.025 \times PvO_2)$$

$$\text{Delivery of oxygen index (DO}_2\text{I)} = CI \times CaO_2$$

$$\text{Oxygen extraction (OE)} = (CaO_2 - C\bar{v}O_2)$$

Oxygen consumption index (VO$_2$I)

$$= CI \times (CaO_2 - C\bar{v}O_2)$$

A database was created in Microsoft excel (Microsoft Corp., WA, USA), and subsequent statistical analysis was performed using JMP Pro

Table 1 Results of the comparison of cardiovascular variables at equivalent anaesthetic depth in pigs anaesthetized with propofol 8 mg kg^{-1} hour^{-1} and ketamine 5 mg kg^{-1} hour^{-1} in combination with either dexmedetomidine at 2, 4 and 8 µg kg^{-1} hour^{-1} or fentanyl at 25, 50 and 100 µg kg^{-1} hour^{-1}. Measurements are presented as median (range).

Parameter	Dexmedetomidine	Fentanyl	p
HR (beats minute^{-1})*	104 (97–125)	95 (85–102)	0.0156
SAP (mmHg)*	132 (114–149)	116 (99–135)	0.0234
DAP (mmHg)*	78 (66–96)	60 (40–83)	0.0078
MAP (mmHg)*	101 (88–117)	81 (61–104)	0.0078
CI (L minute^{-1} m^{-2})	6.39 (5.76–7.82)	6.40 (5.74–7.44)	0.9453
SVI (mL m^{-2} beat^{-1})*	59.1 (48.4–75.9)	69.6 (62.4–76.9)	0.0234
SVR (dynes sec^{-1} cm^{-5-1})*	2125 (1304–2670)	1535 (1112–1950)	0.0391
Hb (g dL^{-1})*	8.9 (7.9–9.4)	7.5 (6.9–8.5)	0.0156
Lactate (mmol L^{-1})	0.6 (0.4–0.8)	0.45 (0.4–0.9)	0.0781
CaO$_2$ (ml dL^{-1})*	13.1 (11.5–14.1)	11.2 (10.4–12.3)	0.0156
CvO$_2$ (ml dL^{-1})*	9.8 (8.5–10.9)	7.5 (6.6–9.0)	0.0156
DO$_2$I (mL minute^{-1} m^{-2})*	81.0 (77.8–101.1)	72.6 (62.5–85.1)	0.0234
VO$_2$I (mL minute^{-1} m^{-2})	22.0 (16.4–23.3)	22.5 (18.2–26.0)	0.3828
OE (mL minute^{-1} m^{-2})	3.2 (2.8–3.8)	3.3 (2.9–4.5)	0.3125

*Statistically significant differences between treatments.

CaO$_2$, content of oxygen in arterial blood; CI, cardiac index; CvO$_2$, content of oxygen in mixed venous blood; DAP, diastolic arterial blood pressure; DO$_2$I, oxygen delivery index; Hb, haemoglobin concentration; HR, heart rate; MAP, mean arterial blood pressure; OE, oxygen extraction; SAP, systolic arterial blood pressure; SVI, stroke volume index; SVR, total peripheral resistance; VO$_2$I, oxygen consumption index.
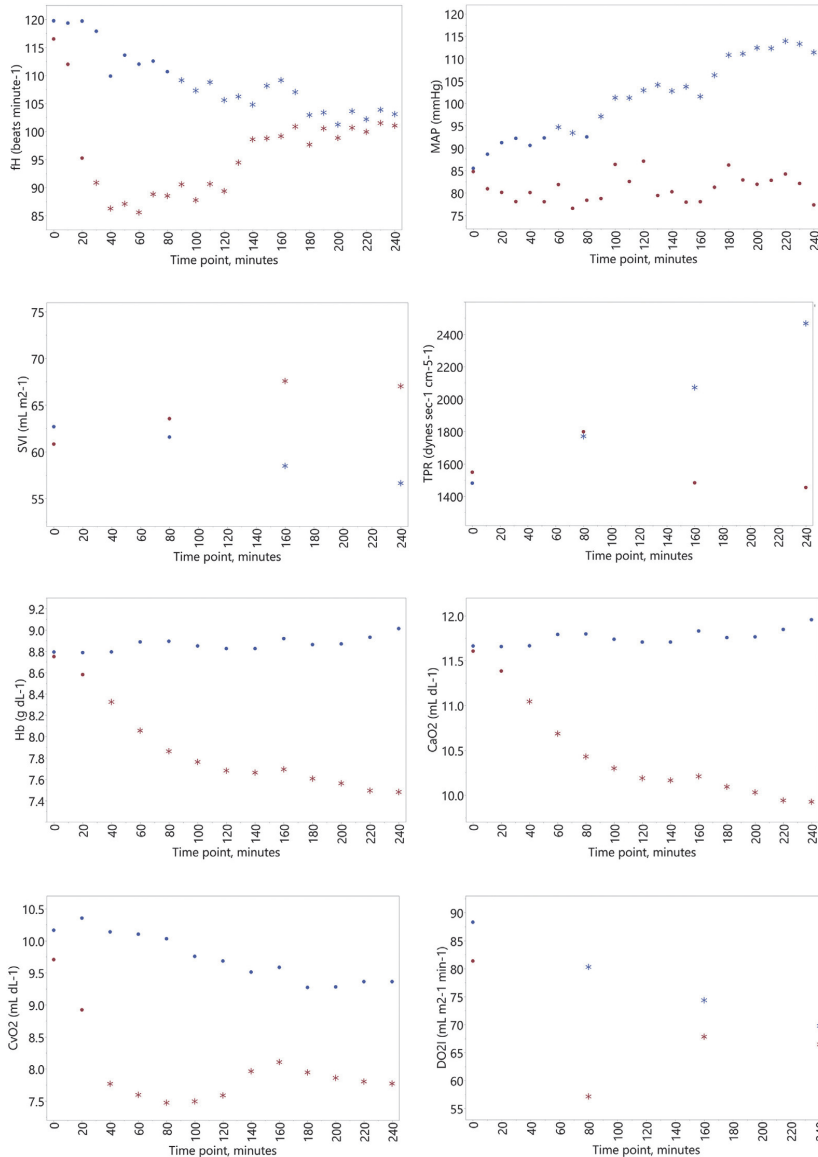
Figure 1 Mean heart rate (HR), mean arterial blood pressure (MAP), stroke volume index (SVI), systemic vascular resistance (SVR), haemoglobin (Hb) concentration, content of oxygen in arterial blood (CaO_2), content of oxygen in mixed venous blood ($C\overline{v}O_2$) and oxygen delivery (DO_2I) plotted against time for pigs anaesthetized with propofol 8 mg kg^{-1} hour^{-1} and ketamine 5 mg kg^{-1} hour^{-1} combined with either dexmedetomidine at 2, 4 and 8 μg kg^{-1} hour^{-1} or fentanyl at 25, 50 and 100 μg kg^{-1} hour^{-1}. Each infusion rate was administered for 80 minutes before commencing the next. Blue marker indicates that a dexmedetomidine infusion was administered, while red indicates fentanyl infusion. A filled circle indicates no significant difference from time point zero, whereas an asterisk indicates that there is a significant difference.

12.1.0 (SAS, NC, USA). Comparison of cardiovascular variables for the two treatments at the time point of equivalent anaesthetic depth was performed using a Wilcoxon matched paired signed rank test. If a significant difference was found at equivalent anaesthetic depth, means for these cardiovascular parameters were calculated for each time point from baseline and onwards, and analyzed within each treatment using a mixed model with time point as fixed and pigs as random effect. When a significant effect of stimulation time point was found, a controlled Dunnett's test was performed comparing each stimulation time point against baseline.

For comparison of blood pressures and HR in response to nociceptive stimulation, means were calculated for the minute preceding and the minute after nociceptive stimulation. Changes in blood pressures and HR were calculated as the difference between prior to and after stimulation. The change within and between treatments were compared using Wilcoxon signed rank and Mann–Whitney test, respectively, at the first time point when there was no response to neither NWR nor mechanical nociceptive stimulation. Two-sided comparison and an alpha value of 5% were used for all statistical tests. Graphs were created in JMP Pro 12.1.0 for visual comparison of the change in blood pressures, HR, the NWR score and mechanical stimulation latency between treatments.

Results

Data are presented as median (range) or mean ± standard deviation. There were missing data for continuous measurement of blood pressures and HR for one pig administered fentanyl. Body weight was 26.4 ± 1.9 kg and 27.5 ± 3.8 kg at the time of dexmedetomidine and fentanyl administration, respectively. Similar anaesthetic depth was reached at time points 100 (80–120) minutes for dexmedetomidine-treated and 220 (100–240) minutes for fentanyl-treated animals. In two fentanyl-treated pigs, a NWR score of 3 was still observed at the time point of 240 minutes.

Results of the comparison of cardiovascular variables at similar anaesthetic depth are summarized in Table 1. Pigs anaesthetized with dexmedetomidine had significantly higher HR, SAP, DAP, MAP, SVR, Hb, CaO_2, $C\overline{v}O_2$ and DO_2I than pigs treated with fentanyl at similar anaesthetic depth. We did not find a difference in CI, VO_2I, OE or lactate between treatments, but SVI was significantly higher in pigs

administered fentanyl. Changes in cardiovascular variables over time are summarized in Fig. 1.

Cardiovascular responses after nociceptive stimulation, NWR scores and latency are presented in Fig. 2. Under fentanyl treatment, all pigs had a NWR score of 2 or more when a latency of 60 seconds was first recorded, whereas for dexmedetomidine treatment, a latency of 60 seconds was first recorded at NWR score 1, 2, 3, and 4 in one, two, two and three pigs, respectively.

At the first time point where no NWR or motoric response to mechanical nociceptive stimulation was observed, blood pressures increased significantly with both treatments. Under dexmedetomidine and fentanyl treatment, respectively, SAP increased 2.5 (-0.4–11.1) mmHg ($p = 0.016$) and 8.2 (1.6–59.0) mmHg ($p = 0.016$), DAP increased 2.1 (-0.1–10.1) mmHg ($p = 0.016$) and 7.2 (1.0–51.8) mmHg ($p = 0.016$) and MAP increased 2.3 (-0.4–10.0) mmHg ($p = 0.016$) and 9.1 (1.4–54.0) mmHg ($p = 0.016$). The HR did not change significantly during dexmedetomidine [0.2 (-0.9–3.1) beats minute^{-1} ($p = 0.74$)] or fentanyl [-2.5 (-6.3–5.9) beats minute^{-1} ($p = 0.25$)] administration. The SAP ($p = 0.043$), DAP ($p = 0.032$), but not the MAP ($p = 0.056$), increased during fentanyl administration compared with dexmedetomidine administration.

Mean plasma concentrations at similar anaesthetic depth were 0.74 ± 0.12 ng mL^{-1} and 19.16 ± 4.29 ng mL^{-1} for dexmedetomidine and fentanyl treatments, respectively (Fig. 3). At similar anaesthetic depth prior to stimulation, four dexmedetomidine-treated and six fentanyl-treated pigs showed burst suppression with a suppression ratio in dexmedetomidine- and fentanyl-treated animals of 0.1 (0.0–20.0)% and 1.6 (0.0–36.0)%, respectively.

Discussion

The two anaesthetic protocols examined in this study induced similar changes in CI, VO_2, OE and lactate at comparable anaesthetic depth. Pigs administered dexmedetomidine had higher HR, blood pressures, SVR, Hb, CaO_2, $C\overline{v}O_2$ and DO_2I than pigs administered fentanyl as an adjunct to propofol–ketamine anaesthesia, while the SVI was significantly higher in fentanyl-treated animals. A reduced cardiovascular response to nociceptive stimulation was also found in the dexmedetomidine-treated animals. In healthy pigs, an anaesthetic protocol containing propofol and ketamine with dexmedetomidine as an adjunct provides stable cardiovascular conditions. When subjected to nociceptive stimulation, less
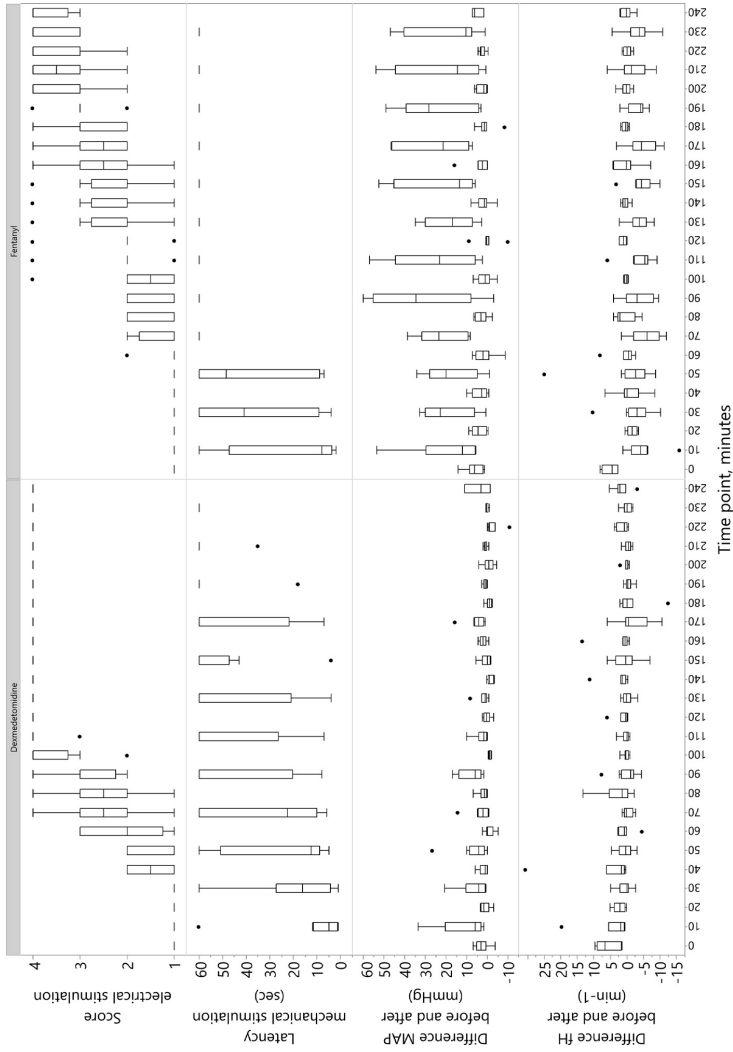
Figure 2 Mean nociceptive withdrawal score (NWR) after electrical stimulation, mean latency time (seconds) until response during mechanical stimulation, difference between mean arterial blood pressure (MAP) and heart rate (HR) 1 minute after and 1 minute before nociceptive stimulation plotted against time from the start of anaesthesia. The pigs were anaesthetized with propofol 8 mg kg^{-1} hour^{-1} and ketamine 5 mg kg^{-1} hour^{-1} in combination with dexmedetomidine at 2, 4 and 8 µg kg^{-1} hour^{-1} or fentanyl at 25, 50 and 100 µg kg^{-1} hour^{-1}. Each infusion rate was administered for 80 minutes before commencing the next.
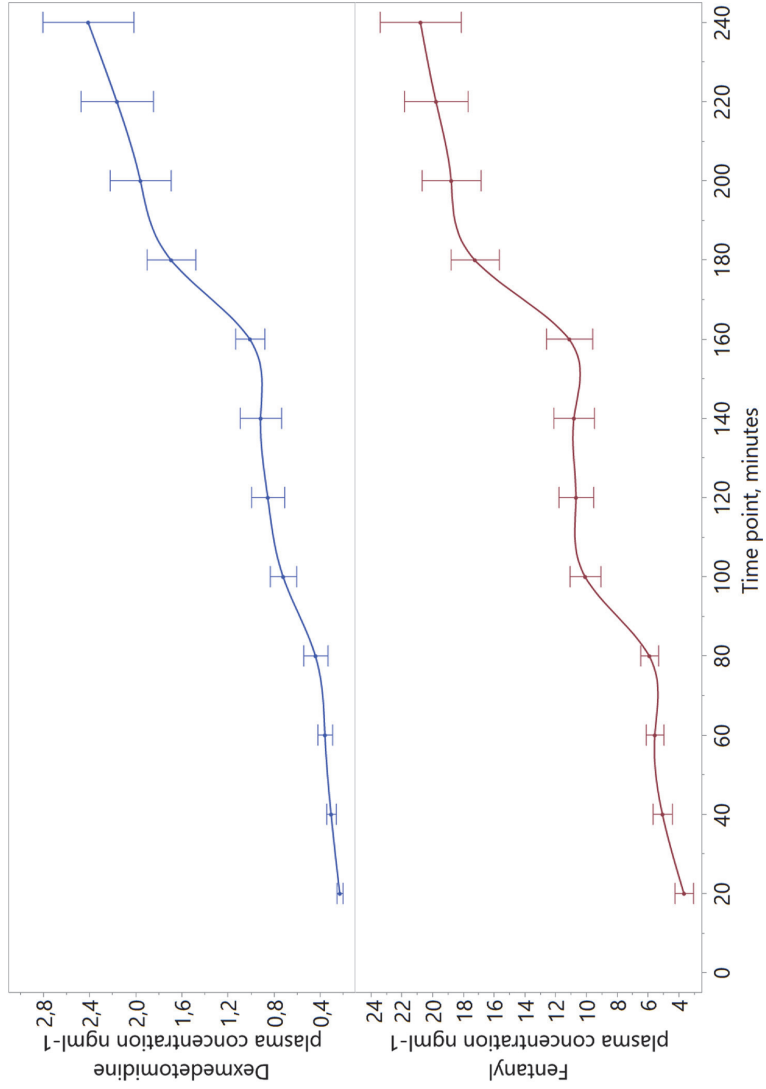
Figure 3 Mean ± standard deviation plasma concentrations in pigs anaesthetized with propofol 8 mg kg^{-1} hour^{-1} and ketamine 5 mg kg^{-1} hour^{-1} and administered dexmedetomidine (blue) at 2, 4 and 8 µg kg^{-1} hour^{-1} or fentanyl (red) at 25, 50 and 100 µg kg^{-1} hour^{-1}. Each infusion rate was administered for 80 minutes before commencing the next.

cardiovascular response was observed with dexmedetomidine compared with fentanyl.

Surprisingly, pigs treated with dexmedetomidine displayed a higher HR than those administered fentanyl at similar anaesthetic depth. This was unexpected as α_2-agonists at a range of doses are known to produce moderate to profound bradycardia in several species including pigs (Tendillo et al. 1996; Sano et al. 2010). Ketamine increases the HR through its sympathomimetic effect (Traber et al. 1971), and a centrally mediated sympatholysis is known to cause bradycardia in α_2-agonist-medicated animals and humans (Hogue et al. 2002; Murrell & Hellebrekers 2005; Feld et al. 2007). In pigs, dexmedetomidine at 0.7 μg kg^{-1} hour^{-1} in combination with propofol produced a marked reduction in $\dot{Q}$t and HR (Sano et al. 2010). The influence of ketamine on sympathetic tone may be a plausible explanation for the relatively high HR found in our study. Opioid-induced bradycardia is commonly attributed to an increase in parasympathetic activity (Reitan et al. 1978); however, this does not seem to be the case with α_2-agonists (Hogue et al. 2002; Feld et al. 2007). The differences in mechanisms leading to bradycardia, and how these are influenced by the other drugs in our study could explain the observations made. Another potential explanation could be differences in circulating blood volume, as described in the next section.

Despite the dissimilar HR between treatments at equivalent anaesthetic depth, CI did not differ significantly, as SVI was higher with fentanyl treatment. This difference in SVI can be attributed to several factors. It seems that intravascular volume was better maintained or even increased in fentanyl-treated animals over time. This is supported by the decrease in haemoglobin concentration observed over time. Increased intravascular volume could increase the left ventricular end diastolic volume and thereby stroke volume. A depression of SVI over time was observed in dexmedetomidine-treated animals. The cause for this may be threefold: 1) a reduction in intravascular volume; 2) an increase in SVR; or 3) a direct effect of dexmedetomidine on myocardial contractility. In an *in vitro* study examining the effects of dexmedetomidine on myocardial contractility in papillary muscles from ferrets, no direct effects of dexmedetomidine were found (Housmans 1990). Similar results were obtained in a model examining the effects of dexmedetomidine on isolated canine hearts (Flacke et al. 1992). This leads us to believe that the effect on SVI with dexmedetomidine mainly

results from increased SVR and afterload. This decrease in SVI will in most situations be of limited clinical relevance.

DO_2I was significantly different between the two treatments at similar anaesthetic depth because the CaO_2 was higher in dexmedetomidine-treated pigs. This higher CaO_2 can be attributed to the drop in haemoglobin concentration over time observed with fentanyl but not with dexmedetomidine treatment. Change in haemoglobin concentration during anaesthesia has previously been reported in mice and sheep (Ceylan et al. 2007; Gothelf et al. 2009); however, the direction and magnitude of this change can result from several factors, including physiological responses to hypovolaemia or the anaesthetic drugs used. Injection of adrenaline was shown to mobilize sequestrated erythrocytes from the porcine spleen (Hannon et al. 1985), but it seems to contract more under the influence of α_1-rather than α_2-receptor stimulation (Barbieri et al. 1998). Dexmedetomidine-induced vascular contraction varies in different models and seems to be dose dependent (Yildiz et al. 2007; Wong et al. 2010). We cannot exclude mobilization of erythrocytes because of splenic contraction in our pigs. Dexmedetomidine increases diuresis (Gellai & Edwards 1988) that theoretically can contribute to haemoconcentration, whereas fentanyl reportedly reduces urine production in dogs (Anderson & Day 2008). Lastly, increased hydrostatic pressure in dexmedetomidine-treated animals could cause extravasation of fluids and increase haemoglobin levels.

The mean plasma concentration of dexmedetomidine at the time point of similar anaesthetic depth was 0.74 ng mL^{-1}, corresponding to an infusion rate of approximately 4 μg kg^{-1} hour^{-1} This is similar to levels that provide analgesia in other species (Pascoe et al. 2006; Risberg et al. 2014), but a direct comparison has limitations as the methodology and aims differ between studies. In combination with propofol and ketamine, dexmedetomidine provided profound analgesia including suppression of the cardiovascular response to nociception; however, motoric response to mechanical stimulation was still present in some pigs. There is scarce information in the literature on the use of low-dose infusions of dexmedetomidine in pigs. A relatively low dose of medetomidine has been used for balanced anaesthesia in pigs undergoing abdominal surgery; however, the study was not specifically standardized to evaluate analgesic efficacy (Calzetta et al. 2014). In other studies, higher doses of medetomidine have suppressed the response to

mechanical nociceptive stimulation (Nishimura et al. 1993; Sakaguchi et al. 1996; Tendillo et al. 1996; Malavasi et al. 2008).

Most pigs in our study reached the point where the NWR was suppressed only at the highest infusion rate of fentanyl (mean plasma concentration of 19.16 ng mL^{-1}). In three animals, the response was never fully depressed. Only in one animal, the NWR disappeared during the second infusion rate, which corresponded to a plasma concentration of around 10 ng mL^{-1}.

Increased blood pressure and decreased pulse rate in response to nociception has previously been described in halothane-anaesthetized piglets (Haga & Ranheim 2005). In Fig. 2, an increase in the mean blood pressure because of nociceptive stimulation is observed under fentanyl treatment but a decrease under dexmedetomidine treatment. At the first time point where no NWR or response to mechanical stimulation was observed, a statistical significant difference in blood pressure, but not in pulse rate, between treatments was found. A comparison in human patients showed that dexmedetomidine depressed cardiovascular response to surgery more than fentanyl during desflurane anaesthesia (Feld et al. 2006). It is unclear whether comparisons were made at a similar depth of anaesthesia as the bispectral index is influenced differently with different anaesthetic regimes. A central sympathetic depression caused by dexmedetomidine cannot be ruled out as an explanation for the lacking cardiovascular response in our study. We still believe our observations to be a result of antinociception as they correspond to a loss of the NWR.

The different motoric response with the two modalities used for nociceptive stimulation was noteworthy. Similar differences have previously been characterized in isoflurane-anaesthetized pigs, where motoric response to dewclaw clamping was lost prior to the NWR (Spadavecchia et al. 2012). The plasma concentration for the loss of motoric response to mechanical stimulation was reached at an earlier time point for fentanyl-than for dexmedetomidine-treated pigs. Taken alone, this might indicate that effective analgesic plasma concentrations were reached earlier for fentanyl than for dexmedetomidine. However, when latency reached 60 seconds under fentanyl treatment, no reduction of NWR was observed in any pig, and in two pigs, NWR was never lost, whereas in most dexmedetomidine-treated pigs, a latency of 60 seconds was reached after a decline in NWR was observed, and NWR was ultimately lost in

all pigs. Our study was not designed to examine the neurophysiological background for the difference in response to nociceptive stimulation, and a discussion of potential mechanisms is beyond the scope of this publication. Taken together with the blunted cardiovascular response to nociception, important goals during general anaesthesia are apparently more readily reached with dexmedetomidine than with fentanyl when used in combination with propofol–ketamine.

The presence of burst suppression in EEG indicates a deep level of cerebrocortical depression ensuring that unconsciousness was achieved (Ching & Brown 2014). Despite loss of NWR and the presence of burst suppression in some pigs, movement in response to mechanical stimulation was still present. Previous experiments show that pigs are less sensitive to the spinal cord motor inhibitory effects of some anaesthetics compared to other species (Haga et al. 2011).

The main aim of the study was to examine the cardiovascular influence of the two anaesthetic protocols at similar anaesthetic depth; however, defining this is challenging, and in some animals administered fentanyl, the NWR was never lost. This was handled by using the evaluation time point at 240 minutes for statistical comparison, leading to some fentanyl-treated animals being lighter anaesthetized than intended at the time of comparison. As expected, there was a more obvious cardiovascular response to combined mechanical and electrical stimulation as opposed to pure electrical stimulation. The intensity of mechanical stimulations only reached maximum when latency became 60 seconds. Since this occurred later than the loss of NWR in several dexmedetomidine-treated pigs, comparison of cardiovascular response to nociceptive stimulation was done at higher dexmedetomidine plasma concentrations than at what we defined as equal anaesthetic depth, skewing the comparison to the benefit of dexmedetomidine. The difference in response to mechanical and electrical stimulation between treatments warrants further investigation.

Based upon the observed plasma concentrations, the fentanyl infusion rates could have been increased at shorter intervals since at the rate of 50 µg kg^{-1} hour^{-1}, plasma concentrations seemed to level out. A higher maximum infusion rate could have been used as the loss of NWR was not achieved in all pigs.

Propofol in combination with ketamine and either fentanyl or dexmedetomidine provided stable

cardiovascular conditions in normovolaemic, healthy pigs, even if a mild cardiovascular depression was observed at the lowest infusion rate of fentanyl. Based upon the cardiovascular response and depression of NWR, dexmedetomidine seems to provide superior analgesia to fentanyl. Both protocols should be studied in pigs subjected to hypovolaemia, and the difference in response to modalities of nociceptive stimulation warrants further investigation.

Authors' contributions

AL: conception and design of study, acquisition of data, analysis and interpretation of data, drafted the article, revised it critically for important intellectual content, approved the final version to be published; JR: conception and design of study, acquisition of data, analysis, drafted the article, revised it critically for important intellectual content, approved the final version to be published; BR: conception and design of study, acquisition of data, revised article critically for important intellectual content, approved the final version to be published; SS: acquisition of data, revised article critically for important intellectual content, approved the final version to be published; SFT: analysis and interpretation of data, revised article critically for important intellectual content, approved the final version to be published; HAH: conception and design of study, acquisition of data, analysis and interpretation of data, drafted the article, revised it critically for important intellectual content, approved the final version to be published.

Conflict of interest

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A comparison of respiratory function in pigs anaesthetised by propofol or alfaxalone in combination with dexmedetomidine and ketamine

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Abstract

Background: General anaesthesia in pigs maintained with intravenous drugs such as propofol may cause respiratory depression. Alfaxalone gives less respiratory depression than propofol in some species. The aim of the investigation was to compare respiratory effects of propofol–ketamine–dexmedetomidine and alfaxalone–ketamine–dexmedetomidine in pigs. Sixteen pigs premedicated with ketamine 15 mg/kg and midazolam 1 mg/kg intramuscularly were anaesthetised with propofol or alfaxalone to allow endotracheal intubation, followed by propofol 8 mg/kg/h or alfaxalone 5 mg/kg/h in combination with ketamine 5 mg/kg/h and dexmedetomidine 4 µg/kg/h given as a continuous infusion for 60 min. The pigs breathed spontaneously with an FIO_2 of 0.21. Oxygen saturation (SpO_2), end-tidal CO_2 concentration ($PE'CO_2$), respiratory rate (f_R) and inspired tidal volume (V_T) were measured, and statistically compared between treatments. If the SpO_2 dropped below 80% or if $PE'CO_2$ increased above 10.0 kPa, the pigs were recorded as failing to complete the study, and time to failure was statistically compared between treatments.

Results: Alfaxalone treated pigs had significantly higher respiratory rates and lower $PE'CO_2$ than propofol treated pigs, with a f_R being 7.3 /min higher (P = 0.01) and $PE'CO_2$ 0.8 kPa lower (P = 0.05). SpO_2 decreased by 0.6% and f_R by 1.0 /min per kg increase in body weight in both treatment groups. Three of eight propofol treated and two of eight alfaxalone treated pigs failed to complete the study, and times to failure were not significantly different between treatments (P = 0.75).

Conclusions: No major differences in respiratory variables were found when comparing treatments. Respiratory supportive measures must be available when using both protocols.

Keywords: Alfaxalone, Dexmedetomidine, Oxygenation, Pigs, Propofol, Respiratory function, TIVA, Ventilation

Background

Anaesthetised pigs are used as live tissue models in several countries for the training of prehospital trauma care providers [1]. Under these circumstances maintenance of anaesthesia with injectable anaesthetics might be necessary, as the provision of inhalational anaesthetic drugs is not possible, or even unwanted due to their effects on cardiovascular function [2]. This might also be the case for pigs used in laboratory investigations. Some of the commonly used intravenous anaesthetic drugs do however influence respiratory function when used for induction and maintenance of general anaesthesia [3], causing hypoventilation, respiratory acidosis and potentially hypoxemia.

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In a previous publication, a total intravenous anaesthetic (TIVA) protocol containing propofol, ketamine and dexmedetomidine was found to provide stable cardiovascular conditions and excellent antinociception in healthy pigs [4]; features that are important when delivering anaesthesia during invasive surgical procedures in experimental animals. The effect on spontaneous ventilation was not examined in that study, but propofol has long been known to cause hypoventilation or even apnoea in several animal species including dogs and sheep when given intravenously [5, 6]. The effects of propofol on ventilation and oxygenation in spontaneously breathing pigs are only sparsely described in the literature, with apnoea and respiratory depression reported in some animals [7–9].

Alfaxalone, a steroid anaesthetic, has been used for induction and maintenance of anaesthesia in pigs without causing apnoea after induction, but hypoventilation can result when it is used for maintenance [10, 11]. At lower doses when alfaxalone was combined with isoflurane and dexmedetomidine little effect on respiration was found in spontaneously breathing pigs, with mild hypercapnia as the major finding [12]. When used to maintain anaesthesia in cats during ovariohysterectomy, alfaxalone was shown to have less effect on respiration when compared to propofol [13], but when a similar comparison was made in canine clinical patients hypoventilation occurred with both drugs [14]. To our knowledge, no comparison has been made between propofol and alfaxalone regarding their effects on ventilation and oxygenation in pigs.

The primary aim of the current study was to determine if pigs anaesthetised with either propofol–ketamine–dexmedetomidine or alfaxalone–ketamine–dexmedetomidine could maintain adequate respiratory function when spontaneously breathing atmospheric air.

Methods

Animals

Sixteen mixed breed (Norwegian land race 50% and Duroc 50%) pigs, 10 castrated males and 6 females, with a median (range) age of 66 (52–73) days and a mean (SD) body weight of 24.9 (4.2) kg in the propofol group and 25.9 (3.7) kg in the alfaxalone group were included. The pigs were acquired from the same breeder and identified using their existing ear tag numbers. They were transported to the research animal facility of the Norwegian University of Life Sciences where they were housed for approximately 14 days prior to the experiment. All pigs were allowed to follow normal light–dark cycles in a room with natural day light and a room temperature kept between 15 and 20 ℃. They were fed a commercial pig diet in combination with free access to hay. Approximately 1 week prior to this experiment the pigs were

used in another anaesthesia study and a minimum wash out period of 7 days was allowed between experiments. Their health status was monitored minimanual breaths in the post induction period duemum once daily during the entire period. The study was approved by the Norwegian National Animal Research Authority (FOTS 14,277).

Study design

A balanced, randomized study design was used. The 16 pigs were randomized in blocks of four to receive either propofol–ketamine–dexmedetomidine or alfaxalone–ketamine–dexmedetomidine by drawing paper notes. None of the investigators were blinded to the given treatment.

Anaesthesia, monitoring and data collection

Food, but not straw and water, was withheld for approximately 12 h before premedication, and all pigs were found healthy based on a clinical examination before each experimental session. Their body weight was measured on the day of the experiment.

Premedication was given using ketamine 15 mg/kg (Ketamine Le Vet 100 mg/mL; Le Vet Beheer B.V., TV Oudewater, Holland) in combination with midazolam 1 mg/kg (Midazolam 5 mg/mL; B. Braun, Melsungen, Germany) administered intramuscularly (IM) in the cervical muscles. After sedation an intravenous catheter (Venflon Pro; Becton Dickinson Infusion Therapy, Franklin Lakes, NJ, USA) was placed in an auricular vein. The pigs were preoxygenated for 4–5 min with 100% O_2 delivered by a face mask, and anaesthesia was thereafter induced by slow intravenous titration of propofol (Propofol–Lipuro 20 mg/mL; B. Braun) or alfaxalone (Alfaxan 10 mg/mL; Jurox, Rutherford, Australia) to allow endotracheal intubation after application of topical lidocaine (Xylocain 100 mg/mL spray; Aspen, Dublin, Ireland). The induction of anaesthesia was always performed by the same investigator (AL), and the total dose needed to allow endotracheal intubation and the internal diameter of the endotracheal tube (Rüschelit super safety clear, Teleflex Medical, Westmeath, Ireland) was noted. The cuff was inflated to a pressure of 40–45 cm H_2O using a manometer syringe (AG Cuffill, Hospitech Respiration, Kfar Sabam, Israel).

After intubation, the pigs were placed in left lateral recumbency, and allowed to breathe atmospheric air spontaneously. Upper airway suction through the endotracheal tube was allowed if excessive airway secretion was detected by auscultation. In addition, single manual breaths with a FIO_2 of 0.21 were allowed to be administered with a manual resuscitation bag (Laerdal Silicone Resuscitator, Laerdal Medical, Stavanger, Norway) if the SpO_2 dropped below 80% during the first

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5 min after intubation. Anaesthesia was maintained with undiluted propofol 8 mg/kg/h or undiluted alfaxalone 5 mg/kg/h in combination with ketamine diluted to 50 mg/mL at 5 mg/kg/h and dexmedetomidine (Dexdomitor 0.5 mg/mL, Orion Corporation, Esbo, Finland) diluted to 50 µg/mL at 4 µg/kg/h intravenously (IV). All drugs were diluted using 0.9% NaCl (Natriumklorid Fresenius Kabi, Fresenius Kabi, Halden, Norway) and were given as a continuous infusion delivered by syringe drivers (Alaris GH Plus, BD Medical, Franklin Lakes, NJ, USA). The doses of propofol, ketamine and dexmedetomidine were chosen based on the results from a previous study [4]. The alfaxalone dose used was based on a dose titration pilot study in four pigs, where the lowest infusion rate abolishing the motor response to the same standardised electrical nociceptive stimulation used in the previous study [4] was determined.

The pigs were monitored using a multiparameter anaesthetic monitor (GE Carescape Monitor B650; GE Healthcare, Helsinki, Finland). Heart rate (f_H), respiratory rate (f_R) based on the capnography trace, 3-lead electrocardiogram, oxygen saturation (SpO_2), end tidal CO_2 ($PE'CO_2$), fractioned inspired oxygen concentration (FIO_2), and oesophageal temperature were recorded and automatically downloaded every 5 s for 60 min using data collection software (iCollect Version 5.0, GE Healthcare). SpO_2 was measured using a pulse oximetry finger sensor (TruSignal finger sensor, GE Healthcare) placed on the lateral digit of right hind- or front limb. The plethysmography trace was continuously inspected by one of the examiners to ensure proper signal quality. If a flattening of trace or a sudden drop in SpO_2 was observed the probe was repositioned, and the trace was assessed again. In addition, a pitot tube with a sample port for gas monitoring (Pedi Lite + Flow Sensor, GE Healthcare) was fitted to the end of the endotracheal tube and connected to the monitor using the manufacturers tubing (Spirometry tube, disposable, yellow, GE Healthcare). The capnography trace was continuously inspected by one of the examiners. According to the manufacturer, the pitot tube allows measurements of tidal volumes from 5 to 300 mL. Inspired tidal volumes (V_T) were recorded every 5 s for 60 min.

If the SpO_2 dropped below 80% for more than 30 s, FIO_2 was increased to 0.5. Similarly, if $PE'CO_2$ increased above 10.0 kPa for more than 30 s, intermittent positive pressure ventilation (IPPV) was instituted for the remaining study period. In either case, the pigs were then recorded as failing to complete the study period, and time to failure was noted. An SpO_2 of 80% represents severe hypoxemia and was chosen as our cut off value for intervention. Using the alveolar oxygen equation and the oxyhaemoglobin dissociation curve for adult miniature pigs

with a P50 of 4.30 kPa [15] this SpO_2 would result from hypoventilation leading to a $PaCO_2$ of approximately 10.5 kPa in a lung with normal gas exchange.

All pigs received a balanced electrolyte solution (Ringers acetate; Fresenius Kabi) intravenously at a rate of 0.33 mL/kg/h delivered by a volumetric infusion pump (Volumat Agilia; Fresenius Kabi). In addition, the total volume of drugs infused was 1.08 mL/kg/h, with a total infusion rate throughout the study of 1.41 mL/kg/h. All fluid and drug infusions were administered through the same intravenous catheter placed in the auricular vein. In addition, the pigs were covered with bubble wrap to avoid heat loss and external heat was provided with a forced air patient warming device (Bair Hugger, 3 M, St. Paul, MN, USA) as long as the body temperature was < 39.5 ℃. After completing a second study performed under the same anaesthetic the pigs where euthanised with pentobarbital (Euthasol vet 400 mg/mL, Le Vet Beheer) given intravenously.

Evaluation of anaesthetic depth and electroencephalography

Sixty-five minutes after intubation a standardised evaluation of anaesthetic depth was performed. Clinical signs of anaesthetic depth were scored by the same investigator as follows: First Eye position (ventral = 0, central = 1), nystagmus (present = 0, absent = 1), palpebral reflex (present = 0, absent = 1), and corneal reflex (present = 0, absent = 1) were assessed. Thereafter mechanical nociceptive stimulation was applied to the lateral dewclaw of the right front limb by a latex-coated forceps with a clamping area 1×1 cm. The applied clamping pressure was monitored with a spring balance attached to one forceps arm at a point an equal distance from, and on the opposite side of the articulation as the clamping jaws. The force applied at clamping was 100 N and maximum clamping time for each stimulus was 59 s. Stimulation was stopped at withdrawal of the limb or if vigorous movement in other limbs or whole-body movement was observed. The withdrawal was scored as present (= 0) or absent (= 1). A summarised score ranging from 0 to 5 for eye reflexes, eye position and response to clamping was noted.

In seven pigs, electroencephalography (EEG) was recorded. For recording of a two-channel referential EEG needle electrodes (Aiglette, Technomed Europe, Maastricht, Netherlands) were used. Electrodes were placed 1 cm caudal to the lateral angle of the eye and 1 cm medial to the temporal line bilaterally, and these electrodes were referred to an electrode placed in the median plane 2 cm caudal to the recording electrodes. A ground electrode was placed caudal to the atlas wing. The resistance of each electrode pair was kept below 3

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kΩ. The electrodes were connected to an EEG monitor (A-1000™, Aspect Medical Systems, Newton, MA, USA). The monitor filters were set as follows—high frequency filter: 50 Hz, 50/60 Hz filter: 50 Hz and low frequency filter: 2.0 Hz. The monitor automatically detected burst suppression and calculated the percentage of epochs in the previous 63 s where the EEG signal was considered suppressed. This percentage is called burst suppression ratio (BSR) and was updated every 5 s.

Statistical analysis

Respiratory data collected from 5 min (time point 0) to 65 min (time point 60) after intubation were used for statistical analysis, with a total observation period of 60 min. V_T was indexed to body weight for statistical analysis (V_T/kg).

For graphical evaluation and a Kaplan–Meier analysis, a database was created in JMP 14.1.0 (SAS Institute Inc., Cary, NC, USA). The mean SpO_2, $PE'CO_2$, f_R and V_T/kg for each minute was calculated and used for graphical evaluation of respiratory parameters. In addition, time to failure was compared between treatments using a Kaplan–Meier analysis including a log-rank test.

Further statistical comparison of respiratory parameters was performed using Stata SE 15 (Stata Corp LLC, Lakeway Drive College Station TX., USA). The data collection time points were reduced to recordings every 10 min, where the mean for 1 min at each evaluation time point was calculated. Seven means per subject (at 0, 10, 20, 30, 40, 50 and 60 min from baseline) were included in the analysis. Distribution of the outcome variables SpO_2, $PE'CO_2$, f_R and V_T/kg were assessed using histograms. Data sampled after a pig failed the study were handled using "last observation carried forward" (LOCF). At time points where FIO_2 was increased to 0.5 due to low SpO_2, a SpO_2 of 79% was used when analysing the data. Similarly, at time points where IPPV was used, $PE'CO_2$ was set to 10.1 kPa; in addition, the last f_R and V_T/kg recorded before instituting IPPV were also used. Missing data were otherwise not imputed. Linear mixed models with individual pig as random effect and a compound symmetry (exchangeable) correlation structure were fitted for all outcome variables to assess the impact of treatment on the outcome in question. Other correlation structures were considered, but not deemed feasible. In the present data with the low sample size, a better fit of the unstructured covariance structure came at a cost of using many degrees of freedom. In addition, the other common matrices would result in overfitting due to the fact that the random effect was at the same level as the repeated measures (pig). The correlation and covariance structure observed in the data supported the compound symmetry.

Sex and body weight were included as fixed effects in all models.

Intraclass correlation coefficients (ICC) were calculated based on the variance estimates from the models to give an estimate of the level of clustering in the data.

$$ICC = \sigma_{pig}^2 / (\sigma^2 + \sigma_{pig}^2).$$

Models with and without pig random effect were compared with likelihood ratio tests (LRT). Assumptions for linear mixed models were evaluated as described in the literature. For all statistical tests, an alpha of 5% was used.
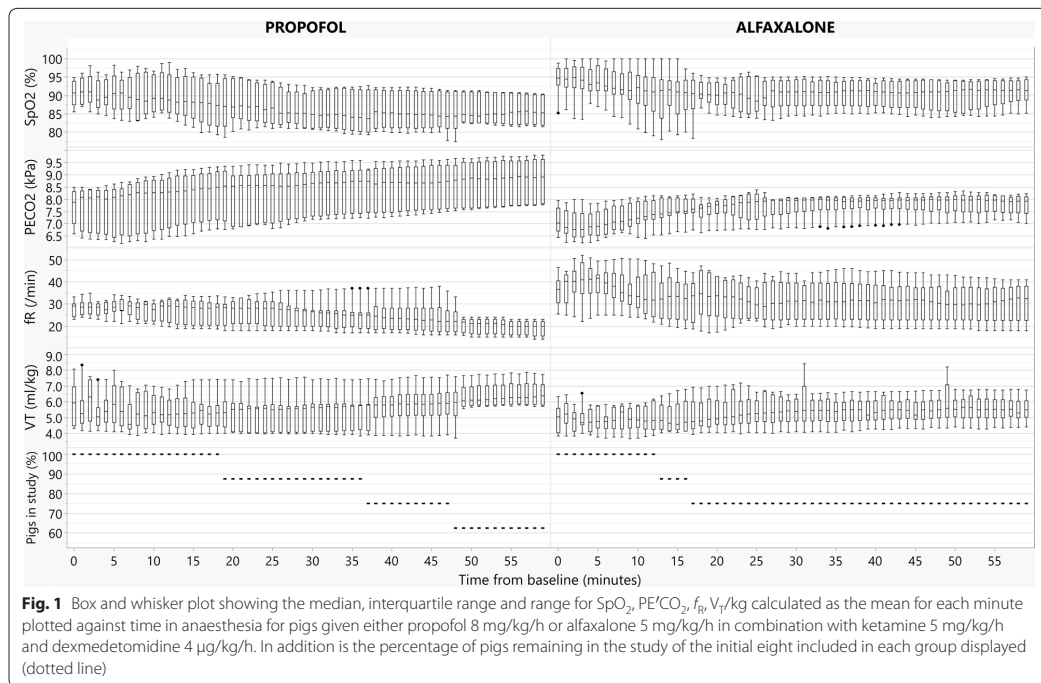
Results

The mean (SD) dose needed to allow sufficient anaesthetic depth for intubation was 2.54 (0.80) and 1.10 (0.23) mg/kg in the propofol and alfaxalone group respectively. Single manual breaths in the post induction period due to low SpO_2 suggesting possible hypoxemia were necessary in two of eight pigs in each group. None of the pigs experienced periods of apnoea in this period.

Measured respiratory parameters for the entire study period and time to failure are summarised in Fig. 1. Three of eight propofol treated and two of eight alfaxalone treated pigs failed to complete the study. All failures where due to low SpO_2 and respiratory parameters at the time of failure are summarised in Table 1. In two propofol treated and one alfaxalone treated pigs IPPV was also necessary due to $PE'CO_2 > 10$ kPa after increasing FIO_2 to 0.5. Results of the Kaplan Meier analysis did not reveal any significant difference in time to failure between treatments (P = 0.750).

Results from the mixed models are shown in Table 2. The results from the mixed models were consistent with the descriptive statistics. Pigs treated with alfaxalone had a higher respiratory rate (P = 0.01) and lower $PE'CO_2$ (P = 0.05) than propofol treated pigs, with a f_R that was 7.3 /min higher and a 0.8 kPa lower $PE'CO_2$ for the study period. Body weight had statistically significant impact on SpO_2 and f_R in both treatment groups, with SpO_2 decreasing 0.6% and f_R decreasing 1.0 /min per kg increase in body weight.

From the linear mixed models calculated ICCs were very high; 53.8% for SpO_2, 57.4% for $PE'CO_2$, 59.7% for f_R and 76.8% for V_T/kg. Results from the LRT gave P-values < 0.0001 for all four models.

The median (range) summarised score for anaesthetic depth after 60 min was 4 (3–4) and 4 (2–5) in the propofol and alfaxalone group respectively. One of eight and three of eight pigs had a positive withdrawal reflex in response to clamping of the dewclaw in the propofol and alfaxalone group respectively. EEG was recorded in

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Fig. 1 Box and whisker plot showing the median, interquartile range and range for SpO$_2$, PE'CO$_2$, f_R, V$_T$/kg calculated as the mean for each minute plotted against time in anaesthesia for pigs given either propofol 8 mg/kg/h or alfaxalone 5 mg/kg/h in combination with ketamine 5 mg/kg/h and dexmedetomidine 4 µg/kg/h. In addition is the percentage of pigs remaining in the study of the initial eight included in each group displayed (dotted line)

Table 1 Respiratory parameters at time point of study failure for pigs failing to complete the study are shown. The pigs are anaesthetised with either propofol 8 mg/kg/h or alfaxalone 5 mg/kg/h in combination with ketamine 5 mg/kg/h and dexmedetomidine 4 µg/kg/h

Treatment	Pig no	Time to failure (min)	SpO$_2$ (%)	PE'CO$_2$ (kPa)	V$_T$/kg (mL/kg)	f_R (/min)
Propofol	1	48	77	8.6	3.7	34
	4	37	79	8.9	3.8	25
	7	19	79	9.3	6.4	20
Alfaxalone	3	13	78	8.2	6.7	23
	6	17	79	8.2	5.2	22

four pigs in the propofol and three pigs in the alfaxalone group. BSR was 0, 0, 2 and 9% and 0, 3 and 33% in the respective group.

Discussion

Pigs anaesthetised with propofol–ketamine–dexmedetomidine or alfaxalone–ketamine–dexmedetomidine both displayed compromised respiratory function when breathing atmospheric air, with 37.5% and 25% respectively needing intervention based on our predetermined cut-off levels. The cut off levels chosen in this study were on purpose set rather liberally to avoid exclusion of too

many subjects during the study period. The time point of failure was evenly distributed over time in both treatment groups. When using these anaesthetic regimes in pigs, supplemental oxygen must be given, as moderate to severe hypoxemia as detected by pulse oximetry was the main cause of failure to complete the study. Even if none of the pigs experienced apnoea, moderate to severe hypoventilation was observed, and the ability to provide intermittent positive pressure ventilation should be available if using these anaesthetic regimes.

It is likely that the main mechanism of hypoxemia in our study is anaesthesia-induced hypoventilation. Our

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Table 2 Results from the linear mixed model analysis on effect of treatment on SpO_2, $PE'CO_2$, f_R and V_T in pigs anaesthetised with either propofol 8 mg/kg/h or alfaxalone 5 mg/kg/h in combination with ketamine 5 mg/kg/h and dexmedetomidine 4 μg/kg/h (n = 16)

Variable and level	Estimate (SE)	P-value	95% Confidence interval
SpO_2			
Treatment			
Propofol	Baseline	–	–
Alfaxalone	3.6 (2.3)	0.12	− 0.87, 8.04
Sex			
Male	Baseline	–	–
Female	3.4 (2.3)	0.14	− 1.13, 8.02
Body weight	− 0.6 (0.3)	0.04	− 1.20, − 0.05
$PE'CO_2$			
Treatment			
Propofol	Baseline	–	–
Alfaxalone	− 0.8 (0.4)	0.05	− 1.62, 0.01
Sex			
Male	Baseline	–	–
Female	− 0.6 (0.4)	0.17	− 1.43, 0.25
Body weight	0.1 (0.05)	0.14	− 0.03, 0.19
f_R			
Treatment			
Propofol	Baseline	–	–
Alfaxalone	7.5 (3.1)	0.01	1.51, 13.51
Sex			
Male	Baseline	–	–
Female	2.3 (3.1)	0.47	− 3.91, 8.41
Body weight	− 1.0 (0.4)	0.02	− 1.74, − 0.18
V_T/kg			
Treatment			
Propofol	Baseline	–	–
Alfaxalone	0.0 (0.5)	0.94	− 1.0, 1.0
Sex			
Male	Baseline	–	–
Female	0.1 (0.5)	0.80	− 0.9, 1.2
Body weight	− 0.1 (0.1)	0.235	− 0.2, 0.1

study was not designed to elucidate the pathophysiological mechanisms of hypoxemia that we observed, but rather to characterise the respiratory function using a particular anaesthetic regime in healthy pigs. After performing a theoretical calculation of the alveolar partial pressure of oxygen when breathing atmospheric air in the anaesthetised pigs, and taking the published P50 for adult miniature pigs [15] and a right shift of the oxy-haemoglobin dissociation curve due to hypercapnia into account, it seems that the drop in SpO_2 is clearly associated with the observed increase in $PE'CO_2$. This is

also supported by the physiological parameters at the time of failure, where moderate to severe hypercapnia is observed in combination with hypoxemia in all pigs. The registered tidal volume was also very low in two of the pigs at this time point, with the likelihood of a high Pa-E'CO_2-difference, supporting that the $PaCO_2$ is even higher than the observed $PE'CO_2$. In addition to this, a study in healthy pigs showed that the degree of venous admixture is low during spontaneous breathing, making this a less likely cause, but a direct comparison to our animals is difficult as these animals where anaesthetised with ketamine, placed in dorsal recumbency and received 100% oxygen [16].

In the current study, a statistically significant difference between treatments was found for respiratory rate and $PE'CO_2$. This stands in contrast to a clinical study in dogs, where no significant differences were found in respiratory rate, tidal volume or $PE'CO_2$ when comparing TIVA with propofol and alfaxalone [14]. A relatively high respiration rate was also found in previous studies in pigs when anaesthesia was induced or maintained with alfaxalone [10, 17]. In a systematic review comparing respiratory rate in dogs and cats after induction with either alfaxalone or propofol no evidence of difference could be found between the two agents [18]. The $PE'CO_2$ in our study was lower in the alfaxalone treated than in propofol treated pigs. The 95% confidence interval for $PE'CO_2$ and the descriptive data also supports that a population of pigs anaesthetised with alfaxalone–ketamine–dexmedetomidine likely will maintain a better alveolar minute ventilation than pigs receiving propofol–ketamine–dexmedetomidine, but the magnitude of this difference might not be of clinical importance. LOCF was used in the statistical comparison in this study, and this approach can be debated. It is traditionally viewed upon as a conservative approach, but this will of course depend on the true missing values [19]. It is plausible that the imputed data in our study represent a truly conservative picture of the reality, and that the difference in $PE'CO_2$ could have been larger without our intervention at a $PE'CO_2$ of 10 kPa.

Arterial blood gas analysis can be considered a gold standard when investigating the effectiveness of alveolar ventilation and gas exchange in the clinical setting [20]. Arterial catheterisation after induction was not performed, as arterial catheterisation would have delayed the documentation of the respiratory effects by several minutes. In addition, the access to patent arteries were important for the second study performed in the same pigs. As such, a major weakness in the current investigation was the use of pulse oximetry, capnography and spirometry as the sole methods for the assessment of oxygenation and alveolar ventilation. These methods

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have their limitations when it comes to both accuracy and precision [21, 22]. Several studies in human patients compare the performance of pulse oximetry to arterial oxygen saturation (SaO_2) showing a varying accuracy, but also that the accuracy is higher in the range from 80–100% [23, 24]. The precision and accuracy of pulse oximetry in newborn piglets has been examined, using an ear clip sensor placed on the thigh. The investigators found a deviation from measured SaO_2, in particular with values below 60% and during periods where hypoperfusion was suspected [25]. The saturations measured in the current study were all in the range from 80–100%. In addition, none of the pigs experienced hypoperfusion or hypothermia that could have influenced pulse oximeter performance. Pulse oximetry plethysmography curves and capnography waveforms were continuously assessed during data sampling in this study to reduce the risk of inaccurate measurements.

A tidal volume of 9 mL/kg has been reported in awake, young tracheotomised pigs [26]. In our study, observed tidal volumes were similar between groups, but lower than reported in awake pigs. Anatomical dead space is a constant factor, and a relatively larger proportion of each breath would be dead space if the tidal volume decreases. This could easily lead to variations in the measured $PE'CO_2$ that does not correspond to the true $PaCO_2$, and thus not reflect the true alveolar ventilation. In addition, one could argue that the higher f_R in the alfaxalone treated animals could influence the measured $PE'CO_2$, introducing a bias towards lower values [27]. The V_T/kg is however very similar in the two groups, thus supporting that alveolar ventilation was higher in the alfaxalone group. Single manually performed positive pressure ventilations could have been performed to reduce the influence of dead space ventilation on the $PE'CO_2$. In the authors experience this would however influence the respiratory pattern of the pigs, and potentially the study result. Despite limitations in the methodology, we are confident that the comparison between treatments and the main conclusion on the suitability of the TIVA protocols in spontaneously breathing pigs is valid. In addition, pulse oximetry and capnography are the commonly used monitoring modalities for clinical decision-making during anaesthesia for live tissue training in pigs. Interpretation of absolute values should on the other hand be made with caution.

Body weight had significant impact on SpO_2 and f_R in our study. One possible explanation for the drop in SpO_2 could be that heavier pigs reached a deeper plane of anaesthesia during the study period, as the body weights in the study population varied from 20 to 31 kg. Increasing body weight could result in higher drug plasma concentrations, and thereby negative impact on respiratory function. An allometric dose effect of medetomidine in pigs has previously been observed by the authors, with heavier animals becoming more sedated than pigs with lower body weight when receiving 80 μg/kg intramuscularly [28], however an effect of age cannot be excluded. In humans given an infusion of 5 mg/kg/h of propofol plasma concentrations increase with bodyweight, also when corrected to lean body weight [29]. Repeated measurements of anaesthetic depth could have elucidated the temporal change in anaesthetic depth during the examination period in this study. Nociceptive stimulation could however potentially influence the pattern of spontaneous ventilation, and the authors decided to delay the assessment of anaesthetic depth to the end of the study period. All pigs were induced to a sufficient depth to allow endotracheal intubation at the start of the study, and the median score for anaesthetic depth was the same in both groups at the end of the study period. We therefore believe that the anaesthetic depth was similar in both groups in the study period.

When comparing the effect of anaesthetic drugs on physiological variables, similar anaesthetic depth should be presumed. However, establishing equipotent doses of anaesthetic drug combinations can be very challenging. At the same time, determining anaesthetic depth is difficult, with physical signs including ocular reflexes, cardiovascular and respiratory response and response to nociceptive stimulation being commonly utilized in clinical veterinary anaesthesia. The median score for anaesthetic depth in the current study was the same in the two groups, with a somewhat larger range in both directions in the alfaxalone group. The score was based on what is typically used during clinical anaesthesia in pigs, with the exception of arterial blood pressure response to nociceptive stimulation that was not evaluated. Arterial cannulation was not performed for reasons mentioned above. Several of the pigs in both treatment groups displayed burst suppression at the evaluation time point after 60 min, leading us to conclude that a relatively profound level of cerebrocortical depression was present [30]. Interestingly, a previous study in isoflurane anaesthetised pigs also shows that burst suppression can be present in the EEG of pigs responding with movement to nociceptive stimulation [31]. In the current study, pigs in both groups moved in response to mechanical nociceptive stimulation. Based on the burst suppression seen in some pigs one could argue that this is probably not a conscious response, but rather spinally mediated. Muscle relaxation and immobility are however important characteristics of general anaesthesia, and it is possible that a dose adjustment must be made when using these anaesthetic protocols during surgery.

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The number of animals included was not based on a previous sample size calculation for this study, rather than on the number of animals needed for another experiment performed under the same anaesthesia. This is not according to the ARRIVE guidelines for animal experimentation [32]. This study may be underpowered, and other statistically significant differences in the examined respiratory parameters might have been found if more animals had been included. The authors still find it highly unlikely that the overall conclusion on the effect of the anaesthetics regimes on respiration would have been different.

Conclusions

The current study shows that pigs anaesthetized with propofol–ketamine–dexmedetomidine or alfaxalone–ketamine–dexmedetomidine can experience hypoxemia and hypercapnia, and that proper supportive measures must be available when using these anaesthetic regimes. Possible allometric dose effects of the drugs used should be considered and warrants further investigation.

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Publisher's Note

Macrocirculatory Parameters and Oxygen Debt Indices in Pigs During Propofol Or Alfaxalone Anesthesia When Subjected to Experimental Stepwise Hemorrhage

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Background: Pigs are anesthetized when used for emergency procedures live tissue training (LTT) of civilian and military medical personnel or for experimental purposes, but there is a paucity in the literature regarding anesthesia of pigs for this purpose.

Objective(s): The main goals of the study were to compare oxygen debt, macrocirculatory parameters, and time to cardiac arrest between pigs in hemorrhagic shock and anesthetized with propofol-ketamine-dexmedetomidine or alfaxalone-ketamine-dexmedetomidine.

Design: A prospective, non-blinded randomized study design was used. Sixteen pigs were randomized in blocks of four to be anesthetized with either propofol-ketamine-dexmedetomidine ($n = 8$) or alfaxalone-ketamine-dexmedetomidine ($n = 8$) as a continuous infusion.

Interventions: Premedication with ketamine 15 mg kg^{-1} and midazolam 1 mg kg^{-1} was given i.m. Anesthesia was maintained with propofol 8 mg kg^{-1} h^{-1} or alfaxalone 5 mg kg^{-1} h^{-1} combined with ketamine 5 mg kg^{-1} h^{-1} and dexmedetomidine 4 μg kg^{-1} h^{-1} i.v. A stepwise, volume-controlled model for hemorrhage was created by exsanguination.

Main Outcome Measures: Indices of oxygen debt (lactate, base excess, and oxygen extraction), macrocirculatory (PR, SAP, DAP, MAP, and *CI*, SVI, and TPR) variables, and time to death was compared between groups.

Results: Pigs in the alfaxalone group had significantly higher SAP than pigs given propofol. No difference in other macrocirculatory variables or indices of oxygen debt could be found. A blood loss of 50% of the total blood volume or more was possible in most pigs with both anesthetic regimes.

Conclusions: Pigs anesthetized with propofol or alfaxalone combined with ketamine and dexmedetomidine tolerated substantial blood loss.

Keywords: pigs, anesthesia, TIVA, propofol, alfaxalone, dexmedetomidine, ketamine, hemorrhage

INTRODUCTION

For emergency procedures live tissue training (LTT) of civilian and military medical personnel *Sus domesticus* is the most commonly used species (1). When anesthetizing pigs for LTT the animal must be unconscious and immobile, and proper antinociception should be provided. Hence the goals of general anesthesia should be fulfilled. To avoid premature death, the anesthetic regime should provide cardiovascular stability during surgery and tolerance to hemorrhage and hypovolemia. This will reduce the total number of animals used, and thereby adhering to the 3 R's principle. For training of human anesthesiologists, the model should ideally mimic a realistic emergency situation, enhancing the learning outcome. This could include using intravenous anesthetic agents to maintain anesthesia and preserving the cardiovascular response during episodes of hemorrhage. A conundrum when anesthetizing pigs for LTT is accomplishing all the above described aspects, as fulfilling one might influence the others.

In a previous study in pigs propofol, ketamine, and dexmedetomidine combined was found to maintain a stable cardiovascular status and provide antinociception (2). As a pilot investigation for the current experiment, 30% of the pigs' blood volume was removed but only a minimal chronotropic response was observed. In two other studies in pigs anesthetized with propofol and remifentanil exsanguination of 35–55% of the blood volumes increased the heart rate only by ∼30% from baseline (3, 4). This is relatively modest compared with findings in pigs anesthetized with pure isoflurane anesthesia (5, 6).

Lack of chronotropic response to hypotension has also been shown after anesthetic induction with propofol in humans. In one study propofol depressed the baroreceptor reflex and sympathetic activity (7) and increases in vagal tone has also been a suggested cause (8). In fentanyl premedicated dogs, induction of anesthesia with alfaxalone decreased the heart rate significantly less than did propofol (9), while when the two drugs were compared for anesthetic maintenance no difference in cardiovascular variables such as, mean arterial blood pressure or heart rate could be found (10). We therefore wanted to compare the chronotropic response in pigs anesthetized by the previous studied anesthetic regime and when replacing propofol with alfaxalone.

Macrocirculatory parameters, including heart rate, arterial blood pressure and cardiac output (*CO*) are often used clinically to assess the severity of hypovolemia during hemorrhage, but are also deemed inadequate when used alone (11). Oxygen debt occurs when insufficient oxygen delivery results in a reduction of oxygen consumption, with subsequent cell death and organ system failure (12, 13). In experimental pigs subjected to hemorrhagic shock, oxygen debt is closely linked to the risk of death. The accumulation of metabolic acids calculated as base excess or specifically measured as lactate may be used to quantify oxygen debt (14).

The main goals of our study were to compare oxygen debt, macrocirculatory parameters, and time to cardiac arrest between pigs anesthetized with propofol-ketamine-dexmedetomidine or alfaxalone-ketamine-dexmedetomidine subjected to stepwise, volume-controlled model of hemorrhagic shock.

MATERIALS AND METHODS

Ethics

Ethical approval was provided by the Norwegian National Animal Research Authority (FOTS ID 14277), Oslo, Norway (Advisor Marianne Waldum Furnes) on the 2nd of January 2018.

Animals

The experiments were performed between the 22nd of January and 23rd of March 2018 at The Research Animal Facility of the Norwegian University of Life Sciences. Sixteen mixed breed pigs (Norwegian land race 50% and Duroc 50%), 10 castrated males and six females, were included. They originated from the Animal Production Experimental Centre of the University, with a median (range) age of 66 (52–73) days and a mean (SD) body weight of 24.9 (4.2) kg in the propofol group and 25.9 (3.7) kg in the alfaxalone group at the time of anesthesia. They were identified by using their existing ear tag numbers and housed with natural light-dark cycles and room temperature between 15 and 20°C and fed a commercial pig diet combined with free access to hay at the Research Animal Facility of the Norwegian University of Life Sciences for 14 days prior to the experiment. Health status was monitored at minimum once daily during the entire period. Prior to the current study, the pigs were used in another anesthesia study with a minimum wash out period of seven days in between.

Study Design

A prospective, non-blinded, balanced, randomized study design was used. The 16 pigs were randomized in blocks of four to receive either propofol-ketamine-dexmedetomidine ($n = 8$) or alfaxalone-ketamine-dexmedetomidine ($n = 8$) by drawing paper notes.

Anesthesia

Grain was withheld for approximately 12 h before premedication, and all pigs were deemed healthy based on a clinical examination before each experimental session.

Premedication with ketamine 15 mg kg^{-1} (Ketamine Le Vet 100 mg ml^{-1}; Le Vet Beheer B.V., Holland) in combination with midazolam 1 mg kg^{-1} (Midazolam 5 mg ml^{-1}; B. Braun, Germany) was given intramuscularly in the cervical muscles behind the ear. An intravenous catheter (Venflon Pro; Becton Dickinson Infusion Therapy, USA) was placed in an auricular

vein. Anesthesia was induced with propofol (Propofol-Lipuro 20 mg ml^{-1}; B. Braun, Germany) or alfaxalone (Alfaxan 10 mg ml^{-1}; Jurox, Rutherford, Australia) to allow endotracheal intubation after topical application of lidocaine (Xylocaine 100 mg/ml spray, Aspen, Denmark) in the laryngeal area. They were placed in left lateral recumbency, covered with bubble wrap and external heat was provided with a forced air patient warming device (Bair Hugger, 3M, MN, USA) if the body temperature was <39.5°C. Volume controlled intermittent positive pressure ventilation was instituted with a rate of 20 breaths min^{-1} and tidal volume adjusted to maintain end tidal CO_2 ($PE'CO_2$) between 5.0 and 6.0 kPa before inducing hemorrhage. Ventilator settings was thereafter kept constant throughout the study.

Anesthesia was maintained with propofol 8 mg kg^{-1} h^{-1} or alfaxalone 5 mg kg^{-1} h^{-1}, ketamine diluted to 50 mg ml^{-1} at 5 mg kg^{-1} h^{-1} and dexmedetomidine (Dexdomitor 0.5 mg ml^{-1}, Orion Corporation, Finland) diluted to 50 µg ml^{-1} at 4 µg kg^{-1} h^{-1} i.v. Each drug was delivered by a separate syringe driver (Alaris GH Plus, BD Medical, Franklin Lakes, NJ, USA). The doses of propofol, ketamine and dexmedetomidine were based on the results from a previous study (2), whereas the alfaxalone dose was based on a pilot study in four pigs, where the infusion rate abolishing the motor response to an electrical nociceptive stimulus used in the previous study (2) was determined.

All pigs received a balanced electrolyte solution (Ringers acetate; Fresenius Kabi, Norway) i.v. at a rate of 0.33 ml kg^{-1} h^{-1} delivered by a volumetric infusion pump (Volumat Agilia; Fresenius Kabi, Norway). The total infused fluid volume including anesthetic drugs was 1.01 and 0.91 ml kg^{-1} h^{-1} in the alfaxalone and propofol group, respectively.

Instrumentation, Monitoring, and Data Collection

A multiparameter anesthetic monitor was used (GE Carescape Monitor B650; GE Healthcare, Finland) to record heart rate, 3-lead electrocardiography, invasive systolic, mean and diastolic arterial blood pressure (SAP, MAP, DAP), pulse rate (PR) taken from the arterial blood pressure trace, arterial oxygen saturation, end-tidal CO_2 ($PE'CO_2$), inspired oxygen fraction, and esophageal temperature. Data were downloaded every 5 s using data collection software (iCollect Version 5.0, GE Healthcare, Finland).

A 10 cm, 6 Fr. introducer sheath (Percutaneous sheath introducer set; Arrow Int. Inc., USA) was placed in the external jugular vein under ultrasound guidance using a 13 MHz linear array transducer (SL 3323, Esaote, Italy) and preprogrammed settings for small parts in the ultrasound system (Esaote MyLab One, Esaote, Italy). A single lumen 60 cm, 6 Fr. balloon pulmonary artery catheter (Balloon Wedge Pressure Catheter; Arrow Int. Inc., USA) (PAC) attached to a pressure transducer (TruWave pressure monitoring transducer; Edwards Lifesciences Corp., USA) was inserted through the introducer sheath, and advanced into the pulmonary artery under observation of the characteristic waveforms and pressures. The pressure

transducer was fixed at the level of the sternum and zeroed to atmospheric pressure.

A 20 cm thermistor tip, 5 Fr. thermodilution catheter (PiCCO Catheter; Pulsion Medical Systems SE, Germany) was placed by a modified Seldinger technique in the left tibial artery, and connected to a pressure transducer set (TruWave pressure monitoring transducer; Edwards Lifesciences Corp., USA) fixed at the level of the sternum and zeroed to atmospheric pressure, allowing continuous arterial blood pressure measurement, in addition to transpulmonary thermodilution measurement of CO.

To allow for rapid blood withdrawal, a 23 cm, 18-gauge catheter (Arterial Catheterization set, Arrow Int. Inc., USA) was placed percutaneously in the right femoral artery under ultrasound guidance using a modified Seldinger technique.

Hemorrhage

Hemorrhagic shock was initiated ~120 min after induction of anesthesia. The total blood volume was estimated as 65 ml/kg body weight (15). The blood volume to be withdrawn was taken from the femoral arterial catheter using a 60 ml syringe, a closed collection system, a 3-way stop-cock and a stopwatch to achieve a steady exsanguination rate. Thirty percent (H30) of the total blood volume was first removed over 10 min, followed by a 20 min period to allow for compensation. Thereafter 10% of the total blood volume (H40) was withdrawn over 10 min, followed by a 10 min period to allow for compensation. Thereafter 5% of the total blood volume was withdrawn every 10 min (H45, H50, and so on), followed by a 10 min period to allow for compensation between each period until cardiac arrest occurred (**Figure 1**). Cardiac arrest was defined as the first time point when $PE'CO_2$ was below 1.5 kPa with a concurrent pulse pressure below 10 mmHg.

Cardiovascular Evaluation

CO was measured prior to and after H30 and H40. Subsequent measurements of CO were then performed only after each period of hemorrhage (**Figure 1**).

For the transpulmonary thermodilution measurement, 10 ml of cold saline was injected as a rapid bolus through an injectate temperature sensor housing (Pulsion Medical System SE, Germany) into the side port of the jugular venous introducer sheath. The CO measurement was repeated three times at each time point. If a measurement deviated more than 10% from the mean of these measurements, additional measurements were performed until there were three measurements within this range. To standardize injected fluid volume between groups all injections were performed even if a CO could not be measured at the low flow states during shock.

Evaluation of Anesthetic Depth

Anesthetic depth was evaluated after the baseline measurement of CO, prior to induction of hemorrhage (**Figure 1**).

Clinical signs of anesthetic depth were evaluated and scored using a binary scoring system by the same investigator, eye position (ventral = 0, central = 1), nystagmus (present = 0, absent = 1), palpebral reflex (present = 0, absent = 1), and corneal reflex (present = 0, absent = 1) were assessed.

FIGURE 1 | Timeline of the experiment. Figure showing timeline of the experiment. Evaluation of anesthetic depth, percentage of total blood volume removed, measurement of cardiac output (CO), and sampling time point for blood gases (BG) are shown. The first box (black) represents the 120 min from induction of anesthesia until start of the experiment. Each additional box represents 10 min.
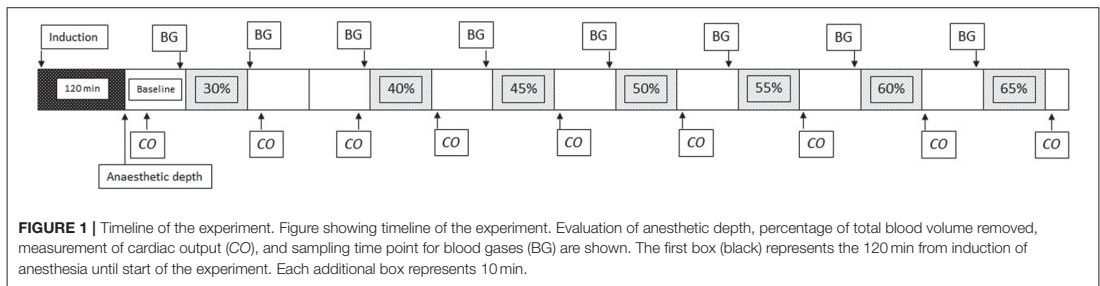
Thereafter mechanical nociceptive stimulation was applied to the lateral dewclaw of the right front limb by a latex-coated forceps with a clamping area 1×1 cm. The applied pressure was monitored with a spring balance attached to one forceps arm at a point equal distance from, and on the opposite side of the articulation as the clamping jaws. The force applied at clamping was 100 N and maximum clamping time for each stimulus was 59 s. Stimulation was stopped at withdrawal of the limb or if vigorous movement in other limbs or whole-body movement was observed. The withdrawal was scored as present (=0) or absent (=1). A summarized anesthetic depth score created for this study ranging from 0 to 5 was then calculated.

In seven pigs a two-channel referential electroencephalogram (EEG) was recorded using needle electrodes (Aiglette, Technomed Europe, Netherlands) placed 1 cm caudal to the lateral angle of the eye and 1 cm medial to the temporal line bilaterally. These two electrodes were referred to an electrode placed in the median plane 2 cm caudal to the recording electrodes. A ground electrode was placed caudal to the atlas wing. The resistance between the recording electrodes was kept below 3 kΩ. The electrodes were connected to an EEG monitor (A-1.000^{TM}, Aspect Medical Systems, USA). The monitor filters were set as follows - high frequency filter: 50 Hz, 50/60 Hz filter: 50 Hz and low frequency filter: 2.0 Hz. The monitor automatically detected burst suppression and calculated suppression ratio (BSR) as the percentage of epochs in the previous 63 s where the EEG signal was considered suppressed as a running average every 5 s.

Blood Sampling and Analysis

One ml of arterial and mixed venous blood was simultaneously sampled from the arterial and PAC before hemorrhage (baseline). Thereafter blood was withdrawn after H30 and then before each period of hemorrhage (**Figure 1**). Samples were drawn into heparinized blood gas syringes (Pico 70; Radiometer, Denmark), and analyzed within 30 min using a bench top blood gas analyzer (ABL 800 Flex; Radiometer, Denmark).

Data Analysis and Statistics

A power analysis was performed prior to the study. A difference in arterial lactate of at least 2.1 mmol/L after H50 was considered as clinically significant since this difference previously has been associated with an increase in mortality from 25 to 50% (16). In

the same study a standard deviation of 1.8 mmol/L was found. Aiming at a beta of 0.8 and an alpha of 0.05, a total number of 26 pigs was needed. Based on this we decided to include a total of 32 pigs, but also to perform an interim statistical analysis including a new sample size calculation based on the observed difference in lactate after the first 16 pigs had been enrolled.

A database was created in Microsoft excel and additional calculations were made (Microsoft Corp., NM, USA):

Haemoglobin concentration (Hb) = (Hb$_{arterial}$ + Hb$_{venous}$)/2
Body surface area (BSA) m^2 = $0.0734 \times$ BW$^{0.656}$
Cardiac index (CI) = CO/BSA
Stroke volume index (SVI) = (CO/HR)/BSA
Total peripheral resistance (TPR) = $80 \times$ MAP/CO
Content of oxygen in arterial blood (CaO$_2$) = $(1.34 \times$ Hb $\times$ SaO$_2$) + $(0.025 \times$ PaO$_2$)
Content of oxygen in mixed venous blood (CvO$_2$) = $(1.34 \times$ Hb $\times$ SvO$_2$) + $(0.025 \times$ PvO$_2$)
Oxygen extraction (OE) = (CaO$_2$-CvO$_2$)

Graphical and further statistical analysis was performed using statistical software (JMP Pro 15.0.0, SAS, NC, USA, and R with the "nlme" package).

For comparison of indices of oxygen debt, including arterial lactate concentration (lactate), arterial base excess (BE) and OE, and macrocirculatory parameters, including PR, SAP, DAP, MAP, CI, SVI, and TPR, linear mixed effect model "lme" with restricted maximum likelihood (REML) was used. All models were fitted with pig with respect to time as a random slope effect, while several covariates were tested against both a null model having only the random slope effect and no covariates and a null model having neither covariates nor random effects. The latter model was fitted using the "gls" function using REML. The Akaike Information Criterion (AIC) was used to examine goodness of fit of all models. Model fit to data and normality of model residuals were examined using distributional plots of the residuals resulting from the models. All model testing statistics, without exception, favored the inclusion of random slope effects.

Time to cardiac arrest was compared between groups using a Kaplan Meier analysis. α was set to 0.05.

RESULTS

Data are given as mean ± SD unless otherwise stated.

Based on the interim analysis after the inclusion of eight pigs in each group, with a difference in lactate of 0.94 ± 3.58 mmol/L at H50, a total number of 458 pigs were needed to find a significant difference in lactate at H50. No further pigs were thus included.

The median (range) summarized anesthetic depth score was 4 (3–5) in both groups, while the median (range) BSR was 3 (0–27) in the propofol group, and 2 (1–53) in the alfaxalone group. EEG was evaluated in the last seven pigs of the experiment. One of eight and three of eight pigs had a positive withdrawal reflex in response to clamping of the dewclaw in the propofol and alfaxalone group, respectively. The one pig in the propofol group displayed a BSR of 3, while the two pigs with a positive withdrawal reflex in the alfaxalone group had a BSR of 1 and 2. In the third pig EEG was not evaluated.

Time until death is shown in **Figure 2**. No significant difference in time to death was found between groups ($P = 0.56$). Withdrawn median (range) blood volume at the time of cardiac arrest was 56.8 (50.5–64.5) and 57.3 (45–65)% of the total blood volume in the propofol and alfaxalone group, respectively.

There was a statistically significant association between MAP and treatment when adjusting for weight (8.54, 95% CI 0.88–16.3, $P = 0.04$, AIC = 6,178), where alfaxalone was associated with higher MAP. Adjusting for sex reduced the effect and AIC slightly (7.32, 95% CI −0.42–15.06, $P = 0.08$, AIC = 6,174), suggesting that the effect of treatment on MAP was weak.

An effect was detected for SAP including weight (13.62, 95% CI 3.72–23.52, $P = 0.02$, AIC = 6,562) and both weight and sex (11.76, 95% CI 2.2–21.32, $P = 0.03$, AIC = 6,557), where treatment with alfaxalone was associated with higher SAP.

For DAP the effect was at best weak when adjusting for weight (6.68, 95% CI −0.1–13.46, $P = 0.07$, AIC = 5,856) and non-significant when adjusting for both weight and sex (5.71, 95% CI −1.25–12.67, $P = 0.13$, AIC = 5,852).
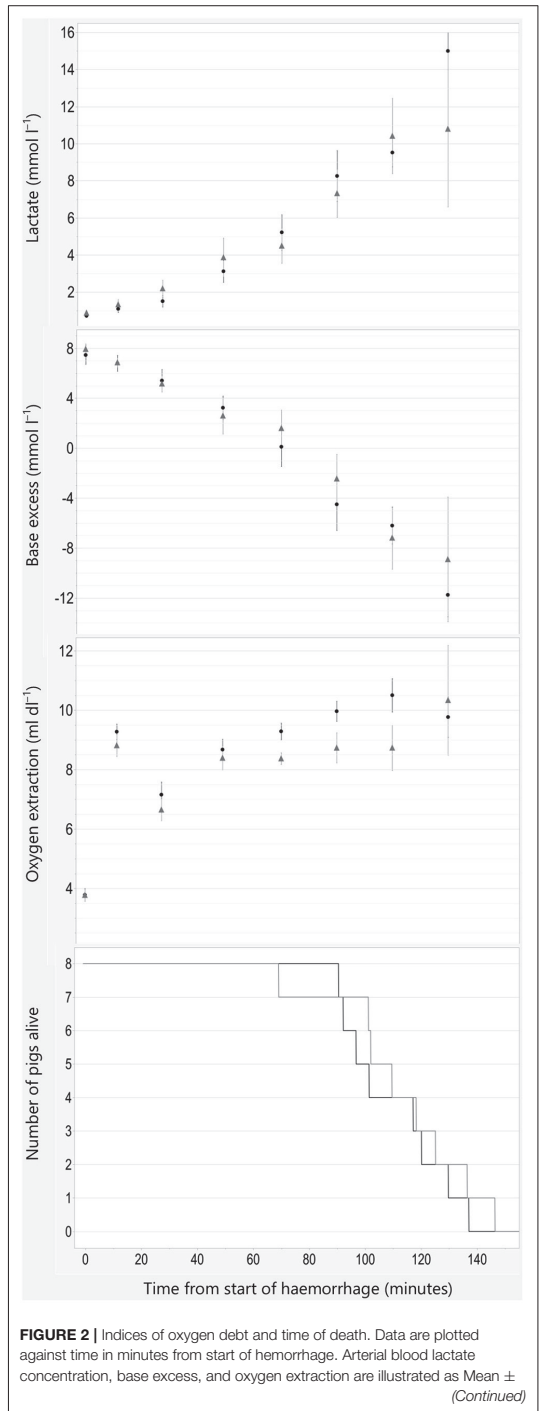
For all other models, including lactate, BE, OE, PR, *CI*, SVI, and TPR the effect of treatment was not significant ($P > 0.05$), regardless of covariates included, and therefor omitted.

Graphical description of oxygen debt indices and macrocirculatory parameters are shown in **Figures 2–4**.

DISCUSSION

Pigs anesthetized with either propofol-ketamine-dexmedetomidine or alfaxalone-ketamine-dexmedetomidine displayed a similar resilience to blood loss. A non-lethal volume loss of 50% or more of the total blood volume was possible in most pigs, even without resuscitation efforts. This volume of blood loss exceeds the volume loss defining class IV hemorrhage in the Advanced trauma life support classification of hypovolemic shock (17); a level of blood loss that is associated with severe physiological derangements and mortality in humans, often reproduced in the laboratory setting (18, 19) and encountered during LTT.

Oxygen debt is strongly associated with cellular damage, apoptosis and mortality in animal models of hemorrhagic shock (14). Both increased lactate and decreased BE correspond well
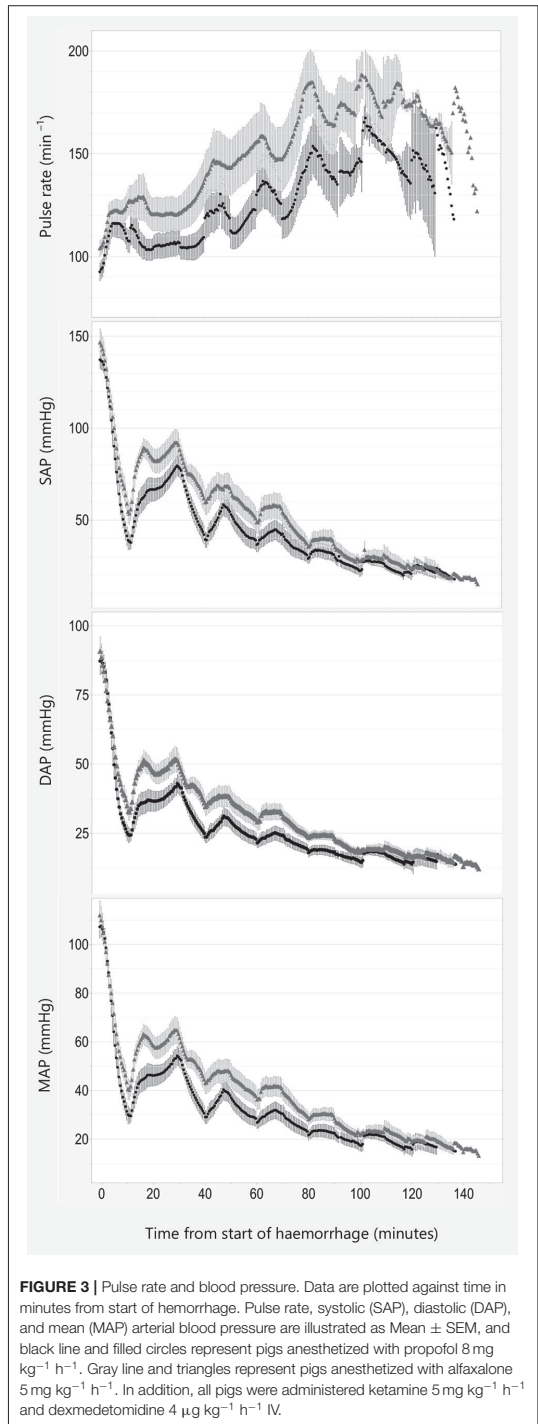

FIGURE 2 | Indices of oxygen debt and time of death. Data are plotted against time in minutes from start of hemorrhage. Arterial blood lactate concentration, base excess, and oxygen extraction are illustrated as Mean ±
(Continued)

FIGURE 2 | SEM, and numbers of pigs alive are illustrated as lines. Black line and filled circles represent pigs anesthetized with propofol 8 mg kg^{-1} h^{-1}. Gray line and triangles represent pigs anesthetized with alfaxalone 5 mg kg^{-1} h^{-1}. In addition, all pigs were administered ketamine 5 mg kg^{-1} h^{-1} and dexmedetomidine 4 μg kg^{-1} h^{-1} IV.
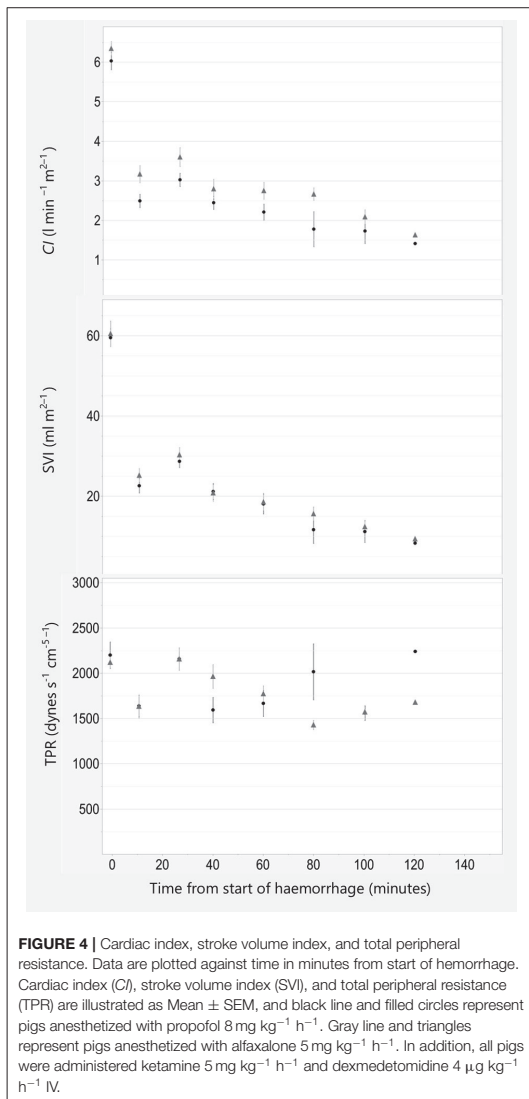
with increased oxygen debt. The LD50 for oxygen debt in pigs subjected to hemorrhage occur at a BE reduction of -15.3 mmol/L and a lactate concentration increase of 7.7 mmol/L (16). This corresponds to values of base excess and plasma lactate where 50% mortality occurred after ∼55% blood loss in the current study. A difference between the two groups was found in blood pressure, but not for base excess or blood lactate. The changes found for indices of oxygen debt are similar to the results in the study by Rixen et al. (16), where anesthesia was maintained with etomidate and fentanyl.

Pigs anesthetized with alfaxalone had a significantly higher systolic blood pressure than pigs given propofol. This could result from higher *CO* due to a higher PR og SVI, or a higher TPR in the alfaxalone group. Concurrently no statistical difference could be established for the other macrocirculatory parameters examined, including *CO* and TPR. Upon visual inspection of the trend curves for these parameters we do however consider the difference in systolic pressure to be a consequence of a higher PR and thus *CO* in the alfaxalone treated pigs, rather than a change in SVI or TPR, as these are more similar between the two groups.

Our observation from the previous pilot study could not be reproduced, as pigs in both groups displayed increasing pulse rate with increasing hemorrhage volume. However, the variation in pulse rate was large in both groups, and pigs anesthetized with alfaxalone seem to maintain a stronger chronotropic response to hypovolemia than pigs given propofol. This is similar to findings in a previous investigation comparing alphaxalone/alphadolone and propofol when used to maintain anesthesia in pigs subjected to hemorrhage, where a higher heart rate was found at baseline after 2 h of drug infusion, and after a blood loss of 40 ml kg^{-1} (20).

Ketamine and dexmedetomidine are drugs known to influence cardiovascular function. Ketamine has been shown to increase heart rate and thereby blood pressure (21), while dexmedetomidine is known to induce bradycardia in several species, including pigs (22–24). In a previous publication in normovolemic pigs dexmedetomidine did not produce the expected decrease in heart rate (2), a finding that was later reproduced with pigs in isoflurane anesthesia (25). The reason for this surprising finding is not known. In the same publication we found a slight increase in TPR when dexmedetomidine was infused, while TPR remained relatively unchanged in the current experiment despite significant blood loss. The cause of this vascular unresponsiveness cannot be elucidated in our study. The cardiac index found at baseline in this experiment was similar in both groups, and also similar to that found in our previous publication in normovolemic pig given either fentanyl or dexmedetomidine in combination with propofol and ketamine (2). This leads us to believe that the cardiovascular effects

FIGURE 3 | Pulse rate and blood pressure. Data are plotted against time in minutes from start of hemorrhage. Pulse rate, systolic (SAP), diastolic (DAP), and mean (MAP) arterial blood pressure are illustrated as Mean ± SEM, and black line and filled circles represent pigs anesthetized with propofol 8 mg kg^{-1} h^{-1}. Gray line and triangles represent pigs anesthetized with alfaxalone 5 mg kg^{-1} h^{-1}. In addition, all pigs were administered ketamine 5 mg kg^{-1} h^{-1} and dexmedetomidine 4 μg kg^{-1} h^{-1} IV.

FIGURE 4 | Cardiac index, stroke volume index, and total peripheral resistance. Data are plotted against time in minutes from start of hemorrhage. Cardiac index (CI), stroke volume index (SVI), and total peripheral resistance (TPR) are illustrated as Mean ± SEM, and black line and filled circles represent pigs anesthetized with propofol 8 mg kg^{-1} h^{-1}. Gray line and triangles represent pigs anesthetized with alfaxalone 5 mg kg^{-1} h^{-1}. In addition, all pigs were administered ketamine 5 mg kg^{-1} h^{-1} and dexmedetomidine 4 μg kg^{-1} h^{-1} IV.

is not linear, and a previous study found that the speed of hemorrhage can influence the physiological response observed (18). A weakness of that study was however the large variation in the isoflurane concentrations used. Despite this, it seems important to interpret results from studies of experimental hemorrhage in the light of the hemorrhage model used. The stepwise model with a decreasing loss of volume over time and time for compensation was used to resemble clinical hemorrhage during for example live tissue training more closely than a continuous hemorrhage model would do.

Defining equipotent doses of anesthetic agents and similar anesthetic depth is challenging. To compare anesthetic depth a simple scoring system was used based on common clinical indicators of anesthetic depth in pigs, in conjunction with EEG in seven of the animals. EEG was added to the study protocol after the inclusion of nine animals as one of the pigs displayed a positive response to mechanical nociceptive stimulation, in addition to a positive corneal reflex being observed in some pigs. EEG indicated that the cerebrocortical depression was profound with both anesthetic regimes, with several pigs displaying burst suppression (26). The use of ketamine in our anesthetic protocol could have influenced the EEG results, as ketamine traditionally has been known to increase EEG activity (27). The combined effects of ketamine and GABA-agonists or inhalational anesthetics on the EEG do however seem to be more complex, as the addition of ketamine also has been shown to induce burst suppression in rats (28). In addition, the anesthetics depth score was similar in both groups before induction of hemorrhage. With progressive hemorrhage plasma concentrations of both propofol and alfaxalone increased in our study, with an increasing variation in plasma concentrations of both propofol and alfaxalone (unpublished observations).

CO measurement using transpulmonary thermodilution has shown good accuracy when compared to techniques using thermistor catheters in the pulmonary artery, and a comparable precision has also been shown in pigs when comparing the two methods (29–31). At the same time variations in agreement was documented in a study comparing the two methods at different levels of CO (32). All measurements in our study were performed in triplicates, and the need for repeated measurements due to variations in the three measurements was very low. We did however encounter difficulties obtaining measurements at very low flow states, resulting in missing CO data toward the end of each experimental session. This was not unexpected and is probably due to recirculation and loss of indicator, leading to overestimations of CO followed by failing measurements (33–35). If a false increase in CO occurred at lower flow states, this could make it harder to detect differences between the anesthetic agents examined.

In this study lactate was considered the primary outcome variable and was used for interim sample size calculation. If more animals had been included a statistical difference may have been found for macrocirculatory variables. However, based on our results, a possible difference would likely have been small. The rationale for an interim analysis was a to minimize the total number of animals being sacrificed, which may have resulted in not detecting subtle statistical differences in other variables.

of ketamine and dexmedetomidine are similar in pigs given either propofol or alfaxalone. At the same time a cardiovascular comparison of the two drugs could very well have been different without the addition of ketamine and dexmedetomidine.

A fixed volume, stepwise hemorrhage model was used in our study. The advantage of fixed volume hemorrhage is that physiological responses and compensation can be compared between groups (15). At the same time the effect of hemorrhage on cardiovascular performance and oxygen debt is not controlled and may vary between individuals. Also, clinical hemorrhage

The reported blood volume in pigs shows some variation in different publications (15, 36, 37), and the reported hemorrhage volume tolerated in our pigs is based on a total blood volume of 65 ml kg^{-1}. Also, the pigs in our study were not splenectomized. Splenic contraction can increase the circulating volume and oxygen carrying capacity in pigs during hemorrhage, and splenectomy may influence the physiological response to blood loss (38). A difference in splenic volume has also been reported with different anesthetic drugs in dogs (39), but to our knowledge the effect of propofol or alfaxalone on splenic size in pigs has not been examined. For the comparison between groups in our study this likely has no consequence. All pigs used were from two litters and were block randomized to reduce the interindividual variation between groups.

An additional limitation of our study could be the transferability into a clinical situation in pigs anesthetized for LTT or experimental surgery. Resuscitation efforts where not performed in our pigs. Hence, the cardiovascular response to interventions such as intravenous fluid therapy or vasoactive drugs remains unknown, and potential differences between treatments remains undiscovered. In addition, only a small volume of fluids where administered not to change the volume status of the pigs during the experiment. One could however argue that the infused fluid volume was to low, and thus not mimicking a realistic clinical situation. Another limitation is that the nociceptive and cardiovascular response to surgical trauma was not examined. The anesthetic regime has been used by the authors in pigs, and we consider the cardiovascular stability to be excellent during invasive experimental surgical procedures (personal observations).

In conclusion, total intravenous anesthesia with either propofol-ketamine-dexmedetomidine or alfaxalone-ketamine-dexmedetomidine allows for substantial blood loss in pigs used in experimental settings. Pigs anesthetized with alfaxalone seem to maintain a higher systolic blood pressure than pigs given propofol, but we could not find differences in other macrocirculatory variables or indices of oxygen debt.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical approval was provided by the Norwegian National Animal Research Authority (FOTS ID 14277), Oslo, Norway (Advisor Marianne Waldum Furnes) on the 2nd of January 2018.

AUTHOR CONTRIBUTIONS

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RESEARCH PAPER

Inhibition and facilitation of nociceptively evoked muscular activity by fentanyl or dexmedetomidine in isoflurane-anaesthetized pigs

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Abstract

Objective To investigate motor and cardiovascular responses to dexmedetomidine or fentanyl in isoflurane-anaesthetized pigs.

Study design Experimental, balanced, block randomized, two-group design.

Animals A group of 16 crossbred pigs, 55 ± 8 days (mean ± standard deviation) old.

Methods Deltoid electromyography (EMG) was recorded during isoflurane anaesthesia. Electrical stimulation using 5, 10, 20 and 40 mA of the distal right thoracic limb elicited a nociceptive withdrawal reflex (NWR), quantified by the area under the curve (AUC) for the simulation intensity *versus* EMG amplitude response curve. Latency to movement evoked by clamping a claw for maximum 60 seconds was noted. Arterial blood pressure and pulse rate were recorded. Data were sampled at baseline and during dexmedetomidine 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 μg kg^{-1} hour^{-1} or fentanyl 5, 10, 20, 40, 80 and 160 μg kg^{-1} hour^{-1} infusions. The influence of infusion rate on NWR AUC and spontaneous EMG was analysed using a mixed model, with $p < 5\%$.

Results NWR AUC increased at fentanyl 5 μg kg^{-1} hour^{-1} but decreased at fentanyl 40, 80 and 160 μg kg^{-1} hour^{-1} and dexmedetomidine 4.0 and 8.0 μg kg^{-1} hour^{-1}. All pigs at fentanyl 80 μg kg^{-1} hour^{-1}, and three pigs at dexmedetomidine 8.0 μg kg^{-1} hour^{-1} had mechanical latencies greater than 60 seconds. Spontaneous EMG activity increased accompanied by visually evident 'shivering' at fentanyl 5, 10 and 20 μg kg^{-1} hour^{-1} but decreased at dexmedetomidine 2, 4 and 8 μg kg^{-1} hour^{-1}. Clinically relevant effects of increasing infusion rates on blood pressure or pulse rate were not observed.

Conclusion and clinical relevance If anaesthetic plane or antinociception is evaluated in pigs, response to claw clamping and NWR will not necessarily give uniform results when comparing drugs. If only one method is used, results should be interpreted cautiously.

Keywords anaesthesia, dexmedetomidine, fentanyl, pigs, reflex, shivering.

Introduction

Pigs are increasingly used for biomedical research and surgical training (Gutierrez et al. 2015). It is commonly assumed that pigs respond in a similar manner to humans and that results can be transferred directly across species boundaries (Rey-Santano et al. 2014). To safeguard animal welfare, secure research quality and to gain knowledge of generalizability, investigations into the specific anaesthetic responses in pigs are needed (Bradbury et al. 2016). In addition to antinociception and unconsciousness, loss of motor activity is one goal of general anaesthesia; however, shivering is a commonly observed phenomenon in anaesthetized pigs (Bradbury & Clutton 2016). Movement after noxious stimulation is a sign of inadequate anaesthetic level and the basis for the minimum alveolar concentration (MAC) concept. In pigs, clamping a claw is commonly used as a nociceptive stimulus in MAC studies and is considered a supramaximal stimulus (Eger et al. 1988). The nociceptive withdrawal reflex (NWR) is a nocifensive spinal reflex in mammals initiated by a noxious insult and it may be elicited electrically by depolarizing a sensory nerve. The response can be evaluated electromyographically (EMG) (Andersen 2007). Both claw clamping and NWR have been used to evaluate anaesthetic depth.

In a previous study, pigs anaesthetized with propofol and ketamine were administered either dexmedetomidine or fentanyl at increasing infusion rates. To determine the infusion rates that could be considered as equipotent, movement response to claw clamping and NWR after electrical stimulation were used (Lervik et al. 2018). However, fentanyl was found to inhibit the movement response to claw clamping at infusion rates where there was little inhibitory effect on the

NWR. For dexmedetomidine, the opposite response was observed; NWR was inhibited, with little effect on response to claw clamping. Infusion rates considered as equipotent would differ to a high degree depending on which stimulation method was used. Therefore, the authors decided to investigate whether the same response pattern could be observed using a stable inhalant anaesthetic background.

The primary aim of the present study was to investigate the effect of increasing fentanyl and dexmedetomidine infusion rates on the motor response to electrical and mechanical nociceptive stimulation against a constant background of isoflurane anaesthesia. The secondary aim was to describe the effect of increasing infusion rates of dexmedetomidine and fentanyl on pulse rate (PR) and mean arterial blood pressure (MAP) in isoflurane-anaesthetized pigs.

Materials and methods

A group of 10 surgically castrated male and six intact female Landrace and Duroc hybrid pigs were included. The pigs originated from the Animal Production Experimental Centre of the Norwegian University of Life Science. On the day of anaesthesia, they were 55 ± 8 days old (mean $\pm$ standard deviation) with a bodyweight of 22 ± 4.0 kg. The number of animals selected was based on data from a previous study in which 16 pigs were used and different response patterns to the two stimulation modalities were identified (Lervik et al. 2018). A minimum 1 week prior to anaesthesia, the pigs were stabled at the research facility in an indoor pen in groups of four with wooden shavings for bedding and fed a commercial slaughter pig diet (Format kvikk 140; Felleskjøpet, Norway) and hay *ad libitum* with free access to fresh water. There were eight pigs in each group using a balanced, two-group design where treatment was randomized in blocks of four prior to the study. During the study, blocks of four pigs were sequentially included. The same researchers, who were unaware of the identity of the administered drugs, performed all procedures. Ethical approval was given by the Norwegian Animal Research Authority, approval number FOTS ID 14629.

Anaesthesia and instrumentation

Hay and water, but no commercial food, were available for 6 hours prior to each study. Anaesthesia was induced with isoflurane (IsoFlo vet.; Orion Pharma Animal Health, Norway) vaporized in 100% oxygen administered by a facemask (Anestesimaske Large; Kruuse, Norway). Induction started between 08:45 and 09:45 hours. The trachea of each pig was intubated using a 5 or 6 mm internal diameter polyvinylchloride tube (Rüschelit super safety clear; Teleflex Medical, Ireland). Anaesthesia was maintained with isoflurane in

oxygen and air administered by a rebreathing circle system (Circle system Q; Anmedic AB, Sweden). Respiratory and anaesthetic gases were sampled at a rate of 200 mL minute^{-1} from a sidestream connector placed in between the endotracheal tube and the rebreathing circuit. A multigas monitor analysed the inspired and exhaled gas samples (GE Carescape Monitor B650; GE Healthcare, Finland). The inspired fraction of oxygen (FiO_2) was maintained between 0.4 and 0.60. The multigas analyser was calibrated according to the manufacturer's instructions weekly. Mechanical ventilation was initiated using a volume-controlled, time-cycled ventilator (MCM 801 Dameca; Rødovre, Denmark) at 20 breaths minute^{-1}. Tidal volume was adjusted to maintain the end-tidal fraction of carbon dioxide ($Fe'CO_2$) between 5.0% and 6.5% (38 and 49 mmHg). After each pig was placed in left lateral recumbency, 22 gauge catheters (Venflon Pro; Becton Dickinson Infusion Therapy, Switzerland) were placed in an auricular vein and a metatarsal artery. The arterial catheter was connected to a pressure transducer (TruWave; Edwards Life Science, UK) level with the thoracic inlet for invasive blood pressure. If MAP decreased below 50 mmHg for more than 3 minutes, or below 45 mmHg, dobutamine (Dobutamine, Hameln, Germany) was infused. These liberal cut-off values were used because the cardiovascular response to the drugs was investigated. Further physiological variables monitored were haemoglobin oxygen saturation (SpO_2), electrocardiography and rectal temperature. Data from the anaesthetic monitor were downloaded to a computer every 5 seconds using commercially available software (iCollect 5.0; GE Healthcare). During instrumentation, the end-tidal isoflurane concentration ($Fe'Iso$) was titrated to the lowest value sufficient for the pig to remain immobile. This was based on clinical judgement and was performed by the same investigator in all pigs. This $Fe'Iso$ was maintained for at least 20 minutes prior to the first stimulation and further throughout the study. If spontaneous movement occurred in this 20 minute period, the $Fe'Iso$ was increased by 0.1% and a new 20 minute equilibration period started.

The skin was shaved and cleansed using ethanol (Antibac, Norway) prior to placing self-adhesive surface electrodes (Neuroline Surface Electrodes; Cephalon, Denmark) 15 mm apart palmar to the metacarpal bone with the anode distally for transcutaneous stimulation of the palmar nerve. The resistance was maintained below 5 kΩ. A ground electrode (Disposable Ground Plate Electrode; Cephalon) was placed over the right lateral neck, and two 10 mm stainless-steel needle electrodes (Aiglette Disposable Needle Electrodes; Cephalon) were placed in the ipsilateral deltoid muscle. After the last stimulation, prior to recovery, 0.4 mg kg^{-1} meloxicam (Metacam, Boehringer Ingelheim Vetmedica Gmbh, Germany) was administered intramuscularly.

Test drug infusion

Fentanyl 50 µg mL^{-1} (Fentanyl; Hameln, Germany) and dexmedetomidine 0.5 mg mL^{-1} (Dexdomitor; Orion Corporation, Finland) were diluted in 0.9% NaCl solution (Baxter, Norway). All drug solutions were prepared by one individual. For each drug, three concentrations were prepared, and each concentration was infused at 1.6 and 3.2 mL kg^{-1} hour^{-1}, resulting in infusion rates of fentanyl at 5, 10, 20, 40, 80 and 160 µg kg^{-1} hour^{-1} and dexmedetomidine at 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 µg kg^{-1} hour^{-1}. The rates were administered for 31 minutes at increasing doses using a syringe driver (Alaris GH Plus; BD Medical, NJ, USA), a 50 mL syringe (Terumo, Belgium) and a low-volume infusion line (Mediplast, Italy). Ringer's acetate (Fresenuis Kabi, Norway) and 500 mg mL^{-1} glucose solution (Braun, Germany) mixed in a 20:1 ratio was infused at 5 mL kg^{-1} hour^{-1} by a volumetric infusion pump (Volumat Agilia; Fresenius Kabi) throughout the study.

Recording motor responses

Time to motor response from mechanical stimulation was recorded manually, whereas response to electrical stimulation was recorded by EMG. Mechanical stimulation was performed by clamping one claw and alternating between the two middle claws of the right pelvic limb, using a latex-shod towel clamp (Nor Stainless, Norway) with a clamping area of 1×1 cm and a force of 100 N controlled through a spring balance (Little Samson; Salter, UK). Withdrawal of the stimulated limb was considered a positive response. Mechanical stimulation was performed for a maximum of 60 seconds, and latency to withdrawal of the limb was manually recorded.

For NWR recording, an electrophysiological monitor system was used (VikingQuest; Cephalon). The EMG filter settings were 20 Hz lower filter and 30 kHz upper filter. Spontaneous EMG was recorded for 480 ms prior to each single stimulation and repeated stimulation series. Each stimulation consisted of a train of five 1 ms constant current, square wave pulses at 200 Hz.

Repeated stimulation consisted of 10 stimulations at 5 Hz. Single stimulation preceded repeated stimulation, both were performed at 5, 10, 20 and 40 mA in order of increasing intensity. The time interval between each stimulation intensity was 60 seconds. Electrical stimulation was initiated 180 seconds after the start of mechanical stimulation. Motor response recording was performed at baseline and subsequently after 20 minutes of drug infusion. Immediately after each stimulation series, the next drug infusion rate was started and maintained for 20 minutes prior to stimulation. This was repeated until all six drug infusion rates were administered (Fig. 1).

The EMG signal was quantified as root mean square (RMS) of recorded voltage. The EMG recording quality was manually controlled after the study. To avoid artefacts caused by electrical cardiac activity, an 80 ms time window was manually placed within the spontaneous baseline EMG recording and RMS calculated. This was done for each single stimulation and each repeated stimulation series. NWR was quantified as RMS voltage of the recorded EMG signal within the time window of 20−100 ms after stimulation. This EMG signal was visually inspected for artefacts; if present, it was not included in further
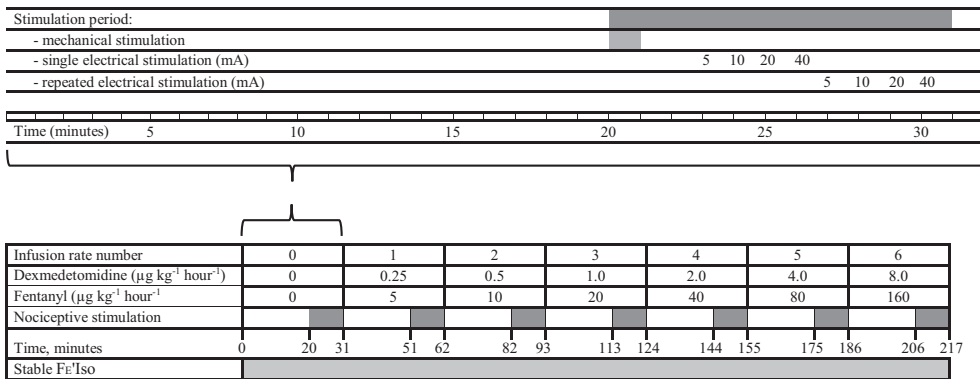
Figure 1 Experimental timeline for the 16 pigs given either fentanyl ($n = 8$) or dexmedetomidine ($n = 8$). From time −20 minutes, end-tidal isoflurane concentration (F$_E'$Iso) was maintained stable at a level adjusted to each individual pig. Nociceptive stimulations started at 20 minutes and lasted for 10 minutes. At time 31 minutes, the first drug infusion rate was started, and nociceptive stimulations started again after 20 minutes of drug infusion. Each drug infusion lasted for 31 minutes before the next rate of infusion was started. This was repeated for six infusion rates. A detailed timeline is given at the top for the 31 minute epochs.

analysis. To eliminate the influence of spontaneous EMG activity on NWR RMS, the 80 ms baseline RMS EMG was subtracted from the NWR RMS, further described as NWR. The area under the curve (AUC) for the dose response curves of single stimulation electrical current *versus* NWR was calculated using the trapezoid method. This AUC is further referred to as NWR AUC. To evaluate temporal summation, the ratios between the mean of the nine last NWR to the first NWR within each stimulation series were calculated, and a ratio above 1.5 was considered temporal summation. To evaluate spontaneous muscular activity, RMS of the 480 ms baseline EMG prior to 5 mA single stimulation was used.

Data management

Commercially available software was used for data analysis (Excel 2016; Microsoft, WA, USA) (JMP Pro 14.1.0; SAS Institute Inc., NC, USA) (Stata SE 14.2 for Windows; StataCorp LLC, TX, USA). Mean rectal temperature, $F_E'CO_2$, $F_E'Iso$, F_IO_2 and SpO_2 were calculated for individual pigs for the study period starting at the beginning of the baseline period. The differences in mean PR and mean MAP from the last minute prior to stimulation to the first 3 minutes of the stimulation period were calculated prior to test drug infusion and at the highest infusion rate. The 95% confidence interval (CI) for these means were calculated using Student t statistics. The 95% CIs were also calculated to evaluate the temporal summation ratios.

The influence of infusion rate on NWR AUC and spontaneous EMG activity as response variables was analysed by a mixed model using infusion rate as fixed effect and pig as random effect. The level of significance was set to 5%, and Bonferroni correction was used for multiple comparisons. The two treatments were analysed separately. The residuals were inspected for normality and homogeneity of variance. For

spontaneous EMG, a box cox transformation had to be performed. The NWR AUC and spontaneous EMG at each infusion rate was compared to baseline with a two-tailed comparison test.

In order to quantify the relationship between NWR AUC response and the likelihood of reaching the mechanical stimulation latency cut-off at 60 seconds, a multilevel logistic regression analysis was performed. The mechanical latency was transformed to a categorical variable, with mechanical latency 60 seconds and mechanical latency less than 60 seconds as the two categories. Drug, NWR AUC and weight were included as fixed effects, and pig as the random effect. Lastly, the interaction between drug and NWR AUC was included in the model. Results from the logistic regression are reported as odds ratios.

Results

All pigs recovered uneventfully from anaesthesia and were included in a subsequent terminal study. During the baseline equilibration period, three pigs in each group moved spontaneously. A new baseline equilibration period was started with 0.1% higher $F_E'Iso$, until immobility was achieved. At fentanyl 10 μg kg^{-1} $hour^{-1}$, one pig moved without being stimulated, necessitating a higher than baseline $F_E'Iso$ until the end of fentanyl 40 μg kg^{-1} $hour^{-1}$ infusion. Data from this period were excluded from further analysis and are not included in the physiologic data listed in Table 1.

Dobutamine was administered to one pig at a fentanyl dose of 160 μg kg^{-1} $hour^{-1}$ and the cardiovascular data were excluded. Shivering, observed as superficial muscle tremors, was noted in one pig at a dexmedetomidine constant rate of infusion of 0.5 μg kg^{-1} $hour^{-1}$, whereas shivering was observed in all but one pig at fentanyl 5, 10 or 20 μg kg^{-1} $hour^{-1}$; this pig exhibited violent movements. To investigate

Table 1 Mean ± standard deviation values of physiologic data recorded from the start of the baseline equilibration period used until the end of the last infusion. Data are given for the two groups to which either fentanyl or dexmedetomidine were administered during isoflurane anaesthesia at an individually stabilized end-tidal isoflurane concentration ($F_E'Iso$). Each group consisted of eight Landrace and Duroc hybrid pigs. The infusion rates were 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 μg kg^{-1} $hour^{-1}$ of dexmedetomidine, or 5, 10, 20, 40, 80 and 160 μg kg^{-1} $hour^{-1}$ of fentanyl. Time from induction of anaesthesia until the individually determined baseline $F_E'Iso$ were reached, are also given

	Dexmedetomidine	Fentanyl
Sex	2 female, 6 castrated male	4 female, 4 castrated male
Bodyweight (kg)	22 ± 4.3	21± 3.9
Age (days)	55 ± 8	55 ± 8
Time from induction to stable $F_E'Iso$ (minutes)	70 ± 19	62 ± 16
Rectal temperature (°C)	38.3 ± 0.3	38.5 ± 0.5
$F_E'CO_2$ (%)	5.3 ± 0.1	5.7 ± 0.2
SpO_2 (%)	99.5 ± 0.4	99.5 ± 0.6
F_IO_2 (%)	52 ± 2	50 ± 4
$F_E'Iso$ (%)	2.0 ± 0.10	1.9 ± 0.16

$F_E'CO_2$, end-tidal carbon dioxide concentration; $F_E'Iso$: End-tidal isoflurane concentration, F_IO_2, inspired fraction of oxygen; SpO_2, arterial haemoglobin saturation.

the cause, arterial blood glucose was measured by a glucometer (Accu-Chek; Roche, Switzerland) in three of these pigs, and the range was 6.0–7.2 mmol L^{-1}.

In Fig. 2, spontaneous EMG activity, NWR AUC and mechanical latency data are illustrated. In the dexmedetomidine group, there was a significant $F_{(6, 42)} = 7.26$; ($p < 0.0001$) overall effect of infusion rate on spontaneous EMG activity, which decreased significantly from baseline at infusion rates 2.0, 4.0 and 8.0 µg kg^{-1} $hour^{-1}$ ($p = 0.0009$, $p < 0.0001$ and $p < 0.0001$, respectively). There was also a significant $F_{(6, 40.09)} = 4.84$; $p = 0.0008$ overall effect of infusion rate on NWR AUC, which decreased from baseline at infusion rates 4.0 and 8.0 µg kg^{-1} $hour^{-1}$ ($p = 0.0033$ and $p = 0.0019$, respectively).

In the fentanyl group, there was a significant $F_{(6, 40.2)} = 17.63$; $p < 0.0001$ overall effect of infusion rate on spontaneous EMG activity, which significantly increased from baseline at infusion rates 5, 10 and 20 µg kg^{-1} $hour^{-1}$ ($p = 0.0003$, $p < 0.0001$ and $p < 0.0001$, respectively). There was also a significant $F_{(6, 39.2)} = 16.92$; $p < 0.0001$ overall effect of infusion rate on NWR AUC, which significantly increased from baseline at infusion rates 5 µg kg^{-1} $hour^{-1}$ ($p = 0.0013$) and decreased at 40, 80 and 160 µg kg^{-1} $hour^{-1}$ ($p = 0.0026$, $p = 0.0003$ and $p = 0.0003$, respectively).

No CI for the temporal summations was outside the range −1.5 to 1.5, and temporal summation was not investigated further.

In Fig. 3, MAP and PR data are shown. In the fentanyl group, the 95% CIs were 0.4–5.2 and 9.8–22.0 mmHg for the nociceptive change in MAP and −0.5 to 1.1 and −5.3 to 0.7 pulses $minute^{-1}$ for PR at infusion rates 0 and 160 µg kg^{-1} $hour^{-1}$, respectively.

In the dexmedetomidine group, the 95% CIs were −3.8 to 2.9 and 1.6 to 13.0 mmHg for the nociceptive change in MAP and −0.9 to 1.3 and −0.8 to 0.0 pulses $minute^{-1}$ for PR at infusion rates 0 and 8 µg kg^{-1} $hour^{-1}$, respectively.

In the logistic regression model, drug, body weight and the interaction between drug and NWR AUC had a significant effect on the log odds of reaching the cut-off latency of 60 seconds during claw clamp stimulation. For a pig of average weight and with an average NWR AUC, the odds of reaching cut-off was 7.5 times higher in the fentanyl group compared with the dexmedetomidine group ($p = 0.01$). The relationship between the odds of having a mechanical latency > 60 seconds and the NWR AUC was stronger in the fentanyl group than in the dexmedetomidine group ($p = 0.007$). A higher NWR AUC was associated with lower odds of not responding, as expected.
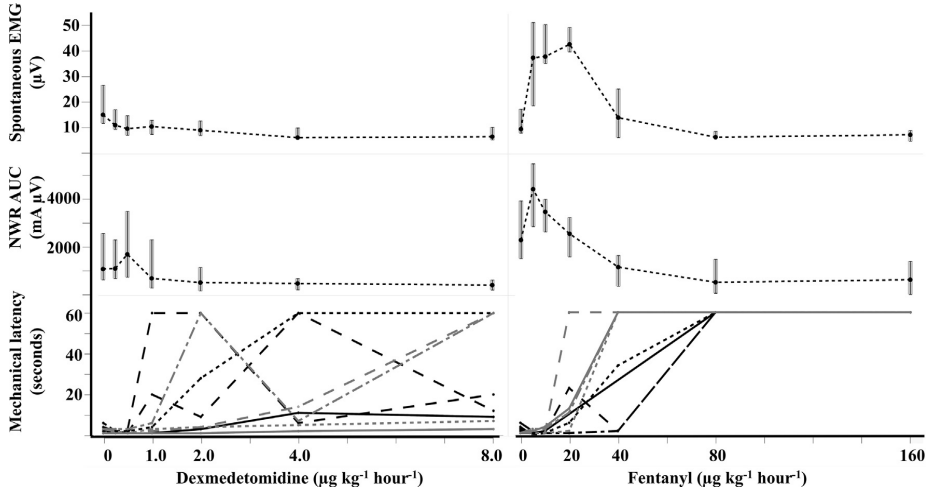
Figure 2 The y-axis represents the results from both groups. The results are given as root mean square of spontaneous electromyography (EMG) recorded from the deltoid muscle. The area under the curve of the nociceptive withdrawal reflex (NWR AUC) evoked by 5, 10, 20 and 40 mA single stimulation of the right distal thoracic limb and latency until withdrawal of limb after the dew claw of a pelvic limb was clamped. All pigs were anaesthetized with isoflurane and one of two test drugs: dexmedetomidine or fentanyl. Each group consisted of eight pigs. Infusion rates of the test drugs are shown on the x-axis. Baseline data are given at infusion rate zero. Spontaneous EMG activity and NWR AUC are given as median and interquartile range, whereas latency is illustrated by a line for each pig.
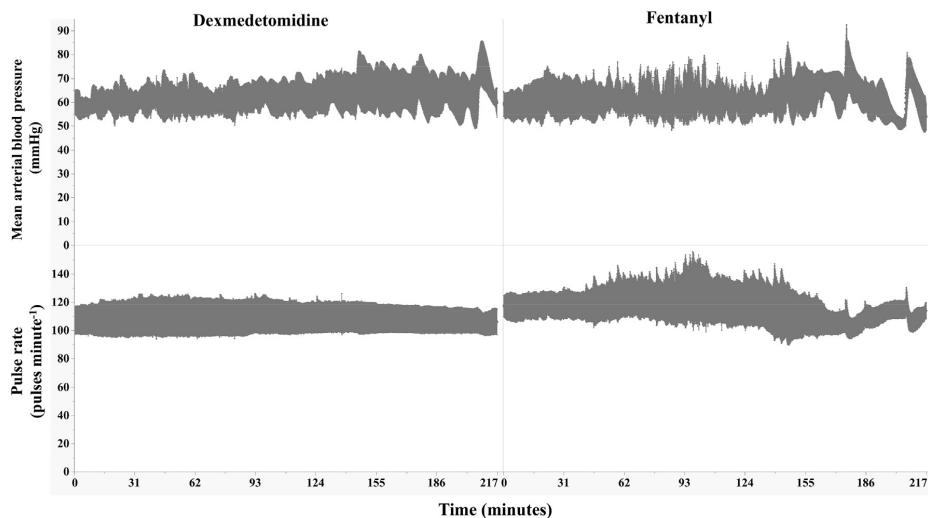
Figure 3 Data are reported as mean ± standard deviation for each 5 seconds of pulse rate and mean invasive arterial blood pressure recorded for isoflurane-anaesthetized pigs. Each group, dexmedetomidine and fentanyl, represent eight pigs that were given dexmedetomidine at 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 μg kg^{-1} hour^{-1} or fentanyl at 5, 10, 20, 40, 80 and 160 μg kg^{-1} hour^{-1}. For the first 31 minutes, no drug infusions were administered. Each drug infusion rate was administered for 31 minutes in increasing order with no interval in between.

Discussion

Both drugs significantly decreased NWR AUC at the higher infusion rates. Fentanyl had a prominent and consistent effect on response to mechanical stimulation. In the dexmedetomidine group, three of the five pigs that reached the 60 second mechanical latency cut-off had a latency far shorter than 60 seconds at a higher infusion rate. In the fentanyl group, all pigs reached the mechanical latency cut-off of 60 seconds and then maintained this latency (Fig. 2). The effect of dexmedetomidine on the mechanically elicited withdrawal response reaches a plateau in dogs (Kuusela et al. 2000). Decreasing F$_E'$Iso in pigs may both increase and decrease the response to claw clamping (Haga et al. 2002). The observation in the current study that the response to mechanical stimulation reappeared after it had been abolished at lower infusion rates may be explained by a plateau effect of dexmedetomidine being insufficiently profound to mask the inconsistent effect of isoflurane on this response.

The response to claw clamping is commonly used for MAC studies and electrically elicited NWR is used to evaluate antinociception in anaesthetized animals (Lervik et al. 2012). The current results demonstrate that these two stimulation modalities can give different results if used to compare antinociceptive effects of drugs. This is illustrated by finding a stronger association between NWR AUC and the odds of having a mechanical latency of 60 seconds in the fentanyl group compared with the dexmedetomidine group. This might be explained by fentanyl having a greater efficacy or a steeper dose response curve than dexmedetomidine for inhibiting motor response to claw clamping.

In humans, postanaesthetic shivering is usually attributed to hypothermia and dexmedetomidine and fentanyl may reduce the incidence of this effect (De Witte & Sessler 2002). In our study, shivering was readily observed at the lower fentanyl infusion rates and quantified by increased spontaneous EMG activity. Dexmedetomidine gradually decreased spontaneous EMG activity. Hypoglycaemia can cause seizures (Brauer et al. 2011); however, hypoglycaemia reduces the temperature threshold for thermogenesis shivering (Gale et al. 1981; Ino et al. 2015). Dexmedetomidine may raise blood glucose, but hypoglycaemia was not found when blood glucose was measured in three pigs in the fentanyl group. Hypothermia or altered plasma glucose levels are probably not the reasons for shivering in this study, because normothermia was maintained and glucose was continuously administered.

In addition to shivering, fentanyl increased NWR AUC at the lowest infusion rate. This was an unexpected finding. Decreasing NWR is commonly taken as evidence of antinociception (Fischer et al. 2017b). Previous studies in other mammals found decreased NWR amplitude after administration of morphine, fentanyl and buprenorphine, but no change in NWR threshold to butorphanol (Chabal et al. 1989;

Spadavecchia et al. 2007; Risberg et al. 2015; Fischer et al. 2017a). The increased NWR AUC could indicate a hyperalgesic effect of fentanyl. There is some evidence for an acute hyperalgesic effect after terminating fentanyl administration in humans (Lyons et al. 2015); however, in the current study, fentanyl plasma concentrations increased when NWR AUC increased, so we find this explanation less plausible. Another possible explanation is a change in the gain of the spinal motor control system, supported by the fact that spontaneous EMG activity increased and decreased following the same overall pattern as the increase and decrease in NWR AUC. In decerebrate cats, the selective μ agonist D-Ala(2)-N-MePhe(4)-Gly-ol(5)-enkephalin (DAMGO) facilitates activity in motor flexor reflex efferent neurons, a possible cause being the descending opioid and monoamine system controlling the gain of the spinal motor system (Steffens & Schomburg 2011). Previous studies show that fentanyl increases the level of serotonin in the central nervous system (Tao et al. 2003; Baldo 2018). Fentanyl has been linked to serotonergic syndrome in humans (Baldo 2018), and this link has been experimentally verified in rats (Kitamura et al. 2016). In humans, serotonin changes the motor gain system (Wei et al. 2014) in a biphasic pattern (Kavanagh et al. 2019) lending support to the hypothesis that the monoamine system is involved in the effects of fentanyl observed in the current study.

No hyperreflexia to claw clamping was found in this study. If present, it would be difficult to observe since baseline latency was short. Mechanical latency probably involves more complex spinal motor neural networks than the electrically elicited NWR since mechanical stimulation commonly elicits withdrawal and repeat rapid extensions. This could be an explanation for the difference in drug effect on the two responses. Another possible explanation is that claw clamping is a continuous stimulation of nociceptors likely resulting in both A-δ and C-fibre nociceptive transmission, whereas NWR AUC from the time window 20–100 ms is mainly a result of electrically elicited A-δ fibre activity.

Fentanyl commonly causes bradycardia in mammals likely due to a parasympathomimetic effect (Bowdle 1998). A fentanyl infusion at 25 μg kg^{-1} $hour^{-1}$ in pigs anaesthetized with propofol–ketamine caused a decrease in heart rate (HR) followed by a normalization (Lervik et al. 2018). In the current study, PR and MAP remained stable at fentanyl infusion rate from 5 to 160 μg kg^{-1} $minute^{-1}$ (Fig. 3).

Decreased HR, increased total peripheral resistance and arterial blood pressures are commonly observed cardiovascular effects of dexmedetomidine in mammals (Vujk J & Reekers 2015; Posner 2018). This has also been observed in isoflurane-anaesthetized pigs; a medetomidine bolus of 40 μg kg^{-1} increased blood pressure and decreased HR (Tendillo et al. 1996). In the current study, such a pattern was not observed.

The discrepancy between previously reported cardiovascular effects of the two drugs and the current study may be that the background anaesthetic drugs differ or that the infusion started at a low rate, thus giving the animal time to compensate for the cardiovascular effects. However, in other mammals, a dose-dependent, bradycardic and hypertensive effect is also seen during intravenous infusion of dexmedetomidine (Lawrence et al. 1997; Kutter et al. 2006; Pypendop et al. 2011; Hector et al. 2017) making this explanation improbable.

The observed decrease in motor response may be caused by an effect on the sensory input or the motor output from the central nervous system. At the highest infusion rate of both drugs, MAP increased during nociceptive stimulation. The 95% CIs for change in MAP were 9.8–22.0 and 1.6–13.0 mmHg for fentanyl and dexmedetomidine infusions, respectively. A reasonable conclusion would be that for both drugs some sensory input still reached the basal brain areas.

Drug infusion rates were doubled every 31 minutes. Based upon a previous study (Lervik et al. 2018), likely no stable plasma concentration was achieved at any of the infusion rates. Thus, the effects observed in the current study were probably less than might be observed if a stable plasma level state were reached. There were probably increasing plasma levels and increasing effect of the drugs within the 11 minutes of nociceptive stimulation. Each infusion rate could have been administered for a longer time prior to evaluation, but then fewer evaluations could have been performed or an even longer anaesthesia would have been needed. Thus, this design was chosen to prioritize more evaluations at a wide range of plasma concentrations rather than at steady state plasma concentration.

Shivering was an incidental finding, and only short periods of spontaneous EMG and no scoring system to obtain a good description of the shivering is available. However, the baseline EMG data illustrates the waxing and waning of shivering in the fentanyl group.

MAC values vary between individual pigs (Haga et al. 2011); therefore, rather than standardizing Fᴇ′Iso, it was individually adapted to achieve a similar anaesthetic plane. No predetermined algorithm was used, but Fᴇ′Iso was adjusted based on clinical judgement by an experienced anaesthesiologist during instrumentation. This may be a weakness of the study, but was done to avoid a prolonged time in anaesthesia for each pig. The interquartile range of NWR AUC in both groups overlapped, and mechanical latency at baseline was similar between pigs indicating a similar anaesthetic depth.

Similar to a previous study in isoflurane-anaesthetized pigs (Spadavecchia et al. 2012), no temporal summation was observed in the current study. Temporal summation has been demonstrated in other isoflurane-anaesthetized mammals (Baars et al. 2009; Spadavecchia et al. 2010; Lervik et al. 2012). Possibly, temporal summation is not present in

isoflurane-anaesthetized pigs at this age; the deltoid muscle may be unsuitable for this purpose or the leg position was not ideal since this may influence the measured response (Andersen 2007).

In conclusion, in pigs if the anaesthetic plane or anti-nociception is evaluated, the two methods, response to claw clamping and NWR, will not give uniform results if dexmedetomidine and fentanyl are compared. If only one method is used, the results should be interpreted cautiously. Fentanyl may induce shivering, NWR hyperreflexia and depression depending on the infusion rate.

Acknowledgements

Authors' contributions

HAH and JN: study design, data collection, data management, statistical analysis, data interpretation, preparation of manuscript. AL: study design, data collection, data interpretation, preparation of manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

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Abstract

Objective

To describe the changes in propofol and alfaxalone plasma concentrations during haemorrhagic shock in pigs.

Study design

A prospective, randomized study design was used.

Animals

Sixteen mixed breed pigs.

Methods

The pigs were premedicated with ketamine 15 mg kg^{-1} and midazolam 1 mg kg^{-1} intramuscularly , and anaesthesia was induced with propofol or alfaxalone for endotracheal intubation, followed by propofol 8 mg kg^{-1} h^{-1} or alfaxalone 5 mg kg^{-1} h^{-1} in combination with ketamine 5 mg kg^{-1} h^{-1} and dexmedetomidine 4 µg kg^{-1} h^{-1}given as a continuous infusion. Haemorrhagic shock was induced by stepwise exsanguination from the femoral artery, and blood samples for measurement of propofol and alfaxalone plasma concentrations were withdrawn at baseline (BL) after loss of 30% (H30) and 50% (H50) of the total blood volume.

Results

The median (range) plasma concentrations (µg/ml) were 5.1 (4.4 – 7.3) at BL, 6.9 (6.1 – 9.2) at H30 and 10.3 (8.8 – 19.4) at H50 for propofol, and 2.7 (1.9 – 4.8) at BL, 3.6 (2.3 – 5.8) at H30 and 4.6 (3.1 – 13.8) at H50 for alfaxalone. The median (range) relative increase (%) in plasma concentration from BL (%) was 45 (-16 - 66) and 27 (-9 - 92) at H30, 117 (25 - 248) and 120 (48 - 355) at H50 in

the propofol and alfaxalone group respectively. The relative increase from BL at H50 was not statistically different between treatments (p = 0.86).

Conclusions and clinical relevance

Plasma concentrations of both propofol and alfaxalone will increase during haemorrhage, with a large individual variation.

Keywords: Pigs, propofol, alfaxalone, haemorrhage, plasma concentration

Introduction

The effects of anaesthetic drugs on homeostasis are strongly associated with plasma concentrations. The plasma concentration of a drug depends on several factors, including the amount of drug infused, volume of distribution and drug clearance. Drug doses are usually based on pharmacokinetic or pharmacodynamic studies performed in healthy subjects. During haemorrhagic shock, changes in drug distribution and elimination may affect the plasma concentration (Neugebauer et al. 1992). In addition, pharmacodynamics may also be influenced by haemorrhage (Johnson et al. 2003), leading to increased anaesthetic effect and impaired cardiorespiratory function.

Haemorrhagic shock studies in pigs are performed under general anaesthesia. Previous studies have examined the effect of haemorrhage upon propofol plasma concentrations in pigs (Kurita et al. 2011), but to the authors knowledge, no such data exist for alfaxalone.

The objective of this investigation was to describe and compare the effect of stepwise haemorrhage on propofol and alfaxalone plasma concentrations in pigs given a constant rate infusion of the two drugs.

Methods

Animals

The study was approved by the Norwegian National Animal Research Authority (FOTS 14629), and the study was performed according to the ARRIVE guidelines. Sixteen mixed breed pigs (Norwegian landrace 50% and Duroc 50%), 10 castrated males and 6 females, were included in the study. All pigs originated from The Livestock Production Research Centre of the University and were 55 ± 8 days with a mean (SD) body weight of 24.9 (4.2) kg in the propofol group and 25.9 (3.7) kg in the alfaxalone group. They were housed in groups at the research animal facility of the veterinary faculty for approximately 14 days prior to the experiment. All pigs followed normal light-dark cycles in a room with day light and a room temperature kept between 15-20 °C. They were fed a commercial pig diet and had free access to hay. Approximately one week prior to this experiment the pigs were used in another anaesthesia study. A minimum wash out period of seven days was allowed between experiments. Their health status was monitored minimum once daily during the entire period.

Study design

A prospective, randomized study design was used. Sixteen pigs were allocated to two groups by permuted block randomization by drawing paper notes with 8 animals in each group. None of the

investigators were blinded to the given treatment. The number of animals included was decided by a sample size calculation performed for another investigation of haemorrhagic shock under the same anaesthesia.

Anaesthesia, monitoring and data collection

Food, but not straw and water, was withheld for 12 hours before premedication. All pigs were found healthy based on a clinical examination, and their body weight was measured on the day of the experiment.

Premedication was given using ketamine 15 mg kg^{-1} (Ketamine Le Vet 100 mg mL^{-1}; Le Vet Beheer B.V., Holland) and midazolam 1 mg kg^{-1} (Midazolam 5 mg mL^{-1}; B. Braun, Germany) administered intramuscularly in the cervical muscles. After sedation an intravenous catheter (Venflon Pro; Becton Dickinson Infusion Therapy, USA) was placed in an auricular vein. The pigs were preoxygenated for 4-5 minutes with 100% O$_2$ delivered by a face mask, and anaesthesia was induced by intravenous titration of propofol (Propofol-Lipuro 20 mg mL^{-1}; B. Braun, Germany) or alfaxalone (Alfaxan 10 mg mL^{-1}; Jurox, Rutherford, Australia) to allow endotracheal intubation after application of topical lidocaine (Xylocain 100 mg mL^{-1} spray; Aspen, Denmark). Volume controlled intermittent positive pressure ventilation (IPPV) was instituted with a respiratory rate of 20 min^{-1}, with the tidal volume adjusted to maintain P$_E$'CO$_2$ between 5.0 (37.5) and 6.0 (45.0) kPa (mmHg) before inducing haemorrhage. Anaesthesia was maintained with propofol 8 mg kg^{-1} h^{-1} or alfaxalone 5 mg kg^{-1} h^{-1} in combination with ketamine diluted to 50 mg mL^{-1} at 5 mg kg^{-1} h^{-1} and dexmedetomidine (Dexdomitor 0.5 mg mL^{-1}, Orion Corporation, Finland) diluted to 50 µg mL^{-1} at 4 µg kg^{-1} h^{-1} intravenously (IV). All drug dilutions were made with 0.9% NaCl (Natriumklorid Fresenius Kabi, Fresenius Kabi, Norway) and delivered by syringe drivers (Alaris GH Plus, BD Medical, Franklin Lakes, NJ, USA). The doses of propofol, ketamine and dexmedetomidine were

based on results from a previous study (Lervik et al. 2018). The alfaxalone dose was based on a dose titration pilot study in four pigs, using the lowest infusion rate abolishing the motor response to a standardised electrical nociceptive stimulation used in the previous study (Lervik et al. 2018).

The pigs were monitored using a multiparameter anaesthetic monitor (GE Carescape Monitor B650; GE Healthcare, Finland). Heart rate, respiratory rate, 3-lead electrocardiogram, oxygen saturation, end tidal CO_2, fractioned inspired oxygen concentration, and oesophageal temperature were recorded and automatically downloaded every 5 seconds to a computer using commercial software (iCollect Version 5.0, GE Healthcare, Finland).

All pigs received a balanced electrolyte solution (Ringers acetate; Fresenius Kabi, Norway) intravenously at a rate of 0.33 ml kg^{-1} h^{-1} delivered by a volumetric infusion pump (Volumat Agilia; Fresenius Kabi, Norway). The total infused fluid volumes including anaesthetic drugs were 1.01 ml kg^{-1} h^{-1} and 0.91 ml kg^{-1} h^{-1} in the alfaxalone and propofol group respectively. All fluid and drug infusions were administered through the same intravenous catheter placed in the auricular vein. The pigs were covered with bubble wrap to avoid heat loss, and external heat was provided with a forced air patient warming device (Bair Hugger, 3M, MN, USA) if the body temperature was < 39.5 °C. After completing the study, the pigs where euthanised with pentobarbital (Euthasol vet 400 mg mL^{-1}, Le Vet Beheer B.V., Holland) given intravenously.

Instrumentation

To allow for rapid blood withdrawal in order to induce haemorrhagic shock a 23 cm, 18G arterial catheter (Arrow Seldinger Arterial Catheter, Teleflex, PA, USA) was placed percutaneously in the

right femoral artery under ultrasound guidance using a modified Seldinger technique. In addition the pigs were instrumented with a pulmonary artery catheter (Arrow Balloon Wedge Pressure Catheter, Teleflex, PA, USA) and a thermistor tip 20 cm, 5 Fr. thermodilution catheter (PiCCO Catheter; Pulsion Medical Systems SE, Germany) as a part of the haemorrhagic shock study performed under the same anaesthesia.

Haemorrhage

Induction of haemorrhagic shock was started approximately 120 minutes after induction of anaesthesia. The total blood volume was calculated, using an estimated total blood volume of 65 ml/kg body weight (Hannon et al. 1990).

After baseline measurements (BL) blood was manually withdrawn at a constant rate from the femoral arterial catheter using a closed collection system with a 3-way stop-cock and a 60 ml syringe. Thirty percent (H30) of the total blood volume was removed over 10 minutes, followed by a 20 minutes period to allow for compensation. Thereafter 10% of the total blood volume (H40) was withdrawn over 10 minutes, followed by a 10 minutes period to allow for compensation. Thereafter 5% of the total blood volume was withdrawn over 10 minutes (H45, H50 and so on), followed by 10 minutes periods to allow for compensation between each period until cardiac arrest occurred.

Blood sampling and analysis of plasma concentrations

Heparinized blood was sampled from the arterial haemorrhage catheter at BL, immediately after H30 and 9 minutes after ending H50. A specific liquid chromatography- tandem mass spectrometry

(LC-MS/MS) method for analysis of alfaxalone and propofol in porcine plasma was used to determine plasma concentrations.

Statistical analysis

A database was created in Microsoft excel (Microsoft Corp., NM, USA). Graphical and further statistical analysis was performed using statistical software (JMP Pro 15.0.0, SAS, NC, USA). The relative increase in plasma concentration from BL was calculated for both treatment groups at H30 and H50. The relative increases from BL at H50 in both groups was visually assessed for normality of distribution, and comparisons between treatments were done with a Wilcoxon signed rank test. Alfa was set at 0.05.

Results

Absolute and relative increase in plasma concentrations are summarised in figure 1. One pig in the alfaxalone group died after H45. Plasma concentration measurements are missing for this animal at H50.

The median (range) plasma concentrations (µg/ml) were 5.1 (4.4 – 7.3) at BL, 6.9 (6.1 – 9.2) at H30 and 10.3 (8.8 – 19.4) at H50 for propofol, and 2.7 (1.9 – 4.8) at BL, 3.6 (2.3 – 5.8) at H30 and 4.6 (3.1 – 13.8) at H50 for alfaxalone.

The median (range) relative increase (%) in plasma concentration from BL (%) was 45 (-16 - 66) and 27 (-9 - 92) at H30, 117 (25 - 248) and 120 (48 - 355) at H50 in the propofol and alfaxalone group respectively.

The relative increase from BL to H50 was not statistically different between treatments (p = 0.86).

Discussion

The major finding in this investigation was that induction of moderate to severe haemorrhage in pigs anaesthetised with either propofol-ketamine-dexmedetomidine or alfaxalone-ketamine-dexmedetomidine will lead to changes in plasma concentrations of both propofol and alfaxalone, with a substantial increase observed in some individuals after 30 and 50% blood loss. However, we could not detect a statistically significant difference between the two drug protocols.

Increases in plasma concentrations during haemorrhagic shock have previously been documented in pigs administered different anaesthetic and analgesic agents, including propofol and fentanyl (Egan et al. 1999; Johnson et al. 2003). Causes of increased plasma concentration during haemorrhagic shock can be altered distribution and elimination of the drug. An earlier study of propofol pharmacokinetics during haemorrhagic shock in pigs found a twofold higher plasma concentrations when propofol was infused after approximately 43% loss in blood volume (Johnson et al. 2003), which is similar to our findings, despite a difference in study design. The major cause of the increase in the study by Johnson et al. was thought to be a decrease in total clearance, both due to a decrease in fast distribution clearance and reduced hepatic perfusion. No similar investigation has been performed for alfaxalone. When alfaxalone is used alone for TIVA in healthy, normovolemic dogs, hepatic blood flow is the most important factor for alfaxalone elimination even after 180 minutes of infusion (Dehuisser et al. 2019). The increase in alfaxalone plasma concentration observed by us is likely linked to a decrease in hepatic perfusion, among other pharmacokinetic parameters affected during haemorrhagic shock.

The median increase after H30 was only 37% on average, with a decrease in plasma concentration observed in some animals. These observations are most likely linked to the fact that blood was sampled immediately after ending this haemorrhage period. A later sampling time point could have given other results.

Another very important aspect of our observations is the variable influence of exsanguination upon plasma concentrations demonstrated by the wide range of increase in propofol and alfaxalone concentrations at H50. This observation could be a result of the chosen model for induction of haemorrhagic shock in our study, where a fixed controlled bleeding volume was used. In this model, the effect on organ function may differ more between individuals compared to when the rate and volume of haemorrhage are based on blood pressure or oxygen debt (Rixen & Siegel 2005). The wide range in plasma concentrations observed implicates that dynamic dose adjustments are needed, rather than using a fixed infusion rate to avoid increasing plasma concentrations and anaesthetic depth when using TIVA-protocols in experimental pigs during haemorrhagic shock.

Several factors may have influenced our results. The anaesthetic regime used contains other drugs that might impact the plasma concentration of propofol and alfaxalone. Dexmedetomidine has traditionally been shown to reduce cardiac output and thereby organ perfusion, but given in low doses in sheep with LPS-induced septic shock, cardiac output was well maintained (Hernández et al. 2016). In addition, we have previously shown that the combination of propofol-ketamine-dexmedetomidine maintains stable cardiovascular condition in experimental pigs (Lervik et al. 2018). This leads us to believe that the observed increase in plasma concentration of both propofol and alfaxalone is mainly a result of haemorrhage.

The blood used for analysis of drug plasma concentrations in the current study was arterial rather than venous. With progressive haemorrhage the arteriovenous difference in drug plasma

concentrations will increase. However, arterial blood samples probably reflect the drug concentration of the central compartment and effect site of the anaesthetic drugs most accurately (Chiou 1989).

In conclusion, plasma concentrations of both propofol and alfaxalone will increase during haemorrhage, with a large individual variation. Dose adjustments are necessary when using these drugs to avoid increasing plasma concentrations under such circumstances.

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<u>**Determination of alfaxalone and propofol in porcine plasma LC-MS/MS**</u>

A specific liquid chromatography- tandem mass spectrometry (LC-MS/MS) method for the simultaneous analysis of alfaxalone and propofol in porcine plasma was developed.

Extraction procedure:

1. In an1.5 mL Eppendorf centrifuge tube 100 µL of each sample, calibrator and QC (control quality sample) were mixed with the internal standard. (Table 1 and Table 2) and vortexed for 15 seconds.

2. Add 300µL of 1% acetic acid in acetonitrile(v/v).

3. Vortex the mixture for 5 minutes.

4. Centrifuge at 3000 rpm for 15 minutes.

5. Transfer the supernatant into a spin-X filter and centrifuge at 10000 rpm for 2 minutes, then transfer the resulting solution subsequently into a high-performance-liquid-chromatography (HPLC) injection vial with insert.

Table 1. Matrix-matched calibration curve of alfaxalone in pig plasma

Conc.alfaxalone (µg mL^{-1}) (spiked drug free porcine plasma)	0	0.25	0.5	2.5	5	10	20
µL added of alfaxalone10 µg mL^{-1} solution	-	2.5	5				
µL added of alfaxalone 100 µg mL^{-1} solution				2.5	5	10	20
µL added of alfaxalone-d5 10 µg mL^{-1}solution	25	25	25	25	25	25	25

Table 2. Matrix-matched calibration curve of propofol in pig plasma

Conc.propofol (µg mL^{-1}) (spiked drug free porcine plasma)	0	1	2.5	5	10	20	30
µL added of propofol 10 µg mL^{-1} solution	-	10	25				
µL added of propofol 100 µg mL^{-1} solution				5	10	20	30
µL added of propofol-d17 10 µg mL^{-1}solution	50	50	50	50	50	50	50

Individual alfaxalone, propofol and the respectively internal standards alafaxalone-d5 and propofol-d17 stock solutions were prepared in methanol to give a final concentration of 1.0 mg mL^{-1}and 0.1mg mL^{-1}.

Working solutions were prepared by diluting stock solutions with methanol to concentrations of 100 µg mL^{-1} and 10 µg mL^{-1}.

Stock solutions were stored at -20^{0}C.Working solutions were prepared freshly for each assay.

Two quality control samples at 1 µg mL^{-1} for alfaxalone and 2.5 µg mL^{-1} for propofol were used for each analysis.

All frozen porcine plasma samples (stored at -80ºC) were thawed at 4°C in refrigerator away from the day light before analysis.

LC –MS/MS analysis

1. LC: Agilent 1100 setup binary pump and autosampler

Mobile phase A: 10mM CH3COONH4 with 10% Acetonitrile (v/v)

Mobile phase B: 10mM CH3COONH4 with 90% Acetonitrile (v/v)

Column: Kinetex C18, 100x21 mm i.d., 2.5µm with C18 guard column

Injection volume: 10µL

Autosampler temperature: 5^0C

Flow rate: 0.2 mLmin^{-1}

Gradient: 30% B to 100% B for 10 minutes, post run for 5 minutes

2. MS/MS: ABSciex API 4000 triple-quadrupole mass spectrometer

Scan Type: MRM

Ion mode: ESI positive/ESI negative

Ion spray voltage: 5000 V/-4500 V

Q1/Q3 resolution: Unit

Curtain gas (N_2):   25 psi

Nebulizer gas (N_2):   50 psi

Gas Temperature: 400^0C

CAD (Collision gas) (N2):   4/10 psi

Table 3. MRM (multiple-reactions monitoring) acquisition table

Compound	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)
Alfaxalone	7.14	333	215,295
Alfaxalone-d5 (internal standard)	7.14	338	215,302
Propofol	8.29	177	177,161
Propofol-d17 (internal standard)	8.29	194	194,174

The ESI (Electrospray ionization) positive /negative ion mode for MS/MS (tandem mass spectrometry) analysis was selected.

Each run analysis (15 minutes) was performed in 2 periods by switching from positive MS/MS scan in 8 minutes to negative MS/MS scan in 7 minutes.

Alfaxalone and alfaxalone-d5 were detected in positive modus in the first period (0-8 minutes). The second period of analysis (8.1-15 minutes) was accomplished in negative electrospray mode with multiple-reactions monitoring (MRM) in order to detect propofol and its deuterated labeled internal standard, propofol-d17.

In the present study, the m/z transition 177/177 for propofol and m/z 194/194 for the internal standard determination in MRM were used because of the low ratio of the intensities of the product to parent ions at the true transition: m/z 177/161 and 194/174 respectively.

The software used for controlling this equipment, acquiring and processing the data was Analyst Version 1.7.

The concentration of alfaxalone and propofol in each sample was calculated by the internal standard method using the peak area ratio and linear regression analysis.

The response was linear and gave correlation coefficients (R^2) of 0.99 or better.

LOD (limit of detection) was based on 3xS/N (signal to noise) ratio and LOQ (limit of quantification) was established as the lowest validated concentration.

LOD for propofol is 0.3μg/mL and LOD for alfaxalone is 0.1μg/mL.

LOQ for propofol is 0.5μg/ml and 0.25μg/mL for alfaxalone.

Chemicals and reagents:

All chemicals were of at least HPLC grade and supplied by VWR International (Fontenay sous Bois, France).
Purified water (18.2 MΩ) was obtained from a Milli-Q water purifying system from Merck Millipore (Bedford, MA, USA).
Spin-X centrifuge tube filter, 0.22µm were purchased from Sigma-Aldrich (Costar, USA) and HPLC vial with insert from Agilent Technologies, (Santa Clara, CA, USA).

Propofol and propofol-d17 were purchased from Cerilliant (Texas, USA).
Alfaxalone-d5 was obtained from TRC (Toronto, Canada).
Alfaxalone was obtained from Jurox (UK).

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