THE UNIVERSITY OF QUEENSLAND

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Selenium and Oxidative Stress Modulation during Extracorporeal Circulation.

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Abstract

Oxidative stress occurs when there is an imbalance between reactive oxygen and nitrogen species (RNOS) and the antioxidant system. In cardiac surgery patients and the critically ill there is an association between oxidative stress and morbidity and mortality. Extracorporeal circulation (ECC) encompasses cardiopulmonary bypass (CPB) which is used for cardiac surgery; extracorporeal membrane oxygenation (ECMO) which is used for cardio-respiratory support in the critically ill and haemodialysis which is used in patients with intermittent or end-stage renal failure.

During ECC, the patient's total blood volume is in direct contact with an artificial surface for extended periods. In addition, there is often pre-existing inflammation, surgical trauma and/or ischemia-reperfusion (I-R) injury, all of which are major activators for the increased production of RNOS. Concurrent decreases in the level of essential antioxidants and trace elements (such as selenium, zinc and copper) occur, which upset redox balance in favour of oxidative stress. The trace element selenium is required for the normal function of the antioxidant enzyme glutathione peroxidase (GPx). Despite *in vitro* and biochemical data suggesting that reinforcement of antioxidant activity by selenium supplementation might reduce excessive oxidative stress damage, there is conflicting evidence in clinical studies. The reason for failure of antioxidant supplementation in the clinical setting is currently unclear.

We postulated that the ECC and its associated interventions such as transfusion, might have a larger impact on trace element loss, antioxidant function and the oxidative stress response than previously recognised. This led to the following questions:

- What is the selenium level in healthy Queensland residents, and is this level similar to that reported in other parts of the world?
- Does a patient's pre-CPB or pre-ECMO circulating selenium level influence their outcome?
- Does the ECC independently alter circulating selenium levels and the oxidative stress response?
- Do other interventions during ECC, such as transfusion, alter circulating selenium and oxidative stress levels?

This thesis focussed on oxidative stress and the trace element selenium, because oxidative stress is associated with poorer outcomes in cardiac and critically ill patients, and decreased selenium is independently associated with poorer outcomes. To answer the research questions outlined above, a series of experiments were designed and conducted, and they generated the following new information.

The average selenium level in a Queensland blood donor population was defined at 1.09 µmol/L (**Chapter 3**). In addition 88.5% of participants had a selenium level lower than the critical level required for maximal glutathione peroxidase activity, which has previously been determined to be between 1.14 – 1.27 µmol/L. Furthermore, this study generated novel information that packed red blood cells and buffy coat pooled platelets have very low levels of selenium (≤ 0.3 µmol/L).

The effect of cardiopulmonary bypass circuits on circulating trace element levels was reported in **Chapter 4**. Reductions in selenium (17.1%), zinc (29%) and copper (18.2%) were recorded over a two-hour period. Coating the CPB circuit with albumin did not reduce the loss of trace elements.

The effect of transfusing fresh or aged packed red blood cells (PRBC) on selenium and oxidative stress levels was compared using an ovine model (**Chapter 5**). Transfusion of PRBC (with low selenium content), into a healthy host reduced selenium and GPx activity and increased oxidative stress markers.

The separate and combined effects of lung injury and ECMO on selenium levels and oxidative stress were investigated using an ovine model (**Chapter 6**). Our results revealed that the combination of acute lung injury and ECMO resulted in a 72% reduction in selenium after 24 hours, (1.36 to 0.38 µmol/L); however, oxidative stress markers were no worse than that associated with each separate intervention.

Finally the association between selenium and post-operative atrial fibrillation (POAF) in low and high-risk cardiac patients was investigated (**Chapter 7**). Selenium levels were significantly lower pre-operatively and at all post-operative time points in high-risk patients and also in those who developed POAF. There was an association between longer intensive care stay at pre-operative selenium levels ≤ 0.7 µmol/L.

Overall the key findings of this thesis were:

- Selenium levels in healthy Queensland blood donors were lower than the previously reported levels for other regions of Australia.
- PRBC have very low levels of selenium.
- CPB and ECMO independently reduced selenium levels.
- The addition of ECMO to a host with lung injury compounded selenium reductions but did not increase oxidative stress.
- Low selenium levels before cardiac surgery were associated with POAF.
- Selenium levels lower than 0.7 μmol/L in cardiac surgical patients were associated with longer ICU stay.

Collectively, the findings of this thesis answered the original questions. The knowledge and experience gathered from this journey has significantly improved my ability to design and conduct research, and my future research aspirations are detailed in **chapter 8**.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer reviewed papers.

1. **Antioxidant trace element reduction in an in vitro cardiopulmonary bypass circuit.** Charles I McDonald, Yoke Lin Fung, John F Fraser. ASAIO 2012; 58(3):217-22. Impact Factor 1.516. Citations- 6
2. **Plasma selenium status in a group of Australian blood donors and fresh blood components.** McDonald C, Colebourne K, Faddy HM, Flower R, Fraser JF. J Trace Elem Med Biol. 2013 Oct;27(4):352-4. Impact Factor 2.491. Citations- 2
3. **Transfusion of packed red blood cells reduces selenium levels and increases lipid peroxidation in an in vivo ovine model.** C. I. McDonald, J. F. Fraser, K. Shekar, K. R. Dunster, O. Thom, Y. L. Fung. Transfusion Medicine 2014;24(1):50-54 Impact Factor 1.647. Citations- 1
4. **Oxidative Stress during Extracorporeal Circulation.** McDonald C.I., Fraser, J.F., Coombes, J. and Fung, Y.L. Eur J Cardiothorac Surg 2014 Dec;46(6):937-40 Impact Factor 3.304. Citations- 2
5. **The impact of acute lung injury, ECMO and transfusion on selenium and oxidative stress status in an ovine model.** McDonald CI, Fung YL, Shekar K, Diab S, Dunster KR, Passmore MR, Simonova G, Foley SR, Platts D and Fraser JF. J Trace Elem Med Biol. 2015 Apr;30:4-10 Impact Factor 2.491.
6. **The association between pre-operative selenium and post-operative atrial fibrillation after high-risk coronary artery surgery.** Charles I McDonald**,** John F Fraser, Kiran Shekar, Andrew JB Clarke, Jeff S Coombes, Adrian G Barnett, Bronwyn L Pearse and Yoke Lin Fung. Eur J Cardiothorac Surg. **Submitted 26th July 2015 (under review).** Impact Factor 3.304

Published Abstracts

1. **Transfusion of fresh or aged red cells after haemorrhagic shock reduces selenium and glutathione peroxidase** Fung, YL., McDonald, CI., Thom, O., Fraser, JF. Vox Sanguis, 2011; 101 (suppl. 2):122

Oral Presentations

1. **Why care about Selenium.** McDonald, C. Invited speaker. Perfusion Down Under Winter Meeting. Hayman Island. 2011 (August 2-5)
2. **Trace Elements and Bypass.** McDonald, C. Invited speaker. Perfusion Down Under. 2011 (August)
3. **The age of packed red blood cells and oxidative stress.** McDonald, C. 28th ASM-ANZCP. 2011 (November)
4. **ECMO and oxidative stress.** McDonald, C. 29th ASM of the Australian and New Zealand Society of Cardiovascular Perfusionists. Uluru. 2012 (November)
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Publications included in this thesis

Chapter 2: Literature Review- Publication incorporated as part of chapter.
Oxidative Stress during Extracorporeal Circulation. McDonald C.I., Fraser, J.F., Coombes, J. and Fung, Y.L. Eur J Cardiothorac Surg 2014 Dec;46(6):937-40.

Contributor	Statement of contribution
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Fung Y.L.	Wrote (20%) and edited paper (75%)

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Chapter 4: Antioxidant trace element reduction in an in vitro cardiopulmonary bypass circuit. Charles I McDonald, Yoke Lin Fung and John F Fraser. ASAIO 2012; 58(3):217-22.

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Chapter 5: Transfusion of packed red blood cells reduces selenium levels and increases lipid peroxidation in an in vivo ovine model. C. I. McDonald, J. F. Fraser, K. Shekar, K. R. Dunster, O. Thom and Y. L. Fung. Transfusion Medicine 2014;24(1):50-54

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Chapter 6: The impact of acute lung injury, ECMO and transfusion on selenium and oxidative stress status in an ovine model. McDonald CI, Fung YL, Shekar K, Diab S, Dunster KR, Passmore MR, Simonova G, Foley SR, Platts D and Fraser JF. J Trace Elem Med Biol. 2015 Apr;30:4-10 IF 2.491

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Chapter 7: The association between pre-operative selenium and post-operative atrial fibrillation after high-risk coronary artery surgery. Charles I McDonald, John F Fraser, Kiran Shekar, Andrew JB Clarke, Jeff S Coombes, Adrian G Barnett, Bronwyn L Pearse and Yoke Lin Fung**.** Submitted 26th July 2015. Eur J Cardio-thorac Surg.

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Contributions by others to the thesis

Kiran Shekar-	statistical analysis, interpretation and critical revision of most chapters.
Sara Diab-	sample collection, conduct of Ovine model studies and general study support.
Margaret Passmore-	sample collection and processing, sample analysis
Kimble Dunster-	technical support on Ovine model studies.
Bronwyn Pearse-	patient consenting, ethics submissions and human sample collection.
Barbara Mathews-	selenium analysis.
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None

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Keywords

Oxidative stress, selenium, cardiopulmonary bypass, extracorporeal circulation, trace elements, antioxidants, glutathione peroxidase.

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Fields of Research (FoR) Classification

FoR code: 1102 Cardiorespiratory Medicine and Haematology, 60%

FoR code: 1199 Other Medical and Health Sciences, 40%

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Abbreviations

ARCBS	*Australian Red Cross Blood Service*	**ECMO**	*Extracorporeal Membrane Oxygenation*
ACT	*Activated Clotting Time*	**EDTA**	*Ethylenediaminetetraacetic acid*
AF	*Atrial Fibrillation*	**ELSO**	*Extracorporeal Life Support Organisation*
AKI	*Acute Kidney Injury*	**FFP**	*Fresh Frozen Plasma*
ALI	*Acute Lung Injury*	**GF-AAS**	*Graphite furnace atomic absorption spectrometry*
ALT	*Alanine transaminase*	**GSH**	*Glutathione (reduced)*
APACHE	*Acute Physiology and Chronic Health Evaluation*	**GS-SG**	*Glutathione (Oxidised)*
APH-Plt	*Apheresis platelet*	**GPx**	*Glutathione Peroxidase*
ARDS	*Acute respiratory distress syndrome*	**GR**	*Glutathione reductase*
BC-Plt	*Buffy coat platelet*	**HD**	*Haemodialysis*
CABG	*Coronary Artery Bypass Graft*	**HPLC**	*High-performance liquid chromatography*
CCO	*Continuous Cardiac Output*	**ICU**	*Intensive Care Unit*
COPD	*Chronic obstructive pulmonary disease*	**IL**	*Interleukin*
cFFP	*Clinical Fresh Frozen Plasma*	**I-R**	*Ischemia reperfusion*
CoHb	*Carboxyhaemoglobin*	**MDA**	*Malondialdehyde*
CPB	*Cardiopulmonary Bypass*	**MECC**	*Mini-extracorporeal circuit*
CRP	*C-reactive protein*	**MOF**	*Multi-organ failure*
CVVH	*Continuous Veno-venous Hemofiltration*	**NADPH**	*reduced form of NADP+*
DNA	*Deoxyribonucleic acid*	**NHMRC**	*National Health and Medical Research Council*
ECC	*Extra-corporeal Circulation.*	**NSR**	*Normal Sinus Rhythm*
ECG	*Electrocardiogram*	**ovPRBC**	*Ovine Packed Red Blood Cells*

PMP	*Polymethylpentene*	**SCUF**	*Slow continuous ultrafiltration*
POAF	*Post-operative Atrial Fibrillation*	**SD**	*Standard Deviation*
POD	*Post-operative Day*	**SIRS**	*Systemic Inflammatory Response Syndrome*
PRBC	*Packed red blood cell*	**SLED**	*Sustained low-efficiency dialysis*
PUFA	*Polyunsaturated fatty acid*	**SOD**	*Superoxide dismutase*
RCT	*Randomised Controlled Trial*	**SOFA**	*Sequential Organ Failure Assessment*
RIJV	*Right Internal Jugular Vein*	**STS**	*Society of Thoracic Surgeons score*
ROS	*Reactive oxygen species*	**TBARS**	*Thiobarbituric acid Reactive Substances*
RNOS	*Reactive Oxygen and nitrogen species*	**VA-ECMO**	*Venoarterial extracorporeal membrane oxygenation*
S-ALI	*Smoke induced Acute Lung Injury*	**VV-ECMO**	*Venoveno extracorporeal membrane oxygenation*

Convention for selenium units in this thesis

The S.I. unit for selenium is µg/L, however, the chemical pathology laboratory that analysed our samples reported the results as µmol/L. To facilitate comparison of results, throughout this thesis and with the wider peer-reviewed clinical literature, selenium concentration is reported as µmol/L in preference to µg/L. Where published data is in the form µg/L it will be converted to µmol/L using the following formula (µg/L x 0.0127 = µmol/L). The exceptions to this are references to daily intake and Chapter 3 where the journal required use of the S.I. unit for selenium (µg/L). For this reason Chapter 3 retains the standard S.I. unit classification.

Chapter 1

Introduction

Chapter 1 Introduction

1.0 Background and rationale.

Oxidative stress occurs when the level of pro-oxidant production exceeds the ability of the antioxidant system to maintain balance.[2] Coupled to this antioxidant system is a select group of essential trace elements, which ultimately determine the ability of the antioxidant system to function normally and maintain redox balance. Cardiac surgery patients and the critically ill experience an oxidative stress response.[3,4] Oxidative stress may be tolerated in patients with low-risk surgery or uncomplicated illness in the intensive care unit (ICU), however in patients with systemic inflammatory response syndrome, sepsis or multiple co-morbidities it can compromise their outcome.[5,6] For patients having cardiac surgery, one of the more common complications is post-operative atrial fibrillation (POAF). Increasing evidence suggests that one of the main factors in its development is increased oxidative stress during the surgical period.[7,8] While blood contact with the cardiopulmonary bypass (CPB) circuit is known to trigger the inflammatory response,[9] there is little information on the contribution of the circuit to the oxidative stress response. In critically ill patients with cardiac and/or respiratory failure and who are refractory to conventional medical therapy with a high risk of death, extracorporeal membrane oxygenation (ECMO) can be considered.[10] Despite the great advantage offered by such therapies, the mortality associated with ECMO remains unacceptably high.[10,11] The contribution of oxidative stress that develops during ECMO to the morbidity and mortality of these patients is poorly understood.

Understanding what modifiable triggers of oxidative stress exist, specifically in the cardiac surgical and ECMO cohorts, may identify strategies that mitigate oxidative stress and potentially improve patient outcomes. An understanding of the oxidative stress response suggests that reinforcement of the antioxidant system should be a significant component of any such strategy. However, while select studies show improved outcomes in cardiac surgery and critically ill patients when antioxidants such as selenium, zinc, vitamin C and vitamin E are used, the majority of studies show no improvement, and the reason for this lack of benefit is unclear.[12] Understanding the impact of CPB and ECMO circuits (and associated interventions such as transfusion) on trace element loss and subsequent oxidative stress responses may improve the design of future studies investigating the benefit of antioxidant supplementation.

The most common forms of extracorporeal circulation (ECC) are CPB, ECMO and haemodialysis (HD). ECC involves the patient's blood being directed through these artificial

circuits, which are known to stimulate the inflammatory response, (through contact activation), and increase oxidative stress.[4,9] Additionally, recent studies in cardiac patients and the critically ill indicate clinically significant reductions in selenium levels, which are associated with increased morbidity and mortality.[13,14] However, for patients having cardiac surgery or requiring ECMO, few studies have defined the interaction between selenium and oxidative stress and its effect on outcomes. Redox control is achieved by an appropriate antioxidant response and some of these antioxidants, such as glutathione peroxidase (GPx) and superoxide dismutase (SOD) require trace elements for normal function. As the focus of this thesis is selenium, the investigations are centred on the antioxidant GPx rather than SOD activity.

Table 1-1: Global selenium levels

Selenium (µmol/L)	Country
0.53	Finland (Helsinki)
0.60	New Zealand (Dunedin)
0.92±0.18	Poland (Gdansk)
0.93	Brazil (Rio de Janeiro)
1.03	W. Germany (Mainz)
1.08	Sweden (Lund)
1.09±0.17	Germany (Dresden)
1.22±0.17 (men)	Switzerland
1.12±0.18 (women)	
1.24	Japan (Hiroshima)
1.27	Iran (Tehran)
1.30	Saudi Arabia
1.47	England (Southampton)
2.01	Canada (Toronto)
2.72±2.08	Pakistan (Islamabad)

(Adapted with permission [16])

Selenium reductions in the surgical and critically ill population are related to pre-, peri- and post-operative factors. The first pre-operative factor concerns dietary intake. Globally there is variation in selenium levels in the soil which influences selenium uptake into the bio-food chain,[15,16] and consequently there is significant variability in the selenium levels of individuals (**Table 1-1**).[17] Demographics on patients with ischemic heart disease reveal that in general they have poorer diets compared to healthy people,[18] and consequently have an inadequate selenium intake. Other lifestyle aspects of cardiac disease also influence selenium levels. Tobacco use, obesity, diabetes, alcohol and high dietary fat intake, (which are cardiac risk factors), also contribute to selenium reductions.[19] These observations suggest that cardiac surgical patients are more likely to present to hospital with low selenium levels and elevated markers of oxidative stress compared to the general population. The variability in soil selenium levels globally and corresponding selenium levels in any population makes interpretation of the impact of selenium levels on oxidative stress challenging. Hence, the first objective of this thesis involved defining selenium levels of the Queensland population.

In the peri-operative period of cardiac surgery a host of factors such as surgical trauma, blood contact with the CPB circuit, hyperoxia and reperfusion of the ischemic myocardium, are associated with increased reactive oxygen species (ROS) production.[4] It has also been demonstrated that ROS levels are elevated at the end of CPB and that these elevated levels precede the peak inflammatory response, (which occurs from 3hr post-CPB).[20] Elevated ROS are associated with a host of post-operative complications, including POAF, reduced pulmonary function, acute kidney injury (AKI) and neurological sequela.[21-24] During this period there are also concurrent reductions in selenium.[13,25] One of the first studies to note such a reduction during CPB was an observational paediatric study of 59 patients.[26] There were no explanations for the selenium reductions given, and the levels were still lower than baseline 48 hours after surgery. In a clinical survey of 197 cardiac patients, investigators determined that pre-operative selenium levels before CPB can be used as a risk predictors of inflammation and mortality.[14] While a prospective observational study of 60 cardiac patients demonstrated that post-operative selenium levels are a predictor of multi-organ failure (MOF).[13] The cause for the intra-operative decrease in selenium likely involves a combination of dilution and acute phase transition. However, an additional explanation may lie within the CPB circuit itself, as these circuits are known to adsorb proteins as well as drugs.[27,28] Given both pre- and post-operative selenium levels appear important in determining outcomes in cardiac patients,[13,14,29] confirming if the CPB circuit adsorbs selenium will provide information to estimate ECC associated selenium loss and also indicate if supplementation should be considered. This question was investigated in chapter 4 of this thesis.

Patients requiring ECMO are a subset of the acutely critically ill and thus exhibit many of the same co-morbidities as this population (i.e. inflammation, sepsis, ischemia), all of which are known triggers of ROS production. While on ECMO support, the patient experiences continuous exposure to the ECC circuit, multiple transfusions and they may also be on HD. Each of these interventions may individually or cumulatively contribute to patient morbidity.[30] There is still significant mortality associated with both veno-arterial (VA) and veno-venous (VV) ECMO, both during the course of ECMO itself and between successful weaning from ECMO and hospital discharge.[11] This thesis applied an *in vitro* model of ECMO (Chapter 6) to elucidate the impact the foreign surface of the ECMO circuit or multiple transfusions have on acute and chronic trace element reductions, oxidative stress and outcomes on the host/patient.

1.1 Hypotheses

The hypotheses of this PhD thesis are:

1: CPB circuits adsorb trace elements and increase oxidative stress.

2: Transfusion of blood products is associated with a reduction in selenium and an increase in oxidative stress.

3: ECMO therapy is associated with significant selenium loss that contributes to increased oxidative stress.

4: High-risk cardiac surgery patient with low pre-operative selenium levels are more likely to develop POAF.

To investigate these hypotheses in both CPB and ECMO the following studies were conducted:

Study 1: The first objective was to determine the selenium level in the healthy Queensland population **(Chapter 3),** to provide an accurate baseline (and local relevance), for the whole thesis.

Study 2: In order to understand the impact of the cardiopulmonary bypass (CPB) circuit on trace element levels **(hypothesis 1)**, an *in vitro* circuit was utilised **(Chapter 4).**

Study 3: In order to investigate the effect of blood transfusion on oxidative stress and trace element levels **(hypothesis 2),** an *in vivo* ovine model was utilised **(Chapter 5)**.

Study 4: In order to investigate the individual and combined effects of acute lung injury, ECMO and transfusion on the host's oxidative stress and selenium level **(hypothesis 3)**, an *in vivo* ovine model of ECMO was used **(Chapter 6).**

Study 5: In order to investigate the association between oxidative stress, selenium levels and POAF **(hypothesis 4)**, a prospective clinical study was conducted on high and low-risk CABG patients **(Chapter 7).**

Chapter 2

Literature Review

Chapter 2 Literature review

2.1. Publication incorporated as section of Chapter: Oxidative Stress during Extracorporeal Circulation

McDonald CI, Fraser JF, Coombes, JS and Fung YL

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ABSTRACT. There is an increased oxidative stress response in patients having cardiac surgery, haemodialysis or extracorporeal membrane oxygenation that is related to poorer outcomes and increased mortality. Exposure of the patient's blood to the artificial surfaces of these extracorporeal devices, coupled with inflammatory responses, hyperoxia and the pathophysiological aspects of the underlying illness itself, all contribute to this oxidative stress response. Oxidative stress occurs when there is a disruption of redox signalling and loss of control of redox balance. Ongoing oxidative stress occurring during extracorporeal circulation results in damage to lipids, proteins and DNA and contributes to morbidity and mortality. This review discusses reactive species generation and the potential clinical consequences of oxidative stress during extracorporeal circulation as well as an overview of some current antioxidant compounds that are available to potentially mitigate the oxidative stress response.

Keywords: Extracorporeal Circulation, Cardiopulmonary bypass, ECMO, dialysis, oxidative stress, antioxidants.

INTRODUCTION

Oxidative stress has been attributed to increases in morbidity and mortality in cardiac surgical patients, the critically ill and patients requiring haemodialysis (HD). [3,31-33] These patients are exposed to various extracorporeal circulatory devices such as cardiopulmonary bypass (CPB), HD and extracorporeal membrane oxygenation (ECMO). Substantive evidence from CPB and HD studies indicates that extracorporeal circulation (ECC) stimulates the inflammatory response generating reactive nitrogen and oxygen species (RNOS) and overwhelming the endogenous antioxidants, resulting in increased oxidative stress.[3,31,34] Through an understanding of the multiple factors that occur during ECC responsible for generating RNOS, strategies may be developed which mitigate or reduce their generation and thereby reduce the associated complications. This review examines oxidative stress during CPB, dialysis and ECMO, as well as common antioxidant supplements, which may mitigate this response leading to improved patient care.

OXIDATIVE STRESS

Definition and basic overview

Oxidative stress can be defined as a "disruption of redox signalling and control".[2] Under normal physiological conditions RNOS play essential roles in cell signalling (secondary messengers), immunity and cellular defense.[3] Both intra and extra-cellular levels of RNOS are controlled by antioxidant moieties to maintain this redox balance.[3] However, during various chronic and acute illnesses, an increase in RNOS production and loss of redox control is responsible for tissue and cellular injury. For instance, disturbance of the intracellular redox environment can result in cell apoptosis, senescence and disrupted differentiation.[35] There are three classical mechanisms by which RNOS exert their damaging effects, (i) peroxidation of lipids, (ii) denaturation of proteins or (iii) damage to deoxyribonucleic acid (DNA).[3] (**Figure 2-1**)

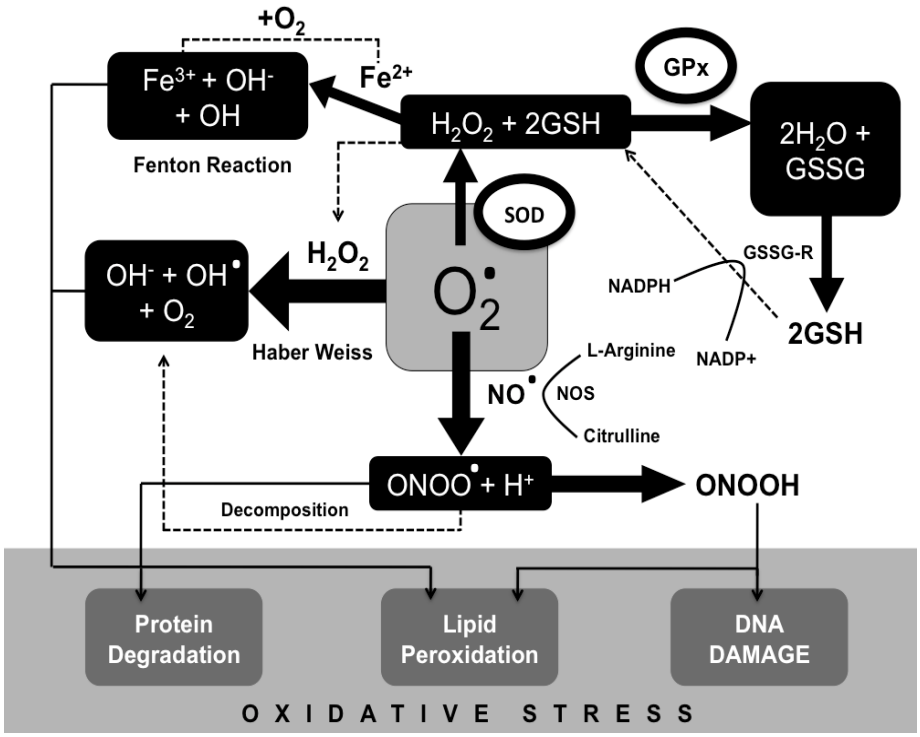

Figure 2-1: Simplified mechanism of oxidative stress, cellular RNOS generation and antioxidant action of SOD and GPx.

Key: O_2•-Superoxide radical; H2O2-Hydrogen Peroxide; ONOO•-peroxy radical; OH•-hydroxyl radical; NO•-nitric radical; SOD-Superoxide dismutase; GPx-Glutathione peroxidase; GSH-Glutathione; GSSG-oxidised glutathione; GSSG-R-Glutathione reductase; NADPH-Nicotinamide adenine dinucleotide phosphate (reduced); NADP+ - Nicotinamide adenine dinucleotide phosphate.

(i) Lipid Peroxidation

Lipid peroxidation is an important component of oxidative stress and is linked to a number of diseases including aging, atherosclerosis, Parkinson's disease, Alzheimer's, diabetes mellitus, cataracts and rheumatoid arthritis.[36,37] Lipid peroxidation alters membrane fluidity and permeability, sometimes irreversibly, resulting in physiologic changes such as altered ion gradients across the membrane.[36] The lipids in cell membranes, particularly poly-unsaturated fatty acids (PUFA's), are susceptible to attack by various RNOS such as hydroxyl and peroxyhydrides.[37] Typically, damage in these conditions is a multi-step sequence of events beginning with RNOS mediated extraction of hydrogen from the lipid molecule, creating a fatty acid radical that itself extracts a hydrogen atom from the neighbouring lipid molecule creating another fatty acid radical. This self-propagating phase no longer requires the presence of the initiating RNOS to continue and expand.[37] Stopping the lipid peroxidation sequence requires the intervention of a "chain breaking" antioxidant,

such as vitamin E, or for the fatty acid radical to react with another radical, “quenching the fire”.[37]

(ii) **Oxidative Protein damage**

Similar to lipids, proteins are also a major component of cell membranes as well as being part of various essential enzymes throughout the body. Proteins are susceptible to both direct and indirect damage from RNOS. The hydroxyl and nitrogen based radicals can damage various amino acids. Additionally proteins that contain sulfur in their structure (such as cysteine and methionine) are also susceptible to oxidative damage.[3] Several RNOS can also interact directly with proteins disrupting membrane ion transport mechanisms or causing the inactivation of enzymes and amino acids.[3]

(iii) Oxidative DNA damage

DNA is normally a stable molecule, however in the presence of high concentrations of the radical hydroxyl, oxidative damage occurs.[38] Oxidative DNA damage is associated with carcinogenesis, neurodegenerative diseases, cardiovascular disease and aging.[36,38] Uncontrolled increases in RNOS result in modification of DNA bases, single and double-DNA breaks, loss of purines, damage to the deoxyribose sugar, DNA-protein cross-linkage and damage to the DNA repair system.[38] Two of the most common base modifications include 8-oxo-7,8-dihydroguanine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine.[39] Some oxidative DNA damage can either be prevented by antioxidants or repaired by various endogenous DNA repair enzymes. Conversely reduced antioxidant or trace elements levels can undermine repair mechanisms leading to permanent damage.[38 40]

ANTIOXIDANT DEFENCE AGAINST OXIDATIVE STRESS

The endogenous antioxidant response is a coordinated effort between enzymes, proteins and vitamins to remove, alter or inactivate excessive build-up of RNOS. This is a multi-layered defence system and the exact nature of the antioxidant response is influenced by (i) the solubility of the antioxidant (lipid or water solubility), (ii) whether it is an enzyme/non-enzyme and (iii) the relative concentration of specific antioxidants in cytosol, plasma or tissue.[3]

From a redox point of view, the enzyme antioxidants represent the first line of defence in cells and plasma.[3] These primary antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase.[3] SOD enzymes are present in most extracellular fluids as well as aerobic cells within the mitochondria and cytosol. The function

of all forms of SOD is the dismutation of the superoxide radical ($O_2^{\bullet}$-) to hydrogen peroxide (H_20_2) (**Figure 2-2**. equation 1).[3] The primary function of GPx is to catalyse the reduction of hydrogen peroxide to water (**Figure 2-2**. equation 2) at the expense of glutathione (GSH). GSH is oxidized in this process, forming GS-SG, and in the presence of glutathione reductase (GR), the GS-SG is reduced back to GSH at the expense of nicotinamide adenine dinucleotide phosphate oxidase (NADPH) (**Figure 2-2**. equation 3).[3] Catalase is present in the peroxisomes of cells and predominantly plays a role in the enzymatic decomposition of hydrogen peroxide when hydrogen peroxide levels are high within the cell (**Figure 2-2**. equation 4).[3]

Equation 1: $2O_2^{\bullet}$-+ 2 $H^+ \rightarrow {}^1 \rightarrow O_2 + H_2O_2$

Equation 2: 2GSH (reduced) + $H_20_2 \rightarrow$ **[GPx]** →GS-SG (oxidized) + $2H_20$

Equation 3: GS–SG + NADPH + $H^+ \rightarrow$ **[GR]** →2 GSH + $NADP^+$

Equation 4: $2H_20_2 \rightarrow$ **Catalase** → $2H_20 + 0_2$

Figure 2-2: Mechanism of action of SOD, GPx and Catalase.

These antioxidant enzymes react directly with RNOS before they can react with lipids or proteins, thus preventing or delaying the generation of secondary and tertiary radicals. Trace metals are required for their normal functioning, i.e. selenium for GPx, copper/zinc or manganese for SOD and iron for catalase.[41] The non-enzyme antioxidants include ß-carotene, Vitamin E, vitamin C, glutathione and Co-enzyme Q, which act primarily as scavenger and chain breaking antioxidants.[36,41] The potential benefits of various antioxidant supplements are discussed below.

CLINICAL SIGNIFICANCE OF OXIDATIVE STRESS DURING ECC

Cardiopulmonary Bypass.

Patients with underlying atherosclerosis, diabetes or chronic kidney disease (with or without dialysis) have increased levels of inflammatory cytokines and oxidative stress before cardiac surgery relative to the normal population.[31,33,36] In addition, cardiac surgery utilizing CPB is associated with systemic inflammation, ischemia-reperfusion and surgical trauma, which are all strong stimulants of RNOS generation resulting in increased oxidative stress during and after surgery (**Figure 2-3**).[31,42] A growing body of evidence indicates that this oxidative stress response is a major contributor to complications such as post-operative

atrial fibrillation (POAF), acute kidney injury (AKI) and acute lung injury (ALI) after cardiac surgery.[32,43,44]

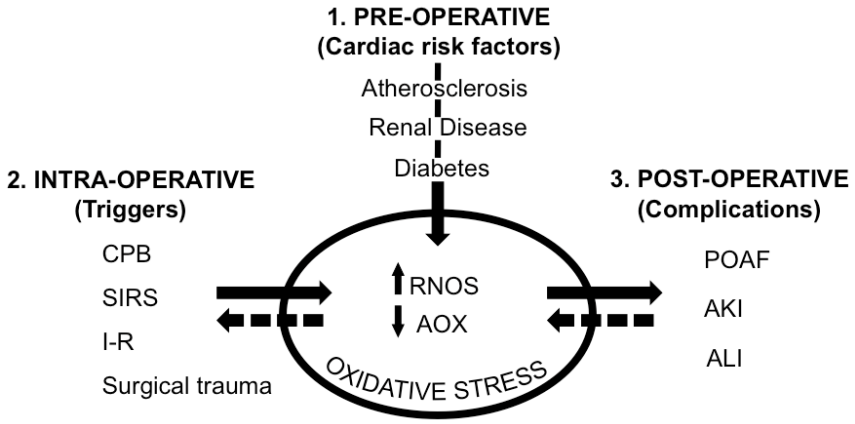

Figure 2-3: Factors involved in oxidative stress during cardiac surgery.

Key: CPB: cardiopulmonary bypass; SIRS: systemic inflammatory response syndrome; I-R: ischemia-reperfusion; POAF: postoperative atrial fibrillation; AKI: acute kidney injury; ALI: acute lung injury; RNOS: reactive nitrogen and oxygen species; AOX: antioxidants.

When CPB is initiated, blood contact with the artificial surface of the ECC circuit elicits a systemic inflammatory response activating neutrophils, NADPH oxidase and xanthine oxidase causing an increased production of RNOS.[31,45] The inflammatory response to the ECC circuit also causes an acute phase redistribution of these trace elements to tissues and away from the intravascular circulation.[46] Concurrently, the ECC circuit adsorbs trace elements vital to the antioxidant response, specifically selenium and zinc.[47] Mini-extracorporeal circuits (MECC) have a smaller surface area relative to standard CPB circuits. Select studies have shown reduced circulating levels of C-reactive protein (CRP), TNFα and (interleukin-6) IL-6 when MECC are used in cardiac surgery.[48,49] Whether these reductions in inflammatory cytokines translate to a reduced oxidative stress response in the patient is unclear. Importantly though, MECC are not suitable for highly complex cardiac surgery, and in these patients inflammation and oxidative stress is expected to be higher when compared to low-risk elective coronary artery surgery patients. Modern CPB circuits are coated with a variety of “biopassive or bioactive” coatings designed to dampen the contact inflammatory response and reduce platelet adhesion. Only one study has detailed the inflammatory and oxidative stress response between seven different biocoatings commonly used in CPB circuits.[50] They determined that patients on CPB circuits using Bioline™ and phosphorylcholine coatings exhibited lower inflammatory and oxidative stress levels than other commonly used coatings.

Increased RNOS production also occurs during reperfusion of the ischemic heart with oxygenated blood following cardioplegic myocardial arrest during surgery.[36,51] During I-R endothelial cells respond by producing xanthine oxidase, generating the superoxide radical.[3,36] In addition I-R results in a decreased function of myocardial antioxidants, especially GPx.[51] Increased myocardial oxidative stress (such as during I-R) causes impaired atrial contraction, altered energetics of the myofibrils and a reduction in the effective refractory period of the atria leading to POAF.[52,53] Cardiac surgical operations are often managed with hyperoxic (pO_2 > 200mmHg) anaesthesia and CPB. Hyperoxia has been shown to stimulate neutrophils and mitochondria to increase production of RNOS, especially in the lungs.[54] While the lungs do receive some blood supply during CPB from the bronchial arteries, they are still at risk of ischemic injury.[55] While there are no controlled studies of hyperoxia vs. normoxia and oxidative stress, it is plausible that hyperoxia, especially during reperfusion of the ischemic lung post aortic clamping, may contribute to the overall oxidative stress profile. Further studies are needed to determine if oxidative stress profiles and clinical outcomes are improved when using a normoxic CPB strategy.

Cardiac surgical patients are frequently transfused with blood products. Packed red blood cell (PRBC) transfusions have been associated with increases in lipid peroxidation and oxidative stress in a small number of studies, presumably the consequence of a combination of the storage lesion, increased iron load and decreased antioxidant profile of the stored red cells.[56,57] Some patients, especially those with more complex procedures such as aortic dissections, re-operations or aortic arch repairs, may require massive transfusion. Thus, the high number of PRBC transfusions may contribute to increased oxidative stress and compromise patient outcome. Platelet transfusions are not uncommon during cardiac surgery and mostly occur at the end of CPB, when the inflammatory response is already activated. Platelets are an important source of RNOS, primarily generating superoxide,[58] and studies indicate that stored platelets similar to stored red cells, will undergo a time dependant storage lesion.[59] Whether the transfusion of these platelets into a patient will affect the oxidative response has not been investigated.

Haemodialysis

HD is the most prevalent form of renal therapy for acute kidney injury in the critically ill/post-surgical setting, or more commonly for chronic end stage renal failure.[60] HD may be applied as conventional HD in the chronic setting or in a variety of modes for the acute setting (i.e. slow low efficient dialysis (SLED), slow continuous ultrafiltration (SCUF) and continuous veno-venous haemofiltration (CVVH)). All forms of HD utilise artificial circuits to remove

uremic waste and restore fluid and electrolyte balance. These circuits can have surface areas approaching mini-CPB circuits, and thus, are also associated with the activation of the coagulation and inflammation systems in a similar fashion, if not magnitude, to CPB.[61]

Evidence that patients with renal failure have increased oxidative stress and decreased antioxidant responses both pre- and post-HD has been reviewed previously.[62] Increases in oxidative stress after HD are associated with increased morbidity, (specifically cardiovascular disease) and mortality. The mechanisms of increased oxidative stress in these patients is multi-factorial and includes the renal disease itself, reduced antioxidant intake associated with malnutrition, loss of antioxidants during HD and the interaction between blood and the HD membrane.[63] Additionally, the overall uremic state of the patient and haemo-incompatibility of circuits (polysulfone vs. cuprophane) are recognised as the major factors associated with both inflammation and oxidative stress.[64]

Attempts to reduce the oxidative stress response due to blood contact with the dialysis circuit have been investigated using dialyser membranes that are more biocompatible. A 2006 meta-analysis of 14 studies using vitamin E coated cellulose filters demonstrated an overall reduction in lipid peroxidation levels as measured by malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS) and low density lipoproteins.[65] More recently, companies have coated polysulfone dialysers with vitamin E. Early findings have demonstrated better reductions in oxidative stress markers as well as reduced markers of inflammation.[66] Further studies are needed to determine if reductions in oxidative stress levels during HD equates to better clinical outcomes.

Extracorporeal Membrane Oxygenation

ECMO is a rescue therapy for critically ill patients with potentially reversible cardiac and/or respiratory failure refractory to maximal conventional medicine.[30] In spite of the potential benefits of ECMO in these patients, the prolonged exposure of the blood to these artificial circuits is associated with a variety of deleterious pathophysiological changes and complications.[30]

There are currently very limited data on oxidative stress or antioxidant changes during ECMO, either clinically or experimentally. In a rabbit model of ECMO, increases in lipid peroxides were measured just after initiation in plasma and lung tissue.[67] However, in a lamb model of short duration ECMO, no increases in lipid peroxides were detected, instead there were decreases in the antioxidant enzymes SOD and GPx.[68] In the only clinical study investigating oxidative changes during ECMO, Hirthler *et al* showed increases in lipid

peroxides for up to 96 hrs after initiation of ECMO in a paediatric population.[69] In this study non-survivors had increasing lipid peroxidation levels, while the levels plateaued after 24 hours in the survivor group. There are significant differences between paediatric and adult ECMO, such as the ratio of ECMO surface area to body size. This aspect limits the ability to translate paediatric or small animal ECMO studies to the adult. To date there are no studies investigating oxidative stress in adult ECMO.

Despite a lack of current data on the oxidative stress response in the adult ECMO population, there is considerable evidence from the critically ill population to indicate that oxidative stress is a major contributor to morbidity and mortality. Specific RNOS triggering events in the critically ill, which are especially relevant to the ECMO patient, are listed in **Table 2-1**. Two of these triggers are specifically relevant in the ECMO patient. The first major trigger, unique to patients on ECMO, is the initial and continued contact of whole blood with the ECMO circuit for days and weeks. Initial contact of blood with the ECMO circuit elicits a systemic inflammatory response (SIRS) as measured by increased production of cytokines such as IL-6.[70] Studies in CPB show that patients with SIRS also have a disturbed redox equilibrium,[71] which is a result of both increased RNOS production and decreased antioxidant levels. Hence, it is reasonable to also expect oxidative injury via this mechanism during ECMO. However, whether prolonged exposure to the ECMO circuit continues to increase oxidative injury is unknown.

Table 2-1: Potential triggers for RNOS during ECMO

Potential triggers for increased RNOS generation during ECMO.
Exposure to ECC (ECMO circuits [72], Dialysis [34])
Systemic Inflammatory Response Syndrome [73]
Acute Respiratory Distress Syndrome [44]
Hyperoxia [54]
Mechanical ventilation [74]
Sepsis [75]
Ischemia-Reperfusion [76]
Transfusion [56,57]
Sequestration of antioxidant trace elements by the ECMO circuit [47]

The second important trigger during ECMO is hyperoxia,[77] which may be particularly relevant in the VV-ECMO patient. During VV-ECMO, hyperoxic blood is returned directly to the venous system where it flows directly to the damaged pulmonary vasculature. Worsening lung function as determined by radiographic evidence of consolidation is evident within the first 24-48 hours of ECMO initiation (**Figure 2-4**). Limited experimental data suggests that hyperoxia in this setting may facilitate neutrophil sequestration to the lungs, leading to increased pulmonary endothelial

RNOS production and lipid peroxidation.[54,67] Given the ability of these oxidative stress triggers to increase ECMO morbidity and mortality, it highlights the need for detailed oxidative stress studies in this cohort.

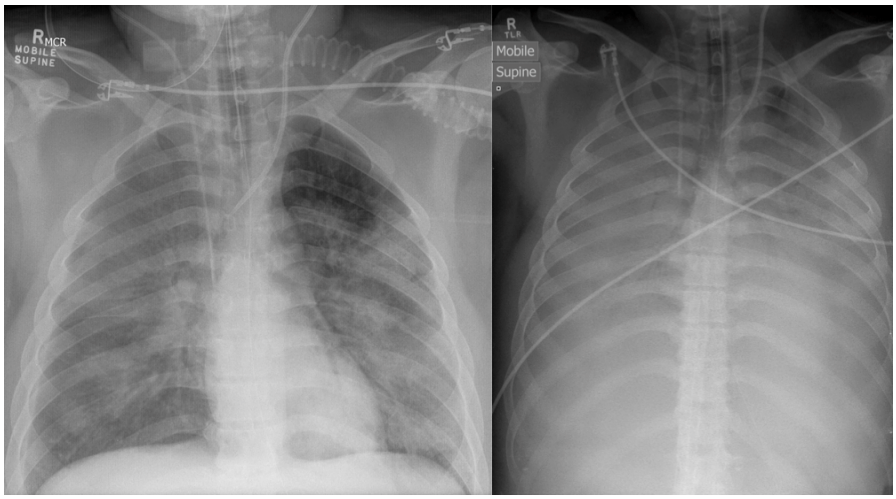

Figure 2-4: Chest X-ray showing lungs before VV-ECMO (L) and 24-h after initiating VV-ECMO (R).

ANTIOXIDANT SUPPLEMENTS TO REDUCE OXIDATIVE STRESS.

The endogenous antioxidant defence system consists of both intra- and extra-cellular antioxidants that regulate the quantity of RNOS. There are considerable disturbances to this antioxidant system during critical illness as well as during cardiac surgery and HD. Specifically, there are reductions in antioxidant micronutrient levels such as selenium, zinc and vitamin C.[78] Some of these reductions during ECC are a due to (i) an acute-phase redistribution as a result of the increased inflammatory response, (ii) adsorption to the ECC circuit and (iii) excretion in bodily fluids/dialysate.[47,78,79] In addition to providing appropriate nutritional support in patients exposed to ECC, replacement of these antioxidant micronutrients may be a relatively low cost and safe intervention to mitigate the effects of oxidative stress.[78,80] Various compounds with antioxidant properties that have been investigated, either as individual supplements or in combination therapy, are discussed in brief below.

Selenium

Selenium is an essential trace element that is incorporated into selenoproteins, some of which have antioxidant properties (i.e. GPx, Selenoprotein P and thioredoxin).[81] There are many potential benefits to selenium supplementation but most are centred around its link with increasing the activity of the antioxidant GPx.[82] The relationship between GPx and

selenium is such that when selenium levels fall below plasma levels of 90 - 100 µg/L there is a direct reduction in GPx activity.[82] Significant reductions in selenium occur after cardiac surgery and dialysis.[13,79] In both these patient groups the reductions are inversely associated with increased complications and morbidity.[13,83] Similarly, in critical illness (without ECMO), selenium reductions occur especially during sepsis, and are associated with adverse outcomes.[84] Selenium supplementation has been investigated in cardiac surgical patients undergoing CPB [25] and in the critically ill.[85] A recent meta-analysis of micronutrient use in the critically ill population (not on ECMO) concluded that the treatment effect of selenium supplementation was greatest in patients with more severe illness.[80] It is worth noting that those benefits were only seen when doses were greater than 500 µg/day, a dose far in excess of the 50 - 70 µg/day recommended dose. Despite the inconsistent beneficial findings Visser *et al* have recommended that micronutrients, including selenium, should at least be given at the current recommended daily dosages in the critically ill as a safe and effective way to prevent clinical deficiencies.[78] Additionally, the 2013 Canadian Clinical Practice Guidelines recommend the use of parenteral selenium in the critically ill.[86]

Vitamin C

Vitamin C (L-ascorbic acid) is an essential nutrient with potent hydrophilic antioxidant properties. It acts as an aqueous phase antioxidant that has the ability to stop the propagation of free radicals that cause lipid peroxidation, termed “chain breaking antioxidant”, and is also required for the regeneration of the oxidised form of a-Tocopherol.[87] Studies in surgical and critically ill patients indicate an acute reduction in plasma vitamin C levels that is explained only partly by increased antioxidant demand in the tissues.[87] Levels are further decreased in patients that develop post-operative complications. The well-established link between oxidative stress and atrial fibrillation (AF) studies has led investigators to assess the preventative effect of vitamin C supplementation (isolated or combination therapy) on the development of POAF.[87,88] Experimental and clinical evidence demonstrates a reduction in POAF following ascorbate supplementation.[89] A recent study demonstrated that the effect of vitamin C supplementation on POAF was more effective when given in association with ß-blockers compared to ß-blockers alone.[90] However, more studies are required to confirm these results.

In dialysis patients, vitamin C deficiency has been reported after extended hours HD. Vitamin C supplementation in these patients has been shown to restore plasma levels and reduce levels of oxidative stress, as evidenced by markers of lipid peroxidation.[91] Caution must be used, however, when considering vitamin C supplementation in patients with renal

disease, as vitamin C is metabolised to oxalate. Oxalate causes the build-up of calcium oxalate in tissues, which can have cardiovascular consequences.[92] While there are no studies investigating the clinical outcome benefits of vitamin C supplementation during ECMO, it has been shown that supranormal doses of I.V. vitamin C in the critically ill can restore endothelial function and thereby modulate redox signalling.[93]

Vitamin E

Vitamin E (□-tocopherol) is a fat-soluble antioxidant that reacts with lipid peroxides to prevent lipid peroxidation. It acts synergistically with vitamin C at the lipid-water interface of cell membranes where vitamin C can act as an electron donor to regenerate vitamin E.[88] Based on its known antioxidant properties, the ability of vitamin E to prevent cardiovascular disease has been investigated in a study of 9551 patients but found no positive effect in this regard.[94] Although vitamin E levels are reduced during cardiac surgery and can be normalised with supplementation, another study showed that there was no reduction in lipid peroxidation after supplementation.[95] Studies that have investigated the ability of prophylactic vitamin E to reduce POAF have also produced conflicting results. When used in isolation the results indicate that vitamin E has no protective effect,[95] while studies using combination antioxidant therapy have shown reductions in POAF in the treatment groups.[96,97] Such studies suggest a synergistic effect, which needs to be confirmed by more rigorous studies.

Vitamin E supplementation has also been investigated during HD as a way of reducing the adverse effects associated with oxidative stress. One of the larger of such studies, the SPACE study,[98] concluded that vitamin E supplementation in HD patients with cardiovascular disease reduced the incidence of myocardial infarction and other composite endpoints, but they did not measure markers of oxidative stress. A review of 25 studies where vitamin E supplementation was used and oxidative stress markers were measured found that in 20 studies there was a reduction in a variety of oxidative stress markers.[63]

Zinc

Zinc is a trace element important to many aspects of human health, including an essential role supporting SOD activity. Supplementation with zinc has been shown to improve plasma zinc levels, but it is unclear if it improves SOD activity. Transient zinc deficiencies occur after cardiac surgery, HD and during critical illness.[99] Zinc levels have been shown to be inversely proportional to SOFA scores and organ failure in critical illness.[100] However, we could find no studies demonstrating a mortality benefit when zinc supplementation was used

alone. Nevertheless, reports of toxic side effects are rare, and given the purported benefits of zinc supplementation in the mentioned patient populations, it is often added to the standard nutritional profile for many critically ill patients.

N-acetylcysteine

N-acetylcysteine (NAC) is a free radical scavenger antioxidant useful in chronic lung disease and has been shown to reduce RNOS and pro-inflammatory cytokine production.[101,102] These properties have led to investigating the benefits of prophylactic NAC supplementation in cardiac surgical patients with specific aims to reduce the incidence of POAF.[102] While selected studies such as this have shown improved outcomes in patients supplemented with NAC, a recent meta-analysis involving 1163 cardiac surgical patients concluded that the evidence did not support the use of NAC for the prevention of AKI.[101]

Glutamine

Glutamine is a non-essential amino acid that is metabolised to glutamate, a precursor to glutathione synthesis. Glutathione is the major endogenous antioxidant produced by cells and it exists mostly in the reduced form (GSH). The body has the capacity to synthesise sufficient glutamine under normal physiological conditions, however, significant reductions in glutamine occur during critical illness, after cardiac surgery and after prolonged dialysis.[103] Despite these reductions, there is sparse data on the beneficial effects of glutamine supplementation during cardiac surgery. In the cardiac surgical population studies have demonstrated that glutamine supplementation maintains GSH activity and increased glutamine levels, but had no effect on organ dysfunction or length of stay in ICU.[104] Although high quality evidence supporting parenteral glutamine supplementation in the critically ill has recently emerged,[105] we could find no studies solely investigating the effects of glutamine in ECMO patients. The recommendations from the Canadian Clinical Practice Guidelines for the critically ill have since updated their support for the “use of parenteral glutamine in the critically ill, except in those with multi-organ failure”.[106]

Conclusion

Oxidative stress has been shown to be clinically relevant to patient outcomes and this review has highlighted the mechanisms leading to increased oxidative stress in patients exposed to ECC, and discussed some antioxidant supplements commonly used to modulate this response. Other avenues for possibly controlling ECC induced oxidative stress include reducing ECC surface areas, designing circuits with better bio-compatibilities, implementing normoxia and investigating the effects of pulsatile flow (during CPB) on

endothelial RNOS production. In addition, there are a range of pharmaceutical compounds beyond the scope of this review (e.g. statins and antifibrinolytics), which also exhibit antioxidant properties. However, many of these interventions require further confirmation of efficacy.

Redox control via antioxidant supplementation presents an attractive low risk and low cost intervention in this assault. Already the benefits of antioxidant supplementation have been reported in critically ill patients, leading to recent recommendations for the use of antioxidant supplements, specifically glutamine (and possibly selenium). Despite the theoretical benefits of antioxidant therapy, the available evidence has challenged the practicality of implementing widespread antioxidant supplementation in patients exposed to ECC. This evidence has suffered from, poor study design, low sample numbers, heterogenous patient groups and a lack of consensus as to the best biomarker for oxidative stress. Thus, improved prospective randomised controlled trials of antioxidant supplementation are needed to define not only the most appropriate patient groups, but also antioxidant dosing and timing.

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2.2. Supplementary review material relevant to thesis not included in publication

2.2.1 Antioxidants (overview)

The human body has an impressive array of defense mechanisms designed to reduce and control RNOS generation and repair oxidative damage (**Figure 2-5**).

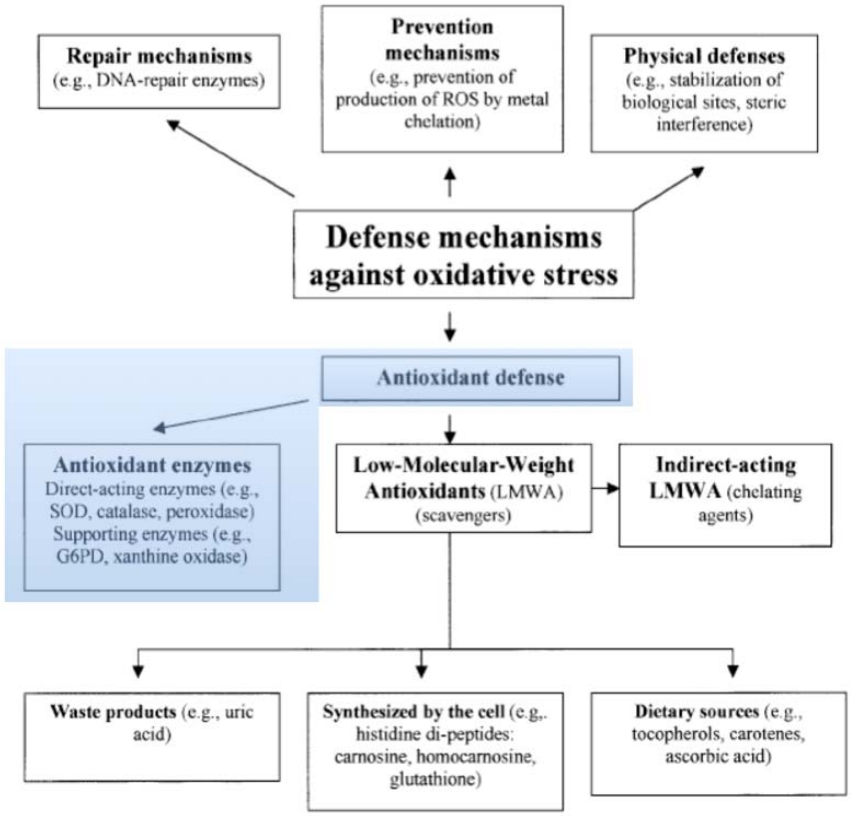

Figure 2-5: Simplified defense mechanisms against oxidative stress.

Highlighted area indicates antioxidant defence against oxidative stress. (reproduced with permission [108])

Antioxidants are critical to this multi-layered defense system and are capable of delaying, preventing or removing oxidative damage.[107] Although there are multiple ways to categorize antioxidants, the more common system categorizes them either as enzyme (direct acting) or non-enzyme antioxidants (indirect acting).[108] Antioxidants have specific functions, but they also retain some degree of redundancy and overlap, e.g. glutathione peroxidase and catalase. Determining which antioxidant responds first to an increase in RNOS generation depends initially on the type of RNOS molecule, then on the relative concentration of each

specific antioxidant in cytosol, plasma or tissue as well as the lipid/water solubility of the antioxidant.

Enzyme antioxidants are also known as primary antioxidants, because they represent the first line of defense in cells and plasma. Under normal physiological conditions they interact directly with RNOS before they can react with lipids or proteins, preventing the generation of secondary and tertiary radicals.[109,110] The three main antioxidant enzymes are superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase. This thesis focused primarily on the enzyme GPx but also discussion of SOD.

2.2.2. Superoxide Dismutase (SOD)

The function of all forms of SOD is the dismutation of the superoxide radical to hydrogen peroxide (equation 1).[109] SOD enzymes are present in most extracellular fluids as well as aerobic cells within the mitochondria and cytosol. Three iso-enzyme forms of SOD exist and all three contain a metal ion co-factor.

(i) Cu-Zn-SOD (SOD-1) is present in the cytosol of cells,
(ii) Mn-SOD (SOD-2) exists exclusively in the mitochondria,
(iii) EC-SOD (extracellular-SOD-3), this is also a Cu-Zn containing SOD and is found mostly in extracellular fluid and tissue interstitial spaces.[111]

In excess, the superoxide radical has the ability to generate hydroxyl (via Haber-Weiss pathways, **Figure 2-1)** or peroxynitrite (through interaction with nitrite radical), therefore any reduction in SOD activity will result in a disproportionate increase in the generation of other RNOS.

It is unclear which form of SOD is of greatest importance, studies have shown that SOD-1 knockout mice age more rapidly and have higher levels of skeletal oxidative stress,[112] while decreases in SOD-3 have been associated with the development of atherosclerosis.[113] More dramatically, mice lacking Mn-SOD die within 10 days of birth from cardiac abnormalities.[114] With the exception of mitochondrial SOD the other two forms of SOD require copper and zinc for normal expression and activity. The copper molecule is required by SOD for the disproportionation of superoxide, while zinc is required to enable the correct folding of the SOD protein complex.[115]

2.2.3. Glutathione peroxidase (GPx).

The glutathione peroxidases are a group of selenium dependent enzymes with antioxidant activity. Their primary function is to catalyse the reduction of hydrogen peroxide to water (**Figure 2-2** equation 2) at the expense of glutathione (GSH).[109,116] GSH is oxidized in this process, forming GS-SG, and in the presence of GR, the GS-SG is reduced back to GSH at the expense of NADPH (**Figure 2-2** equation 3).

There are at least 4 identified peroxidase enzymes in this family. GPx-1 is present in nearly all mammalian cells representing nearly 50% of total body selenium, and resides in the cytosol. GPx-2 lies in the gastro-intestinal tract, GPx-3 exists as the second most abundant selenoprotein in plasma and GPx-4 is a monomer, as opposed to a tetramer like GPx-1, -2, -3, which contain 4 selenium molecules at the active site of the 4 protein subunits.[116,117] The activity of these enzymes is dependent upon appropriate levels of selenium.[82,117,118] Previous bovine and human studies report a linear relationship between selenium levels and GPx activity up to a selenium level of 1.14 - 1.27 μmol/L.[82,119] Levels above this do not appear to increase GPx activity. This relationship has formed the basis of determining the recommended daily intake for men and women. Even though each isoenzyme of GPx responds at slightly different rates to reductions in selenium, plasma GPx activity has often been used as a surrogate marker of selenium status and vice versa.[82] Reductions in GPx activity are also associated with increased oxidative stress.[120,121] Though GPx activity may be a convenient indicator of oxidative stress,[122] the limitations of its use cannot be ignored. For example, it is unclear if plasma GPx activity reliably reflects oxidative stress in populations with high selenium intakes.

2.3 Selenium and Oxidative stress modulation during Cardiac Surgery

2.3.1 Pre-operative influences.

In many instances, patients requiring cardiac surgery and CPB have an altered oxidant-antioxidant balance before surgery due to a combination of lifestyle choices (poor diet, obesity, smoking and alcohol use)[123-126] and underlying non-cardiac illnesses (diabetes, renal disease, lung disease).[3,36] The degree to which chronic oxidant-antioxidant imbalance prior to surgery will influence peak oxidative stress levels during CPB is not known, because oxidative stress and selenium are not routinely assessed prior to surgery. Chronic oxidative stress has been shown to reduce endogenous antioxidant capacity.[127] Therefore, it is plausible that subsequent triggers of ROS production may overwhelm the antioxidant system, resulting in increased oxidative stress mediated morbidity.

2.3.2. Intra-operative influences

Observational studies have demonstrated significant selenium reductions during cardiac surgery.[13,14,29] Likely causes for this reduction include an acute phase response (due to the ECC-stimulated inflammatory response) that results in a redistribution of antioxidant trace elements (such as selenium) to tissues and away from the intravascular circulation.[46] We theorize that the ECC circuit may also adsorb some of these trace elements, which are key components of the antioxidant response. Such reductions have a high degree of clinical importance due to their dependent and independent associations with multi-organ dysfunction and mortality. Studies in the critically ill suggest that selenium should be administered prior to injury, as giving it after injury seems to have little effect on oxidative injury.[128] Given that the onset of injury in cardiac surgical patients is predictable (initiation of CPB plus removal of aortic cross clamp) the cardiac surgical patient may be the ideal patient to benefit from selenium supplementation. However, to understand which patients may potentially benefit from supplementation it is first important to know (i) background levels in the local healthy population, (ii) what are the clinical consequences of low selenium levels.

The oxidative stress response during CPB is due to a combination of material dependent (exposure of blood to non-physiologic surfaces) and material independent triggers (surgical trauma, ischemia-reperfusion, hyperoxia).[129] Blood contact with the artificial surface of the ECC circuit activates neutrophils, NADPH oxidase and xanthine oxidase, causing an increased production of RNOS.[31,45] Acute increases in oxidative stress are associated with increased morbidity (such as post-operative atrial fibrillation (POAF), acute kidney injury (AKI) and acute lung injury),[32,43,44] and mortality. However, while all patients exposed to CPB experience an inflammatory and oxidative stress response,[20,45,130] not all patients suffer the negative effects of these responses. However, rather than requesting costly blood tests for pre-operative oxidative stress levels (that may not get to be analysed prior to surgery), it would be beneficial to categorise patients into “at risk” groups pre-operatively. This may be based upon known risk factors for either increased oxidative stress or decreased selenium, and would add value to any prospective study investigating the benefits of antioxidant supplementation. To our knowledge no studies have done this.

2.3.3 Association of oxidative stress and selenium on POAF

POAF is the most common and significant sustained arrhythmia after cardiac surgery with an incidence ranging from 5 - 70%.[131,132] It is thought to be closely associated with the

oxidative stress response and inflammation,[132-135] and while it can be self-limiting, there are numerous post-operative complications associated with AF including; hypotension and shock; thromboembolic events; renal failure; prolonged ICU stay[136] and higher long-term health care costs.[137,138]

The standard course of treatment for AF in general includes rate and rhythm control and/or anti-coagulation.[131,132,139] While there is success with either modality there are also significant failures and risks associated with long-term pharmacotherapy and anticoagulation as well as inconsistency with surgical ablation.[131] Contributing to the failure rates of pharmacotherapy and/or surgical intervention is the lack of a full understanding of the precise mechanisms involved in the development of POAF.[134,140] Current data suggests that the development of POAF is multi-factorial involving both electrical and structural remodelling. Within this context, inflammation and oxidative stress are two key triggers [132-135] identified as being important components in its development; (although this study will not be examining the role of inflammation).

In the cardiac surgical patient, a host of pre- and intra-operative factors contribute to increasing oxidative stress levels. Intra-operatively the important triggers to oxidative stress are myocardial ischemia-reperfusion,[141] inflammation,[20] hyperoxia,[142] PRBC transfusion[57] and blood contact with the CPB circuit.[47] Levels of lipid peroxidation and protein carbonyl products are elevated in the myocardium, especially after ischemic reperfusion at the end of surgery.[51] Mitochondria, NAPH oxidase and uncoupled NOS are important sources of oxidative species in the myocardium at this time.[143] While the exact mechanism of how oxidative stress induces POAF has not been determined, animal and experimental studies indicate that there is electro-anatomical remodelling with a down-regulation of Na+ channels and abnormal Ca^{2+} cycling within the myocyte.[144]

An appropriate antioxidant response balances the generation of RNOS and reduces oxidative stress. Patients who develop POAF have been shown to have lower plasma and myocardial levels of the two main antioxidants, superoxide dismutase and glutathione peroxidase. Both of these antioxidants rely on specific trace elements for normal activity and their activities can be both decreased by intra-operative reductions and increased through appropriate supplementation.

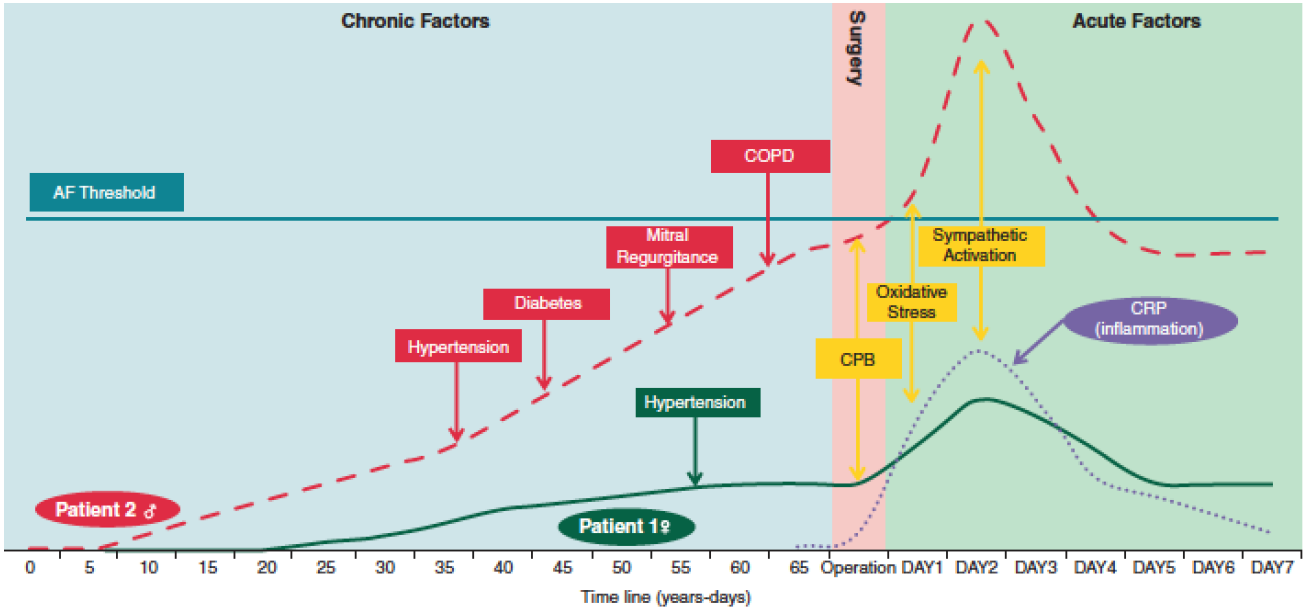

Figure 2-6: Time course of substrate development, oxidative stress and surgery-related factors in the occurrence of atrial fibrillation.

Time course of pro-arrhythmic mechanisms is depicted in two hypothetical patients undergoing cardiac surgery. Both chronic as well as acute factors related to the operation on day 0 are shown. When the intensity of pro-arrhythmic factors reaches a certain threshold, atrial fibrillation will occur. Patient 1 has no relevant cardiovascular history, only hypertension (green) at the age of 57. Patient 2 already developed hypertension (red) at a younger age, followed by diabetes (red), mitral regurgitation (red), and COPD (red) at an older age, respectively. Both patients have no history of AF and undergo on-pump coronary artery bypass grafting at the same age. However, patient 2 has developed an AF substrate by the time of operation due to above mentioned cardiovascular diseases. Acute, surgery-related factors occur in both patients: cardiopulmonary bypass (CPB, yellow), inflammation (CRP, purple), oxidative stress (yellow), and sympathetic activation (yellow). Patient 2 develops postoperative atrial fibrillation (exceeds the 'AF threshold'), while patient 1 remains with sinus rhythm. AF, atrial fibrillation; COPD, chronic obstructive pulmonary disease. (reproduced with permission [241])

These observations suggest that strategies to minimise oxidative stress or inflammation may reduce the susceptibility to POAF. So far several studies have demonstrated that antioxidant supplementation in the forms of Vitamin C and Vitamin E were successful as both prophylactic and therapeutic agents to reduce the reactive products of oxygen and nitrogen produced during surgery. A meta-analysis by Harling *et al* [87] in 2011 reviewed 6 studies (5 were randomised controlled trials (RCT)) and concluded that despite poor overall quality, antioxidant vitamin therapy reduced POAF (OR 0.43, 95% CI 0.21 to 0.89) and all-cause arrhythmias (OR 0.54, 95% CI 0.29 to 0.99). Another study suggested that this effect might be even greater when combined with ß-blockers.[90] It is worth noting that both these compounds are "chain breaking" antioxidants—working to stop lipid peroxidation after it has begun, and they do not interact with RNOS directly. Selenium on the other hand is directly associated with the primary antioxidant GPx which interacts directly with hydroperoxides

before excessive lipid peroxidation damage occurs. With its links to GPx and thyroid function, selenium supplementation may provide earlier and more direct intervention to restore GPx activity and reduce oxidative stress.

It is acknowledged that oxidative stress contributes to POAF, and that administration of antioxidants may reduce its incidence.[145] However, no studies have determined: firstly if selenium reductions are associated with POAF and secondly if selenium supplementation might reduce its incidence more effectively compared to antioxidants such as vitamin E and C. As a first step, this thesis monitored selenium levels of cardiac surgery patients before and up to five days post-surgery to investigate the association between selenium levels and POAF as well as the association with levels of oxidative stress.

2.4 Selenium and Oxidative stress modulation during ECMO

ECMO is used to support cardiac (**Figure 2-7** VA-ECMO) or respiratory (**Figure 2-7** VV-ECMO) function in patients where maximal conventional medicine has failed.[146]

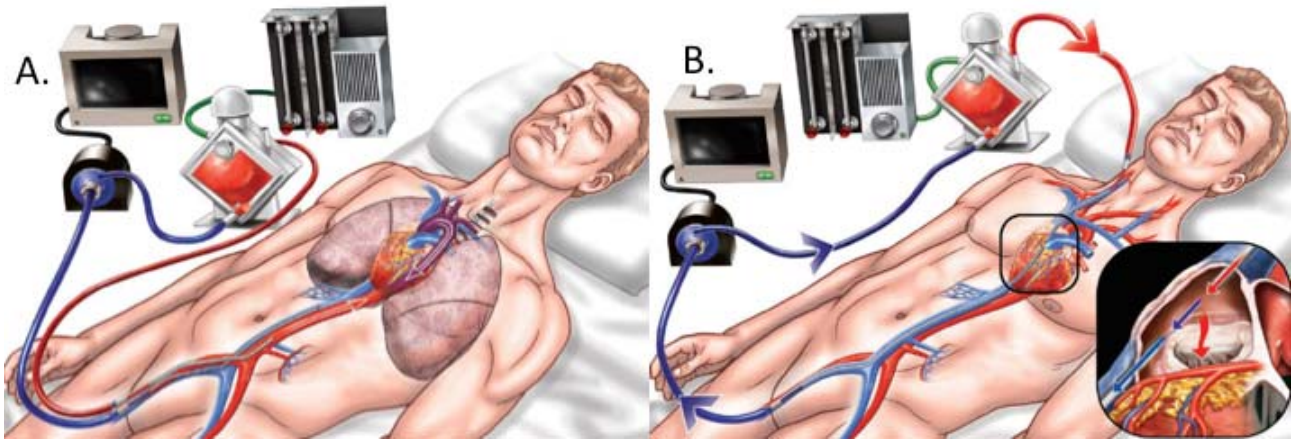

Figure 2-7: Schematic of the two common forms of ECMO

(A) depicts VA-ECMO, (B) depicts VV-ECMO. Oxygenator (red) and pumphead (blue).
(reproduced with permission [144])

ECMO patients are critically ill and the initiation of ECMO is seen as a potentially lifesaving intervention. However, even in 2015 the benefit of ECMO is not definitively established, as there is still significant morbidity and mortality associated with its use.[147,148] Despite purported improvements in the manufacture of ECMO systems over the last 40 years, there is still very limited data on whether smaller, more biocompatible ECMO circuits reduce the inflammatory/oxidative stress response due to contact activation and whether these circuits adsorb less trace elements.

2.4.1 Animal models of ECMO and oxidative stress

Studies in rabbits, pigs and lambs have produced conflicting results regarding increased lipid peroxidation (and the possible influence of hyperoxia). Rabbits exposed to VV and VA ECMO demonstrated increased lipid peroxidation, which was dependent on PaO_2 during VV-ECMO.[67] Yet in contrast, investigators were unable to demonstrate significant increases in lipid peroxidation, despite high PaO_2 levels, in a healthy lamb model.[68] In a healthy porcine model of VV-ECMO, it was recently been demonstrated that significant increases in lipid peroxidation levels occur (as measured by MDA) as well as concurrent decreases in SOD activity.[149] The only animal study to investigate oxidative stress in a clinically relevant model of organ injury was conducted more than 30 years ago. In this dated study it was demonstrated that significant increases in lipid peroxidation occurred after smoke induced acute lung injury.[150] It is unclear how relevant this study is in the context of the modern ECMO circuit, which constitutes a polymethylpentene (PMP) hollow fibre oxygenator with small surface area of $1.8m^2$. This is in stark contrast to the ECMO circuits of the 1990's which were silicon membrane oxygenators with large surface areas (approximately $4.5m^2$).[151]

2.4.1 Human studies of ECMO and oxidative stress

To date there has only been a single human study investigating oxidative stress during ECMO. In a study of 16 infants supported on ECMO, lipid peroxidation levels were significantly lower in the survivors (n = 12) compared to the non survivors.[69] Unfortunately that study did not detail the type of ECMO circuit used. As the study was conducted more than 30 years ago, the ECMO circuit was likely a silicon oxygenator with a large surface area.[151] This large surface area would have contributed significantly to the elevated levels of lipid peroxides.

There have been no studies investigating selenium modulation during ECMO. Data on the critically ill, documents significant reductions in selenium, especially in those with sepsis and/or inflammatory syndromes. The primary causes for such reductions include chronic deficiency in the general population, reduced nutritional intake, hemodilution, acute phase response and loss in body fluids.[152] In fact, Sakr and colleagues have demonstrated that up to 92% of patients admitted to ICU with sepsis and SIRS have low levels of selenium.[153] Many studies have also demonstrated that selenium reductions in these patients continue despite modest supplementation.[5,153] These reductions are closely associated with Acute Physiology and Chronic Health Evaluation (APACHE) and sequential organ failure

assessment (SOFA) scores, as well as increased oxidative stress; increased organ dysfunction and increased mortality. Some studies suggest that when selenium reductions fall as low as 36 μg/L there is a high degree of sensitivity and specificity to predicting ICU mortality.[153] Understanding the separate and combined effects of critical illness and ECMO on oxidative stress and selenium, may well give guidance to future studies aimed at reducing ECMO related morbidity and mortality.

This thesis investigated the effect of ECMO and transfusion on oxidative stress, antioxidant and trace element status, using a range of healthy and critically ill controls in an *in vivo* ovine model using current low-prime ECMO circuits.

Chapter 3

Chapter 3 Plasma selenium status in a group of Australian blood donors and fresh blood components.

McDonald C, Colebourne K, Faddy HM, Flower R and Fraser JF.

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Rationale

Globally there is considerable variation in selenium levels in soil and food, resulting in differences in total plasma selenium levels in humans.[17,154] The effect of this variation across populations is unclear, however low plasma selenium levels have been associated with chronic illnesses and acute complications in critically ill patients.[16,155] To determine associations of selenium levels with morbidity in the surgical and ill population, accurate measurements of normal selenium levels in the healthy population of the region being investigated are first needed. In 2012 there was limited current information on selenium levels in Australia, and no information on selenium levels in Queensland. In addition, some patients having cardiac surgery and most patients having ECMO therapy receive a considerable number of blood product transfusions resulting in a temporary dilution of the recipient's own blood cells and plasma proteins. While deficiency in some micronutrients has been observed after PRBC transfusion in paediatric[156] and Beta Thalassemia Major[157] patients, no studies have investigated whether commonly used blood products are deficient in selenium, and thereby increasing the risk of a dilutional reduction of selenium in the recipient.

To address these information gaps this study:

- determined the normal variance of selenium within a healthy blood donor population.
- assessed if there were regional differences in selenium levels across Queensland.
- measured selenium levels in four commonly used blood products.

The results from this study were initially presented as a poster at the Australian and New Zealand Intensive Care Society (ANZICS) annual meeting in October 2010, where it was awarded the "Best Medical Poster". That poster abstract was published in *Anaesthesia and*

Intensive Care. 2010 Volume 38, Issue 6. The full results were subsequently published in Journal of Trace Elements in Medicine and Biology.[158]

Journal requirements for our publication where to use µg/L. For this reason Chapter 3 retains the standard S.I. unit classification.

Significance

This study determined that plasmapharesis donors had an average plasma selenium level of 85.6 ± 0.5 µg/L. Of those tested, 88.5% had a level below the 90 - 100 µg/L range (1.14 - 1.27 µmol/L), a level that has previously been shown to be required for maximal GPx activity. Selenium levels are profoundly reduced during critical illness, especially when sepsis or inflammation is involved. Thus, low levels of selenium in the healthy population places the surgical or critically ill patient at increased risk of selenium related morbidities.

This study also reported for the first time the selenium levels in a range of blood products. The extra-cellular selenium levels in clinical fresh frozen plasma (FFP) and apheresis-derived platelets (APH-Plt) were within the Australian reference range (0.7 – 1.4 µmol/L). However, in packed red blood cells (PRBC) and pooled buffy coat-derived platelets (BC-Plt), selenium levels were below the lower limit of detection of 23.7 µg/L. This new information indicates that the transfusion of PRBC and BC-Plt could potentially deplete the recipient's selenium level, particularly in those with low body weight or those receiving multiple units of blood products either acutely (i.e. massive transfusion) or over many days such as during ECMO. Investigations into transfusion related dilution of circulating selenium levels were subsequently conducted in Chapter 5.

Plasma selenium status in a group of Australian blood donors and fresh blood components.

Summary

The purpose of this study was to assess plasma selenium levels in an Australian blood donor population, and measure extra-cellular selenium levels in fresh manufactured blood components. Selenium levels were measured using graphite furnace atomic absorption spectrometry (GF-AAS) with Zeeman background correction. The mean plasma selenium level in healthy plasmapheresis donors was 85.6 µg/L, and a regional difference was observed between donors in South East Queensland and Far North Queensland. Although participants had selenium levels within the normal range (55.3 - 110.5 µg/L), 88.5% had levels below 100 µg/L, a level that has been associated with sub-optimal activity of the antioxidant enzyme glutathione peroxidase (GPx). Extra-cellular selenium levels in clinical fresh frozen plasma (cFFP) and apheresis-derived platelets (APH-Plt) were within the normal range. Packed red blood cells (PRBC) and pooled buffy coat-derived platelets (BC-Plt) had levels at the lower limit of detection, which may have clinical implications for the massively transfused patient.

Introduction

Selenium, a trace element essential for optimal human health, is incorporated in the form selenocysteine into selenoproteins.[159] There are more than 30 selenoproteins functionally important for human health, including the antioxidant enzyme glutathione peroxidase (GPx), selenoprotein P, thioredoxin reductase and iodothyronine 5'-deiodinase.[159]

Selenium, in the form of amino acids selenocysteine and selenomethionine, is sourced from the diet.[160] There is significant variation in the amount of selenium present in the soil globally (ranging from <0.01 µg/g to > 1000 µg/g)[161] and plant food, such as brazil nuts, rice and wheat, as well as higher chain animal products, will reflect this variation. The result being significant differences in total plasma selenium levels in humans.[17,154] Low plasma selenium levels have been associated with chronic illnesses[16] and acute complications in critically ill patients.[155] Therefore, monitoring selenium levels in a country or region is relevant to community health and possibly the outcomes of blood transfusion.

Donated blood is separated into fresh blood components or manufactured into at least 20 different plasma-derived blood products.[162] The concentration of various micronutrients,

including selenium, in fresh components depends on the level in the donor at the time of donation. It is plausible that large volume blood transfusions may result in depletion of micronutrients in the recipient to levels similar to those in the transfused component.[163] The level of extra-cellular selenium in manufactured fresh blood components issued by the Australian Red Cross Blood Service (ARCBS) is currently unknown. The aim of this study was to measure plasma selenium levels, by graphite furnace atomic absorbance spectrometry (GF AAS), in an Australian plasmapharesis donor population, as well as in supernatant fluids from manufactured fresh blood components.

Materials and Methods

A total of 592 plasmapheresis donors from three regions in Queensland (Toowoomba (221), Rockhampton (220) and Cairns (151)) were enrolled in the study between 20 January 2010 and 29 October 2010. Written consent was obtained from all participants and a study-specific questionnaire (asking about history of thyroid or celiac disease, diabetes, smoking and multivitamin use) was completed prior to sample collection. Additional demographic information was obtained retrospectively from ARCBS records. This study was conducted under approval from the ARCBS Human Research Ethics Committee.

Samples (6 ml) were collected from either donor whole blood (n = 592) or fresh manufactured blood components PRBC (n = 8), cFFP (n = 8), APH-Plt (n = 7) and BC-Plt (n = 3) into trace element-free tubes (Greiner Bio-One, Monroe, North Carolina, United States). Samples were processed and stored as per Queensland Health protocols. Specifically, samples were stored at 4 ˚C until cold centrifugation (4 ºC) at 3000 rpm (1683g) for 15 minutes. Plasma was transferred to plastic Eppendorf tubes® and transported at 4 ˚C to a central laboratory where plasma selenium was measured by GF-AAS with Zeeman background correction using a Varian AA280Z analyser (Agilent Technologies Inc, Santa Clara CA, United States). Internal quality controls were included with each run using commercial serum control material (UTAK Laboratories, Valencia, CA, U.S.A.). Inter-assay imprecision was 5.5%. Analysis of covariance (ANCOVA- GLIM Genstat: VSN International Ltd., Hemel Hempstead, United Kingdom) was performed to identify factors important in explaining selenium levels and also to identify interactions between significant factors.

Table 3-1: Selenium levels in Queensland plasmapheresis donors

	N	Average ± SD(µg/L)
Combined Total	**592**	**85.6±0.5**
Location		
Cairns	171	87.2 ± 13.0
Rockhampton	200	86.3 ± 9.8
Toowoomba #	221	83.7 ± 15.0
Gender #		
Male	371	86.7 ± 12.4
Female	221	83.7 ± 13.6
BMI category		
Underweight (<18.5)	1	94.8
Normal (18.5 – 24.9)	140	84.6 ± 12.8
Overweight (25 – 29.9)	267	86.2 ± 13.7
Obese (>30)	181	85.4 ± 11.9
Multivitamin use* #		
No	324	84.8 ± 12.4
Yes	181	87.2 ± 13.4
Thyroid medication* #		
No	498	85.9 ± 12.8
Yes	8	74.0 ± 10.3
Smoker		
No	472	85.8 ± 12.9
Yes	35	85.3 ± 11.5
Diabetic		
No	499	85.6 ±12.8
Yes	8	90.8 ± 14.0
Coeliac disease		
No	506	85.8 ± 12.8
Yes	1	63.2

* Indicates incomplete responses to questionnaire.
# Indicates significant difference ($p < 0.05$) for values in category.

Results

The mean plasma selenium level in study participants was 85.6 ± 0.5 µg/L (SD,12.9; range 55.2 – 173.7 µg/L). Selenium levels were within the normal reference range (55.0 - 110.5 µg/L) for 98% of participants (581/592), the remaining 11 participants had selenium levels greater than 110.5 µg/L (0.2%). Selenium levels were equal to or below 100 µg/L in 88.5% (524/592) of donors, while 43.4% (257/592) had levels below 80 µg/L.

Predictor variables significant in explaining serum selenium levels included: region ($p = 0.005$), gender ($p = 0.014$), multivitamin use ($p = 0.01$) and thyroid medication ($p = 0.02$). No significant interaction between any of these factors was observed ($p \geq 0.35$ in all analyses). Neither BMI, number of prior donations, smoking, diabetes nor celiac disease were significant predictors ($p \geq 0.33$) of lower selenium levels. Mean plasma selenium concentrations in donors from South East Queensland (Toowoomba) were significantly lower than donors from Far North Queensland (Cairns) ($p = 0.002$) and donors from Central Queensland (Rockhampton) ($p = 0.04$).

No significant difference ($p = 0.14$) between donors from Far North Queensland and Central Queensland was observed. The average plasma selenium level in female plasmapheresis donors was significantly ($p = 0.01$) lower than in males. The average plasma selenium concentration in donors taking multivitamins at the time of donation was significantly (p =

0.01) higher than in those not taking multivitamins (multivitamin brand not specified). A small number of donors (8/592) reported taking thyroid medication (medication not specified) at the time of donation. They had significantly (p = 0.02) lower average selenium concentrations than donors not taking thyroid medication. One donor who had a history of coeliac disease had serum selenium levels towards the lower end of the normal range. Some participants did not complete all answers in the questionnaire, however, considering the non-recording rate was low, we do not believe it alters validity of these observations.
Selenium levels in PRBC and BC Plt's were at, or below, the limit of detection by GF AAS analysis (<23.7 µg/L). Conversely, cFFP (71.1 µg/L, SD 7.3) and APH-Plt (92.1 µg/L, SD 9.2) components had selenium levels that were comparable to plasma selenium levels in donors.

Discussion

Selenium is a micronutrient essential to human health and while significant global variation in plasma selenium levels has been reported, there are limited studies for the Australian population (**Table 3-2**).[164-170] The present study assessed plasma selenium levels in donors from three geographically distinct regions and demonstrated that selenium levels in donors from South East Queensland (Toowoomba) were lower than those from Far North Queensland (Cairns) and Central Queensland (Rockhampton). These data, together with previous studies in South Australia [167,168,170] and Tasmania [164,166] (**Table 3-2**), highlight regional variation in levels of this micronutrient in the Australian population.

Table 3-2: Published selenium levels in Australia

Author	Year	Sample Size	Location	Selenium Level (µg/L, mean (SD))	Method Used
Judson *et al* [168]	1978	116	South Australia	152 (nr)	nr
Judson *et al* [167]	1982	30	South Australia	143.3 (nr)	Flurometric
Dhindsa *et al* [165]	1998	196	New South Wales	91.8 (15)	ICP-MS
Lyons *et al* [170]	2004	288	South Australia	103 (11)	ICPOES
Jacobson *et al* [166]	2007	335	Tasmania	110 (19)	ICP-MS
Lymbury *et al* [169]	2008	140	Australia	100.2 (SEM 1.3)	ICP-MS
Beckett *et al* [164]	2011	498	Tasmania	89.1 (15.1)	GF-AAS

nr- not recorded; ICP-MS, inductively coupled plasma mass spectrometry; ICPOES, inductively coupled plasma optical emission spectrometry; GF-AAS, graphite furnace atomic absorbance spectrometry.

The average plasma selenium level in the Queensland population studied was within the normal reference range currently used in Australia (55 - 110 µg/L). However, the majority of donors (88.5%) had levels that were below 100 µg/L and 43.4% had levels below 80 µg/L. Some studies report that a plasma selenium level of at least 80 - 90 µg/L is needed for maximal antioxidant GPx activity,[171,172] while others report a level at or near 100 µg/L.[159,173] Our study demonstrated that a relatively high proportion of participants in Queensland had plasma selenium levels that were below a level associated with reduced GPx activity. In previous Australian studies, plasma selenium levels below 100 µg/L were reported for 29% of Tasmanians [166] and 39% of South Australians.[170] In this study we demonstrate that 88.5% of volunteer donors had plasma selenium below 100 µg/L, a considerably larger proportion than those previously reported. Further analysis using identical methodology and similar inclusion criteria are required to confirm these interstate differences.

This study demonstrated significantly higher selenium levels in donors who reported multivitamin use. In Australia, the recommended daily intake of selenium is 70 µg for men and 60 µg for women.[161] Commonly available multivitamins in Australia contain between 20-65 µg of selenium per tablet, which, if used on a daily basis, would constitute a significant portion of the recommended daily intake for both males and females. While the brand of multivitamin used by study participants, and its selenium content was not recorded, our study suggests that regular multivitamin use increases plasma selenium levels.

A novel aspect of this study was the measurement of extra-cellular selenium levels in manufactured fresh blood components. PRBC and BC-Plt's had selenium levels at the lower limit of the detection by GF-AAS, which is to be expected given these components are manufactured in an additive solution (SAGM for PRBC's and SSP+ for BC-Plt's) rather than plasma. Conversely, APH-Plt and cFFP components had levels comparable to the plasmapheresis donor average, and within the normal range. It is currently unclear if there are clinical implications of transfusing selenium depleted fresh blood components. Deficiency in other micronutrients has been observed after significant PRBC transfusion in paediatric[156] and Beta Thalassemia Major[157] patients. In certain patient populations, reduced plasma selenium levels have been associated with increased morbidity and mortality,[13,128,174] therefore it may be prudent to further assess plasma selenium levels in transfusion-dependent and massively transfused patients and if indicated, to consider supplementation.

Conclusion

The results from this study add, for the Australian donor population, current and much needed data regarding plasma selenium levels. Studies examining the benefit of selenium supplementation for various diseases and illnesses have increased in the past decade. Further research is required to determine whether anti-oxidant pathways are compromised in massively transfused patients, in part as a result of lowered selenium levels, and whether this could be ameliorated by selenium supplementation.

Acknowledgements

The authors thank the donors who consented to participate in this study, the Donor Services staff that were involved in the recruitment of donors and also L Johnson, B Mathews, K Davenport, K Rooks, J Fryk and M Faddy. We acknowledge Australian Governments that fully fund the Australian Red Cross Blood Service for the provision of blood products and services to the Australian community.

Chapter 4

Chapter 4 Antioxidant trace element reduction in an *in vitro* cardiopulmonary bypass circuit.

Charles I McDonald, Yoke Lin Fung and John F Fraser.

American Society for Artificial Organs. 2012; 58(3):217-22. IF 1.516

Accepted for publication January 2012.

Rationale

During CPB there is increased production of reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS) due to blood contact with the ECC initiating inflammation, ischemia/reperfusion, hyperoxia and shear stresses.[31,175] These reactive species create an environment of oxidative stress, which have been shown to contribute to myocardial depression, cardiac arrhythmias, pulmonary dysfunction and acute kidney injury.[4] The oxidative stress response is mitigated by an appropriate antioxidant response.[36] Some trace elements, such as selenium, copper and zinc, are critical to this antioxidant response, (either directly or by being an essential part of antioxidant enzymes). Selenium in particular, has been shown to be reduced after cardiac surgery and low levels are associated with poorer outcomes. This study was designed to:

- determine if the CPB circuit is capable of adsorbing essential trace elements.
- determine if priming the CPB circuit with albumin would mitigate the loss of trace elements.

The results from this study were presented at the 27th ASM of the Australian and New Zealand Society of Cardiovascular Perfusionists in 2010 and awarded "*Best Scientific Presentation"*.

Significance

The findings of this study are clinically relevant as the data demonstrated for the first time that the CPB circuit is capable of adsorbing essential trace elements, and that simple measures to try an prevent this (i.e. priming CPB circuit with albumin) are ineffective. As reductions in essential trace elements occur after cardiac surgery and are associated with poor outcomes, particularly for selenium, we can now show a mechanism for losses that may be as high as 29% of some essential trace elements. These results will help clinicians and nutritionists decide whether acute reductions after surgery are real deficiencies (as

opposed to acute phase shifts, where levels will probably normalise over time) and therefore whether these patients should require, and receive supplements.

Antioxidant trace element reduction in an in vitro cardiopulmonary bypass circuit.

Abstract

Many complications occurring after cardiac surgery are attributed to an acute increase in reactive oxygen and reactive nitrogen species, which under normal conditions are balanced by the antioxidant response. Two key enzymes of the antioxidant response, glutathione peroxidase (GPx) and superoxide dismutase (SOD) rely on trace elements for normal function.

It was hypothesized that circulation of blood through the cardiopulmonary bypass circuit (CPB) would (i) reduce trace element levels and antioxidant function (ii) increase oxidative stress and that (iii) pre-priming circuits with albumin would ameliorate trace element loss. This hypothesis was investigated by circulating fresh human whole blood in an *in vitro* CPB circuit. Plasma selenium, copper and zinc levels were measured, as were SOD and GPx and oxidative stress by TBARS. In spite of significant decreases in copper and zinc levels, SOD levels increased with time. Significant decreases in selenium were associated with a trend to increase TBARS but no change in GPx. Pre-priming with albumin provided no benefit in reducing trace elements loss nor in lowering levels of oxidative stress. This study confirms that CPB circuits cause significant depletion of the trace elements selenium, copper and zinc, which are necessary to maintain redox balance. The loss of trace elements is a potential contributor to cardiac surgical morbidities and further studies in the cardiac patient population are warranted to investigate this.

Introduction

Despite advances in cardiac surgery, notably from improvements in the biocompatibility of cardiopulmonary bypass (CPB) components, morbidity after cardiac surgery remains an area of great concern. During CPB there is an increased production of reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS) initiated by inflammation,[20] ischemia/reperfusion,[176] hyperoxia[142] and shear stresses.[177] These reactive species cause oxidative stress which contributes to myocardial depression, cardiac arrhythmias,[178,179] pulmonary dysfunction[180,181] and acute kidney injury.[182] Under normal physiological conditions redox homeostasis is maintained by an appropriate antioxidant response. The antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase[179] are two primary antioxidants fundamental in maintaining this redox homeostasis,[179] preventing an excessive build-up of SOD and hydrogen peroxide

(**Figure 4-1**). Their activity is dependent on appropriate levels of essential trace elements such as selenium for GPx activity,[183] and copper and zinc for SOD activity.[184,185]

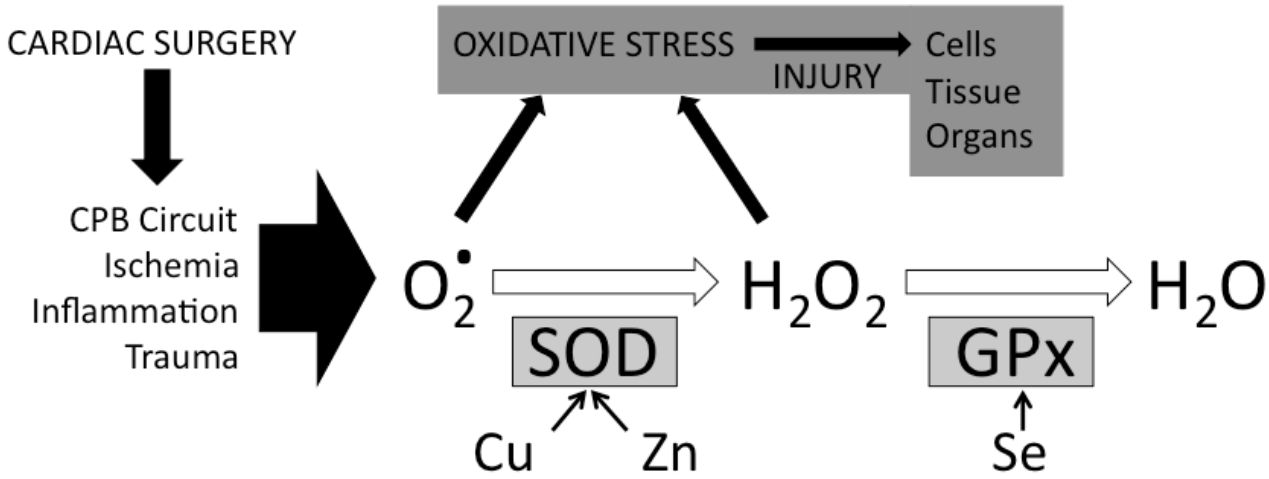

Figure 4-1: Cardiac surgery and cardiopulmonary bypass (CPB) increase production of reactive oxygen species like superoxide (O2•).

The antioxidant enzyme superoxide dismutase (SOD) converts superoxide to hydrogen peroxide (H_2O_2). SOD requires Cu and Zn for normal function. Glutathione peroxidase (GPx) catalyses conversion of H_2O_2 to water. GPx requires selenium for normal function. Oxidative stress and injury occurs when superoxide and hydrogen peroxide levels increase above capacity of SOD and GPx to maintain balance.

Reductions of selenium in non-cardiac critically ill patients have been associated with increases in mortality and morbidity,[5,128] while a recent study of adult cardiac surgical patients indicated that decreases in selenium were associated with increased post-operative multi-organ failure (MOF).[13] Hence, understanding the etiology of trace element reductions and their influence on oxidative stress during cardiac surgery may inform improvements to CPB circuits, and identify ways to reduce morbidities. This study used a closed loop *in vitro* CPB model to test the hypothesis that (i) the large surface area of the CPB circuit can reduce trace elements, which decreases antioxidant activity and increases oxidative stress; (ii) priming the CPB circuit with albumin would mitigate loss of trace elements.

Materials and Methods

Circuits

The *in vitro* closed-loop CPB model consisted of a Trillium-coated Affinity NT oxygenator with hard shell reservoir (Medtronic Pty Ltd, Minneapolis, MN, USA), Affinity NT arterial filter and Trillium-coated tubing. A roller-pump (Jostra HL-20 HLM, Maquet, Germany),

Jostra heater-cooler and in-line blood gas analyser (CDI500, Terumo Cardiovascular, Tustin, CA, USA) were used.

Five CPB circuits (PR) were primed with one liter of crystalloid (Plasma-lyte P-148, Baxter Healthcare Pty Ltd, Toongabbie, NSW, Australia). This was exchanged in part for two units of type-matched fresh human whole blood followed by porcine mucosal heparin (5000 IU) (Pfizer, Pty Ltd, Perth, Australia), 30 ml sodium bicarbonate 8.4% (Pfizer, Pty Ltd, Perth, Australia) and 2.7 mmol calcium chloride (Phebra Ltd, Lane Cove, Australia). Another five circuits (AR) were primed with one liter of 4% human albumin (Albumex 4™, CSL Ltd, Bioplasma division, Broadmeadows, Vic, Australia) and circulated for one hour at 36 °C. Albumin was exchanged with 2L Plasma-lyte at a rate of 50 ml/min. Blood and other additives were added as described for PR circuits. The blood was circulated at 3.0 L/min, warmed (36 °C) and oxygenated (pO_2 200 – 300 mmHg). To minimize the potential for haemolysis, roller pump head occlusion was adjusted using the standard static drop method.[186]

Fresh human whole blood

The Australian Red Cross Blood Service (10-01QLD-09) provided 20 units of fresh non-leukodepleted whole blood (< 5 days old). Two units of group-matched whole blood were used per circuit. Both units were connected via a perfusion priming line and mixed from one bag to another prior to addition to the circuit.

Blood samples

Baseline samples were collected prior to blood being introduced to the circuit (T0) and from the post-oxygenator sample line at 10 min (T1) and 120 min (T2) of circulation. Haemoglobin levels, platelet and white blood cell counts were measured (Abbott Cell-Dyne 3200 Multiparameter Haematology analyser. Abbott Diagnostics Division, North Ryde, Australia). Haemoglobin levels at T1 and T2 were compared with T0 for each circuit to determine the haemodilution factor.* Reported results were corrected for haemodilution.

* Addendum to publication:
For each circuit a dilution factor was calculated based upon the measured haematocrit of the combined blood bags ($Hct_{blood\ bag}$) divided by the haematocrit at T1 and T2 ($Hct_{time\ x}$).

$$\text{Dilution factor} = Hct_{time\ x} / Hct_{blood\ bag}$$

The measured value for each of the trace elements was then divided by this dilution factor.

Analytical methods.

Blood was collected into trace element sodium heparin tubes (Greiner Bio-one GmbH, Kremsmunster, Austria), plasma separated and stored at -80 °C until analysis. Selenium and copper were analysed using graphite furnace atomic absorbance spectrophotometry (GF AAS; Varian AA280Z, Agilent Technologies Inc, Santa Clara, CA, USA); zinc was analysed by colorimetric assay (ABX Pentra 400, Horiba ABX Inc, Kyoto, Japan). Ethylenediaminetetraacetic (EDTA) plasma for oxidative stress and antioxidant analysis was separated and stored at -80 °C until analysis.

Thiobarbituric acid reactive substances (TBARS) were measured using the Cayman TBARS assay (Cayman Chemical Company, Ann Arbor, MI, USA). Results were expressed as units of malondialdehyde (MDA).

Glutathione peroxidase (GPx) activity in plasma was measured using a GPx assay kit (Cayman Chemical Company, Ann Arbor, MI, USA), which measures GPx activity in extracellular fluid indirectly by a coupled reaction with glutathione reductase (GR).

Superoxide dismutase activity in plasma was measured using Cayman SOD assay kit (Cayman Chemical Company, Ann Arbor, MI, USA), which measures all three isotopes of SOD (SOD1, SOD2, SOD3).

Serum albumin was measured on a Beckman DxC600 clinical chemistry analyser (Beckman Coulter, Inc., Brea, CA, USA).

Statistical analysis

Within each group of circuits, changes from baseline were assessed using a one-way ANOVA with Tukey post-hoc analysis (Graphpad Prism 5, version 5.0d). Using a 95% confidence interval (CI), results were considered statistically significant if $p < 0.05$. An independent t-test was used to identify significant differences of similar time points between the PR and AR circuits. Results are reported as mean ± standard deviation.

Results

The mean age of whole blood (n = 20) was 4.0 ± 1.0 days, and the volume added to each circuit was 1065 ± 1.1 ml. The minimum circulating volume of the CPB circuits was 1500 ml. Platelet counts at T0 were comparable between the circuits (188.2 ± 23.6 x 10^9/L vs. 186 ± 31.0 x 10^9/L, PR:AR). At T2 there were profound reductions in both circuits (92.8 ± 19.7 x 10^9/L vs. 103.0 x 10^9/L, PR:AR). No differences existed between the PR or AR circuits at these time points. White blood cell counts were also comparable at T0 in both

circuits (4.55 x 10^9/L vs. 4.11 x 10^9/L, PR:AR), with profound reductions at T2 in both circuits (1.12 x 10^9/L vs. 1.51 x 10^9/L, PR:AR). No differences existed between the PR or AR circuits at these time points.

Trace Elements

Zinc levels were at the upper range of normal at T0 (**Figure 4-2A**) (PR-21.8 ± 2.5 µmol/L, AR-20.4 ± 2.9 µmol/L). Zinc levels in both circuits were significantly reduced at T1, but remained within the normal range, (PR-15.4 ± 3.5 µmol/L, AR-15.2 ± 1.6 µmol/L), representing a 29% reduction. There were no further reductions at T2 (PR- 15.4 ± 1.1, AR-15.2 ± 1.9 µmol/L). No significant differences were detected between corresponding time points in the PR and AR circuits.

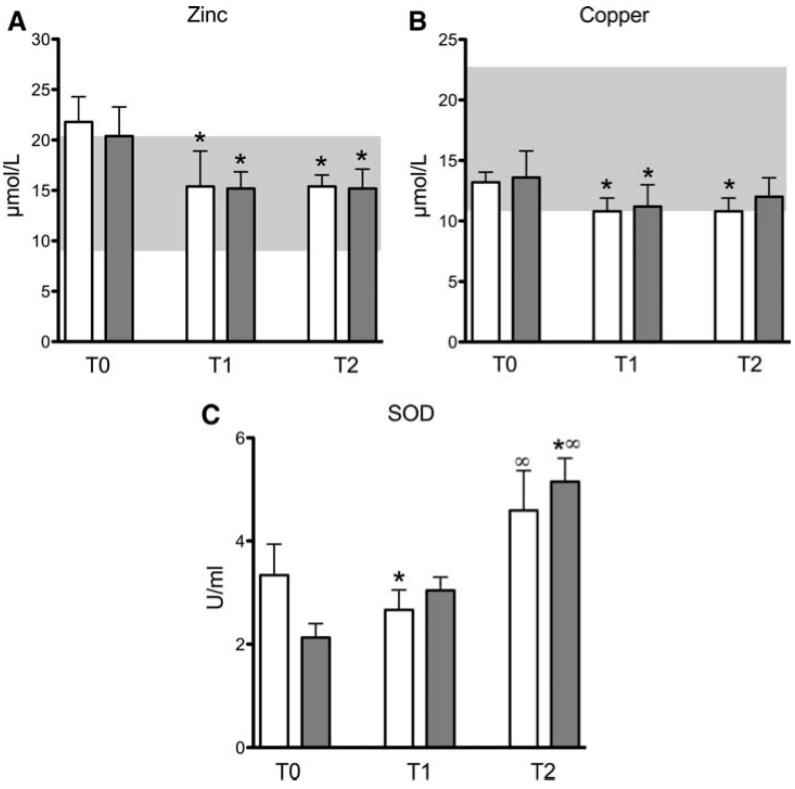

Figure 4-2: Plasma zinc (A), copper (B), and superoxide dismutase (SOD) (C) levels pre-cardiopulmonary bypass circuit (T0), at 10 min (T1) and 120 min (T2) of whole blood circulation.

Circuits were preprimed with Plasma-lyte (PR, n = 5, white) or Albumin (AR, n = 5, grey). Normal reference ranges for copper and zinc are indicated by grey background box. Significant differences ($p < 0.05$) with respect to T0 are marked with asterisks. Infinity (∞) indicates a significant difference with respect to T1.

Copper levels were at the lower limit of the normal range for both AR and PR circuits at T0 (**Figure 4-2B**). Mean copper levels were reduced by 18.2% at T1 in the PR circuits

(13.2 ± 0.8 µmol/L (T0) to 10.8 ± 1.1 µmol/L (T1)), remaining unchanged at T2 (10.8 ± 1.1 µmol/L). In the AR circuits there was a reduction of 17.6% at T1 (13.6 ± 2.2 µmol/L (T0) to 11.2 ± 1.8 µmol/L (T1)). Levels at T2 rose marginally (12 ± 1.6 µmol/L). Although this was an 11.7% reduction, it was not statistically significant. Levels at T1 and T2 in the PR circuits were below the lower limit for normal. No significant differences were detected between corresponding time points in the PR and AR circuits.

The level of selenium at baseline (T0) was 0.82 ± 0.11 µmol/L for both circuits (**Figure 4-3A).** Levels declined equally in both the PR and AR circuits at T1 (0.68 ± 0.08 µmol/L), representing a 17.1% reduction. Selenium levels were similarly reduced in both circuit groups at T2 (PR- 0.68 ± 0.08 µmol/L. AR- 0.68 ± 0.04 µmol/L). Mean levels at T1 and T2 were just below the lower limit of the defined normal range of 0.7 - 1.4 µmol/L. No significant differences were detected between the PR and AR circuits at corresponding time points.

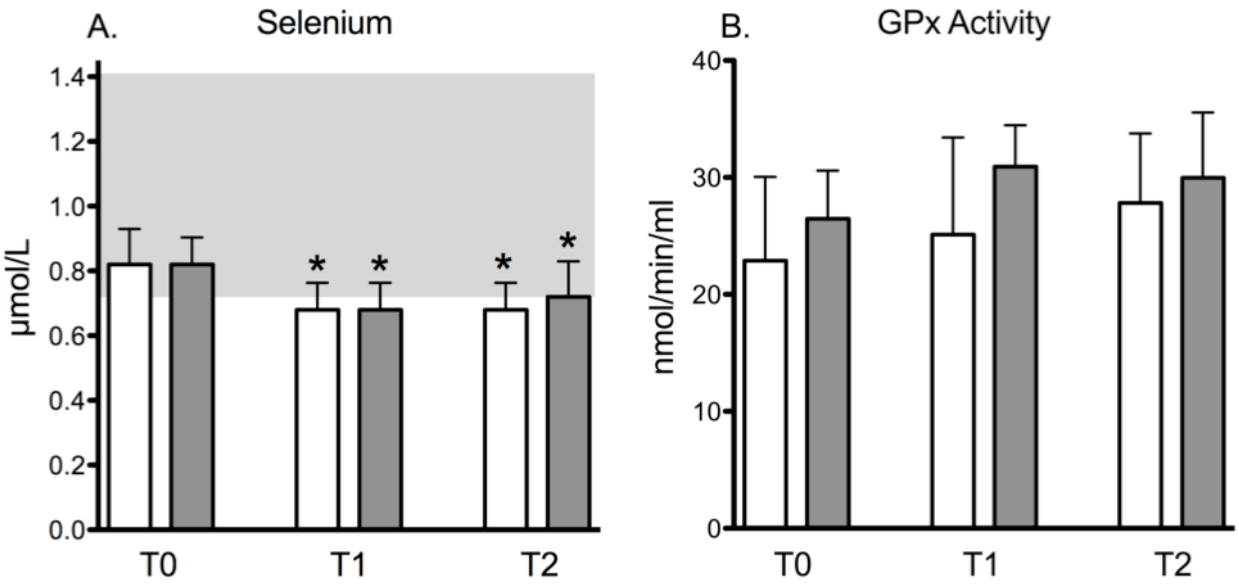

Figure 4-3: Selenium levels (A) and glutathione peroxidase (GPx) activity (B)

Selenium levels (**A**) and glutathione peroxidase (GPx) activity (**B**) precardiopulmonary bypass circuit (T0), at 10 min (T1) and 120 min (T2) of whole blood circulation. Circuits were preprimed with Plasma-lyte (PR, n = 5, white) or Albumin (AR, n = 5, grey). Normal selenium reference range is indicated by grey background box. Significant differences ($p < 0.05$) with respect to T0 are marked with asterisks.

Superoxide dismutase levels

There was a significant increase in SOD activity in the AR circuits at T1 compared to T0 (2.13 ± 0.61 to 3.04 ± 0.57 U/ml) but levels in the PR circuits decreased (3.34 ± 1.35 to 2.67 ± 0.86 U/ml), (**Figure 4-2**). At T2 there was a significant increase in activity in the PR circuits with respect to T1 (2.67 ± 0.86 to 4.60 ± 1.72 U/ml) but not when compared to

T0 (3.34 ± 1.35 U/ml). In the AR circuits SOD activity at T2 (4.42 ± 0.25 U/ml) was significantly increased when compared to T1 and T2. No significant differences were detected between the PR and AR circuits at corresponding time points.

Glutathione Peroxidase (GPx) activity.

GPx activity did not increase significantly at T1 ($p > 0.05$) (**Figure 4-3B**) in either the PR or AR circuits (25.12 ± 8.30 nmol/min/ml, 30.91 ± 3.55 nmol/min/ml), or at T2 (27.81 ± 5.96 nmol/min/ml, 29.98 ± 5.58 nmol/min/ml). However, there were no significant differences between the PR and AR circuits at any of the same time points.

Thiobarbituric Acid Reactive Substances (TBARS) activity.

No difference was detected between the PR and AR circuits at the same time points (**Figure 4-4**). There was a trend towards an increase from T0 to T1 (PR- 2.19 ± 0.60 to 2.75 ± 0.78 µM, AR- 2.11 ± 0.75 to 3.14 ± 0.79 µM), and also at T2 (3.48 ± 1.05 µM, 3.49 ± 1.81 µM. PR and AR respectively) but these increases did not prove to be statistically significant.

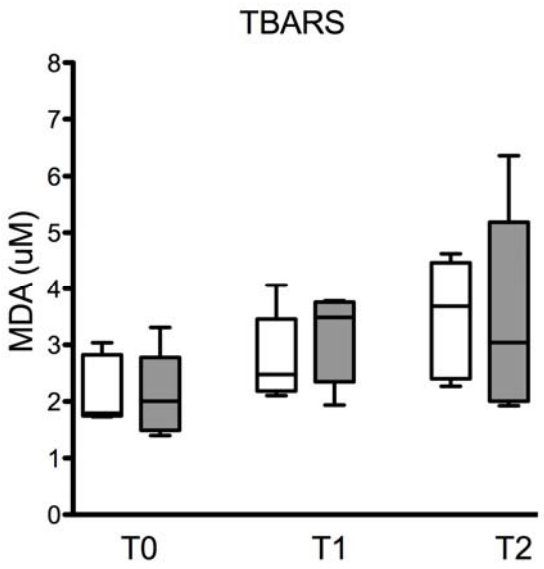

Figure 4-4: Thiobarbituric reactive substances (TBARS) activity

Thiobarbituric reactive substances (TBARS) activity in plasma precardiopulmonary bypass circuit (T0) and at 10 min (T1) and 120 min (T2) of whole blood circulation. Circuits were preprimed with Plasma-lyte (PR, n = 5, white) or Albumin (AR, n = 5, grey). MDA, malondialdehyde.

Albumin

In the PR circuits, albumin levels fell significantly by 19.5% from 33.8 ± 0.8 g/L at T0 to 27.2 ± 1.6 g/L at T1 (**Figure 4-5**) and remained unchanged at T2 (27.4 ± 1.5 g/L). In the AR circuits there were also significant reductions (15.7%) in serum albumin from 34.2 ± 2.5 g/L (T0) to 28.8 ± 3.8 g/L (T1). Mean levels at T2 were 30.0 ± 2.0 g/L, which was also a significant reduction. There were no differences between the two circuits at similar time points.

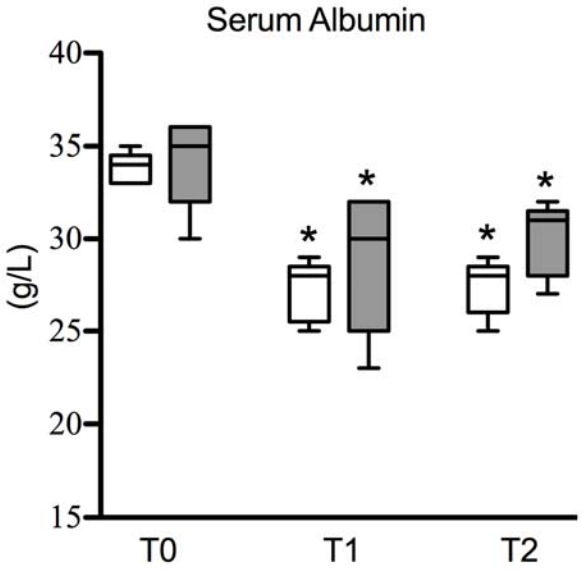

Figure 4-5: Albumin levels in serum

Albumin levels in serum precardiopulmonary bypass circuit (T0) and at 10 min (T1) and 120 min (T2) of whole blood circulation. Circuits were preprimed with Plasma-lyte (PR, n = 5, white) or Albumin (AR, n = 5, grey). Asterisk (*) indicates a significance difference ($p < 0.05$) with respect to T0.

Discussion

Profound reductions of key trace elements have been shown to occur after CPB,[13] however, the reasons for these reductions are unclear. Results from this study confirm that significant quantities of trace elements; copper, zinc and selenium are lost when whole blood is exposed to the CPB circuit.

There was a rapid and significant reduction of copper, zinc and selenium in whole blood contact with CPB circuit, irrespective of whether the circuits were pre-primed with plasma-lyte or albumin. Plasma levels of copper were reduced from pre-circuit baseline levels by 18.2%, zinc by 29% and selenium by 17.1%, mirroring results from another clinical study.[13] These reductions have compelling clinical implications, as CPB is one of the major triggers for an increased production of ROS/RNOS, which causes oxidative stress and contributes significantly to post-operative mortality and morbidity.[179,187]

Copper, zinc and selenium are critical to the activity of several key antioxidant enzymes (SOD and GPx) that help maintain redox homeostasis (**Figure 4-1**). Oxidative stress during cardiac surgery is triggered by multiple events, (inflammation,[20] ischemia/reperfusion,[176] hyperoxia[142] and shear stresses[177]), which then contributes to the development of post-operative morbidities. To determine if reductions in copper, zinc or selenium might contribute to the increase in oxidative stress, we measured TBARS, as well as the activity of the antioxidant enzymes GPx and SOD.

As copper and zinc levels can determine SOD levels,[185] we hypothesized reductions in copper and zinc associated with CPB would result in a reduction of SOD activity. Despite significant reductions in copper and zinc we demonstrated a transient marginal reduction in SOD at T1 in the PR circuits but increased SOD in the AR circuits (**Figure 4-2C**). At T2 SOD levels in both circuits had increased significantly from T1 and were comparable. SOD exists in three distinct forms– SOD1 (cytosol), SOD2 (mitochondria) and SOD3 (extracellular) and the assay used in our study measured all three forms. Given the rapid rise in SOD activity at T2, we propose that some of this increase may be due to mitochondrial SOD (SOD2) originating from disrupted platelets and white blood cells. Previous studies have shown that both roller and centrifugal pumps used in CPB can damage platelets [188] and white blood cells.[189] These results can be supported in our study by profound reductions of platelet and white blood cell counts at T2. Therefore, while CPB caused decreases in circulating copper and zinc levels, this did not curb the net SOD response, probably due to increased availability of mitochondrial SOD from cell damage.

Published literature suggests that a reduction in selenium levels has clinical implications. A study of critically ill patients has shown that selenium levels below 0.7 μmol/L were independently associated with a three fold increase in morbidity and mortality.[5] Recently low levels of selenium after cardiac surgery have also been found to be an independent predictor of MOF.[13] The average selenium level in the WB units used prior to contact with the circuit was 0.82 ± 0.11 μmol/L, and 10 minutes after contact with the CPB circuit selenium levels in both the PR and AR circuits were 0.68 ± 0.08 μmol/L. This loss of selenium has clinical implications, as an equivalent reduction in a cardiac surgical patient would place their selenium levels at levels previously associated with increases in mortality and morbidity.[5]

Selenium is key to GPx activity, but variations in selenium levels delay the definition and international reference ranges for GPx.[190] Some studies report that a minimum plasma

selenium level of 1.13 μmol/L is needed for maximal expression of GPx activity.[191] In this study, the additional 17% loss in selenium due to the circuit did not change the measured GPx activity. This reflects a limitation of this study, because the baseline whole blood selenium level (0.82 ± 0.11 μmol/L) was already on the lower end of the normal range. Hence, the GPx activity was correspondingly low. Because GPx activity was already reduced, further decreases in selenium caused no significant change in activity. Had selenium levels and GPx activity been optimal at the start (baseline), the subsequent 17% selenium loss may have produced a detectable decrease in GPx activity.

As the three trace elements studied are antioxidant trace elements,[192] their reduction would be expected to increase oxidative stress. To measure oxidative stress in our circuits we used the TBARS assay, which assesses lipid peroxidation. Although not statistically significant, there was a trend to increased levels of TBARS above baseline at T1 and T2 in both PR and AR circuits suggesting an increase in oxidative stress (**Figure 4-4**). It is possible that statistical significance may have been reached had more circuits been done, but availability of fresh whole blood prevented us from running more circuits. This is a limitation of this study. The suggestion of an increase in oxidative stress due to the CPB circuit is worthy of further investigation.

The binding of whole blood proteins to the surface of uncoated CPB circuits has been demonstrated previously.[193,194] Based on these earlier studies, it is still common practice that CPB circuits are primed with albumin, believing it will bind to the circuit and reduce the binding of other physiologically important proteins such as fibrinogen, and preserving platelet function.[193,194] Despite albumin priming of one group (AR), we reported similar reductions in circulating albumin from the whole blood in both the PR and AR circuits at T1 and T2. Our circuits were Trillium coated, and studies have shown minimized protein binding with this surface coating.[195] Low binding avidity of albumin may have reduced any benefit of albumin priming and contributed to both groups showing equal reductions in albumin after the addition of blood. A comparison with non-coated circuits may have produced a different result, as might a comparison between the various brands of surface coatings and CPB oxygenator types.

Findings from this study show that albumin priming does not reduce trace element loss in CPB circuits. However, it is important to keep in mind that although albumin is an important transport protein for trace elements, other important transport proteins such as ceruloplasmin,[196] selenoprotein-P,[183] and GPx-3[183] may still bind to the circuit in significant quantities. Thus, there are other avenues where trace elements may be lost to

the circuit. The contribution of these other proteins to trace element loss and their impact on CPB patient morbidity remain to be investigated.

Conclusion

In this *in vitro* CPB model there was a trend to increased oxidative stress levels, consistent with previous studies that show an acute increase in oxidative stress occurring during cardiac surgery as a result of ROS/RNOS generation. Redox homeostasis only occurs if there is an adequate antioxidant response, a response somewhat dependent on appropriate levels of trace elements. This study confirms that CPB circuits deplete circulating levels of antioxidant trace elements copper, zinc and selenium. Priming of circuits with albumin did not reduce trace element loss. These results imply that CPB patients with low selenium levels may have increased vulnerability to oxidative stress.

Chapter 5

Chapter 5 Transfusion of packed red blood cells reduces selenium levels and increases lipid peroxidation in an in vivo ovine model.

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Rationale

While much evidence demonstrates that the transfusion of packed red blood cells (PRBC) is associated with increased morbidity and mortality in some patient groups,[197,198] the mechanisms by which this occurs after transfusion remain elusive. A limited number of studies indicate an increased oxidative stress response following transfusion, especially in neonates.[56,57,199] Prolonged oxidative stress results in tissue injury and organ failure leading us to speculate that this might be one mechanism for the morbidity associated with PRBC transfusion. The primary aim of this study was to

- investigate if transfusion of aged PRBC might invoke a greater oxidative stress response compared to fresh PRBC.

Having previously determined that PRBC products have very little extracellular selenium (**Chapter 3**) we also investigated if

- transfusion of a selenium poor blood product results in a reduction of selenium in the recipient

We also measured GPx activity, copper, zinc levels and SOD activity in an *in vivo* ovine model of transfusion. Preliminary results from this study were published in abstract form in *Vox Sanguis, 2011; 101 (suppl. 2):122*. The results from this study were presented at the 28th ASM of the Australian and New Zealand Society of Cardiovascular Perfusionists in 2011, where it was awarded the *"Encouragement award for Scientific Development"*.

Significance

Transfusion of PRBC are used for the management of anaemia in both acute, (the critically ill, neonates, paediatrics and the elderly having major surgery), and chronic settings (e.g. cancer, ß-thalassemia). Though PRBC transfusion often benefits the recipient, they are not

risk free, and have been associated with patient morbidity and mortality. Some data also suggests that the age of the PRBC product may contribute to these adverse outcomes. The results from this study confirm that transfusion with a trace element poor blood product, such as PRBC, resulted in a dilutional decrease in the recipient's trace element levels (especially selenium). We also demonstrated that there was an increase in oxidative stress after transfusion, and that there were no differences between fresh and aged PRBC. These results have yet to be replicated in human studies.

Transfusion of packed red blood cells reduces selenium levels and increases lipid peroxidation in an *in vivo* ovine model

Abstract:

Background: Oxidative stress from surgery or critical illness has been shown to adversely contribute to morbidity and mortality. As the stored PRBC ages, products of oxidative stress accumulate; recent studies also record that oxidative stress is heightened following packed red blood cell (PRBC) transfusions, and there are no studies that investigate if transfusion of aged PRBC actually increases the recipient's oxidative stress profile more than fresh PRBC.

Objective: To compare the effect of fresh versus aged PRBC transfusions on the recipient's oxidative stress using an ovine model.

Methods and Materials: Male sheep were transfused with either fresh (n = 6) or aged (n = 6) ovine PRBC, and serial blood samples taken. Plasma samples were analysed for lipid peroxidation using the thiobarbituric acid reactive substances (TBARS) assay. This served as an indicator of oxidative injury. Antioxidant function and trace elements levels were also measured.

Results: Like human PRBC the ovine PRBC had negligible selenium levels. Irrespective of age, PRBC transfusions were associated with reduced selenium levels and antioxidant function, which correlated with increased markers of lipid peroxidation.

Conclusion: Transfusion of selenium poor PRBC can dilute recipient plasma selenium levels and compromise glutathione peroxidase antioxidant activity and thereby allowing lipid peroxidation. As there was no evidence that aged PRBC induced more severe oxidative injury, this suggests that selenium dilution is a key underlying mechanism. Further studies are needed to assess the impact of transfusion related oxidative stress in massive transfusions.

Key words: oxidative stress, transfusion, glutathione peroxidase, selenium.

Introduction

The transfusion of packed red blood cells (PRBC) has been associated with increased morbidity and mortality.[200,201] Several clinical studies have documented increased oxidative damage to both lipids and proteins following PRBC transfusions.[56,57,199] Antioxidants are required to neutralize and control levels of oxygen free radicals before they exert damage. Glutathione peroxidase (GPx) is a primary antioxidant enzyme present in the plasma, and in both the cytosol and membrane of erythrocytes. There is evidence that the antioxidant

function of PRBC, particularly GPx, is reduced over time.[202,203] GPx function is dependent on the trace element selenium[82] and our studies show that PRBC have very low extracellular selenium.[158] Hence transfusion of PRBC could dilute selenium levels, reduce GPx activity and consequently promote oxidative injury. Thus we hypothesized that aged PRBC would induce more oxidative injury compared to fresh PRBC, and investigated this in an *in vivo* ovine model.

Material and Methods

Study approval was granted by the Animal Research Ethics Committees of the Queensland University of Technology and University of Queensland. Healthy 1–3 year old male sheep (n = 12) with an average (± SD) weight of 40.2 ± 2.9 kg were fasted overnight, anaesthetised and intubated. Ovine normothermia (39 ºC) was maintained, with continuous hemodynamic monitoring and regular blood gas analysis (ABL825 blood gas analyser, Radiometer, Copenhagen, Denmark).

Sheep were initially haemorrhaged 30% of estimated blood volume into Leukotrap whole blood (WB) bags (Pall Medical, UK). After leukodepletion, the blood was centrifuged and the plasma removed. Saline-adenine-glucose-mannitol (SAGM) was added and the units were stored at 4^{o}C till required. Ovine PRBC (ovPRBC) compatibility with recipient sheep was confirmed prior to transfusion as previously described.[204] Sheep were kept hypovolemic for thirty minutes after haemorrhage, and then transfused with two units of compatible fresh (less than 7 day old (do); n = 6 sheep) or aged (30 - 42 (do); n = 6 sheep) ovPRBC, followed by human albumin 4% (CSL inc, Broadmeadows, Vic, Australia) to replace the remaining haemorrhage volume. Physiological monitoring continued for four hours followed by euthanasia (pentobarbitone-15ml of 325 mg/ml).

Blood samples were collected prior to anaesthesia (B), post-haemorrhage (H), Four hours after fresh (TF) or aged (TA) transfusions and from ovPRBC bags (trace element analysis only). Blood was collected in trace element-free tubes (Greiner Bio-One, Monroe, North Carolina, United States) for selenium, copper and zinc analysis, and EDTA tubes for thiobarbituric acid reactive substances (TBARS), GPx and superoxide dismutase (SOD) analysis. Samples were immediately cold centrifuged at 4 ºC and the separated plasma stored at -80 ºC until analysis. TBARS, GPx and SOD were analysed in plasma using spectrophotometric assays (Cayman Chemical Company, Ann Arbor, MI, USA). The TBARS assay measures malondialdehyde (MDA- an end product of lipid peroxidation)

which is a widely used measure of oxidative stress.[205] The MDA in a sample reacts with the thiobarbituric acid in the TBARS assay at high temperature (90 - 100 ^{o}C) to yield a fluorescent product detectable at 530nm using a colorimetric assay with the result expressed in units of MDA. The GPx assay measured GPx activity indirectly through a coupled reaction with glutathione reductase (GR). The SOD assay utilized a tetrazolium salt to measure all three isotopes of SOD (SOD1, SOD2, SOD3). Plasma selenium and copper were measured by graphite furnace atomic absorption spectrometry with Zeeman background correction (GF-AAS) using a Varian AA280Z analyser (Agilent Technologies Inc, Santa Clara CA, United States). Plasma zinc was measured by colorimetric assay (ABX Pentra 400, Horiba ABX Inc, Kyoto, Japan).

Statistical analysis

Data was analysed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test (Graphpad Prism 5, version 6.0a, San Diego, CA, USA). Statistical significance was $p < 0.05$ using a 95% confidence interval. Data reported (table 1) as mean ± standard deviation. Box-whisker plots (Fig. 1) show minimum and maximum values, box is 25th and 75th percentiles with median.

Results

Sheep were haemorrhaged a total of 881.1 ± 52.0 ml. The mean age and volume of transfused fresh ovPRBC was three days (2 - 7 days) and 422.5 ± 40.1 ml, and 37 days (33 - 42 days) and 403.2 ± 52 ml for aged ovPRBC. Mean volume of albumin infused was 468 ± 42 ml in all sheep. Haemorrhage induced reductions in MAP, CI and SVO_2, which recovered immediately following transfusion resuscitation (**Table 5-1**).

Prior to transfusion the sheep had normal whole blood levels of selenium, copper and zinc. In contrast, these levels were all at the lower limit of detection in the ovPRBC product (**Table 5-1**). Haemorrhage induced no significant changes to TBARS, GPx, or selenium levels (**Figure 5-1** A-C). Transfusion of both fresh and aged ovPRBC induced similar increases in TBARS levels (**Figure 5-1**A, $p < 0.05$), while selenium levels decreased ($p < 0.05$) with a corresponding decrease in GPx activity (**Figure 5-1**B and C, $p < 0.05$). There were no significant changes in SOD activity after either fresh or aged ovPRBC transfusions (**Figure 5-1**D). Compared to baseline and post-haemorrhage, zinc levels were significantly reduced in both the fresh and aged ovPRBC transfusion groups, ($p < 0.05$, **Figure 5-1**E), but no difference was detected between fresh or aged levels. Copper levels were significantly reduced compared to baseline levels after both fresh and aged transfusion, (p <

0.05), but only the aged ovPRBC transfusions post-haemorrhage levels were significantly reduced, ($p < 0.05$, **Figure 5-1**F).

Table 5-1: Physiologic data of sheep and trace element content of transfused ovPRBC

	Baseline (n = 12)	**Post-Haemorrhage** (n = 12)	**Post-Fresh ovPRBC** (n = 6)	**Post-Aged ovPRBC** (n = 6)
Hb (g/L)	80.6 ± 12.2	83.6 ± 12.6	83.0 ± 12.6	78.8 ± 12.6
Lactate (mmol/L)	0.85 ± 0.33	1.06 ± 0.39	0.40 ± 0.06	0.38 ± 0.15
pH	7.43 ± 0.03	7.46 ± 0.04	7.46 ± 0.04	7.45 ± 0.02
MAP (mmHg)	104 ± 10	35 ± 9 *****	82 ± 14	90 ± 13
HR	132 ± 23	133 ± 18	113 ± 21	118 ± 20
CI (L/min)	4.9 ± 1.7	2.0 ± 0.5 *****	3.9 ± 1.4	4.6 ± 1.2
SVO_2 (%)	77 ± 8	56 ± 14 *****	76 ± 8	73 ± 13
Trace Element content	**Whole blood at donation**		**Fresh ovPRBC**	**Aged ovPRBC**
Selenium (µmol/L)	1.35 ± 0.29		0.22 ± 0.13 *****	0.15 ± 0.12 *****
Copper (µmol/L)	17.4 ± 3.3		3.0 ± 2.4 *****	2.8 ± 1.6 *****
Zinc (µmol/L)	11.8 ± 1.9		24.7 ± 2.3 *****	26.3 ± 5.7 *****

Hb, haemoglobin; HR, heart rate; MAP, mean arterial pressure; CI, cardiac index; SVO_2; mixed venous saturation. Physiologic data from sheep at baseline, post-haemorrhage and 4 h post-transfusion with fresh and aged ovPRBC. Trace element levels in ovPRBC are reported and compared to levels in whole blood prior to processing. Data is mean ± standard deviation. Statistical comparisons made between groups using t-test. Asterisk (*) indicates statistically significant change compared to baseline data ($p < 0.05$).

Discussion

Oxidative stress and injury are terms used to describe the deleterious effect of free radicals on lipids, proteins and DNA. This mechanism of injury is clinically relevant especially in the critically ill.[3,206] While there have been reports of transfusion related oxidative stress,[56,57,199] it is unclear if the age of the transfused PRBC determines the severity of oxidative injury. An ovine model was employed to address this question, as ovine RBC have a similar survival time (114 - 120 days) to human RBC,[207] and ovPRBC, like human PRBC are selenium deplete.[158] The findings from this study show that PRBC transfusions following controlled haemorrhage resulted in reduced selenium and an increase in lipid peroxidation. However, age of the PRBC transfused had no influence on either selenium or lipid peroxidation levels.

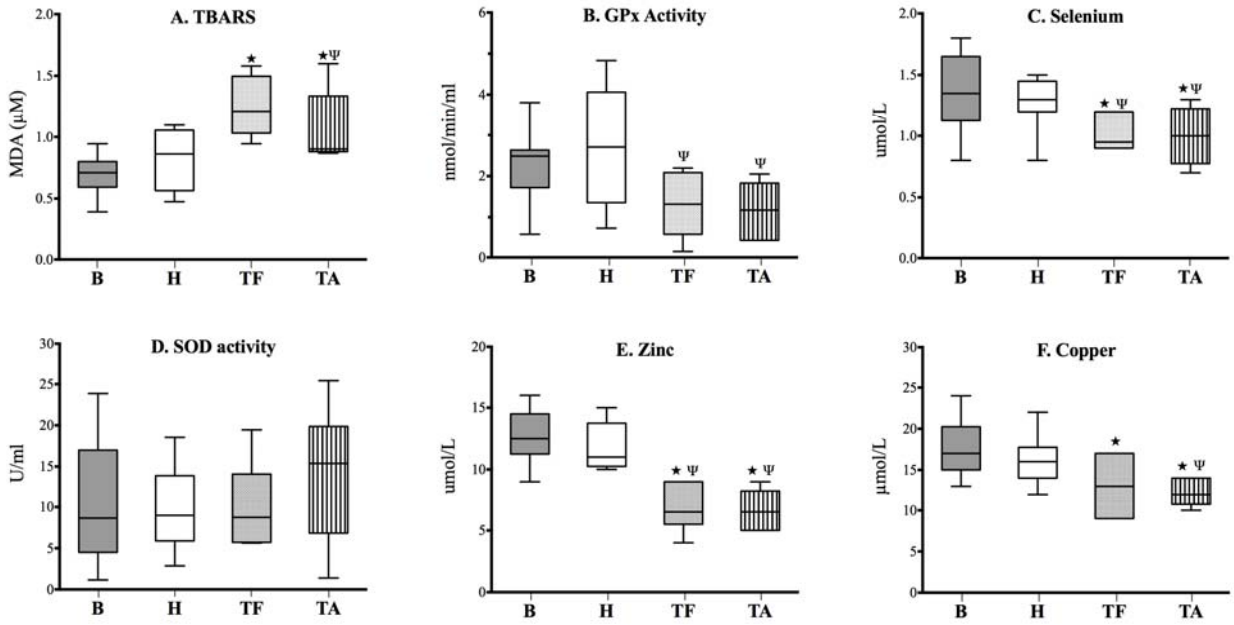

Figure 5-1: TBARS, GPx activity, Selenium, SOD activity, Zinc, Copper in sheep

TBARS (a), GPx activity (b), Selenium (c), SOD activity (d), Zinc (e), Copper (f) in sheep at baseline (B), after haemorrhage (H) and 4 h after transfusion with fresh ovPRBC (TA, n = 6) or aged ovPRBC (TF, n = 6). *-significant difference ($p < 0.05$) with respect to baseline, ψ-significant difference ($p < 0.05$) with respect to haemorrhage.

To achieve redox control, antioxidants counteract the effects of oxygen and nitrogen free radicals. Consequently, any loss of antioxidant function produces an environment conducive to oxidative stress/injury. Three of the most important antioxidants in humans are GPx, SOD and catalase.[3] GPx exists in several forms with GPx-1 occurring in the erythrocyte cytosol and membrane, and GPx-3 is present in extracellular fluid such as plasma.[208] The function of GPx is exclusively reliant on the trace element selenium,[82] while SOD relies on copper and zinc for its function.[209] In this controlled ovine haemorrhage and transfusion model, we demonstrated that the transfusion of as little as two units of ovPRBC caused a net reduction in circulating levels of all three trace elements in the recipient.

Despite reductions in plasma copper after transfusion, no reduction in SOD activity was detected (**Figure 5-1**D). Two forms of the SOD enzyme (SOD1 and SOD3) require zinc and copper for antioxidant activity, even though copper appears more important.[209] Because unbound copper in cells is toxic, it is bound and transported by the copper chaperone for SOD.[210] Therefore, we speculate that copper from this reservoir maintained the observed SOD activity.

Previous studies have shown that human PRBC have negligible levels of selenium[158] and ovPRBC demonstrated similar low levels irrespective of the age of the product (**Table 5-1**).

Given that GPx function is dependent on selenium,[82] we propose that the observed reduction in GPx function in the transfused sheep (**Figure 5-1**B) was a result of selenium dilution. Firstly, the haemorrhage would have caused a net selenium loss. The subsequent resuscitation to replacement volume with two units of selenium poor ovPRBC and human albumin 4% would then have diluted the recipient's remaining selenium ration, which in turn compromised the antioxidant function of GPx. The consequence of the diminished antioxidant function is reflected by the increase in lipid peroxidation (as measured in the TBARS assay). Using albumin for volume replacement is a limitation of the study, as albumin itself has significant antioxidant properties.[211] Hence, it is possible that the increase in lipid peroxidation may have been greater if a different colloid was used.

Studies have reported that the antioxidant function of the PRBC product, particularly GPx, is reduced as storage time increases.[202,203] Therefore, we considered it useful to determine if aged PRBC was associated with more severe oxidative injury than fresh PRBC transfusion. Serial measurements of aging human PRBC document a direct correlation of MDA and haemoglobin oxidation with RBC membrane damage.[212] Other studies have demonstrated that as PRBC age there is a progressive increase of protein carbonyl and lipid peroxidation products which compromise function.[212-215] However, in this ovine model there was no evidence for increased lipid peroxidation with aged PRBC transfusion adding support to the selenium dilution mechanism.

Some attempts have been made to counteract PRBC transfusion related oxidative stress. A small study indicated that pre-donation supplementation with selenium, beta-carotene, vitamin C or E reduced lipid peroxidation in stored PRBC.[216] Alternatively, the addition of antioxidant compounds, such as glutathione, to the PRBC product after donation has been shown to reduce oxidative damage to the red cell.[217] Selenium supplementation has been shown to increase GPx activity,[82] and supplementing critically ill patients with selenium has resulted in reduced mortality.[80] However, no studies have investigated this effect solely after transfusion. While further data is needed, selenium supplementation may provide a potentially safe and economical intervention to increase GPx function and improve redox control especially in cases of massive transfusion. Animal models may provide the means for investigating the effect of antioxidant supplementation on oxidative stress levels following massive transfusion.

Acknowledgements

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Chapter 6

Chapter 6 The impact of Acute Lung Injury, ECMO and transfusion on selenium and oxidative stress status in an ovine model.

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Rationale

While ECMO is commonly used for the treatment of acute respiratory distress syndrome (ARDS) that is refractory to conventional medicine, the morbidity and mortality rate in this cohort is still high.[218] It is unclear from the literature whether the morbidity and mortality associated with this intervention is a result of overwhelming aspects of the underlying illness or if the ECMO circuit significantly contributes by some, as yet, undefined mechanism. While limited animal studies have demonstrated an increase in oxidative stress when ECMO is used in healthy animals,[67-69,150] many of these studies have used ECMO circuits that are no longer available, thus questioning the relevance of the "old" published data to today's practice. Additionally, given that low selenium levels in the critically ill patient, independent of oxidative stress, are associated with adverse outcomes,[128] it is plausible that ECMO might also be associated with significant reductions in selenium and thus contribute to ECMO related complications and adverse outcomes. Therefore, the primary aims of this study were to investigate in an *in vivo* ovine model:

- the effect of an acute lung injury on oxidative stress, antioxidant and selenium status,
- the additional effect of ECMO and transfusion on oxidative stress, antioxidant and selenium status using a range of healthy and critically ill controls.

The results of this study were initially presented at the 29th ASM of the Australian and New Zealand Society of Cardiovascular Perfusionists in 2012 where it was awarded the *"Best Scientific Presentation".* This study was also presented at The Prince Charles Hospital Research Forum in 2014 where it won the *"Michael Ray Best Basic/Translational Research Project."*

Significance

As the therapeutic role of ECMO is expanding across various areas of critical illness, the need to understand the acute and chronic interactions between patient and ECMO circuit is never more present. For the first time, we have demonstrated that the combination of acute injury and ECMO results in significant disturbances to selenium compared to either intervention alone. However, as far as oxidative stress is concerned, the addition of ECMO did not result in a greater oxidative stress response compared to the response associated with acute injury. We were unable to determine any effect on either parameter as a result of transfusion, but this may be due to the small transfusion volume. As this was in an ovine model, clinical studies are needed to confirm if ECMO patients experience similar effects. Patients often remain on ECMO support for weeks to months, and the longer term effects of this intervention remains to be investigated. In addition the influence of selenium on immune, inflammatory and thyroid function open many more avenues of investigation in this patient population.

The impact of Acute Lung Injury, ECMO and transfusion on oxidative stress and plasma selenium levels in an ovine model.

Abstract:

The purpose of this study was to determine the effects of smoke induced acute lung injury (S-ALI), extracorporeal membrane oxygenation (ECMO) and transfusion on oxidative stress and plasma selenium levels. Forty ewes were divided into (i) healthy control (n = 4), (ii) S-ALI control (n = 7), (iii) ECMO control (n = 7) (iv) S-ALI + ECMO (n = 8) and (v) S-ALI + ECMO + packed red blood cell (PRBC) transfusion (n = 14). Plasma thiobarbituric acid reactive substances (TBARS), selenium and glutathione peroxidase (GPx) activity were analysed at baseline, after smoke injury (or sham) and 0.25, 1, 2, 6, 7, 12 and 24 hours after initiation of ECMO. Peak TBARS levels were similar across all groups. Plasma selenium decreased by 54% in S-ALI sheep (1.36 ± 0.20 to 0.63 ± 0.27 μmol/L, $p < 0.0001$), and 72% in sheep with S-ALI + ECMO at 24hr (1.36 ± 0.20 to 0.38 ± 0.19, $p < 0.0001$). PRBC transfusion had no effect on TBARS, selenium levels or glutathione peroxidase activity in plasma. While ECMO independently increased TBARS in healthy sheep to levels, which were similar to the S-ALI control. The addition of ECMO after S-ALI caused a negligible increase in TBARS. This suggests that the initial lung injury was the predominant feature in the TBARS response. In contrast, the addition of ECMO in S-ALI sheep exacerbated reductions in plasma selenium beyond that of S-ALI or ECMO alone. Clinical studies are needed to confirm the extent and duration of selenium loss associated with ECMO.

Introduction

Patients with acute respiratory distress syndrome (ARDS) are critically ill and exhibit increased oxidative stress and reduced selenium levels compared to other hospital in-patients.[219,220] Regardless of the cause of lung injury, oxidative stress is thought to be a major contributor to the pathogenesis and progression of ARDS.[44,219] The mortality among patients with acute lung injury (ALI) and ARDS may be as high as 41%, and in severe cases of ARDS, veno-venous extracorporeal membrane oxygenation (VV-ECMO) provides a rescue therapy for temporary respiratory support.[218,221]

VV-ECMO involves the insertion of large cannulae into the central venous circulation, redirecting the blood through an oxygenator (artificial lung), where carbon dioxide is removed and oxygen is added. (**Figure 6-1**) Oxygenated blood is then returned through a cannula to the right side of the heart before passing through the lungs. ECMO circuits have

a large surface area to enable efficient gaseous exchange, however exposure of the patient's blood to these foreign surfaces initiates an inflammatory response[222] as well as adsorbing trace elements and drugs.[47,223]

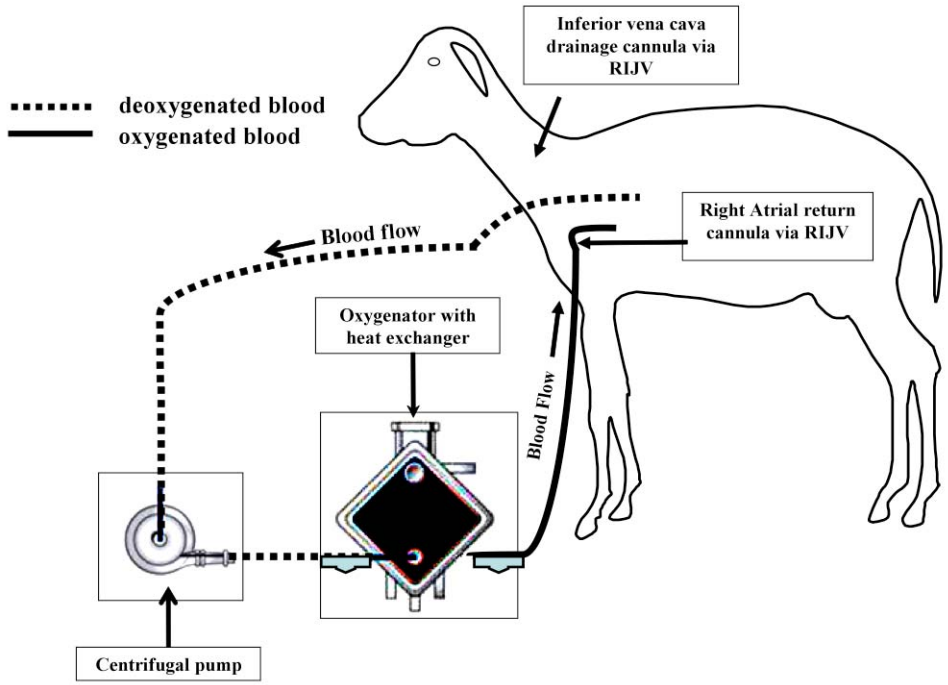

Figure 6-1: Schematic diagram of sheep on VV-ECMO.

ECMO cannulae are inserted via right internal jugular vein (RIJV).

Oxidative stress occurs when excessive production of reactive species of oxygen (ROS) overwhelms the antioxidant system.[36] Excessive ROS production during sepsis, systemic inflammation and/or ischemia-reperfusion injury has been associated with cellular, tissue and ultimately organ injury.[36] Additionally, extracorporeal circuits (ECMO, dialysis, CPB) involve extensive blood contact with a foreign surface, and this coupled with hyperoxia and massive blood transfusions have the potential to augment oxidative stress.[77,224] Whether oxidative stress in this setting contributes to the morbidity/mortality risk of patients on ECMO is unclear.

Selenium is a trace element incorporated into a variety of selenoproteins involved in antioxidant, thyroid, immunological and inflammatory regulation.[225] While some studies have demonstrated a negative correlation between selenium and oxidative stress,[120,226] reduced plasma selenium levels have also been independently associated with worse outcomes in some critically ill patients.[153] Glutathione peroxidase (GPx), a primary antioxidant enzyme, requires selenium for normal functioning.[84] The reduction of serum selenium levels during

critical illness and inflammatory syndromes may compromise GPx activity.[152] Despite its importance to antioxidant function (as well as thyroid and immune function), the impact of ECMO on selenium levels has not been previously investigated. We hypothesised that the addition of an ECMO circuit to a critically ill host would result in selenium reductions, and exacerbate oxidative stress levels. We sought to investigate the impact of acute lung injury, (i.e. critical injury), ECMO and transfusion, individually and in combination on oxidant status and plasma selenium levels using an ovine model of smoke induced acute lung injury (S-ALI).

Materials and Methods

The animal ethics committees of The University Of Queensland (QUT/194/12) and The Queensland University of Technology (1100000053) approved this study that adhered to the National Health and Medical Research Council (NHMRC) Code of Practice for the Care and Use of Animals for Scientific Purposes.[227] Forty Australian Samm Border Leicester Cross ewes (1 - 3 yr old) were divided into the following groups: (i) healthy control (n = 4), (ii) ECMO control (healthy sheep + ECMO, n = 7), (iii) S-ALI control (n = 7), (iv) S-ALI + ECMO (n = 8) and (v) S-ALI + ECMO + PRBC transfusion (n = 14). Half of the transfused group received fresh PRBC (< 5 days old) and the other half received aged PRBC (> 35 days old).

Animal Preparation:

Sheep were allocated to five different groups. Each sheep was anaesthetised and instrumented as previously detailed.[228] Briefly, standard monitoring was utilised, including-arterial blood pressure; three-lead electrocardiograph (ECG); oxygen saturation monitoring; continuous hemodynamic monitoring; regular blood gas analysis (ABL825 blood gas analyser, Radiometer, Copenhagen, Denmark); and continuous cardiac output (CCO) and SvO_2 monitoring (Swan-Ganz CCOmbo, Edwards Lifesciences, California, USA). Sheep were ventilated through a 10Fr tracheostomy tube. Normothermia (39 ºC) was maintained with a warming blanket.

Experimental Protocol

Acute lung injury was delivered as previously described.[229] Cooled cotton smoke was administered through a custom designed hand ventilator. Animals received a total of 14 breaths of cotton smoke (or sham for controls) to achieve a carboxyhaemoglobin (COHb) level of 35-45%. A two-hour lung injury development period was allowed prior to initiating ECMO.

ECMO Circuits

The ECMO circuits comprised a Quadrox PLS oxygenator, Bioline tubing and Rotaflow pump head (Maquet Cardiopulmonary AG, Hechinger Strabe Germany). Circuits were primed with Plasma-lyte P-148 (Baxter Healthcare Pty Ltd. Toongabbie, NSW, Australia), 4% human albumin (CSL Behring. Broadmeadows, Vic, Australia), 1000IU porcine heparin (Pfizer Australia Pty Ltd. West Ryde, NSW, Australia) and warmed to 38 ºC. Target ECMO flows were calculated at two thirds of the pre-ECMO cardiac output. Sodium chloride (0.9%) (Baxter Healthcare Pty Ltd. Toongabbie, NSW, Australia) was administered intravenously as needed to maintain the target ECMO flows. FiO2 was set at 100% and ECMO gas flows set to a ratio 0.8:1 of blood flow. Anticoagulation was achieved with 3000 IU of heparin given to the sheep prior to ECMO cannulation and the activated clotting time (ACT) was targeted between 200 - 300sec. Cannulae were a 19 Fr (return cannula) and a 21 Fr (access cannula) Carmeda™ bonded femoral venous cannula (Medtronic Pty Ltd, Minneapolis, MN) positioned via the right external jugular vein. Final cannulae position was confirmed using a technique of intra-cannula echo using an intra-cardiac echocardiography probe.[230]

Bilateral 20 Fr intercostal catheters were inserted two hours after commencement of ECMO to collect pleural effusion fluid. After 24 hours sheep were euthanized using sodium pentobarbitone (100 mg/kg).

Ovine PRBC preparation

Sheep in the transfusion arm received two units of leukofiltered cross-match compatible ovine PRBC that were (i) less than 5 days (fresh) or (ii) 35 - 42 days (aged) old using a previously described protocol.[204] Transfusion of the PRBC commenced after the 6 hr samples had been collected.

Blood Sample Collection

Arterial blood samples were collected at anaesthetic induction (baseline), after smoke injury (PS), 15 minutes after ECMO commencement (0.25 hr) then at 1, 2, 6, 7, 12, and 24 hours. Blood for selenium analysis was collected into trace free element tubes (Greiner Bio-One, Monroe, North Carolina, United States), and the separated plasma was stored at 4 °C until analysis. EDTA blood samples were immediately centrifuged 3000g x 10min @ 4 °C and plasma stored at -80 °C for analysis of thiobarbituric acid reactive substances (TBARS) and GPx.

Measurement of Selenium, GPx and TBARS

Plasma selenium levels were analysed by graphite furnace atomic absorption spectrometry with Zeeman background correction (GF-AAS) using a Varian AA280Z analyser (Agilent Technologies Inc, Santa Clara CA, United States) as previously described.[231] Inter-assay imprecision was 5.5%. Plasma TBARS and GPx were analysed using commercially available assay kits (Cayman Chemical Company, Ann Arbor, MI).

Baseline data depicted in **Figure 6-3** (A, B and C) include baseline data from all groups (i.e. n = 40).

Statistical Analysis

Summary data are displayed as mean ± standard deviation with associated 95% C.I. (difference of the mean) unless otherwise stated. A two-way ANOVA with unmatched pairs and a Tukey *post hoc* correction was used to compare means within and between groups (Graphpad Prism 6.0a). Statistical significance was assumed when $p < 0.05$. A linear regression was performed to determine the relationship between selenium and GPx or selenium and TBARS (Graphpad Prism 6.0a).

Results

The sheep in this study weighed an average of 48.6 ± 6.0 kg. **Table 6-1** summarises PaO_2, and COHb levels, heart rate, mean arterial pressure (MAP), inotrope use (noradrenaline, dopamine and vasopressin) and fluid requirements across the various study groups. COHb levels ranged from 25.3 - 53.6% after smoke injury, while PaO_2 levels progressively declined in the S-ALI control sheep over the 24 hours period (**Table 6-1**). Heart rate was similar across all the groups, however the average MAP over the study period was lower in the S-ALI control, S-ALI + ECMO and S-ALI + ECMO + Tf sheep compared to healthy control and ECMO controls. Inotropes (noradrenaline and dopamine) were required in the S-ALI control, S-ALI + ECMO and S-ALI + ECMO + Tf sheep to maintain cardiac function and blood pressure, but not in the healthy control or ECMO control sheep. Vasopressin was required in 5/8 of the S-ALI + ECMO and 4/14 of the S-ALI + ECMO + Tf sheep as they were mildly vasoplegic and non-responsive to noradrenaline (**Table 6-1**). Saline was administered intravenously as required to maintain ECMO flows at two-thirds cardiac output. As a result sheep in the S-ALI control, S-ALI + ECMO and S-ALI + ECMO + Tf groups received significantly more fluids and thus had a positive fluid balance at 24hr. Despite these sheep having a higher positive fluid balance, there was no significant difference in Hb compared to the ECMO control sheep. (**Figure 6-2**A).

Serial changes to relevant biochemical parameters are detailed in **Figure 6-2**. The pH levels remained consistent throughout the study (**Figure 6-2**B). Albumin levels were reduced significantly ($p < 0.05$) in the S-ALI control and S-ALI + ECMO groups after 6 hours **Figure 6-2**C). Bilirubin levels rose significantly in the healthy control and ECMO control sheep ($p < 0.05$) after 12 and 18 hours respectively but remained unchanged in the S-ALI control and S-ALI + ECMO groups (**Figure 6-2**F). Samples in S-ALI + ECMO +Tf group were unavailable for albumin, creatinine, alanine transaminase (ALT) and bilirubin analysis. As no significant differences were detected between fresh and aged PRBC transfused sheep for plasma TBARS, selenium and GPx, the data was combined into a single transfusion group (n = 14) and henceforth labelled S-ALI + ECMO + Tf.

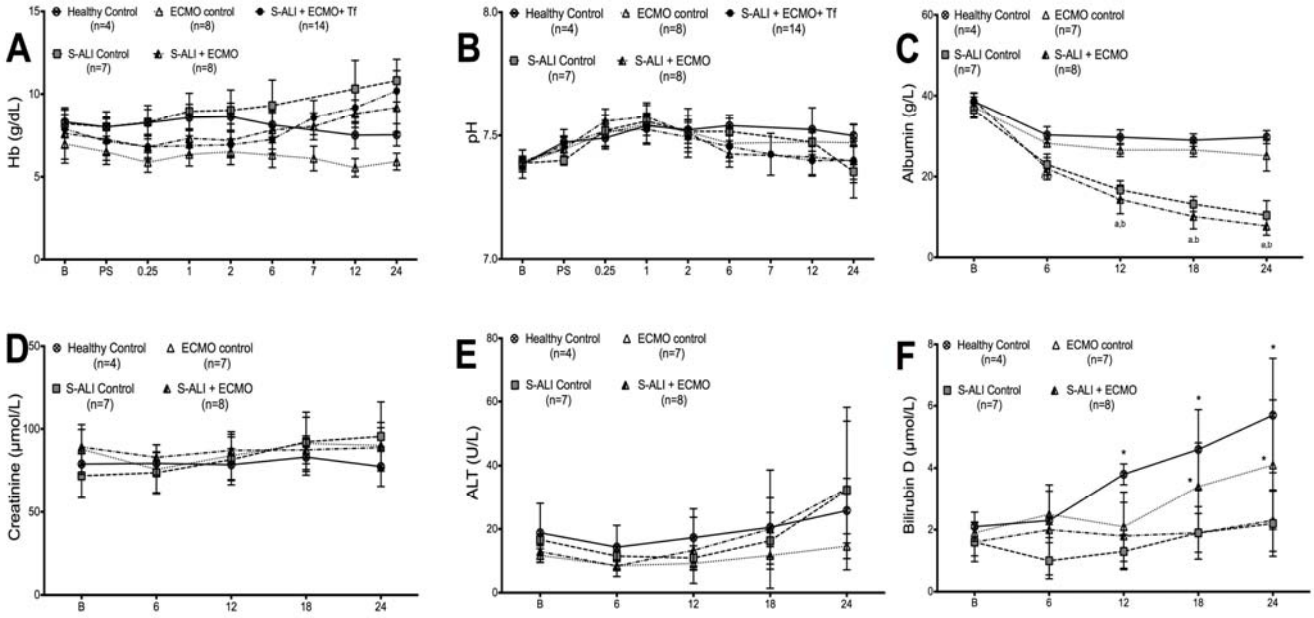

Figure 6-2: Effect of ECMO and/or S-ALI on parameters of haemoglobin (A), pH (B), albumin (C), creatinine (D), alanine transaminase (E) and bilirubin (F).

PS, post-smoke injury; ALT, alanine transaminase. Numbers represent hours of ECMO support. a and b in C indicate significant difference ($p < 0.05$) with respect to healthy control and ECMO control. Asterisks indicate significant difference ($p < 0.05$) with respect to baseline.

Plasma Thiobarbituric Acid Reactive Substances (TBARS)

Plasma TBARS levels remained unchanged in the healthy control sheep (**Figure 6-3**A). Compared to the healthy control sheep, plasma TBARS were significantly higher at corresponding time points between 0.25 hr and 24 hr for all other groups ($p < 0.001$). Plasma TBARS were significantly elevated in S-ALI control sheep, peaking at 2 hr (1.32 ± 0.40 to 2.90 ± 0.35 μM, 95% CI -2.78 to -1.16; $p < 0.001$). Plasma TBARS rose rapidly with

the initiation of ECMO in ECMO control sheep, peaking at 6 hr (1.32 ± 0.40 to 3.11 ± 0.70 μM, 95% CI -2.60 to -0.98; p < 0.0001). In S-ALI + ECMO sheep plasma TBARS were 155% above baseline (1.32 ± 0.40 vs 3.37 ± 0.35 μM, 95% CI -2.82 to -1.29; p < 0.0001) and 200% above baseline in S-ALI + ECMO + Tf sheep at 24 hr (1.32 ± 0.40 vs. 3.97 ± 0.44 μM, 95% CI -3.27 to -2.04; p < 0.0001), but this was not significantly higher than S-ALI control or ECMO control. No differences were detected due to PRBC transfusion between 7 hr and 24 hr in the S-ALI + ECMO + Tf group.

Table 6-1: Comparison of basic physiological variables

	Healthy Control (n = 4)	ECMO control (n = 7)	S-ALI Control (n = 7)	S-ALI + ECMO (n = 8)	S-ALI + ECMO + Tf (n = 14)
PaO_2 (mmHg)					
Pre-ECMO	533 ± 39	395 ± 162	562 ± 27	409 ± 120	403 ± 172
24 hr	526 ± 23	148 ± 40	169 ± 148	161 ± 48	139 ± 24
COHb (%)	4.0 ± 0.4	4.2 ± 0.6	43.3 ± 8.8	44.2 ± 5.6	39.35 ± 6.40
[range]	[3.6 – 4.3]	[3.5 – 4.7]	[30.5 – 53.6]	[35.0 – 51.4]	[25.3 – 48.4]
Heart Rate	108 ± 16	102 ± 17	114 ± 17	103 ± 17	100 ± 15
MAP (mmHg)	120 ± 15	108 ± 18	85 ± 22	81 ± 22	89 ± 22
Inotropes					
Noradrenaline (μg/min)	0	0	11.9 ± 9.3 (n = 5/7)	11.6 ± 4.4 (n = 8/8)	9.17 ± 3.9 (n = 13/14)
Dopamine (μg/min)	0	0	220 ± 92 (n = 4/7)	163 ± 103 (n = 7/8)	136 ±49 (n = 9/14)
Vasopressin (unit/hr)	0	0	0	3.3 ± 1.1 (n = 5/8)	3.4 ± 1.3 (n = 4/14)
Fluid Balance @ 24hr (ml)	378 ± 251	1289 ± 820	4346 ± 1270	9226 ± 2244*	9698 ± 2514*

COHb, carboxyhemoglobin; Tf, transfusion; MAP, mean arterial pressure. COHb values are after smoke breath cycles completed. Heart rate, MAP and Inotrope values are mean ± S.D. for the period between smoke injury/SHAM (2 h prior to ECMO) and 24 h ECMO. Asterisk denotes comparison between S-ALI control and S-ALI + ECMO/SALI + ECMO + transfusion (p < 0.05).

Plasma Selenium levels

Baseline plasma selenium levels were similar amongst all sheep. In ECMO control sheep, plasma selenium levels declined at 2 hr to 46% (1.36 ± 0.20 to 0.73 ± 0.21 μmol/L. 95% CI 0.29 to 0.97; p < 0.0001). They rose after 6 hr but were still below baseline at 24 hr (0.96 ±

0.17 µmol/L. 95% CI 0.06 to 0.74; p < 0.01). In S-ALI control sheep plasma selenium began to decline at 6 hr, and were significantly below baseline levels at 24 hr (54% reduction; 1.36 ± 0.20 to 0.63 ± 0.27 µmol/L at 24h. 95% CI, 0.39 to 1.07; p < 0.0001) (**Figure 6-3**B). The double insult of S-ALI followed by ECMO produced the greatest decline in plasma selenium of 72% at 24 hr (1.36 ± 0.20 to 0.38 ± 0.19, 95% CI 0.66 to 1.3; p < 0.0001). Between group comparisons of plasma selenium results detected a significant difference between S-ALI + ECMO vs. ECMO control at 24 hr (0.38 ± 0.19 vs. 0.96 ± 0.17 µmol/L, 95% CI 0.15 to 1.01; p < 0.001). PRBC transfusions did not cause any change.

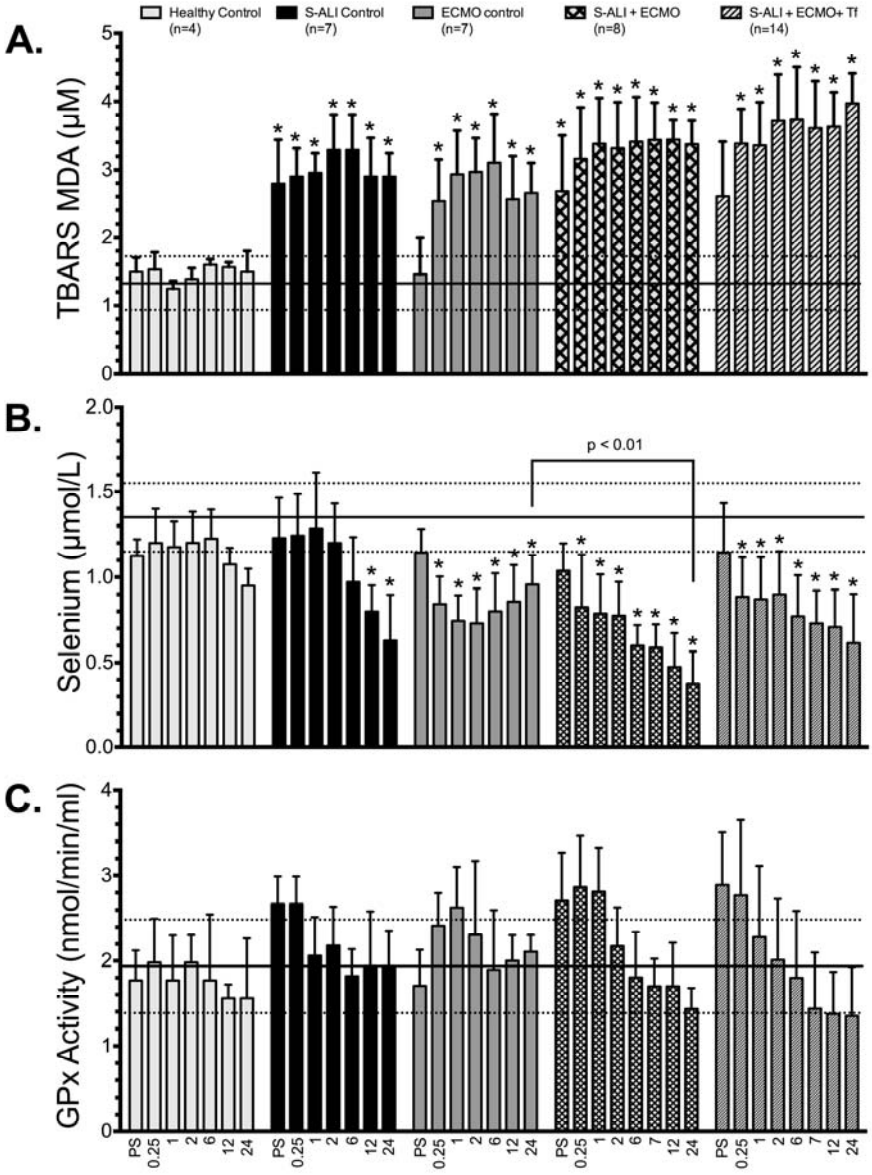

Figure 6-3: Effects of smoke injury (S-ALI) ± ECMO ± transfusion (Tf) on (A) plasma thiobarbituric acid reactive substances (TBARS), (B) selenium and (C) glutathione peroxidase (GPx) levels.

The mean baseline data is represented by a full horizontal line. Dashed horizontal line is ± 1SD. PS-2h post smoke/sham injury. Numbers represent hours after initiation of ECMO support. Data are mean ± SD. Asterisks indicate significant within group difference compared to baseline levels.

Pleural effusion fluid collected from S-ALI+ ECMO sheep had selenium levels of 0.5 ± 0.1 μmol/L (n = 16). Linear regression revealed that decreased plasma selenium was associated with increased plasma TBARS for S-ALI + ECMO sheep (r^2 = 0.50, $p < 0.0001$). This association was reduced when data from all sheep on ECMO was combined (healthy and S-ALI; r^2 = 0.39, $p < 0.001$).

Plasma Glutathione Peroxidase activity

No changes in plasma GPx activity occurred in healthy control sheep (**Figure 6-3**C). Though not statistically significant, the following trends were observed: (i) Smoke injury (S-ALI control) induced a rapid but transient increase in plasma GPx, (1.93 ± 0.55 to 2.67 ± 0.32 nmol/min/ml), returning to baseline levels at 24 hr (1.94 ± 0.41 nmol/min/ml). (ii) ECMO-control experienced a similar increase in plasma GPx (1.93 ± 0.55 to 2.63 ± 0.47 nmol/min/ml) after ECMO initiation, trending back to baseline levels at 24 hr. (iii) S-ALI + ECMO produced peak plasma GPx responses similar to ECMO control (2.86 ± 0.6 nmol/min/ml), which then declined 1h after ECMO began, being below baseline at 24 hr (1.43 ± 0.24 vs. 1.93 ± 0.55 nmol/min/ml). (iv) plasma GPx activity was elevated after smoke injury and during the initial stages of ECMO in ECMO + S-ALI + Tf group. No effect of PRBC transfusion was detected.

Discussion

This ovine model has generated two significant findings. First, we found that both S-ALI and ECMO independently increased oxidative stress, but when conducted sequentially (i.e. S-ALI followed by ECMO) the latter caused no additional impact on oxidative stress. Secondly, S-ALI caused significant reductions in plasma selenium, and subsequent ECMO support exacerbated this loss.

ARDS is associated with a dramatic and uncontrolled increase in ROS production that contributes to morbidity and mortality.[44,232] When conventional medicine fails in these patients, ECMO is one of the few remaining life-saving options.[218] Previous ECMO studies in paediatrics,[69] rabbits,[67] lambs,[68] sheep[150] and pigs[149] have shown significant elevations in lipid peroxides. However, it is difficult to translate these findings to critically ill adult patients as (i) paediatric patients have significantly different bodyweight to ECMO surface area ratios and (ii) (with the exception of the sheep study[150]) in all the other animal models ECMO was initiated in a healthy host. Thus, the impact of acute illness on overall oxidative stress could not be determined.

We demonstrated that both S-ALI and ECMO independently increased lipid peroxidation in plasma. Over the 24 hr study period, we found that while ECMO use in sheep with S-ALI resulted in an increase in lipid peroxides, the levels were not significantly greater than for S-ALI or ECMO alone. Our results contradict the findings of an earlier similar study,[150] which measured lipid peroxides in a model of S-ALI and veno-arterial ECMO, and demonstrated significant increases in lipid peroxides after ECMO was initiated in a S-ALI animal. As that study was conducted 20 years ago, their ECMO circuit was significantly different, which may account for the disparity in results to our study. Their circuit included a silicon membrane oxygenator with a large surface area (4.5 m^2), a servo controlled roller pump and venous blood drainage reservoir in a venous arterial ECMO configuration. In comparison, our study utilised a low volume closed circuit, PMP fibre oxygenator, (surface area 1.8 m^2), with centrifugal pump and coated tubing in a VV-ECMO configuration. We speculate that the improvements in ECMO technology and circuit biocompatibility have reduced the trauma associated with continual blood exposure to the foreign circuit and consequently dampened any increase in lipid peroxidation.

Independent of oxidative stress, reductions in serum or plasma selenium during sepsis and ARDS are associated with multi-organ failure, inflammatory syndromes, increased infections and increased mortality.[13,44,153,232,233] Some studies have shown that selenium replacement in critically ill patients is beneficial in reducing infections (such as ventilator acquired pneumonia) and mortality.[128,234] This led us to investigate the impact of ECMO on plasma selenium levels. We demonstrated that S-ALI and ECMO independently led to significant reductions in plasma selenium (54 and 46% respectively). Notably the “two-hit” combination of S-ALI + ECMO resulted in a greater loss (72%). We postulate that the observed plasma selenium reductions may be attributed to an acute phase response, circuit adsorption and haemodilution (due to the ECMO priming solution and ongoing fluid replacement). The acute phase response seen during inflammatory syndromes increases the permeability of cellular membranes to proteins and micronutrients, that are redistributed to tissues.[235] Hypoalbuminaemia, ascites, high volume requirement and significant pleural effusions confirm the presence of an acute phase response in the sheep of this current study. Measurement of the selenium concentration in the pleural effusion fluid also confirms this fluid to be one avenue of significant selenium loss. This may have some clinical relevance, as pleural effusions are not uncommon in VV-ECMO patients.

We have previously demonstrated that CPB circuits adsorb trace elements.[47] Given the similarities between CPB and ECMO circuits this presents another mechanism for the loss

of trace elements such as selenium. In addition, because patients on ECMO are critically ill, the associated altered gut motility and absorption may compromise dietary intake of trace elements.[236] Since ECMO support often lasts for over a week, the combination of acute phase redistribution, decreased dietary intake and loss through circuits adsorption could lead to a true selenium deficiency.

While many studies cite plasma or serum selenium reductions in the critically ill as an independent predictor of poor outcome, it is important to recognise that plasma selenium levels provide limited information regarding functional selenium status. Selenium in plasma exists in several forms, 40 - 70% is in the functional form selenoprotein P, GPx-3 accounts for another 20 - 40% and 6 - 10% is bound to albumin.[237] Unfortunately, in this present study we did not have the capacity to measure selenoprotein P. Plasma GPx (GPx-3) is an important extracellular antioxidant and can be used at times as a surrogate marker of selenium status. GPx is produced primarily in the kidneys and secondarily in the liver,[238] and while the association between selenium and GPx activity is well established, how rapidly GPx synthesis in the kidneys and liver is altered by acute reductions in selenium is largely unknown. We attempted to measure kidney (creatinine) and liver function (bilirubin and ALT). Creatinine levels remained stable in all groups, however there were increased bilirubin levels in the healthy control and ECMO control sheep but these were stable in the S-ALI control and S-ALI + ECMO sheep (**Figure 6-2**E). In contrast ALT levels were similar across the study groups (**Figure 6-2**F). The inconsistency between bilirubin and ALT levels suggests that the high positive fluid balance in the S-ALI control and S-ALI + ECMO sheep has possibly diluted both bilirubin and ALT levels. For GPx activity our data suggests a trend towards GPx reductions after 6 hours in S-ALI + ECMO sheep, which may be indicative of a delayed response to the reductions in plasma selenium.

ECMO patients often required multiple transfusions,[239,240] which may convey an increased risk of transfusion related complications. Previous studies in neonates and animals have demonstrated increases in lipid peroxidation after PRBC transfusions.[56,57,199,241] We and others have shown that PRBC contain low selenium levels[158] and reduced antioxidant capacity.[242] Despite these observations, in this model PRBC transfusions had no significant impact on oxidative stress, selenium or GPx. One reason for the lack of response may be because of the small volume of PRBC transfused and the period of observation was too short. In addition, the magnitude of the S-ALI induced oxidative stress and selenium alterations could have masked any transfusion related effect. Thus the effect of massive PRBC transfusion during a prolonged ECMO course remains to be investigated.

Investigations into the *in vivo* impact of ECMO using modern adult ECMO cannulae and circuits can only be performed with a large animal model. Sheep models have the advantage of being similar to humans in weight ranges, cardiopulmonary physiology, coagulation and inflammatory systems.[228] However, large animal models are expensive and this limitation restricted the sample size of each group in our study. The incremental design of this *in vivo* study enabled the differentiation of the effect of ECMO from that of other variables such as S-ALI and transfusion, but also had several limitations. The complexity of maintaining a critically ill animal restricted the duration of the experiment to 24 hours, thus preventing the study of longer-term effects on oxidative stress. While this study utilised a common measure of lipid peroxidation (TBARS), it may have benefited from analysing F_2-isoprostanes or protein carbonyl measurements. The analysis of tissue samples from specific organs may have also revealed localized decreases in selenium as well as the consequences of oxidative stress, that were not apparent using systemic blood samples. However, in an ECMO model where sheep are anticoagulated, serial tissue samples are not practical due to the risk of catastrophic bleeding.

During VV-ECMO, blood flow is a key determinant of oxygenation as significant shunting can occur in injured lungs, and suboptimal ECMO flows may result in systemic hypoxia. Clinically, patients with a high cardiac output who are dependent on higher ECMO flows for oxygenation require a degree of fluid resuscitation. Alternatives to optimise oxygenation and minimize fluid resuscitation in these patients include pharmacological interventions to reduce cardiac output, deep sedation, paralysis or induced hypothermia. To avoid additional confounders in this study, we chose to maintain ECMO flows to 2/3 of cardiac output across all sheep and to only use fluids and vasoactive agents to achieve this. Despite the high dose vasopressor and inotrope drugs in the S-ALI sheep (**Table 6-1**), the excessive capillary leak necessitated significant fluid resuscitation to maintain target ECMO blood flows. Notwithstanding this effort, we acknowledge that the resulting higher fluid balance in S-ALI + ECMO ± Tf sheep, (**Table 6-1**) may have contributed to lower selenium levels and a blunted TBARS response.

Conclusions

We have demonstrated that 24 hours of ECMO induced increased plasma TBARS in a healthy host. However, in a S-ALI animal, ECMO did not cause any additional TBARS increase. Nevertheless, the combination of S-ALI and ECMO lead to profound reductions in plasma selenium. It is unclear if this represents a true deficiency or is a result of fluid shifts associated with the acute phase response. In addition to influencing antioxidant function,

reductions in plasma selenium may also compromise the function of thyroid and immune function. Clinical studies are now needed to confirm if these effects, on selenium and oxidative stress, are augmented or diminished with longer periods of ECMO support, and to assess if normalisation of selenium through supplementation is beneficial.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Chapter 7

Chapter 7 The association between pre-operative selenium and post-operative atrial fibrillation after high-risk coronary artery surgery.

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Rationale

Atrial fibrillation after cardiac surgery is one of the most common complications and its impact on the patient involves quantifiable aspects (hemodynamic instability, stroke risk) as well as non-quantifiable aspects (stress). In addition, there is an economic burden on the health system as patients stay longer in hospital after surgery, or require ongoing medication. There is a reduction in POAF when prophylactic strategies such as ß-blockers, sotalol, amiodarone, magnesium, and atrial pacing are used. However, these strategies are not always successful or appropriate for everyone. In addition to certain recognized risk factors, evidence indicates that oxidative stress and inflammation are key aspects to the development of POAF, with both of these elevated during cardiac surgery involving CPB. This knowledge has led a relatively small number of studies to investigate whether prophylactic antioxidant supplementation reduces the incidence of POAF. There has been some success with vitamins C and E. Both of these antioxidants however, are classified as non-enzyme “chain breaking” antioxidants, meaning they stop lipid peroxidation after the process has started and some damage has already occurred. Primary enzyme antioxidants, such as GPx, have the ability to directly interact with and neutralize ROS before damage occurs, and therefore have a greater potential to maintain redox balance. As selenium is key to GPx activity, we theorized that selenium supplementation might be of significant prophylactic value in reducing the incidence of POAF. However, as many selenium supplementation studies in the critically ill have failed to actually show a clinical benefit, this study was designed to:

- determine if low pre-operative selenium levels increased the risk (or incidence) of POAF in the cardiac surgical population,
- determine if low pre-operative selenium levels increased post-operative ICU stay.

The results of this investigation would help determine if pre-operative selenium assessment and supplementation may be beneficial.

Significance

Several significant findings arose from this study. The first was that low pre-operative selenium levels were independently associated with POAF in patients who had a high surgical risk score. In these patients, mean pre-operative selenium levels were 0.73 ± 0.16 μmol/L, which was significantly lower than levels in those who did not develop POAF and had normal sinus rhythm (0.89 ± 0.13 μmol/L, $p = 0.005$). Half of the patients (53%) with a high surgical risk score had pre-operative selenium levels below the normal reference range.

Secondly, this study observed that patients with a pre-operative selenium level below 0.7 μmol/L had significantly longer ICU stay compared to patients whose pre-operative level was ≥ 0.8 μmol/L.

The results of this study indicates that pre-operative selenium level assessment in high-risk patients, and supplementing patients with levels ≤ 0.7 μmol/L may be beneficial. Whether supplementation can normalise selenium levels and lead to a reduced incidence of adverse complications after cardiac surgery (including POAF) remains to be investigated.

The association between pre-operative selenium and post-operative atrial fibrillation after high-risk coronary artery surgery.

Abstract

Objectives: The primary objective of this study was to investigate whether an association exists between low selenium levels and the development of post-operative atrial fibrillation (POAF) in cardiac surgical patients. POAF is one of the most frequent complications after cardiac surgery, with oxidative stress and reduced antioxidant function playing major roles in its development. Selenium is a key trace element with antioxidant properties and studies document significant reductions during cardiac surgery. Secondary aspects investigated were antioxidant function, oxidative stress levels and intensive care length of stay.

Methods: Fifty patients having primary coronary artery bypass (CABG) surgery with cardiopulmonary bypass (CPB) were divided into two groups based on their Society of Thoracic Surgeons Mortality risk (STS) score: (i) low-risk group with STS ≤ 0.5% (n = 26) or (ii) high-risk group with STS ≥ 2.0% (n = 24). Blood samples were taken at anaesthetic induction, after aortic cross clamp removal, 3hr post CPB and post-operative day 1 and 5. Samples were analysed for selenium, glutathione peroxidase (GPx) and malondialdehyde (MDA). Patients had ECG monitoring in the post-operative period. Bayesian multiple logistic regression analysis was used to estimate the independent association of selenium from pre-operative variables with possible associations to the development of POAF ($p < 0.05$).

Results: Seventeen patients developed POAF (14 patients in the high-risk group and 3 in the low-risk). Pre-operative selenium was lower in patients who developed POAF compared to those with normal sinus rhythm (0.73 ± 0.16 vs. 0.89 ± 0.13 μmol/L, $p = 0.005$) and this was independently associated with POAF in high-risk patients ($p = 0.016$). Selenium remained lower at all time points in those that developed POAF. Lower GPx activity and MDA levels were not independently associated with POAF. Independent of POAF, high-risk patients had lower pre-operative selenium than those with an STS ≤ 0.5% (0.77 ± 0.15 vs. 0.89 ± 0.14 μmol/L; $p = 0.004$).

Conclusion: High-risk patients with lower pre-operative selenium levels may be at greater risk of developing POAF following CABG. The association between selenium concentrations, operative risk and POAF demonstrated in this study raises questions of whether selenium supplementation in cardiac surgical patients may reduce their POAF risk.

Trial Registration: Australia and New Zealand Clinical Trials Registry: ACTRN12612000934842

Introduction:

Post-operative atrial fibrillation (POAF) is one of the most frequent complications after cardiac surgery with an incidence of 10-60%.[131,243] It significantly contributes to patient morbidity with direct translation to higher health care costs.[137,244] While the pathogenesis of POAF itself is still not clearly defined, currently accepted risk factors for the development of POAF include age, type of cardiac surgery, prolonged perioperative ischemia, chronic obstructive pulmonary disease (COPD) and postoperative pneumonia.[136,245] Current evidence suggests the development of POAF is multi-factorial involving a trigger and an electrophysiologic/structural substrate,[137,243,244] with inflammation and oxidative stress also contributing to this pathology.[7,8,243] Pharmacological prophylactic therapies and/or surgical correction for POAF are not appropriate for all patients. Current treatment options have variable efficacy and are associated with frequent and significant side effects.[244] The variability in current prevention regimens for POAF has lead to research investigating alternative or adjunctive strategies, including the use of antioxidants.[145] Antioxidants may be used as prophylactic and/or therapeutic agents to reduce levels of reactive oxygen species (ROS), which contribute to increased myocardial oxidative stress and subsequently POAF. However, while few studies have investigated the association between circulating antioxidants and POAF, reductions in antioxidant trace elements such as zinc and copper have been associated with a higher incidence of POAF.[246] Evidence is also emerging documenting that a reduction in POAF occurs after administration of the antioxidant vitamins C and E.[87,145]

The trace element selenium is an essential component of at least 25 selenoproteins, which play a role in the cellular redox processes, and thus has been considered as the cornerstone of antioxidant defence.[247] One of the key seleno-enzymes in the antioxidant process is the enzyme glutathione peroxidase (GPx), which can be used as a surrogate marker for both selenium and oxidative stress status.[247] This enzyme acts as a catalyst for the reduction of lipid hydroperoxides, thereby protecting cells and tissue from the deleterious effects of lipid peroxidation. Adequate levels of selenium are required for normal GPx activity, and maximal GPx activity occurs only when plasma selenium levels exceed 1.0 µmol/L.[248] In cardiac surgical patients reduced selenium levels and GPx activity are sometimes associated with worse outcomes.[14] Some studies have reported that a majority of cardiac surgical patients present to theatre with lower selenium levels,[249] and that low selenium levels may be inversely related to surgical risk.[14] A recent study has documented that pre-operative selenium levels are weakly associated with post-operative mortality, and

that patients who had chronic AF had lower selenium levels before surgery.[14] However, this study did not differentiate patients according to surgical risk, assess whether selenium levels were associated with the development of POAF, or investigate aspects of oxidative stress. Therefore we investigated if low pre-operative selenium levels in cardiac surgical patients are associated with either POAF or increased length of stay in the intensive care unit (ICU), and whether this association was stronger in patients with more pre-operative co-morbidities.

Materials and Methods

Patient Population

This was a prospective observational study of patients having primary CABG at The Prince Charles Hospital, Brisbane Australia. After Human Research Ethics Committee approval (HREC/11/QPCH/138) prospective patients were initially screened using the Society of Thoracic Surgeons predicted risk of mortality (STS) score.[250] To investigate the association between selenium levels, POAF and length of time in ICU in high-risk patients, fifty cardiac surgical patients having primary CABG surgery were recruited to the study and divided into two groups based on their STS score: (i) low-risk group with STS ≤ 0.5% (n = 26) or (ii) high-risk group with STS ≥ 2.0% (n = 24). Patients were excluded if they had a history of previous cardiac surgery, atrial fibrillation, permanent pacemaker or STS score 0.6 - 1.9%.

Anaesthesia protocol

All patients were anaesthetised according to institutional guidelines. Premedication included oral temazepam, ranitidine or metoclopramide. General anaesthetic induction was obtained with intravenous midazolam, fentanyl and propofol titrated to effect and endotracheal intubation was facilitated with intravenously administered rocuronium or pancuronium. Anaesthesia was maintained pre-surgery with intravenous propofol and inhalational sevoflurane. During CPB, and post-CPB, anaesthesia was maintained with propofol infusion and intermittent dosing of fentanyl. Standard cardiac anaesthetic monitoring consisting electrocardiogram (ECG), pulse oximetry and bispectral index monitoring, was used. Patients were systemically heparinised prior to aortic cannulation and activated clotting time (ACT) was confirmed to be > 480 seconds before commencing CPB. At the end of CPB, heparin was reversed with protamine sulphate at a 1:1 ratio of the loading dose.

Cardiopulmonary bypass protocol

A standardised CPB protocol was used and involved the use of a coated hollow fibre open reservoir oxygenator (Medtronic Fusion Oxygenator, “Balance” coated tubing. Medtronic Pty Ltd, Minneapolis, MN, USA), priming solution was crystalloid solution (P-148; Baxter

Healthcare Pty Ltd, Toongabbie, NSW, Australia), 100 IU heparin (Pfizer, Pty Ltd, Perth, Australia), and buffered with sodium bicarbonate (Pfizer, Pty Ltd, Perth, Australia). CPB flows were targeted to a cardiac index of 2.5 l/min/m^2, coupled with moderate hypothermia (32-34°C) and alpha-stat blood gas management. Myocardial arrest was achieved using an induction dose of 1 litre cold crystalloid cardioplegia (antegrade and retrograde) followed by subsequent 500 ml cold blood cardioplegia every 20 minutes. Patients were rewarmed to 36-37°C prior to separation from CPB.

Intensive Care Protocol

Protocolised post-operative care was provided. Where possible, patients were routinely fast-tracked for extubation and early mobilisation. Routine ICU monitoring comprised a continuous 5 lead ECG, invasive arterial blood pressure, central venous pressure, pulse oximetry and arterial blood gases as required. Hemodynamic management was guided by a combination of static and dynamic hemodynamic indices and involved optimisation of patient's heart rate and rhythm, judicious use of fluids (0.9% saline or 4% albumin) and the use of inotropes, inodilators and vasopressors as required to maintain a mean arterial pressure of > 65 mm Hg. Discharge from ICU followed unit protocols and the time ready for discharge was documented rather than actual discharge time to avoid inaccurate data recording associated with "bed-block" in the ward. As per unit protocol, patients were monitored by continuous electrocardiography until post-operative day 5 (or they were free of POAF). Patient demographics and relevant variables were collected for comparison.

Blood Sampling and analysis

Blood samples were collected from existing arterial lines at the following times:
1) anaesthetic induction (B), 2) after the aortic x-clamp was removed (XC), 3) 3 hours post-CPB, 4) post-operative day 1 (PD1) and 5) post-operative day 5 (PD5).
At each time point blood was collected into a trace free elements tube (Greiner Bio-one GmbH, Kremsmunster, Austria) and an EDTA tube, and processed within 30 min of collection. The separated plasma was stored at -80 °C until analysis. Plasma from trace free element tubes was analysed for selenium using graphite furnace atomic absorption spectrometry with Zeeman background correction (Varian AA280Z, Agilent Technologies Inc, Santa Clara, CA, USA). EDTA plasma was batch analysed for glutathione peroxidase (GPx) activity by colorimetric assay. Using this method the predominant variant of GPx measured is GPx-3. Malondialdehyde (MDA) by high performance liquid chromatography (HPLC) using Waters 2695 Separation Module with a Waters 2474 Fluorescence Detector (Waters Corporation-Micromass UK Ltd, Manchester. UK). These tests were conducted in a

National Association of Testing Authorities (NATA) accredited pathology laboratory. The reference ranges used by this laboratory for selenium and GPx are 0.7 – 1.4 µmol/L and 210 - 540 U/L respectively. A reference range for MDA has not been determined.

Statistical analysis

Continuous variables are presented as mean ± standard deviation **(**SD) unless otherwise stated. Categorical variables are presented as number and percentages. Differences in baseline characteristics between the normal sinus rhythm (NSR) and POAF groups were evaluated by the student t-test, Chi-squared or Fisher exact test, where appropriate. The independent association between significant pre-operative variables and the development of POAF identified in the analyses above was tested using a Bayesian multiple logistic regression model. A prevalence ratio (PR) and 95% confidence credible interval (95%cI) were reported [251]. Univariate linear regression and correlation analysis was used to analyse the association between selected continuous variables, and results reported as correlation coefficient (r) and 95% confidence interval (95%CI). Statistical analysis was carried out using Prism (version 6.0a) for the t-test, Chi-squared and Fischer exact tests as well as correlation and linear regression. WinBUGS software (version 1.4.3) was used for the Bayesian multiple logistic regression[252] in Table 2 and 3. A p value of < 0.05, by a two-sided test, was considered statistically significant.

Results

Patient Characteristics

Fifty patients were enrolled in the study with an average age 69.8 ± 10.8 years and a male dominance (39/50) (**Table 7-1**). Twenty-four patients were in the high-risk group (STS score 3.1 ± 0.97) and 26 in the low-risk group (STS score 0.37 ± 0.09).

In the high-risk group, 14 patients developed POAF (58%) versus three in the low-risk group (12%). Analysis of all patients found that patients who developed POAF were older ($p < 0.05$), had more co-morbidities ($p < 0.05$) and lower pre-operative ejection fraction ($p < 0.05$). Of the pre-operative biochemical variables creatinine, albumin, selenium and GPx were also lower in patients who developed POAF ($p < 0.05$). Patients who developed POAF had a higher sequential organ failure assessment (SOFA) score ($p < 0.05$) and tended to stay longer in ICU (104.5 ± 235.3 vs. 29.5 ± 13.2 hrs), though this extra stay was not statistically significant ($p = 0.07$). No differences between the POAF and NSR groups were noted for gender, body mass index (BMI), smoking history, CPB and X-clamp time or length of hospital stay ($P > 0.05$).

Table 7-1: Patient characteristics, baseline biochemical data, intra- and post-operative variables.

	NSR (n=33)	POAF (n=17)	P Value
Age (years ± SD) *	66.4±10.8	76.2±7.1	0.0014
Male gender [n (%)]	26 (79)	13 (77)	0.85
STS <0.5 [n (%)]	23 (78)	3 (12)	0.0001
STS >2.0 [n (%)] *	10 (42)	14 (58)	
BMI (kg/m^2)	28.4±4.9	29.5±5.0	0.47
Smoker [n (%)]	21 (64)	12 (71)	0.72
LVEF [n (%)]	58.1±11.3	45.3±16.6	0.002
DM [n (%)]	6 (18)	9 (53)	0.021
Previous MI			
STEMI [n (%)]	1 (3)	2 (11.7)	0.74
NSTEMI [n (%)]	20 (61)	15 (88)	
HTN [n (%)]	25 (76)	13 (76)	0.96
Pre-op Creatinine (µmol/L) *	93.1±22.0	175.0±205.3	0.035
Pre-op Albumin (g/L)	36.7±3.2	33.5±2.3	0.001
Pre-op Selenium (µmol/L) *	0.89±0.13	0.73±0.16	0.005
Pre-op GPx (U/L) *	328.8±73.2	268.9±83.8	0.012
Pre-op MDA (µmol/L)	0.105±0.03	0.104±0.03	0.923
CPB (min)	66.9±25.7	73.7±29.0	0.383
X-clamp (min)	48.3±21.0	54.6±23.8	0.334
ICU (hrs)	29.5±13.2	104.5±235.3	0.072
APACHE	53.2±12.9	60.2±14.7	0.088
SOFA	4.8±1.9	6.1±2.1	0.026
Hospital Stay (days)	11.2±7.7	13.9±12.6	0.36
Mortality (28d)	0/33	0/17	

[NSR–normal sinus rhythm; POAF–post operative atrial fibrillation; STS–Society of Thoracic Surgeons Mortality Risk score; BMI–body mass index; LVEF–left ventricular ejection fraction; DM– diabetes mellitus; MI– myocardial infarction; HTN–hypertension; GPx–glutathione peroxidase; MDA–malondialdehyde; CPB–cardiopulmonary bypass; ICU–intensive care unit; APACHE–Acute physiology and Chronic Health Evaluation ; SOFA–Sequential Organ Failure Assessment score.

[Asterisk indicates significant pre-operative variables used in the multivariate analysis.]

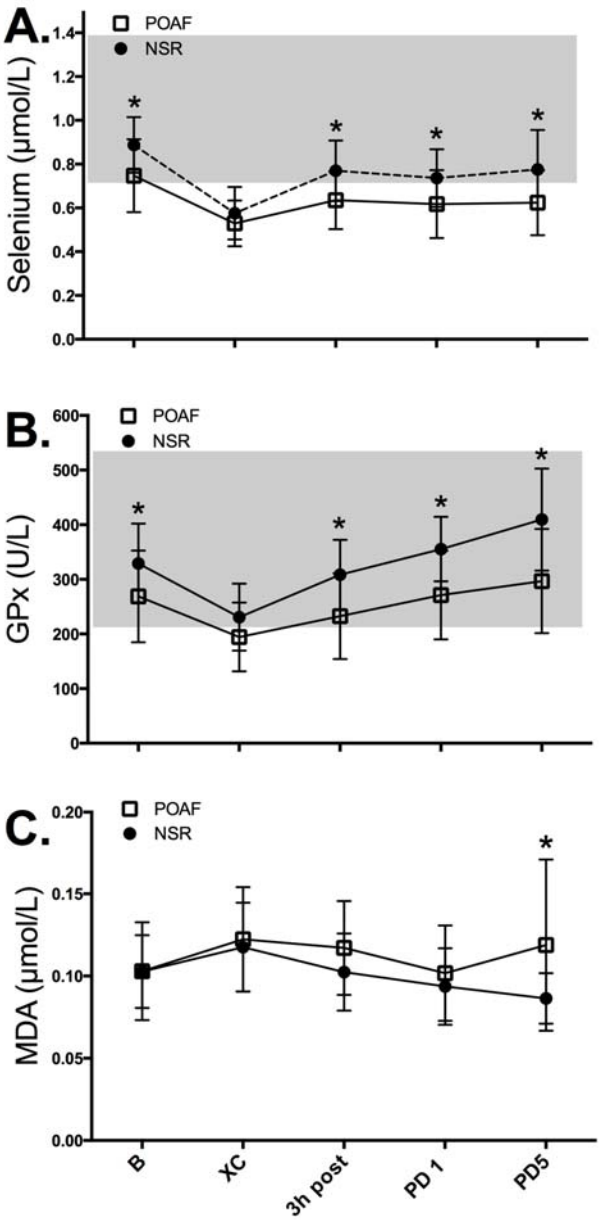

Figure 7-1: Serial changes in (A) selenium, (B) Glutathione peroxidase (GPx) and (C) Malondialdehyde (MDA).

B; pre-operative, XC; after aortic cross-clamp, PD; post-operative day 1 or 5. GPx; glutathione peroxidase, MDA; malondialdehyde. Shaded area represents common Australian reference range. Asterisk indicates significant difference between groups ($p < 0.05$).

Pre-, intra- and post-operative changes in plasma selenium.

Of the 50 patients, 34% (17/50) had pre-operative selenium levels below the reference range of 0.7 – 1.4 µmol/L used in our chemical pathology laboratory. Patients who developed POAF had lower pre-operative selenium levels compared to those in the NSR group (**Table 7-1**; $p < 0.005$). Of the POAF patients 53% (9/17) had selenium levels ≤ 0.7 µmol/L, while only 24% (8/33) of NSR patients were ≤ 0.7 µmol/L. Both POAF and NSR patients demonstrated a decrease in selenium levels at cross clamp, with levels recovering during and after surgery (**Figure 7-1**A). However, patients who developed POAF still had significantly lower selenium levels than NSR patients at 3hrs, PD 1 and 5 ($p < 0.05$). Furthermore, mean selenium levels of patients who developed POAF remained below the reference range from XC onwards.

High-risk patients with an STS ≥ 2.0% had lower pre-operative selenium than low-risk patients (STS ≤ 0.5%) (0.77 ± 0.15 vs. 0.89 ± 0.14 µmol/L; $p = 0.004$). Selenium levels in the high-risk patients stayed significantly below low-risk patients 3 hr after CPB and at PD1 and 5 ($p < 0.01$).

There was a positive correlation between selenium level and GPx activity (**Figure 7-2**A. $r = 0.55$; 95%CI 0.46 to 0.63, $p < 0.0001$) and a weak but significant negative correlation between selenium and age (**Figure 7-2**B, $r = -0.28$; 95%CI -0.52 to -0.03, $p < 0.048$) and selenium and MDA levels (**Figure 7-2**C. $r = -0.3$; 95%CI -0.41 to -0.18, $p < 0.0001$).

To determine if there was an independent association between selenium and the development of POAF the significant pre-operative variables from **Table 7-1**, (indicated by asterisk), were analysed by Bayesian multiple logistic regression. Pre-operative selenium was not associated with POAF when comparing both low and high-risk patients as a single group (**Table 7-2**; PR 0.77, 95%cI 0.37 to 1.35, p = 0.32). However, when analysing data from the high-risk group (n = 24), there was a statistically significant association with selenium and the development of POAF (**Table 7-3**; PR 0.32; 95%cI 0.06 to 0.85, p = 0.016). Comparing pre-operative selenium from all patients revealed that selenium was not associated with an increase length of stay in ICU. However, there was a statistically significant association with increased length of stay in ICU with low pre-operative selenium levels (threshold of ≤ 0.7 μmol/L) irrespective of the presence of POAF (r = -0.56; CI -0.82 to -0.11, p = 0.02). This association was not an independent predictor of ICU stay when other variables of pre-operative creatinine and define LVEF were considered (p > 0.05).

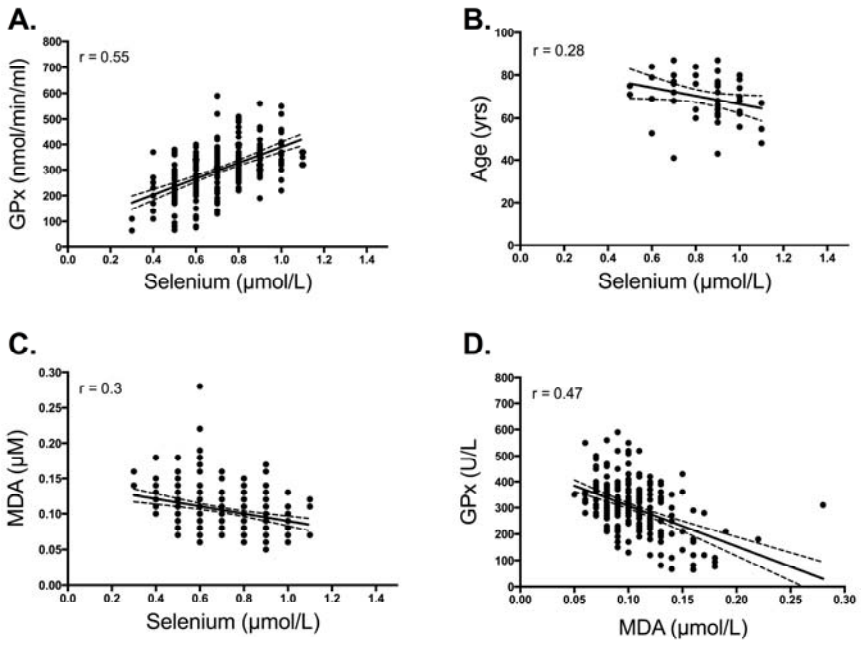

Figure 7-2: Correlations between (A) selenium and GPx, (B) pre-operative selenium and age, (C) selenium and MDA and (D) GPx and MDA.

Pre-, Intra and post-operative changes in plasma glutathione peroxidase

Pre-operative activity of the antioxidant GPx was significantly lower in the POAF group (**Table 7-1**; p < 0.05). GPx activity decreased similarly in patients with POAF and NSR after X-clamp removal and then recovered post-operatively at 3 hr, PD 1 and PD 5 (**Figure 7-1**B). GPx activity remained within the reference range throughout recovery; however, patients

who developed POAF continued to have significantly lower GPx activity than the NSR group at all time points ($P < 0.05$).

Table 7-2: Variables associated with POAF in low (n = 26) and high-risk (n = 24) patients.

Variable	Prevalence ratio	95% credible Interval	P
Risk (STS)	2.94	0.11 - 16.36	0.899
Age	4.86	0.95 - 19.26	0.072
EF (%)	0.54	0.23 - 0.94	0.029
Pre-op selenium	0.77	0.37 - 1.35	0.321
Pre-op creatinine	4.45	0.30 - 21.92	0.414
Pre-operative GPx	0.47	0.12 - 1.10	0.081

Table 7-3: Variables associated with POAF in high-risk patients (n = 24). Estimates from multiple logistic regression.

Variable	Prevalence ratio	95% credible interval	P
Age	26.22	0.61 - 160.9	0.182
EF (%)	0.40	0.10 - 0.91	0.013
Pre-op selenium	0.32	0.06 - 0.85	0.016
Pre-op creatinine	78.36	0.28 - 304.5	0.266
Pre-operative GPx	1.33	0.10 - 5.43	0.846

Pre-, Intra and post-operative changes in plasma MDA

Levels of MDA, (a by-product of lipid peroxidation), were similar in patients with POAF and NSR at baseline (**Table 7-1**). MDA levels increased in both groups after the X-clamp was removed, but this was not different between the two groups or significantly above baseline. At PD 5 MDA levels were significantly higher in patients with POAF (**Figure 7-1**C $p < 0.05$). There was a significant negative relationship between MDA levels and GPx activity (**Figure**

7-2D: r = -0.47; 95%CI -0.56 to -0.37, p < 0.0001), such that as GPx activity increased, MDA levels decreased.

Discussion

POAF is a common complication after cardiac surgery and the overall incidence of POAF in this present study (34%) was similar to other published studies with similar patients risk profiles (10 - 60%).[131,243] POAF usually presents within the first few days following surgery and by PD 5 the incidence of new onset POAF has dropped to around 2%.[243] Equally POAF has often resolved within this time period, suggesting a reversible nature to the condition. Cardiac surgery with CPB is known to induce an oxidative stress response, [4] as well as to decrease selenium levels.[13] Thus it is plausible that these two responses may be linked. In addition to intraoperative decreases in selenium, other studies have shown that cardiac surgical patients often present for surgery with lower than normal selenium levels.[29] Lower selenium levels are also predictive of post-operative complications and mortality. A recent study has serendipitously recorded lower pre-operative selenium levels in cardiac patients with chronic AF;[14] however, no studies have thus far investigated what association exists between low pre-operative selenium and the development of POAF.

Data from this study found that CABG patients who developed POAF had significantly lower pre-operative selenium levels than those with NSR; furthermore, their post-operative selenium levels remained significantly lower. Patients assessed as high-risk (STS ≥ 2.0%) also recorded lower pre-operative selenium levels and demonstrated a higher incidence of POAF than their low-risk counterparts (STS ≤ 0.5%). Analysis confirms that preoperative selenium levels of ≤ 0.7 μmol/L are associated with an increased length of ICU stay (R^2 = 0.31; CI95% -0.82 to -0.11, p = 0.0196), but the association is not present at levels ≥ 0.8 μmol/L. Thus a pre-operative plasma selenium concentration of ≤ 0.7 μmol/L appears to be a useful threshold for assessing POAF risk.

As has been previously reported, this study also found a linear relationship between selenium levels and antioxidant GPx activity.[173] However, our results unexpectedly determined that lower GPx activity and increased oxidative stress were not independently associated with POAF. It is plausible that with a larger sample size an association between GPx and POAF may have been detected. The sensitivity of GPx measurement may have been enhanced with measurements of intracellular levels of GPx (i.e. GPx-1) instead of only GPx-3 (the predominant plasma variant of GPx). In addition, as nearly half of the identified selenoproteins in humans have antioxidant activity,[81] the possibility remains that

selenoproteins other than plasma GPx may have been critically altered during the operative and ischemia-reperfusion period.

Oxidative stress is the imbalance between the production of reactive oxygen species and the ability of antioxidants to maintain redox balance. There is compelling evidence to suggest that oxidative stress and inflammation are key components to the development of POAF.[7,8] Increases in myocardial oxidation and systemic serum peroxides have been demonstrated in cardiac surgical patients with POAF compared to those with NSR.[1] In our study we demonstrated a small but significant increase in plasma MDA over the study period in those with POAF. While we recorded associations between MDA, selenium and GPx (**Figure 7-2**), we could show no direct association between MDA and POAF. It is possible that the measurement of plasma MDA levels may not have been specific enough to measure local changes that were present in the myocardium. Ramlawi *et al* demonstrated the value in assessing oxidative stress in myocardial tissue,[1] however, we did not have access to right atrial tissue in this study. In this study we measured lipid peroxidation via MDA but did not measure oxidative stress damage to proteins and DNA, which may have given additional supportive evidence of the relationship between selenium and POAF.

Significant reductions of the antioxidant trace element selenium occur during cardiac surgery, and these have been associated with various adverse outcomes.[14,29] Given the link between oxidative stress and POAF it is plausible that reinforcement of the antioxidant response might reduce myocardial oxidative stress, and consequently reduce susceptibility to POAF. Antioxidants investigated thus far are vitamin C and E and N-acetylcysteine (NAC), yet few studies have shown that the use of antioxidants is associated with a reduced incidence of POAF, and the results are variable in those that have. A recent meta-analysis and systematic review examined 23 studies and determined that antioxidants such as vitamin C have significant capacity to reduce POAF.[145] To date, the association between selenium and the development of POAF has not been investigated. Our results show that low pre-operative selenium levels and high STS scores are associated with post-operative POAF. This suggests that correction of low selenium levels prior to cardiac surgery may lessen the risk of development of POAF.

Selenium supplementation in cardiac surgical patients may provide many antioxidant benefits, which ultimately result in an attenuated oxidative stress response. Presently there is only one study that has investigated the use of selenium supplementation (as a monotherapy) in cardiac surgical patients.[25] One hundred low-risk cardiac surgical patients received a 2000μg dose of selenium followed by 1000 μg I.V. boluses per ICU day and

were compared with matched historical controls. The pre-operative selenium levels of 75% of their patients were below the normal German reference range (127 - 178 μmol/L). Although intravenous selenium was given to raise selenium to normal levels there was no improvement in clinical outcome.[25] The authors suggest that the administration of intravenous selenium just prior to CPB may have been too late to have a protective effect. POAF was not documented in that study.

Our study found that low pre-operative selenium levels of ≤ 0.7 μmol/L is associated with increased length of ICU stay, and when combined with a high STS score, is associated with a higher risk of developing POAF. This data suggests that pre-operative assessment of selenium levels coupled with pre-operative STS risk assessment may identify patients susceptible to POAF who may benefit from selenium supplementation.

Limitations in this study are as follows. Age is an important risk factor for POAF independent of surgery,[131] and is also associated with a decline in selenium.[253] In our study patients who developed POAF were older than those with NSR and we demonstrated a small association between age and declining pre-operative selenium levels. Additionally, even in the absence of co-morbidities, a patient with uncomplicated three vessel disease would achieve an STS score > 0.5% simply because they were older than 73 (male) or 68 (female). Therefore our patient groups were biased towards older males. It may be no coincidence that age is the predominant risk factor for POAF and that age is also related to a reduction in selenium. The factors of age and sex on selenium and POAF warrant further investigation. Lastly, the study population only represented patients undergoing CABG and whether the findings may be extrapolated to patients undergoing valve procedures and combined valve and graft procedures remains uncertain. Despite these limitations we believe that the data collected during this study will inform future prospective studies investigating the role of selenium in POAF vulnerability and whether selenium might be a reasonable prophylactic antioxidant supplement in select cardiac surgical patients as an additional adjunct to POAF prevention.

Conclusion

High-risk patients with low pre-operative selenium levels may be at greater risk of developing POAF following CABG. Patients with selenium below 0.7 μmol/L may also have an increased ICU length of stay, a finding that needs further confirmation in future studies.

The assessment of selenium levels pre-operatively in the cardiac patient is not part of current practice, nor is the routine normalisation of marginal or low selenium levels. The association between selenium concentrations, operative risk and POAF demonstrated in

this study suggest that there may be value in pre-operative selenium screening to help guide supplementation studies investigating whether selenium normalisation reduces the incidence of adverse complications after cardiac surgery, including POAF.

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Chapter 8

General Discussion and Conclusion

Chapter 8 : General Discussion and Conclusion

There were four main hypotheses to this thesis, which examined key aspects of selenium and oxidative stress modulation during extracorporeal circulation. The main findings were that low pre-operative selenium levels in high-risk patients having cardiac surgery were independently associated with POAF. Pre-operative selenium levels below 0.7 μmol/L were also predictive of a longer ICU stay irrespective of surgical risk or the development of POAF. As a similar threshold has been associated with SIRS,[5] it is plausible that selenium levels below 0.7 μmol/L may prove to be a useful threshold in future Australian clinical studies investigating the benefits of selenium supplementation.

The ovine ECMO model determined that in the first 24 hours, both lung injury and ECMO independently increased oxidative stress, but when conducted sequentially i.e. lung injury followed by ECMO, there was no additional impact on oxidative stress. Lung injury caused significant reductions in plasma selenium and subsequent ECMO support exacerbated this loss.

In 2010 there was scant literature describing the influence of selenium reductions on outcomes in cardiac surgery and critical illness. Despite subsequent studies reporting low selenium levels during cardiac surgery and critical illness, there is still a paucity of data regarding clinical relevance of these reductions.[5,13,14,26,29,254,255] To better understand and interpret the data that existed in 2010 and subsequently the data of 2015, several demographic, animal and laboratory studies were required to define selenium levels of Queensland blood donors, and investigate the effects of PRBC transfusion and the relevance of the artificial ECC circuit on selenium and oxidative stress levels.

Due to significant global variation in selenium there is no universal reference range.[248] For example in Finland the mean plasma levels is 0.53 μmol/L, while in Pakistan it is 2.72 μmol/L (**Table 1-1**).[17] Furthermore, even within some countries (such as Pakistan) there are huge local variations (range 0.38 – 7.37 μmol/L).[17] There are also considerable differences across the various regions and states of Australia[164-170] (**Table 3-2**). These variations are largely due to variations in soil selenium levels and ultimately the availability of selenium in foods.[16] Large variations across various populations make it difficult to interpret the literature reported effect of low levels on clinical outcome, as the selenium level in the relevant local population is required to provide context.

In the cardiac surgical and critically ill populations there appears to be significant variability between reported selenium levels and their clinical consequences. For example, in 1990 Antilla *et al* were the first to demonstrate reductions in selenium in Finnish adult cardiac surgical patients undergoing CPB. Levels fell from pre-operative levels of 1.35 μmol/L to 1.09 μmol/L at POD 4, though they did not record outcomes.[254] Similarly, in a U.K. paediatric cardiac surgery population selenium levels were reduced from 0.61 μmol/L to 0.51 μmol/L 48 hrs after surgery.[26] Despite these reductions in selenium, no reduction in GPx activity was recorded. Lower selenium levels were nevertheless associated with increased ICU stay and low circulating levels of the thyroid hormone triiodothyronine (T3). More recently Stoppe *et al* recorded reductions in selenium from pre-surgical levels of 1.13 to 0.9 μmol/L after surgery in German cardiac patients and determined that low levels were independently associated with, and predictive for, the development of MOF.[13] In a 2012 study of 197 Hungarian cardiac patients Koszta *et al* determined selenium levels after cardiac surgery in survivors were significantly higher (1.41 μmol/L) compared to non-survivors (1.30 μmol/L). They had previously determined the healthy level in the Hungarian population to be 1.57 μmol/L.[14] Finally in a German study comparing off-pump and on-pump cardiac surgical patients, Stevanovic *et al* determined that a post-operative selenium level of 0.55 μmol/L in on-pump patients was associated with a decrease in GPx activity as well as being associated with the development of various complications.[29] These few studies reveal the difficulty in interpreting the clinical relevance of selenium reductions from countries that have significantly different selenium levels in their healthy populations.

The data shows that a selenium level may be associated with adverse outcomes in one population but not with another. For example, the selenium level of the non-survivors in Hungarian cardiac patients (1.30 μmol/L)[14] is already higher than the pre-operative levels in German study of similar patient population (1.13 μmol/L) who clearly were alive prior to surgery.[13] While the mean post-operative level associated with MOF in the same German study (0.9 μmol/L)[13] is significantly higher than the pre-surgical levels in the study investigating thyroid dysfunction in a paediatric cardiac surgical population (0.61 μmol/L); who also did not present to surgery with MOF or signs of thyroid dysfunction. The data available suggests that the measured level of selenium associated with adverse outcomes appears less relevant than the percentage change in selenium from pre-surgical or background healthy levels, hence the importance of understanding the selenium level in the local population. For the Australian cardiac surgical population, our data seems to suggest a level lower than 0.7 μmol/L as the level associated with increased ICU stay and (in general) a higher incidence of POAF.

In the heterogenous population of the critically ill, low selenium levels have also been associated with increased rates of infection, increased ICU stay and increased morbidity.[5,128] The effects of critical illness on selenium levels were first reported in an Australian study in 1990. Hawker *et al* documented low levels of selenium (0.49 μmol/L) in 175 patients who had been in ICU for 4 weeks, noting that this was considerably lower than that of healthy volunteers where the level was 1.05 μmol/L.[255] While these levels are truly low compared to the Australian reference range, they were not correlated with outcome. Forceville *et al* examined 134 French ICU patients and determined that mean selenium levels were lower at admission than the healthy reference mean, and that patients with SIRS had significantly lower levels than patients without SIRS (0.62 vs. 0.83 μmol/L).[5] They also determined that selenium levels lower than 0.7 μmol/L were associated with more complications and greater mortality.[5] Another study of German ICU patients examined the time-course changes of selenium levels, looking for associations with post-operative outcome. Nearly all patients had selenium levels below the standard mean healthy value of 0.94 μmol/L. Patients with SIRS and sepsis had the lowest selenium levels, which correlated with APACHE and SOFA scores. A selenium level lower than 0.46 μmol/L was also predictive of mortality.[153] These variations in selenium level and associated adverse outcomes mirror the cardiac surgical population. Unless clinicians can contextualize those levels with respect to the normal healthy range of the local population, understanding the relevance of certain selenium levels being associated with a reported adverse outcome is difficult.

Objective 1: Define the Queensland Selenium level

In **Chapter 3** the selenium level in a healthy Queensland population was measured in order to understand where Queensland levels were situated compared to the global setting. The results of this study provided several points of interest that proved useful for other sections of this thesis. The average plasma selenium level across the three Queensland regions studied was 1.09 μmol/L [85.6 μg/L] (SD 12.9; range 0.7 – 2.21 μmol/L).[158] This was considerably lower than previously reported levels for Australia,[164-170] (**Table 3-2**) with the majority of patients having a level below the minimum level of 1.14 – 1.27 μmol/L required for maximal GPx activity.[171,173] An additional finding from our study was that cellular components i.e. PRBC and pooled buffy coat derived platelets, had very low selenium levels.

This newly defined selenium level for healthy Queensland blood donors sits in the middle of reported global levels, (**Table 1-1**)[17] being similar to Germany,[29,13] and potentially giving the findings of studies from there more relevance to the results observed in our studies.

Hypothesis 1: CPB circuits will adsorb trace elements and increase oxidative stress.

As intra-operative reductions in various trace elements have been demonstrated to occur during CPB,[26,184,246,256,257] we postulated that adsorption to the CPB circuit (either directly or via carrier proteins) may explain part or all of these reductions. In **Chapter 4** an *in vitro* CPB model was used to test this hypothesis. CPB circuits were primed with fresh whole blood, and significant reductions in selenium, zinc and copper were measured.[47] These three trace elements are required by antioxidant enzymes.[183,184,185] Given the closeness of CPB and ECMO circuits in design and materials, a similar reduction might be expected to occur during ECMO, although this has not yet been demonstrated in any study. Modern CPB and ECMO circuits are coated with artificial substances, such as heparin, heparin substitutes and phosphorylcholine derivatives[258,259] that attempt to mimic the surface of the endothelium, reducing protein adsorption and improving the biocompatibility of the artificial surface. Despite this there is still evidence of an immediate adherence of plasma proteins [28] and activation of the inflammatory and coagulation systems after blood contact with the surface of the ECC.[27,260] Additionally the tubing and fibres of ECC have been shown to adsorb an assortment of drugs.[261-263]

What is unclear from our current study and that of others is whether these trace elements return to circulation after some period of time, or remain bound indefinitely. In cardiac surgical patients, where the time spent exposed to the CPB circuit is short, the selenium bound to the circuit during CPB will likely be lost from the patient after surgery is complete. However, as ECMO typically lasts several days to weeks, it is plausible that selenium (and other trace elements) may only be bound to the circuit temporarily as the surface environment of the ECMO circuit changes and pacifies over time.[27] It was demonstrated that coating the circuit with albumin, which preferentially binds to the tubing surface in competition with fibrinogen,[27] provided no protection in reducing trace element loss. Undoubtedly modern coated CPB and ECMO circuits are an improvement to older generation circuits (which had no coating), with respect to inflammation and platelet activation. However, our results raise the question of whether preloading the circuits with key trace elements might be clinically beneficial from this respect.

Hypothesis 2: Transfusion of blood products is associated with a reduction in selenium and an increase in oxidative stress.

We had previously demonstrated that PRBC contains very little extracellular selenium[158] and postulated that the transfusion of significant volumes of PRBC would have a dilution effect on the selenium levels of the recipient, and thereby compromise the antioxidant response leading to an increase in oxidative stress. We investigated this hypothesis using an ovine model (**Chapter 5**) and confirmed a reduction in selenium and an increase in lipid peroxidation levels after only two units PRBC for both fresh and aged products.[241] This effect has previously been reported in paediatric patients.[56,57,199]

By using a healthy animal model our data confirms that PRBC transfusion dilutes the recipients circulating selenium levels and compromises antioxidant capacity. Previously the transfusion of as little as 1-2 units of PRBC has been associated with adverse outcomes in cardiac patients.[198] Prior to 2015 a number of reports suggested that aged PRBC was associated with worse outcomes compared to fresh PRBC.[200,264,265] This year (2015) results from a multi-centered study of 1098 cardiac patients found that fresh PRBC transfusion was not associated with less MOF than aged PRBC transfusion.[266] Another multi-centered study of 2430 critically ill patients also reported that fresh PRBC transfusion was not superior to standard issue PRBC units in reducing 90-day mortality.[267] Similar to our findings with the ovine model, these studies show that aged PRBC transfusion is not associated with more adverse outcomes. Both studies did still document significant adverse outcomes associated with transfusion in general, however, there is little consensus on the mechanisms behind these adverse outcomes. Further investigation is needed to determine if reduced selenium levels and increases in oxidative stress (transfusion related oxidative stress (TROS)) may contribute to adverse outcomes after transfusion, especially in cases of massive transfusion (> 1 blood volume in 24 hours or > 50% of blood volume in 4 hours[268]). Other adverse aspects of transfusion such as TRIM (transfusion related immuno-modulation), TRALI (transfusion related acute lung injury) and TACO (transfusion associated circulatory overload),[269] which were unrecognized complications a decade ago, are now recognized to be part of the adverse outcomes associated with transfusion. Only well designed studies in select patient populations will be able to determine the relevance of our results from the ovine model.

Hypothesis 3: ECMO therapy is associated with significant selenium loss that contributes to increased oxidative stress.

To test the hypothesis that ECMO therapy is associated with significant selenium loss and increased oxidative stress, an ovine model of lung injury and ECMO was used to investigate the separate and combined effects of each intervention (**Chapter 6**). We discovered that similar to CPB, the introduction of ECMO support in a healthy animal resulted in reductions in selenium and increases in oxidative stress. When ECMO therapy followed acute lung injury, there was an additive effect of ALI and ECMO on selenium reductions but no additional effect on oxidative stress levels.[270]

ECMO is used in critically ill patients who are in refractory respiratory and/or cardiac failure that has not responded to conventional medical intervention.[146,218] While the absolute benefit of ECMO in critical illness with refractory cardiorespiratory failure is yet to be determined, radiographic evidence suggests that VV-ECMO may temporarily exacerbate lung injury (**Figure 2-4**). In our study we postulated that the additional oxidative stress response stimulated by the ECMO circuit was a contributor to this apparent exacerbation. Our results did not find this, and this may be because only systemic levels of lipid peroxidation were measured, and unfortunately, we did not have the ability to directly sample or test pulmonary tissue. In future investigations regarding early and late deleterious effects of ECMO therapy (includes ECMO, dialysis and transfusion aspects) additional markers of lipid and protein oxidation (such as isoprostanes or protein carbonyls), may reveal additional information about this response.

The selenium reductions noted in the first 24 hours of this animal study are concerning, as significant selenium reductions in critically ill patients are associated with worse outcomes, independent of oxidative stress. It would be worthwhile to determine if similar reductions occur in patients, and to consider selenium supplementation to correct this depletion. Such studies would need to consider the metabolic requirements of critically ill patients on ECMO as it has been suggested that these patients may be receiving as little as 50% of their nutritional requirements.[271] While large doses of selenium (well in excess of recommended daily allowances [161]) seem well tolerated in the critically ill patient,[128,272,273] it is unclear whether such doses would be tolerated in the ECMO patient over the long term.

The results of our study have provided considerable additional information regarding the isolated and combined effects of acute illness and ECMO on oxidative stress and selenium levels. A small number of animal studies were conducted in the late 1990's and documented

increases in lipid peroxidation; however, these studies utilized circuits that had little clinical relevance to the modern ECMO circuit.[67,68,150] A clinical study investigated oxidative stress in paediatric patients on ECMO and also noted increases in lipid peroxides, but that study was also conducted in 1990 and also is of little clinical relevance to modern ECMO.[69] In 2014 a porcine study demonstrated significant increases in lipid peroxides in blood and tissue after initiating ECMO using current modern circuits.[149] However, only one of these studies investigated the combined aspects of injury and ECMO[150] whereas the other studies used healthy animals. Our results suggest the short term effect of ECMO and transfusion is not additive in the first 24 hours. What subsequently happens, as time on ECMO increases in these patients is, however, unknown. Clinical studies are needed to determine the effects of illness, ECMO, dialysis and multiple transfusions over the entire hospital stay and investigate aspects of oxidative stress, inflammation and immunity against clinical outcomes such as infection, MOF and mortality.

Hypothesis 4: High-risk cardiac surgery patient with low pre-operative selenium levels in are more likely to develop POAF.

POAF is the most common complication after cardiac surgery, increasing operative mortality, stroke risk, length of stay in ICU and requires more healthcare dollars to treat.[243,274] Understanding the mechanisms behind its development and developing protocols to reduce its incidence would not only improve patient outcomes, but also produce a more cost-effective healthcare service. The development of POAF is multi-factorial[274] but includes oxidative stress.[275] As normal selenium levels are required for appropriate antioxidant function to combat the oxidative stress response, it was hypothesized that patients with low pre-operative selenium levels were more likely to develop POAF. This hypothesis was tested in a group of twenty-six low-risk and twenty-four high-risk patients having primary CABG (**Chapter 7**). Similar to previous studies in cardiac surgical patients,[13,14] we also determined that more than half of patients with an STS score ≥ 2.0 had selenium levels below the Australian reference range. Previously a study of 197 consecutive cardiac surgical patients had documented lower pre-operative selenium levels in patients with chronic AF,[14] however no studies had investigated if an association between pre-operative selenium levels and POAF in cardiac surgical patients existed. We discovered that patients with an STS score ≥ 2.0 (i.e. high-risk group) had multiple co-morbidities and risk factors for POAF. In these patients pre-operative selenium was independently associated with POAF. We also determined that patients stayed longer in ICU when

selenium was ≤ 0.7 μmol/L compared to those with a level ≥ 0.8 μmol/L (irrespective of STS score or POAF). While such a threshold may not be applicable globally (due to variability in selenium levels), at least one other study has identified the same threshold in a critically ill population. In the French study, organ failure and mortality was three-fold higher in those with selenium ≤ 0.7 μmol/L.[5] We speculate that this threshold, at least in the Australian population, may be key to future studies attempting to determine the benefits of selenium supplementation in surgical and critically ill populations.

As described earlier, cardiac surgery with the use of CPB is associated with reductions in selenium that are associated with adverse outcomes such as MOF and increased length of stay in ICU.[13,29] Concurrently there are also increases in oxidative stress,[175] although these alterations have largely been identified in separate studies to that of selenium investigations. That oxidative stress and inflammation are important components to the development of POAF, and the realization that many cardiac patients present to surgery with suboptimal selenium levels, lends itself to the hypothesis that low pre-operative selenium levels may increase the risk of POAF in those patients who had other risk factors favoring the development of POAF such as proposed in **Figure 2-6**. In our study GPx activity was lower in patients who developed POAF over the 5-day study period (and presumably this was related to the measured selenium reductions); however, our study did not measure differences in oxidative stress (as measured by plasma MDA) between those with POAF and those with NSR. It is plausible that measuring MDA, isoprostanes or protein carbonyls directly from atrial tissue may have revealed a different result. Nevertheless, our results appear to be the ideal segue to the next stage of investigations into the benefit of selenium supplementation in the cardiac patient.

Limitations

Each of the studies that constitute this thesis had their limitations, most of which have been acknowledged within the discussion of each chapter publication. This section discusses limitations that could not be included in the manuscripts due to word limits.

In attempting to define a selenium level for the Queensland population we chose three broad regions across the state. However, within these regions there was only one ARCBS collection centre. So in addition to our choice of geographic regions not being representative of the whole state it is also plausible that with only a single collection centre our patients

were not representative of that region, some of which are quite large and possibly remote. Equally, we did not try to discern if there were selenium differences between indigenous and non-indigenous Australians. Indigenous Australians suffer from high rates of diabetes and cardiovascular disease,[276] both of which have been shown to influence selenium levels. While no indigenous patients were recruited in this study, understanding selenium levels in this sub-population may be highly relevant to future cardiac surgical studies.

Throughout this study we applied TBARS and MDA as our measures of oxidative stress. While these are certainly valid, and popular, measures of lipid peroxidation within the literature, they are prone to error.[36] As a colorimetric assay, the measurement of TBARS is easy to perform for a skilled operator. In one of the first studies of this thesis (**Chapter 4**) there was a steep learning curve to this analysis that resulted in a series of erroneous measurements. The limitation can be attributed to the inexperience of the operator (this candidate). As a result, some circuit experiments had to be repeated, and extra assay kits purchased. In one of the first ovine studies (**Chapter 5**) the decision was made to move to a different brand of TBARS assay that utilized a significantly smaller volume of blood (R&D Systems Inc, Minneapolis, MN. USA). However, we were unable to achieve reproducible results with ovine blood and the assays were all repeated using the Cayman TBARS assay. This experience highlights the potential variability in results achieved between different manufacturers even when assessing the same metabolites. A disadvantage of the TBARS colorimetric assay is that it can measure aldehydes other than MDA, and degrading fatty acids within a sample can influence the amount of MDA measured.[36] Drawing on this knowledge and the earlier experiences with the TBARS assay we funded the state pathology service (Queensland Health Pathology Service) to setup and validate a HPLC column specifically for the analysis of MDA. The HPLC method allows the separation of the MDA-TBA adduct from interfering chromophores, producing results which are more specific for MDA.[36] In addition, our studies may have been improved by incorporating additional measures of lipid, and protein, such as F_2-isoprostanes and protein carbonyls.

One of the more significant limitations of this thesis occurred in the ovine ECMO study and these have been touched on briefly in **Chapter 6**. ECMO typically lasts several days to several weeks, during which time there will be changes in the proteins and cells adhering to the ECMO circuit.[27] Some of these proteins (e.g. albumin, selenoprotein P) are thought to be transport proteins for selenium,[277] and certainly for albumin, are quickly deposited on the ECMO circuit surface upon first contact with blood. However, beyond 24 hrs there is some evidence that these proteins are no longer deposited on the circuit surface and blood levels

will remain stable.[27] Equally, as the population of inflammatory cells adhering to the circuit surface changes over time this will ultimately change the level of ROS generation and the chronic redox status of the patients. As our study was only 24 hrs in duration we are unable to comment on the effects of ECMO on selenium and redox status in the long term. While the practicalities of running a longer animal model of ECMO were beyond the abilities of this research institution, this has to be recognized as a significant limitation of the study with respect to the clinically relevant conclusions that could be drawn. Despite the limitations in this study, the results do support conducting a similar clinical study over the full duration of ECMO and even into the rehabilitation period. Only in this manner will we gain a better understanding as to whether selenium levels (and redox status) are altered in a negative manner, or if they wax and wane with various interventions such as transfusion and dialysis, and if opportunistic infections occur only when selenium levels are at their lowest.

The final limitation, which applies to the studies in chapter 5 and 6 is the use of human albumin as volume replacement in the ovine studies. There is no data comparing the use of human albumin in ovine studies and its potential effect on the inflammatory or oxidative stress response. In studies comparing human and bovine albumin the physical properties appear to be indistinguishable, yet despite this similarity there are differences regarding electrophoretic behaviour, thermal and chemical stability and some binding properties.[278] Equally albumin has been shown to have pro-inflammatory effects *in vitro* as well as immunomodulatory properties.[279] The possibility that human albumin transfusion in an ovine model causes an inflammatory response (and therefore influencing oxidative stress) cannot be excluded and presents itself as a minor limitation in the studies of chapter 5 and 6.

Considerations and Future Directions

While the results from the collective studies of this thesis are clinically interesting, the challenge is in translating them into clinical practice. Currently selenium is not a routine test in the cardiac surgical or intensive care setting. The knowledge that low pre-operative selenium levels before cardiac surgery might increase risk of POAF or result in a longer ICU stay suggests that high-risk cardiac surgical patients may benefit from earlier screening of selenium levels. Hence, one of the post-PhD aims is to conduct a randomized controlled trial (n = 200) to investigate the potential benefits of high-dose selenium supplementation in patients with (pre-operatively) selenium levels ≤ 0.7 µmol/L. High doses of selenium have

been determined to be safe in this patient population[25], but it remains to be confirmed if such high dose selenium supplementation might be associated with reductions in POAF.

Despite the postulated benefits of selenium supplementation in the cardiac surgical and critically ill patient, the reported benefits of selenium as a monotherapy are variable,[80,280] (despite documented improvement in GPx activity). The latest Cochrane review in 2008 regarding selenium supplementation in the critically ill noted that "no clear evidence emerged for ventilator time, length of intensive care unit stay, length of hospital stay or quality of life".[281] One problem cited in the review was poor methodological design, which involved deciding on the patient group to supplement, when to give the supplementation and at what dose. In 2009, the Canadian Critical Care Nutrition Guidelines reported that there was insufficient evidence to recommend the use of selenium supplements in the critically ill, even in combination with other antioxidants. A 2011 meta-analyses examined the benefit of selenium supplementation across 12 publications involving critically ill patients, concluding that selenium supplementation significantly reduced the risk of mortality among critically ill patients with SIRS or sepsis without evidence of adverse effects.[234] In 2013, the Canadian Critical Care Nutrition Guidelines were updated upgraded to say that "selenium supplementation in critically ill patients should be considered, alone or in combination."[86] However, the latest updated review in 2015 by the same committee reversed this decision, stating that "the use of IV/PN selenium supplementation, alone or in combination with other antioxidants, is not recommended in critically ill patients. The change in decision was based upon two additional studies, [282] and indicates how quickly recommendations can change based upon relatively small studies. One of these studies was under submission review (Bloos 2015) and was a multi-centre study of 33 ICU's concluding that selenium had no effect on mortality, length of stay (ICU or hospital). The other single centre study randomized 40 severe septic patients to high dose Na-selenite or placebo. No beneficial effect was noted on clinical outcome, although selenium levels were restored.[283] Despite this change of recommendation, the committee still expressed concern over the variability in study designs, selenium doing and patient populations. [282] It is therefore likely that as more evidence comes to light there will be further changes to the recommendations. As an example of how rapidly the position might change regarding selenium use in the critically ill, a recent study of 54 septic patients, (not reviewed by the Canadian Critical Care Nutrition committee), has shown a reduction in ventilator acquired pneumonia in mechanically ventilated patients with sepsis, although there was no change in 28 day mortality.[284]

Many studies demonstrate that critically ill patients are hyper-metabolic, and it has even been suggested that as many as 50% of ECMO patients do not reach nutritional adequacy during treatment.[271] Presumably the requirements for trace elements in these patients are elevated and indeed critically ill patients supplemented with a level of selenium far in excess of the daily upper limit exhibit no negative effects at all.[273] The first step towards investigating the benefits of supplementation in the ECMO patient is to establish the time-course changes in selenium over the duration of ECMO therapy. Indeed, in this thesis the ECMO studies in sheep identified profound early reductions of selenium,[270] so now studies are needed to determine if the same occurs in patients over the duration of their ECMO support and hospital stay. In addition there are preliminary plans to investigate changes to the inflammatory and immune systems in ECMO patients, beginning the sampling at the initiation of ECMO and continuing to hospital discharge (in the survivors).

Selenium, as an antioxidant supplement, is certain to remain of research interest in these patients groups. By incorporating the findings of this thesis to select and specifically target supplementation at patients with low selenium levels, it may be possible, to determine if there really is a clinical benefit to normalizing levels in cardiac surgical and ECMO patients. Therefore, this body of work has led to the following new research questions:

- Do plasma selenium levels in the normal reference range provide adequate oxidative stress protection for cardiac surgery or ECMO patients, and do such levels also equate to optimal immune function and thyroid activity?
- If not, what target plasma selenium level is required to protect cardiac surgical or ECMO patients from oxidative stress injury?
- What dose of supplemental selenium is required to reach this target plasma selenium level?
- When is the optimal time to give supplemental selenium in cardiac and ECMO patients?

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Appendices

10.0 Appendices

A. Ethics approvals.

Animal Ethics.

Chapter 5: Transfusion of packed red blood cells reduces selenium levels and increases lipid peroxidation in an in vivo ovine model. **QUT animal ethics 1100000053**

Chapter 6: The impact of Acute Lung Injury and ECMO on selenium and oxidative stress status in an ovine model. **Animal ethics committees of The Queensland University of Technology (1100000053) and The University Of Queensland (QUT/194/12).**

Human Ethics

Chapter 3: Plasma selenium status in a group of Australian blood donors and fresh blood components. ARCBS (blood supply). **Australian Red Cross Blood Service ethics committee 2010**

Chapter 4: Antioxidant Trace Element Reduction in an *In Vitro* Cardiopulmonary Bypass Circuit. **Australian Red Cross Blood Service ethics committee 10-01QLD-09**

Chapter 7: The association between pre-operative selenium and post-operative atrial fibrillation after high-risk coronary artery surgery. **The Prince Charles Hospital Human Research Ethics Committee HREC/11/QPCH/138:**

B. Journal Permissions

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Chapter 3

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Chapter 6

The impact of Acute Lung Injury, ECMO and transfusion on selenium and oxidative stress status in an ovine model.[270] Reproduced in full with permission. Date granted: 28/7/2014

Chapter 7

Journal Publication. (not yet accepted)

C. Funding received during Candidature

PhD related grants

- 2009 Australian Red Cross Blood Service $15,500. **Selenium levels in the healthy blood donor population of Queensland.** (Chief Investigator)
- 2012-13 HPRGS (Health Practitioner Research Grant Scheme) $48,800 **Low levels of Selenium are associated with Atrial Fibrillation following cardiac surgery.** (Chief Investigator)
- 2011-13 NHMRC grant $929,299 **The unholy alliance between extracorporeal circuitry and transfusion medicine: characterising inflammation, tissue injury and trace elements**
- 2010 TPCH Foundation $9990 **Changes in Trace Elements as a result of adsorption by the Cardiopulmonary Bypass circuit.** (Associate Investigator)
- 2010 TPCH Foundation $101 000 **The unholy alliance between extracorporeal circuitry and transfusion medicine: characterising inflammation, tissue injury and trace elements.** (Associate Investigator)

Non-PhD related grants

- 2011 The Prince Charles Hospital Foundation (2011) $9997.84. Shekar K, Roberts JA, Smith MT, Fraser JF, McDonald C. **Sedative and analgesic drug disposition during simulated extracorporeal membrane oxygenation.** (Co-Investigator)
- 2011 Intensive Care Foundation $10,909.09. Shekar K, Roberts JA, Smith MT, Fraser JF, McDonald C. **Disposition of sedative, analgesic and antibiotic drugs during simulated extracorporeal membrane oxygenation.** (Co-investigator)
- 2012-13 TPCH Foundation $91,899.95 **Antibiotic, sedative and analgesic drug pharmacokinetics during ovine extracorporeal membrane oxygenation (ECMO) - understanding altered pharmacokinetics to improve patient outcomes.** (Associate Investigator)
- 2011-12 Intensive Care Foundation Research grant $12,000 **Disposition of sedative, analgesic and antibiotic drugs during simulated extracorporeal membrane oxygenation.** (Co-investigator)

D. Awards received during candidature.

- 2010 (Oct) ANZICS, Melbourne. Best Medical Poster. **"Selenium levels in Queensland donors and red cell concentrates: Does a Selenium replete donor mean a Selenium replete product?"** Kathryn Colebourne, Helen Faddy, Charles McDonald, Robert Flower and John F Fraser."
- 2010 (Oct) 27th ASM-ANZCP, Gold Coast. Best Scientific Presentation. For the presentation **"Trace Elements and the Cardiopulmonary Bypass Circuit."** Charles McDonald, Lin Fung and John Fraser. $5000 monetary prize.
- 2011 (Nov) 28th ASM-ANZCP, Sydney. Encouragements award for Scientific Development. For the presentation **"The age of packed red blood cells and oxidative stress in a sheep model."** Charles McDonald, Lin Fung, Ogilvie Thom and John Fraser. $2500 monetary prize.
- 2012 (Nov) 29th ASM-ANZCP, Uluru. Best Scientific Presentation. For the presentation **"ECMO and Oxidative stress."** Charles McDonald, Lin Fung, Kiran Shekar, Margaret Passmore, Sara Diab, Kimble Dunster and John Fraser. $5000 monetary prize.
- 2014 Michael Ray Best Basic/Translational Research Project. The Prince Charles Hospital Research Forum. For the presentation of **"The impact of acute lung injury, ECMO and transfusion on selenium and oxidative stress status in an ovine model."** Charles McDonald, Yoke Lin Fung, Kiran Shekar, Sara Diab, Kimble Dunster, Margaret Passmore, Samuel Foley, Gabriela Simonova, David Platts and John Fraser.
- 2015 (Nov) 32nd ASM-ANZCP, Sydney. Best Scientific Presentation. For the presentation **"Hydrodynamic Evaluation of Aortic Cardiopulmonary BypassCannulae using Particle Image Velocimetry."** Cl McDonald, E Bolle, HF Lang, C Ribolzi, B Thomson, GD Tansley, JF Fraser and SD Gregory. $5000 monetary prize.

E. Additional Peer Reviewed Papers accepted during candidature.

1. **In vitro evaluation of a compliant inflow cannula reservoir to reduce suction events with extracorporeal rotary ventricular assist device.** Shaun David Gregory, Daniel Timms, Nicholas Richard Gaddum, Charles McDonald, Mark John Pearcy, John F Fraser. Artificial Organs 2011; 35(8):765-72.

2. **246 BiVACOR® – A Magnetically Levitated Rotary Total Artificial Heart.** B. Thomson, J. Choudhary, N. Greatrex, S. Gregory, M. Stevens, S. Diab, C. McDonald, K. Dunster, N. Kurita The Journal of Heart and Lung Transplantation 2012; 31(4):S89.
3. **Altered Antibiotic Pharmacokinetics during Extracorporeal Membrane Oxygenation may Cause Therapeutic Failure.** K. Shekar, S. Diab, C. McDonald, S. Fisquet, K. Dunster, L. Fung, D. Platts, S. Ghassabian, A. Barnett, J. Roberts, M. Smith, J. Fraser. Heart Lung & Circulation 2012; 21:S235-S236.
4. **Development of simulated and ovine models of extracorporeal life support to improve understanding of circuit-host interactions.** Kiran Shekar, Yoke L Fung, Sara Diab, Daniel V Mullany, Charles I McDonald, Kimble R Dunster, Stephanie Fisquet, David G Platts, David Stewart, Steven C Wallis, Maree T Smith, Jason A Roberts, John F Fraser. Critical Care and Resuscitation. 2012; 14(2):105-11.
5. **Is Morphine Superior to Fentanyl for Analgesia during Extracorporeal Membrane Oxygenation in Adult Patients?** K. Shekar, C. McDonald, S. Fisquet, A. Barnett, S. Ghassabian, L. Fung, J. Roberts, M. Smith, J. Fraser. Heart Lung and Circulation 2012; 21:S237-S238. ·
6. **Circuit sequestration of meropenem and vancomycin during extracorporeal membrane oxygenation may lead to therapeutic failure.** Shekar, K., McDonald, C.I., Fisquet, S., Barnett, A.G., Mullany, D.V., Fung, Y.L., Wallis, S.C., Smith, M.T., Roberts, J.A., and Fraser, J.F. 2012. Critical Care 2012;16(5):R194.
7. **Extracorporeal Lessons from Sheep.** Fung YL, Diab S, Dunster K, Foley SR, McDonald CI, Passmore M, Platts D, Simonova G, Shekar K, Stewart D, et al. ISBT Science Series 2012:7(1);92-95.
8. **ECMO – The Clinician's View.** Fraser JF, Shekar K, Diab S, Dunster K, Foley SR, McDonald CI, Passmore M, Simonova G, Roberts J, Platts D, Fung YL. ISBT Science Series 2012:7(1);82-88.
9. **Feasibility of a Novel Echocardiographic Imaging Technique, Intracatheter Echocardiography, to Guide Venovenous Extracorporeal Membrane Oxygenation Cannulae Placement in a Validated Ovine Model.** D. Platts, A. Hilton, S. Diab, C. McDonald, M. Tunbridge, S. Chemonges, K. Dunster, K. Shekar, D. Burstow, J. Fraser. Heart Lung and Circulation 2013; 22;(Supp 1):S180. ·
10. **The Impact of Continuous Flow From Venovenous Extracorporeal Membrane Oxygenation Cannulae on Tricuspid Valve Geometry and Function.** D. Platts, S.

Diab, C. McDonald, M. Tunbridge, S. Chemonges, K. Dunster, K. Shekar, D. Burstow, D. Mullany, J. Fraser. Heart Lung and Circulation 2013; 22 (Supp 1):S199. ·

11. **Management of Exsanguination During Laser Lead Extraction.** Smith I, Rapchuk I, Macdonald C, Thomson B, Pearse B. J Cardiothorac Vasc Anesth. 2014; 28(6):1575-9
12. **Feasibility of a Novel Echocardiographic Imaging Technique, Intracatheter Echocardiography, to Guide Venovenous Extracorporeal Membrane Oxygenation Cannulae Placement in a Validated Ovine Model.** D. Platts, A. Hilton, S. Diab, C. MacDonald, M. Tunbridge, S. Chemonges, K. Dunster, K. Shekar, D. Burstow and J. Fraser. Heart Lung and Circulation 2013; 22(supp 1): S180
13. **A Novel Echocardiographic Imaging Technique, Intracatheter Echocardiography, to Guide Venovenous Extracorporeal Membrane Oxygenation Cannulae Placement in a Validated Ovine Model** David Platts, Andrew Hilton, Sara Diab, Charles McDonald, Kiran Shekar, Saul Chemonges, Kimble Dunster, Matthew Tunbridge, Darryl J Burstow, John F Fraser. Intensive Care Medicine Experimental. 2014; 2:2
14. **Optimal management of the critically ill: Anaesthesia, monitoring, data capture and point-of-care technological practices in ovine models of critical care.** Saul Chemonges, Kiran Shekar, John-Paul Tung, Kimble R. Dunster, Sara Diab, David Platts, Ryan P. Watts, Shaun D. Gregory, Samuel Foley, Gabriela Simonova, Charles McDonald, Rylan Hayes, Judith Bellpart, Daniel Timms, Michelle Chew, Yoke L. Fung, Michael Toon, Marc O. Maybauer, and John F. Fraser. BioMed Research International. 2014. Article ID 468309 (17 pages).
15. **Macro and micronutrient disposition in an *ex vivo* model of extracorporeal membrane oxygenation.** Estensen K, Shekar K, Robins E, McDonald C, Barnet A and Fraser J. Intensive Care Medicine Experimental. 2014; 2(1):29
16. **The link between physicochemistry and drug sequestration in an ECMO circuit. Results from an ex vivo study.** Shekar K, McDonald CI, Ghassabian, Wallis, Roberts, Fraser and Barnett. Critical Care submitted 2015
17. **Feasibility of Perflutren Microsphere Contrast Transthoracic Echocardiography in the Visualisation of Ventricular Endocardium During Venovenous Extracorporeal Membrane Oxygenation in a Validated Ovine Model.** Platts, D et al Echocardiography 2015; 32(3):548-556

18. **Protein bound drugs are prone to sequestration in the ECMO circuit: Results from an *ex vivo* study.** Shekar K, Roberts JA, McDonald CI, Ghassabian S**,** Anstey C, Wallis SC, Mullany DV, Fung YL and Fraser JF. Critical Care 2015;19(1):164.

19. **Ovine platelet function is unaffected by extracorporeal membrane oxygenation within the first 24 h.** Hayes RA, Foley S, Shekar K, Diab S, Dunster KR, McDonald C, Fraser JF. Blood Coagul Fibrinolysis. 2015 Jul 20. [Epub ahead of print]

F. Additional Conference Abstracts during candidature

1. **Sequestration of up to eighty percent meropenem in the circuit may cause treatment failure in patients receiving extracorporeal membrane oxygenation**. Shekar K, McDonald C, Fisquet S, Mullany D, Barnett A, Wallis S, Ghassabian S, Fung L, Roberts J. Medicines Management 2011, the 37th Society of Hospital Pharmacists of Australia National Conference, Hobart, Australia. 10-13 November. 2011
2. **Sequestration of Meropenem in the circuit may cause treatment failure in patients receiving Extra-corporeal Membrane Oxygenation** Shekar K, McDonald C, Fisquet S, Mullany D, Barnett A, Wallis S, Ghassabian S, Fung L, Roberts J..28th Annual Scientific Meeting of the Australian and New Zealand College of Perfusion, Sydney, Australia. 3-5 November 2011
3. **Development of simulated and ovine models of extracorporeal life support to improve understanding of circuit-host interactions.** Shekar K, Fung YL, Diab S, Mullany DV, McDonald CI, Dunster KR, Fisquet S, Platts DG, Stewart D, Wallis S. College of Intensive Care Medicine. Annual Scientific Meeting 2012
4. **Extracorporeal lessons from sheep.** Fung YL, Diab S, Dunster K, Foley SR, McDonald CI, Passmore M, Platts D, Simonova G, Shekar K, Stewart D, Fraser JF. 32nd International Congress of the International Society of Blood Transfusion, Cancun, Mexico. 7-12 July 2012
5. **Stored transfusion transiently affects pulmonary haemodynamics in an ovine model of ECMO.** Tunbridge, M., Sim, B., Diab, S., Dunster, K., McDonald, C., Platts, D., Foley, S., Simonova, G., Tung, J., Shekar, K. and Fraser, J. Poster: ASPELSO Beijing October 2013.
6. **Extracorporeal circuit may not short change nutrient bioavailability during ECMO** Estensen, K., Shekar, K., Robins, E., McDonald, C. and Fraser, J.. Poster: ASPELSO Beijing October 2013.

7. **ECMO has more profound influence on Ciprofoxacin pharmacokinetics in critically ill sheep when compared to healthy sheep.** Shekar, K., Roberts, J., Diab, S., Dunster, K., McDonald, C., Chemonges, S., Simonova, G., Foley, S., Wallis, S., Platts, D., Fung, Y., Smith, M. and Fraser, J. Poster: ASPELSO Beijing October 2013.
8. **Quantification of perflutren microshpere destruction during transit through an ex-vivo ECMO circuit.** Platts, D., McDonald, C., Shekar, K., Diab, S., Dunster, K., Burstow, D., Chan, J. and Fraser, J. Poster: ASPELSO Beijing October 2013.
9. **Acute lung injury compounds ECMO-induced changes to haemostasis in an ovine model.** Foley, S., Simonova, G., Diab, S., Dunster, K., McDonald, C., Shekar, K., Fung, Y. and Fraser, J. Poster: ASPELSO Beijing October 2013.
10. **Does tricuspid valve geometry and function alter during exposure to continuous high blood flow return from venovenous extracorporeal membrane oxygenator cannulae.** Platts, D., Diab, S., McDonald, C., Tunbridge, M., Chemonges, S., Dunster, K., Shekar, K., Burstow, D., Mullaney, D. and Fraser, J. Poster: ASPELSO Beijing October 2013.
11. **Successful use of pre and post operative ECMO for pulmonary thromboendarterectomy, mitral valve replacement and myomectomy in a patient with chronic thromboembolic pulmonary hypertension and hypertrophic cardiomyopathy** Williams, L., Kermeen, F., Ziegenfuss, M., Bull, T., McDonald, C., Mullany, D., Fraser, J. and Thomson, B. Poster: ELSO Philadelphia October 2013.
12. **Successful use of pre and post operative ECMO for pulmonary thromboendarterectomy, mitral valve replacement and myomectomy in a patient with chronic thromboembolic pulmonary hypertension and hypertrophic cardiomyopathy.** Williams, L., Kermeen, F., Ziegenfuss, M., Bull, T., McDonald, C., Mullany, D., Fraser, J. and Thomson, B. Abstract: ANZCP Melbourne 2013.
13. **A novel ovine model to investigate the tissue effects of fluid and blood resuscitation in sepsis.** Shekar, K., Chew, M., Diab, S., Dunster, K., McDonald, C., Simonova, G., Foley, S., Passmore, M., Fung, L., Reade, M. and Fraser, J. Poster: TPCH Research Forum. October 2013.
14. **Evidence of ECMO induced changes to haemostasis in an ovine model.** S.R. Foley, Y.L. Fung, G. Simonova, C. Solano, S. Diab, K.R. Dunster, C.I. McDonald, K. Shekar, J.F. Fraser. Poster : ANZICS, Hobart 2013.

15. **Transfusion of fresh or aged red cells after haemorrhagic shock reduces selenium and glutathione peroxidase** Fung, YL., McDonald, CI., Thom, O., Fraser, JF. Vox Sanguis, 2011; 101 (suppl. 2):122.
16. **Critical illness has more profound effects on ciprofloxacin PK when compared to independent effects of ECMO**. Kiran Shekar, Sara Diab, Kimble R Dunster, Jason A Roberts, Charles I McDonald, Margaret Passmore, David G Platts, Gabriella Simonova, Sam Foley, Steven C Wallis, Fung YL, Smith MT, Fraser JF. Australia and New Zealand Society of Intensive Care Medicine. 2013.
17. **Critical illness has more profound effects on ciprofloxacin PK when compared to independent effects of ECMO.** Kiran Shekar, Sara Diab, Kimble R Dunster, Jason A Roberts, Charles I McDonald, Margaret Passmore, David G Platts, Gabriella Simonova, Sam Foley, Steven C Wallis, Fung YL, Smith MT, Fraser JF. Asia-Pacific ELSO meeting, Beijing. October 2013.

G. Additional Oral Presentations during candidature

1. **Successful use of pre and post-operative ECMO for pulmonary thromboendarterectomy, mitral valve replacement and myomectomy in a patient with chronic thromboembolic pulmonary hypertension and hypertrophic cardiomyopathy.** 30th ASM of the Australian and New Zealand Society of Cardiovascular Perfusionists. Melbourne. 2013 (Nov).