STATEMENT OF ORIGINALITY

The work presented within this thesis was performed in the Heart Foundation Research Centre, School of Health Science, Griffith University Gold Coast. The research was carried out under the supervision of Dr Anthony Perkins, Professor John Headrick and Dr Glenn Harrison.

To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself. This work has not previously been submitted for a degree or diploma in any other university.

........................................

Kylie M. Venardos
December 2004
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>I</td>
</tr>
<tr>
<td>PUBLICATIONS &amp; PRESENTATIONS</td>
<td>VI</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>X</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>XVI</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>XVII</td>
</tr>
<tr>
<td>COMPOUND ABBREVIATIONS</td>
<td>XXIII</td>
</tr>
<tr>
<td><strong>CHAPTER 1: GENERAL INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 CARDIOVASCULAR DISEASE</td>
<td>2</td>
</tr>
<tr>
<td>1.2 MYOCARDIAL ISCHEMIA</td>
<td>2</td>
</tr>
<tr>
<td>1.3 REPERFUSION INJURY</td>
<td>7</td>
</tr>
<tr>
<td>1.4 MECHANISMS OF REVERSIBLE ISCHEMIA-REPERFUSION INJURY</td>
<td>8</td>
</tr>
<tr>
<td>1.4.1 MYOCARDIAL STUNNING</td>
<td>8</td>
</tr>
<tr>
<td>1.5 MECHANISMS OF IRREVERSIBLE ISCHEMIA-REPERFUSION INJURY</td>
<td>12</td>
</tr>
<tr>
<td>1.5.1 IMMEDIATE LETHAL REPERFUSION INJURY</td>
<td>13</td>
</tr>
<tr>
<td>1.6 GENERATION OF REACTIVE OXYGEN SPECIES DURING MYOCARDIAL ISCHEMIA-REPERFUSION</td>
<td>22</td>
</tr>
<tr>
<td>1.7 OXIDATIVE DAMAGE CAUSED BY REACTIVE OXYGEN SPECIES</td>
<td>27</td>
</tr>
<tr>
<td>1.8 MYOCARDIAL ANTIOXIDANT DEFENCE SYSTEMS</td>
<td>31</td>
</tr>
<tr>
<td>1.9 GLUTATHIONE / GLUTATHIONE PEROXIDASE RELATED SYSTEM</td>
<td>35</td>
</tr>
<tr>
<td>1.10 THIOREDOXIN / THIOREDOXIN REDUCTASE RELATED SYSTEM</td>
<td>38</td>
</tr>
<tr>
<td>1.11 SELENIUM AND SELENOCYSTEINE</td>
<td>45</td>
</tr>
<tr>
<td>1.12 INHIBITION OF SELENOCYSTEINE</td>
<td>49</td>
</tr>
<tr>
<td>1.13 AIMS AND OBJECTIVES</td>
<td>52</td>
</tr>
<tr>
<td><strong>CHAPTER 2: GENERAL METHODS AND MATERIALS</strong></td>
<td>53</td>
</tr>
<tr>
<td>2.1 MODELS EMPLOYED</td>
<td>54</td>
</tr>
<tr>
<td>2.2 MATERIALS</td>
<td>56</td>
</tr>
</tbody>
</table>
CHAPTER 3:

ISCHEMIA vs REPERFUSION: EFFECTS ON EXPRESSION & ACTIVITY OF MYOCARDIAL ANTIOXIDANT ENZYME SYSTEMS, & OXIDATIVE DAMAGE

3.1 ABSTRACT
3.2 INTRODUCTION
3.3 MATERIALS AND METHODS
3.4 RESULTS
3.5 DISCUSSION

CHAPTER 4:

SELENIUM SUPPLEMENTATION & MYOCARDIAL ISCHEMIA-REPERFUSION INJURY

4.1 ABSTRACT
4.2 INTRODUCTION
4.3 MATERIALS AND METHODS
4.4 RESULTS
4.5 DISCUSSION

CHAPTER 5:

EFFECTS OF DIETARY SELENIUM ON POST-ISCHEMIC EXPRESSION OF MYOCARDIAL ANTIOXIDANT mRNA

5.1 ABSTRACT
5.2 INTRODUCTION
5.3 MATERIALS AND METHODS
CHAPTER 6:
EFFECTS OF AURANOFIN ON MYOCARDIAL ISCHEMIA-REPERFUSION INJURY

6.1 ABSTRACT
6.2 INTRODUCTION
6.3 MATERIALS AND METHODS
6.4 RESULTS
6.5 DISCUSSION

CHAPTER 7:
THE EFFECT OF AGEING ON EXPRESSION & ACTIVITY OF MYOCARDIAL ANTIOXIDANT ENZYME SYSTEMS, & OXIDATIVE DAMAGE

7.1 ABSTRACT
7.2 INTRODUCTION
7.3 MATERIALS AND METHODS
7.4 RESULTS
7.5 DISCUSSION

CHAPTER 8: GENERAL DISCUSSION

REFERENCE LIST
Coronary heart disease remains the greatest killer of Australian’s, and given our ageing population, along with increasing risk factors, it is predicted to become an even more significant problem worldwide over the next 20 years. Reperfusion, without doubt is the most effective treatment for ischemic myocardium. However, this produces deleterious effects upon cells, and depending on the severity, may ultimately lead to cell death. While the pathogenesis of ischemia-reperfusion is not completely understood, there is considerable evidence implicating reactive oxygen species (ROS) as an initial cause of the injury. ROS formed during oxidative stress can initiate lipid peroxidation, oxidize proteins to inactive states and cause DNA strand breaks, all potentially damaging to normal cellular function. ROS have been shown to be generated following routine clinical procedures such as coronary bypass surgery and thrombolysis, due to the unavoidable episode of ischemia-reperfusion. Furthermore, they have been associated with poor cardiac recovery post-ischemia, with recent studies supporting a role for them in infarction, necrosis, apoptosis, arrhythmogenesis and endothelial dysfunction following ischemia-reperfusion. In normal physiological condition, ROS production is usually homeostatically controlled by endogenous free radical scavengers such as SOD, catalase, and the glutathione peroxidase and thioredoxin reductase systems. Targeting the generation of ROS with various antioxidants has been shown to reduce injury following oxidative stress, and improve recovery from ischemia-reperfusion injury.

This thesis investigates the role of myocardial antioxidant enzymes in ischemia-reperfusion injury, particularly the glutathione peroxidase (GPX) and the thioredoxin reductase (TxnRed) systems. GPX and TxnRed are selenocysteine dependent enzymes, and their activity is known to be dependent upon an adequate supply of dietary selenium and selenocysteine. In mammalian cells, the generation of selenocysteine occurs during amino acid biosynthesis and the degree of selenium (Se) incorporation into the cysteine residue is concentration dependent. Previous studies have found that up-regulation of these systems is cardioprotective and down-regulation is detrimental following ischemia-reperfusion. This thesis attempts to extend these observations by increasing not only our understanding of the roles of myocardial antioxidant enzymes in ischemia-reperfusion injury, but also the effect
of dietary selenium on these systems. Furthermore, it investigates the effects of ischemia, reperfusion, and ageing on myocardial antioxidant enzymes.

Chapter 3 examines the effect of ischemia alone and with reperfusion on the expression and activity of antioxidant proteins and on cellular injury in the isolated rat heart. The results of this chapter suggest the TxnRed and GPX systems may complement each other, and respond to acute injurious stimuli to aid in limiting oxidant stress, as both were found to be up-regulated during periods of ischemia and reperfusion. There were no changes in SOD activity during ischemia or reperfusion, suggesting the GPX and TxnRed systems are more finely ‘in tune’ with the oxidative status of cardiac cells. Expression and activity of the TxnRed system was up-regulated during ischemia suggesting it may contain a hypoxic responsive element in its promoter, whereas GPX system expression and activity was only up-regulated after reperfusion. Protein oxidation was increased during reperfusion, and although LPO was also significantly increased in reperfusion, it was greater during ischemia, indicating oxidative damage occurs at various oxygen levels. This also shows that some of the oxidative damage associated with ischemia-reperfusion injury actually occurs during the ischemic episode itself, and before the re-introduction of oxygen and the associated oxidative burst. Caspase-3 was found to be elevated during reperfusion but not ischemia indicating it is not a major process for cell death during ischemia. Thus, it is concluded that the GPX system may be responsible for protecting the heart against LPO and removing ROS such as hydroperoxides and lipid hydroperoxides, whilst the TxnRed system may provide more protection against protein oxidation and apoptosis.

Chapter 4 examines the effects of dietary selenium on the activity of GPX and TxnRed in rats, and the ability of selenium to modulate myocardial function during ischemia-reperfusion in isolated hearts. Selenium deficiency reduced the activity of both glutathione peroxidase and thioredoxin reductase systemically. Hearts from selenium deficient animals were more susceptible to ischemia-reperfusion injury when compared to normal controls, and had higher levels of lipid peroxidation and protein oxidation. Selenium supplementation increased the endogenous activity of TxnRed and GPX and resulted in improved recovery of cardiac function post ischemia-reperfusion. This correlated to lower levels of LPO, and oxidized protein products such as PCO. Myocardial SOD activity was not affected by dietary selenium. These results indicate the endogenous
activity of GPX and TxnRed is dependent on an adequate supply of the micronutrient selenium. Reduced activity of these antioxidant enzymes leaves hearts more susceptible to oxidative damage leading to significant reductions in myocardial function post ischemia-reperfusion.

Following Chapter 4, which demonstrated a key role for dietary selenium, and the GPX and TxnRed systems in protecting against ischemia-reperfusion injury, Chapter 5 investigates the role of selenium in the post-ischemic cardiac mRNA expression of these systems, and related antioxidant proteins. Selenium deficiency produced significant reductions in Gpx-1, Gpx-4, Prdx2, Txnrd-1 and Txnrd-2 mRNA expression following ischemia-reperfusion, potentially leaving hearts more susceptible to oxidative damage. Conversely, selenium supplementation of 1000 µg/kg significantly up-regulated Gpx-1, Gpx-4, Txn, Txnrd-1 and Txnrd-2 gene transcription. These results show selenium modulates the cardiac mRNA expression of thioredoxin and glutathione related enzymes post ischemia-reperfusion.

Given previous findings on poor functional recovery after down-regulation of the GPX and TxnRed systems, Chapter 6 attempts to investigate the relative importance of myocardial TxnRed alone, and how its inhibition effects functional recovery, oxidative damage and apoptosis following ischemia-reperfusion. In this study auranofin (an anti-rheumatic gold compound) was used to selectively inhibit TxnRed in rats without altering GPX activity. There was significantly less TxnRed activity in auranofin treated rat liver extracts and auranofin treated normoxic hearts, whilst GPX activity remained unaffected, demonstrating the dose of auranofin used was able to selectively inhibit one of these enzymes. Hearts treated with auranofin display significantly impaired recovery from ischemia-reperfusion injury with lower contractile function and greater diastolic dysfunction at the end of reperfusion. This poor functional recovery correlated with elevated levels of lipid peroxidation and apoptotic activity, which may have contributed to reduced recovery. This chapter shows that whilst both antioxidants are important in preventing oxidative stress, TxnRed activity is critical for recovery from reperfusion injury.

Given the increased incidence and severity of CVD in aged hearts, along with the increased susceptibility to oxidative stress in ageing, Chapter 7 examined the effect of ageing on cardiac expression of the TxnRed and GPX systems, and levels of naturally
occurring oxidative damage. The results of this chapter indicate that the ratio of antioxidant enzyme activity to transcription decreases in aged hearts. This chapter found significantly lower GPX activities in aged rat hearts when compared to younger hearts without significant changes in GPX mRNA expression, and significantly higher mRNA expression of the TxnRed system in aged hearts without any changes in TxnRed activity. These lower activity-to-transcription ratios may be due to inefficient or ineffective translation, lower levels of selenocysteine and/or increased protein turnover or degradation. Although no changes in baseline LPO, protein carbonyl or 3-nitrotyrosine levels were found in aged hearts, increased levels of di-Tyr and o-Tyr were found. These results indicate that aged hearts are oxidatively stressed under normal conditions, which may be due to an increased production of ROS and/or decreased antioxidant status, and suggests aged hearts would not recover well from an oxidative insult such as ischemia-reperfusion, as has been shown previously by others. This chapter also suggests that antioxidant status should be monitored in elderly patients prior to clinical interventions involving ischemia-reperfusion. Increasing antioxidant defences, possibly with selenium supplementation, may be a relatively easy and effective mechanism for improving the heart health of ageing populations.

In summary, these studies show that the TxnRed and GPX systems have a significant role in protecting against myocardial ischemia-reperfusion injury. Up-regulation of the GPX and TxnRed systems improved contractile recovery of hearts post ischemia-reperfusion, by reducing the amount of oxidative stress generated. By reducing the amount of LPO and protein oxidation occurring in the heart, less damage, including apoptosis, is caused to cells allowing greater recovery. Given that we have shown oxidative damage occurs during both the ischemic episode and reperfusion, it is important to have hearts prepared for such an insult by increasing the available antioxidant defences prior to the initiation of ischemia-reperfusion. This thesis supports a positive role for both dietary selenium and the TxnRed and GPX systems in myocardial ischemia-reperfusion injury. Dietary selenium supplementation may provide a safe and convenient method for increasing antioxidant protection in aged individuals, those at risk of ischemic heart disease, or those expecting to undergo clinical procedures involving transient periods of hypoxia. Enhanced selenium intake may be a useful way of preconditioning, and this may
represent a new strategy for directly protecting against oxidative stress, and improving recovery rates following myocardial ischemic insults.
PUBLICATIONS & PRESENTATIONS

PUBLISHED MANUSCRIPTS


PUBLISHED ABSTRACTS


**SUBMITTED MANUSCRIPTS**


**ABSTRACTS PRESENTED AT NATIONAL AND INTERNATIONAL MEETINGS**


**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Cellular and metabolic alterations induced by ischemia.</td>
</tr>
<tr>
<td>1.2</td>
<td>Proposed mechanism of myocardial stunning following ischemia-reperfusion.</td>
</tr>
<tr>
<td>1.3</td>
<td>Potential initial causes of immediate lethal reperfusion injury.</td>
</tr>
<tr>
<td>1.4</td>
<td>The effect of rapid normalisation of intracellular H(^+) concentration in cardiomyocytes upon reperfusion.</td>
</tr>
<tr>
<td>1.5</td>
<td>Cell swelling and rupture as a result of normalisation of tissue osmolality following reperfusion of ischemic myocytes.</td>
</tr>
<tr>
<td>1.6</td>
<td>Schematic diagram of the major cation transport mechanisms activated upon reoxygenation of the myocyte.</td>
</tr>
<tr>
<td>1.7</td>
<td>Cellular mechanisms for reactive oxygen species generation during ischemia-reperfusion.</td>
</tr>
<tr>
<td>2.1</td>
<td>Langendorff perfusion apparatus (A) and isolated Langendorff perfused rat heart (B).</td>
</tr>
<tr>
<td>2.2</td>
<td>Formaldehyde agarose gel of total RNA isolated from rat hearts.</td>
</tr>
<tr>
<td>2.3</td>
<td>Elution profiles for standards of DOPA, tyrosine, di-Tyrosine, o-Tyrosine and 3-nitrotyrosine by 12 channel electrochemical detector.</td>
</tr>
<tr>
<td>2.4</td>
<td>Real-time PCR amplification plot for a test gene (A) and 18S (B).</td>
</tr>
<tr>
<td>2.5</td>
<td>Melt (dissociation) curve used to assess PCR product purity and ensure no primer dimer formation.</td>
</tr>
<tr>
<td>3.1</td>
<td>Recovery of Rate Pressure Product (RPP) in the Langendorff perfused isolated rat heart following different lengths of zero-flow global ischemia.</td>
</tr>
<tr>
<td>3.2</td>
<td>Effect of ischemia and reperfusion on lipid peroxidation in the isolated rat heart.</td>
</tr>
<tr>
<td>3.3</td>
<td>Effect of ischemia and reperfusion on the formation of oxidised protein carbonyl derivatives in the heart.</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>3.4</td>
<td>Effect of ischemia and reperfusion on the oxidation of tyrosine to DOPA in the heart. 89</td>
</tr>
<tr>
<td>3.5</td>
<td>Effect of ischemia and reperfusion on tyrosine oxidation to di-Tyrosine in the isolated heart. 89</td>
</tr>
<tr>
<td>3.6</td>
<td>Effect of ischemia and reperfusion on phenylalanine oxidation to o-Tyrosine in the heart. 90</td>
</tr>
<tr>
<td>3.7</td>
<td>Effect of ischemia and reperfusion on tyrosine nitration to the oxidation product 3-nitrotyrosine. 90</td>
</tr>
<tr>
<td>3.8</td>
<td>Effect of ischemia and reperfusion on apoptotic activity in the heart. 91</td>
</tr>
<tr>
<td>3.9</td>
<td>Effect of ischemia and reperfusion on myocardial transcription levels of thioredoxin reductase-1. 93</td>
</tr>
<tr>
<td>3.10</td>
<td>Effect of ischemia and reperfusion on thioredoxin reductase-2 expression in the heart. 94</td>
</tr>
<tr>
<td>3.11</td>
<td>Effect of ischemia and reperfusion on myocardial mRNA levels of thioredoxin peroxidase-2 or peroxiredoxin-2. 94</td>
</tr>
<tr>
<td>3.12</td>
<td>Effect of ischemia and reperfusion on thioredoxin transcription levels in the heart. 95</td>
</tr>
<tr>
<td>3.13</td>
<td>Effect of ischemia and reperfusion on myocardial transcription levels of glutathione peroxidase-1. 97</td>
</tr>
<tr>
<td>3.14</td>
<td>Effect of ischemia and reperfusion on myocardial mRNA levels of glutathione peroxidase-4. 97</td>
</tr>
<tr>
<td>3.15</td>
<td>Effect of ischemia and reperfusion on glutathione reductase expression in the heart. 98</td>
</tr>
<tr>
<td>3.16</td>
<td>Effect of ischemia and reperfusion on thioredoxin reductase activity in the rat heart. 100</td>
</tr>
<tr>
<td>3.17</td>
<td>Effect of ischemia and reperfusion on myocardial glutathione peroxidase activity. 101</td>
</tr>
<tr>
<td>3.18</td>
<td>Effect of ischemia and reperfusion on superoxide dismutase activity in the rat heart. 102</td>
</tr>
<tr>
<td>4.1</td>
<td>Effect of dietary selenium on thioredoxin reductase activity in rat liver extracts (A) and post-ischemic heart extracts (B). 121</td>
</tr>
</tbody>
</table>
4.2 Effect of dietary selenium on glutathione peroxidase activity in rat liver extracts (A) and post-ischemic heart extracts (B). 123

4.3 Effect of dietary selenium on superoxide dismutase activity in the rat heart following 22.5 mins ischemia and 45 mins reperfusion. 124

4.4 Effect of dietary selenium on pre-ischemic heart function. 126

4.5 Effect of dietary selenium on ischemic contracture after 22.5 mins ischemia in the isolated perfused rat heart. 127

4.6 Effect of dietary selenium on contractile function in the isolated rat heart during reperfusion. 131

4.7 Effect of dietary selenium on the recovery of Rate Pressure Products (RPPs) after 22.5 mins ischemia and 45 mins reperfusion in the Langendorff perfused isolated rat heart. 132

4.8 Effect of dietary selenium on end diastolic pressure (EDP) after 22.5 mins ischemia and 45 mins reperfusion in the isolated rat heart. 133

4.9 Effect of dietary selenium on the rate of ventricular contraction after ischemia-reperfusion. 134

4.10 Effect of dietary selenium on the recovery of coronary flow after 22.5 mins ischemia and 45 mins reperfusion in the Langendorff perfused isolated rat heart. 135

4.11 Effect of dietary selenium on lipid peroxidation in the isolated rat heart following ischemia-reperfusion. 137

4.12 Effect of dietary selenium on formation of oxidised protein carbonyl derivatives in the heart during ischemia-reperfusion. 138

4.13 Effect of dietary selenium on the oxidation of tyrosine to DOPA in the heart during ischemia-reperfusion. 139

4.14 Effect of dietary selenium on phenylalanine oxidation to o-Tyrosine in the heart during ischemia-reperfusion. 139

4.15 Effect of dietary selenium on tyrosine nitration to the oxidation product 3-nitrotyrosine. 140

4.16 Effect of dietary selenium on tyrosine oxidation to di-Tyrosine in the isolated heart during ischemia-reperfusion. 140
4.17 Effect of dietary selenium on apoptotic activity in the heart post-ischemia-reperfusion.

5.1 Effect of dietary selenium on post-ischemic myocardial transcription levels of thioredoxin reductase-1.

5.2 Effect of dietary selenium on thioredoxin reductase-2 expression in the post-ischemic rat heart.

5.3 Effect of dietary selenium on post-ischemic myocardial mRNA levels of thioredoxin peroxidase-2 or peroxiredoxin-2.

5.4 Effect of dietary selenium on post-ischemic thioredoxin transcription levels in the rat heart.

5.5 Effect of dietary selenium on post-ischemic myocardial transcription levels of glutathione peroxidase-1.

5.6 Effect of dietary selenium on post-ischemic myocardial mRNA levels of glutathione peroxidase-4.

5.7 Effect of dietary selenium on post-ischemic glutathione reductase expression in the rat heart.

6.1 Effect of auranofin (100 mg/kg bodyweight) on thioredoxin reductase activity in rat liver extracts (A), normoxic heart extracts (B) and post-ischemic heart extracts (C).

6.2 Effect of auranofin (100 mg/kg bodyweight) on glutathione peroxidase activity in rat liver extracts (A), normoxic heart extracts (B) and post-ischemic heart extracts (C).

6.3 Effect of auranofin (100 mg/kg bodyweight) on superoxide dismutase activity in heart extracts following normoxic perfusion (A) and 22.5 mins ischemia and 45 mins reperfusion (B).

6.4 Effect of auranofin on pre-ischemic heart function.

6.5 Effect of auranofin on ischemic contracture after 22.5 mins ischemia in the isolated perfused rat heart.

6.6 Effect of auranofin on contractile function in the isolated rat heart during reperfusion.

6.7 Effect of auranofin on the recovery of Rate Pressure Products (RPPs) after 22.5 mins ischemia and 45 mins reperfusion in the Langendorff perfused isolated rat heart.
6.8  Effect of auranofin on end diastolic pressure (EDP) after 22.5 mins ischemia and 45 mins reperfusion in the isolated rat heart. 198

6.9  Effect of auranofin on the rate of ventricular contraction after ischemia-reperfusion. 199

6.10 Effect of auranofin on the recovery of coronary flow after 22.5 mins ischemia and 45 mins reperfusion in the Langendorff perfused isolated rat heart. 200

6.11 Effect of auranofin on lipid peroxidation in the isolated rat heart following ischemia-reperfusion. 202

6.12 Effect of auranofin on the formation of oxidised protein carbonyl derivatives in the heart during ischemia-reperfusion. 203

6.13 Effect of auranofin on the oxidation of tyrosine to DOPA in the heart during ischemia-reperfusion. 204

6.14 Effect of auranofin on tyrosine oxidation to di-Tyrosine in the isolated heart during ischemia-reperfusion. 205

6.15 Effect of auranofin on tyrosine nitration to the oxidation product 3-nitrotyrosine. 206

6.16 Effect of auranofin on phenylalanine oxidation to o-Tyrosine in the heart during ischemia-reperfusion. 207

6.17 Effect of auranofin on apoptotic activity in the heart post ischemia-reperfusion. 208

7.1  Effect of ageing on myocardial transcription levels of thioredoxin reductase-1 (Txnrd-1) in the rat. 224

7.2  Effect of ageing on thioredoxin reductase-2 (Txnrd-2) expression in the rat heart. 225

7.3  Effect of ageing on rat myocardial mRNA levels of thioredoxin peroxidase-2 (or peroxiredoxin-2, Prdx-2). 225

7.4  Effect of ageing on thioredoxin (Txn) transcription levels in the rat heart. 226

7.5  Effect of ageing on myocardial transcription levels of glutathione peroxidase-1 (Gpx-1) in the rat. 228
7.6 Effect of ageing on myocardial mRNA levels of glutathione peroxidase-4 (or phospholipid hydroperoxidase,Gpx-4) in the rat.  
7.7 Effect of ageing on glutathione reductase (Gsr) expression in the heart.  
7.8 Effect of ageing on thioredoxin reductase activity in the rat heart.  
7.9 Effect of ageing on myocardial glutathione peroxidase activity in the rat.  
7.10 Effect of ageing on superoxide dismutase activity in the rat heart.  
7.11 Effect of ageing on lipid peroxidation in the rat heart.  
7.12 Effect of ageing on the formation of oxidised protein carbonyl derivatives in the heart.  
7.13 Effect of ageing on the oxidation of tyrosine to DOPA in the heart.  
7.14 Effect of ageing on tyrosine oxidation to di-Tyrosine in the rat heart.  
7.15 Effect of ageing on phenylalanine oxidation to o-Tyrosine in the rat heart.  
7.16 Effect of ageing on tyrosine nitration to the oxidation product 3-nitrotyrosine.
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Primer sequences and expected amplicon lengths for 7 genes within the glutathione peroxidase and thioredoxin reductase antioxidant systems, and the internal housekeeping gene 18S.</td>
</tr>
<tr>
<td>4.1</td>
<td>Effects of dietary selenium on myocardial function in the isolated rat heart, both pre- and post ischemia-reperfusion.</td>
</tr>
<tr>
<td>6.1</td>
<td>Effects of auranofin on myocardial function in the isolated rat heart, both pre- and post ischemia-reperfusion.</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5’ phosphate</td>
</tr>
<tr>
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<td>alanine</td>
</tr>
<tr>
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<td>adenosine 5’ monophosphate</td>
</tr>
<tr>
<td>ANT</td>
<td>adenine nucleotide translocase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptotic protease activating factor-1</td>
</tr>
<tr>
<td>ASK1</td>
<td>apoptosis signalling kinase-1</td>
</tr>
<tr>
<td>Asn</td>
<td>asparagine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartate</td>
</tr>
<tr>
<td>ATG</td>
<td>aurothioglucose</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’ triphosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CK</td>
<td>creatine kinase</td>
</tr>
<tr>
<td>CoQ10</td>
<td>coenzyme Q10</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>Ct</td>
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<tr>
<td>Cys</td>
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</tr>
<tr>
<td>di-Tyr</td>
<td>3,3’-di-tryosine</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>dNTPs</td>
<td>nucleotide mix</td>
</tr>
<tr>
<td>DOPA</td>
<td>3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>+dP/dt</td>
<td>first derivative of positive pressure development over time (rate)</td>
</tr>
<tr>
<td>-dP/dt</td>
<td>first derivative of negative pressure development over time (rate)</td>
</tr>
<tr>
<td>EBV</td>
<td>Epsteine-Barr virus</td>
</tr>
<tr>
<td>EDP</td>
<td>end diastolic pressure</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<td>FAD</td>
<td>flavin adenine dinucleotide</td>
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<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>GPX</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>GPX-1</td>
<td>classical / cytosolic and mitochondrial glutathione peroxidase</td>
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<td>GPX-2</td>
<td>gastrointestinal glutathione peroxidase</td>
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<tr>
<td>GPX-3</td>
<td>plasma or extracellular glutathione peroxidase</td>
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<tr>
<td>GPX-4</td>
<td>phospholipid hydroperoxide glutathione peroxidase</td>
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<td>GPX-5</td>
<td>selenium-independent glutathione peroxidase</td>
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<td>GSH</td>
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<td>GSSG</td>
<td>glutathione (oxidised form) / glutathione disulfide</td>
</tr>
<tr>
<td>Gsr</td>
<td>glutathione reductase</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
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<td>HDL</td>
<td>high-density lipoprotein</td>
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<td>HOCl</td>
<td>hypochlorous acid</td>
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<td>HPETEs</td>
<td>hydroperoxyeicosatetraenoic acids</td>
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<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Ki</td>
<td>dissociation constant of the enzyme-inhibitor complex</td>
</tr>
<tr>
<td>$K_m$</td>
<td>substrate concentration at which the reaction rate is half its maximal value</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>LPO</td>
<td>lipid peroxidation</td>
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<td>LVDP</td>
<td>left ventricular developed pressure</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen activated protein</td>
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<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>MPT</td>
<td>mitochondrial permeability transition</td>
</tr>
<tr>
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<td>mitochondrial permeability transition pore</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>$m$-Tyr</td>
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<td>NAD$^+$</td>
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<td>3-nitrotyr</td>
<td>3-nitrotyrosine</td>
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<tr>
<td>-----------</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NTC</td>
<td>non template control</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>'O$_2$</td>
<td>singlet oxygen</td>
</tr>
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<td>OH$^+$</td>
<td>hydroxyl radical</td>
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<tr>
<td>-OO$^+$</td>
<td>peroxyl</td>
</tr>
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<td>peroxynitrite</td>
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<tr>
<td>ONOOOH</td>
<td>peroxynitrous acid</td>
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<td>Osm</td>
<td>osmolality</td>
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<td>protein carbonyl</td>
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<td>polymerase chain reaction</td>
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<td>PHGPX</td>
<td>phospholipid hydroperoxide glutathione peroxidase</td>
</tr>
<tr>
<td>Pro</td>
<td>proline</td>
</tr>
<tr>
<td>Prdx</td>
<td>peroxiredoxin or thioredoxin peroxidase</td>
</tr>
<tr>
<td>Prdx-2</td>
<td>mitochondrial peroxiredoxin</td>
</tr>
<tr>
<td>PSVP</td>
<td>peak systolic ventricular pressure</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative (real-time) reverse transcriptase-polymerase chain reaction</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RNasin</td>
<td>ribonuclease inhibitor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>RPP</td>
<td>rate pressure product</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>Se</td>
<td>selenium</td>
</tr>
<tr>
<td>Sec</td>
<td>selenocysteine</td>
</tr>
<tr>
<td>SECIS</td>
<td>selenocystein insertion sequence</td>
</tr>
<tr>
<td>Sec-tRNA&lt;sup&gt;sec&lt;/sup&gt;</td>
<td>selenocysteine esterified to transfer ribonucleic acid</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>Tm</td>
<td>temperature of melting</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>Txn</td>
<td>thioredoxin (reduced form)</td>
</tr>
<tr>
<td>Txn-1</td>
<td>thioredoxin-1 or cytosolic thioredoxin</td>
</tr>
<tr>
<td>Txn-2</td>
<td>thioredoxin-2 or mitochondrial thioredoxin</td>
</tr>
<tr>
<td>Txn-S&lt;sub&gt;2&lt;/sub&gt;</td>
<td>thioredoxin (oxidised form) / thioredoxin disulfide</td>
</tr>
<tr>
<td>Txnrd-1</td>
<td>thioredoxin reductase-1 (gene)</td>
</tr>
<tr>
<td>Txnrd-2</td>
<td>thioredoxin reducatase-2 (gene)</td>
</tr>
<tr>
<td>TxnRed</td>
<td>thioredoxin reducatse (enzyme)</td>
</tr>
<tr>
<td>TxnRed-1</td>
<td>thioredoxin reductase-1 or cytosolic thioredoxin reductase (enzyme)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TxnRed-2</td>
<td>thioredoxin reductase-2 or mitochondrial thioredoxin reductase (enzyme)</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>Val</td>
<td>valine</td>
</tr>
<tr>
<td>XD</td>
<td>xanthine dehydrogenase</td>
</tr>
<tr>
<td>XO</td>
<td>xanthine oxidase</td>
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**COMPOUND ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFC</td>
<td>7-amino-4-trifluoromethyl coumarin</td>
</tr>
<tr>
<td>Auranofin</td>
<td>2,3,4,6-tetra-o-acetyl-1-thio-β-D-glucopyranoside-S-Triethylphosphine gold</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNP</td>
<td>2,4-dinitrophenylhydrazine</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-dithio-2-nitrobenzoic acid</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>FA</td>
<td>formaldehyde agarose</td>
</tr>
<tr>
<td>4-HNE</td>
<td>4-hydroxynonenal</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>KP&lt;sub&gt;i&lt;/sub&gt;</td>
<td>potassium phosphate buffer</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-morpholino]propanesulfonic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>R1</td>
<td>N-methyl-2-phenylindole in acetonitrile</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TNB</td>
<td>5-thio-trinitrobenzoic acid</td>
</tr>
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</table>
CHAPTER 1

General Introduction
1.1 **CARDIOVASCULAR DISEASE**

Cardiovascular Disease (CVD) is the most common cause of morbidity and mortality in economically developed countries. In Australia, CVD is responsible for approximately 38% of all deaths, with coronary (or ischemic) heart disease accounting for 20% of all deaths in 2002 [1-3]. CVD is the most costly disease for the Australian health system, consuming 11.2% of the total health budget, with coronary heart disease costing an estimated $894 million per year [4]. This is a significant burden on the community, and as a result CVD has been identified as a “National Health Priority Area” requiring greater health promotion, prevention and research. Over the next 20 years these issues are expected to become more significant, as the risk of suffering from coronary heart disease for people over the age of forty has risen to one in two for men and one in three for women [1,5]. While these risk factors are increasing, there is also a growing number of elderly Australians among whom CVD is most common.

These statistics emphasize the importance of research into the prevention or intervention of CVD, and in particular coronary or ischemic heart disease. Whilst increasing numbers of patients with ischemic heart disease are treated with thrombolytic agents, coronary angioplasty, intra-coronary stents, and coronary bypass surgery, there is a growing need to understand the role of reactive oxygen species and antioxidant enzymes during ischemia and after reperfusion of these tissues, if recovery is to be achieved.

1.2 **MYOCARDIAL ISCHEMIA**

Ischemic heart disease accounts for approximately 80 deaths per day in Australia, and is believed to start developing during childhood [1-3]. There are many risk factors for ischemic heart disease including hyperlipidemia, hypertension, obesity, smoking,
diabetes, inactivity, family history, stress, age and sex, with the disease being more prevalent amongst males [1]. However, ischemic heart disease rates have decreased over the last 20 years probably due to changes in lifestyle caused by an increased awareness of risk factors [1].

Ischemic heart disease arises when there is an imbalance between the myocardial oxygen demand and blood supply. The wall of the myocardium contains its own vasculature known as the coronary circulation. Faulty functioning of this circulation, most commonly due to fatty atherosclerotic plaques or blood clots, causes a reduction in blood flow, and subsequently ischemia and/or myocardial infarction (MI) [6-9].

Atherosclerosis is characterized by the formation of lipid filled fibrous plaques that collect on the inner surface (tunica intima) of the coronary arteries [10]. Features of the disease process include fatty streaking of the intima, injury to the endothelial cells of the intima caused from mechanical stress and inflammation, migration of leukocytes and platelets to site of injury, smooth muscle proliferation, lipid accumulation and plaque maturation [10-14]. Eventually the plaque calcifies and connective tissue forms producing a narrowed, rigid blood vessel. This impairs or even blocks the blood flow. The maturation of the fibrous plaque is associated with a loss of cells through necrosis (cell death), and a weakening of the arterial lining [10]. As the disease progresses, the weak fibrous plaque can rupture or ulcerate which leads to thrombosis [10,15]. Over 90% of major myocardial infarctions are linked to clot formation where a plaque has ruptured [16]. Early symptoms of ischemic heart disease include angina that gradually increases in severity (or becomes unstable) as the disease progresses, leading to myocardial infarction, which typically begins after the artery is 60% occluded [16].

Ischemia usually progresses from hypoxia (reduced oxygen supply), and results in a condition in which the heart is unable to maintain its rate of cellular oxidation leading to metabolic imbalances [9,17,18]. Following total or partial occlusion of a coronary artery, metabolic and functional changes are initiated within seconds. These changes are initially of a reversible nature (myocardial stunning), however, if oxygen is deprived for an extended period of time, these changes progressively become more severe, leading to tissue damage and eventually irreversible injury (or infarction). Conversely, if the supply of blood is quickly restored, readmission of oxygen promotes the re-establishment of
normal aerobic metabolism [9,17,18]. Furthermore, the severity and progression of ischemia is not solely determined by the extent of oxygen deprivation but by many other factors including the relative accumulation of toxic metabolites. The reduction in blood supply during ischemia also limits the removal of these metabolites further contributing to the severe metabolic injury [9,18,19].

The main source of ATP in the heart muscle is oxidative phosphorylation via the respiratory chain located within the mitochondria. However, during ischemia the heart almost instantaneously undergoes several metabolic changes that allow for a reduction in oxygen demand and a more efficient utilization of substrates. This results in cardiomyocytes switching from aerobic fatty acid metabolism to anaerobic glucose metabolism. Under severe ischemic conditions, the sole source of glycolytic substrate becomes glycogen since there is no blood flow to deliver glucose to the tissue, which leads to a rapid breakdown of glycogen and an increase in the number of glucose transporters [9,19-23].

During ischemia the rate of glycolysis increases. This is believed to be important during ischemia because glycolytic ATP is located adjacent to the sarcolemma and sarcoplasmic reticulum (SR), allowing for preferential use in support of ion pumps (Na\(^+\)/K\(^+\) ATPase pump in the sarcolemma and Ca\(^{2+}\) ATPase pump in the SR). Furthermore, it has a role in maintaining membrane integrity and limiting ischemic contracture (which is characterised by the formation of rigor complexes between actin-myosin cross bridges due to a decrease in glycolytic ATP flux rate). Therefore, continued production of glycolytic ATP undoubtedly limits the degree of injury and determines the potential viability of the myocardium [9,17-19,24-30].

However, as ischemia becomes more severe, lactate and other reducing equivalents accumulate due to their decreased washout, and the myocardium becomes more acidic due to H\(^+\) generation. The decrease in intracellular pH, along with the accumulation of NADH, protons, lactate, succinate and alanine inhibits ATP production via inhibition of enzymes such as pyruvate dehydrogenase, phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase as well as inhibition of the malate-aspartate shuttle and the citric acid cycle. This ultimately depresses contractile function and alters ionic homeostasis [9,17-19,24-30].
Since the rephosphorylation of ADP to ATP occurs at a much slower rate during anaerobic glycolysis and therefore ischemia, the adenine nucleotide pool is also degraded during ischemia as the energy bond of ADP is captured via the action of adenylate kinase. In the process, AMP is formed which accumulates intracellularly and is degraded to adenosine. Adenosine is subsequently lost from the adenine nucleotide pool as it is catabolized to inosine and hypoxanthine, both of which potentially contribute to free radical generation [18,20,25,29].

Under conditions of energy depletion (i.e. during anaerobic metabolism), the cytosol and mitochondria of myocardial cells also become loaded with catabolites such as $\text{Na}^+$ and $\text{Ca}^{2+}$. As intracellular pH drops, the $\text{Na}^+/\text{H}^+$ exchanger (NHE) becomes progressively more active due to stimulation of the $\text{H}^+$ sensor site of the exchanger protein [31]. This results in intracellular accumulation of $\text{Na}^+$ since the $\text{Na}^+$ extrusion pathway ($\text{Na}^+/\text{K}^+$ ATPase pump) becomes inactivated due to decreased ATP levels [32, 33]. Accumulation of $\text{Na}^+$ can drive the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, removing $\text{Na}^+$ in exchange for extracellular $\text{Ca}^{2+}$, leading to a $\text{Ca}^{2+}$ influx and subsequent $\text{Ca}^{2+}$ overload. These ionic imbalances lead to inhibition of the $\text{H}^+$ electrochemical gradient required for energy production, altered permeability of the inner mitochondrial membrane (which causes it to swell and the permeability transition pore to open), and also contributes to the deleterious sustained contracture of the cell [9,18,34-36].

Another component of ischemic damage is caused by accumulation of substances such as $\text{K}^+$ and catecholamines in the extracellular space of acutely ischemic myocardium. Catecholamines are released after 10 or more minutes of ischemia, and the resulting increase in extracellular catecholamine concentration modifies the accumulation of extracellular $\text{K}^+$. This plays a pivotal role in the development of electrophysiological changes, which often leads to severe ventricular arrhythmia’s [17,37].

The release of catecholamines from cardiac nerve terminals during ischemia may also cause platelet aggregation within the ischemic myocardium and stimulate $\beta$-adrenoceptors. Stimulation of $\beta$-adrenoceptors within the heart leads to inotropic and vasodilatory responses unlike $\alpha$-adrenoceptor stimulation, which causes vasoconstriction [38].
Myocardial ischemia and the resultant metabolic derangements are shown in Figure 1.1. These changes are linked to various stages of cellular damage including the oxidation of proteins, loss of enzymes, cellular membrane disruption, mitochondrial and myofibril ultrastructural changes, depressed contractile function and ultimately cell death [9,20,25,39-41].

Fig 1.1: Cellular and metabolic alterations induced by ischemia [9].
Therapeutic interventions for patients with ischemic heart disease involve the reopening of occluded coronary arteries. Occlusion may be prevented pharmacologically with antihypertensive or anti-cholesterol agents, or if occlusion does occur, treated pharmacologically with anti-thrombolytics, vasodilators, inotropic agents, and in more severe cases with invasive procedures. It is well known that the salvage of ischemic tissue is most successful when interventions are initiated as soon as possible after vessel occlusion, resulting in the restoration of blood flow (reperfusion) to the affected myocardium. Reperfusion injury is cellular damage and dysfunction that occurs during the re-establishment of blood flow, and may possibly lead to lethal arrhythmias and/or the death of cells that were weakened but potentially functional prior to reflow [18,41-43].

Existence of reperfusion injury has clinical implications in the recovery of cardiac function to preischemic levels, particularly following invasive procedures such as the application of stents, coronary bypass surgery, coronary angioplasty, heart transplants or following MI. After MI left ventricular remodelling occurs as an adaptive process by which myocardium changes shape, size and function in response to stress. These deleterious adaptations include the formation of scar tissue and fibrosis in infarct zones through the proliferation of myofibroblasts and increased deposition of extracellular matrix components along with pathological hypertrophy. These changes contribute to increased myocardial stiffness, contractile dysfunction, reduced cardiac output, diastolic dysfunction, ventricular fibrillation and left ventricular failure which are seen after MI. Eventually this can lead to ventricular dilatation, heart failure and even sudden death [10,16,20,24,42,43]. If the mechanisms underlying reperfusion injury can be understood, there may be the possibility of improving recovery from these procedures through the application of specific interventions prior to or at the time of onset of reperfusion.

1.3 REPERFUSION INJURY

Reperfusion, without doubt is the most effective treatment for ischemic myocardium, and the more severe the ischemia, the earlier reperfusion is required to
ensure tissue survival. However late reperfusion has also been referred to as a “double-edged sword” in that it directly damages previously ischemic tissue, when in theory it should be cell-salvaging flow [18,44-46].

The existence of lethal reperfusion injury has been debated for years by researchers, and due to the technical difficulty in determining whether cell death is caused entirely by the ischemic insult or by reperfusion, the area is still controversial. It has however been suggested that ischemia essentially sets the stage for the damage induced by reperfusion, with ischemia causing cellular alterations that are necessary prerequisites for reperfusion injury to occur, but themselves are insufficient to cause cell death [18,20,46].

Injury of ischemic-reperfused myocardium is complex, involving injury of endothelial cells, vascular smooth muscle and conducting tissue as well as cardiomyocytes. Regional differences in metabolism and energy requirements render the endocardium most vulnerable to injury. For this reason, myocardial injury and tissue necrosis usually originate in the endocardium and with time migrate ‘as a wavefront of cell death’ towards the epicardial surface [18,44-46].

Ischemia-reperfusion in the myocardium generates both irreversible (lethal) injury, marked by necrotic or apoptotic tissue, and also reversible injury, manifested by contractile dysfunction (known as myocardial stunning). Immediate lethal reperfusion injury (caused by necrosis) occurs immediately upon reperfusion and can be distinguished from delayed lethal reperfusion injury, which may be delivered to the cardiomyocytes by activated neutrophils or from the induction of apoptosis [18,34,45-48].

1.4 MECHANISMS OF REVERSIBLE ISCHEMIA-REPERFUSION INJURY

i) MYOCARDIAL STUNNING

The delayed recovery of viable myocytes after reperfusion is known as
myocardial stunning. This is the mechanical dysfunction that persists despite the absence of irreversible damage, and despite restoration of normal (or near normal) coronary flow. Myocardial stunning was first described in 1975 by Heyndrickx et al who reported prolonged contractile dysfunction in the absence of necrosis, following brief coronary occlusion [49]. After initially being thought to be a syndrome resulting from experimental conditions, it was given the term “myocardial stunning” in 1982 by Braunwald and Kloner [50].

Myocardial hibernation is characterized by persistent or chronic stunning, and is associated with reduced coronary flow but preserved myocardial viability. This phenomenon is believed to be an adaptive response of the heart to low flow, whereby oxygen demands are down-regulated to the point where reduced oxygen can be tolerated for extended periods of time without cell death, and once flow is restored the dysfunction is reversed. These conditions are observed clinically as depressed ventricular function that eventually recovers to normal or near normal function over a period of hours to days. It is not uncommon to observe myocardial stunning in postoperative patients, particularly those who have undergone coronary bypass surgery, valve replacement or heart transplant. Stunned myocardium is however responsive to inotropic agents such as dopamine, adrenaline and calcium making it possible to manage stunning [47-53].

Whilst myocardial stunning is defined by a mild or sublethal injury, it is still not known whether stunning and necrosis share the same mechanisms. Several different hypotheses have been proposed for myocardial stunning, however the generation of reactive oxygen species (ROS) and disturbance of Ca^{2+} homeostasis appear to be the most likely instigators [54-58]. These hypotheses, summarised below in Figure 1.2, may not be mutually exclusive, and may actually represent different facets of the same pathophysiological cascade.

The generation of ROS in stunned myocardium has been directly demonstrated using spin traps, and further evidence implicating ROS as a cause of stunning is attenuation of contractile dysfunction following the attenuation of ROS generation [57-61]. By nature, ROS can non-specifically attack virtually any cellular targets, and can lead to protein oxidation, enzyme inactivation as well as membrane peroxidation [52,62].
ROS have also been shown to disrupt sarcolemma, and more specifically, ATPase Ca\textsuperscript{2+} transport activity [54,63]. In addition, ROS have also been demonstrated to interfere with the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, and to inhibit the Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity [54,64-66]. Inhibition of Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity results in Na\textsuperscript{+} overload, which subsequently leads to activation of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger and Ca\textsuperscript{2+} overload [34,67]. ROS may also reduce the responsiveness of myofilaments to Ca\textsuperscript{2+} by damaging contractile proteins. Evidence exists showing exposure of myofilaments to superoxide anions results in a dose-dependent reduction in maximal Ca\textsuperscript{2+} -activated force, without alterations in Ca\textsuperscript{2+} sensitivity [54,68,69]. Furthermore, ROS are also reported to impair sarcoplasmic reticulum function, which may be detrimental given that recent studies have highlighted a role for SR modulation in stunning [54,70-72]. Although the exact mechanism by which ROS depress contractile function remains to be elucidated, these studies do support a role for ROS in the genesis of myocardial stunning. Moreover, these studies also suggest that ROS injury is not the sole mediator of stunning, and it would appear that much of the ROS hypothesis involves ROS disruption of Ca\textsuperscript{2+} homeostasis.

Numerous studies have shown that Ca\textsuperscript{2+} availability is not limited in stunned myocardium despite a decreased in contractile force [48,54,73-75]. This therefore suggests that the contractile dysfunction observed in myocardial stunning occurs not due to reduced availability of Ca\textsuperscript{2+} but rather impaired responsiveness of myofilaments to Ca\textsuperscript{2+}. Today there are three proposed mechanisms that involve alterations in Ca\textsuperscript{2+} homeostasis in myocardial stunning; firstly, excitation-contraction uncoupling arising due to sarcoplasmic reticulum dysfunction; secondly, decreased responsiveness of the contractile apparatus to Ca\textsuperscript{2+}; and thirdly, Ca\textsuperscript{2+} overload [54,76].
As summarised above, it is most probable that ROS generation, Ca\(^{2+}\) overload and decreased myofilament Ca\(^{2+}\) responsiveness all have individual and overlapping roles in the pathogenesis of myocardial stunning [54]. It is also possible that ROS generation is the principle “initiator” of myocardial stunning whilst decreased Ca\(^{2+}\) responsiveness of myofilaments is the principle “mediator” of myocardial stunning, as suggested by Shattock [53].
1.5 MECHANISMS OF IRREVERSIBLE ISCHEMIA-REPERFUSION INJURY

The major indicator of the transition from reversible to irreversible ischemia-reperfusion injury is the release of intracellular cardiac enzymes (such as creatine kinase, troponin-T and lactate dehydrogenase) into the circulation. This reflects major cellular membrane damage and/or death of cardiomyocytes (specifically by necrosis) [77-79].

Experimental studies have shown that myocytes undergo cell death during ischemia and reperfusion by two mechanisms: necrosis (or oncosis) and apoptosis.

Necrosis, or ‘cell murder’ is often described as “accidental collapse of cellular homeostasis, compartmentalisation and cell membrane integrity with the release of cytosolic material and with random nuclear DNA fragmentation” [80]. Apoptosis on the other hand is considered to be ‘cell suicide’ and has two major morphological differences from necrosis: firstly cell shrinkage with a functionally intact membrane, and secondly, nuclear condensation (which consists of chromatin condensation then nuclear fragmentation) [20,25,40,41,80,81]. These characteristic signs of apoptosis appear in ischemic myocardium after only short periods of ischemia, and may result from activation of killer genes, death programs, mitotic catastrophe or the effects of reactive oxygen species. As apoptosis is an integral part of a cell’s developmental program, and is frequently the end-point of a temporal course of cellular events, it is difficult to say why a particular cell will undergo apoptosis. It has been suggested however that it is not oxygen free radical mediated damage itself which results in apoptosis, but rather an oxidative shift in the cellular redox state which modifies the nature of the stimulatory signal, and ultimately results in cell death rather than proliferation. A similar theory has been postulated as to why a particular cell undergoes apoptosis rather than necrosis [18,20,25,40,41,80,81].

Whilst the process of necrosis and apoptosis may differ in a number of ways, and may appear to proceed along separate paths, apoptosis and necrosis are unlikely to be two separate, mutually exclusive forms of cell death, and it is becoming increasingly evident that there is overlap or cross-over between these two types of cell death [80,82,83].
i) IMMEDIATE LETHAL REPERFUSION INJURY

Immediate lethal reperfusion injury occurs immediately upon reperfusion of ischemic myocardium (i.e. within the first few minutes to first few hours of reperfusion), and usually manifests itself as necrosis. Necrotic cell death, marked by the release of intracardiac enzymes (such as creatine kinase and troponin-T), is believed to be responsible for the cell death observed in permanently ischemic tissue [77-79]. The end point of this injury is mechanical disruption of the sarcolemma, which is most likely initiated by severe hypercontracture of the myofibrils.

The potential initial causes of immediate reperfusion injury are shown in Figure 1.3, and include: i) rapid normalization of tissue pH; ii) rapid normalization of tissue osmolality; and most importantly iii) re-energisation (oxygen and calcium paradox); and, iv) oxygen radical generation [34,84,85]. These potential initial causes are not entirely independent, and when they occur in conjunction with one another, mechanical disruption of the sarcolemma, hypercontracture of myofibrils and subsequent immediate lethal reperfusion injury appears to be the endpoint [34].
Fig 1.3: Potential initial causes of immediate lethal reperfusion injury (modified from Piper et al.[34]).
Tissue pH

Rapid normalization of tissue pH is the first potential cause of immediate lethal reperfusion injury (Fig 1.4). After prolonged ischemia the cytosolic pH is markedly lowered because anaerobic metabolism and the breakdown of ATP produce an excess of H⁺, resulting in acidification of both the intracellular and interstitial spaces. Upon reperfusion the pH in the interstitial space is rapidly normalized, and a gradient is therefore generated between the cytosol (as it still contains a high H⁺ concentration) and the interstitium. This causes activation of the Na⁺/H⁺ exchanger and the Na⁺/HCO₃⁻ symporter which are required for the extrusion of H⁺ from cardiomyocytes. As a result intracellular acidosis is reduced and there is an influx of Na⁺ into the cytosol [34,86-88].

This rapid removal of excess H⁺ from the re-oxygenated cell may however remove a potentially protective agent. Intracellular acidosis may be beneficial during ischemia as it impairs the contractile machinery, thereby aiding in the prevention of hypercontracture if maintained during early reperfusion. However, high intracellular acidosis leads to excess Na⁺ which can be detrimental as it may lead to a secondary activation of the Na⁺/Ca²⁺ exchange mechanism transporting Na⁺ out and Ca²⁺ into the cell, enhancing the pre-existing Ca²⁺ overload in cells [34,88,89]. This rapid removal of H⁺ and secondary uptake of Ca²⁺ thus favours the development of hypercontracture if ischemia-reperfused cardiomyocytes are allowed to restore a normal intracellular acid-base balance.
Fig 1.4: The effect of rapid normalisation of intracellular $H^+$ concentration in cardiomyocytes upon reperfusion (modified from Piper et al [34]).
**Tissue Osmolality**

Rapid normalisation of tissue osmolality is the second potential cause of immediate lethal reperfusion injury. Ischemia creates an intracellular and extracellular increase in osmotic load due to accumulation of Na\(^+\) and the end products of anaerobic metabolism. Reperfusion normalizes the extracellular osmolality by washing out these excess molecules, creating a transsarcolemmal osmotic gradient across the membrane. This ultimately leads to a cellular uptake of water and an increase in intracellular pressure and mechanical stretch [34,89-92].

During ischemia-reperfusion there is also an increase in sarcolemmal fragility. This results from changes in membrane proteins and sarcolemmal-cytoskeleton anchorage, as well as alterations in the lipid composition of the membrane caused by an increased phospholipid turnover during ischemia, and oxygen radical generation during reperfusion. Under normal conditions cell swelling isn’t capable of disrupting the sarcolemma, however this increase in cell fragility in conjunction with the mechanical stress produced by cell swelling results in rupture of the sarcolemma and cell deterioration as is displayed in *Figure 1.5* [34,90,93-95].
Fig 1.5: Cell swelling and rupture as a result of normalisation of tissue osmolality following reperfusion of ischemic myocytes (modified from Piper et al [34]).
Calcium Paradox

Whilst other pathways may provide the means (i.e. Ca\(^{2+}\) overload), it is the second pathway which leads to the generation of sustained hypercontracture brought about by this loss of calcium homeostasis. Cytosolic calcium overload may result from impairment of energy-requiring calcium extrusion processes, dysfunction in Na\(^+\)/Ca\(^{2+}\) exchangers or from gross disruption in cellular membranes. The reactivation of oxidative phosphorylation in the mitochondria not only supplies energy to the cation pumps, but also to the myofibrillar contractile elements, and under these conditions this is deleterious for the reoxygenated cell [20,25,34,96,97]. This is illustrated in Figure 1.6 below.

Upon reperfusion excess cytosolic calcium is taken up by intracellular compartments. However, abnormalities in calcium ion re-uptake into the sarcoplasmic reticulum causes excessive calcium to be sequestered within the mitochondria, significantly affecting ATP formation [20,24,25,96]. Depletion of ATP further disrupts cytosolic calcium regulation and excitation-contraction coupling, and causes a loss of cellular energy and acidosis thereby creating a vicious cycle [20,96,97].

Pathological increases in cytosolic calcium activate proteases, lipases and phospholipases, which all increase membrane and protein destruction, and result in increased intracellular enzyme release. Contractile machinery is also activated with the actin-myosin crossbridges becoming irreversibly deformed by the cell's inability to relax. Hypercontracture of adjacent cells then leads to mutual cellular disruptions and necrosis [9,20,34,96-98].
Oxygen (or re-energisation) Paradox

The injury provoked by restoration of oxygen and energy to the ischemic myocardium has been termed ‘oxygen paradox’[25,34,42] However researchers have long debated whether “the oxygen paradox represents a genuine reoxygenation injury or just a dramatic manifestation of injury that had developed during the oxygen depletion period”[42].

The resupply of oxygen to the mitochondria provides a source for free radical production (discussed later), and reactivates oxidative phosphorylation providing energy to the cation pumps which initiates recovery of the cellular cation balance. This also supplies the myofibrillar elements with energy inducing contractile activation possibly leading to hypercontracture [34,99]. Under conditions of energy depletion (i.e. in ischemic or hypoxic myocardium), the cytosol of the myocardial cells becomes loaded with Na⁺ and Ca²⁺ which leads to sustained contracture of the cell. Recovery of energy production under these conditions can promote hypercontracture via one of two pathways that are activated simultaneously (Fig 1.6) [42].

The first pathway promotes cell recovery by reactivating two major cation pumps, the Ca²⁺ (Ca²⁺-ATPase) of the sarcoplasmic reticulum and the Na⁺ pump (Na⁺/K⁺-ATPase) of the sarcolemma [34,96,99,100]. This produces a continual cyclic effect where excess cytosolic Ca²⁺ release and re-uptake by the sarcoplasmic reticulum occurs. These cycles come to an end only if the major mechanism for Ca²⁺ extrusion from the cytosol is sufficiently activated, i.e. the Na⁺/Ca²⁺ exchanger. The ability of this exchanger to remove Ca²⁺ depends on the magnitude of the Na⁺ gradient across the sarcolemma, with a large gradient being the pre-requisite for Ca²⁺ extrusion [34,96,99,100]. Since Na⁺ has built up in the cytosol during ischemia (and for the extrusion of Ca²⁺) it is also essential that the Na⁺-ATPase pump of the sarcolemma is rapidly reactivated to remove excess Na⁺ from the interior of the cell [34,96,99,100].
Fig 1.6: Schematic diagram of the major cation transport mechanisms activated upon reoxygenation of the myocyte. On reactivation of oxidative phosphorylation in the mitochondria, energy is supplied to the cation pumps and results in an overload of calcium in the sarcoplasmic reticulum. At the same time, energy is supplied to the contractile machinery, and in the presence of high cytosolic calcium this leads to hypercontracture (modified from Piper et al [34]).
Free Radical (Oxidant) Injury

There is substantial evidence that reactive oxygen species (ROS) are generated during myocardial ischemia-reperfusion. Furthermore, there is increasing evidence that this is a major cause of both immediate and delayed reperfusion injury, and that ROS contribute to the oxygen paradox. This free radical induced injury will be discussed in detail below.

1.6 GENERATION OF REACTIVE OXYGEN SPECIES DURING MYOCARDIAL ISCHEMIA-REPERFUSION

A free radical is simply any organic or inorganic molecule or atom that contains an unpaired electron. This electron configuration renders free radicals highly unstable and chemically reactive. Whilst free radicals possess an extremely short half-life, making them difficult to study, there is strong evidence for roles of free radicals in a wide variety of diseases and degenerative conditions including cardiac pathologies, cancer, rheumatoid arthritis, inflammation and ageing [20,40,44,57,101-104].

The oxygen centred free radicals are the most frequently occurring species in biological systems. This is because the molecular oxygen required for aerobic metabolism has a strong affinity for electrons, and as a result, the partial reduction of oxygen leads to the formation of reactive oxygen intermediates [20,40,44].

In the myocardium, the reduction of oxygen to water proceeds by two pathways. The major pathway that accounts for 95% of the oxygen consumption of tissues involves the tetravalent reduction of oxygen by the mitochondrial cytochrome oxidase, and is devoid of any intermediates. The remaining 5% of oxygen is reduced through a univalent pathway in which several intermediates are produced. These intermediates include superoxide anions (O$_2$$^\cdot$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^\cdot$) and singlet oxygen (O$_2^\cdot$). These are all activated oxygen species and are collectively known as...
reactive oxygen species (ROS). Generally ROS are all oxidants, but by definition only \( \text{O}_2^\cdot \), \( \text{HO}_2^\cdot \) and \( \text{OH}^\cdot \) are free radicals [23,40,44,102,103]. The step wise reduction of molecular oxygen via 1-electron transfers can be summarised as follows:

\[
\begin{align*}
\text{e}^- & \rightarrow \text{O}_2 \rightarrow \text{O}_2^\cdot \rightarrow \text{H}_2\text{O}_2 \rightarrow \text{OH}^\cdot + \text{OH}^- \rightarrow 2\text{H}_2\text{O}_2 \\
2\text{H}^+ & 
\end{align*}
\]

The superoxide anion is comparatively unreactive and in physiological conditions is converted, by dismutation, to hydrogen peroxide. This is less reactive, longer lived and more lipophillic than the superoxide anion, and as a result can diffuse considerable distances from its site of generation. The critical problem with increased hydrogen peroxide production is the generation of the hydroxyl radical Haber-Weiss or Fenton reactions as can be seen below:

\[
\begin{align*}
\text{H}_2\text{O}_2 + \text{Cu}^+ & \rightarrow \text{OH}^\cdot + \text{OH}^- + \text{Cu}^{2+} \quad \text{(Fenton reaction)} \\
\text{H}_2\text{O}_2 + \text{Fe}^{2+} & \rightarrow \text{OH}^\cdot + \text{OH}^- + \text{Fe}^{3+} \quad \text{(Haber-Weiss reaction)}
\end{align*}
\]

The hydroxyl radical has a very short half–life, but is extremely reactive and will rapidly react with unsaturated fatty acid side chains resulting in lipid peroxidation and disruption of cell membranes [12,40,44,57,105].

Another group of ROS generated during ischemia-reperfusion are the reactive nitrites and nitrates. Peroxynitrite, peroxynitrous acid and nitrogen dioxide are generated from an iron-independent reaction involving the interaction of \( \text{O}_2^\cdot \) and nitric oxide as shown in the following equations [106-107]:

\[
\begin{align*}
\text{NO} + \text{O}_2^\cdot & \rightarrow \text{ONO}^- \quad \text{(peroxynitrite anion)} \\
\text{ONO}^- + \text{H} & \leftrightarrow \text{ONO}^- \quad \text{(peroxynitrous acid)} \\
\text{ONO}^- & \leftrightarrow \text{OH}^\cdot + \text{NO}_2 \quad \text{(nitrogen dioxide)}
\end{align*}
\]
During reperfusion a large burst of NO is produced, and during the same time frame large amounts of \(O_2^*\) are also generated [107]. The overwhelming affinity of NO and \(O_2^*\) ensures their rapid reactivity during the initial moments of reperfusion to form ONOO\(^-\) [107].

There are many possible sources of ROS production in the myocardium, and of greater interest is the fact that during ischemia when oxygen availability is decreased, ROS production can still occur. This issue becomes more complex when you take into consideration that oxygen-derived free radicals may be produced from different sources after different periods of ischemia and reperfusion. The mitochondria, myocardial cell membranes and endothelial cells are all potential sites of free radical production [12,40,44,101-103,106-109].

The major source of superoxide in postischemic tissue is the enzyme xanthine oxidase. Whilst it is widely distributed among tissues, it appears to be somewhat species specific, and is not present in the myocardium of humans or rabbits. It is however present in their endothelial cells and may therefore be a source of free radical generation. Although humans and rabbits lack xanthine oxidase (XO) in their cardiomyocytes, XO may still contribute to some superoxide production in their hearts through its presence in endothelial cells of the coronary circulation [101,109,110].

This enzyme is synthesised as xanthine dehydrogenase (XD), and is converted to XO by proteases. During ischemia there is a loss of ionic homeostasis, and as a result cytosolic \(Ca^{2+}\) concentrations increase. This calcium overload activates proteases capable of converting the dehydrogenase to the oxidase. At the same time depletion of the cells ATP results in elevated levels of AMP which is catabolized to adenosine, inosine and then hypoxanthine. Hypoxanthine is an oxidizable purine substrate for xanthine oxidase. The remaining substrate required is oxygen, which is supplied by residual oxygen reserves in the mitochondria, or by reperfusion, as is shown below in Figure 1.7. This ultimately results in a burst of superoxide and hydrogen peroxide production [40,44,101,103,108-112].
Fig 1.7: Cellular mechanisms for reactive oxygen species generation during ischemia-reperfusion [112].
Degradation of the adenine nucleotide pool also leaves the mitochondrial electron carriers in a reduced state. This leads to an increase in electron leakage from the respiratory chain, that in turn reacts with the residual oxygen trapped in the inner membrane of the mitochondria to produce superoxide anions [40,44,102,111]. Ubisemiquinone and ubiquinol are the main sources of mitochondrial \( \text{O}_2^{\cdot -} \), although nicotinamide adenine dinucleotide dehydrogenase (NADH) and dehydroorotate dehydrogenase also produce \( \text{O}_2^{\cdot -} \). Amine oxidase, located in the outer mitochondrial membrane is another source of mitochondrial ROS, however it produces hydrogen peroxide [40,44,102,111].

Other potential sources of oxygen radicals include superoxide production from the cyclooxygenase pathway of arachidonic acid metabolism, adrenochrome formation from the autooxidation of catecholamines and superoxide production by activated neutrophils [40,44,101-103,113].

Calcium activation of phospholipases degrades cell membrane phospholipids which in turn releases arachidonic acid. This is subsequently metabolised to prostaglandins and leukotrienes via the cyclooxgenase and lipoxygenase pathways during ischemia. However these metabolic pathways involve electron transfers that can also initiate the formation of free radicals [40,44,103].

Increased plasma levels of catecholamines during stressful events such as ischemia, can lead to oxidation products consisting of quinone derivatives as well as electrons leading to the formation of ROS and potentially myocardial damage [113]. Furthermore, auto-oxidation of catecholamines, which are abundantly released from the ischaemic myocardium, could provide ROS through the formation of adrenochromes[40,44,103,113].

Neutrophil migration to injured tissue during reperfusion is another potential source of reactive oxygen species. Neutrophils infiltrate because of the chemotactic activity of substances involved in the inflammatory response to ischemia-reperfusion. They then become activated and adhere to damaged endothelium where they initiate further damage through the generation of oxygen free radicals. Neutrophils generate reactive oxygen species via activation of NADPH oxidase on their cell membrane, which reduces molecular oxygen to superoxide anion while at the same time oxidizing NADPH
to NADP⁺. In addition, myeloperoxidases found within the granules of the neutrophil can convert hydrogen peroxide to the toxic hypochlorous acid (HOCl) [40,102,114].

Although neutrophils may be an important source of free radicals in certain systems, it is clear that their presence isn’t always necessary for free radical generation during ischemia-reperfusion of myocardium. This has been proven by numerous studies in which oxygen free radicals have been detected in in vitro models using isolated hearts that were perfused with neutrophil-free solutions. This also suggests that the initial burst of free radicals within the first few minutes of reperfusion isn’t from neutrophils, and that they don’t become an important source of free radicals until later in reperfusion (delayed reperfusion injury) [40,102,115].

1.7 OXIDATIVE DAMAGE CAUSED BY REACTIVE OXYGEN SPECIES

Oxidative stress is said to occur when there is “an increased generation of superoxide anion and hydrogen peroxide (ROS) which overwhelms the normal cellular defence mechanisms”[20], and this results in oxidative damage. By nature ROS can attack virtually all cellular targets nonspecifically. In addition to altering membrane integrity and permeability, ROS also denature proteins causing a loss in normal enzyme activity. As well as this they interfere with the sarcoplasmic reticulum calcium transport and potentiate inflammatory responses by acting as chemotactic agents. Changes in cellular oxidative status can alter signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation, and even regulation of the cell cycle. [21,102,114-118].

One of the main targets of OH• and ONOO⁻ are polyunsaturated fatty acids of membrane phospholipids leading to a chain reaction involving the formation and propagation of lipid radicals [112,113,117,119-121]. The process is initiated when free radicals abstract hydrogen from polyunsaturated fatty acids to form fatty acid radicals with a carbon-center [117,119-121]:

\[
\text{Lipid-H} + \text{OH}^{\bullet} \rightarrow \text{H}_2\text{O} + \text{Lipid}^{\bullet}
\]
Lipid peroxyl radicals (Lipid-OO•) are formed by the molecular rearrangement of lipid• when they react with O₂ to form a conjugated diene:

\[
\text{Lipid}^• + O_2 \rightarrow \text{Lipid-OO}^•
\]

These biologically active lipid peroxy radicals are then capable of reacting with other lipids (extracting H+), proteins, or nucleic acids and thereby propagate the transfer of electrons and subsequent oxidation of various biological molecules [117-119-121]:

\[
\text{Lipid-OO}^• + \text{Lipid-H} \rightarrow \text{Lipid-OOH + Lipid}^•
\]

Peroxidation of membrane lipids alters membrane fluidity, impairs the function of membrane proteins and alters membrane permeability. This contributes to the calcium overload in ischemia-reperfusion, and ultimately leads to the death of cardiomyocytes.

ROS generation during the early stages of reperfusion are also believed to be involved in reperfusion arrhythmias. Lipid peroxidation of myocardial cell membranes has been implicated as a potential cause of ventricular fibrillation, tachycardia and premature beating, which are all associated with reperfusion. [21,102,116-118].

As discussed earlier, ROS have also been implicated in contractile dysfunction and hypercontracture. They have been shown to disrupt sarcolemma and ATPase Ca²⁺ transport activity, in addition to interfering with the Na⁺/Ca²⁺ exchanger, and inhibiting the Na⁺/K⁺ ATPase activity [54,63-66]. Inhibition of Na⁺/K⁺ ATPase activity results in Na⁺ overload, which subsequently leads to activation of Na⁺/Ca²⁺ exchanger and Ca²⁺ overload [34,67]. Furthermore, ROS may reduce the responsiveness of myofilaments to Ca²⁺ by damaging contractile proteins, and they have also been reported to impair sarcoplasmic reticulum function. [54,68,69]. Although the exact mechanism by which ROS depress contractile function, or initiates hypercontracture remains to be elucidated, these studies do support a role for ROS in the genesis of this dysfunction.

Both endothelial cells and vascular smooth muscle cells are capable of generating ROS from various enzymatic sources. Hydrogen peroxide has recently been recognised as a second messenger regulating the proliferation of various mammalian cells. At low concentrations it can stimulate endothelial cell proliferation and migration, regulate smooth muscle tone and mediate endothelial-dependent relaxation in some vascular beds. However in disease states such as atherosclerosis and hypertension, vascular production of these ROS increases substantially to cytotoxic levels. Oxidative stress has also been
implicated as a mediator of endothelial dysfunction, both in the absence or presence of blood [122-127]. ROS generated on reperfusion can potentially inactivate NO, reduce NO-synthase activity, and attenuate agonist-stimulated NO release, all of which contributes to reduced coronary flow [128-132]. Furthermore, the proximity of endothelial cells to sites of reoxygenation and subsequent ROS generation renders them particularly susceptible damage.

Numerous studies have found free radicals to be involved in apoptosis or ‘programmed cell death’ in a number of different ways. Apoptosis eliminates cells no longer necessary, or altered, and plays an important role during embryonic development, in tissue remodeling and in the host defence system. One model proposed for free radical induction of apoptosis is up-regulation of the Fas-FasL system leading to activation of caspase-8. Caspase-8 activates caspase-3 that in turn activates other caspases downstream which ultimately cleave various substrates. One of the substrates is a caspase-dependent endonuclease in the cytoplasm which can subsequently enter the nucleus where it cuts DNA into oligonucleosomal fragments [133-136]. Hydrogen peroxide can also cause the release of cytochrome c from the mitochondria into the cytoplasm. Here cytochrome c binds to apoptotic protease activating factor-1, Apaf-1, to form a apoptosome. The apoptosome complex activates caspase-9 which then activates caspase-3 and so the caspase cascade continues. ROS also activate nuclear transcription factors such as NFκB and AP-1 which may up-regulate death proteins or produce inhibitors of survival proteins. Oxidative damage to DNA also leads to an increased transcription of genes which assist DNA repair, resulting in increased levels of p53 protein and increased ADP-ribosylation. Both of these aid DNA repair and prevent progression through the cell cycle until DNA repair is complete. However if there is excessive DNA damage, gene transcription increases and the apoptotic pathways are induced. This is believed to be a survival mechanism of organisms so that individual cells are sent to death to prevent mutagenic replication and cancer [40,133-136].

Mitochondrial damage arising from oxidative stress also allows for calcium release from it into the cytosol. Here calcium activates phospholipases resulting in the release of arachidonic acid from membranes. Oxidized lipids from cell membranes, especially hydroperoxyeicosatetraenoic acids (HPETEs), are also capable of directly
inducing apoptosis. HPETEs are oxygenated derivatives of arachidonic acid and have been implicated in TNF-mediated apoptosis [40,133,134,137,138]. It is also possible that intracellular ROS such as hydrogen peroxide activate the genes responsible for apoptosis through an oxidative stress-responsive nuclear transcription factor or down-regulate anti-death genes such as \( Bcl-2 \). Decreases in \( Bcl-2 \) also causes a decrease in mitochondrial membrane potentials leaving cells more sensitive to TNF which can also induce apoptosis [40,134,139].

To make the link between apoptosis and oxidative stress even more complex, when cells do undergo apoptosis they too hyperproduce superoxide anions, therefore suggesting that apoptosis can also induce oxidative stress [40,138,140].

Oxidative stress has also been implicated in the opening of the mitochondrial permeability transition pore (MPTP). The primary role of mitochondria in healthy cells in the provision of ATP to support cell function. However, they also play a critical role in initiating both apoptotic and necrotic cell death. A major player in this process is the mitochondrial permeability transition (MPT) [141-143]. The phenomenon of MPT refers to the massive swelling, depolarisation and uncoupling of mitochondria that occurs under certain conditions, most notably as a result of supraphysiological matrix concentrations of \( \text{Ca}^{2+} \) and oxidative stress. The cause of MPT is the opening of a non-specific pore in the inner mitochondrial membrane, the MPTP [141-144]. Opening of the MPTP is known to be a result of a conformational change in the membrane protein adenine nucleotide translocase (ANT), whose normal function is to catalyse the transport of ADP into and ATP out of the mitochondria [141,145,146]. The selective permeability of the mitochondrial inner membrane is essential for the maintenance of the membrane potential and pH gradient that drive ATP synthesis during oxidative phosphorylation. Therefore, when conditions are optimal for MPTP opening, mitochondria become depolarized and the proton-translocating ATPase actively hydrolyses ATP rather than synthesizing it [141-144]. If MPTP opening is extensive and prolonged, mitochondria remain depolarized and are unable to produce the ATP required for maintaining ionic homeostasis and repairing tissue damage. This inevitably leads to necrotic cell death. In addition to its role in necrosis, opening of the MPTP releases cytochrome c and other pro-
apoptotic molecules from the mitochondria, which go on to activate the caspase cascade leading to apoptosis [141,144,147,148].

It has been proposed that opening of the MPTP plays a major role in reperfusion injury since the conditions required for MPTP opening are those created during ischemia-reperfusion [142,148]. Studies by Kerr and colleagues have shown a strong correlation between the amount of MPTP opening and the degree to which the heart recovers after ischemia-reperfusion, and that full recovery is possible providing the MPTP closes within minutes of opening [149]. Calcium is known to start accumulating during ischemia, and upon reperfusion excess calcium is taken up by intracellular compartments. Several factors are known to increase the sensitivity of the MPTP to calcium, including oxidative stress, adenine nucleotide depletion and increased inorganic phosphate concentrations, all of which occur during ischemia-reperfusion [141,142,144,148,150,151]. Furthermore, the ANT is known to have three cysteine residues that show differential reactivity towards various oxidizing agents in a conformational dependent manner. Oxidative stress leads to the cross-linking of these cysteines and activation of MPT. In addition, oxidative stress also displaces adenine nucleotides from the ANT, which enhances the sensitivity of the pore to calcium [141, 152-154]. These suggest that oxidative stress plays a role in MPT, which is a critical factor in mediating reperfusion injury, and is not only associated with contractile dysfunction and ionic imbalances, but also cell death.

From all of the above it may be postulated that ROS production and oxidative stress are a major cause of the cellular dysfunction and death that occurs during ischemia-reperfusion. Therefore, inhibition of ROS generation, or the scavenging of them once they are produced may be a possible way to protect tissue from ischemia-reperfusion induced damage. This will be the main focus of this thesis.

1.8 MYOCARDIAL ANTIOXIDANT DEFENCE SYSTEMS

In normal physiological conditions, oxygen free radical production is usually kept
under homeostatic control by endogenous free radical scavengers known as antioxidants. A complex system of antioxidant proteins have evolved in eukaryotes indicating the importance of maintaining redox balance within cells. Normally these antioxidant enzymes are only found in the mitochondria or cytosol. However, during ischemia there is a loss of antioxidant enzyme function, together with leakage of these enzymes into the extracellular fluid. On reperfusion, the enzymes are then washed out, further depleting the available control over free radical production, and as a result, the unbalanced burst of free radicals on reperfusion (‘respiratory burst’) easily overwhelms the available counteractive enzymes so control of reactive oxygen species generation is lost [20].

Although the exact mechanisms and interactions among various antioxidants are not fully understood, it is possible that one antioxidant may “equilibrate with another to establish a cellular redox potential” and that all endogenous antioxidants may work in conjunction with one another to protect against oxidative stress [110]. It is also possible that certain antioxidants act as a first line of defence against oxidative stress in ischemia-reperfusion while other antioxidants may only act later on during severe oxidative stress [110]. There are many mechanisms through which antioxidants may act such as: (1) scavenging reactive oxygen species or their precursors, (2) inhibiting the formation of reactive oxygen species, (3) attenuating the catalysis of reactive oxygen species generation via binding to metal ions, (4) enhancing endogenous antioxidant generation and (5) reducing apoptotic cell death by up-regulating the anti-death gene Bcl-2 [110,139].

Many substances have been suggested to act as endogenous antioxidants, and in the heart these include the enzymes superoxide dismutase (SOD), catalase, glutathione peroxidase, thioredoxin peroxidase plus non enzymatic antioxidants such as vitamin E, ascorbic acid, vitamin A, coenzyme Q10 and cysteine.

The longest known biological antioxidant is the lipid soluble vitamin E (α-tocopherol), and it has been identified in both cell membranes and lipoproteins. Vitamin E acts as a potent peroxyl radical scavenger and protects against lipid peroxidation of polyunsaturated fatty acids by acting as a chain breaker. It works synergistically with ascorbic acid (vitamin C), which can react with tocopheroxyl radicals to regenerate vitamin E. Ascorbic acid radicals are then reduced by NADH reductase. Due to its
lipophillic nature, vitamin E is likely to work within membranes whereas the watersoluble vitamin C is likely to work in the cytosol or extracellular fluid as an electron transport system [40,44,110,155-157]. Vitamin A, or β-carotene is present in plasma, and also serves as an antioxidant by inhibiting the oxidation of LDL [110,158].

Coenzyme Q10 (CoQ10) in its fully reduced form (ubiquinol) is a powerful antioxidant. In the mitochondria CoQ10 is a component of the respiratory electron transfer chain where it transports electrons from NADH and succinate dehydrogenase to the cytochrome system, as well as moving protons across the inner membrane coupling respiration to oxidative phosphorylation [159,160]. Mitochondrial free radical generation is increased during ischemia-reperfusion, with specific segments of the respiratory chain being primarily responsible. The lipid soluble ubiquinol is however located at the main sites of this oxygen radical generation making it a very useful antioxidant. There are two mechanisms for CoQ10 antioxidant functions: 1) by providing hydrogen equivalents to reduce peroxyl and/or alkoxyl radicals preventing both the initiation and propagation of lipid peroxidation, and 2) regenerating the reduced form of vitamin E from the tocopheroxyl radical. Ubiquinol is also continuously regenerated from the semiquinone radical by complex III providing an endless supply of antioxidant activity at a major site of radical generation [160-164].

N-acetylcysteine (NAC) is another naturally occurring antioxidant which has been researched as a possible protective agent in the myocardium. NAC is a sulfhydryl group donor which is easily transported into cells where it is deacetylated to cysteine resulting in an increased thiol pool (primarily reduced glutathione) and greater reducing capacity. It can scavenge various ROS such as hydroxyl radicals, hypochlorous acid as well as peroxynitrite [165,166].

Superoxide dismutase (SOD) is the enzyme primarily responsible for the clearance of superoxide anions. It is present in the cytoplasm as well as on cell surfaces with either copper (CuSOD) or zinc (ZnSOD), and in the mitochondria with manganese (MnSOD). SOD catalyzes the dismutation of superoxide anion (O₂⁻) to hydrogen peroxide (H₂O₂) by the following reaction:

\[
O_2^{•−} + 2H^+ \rightarrow H_2O_2 + O_2
\]
This reaction can also proceed spontaneously, but SOD is able to increase the rate of cellular dismutation by a factor of $10^9$ [40,44,110,167].

However, increasing the duration of ischemia leads to a progressive decline of SOD and CoQ10 activity leaving the mitochondria less well equipped to deal with the increased free radical flux during reperfusion [44,160,168].

Catalase, glutathione peroxidase and thioredoxin peroxidase are the enzymes primarily responsible for the metabolism of the hydrogen peroxide produced by SOD[110,169].

Catalase is a membrane bound enzyme present in peroxisomes. It catalyzes the reduction of hydrogen peroxide to water by the following reaction:

$$\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$$

While catalase may function in the metabolism of intraperoxisomal hydrogen peroxide, the role of this enzyme in detoxifying extraperoxisomal appears to be minimal. It is also well known that the specific activity of catalase relative to glutathione peroxidase is considerably lower in the myocardium than in other organs such as the liver. This relative difference in activity can be explained by two factors: (1) the $K_m$ of catalase for $\text{H}_2\text{O}_2$ is around 1 mM whereas glutathione peroxidase has a $K_m$ for $\text{H}_2\text{O}_2$ of approximately 1 $\mu$M, indicating a higher affinity of glutathione peroxidase for the substrate, and (2) the fact that catalase is localised in peroxisomes whereas glutathione peroxidase is distributed in both cytosol and mitochondria. Since mitochondria are often the site of oxidative stress, glutathione peroxidase and thioredoxin peroxidase provide an immediate mechanism for the metabolism of hydrogen peroxides without having to re-locate the radical to the peroxisome, suggesting that they may be the predominate $\text{H}_2\text{O}_2$ scavengers in the heart. Also of particular importance is that they can be regenerated by glutathione and thioredoxin respectively, meaning these enzymes can be repeatedly recycled [40,160,169-174].
Glutathione peroxidases (GPX) are selenocysteine containing enzymes that are expressed in most tissues. To date, five GPX isoenzymes have been identified; Cytosolic and mitochondrial GPX (GPX-1) was the first mammalian selenoprotein to be identified in 1957, and being expressed ubiquitously suggests it plays a central role in cellular defence against hydroperoxides. Phospholipid hydroperoxide GPX (GPX-4 or PHGPX) was first described in 1982 and is also found in most tissues whereas gastrointestinal GPX (GPX-2) and extracellular/plasma GPX (GPX-3) are localised in the gastrointestinal tract and plasma respectively. GPX-5 is a selenium-independent GPX specifically expressed in mouse epididymis [175-179].

All GPX isoenzymes reduce hydrogen peroxide and alkyl hydroperoxides, however their specificities for hydroperoxide substrates differ markedly. GPX-1 and GPX-2 reduce only soluble hydroperoxides such as H₂O₂, and some organic hydroperoxides such as hydroperoxy fatty acids, cumene hydroperoxide or t-butyl hydroperoxide whereas GPX-4 and to some extent GPX-3 also reduce hydroperoxides of more complex lipids such as phosphatidylcholine hydroperoxide. GPX-4 is also capable of reducing hydroperoxide groups of thymine, lipoproteins and cholesterol esters, and is unique in acting on hydroperoxides integrated in membranes [175,180-183].

Under adequate selenium supply, all cells express at least some GPX-1. Particularly high levels are found in tissues with a high rate of peroxide production, such as erythrocytes, liver, kidney and lung [175,184]. Developmental changes in GPX-1 expression have also been observed in rat lungs where it increases after birth, especially when exposed to high oxygen tensions [185]. Thus it would appear GPX-1 expression parallels metabolic activity, which complies with an antioxidant function of the enzyme [175]. Increased GPX-1 activity has also been shown to inhibit apoptosis, even under conditions of low ROS production, demonstrating GPX has the ability to modulate apoptotic responses to a variety of stimuli [175].

The expression of GPX-2 appears to be limited to the epithelium of the gastrointestinal tract, but in humans it is also found in the liver [175,186]. This unusual
distribution has lead to the hypothesis that GPX-2 represents a first line of defence against ingested lipid hydroperoxides. It has also been proposed that it defends against hydrogen peroxide generated from the oxidative metabolism of xenobiotics by the liver [186].

GPX-3 is expressed in various tissues in contact with body fluids, including kidney, ciliary body and the maternal/fetal interface. From its site of production, GPX-3 is then secreted into the surrounding extracellular environment. Its presence on these border lines suggests it functions as a barrier for hydroperoxide transfer [175].

The activity of GPX-4 is generally lower than that of GPX-1 in all organs except the testis, which has the highest GPX-4 expression of all tissues studied [175]. Originally presumed to be a universal antioxidant protecting membrane lipids, GPX-4 appears to also have roles in redox regulation, silencing lipoxygenases, sexual maturation and differentiation [175,179,187,188]. It also has the ability to reduce hydroperoxides in HDL and LDL suggesting it may play a role in atherogenesis [181].

GPX-1 exists as a homotetramer with each of its subunits containing a selenium atom incorporated within a catalytic active selenocysteine residue. This amino acid is sterically exposed at a flat lipophilic surface on the protein allowing it to become oxidized by any hydroperoxide that can approach it [175,189,190]. Although GPX-1, GPX-2 and GPX-3 are all homotetramers, GPX-4 is a monomer with a molecular size smaller than the subunits of the other isoenzymes. Its small size and hydrophobic surface has been implicated in its ability to react with complex hydroperoxides and lipids in membranes [175].

The catalytic mechanism proposed for reduction of hydroperoxide substrates by GPX involves the oxidation of the active site selenolate to selenoic acid. This results in GPX being oxidized to an inactive state, which then requires glutathione (GSH) for regeneration of its active state. Glutathione binds to two arginine residues adjacent to the catalytic centre causing its SH group to be directed towards the oxidized selenium (selenic acid), consequently forming a selenosulfide bridge. This bridge is split by a second GSH molecule regenerating selenolate and reduced (active) GPX. In the process two molecules of GSH are oxidized to glutathione disulfide (GSSG). These two molecules of oxidized glutathione (GSSG) are converted back to their reduced state,
Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) which forms the largest pool of non-protein thiols in cells. On average, the concentration of GSH in the cytosol of most cells is 1-11 mM, which is much higher than most other redox active compounds [195,196]. GSH is only taken up in small amounts by most cell types, and when it has been fed to rodents, GSH levels increase in their blood and urine but not tissues [195]. It has to be broken down into its amino acids, transported into the cell and then re-synthesised, therefore very little GSH is imported into cells, and the majority of it is produced within the cytosol of cells. GSH is synthesized from L-glutamate by γ-glutamylcysteine synthetase and glutathione synthetase [195]. The cytosol then exchanges GSH with intracellular compartments, however some organelles appear to have their own independent GSH pool. For example, part of the GSH pool in the nucleus is independent from the cytosol, and it maintains the redox state of critical protein sulphydryls which are necessary for DNA repair and expression [197,198]. Some cells also export GSH, for example the liver is a major organ for the synthesis and export of GSH into the plasma (extracellular GSH). Whilst GSH is found outside cells, it is in much lower concentrations (100 to 1000 times less than intracellular GSH) [195,199].
Glutathione is believed to function in detoxification processes in addition to providing protection against oxidant injury. As well as being a cofactor for GPX, GSH can also directly reduce reactive oxygen species such as the hydroxyl radical, N$_2$O$_3$ and peroxynitrite. It also functions in the reduction of disulfide linkages between proteins and molecules, and in the synthesis of deoxyribonucleotide precursors of DNA. Glutathione, whilst being used as a thiol substrate by all GPX isoenzymes, isn’t the only available reductant, with GPX-3 having been shown to also use thioredoxin [175,177,192-194,199-202].

Changes in glutathione status provide important information on cellular oxidative events, with tissue accumulation or effluent release of GSSG being an accurate index of oxidative stress [166,195]. When cells or tissues are oxidatively stressed, they have been shown to export GSSG. The shift in ratio of [GSH]/[GSSG] changes the redox state to a more positive potential, and if the potential rises too much it is detrimental to the cell. The export of GSSG prevents this shift, and helps maintain the redox balance of cells [195,203]. This appears to be a part of the protection of cells and tissues from oxidative stress, and has been observed in hearts perfused with tert-butylhydroperoxide [204,205]. Although the efflux of GSSG does maintain the redox status during oxidative stress, it also results in the loss of GSH from the cell thereby decreasing the reducing capacity of the cell, which can only be replaced by synthesis of new GSH [195].

1.10 THIOREDOXIN / THIOREDOXIN REDUCTASE RELATED SYSTEM

Thioredoxin (Txn) was first described in 1964 by Laurent et al as a small redox protein from E. coli, but it was in 1967 that a mammalian Txn was reported by Moore [206,207].

The mammalian thioredoxins are a family of small (approximately 12kDa) proteins with oxidoreductase activity that are ubiquitously expressed. Intracellular
concentrations of Txn range from approximately 1-10 µM in bovine tissues and up to 15 µM in bacteria [195]. They are compact globular proteins with 5-stranded β-sheets forming a hydrophobic core surrounded by 4 α helices on the external surface. They have a conserved active site that links the second beta strand to the second alpha helix forming the first turn of the second helix. This tertiary structure is known as the thioredoxin fold. Residues in the highly conserved active site include Asp-26, Ala-29, Trp-31, Cys-32, Gly-33, Pro-34, Cys-35, Asp-61, Pro-76 and Gly-92 [208-210].

The two main thioredoxins are Txn-1, a cytosolic and nuclear form, and Txn-2, a mitochondrial form. Txn-1 acts as a cofactor providing reducing equivalents to other redox enzymes, and reduces cysteine groups on proteins altering binding and enzymatic activity. Txn-1 is also a secreted protein acting outside the cell to stimulate cell growth and inhibit apoptosis. The newly identified Txn-2 functions remain unknown but it contains a 33 amino acid N-terminal extension which serves as a mitochondrial import sequence suggesting it may play a role in protecting the cell against mitochondrial-mediated oxidative stress [201,210,211].

Thioredoxin is the major disulfide reductase responsible for maintaining proteins in their reduced state. Thiol-disulfide exchange reactions are efficient for electron transport, and are used in the mechanism of essential enzymes such as ribonucleotide reductase, which is required to provide deoxyribonucleotides for DNA synthesis. These exchange reactions are also used to control protein function via the redox state of structural or catalytic SH groups. Oxidation of a critical SH group will generally lead to a changed biological function. This stands true for Txn itself, which in reducing protein disulfides becomes oxidized to an inactive state [212-215].

The catalytic site of Txn undergoes reversible oxidation to cysteine disulfide (Txn-S$_2$) through the transfer of reducing equivalents from the catalytic site cysteine residues to a disulfide substrate (X-S$_2$). The mechanism for this action is that the substrate X-S$_2$ binds to a conserved hydrophobic surface where the thiolate of Cys-32 (acting as a nucleophile) combines with the protein substrate to form a covalently linked mixed disulfide Cys$_{32}$-S-S-protein. The now deprotonated Cys-35 attacks the cys-protein disulfide bond releasing the reduced protein substrate and forming Txn-Cys$_{32}$-Cys$_{35}$-disulfide. This oxidized form of Txn is then reduced back to the cysteine form (Txn-
(SH)₂) by thioredoxin reductase (TxnRed) [208,210,213]. This system is illustrated below:

Mammalian thioredoxin reductases are homodimeric flavin adenine dinucleotide (FAD) containing proteins. Each monomer also contains an NADPH binding site and an active site containing a redox-active disulfide. Electrons are transferred from NADPH via FAD to the active site disulfide of TxnRed which then reduces Txn. The catalytic site of human TxnRed, Cys-Val-Asn-Val-Gly-Cys, is also found in human glutathione reductase, and it is located in the FAD domain of these enzymes [211].

Thioredoxin reductase contains selenium in the form of selenocysteine. This residue is located at the very C-terminus of the protein within a tetrapeptide motif that is conserved in all isoenzymes and between species. The selenocysteine is essential for the activity of mammalian TxnRed, and its activity is severely impaired in selenium deficient animals and cells [211,216-219].

Mammalian TxnRed reduces not only the disulfide in oxidized Txn, but also some other protein disulfides and oxidized low molecular weight compounds [220]. Peroxides including lipid hydroperoxides and hydrogen peroxide can be directly reduced by TxnRed. Thioredoxin reductase can therefore function as an alternative enzymatic pathway for the detoxification of these molecules otherwise normally managed by GPX. However the high \( K_m \) of TxnRed for \( \text{H}_2\text{O}_2 \) (2.5mM) suggests TxnRed only plays a role when levels are high [210,217,221].
Another thioredoxin related protein capable of directly reducing peroxides is thioredoxin peroxidase. The thioredoxin peroxidases (Prdx) belong to a conserved family of antioxidant proteins, the peroxiredoxins which use thyl groups as reducing equivalents to scavenge oxidants. The reduced form of Prdx scavenges oxidant species such as hydrogen peroxide and alkyl peroxides. In the process they are oxidized as they homo or heterodimerize with other family members through disulfide bonds formed between conserved cysteine residues. Txn then reduces the oxidized Prdx back to the active monomeric form. In the process Txn becomes oxidized and is reduced by TxnRed as previously described [201,194,210,215,222,223]. The thioredoxin peroxidase system is shown below:

Several human Txn peroxidases have been identified including Prdx-1 which has been shown to protect endothelial cells from H$_2$O$_2$–induced cytotoxicity and inflammation-induced monocyte adhesion. Another isoenzyme, Prdx-2 protects cells from apoptosis and inhibits the release of cytochrome c from mitochondria during apoptosis [210,224].

Whilst the general oxidoreductase activity of thioredoxins play three well known roles as: i) electron carriers necessary for the catalytic cycles of biosynthetic enzymes (such as ribonucleotide reductase, methionine sulfoxide reductases and sulfate reductases), ii) in protecting cytosolic proteins from aggregation or inactivation via
oxidative formation of intra- or inter molecular disulfides, and iii) as direct free radical scavengers, they also have numerous other physiological functions. Some of these functions include serving as a growth factor, activation/inactivation of transcription factors and protein refolding [210,213,225].

Thioredoxin-1 acts as a growth factor and is produced by a variety of cells. It is secreted by lymphocytes, hepatocytes, fibroblasts and numerous cancer cells although the mechanism by which secretion occurs is not known. It stimulates the growth of these same cells by an atypical mechanism. Thioredoxin is also known as adult T-cell leukemia-derived factor because this was one of the first cell types known to secrete the protein, which then acts as an autocrine growth factor. Txn-1 is believed to play a role in a number of cancers, as increased levels are found in many tumours where it has also been associated with aggressive growth [21-,211,222,226-228].

Many transcription factors have been shown to be redox regulated by Txn-1, with either their activation or inactivation dependent on thioredoxin-catalyzed reduction. This includes the transcription factor NF-κB that is important for the cell’s response to oxidative stress, apoptosis and tumorigenesis. The binding of NF-κB to DNA is inhibited under oxidizing conditions, however reduced Txn-1 increases its binding to DNA thereby increasing the expression of a number of genes under NF-κB regulation. A second transcription factor whose activity is regulated by Txn is the glucocorticoid receptor, which has its DNA binding activity increased by Txn-1. The transcription factor AP-1 whose activation is closely correlated with increased cell growth and DNA repair is also redox regulated. Txn-1 increases the DNA binding activity of AP-1 via Ref-1 in the nucleus. Other transcription factors whose binding to DNA in increased by Txn-1 are AP-2, the estrogen receptor, and transcription factor PEBP2/CBF [201,210,228-231].

Reduced Txn-1 binds to a variety of cellular proteins including apoptosis signal-regulating kinase 1 (ASK1). The mechanism of this binding has not been elucidated but may involve mixed disulfide formation between a catalytic site cysteine residue and a cysteine on ASK1. ASK1 is an activator of the c-Jun N-terminal kinase (JNK) and p36 MAP kinase pathways and is required for TNF-α-induced apoptosis. However Txn-1 binds to the N-terminal portion of ASK1 inhibiting its activity, therefore preventing downstream signaling for apoptosis [136,210,229,231-233].
At least one of the thioredoxin peroxidases, human Prdx-2, has also been demonstrated to inhibit the induction of apoptosis by decreasing H$_2$O$_2$ levels. This reduction in H$_2$O$_2$ therefore not only prevents oxidative stress, but also constitutes a Txn-dependent regulatory step of apoptosis upstream that of Bcl-2 [215,232,233].

Txn-1 can act as a catalyst of protein folding because of its relatively weak protein disulfide bond isomerase activity. It can directly reactivate proteins such as glyceraldehyde-3-phosphate dehydrogenase, iodothyronine 5’-deiodinase, and ornithine decarboxylase. Whilst these repair or regeneration mechanisms may play an important role in the cellular defence against oxidative stress, little is known about the actual cellular mechanisms responsible although protein disulfide isomerase has two regions homologous to the Txn catalytic site suggesting they may share a similar mechanism [210,223,234,235].

Thioredoxin reductase in its reduced state may play an additional role in the recycling of another antioxidant, ascorbate. TxnRed can reduce the ascorbic acid free radical to ascorbate suggesting an alternative way that TxnRed can protect the cell from oxidative stress and replenish the available antioxidant pool [201,211,213].

Txn-1 may also be a component of the immune system. Extracellular Txn is both a co-cytokine and chemokine, and a truncated form of Txn stimulates eosinophils. Eosinophils are inflammatory cells associated with inflammatory responses, allergic diseases and tumour cytotoxicities, Txn-1 increases eosinophil migration and release of their major basic proteins. Due to its increased expression in a number of autoimmune diseases such as Sjogren’s syndrome and rheumatoid arthritis, Txn-1 is thought to play a pathogenic role and be a mediator of inflammation by enhancing eosinophil infiltration and interleukin-1 (IL-1) activity [210,231,236-242].

Mice with targeted disruption of the Txn-1 gene show that homozygous embryos die shortly after implantation, whereas heterozygous embryos are viable, fertile and develop normally. These lethal effects are consistent with the finding that Txn is widely distributed in different organs and tissues during early fetal development. Txn is believed to have another role in pregnancy by assisting in implantation. Cytotrophoblasts synthesize both intracellular and extracellular Txn which is used in the establishment of pregnancy. Txn localised in the placenta is also believed to protect the developing fetus
and placental trophoblasts from oxidative stress induced by free radicals [210,231,243-247].

Expression of Txn is induced by oxidative stress and this induction most likely involves an antioxidant responsive element present in the Txn promoter. This is further supported by the induction of Txn and TxnRed gene expression in the lungs of newborns by oxygen. Txn-1 is believed to protect the newborn from hyperoxia at birth. Increased Txn expression is thought to be aimed at increasing the reduction of intracellular proteins and other biomolecules as part of the body’s antioxidant defence [201,194,216,231,248,249].

Of particular interest to this research is that recombinant Txn-1 has been shown to exert a protective effect against ischemia-reperfusion damage in a number of tissues. This includes protection from reperfusion-induced arrhythmias in isolated rat hearts and improved lung function in an in-vivo rat and rabbit model of lung ischemia-reperfusion injury. Thioredoxin-1 transgenic mice display smaller infarct sizes following cerebral artery occlusion, and Txn secreted from glial cells has been found to promote neuronal survival from ischemia-reperfusion. Txn-1 also protects endothelial cells and retinal cells against reperfusion injury. These studies suggest the thioredoxin system may be a potential therapeutic target in the treatment of ischemic heart disease [210,231,233,250-254].

These studies suggest that modulation of this antioxidant enzyme system and/or the GPX system may provide ways of protecting various tissues and cells from oxidative stress, and therefore justifies further investigation. Up-regulation of either or both of these enzyme systems may be protective against the oxidative damage incurred during ischemia-reperfusion of the heart, and down regulation may also prove to be detrimental and exacerbate the injury.
1.11 **SELENIUM AND SELENOCYSTEINE**

Selenoproteins are proteins that contain selenium in one or all of their cysteine and/or methionine residues. Selenocysteine (Sec), which is now recognised as the 21st amino acid, is the more biologically active of the two. Glutathione peroxidases and thioredoxin reductases are selenocysteine containing enzymes. They are two of only three classes of selenocysteine enzymes, the third being the iodothyronine deiodinases. Levels of all of these selenoproteins in various tissues are affected to different extents by selenium availability [255,256].

Selenium (Se) is a trace element whose role in mammalian biochemistry was described in 1957. However it wasn’t until 1979 that the needs for selenium in human nutrition were found when a Chinese research group discovered a relationship between the low concentration of selenium in the geographical area of Keshan in China and Keshan disease. Keshan disease is a congestive cardiomyopathy that primarily affects children aged between 2 and 10 years, and premenopausal women. This disease affected almost exclusively the towns of the mountainous zones of the Keshan area in which the soya cultivated had a very low selenium content, and consequently all food produced there had minimal concentrations of the element [256-259].

Selenium has a large number of biological functions, the most important is its antioxidant effect because it forms selenocysteine which is present in the active sites of Txn reductases and GPXs. Under conditions of low selenium, the activity of these enzymes is therefore reduced. Protein levels are also decreased but not to the same extent as activity suggesting that the activity of Txn reductases and GPXs are not only related to their protein levels but rather the amount of selenium found within them [255,259,260].

Whilst all forms of selenium are absorbed through the intestine, different types then follow different metabolic pathways for their conversion to a biologically active form. In general, the bioavailability of organic forms of selenium (ie. Se-methionine and Se-yeast) is higher than that obtained for inorganic forms (ie. selenite and selenate). Although selenomethionine is more effectively utilized by cells due to its nonspecific incorporation into proteins in place of methionine it has no real biological benefit. It is
the inorganic forms of selenium that are incorporated into selenoproteins. Selenite and selenate go directly into a protein pool, from which independent of its origin, all the selenium is used in the synthesis of selenoproteins. These inorganic forms are incorporated into cysteine in much the same way that sulfur is incorporated during biosynthesis. It has also been shown that at high selenium levels, selenium is incorporated in place of sulfur leading to an abundance of selenocysteine available for use in enzymes such as Txn reductases and GPXs. Consequently, Txn reductase and GPX levels are mainly regulated by levels of inorganic forms of selenium rather than the more abundant organic forms. Consumption of animal tissues containing selenium dependent enzymes are also sources of selenium in the readily available form of selenocysteine. This selenocysteine is not stored but directly incorporated into selenoproteins and the excess catabolized with the resulting selenium going into a pool for later use [255,256,261-263].

In eukaryotes, translation of selenocysteine requires misreading of the UGA codon (instead of its normal usage in terminating protein synthesis), the presence of a selenocysteine insertion sequence (SECIS) element in the 3’-untranslated region of mRNA where it forms a hairpin secondary structure and the involvement of a SECIS binding protein, a selenocysteine elongation factor, and selenocysteine tRNA [264-268]. Sec is synthesized while esterified to its tRNA, tRNA\textsuperscript{Sec}, using inorganic selenophosphate and serine. Its position in the peptide backbone of selenoproteins is encoded by in-frame UGA codons in the mRNA [266,267]. The dietary supply of selenium modulates turnover of Sec-tRNA\textsuperscript{Sec}, limiting effectiveness of the translational machinery. With adequate selenium and all the other necessary components, Sec-tRNA\textsuperscript{Sec} will compete sufficiently well with termination release factors for binding at UGA, such that full length selenoproteins are synthesized. However, without adequate selenium and therefore insufficient Sec-tRNA\textsuperscript{Sec}, UGA is interpreted as a stop codon limiting selenoprotein translation [265-268].

Studies in rats show selenium deficiency has a dramatic effect on Gpx-1 mRNA in the liver. Sunde and colleagues found selenium deficiency reduces Gpx-1 mRNA levels in male rat livers to be one-tenth of those found in selenium adequate animals [269,270]. They have also shown both Gpx-1 activity and mRNA respond sigmoidally to
increasing selenium concentrations, but begin to plateau at 0.1 µg/g food and 0.05 µg/g food respectively [271,272]. Taken together, their results show Gpx-1 mRNA levels respond quicker to selenium status than Gpx-1 activity [273]. In addition to this, they also found female rats respond similarly to males although they have more than twice the level of Gpx-1 mRNA and activity [272]. Similar results have also been reported in erythrocytes, hearts, kidneys and lungs from selenium deficient rats along with cells cultured in selenium deficient media [271,274]. Previous studies in porcine kidney epithelial cells show levels of Gpx-1 mRNA increased with continued addition of selenite whilst Gpx-1 activity plateau’s above 50nM [275]. For selenium repletion, Gpx-1 protein and activity requires larger doses and longer time periods than for maintenance, and this is believed to occur because other selenoproteins have first priority for selenium during selenium deficiency [276].

Along with its function in the synthesis of GPXs and Txn reductases, and crucial role in their active sites, selenium has also been suggested to stabilize the mRNA of these enzymes. Bermano et al. showed that selenium deficiency in H4 hepatoma cells effected the stability of translated mRNA resulting in increased degradation, and that this is an important factor in the regulation of selenoprotein levels [255,265].

Numerous studies have shown that selenium supplementation is able to up-regulate the activity of selenocysteine containing enzymes in both whole animals and cells. This has been demonstrated for both GPX and Txn reductase in various rat organs, and for GPX in LLC-PK₁ epithelial cells. Powis et al. have shown a nearly 30-fold increase in Txn reductase activity and protein expression in MCF-7 breast cancer cells with as little as 1µM selenium supplementation in the form of sodium selenite. Supplementing rats with 1.5mg Se/kg diet, also in the form of sodium selenite, has been found to significantly decrease the occurrence of reperfusion induced arrhythmias, and this has been hypothesised to be linked to an increase in GPX activity in their hearts [255,260,262,271,277-279].

Selenium is consumed through a variety of foods with the recommended dietary intake being 50-70 µg/day. It is associated with protein in animal tissue, and as a result muscle meats, organ meats and seafood are dependable dietary sources. Grains and seeds however vary in selenium content depending on the selenium content of the soils in
which they were grown. This in turn also effects the selenium content of livestock and some meats since the livestock also graze off certain grasses whose selenium content is reliant on the soil [255,257,280].

While severe human selenium deficiencies are rare and only occur in response to a very low (less than 20 µg/day) selenium supply, mild selenium deficiencies are much more common, and usually arise due to low selenium contents of soil [255,257,262]. However, even mild selenium deficiencies have been implicated in the etiology of some disease processes including cardiovascular disease, cancer, diabetes mellitus, liver disease and other degenerative conditions associated with aging [256,257].

Selenium may be protective against cardiovascular disease. In theory this hypothesis is supported by the ability of GPX to combat the oxidative modification of lipids and to reduce platelet aggregation. In selenium deficiency, a build up of hydroperoxides may attack the vascular endothelium and promote the production of thromboxane which is associated with vasoconstriction and platelet aggregation. This may be the mechanism by which low plasma selenium levels that are found in patients with atherosclerosis act [256,257,281-283].

Numerous studies have shown a significant decrease of serum or plasma selenium concentrations in patients with different cardiopathies including acute myocardial infarction (MI), congestive heart failure, cardiomyopathy, hypertension, chronic heart disease, ischemic heart disease as well as atherosclerosis. Whilst it is not completely clear whether these decreased selenium levels are etiological factors or biological consequences, it is clear that the selenium deficiency does enhance the risks associated with these conditions. Another factor to take into consideration is the decreased antioxidant activity of GPX and Tnx reductase that occurs along with selenium deficiency. Selenium deficiencies may therefore contribute to the ischemia-reperfusion injury that is seen following acute myocardial infarction, coronary bypass surgery, heart transplants and coronary angioplasty. In the same respect selenium supplementation may be cardioprotective against ischemia-reperfusion injury, as has been shown by Toufektsian et al., Poltronieri et al. and Sinci et al [256,262,281,284-291]. A small human study of 46 patients also demonstrated that selenium supplementation (400 µg/day for 7 days) improved outcome in patients undergoing corrective heart surgery involving
ischemic insult. Interestingly, this study also noted increased glutathione peroxidase mRNA expression in selenium supplemented patients [292].

Selenium supplementation is a convenient and practical method of up-regulating the activity of selenocysteine containing enzymes both in vitro and in vivo without having to go to the expense and difficulty of genetic modifications through gene transfection or the generation of transgenic animals. There is a narrow concentration range between what causes deficiency and that which causes toxicity in humans and animals, therefore it is critical that an optimal amount of selenium is used in dietary supplementation so as to increase the activity of Txn reductases and GPXs without leading to selenium toxicity [255,256].

1.12 INHIBITORS OF SELENOCYSTEINE

Gold containing compounds such as auranofin are potent inhibitors of selenocysteine containing enzymes. These organic gold compounds are frequently prescribed in the treatment of rheumatoid arthritis although their exact mechanism of action is not clear [293].

Txn reductase has been implicated in various auto-immune diseases including rheumatoid arthritis. Rheumatoid arthritis is believed to be initiated by a variety of agents including the Epstein-Barr virus (EBV). Lymphocytes infected with EBV or other viruses have been shown to secrete Txn. This suggests that the Txn redox system plays a cytokine role in rheumatoid arthritis, and that auranofin and other gold containing compounds exert some of their pharmacologic effects by inhibiting the selenoenzyme Txn reductase [237,293,294].

The use of gold containing compounds to selectively down-regulate the activity of Txn reductase and GPX in both cells and systemically in whole animals has been demonstrated. In a study by Gromer et al., they effectively knocked out the placental thioredoxin reductase system by reducing the activity of Txn reductase by 90%. This was achieved with only 100nM auranofin. Increasing the concentration of auranofin
1000-fold to 100µM results in inhibition of GPX and a shut down of Txn reductase [294]. Similar results have been found by Smith et al. in mice with injection of 0.025mg aurothioglucose/g bodyweight. This resulted in sustained inhibition of Txn reductase activity in tissue extracts of heart, liver, kidney and pancreas. They found no significant decrease in GPX activity in the heart, although 2 hours after the injection of 0.2mg/g bodyweight there was a 25% decrease in GPX activity. There was however a significant dose-dependent decrease in GPX activity in the pancreas and kidney 2 hours after treatment, but this inhibitory effect was lost by 24 hours [293]. These results are also supported by another study by Hu and colleagues in rats [295].

These findings suggest that administration of gold containing compounds such as auranofin have the same effect on selenocysteine containing enzymes as selenium deficiency, and that these compounds may in fact act as selenocysteine antagonists [296].

While it is unknown how gold compounds inhibit the activity of selenocysteine containing enzymes, it has been proposed that it is the gold content that is responsible. Gromer et al. looked at the effects of the thioglucose moiety that is found in both auranofin and aurothioglucose (ATG), and found it to have no inhibitory effects on Txn reductase. In the same study they also used a gold chelating agent, BAL, and found it was capable of preventing and reversing the inhibition of Txn reductase caused by auranofin and ATG [294]. Since selenols have a greater tendency to bind heavy metal ions (such as gold) than thiols, they have speculated that the C-terminal redox active selenocysteine centre of Txn reductase is the target of these gold inhibitors [216,294,297,298].

This hypothesis is supported by the finding that glutathione reductase, which is structurally and mechanistically similar to Txn reductase, is far less sensitive to auranofin and ATG. This may be due to Gsr containing only cysteine rather than selenocysteine in its active site [216,295,299].

These studies suggest that antioxidant defence in patients being treated with auranofin (or ATG) may in fact be impaired, sinceTxnRed and GPX have crucial roles in the protecting against oxidative stress. There is also the possibility that these gold compounds potentiate the virulence of several viruses, including the selenium responsive cardiomyopathy Keshan disease [237,293,296,299] Whilst these gold compounds provide
a simple method to down-regulate the activities of both TxnRed and GPX, it is important that their function *in vivo* be investigated further, along with their roles in oxidative stress, both *in vivo* and *in vitro*. 
1.13 **AIMS AND OBJECTIVES**

In reference to the previous review describing the role of oxidative damage and antioxidant enzymes in myocardial ischemia-reperfusion injury, this thesis aimed to investigate the following:

i) assess the effect of ischemia and ischemia-reperfusion on the mRNA expression and activity of the TxnRed and GPX systems in the rat heart

ii) to elucidate the mechanisms by which the activity of the TxnRed and/or GPX systems may effect the recovery of the rat heart from ischemia-reperfusion injury

iii) examine the effect of dietary selenium levels on the activity and mRNA expression of the TxnRed and GPX systems in the rat

iv) determine whether selenium deficiency or supplementation effects cardiac performance both before and after ischemia-reperfusion injury, and elucidate the mechanism by which selenium levels may effect the recovery of the rat heart from ischemia-reperfusion injury

v) determine whether gold containing compounds such as auranofin decreases the activity of the TxnRed and/or GPX systems in the rat, and examine the effect this may have on recovery from myocardial ischemia-reperfusion injury

vi) investigate the effect of ageing on myocardial oxidative damage, along with mRNA expression and activity of the TxnRed and GPX systems in the rat
CHAPTER 2

General
Methods & Materials
2.1 MODELS EMPLOYED

The main experimental model employed in the following studies was the rat model. Using an animal model allowed us to conduct studies on how aging, ischemia and ischemia-reperfusion effects myocardial antioxidant enzyme systems, and to investigate the effects of modulating antioxidant enzymes, by altering dietary selenium or treating with auranofin. The rat model made it possible to study antioxidant enzyme activity and transcription as well as functional and biochemical parameters. Studying the whole heart also allows for all critical interactions between multiple cell types (myocytes, smooth muscle and endothelium) to occur.

Cardiovascular function was studied using the perfused isolated rat heart. This is a valuable and reproducible model for the study of contractile function, metabolism and tissue injury over a range of experimental conditions [300]. The advantage of using this model is it permits functional performance to be assessed in a standardised manner without the interference of blood borne elements, sympathetic stimulation, peripheral resistance and circulating hormones. Standardising these experimental conditions also ensures that the tissues all receive the same metabolite, oxygen and energy supplies. This model allowed us to investigate the actual cardioprotective effects of the TxnRed and GPX antioxidant systems in the whole heart, and also allowed us to eliminate some of the other factors that contribute to ischemia-reperfusion injury and inflammation.

Isolated rat hearts were perfused on a Langendorff perfusion apparatus as designed by Oscar Langendorff between 1895-1897 [300]. This is a retrograde perfusion in which an oxygenated buffer is forced into the heart via a cannula that is inserted in the aorta. This perfusion closes the aortic valve and allows the perfusate to flow into the coronary arteries, supplying the heart with all the substrates and oxygen it needs to sustain itself. To prevent the build up of perfusate in the heart, the left ventricle contains an opening for Thebesian (or coronary perfusion) drainage. The actual Langendorff perfusion method used throughout these studies will be described in further detail below.

Whilst there are two methods used in the study of ischemia-reperfusion injury in perfused isolated hearts, we chose to use the Langendorff preparation over the antegrade perfused working heart preparation even though it is considered to have
greater resemblance to the physiological situation [300]. Whilst both methods are equally valuable in assessing tissue injury and cardioprotection, and ischemic insult is identical in both, the working heart can present difficulties during reperfusion. The major issue with using the working heart to study ischemia-reperfusion is the inter-dependence of ventricular function and coronary perfusion post-ischemia. The fluid column that drives coronary perfusion in the working heart is driven by the heart, so depending on the extent of injury to the heart (i.e. if it is unable to pump against a normal afterload due to contractile dysfunction) it may have limited ability to maintain the fluid column, so aortic pressure drops along with coronary perfusion pressure. This results in reduced coronary perfusion, and hence during reperfusion the heart doesn’t receive adequate flow so is still partially ischemic. With the Langendorff model however, the extent of post-ischemic dysfunction doesn’t directly impact on the ability to perfuse the heart as the aortic pressure is constant and it is driving the coronary flow. Given the complexity of the working heart together with the problems associated with ischemia-reperfusion studies, we chose to use the Langendorff method of perfusion.
2.2 MATERIALS

All reagents used were of analytic reagent grade (AR) and were obtained from Sigma (St Louis, MO, USA) unless otherwise specified.

2.3 ANIMALS

Adult male wistar rats were randomly assigned to experimental groups. They were age matched, and all rats were weighed prior to sacrifice to ensure there were no differences in body weights or growth rates between animals. To ensure appropriate animal numbers were used whilst still maintaining statistical power, (such that type 1 and 2 errors in which false differences are detected or differences are not detected due to low power were avoided) we chose an $\alpha$ level of 0.05 (ie. a 95% confidence of significance) and a $\beta$ level of 0.10 (ie. a 90% assurance that the confidence interval will be no larger than 95%), and calculated experimental sample size using variances (errors) from preliminary experiments. We chose a detectable difference of 20% between treatment groups, and calculated sample size from data from previous experiments. This gave us an $n$ value between 6-10 depending on the experiment.

All rats were fed standard rat pellets unless otherwise specified. The research projects were approved by the Griffith University Animal Ethics Committee. Rats were housed in an animal facility complying with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC) under conditions of constant temperature and humidity with 12 hour light-dark cycles. They had free access to water and food, and diets were replaced daily.
2.4 FUNCTIONAL ANALYSIS OF CARDIAC PERFORMANCE

i) LANGENDORFF ISOLATED HEART PREPARATIONS

Animals were anaesthetised with 60 mg/kg sodium pentobarbitone (Nembutal, Merial, Parramatta, Australia) via an intraperitoneal injection. A thoracotomy was performed, hearts rapidly removed and immersed in ice-cold perfusion fluid. The aorta was cannulated with a glass cannula and coronary circulation perfused at a hydrostatic pressure of 100 mmHg (as shown in Fig. 2.1) with modified Krebs-Henseleit buffer containing (in mM): NaCl, 119; NaHCO₃, 22; KCl, 4.7; KH₂PO₄, 1.2; MgCl₂, 1.2; CaCl₂, 1.75; EDTA, 0.5 and glucose, 11. Perfusion buffer was delivered through the compliance chamber by a peristaltic pump at a rate exceeding the basal coronary flow. The perfusion buffer was initially passed through 5.0 µm filter on preparation and all perfusate delivered to the heart was filtered via an in-line 0.45 µm Sterivex-HV filter (Millipore, Bedford, MA, USA). Buffer was equilibrated with 95% O₂ and 5% CO₂ at 37°C to give a pH of 7.4 and PO₂ ≥ 600 mmHg. Perfusion buffer was maintained at 37°C and hearts were constantly bathed in perfusate within a water-jacketed organ chamber also maintained at 37°C. Temperature of perfusion buffer was constantly monitored by a needle thermistor at the entry of the aortic cannula, and temperature measured using a Physitemp TH-8 digital thermometer (Physitemp Instruments Inc, Clifton, NJ, USA).

The left ventricle was vented with a polyethylene tube for drainage of venous flow. A fluid-filled balloon was inserted into the left ventricle via the mitral valve for assessment of isovolumic function. Balloons were constructed of latex and were tied to a short segment of polyethylene tubing with 3-0 suture. The balloon was connected to a pressure transducer (ADIInstruments, Castle Hill, Australia) by a fluid-filled polyethylene tube permitting continuous measurement of left ventricular pressure. Balloon volume was controlled by a 1 ml syringe, and was initially increased to an end-diastolic pressure of approximately 5 mmHg. Aortic perfusion pressure was monitored using a second pressure transducer connected via fluid-filled polyethylene tubing to a needle probe inserted in the perfusion line just above the cannula. Both pressure transducers were connected to a 4/s MacLab data acquisition unit (ADIInstruments, Castle Hill, Australia).
These were connected to an Apple computer, and all functional data was recorded at 1 KHz.

Functional parameters measured include peak systolic left ventricular pressure (PSVP), left ventricular developed pressure (LVDP) and its derivatives $+dP/dt_{\text{max}}$, end diastolic pressure (EDP), heart rate (HR), rate pressure product (RPP) and ischemic contracture. Coronary flow rates were measured manually by timed collection of effluent from the heart.

Global ischemia (100 % ischemia, no-flow) was generated in hearts by stopping coronary flow. This was achieved by turning off the flow into the heart using a tap situated above the cannula. Hearts remained bathed in perfusate, and were maintained at 37 °C in a temperature controlled organ bath during ischemia. Reperfusion was generated by re-introducing coronary flow into the heart, and this was achieved by turning the tap back on. Actual experimental time courses and ischemic protocols are explained in each of the experimental chapters. The length of ischemia used in these studies was determined in pilot experiments to be a period which resulted in ~50% recovery of contractile function. This provided the potential to measure both improved and reduced recoveries.

At the end of perfusion studies, hearts were removed from the cannula, rapidly blotted, weighed and then frozen at –80 °C until subsequent biochemical analysis.
Fig 2.1: Langendorff perfusion apparatus (A) and isolated Langendorff perfused rat heart (B)
2.5 **TISSUE PREPARATION**

Livers were also removed from rats at the time of sacrifice, weighed and kept at –80 °C until analysis.

**i) PROTEIN EXTRACTION**

Tissues were homogenized in 4 volumes of protein extraction buffer containing 50 mM Tris-Cl pH 7.5 and 2 mM EDTA using an Ultra Turax homogeniser (Heidolph IKA-Werke GMBH KG, Staufen, Germany). Samples were then centrifuged for 30 minutes at 3500 g to separate proteins from cellular debris. Supernatants were collected into 1 mL aliquots, and stored at –80 °C until use. Prior to use, tissue extracts were thawed and centrifuged for 15 minutes at 14 500 g.

**ii) PROTEIN ESTIMATIONS**

Protein estimations were performed using a BCA Protein Assay reagent kit (Pierce, Rockford, USA), following the manufacturers recommended protocol. The method relies on bicinchoninic acid (BCA) for colourimetric detection and quantitation of total protein. The chromophore produced exhibits a strong absorbance at 540 nm and is linear with increasing protein concentrations. Bovine serum albumin (BSA) was used as the standard and assayed alongside the unknown samples allowing for the construction of a standard curve and quantitation of the unknowns. Protein concentrations were expressed as mg protein per mL of tissue extract (mg/mL).
iii) RNA EXTRACTION

Total RNA was extracted from individual hearts by the monophasic phenol/guanidine isothiocyanate reagent, TRIzol (Invitrogen, Carlsbad, CA, USA), which maintains the integrity of RNA during isolation whilst disrupting cells and dissolving cellular components. TRIzol also contains RNase Inhibitors to prevent RNA degradation during and after isolation. RNA was isolated according to the manufacturer’s protocol by homogenising hearts with an Ultra Turax homogeniser in TRIzol (the homogeniser was previously treated with the RNase decontaminate RNA zap (Ambion, Austin, TX, USA)). The samples were incubated at room temperature for 10 minutes to allow complete disruption of cells. Samples were centrifuged at 12 000 g for 5 minutes to pellet insoluble material, and the supernatant transferred to a new tube. The supernatant was left to stand at room temperature for 5 minutes to permit complete dissociation of nucleoprotein complexes, before adding chloroform (0.2x volumes) and shaking vigorously by hand. Samples were incubated at room temperature for 5 minutes then centrifuged at 12 000 g for 5 minutes. This separates the sample into 3 layers; upper clear aqueous phase containing RNA, a white interphase containing DNA, and a lower red organic phenol/chloroform phase containing proteins. The upper phase was transferred to another tube, and RNA precipitated by adding isopropyl alcohol (0.5x volumes), rotating tubes several times and incubating at room temperature for 1-2 hours. RNA was pelleted by centrifugation at 12 000 g for 10 minutes, and the supernatant removed without disturbing the gel-like RNA pellet. RNA was then washed twice with 70 % ethanol in nuclease-free water (1x volume) then centrifuged at 7500 g for 5 minutes to pellet the RNA. The supernatant was removed, the pellet air-dried for 5 minutes then resuspended in nuclease-free water. To prevent RNA degradation, 40 U of RNasin (Promega, Madison, WI, USA) were added to each sample and they were incubated at 55°C for 10 minutes.

Extracted total RNA was further treated with 50 U DNase I (Promega, Madison, WI, USA) for 15 minutes at 37°C to degrade traces of contaminating genomic DNA.

DNase treated total RNA was then spin column purified using an RNeasy kit (Qiagen, Maryland, USA). Firstly, 200 µL of 100% ethanol was added to the samples to
precipitate the RNA, which can then bind to the silica-gel-based membrane of the spin column. Contaminants are then removed with wash spin steps using specialized buffers supplied in the kit, and high-quality RNA is then eluted in nuclease-free water. Total RNA was stored at -80°C until use.

**iv) RNA QUANTITATION AND DETERMINATION**

Yield and purity was determined spectrophotometrically at 260/280 nm. RNA concentration was determined by diluting total RNA 1/20 with nuclease-free water and measuring the absorbance at 260 nm. An absorbance reading of 1 unit corresponds to 40 µg RNA/mL, and RNA concentrations of 0.5 µg/µL and above were considered acceptable. The purity of RNA was determined using the ratio of the absorbance readings at 260 nm and 280 nm (A_{260}/A_{280}). Contaminants such as protein have an absorbance maxima of 280 nm lowering the A_{260}/A_{280}. Partially dissolved RNA also has an A_{260}/A_{280} ratio lower than 1.6. Ideally, pure RNA should have an A_{260}/A_{280} ratio of 1.9-2.1. A_{260}/A_{280} ratios of 1.6-2.1 were considered acceptable.

RNA integrity was determined by the appearance of sharp 18S and 28S ribosomal RNA bands on denaturing formaldehyde agarose gel electrophoresis and ethidium bromide staining (Fig. 2.2). Degraded RNA will not present as sharp ribosomal bands but rather a smear of smaller RNAs. Formaldehyde agarose (FA) gels were prepared with 0.6 g agarose (1.2%), 5 mL 10x FA gel buffer (containing 200 mM MOPS free acid, 50 mM sodium acetate and 10 mM EDTA, pH 7.0), 0.9 mL 37% formaldehyde and 1 µL ethidium bromide (10 mg/mL) in a final volume of 50 mL. The FA gel was left to equilibrate in 1x FA gel running buffer (containing 1x FA gel buffer, 250 mM formaldehyde and nuclease-free water) for 30 minutes prior to running. Total RNA was diluted 1/5 with nuclease-free water and kept on ice. 1 µL of 5x RNA loading buffer, containing saturated aqueous bromophenol blue solution, 4 mM EDTA, 885 mM formaldehyde, 100% glycerol, 3.1% formamide and 4x FA gel buffer, was added to the diluted RNA samples. These were then incubated at 65°C for 5 minutes, chilled on ice and loaded onto the equilibrated gel. The gel was run from cathode to anode at 85 V (5-7
V/cm) for 20 minutes in 1x FA gel running buffer. At the end of electrophoresis, the gel was viewed and photographed under UV illumination which causes any RNA bands to fluoresce due to ethidium bromide staining.

**Fig 2.2:** Formaldehyde agarose gel of total RNA isolated from rat hearts. RNA integrity is assessed by the presence of 28S (~5kb) and 18S (~2kb) ribosomal RNA bands.
2.6 BIOCHEMICAL ANALYSIS

i) GLUTATHIONE PEROXIDASE ASSAY

Glutathione peroxidase activity was determined by a modified method of Flohe and Gunzler [301]. Activity was assessed spectrophotometrically by measuring the rate of decrease in absorbance at 340 nm over 3 minutes. 50 µL of 1/10 diluted tissue extracts were added to 900 µL of assay mix containing 0.1 M KPi pH 7.0, 2 mM EDTA, 0.5 U/mL glutathione reductase, 10 mM glutathione and 0.3 mM NADPH. 50 µL of 20 mM tert-butyl hydroperoxide was added as the substrate for glutathione peroxidase. The GPX present in the tissues reduces this hydroperoxide substrate and in the process becomes oxidized itself. Glutathione regenerates GPX and in doing so becomes oxidized to glutathione disulfide. Glutathione reductase reduces it back to an active state by transferring electrons from NADPH which becomes oxidized to NADP⁺ and no longer absorbs light. Hence the greater the amount of GPX present, the greater the decrease in absorbance over time. After performing rate calculations and standardizing activity to protein concentrations, glutathione peroxidase activity was expressed as mmoles per minute per milligram of extracted protein (mmoles/min/mg).

ii) THIOREDOXIN REDUCTASE – INSULIN REDUCTION ASSAY

Thioredoxin reductase activity was determined by a modified method of Arner, Zhong and Holmgren [220]. Tissue extracts (20 µL of 1/10 dilution) were incubated for 20 minutes at 37 °C in 120 µL of assay mix containing 50 mM KPi, 5 mM EDTA, 0.66 mg/mL NADPH and 2.16 mg/mL insulin in a 96 well plate. Thioredoxin (10 µL of 1.4 mg/mL) was added to duplicate wells of each sample, while a further 10 µL of assay mix was added to a second series of wells to measure background absorbance. After incubation, reactions were stopped by adding 50 µL of 0.4 mg/mL DTNB in 6 M guanidine HCl. The plates were placed in the dark and left to react at room temperature
for 10 minutes, then read on a platereader at 412nm. The activity of thioredoxin reductase is detected by the ability of thioredoxin to reduce protein disulfides present in insulin. Thioredoxin reductase reduces thioredoxin, which is present in excess, using electrons from NADPH. The reduced thioredoxin then reduces insulin disulfides to SH groups, which are detected by DTNB. This results in the formation of the bright yellow product, TNB, which has a maximum absorbance at 412 nm, therefore the greater the amount of thioredoxin reductase present, the greater the production of TNB, and the greater the absorbance at 412 nm. Protein estimations were carried out on the samples as detailed above, and thioredoxin reductase activity expressed as moles per minute per milligram of protein (moles/min/mg).

### iii) SUPEROXIDE DISMUTASE ASSAY

Superoxide dismutase (SOD) activity was determined using the pyrogallol oxidation method by Ahmed, Schott *et al.* [302]. Pyrogallol autooxidises at alkaline pH’s producing a yellow-brown product that can be measured at 405 nm. SOD activity can be measured indirectly by its ability to inhibit this autooxidation, and it does so in a concentration dependent manner. 10 µL of 1/10 diluted heart extracts were placed in triplicate in wells of 96 wells plates. 180 µL of air equilibrated pyrogallol assay buffer containing 0.2mM pyrogallol (previously flushed with nitrogen for 1 hour to remove oxygen), 50 mM Tris-cacodylic acid, pH 8.2 and 1 mM DTPA was then added to each well. Plates were read immediately at 405 nm and then every 5 minutes for 1 hour. Inhibition of pyrogallol oxidation was determined graphically using the rate of change in absorbance over the hour (gradient). The greater the amount of SOD present, the greater the inhibition of pyrogallol oxidation so the lower the amount of chromophore produced resulting in a lower rate of change in absorbance (flatter curve). SOD standards, ranging from 20 µg/mL to 0.01 µg/mL, were assayed alongside the unknown samples allowing for the construction of a standard curve. A control well containing water in place of heart extracts or SOD standards was used also, and it showed the largest gradient (therefore least amount of SOD) as expected. 1 unit of SOD activity is defined as the amount of
SOD that inhibits pyrogallol oxidation by 50 % relative to the water control. Protein estimations were performed on samples as previously described, and superoxide dismutase activity expressed as units per milligram of protein (U/mg).

iv) CASPASE-3 ASSAY

Apoptotic activity in the heart tissue extracts was measured using a BD ApoAlert™ Caspase-3 Fluorescent Assay Kit (Clontech, Palo Alto, USA) using the manufacturers protocol. Caspase-3 is an active cell-death cysteine protease involved in the execution phase of apoptosis, and is activated by other upstream caspases, such as caspase-8 and caspase-9. It is a central checkpoint for signals triggered by death receptors, mitochondria (e.g. cytochrome c release) and other stresses. Measurement of caspase-3 activation correlates strongly with TUNEL staining and DNA laddering and is an accepted marker for apoptosis [303,304]. The kit detects the emission shift of 7-amino-4-trifluoromethyl coumarin (AFC). The AFC-substrate conjugate usually emits blue light ($\lambda_{\text{max}} = 400$ nm), however cleavage of the substrate by caspase-3 in the samples liberates AFC, which fluoresces yellow-green ($\lambda_{\text{max}} = 505$ nm). Each kit includes a specific caspase inhibitor that was used as a negative control. Samples were centrifuged at 48 000 g for 10 minutes at 4 °C then diluted 2/3 with protein extraction buffer, and 30 µL of each sample was then used in the assay. Quantification of caspase-3 activity was determined by constructing an AFC calibration curve, and using protein extraction buffer for background calibration. Protein estimations were carried out on the samples, and activity expressed as picomoles AFC per milligram of protein (picomoles AFC/mg).

v) PROTEIN CARBONYL ELISA

Oxidative stress leads to the formation of ROS and the oxidation of a range of molecules including proteins, lipids and DNA, all of which are potentially damaging to normal cellular function. Oxidation of proteins leads to their denaturation and subsequent
loss of biological activity (e.g. enzyme inactivation). Protein oxidation gives rise to protein carbonyl derivatives (PCO) via a variety of mechanisms including fragmentation and amine oxidation by either metal catalysis or hypochlorous acid (HOCl) [305-308].

Protein carbonyls were measured as a marker of oxidative injury using a quantitative immunoassay as described by Buss et al [305]. This ELISA method is more sensitive than the colourimetric assay, requiring only micrograms of protein. Protein derivatization was performed using dintrophenylhydrazine (DNP), then the protein nonspecifically adsorbed to wells of an ELISA plate before probing with a biotinylated antibody raised against protein-conjugated DNP. The biotinylated antibody was then reacted with streptavidin- horseradish peroxidase (Molecular Probes Inc, Eugene, OR, USA) for quantification. 15 µL of heart extract was reacted with 45 µL of freshly made 10 mM DNP in 6 M guanidine hydorchloride, 0.5 M potassium phosphate buffer pH 2.5 for 45 minutes to give a final protein concentration of approximately 1 mg/mL. 2.5 µL was then transferred to 1 mL of coating buffer (10 mM sodium potassium buffer containing 140 mM NaCl, pH 7.0). Triplicate 200 µL aliquots were added to wells of a Nunc Immuno Maxisorp plate (Nalge Nunc International, Roskildo, Denmark), and these were incubated overnight at 4°C to allow protein to adsorb to the wells. The following day plates were washed five times with 1x PBS and wells blocked with 250 µL 0.1% reduced BSA in PBS for 1.5 hours at room temperature. BSA contains carbonyls that can be easily reduced by sodium borohydride, and reduced BSA was prepared by reacting 1% BSA in PBS with 2% sodium borohydride for 30 minute and dialyzing overnight against 1x PBS. After blocking, wells were washed five times with 1X PBS, then 200 µL of biotinylated anti-DNP antibody (Molecular Probes Inc, Eugene, OR, USA) diluted 1/1000 with 0.1% reduced BSA, 0.1% Tween 20 solution was added to each well. The anti-DNP antibody was a biotin-conjugated rabbit IgG polyclonal antibody raised against a DNP conjugate of keyhole limpet hemocyanin. Plates were incubated with anti-DNP for 1 hour at 37°C then washed five times with 1x PBS to remove any unbound antibody. 200 µL of streptavidin- horseradish peroxidase (HRP) (diluted 1/3000 with 0.1% reduced BSA, 0.1% Tween 20 solution) was added to each well and plates incubated at room temperature for 1 hour. The streptavidin present on the HRP binds to the biotin on the protein bound antibody, and any unbound streptavidin- HRP is removed during the five
washes with 1x PBS at the end of the 1 hour incubation. 200 µL of a substrate solution containing o-phenylenediamine (0.6 mg/mL) and hydrogen peroxide (stock diluted 1/2500) in 50 mM Na₂HPO₄ plus 24 mM citric acid was added to each well. Colour was allowed to develop for 25 minutes through the reaction of hydrogen peroxide with HRP, then the reaction was stopped by adding 100 µL of 2.5 M sulfuric acid. Absorbance was measured on a plate reader at 490 nm. Oxidised BSA containing additional carbonyls was prepared for use as a reference by reacting 50 mg/mL BSA in PBS with 5mM hypochlorous acid. A seven-point standard curve of reduced and oxidized BSA (ranging from 0-40% oxidized BSA) was included in the assay, and a blank for the DNP solution containing only extraction buffer with no protein was subtracted from all absorbance readings [307]. Protein carbonyl levels were standardised to protein concentrations, and are expressed as protein carbonyl units per milligram of protein (units/mg).

vi) LIPI D PEROXIDATION ASSAY

Lipid peroxidation (LPO) is a marker of biological oxidation and occurs due to the generation of reactive oxygen species. It is a well established mechanism of cellular injury and ultimately leads to loss of membrane integrity and function [309]. A colourimetric LPO assay kit (Calbiochem, La Jolla, CA, USA) was used to measure malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) levels in heart extracts. MDA and 4-HNE are end products derived from peroxidation of polyunsaturated fatty acids and esters, and measurement of these aldehydes provides an index of lipid peroxidation.

This assay used the chromogenic reagent, 10.3 mM N-methyl-2-phenylindole in acetonitrile (R1), which reacts with MDA and/or 4-HNE present within the samples at 45°C. Condensation of one molecule of MDA or 4-HNE with 2 molecules of R1 yields a chromophore with maximal absorbance at 586 nm. Tissue extracts were diluted to 10% w/v with protein extraction buffer. Although it has been recommended to add butylated hydroxytoluene to samples during storage to prevent sample oxidation, our tissue extracts were stored at -80°C thereby minimizing the amount of autooxidation occurring [309]. All samples were treated identically, so autooxidation should be constant between
samples, and as we are only looking for differences between groups rather than exact ‘numbers’, we don’t believe autooxidation will impact on these studies. 200 µL of diluted heart extracts were reacted with 650 µL of methanol diluted R1 in a glass test tube. 150 µL of 15.4 M methane-sulphonic acid was then added, the solution mixed and rubber stoppers placed on top of the tubes. Due to the build up of pressure in the tubes, stoppers had to be parafilmed onto tubes to prevent them popping off during heating. Tubes were incubated in a waterbath at 45 °C for 40 minutes after which the reactions reach a plateau. Reactions were stopped by chilling samples on ice. The purple chromophore produced in the reaction is stable for at least 2 hours at 4°C, so samples were left on ice prior to measuring their absorbance at 586 nm. Absorbance at 586 nm is a linear function of both MDA and 4-HNE concentrations, and the extinction coefficients for MDA and 4-HNE do not differ significantly at 586 nm. MDA and 4-HNE standards were assayed alongside the unknown samples to allow construction of standard curves. Protein extraction buffer was used as the blank. Lipid peroxide levels are expressed as nmoles MDA and 4-HNE per milligram of extracted protein (nmoles MDA & 4-HNE/mg).

vii) **AMINO ACID OXIDATION**

Oxygen-derived radicals are capable of oxidizing amino acids present in the side-chains of proteins. Tyrosine is one such amino acid that readily undergoes oxidation by ROS such as the hydroxyl radical and peroxynitrite to form the well characterized products; 3,4-dihydroxyphenylalanine (DOPA); 3,3'-di-tryosine (di-Tyr); 3-hydroxyphenylalanine (m-Tyr) and 3-nitrotyrosine (3-nitrotyr). Phenylalanine can also undergo oxidation to form 2-hydroxyphenylalanine (o-Tyr). These oxidation products can also be used to assess the degree of oxidative stress within a sample, and can all be detected in sample homogenates using high pressure liquid chromatography (HPLC) combined with UV detectors, fluorescence detectors and electrochemical detectors.

Prior to HPLC analysis, protein extractions had to be delipidated and hydrolysed, and this was done according to the method described by Woods et al [310]. 200 µL of each tissue homogenate was transferred to chilled 1 mL brown glass autosampler vials.
(Alltech Associates, Baulkham Hills, NSW, Australia), and 400 µL of 100 mM phosphate buffer was added to each. Phosphate buffer was used rather than PBS incase we chose to measure 3-chlorotyrosine (another tyrosine oxidation product generated by HOCl and Cl₂) in the hydrolysed samples in the future. Sodium borohydride (10 µL of 10 mg/mL in nanopure water) was added to each sample to reduce hydroperoxyl groups (-OOH) within the proteins to their corresponding hydroxyl group (-OH) for detection by HPLC. 50 µL of sodium deoxycholate (0.3% stock) and 100 µL of trichloroacetic acid (TCA, 50% stock) was then added to the vials. Deoxycholate was used for delipidation of the samples as it solubilises lipids and holds them in the aqueous layer, and TCA was then used to precipitate the protein. Samples were centrifuged at 7000 rpm for 2 minutes at 4°C to pellet the protein, and the pellet washed twice with 500 µL cold acetone and once with 500 µL diethyl ether. The washed delipidated samples were then freeze-dried and hydrolysed using a standard gas-phase acid-catalysed method for 16 hours at 110°C under vacuum [310-312]. This was achieved by adding 50 µL of mercaptoacetic acid and 1 mL of HCl to Pico-Tag reaction vessels (Waters Corporation, Milford, MA, USA). Up to 8 brown glass vials containing the protein pellets were placed in each vessel, and the vessels completely deoxygenated using a vacuum pump. These vessels were then incubated at 110°C overnight (for 16 hours). These reaction vessels allow hydrolysis of the protein samples to occur in the absence of oxygen, and without diluting the samples (particularly during heating, the acid fumes are able to react with the protein samples without diluting them). Following incubation, reaction vessels were removed from the oven and allowed to cool for 30 minutes in the fumehood. The vacuum was released immediately to reduce the amount of acid condensing on the hydrolysate samples. The brown glass vials containing the hydrolysed proteins were removed from the reaction vessels, rinsed with acetone to remove all traces of acid from the outside of the vials, and placed in a pre-cooled Speedi-vac (Savant, Farmingdale, NY, USA) for 60 minutes. The Speedi-vac freeze dries the samples by centrifuging under vacuum using two cold traps, the first is at -60°C and collects most solutions whilst the second cold trap contains liquid nitrogen and collects volatile solutions. The dried hydrolysates were then rehydrated in 200 µL of nanopure water, and filtered by centrifuging at 14 000 rpm for 2 minutes through a 0.45 µm-pore size filter (Nanosep; Pall Life Sciences, Ann Arbor, MI,
USA). The cleared samples were then transferred into 200 µL glass inserts (Alltech Associates, Baulkham Hills, NSW, Australia) within HPLC vials (with teflon septum-containing caps, Supelco, Bellafonte, PA, USA). These samples were stored at -80°C prior to HPLC analysis. Previous studies by Woods et al, Davies et al and Fu et al have confirmed that no detectable artifactual oxidation occurs during the described processes [310-312].

Samples were chromatographed on an SCL-10A HPLC system (Shimadzu, South Rydalmere, NSW, Australia) which was equipped with a column oven set at 30°C (Waters Corporation, Milford, MA, USA). Using a column oven allows samples to elute in the same position with each run regardless of room temperature making the HPLC more efficient and reliable. Peak areas were determined using Class SCL-10 software (Shimadzu). DOPA, di-Tyr, 3-nitrotyrosine, m-Tyr, o-Tyr and parent tyrosine were assayed in the protein hydrolysates by separation on a Luna 5U C18 (2) column (250 x 4.6 mm, 5 µm particle size; Phenomenex, Torrance, CA, USA). The mobile phase (0.8 mL/min) consisted of a two-solvent system; Solvent A being 50 mM citric acid buffer (pH 4.3) and Solvent B being 20% (v/v) acetonitrile in 50 mM citric acid buffer. Both solvents were filtered using 0.2 µm-pore sized disposable filters and bubbled helium to remove any air. The column was equilibrated with 100 % Solvent A prior to the injection of any samples. 20 µL of each sample or standard was then injected into the column. The gradient used was a linear gradient with 0% Solvent B for 35 minutes, then a linear increase to 50% Solvent B over the next 20 minutes before washing with 100% Solvent B for 20 minutes and re-equilibrating with 0% Solvent B (100% Solvent A) for 10 minutes. The organic phase of the solvent-system is required to elute hydrophobic products such as 3-nitrotyrosine. The elution profile was monitored in series with a UV detector (λ 280 nm; SPD-M10A, Shimadzu), a fluorescence detector (RF-10A XL, Shimadzu) and a 12-channel (400-950 mV) Coularray electrochemical detector (ESA, Chelmsford, MA, USA). Parent Tyr (retention time = 11.9 min) was quantified by UV absorbance. Fluorescence detection was carried out (for t = 0-45 min) using a λexcitation of 280 nm and λemission of 320 nm for the detection of DOPA (8 min), m-Tyr (17.7 min) and o-Tyr (26.6 min), and then an λemission of 410 nm for the detection of di-Tyr (17.6 min). 3-nitrotyrosine (51.1 min) was measured by oxidation at +950 mV (twelfth electrode) on the
electrochemical detector (for $t = 0$-60 min). The Coularray electrochemical detector is last detector in system as it destroys the sample by oxidizing it. Any interfering co-eluting peaks were first oxidized at +960 mV in the guard cell to allow accurate detection of 3-nitrotyrosine. All cells/electrodes were cleaned between samples by running them at 1000 mV to oxidize any remaining contaminants.

Elution positions were determined with standards, the identities of compounds being confirmed previously by Davies et al. by UV absorption and fluorescence spectra [311]. The standard contained 10 $\mu$M of each DOPA, tyrosine, $m$-Tyr, $o$-Tyr and 3-nitrotyr with 1 $\mu$M di-Tyr, and elution profiles of each are shown below in Fig. 2.3. Accurate quantification of $m$-Tyr could not be achieved due to overlap with di-Tyr as it eluted immediately after di-Tyr, and hence could not be quantitated (Fig. 2.3). Levels of DOPA, di-Tyr, 3-nitrotyr and $o$-Tyr are expressed as molar ratios relative to the amount of parent tyrosine present within the protein sample ($\mu$mole oxidized product/mole tyrosine). This is to compensate for any loss during sample processing or analysis.

![Fig 2.3](image)

**Fig 2.3:** Elution profiles for standards of DOPA (A), tyrosine (B), di-Tyr (C), $o$-Tyr (D) and 3-nitrotyrosine (E) by 12 channel electrochemical detector. Accurate quantification of $m$-Tyr (F) could not be achieved due to overlap with di-Tyr as it eluted immediately after di-Tyr.
2.7 GENE EXPRESSION ANALYSIS

i) cDNA SYNTHESIS

First strand cDNA was synthesised from 5µg of total RNA using 200 units Superscript III H- reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with 250 ng of random hexamers (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. Random hexamer primers (250 ng) were added to 5 µg total RNA with 10 nmoles PCR nucleotide mix (dNTPs), and the final volume made up to 12 µL with nuclease-free water. This was incubated at 65°C for 5 minutes then chilled on ice for 1 minute. First strand buffer (1x), 10mM DTT, and 40 units of RNasin (Promega, Madison, WI, USA) were added and the reaction mix incubated at 25 °C for 2 minutes before adding 200 units of Superscript III H- reverse transcriptase. cDNA was then synthesised by incubating at 25°C for 10 minutes and 50°C for 60 minutes. Superscript III was inactivated by heating at 70°C for 15 minutes. Random hexamers were used as they bind to any region/sequence of RNA giving greater amplification, and enable the use of 18S rRNA as the invariant endogenous control. Synthesised cDNA was diluted 1/400 with nuclease-free water and stored at -20°C before use.

ii) PRIMER DESIGN

Primers Express software (Applied Biosystems, Foster City, CA, USA) was used to design primer sequences (Table 2.1) with primer pairs for each transcript designed to span an exon-intron-exon boundary where possible. This stops amplification of any possible contaminating DNA including genomic DNA. A BLAST search was also performed on primer sequences to ensure oligos didn’t hybridize to unintended targets. Primers were designed following these basic guidelines [313]:

* Amplicon should be between 75-150 bp in size
* Optimal primer length is between 17-25 bp’s
* Tm of primers should be within 58-60°C with 1°C difference between them
* GC content within 30-80%
* Last 5 bases at the 3’ terminal of each primer should have no more than 2 G’s or C’s
* Avoid runs of more than 3 identical nucleotides, especially for G
* Avoid secondary structures (e.g. primer dimers and hairpins)

**Table 2.1:** Primer sequences and expected amplicon lengths for 7 rat genes within the glutathione peroxidase and thioredoxin reductase antioxidant systems, and the internal housekeeping gene, 18S.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>5’ → 3’ sequence</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gpx-1</td>
<td>Forward</td>
<td>CAGTTCCGGACATCAGGGAGAAT</td>
<td>139</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGAGCAGGGTGAGCCTTCT</td>
<td></td>
</tr>
<tr>
<td>Gpx-4</td>
<td>Forward</td>
<td>AACGTTGCCCTCAGCAATGA</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGAGAAGGAGCAGGAGTTCGATTA</td>
<td></td>
</tr>
<tr>
<td>Gsr</td>
<td>Forward</td>
<td>GGGCAAGAGAGATTTCCAGGT</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGACGGCTTTCCATCTTCAGTGA</td>
<td></td>
</tr>
<tr>
<td>Txnr-1</td>
<td>Forward</td>
<td>CGCTAAATCCACAAAACAGTGA</td>
<td>141</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGTATCTTCAGCCGTTCTTTTCAT</td>
<td></td>
</tr>
<tr>
<td>Txnr-2</td>
<td>Forward</td>
<td>CAACGTCCCCACAACTGTC</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATCCCGATCTGCCACTGT</td>
<td></td>
</tr>
<tr>
<td>Prdx-2</td>
<td>Forward</td>
<td>GATGCCAAAGGGTGCTCTTC</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ACAGACTTTCCCATGCTCA</td>
<td></td>
</tr>
<tr>
<td>Txn</td>
<td>Forward</td>
<td>TTCCCTCTGTGACAGTATTCCAA</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGGTCGGCATGCTTTGACT</td>
<td></td>
</tr>
<tr>
<td>18S-A</td>
<td>Forward</td>
<td>CTCAACACGGGAAACCTC</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAATCGCTCCACCAACTA</td>
<td></td>
</tr>
</tbody>
</table>
iii) QUANTITATIVE REAL-TIME PCR

Quantitative real-time PCR (qRT-PCR) was used to measure the expression of antioxidant proteins in the glutathione peroxidase and thioredoxin reductase systems in response to various experimental factors. It relies on the ability to measure the accumulation of PCR product during each amplification cycle, and this is achieved by measuring the increase in a fluorescent signal which is proportional to the amount of double stranded DNA present. SYBR Green 1 (Molecular Probes, Eugene, OR, USA) is a non-specific intercalating fluorescent dye which binds to the double-stranded DNA PCR amplicon allowing the real-time measurement of PCR products. The qRT-PCR method used in the following studies measured two transcripts, the target gene and an invariant control as a singleplex. The choice of invariant control (also referred to as housekeeping gene or endogenous control) is important as its expression must be virtually identical across all samples and remain constant over the conditions to which the target gene is tested. The expression of the invariant control is used to normalize expression values for the target gene, and correct for any variations in reaction kinetics, experimental conditions and cDNA quantity [313]. 18S rRNA was used as the invariant internal control in these studies as our laboratory have previously shown ischemia induces differential expression of other widely used housekeeping genes such as GAPDH and β-actin, whilst 18S rRNA is more stable [314].

Quantitative real-time PCR was performed in triplicate on an ABI PRISM 7700 Detection System (Applied Biosystems, Foster City, CA, USA). All PCR reagents were purchased from Ambion, (Austin, TX, USA) unless otherwise noted. The final reaction volume (20 µL) contained 1x PCR buffer, 3 mM MgCl₂, 0.2 mM dNTPs, 0.5x SYBR Green 1 (Molecular Probes, Eugene, OR, USA), 1x ROX, 0.5 U/µL Supertaq, 50 nM of each primer (Geneworks, Adelaide, Aust) and 5 µL of 1/400 diluted cDNA. Due to the considerably lower expression of Gpx-4 in the heart, a greater amount of primer was needed for PCR amplification. Increasing the primer concentration (400nM) ensured that the signal-to-noise ratio was sufficient to confidently measure Gpx-4 expression. Primer limiting concentrations for 18S rRNA were used (10 nM forward primer and 5 nM reverse primer) due to the abundance of 18S rRNA within the samples. These
concentrations had been previously optimised in earlier experiments. The PCR protocol consisted of a 95°C step for 3 minutes followed by 40 cycles consisting of 15 seconds at 95°C and 1 minute at 60°C. All reaction plates contained a serial dilution series (1/200, 1/400, 1/800) of pooled cDNA to test the PCR amplification efficiency of all assays and enable normalisation of $C_T$ values between plates (cross-plate calibrator). Non-template controls (NTC) were also added to determine baseline noise.

Following baseline correction, the threshold level was set during the geometric (exponential) phase of PCR amplification to generate the threshold cycle ($C_T$) value for each amplification curve (Fig. 2.4). This was performed separately for each gene and 18S rRNA, (on each triplicate of each sample, and only accepted if the standard deviation within the one sample was < 0.16). Average gene $C_T$ values were normalized to the average 18S rRNA $C_T$ values from the same cDNA sample.

A melt (dissociation) curve was run on PCR products to assess product purity and ensure no primer dimer had been formed. This is run independent of the PCR by heating PCR products to 95°C for 15 seconds, cooling to 60°C for 20 seconds (to equilibrate) before gradually re-heating (dissociating) to 95°C over 20 minutes. Melt curves (Fig. 2.5) were analysed to ensure only one peak was present for each PCR product and that the product melted at the predicted temperature of melting ($T_m$). Using a primer concentration between 200 – 500 nM causes the $T_m$ of the PCR amplicon to increase by 3°C. This supports our melt curve analysis for Gpx-4 which showed a $T_m$ of 84°C, 3°C higher than the predicted $T_m$ of 81°C. Although a greater amount of primer was used, no primer dimer was produced in any samples, and the non-template control was clear of any products in both the amplification plot and the melt curve.

Gene expression changes per group are expressed as a % relative to control hearts using a modification of the $2^{ΔΔC_T}$ method, taking reaction efficiency into account [315,316].
Fig 2.4: Real-time PCR amplification plot for test gene (Gpx-4 in this example) (A) and 18S (B). Non-template controls demonstrate no amplification ($C_T = 40$) for both Gpx-4 (C) and 18S (D) indicating no products or primer dimer were formed. The threshold level was set during the geometric (exponential) phase of PCR amplification as can be seen above on the test gene plots. This was to generate the threshold cycle ($C_T$) value for each amplification curve, and was performed separately for each gene and 18S rRNA.

Fig 2.5: Melt (dissociation) curve used to assess PCR product purity and ensure no primer dimer formation. Peaks occur on the melt curve at the specific temperature the PCR product (amplicon) melts at ($T_m$). Melt curves are analysed to ensure only one peak is present for each amplicon and that it occurs at the predicted $T_m$. This plot demonstrates a single peak for the test gene amplicon (Txnrd1 in this example, $T_m = 82.3°C$) and a single peak for the 18S amplicon ($T_m = 85°C$) indicating pure PCR product. The non-template controls contain no peaks indicating no products (including primer dimer) were formed and no contaminations are present.
2.8 **STATISTICAL ANALYSIS**

All data was analysed using either unpaired two-tailed Student t-tests (for comparison of 2 experimental groups), or one-way analysis of variance (ANOVA) (comparison of more than 2 experimental groups) followed by Newman-Kuels and Bonferroni post-hoc tests for multiple comparisons when initial differences were detected. All statistical tests were performed using GraphPad Prism version 3.0a (GraphPad Software Inc., San Diego, USA) and p<0.05 was considered indicative of statistical significance. All data values are reported as mean ± standard error of the mean (SEM).
CHAPTER 3

Ischemia vs Reperfusion: Effects on expression & activity of myocardial antioxidant enzyme systems, and oxidative damage.
3.1 **ABSTRACT**

Reperfusion of ischemic tissue leads to generation of reactive oxygen species (ROS) which are important in the pathogenesis of ischemic/reperfusion cellular damage. ROS formed during oxidative stress can initiate lipid peroxidation, oxidize proteins to inactive states and cause DNA strand breaks, all potentially damaging to normal cellular function. In normal physiological conditions ROS production is usually homeostatically controlled by endogenous free radical scavengers such as SOD, catalase, and the GPX and TxnRed systems. This chapter examines the effect ischemia alone and with reperfusion, has on the activity and transcription of antioxidant proteins (such as those in the GPX and TxnRed systems), and on cellular injury. The levels of various lipid and protein oxidation products were measured in heart extracts as an indicator of oxidative stress, along with activity of the apoptotic protease caspase-3. Hearts were isolated from male wistar rats and subjected to 22.5 min global ischemia ± 45min reperfusion or 87.5 min normoxic perfusion using a Langendorff perfusion apparatus. Expression of the TxnRed system and activity of TxnRed was found to be up-regulated during ischemia suggesting it may contain a hypoxic responsive element in its promoter, whereas GPX system expression and activity was only up-regulated after reperfusion. There were no changes in SOD activity during ischemia or reperfusion suggesting the GPX and TxnRed systems are more finely ‘in tune’ with the oxidative status of cardiac cells. Protein oxidation was increased during reperfusion with significantly higher levels of protein carbonyls (PCO), DOPA, di-Tyrosine and o-Tyrosine. Although lipid peroxidation was also significantly increased in reperfusion, it was greater during ischemia, indicating oxidative damage occurs both in the absence and presence of oxygen. Apoptosis was found to be elevated during reperfusion but not ischemia indicating it is not a major process for cell death during ischemia. These results suggest GPX plays a major role in myocardial defence against ROS during reperfusion whilst TxnRed may play a role in providing protection during periods of ischemia.
3.2 **INTRODUCTION**

During clinical procedures such as by-pass surgery, transplantation and angioplasty, the heart experiences transient periods of ischemia followed by reperfusion with oxygenated fluids. The re-introduction of oxygen has been shown to increase the generation of reactive oxygen species (ROS) that are capable of damaging lipids, proteins and DNA [317]. Generation of ROS has been associated with infarction, necrosis, apoptosis, arrhythmogenesis and endothelial dysfunction that all lead to poor cardiac recovery post ischemia [40,102,108,118,174,317].

Targeting free radical generation with various antioxidants has been shown to reduce injury following oxidative stress. The major endogenous antioxidants that counteract ROS are superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione, thioredoxin peroxidase and thioredoxin [40,166]. Glutathione reductase and thioredoxin reductase also play a major role in shuffling electrons from NADPH to glutathione and thioredoxin respectively thereby maintaining the activity of these important antioxidant enzyme systems [110,213,318]. Both the GPX and TxnRed systems are expressed in the mammalian heart, and it has been shown that up-regulation of these systems is cardioprotective and down-regulation is detrimental following ischemia-reperfusion [257,277,289,290,319,320].

This chapter examines the effect ischemia and reperfusion has on the activity and expression of these antioxidant systems, and on oxidative damage. It is well known ischemia-reperfusion leads to cellular dysfunction but the relative contribution of each to cellular damage is unknown. Whether ischemia and/or reperfusion induces the expression and/or activity of antioxidants is also unknown. Up-regulation of antioxidants may be a ‘self protection’ mechanism thereby limiting the oxidative injury caused by ischemia and/or reperfusion. Levels of various markers of biological oxidation will also be measured in hearts subjected to ischemia with and without reperfusion to determine whether oxidative damage can only occur in the presence of oxygen. Apoptosis will be examined to establish if it is a major contributing process for cell death during ischemia and/or reperfusion.
3.3 MATERIALS AND METHODS

i) EXPERIMENTAL GROUPS AND ISOLATED HEART PROTOCOL

Male wistar rats (10 weeks of age), as described in Section 2.2 of Chapter 2 (General Methods), were randomly assigned to one of 3 groups: normoxic rats (n=10) underwent normoxic perfusion only; ischemic rats (n=8) underwent ischemia without reperfusion; and reperfused rats (n=10) were subjected to ischemia and reperfusion.

Animals were anaesthetised and hearts removed as described in Chapter 2 (Section 2.4, General Methods). Hearts were then perfused using the Langendorff isolated heart preparation as explained in Section 2.4 of Chapter 2 (General Methods). Normoxic hearts were subjected to 87.5 minutes of normoxic perfusion only (i.e. hearts received adequate (100%) perfusion with 37°C Krebs-Henseleit buffer equilibrated with 95% O₂ and 5% CO₂ for the entire experimental time course); ischemic hearts underwent a 20 minute equilibration period of normoxic perfusion followed by 22.5 minutes of global ischemia (zero flow, 100% ischemia); and reperfused hearts underwent 20 minutes of normoxic perfusion (equilibration), 22.5 minutes global ischemia and 45 minutes reperfusion. The length of ischemia used in these and subsequent studies (22.5 minutes) was determined in pilot experiments to be a period which resulted in ~50% recovery of contractile function. This provided the potential to measure both improved and reduced recoveries. At the end of perfusion/ischemia/reperfusion, hearts were removed from the cannula, rapidly blotted and weighed, then frozen at -80°C until subsequent analysis.

ii) BIOCHEMICAL ANALYSIS

Protein was extracted from hearts as described in Section 2.5 i of Chapter 2 (General Methods), and protein estimations performed on extracts as detailed in Section 2.5 ii of Chapter 2.

Activity of the antioxidant enzymes, glutathione peroxidase (GPX), thioredoxin reductase (TxnRed) and superoxide dismutase (SOD) were measured in protein extracts as described in Section 2.6 (methods i, ii and iii) of Chapter 2.
To assess the level of oxidative stress within these tissues, lipid peroxidation was measured in heart extracts along with protein carbonyls and the tyrosine oxidation products DOPA, di-Tyr, o-Tyr and 3-nitrotyrosine. These methods are explained in detail in Section 2.6 (methods v, vi, and vii) of Chapter 2 (General Methods).

Caspase-3 is a marker of apoptosis common to both the caspase-6/9 and caspase-8 pathways. Its activity was measured as an indicator of apoptosis in heart samples using the method described in Chapter 2, Section 2.6 iv.

**iii) GENE EXPRESSION ANALYSIS**

RNA extraction, quantitation and determination were performed on heart samples (n=6) according to methods iii and iv in Section 2.5 of Chapter 2 (General Methods).

The mRNA expression of the glutathione related and thioredoxin related genes was measured by firstly synthesising cDNA from total RNA, then performing quantitative real-time PCR with primers for Gpx-1, Gpx-4, Gsr, Txnrd-1, Txnrd-2, Txn and Prdx-2. These methods are explained in full in Section 2.7 of Chapter 2 (General Methods). Gene expression changes per group are expressed as a % relative to normoxic control hearts.

**iv) DATA ANALYSIS**

All data was analysed using one-way ANOVA’s as described in Section 2.9 of Chapter 2 (General Methods).
3.4 RESULTS

i) PHYSIOLOGICAL AND FUNCTIONAL ANALYSIS

There were no differences in body weights between the experimental groups at the time of sacrifice, with all animals weighing between 365-485 grams. Wet heart weights were also similar between all groups. Whilst heart rates were similar between all experimental groups during equilibration, hearts were not paced and as a result we have chosen to monitor RPP as opposed to developed pressure as an indicator of myocardial function. All hearts displayed an initial RPP of between 46 and 52 x10³ mmHg/min, whereas coronary flow was approximately 24-27 ml/min. After 20 minutes equilibration, dP/dt’s were similar between all groups, with +dP/dt ranging between 3100-3400 mmHg/min and –dP/dt between –3000 and –3400 mmHg/min.

In initial studies, stability of rat hearts under normoxic conditions were assessed. To assess functional stability in the Langendorff perfused rat heart, a group of hearts were perfused under normoxic conditions for 120 minutes. Function was monitored over this period and the decline in developed pressure, heart rate and coronary flow assessed relative to function during equilibration. Heart rate and coronary flow remained stable throughout the perfusion time-course. There was a gradual fall in developed pressure and therefore RPP (~23%) over 120 minutes of normoxic perfusion (represented on Fig 3.1 as 90 min reperfusion). However this decline was not significant and is not believed to impact on functional recovery during ischemia-reperfusion.

The length of ischemia used in this study and subsequent studies was determined in preliminary experiments by examining functional recovery of hearts following different lengths of zero flow 100 % ischemia. 22.5 minutes ischemia resulted in approximately 48 % recovery of contractile function (RPP) after 45 minutes reperfusion whereas 20 minutes ischemia resulted in ~55 % recovery of RPP and 25 minutes of ischemia reduced contractile recovery to ~43 % (Fig 3.1). We chose to use 22.5 minutes ischemia as recoveries were ~50%, providing the potential to measure both improved and reduced recoveries.
**Fig 3.1:** Recovery of Rate Pressure Product (RPP) in the Langendorff perfused isolated rat heart following different lengths of zero flow global ischemia. Time 0 min represents the end of ischemia and start of reperfusion. Time -10 min signifies function during the equilibration period. Normoxic hearts were not subjected to ischemia-reperfusion and were used to test the stability of Langendorff perfused hearts over the experimental time-course. Data is expressed as mean ± SEM, x1000 mmHg/min.
ii) **OXIDATIVE DAMAGE**

Markers of oxidative damage were increased in both ischemic and reperfused isolated rat hearts when compared to levels in normoxic hearts.

Lipid peroxidation (*Fig 3.2*), measured as 4-HNE and MDA, was significantly increased by both ischemia (*p*<0.001) and reperfusion (*p*<0.05). However, ischemic levels of 4-HNE and MDA were greater than the levels generated during reperfusion (4.86 ± 0.08 vs 1.90 ± 0.1 nmoles MDA & 4-HNE/mg protein respectively) indicating a greater amount of lipid peroxidation occurred in the presence of reduced tissue oxygen. LPO levels in normoxic hearts were 1.52 ± 0.1 nmoles MDA & 4-HNE/mg protein.

All markers of protein and amino acid oxidation measured were increased during reperfusion. Oxidised protein carbonyl derivatives (*Fig 3.3*) were significantly increased in both reperfusion (*p*<0.001) and ischemia (*p*<0.05), however reperfused levels of PCO were higher than ischemic levels (102.6 ± 4.4 and 80.2 ± 2.1 respectively, *vs* 58.5 ± 8.1 units/mg protein for normoxic hearts). Normoxic levels of DOPA (1253 ± 63.1 µmol/mol Tyr) were significantly lower than DOPA levels in both reperfused (1729 ± 71.3 µmol/mol Tyr, *p*<0.01) and ischemic (1592 ± 82.5 µmol/mol Tyr, *p*<0.05) hearts (*Fig 3.4*). Reperfused hearts also contained significantly (*p*<0.05) higher levels of di-tyrosine (*Fig 3.5*) when compared to normoxic hearts (51.7 ± 5.2 *vs* 33.5 ± 1.7 µmol/mol Tyr). Ischemic levels of di-Tyr (39.4 ± 3.8 µmol/mol Tyr) were also slightly higher than the normoxic levels. There was a slight increase in o-Tyr in ischemic hearts when compared to normoxic hearts (1121 ± 30.8 *vs* 978 ± 41.7 µmol/mol Tyr), however there was a large significant (*p*<0.05) increase in o-Tyr in reperfused hearts as can be seen in *Fig 3.6* (1898 ± 292.6 µmol/mol Tyr). There were no significant differences in 3-nitrotyrosine levels in normoxic, ischemic or reperfused hearts, however levels were increased during both ischemia and reperfusion (*Fig 3.7*, 1558 ± 61.1, 1729 ± 77.7 and 1908 ± 157.5 µmol/mol Tyr respectively).
Fig 3.2: Effect of ischemia and reperfusion on lipid peroxidation in the isolated rat heart. LPO was measured as 4-HNE & MDA in heart extracts following 22.5 mins ischemia ± 45 mins reperfusion. Data is expressed as mean ± SEM, nmoles MDA & 4-HNE/mg protein (*p<0.05, #p<0.001 vs normoxics).
**Fig 3.3:** Effect of ischemia and reperfusion on the formation of oxidised protein carbonyl derivatives in the heart. PCO levels were measured in protein extracts from isolated rat hearts following 22.5 mins ischemia ± 45 mins reperfusion. Data is expressed as mean ± SEM, arbitrary units PCO/mg protein (*p<0.05, #p<0.001 vs normoxics).
**Fig 3.4:** Effect of ischemia and reperfusion on the oxidation of tyrosine to DOPA in the heart. DOPA levels were measured in protein extracts from isolated rat hearts subjected to 22.5 mins ischemia + 45 mins reperfusion. Data is expressed as mean ± SEM, µmol DOPA/mol Tyr (*p<0.05, **p<0.01 vs normoxics).

**Fig 3.5:** Effect of ischemia and reperfusion on tyrosine oxidation to di-Tyrosine in the isolated heart. di-Tyr was isolated and measured from rat hearts following 22.5 mins ischemia + 45 mins reperfusion. Data is expressed as mean ± SEM, µmol di-Tyr/mol Tyr (*p<0.05 vs normoxics).
**Fig 3.6:** Effect of ischemia and reperfusion on phenylalanine oxidation to o-Tyrosine in the heart. o-Tyr levels were measured in protein extracts from isolated rat hearts following 22.5 mins ischemia + 45 mins reperfusion. Data is expressed as mean ± SEM, µmol o-Tyr/mol Tyr (*p<0.05 vs normoxics).

**Fig 3.7:** Effect of ischemia and reperfusion on tyrosine nitration to the oxidation product 3-nitrotyrosine. 3-nitrotyr levels were measured in protein extracts from isolated rat hearts subjected to 22.5 mins ischemia + 45 mins reperfusion. Data is expressed as mean ± SEM, µmol 3-nitrotyr/mol Tyr.
iii) APOPTOSIS

Apoptotic activity in the heart (Fig 3.8) was measured by caspase-3 activity, a marker of apoptosis common to both the caspase-6/9 and caspase-8 pathways. Caspase-3 activity was not affected by ischemia (439.6 ± 46.2 vs 537.2 ± 131.6 picomoles AFC/mg protein for normoxic hearts), however it was significantly (p<0.05) increased during reperfusion (951.9 ± 139.6 picomoles AFC/mg protein). This was also significantly (p<0.05) greater than the caspase-3 activity in ischemic hearts, indicating a significantly greater level of apoptosis occurs in hearts during reperfusion. This may contribute to, or may result from, the reduced functional recovery observed during reperfusion of ischemic hearts.

![Graph showing caspase-3 activity](image)

**Fig 3.8:** Effect of ischemia and reperfusion on apoptotic activity in the heart. Apoptosis was measured as caspase-3 activation in protein extracts from isolated rat hearts following 22.5 mins ischemia ± 45 mins reperfusion. Data is expressed as mean ± SEM, picomoles AFC/mg protein (*p*<0.05 vs normoxics).
iv) ANTIOXIDANT mRNA EXPRESSION

Quantitative real-time PCR was used to measure the myocardial expression of antioxidant proteins in the glutathione peroxidase and thioredoxin reductase systems in response to ischemia and reperfusion. However, due to the considerably lower expression of Gpx-4, a greater amount of primer was needed for PCR amplification. Increasing the primer concentration (400nM) ensured that the signal-to-noise ratio was sufficient to confidently measure Gpx-4 expression. Using a primer concentration between 200 – 500 nM also causes the T_m of the PCR amplicon to increase by 3°C. This supports our melt curve analysis for Gpx-4 which showed a T_m of 84°C, 3°C higher than the predicted T_m of 81°C. Although a greater amount of primer was used, no primer dimer was produced in any samples, and the non-template control was clear of any products in both the amplification plot and the melt curve. Since the endogenous internal control is important for standardising results, 18S rRNA was chosen as the invariant internal control, as others in our laboratory have previously shown ischemia induces differential expression of other widely used housekeeping genes such as GAPDH and β-actin [314].

Expression of Thioredoxin Related Genes

The mRNA expression of thioredoxin related genes (Txnrd-1, Txnrd-2, Prdx-2 and Txn) in ischemic and reperfused hearts is expressed relative to expression in normoxic hearts, which was set as the baseline (or 100 %) expression in rat hearts.

Ischemia alone was found to up-regulate transcription of thioredoxin related genes in isolated rat hearts. Whilst expression of thioredoxin related genes then decreased during reperfusion, it still remained higher than expression during normoxia.

Expression of Txnrd-1 (Fig 3.9) was significantly (p<0.001) increased by ~170 % during ischemia to 269 ± 14.7 % (relative to normoxic controls). Although Txnrd-1 expression wasn’t as high during reperfusion, expression was still significantly (p<0.01) higher than that of normoxic hearts (170 ± 5.9 % relative to normoxics).

When compared to expression in normoxic hearts, expression of Txnrd-2, or mitochondrial TxnRed, was significantly (p<0.001) higher in ischemic hearts (Fig 3.10).
Expression was also significantly (p<0.001) increased by 300 % in reperfused hearts when compared to normoxic hearts. Txnrd-2 expression was 1274 ± 13.5 % in ischemic hearts, and 400 ± 16.3 % in reperfused hearts, relative to normoxic controls.

Ischemia significantly (p<0.001) increased Prdx-2 transcription in the rat heart by 150 % to 253 ± 25.6 % when compared to normoxic hearts. Expression then decreased during reperfusion to 138 ± 21.1 % (Fig 3.11), which was still ~40 % higher than expression in normoxic hearts.

Txn expression (Fig 3.12) was significantly (p<0.001) increased ~600 % in ischemic hearts (724 ± 13.8 % relative to normoxics ). Txn expression also remained significantly (p<0.05) greater in reperfused hearts (179 ± 17.2 %) being ~80 % higher than that in normoxic hearts.

**Fig 3.9:** Effect of ischemia and reperfusion on myocardial transcription levels of thioredoxin reductase-1. Txnrd-1 gene expression was measured in isolated rat hearts following 22.5 mins ischemia ± 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to normoxic controls, with normoxic expression set at 100%. (**p<0.01, # p<0.001 vs normoxics).
Fig 3.10: Effect of ischemia and reperfusion on thioredoxin reductase-2 expression in the heart. Txnrd-2 gene expression was measured in isolated rat hearts following 22.5 mins ischemia + 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to normoxic controls, with normoxic expression set at 100%. (# p<0.001 vs normoxics).

Fig 3.11: Effect of ischemia and reperfusion on myocardial mRNA levels of thioredoxin peroxidase-2, or peroxiredoxin-2. Prdx-2 gene expression was measured in isolated rat hearts following 22.5 mins ischemia + 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to normoxic controls, with normoxic expression set at 100%. (# p<0.001 vs normoxics).
Fig 3.12: Effect of ischemia and reperfusion on thioredoxin transcription levels in the heart. Txn gene expression was measured in isolated rat hearts following 22.5 mins ischemia ± 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to normoxic controls, with normoxic expression set at 100%. (* p<0.05, # p<0.001 vs normoxics).
Expression of Glutathione Related Genes

The mRNA transcription of glutathione related genes (Gpx-1, Gpx-4 and Gsr) in ischemic and reperfused hearts is also expressed relative to the expression in normoxic hearts, which was set as the baseline (or 100%) expression in rat hearts.

Unlike the TxnRed system, expression of glutathione related genes in the rat heart was found to significantly increase during reperfusion, with only smaller changes in expression during ischemia.

There was a significant (p<0.001) 100% increase in Gpx-1 expression (Fig 3.13) in hearts following reperfusion (200 ± 39.0 % relative to normoxic hearts). Ischemia alone induced a 30 % increase in Gpx-1 expression in rat hearts when compared to expression in normoxic hearts (130 ± 6.6 %).

Myocardial Gpx-4 (or phospholipid hydroperoxidase) transcription was significantly (p<0.001) up-regulated ~700 % following reperfusion (846 ± 21.1 % relative to normoxic controls). Although Gpx-1 expression was only slightly increased during ischemia, Gpx-4 expression was significantly (p<0.001) increased during ischemia to 277 ± 20.23 when compared to normoxics (Fig 3.14).

Glutathione reductase mRNA transcription was not significantly affected by myocardial ischemia or reperfusion with expression only increasing by ~20 and ~25 % respectively when compared to normoxic levels (Fig 3.15). Gsr expression was 118 ± 18.42 % in ischemic hearts and 126 ± 19.17 % in reperfused hearts (relative to normoxics).
Fig 3.13: Effect of ischemia and reperfusion on myocardial transcription levels of glutathione peroxidase-1. Gpx-1 gene expression was measured in isolated rat hearts following 22.5 mins ischemia + 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to normoxic controls, with normoxic expression set at 100%. ( # p<0.001 vs normoxics).

Fig 3.14: Effect of ischemia and reperfusion on myocardial mRNA levels of glutathione peroxidase-4. Gpx-4 (or phospholipid hydroperoxidase) gene expression was measured in isolated rat hearts following 22.5 mins ischemia + 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to normoxic controls, with normoxic expression set at 100%. ( # p<0.001 vs normoxics).
**Fig 3.15:** Effect of ischemia and reperfusion on glutathione reductase expression in the heart. Gsr gene expression was measured in isolated rat hearts following 22.5 mins ischemia + 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to normoxic controls, with normoxic expression set at 100%.
v) **ANTIOXIDANT ENZYME ACTIVITIES**

Ischemia induced a significant (p<0.05) increase in thioredoxin reductase activity *(Fig 3.16)* when compared to the activity of normoxic hearts (121.7 ± 5.0 vs 87.79 ± 4.8 moles/min/mg protein). Thioredoxin reductase activity in reperfused hearts was 105.5 ± 5.3 moles/min/mg protein. This was also higher than activity in normoxic hearts, however it was lower than TxnRed activity in ischemic hearts.

There was no real change in glutathione peroxidase activity in hearts during ischemia when compared to normoxic hearts, with activity being 310.5 ± 50.7 mmoles/min/mg protein, only slightly higher than activity in normoxic controls (295 ± 21.5 mmoles/min/mg protein). Glutathione peroxidase activity *(Fig 3.17)* in reperfused hearts was significantly (p<0.05) higher than activity in normoxic hearts (437 ± 26.4 vs 295 ± 21.5 mmoles/min/mg protein). When compared to the ischemic hearts, glutathione peroxidase activity in the reperfused hearts was also significantly (p<0.05) increased.

Superoxide Dismutase activity was not affected by myocardial ischemia and/or reperfusion, with activities being very similar to that in normoxic hearts *(Fig 3.18)*. Superoxide dismutase activities for normoxic, ischemic and reperfused isolated rat hearts were 45.6 ± 5.9, 42.2 ± 5.0 and 50.0 ± 8.4 units SOD/mg protein respectively.
Fig 3.16: Effect of ischemia and reperfusion on thioredoxin reductase activity in the rat heart. TxnRed activity was measured in protein extracts from isolated rat hearts following 22.5 mins ischemia + 45 mins reperfusion. Values are presented as mean ± SEM, moles TxnRed/min/mg protein. (* p<0.05 vs normoxics).
**Fig 3.17:** Effect of ischemia and reperfusion on myocardial glutathione peroxidase activity. GPX activity was measured in protein extracts from isolated rat hearts following 22.5 mins ischemia + 45 mins reperfusion. Values are presented as mean ± SEM, mmoles GPX/min/mg protein. (*p<0.05 vs normoxics).
**Fig 3.18:** Effect of ischemia and reperfusion on superoxide dismutase activity in the rat heart. SOD activity was measured in protein extracted from isolated rat hearts following 22.5 mins ischemia ± 45 mins reperfusion. Values are presented as mean ± SEM, units SOD/mg protein.
3.5 **DISCUSSION**

Ischemia-reperfusion has been shown to generate reactive oxygen (ROS) species in cardiac tissues [102,317]. Controlling this damaging process has been the subject of considerable research in recent years as the degree of ROS generation and biological oxidation has been correlated with poor functional cardiac recovery post ischemia-reperfusion [102,105,108,118]. This chapter has extended these observations by examining the degree of biological oxidation in hearts during ischemia alone and reperfusion, and has shown that oxidative damage occurs during both the ischemic episode (i.e. in the absence of oxygen) and reperfusion (i.e. the re-introduction of oxygen). Due to the short half life, lipophillic nature and high reactivity of ROS, it is difficult to measure them directly, however it is possible to measure their products which are more stable and an indicator of cellular damage. This study has shown that the tissue concentrations of both oxidized proteins and lipid peroxides increases during and following ischemic insults, presumably in response to the production of ROS.

Protein oxidation was measured as protein carbonyl derivatives (PCO), DOPA, di-Tyr, o-Tyr and 3-nitrotyr. DOPA, di-Tyr and 3-nitrotyr are formed from the oxidation of tyrosine whilst o-Tyr is an oxidation product of phenylalanine. The oxidation of tyrosine by hydroxyl radicals or HOCl produces tyrosyl radicals. DOPA is then formed by the direct addition of another hydroxyl radical to tyrosyl radicals, whilst di-Tyr is formed when tyrosyl radicals dimerise, causing protein dimerisation and subsequent inactivation. 3-nitrotyrosine is produced from the nitration of tyrosine by peroxynitrite. Peroxynitrite is generated by the reaction of superoxide with nitric oxide, and does not only oxidize tyrosine but also sulphhydyl groups and inhibits mitochondrial electron transport chain components [313]. All of these markers were found to be significantly increased during reperfusion except for 3-nitrotyr which also showed higher levels after reperfusion.

The oxidation of proteins can occur via two broad oxidative mechanisms, one involving oxygen-derived radicals catalysed by trace transition metal ions, and a second involving chlorinating species such as HOCl or Cl₂, generated by myeloperoxidases.
Given that MPO is released extracellularly by activated leukocytes, it is unlikely that this would be the major cause of protein oxidation in these studies as we have used a cell free model. However, it is impossible to remove all leukocytes from the heart by perfusing with Krebs-Henseleit buffer, so it is possible that some MPO may have been released by residual leukocytes present within the heart. This may also contribute to some of the protein oxidation which occurred during ischemia, when ROS generation would not have been as high as during reperfusion. The oxidative burst of ROS seen in early reperfusion, however would presumably be the major source of protein oxidation in these studies.

Oxidation of proteins results in their denaturation leading to the loss of enzyme activity, reduced energy production, mechanical dysfunction, altered calcium transport in the sarcoplasmic reticulum and structural damage, all of which lead to cellular dysfunction [102,21]. Increases in protein oxidation during reperfusion may therefore be a major contributor to the cellular dysfunction and death observed following reperfusion of ischemic myocardium. Protein oxidation in ischemic hearts, although lower than levels in reperfused hearts, was higher than levels observed in normoxic hearts. This indicates oxidative damage can occur in the myocardium when oxygen levels are depleted. The increase in protein oxidation may also be a cause of cell death seen in ischemia.

Lipid peroxidation, another marker of biological oxidation, was also measured in hearts post-ischemia and post-reperfusion. MDA and 4-HNE are end products derived from peroxidation of polyunsaturated fatty acids and esters, and measurement of these aldehydes provides an index of lipid peroxidation. There was a significant increase in LPO in both ischemic and reperfused hearts, however there was a greater increase in ischemic hearts. LPO are produced by the oxidation of lipids and fatty acids within cell membranes, leading to disruptions in membrane integrity, altered permeability as well as the leakage of cellular contents [102,105,117,174,317]. Their increase in ischemia and reperfusion may also be a cause of cellular dysfunction and death that is associated with myocardial ischemia-reperfusion injury. The significantly higher levels of LPO during reperfusion further supports an increase in ROS generation during reperfusion. Lipid peroxidation of myocardial cell membranes has also been implicated as a potential cause
of ventricular fibrillation, tachycardia, premature beating and arrhythmias, which are all associated with reperfusion [21,40,108,116,118].

These lower levels of LPO in reperfusion when compared to ischemic levels may not however be a true indicator of lipid oxidation in reperfusion, as some LPO products may have been washed out from hearts with perfusate. Given that there is an initial burst of ROS within the first few minutes of reperfusion, which may potentially lead to LPO, it is possible that during the 45 minutes of reperfusion, some LPO products were carried away with buffer as it was perfused through the heart. Since LPO are generated by oxidation of fatty acids and lipids within cellular membranes, it is possible for LPO to be released from cell membranes into the coronary circulation, where they can be removed in perfusate. Since no buffer was perfused through the heart during ischemia (zero flow 100% ischemia), any LPO released from cells would not have been removed from hearts with perfusate.

The build up of calcium during ischemia activates phospholipases, which degrade phospholipids in cell membranes [40,44,103]. This also leads to a release of arachidonic acid which is metabolised to prostaglandins and leukotrienes during ischemia. These metabolic pathways however require electron transfers that can also initiate the formation of free radicals [40,44,103]. These processes may be possible causes of the increased LPO observed during ischemia.

There are many potential sites of ROS production in the myocardium including mitochondria, myocardial cell membranes and endothelial cells, and the major sources of ROS in post-ischemic tissues are the enzyme xanthine oxidase present within endothelial cells of the coronary circulation, electron leakage from damaged mitochondrial respiratory chains as well as activated neutrophils [21,40,44,101,103,108,110,111,114]. Although neutrophils may be an important source of ROS in certain systems, it is clear their presence is not always necessary for ROS generation during myocardial ischemia-reperfusion. This has been proven by numerous studies in which ROS have been detected in \textit{in vitro} models using isolated hearts perfused with cell-free solutions [102,110,115]. Our results further support this as we have shown significant increases in biological oxidation during reperfusion in the absence of neutrophils. This also suggests that the initial burst of ROS within the first few minutes of reperfusion is not from neutrophils but
rather another source such as the respiratory chain, and that neutrophils do not become an important source of ROS (in vivo) until later in reperfusion.

The increase in biological oxidation in ischemic hearts indicates oxidative damage can occur when oxygen is low or absent, as 100% global ischemia was used in these studies, so the only oxygen present would have been from residual supplies trapped within cells. It has been shown previously that free radical production in the myocardium can occur from many different sources after different periods of ischemia and reperfusion, and that ROS are generated during ischemia when oxygen availability is diminished [20,40,44,101-103,109,110]. Although it was found that myocardial oxidative injury occurs during both the ischemic insult and reperfusion, the mechanism of ROS production is highly likely to be different in ischemia than reperfusion. During ischemia, the adenine nucleotide pool becomes degraded leaving the mitochondrial electron carriers in a reduced state. This leads to an increased leakage of electrons from the respiratory chain which react with residual oxygen trapped in the inner membrane of the mitochondria to produce superoxide [21,44,111]. Residual neutrophils trapped within the coronary circulation may also be a source of ROS generation during ischemia. Once activated, neutrophils generate ROS via NADPH oxidase on their cell membranes [21,44,114]. These results indicate that some of the oxidative damage associated with ischemia-reperfusion injury actually occurs during the ischemic episode itself, and before the re-introduction of oxygen and the associated oxidative burst.

Oxidative stress has also been implicated as a mediator of apoptosis with numerous studies having found ROS to be involved in apoptosis in a number of different ways [133-136,140]. Apoptotic activity was measured in hearts during ischemia and reperfusion, and was found it to be increased during reperfusion only. The higher levels of protein oxidation and lipid peroxidation observed in these hearts indicates elevated ROS production during reperfusion. The correlation between increased apoptosis and increased biological oxidation in reperfused hearts suggests the increase in apoptosis may be mediated by ROS. ROS can induce apoptosis by up-regulating the Fas-FasL system, causing mitochondrial cytochrome c release, activating transcription factors such as AP-1 and NFκB, inhibiting the anti-death gene Bcl-2, and damaging DNA [133,134,137,139,140]. Apoptosis, or “programmed cell death” is also an energy-
dependent process, which supports its increase observed during reperfusion when cellular energy levels are increased [133]. These results indicate apoptosis is not a major process for cell death during ischemia, suggesting the cell death that does result during ischemia must occur via necrosis. During reperfusion however, apoptosis is a major contributor to cell death, possibly due to restored energy levels and ROS generation.

From the results of this study, it may be postulated that ROS are a major cause of the cellular dysfunction and death that occurs during ischemia as well as reperfusion. Therefore, inhibition of ROS generation or scavenging them once they are produced may be a possible way to protect tissues from ischemia-reperfusion induced damage.

Under normal physiological conditions, ROS production is kept under homeostatic control by endogenous free radical scavengers or antioxidants. However, whether these antioxidants are up-regulated in times of oxidative stress is unclear. Oxidative stress is said to occur when there is an increased generation of ROS which overwhelms the normal cellular defence mechanisms [20], so up-regulation of antioxidants may be a ‘self protection’ mechanism to limit oxidative injury caused by ischemia and/or reperfusion.

Catalase, GPX and Prdx are enzymes primarily responsible for the metabolism of hydrogen peroxide [169,174]. Given that ROS generation is increased during ischemia-reperfusion, with specific segments of the mitochondrial respiratory chain being major sources, we chose to study GPX and Prdx as they are found in both the cytosol and mitochondria of cells whereas catalase is localized in peroxisomes [161]. GPX and Prdx therefore provide an immediate mechanism for the metabolism of ROS generated in mitochondria, thereby protecting not only the whole cell but the mitochondria too. It is also well known that the specific activity of catalase relative to GPX is considerably lower in the heart than other organs such as the liver. This is due partly to differences in $K_m$ as GPX has a $K_m$ for hydrogen peroxide 1000 times lower than catalase indicating it has a greater affinity for the substrate than catalase [174,161,169,171,172]. For these reasons plus their ability to reduce other peroxides/proteins we have chosen to study glutathione and thioredoxin related genes within the heart.

Expression of thioredoxin related genes was found to be induced by ischemia, with significantly greater transcription of Txnrd-1, Txnrd-2, Prdx-2 and Txn. Txnrd-1
expression was increased 170% and Txnrd-2 expression by over 1000 % in ischemic hearts. This corresponded to significantly higher TxnRed activity in ischemic hearts.

TxnRed is an important antioxidant as it reduces not only oxidized Txn, but can regenerate other proteins by reducing protein disulfides and also reduce peroxides such as lipid hydroperoxides and hydrogen peroxide (although the $K_m$ is much higher than GPX so it only plays a role when levels are high). TxnRed can also reduce the ascorbic acid free radical to ascorbate, and reduce oxidized glutathione thereby replenishing the available antioxidant pool [201,213,221]. Whilst Txnrd-1 is found only within the cytosol, Txnrd-2 contains a mitochondrial import sequence allowing it into the mitochondria where it can protect cells from mitochondria-mediated oxidative stress [201,213,221]. The large increase in Txnrd-2 expression observed in ischemic hearts may be an attempt to combat ROS generation through the reaction of residual oxygen within the mitochondrial membrane with electrons that have leaked from the respiratory chain.

Although there was not a significant increase in TxnRed activity in reperfused hearts, activity was increased above that of normoxic hearts, and Txnrd-1 and-2 mRNA was significantly increased. Expression in reperfused hearts was however lower than that in ischemic hearts suggesting TxnRed may contain a hypoxic response element in its promoter. This may be true for other components of the TxnRed system too, as Txn and Prdx-2 expression was also increased during ischemia, and although transcription was higher in reperfused hearts than normoxic hearts, it was still lower than that in ischemic hearts.

It is also possible that components of the TxnRed may up-regulate each other. Txn expression has previously been shown to be induced by oxidative stress, and this induction may involve an antioxidant or oxidative stress responsive element in its promoter [201,194,245,248,253]. The promoter region of Txn-1 contains many possible regulatory binding motifs compatible with constitutive expression, including GCF, SP1 and WT-ZFP; with inducible expression, including AP-1, AP-2 and NF-κB; and with an oxidative stress response element [248,321,322]. A variety of stress stimuli increase Txn-1 expression in cells, including hypoxia [323,324], lipopolysaccharide [325], oxygen [245,326], hydrogen peroxide [327-329] and photochemical oxidative stress [330]. This is further supported by the induction of Txn gene expression in the lungs of newborns by
oxygen, where it is believed to protect the newborn from hyperoxia at birth [245,326]. Increased Txn expression is thought to be aimed at increasing the reduction of intracellular proteins and other biomolecules as part of the body’s antioxidant defence. Txn not only reduces cysteine groups on proteins but also reduces Gpx-3, inhibits apoptosis and acts as a cofactor by providing reducing equivalents to redox enzymes, thereby providing protection to cells in a number of ways during times of increased stress [248,193,194,210,212,213,232]. It is therefore highly likely Txn is finely tuned to the overall oxidative status and/or oxygen levels in cells [213,245,248,323,324,326-329]. Considering its regulation by the oxidative state of cells, it is possible Txn itself may induce Txnrd and Prdx.

Previous studies in Txn transfected cells have found Prdx2 expression is increased as a result of Txn overexpression [331]. Increases in Prdx-2 during ischemia may protect cells from oxidative injury and apoptosis, as Prdx-2 directly reduces peroxides such as hydrogen peroxide and alkyl peroxides as well as inhibiting mitochondrial cytochrome c release [210,215,223,331].

The low level of apoptosis in ischemia and its increase in reperfusion may also be related to expression on the TxnRed system, as both Txn and Prdx protect cells from apoptosis. Prdx-2 inhibits release of cytochrome c from mitochondria (a known cause of apoptosis), reduces hydrogen peroxide thereby preventing it initiating apoptosis and regulates the anti-death gene Bcl-2 [177,215,223,331]. Txn inhibits apoptosis by increasing DNA binding of the transcription factor AP-1 and inhibiting ASK1 activity [136,177,210,229-231,243]. Txn-1 is the cytosolic and nuclear form of the protein, so its presence within the nucleus may also protect DNA from damage which can lead to apoptosis.

GPX transcription and activity was not found to be significantly increased until reperfusion suggesting GPX plays a major role in cellular defence against hydroperoxides generated during reperfusion. Gpx-1 expression was increased 100% and Gpx-4 expression by more than 700% during reperfusion, and this corresponded to significantly higher GPX activities in reperfused hearts.

Whilst lipid peroxidation was found to be increased during reperfusion, it was found to be greater in ischemic hearts. This may be partially due to the increased GPX
activity/expression that occurs during reperfusion, particularly the increased Gpx-4 expression. Gpx-4, also known as phospolipid hydroperoxide, is a smaller Gpx isoenzyme with a hydrophobic surface capable of reacting with complex hydroperoxides and lipids in membranes, including lipid hydroperoxides [193,213,232,331]. Thus, Gpx-4 may inhibit the production of LPO, or may metabolise lipid hydroperoxides produced by lipid peroxidation. Its increased expression in reperfusion, along with the potential wash out of some LPO products may therefore contribute to the lower LPO levels observed in reperfused hearts.

Gpx-1 is a cytosolic and mitochondrial form of GPX, and its increased activity in reperfusion may provide an immediate mechanism for removal of mitochondria generated ROS [172,175,178,193]. ROS generation from the mitochondria is increased during reperfusion due to electron leakage from respiratory chains which then react with oxygen supplied by reperfusion, leading to a ‘respiratory burst’ [21,40,44,114,161]. Increasing GPX activity in the mitochondria may therefore be one way cells combat this surge and protect themselves. Glutathione reductase expression was not significantly different during ischemia or reperfusion, so whilst increased GPX will provide additional protection against ROS, once this extra GPX becomes oxidized by ROS there will be no additional supply of Gsr to reduce it back to an active form. This however may not be necessary as myocardial Gsr may already be present in excess, especially since GPX activity in the heart, relative to catalase, is considerably higher than in other tissues [169,172], so normal myocardial Gsr levels may already be quite high. It is also possible that GPX expression is so high during reperfusion that enough GPX is produced to protect cells without Gsr regenerating more GPX than normal.

It has been found previously that increasing the duration of ischemia leads to a progressive decline in SOD and CoQ10 activity which leaves mitochondria less well equipped to deal with the increased free radical flux during reperfusion [44,110,159,160,168]. A slight reduction in myocardial SOD activity after 22.5 minutes ischemia was found, which supports these previous findings. Given that there were no significant changes in SOD activity during reperfusion, and a slight decline in activity during ischemia, suggests the GPX and TxnRed systems are more finely ‘in tune’ with
the oxidative status of cardiac cells, and they may be the predominate ROS scavengers in the heart in times of increased stress.

The results of this chapter suggest the GPX system may play a major role in protecting the heart against LPO and removing ROS such as hydroperoxides and lipid hydroperoxides whilst the TxnRed system may provide more protection against protein oxidation. LPO was at its greatest when TxnRed expression was at its peak and before GPX expression was at its highest, whilst protein oxidation was at its greatest during reperfusion when GPX expression was at its peak and TxnRed expression had started to decrease. These two systems may therefore complement each other in protecting cells against oxidative damage. Therefore increasing expression and/or activity of BOTH systems, during ischemia AND reperfusion, may reduce cellular dysfunction and death post-ischemia, and improve myocardial recovery.
LIMITATIONS

There are a few experimental limitations that deserve mention in this chapter. The first has already been discussed and was the potential washout of some LPO products from hearts in their perfusates. Although this would not have been an issue for ischemic hearts, it may have been for other hearts, although significant levels were still detected in reperfused hearts when compared to normoxic controls.

Due to the changes in Txn expression observed, it would have been interesting to see if this up-regulation was translated into changes in Txn protein levels. This was attempted, however the antibodies used were generated for Human Txn, and although Txns are >80% homologous between species, the human monoclonal antibodies were unable to recognise rat Txn. We found similar results for our qRT-PCR primers when the Txn primer, which was designed for rat Txn, was tested on human samples. There were no antibodies available for rat Txn at the time of these studies.

Whilst we chose to the study direct effects of ischemia and reperfusion on hearts, the use of a cell free model could also be considered a limitation since no circulating leukocytes were present. Neutrophils are another source of ROS and inflammation following ischemia-reperfusion. However, given that cellular dysfunction and death still occur, and ROS have previously been detected in hearts perfused with cell free solutions, it is also important to investigate other direct effects of ischemia-reperfusion on the heart, and other sources of ROS.

We don’t believe using SYBR green for our real-time PCR rather than flurogenic probes had a significant effect on the low expression of Gpx-4. We have experience with both fluorogenic probes and SYBR Green, and have only ever noticed a difference in sensitivity of 2-3 cycles for the same gene. Therefore we don’t believe this would have a significant effect on the low expression of Gpx-4, and we are certain the signal-to-noise ratio was sufficient to measure Gpx-4 expression. We also run melt curves on all our PCR products to ensure the $T_m$ is correct for the product and that the non template control (NTC) is clear of products or primer dimer. Both the melt curve and agarose gel gave the correct $T_m$ and product size for Gpx-4 whilst all NTCs were clear therefore we are confident we are detecting Gpx-4.
CONCLUSIONS

The results of this chapter indicate the TxnRed and GPX systems may respond to acute injurious stimuli to aid in limiting oxidant stress, as both were found to be up-regulated during periods of ischemia and reperfusion. There were no changes in SOD activity during ischemia or reperfusion which suggests the GPX and TxnRed systems are more finely ‘in tune’ with the oxidative status of cardiac cells. It can also be concluded that GPX plays a major role in myocardial defence against ROS during reperfusion whilst TxnRed plays a role in providing protection during periods of ischemia. The GPX system may also be responsible for protecting the heart against LPO and removing ROS such as hydroperoxides and lipid hydroperoxides, whilst the TxnRed system may provide more protection against protein oxidation.

Protein oxidation was increased during reperfusion with significantly higher levels of PCO, DOPA, di-Tyr and o-Tyr. Although LPO was also significantly increased in reperfusion, it was greater during ischemia, indicating oxidative damage occurs at various oxygen levels. This also shows that some of the oxidative damage associated with ischemia-reperfusion injury actually occurs during the ischemic episode itself, and before the re-introduction of oxygen and the associated oxidative burst. From these results it may be postulated that ROS are a major cause of cellular dysfunction and death that occurs during ischemia as well as reperfusion.

Caspase-3 was found to be elevated during reperfusion but not ischemia indicating it is not a major process for cell death during ischemia. The increase in apoptosis during reperfusion may be due to it being an energy-dependent process combined with an increase in ROS, a known mediator of apoptosis. Reduced expression of the TxnRed system during reperfusion may contribute to ROS-mediated apoptosis as Tnx and Prdx2 are known inhibitors of apoptosis.

It is possible the GPX and TxnRed systems complement each other in protecting hearts from oxidative stress and ischemia-reperfusion injury. Therefore increasing expression and/or activity of BOTH systems, during ischemia AND reperfusion, may reduce cellular dysfunction and death post-ischemia, and improve myocardial recovery.
**PUBLICATIONS ARISING FROM THIS CHAPTER**


*In Press*

CHAPTER 4

Effects of dietary selenium on myocardial antioxidant enzyme systems, & ischemia-reperfusion injury.
4.1 **ABSTRACT**

Glutathione peroxidase and thioredoxin reductase are selenocysteine dependent enzymes that protect against oxidative injury. In the previous chapter it was found that these antioxidant enzymes are up-regulated during periods of ischemia and reperfusion whilst SOD is unaffected, suggesting GPX and TxnRed are important in protecting cardiac cells from oxidative damage. It is also possible the GPX and TxnRed systems complement each other in protecting hearts from ischemia-reperfusion injury, therefore up-regulation of both systems during the ischemic insult and reperfusion may limit the extent of oxidative damage and cellular dysfunction/death that occurs, and improve myocardial recovery. This chapter examines the effects of dietary selenium on the activity of these two enzymes in rats, and investigates the ability of selenium to modulate myocardial function post ischemia-reperfusion. Male wistar rats were fed diets containing 0, 50, 240 and 1000 µg/kg sodium selenite for 5 weeks. Hearts were then isolated and perfused using the Langendorff model where they were subjected to 22.5 min global ischemia and 45 min reperfusion, with functional recovery assessed. Liver samples were collected at the time of sacrifice, and heart and liver tissues assayed for TxnRed, GPX and SOD activity. Markers of oxidative damage were also measured in heart extracts post ischemia-reperfusion. Selenium deficiency reduced the activity of both glutathione peroxidase and thioredoxin reductase systemically. Hearts from selenium deficient animals were more susceptible to ischemia-reperfusion injury when compared to normal controls (38% recovery of RPP vs 47% RPP), and had higher levels of lipid peroxidation and protein oxidation. Selenium supplementation increased the endogenous activity of TxnRed and GPX and resulted in improved recovery of cardiac function post ischemia-reperfusion (57% recovery of RPP). This correlated to lower levels of LPO, and oxidized protein products such as PCO. Myocardial SOD activity was not effected by dietary selenium. These results indicate the endogenous activity of GPX and TxnRed is dependent on an adequate supply of the micronutrient selenium. Reduced activity of these antioxidant enzymes leaves hearts more susceptible to oxidative damage leading to significant reductions in myocardial function post ischemia-reperfusion.
4.2 **INTRODUCTION**

Reperfusion of ischemic tissue generates reactive oxygen species (ROS) which lead to lipid, protein and DNA oxidation, cellular damage and apoptosis. Generation of ROS has been associated with poor cardiac recovery post ischemia and many studies have sought to control their production or increase cellular defences against their damaging effects [40,102,108,118,174,317].

The production of ROS is controlled by endogenous antioxidants such as superoxide dismutase (SOD), catalase, glutathione peroxidase and thioredoxin reductase [40,166]. Glutathione peroxidase and thioredoxin reductase are selenocysteine dependent enzymes which play a critical role in protecting cardiac tissue from ischemia-reperfusion injury [213,256,318].

In mammalian cells, the generation of selenocysteine occurs during amino acid biosynthesis and the degree of selenium (Se) incorporation into the cysteine residue is concentration dependent [255,256]. The incorporation of selenocysteine into proteins is regulated by the misreading of the UGA codon, where instead of a stop signal being recognised, selenocysteine is incorporated during translation [264,265]. Enzymes that have been shown to be critically dependent on the incorporation of selenocysteine include glutathione peroxidases, thioredoxin reductases and the thyroid specific deiodinases. Of these glutathione peroxidase and thioredoxin reductase are produced by cardiac tissues [110,256].

Previous studies have reported poor recoveries from ischemia-reperfusion with selenium deficiency and this was correlated to significantly decreased activity of both cytosolic and mitochondrial glutathione peroxidase [289,319,320]. Furthermore, selenium supplementation has been shown increase glutathione peroxidase activity in cardiac tissues and decrease the incidence of reperfusion induced arrhythmias [277,290,291,320]. These observations have been extended in this chapter by examining post-ischemic cardiovascular function and oxidative damage in rats subjected to selenium supplementation or elimination. Furthermore, the activity of TxnRed and GPX as well as the selenium independent enzyme SOD have been studied post selenium manipulation.
4.3 MATERIALS AND METHODS

i) ANIMALS AND DIETS

Male wistar rats (6 weeks of age), as described in Section 2.2 of Chapter 2 (General Methods), were randomly assigned to one of 4 groups and fed an experimental diet for 5 weeks. The basal diet was selenium free - containing 30% torula yeast, 59% sucrose, 5% coconut oil (vitamin E free), 5% premixed minerals (Hubbel, Mendel & Wakeman salt mix, ICN, Seven Hills, Australia) and 1% premixed vitamins (vitamin diet fortification mixture, ICN, Seven Hills, Australia). Group one (n=10) were fed the basal selenium free diet, whilst group two and three received diets supplemented with different doses of selenium in the form of sodium selenite (Sigma, Castle Hill, Australia): 50 µg Se/kg food (n=10) and 1000 µg Se/kg (n=10). Control rats (n=12) were fed standard rat pellets, which contains approximately 240 µg Se/kg food. All rats had free access to water and food, and diets were replaced daily.

ii) ISOLATED HEART PROTOCOL

Animals were anaesthetised and hearts removed as described in Chapter 2 (Section 2.4, General Methods). Hearts were then perfused using the Langendorff isolated heart preparation as explained in Section 2.4 of Chapter 2 (General Methods). Hearts were perfused for an initial 20 minute normoxic period to allow them to stabilize. Coronary flow was then stopped to generate zero flow global ischemia. Ischemia was maintained for 22.5 minutes and hearts were maintained at 37°C in the temperature controlled organ chamber. Flow was then returned to the heart and reperfusion continued for 45 minutes. The length of ischemia used in this study (22.5 minutes) was determined in pilot experiments to be a period which resulted in ~50% recovery of contractile function. This provided the potential to measure both improved and reduced recoveries. At the end of reperfusion, hearts were removed from the cannula, rapidly blotted and weighed, then frozen at –80°C until subsequent biochemical analysis.
iii) BIOCHEMICAL ANALYSIS

Liver samples were also removed from the rats at the time of sacrifice, weighed and kept at –80 °C until use. Protein was extracted from hearts and livers as described in Section 2.5 i of Chapter 2 (General Methods), and protein estimations performed on extracts as detailed in Section 2.5 ii of Chapter 2.

Activity of the antioxidant enzymes, glutathione peroxidase (GPX), thioredoxin reductase (TxnRed) and superoxide dismutase (SOD) were measured in protein extracts as described in Section 2.6 (methods i, ii and iii) of Chapter 2. GPX and TxnRed were measured in both heart and liver extracts to determine systemic effects of selenium modulation on selenocysteine containing enzymes, whilst SOD was measured in heart extracts only.

To assess the level of oxidative stress within these tissues, lipid peroxidation was measured in heart extracts along with protein carbonyls and the tyrosine oxidation products DOPA, di-Tyr, o-Tyr and 3-nitrotyrosine. These methods are explained in detail in Section 2.6 (methods v, vi, and vii) of Chapter 2 (General Methods).

Caspase-3 is a marker of apoptosis common to both the caspase-6/9 and caspase-8 pathways. Its activity was measured as an indicator of apoptosis in hearts using the method described in Chapter 2, Section 2.6 iv.

iv) DATA ANALYSIS

All data was analysed using one-way ANOVA’s as described in Section 2.9 of Chapter 2 (General Methods).
4.4 RESULTS

i) EFFECT OF SELENIUM DIETS ON BODY WEIGHTS

The 5 week Torula yeast diet with and without selenium supplementation did not affect the appetite nor growth rate of the animals. The increase in body weight over the study period was similar in all experimental groups and was consistent with the growth of control rats. There were no significant differences in body weights at the time of sacrifice and all animals weighed between 435-495 grams (Table 4.1).

ii) ANTIOXIDANT ENZYME ACTIVITIES

Thioredoxin Reductase activity

Total selenium depletion induced a significant decrease (p<0.001) in thioredoxin reductase activity in rat livers when compared to all other groups (Fig 4.1a). The Se free animals displayed a TxnRed activity of only 5.1 ± 1.4 moles/min/mg protein compared to the control level of 70.4 ± 5.6 moles/min/mg protein for the animals receiving the normal diet containing 240 µg/kg Se. A significant decrease (p<0.001) was also observed in the 50 µg/kg selenium group (28.9 ± 3.2 mole/min/mg protein). There was however only a slight increase in activity between the control and 1000 µg/kg selenium group (70.4 ± 5.6 vs 72 ± 3.5 moles/min/mg protein).

Similar results were also observed in the rat hearts post ischemia-reperfusion (Fig 4.1b), with a significant decrease (p<0.01) in thioredoxin reductase activity in those animals receiving a selenium free diet when compared to those receiving a normal diet (76.6 ± 4.3 vs 105.5 ± 5.3 moles/min/mg protein). Myocardial TxnRed activity in selenium deficient animals was 98.7 ± 3.9 moles/min/mg. Again, there was only a marginal increase in TxnRed activity in the 1000 µg/kg group (111.7 ± 6.7 moles/min/mg protein) compared to controls.
Fig 4.1: Effect of dietary selenium on thioredoxin reductase activity in rat liver extracts (A) and post-ischemic heart extracts (B). Data is expressed as mean ± SEM. (moles TsnRed/min/mg protein). (** p<0.01, #p<0.001 vs controls).
Glutathione Peroxidase activity

When compared to the control group consuming a normal diet containing 240 μg/kg of selenium, the glutathione peroxidase activity in rat livers from the selenium free group and the 50 μg/kg group was significantly reduced (p<0.001) (Fig 4.2a). Significant differences (p<0.001) were also found between the 1000 μg/kg group and both the selenium free and 50 μg/kg groups. The activity in control liver extracts was 407.9 ± 19.9 mmole/min/mg protein compared to 79.4 ± 7.9 and 211.5 ± 12.4 mmole/min/mg for the Se free and 50 μg/kg groups respectively. There was no significant difference in GPX activity observed between the control group and the 1000 μg/kg selenium group (407.9 ± 19.9 vs 397.3 ± 10.6 mmole/min/mg protein) suggesting a saturation point above which a significant increase in selenium results in only a marginal increase in enzyme activity.

Similar observations were made in hearts following ischemia-reperfusion (Fig 4.2b). There was a significant decrease (p<0.05) in myocardial GPX activity in rats that consumed selenium free diets (507.8 ± 21.5 mmole/min/mg protein) compared to controls rats fed a normal diet containing (615.6 ± 20.8 mmole/min/mg). A diet of 1000 μg/kg did not result in a significant increase in GPX activity above that observed in the control group (624.9 ± 37.8 vs 615.6 ± 20.8 mmole/min/mg protein). Myocardial GPX activity in selenium deficient animals was 519 ± 33.4 mmole/min/mg.
**Fig 4.2:** Effect of dietary selenium on glutathione peroxidase activity in rat liver extracts (A) and post-ischemic heart extracts (B). Data is expressed as mean ± SEM. (mmoles Gpx/min/mg protein). (* p<0.05, #p<0.001 vs controls).
**Superoxide Dismutase activity**

Superoxide Dismutase activity was not affected by dietary selenium levels, with activities being very similar to that in control hearts (*Fig 4.3*). Superoxide dismutase activities in post-ischemic rat hearts were $53.6 \pm 11.8$, $53.9 \pm 15$ and $50.8 \pm 6.9$ units SOD/mg protein for Se free, $50 \, \mu\text{g/kg}$ and $1000 \, \mu\text{g/kg}$ groups respectively. Control hearts had a SOD activity of $54.3 \pm 8.7$ units/mg protein.

*Fig 4.3: Effect of dietary selenium on superoxide dismutase activity in the rat heart following 22.5 mins ischemia and 45 mins reperfusion. Values are presented as mean $\pm$ SEM, units SOD/mg protein.*
iii) MYOCARDIAL FUNCTIONAL ANALYSIS

Pre-ischemic heart function

Selenium supplementation or deficiency had no effect on initial heart function (Fig 4.4a-f) in relation to left ventricular developed pressure, heart rate, dP/dt and coronary flow (Table 4.1). Whilst heart rates were similar between all experimental groups during equilibration (265-295 bpm), hearts were not paced and as a result we have chosen to monitor rate pressure product as opposed to developed pressure only. All hearts displayed an RPP of between 53-56 x10³ mmHg/min, +dP/dt between 3300-3600 mmHg/sec, and –dP/dt from –3250 to –3550 mmHg/sec. Coronary flow during equilibration was approximately 25–28 ml/min.
**Fig 4.4:** Effect of dietary selenium on pre-ischemic heart function. Rats were fed an experimental diet for 5 weeks then hearts were isolated and perfused on a Langendorff perfusion apparatus. Left Ventricular Developed Pressure (LVDP, mmHg, 4.4A), heart rate (bpm, 4.4B), rate pressure product (RPP, mmHg/min, 4.4C), rate of contraction (+dP/dt, mmHg/sec, 4.4D), rate of relaxation (-dP/dt, mmHg/sec, 4.4E) and coronary flow (mL/min, 4.4F) were measured during normoxic equilibration. Values are presented as mean ± SEM.
**Ischemic contracture**

Ischemic contracture is an indicator of the severity of ischemic damage, and arises due to sustained contraction of the ventricle. This inability of the ventricle to relax is measured as an increase in end diastolic pressure (EDP) at the end of the ischemic episode. Contracture was significantly greater (p<0.05) in the selenium free group (87.2 ± 4.2 mmHg) when compared to controls (*Fig 4.5*), and decreased as the dietary selenium level increased (83.7 ± 3.6 mmHg for 50 µg Se/kg group, 72.4 ± 4.7 mmHg for the control group and 71.2 ± 2.3 mmHg for the 1000 µg Se/kg group). These results indicate a greater tolerance to ischemia occurs with increasing dietary selenium levels.

*Fig 4.5: Effect of dietary selenium on ischemic contracture after 22.5 minutes of ischemia in the isolated perfused rat heart. Data is expressed as mean ± SEM, mmHg. (*p<0.05 vs controls).*
Post-ischemic heart function

The contractile function following ischemia-reperfusion is shown in Table 4.1 and Figures 4.6-4.10. Contractile function was measured as left ventricular developed pressure (LVDP), rate pressure product (RPP), % recovery of RPP after 45 minutes reperfusion, as well as end diastolic pressure (EDP) and rate of left ventricular pressure development (+/- dP/dt) at the end of 45 minutes reperfusion.

As hearts were not paced during equilibration or reperfusion, RPP was used as an indicator of contractile function during reperfusion rather than developed pressure alone. This is because left ventricular pressure development (LVPD) is somewhat dependent upon heart rate, so any changes in heart rate caused by dietary selenium or ischemia-reperfusion may effect LVDP, whereas RPP accounts for changes in both heart rate and LVDP. Following ischemia, RPPs were lower in all experimental groups than during equilibration. These did however increase in all groups during the time course of reperfusion. Selenium supplementation of 1000 µg/kg resulted in greater RPPs at all measured time points during reperfusion, whilst hearts from selenium free animals displayed the lowest RPPs at all time points during reperfusion (Fig 4.6). Although RPPs for the 50 µg Se/kg group were higher than those for the selenium free hearts, they were lower than those of the controls receiving 240 µg Se/kg.

At the end of 45 minutes reperfusion, recovery of RPP had started to plateau in the selenium free, 50 µg Se/kg and control groups (Fig 4.6). Rate pressure products were 22.5 ± 1.6, 23.7 ± 1.6 and 26.2 ± 2 mmHg/min for the selenium free, 50 µg Se/kg and control groups respectively. Hearts from animals supplemented with 1000 µg Se/kg showed the highest RPPs after 45 minutes reperfusion (33.4 ± 2.3 mmHg/min) when compared to all other groups (Fig 4.7a).

This is further supported with the percent (%) recovery of RPP after 45 minutes reperfusion (Fig 4.7b). These results show selenium supplementation of 1000 µg/kg significantly improved heart recovery (p<0.05) with the RPP recovering to 57 ± 4.8 % of pre-ischemic levels, compared to 46.6 ± 2.2 % recovery for the control group. There was a significant decrease (p<0.05) in % recovery of RPP for the selenium free group (38 ± 2.6 %), and significant differences (p<0.05) also between the 1000 µg Se/kg and 50 µg
Se/kg groups (44.4 ± 4 %). The control group (which received 240 µg Se/kg food) recovered to ~47% of the pre-ischemic value, and this fell between the recovery levels for the 50 µg Se/kg and 1000 µg Se/kg treated groups suggesting a dose-dependent relationship between selenium supplementation and recovery from ischemia-reperfusion.

Increased EDP (Fig 4.8) reflects residual diastolic dysfunction and is representative of the left ventricle’s inability to relax during diastole. EDP was significantly greater (p<0.01) in the selenium free group when compared to controls (73.3 ± 4.9 vs 51.6 ± 3.4 mmHg), and decreased with each increasing concentration of selenium. There were significant differences (p<0.05) also between the 1000 µg Se/kg treated group (44.1 ± 3.8 mmHg) and the 50 µg/kg group (61.4 ± 5.5 mmHg).

The rate of pressure development over time was also measured in hearts at the end of reperfusion (Table 4.1). The rate of left ventricular positive pressure development (+dP/dt, or rate of contraction), was found to be significantly (p<0.05) lower in selenium free hearts (1791 ± 117 mmHg/sec) when compared to controls, and higher in hearts supplemented with 1000 µg Se/kg (2200 ± 174 mmHg/sec, Fig 4.9a). This was the same for the rate of left ventricular negative pressure development (–dP/dt, or rate of relaxation) where selenium free hearts had a –dP/dt of –1992 ± 138 and 1000 µg Se/kg treated hearts –2312 ± 190 (Fig 4.9b). Selenium deficiency of 50 µg Se/kg resulted in positive and negative rates of contraction lower than controls but higher than selenium free hearts. These results also show a dose-dependent relationship to dietary selenium.

Differences in the recovery of coronary flow after 45 minutes reperfusion were also observed (Table 4.1). In the control and 1000 µg Se/kg treated groups, flow recovered to ~79% of the starting rate as opposed to ~71% for the selenium free and 50 µg Se/kg groups (Fig 4.10). Although these results did not reach statistical significance, they may suggest a protective effect of selenium supplementation on vascular endothelial cells permitting greater flow through the coronary circulation.
Table 4.1: Effect of dietary selenium on myocardial function in the isolated rat heart, both pre- and post ischemia-reperfusion. Male rats were fed varying levels of selenium for 5 weeks, then hearts were isolated and subjected to ischemia-reperfusion. Data is presented as mean ± SEM. (*p<0.05, **p<0.01) (HR - heart rate, LVDP - left ventricular developed pressure, RPP - rate pressure product, +dP/dt – rate of LV positive pressure development (contraction) over time, -dP/dt – rate of LV negative pressure development (relaxation) over time, Flow - coronary flow, EDP - LV end diastolic pressure).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Se Free</th>
<th>50 µg Se/kg food</th>
<th>Controls (240 µg/kg)</th>
<th>1000 µg Se/kg food</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>460 ± 11.7</td>
<td>485.2 ± 10.3</td>
<td>451.3 ± 13.4</td>
<td>463.4 ± 14</td>
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<tr>
<td><strong>Pre-Ischemia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>276 ± 9.8</td>
<td>272 ± 9.4</td>
<td>287 ± 8.5</td>
<td>270 ± 2.4</td>
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<tr>
<td>LVDP (mmHg)</td>
<td>207.5 ± 5.2</td>
<td>202.4 ± 4.9</td>
<td>200 ± 5.6</td>
<td>191.1 ± 9.7</td>
</tr>
<tr>
<td>RPP (x10³ mmHg/min)</td>
<td>54.6 ± 1.6</td>
<td>53.6 ± 1.4</td>
<td>55.9 ± 2.5</td>
<td>55 ± 1.4</td>
</tr>
<tr>
<td>+dP/dt (mmHg/sec)</td>
<td>3574 ± 153.7</td>
<td>3544 ± 130.4</td>
<td>3498 ± 228.8</td>
<td>3528 ± 127.8</td>
</tr>
<tr>
<td>-dP/dt (mmHg/sec)</td>
<td>-3454 ± 117.0</td>
<td>-3427 ± 154.3</td>
<td>-3431 ± 157.5</td>
<td>-3460 ± 120.0</td>
</tr>
<tr>
<td>Flow (ml/min)</td>
<td>28.1 ± 0.7</td>
<td>25.9 ± 0.8</td>
<td>26 ± 1.3</td>
<td>27.3 ± 1.7</td>
</tr>
<tr>
<td><strong>Post-Ischemia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contracture (mmHg)</td>
<td>87.2 ± 4.2*</td>
<td>83.7 ± 3.6</td>
<td>72.4 ± 4.7</td>
<td>71.2 ± 2.3</td>
</tr>
<tr>
<td>45 mins reperfusion</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>EDP (mmHg)</td>
<td>73.3 ± 4.9**</td>
<td>61.4 ± 5.5</td>
<td>51.6 ± 3.4</td>
<td>44.1 ± 3.8</td>
</tr>
<tr>
<td>HR (bpm)</td>
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<td>264.3 ± 6.1</td>
<td>277 ± 5.3</td>
<td>265.3 ± 4.3</td>
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<tr>
<td>LVDP (mmHg)</td>
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<td>94.2 ± 6.9</td>
<td>96.4 ± 7.3</td>
<td>112.9 ± 10.4</td>
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<td>RPP (x10³ mmHg/min)</td>
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<td>23.7 ± 1.6</td>
<td>26.2 ± 2</td>
<td>33.4 ± 2.3</td>
</tr>
<tr>
<td>% Recovery RPP</td>
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<td>46.6 ± 2.2</td>
<td>57 ± 4.8*</td>
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<tr>
<td>+dP/dt (mmHg/sec)</td>
<td>1172 ±239.6*</td>
<td>1370 ± 301.6</td>
<td>1791 ± 116.9</td>
<td>2200 ± 174.6</td>
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<tr>
<td>-dP/dt (mmHg/sec)</td>
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<td>-1450 ± 405.2</td>
<td>-1992 ± 138.2</td>
<td>-2312 ± 190.0</td>
</tr>
<tr>
<td>Flow (ml/min)</td>
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<td>20.5 ± 2.2</td>
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</tr>
<tr>
<td>% Recovery flow</td>
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<td>71.1 ± 2.0</td>
<td>79 ± 3.5</td>
<td>78.7 ± 4.5</td>
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</tbody>
</table>
Fig 4.6: Effect of dietary selenium on contractile function in the isolated rat heart during reperfusion. Male wistar rats were fed an experimental diet for 5 weeks, then hearts were removed and subjected to 22.5 mins ischemia and 45 mins reperfusion on a Langendorff perfusion apparatus. Rate pressure product (RPP) was measured as opposed to left ventricular developed pressure as hearts were not paced. –10 mins represents pre-ischemic heart function and time 0 mins represents the start of reperfusion after 22.5 mins global ischemia. Values are presented as mean ± SEM, x10⁴ mmHg/min.
**Fig 4.7:** Effect of dietary selenium on the recovery of rate pressure products (RPP, 4.7A) after 22.5 mins ischemia and 45 mins reperfusion in the Langendorff perfused isolated rat heart. RPPs at the end of reperfusion were also compared to pre-ischemic RPPs to determine the % recovery of contractile function after ischemia-reperfusion (4.7B). Values are presented as mean ± SEM. (⁎p<0.05 vs controls).
**Fig 4.8:** Effect of dietary selenium on end diastolic pressure (EDP) after 22.5 mins ischemia and 45 mins reperfusion in the isolated rat heart. Data is presented as mean ± SEM, mmHg. (**p<0.01 vs controls).
**Fig 4.9:** Effect of dietary selenium on the rate of ventricular contraction after ischemia-reperfusion. Both positive (+dP/dt, 4.9A) and negative (-dP/dt, 4.9B) rates of pressure development (representing the rate of contraction and relaxation respectively) were measured in hearts after 22.5 mins ischemia and 45 mins reperfusion. Data is presented as mean ± SEM, mmHg/sec. (*p<0.05 vs controls).
Fig 4.10: Effect of dietary selenium on the recovery of coronary flow after 22.5 mins ischemia and 45 mins reperfusion in the Langendorff perfused isolated rat heart. Values are presented as mean ± SEM, % recovery compared to pre-ischemic coronary flow rates.
**iv) OXIDATIVE DAMAGE**

Markers of oxidative damage were increased in selenium deficient hearts when compared to control hearts, and reduced in hearts supplemented with 1000 µg Se/kg food following ischemia-reperfusion.

Lipid peroxidation (*Fig 4.11*), measured as 4-HNE and MDA was significantly (p<0.05) increased in selenium free hearts (2.51 ± 0.2 nmoles 4-HNE & MDA/mg protein) compared to control hearts (1.80 ± 0.1 nmoles 4-HNE & MDA/mg protein). Selenium deficient hearts also had higher levels of LPO (1.95 ± 0.3 nmoles 4-HNE & MDA/mg protein) whilst hearts supplemented with 1000 µg Se/kg showed lower levels (1.53 ± 0.1 nmoles 4-HNE & MDA/mg protein) than control hearts.

Oxidised protein carbonyl derivatives (*Fig 4.12*) were also significantly higher (p<0.05) in selenium free hearts than control hearts (117.2 ± 3.7 vs 102.6 ± 4.4 units/mg protein for controls). Significantly (p<0.05) lower levels of PCO were found in hearts from animals treated with 1000 µg Se/kg (88 ± 3.7 units/mg protein). PCO levels in 50 µg Se/kg treated hearts were 113.9 ± 3.8 units/mg protein, being lower than the selenium free but higher than control hearts.

DOPA, o-Tyr, di-Tyr and 3-nitrotyrosine all appear to be unaffected by dietary selenium levels with no significant differences in their levels after ischemia-reperfusion. Although levels of DOPA were similar between all groups, there was a slight inverse relationship between levels of DOPA and dietary selenium. DOPA levels were 1543 ± 111, 1632 ± 96, 1729 ± 71 and 1742 ± 71 µmol/mol Tyr for 0, 50, 240 (controls) and 1000 µg Se/kg groups respectively (*Fig 4.13*).

o-Tyr levels decreased with increased dietary selenium, however this was not significant (*Fig 4.14*). Selenium supplementation of 1000 µg Se/kg reduced o-Tyr levels to 1609 ± 291 µmol/mol Tyr compared to controls (1723 ± 277µmol/mol Tyr), whilst selenium deficient and selenium free hearts had higher levels of o-Tyr (1793 ± 338 and 2247 ± 176 µmol/mol Tyr respectively).

Similar results were observed for 3-nitrotyrosine (*Fig 4.15*) where levels were increased in the selenium free group (1968 ± 91 µmol/mol Tyr) and decreased in the high selenium group (1419 ± 65 µmol/mol Tyr). There were however no differences in 3-
nitrotyrosine levels between the control and 50 µg Se/kg treated groups (1736 ± 164 vs 1733 ± 225 µmol/mol Tyr).

There were no changes or trends in di-Tyr levels with selenium supplementation or deficiency. di-Tyr levels were 49.5 ± 4 µmol/mol Tyr for selenium free hearts, 62.6 ± 5 µmol/mol Tyr for 50 µg Se/kg hearts, 51.65 ± 5 µmol/mol Tyr for controls consuming 240 µg Se/kg, and 66.7 ± 1 µmol/mol Tyr for 1000 µg Se/kg treated hearts.

Fig 4.11: Effect of dietary selenium on lipid peroxidation in the isolated rat heart following ischemia-reperfusion. LPO was measured as 4-HNE & MDA in heart extracts following 22.5 mins ischemia ± 45 mins reperfusion. Data is expressed as mean ± SEM, nmoles MDA & 4-HNE/mg protein (*p<0.05 vs controls).
Fig 4.12: Effect of dietary selenium levels on the formation of oxidised protein carbonyl derivatives in the heart during ischemia-reperfusion. PCO levels were measured in protein extracts from isolated rat hearts following 22.5 mins ischemia ± 45 mins reperfusion. Data is expressed as mean ± SEM, arbitrary units PCO/mg protein (*p<0.05 vs controls).
Fig 4.13: Effect of dietary selenium on the oxidation of tyrosine to DOPA in the heart during ischemia-reperfusion. DOPA levels were measured in protein extracts from isolated rat hearts subjected to 22.5 mins ischemia + 45 mins reperfusion. Data is expressed as mean ± SEM, μmol DOPA/mol Tyr.

Fig 4.14: Effect of dietary selenium levels on phenylalanine oxidation to o-Tyrosine in the heart during ischemia-reperfusion. o-Tyr levels were measured in protein extracts from isolated rat hearts following 22.5 mins ischemia + 45 mins reperfusion. Data is expressed as mean ± SEM, μmol o-Tyr/mol Tyr.
Fig 4.15: Effect of dietary selenium on tyrosine nitration to the oxidation product 3-nitrotyrosine. 3-nitrotyr levels were measured in protein extracts from isolated rat hearts subjected to 22.5 mins ischemia + 45 mins reperfusion. Data is expressed as mean ± SEM, µmol 3-nitrotyr/mol Tyr.

Fig 4.16: Effect of dietary selenium on tyrosine oxidation to di-Tyrosine in the isolated heart during ischemia-reperfusion. di-Tyr was isolated and measured from rat hearts following 22.5 mins ischemia + 45 mins reperfusion. Data is expressed as mean ± SEM, µmol di-Tyr/mol Tyr.
v) **APOPTOSIS**

Apoptotic activity in the heart (*Fig 4.17*) was measured by caspase-3 activity, a marker of apoptosis common to both the caspase-6/9 and caspase-8 pathways. Caspase-3 activity was not significantly affected by dietary selenium levels, however there was a trend for increasing caspase-3 activity as dietary selenium levels decreased. Caspase-3 activities were 1178 ± 79, 1112 ± 103, 951 ± 139 and 936 ± 172 picomoles AFC/mg protein for selenium free, selenium deficient, control and selenium supplemented hearts respectively. Although not significant, these results do suggest greater apoptotic activity occurs in hearts from selenium deficient animals.

![Fig 4.17: Effect of dietary selenium on apoptotic activity in the heart post ischemia-reperfusion. Apoptosis was measured as caspase-3 activation in protein extracts from isolated rat hearts following 22.5 mins ischemia ± 45 mins reperfusion. Data is expressed as mean ± SEM, picomoles AFC/mg protein.](image-url)
4.5 DISCUSSION

It is now generally accepted that reperfusion of ischemic tissue leads to the generation of oxygen derived free radicals which play an important role in cellular damage [102,317]. Numerous studies to date suggest that antioxidants alleviate mechanical dysfunction after global ischemia and reperfusion [104,109,110,156,158,160,277,289,290,291,319,320,332]. In this chapter we have used a dietary method to decrease the activity of two important antioxidant proteins, thioredoxin reductase and glutathione peroxidase. Diets devoid of selenium resulted in substantial reductions in antioxidant activity in rats, and this led to impaired cardiac contractile recovery post ischemia-reperfusion. Selenium supplementation improved cardiac function post ischemia-reperfusion and this correlated with increased tissue concentrations of GPX and TxnRed, and lower levels of lipid and protein oxidation.

Animal diseases associated with selenium deficiency have been identified in livestock from the mid 1950’s and more recently human pathologies associated with selenium poor diets have been described [282,333]. Amongst these are Keshan disease, an endemic cardiomyopathy, Kashin-Beck syndrome, a form of arthritis and an increased incident of pregnancy-induced hypertension, which have all been described in selenium deficient areas in China [259,282,333,334]. The molecular mechanisms underpinning these pathologies are emerging as more is learned regarding the importance of selenium, in particular selenocysteine, in biological systems.

During cysteine metabolism, elemental selenium can be incorporated instead of sulfur in a concentration dependent manner to yield selenocysteine [255,256]. The incorporation of selenocysteine into proteins is a more regulated process with the involvement on a 3’mRNA selenocysteine insertion sequence (SECIS) and the misreading of a UGA codon to insert selenocysteine rather than the usual stop signal inferred by this codon [256,264,265]. In a limited number of proteins the insertion of selenocysteine has been shown to be critical for catalytic function. These include glutathione peroxidase and thioredoxin reductase [255,256]. In this chapter we have reinforced observations made by others and have shown a correlation between dietary levels of selenium and tissue concentrations of these two important antioxidant proteins.
In both liver and heart tissue there was a dose dependent increase in GPX and TxnRed activity with increasing selenium content in the diet, indicating an essential role of this micronutrient in endogenous antioxidant enzyme activity.

Ischemia-reperfusion results in the generation of reactive oxygen species which, if not counteracted, will lead to oxidative damage to lipids, proteins and DNA [102,317]. Superoxide dismutase provides a first line of defense against superoxide, but the resulting generation of hydrogen peroxide can be just as threatening to cell function [44,105,108,335]. In the mitochondria, H$_2$O$_2$ can migrate into the mitochondrial membrane and draw electrons from Fe$^{3+}$ centered proteins to generate the highly reactive hydroxyl radical (OH$^-$) that can initiate lipid peroxidation [102,105,112,201,317]. If unchecked lipid peroxidation results in a positive cascade which eventually compromises mitochondrial membrane integrity, resulting in cytochrome c leakage and apoptosis [40,110,112,166,201,210].

Others in our laboratory have previously shown that hydrogen peroxide infusion in hearts reduces the rate pressure product by 42% and causes a fivefold increase in EDP, both of which are seen in ischaemia-reperfusion [105]. They have also shown similar changes using oxidized low-density lipoproteins [336]. This would suggest that the removal of H$_2$O$_2$ is fundamentally important to cell recovery from periods of ischemia when the re-introduction of oxygen leads to ROS generation. In the mitochondria this is achieved by glutathione peroxidase and thioredoxin peroxidase, whereas in the cytoplasm, these two enzymes are joined by the actions of catalase, an effective reducer of hydrogen peroxide [40,44,335]. Both GPX and thioredoxin peroxidase rely on a regeneration system, with electrons being drawn from NADPH through glutathione reductase and glutathione, along with TxnRed and thioredoxin respectively. Without this regeneration, the active site of both GPX and thioredoxin peroxidase remains oxidized and the enzymes are inactive [166,210,213,318].

The direct involvement of glutathione peroxidase in reducing both hydrogen peroxide (H$_2$O$_2$) and lipid peroxides, thereby limiting the detrimental effects of oxidative stress, has been well documented [166,172,175,177,215,318,337]. Thioredoxin reductase displays a broad specificity and plays an important antioxidant role, not only by supplying reducing equivalents to the thioredoxin/thioredoxin peroxidase systems but
also in directly reducing $H_2O_2$ and lipid peroxides as well as protein disulfides including oxidized glutathione [166,193,194,201,210,211,213]. Furthermore thioredoxin reductase is involved in recycling the antioxidant vitamins C and E. Vitamin C reacts directly with ROS such as superoxide and peroxides, as well as recycling oxidized Vitamin E, but in the process becomes oxidized to dihydroascorbate. Dihydroascorbate is a substrate of thioredoxin reductase leading to the regeneration of ascorbic acid [166,193,194,201,210,211,213]. Therefore thioredoxin reductase is critical in defending against oxidative injury by shifting electrons from NADPH and providing reducing power to both protein and non-protein based antioxidants.

This chapter examined a scenario of oxidative stress in cardiac tissue where rat hearts were subjected to global ischemia and reperfusion resulting in a substantial (47%) reduction in contractile function. It was found that reduced dietary selenium not only impaired post-ischemic recovery of contractile function, indicated by lower RPPs at all measured time points during reperfusion, reduced rates of pressure development, higher EDP during reperfusion and lower % recovery of RPP to pre-ischemic levels, but also exacerbated the development of ventricular contracture during the ischemic insult. Recovery of all functional parameters measured improved with increasing dietary selenium whilst ischemic contracture decreased indicating dietary selenium is protective against myocardial ischemia-reperfusion injury. If selenium deficiency functions through limiting antioxidant status, as supported by the findings for liver and heart GPX and TxnRed activities, then these findings imply antioxidant status is important during the ischemic insult itself. This is consistent with the previous chapter which found ischemia up-regulated the TxnRed system, as well as the increasing evidence supporting injurious oxidant stress during the ischemic episode, additional to the conventional oxidant injury invoked upon reperfusion [110].

These results indicate that selenium deficiency may limit tolerance through diminished cellular levels of the antioxidants GPX and TxnRed. This is in agreement with other reports suggesting a role for GPX in limiting ischemia-reperfusion injury [277,290,291,320]. These observations have been further extended in this chapter by investigating the role of reduced GPX and/or TxnRed activities in impaired tolerance to
ischemia-reperfusion. Reductions in GPX and/or TxnRed deplete the pool of antioxidants available to combat oxidative stress leaving tissues more susceptible to oxidative damage.

Since ROS generation is increased during ischemia-reperfusion, reducing the antioxidant status of tissues will be detrimental to their recovery. Given that specific segments of the mitochondrial respiratory chain are major sources of ROS, decreasing GPX and TxnRed activities removes an immediate mechanism for the metabolism of ROS generated there. This will damage not only the mitochondria, but the entire cell leading to cellular dysfunction and/or death.

Selenium free hearts were found to not only have lower GPX and TxnRed activities, but significantly higher levels of lipid peroxidation (LPO, measured as 4-HNE and MDA) and protein carbonyls (PCO) post ischemia-reperfusion. Lipid peroxidation of myocardial cell membranes has also been implicated as a potential cause of ventricular fibrillation, tachycardia, premature beating and arrhythmias, which are all associated with reperfusion [40,102,105,108,118,174,317] Given that GPX and TxnRed are known to reduce hydrogen peroxide, which can initiate lipid peroxidation and damage proteins, lower levels of these antioxidants mean less hydrogen peroxide is scavenged leaving more available to damage lipids in membranes and proteins. These increases in LPO and PCO may therefore contribute to the poorer recoveries seen in selenium free and selenium deficient hearts.

TxnRed and GPX are also capable of directly reducing oxidized proteins and lipid peroxides, therefore increased levels of TxnRed and GPX (through increased dietary selenium) will not only reduce the amount of damaging hydrogen peroxide present in cells, but remove lipid peroxides and reduce oxidized proteins back to active states. This will also limit the extent of injury to the cells and help improve their tolerance to ischemia-reperfusion. Increased TxnRed activity will also help restore the supply of antioxidants available to combat the surge in oxidative stress seen with ischemia-reperfusion, as it regenerates not only thioredoxin (and thioredoxin peroxidase) but also glutathione and vitamins E and C. These results indicate that limiting the amount of oxidative damage occurring within cells through an increase in the antioxidants GPX and TxnRed, leads to reduced cellular dysfunction and death. This thereby protects tissues from ischemia-reperfusion, and improves myocardial functional recovery.
Although increased dietary selenium and the subsequent increase in GPX and TxnRed was found to decrease levels of LPO and PCO, there were no significant changes in DOPA, 3-Tyr, 3-nitrotyrosine or di-Tyr. Tyrosyl radicals are produced by the oxidation of tyrosine by hydroxyl radicals. DOPA is then formed by the direct addition of another hydroxyl radical to tyrosyl radicals, whilst di-Tyr is formed when tyrosyl radicals dimerise. 3-Tyr is formed via the hydroxyl mediated oxidation of phenylalanine. 3-nitrotyrosine is produced from the nitration of tyrosine by peroxynitrite, which is generated by the reaction of superoxide with nitric oxide [313]. Given that DOPA, di-Tyr and 3-Tyr are produced via the hydroxyl radical, it is possible changes in GPX and TxnRed will have little effect on the generation of these products. Although GPX and TxnRed reduce hydrogen peroxide to water, any hydrogen peroxide not scavenged will produce hydroxyl radicals which are extremely reactive and as such have extremely short half-lives [40,102,118,166,174,317]. Neither GPX or TxnRed can act directly on hydroxyl radicals or superoxide, and as such won’t protect molecules from damage caused by them. Although they can reduce hydrogen peroxide to prevent it going on to form hydroxyl radicals, it is possible the surge in ROS during reperfusion means some hydrogen peroxide escapes the antioxidant defence and does go onto to produce hydroxyl radicals. Even though it is not significant, there is a downward trend in 3-Tyr levels with increasing dietary selenium. A similar trend was also observed for levels of 3-nitrotyrosine in post-ischemic hearts. Given that GPX has been reported to detoxify peroxynitrite, it is possible this trend arises from increased activity of GPX [337]. There were no real changes or trends observed for DOPA or di-Tyr with the various levels of dietary selenium which may indicate a different oxidative mechanism for them.

Oxidative stress has also been implicated as a mediator of apoptosis, with numerous studies having found ROS to be involved in apoptosis in a number of different ways [135,136]. Apoptotic activity was measured in the hearts as caspase-3 activity, a marker of apoptosis common to both the caspase-6/9 and caspase-8 pathways. Whilst caspase-3 activity was not significantly affected by dietary selenium levels a trend for increasing caspase-3 activity with decreasing selenium levels was observed, suggesting greater apoptotic activity occurs in hearts from selenium deficient animals. This may result from an increase in oxidative stress caused by decreased GPX and TxnRed.
activities, or from the reduced activity of the TxnRed system. Both thioredoxin (Txn) and thioredoxin peroxidase (Prdx) protect cells from apoptosis. Prdx-2 inhibits release of cytochrome c from mitochondria (a known cause of apoptosis), reduces hydrogen peroxide thereby preventing it initiating apoptosis and regulates the anti-death gene Bcl-2 [177,215,223,331]. Txn inhibits apoptosis by increasing DNA binding of the transcription factor AP-1 and inhibiting ASK1 activity [136,210,229-231,243]. Although it is not known whether dietary selenium and/or changes in TxnRed regulate Txn or Prdx, it is highly possible given the results of previous studies. In the previous chapter, TxnRed, Txn and Prdx were all up-regulated by ischemia, and studies in Txn transfected cells have found Prdx2 expression is increased as a result of Txn overexpression [331]. Increases in Txn and/or Prdx may therefore limit not only oxidative stress but also apoptosis. Hence, further research is warranted to assess whether dietary selenium and/or changes in TxnRed effect other components of the TxnRed system post ischemia-reperfusion.

Superoxide dismutase activity was not effected by dietary selenium levels, with no changes in activity between groups. Whilst this was to be expected as SOD does not contain selenocysteine in its active site, it does indicate that GPX and TxnRed do not regulate SOD. This is also supported by the previous chapter in which increases in TxnRed and GPX caused by ischemia and reperfusion did not alter SOD activity. Taken together, it can be concluded SOD activity is not induced by ischemia, reperfusion or changes in antioxidant status.

Whilst the activity of GPX and TxnRed is dependent upon the amount of selenium available, it does appear this relationship is sigmoidal and that there is a saturation point above which a significant increase in selenium results in only a marginal increase in enzyme activity. This was found for both GPX and TxnRed in hearts. This supports previous studies in rats which found liver GPX-1 activity begins to plateau above 100 µg Se/kg food, liver GPX-4 at 65 µg Se/kg food and plasma GPX-3 around 70 µg Se/kg food [271,273]. They also found similar responses in rat kidney, lung and erythrocytes. Cells cultured in selenium-deficient media, and supplemented with graded levels of selenium also show similar responses for GPX-1 activity [274]. This was also found in porcine kidney epithelial cells, where the activity of GPX-1 plateau’s above
Whilst results in this chapter support these findings for GPX activity, they also indicate TxnRed responds in a similar way to dietary selenium levels. LPO and PCO were both reduced in the 1000 µg Se/kg group indicating the additional selenium did provide some protection against oxidative stress.

The recommended daily intake of selenium for humans is ~75 µg per day for males and ~60 µg per day for females, however in many countries the daily selenium intake has been shown to much less than this [255,282]. Saturation of selenocysteine based enzymes in humans has been demonstrated at 80-100 µg /day, however higher doses have been shown to have anti-cancer effects and some experimental evidence has suggested that increased selenium intake induces the expression of antioxidant proteins [255,256,271,274,282]. Hence, it may be important to assess the selenium status of individuals at risk of ischemic heart disease or those expecting to undergo clinical procedures that involve transient periods of cardiac hypoxia and/or anoxia. Dietary selenium supplementation may therefore provide a safe and convenient way of increasing antioxidant protection in these individuals.

The results of this chapter support a role for both selenium, and the thioredoxin reductase and glutathione peroxidase antioxidant systems in ischemia-reperfusion injury. Decreasing dietary selenium significantly reduces GPX and TxnRed activity impairing tolerance to ischemia-reperfusion. This results in increased lipid and protein oxidation, and reduced functional recoveries. Both functional recovery and biological oxidation improved with increasing dietary selenium. These findings may lead to advances in medical interventions related to oxidative stress and improved morbidity and mortality rates following ischemic insult.
LIMITATIONS

There are several experimental limitations in this chapter that deserve to be mentioned.

The first is an obvious limitation for the Langendorff isolated heart preparation, and that is *ex vivo*. There is an absence of variable factors such as sympathetic control, peripheral resistance, substrate supply and circulating hormones, all of which may alter the response of the heart *in vivo*. However, while this may be a limitation, it also presents an advantage, as the model can be structured to examine direct effects upon the heart during ischemia-reperfusion.

The second limitation is the absence of blood borne elements, specifically those involved with ROS generation and inflammation. Whilst the presence of these elements may alter the response to injurious stimuli, we and others have previously shown ROS are generated in the absence of these elements, and that cellular dysfunction/death still occur. Once again it is also important to investigate the other sources of ROS and the direct effects of ischemia-reperfusion on the heart.

Although our control rats received a different diet to the experimental groups, we don’t believe the diet of the control rats is a factor in the results observed. The functional data obtained for the control hearts falls between that of the 50 µg Se/kg food and the 1000 µg Se/kg food treated groups. The growth rate and body weights at the time of sacrifice were also the same between groups and there were no differences in pre-ischemic heart function. This basal diet is the standard selenium free diet used by numerous other research groups, including de Leiris, Tanguy and Boucher’s group [289,319,320].

Due to the changes in expression for various components of the TxnRed system in the previous chapter, it would have been interesting to see if thioredoxin itself was altered by dietary selenium levels and/or changes in TxnRed activity. Measurement of Txn protein levels was attempted, however, as per the previous chapter, the antibodies used were generated for human Txn. Whilst Txns are <80% homologous between species, the human monoclonal antibodies were unable to recognise rat Txn. There were no antibodies available for rat Txn at the time of these studies. Whether dietary selenium
and/or changes in TxnRed activity alter Txn expression will be examined in the next chapter, where the effect of dietary selenium on post-ischemic expression of the TxnRed and GPX systems will be investigated.
CONCLUSIONS

In summary, this chapter reveals that a reduction in dietary selenium can significantly impair intrinsic myocardial tolerance to ischemic insult. Moreover, enhanced dietary selenium has a modest protective action. These effects may be related to parallel changes in tissue activities of GPX and/or TxnRed. Selenium deficiency produced parallel reductions in GPX and TxnRed activity along with poorer recoveries from ischemia-reperfusion. This was also associated with increased levels of biological oxidation including lipid peroxides and protein carbonyls, as well as a higher level of apoptotic activity. Conversely, selenium supplementation of 1000 µg/kg food significantly improved recovery from ischemia-reperfusion however there was no real increase in enzyme activities above that of the controls. This supports previous studies showing GPX activity in rat tissues begins to plateau above 100 µg Se/kg, and also indicates TxnRed responds in a similar way. Lipid peroxidation and protein oxidation were however both lower in the 1000 µg Se/kg group indicating the additional selenium does provide some protection against oxidative stress.

The results of this chapter indicate dietary selenium protects the heart against ischemia-reperfusion injury by increasing the activity of both TxnRed and GPX (but not SOD) which protect against the oxidative stress generated during ischemia-reperfusion. By reducing the amount of lipid peroxidation and protein oxidation occurring in the heart, less damage, including apoptosis, is caused to cells allowing a greater recovery from ischemia-reperfusion. These results also support a role for both selenium and the TxnRed and GPX antioxidant systems in ischemia-reperfusion injury. Given the many functions of thioredoxin related proteins, further research is warranted to assess the relative contribution of this system to the endogenous antioxidant status of reperfused cardiac tissue. The precise role of dietary selenium in modulating the activity of TxnRed and GPX (and any related proteins) also remains to be determined, and whether it alters post-ischemic mRNA expression or just activity will be investigated in the next chapter. These results may lead to new advances in medical interventions related to oxidative stress, and improved recovery rates following myocardial ischemic insults.
PUBLICATIONS ARISING FROM THIS CHAPTER


CHAPTER 5

Effects of dietary selenium on post-ischemic expression of myocardial antioxidant mRNA
5.1 **ABSTRACT**

It is well documented that cardiac ischemia-reperfusion leads to oxidative stress and poor physiological recovery. In the previous chapter it was found that selenium deficiency down-regulates thioredoxin reductase (TxnRed) and glutathione peroxidase (GPX) activity, impairing recovery from ischemia-reperfusion. Furthermore, selenium supplementation was shown to be cardioprotective and lessen oxidative damage in reperfused rat hearts. The activity and expression of these antioxidant systems has also previously been shown to be regulated by myocardial ischemia with and without reperfusion. As an extension to these previous studies, this chapter investigates the role of selenium in the mRNA expression of these, and related antioxidant proteins, post ischemia-reperfusion. Male rats were fed varying doses of selenium for five weeks. Hearts were isolated and perfused using the Langendorff method with 22.5 minutes of global ischemia and 45 minutes reperfusion. RNA was extracted for quantitative real-time PCR analysis of glutathione peroxidase (Gpx)-1 and 4, glutathione reductase (Gsr), thioredoxin peroxidase-2 (Prdx2), thioredoxin (Txn) and thioredoxin reductase (Txnrd)-1 and 2 gene expression. Selenium deficiency produced significant reductions in Gpx-1, Gpx-4, Prdx2, Txnrd-1 and Txnrd-2 mRNA expression. Conversely, selenium supplementation of 1000 µg/kg significantly up-regulated Gpx-1, Gpx-4, Txn, Txnrd-1 and Txnrd-2 gene transcription. These results show selenium modulates the cardiac mRNA expression of thioredoxin and glutathione related enzymes post ischemia-reperfusion. Taken with the results of the previous chapter, this impacts on tolerance to ischemia-reperfusion.
5.2 **INTRODUCTION**

It is now generally accepted that reperfusion of ischemic tissue leads to the generation of oxygen derived free radicals which play an important role in cellular damage [102]. Under normal physiological conditions, the production of reactive oxygen species (ROS) is controlled by endogenous free radical scavengers such as superoxide dismutase (SOD), along with the glutathione peroxidase/glutathione/glutathione reductase and thioredoxin peroxidase/thioredoxin/thioredoxin reductase antioxidant systems [317].

Glutathione peroxidase and thioredoxin reductase contain selenocysteine in their active sites and previously, ourselves [338] and others [289] have shown that selenium deficiency down regulates thioredoxin reductase (TxnRed) and glutathione peroxidase (GPX) activity, impairing the recovery of hearts from ischemia-reperfusion. Conversely, selenium supplementation has been shown to improve both tolerance to ischemia and recovery following reperfusion [290,338].

Whilst selenium (Se) has been shown to have a role in selenoprotein synthesis (following its incorporation into selenocysteine in place of sulfur) less is known about the effects of selenium on the transcription of these enzymes. Previous studies have shown that dietary selenium enhances the mRNA expression of glutathione peroxidase [272] and thioredoxin reductase [339] in the liver of rats. However, the effects of selenium on antioxidant expression in cardiac tissues remains to be investigated. One study has suggested that selenium supplementation in humans leads to enhanced cardiac expression of glutathione peroxidase mRNA and improved recovery post-ischemia [292]. Due to the increased oxidative stress generated during ischemia-reperfusion, and the importance of endogenous antioxidants in determining recovery, the experiments described in this chapter were designed to investigate whether selenium modulates expression of GPX and TxnRed mRNA post ischemia-reperfusion.
5.3 MATERIALS AND METHODS

i) ANIMALS AND DIETS

Male wistar rats (6 weeks of age), as described in Section 2.2 of Chapter 2 (General Methods), were randomly assigned to one of 4 groups and fed an experimental diet for 5 weeks. The basal diet was selenium free - containing 30% torula yeast, 59% sucrose, 5% coconut oil (vitamin E free), 5% premixed minerals (Hubbel, Mendel & Wakeman salt mix, ICN, Seven Hills, Australia) and 1% premixed vitamins (vitamin diet fortification mixture, ICN, Seven Hills, Australia). Group one (n=10) were fed the basal selenium free diet, whilst groups two and three received diets supplemented with different doses of selenium in the form of sodium selenite (Sigma, Castle Hill, Australia): 50 µg Se/kg food (n=10) and 1000 µg Se/kg (n=10). Control rats (n=12) were fed standard rat pellets, which contains approximately 240 µg Se/kg food. All rats had free access to water and food, and diets were replaced daily.

ii) ISOLATED HEART PROTOCOL

Animals were anaesthetised and hearts removed as described in Chapter 2 (Section 2.4, General Methods). Hearts were then perfused using the Langendorff isolated heart preparation as explained in Section 2.4 of Chapter 2 (General Methods). Hearts were perfused for an initial 30 minute normoxic period to allow them to stabilize. Coronary flow was then stopped to generate zero flow global ischemia. Ischemia was maintained for 22.5 minutes and hearts were maintained at 37°C in the temperature controlled organ chamber. Flow was then returned to the heart and reperfusion continued for 45 minutes. The length of ischemia used in this study (22.5 minutes) was determined in pilot experiments to be a period which resulted in ~50% recovery of contractile function. At the end of reperfusion, hearts were removed from the cannula, rapidly blotted and weighed, then frozen at –80 °C until subsequent biochemical analysis.
iii) **GENE EXPRESSION ANALYSIS**

RNA extraction, quantitation and characterisation were performed on heart samples \( n=6 \) according to methods *iii* and *iv* in *Section 2.5* of Chapter 2 (General Methods).

The mRNA expression of the glutathione related and thioredoxin related genes was measured by firstly synthesising cDNA from total RNA, then performing quantitative real-time PCR with primers for Gpx-1, Gpx-4, Gsr, Txnrd-1, Txnrd-2, Txn and Prdx-2. These methods, along with primer sequences, are explained in full in *Section 2.7* of Chapter 2 (General Methods). Gene expression changes per group are expressed as a % relative to control (i.e. those animals receiving 240 µg Se/kg food) hearts.

*iv) DATA ANALYSIS*

All data was analysed using one-way ANOVA’s as described in *Section 2.9* of Chapter 2 (General Methods).
5.4 RESULTS

i) PHYSIOLOGICAL AND FUNCTIONAL ANALYSIS

As discussed in the previous chapter, the 5 week Torula yeast diet with and without selenium supplementation did not affect the appetite nor growth rate of the animals. Selenium supplementation or deficiency also had no effect on initial heart function in relation to coronary flow, heart rate and developed pressure, however hearts from selenium deficient animals were more susceptible to ischemia-reperfusion injury when compared to controls. Selenium supplementation significantly improved recovery of cardiac function and limited oxidative damage post ischemia-reperfusion.

ii) ANTIOXIDANT mRNA EXPRESSION

Quantitative real-time PCR was used to measure the post-ischemic expression of antioxidant proteins in the glutathione peroxidase and thioredoxin reductase systems in response to dietary selenium supplementation and deficiency. Since the endogenous internal control is important for standardising results, 18S rRNA was chosen as the invariant internal control, as others in our laboratory have previously shown ischemia induces differential expression of other widely used housekeeping genes such as GAPDH and β-actin [314]. Due to the considerably lower expression of Gpx-4, a greater amount of primer was needed for PCR amplification. Increasing the primer concentration (400nM) ensured that the signal-to-noise ratio was sufficient to confidently measure Gpx-4 expression. Using a primer concentration between 200 – 500 nM causes the T_m of the PCR amplicon to increase by 3°C. This supports the melt curve analysis for Gpx-4 which showed a T_m of 84°C, 3°C higher than the predicted T_m of 81°C. Although a greater amount of primer was used, no primer dimer was produced in any samples, and the non-template control was clear of any products in both the amplification plot and the melt curve.
Expression of Thioredoxin Related Genes

The mRNA expression of thioredoxin related genes (Txnrd-1, Txnrd-2, Prdx-2 and Txn) is expressed relative to expression in control (240 µg Se/kg food) hearts, which was set as the baseline (or 100%) expression in post-ischemic rat hearts.

Dietary selenium deficiency was found to down-regulate transcription of thioredoxin related genes in post-ischemic rat hearts. Conversely, selenium supplementation of 1000 µg Se/kg food was found to up-regulate post-ischemic mRNA expression of this antioxidant system.

Thioredoxin reductase-1 expression was significantly (p<0.001) reduced to 43 ± 7 % in selenium free hearts relative to control hearts. While 50 µg Se/kg food did not alter Txnrd-1 expression significantly (76 ± 12 %), selenium supplementation of 1000 µg Se/kg food significantly (p<0.001) increased Txnrd-1 expression to 153 ± 3 % when compared to controls receiving 240 µg Se/kg food (Fig 5.1).

Thioredoxin reductase-2 mRNA expression (Fig 5.2) responded similarly to selenium manipulation, with significantly (p<0.001) ~90 % lower levels in selenium free hearts (11 ± 5 %) and significantly (p<0.01) ~70% higher levels in 1000 µg Se/kg supplemented group (168 ± 14 %), relative to control levels. Although 50 µg Se/kg did not alter Txnrd-1 significantly, Txnrd-2 was significantly (p<0.001) reduced to 38 ± 11% compared to control expression.

The selenium free diet significantly (p<0.05) reduced thioredoxin peroxidase-2 (or peroxiredoxin-2, Prdx-2) transcription by 35 % to 65 ± 8 % compared to control hearts. Post-ischemic Prdx-2 expression was comparable in all other groups, with expression being 79 ± 10, 100 ± 21 and 119 ± 10 % for 50, 240 (control) and 1000 µg Se/kg food treated hearts respectively (Fig 5.3).

Post-ischemic thioredoxin expression (Fig 5.4) was significantly (p<0.001) up-regulated by both selenium supplementation of 1000 µg Se/kg food and total selenium deficiency (235 ± 25 and 258 ± 27 % respectively, compared to controls). Txn mRNA transcription was 93 ± 18 % in 50 µg Se/kg treated hearts and 100 ± 19 % in control hearts.
Fig 5.1: Effect of dietary selenium on post-ischemic myocardial transcription levels of thioredoxin reductase-1. Tnrrd-1 gene expression was measured in isolated rat hearts following 22.5 mins ischemia and 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to controls receiving 240 µg Se/kg food, with control expression set at 100%. (# p<0.001 vs controls).

Fig 5.2: Effect of dietary selenium on thioredoxin reductase-2 expression in the post-ischemic rat heart. Tnrrd-2 gene expression was measured in isolated hearts following 22.5 mins ischemia and 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to controls receiving 240 µg Se/kg food, with control expression set at 100%. (∗∗p<0.01, # p<0.001 vs controls).
**Fig 5.3:** Effect of dietary selenium on post-ischemic myocardial mRNA levels of thioredoxin peroxidase-2, or peroxiredoxin-2. Prdx-2 gene expression was measured in isolated rat hearts following 22.5 mins ischemia and 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to controls receiving 240 µg Se/kg food, with control expression set at 100%. (*p<0.05 vs controls).

**Fig 5.4:** Effect of dietary selenium on post-ischemic thioredoxin transcription levels in the heart. Txn gene expression was measured in isolated rat hearts following 22.5 mins ischemia and 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to controls receiving 240 µg Se/kg food, with control expression set at 100%. (#p<0.001 vs controls).
Expression of Glutathione Related Genes

The mRNA transcription of glutathione related genes (Gpx-1, Gpx-4 and Gsr) is also expressed relative to the expression in control (240 µg Se/kg food) hearts, which was set as the baseline (or 100 %) expression in post-ischemic rat hearts.

Post-ischemic expression of the GPX system responded similarly to dietary selenium manipulation as the TxnRed system. Selenium deficiency was found to down-regulate transcription of glutathione related genes in post-ischemic rat hearts whilst selenium supplementation of 1000 µg Se/kg food was found to up-regulate post-ischemic mRNA expression.

Glutathione peroxidase-1 expression increased in a “dose-dependent” manner with dietary selenium, being significantly (p<0.001) 50 % lower (50 ± 20 %) in selenium free and significantly (p<0.05) ~100 % higher (202 ± 9%) in 1000 µg Se/kg supplemented groups, relative to expression in control hearts (Fig 5.5). Post-ischemic Gpx-1 expression was 66 ± 19 % in 50 µg Se/kg food treated hearts compared to 100 ± 39 % for controls.

Expression of Gpx-4 (or glutathione phospholipid hydroperoxidase) followed a similar pattern to Gpx-1, with significantly (p<0.001) ~60 % lower expression (41 ± 17 %) in selenium free and significantly (p<0.05) ~65 % higher expression (163 ± 16 %) in 1000 µg Se/kg supplemented groups (Fig 5.6). Post-ischemic Gpx-4 expression was also lower in 50 µg Se/kg food treated hearts (64 ± 22 %) when to compared to controls (100 ± 21 %), although this was not significant.

Post-ischemic expression of glutathione reductase (Gsr) in rat hearts was unaltered by selenium modification (Fig 5.7). Gsr mRNA expression was 100 ± 13, 98 ± 25, 100 ± 19 and 110 ± 14 % in 0, 50, 240 (control) and 1000 µg Se/kg food treated hearts, following 22.5 minutes ischemia and 45 minutes reperfusion.
**Fig 5.5:** Effect of dietary selenium on post-ischemic myocardial transcription levels of glutathione peroxidase-1. Gpx-1 gene expression was measured in isolated rat hearts following 22.5 mins ischemia and 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to controls receiving 240 µg Se/kg food, with control expression set at 100%. (∗p<0.05, #p<0.001 vs controls).

**Fig 5.6:** Effect of dietary selenium on post-ischemic myocardial mRNA levels of glutathione peroxidase-4. Gpx-4 (or phospholipid hydroperoxidase) gene expression was measured in isolated rat hearts following 22.5 mins ischemia and 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to controls receiving 240 µg Se/kg food, with control expression set at 100%. (∗p<0.05, #p<0.001 vs controls).
Fig 5.7: Effect of dietary selenium on post-ischemic glutathione reductase expression in the heart. Gsr gene expression was measured in isolated rat hearts following 22.5 mins ischemia and 45 mins reperfusion. Values are presented as mean ± SEM. % expression is relative to controls receiving 240 µg Se/kg food, with control expression set at 100%.
5.5 DISCUSSION

Ischemia and reperfusion results in generation of oxidative stress due to ROS production [102]. Previous studies by ourselves and others have demonstrated that reperfused mammalian cardiac tissue up-regulates expression/activity of key antioxidants to combat this surge in oxidative stress [340,341]. Enhanced expression also plays a key role in pre-conditioning, a phenomenon in which a brief bout of ischemia protects the heart from a more prolonged ischemic insult [340]. In effect the heart is “pre-armed” to counteract impending oxidative stress.

Two key players in antioxidant protection are glutathione peroxidase and thioredoxin reductase. Both enzymes have a well established role as direct antioxidants or as electron donors for other antioxidant systems. GPX and TxnRed are selenocysteine dependent enzymes and require an adequate supply of selenium for activity [256]. It has been shown by various groups that elimination of dietary selenium is an effective way of limiting the endogenous activity of these proteins, and this also limits recovery from ischemia and reperfusion [289,338]. Humans with low selenium intake, as in Keeshan province of China, have poor cardiovascular performance and often develop cardiomyopathies such as Keeshan disease [259]. Larger animals such as pigs and sheep develop a syndrome termed mulberry heart disease due to lack of selenium, with blood vessels surrounding the heart degenerating [342]. It is now accepted that very low levels of selenium lead to poor cardiovascular physiology, and this can be directly linked to reduced endogenous production of antioxidants such as GPX and TxnRed.

Less is known about the role of selenium supplementation in regulating activity and expression of antioxidants during cardiac ischemia and reperfusion. Previous studies from this laboratory and others have shown selenium supplementation increases tissue activity of glutathione peroxidase and thioredoxin reductase, and reduces ischemia-reperfusion injury [289,290,338]. A small human study of 46 patients demonstrated that selenium supplementation (400 µg/day for 7 days) improved outcome in patients undergoing corrective heart surgery involving ischemic insult. Interestingly, this study also noted increased glutathione peroxidase mRNA expression in selenium supplemented patients [292]. Given these observations and our previous work in rats, mRNA expression
of various antioxidant proteins was examined in post-ischemic rats supplied with varying amounts of dietary selenium.

The expression of Gpx-1, Gpx-4, Txnrd-1 and Txnrd-2 were all directly dependent on selenium intake. Interestingly, expression of thioredoxin peroxidase, a protein not known to contain selenium, displayed a similar pattern although this only achieved significance in selenium free animals. Previous studies in Txn transfected cells have found Prdx-2 expression is increased as a result of Txn overexpression [331]. Paradoxically, thioredoxin displayed enhanced expression in the high selenium group and selenium free group. Txn expression has previously been shown to be induced by oxidative stress, possibly involving a hypoxic or antioxidant responsive element in its promoter [194,201,213,245,248,253,341]. This is supported by the induction of Txn gene expression in the lungs of newborns by oxygen, where it is believed to protect the newborn from hyperoxia at birth [245]. Given that Txn appears to be finely tuned to the overall oxidative status in cells, and that selenium free hearts have less GPX and TxnRed protection, Txn expression may be up-regulated due to a general decrease in antioxidant status. Increased Txn expression in selenium free hearts may be aimed at increasing the reduction of intracellular proteins and other biomolecules as part of the heart’s antioxidant defence. Txn not only reduces cysteine groups on proteins, but also reduces Gpx-3, inhibits apoptosis and acts as a cofactor by providing reducing equivalents to redox enzymes, thereby providing protection to cells in a number of different ways during times of increased oxidative stress [210,213,245,248,253].

It is possible that components of the TxnRed system may up-regulate each other, and therefore, the increase in Txnrd expression seen in post-ischemic high dose selenium hearts may induce the increased Txn expression observed in these hearts. Furthermore, it is also possible that changes in Txn and/or Txnrd expression may regulate Prdx expression in the heart. The up-regulation of post-ischemic Prdx-2 expression seen with increased dietary selenium may contribute to the lower levels of lipid peroxidation, protein oxidation and apoptosis seen in these hearts in the previous chapter, as it directly reduces peroxides such as hydrogen peroxide and alkyl peroxides as well as inhibiting mitochondrial cytochrome c release [210,215,223,245].

There was no change in the post-ischemic expression of glutathione reductase in
response to dietary selenium. In chapter 3, it was also found that neither ischemia or reperfusion induced Gsr expression either, whilst Gpx-1 and –4 was up-regulated. Taken together, these results suggest Gsr is not regulated by the oxidative status of cells or dietary selenium levels, and that components of the GPX system do not up-regulate each other.

Previous studies in rats show selenium deficiency has a dramatic effect on Gpx-1 mRNA in the liver. Sunde and colleagues found selenium deficiency reduces Gpx-1 mRNA levels in male rat livers to be one-tenth of those found in selenium adequate animals [269,270]. They have also shown both Gpx-1 activity and mRNA respond sigmoidally to increasing selenium concentrations, but begin to plateau at 0.1 µg/g food and 0.05 µg/g food respectively [271,272]. Taken together, their results show Gpx-1 mRNA levels respond quicker to selenium status than Gpx-1 activity [273]. In addition to this, they also found female rats respond similarly to males although they have more than twice the level of Gpx-1 mRNA and activity [272]. Similar results have also been reported in erythrocytes, hearts, kidneys and lungs from selenium deficient rats along with cells cultured in selenium deficient media [271,274]. Previous studies in porcine kidney epithelial cells show levels of Gpx-1 mRNA increased with continued addition of selenite whilst Gpx-1 activity plateau’s above 50nM [275]. For selenium repletion, Gpx-1 protein and activity requires larger doses and longer time periods than for maintenance, and this is believed to occur because other selenoproteins have first priority for selenium during selenium deficiency [276].

Although Gpx-1 and –4 have 40% nucleotide and amino acid identity, they do respond differently to selenium deficiency [343,344]. Gpx-4 activity in the liver has been found to decrease to 40% of that in selenium adequate animals, whilst Gpx-4 mRNA decreased to 60%. This is different to the changes in Gpx-1 activity and mRNA which fall to 1-2% and 10% respectively [271]. Although the response curve for Gpx-4 activity is similar to that for Gpx-1, activity does begin to plateau at lower selenium concentrations (0.065 µg/g food). Liver Gpx-4 mRNA has been reported to be relatively unaffected by dietary selenium, decreasing by only 40% in selenium deficient animals and beginning to plateau at 0.013 µg/g food [271]. These results suggest Gpx-1 and Gpx-4 are regulated differentially and individually within a given tissue. Given that we found
Gpx-4 mRNA to be reduced by 60% in post-ischemic selenium free rat hearts also suggests there is tissue specific regulation of these selenoenzymes. Liver TxnRed activity in selenium deficient animals has been reported to fall to 10% of selenium adequate animals, whilst Txnrd only falls by 30%. This indicates the pattern of Txnrd mRNA regulation is more similar to Gpx-4 than Gpx-1 [339].

Taken together, these results suggest that it is more than just a loss of the selenium cofactor that is responsible for the loss in activity of these antioxidants in selenium deficient cells and animals. They also indicate that selenium regulation of translation is important for Gpx-4 and Txnrd whereas mRNA stability as well as translation is important for selenium regulation of Gpx-1 [266].

This chapter found cardiac Gpx-1 expression to decrease by 50%, Gpx-4 by 60%, Txnrd-1 by 55% and Txnrd-2 by 90% in selenium free animals. These results, although showing similar trends to other studies, do show different mRNA levels to previous studies. This may be due to a number of factors, including the actual dietary selenium levels, the tissue studied, as well the oxidative insult resulting from ischemia-reperfusion. Sunde and colleagues chose to use a selenium deficient diet of 2 µg /kg food whilst animals in the current study received selenium free diets and the selenium deficient animals, 50 µg /kg food. Post-ischemic Gpx-4 and Txnrd-1 expression in our selenium deficient animals display similar mRNA levels to those previously reported, however further down-regulation was observed in the selenium free animals. Gpx-1 expression in both the selenium free and selenium deficient animals was less affected by reduced dietary selenium than previous studies. This may be due to tissue specific regulation (heart versus liver), and may also result from ischemia-reperfusion. In chapter 3, reperfusion was found to up-regulate both Gpx-1 and Gpx-4 expression, therefore it is possible the reduced response in Gpx-1 expression results from this up-regulation. Owing to the increased oxidative stress generated during ischemia-reperfusion, and the importance of endogenous antioxidants in determining recovery, this study aimed to investigate whether dietary selenium levels effect post-ischemic myocardial expression of the GPX and/or TxnRed systems. Previous studies investigating Txnrd mRNA have not separated the Txnrd’s. This may therefore account for the differences observed in this
study for Txnrd-2, which appears to be more sensitive to selenium deficiency than the more abundant Txnrd-1.

Selenium supplementation of 1000 µg/kg food increased expression of Gpx-1, Gpx-4, Txnrd-1 and Txnrd-2 although previous reports suggest liver mRNA begins to plateau above ~50 µg Se/kg food. In contrast, this study found Gpx-1 to increase 100 % with 1000 µg Se/kg food, Gpx-4 63 %, Txnrd-1 55 % and Txnrd-2 70 % relative to controls. This up-regulation may also involve tissue specific regulation and/or ischemia-reperfusion. One study of patients undergoing heart surgery has demonstrated that selenium supplementation in humans (400 µg/day for 7 days) leads to enhanced cardiac expression of glutathione peroxidase mRNA and improved recovery, which does support the findings of this chapter [292].

Selenium regulation of gene expression can potentially occur at 6 main points: transcription, nuclear processing, nuclear export, translation, mRNA stability and protein turnover [266]. Whilst there is currently no evidence that selenium status has any effect on transcription initiation rates of any genes, protein degradation, rates of nuclear processing or nuclear export of mRNA, it is clear all selenoproteins are regulated by selenium at a translational level [266,345-349].

In eukaryotes, translation of selenocysteine (Sec) requires misreading of the UGA codon, the presence of a selenocysteine insertion sequence (SECIS) element in the 3’-UTR and the involvement of a SECIS binding protein, a selenocysteine elongation factor, and selenocysteine tRNA [267,268]. Sec is synthesized while esterified to its tRNA, tRNA→Sec, using inorganic selenophosphate and serine. Its position in the peptide backbone of selenoproteins is encoded by in-frame UGA codons in the mRNA [266,267]. The dietary supply of selenium modulates turnover of Sec-tRNA→Sec, limiting effectiveness of the translational machinery. With adequate selenium and all the other necessary components, Sec-tRNA→Sec will compete sufficiently well with termination release factors for binding at UGA, such that full length selenoproteins are synthesized. However, without adequate selenium and therefore insufficient Sec-tRNA→Sec, UGA is interpreted as a stop codon limiting selenoprotein translation [266-268]. Differences in SECIS elements, UGA context and/or position, or UGA/SECIS separation have been suggested to be the likely causes of differential translation. Differences in selenoprotein
mRNA SECIS elements alter the relative affinity for the Sec translation complex resulting in differential translation [265,266,350,351]. The position and context of the UGA codon is another potential cause of differential translation. UGA located close to the 5'-start or 3'-termination codons are much less efficient, with one study suggesting UGA must be >21 nt from AUG start and >204 nt from the SECIS element for optimum Sec incorporation [266,352].

Previous studies have found selenium status has dramatic effects on mRNA levels of Gpx-1 [274,278,348,353,354]. One study in H4 hepatoma cells showed selenium effects the stability of Gpx-1 but not Gpx-4 mRNA. Selenium deficiency resulted in rapid degradation of Gpx-1 mRNA, however there was no effect on Gpx-4 mRNA degradation supporting the higher mRNA levels and activity [278]. Another study by Maquat and colleagues found Gpx-1 degradation in hepatocytes cultured in selenium deficient media. They demonstrated Gpx-1 degradation occurs in the cytoplasm, and that it is likely to occur via nonsense-mediated decay of mRNA [348]. Nonsense codons are known to destabilize many different mRNA species, particularly when located upstream from an intron [266,354,355]. Further studies by Weiss and Sunde have shown that selenium regulation of of Gpx-1 mRNA requires a functional SECIS in the 3'-UTR and a Sec codon followed by an intron [353]. Collectively, these previous results establish that Gpx-1 mRNA is degraded by a nonsense mediated mRNA decay mechanism when Sec is not available for translation [266,348,352]. Selenium deficiency and therefore insufficient Sec-tRNA → Sec results in UGA being interpreted as a nonsense codon rather than Sec. This in turn leads to increased mRNA turnover and has been proposed as the putative mechanism linking selenium intake to mRNA expression of selenocysteine dependent enzymes. Therefore, Sec-tRNA → Sec concentrations control gene expression of Gpx-1 not only by limiting translation but also by modulating Gpx-1 mRNA stability [268,348,353].

Selenium specific down-regulation of Gpx-4 and Txnrd mRNA levels may be explained by ribosomal pausing that must occur when Sec-tRNA → Sec concentrations are limiting protein synthesis. The competition between termination factors and a limited supply of Sec-tRNA → Sec would predispose these proteins for early termination. This idle mRNA could then be subjected to more rapid degradation via normal decapping and exonuclease hydrolysis [266].
Post-ischemic thioredoxin peroxidase expression was also found to correlate with selenium intake yet is not a selenium containing protein. This suggests that other selenium dependent or independent mechanisms also control expression of antioxidant mRNAs. Given the marked differences in tissue distribution of selenoproteins, and even the gender differences that occur within some species, it is likely tissue specific transcription factors also exist [356]. Considering there are differences in tissue distribution of selenoproteins and differences in their responsive to dietary selenium, it may be worthwhile investigating the relative importance of each system in the heart during ischemia-reperfusion.

The results of this chapter support a role for both selenium, and the thioredoxin reductase and glutathione peroxidase antioxidant systems in ischemia-reperfusion injury. Decreasing dietary selenium results in reduced antioxidant expression following ischemia-reperfusion, leaving hearts more susceptible to oxidative damage. In most species a selenium intake of 100 µg/kg food is sufficient to maximally express Gpx and Txnrd mRNA and activity. However, this study found a significant difference in Gpx and Txnrd mRNA expression between animals consuming a standard diet (240 µg Se/kg food) and those consuming 1000 µg Se/kg food. This raises the possibility that enhanced selenium intake may be a useful way of preconditioning, by increasing antioxidant expression and activity in cardiac tissues post-ischemia.
LIMITATIONS

Although some may consider the fact we have studied the effect of dietary selenium on myocardial antioxidant expression post ischemia-reperfusion to be a limitation of this study we do not believe it is. Oxidative stress is increased during ischemia-reperfusion, and endogenous antioxidants are important in determining recovery from such insults. This study was therefore designed to investigate whether selenium modulates expression of GPX and TxnRed mRNA post ischemia-reperfusion, when down-regulation could be detrimental to the heart, and up-regulation protective. Since others had previously found selenium alters mRNA levels in rat tissues in the absence of ischemia-reperfusion [269-271,339], we chose to extend these observations by studying cardiac expression following an oxidative insult.

As discussed in the previous chapter, we don’t believe the actual base diet of the rats is a factor involved in the results observed. Whilst control rats received a different diet to the experimental groups (containing 240 µg Se/kg food), we don’t believe this influenced the results. The functional data obtained in the previous chapter for the control hearts fell between that of the 50 µg Se/kg food and the 1000 µg Se/kg food treated groups. The growth rate and body weights at the time of sacrifice were the same between groups and there were no differences in pre-ischemic heart function. GPX and TxnRed expression show a dose-dependent effect indicating the actual base diet did not alter expression of these antioxidants. This basal diet is the standard selenium free diet used by numerous other research groups, including de Leiris, Tanguy and Boucher’s group [289].

As previously discussed in chapter 3, we don’t believe using SYBR green for our real-time PCR rather than fluogenic probes had a significant effect on the low expression of Gpx-4. We have experience with both fluorogenic probes and SYBR Green, and have only ever noticed a difference in sensitivity of 2-3 cycles for the same gene. Therefore we don’t believe this would have a significant effect on the low expression of Gpx-4, and we are certain the signal-to-noise ratio was sufficient to measure Gpx-4 expression. We also run melt curves on all our PCR products to ensure the T_m is correct for the product and that the non template control (NTC) is clear of products or primer dimer. Both the
melt curve and agarose gel gave the correct $T_m$ and product size for Gpx-4 whilst all NTCs were clear therefore we are confident we are detecting Gpx-4.
CONCLUSIONS

In summary, this chapter indicates dietary selenium modulates the cardiac mRNA expression of thioredoxin and glutathione related enzymes post ischemia-reperfusion. This supports the changes seen in the previous chapter in tolerance to ischemia-reperfusion with dietary selenium manipulation. Selenium deficiency resulted in decreased mRNA expression of Txnrd-1, Txnrd-2, Prdx-2, Txn, Gpx-1 and Gpx-4 following ischemia-reperfusion, potentially leaving hearts more susceptible to oxidative damage. These results support previous studies which found selenium deficiency reduces GPX and TxnRed expression in various rat tissues. This chapter extends these findings by looking at the effect dietary selenium has on myocardial antioxidant mRNA expression after an oxidative insult, and found similar trends in expression do occur post ischemia-reperfusion.

In most species, a selenium intake of 100 µg/kg food is sufficient to maximally express GPX and TxnRed mRNA and activity. However, we found significant up-regulation of Gpx-1, Gpx-4, Txnrd-1, Txnrd-2 and Txn mRNA expression in animals consuming 1000 µg Se/kg compared to animals consuming a standard diet (240 µg Se/kg). This raises the possibility that enhanced selenium intake may be an effective method of preconditioning by increasing antioxidant expression and activity in cardiac tissues post-ischemia.

Human selenium supplementation trials use 200-400 µg Se/day which equates to approximately 2.8-5.6 µg Se/kg body weight/day in addition to the selenium consumed in their diets (recommended dietary intake is 1-2 µg Se/kg body weight/day). The control rats in this study received approximately 9 µg Se/kg bodyweight/day whilst the high dose experimental group, which had higher GPX and TxnRed expression, received approximately 36 µg Se/kg bodyweight/day. Although the high dose used in these studies is greater than those used currently in human clinical trials, these findings do suggest humans may benefit from higher selenium levels, particularly those susceptible to ischemia-reperfusion injury or other conditions involving any oxidative stress. Dietary selenium manipulation may ultimately prove beneficial to patients undergoing clinical interventions which incur myocardial ischemia and reperfusion.
PUBLICATIONS ARISING FROM THIS CHAPTER

CHAPTER 6

Effects of auranofin on myocardial antioxidant enzyme systems, & ischemia-reperfusion injury.
6.1 **ABSTRACT**

Auranofin, an anti-rheumatic gold compound is an inhibitor of selenocysteine enzymes such as thioredoxin reductase and glutathione peroxidase. These antioxidant enzymes play an important role in protecting cardiac tissue from oxidative stress generated during ischemia-reperfusion, as shown in chapters 3 and 4. This chapter investigates the relative importance of myocardial TxnRed alone, and how its inhibition effects functional recovery, oxidative damage and apoptosis following ischemia-reperfusion. Auranofin was administered to rats by suspension in corn oil and gavaging with an oral dosing needle 24 hours prior to sacrifice. The dose chosen was 100 mg auranofin/kg bodyweight, as this concentration has previously been shown to selectively down-regulate thioredoxin reductase activity without altering glutathione peroxidase. Vehicle controls were gavaged with corn oil only. Hearts were then isolated and perfused using the Langendorff model where they were subjected to either normoxic perfusion or 22.5 minutes ischemia and 45 minutes reperfusion. The activity of thioredoxin reductase and glutathione peroxidase was determined in heart extracts subjected to normoxic perfusion and ischemia-reperfusion. Caspase-3, a marker of apoptosis, was also measured in heart extracts post ischemia-reperfusion, along with markers of lipid and protein oxidation. There was significantly less thioredoxin reductase activity in auranofin treated rat liver extracts and auranofin treated normoxic hearts, whilst glutathione peroxidase activity remained unaffected. This demonstrated that the dose of auranofin used was able to selectively inhibit one of these enzymes. Rats treated with auranofin displayed significantly impaired recovery from ischemia-reperfusion with significantly elevated end diastolic pressures and decreased contractile function. This corresponded to greater levels of lipid peroxidation, however there were no differences in protein oxidation. The level of caspase-3 activity was also significantly increased in the auranofin treated hearts suggesting auranofin administration is pro-apoptotic. Given that auranofin is prescribed as an anti-rheumatic drug, often to older patients at risk of cardiovascular disease, the results described in this chapter suggest that these patients would recover poorly from an ischemic insult such as myocardial infarction or related clinical procedures.
6.2 **INTRODUCTION**

Cardiac tissues are subjected to ischemia–reperfusion injury during clinical procedures such as angioplasty, by-pass surgery and transplantation [102]. The re-introduction of oxygen into transiently ischemic tissue results in the generation of reactive oxygen species (ROS) and oxidative stress. The degree of oxidative stress and hence tissue damage is dependent on the ability of the cellular antioxidant defences to cope with this oxidative burden [102].

Glutathione peroxidase is an important antioxidant enzyme, present in the cytosol and mitochondria of most mammalian cells, capable of converting hydrogen peroxide to water. During this reaction GPX is oxidized to an inactive state and must be re-generated by electron shuffling through glutathione, glutathione reductase and NADPH. GPX has a selenocysteine residue within the active site that is essential for catalytic activity [357]. Thioredoxin reductase also contains selenocysteine in its active site, and its activity is also dependent upon the presence of this amino acid [201,338]. TxnRed is an antioxidant protein, which can act through the thioredoxin peroxidase/thioredoxin system or act directly to reduce lipid hydroperoxides. TxnRed helps in recycling vitamin C, which is oxidised when reducing vitamin E, and is also able to reduce dihydroascorbate to ascorbic acid. Therefore, TxnRed acts both directly as an antioxidant, as well as having a secondary role in the regeneration of exogenous antioxidants such as Vitamin C and E [201].

Chapter 4 demonstrated that removing selenium from the diet of rats down-regulates the endogenous activity of these two enzymes and this correlated with poor cardiac recovery following ischemia reperfusion [338]. This chapter examines the functional recovery of hearts from rats treated with auranofin, an inhibitor of selenocysteine based enzymes. Auranofin (2,3,4,6-tetra-o-acetyl-1-thio-β-D-glucopyranoside-S-Triethyl-phosphine gold) is a lipophilic gold compound often prescribed in the treatment of rheumatoid arthritis [358]. It has anti-inflammatory properties and is thought to act through the regulation of NF-κB [359]. Gold compounds such as auranofin are also potent inhibitors of selenocysteine containing enzymes such as GPX and TxnRed. GPX is inhibited by auranofin with a Ki of 11.7uM, whereas TxnRed
has a Ki of 4nM [294,360]. This substantial difference in Ki provides a mechanism whereby one enzyme can be inhibited at certain concentrations of auranofin whilst the other remains active. Given our previous findings on the poor functional recovery after reducing the activity of both enzymes [338,361], in this study auranofin was used to selectively inhibit TxnRed in rats without altering GPX activity. Hearts from these animals were subjected to an experimental model of ischemia-reperfusion, and a variety of physiological functions were measured to assess post-ischemic cardiac recovery. These tissues were examined post ischemia-reperfusion for evidence of oxidative damage and apoptosis.
6.3 MATERIALS AND METHODS

i) ANIMALS AND TREATMENTS

Adult male wistar rats (10 weeks of age), as described in Section 2.2 of Chapter 2 (General Methods), were randomly assigned to one of 5 experimental groups; controls (n=12), auranofin treated (n=7), vehicle controls (n=4), normoxics n=10 and auranofin treated normoxics (n=6). The auranofin groups were administered 100 mg auranofin/kg bodyweight via an oral gavage needle (Provet, Brisbane, Australia). Auranofin was suspended in 1 mL of corn oil and was administered to rats 24 hours before sacrifice. A group of sham treated vehicle controls were gavaged with corn oil only, 24 hours before sacrifice. The control group received no treatment prior to sacrifice.

ii) ISOLATED HEART PROTOCOL

Animals were anaesthetised and hearts removed as described in Chapter 2 (Section 2.4, General Methods). Hearts were then perfused using the Langendorff isolated heart preparation as explained in Section 2.4 of Chapter 2 (General Methods). Hearts were perfused for an initial 20 minute normoxic period to allow them to stabilize. Coronary flow was then stopped to generate zero flow global ischemia. Ischemia was maintained for 22.5 minutes and hearts were maintained at 37°C in the temperature controlled organ chamber. Flow was then returned to the heart and reperfusion continued for 45 minutes. The length of ischemia used in this study was determined in pilot experiments to be a period which resulted in ~50% recovery of contractile function. This provided the potential to measure both improved and reduced recoveries. Both normoxic groups were subjected to 87.5 minutes of normoxic perfusion only (i.e. hearts received adequate (100%) perfusion with 37°C Krebs-Henseleit buffer equilibrated with 95% O₂ and 5% CO₂ for the entire experimental time course). At the end of reperfusion/normoxic perfusion, hearts were removed from the cannula, rapidly blotted and weighed, then frozen at −80 °C for subsequent biochemical analysis.
iii) **BIOCHEMICAL ANALYSIS**

Liver samples were also removed from the rats at the time of sacrifice, weighed and kept at –80 °C until use. Protein was extracted from hearts and livers as described in *Section 2.5 i* of Chapter 2 (General Methods), and protein estimations performed on extracts as detailed in *Section 2.5 ii* of Chapter 2.

Activity of the antioxidant enzymes, glutathione peroxidase (GPX), thioredoxin reductase (TxnRed) and superoxide dismutase (SOD) were measured in protein extracts as described in *Section 2.6* (methods *i, ii* and *iii*) of Chapter 2. GPX and TxnRed were measured in both heart and liver extracts to determine systemic effects of auranofin on selenocysteine containing enzymes, whilst SOD was measured in heart extracts only.

To assess the level of oxidative stress within these tissues, lipid peroxidation was measured in heart extracts along with protein carbonyls and the tyrosine oxidation products DOPA, di-Tyr, o-Tyr and 3-nitrotyrosine. These methods are explained in detail in *Section 2.6* (methods *v, vi*, and *vii*) of Chapter 2 (General Methods).

Caspase-3 is a marker of apoptosis common to both the caspase-6/9 and caspase-8 pathways. Its activity was measured as an indicator of apoptosis in hearts using the method described in Chapter 2, *Section 2.6 iv*.

iv) **DATA ANALYSIS**

All data was analysed using one-way ANOVA’s as described in *Section 2.9* of Chapter 2 (General Methods).
6.4 RESULTS

i) ANTIOXIDANT ENZYME ACTIVITIES

Thioredoxin Reductase activity

Auranofin (100mg/kg) induced a significant decrease (p<0.001) in thioredoxin reductase activity in rat livers when compared to vehicle and control groups (Fig 6.1a). The auranofin treated animals displayed a TxnRed activity of only $47.6 \pm 2.7$ moles/min/mg protein compared to the control level of $70.4 \pm 5.6$ moles/min/mg protein. There were no differences in TxnRed activity in livers from control animals and vehicle (corn oil) treated animals, whose activity was $66.0 \pm 4.8$ moles/min/mg protein.

Similar results were also observed in the heart following normoxic perfusion (Fig 6.1b). There was a significant (p<0.01) decrease in thioredoxin reductase activity in those animals treated with auranofin when compared to controls following normoxic perfusion. TxnRed activity was reduced to $18.7 \pm 4.2$ moles/min/mg in the auranofin group versus $53.8 \pm 7$ moles/min/mg in the control group.

However, following ischemia-reperfusion, thioredoxin reductase activity in the heart was similar in all three groups (Fig 6.1c). TxnRed activity was $106.8 \pm 6$, $105.5 \pm 5.3$ and $102.5 \pm 3.5$ moles/min/mg protein for the auranofin treated, control and vehicle groups respectively. Although there are no differences in activities between groups, activity has increased significantly from the levels seen during normoxic perfusion. Given that there were no differences in TxnRed activity in either heart or liver samples between controls and vehicle treated animals indicates corn oil and/or gavaging with an oral dosing needle does not effect TxnRed activity.
**Fig 6.1:** Effect of auranofin (100 mg/kg bodyweight) on thioredoxin reductase activity in rat liver extracts (A), normoxic heart extracts (B) and post-ischemic heart extracts (C, over page). Auranofin treated animals were gavaged 24 hours prior to sacrifice with auranofin suspended in corn oil via an oral dosing needle whilst vehicle treated animals were gavaged with corn oil only. Normoxic hearts (b) received adequate (100%) perfusion for 87.5 minutes. Data is presented as mean ± SEM. (moles TxnRed/min/mg protein). (∗∗p<0.01, #p<0.001 vs controls).
Fig 6.1 cont': Effect of auranofin (100 mg/kg bodyweight) on thioredoxin reductase activity in rat liver extracts (A, previous page), normoxic heart extracts (B, previous page) and post-ischemic heart extracts (C). Auranofin treated animals were gavaged 24 hours prior to sacrifice with auranofin suspended in corn oil via an oral dosing needle whilst vehicle treated animals were gavaged with corn oil only. Normoxic hearts (b) received adequate (100%) perfusion for 87.5 minutes, whilst post-ischemic hearts (c) underwent 22.5 mins ischemia and 45 mins reperfusion. Data is presented as mean ± SEM. (moles Tnnred/min/mg protein). (**p<0.01, #p<0.001 vs controls).
**Glutathione Peroxidase activity**

Glutathione peroxidase activity in rat livers was not affected by auranofin when administered at a dose of 100mg/kg (Fig 6.2a). There was no difference in GPX activity observed between the control group and either the auranofin treated or the corn oil treated groups (397.3 ± 10.9 vs 411.6 ± 7.2 and 399.4 ± 22.5 mmoles/min/mg protein respectively).

Similar results were also found in tissue homogenates from hearts following both normoxic perfusion (Fig 6.2b) and ischemia-reperfusion (Fig 6.2c). GPX activity in normoxic hearts was 352.1 ± 30.6 mmoles/min/mg protein compared to 345.6 ± 25.4 mmoles/min/mg protein in auranofin treated normoxic hearts. These results were predicted as the dose of auranofin chosen had been shown in previous studies to be insufficient to inhibit glutathione peroxidase [10]. GPX activity in the control and auranofin treated groups was also greater in hearts following ischemia-reperfusion when compared to normoxic perfusion (607.2 ± 20.7 and 586.8 ± 24.3 mmoles/min/mg protein respectively). Activity in vehicle treated post-ischemic hearts was 575.4 ± 45.6 mmoles/min/mg protein, showing that corn oil and/or gavaging with an oral dosing needle has no effect on GPX activity.
Control Auranofin Vehicle

A

Gpx activity (mmoles/min/mg prot)

Group

B

Gpx activity (mmoles/min/mg prot)

Group
**Fig 6.2:** Effect of auranofin (100 mg/kg bodyweight) on glutathione peroxidase activity in rat liver extracts (A), normoxic heart extracts (B) and post-ischemic heart extracts (C). Auranofin treated animals were gavaged with auranofin suspended in corn oil via an oral dosing needle 24 hours prior to sacrifice whilst vehicle treated animals were gavaged with corn oil only. Normoxic hearts (b) received adequate (100%) perfusion for 87.5 minutes, whilst post-ischemic hearts (c) underwent 22.5 mins ischemia and 45 mins reperfusion. Data is expressed as mean ± SEM. (mmoles GPX/min/mg protein).
Superoxide Dismutase activity

Superoxide Dismutase activity was not affected by auranofin, with activities being similar to that in control hearts following both normoxic perfusion (Fig 6.3a) and ischemia-reperfusion (Fig 6.3b). SOD activities in normoxic hearts were 45.6 ± 5.9 units/mg protein for controls versus 41.8 ± 2.7 units/mg protein for auranofin treated. Similar results were found in post-ischemic hearts indicating neither auranofin or ischemia-reperfusion has an effect on SOD activity. There were no differences in activity between controls and vehicle treated hearts either indicating corn oil and/or gavaging has no effect on SOD activity. SOD activities following ischemia-reperfusion were 49.9 ± 8.4, 42.7 ± 3.8 and 40.3 ± 4.6 units/mg protein for control, auranofin treated and vehicle treated hearts respectively.
Fig 6.3: Effect of auranofin (100 mg/kg bodyweight) on superoxide activity in heart extracts following normoxic perfusion (A) and 22.5 mins ischemia and 45 mins reperfusion (B). Auranofin treated animals were gavaged with auranofin via an oral dosing needle 24 hours prior to sacrifice whilst vehicle treated animals were gavaged with corn oil only. Data is presented as mean ± SEM. (units/mg protein).
ii) MYOCARDIAL FUNCTIONAL ANALYSIS

Pre-ischemic heart function

There were no differences in body weights between the experimental groups at the time of sacrifice, with all animals weighing between 400-465 grams. Auranofin, corn oil and/or oral gavage had no effect on initial heart function (Fig 6.4a-f) in relation to coronary flow, heart rate, left ventricular developed pressure and dP/dt (Table 6.1). Whilst heart rates were similar between all experimental groups during equilibration (260-300 bpm), hearts were not paced and as a result we have chosen to monitor RPP as opposed to developed pressure as an indicator of myocardial function. All hearts displayed an initial RPP between 54 and 59 x10^3 mmHg/min, +dP/dt between 3400-3900 mmHg/sec, and –dP/dt from –3200 to –3600 mmHg/sec. Coronary flow during equilibration was approximately 23-26 mL/min.
Fig 6.4: Effect of auranofin on pre-ischemic heart function. Auranofin treated rats were gavaged 24 hours prior to sacrifice with 100 mg auranofin/kg body weight, whilst vehicle treated animals were gavaged with corn oil only. Hearts were isolated and perfused on a Langendorff perfusion apparatus. Left Ventricular Developed Pressure (LVDP, mmHg, 6.4A), heart rate (bpm, 6.4B), rate pressure product (RPP, mmHg/min, 6.4C), rate of contraction (+dP/dt, mmHg/sec, 6.4D), rate of relaxation (-dP/dt, mmHg/sec, 6.4E) and coronary flow (mL/min, 6.4F) were measured during normoxic equilibration. Values are presented as mean ± SEM.
Ischemic contracture

Figure 6.5 illustrates the degree of ischemic contracture sustained by each experimental group at the end of the 22.5-minute ischemic period. Ischemic contracture arises due to sustained contraction of the ventricle and is an indicator of severity of ischemic damage. The degree of contracture was greater in the auranofin treated group (77.3 ± 5.4 mmHg), indicating reduced tolerance to the ischemic insult, however this did not reach statistical significance. There were no differences in contracture between the controls (68.3 ± 4.8 mmHg) and the vehicles (66.2 ± 6.2 mmHg).

**Fig 6.5:** Effect of auranofin on ischemic contracture after 22.5 minutes of ischemia in the isolated perfused rat heart. Auranofin treated rats were gavaged with 100 mg auranofin/kg body weight using an oral dosing needle 24 hours prior to sacrifice, whilst vehicles received corn oil only. Data is presented as mean ± SEM, mmHg.
Post-ischemic heart function

The contractile function for each experimental group following ischemia-reperfusion is shown in Table 6.1. Contractile function was measured as left ventricular developed pressure (LVDP), rate pressure product (RPP), % recovery of RPP after 45 minutes reperfusion, as well as end diastolic pressure (EDP) and the rate of left ventricular pressure development (+/- dP/dt) at the end of 45 minutes reperfusion.

As hearts were not paced during equilibration or reperfusion, RPP was used as an indicator of contractile function during reperfusion rather than left ventricular developed pressure alone. This is because left ventricular pressure development (LVPD) is dependent upon heart rate, so any changes in heart rate caused by auranofin or ischemia-reperfusion may effect LVDP, whereas RPP accounts for changes in both heart rate and LVDP. Following ischemia, RPPs were lower in all experimental groups than during equilibration. RPPs did increase though in the control and vehicle treated groups during the time course of reperfusion however auranofin treated hearts failed to recover as well (Fig 6.6). At the end of 45 minutes reperfusion, rate pressure products were significantly (p<0.001) lower in auranofin treated hearts (3.3 ± 1.2 mmHg/min) compared to controls (29.2 ± 2.04 mmHg/min). There were no differences in RPPs between control hearts and vehicle treated hearts (29.5 ± 2.1 mmHg/min) at the end of reperfusion (Fig 6.7a).

This data is further supported with the percent (%) recovery of RPP after 45 minutes reperfusion (Fig 6.7b). These results show auranofin significantly (p<0.001) reduces functional recovery of hearts following ischemia-reperfusion, with the RPP recovering to 8.8 ± 4.1 % of pre-ischemic levels, compared to 50 ± 2.8 and 50.7 ± 5 % recovery for control and vehicle treated groups. The period of ischemic insult used in this study ensured an expected recovery of approximately 50% for control experiments and the administration of auranofin significantly inhibited this recovery.

Increased EDP (Fig 6.8) reflects residual diastolic dysfunction and is representative of the left ventricle’s inability to relax during diastole. EDP was significantly greater (p<0.001) in the auranofin treated group (80.6 ± 6.3 mmHg) when compared to controls (48.5 ± 3.8 mmHg). There were no differences in EDP between the controls and the vehicle treated group (48.5 ± 3.8 vs 47.5 ± 3.0 mmHg).
The rate of pressure development over time was also measured in hearts at the end of reperfusion (Table 6.1). The rate of left ventricular contraction (+dP/dt, or rate of positive pressure development) was significantly (p<0.001) lower in auranofin treated hearts during reperfusion when compared to controls. +dP/dt after 45 minutes reperfusion was 383.7 ± 46.7 vs 1886 ± 87.9 mmHg/sec for auranofin treated and control hearts respectively (Fig 6.9a). There were no differences between control and vehicle treated hearts (1896 ± 256.5 mmHg/sec). Similar results were observed for the rate of left ventricular negative pressure development (-dP/dt, or rate of relaxation). –dP/dt in auranofin treated hearts was –355.4 ± 66.7 mmHg/sec, which was significantly (p<0.001) lower than –dP/dt in control hearts (-1992 ± 138.2 mmHg/sec, Fig 6.9b). The rate of left ventricular relaxation at the end of reperfusion in vehicle treated hearts was –2067 ± 301 mmHg/sec, which was no different to the rates in control hearts, indicating neither + or – dP/dt are effected by corn oil or oral gavage.

Although functional recovery was dramatically reduced in auranofin treated hearts, there were no differences in the recovery of coronary flow after 45 minutes reperfusion (Fig 9.10 and Table 6.1). Coronary flow recovered to 80 ± 3.8, 84.4 ± 2.8 and 79.9 ± 5.5 % of pre-ischemic rates for control, auranofin treated and vehicle treated hearts, indicating that auranofin does not effect coronary flow rates after ischemia-reperfusion.
Table 6.1: Effect of auranofin on myocardial function in the isolated rat heart, both pre- and post ischemia-reperfusion. Male rats were given 100 mg/kg auranofin by oral gavage 24 hrs prior to sacrifice. Hearts were then isolated and subjected to ischemia-reperfusion. Data is presented as mean ± SEM. (*p<0.001 vs controls) (HR - heart rate, LVDP - left ventricular developed pressure, RPP - rate pressure product, +dP/dt – rate of LV positive pressure development (contraction) over time, -dP/dt – rate of LV negative pressure development (relaxation) over time, Flow - coronary flow, EDP - LV end diastolic pressure).

<table>
<thead>
<tr>
<th>GROUP</th>
<th>Controls</th>
<th>Auranofin</th>
<th>Vehicle</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Body Weight (g)</td>
<td>434.8 ± 17.5</td>
<td>441.3 ± 19.9</td>
<td>418.5 ± 1.5</td>
</tr>
<tr>
<td><strong>Pre-Ischemia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>287 ± 8.5</td>
<td>267.4 ± 5</td>
<td>273 ± 17.7</td>
</tr>
<tr>
<td>LVDP (mmHg)</td>
<td>200 ± 5.6</td>
<td>197.5 ± 13.0</td>
<td>215.1 ± 6.6</td>
</tr>
<tr>
<td>RPP (x10³ mmHg/min)</td>
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<td>56.5 ± 2.6</td>
<td>58.9 ± 4.0</td>
</tr>
<tr>
<td>+dP/dt (mmHg/sec)</td>
<td>3646 ± 206.9</td>
<td>3715 ± 76.0</td>
<td>3826 ± 51.0</td>
</tr>
<tr>
<td>-dP/dt (mmHg/sec)</td>
<td>-3431 ± 157.5</td>
<td>-3484 ± 272.4</td>
<td>-3402 ± 16.0</td>
</tr>
<tr>
<td>Flow (ml/min)</td>
<td>25.1 ± 1.2</td>
<td>25.1 ± 2.0</td>
<td>24.9 ± 1.6</td>
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<tr>
<td><strong>Post-Ischemia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contracture (mmHg)</td>
<td>68.3 ± 4.8</td>
<td>77.3 ± 5.4</td>
<td>66.2 ± 6.2</td>
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<td><strong>45 mins reperfusion</strong></td>
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<tr>
<td>EDP (mmHg)</td>
<td>48.5 ± 3.8</td>
<td>80.6 ± 6.3 #</td>
<td>47.5 ± 3.0</td>
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<td>HR (bpm)</td>
<td>271.5 ± 4.8</td>
<td>233.7 ± 11.8 #</td>
<td>261.4 ± 4.9</td>
</tr>
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<td>LVDP (mmHg)</td>
<td>103.4 ± 5.6</td>
<td>15.1 ± 5.6 #</td>
<td>118 ± 8.7</td>
</tr>
<tr>
<td>RPP (x10³ mmHg/min)</td>
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<td>3.3 ± 1.2 #</td>
<td>29.5 ± 2.1</td>
</tr>
<tr>
<td>% Recovery RPP</td>
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<td>8.8 ± 4.1 #</td>
<td>50.7 ± 5.0</td>
</tr>
<tr>
<td>+dP/dt (mmHg/sec)</td>
<td>1886 ± 87.9</td>
<td>383.7 ± 46.7 #</td>
<td>1896 ± 256.5</td>
</tr>
<tr>
<td>-dP/dt (mmHg/sec)</td>
<td>-1992 ± 138.2</td>
<td>-355.4 ± 66.7 #</td>
<td>-2067 ± 301</td>
</tr>
<tr>
<td>Flow (ml/min)</td>
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<td>21.2 ± 1.3</td>
<td>19.9 ± 1.9</td>
</tr>
<tr>
<td>% Recovery flow</td>
<td>80 ± 3.8</td>
<td>84.4 ± 2.8</td>
<td>79.9 ± 5.5</td>
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</tbody>
</table>
Fig 6.6: Effect of auranofin on contractile function in the isolated rat heart during reperfusion. Male wistar rats were gavaged with auranofin (100 mg/kg body weight) suspended in corn oil 24 hours prior to sacrifice. Hearts were then removed and subjected to 22.5 mins ischemia and 45 mins reperfusion on a Langendorff perfusion apparatus. Rate pressure product (RPP) was measured as opposed to left ventricular developed pressure as hearts were not paced. −10 mins represents pre-ischemic heart function and time 0 mins represents the start of reperfusion after 22.5 mins global ischemia. Values are presented as mean ± SEM, x10³ mmHg/min (*p<0.05, #p<0.001 vs controls).
Fig 6.7: Effect of auranofin on the recovery of rate pressure products (RPP, 6.7A) after 22.5 mins ischemia and 45 mins reperfusion in the Langendorff perfused isolated rat heart. RPPs at the end of reperfusion were also compared to pre-ischemic RPPs to determine the % recovery of contractile function after ischemia-reperfusion (6.7B). Values are presented as mean ± SEM. (#p<0.001 vs controls).
**Fig 6.8:** Effect of auranofin on end diastolic pressure (EDP) after 22.5 mins ischemia and 45 mins reperfusion in the isolated rat heart. Data is presented as mean ± SEM, mmHg. (#p<0.001 vs controls).
**Fig 6.9:** Effect of auranofin on the rate of ventricular contraction after ischemia-reperfusion. Both positive (+dP/dt, 6.9A) and negative (-dP/dt, 6.9B) rates of pressure development (representing the rate of contraction and relaxation respectively) were measured in hearts after 22.5 mins ischemia and 45 mins reperfusion. Data is presented as mean ± SEM, mmHg/sec. (#p<0.001 vs controls).
Fig 6.10: Effect of auranofin on the recovery of coronary flow after 22.5 mins ischemia and 45 mins reperfusion in the Langendorff perfused isolated rat heart. Values are presented as mean ± SEM, % recovery compared to pre-ischemic coronary flow rates.
iii) **OXIDATIVE DAMAGE**

Lipid peroxidation (*Fig 6.11*), measured as 4-HNE and MDA was significantly (p<0.001) increased in auranofin treated hearts (2.9 ± 0.1 nmoles 4-HNE & MDA/mg protein) compared to control hearts (1.8 ± 0.1 nmoles 4-HNE & MDA/mg protein). There were no differences in LPO levels in vehicle treated hearts (1.7 ± 0.2 nmoles 4-HNE & MDA/mg protein) compared to controls.

There were no significant differences in any markers of protein or amino acid oxidation measured between any groups. Oxidised protein carbonyl derivatives (*Fig 6.12*) were similar in all groups, with levels being 101.0 ± 5.0, 103.2 ± 1.8 and 96.4 ± 7.3 units/mg protein in control, auranofin treated and vehicle treated hearts respectively. Auranofin treatment did not affect tyrosine oxidation during ischemia-reperfusion with similar levels of DOPA, di-Tyr and 3-nitrotyrosine as those found in controls. DOPA levels were 1714 ± 13.4 µmol/mol Tyr in auranofin treated hearts versus 1729 ± 71.3 µmol/mol Tyr in control hearts (*Fig 6.13*). There were also no differences in DOPA levels in vehicle treated hearts (1692 ± 24.9 µmol/mol Tyr). Comparable results were observed for di-tyrosine (*Fig 6.14*) post ischemia-reperfusion, with levels being 48.6 ± 4.3, 49.2 ± 3 and 44.2 ± 3.5 µmol/mol Tyr for control, auranofin treated and vehicle treated hearts respectively. 3-nitrotyrosine levels followed the same trend, with no changes in post-ischemic levels between any groups (*Fig 6.15*). 3-nitrotyrosine levels in auranofin treated hearts were 1728 ± 269 µmol/mol Tyr, compared to 1736 ± 164 and 1698 ± 478 µmol/mol Tyr for control and vehicle treated hearts respectively. Levels of o-tyrosine, a marker of phenylalanine oxidation, were equal in all groups following ischemia-reperfusion (*Fig 6.16*). o-Tyr levels were 1898 ± 292.6, 1903 ± 210.1 and 1807 ± 148.0 µmol/mol Tyr in control, auranofin treated and vehicle treated hearts respectively.

These results indicate auranofin treatment does increase oxidative damage generated during ischemia-reperfusion as evidenced by increased LPO, however protein oxidation is unaffected.
Fig 6.11: Effect of auranofin on lipid peroxidation in the isolated rat heart following ischemia-reperfusion. LPO was measured as 4-HNE & MDA in heart extracts following 22.5 mins ischemia ± 45 mins reperfusion. Data is expressed as mean ± SEM, nmoles MDA & 4-HNE/mg protein (#p<0.001 vs controls).
Fig 6.12: Effect of auranofin on the formation of oxidised protein carbonyl derivatives in the heart during ischemia-reperfusion. PCO levels were measured in protein extracts from isolated rat hearts following 22.5 mins ischemia + 45 mins reperfusion. Data is expressed as mean ± SEM, arbitrary units PCO/mg protein.
**Fig 6.13:** Effect of auranofin on the oxidation of tyrosine to DOPA in the heart during ischemia-reperfusion. DOPA levels were measured in protein extracts from isolated rat hearts subjected to 22.5 mins ischemia + 45 mins reperfusion. Data is expressed as mean ± SEM, μmol DOPA/mol Tyr.
**Fig 6.14**: Effect of auranofin on tyrosine oxidation to di-Tyrosine in the isolated heart during ischemia-reperfusion. di-Tyr was isolated and measured from rat hearts following 22.5 mins ischemia ± 45 mins reperfusion. Data is expressed as mean ± SEM, µmol di-Tyr/mol Tyr.
Fig 6.15: Effect of auranofin on tyrosine nitration to the oxidation product 3-nitrotyrosine. 3-nitrotyr levels were measured in protein extracts from isolated rat hearts subjected to 22.5 mins ischemia + 45 mins reperfusion. Data is expressed as mean ± SEM, µmol 3-nitrotyr/mol Tyr.
Fig 6.16: Effect of auranofin on phenylalanine oxidation to o-Tyrosine in the heart during ischemia-reperfusion. o-Tyr levels were measured in protein extracts from isolated rat hearts following 22.5 mins ischemia ± 45 mins reperfusion. Data is expressed as mean ± SEM, µmol o-Tyr/mol Tyr.
iv) **APOPTOSIS**

Apoptotic activity in the heart was measured as caspase-3 activity, a marker of apoptosis common to both the caspase-8 and caspase 6/9 pathways. Caspase-3 activity in heart tissue homogenates was significantly increased (p<0.05) in the auranofin treated group to $2493.0 \pm 502.0$ picomoles AFC/mg protein, compared to $951.9 \pm 139.6$ picomoles AFC units/mg protein in the controls *(Fig 6.17)*. There were no differences in caspase-3 activity between the controls and the vehicle treated group ($951.9 \pm 139.6$ vs $1041.0 \pm 162.3$ picomoles AFC units/mg protein). This indicates a greater level of apoptosis in the auranofin treated hearts, and correlates with their poor recovery from ischemia-reperfusion injury.

![Bar chart showing caspase-3 activity](chart.png)

*Fig 6.17: Effect of auranofin on apoptotic activity in the heart post ischemia-reperfusion. Apoptosis was measured as caspase-3 activation in protein extracts from isolated rat hearts following 22.5 mins ischemia ± 45 mins reperfusion. Data is expressed as mean ± SEM, picomoles AFC/mg protein (*p<0.05 vs controls).*
6.5 DISCUSSION

In previous studies by ourselves and others, it has been demonstrated that decreases in endogenous antioxidant enzyme activity leads to poor functional recovery of cardiac tissue post ischemia reperfusion [277,289,290,319,320,338]. This was shown in Chapter 4 to be due to the decreased capacity of these tissues to counteract the damaging effects of reactive oxygen species generated post reperfusion [361].

In this chapter, auranofin, an inhibitor of glutathione peroxidase and thioredoxin reductase, was used to study the role that these enzymes may play in ischemia-reperfusion injury. Gold containing compounds such as auranofin are potent inhibitors of selenocysteine containing enzymes. These organic gold compounds are frequently prescribed in the treatment of rheumatoid arthritis although their exact mechanism of action is not clear [293]. TxnRed has been implicated in various auto-immune diseases including rheumatoid arthritis, and inhibition of its activity with drugs such as auranofin are therefore often prescribed as treatment for rheumatoid arthritis [237,293,294].

While it is unknown how gold compounds inhibit the activity of selenocysteine containing enzymes, it has been proposed that it is the gold content that is responsible. Gromer et al. examined the effects of the thioglucose moiety that is found in both auranofin and aurothioglucose (ATG), and found it to have no inhibitory effects on TxnRed. In the same study, they also used a gold chelating agent, BAL, and found it was capable of preventing and reversing the inhibition of TxnRed caused by auranofin and ATG [294]. Since selenols have a greater tendency to bind heavy metal ions (such as gold) than thiols, they have speculated that the C-terminal redox active selenocysteine centre of TxnRed is the target of these gold inhibitors [216,294,297,298].

The use of gold containing compounds to selectively down-regulate the activity of TxnRed and GPX in both cells and whole animals has been demonstrated [293-296]. These studies suggest that administration of gold containing compounds such as auranofin have the same effect on selenocysteine containing enzymes as selenium deficiency, and that these compounds may in fact act as selenocysteine antagonists [296]. In a study by Gromer et al., they effectively knocked out the placental TxnRed system, reducing the activity of TxnRed by 90%. This was achieved with only 100nM auranofin.
Increasing the concentration of auranofin 1000-fold to 100µM results in inhibition of GPX and a complete down-regulation of TxnRed [294]. Similar results have been found in mice with injection of 0.025mg aurothioglucose/g bodyweight, which caused sustained inhibition of TxnRed activity in tissue extracts of heart, liver, kidney and pancreas. They found no significant decrease in GPX activity in the heart, although 2 hours after the injection of 0.2mg/g bodyweight there was a 25% decrease in GPX activity [293]. These results are also supported in a previous study by Hu and colleagues in rats [295].

This study used auranofin at a dose of 100mg/kg of bodyweight and found it significantly decreased the systemic level of thioredoxin reductase activity. This was also observed in heart tissues during normoxic perfusion, where auranofin significantly depressed TxnRed activity. There was no significant change in the endogenous activity of glutathione peroxidase in either heart or liver tissues. Using this dose of auranofin (100mg/kg), it was possible to selectively alter TxnRed activity due to the differential responses of these two enzymes. GPX is inhibited by auranofin with a Ki of 11.7uM, whereas TxnRed has a Ki of 4nM [294,360]. In pilot experiments, it was found that using a higher dose of auranofin depressed the endogenous activity of both enzymes whereas less auranofin did not adequately suppress the activity of TxnRed.

In this chapter, a change in thioredoxin reductase activity following ischemia-reperfusion was observed. The baseline level of TxnRed activity in perfused hearts was 53.8 ± 7 moles/min/mg of protein, which decreased to 18.7 ± 4 moles/min/mg protein following administration of auranofin. However, when all hearts were subjected to a period of ischemia and reperfusion, the endogenous activity of TxnRed rose significantly to over 100 moles/min/mg. This increase was further investigated in Chapter 3, where a 70% increase in thioredoxin reductase-1 mRNA expression and a 200 % increase in thioredoxin reductase-2 expression post ischemia-reperfusion was observed [341]. This explains the result observed in this chapter where it would appear that auranofin treated hearts subjected to ischemia-reperfusion do not display significantly reduced levels of TxnRed. This is because there is a dramatic increase in TxnRed expression at this time overcoming the effect of the drug.

Thioredoxin reductase displays a broad specificity and plays an important antioxidant role, not only by supplying reducing equivalents to the
thioredoxin/thioredoxin peroxidase systems but also in directly reducing H2O2 and lipid peroxides as well as protein disulfides including oxidized glutathione [194,201,210,211,213,221]. Furthermore thioredoxin reductase is involved in recycling the antioxidant vitamins C and E. Vitamin C reacts directly with ROS such as superoxide and peroxides, as well as recycling oxidized Vitamin E, but in the process becomes oxidized to dihydroascorbate. Dihydroascorbate is a substrate of thioredoxin reductase leading to the regeneration of ascorbic acid [194,201,210,211,213,221]. Therefore thioredoxin reductase is critical in defending against oxidative injury by shifting electrons from NADPH and providing reducing power to both protein and non-protein based antioxidants.

Although the dose of auranofin used did not affect GPX activity, there was an increase in GPX activity post ischemia-reperfusion. This was similar to the results obtained in Chapter 3 where an increase in Gpx-1 and Gpx-4 expression was also observed [341]. This increase in GPX and TxnRed activity following myocardial ischemia-reperfusion may be an attempt to combat increased ROS generation and subsequent oxidative stress associated with ischemia-reperfusion.

Superoxide dismutase activity was not effected by auranofin (100 mg/kg), with no changes in activity between groups following normoxic perfusion or ischemia-reperfusion. This was however expected as SOD does not contain selenocysteine in its active site. There were also no differences in SOD activity during normoxic perfusion when compared to activity post ischemia-reperfusion. This is also supported by Chapter 3, which showed no differences in SOD activity during ischemia or reperfusion. Taken together, it can be concluded SOD activity is not effected by auranofin, ischemia-reperfusion or changes in antioxidant status.

There were significant changes in the ability of cardiac tissue to recover from ischemia-reperfusion following administration of 100mg/kg auranofin. This was indicated by significantly lower rates of pressure development (+ and –dP/dt) and significantly higher EDPs after 45 minutes reperfusion. This was associated with a significant decrease in RPPs for auranofin treated hearts at all time points time points measured during reperfusion, and significantly lower % recovery of RPP. RPP recovered to less than 10% of pre-ischemic levels in auranofin treated hearts versus 50% in both the
control and vehicle treated groups. An ischemic period of 22.5 minutes was used, which under our experimental conditions results in approximately 50% recovery of RPP allowing for increases and decreases in recovery to be observed. Under these conditions, our results clearly indicate that auranofin negatively impacts on the ability of cardiac tissue to recovery from ischemic insult.

In an attempt to investigate the molecular mechanism underlying this change in functional recovery, indicators of myocardial oxidative damage and apoptosis were examined in hearts. Tissue levels of caspase-3 post-ischemia reperfusion were found to be substantially elevated in the auranofin treated group. This observation suggests auranofin is directly pro-apoptotic or counters the effects of endogenous anti-apoptotic processes (such as antioxidant enzyme systems). Lipid peroxidation was found to be significantly increased in auranofin treated hearts, although there were no changes in any markers of protein oxidation. The up-regulation of TxnRed in hearts following ischemia-reperfusion may have protected the heart from protein oxidation. TxnRed is known to regenerate proteins and other antioxidants such as ascorbic acid and glutathione, thereby increasing the available antioxidant pool, all of which may potentially limit protein oxidation [201,210,213,221]. Given that there were no differences in TxnRed activity between any treatments post ischemia-reperfusion, it is likely this is the reason for equal levels of protein oxidation in all groups.

Several reports have now confirmed that apoptosis is initiated by oxidative stress, and apoptosis in cardiomyocytes is induced during ischemia-reperfusion, contributing to poor cardiac recovery [82,133-136,139,140,362]. Several mechanisms have been suggested to explain this observation including the release of pro-apoptotic factors from neutrophils and macrophages during reperfusion [362]. Since a cell free model was used in these studies, it is probable intrinsic activators of apoptosis are involved as opposed to extrinsic processes.

Changes in intracellular oxygen availability can radically affect the way in which mitochondria function. Periods of ischemia or hypoxia followed by re-oxygenation have been shown to generate superoxide and other free radicals, which are damaging to normal cellular function and structure [40,101,102,111,114-117,157,317]. The production of H$_2$O$_2$ within the mitochondria poses a second threat, with H$_2$O$_2$ being able to diffuse into
the mitochondrial membrane where it abstracts electrons from Fe$^{2+}$ centered electron transporters. This results in the production of hydroxyl radicals (OH$^-$), which initiates lipid peroxidation within the mitochondrial membrane [115,117,317]. The production of hydroxyl radicals within the mitochondrial membrane, and the subsequent oxidation of membrane lipids may therefore explain the significant increase in lipid peroxidation observed in auranofin treated hearts. Oxidised lipids from cell membranes, especially hydroperoxyeicosatetraenoic acids (HPETEs), are also capable of directly inducing apoptosis, therefore the increase in LPO seen in auranofin treated hearts may contribute to the increased apoptotic activity observed in these hearts. In addition to this, loss of integrity of the mitochondrial membrane results in leakage of cytochrome c and the initiation of apoptosis [135,136,140,362].

The mitochondrial permeability transition pore (MPTP) is a non-specific pore in the inner membrane permeant to any molecule <1.5 kDa, that opens in response to increased Ca$^{2+}$, especially at times of high oxidative stress [141,144]. Opening of the MPTP causes massive swelling of mitochondria, rupture of the outer membrane and release of intermembrane components that induce apoptosis. In addition, mitochondria become depolarized causing inhibition of oxidative phosphorylation and loss of ATP production [141,144]. The MPTP and its role in ischemia-reperfusion has been the subject of considerable research, since the conditions required for opening of the MPTP in isolated mitochondria are those that occur upon reperfusion following ischemia (i.e. high [Ca$^{2+}$], ATP depletion, increased P$_i$ and oxidative stress [171,142,144,148,363]. Halestrap and colleagues have proven this correct, by showing the MPTP does not open during ischemia but opens between 2 and 5 minutes after the onset of reperfusion, and they were also able to show that myocardial recovery post ischemia-reperfusion correlated well with the amount of MPTP opening [146,148,363,364].

Ischemia-reperfusion and the accompanying oxidative stress that cause these changes in the MPTP also lead to a loss of cytochrome c and the induction of apoptosis [82,140,141,148,362,363]. Interestingly, auranofin has also been shown to induce the opening of the MPTP, resulting in mitochondrial swelling and loss of membrane potential [365]. Such a process could explain the enhanced caspase-3 activity observed in auranofin treated hearts in this study, and lead to the impaired contractile recovery seen
in these hearts. Inhibition of thioredoxin with various compounds such as AW 464 and diamide has also been shown previously to induce apoptosis without increasing oxidative stress [366]. Inhibition of Txn with these compounds was found to induce Bak activation, cytochrome c release, decrease mitochondrial membrane potentials, cause chromatin condensation as well as DNA nicks, all of which are known components of apoptotic cascades [366]. Reduced Txn also binds to apoptosis signal-regulating kinase 1 (ASK1), which is an activator of the c-Jun N-terminal kinase (JNK) and p36 MAP kinase pathways, and is also required for TNF-α induced apoptosis. However Txn binds to the N-terminal portion of ASK1, inhibiting its activity and therefore preventing downstream apoptotic signaling [210,213,367]. Whether auranofin inhibits Txn along with TxnRed remains to be investigated, however if it does inhibit Txn either directly or via the inhibition of TxnRed, this may be another cause of the increased caspase-3 activity in auranofin treated hearts.

Given that auranofin is prescribed as an anti-rheumatic drug, often to older patients at risk of cardiovascular disease, this chapter suggests these patients would recover poorly from an ischemic insult such as myocardial infarction. This may also be particularly important in clinical settings, where such patients may be exposed to higher levels of oxidative stress following cardiac surgery.
LIMITATIONS

The main experimental limitations in this chapter have been discussed in previous chapters, including limitations for the Langendorff isolated heart preparation (i.e. it is \textit{ex vivo}) and the absence of blood borne elements. However, once again this allows us to study the direct effects upon the heart during ischemia-reperfusion.

Auranofin was suspended in corn oil due its insoluble nature. Auranofin is not soluble in any of the typical vehicles used for drug administration, including water, PBS, saline or ethanol. It is however soluble in DMSO which we have used in previous cellular studies. However, intraperitoneal injection of this solution in rats was lethal, and they did not respond well to receiving this solution via oral gavage either. Although auranofin was not soluble in corn oil, it was possible to administer the drug suspended in corn oil using an oral dosing needle.

Although patients that are prescribed auranofin for the treatment of rheumatoid arthritis may be on the drug for extended periods of time, it was deliberately chosen to only study the immediate or acute effects of the drug. This is because the aim of the study was to knock out TxnRed activity without effecting GPX, to investigate its relative role in myocardial ischemia-reperfusion injury, and this was achieved using 100 mg auranofin/kg bodyweight administered 24 hours prior to sacrifice.

Given the changes in expression of various components of the TxnRed system in response to ischemia, reperfusion and dietary selenium levels, it would have also been interesting to measure thioredoxin levels in these hearts to see if auranofin down-regulates other proteins within the system. This would have been particularly interesting given Txn’s role in inhibition of apoptosis, and although measurement of Txn was attempted, once again the monoclonal antibody was raised against human Txn and did not recognize rat Txn.
CONCLUSIONS

This chapter has shown auranofin can be used to selectively inhibit the *in vivo* activity of thioredoxin reductase whilst leaving the activity of glutathione peroxidase unaltered. This was shown by decreased TxnRed activity in liver and normoxic heart extracts using auranofin at a dose of 100 mg/kg bodyweight.

Hearts depleted of thioredoxin reductase activity display impaired recovery from ischemia-reperfusion injury with lower contractile function and greater diastolic dysfunction at the end of reperfusion. This poor functional recovery correlated to elevated levels of lipid peroxidation and apoptotic activity, which may have contributed to their reduced recovery. These results also suggest that auranofin may effect mitochondrial function along with TxnRed activity, possibly by opening the mitochondrial permeability transition pore [146]. Since TxnReds are the only class of enzymes known to reduce Txn, it is possible that alterations in TxnRed activity may also regulate some of the activities of Txn including the inhibition of apoptosis [211].

This chapter shows that whilst both antioxidants are important in preventing oxidative stress, TxnRed activity is critical for recovery from reperfusion injury. It also suggests that antioxidant defences in patients being treated with auranofin may infact be impaired, which could potentially be detrimental at times of high oxidative stress. This is particularly important for older patients who are more prone to ischemic heart disease and myocardial infarction, especially if antioxidant levels are already reduced as a result of the aging process.
PUBLICATIONS ARISING FROM THIS CHAPTER

CHAPTER 7

The effect of ageing on expression & activity of myocardial antioxidant enzyme systems, and oxidative damage.
It is well established that ageing is associated with an increased cardiac sensitivity to oxidative stress, however the exact mechanisms responsible for this deterioration are yet to be elucidated. However, there is much evidence indicating most age-related changes are linked to reactive oxygen species generation during aerobic metabolism. Previous studies by ourselves and others on young hearts have shown up-regulation of TxnRed and GPX systems to be cardioprotective, and inhibition of these antioxidant systems to be detrimental during ischemia-reperfusion. Given these findings, and the increased susceptibility to oxidative stress in ageing, this chapter examined the effect of ageing on cardiac expression and activity of the TxnRed and GPX systems, and levels of naturally occurring oxidative damage. Hearts from male Wistar rats at 10 weeks (young) and 15 months (aged) of age were isolated for biochemical analysis. GPX activity was found to be significantly reduced in aged hearts when compared to young hearts, however there were no differences in Gpx-1, Gpx-4 or Gsr gene expression. TxnRed activity was only marginally reduced, whilst Txnrd-1, Txnrd-2, Txn and Prdx-2 gene expression was significantly increased. No differences in SOD activity were observed with aging. These results show a reduced activity to transcription ratio occurs with ageing, which suggests possible translational problems, reduced selenocysteine incorporation or increased protein turnover / degradation occurs with ageing. These results were also associated with significantly higher levels of di-Tyr and o-Tyr in hearts from aged animals. Although there were no changes in lipid peroxidation or protein carbonyls in these hearts, these results do suggest naturally occurring oxidative stress is increased in aged hearts. This indicates aged hearts would not recover well from an oxidative insult such as ischemia-reperfusion, as has been shown previously by others. Dietary selenium supplementation may therefore provide a safe and convenient method for increasing antioxidant protection in aged individuals, particularly those at risk of ischemic heart disease or those expecting to undergo clinical procedures involving transient periods of hypoxia.
7.2 **INTRODUCTION**

In humans, myocardial ageing appears to be associated with both an increase in the incidence and a worsening of the consequences of cardiovascular diseases, with numerous studies reporting increased mortality following myocardial infarction in aged patients [368-370]. Ageing (> 70 years) has been identified as a significant risk factor for mortality during cardiac surgery in coronary artery bypass in humans, whilst Ataka *et al* has shown aged rabbit hearts exhibit less functional recovery during reperfusion than young hearts [371,372].

Although it has been well established that ageing is associated with a major increase in sensitivity to pathophysiological situations involving an oxidative component, the exact mechanisms responsible for this deterioration have not been fully elucidated [373-375]. Several studies have shown that biological and morphological changes that occur in old rats are associated with increased susceptibility to myocardial ischemia-reperfusion, and there is increasing evidence linking this to an overproduction of ROS which overwhelms the natural defences against these species [368,375-380].

Given that down-regulation of the TxnRed and/or GPX systems has previously been shown to reduce recovery from myocardial ischemia-reperfusion injury in young animals [277,289,290,319,320,338,361,381], it is possible that the increased sensitivity to oxidative insult in aged hearts arises from reduced antioxidant status. This hypothesis is further supported by decreased selenium status [382] and increased cytosolic density during ageing, which could represent a basis for down-regulation of cellular enzymes involved in ROS elimination [368,383,384]. Given that we have also previously shown that ischemia and reperfusion alone regulate the TxnRed and GPX systems, any changes in their expression caused by ageing may potentially impact on the heart’s ability to cope during such insults [341]. Therefore, the aim of this chapter was to examine the expression and activity of these antioxidant systems in young and aged hearts, and measure the levels of naturally occurring oxidative stress in these hearts. This will then provide more information regarding the vulnerability of aged hearts to ROS, which are suspected to be involved in the pathogenesis of ischemia-reperfusion.
7.3 MATERIALS AND METHODS

i) EXPERIMENTAL GROUPS

Adult male wistar rats, as described in Section 2.2 of Chapter 2 (General Methods), were randomly assigned to one of 2 age groups: young rats, 10 weeks of age (n= 6) and aged rats, 15 months of age (n=6). All rats were fed standard rat pellets, and they had free access to food and water. Animals were anaesthetised and hearts removed as described in Chapter 2 (Section 2.4, General Methods). Hearts were then flushed quickly in ice-cold saline, weighed and immediately frozen at -80°C until subsequent analysis.

Most of the experimental approaches to cardiovascular aging have been performed in the rat on the basis that the modifications to its cardiovascular system during the 30-36 months of its normal life span are comparable to the effects of cardiovascular ageing in humans [1]. Generally, 2-4-month-old male wistar rats are considered as young mature rats, 15-month-old rats as old rats and 24-month-old rats as senescent [1,9]. Given that cardiovascular disease is increased in aged patients as well as senescent patients, we chose to study old rats as opposed to senescent rats, to investigate changes that may occur earlier on in the aging process which may contribute to this increased risk.

ii) BIOCHEMICAL ANALYSIS

Protein was extracted from hearts as described in Section 2.5 i of Chapter 2 (General Methods), and protein estimations performed on extracts as detailed in Section 2.5 ii of Chapter 2.

Activity of the antioxidant enzymes, glutathione peroxidase (GPX), thioredoxin reductase (TxnRed) and superoxide dismutase (SOD) were measured in protein extracts as described in Section 2.6 (methods i, ii and iii) of Chapter 2.
To assess the level of oxidative stress within these tissues, lipid peroxidation was measured in heart extracts along with protein carbonyls and the tyrosine oxidation products DOPA, di-Tyr, o-Tyr and 3-nitrotyrosine. These methods are explained in detail in Section 2.6 (methods v, vi, and vii) of Chapter 2 (General Methods).

iii) GENE EXPRESSION ANALYSIS

RNA extraction, quantitation and determination were performed on heart samples according to methods iii and iv in Section 2.5 of Chapter 2 (General Methods).

The mRNA expression of the glutathione related and thioredoxin related genes was measured by firstly synthesising cDNA from total RNA, then performing quantitative real-time PCR with primers for Gpx-1, Gpx-4, Gsr, Txnrd-1, Txnrd-2, Txn and Prdx-2. These methods are explained in full in Section 2.7 of Chapter 2 (General Methods). Gene expression changes in aged hearts are expressed as a % relative to young hearts.

iv) DATA ANALYSIS

All data was analysed using two-tailed unpaired student t-test’s as described in Section 2.9 of Chapter 2 (General Methods).
7.4 RESULTS

i) ANTIOXIDANT mRNA EXPRESSION

Quantitative real-time PCR was used to measure the myocardial expression of antioxidant proteins in the GPX and TxnRed systems in response to ageing. However, due to the considerably lower expression of Gpx-4, a greater amount of primer was needed for PCR amplification. As discussed previously, increasing the primer concentration (to 400nM) ensured the signal-to-noise ratio was sufficient to confidently measure Gpx-4 expression. Using this primer concentration also causes the T<sub>m</sub> of the PCR amplicon to increase by 3°C, which supported our melt curve analysis for Gpx-4 which showed a T<sub>m</sub> of 84°C, 3°C higher than the predicted T<sub>m</sub> of 81°C. Although a greater amount of primer was used, no primer dimer was produced in any samples, and the non-template control was clear of any products in both the amplification plot and the melt curve.

Expression of Thioredoxin Related Genes

The mRNA expression of thioredoxin related genes (Txnrd-1, Txnrd-2, Prdx-2 and Txn) in aged (15 month) hearts is expressed relative to expression in young (10 week) hearts, which was set as the baseline (or 100 %) expression in rat hearts. Expression of thioredoxin related genes was found to be up-regulated in aged hearts when compared to the expression in young rat hearts, with higher transcription levels of Txnrd-1, Txnrd-2, Txn and Prdx-2.

When compared to young (10 week) hearts, expression of Txnrd-1 was slightly (~20%) higher in aged (15 month) hearts (Fig 7.1). Txnrd-1 expression was 122 ± 21.7 % in aged hearts relative to expression in young hearts (100 ± 27 %). Expression of Txnrd-2, or mitochondrial TxnRed, was significantly (p<0.001) increased by ~560 % in aged hearts (Fig 7.2). Txnrd-2 expression was 658 ± 48 % compared to 10 week hearts (100 ±
18.9 %). Ageing significantly (p<0.01) increased Prdx-2 transcription in the rat heart by 
~250 % to 355 ± 37.7 % when compared to young hearts (Fig 7.3). Prdx-2 expression in 10 week hearts was 100 ± 17.2 %. Txn mRNA expression (Fig 7.4) was 157 ± 7.9 % in 15 month aged hearts. This was significantly (p<0.001) ~60 % higher than Txn expression in young 10 week hearts (100 ± 3.3%).

**Fig 7.1:** Effect of ageing on myocardial transcription levels of thioredoxin reductase-1 (Txnrd-1) in the rat. Values are presented as mean ± SEM. % expression is relative to 10 week (young) hearts, with 10 week expression set at 100%.
**Fig 7.2:** Effect of ageing on thioredoxin reductase-2 (Txnrd-2) expression in the rat heart. Values are presented as mean ± SEM. % expression is relative to 10 week (young) hearts, with 10 week expression set at 100%. (# p<0.001).

**Fig 7.3:** Effect of ageing on rat myocardial mRNA levels of thioredoxin peroxidase-2, (or peroxiredoxin-2, Prdx-2). Values are presented as mean ± SEM. % expression is relative to 10 week (young) hearts, with 10 week expression set at 100%. (** p<0.01).
Fig 7.4: Effect of ageing on thioredoxin (Txn) transcription levels in the rat heart. Values are presented as mean ± SEM. % expression is relative to 10 week (young) hearts, with 10 week expression set at 100%. (# p<0.001).
Expression of Glutathione Related Genes

The mRNA transcription of glutathione related genes (Gpx-1, Gpx-4 and Gsr) in aged hearts is also expressed relative to the expression in young hearts, which was set as the baseline (or 100%) expression in rat hearts.

Unlike the TxnRed system, expression of glutathione related genes in the rat heart did not change significantly during ageing, with similar levels of Gpx-1, Gpx-4 and Gsr expression in 15 month aged hearts compared to young 10 week hearts.

There was a slight (10%) increase in Gpx-1 expression (Fig 7.5) in aged hearts (110 ± 15.4%) relative to young hearts (100 ± 30%). Myocardial Gpx-4 (or phospholipid hydroperoxidase) transcription was up-regulated ~25% in 15 month aged hearts to 124 ± 18.9% relative to 10 week hearts (100 ± 20%). These results are shown in Fig 7.6. Glutathione reductase mRNA transcription was not affected by ageing (Fig 7.7). There were no differences observed in myocardial Gsr expression in 15 month aged hearts compared to young (10 week) hearts (100 ± 33 vs 100 ± 18.7% respectively).
**Fig 7.5:** Effect of ageing on myocardial transcription levels of glutathione peroxidase-1 (Gpx-1) in the rat. Values are presented as mean ± SEM. % expression is relative to 10 week (young) hearts, with 10 week expression set at 100%.

**Fig 7.6:** Effect of ageing on myocardial mRNA levels of glutathione peroxidase-4 (or phospholipid hydroperoxidase, Gpx-4) in the rat. Values are presented as mean ± SEM. % expression is relative to 10 week (young) hearts, with 10 week expression set at 100%.
Fig 7.7: Effect of ageing on glutathione reductase (Gsr) expression in the heart. Values are presented as mean ± SEM. % expression is relative to 10 week (young) hearts, with 10 week expression set at 100%.
ii) ANTIOXIDANT ENZYME ACTIVITIES

Thioredoxin reductase activity in the rat heart was relatively unaffected by ageing (Fig 7.8), with activity being $90.5 \pm 4$ moles/min/mg protein in 15 month aged hearts. This was slightly lower than TxnRed activity in young (10 week) hearts, which was $96 \pm 5.1$ moles/min/mg protein.

Glutathione peroxidase activity (Fig 7.9) in aged hearts was significantly ($p<0.05$) lower than the activity in young hearts. GPX activity was $256.4 \pm 36$ mmoles/min/mg protein in 15 month aged hearts compared to $416.5 \pm 5.1$ mmoles/min/mg protein in 10 week hearts.

There was no real change in superoxide dismutase activity in rat hearts with ageing compared to young hearts (Fig 7.10). SOD activity was $36 \pm 2.2$ units/mg protein in 15 month aged hearts, which was only slightly lower than the activity in young (10 week) hearts ($40.9 \pm 3.8$ units SOD/mg protein).
Fig 7.8: Effect of ageing on thioredoxin reductase activity in the rat heart. TxnRed activity was measured in heart extracts from young (10 week) and aged (15 month) rats. Values are presented as mean ± SEM, moles TxnRed/min/mg protein.
Fig 7.9: Effect of ageing on myocardial glutathione peroxidase activity in the rat. GPX activity was measured in heart extracts from young (10 week) and aged (15 month) rats. Values are presented as mean ± SEM, mmoles GPX/min/mg protein. (* p<0.05).
Fig 7.10: Effect of ageing on superoxide dismutase activity in the rat heart. SOD activity was measured in heart extracts from young (10 week) and aged (15 month) rats. Values are presented as mean ± SEM, units SOD/mg protein.
iii) OXIDATIVE DAMAGE

Lipid peroxidation (Fig 7.11), measured as 4-HNE and MDA, was not affected by ageing, with similar levels of LPO observed in both young (10 week) and 15 month aged rat hearts. LPO in aged hearts was $2.94 \pm 0.2$ vs $2.93 \pm 0.2$ nmoles MDA & 4-HNE/mg protein in 10 week hearts.

Similar results were observed for oxidised protein carbonyls, which were also unaffected by ageing (Fig 7.12). PCO levels were $4.26 \pm 0.3$ units/mg protein in young hearts, which was only slightly lower than that in aged hearts ($4.66 \pm 0.2$ units/mg protein).

Amino acid oxidation however does appear to be affected by ageing, with differences detected in levels of DOPA, di-Tyr and o-Tyr in rat hearts. DOPA, a product of tyrosine oxidation by hydroxyl radicals, was significantly ($p<0.001$) lower in aged hearts ($932.9 \pm 34.7 \mu\text{mol/mol Tyr}$) than in young hearts ($1293 \pm 59 \mu\text{mol/mol Tyr}$, Fig 7.13). di-Tyr and o-Tyr however were both significantly increased in aged hearts. di-Tyr levels were significantly ($p<0.05$) higher in 15 month rat hearts ($206.7 \pm 21 \mu\text{mol/mol Tyr}$) compared to $126.7 \pm 11 \mu\text{mol/mol Tyr}$ in 10 week rat hearts (Fig 7.14). Similar results were observed for o-Tyr, an oxidised phenylalanine product (Fig 7.15). o-Tyr levels were $759.3 \pm 40.9 \mu\text{mol/mol Tyr}$ in young hearts, which was significantly ($p<0.05$) lower than levels in 15 month aged hearts ($1012 \pm 84.2 \mu\text{mol/mol Tyr}$). Levels of 3-nitrotyrosine were relatively unaffected by ageing with similar levels observed in both aged and young rat hearts (Fig 7.16). 3-nitrotyrosine levels were $1004 \pm 130 \mu\text{mol/mol Tyr}$ in 15 month hearts versus $1078 \pm 83.5 \mu\text{mol/mol Tyr}$ in 10 week hearts.
Fig 7.11: Effect of ageing on lipid peroxidation in the rat heart. LPO was measured as 4-HNE & MDA in heart extracts from young (10 week) and aged (15 month) rats. Data is expressed as mean ± SEM, nmoles MDA & 4-HNE/mg protein.

Fig 7.12: Effect of ageing on the formation of oxidised protein carbonyl derivatives in the heart. PCO levels were measured in protein extracts from young (10 week) and aged (15 month) rat hearts. Data is expressed as mean ± SEM, arbitrary units PCO/mg protein.
**Fig 7.13:** Effect of ageing on the oxidation of tyrosine to DOPA in the heart. DOPA levels were measured in protein extracts from young (10 week) and aged (15 month) rat hearts. Data is expressed as mean ± SEM, μmol DOPA/mol Tyr (#p<0.001).

**Fig 7.14:** Effect of ageing on tyrosine oxidation to di-Tyrosine in the rat heart. di-Tyr was isolated and measured from young (10 week) and aged (15 month) rat hearts. Data is expressed as mean ± SEM, μmol di-Tyr/mol Tyr (*p<0.05).
**Fig 7.15:** Effect of ageing on phenylalanine oxidation to o-Tyrosine in the rat heart. o-Tyr levels were measured in protein extracts from young (10 week) and aged (15 month) rat hearts. Data is expressed as mean ± SEM, µmol o-Tyr/mol Tyr (*p < 0.05).

**Fig 7.16:** Effect of ageing on tyrosine nitration to the oxidation product 3-nitrotyrosine. 3-nitrotyr levels were measured in protein extracts from young (10 week) and aged (15 month) rat hearts. Data is expressed as mean ± SEM, µmol 3-nitrotyr/mol Tyr.
Nearly every organ and tissue of the body undergoes age-related restructuring, which leads to functional changes [385-387]. More specifically, these age-related changes have been associated with extensive alterations in myocardial biochemistry, structure and function [375,388]. It has been well reported that the incidence and mortality of cardiovascular diseases are significantly higher in the elderly than in young adults [368-371,385,389-391]. In addition to this, it is also well known that the number of people over the age of 65 years will increase dramatically this century [385,390,392]. Taken together, these justify the importance of investigating why the elderly exhibit a higher incidence of these diseases and are more susceptible to oxidative stress. Moreover, the study of age-related disease must include the investigation of both baseline and diseased states if the cardiovascular health of an ageing population is to be improved.

Cardiovascular ageing is a complex event that is likely to include alterations in the expression or regulation of genes, particularly those genes involved in cellular injury prevention and/or cellular repair processes. These genetic alterations could then in turn lead to decreased injury threshold [385,386,390]. It is possible the changes in gene expression associated with ageing may result from, or be the cause of, the imbalances in redox status which have also been linked to ageing [393].

Oxidative stress is said to occur when there is an increased generation of ROS which overwhelms the normal cellular defence mechanisms [20]. It causes wide-ranging damage to macromolecules, and has been linked to various chronic degenerative disease processes including vascular disease [393-397]. Besides their role in the development of cellular alterations, ROS are widely suspected to be involved in ischemia-reperfusion injury [21,40,102]. Protein oxidation results in loss of enzyme activity, reduced energy production, mechanical dysfunction and altered calcium transport in the sarcoplasmic reticulum, all of which contribute to myocardial dysfunction [21,40,102]. Lipid peroxidation of myocardial cell membranes has also been implicated as a potential cause of ventricular fibrillation, tachycardia, premature beating and arrhythmias, which are all associated with reperfusion [40,108,116-118]. Controlling these damaging processes has been the subject of considerable research in recent years, as the degree of ROS generation
and biological oxidation has been correlated with poor functional cardiac recovery post ischemia-reperfusion [21,40,102,118]. Any changes in ROS generation or antioxidant status that occur during ageing may therefore impact on the heart’s ability to cope at times of increased stress, or may even contribute to the increased oxidative stress associated with ageing.

It is possible that the increased sensitivity to oxidative insult in aged hearts arises from reduced antioxidant status. It has been demonstrated that the susceptibility of tissues to undergo protein oxidation in response to experimentally induced acute oxidative stress increases with age [398]. Furthermore, it has been demonstrated that infusion of hydrogen peroxide results in significantly larger impairment of mechanical parameters in aged hearts compared to younger hearts [399]. This hypothesis is further supported by decreased selenium status [382] and increased cytosolic density during ageing, which could represent a basis for down-regulation of cellular enzymes involved in ROS elimination [368,383,384]. Given that we have also previously shown that ischemia and reperfusion alone regulate the TxnRed and GPX systems, any changes in their expression caused by ageing may potentially impact on the heart’s ability to cope during such insults [341].

This chapter found significantly lower GPX activities in aged rat hearts when compared to younger hearts without significant changes in GPX mRNA expression. Furthermore, mRNA expression of the TxnRed system was found to be significantly increased without any changes in TxnRed activity. These results indicate that the ratio of antioxidant enzyme activity to transcription decreases in aged hearts. This may be due to a number of reasons, including inefficient or ineffective translation, mRNA instability, increased protein turnover, or increased protein degradation.

Translational machinery may not be as effective in aged hearts due to age-related damage accumulated over the years. Another possibility is related to the selenium status of these tissues. It has also been previously shown that selenium status decreases with ageing [382], therefore it is possible that there is an inadequate supply of selenium in aged tissues which limits the effectiveness of translational machinery. Without sufficient selenium there is an increased turnover of Sec-tRNA^{Sec}, and UGA is interpreted as a stop codon thereby limiting selenoprotein translation [265-268]. This may particularly
explain the changes seen in the TxnRed system, where Txnrd-1 expression was increased ~20 % and Txnrd-2 ~560 % in aged hearts, without changes in TxnRed activity. When Sec-tRNA→Sec concentrations are low and protein synthesis is thereby limited, ribosomal pausing occurs which leads to competition between termination factors and the limited supply of Sec-tRNA→Sec. This results in the early termination of selenoproteins, and the idle mRNA can then be subjected to more rapid degradation via normal decapping and exonuclease hydrolysis [266]. Therefore, it is also possible that Txnrd mRNA stability is decreased or its degradation increased in aged hearts, which would also account for the large increases in mRNA expression without changes in activity. Whether translation of these selenoproteins is somehow being blocked, terminated earlier and/or degraded quicker (possibly due to low selenium levels) requires further investigation, and this may be relevant to numerous proteins that contribute to, or are altered by ageing.

Lower selenium levels may also lead to decreased levels of selenocysteine, and therefore reduced Sec incorporation. The generation of selenocysteine occurs during amino acid biosynthesis, and the degree of selenium incorporation into the cysteine residue is concentration dependent [255-257,264]. The incorporation of Sec into GPX and TxnRed during translation is critical for their activity [255-257,264]. Therefore, if Sec levels are low, reduced incorporation into these selenoprotein active sites will lead to inactive enzymes. This could explain the lower activity to transcription ratios for GPX and TxnRed observed in aged hearts, and furthermore, this would not affect SOD activity in aged hearts, as was also observed in this chapter.

Existing data on the status of antioxidants during ageing is somewhat conflicting, suggesting that age-related changes in antioxidants may be tissue and/or species specific. For instance, it has been reported by Rikans et al that TxnRed activity in the livers of male and female rats is unchanged with ageing, but data by Santa Maria and Machado show decreased activity of TxnRed in the kidney and lung [400-402]. The latter report is also in agreement with another study by Cho et al, which found age-related decreases in renal TxnRed, Txn and glutathione protein levels, along with decreased GPX, glutathione reductase and glutathione S-transferase activities [393]. Data on glutathione and GPX status during ageing is also conflicting. Age-related decreases in glutathione have been reported in the rat brain and mouse liver, but others also report no changes in glutathione.
content occurs in rats during ageing [403-405]. A study by Boucher and colleagues found decreased catalase activity in 16 month aged rat hearts post ischemia-reperfusion compared to 4 month hearts, but no differences in SOD activity or GPX activity [375]. While their SOD data supports findings in this chapter, their GPX data conflicts the results observed in the current study. These differences may arise because the hearts assayed by Boucher et al had previously been subjected to ischemia-reperfusion, and we have shown previously that ischemia-reperfusion up-regulated the GPX system.

This same study by Boucher and colleagues also reported no changes in LPO during ischemia-reperfusion in 16 month aged hearts compared to younger hearts [375]. Although the hearts used in this chapter were not oxidatively stressed, the baseline LPO levels that occur naturally during the ageing process do support their study. Although no changes in baseline LPO, PCO, or 3-nitrotyrosine were found in aged hearts, increases in di-Tyr and o-Tyr were found in aged hearts. These results indicate that aged hearts are more susceptible to oxidative stress, which may be due to an increased production of ROS and/or decreased antioxidant status.

These results are supported by several studies which demonstrate that ageing cells accumulate oxidative damage [406,407]. Because of the presence of oxidative metabolism, mitochondria accumulate age-related damage, which in turn release more ROS exacerbating the process [406,407]. This process may be particularly relevant to cells such as cardiomyocytes, which do not proliferate or regenerate themselves. It is also possible that this process may lead to increased protein degradation in aged cells, which may contribute to the reduced activity to transcription ratios observed for TxnRed and GPX. The results of this chapter, along with these processes, also supports previous reports of increased sensitivity of aged hearts to hydrogen peroxide, and increased susceptibility of aged tissues to undergo protein oxidation in response to experimentally induced oxidative stress [398,399].

Increased mRNA expression of the TxnRed system in aged hearts may account for the lower levels of DOPA observed in these hearts. This may also contribute to the reduced activity to transcription ratio of TxnRed observed in these hearts. It is possible the lower activity to transcription ratios in aged hearts is caused by an increased turnover
of selenoproteins. If aged hearts are in a more vulnerable oxidative state there may be greater demand placed on these enzymes leading to an increased turnover of them.

Taken together, the reduced antioxidant status found in this chapter, along with the reported increase in ROS production in aged cells, may lead to an increase in oxidative stress that is thought to cause much of the cellular changes associated with ageing. This would also contribute to the increased incidence and mortality, and lower recovery rates reported in elderly patients from CVD.

Numerous studies have demonstrated reduced tolerance and poorer recovery from myocardial ischemia-reperfusion in aged hearts compared to adult hearts. This has been shown by lower recoveries of developed pressure and dP/dt, increased incidence of arrhythmias, increased LDH and CK release (signifying necrosis), greater EDPs and higher levels of apoptosis [375,385,389,399,408-410]. Although the exact mechanism for this phenomenon is yet to be elucidated, several hypotheses other than increased oxidative stress have been postulated.

It has been suggested that a greater decrease in coronary flow is responsible for reduced cardiac performance in aged hearts [410-412]. However a study by Abete and colleagues showed no differences in coronary flow rates between adult and aged hearts at any level of perfusion pressure used [410]. Another theory is that myocardial oxygen consumption (which is coupled to energy production and mechanical work) differs between aged and adult hearts during ischemia [410, 413-415]. However, in another study by Abete and colleagues, they found equal myocardial oxygen consumption in aged and adult hearts during ischemia and reperfusion [410,416]. This suggests the increased mechanical dysfunction during ischemia in the ageing heart is independent of oxygen consumption, and that aerobic metabolism is unaltered by the ageing process [410]. They have also reported no changes in anaerobic metabolism in aged hearts during ischemia, indicating the increased impairment observed in ageing hearts during ischemia cannot be explained by age-related metabolism modifications [410]. Therefore, given the changes in GPX and TxnRed activity and expression observed in this chapter, along with the reported increased sensitivity to ROS, it is likely that reduced cardiac tolerance to oxidative stress is the cause of these age-related decreases in functional recovery. Hence,
increasing antioxidant defences in these patients may be a relatively easy and effective mechanism for improving the heart health of ageing populations.

This chapter highlights the importance of preserving the natural antioxidant balance during ageing, and suggests the ageing population may benefit from increased dietary intake of antioxidants and/or selenium. These results also suggest elderly hearts would not tolerate an oxidative insult very well, and that antioxidant status should be monitored in these patients prior to clinical interventions that involve ischemia-reperfusion.
LIMITATIONS

There are no really obvious experimental limitations to this chapter, other than those already discussed in previous chapters regarding the measurement of Txn protein levels and the use of SYBR green for our real-time PCR.

Given the significant increase in Txn mRNA expression, it would have been interesting to measure Txn protein levels, especially given the low activity-to-expression ratios for TxnRed and GPX. This was attempted, however the antibodies used were generated for Human Txn, and although Txns are >80 % homologous between species, the human monoclonal antibodies were unable to recognise rat Txn. We found similar results for our qRT-PCR primers when the Txn primer, which was designed for rat Txn, was tested on human samples. There were no antibodies available for rat Txn at the time of these studies.

We don’t believe using SYBR green for our real-time PCR rather than flurogenic probes had a significant effect on the low expression of Gpx-4. We have experience with both fluorogenic probes and SYBR Green, and have only ever noticed a difference in sensitivity of 2-3 cycles for the same gene. Therefore we don’t believe this would have a significant effect on the low expression of Gpx-4, and we are certain the signal-to-noise ratio was sufficient to measure Gpx-4 expression. We also run melt curves on all our PCR products to ensure the T_m is correct for the product and that the non template control (NTC) is clear of products or primer dimer. Both the melt curve and agarose gel gave the correct T_m and product size for Gpx-4 whilst all NTCs were clear therefore we are confident we are detecting Gpx-4.

Although this is not an experimental limitation of this chapter, as a future direction it may be interesting to study senescent rats along with aged rats. Furthermore, it may be useful to study diseased aged hearts as well as healthy, and aged hearts following an oxidative insult such as ischemia-reperfusion.
CONCLUSIONS

The results of this chapter indicate that the ratio of antioxidant enzyme activity to transcription decreases in aged hearts. This chapter found significantly lower GPX activities in aged rat hearts when compared to younger hearts without significant changes in GPX mRNA expression, and significantly higher mRNA expression of the TxnRed system in aged hearts without any changes in TxnRed activity. These lower activity to transcription ratios may be due to inefficient or ineffective translation, increased protein turnover and/or increased protein degradation.

Although this chapter found no changes in baseline lipid peroxidation, protein carbonyl or 3-nitrotyrosine levels in aged hearts, increased levels of di-Tyr and o-Tyr were found. These results indicate that aged hearts are oxidatively stressed under normal conditions, and this may be due to an increased production of ROS and/or decreased antioxidant status.

Taken together, the reduced antioxidant status found in this chapter, along with the reported increase in ROS production in aged cells, may lead to an increase in oxidative stress that is thought to cause cellular changes associated with ageing. These results also suggest elderly hearts would not tolerate an oxidative insult as well and may be a major cause of the increased incidence and mortality, and lower recovery rates from CVD reported in elderly patients. This chapter also indicates that antioxidant status should be monitored in elderly patients prior to clinical interventions that involve ischemia-reperfusion. Increasing antioxidant defences may be a relatively easy and effective mechanism for improving the heart health of ageing populations.
PUBLICATIONS ARISING FROM THIS CHAPTER

CHAPTER 8

General Discussion
Cardiovascular disease is the most common cause of morbidity and mortality in economically developed countries [1]. In Australia, it was responsible for approximately 38% of all deaths in 2002 with coronary/ischemic heart disease leading to myocardial infarction being our biggest killer [1,2,417,418]. Coronary heart disease is also the most costly disease for the Australian health system, costing an estimated $894 million per year [1,418]. Over the next 20 years these issues are expected to become more significant worldwide, as risks of suffering from coronary or ischemic heart disease (CHD) for people over the age of 40 have risen to one in two for men, and one in three for women. While these risk factors are increasing, there is also a growing number of elderly Australians, among whom CHD is most common [1,417]. These statistics emphasize the importance of research into the prevention and/or intervention of CHD, in particular ischemic heart disease.

Whilst increasing numbers of patients with CHD are treated with thrombolytic agents, coronary angioplasty, and coronary bypass surgery, there is a growing need to understand the mechanisms involved in the pathogenesis of coronary heart disease if the number of deaths, and the cost to the healthcare system is to be reduced. Importantly, reactive oxygen species and oxidative stress have been shown to play a major role in ischemic heart disease [20,40,44,101,102,103,111,114,118,317]. In addition, ROS have been shown to be generated following routine clinical procedures such as coronary bypass surgery and thrombolysis due to the unavoidable episode of ischemia-reperfusion [20,40,101,102,317]. Therefore, understanding the role of antioxidant enzymes and reactive oxygen species/oxidative stress in ischemia and reperfusion may be the foundation for effective treatment and management of coronary heart disease.

The studies presented within this thesis attempted to not only increase our understanding of myocardial antioxidant enzymes in ischemia-reperfusion injury, but also the effects of dietary selenium levels on them. Additional studies also assessed the role of the TxnRed system alone, by successfully inhibiting its activity within the heart without affecting GPX. Furthermore, the effect of ageing on these antioxidant systems and oxidative damage was investigated.

The studies described in this thesis demonstrate that the TxnRed and GPX systems play a significant role in protecting against myocardial ischemia-reperfusion
injury in the rat. Both systems were found to be up-regulated during periods of ischemia and reperfusion indicating these systems may respond to acute injurious stimuli to aid in limiting oxidant damage. It is also possible the GPX and TxnRed systems complement each other in protecting hearts from oxidative stress and ischemia-reperfusion injury since GPX expression was increased during reperfusion whilst expression of the TxnRed system was highest during reperfusion. These findings suggest the GPX system may be responsible for protecting the heart against LPO and removing ROS such as hydroperoxides and lipid hydroperoxides generated during reperfusion, whilst the TxnRed system may provide more protection against protein oxidation and apoptosis. Interestingly, there were no significant changes in SOD activity found throughout any of these studies which suggests the GPX and TxnRed systems are more finely ‘in tune’ with the oxidative status of cardiac cells. Therefore, increasing the expression and/or activity of BOTH the GPX and TxnRed systems during ischemia AND reperfusion may reduce cellular injury and death, and improve post-ischemic myocardial recovery.

Subsequent studies described in this thesis found that dietary selenium levels modulate both the activity and mRNA expression of the TxnRed and GPX systems, and importantly, this was post ischemia-reperfusion. Dietary selenium protects the heart against ischemia-reperfusion injury by increasing not only the activity of these selenocysteine containing enzymes, but also increasing the post-ischemic mRNA expression. Interestingly, expression of thioredoxin peroxidase-2, a protein not known to contain selenium, also showed a dose-dependent relationship with dietary selenium levels, suggesting components of the TxnRed system may up-regulate each other. This is further supported by changes in Txn expression in hearts supplemented with higher doses of selenium. Txn expression has previously been shown to be induced by oxidative stress, possibly through a hypoxic or antioxidant responsive element in its promoter, and this may also be responsible for the up-regulation of Txn in hearts during ischemia [194,201,213,245,248,253]. Txn mRNA expression was also increased in selenium-free hearts, which may have been induced by a general decrease in antioxidant status or increase in oxidative stress. Furthermore, changes in Txnrd and/or Txn expression may regulate Prdx expression within the heart, which may therefore account for changes in its expression during ischemia and in response to dietary selenium levels.
Data acquired during these studies also found that up-regulation of the GPX and TxnRed systems improved contractile recovery of hearts post ischemia-reperfusion, by reducing the amount of oxidative stress generated. By reducing the amount of lipid peroxidation and protein oxidation occurring in the heart during ischemia-reperfusion, less damage, including apoptosis, is caused to cells allowing greater recovery. Given that we have shown oxidative damage occurs during both the ischemic episode and reperfusion, it is important to have hearts prepared for such an insult by increasing the available antioxidant defences prior to the initiation of ischemia-reperfusion. Therefore, this thesis supports a positive role for both dietary selenium and the TxnRed and GPX systems in myocardial ischemia-reperfusion injury.

Whilst this thesis has shown that both the GPX and TxnRed systems are important in preventing oxidative stress within the heart, subsequent studies identified TxnRed as being critical for recovery from ischemia-reperfusion injury. Auranofin was used to selectively inhibit the *in vivo* activity of TxnRed whilst leaving the activity of GPX unaltered. Hearts depleted of TxnRed activity displayed impaired functional recovery from ischemia-reperfusion injury with lower contractile function, and elevated levels of lipid peroxidation and apoptotic activity. Given these results, it is likely auranofin affects not only TxnRed activity but also mitochondrial function, which will impact on myocardial recovery from oxidative insults as well. Since TxnReds are the only class of enzymes known to reduce Txn, it is possible that alterations in TxnRed activity may also regulate some of the activities of Txn including the inhibition of apoptosis [211]. Whilst auranofin treated hearts subjected to ischemia-reperfusion did not display significantly lower levels of TxnRed, this does support a role for the oxidative stress induced regulation of this system. During ischemia-reperfusion, auranofin treated hearts may have been subjected to a higher level of oxidative stress and/or mitochondrial dysfunction which may have led to a dramatic increase in TxnRed expression at this time, overcoming the effect of the drug. These hearts did however display significantly higher amounts of damage, and significantly lower recoveries post ischemia-reperfusion, which may have important implications given that auranofin is prescribed as an anti-rheumatic drug, often to older patients at risk of cardiovascular disease.
This may also have further implications given that further studies found lower activity to transcription ratios for both the TxnRed and GPX systems in aged hearts. Lower GPX activities were found in aged hearts without significant changes in mRNA expression, along with significantly higher mRNA expression of the TxnRed system without any changes in TxnRed activity. Whether this results from inefficient/ineffective translation, increased protein turnover and/or increased protein degradation still needs to be investigated, however these findings do indicate that aged hearts do have a lower antioxidant status than young hearts. Given that the down-regulation of the GPX and TxnRed systems was shown to reduce recovery from myocardial ischemia-reperfusion injury in young animals, it is possible that the increase in the incidence of and worsening of the consequences of CVD associated with ageing are due to reduced antioxidant defences. This also suggests aged hearts would not tolerate any type of oxidative insult, including various surgical interventions very well. These findings indicate that antioxidant status should be monitored in elderly patients, particularly prior to clinical procedures that involve ischemia-reperfusion. Any further reductions in antioxidant or cellular defences, whether it is by reduced dietary selenium intake or treatment with auranofin, may therefore be particularly detrimental to elderly patients. Given that there is also a decrease in selenium status with ageing, elderly patients may particularly benefit from dietary selenium supplementation [382]. Increasing antioxidant levels may be a relatively simple and effective mechanism for slowing down myocardial ageing, by reducing naturally occurring oxidative damage within the heart, and improving recovery from surgical procedures involving oxidative stress. This may ultimately improve the heart health of ageing populations.

In most species, a selenium intake of 100 µg/kg food is sufficient to maximally express GPX and TxnRed. However, we found significant cardioprotection along with up-regulation of GPX and TxnRed expression in animals consuming 1000 µg Se/kg food compared to animals consuming a normal diet containing 240 µg Se/kg food. Human selenium supplementation trials use 200-400 µg Se/day which equates to approximately 2.8-5.6 µg Se/kg bodyweight/day. This is in addition to selenium consumed within their diets, with the current recommended dietary intake being 1-2 µg Se/kg bodyweight/day. The control rats used in these studies received approximately 9 µg Se/kg bodyweight/day
whilst the high dose experimental group, which had higher GPX and TxnRed expression, together with greater tolerance to oxidative damage, received approximately 36 µg Se/kg bodyweight/day. Although the high dose used in these studies is greater than those used currently in human clinical trials, these findings do suggest humans may benefit from higher selenium levels, particularly those susceptible to ischemia-reperfusion injury or other conditions involving oxidative stress. This also raises the possibility that enhanced selenium intake may be an effective method of preconditioning by increasing antioxidant expression and activity in cardiac tissues.
**SUMMARY**

In summary, these studies show that the TxnRed and GPX systems have a significant role in protecting against myocardial ischemia-reperfusion injury. Ischemia, with and without reperfusion, regulates the expression of these antioxidant enzyme systems, most likely as a self-protection mechanism aimed at increasing endogenous antioxidant protection against impending oxidative stress. Oxidative damage was also found to occur during both the ischemic insult and during reperfusion. Dietary selenium levels were found to regulate the post-ischemic expression and activity of both the TxnRed and GPX systems, and this subsequently impacted on myocardial recovery and oxidative damage. Reduced activity of these antioxidants, by either selenium deficiency or treatment with auranofin, leaves hearts more susceptible to oxidative damage and apoptosis, which leads to significant reductions in myocardial contractile function post ischemia-reperfusion. Aged hearts were found to have lower activity to transcription ratios for GPX and TxnRed, which suggests aged hearts would not tolerate an oxidative insult such as ischemia-reperfusion very well. Dietary selenium supplementation may therefore provide a safe and convenient method for increasing antioxidant protection in aged individuals, those at risk of ischemic heart disease, or in those expecting to undergo clinical procedures involving transient periods of hypoxia. Enhanced selenium intake may be a useful way of preconditioning, and this may represent a new strategy for directly protecting against oxidative stress, and improving recovery rates following myocardial ischemic insults.
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