Canola oil intake, oxidative status and lifespan in SHRSP rats

by

Annateresa Papazzo, BSc (Hons)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University

October, 2011
I am the author of the thesis entitled Canola Oil Intake, Oxidative Status and Lifespan in SHRSP rats submitted for the degree of Doctor of Philosophy

This thesis may be made available for consultation, loan and limited copying in accordance with the Copyright Act 1968.

'I certify that I am the student named below and that the information provided in the form is correct'

Full Name  .......Annateresa Papazzo........................................
(Please Print)
Signed ..................................................
Date ........16/03/2012..........................................................
I certify that the thesis entitled

Canola oil intake, oxidative status and lifespan in SHRSP rats

submitted for the degree of

Doctor of Philosophy

is the result of my own work and that where reference is made to the work of others, due acknowledgment is given.

I also certify that any material in the thesis which has been accepted for a degree or diploma by any university or institution is identified in the text.

'I certify that I am the student named below and that the information provided in the form is correct'

Full Name..........Annateresa Papazzo........................................

(Please Print)

Signed .................................................................

Date............16/03/201.................................................................
Acknowledgements

Firstly, I would like to sincerely thank my supervisors, Paul Lewandowski and Louise Lexis for their endless support, help and guidance throughout my PhD. At times it was not easy, and you always encouraged me to keep going and to think positive. Thank you for all your help with reading through drafts and for your positive feedback. You have taught me to be more confident, and that there is a light at the end of the tunnel.

Also, thank you to the Lewandowski lab group for their help and support throughout my PhD. I would like to thank the Deakin University Animal House Staff for their help and support with the animal studies. Also, thank you to Xavier Conlan for your help and assistance with the HPLC, it really meant a lot you coming in over the Easter break to help me run my final samples. Thank you to Lindsay Brown for allowing me to come to your lab and to learn the isolated aortic rings technique. Also, thank you to Abishek Iyer for teaching me the isolated aortic rings technique, and for all your help and feedback with the organ bath set up. Finally, thank you to Peter Slaviero for your help with the organ bath set up, and for constructing some of the equipment required.

Thank you to Sandra and Laura for your support and friendship. Sandra, even though we were at different institutes doing our PhDs, you were always on email or a call away and always provided me with words of encouragement. Thank you to Mile for your help, support and friendship throughout my PhD. Thanks for always listening to my complaints and worries, and for helping me through them. Also, thank you to Melanie, Kathryn and Monique for your friendship and support throughout my PhD. Finally, thank you to Amit for all the chats, laughs and long coffee breaks. My experience in Geelong and at Deakin University wouldn’t be the same without you all.

I would like to take the opportunity to thank my Mum and Dad for all their support, love and encouragement throughout my PhD. Thank you for believing in
me, for always keeping me going and for your words of wisdom. Thank you to my sister Lisa and to my brother Ilario, for all their support and help throughout my PhD. Lisa, you were always a call away providing me with words of encouragement and advice when times were difficult. Finally, thank you to Michael, my fiancé, for all your help and support. You encouraged me to keep going and to always think positive. I am very blessed to have you in my life.
# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title page</td>
<td>i</td>
</tr>
<tr>
<td>Access to Thesis A</td>
<td>ii</td>
</tr>
<tr>
<td>Candidate Declaration</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>iv</td>
</tr>
<tr>
<td>Table of contents</td>
<td>vi</td>
</tr>
<tr>
<td>List of tables</td>
<td>xi</td>
</tr>
<tr>
<td>List of figures</td>
<td>xii</td>
</tr>
<tr>
<td>Publications</td>
<td>xiii</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xv</td>
</tr>
<tr>
<td>Abstract</td>
<td>1</td>
</tr>
</tbody>
</table>

## Chapter 1 – Overview and thesis rationale

1.1 Aims                                                                  | 5    |
2.1 Research questions                                                   | 6    |

## Chapter 2 – Literature Review

2.1 Cardiovascular health                                                 | 7    |
2.1.1 Endothelial and vascular smooth muscle function                    | 7    |
2.2 ROS, oxidative stress and endogenous antioxidants in the vasculature | 9    |
2.2.1 NADPH oxidase                                                      | 12   |
2.2.2 Antioxidant defence system against ROS                             | 15   |
2.2.2.1 Enzymatic antioxidants                                           | 15   |
2.3 Erythrocytes antioxidant defence against oxidative stress            | 17   |
2.4 Oxidative stress, hypertension and endothelial dysfunction           | 20   |
2.4.1 Assessment of endothelial function                                 | 24   |
2.5 Stroke-prone spontaneously hypertensive (SHRSP) rat model            | 25   |
2.5.1 Canola oil intake and SHRSP rats                                   | 27   |
2.5.2 Canola oil and human health                                        | 36   |
# Chapter 3 – Differential effects of dietary canola and soybean oil intake on oxidative stress in stroke-prone spontaneously hypertensive rats

## 3.1 Abstract

## 3.2 Introduction

## 3.3 Materials and methods

- **3.3.1 Animal husbandry and study design**
- **3.3.2 Measurement of blood pressure**
- **3.3.3 Blood collection and processing**
- **3.3.4 Erythrocyte antioxidant enzymes**
  - **3.3.4.1 SOD assay**
  - **3.3.4.2 Catalase assay**
  - **3.3.4.3 GPx assay**
- **3.3.5 Haemoglobin assay**
- **3.3.6 Lipid peroxidation analysis**
  - **3.3.6.1 MDA**
  - **3.3.6.2 8-isoprostane**
- **3.3.7 Plasma lipids analysis**
  - **3.3.7.1 Triglycerides**
  - **3.3.7.2 Total cholesterol**
  - **3.3.7.3 HDL-C**
  - **3.3.7.4 LDL-C**
- **3.3.8 Statistical analysis**

## 3.4 Results

- **3.4.1 Establishment of canola-induced life shortening model**
- **3.4.2 Body weight, food intake and water intake**
- **3.4.3 Blood pressure**
- **3.4.4 Antioxidant enzymes and oxidative damage**
- **3.4.5 Plasma lipids**

## 3.5 Discussion
Chapter 4 – The effect of short-term canola oil ingestion on oxidative stress in the vasculature of stroke-prone spontaneously hypertensive rats

4.1 Abstract

4.2 Introduction

4.3 Materials and methods
  4.3.1 Animal husbandry and study design
  4.3.2 Measurement of blood pressure
  4.3.3 Blood collection and processing
  4.3.4 Erythrocyte antioxidant enzymes
    4.3.4.1 SOD assay
    4.3.4.2 Catalase assay
    4.3.4.3 GPx assay
  4.3.5 Haemoglobin assay
  4.3.6 Lipid peroxidation analysis
    4.3.6.1 MDA
    4.3.6.2 8-isoprostane
  4.3.7 Plasma lipids analysis
    4.3.7.1 Triglycerides
    4.3.7.2 Total cholesterol
    4.3.7.3 HDL-C
    4.3.7.4 LDL-C
  4.3.8 mRNA gene expression analysis
    4.3.8.1 RNA extraction
    4.3.8.2 Reverse transcription
    4.3.8.3 Reverse transcription-real-time PCR measurement of mRNA
  4.3.9 Statistical analysis

4.4 Results
  4.4.1 Body weight, food intake and water intake
  4.4.2 Blood pressure
  4.4.3 Antioxidant enzymes and oxidative damage
  4.4.4 Plasma lipids
  4.4.5 mRNA gene expression

4.5 Discussion
Chapter 5 – The effect of 50 days of canola oil ingestion on oxidative stress in the vasculature and endothelial dysfunction in SHRSP rats

5.1 Abstract

5.2 Introduction

5.3 Materials and methods

5.3.1 Animal husbandry and study design

5.3.1.1 Fifty day treatment experiment

5.3.1.2 Vascular function experiment

5.3.2 Measurement of blood pressure

5.3.3 Blood collection and processing

5.3.4 Erythrocyte antioxidant enzymes

5.3.4.1 SOD assay

5.3.4.2 Catalase assay

5.3.4.3 GPx assay

5.3.5 Haemoglobin assay

5.3.6 Lipid peroxidation analysis

5.3.6.1 MDA

5.3.6.2 8-isoprostane

5.3.7 Plasma lipids analysis

5.3.7.1 Triglycerides

5.3.7.2 Total cholesterol

5.3.7.3 HDL-C

5.3.7.4 LDL-C

5.3.8 mRNA gene expression analysis

5.3.8.1 RNA extraction

5.3.8.2 Reverse transcription

5.3.8.3 Reverse transcription-real-time PCR measurement of mRNA

5.3.9 Isolated aortic rings

5.3.10 Statistical analysis

5.4 Results

5.4.1 Body weight, food intake and water intake

5.4.2 Blood pressure

5.4.3 Antioxidant enzymes and oxidative damage

ix
### List of tables

Table 2.1: Summary of the lifespan studies examining canola oil fed SHRSP rats compared to soybean oil  
Table 3.1: Fatty acid composition of canola oil and soybean oil diets  
Table 3.2: SOD standards  
Table 3.3: Formaldehyde standards  
Table 3.4 MDA standards  
Table 3.5 Antioxidant status and oxidative damage in SHRSP rats fed canola oil compared with soybean oil diet  
Table 3.6: Plasma lipids in SHRSP rats fed canola oil compared with soybean oil diet  
Table 4.1 Real-time PCR primer sequences for genes of interest  
Table 4.2: Oligo standards  
Table 4.3: Antioxidant status and oxidative damage in SHRSP rats fed 25 days of canola oil compared with soybean oil diet in the absence or presence of NaCl loading  
Table 4.4: Plasma lipids in SHRSP rats fed 25 days of canola oil compared with soybean oil diet in the absence or presence of NaCl loading  
Table 4.5: Effect of 25 days of canola oil intake compared with soybean oil intake on mRNA expression in the aorta of SHRSP rats in the absence or presence of NaCl loading  
Table 5.1: Antioxidant status and oxidative damage in SHRSP rats fed 50 days of canola oil compared with soybean oil diet in the absence or presence of NaCl loading  
Table 5.2: Plasma lipids in SHRSP rats fed 50 days of canola oil compared with soybean oil diet in the absence or presence of NaCl loading  
Table 5.3: Effect of 50 days of canola oil intake compared with soybean oil intake on mRNA expression in the aorta of SHRSP rats in the absence or pressure of NaCl loading
# List of figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>ROS and enzymatic antioxidants in vascular cells</td>
<td>12</td>
</tr>
<tr>
<td>2.2</td>
<td>Structure of the NADPH oxidase complex</td>
<td>15</td>
</tr>
<tr>
<td>2.3</td>
<td>Functional and structural consequences of ROS in hypertension</td>
<td>21</td>
</tr>
<tr>
<td>3.1</td>
<td>Survival curves of SHRSP rats fed a diet containing 10% soybean oil or canola oil with 1% NaCl loading</td>
<td>56</td>
</tr>
<tr>
<td>3.2</td>
<td>Mean body weight of SHRSP rats fed canola oil compared with soybean oil diet</td>
<td>57</td>
</tr>
<tr>
<td>3.3</td>
<td>Food and water intake of SHRSP rats fed canola oil compared with soybean oil diet</td>
<td>58</td>
</tr>
<tr>
<td>3.4</td>
<td>Mean systolic blood pressure of SHRSP rats fed canola oil compared with soybean oil diet</td>
<td>59</td>
</tr>
<tr>
<td>4.1</td>
<td>Mean body weight of SHRSP rats fed canola compared with soybean oil diet in the absence or presence of NaCl loading</td>
<td>82</td>
</tr>
<tr>
<td>4.2</td>
<td>Food and water intake of SHRSP rats fed canola oil compared with soybean oil diet in the absence or presence of NaCl loading</td>
<td>83</td>
</tr>
<tr>
<td>4.3</td>
<td>Mean systolic blood pressure of SHRSP rats fed 25 days of canola oil compared with soybean oil in the absence or presence of NaCl loading</td>
<td>85</td>
</tr>
<tr>
<td>5.1</td>
<td>Mean body weight of SHRSP rats fed canola compared with soybean oil diet in the absence or presence of NaCl loading</td>
<td>105</td>
</tr>
<tr>
<td>5.2</td>
<td>Food and water intake of SHRSP rats fed canola oil compared with soybean oil diet in the absence or presence of NaCl loading</td>
<td>106</td>
</tr>
<tr>
<td>5.3</td>
<td>Mean systolic blood pressure of SHRSP rats fed 50 days of canola oil compared with soybean oil in the absence or presence of NaCl loading</td>
<td>108</td>
</tr>
<tr>
<td>5.4</td>
<td>Cumulative concentration-response curves for norepinephrine (A), SNP (B) and ACh (C) in aortic rings from SHRSP and WKY rats</td>
<td>114</td>
</tr>
</tbody>
</table>
Publications

Peer-reviewed publications from this thesis


Published abstracts

Conference presentations


Other peer-reviewed publications

Other published abstracts
Abbreviations

ACh  Acetylcholine
AChE  Acetylcholinesterase
CVD  Cardiovascular disease
CuZn-SOD  Copper/zinc superoxide dismutase
DNPH  2,4-di-nitrophenylhydrazine
cGMP  Cyclic guanosine monophosphate
eNOS  Endothelial nitric oxide synthase
Ec-SOD  Extracellular superoxide dismutase
G6PD  Glucose-6-phosphate dehydrogenase
GPx  Glutathione peroxidase
GSSG  Glutathione disulfide
Hb  Haemoglobin
HDL-C  High density lipoprotein cholesterol
HPLC  High performance liquid chromatography
H$_2$O$_2$  Hydrogen peroxide
LDL-C  Low density lipoprotein cholesterol
MDA  Malondialdehyde
metHb  Methaemoglobin
Mn-SOD  Mitochondrial superoxide dismutase
NADPH  Nicotinamide adenine dinucleotide phosphate
NaCl  Sodium chloride
NO  Nitric oxide
ONOO$^-$  Peroxynitrite
PMA  Phorbol myristate acetate
ROS  Reactive oxygen species
RBC  Red blood cell
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acids</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>SHR</td>
<td>Spontaneously hypertensive rat</td>
</tr>
<tr>
<td>SHRSP rat</td>
<td>Stroke-prone spontaneously hypertensive rat</td>
</tr>
<tr>
<td>( \cdot O_2^- )</td>
<td>Superoxide</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>VSMCs</td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td>WKY rat</td>
<td>Wistar-Kyoto rat</td>
</tr>
</tbody>
</table>
Abstract

Canola oil intake has been found to shorten the lifespan of stroke-prone spontaneously hypertensive (SHRSP) rats. Oxidative stress may be a possible mechanism leading to the acceleration of hypertension-related deterioration of organs in canola oil fed SHRSP rats. Oxidative stress leads to vascular damage, and plays a role in the pathogenesis of cardiovascular diseases. In vascular cells, NADPH oxidase is a major source of reactive oxygen species (ROS). An increase in ROS can result in endothelial dysfunction, which is an independent predictor of cardiovascular disease. The overall objectives of the thesis were to determine the effect of canola oil ingestion on oxidative stress in the vasculature and endothelial function in SHRSP rats.

The study described in Chapter 3 aimed to determine if the life shortening effect of canola oil intake is associated with an increase in oxidative stress in SHRSP rats. The results found that canola oil ingestion reduced the lifespan of SHRSP rats compared to soybean oil following 1% NaCl loading, 85.8 ± 1.1 and 98.3 ± 3.4 days, respectively. These results strengthen the finding that canola oil intake, as the sole dietary fat source, reduces the lifespan of SHRSP rats. Furthermore, this study also found that canola oil ingestion leads to changes in oxidative status, despite an improvement in the plasma lipids. In addition, NaCl loading may be masking the effects of canola oil in SHRSP rats.

The study described in Chapter 4 examined the effect of short-term ingestion of canola oil in the absence or presence of salt loading on oxidative stress. The
results indicate that canola oil reduces antioxidant status and increases plasma lipids, which are risk factors for cardiovascular disease. However, canola oil in combination with salt intake increases malondialdehyde, a marker of lipid peroxidation and decreases NAPDH oxidase subunits and superoxide dismutase aortic mRNA expression.

The study described in Chapter 5 investigated the effect of 50 days of canola oil intake on oxidative stress and endothelial function. The results from this study showed that 50 days of canola oil ingestion increases p22phox and NOX2 mRNA expression, suggesting an increase in superoxide generation. However, canola oil ingestion in combination with salt leads to impaired endothelium-dependent vasorelaxation and reduced smooth muscle contractile function.

In conclusion, the results from the thesis confirm that canola oil ingestion shortens the lifespan of SHRSP rats. In addition, the results have shown that canola oil ingestion alone leads to changes in oxidative status and plasma lipids in SHRSP rats. Furthermore, canola oil in combination with salt leads to endothelial dysfunction.
The stroke-prone spontaneously hypertensive (SHRSP) rat is a model of human essential hypertension and stroke, and is derived from the spontaneously hypertensive rat (SHR) and the Wistar-Kyoto (WKY) rat strain (Huang et al. 1997; Ogawa et al. 2003; Okamoto et al. 1974; Tatematsu et al. 2004). Canola oil ingestion, as the sole dietary fat source, has been found to shorten the lifespan of SHRSP rats by 15-20% following 1% NaCl loading (Huang et al. 1996 & 1997; Natio et al. 2003; Ogawa et al. 2003; Ohara et al. 2006; Ratnayake et al. 2000a & 2000b). NaCl loading is used to accelerate the development of hypertension. The mechanism by which canola oil shortens the lifespan of SHRSP rats is still unknown. The concentration of phytosterols in canola oil has been suggested to be a contributing factor. However, conflicting results have shown no clear correlation between the content of phytosterols in the diet and survival time observed (Ohara et al. 2006; Tatematsu et al. 2004). In addition, NaCl loading may be masking the effects of dietary phytosterols and canola oil in the SHRSP rat. A recent study showed that dietary phytosterols and phytostanols increase blood pressure in WKY rats in the absence of NaCl loading (Chen et al. 2010). It has been proposed that a key mechanism leading to the shortened lifespan in SHRSP rats is via the acceleration of hypertension-related deterioration of organs (Natio et al. 2003; Ohara et al. 2006). This damage and deterioration of organs may be exacerbated by oxidative stress due to canola oil ingestion, which has not been examined
previously in SHRSP rats. Furthermore, oxidative stress may be increasing overtime with continued ingestion of canola oil.

Growing evidence has found that oxidative stress leads to vascular damage and plays a critical role in the pathogenesis of cardiovascular disease (CVD) such as hypertension, and is elevated in SHRSP rats (Berry et al. 2001). It has been found that reactive oxygen species (ROS) production is increased in both experimental and clinical hypertension, with a decrease in the antioxidant reserve in vascular tissues (Touyz & Schiffrin 2004). Furthermore, in both experimental and clinical hypertension, ROS and oxidative stress have been found to be associated with end-stage organ damage (Touyz & Schiffrin 2004). ROS are produced by all cells within the vascular wall (endothelial, smooth muscle and adventitial cells). In the vasculature, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has been found to be the major producer of ROS (Fortuno et al. 2005). In addition, ROS leads to a decrease in nitric oxide (NO), resulting in endothelial dysfunction. Research has shown that endothelial dysfunction is an independent predicator of CVD (Davies 2009).

The body has a highly structured antioxidant system, which protects and prevents oxidative damage to cellular structures (Sies 1997). Red blood cells (RBCs) have protective mechanisms to detoxify and scavenge ROS, and protect endothelial cells against free radical damage (Richards et al. 1998b). In CVD, evidence has shown that the activities of the antioxidant enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) are reduced, with an increase in the markers of oxidative stress, malondialdehyde (MDA) and 8-isoprostanate (Redon et
al. 2003; Tsuda 2011). Studies have shown that canola oil intake leads to changes in antioxidant status in SHR and WKY rats (Ohara et al. 2008a & 2009). In addition, evidence has shown a clear association between salt intake and hypertension (Bragulat & de la Sierra 2002). Furthermore, high salt intake has been shown to induce oxidative stress in salt sensitive hypertensive individuals (de Cavanagh et al. 2010), increase superoxide (\(O_2^-\)) production in SHR (Kitiyakara et al. 2003) and lead to endothelial dysfunction in Sprague-Dawley rats (Datla & Griendling 2010).

1.1 Aims

The overall objectives of the thesis were to determine the effect of canola oil ingestion on oxidative stress in the vasculature and endothelial function in SHRSP rats.

The first study aimed to determine if the life shortening effect of canola oil intake in SHRSP rats affects:

(1) the antioxidant and oxidative stress markers in the circulation compared to soybean oil and pre-treatment

(2) the plasma lipids compared to soybean oil and pre-treatment

The second study examined the effects of short-term ingestion of canola oil in SHRSP rats. This study aimed to determine if 25 days of canola oil intake in the absence of excess dietary salt or together with salt loading effects:

(1) the antioxidant and oxidative stress markers in the circulation

(2) aortic mRNA expression of NADPH oxidase subunits and SOD isoforms
The third study aimed to determine if 50 days of canola oil intake in the absence of excess dietary salt or together with salt loading effects:

(1) the antioxidant and oxidative stress markers in the circulation

(2) aortic mRNA expression of NADPH oxidase subunits and SOD isoforms

(3) endothelial function in SHRSP rats

1.2 Research questions

Research has found that canola oil ingestion as the sole dietary fat source shortens the lifespan of SHRSP rats by 15-20% following 1% NaCl loading. It has been proposed that a key mechanism leading to the shortened lifespan in SHRSP rats is via the acceleration of hypertension-related deterioration of organs (Natio et al. 2003; Ohara et al. 2006). This damage and deterioration of organs may be exacerbated by oxidative stress due to canola oil ingestion. Therefore, the following research questions were developed:

(1) Is the life-shortening effect of canola oil in SHRSP rats caused by an increase in oxidative stress and altered antioxidant status in plasma and erythrocytes?

(2) Is the life-shortening effect of canola oil in SHRSP rats caused by endothelial dysfunction and enhanced aortic oxidative stress?

(3) Is salt loading masking the effects of canola oil-induced changes in antioxidant status and oxidative stress in the plasma, erythrocytes, and aorta?
CHAPTER 2
Literature Review

2.1 Cardiovascular health

CVD is one of the leading causes of premature death in Australia and is a major health problem (AIHW 2011). CVD refers to conditions related to the heart and blood vessels, such as hypertension, stroke, atherosclerosis, myocardial infarction and angina. Approximately 18% (3.5 million) of Australians reported having a long-term CVD related condition in 2007-08, of which 11% (2.1 million) reported having hypertension (AIHW 2011). There are a number of risk factors that increase the development of CVD, and include hypertension, high blood cholesterol, obesity, diabetes, poor nutrition and inadequate physical activity. Recent research has shown that endothelial dysfunction is an early sign of CVD, such as hypertension and diabetes (Davies 2009).

2.1.1 Endothelial and vascular smooth muscle function

Two major components of blood vessels are endothelial cells and vascular smooth muscle cells (VSMCs) (Hsueh & Anderson 1992). The cellular lining of all blood vessels in the circulatory system is made up of endothelium. It forms a structural barrier between the blood stream and the underlying smooth muscle cells (Fitridge & Thompson 2007). Vascular homeostasis is maintained by the endothelium through complex interactions between the cells in the vessel wall and the lumen (Widlansky et al. 2003). Normal functions carried out by the endothelium to maintain vascular homeostasis include: maintenance of vascular tone; balancing
blood fluidity and thrombosis; and control of the vascular inflammatory process (Dworakowski et al. 2008; Widlansky et al. 2003). These functions are mediated by the synthesis and release of paracrine factors (nitric oxide [NO], endothelin, growth factors and chemotactic molecules) and the expression of surface molecules (Dworakowski et al. 2008; Chhabra 2009).

NO is one of the most significant vasodilating substances released by the endothelium and plays an important role in regulating vascular tone and structure (Endemann & Schiffrin 2004). In the vessel wall NO is produced from L-arginine by endothelial nitric oxide synthase (eNOS) (Endemann & Schiffrin 2004; Stanevicius et al. 2003). The release of NO from the endothelium activates guanylyl cyclase and increases cyclic guanosine monophosphate (cGMP) concentrations in smooth muscles cells. This increase in cGMP concentrations leads to a decrease in calcium levels, which results in vasorelaxation of smooth muscles cells (de Champlain et al. 2004; Stanevicius et al. 2003). NO is also an anti-inflammatory mediator, inhibits platelet and leukocyte aggregation and smooth muscle cell proliferation (Fortuno et al. 2006). Mounting evidence indicates that oxidative stress results in a loss of NO bioactivity, resulting in endothelial dysfunction (Ferroni et al. 2006). Endothelial dysfunction involves alteration in endothelial cell function by irregular vasodilatory responses, enhanced generation of vasoconstricting substances, impaired control of inflammatory processes and increased expression of adhesion molecules (Cai & Harrison 2000; Chhabra 2009).
The endothelium produces vasoconstrictor substances such as endothelin. The vasoconstricting actions are balanced by the production of endothelium-derived relaxing factor, NO (Hsueh & Anderson 1992). The endothelium prevents the proliferation of smooth muscle in its basal unactivated state, through the production of molecules such as transforming growth factor-β (TGF β) and NO. When activated, cytokine/growth factor production by the unstable endothelium or vasoconstrictor substances leads to the proliferation of VSMCs. Thus, the endothelium plays an important role in regulating vascular tone and the growth of surrounding connective tissue (Hsueh & Anderson 1992; Limaye & Vadas 2007).

VSMCs make up the medial layer of blood vessels and in normal physiology they have a differentiated, contractile phenotype (Clempus & Griendling 2006). VSMCs can undergo hypertrophy and lose their specialised form and function under pathological conditions. With pathological stimuli, VSMCs produce inflammatory cytokines, excess extracellular matrix, and divide and migrate towards the intima (Clempus & Griendling 2006). ROS have been associated with all the changes that occur to VSMCs in pathological conditions (Clempus & Griendling 2006).

### 2.2 ROS, oxidative stress and endogenous antioxidants in the vasculature

Molecular oxygen is vital for cellular metabolism and for energy production. However, during the reduction of molecular oxygen to water, ROS are generated (Izawa et al. 1996). The body has a highly structured antioxidant defence system that protects the cells from oxidative damage caused by ROS (Clarkson &
Thompson 2000). An imbalance in excess ROS generation and limited antioxidant protection results in oxidative stress (Sies 1997). Stable concentrations of these oxidants are needed, as they may engage in intracellular signalling. However, an immoderate increase of these oxidants leads to oxidative damage to cellular structures such as lipids, proteins and DNA (Turrens 2003).

ROS are produced by all cells within the vascular wall (endothelial, smooth muscle and adventitial cells) and in both vascular physiology and pathophysiology; ROS plays a central role (Fortuno et al. 2005; Touyz & Briones 2011; Zalba et al. 2001). Growing evidence suggests that in normal physiology, ROS play an important role in vascular and cardiac cell function, and regulate processes involved in gene control and cell to cell interactions (Lyle & Griendling 2006; Zalba et al. 2005). ROS play a role in intracellular signalling that mediates vascular cell growth and survival, differentiation and apoptosis (Berry et al. 2001; Griendling & Fitzgerald 2003). In addition, ROS control numerous classes of genes, such as antioxidant enzymes (SOD and catalase), adhesion molecules, chemotactic molecules and vasoactive substances (Griendling & Fitzgerald 2003). In vascular pathophysiology, the upregulation of adhesion molecules and chemotactic molecules by ROS promote adhesion and attract the migration of monocytes into the vessel wall (Griendling & Fitzgerald 2003). An excess of ROS activity and limited antioxidant protection results in oxidative stress, and as a consequence oxidative damage occurs to cellular structures (Berry et al. 2001). Increased ROS production in the vasculature under pathological conditions leads to vascular remodelling, endothelial dysfunction, increased contractility, lipid peroxidation and inflammation (Touyz & Briones 2011).
In cells there are several enzymatic sources of ROS which include: mitochondrial respiration, xanthine oxidase, NADPH oxidase, uncoupled nitric oxide synthase, lipoxygenases and cyclooxygenases (Jiang et al. 2004). However, in the vasculature NADPH oxidase has been found to be the major producer of ROS (Fortuno et al. 2005). The most significant ROS in vascular cells are superoxide (\( \cdot O_2^- \)), hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) and peroxynitrite (\( \text{ONOO}^- \)) (Figure 2.1). During oxidative stress, when \( \cdot O_2^- \) is formed simultaneously with NO, they react with each other to form \( \text{ONOO}^- \). \( \text{ONOO}^- \) is a highly reactive molecule and is an important mediator of lipid peroxidation and protein nitration (Sachidanandam et al. 2005). \( \text{ONOO}^- \) can act as a NO antagonist and affect its vasodilatory function. This reaction decreases NO bioavailability in the blood vessel (de Champlain et al. 2004). \( \cdot O_2^- \) is produced when oxygen is reduced by one electron. This oxygen radical is the ancestor of the majority of ROS and an intermediate in the oxidative chain reactions (Turrens 2003). In the absence of immediate NO availability, \( \cdot O_2^- \) becomes altered either through a reaction or spontaneously to form \( \text{H}_2\text{O}_2 \) by SOD. \( \text{H}_2\text{O}_2 \) is then converted to water (\( \text{H}_2\text{O} \)) by either catalase or GPx (de Champlain et al. 2004). Potent enzymatic and non-enzymatic antioxidants which are present both at the intracellular and extracellular levels inactivate ROS and help protect the cells against oxidative stress (de Champlain et al. 2004).
2.2.1 NADPH oxidase

NADPH oxidase is a major source of ROS in vascular cells and is involved in endothelial dysfunction, activation and redox signalling (Dworakowski et al 2008; Wassmann et al. 2004). ROS derived from NADPH oxidase has been associated with regulating vascular tone by directly modulating vasodilation or indirectly by reducing NO bioavailability through the formation of ONOO⁻ by \( \cdot O_2^- \) (Paravicini & Touyz 2008). NADPH oxidase is an enzymatic complex that uses NADPH or NADH to catalyse \( \cdot O_2^- \) production by the one electron reduction of oxygen as the electron donor (Paravicini & Touyz 2008; Touyz & Schiffrin 2004):

\[
2O_2 + \text{NAD(P)H} \rightarrow 2\cdot O_2^- + \text{NAD(P)H} + H^+
\]

NADPH oxidase is functionally active within all the layers of the vessel wall: in the endothelium, the media and the adventitia (Touyz & Schiffrin 2004). NADPH oxidase is activated by many factors such as cytokines, shear stress, growth...
factors, hypoxia-reoxygenation and G-protein coupled receptor agonists (Frey et al. 2009).

NADPH oxidase was originally described in phagocytes and is made up of a membrane bound cytochrome b558 reductase domain, three cytosolic subunits (p47<sup>phox</sup>, p67<sup>phox</sup>, and p40<sup>phox</sup>) and a regulatory G-protein (Rac) (Figure 2.2). The cytochrome b558 domain is made up of two subunits: a small α-subunit, p22<sup>phox</sup> and a large β-subunit, gp91<sup>phox</sup> (Nox2). The p22<sup>phox</sup> subunit is bound to the catalytic gp91<sup>phox</sup> subunit (Clempus & Griendling 2006; Jiang et al. 2004). Gp91<sup>phox</sup> contains binding sights for NADPH, molecular oxygen, heme and flavin groups to allow electron transport between the two subunits. On stimulation, p47<sup>phox</sup> becomes phosphorylated and the cytosolic subunits form a complex which is then translocated to the membrane bound cytochrome b558. The enzyme becomes active and electrons are transferred from the substrate to O<sub>2</sub>, forming ·O<sub>2</sub>~ (Dworakowski et al. 2008; Fortuno et al. 2005; Lyle & Griendling 2006; Paravicini & Touyz 2008).

In nonphagocytic cells, several gp91<sup>phox</sup> homologues (referred to as the Nox family) have been identified, each encoded by different genes. These genes are expressed in many tissues and mediate diverse biological functions (Dworakowski et al. 2006; Paravicini & Touyz 2008). The family consists of seven members: Nox1, Nox2 (previously termed gp91<sup>phox</sup>), Nox3, Nox4, Nox5, Duox1 and Duox2 (Paravicini & Touyz 2008). Nox1 is expressed in VSMCs as well as in epithelia; Nox2 is expressed in endothelial cells, cardiomyocytes and fibroblasts; Nox4 is expressed in many tissues such as endothelial cells, VSMCs, cardiomyocytes,
fibroblasts and skeletal muscle and Nox5 is found in lymphoid tissues, testis and pancreas (Dworakowski et al. 2006; Paravicini & Touyz 2008).

In vascular cells all the subunits present on phagocytic NADPH oxidase have been identified, including p47\textsuperscript{phox}, p67\textsuperscript{phox} and Nox2, Nox1, Nox4 and Nox5. Many studies have found that p22\textsuperscript{phox} is present in all NADPH oxidase complexes and is required by all Nox enzymes for activity, except Nox5 (Clempus & Griendling 2006; Fortuno et al. 2005). In all VSMCs p22\textsuperscript{phox} is expressed while the gp91\textsuperscript{phox} homologue present is Nox1, Nox2, Nox4 and/or Nox5. The distribution of the gp91\textsuperscript{phox} homologues in VSMCs is tissue and species specific (Clempus & Griendling 2006). Nox1, Nox 4 and p47\textsuperscript{phox} have been shown to be coexpressed in rat aortic VSMCs, whereas in humans all the major subunits including gp91\textsuperscript{phox} are present in arteries (Touyz & Schiffrin 2004).

In CVD, both vascular and phagocytic NADPH oxidase play a significant role in the production of \textsuperscript{•}O\textsubscript{2}, which may result in the infiltration of monocytes and lymphocytes in cardiovascular tissues (Fortuno et al. 2005). This is supported by studies in hypertensive patients showing an increase in \textsuperscript{•}O\textsubscript{2} production derived by phagocytic NADPH oxidase. In adult SHR, which had endothelial dysfunction and vascular wall hypertrophy, there was an enhanced production of O\textsubscript{2} derived by NADPH oxidase. This was associated with the upregulation of p22\textsuperscript{phox} mRNA expression in the aorta (Zalba et al. 2001).
2.2.2 Antioxidant defence system against ROS

Antioxidants provide protection against ROS and oxidative damage through preventing the formation of ROS, to intercept any further deleterious reactions of damaging species once formed and to repair damage that has occurred (Sies 1997).

2.2.2.1 Enzymatic antioxidants

The major enzymatic antioxidants include SOD, catalase and GPx (Figure 2.1) (Paravicini & Touyz 2008). In vascular cells, SOD is a major cellular antioxidant that provides defence against ·O$_2^-$, by converting ·O$_2^-$ to O$_2$ and H$_2$O$_2$. Three different SOD isoforms have been identified: mitochondrial SOD (MnSOD,
SOD2), the cytosolic copper/zinc-containing SOD (CuZn-SOD, SOD1) and extracellular SOD (eSOD, SOD3). The main vascular SOD is eSOD and is produced and secreted by VSMCs. In the vascular extracellular matrix, it attaches to glycosaminoglycans on the endothelial cell surface (Paravicini & Touyz 2008; Wassmann et al. 2004). Within the vascular interstitium, eSOD plays an essential role in regulating oxidant status (Wassmann et al. 2004).

GPx reduces hydrogen peroxide and lipid peroxides to water and lipid alcohols, respectively, by oxidising reduced glutathione (GSH) to glutathione disulfide (GSSG):

\[ \text{H}_2\text{O}_2 + 2\text{GSH} \xrightarrow{\text{GPx}} \text{GSSG} + 2\text{H}_2\text{O} \]

In the absence of sufficient GPx activity or GSH levels, \( \text{H}_2\text{O}_2 \) and lipid peroxides are not detoxified and can be converted to hydroxyl radicals and lipid peroxyl (Wassmann et al. 2004). In low-level oxidative stress, the GPx/GSH system is considered to be a major defence (Paravicini & Touyz 2008; Wassmann et al. 2004).

Catalase is primarily located in cellular peroxisomes and to some degree in the cytosol (Paravicini & Touyz 2008; Wassmann et al. 2004). It breaks down \( \text{H}_2\text{O}_2 \) to water and molecular oxygen:

\[ 2\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2\text{H}_2\text{O} + \text{O}_2 \]

\( \cdot\text{O}_2^- \) radicals are indirectly detoxified by the removal of \( \text{H}_2\text{O}_2 \) by catalase as \( \cdot\text{O}_2^- \) is converted into \( \text{H}_2\text{O}_2 \) by SOD (Wassmann et al. 2004). Catalase is also able to react with organic peroxides and hydrogen donors and convert them into water.
and organic alcohols. In high level oxidative stress catalase is very efficient and protects the cells from H$_2$O$_2$ made within the cell (Wassmann et al. 2004). In situations of limited concentrations of glutathione and decreased GPx activities, catalase is very important in providing protection (Paravicini & Touyz 2008). Also, catalase plays an important role in the development of tolerance to oxidative stress (Wassmann et al. 2004).

2.3 Erythrocytes antioxidant defence against oxidative stress

Erythrocytes or RBCs are under constant exposure to ROS and oxidative stress due to their role in transporting oxygen and carbon dioxide, and their increased content of heme iron (Cimen 2008). Haemoglobin (Hb) is the source of ROS in RBCs, which undergoes autoxidation to generate ·O$_2^-$ (Cimen 2008). In biochemical reactions, iron is the most important and abundant transition metal. Iron binds oxygen and is also involved in several fundamental oxidation-reduction reactions (Cimen 2008). The haem iron must be maintained in the reduced state in order to bind oxygen. Hb can become non-functional if this mechanism fails. The consequence of this process results in iron release from Hb, which leads to methaemoglobin (metHb) formation. Lipid peroxidation and haemolysis can accompany iron release if RBC GSH is depleted (Cimen 2008).

In cells and tissues, oxidative stress occurs when ROS levels exceed antioxidant protection. In several diseases, the oxidant/antioxidant balance can become altered in RBCs. Within RBCs, protective mechanisms exist to detoxify and scavenge ROS, and protect endothelial cells against free radical damage (Richards et al. 1998b). RBCs have an antioxidant system made up of enzymatic and non-
enzymatic antioxidants that help prevent oxidative damage (Cimen 2008). The enzymatic antioxidants present in RBCs include SOD, catalase, GPx and NADH-metHgb reductase. As mentioned earlier, SOD converts ∙O$_2^-$ to O$_2$ and H$_2$O$_2$. Cytosolic CuZn-SOD is the major form of SOD present in RBCs. The abundance of CuZn-SOD protects RBCs from ROS by scavenging free radicals, which prevents metHgb generation (Cimen 2008). Catalase and GPx are two enzymes present at high levels within RBCs that break down H$_2$O$_2$. GPx is very important in dealing with endogenous H$_2$O$_2$ generated by Hb autoxidation, while catalase plays an important role in dealing with increased H$_2$O$_2$ exposed to RBCs (Cimen 2008). Previous research has shown an inverse relationship between erythrocyte GPx activity and the incidence of CVD (Blankenberg et al. 2003). It has been shown that in patients with coronary artery disease the activity of RBC GPx is significantly reduced (Blankenberg et al. 2003). The reduced activity of RBC GPx may identify the patients who are at risk of cardiovascular events (Blankenberg et al. 2003).

RBCs consist of several non-enzymatic antioxidants, which include vitamin E, C, A and GSH (Cimen 2008). Vitamin E acts as an antioxidant in the lipid phase whereas vitamin C acts in the aqueous phase. Vitamin C reduces ∙O$_2^-$ and lipid peroxyl radicals, and has been shown to have a protective role against peroxidation of membrane lipids in RBCs and tocopherols from t-butylhydroperoxide (Cimen 2008). Vitamin E is one of the most distributed antioxidants within the body. Evidence suggests that RBC vitamin E concentrations are more suitable to evaluate the risk of atherosclerosis (Simon et al. 2001). It has been found that reduced levels of RBC vitamin E are involved in
the early phases of atherosclerosis (Bonithon-Kopp et al. 1997). *In vitro*, vitamin E has protective effects on the early stages in the atherosclerotic process, such as low density lipoprotein (LDL) oxidation, leukocyte adhesion to endothelial cells, VSMC migration and proliferation and platelet aggregation (Simon et al. 2001). Vitamin E and C work together to prevent lipid peroxidation in membranes and plasma lipoproteins. In RBCs, GSH is the most prominent non-enzymatic water soluble antioxidants present (Carroll et al. 2006). It can protect vital proteins such as spectrin, as the oxidation of spectrin can lead to membrane stiffness (Cimen 2008). GSH can directly scavenge oxygen radicals and can act as a substrate for GPx during the detoxification of H₂O₂. It has been shown that GSH provides protection to cells against damage induced by ROS and oxidative stress (Cimen 2008). Vitamin E and GSH are able to prevent the oxidation of the iron haem group on the Hgb molecule and on the RBC membrane (Richards et al. 1999a).

RBCs can provide protective mechanisms against oxidative damage to endothelial cells. It has been shown that RBCs are able to take up ·O₂⁻ through an ion channel present within the membrane (Richards et al. 1998b). *In vitro*, it has been demonstrated that damage to endothelial cells is initiated by neutrophils that have been activated with phorbol myristate acetate (PMA), a process linked to the release of ROS. *In vivo*, endothelial cell injury may also take place due to the production of ROS by neutrophils. Richards et al. (1998b) incubated RBCs and neutrophils that were activated with PMA with radiolabelled human umbilical vein endothelial cells. Cells were treated with inhibitors of either ·O₂⁻ transport (4-acetamido-4'-isothiocyanato-2,2'- disulphonic acid stilbene) or GSH system (N-ethylmaleimide). When ·O₂⁻ transport or the GSH system was inhibited,
endothelial cell damage increased. Therefore, RBCs may provide protection to endothelial cells and other tissues against oxidative damage via importing \( \cdot \text{O}_2^- \) through an ion channel present within the membrane and deactivating them by the antioxidant system present Richards et al. (1998b).

Several cell types are able to synthesis and release NO, which include endothelial cells, neutrophils, macrophages and platelets (Chen & Mehta 1998). RBCs have been recognised to inactivate free NO. Released NO from the endothelium is taken up by RBCs and inactivated by oxyhaemoglobin. During this process, Hgb is changed to methaemoglobin and NO is changed to nitrate, which is removed by the kidneys (Richards et al. 1998a). Evidence suggests that NOS is present within RBCs and they are able to synthesis their own NO. RBC-derived NO has been shown to provide cardioprotective effects in isolated rat heart from ischemia-reperfusion injury (Chen & Mehta 1998). RBC-derived NO may be significant within vascular physiology as it may help regulate vascular tone and platelet aggregation (Chen & Mehta 1998).

2.4 Oxidative stress, hypertension and endothelial dysfunction

Growing evidence has found that oxidative stress leads to vascular damage and plays a critical role in the pathogenesis of CVD such as hypertension (Berry et al. 2001). It has been found that ROS production is increased in both experimental and clinical hypertension, with a decrease in the antioxidant reserve in vascular tissues (Touyz & Schiffrin 2004). In both experimental and clinical hypertension, ROS and oxidative stress have been found to be associated with end-stage organ damage (Touyz & Schiffrin 2004). An increase in ROS activity contributes to the
impaired regulation of physiological processes within the vascular wall, which leads to structural and functional changes observed in hypertension (Figure 2.3) (Zalba et al. 2001). Endothelial dysfunction and VSMC hypertrophy are two characteristic changes that take place within the vascular wall in hypertension (Zalba et al. 2001).

Figure 2.3: Functional and structural consequences of ROS in hypertension (adapted from Fortuno et al. 2005).
In genetic models of hypertension, such as SHR and SHRSPr rats, the production of \( \cdot O_2^- \) derived by NADPH oxidase is increased in the aorta, mesenteric arteries and kidneys. This is linked with the increased expression of NADPH oxidase subunits, mainly p22phox and p47phox (Touyz & Schiffrin 2004). It has been found that vascular \( \cdot O_2^- \) production was greater in SHRSPr rats when compared with WKY rats; and that superoxide production and NADPH oxidase is increased in cultured VSMCs of SHR (Berry et al. 2001; de Champlain et al. 2004). In human hypertension, there is a decrease in NO bioavailability and enhanced oxidative stress levels (Ferroni et al. 2006). In hypertensive patients, VSMCs from resistance arteries have increased ROS generation, and this increase is linked to NADPH oxidase (Paravicini & Touyz 2008). As well as increased ROS production, hypertensive patients have reduced levels of antioxidant. In newly diagnosed untreated hypertensive patients, the levels of the antioxidant vitamins, A, C and E have been found to be reduced compared to the control subjects in plasma (Paravicini & Touyz 2008). It has been reported that low plasma concentrations of vitamin C can predict an increased risk of cardiovascular disease (Berry et al. 2001). The activities and content of the antioxidant enzymes, SOD, catalase and GPx have been found to be reduced both in whole blood and peripheral mononuclear cells (Redon et al. 2003). In addition, lipid peroxidation is a major consequence of oxidative stress. 8-isoprostane and MDA are two biomarkers of lipid peroxidation, which are elevated in CVD (Block et al. 2008). Evidence has shown that the concentration of plasma 8-isoprostane and whole blood MDA are elevated in subjects with hypertension compared with normotensive subjects (Redon et al. 2003; Tsuda 2011). The increase in oxidative stress and reduced levels of antioxidants disrupts the anti-oxidative metabolism in
blood and circulating mononuclear cells. This may affect the functioning of endothelial cells and contributes to the development of CVD (Redon et al. 2003). In both animal models and in human hypertension improvement in vascular function and structure, prevention of organ damage and reduced blood pressure occurs following antioxidant treatment (Touyz & Schiffrin 2004). Several studies have shown that treatment with antioxidant vitamins or SOD mimetics reduce or prevent the development of hypertension and related organ damage (Paravicini & Touyz 2008). Also, in hypertensive animals, treatment with antioxidants or agents that inhibit the production of \( \cdot \text{O}_2^- \), reduces and prevents blood pressure elevation, supporting the concept that \( \cdot \text{O}_2^- \) contributes to the pathogenesis of hypertension (Touyz & Schiffrin 2004; Zhou et al. 2006).

Furthermore, many interventional and epidemiological studies have shown a clear association between salt intake and hypertension (Bragulat & de la Sierra 2002). Evidence has shown that high salt intake can induce oxidative stress in salt sensitive hypertensive individuals and in Dahl salt sensitive rats (de Cavanagh et al. 2010). A recent study by de Cavanagh et al. (2010) found an increase in \( \cdot \text{O}_2^- \) production in aortic and renal arteries in SHR following 1.5% NaCl loading. Furthermore, it has been shown that salt intake in Sprague-Dawley rats lead to an increase in \( \text{O}_2^- \) production (Kitiyakara et al. 2003). This was accompanied by an increase in renal activity and mRNA expression of NADPH oxidase, and a decrease in CuZn-SOD and MnSOD mRNA expression (Kitiyakara et al. 2003). In addition, high salt intake resulted in an increase in vascular \( \text{O}_2^- \) production in apoE-deficient mice, a mouse model of atherosclerosis (Ketonen et al. 2005).
Increased ROS activity in the vasculature can lead to a decrease in NO bioavailability and impaired endothelium-dependent vasorelaxation, which results in endothelial dysfunction (Berry et al. 2001; Datla & Griendling 2010; Zalba et al. 2001). Evidence suggests that endothelial dysfunction is associated with hypertension (Ferroni et al. 2006). An excess of vascular ROS generation or reduced levels of antioxidants to prevent ROS metabolism has recently been associated in the pathogenesis of endothelial dysfunction in SHRSP rats (Ulker et al. 2003). Studies have shown an association between high salt intake and endothelial dysfunction (Bragulat & de la Sierra 2002). Evidence has shown that in Sprague-Dawley rats, high salt intake leads to endothelial dysfunction by reducing the concentration of NO and eNOS activity (Datla & Griendling 2010). In addition, studies suggest that high salt intake and salt sensitivity are associated with endothelial dysfunction in essential hypertensive individuals (Bragulat & de la Sierra 2002).

### 2.4.1 Assessment of endothelial function

Endothelial function can be measured by assessing the vasodilating response to physical or pharmacological stimuli such as shear stress, acetylcholine (ACh), bradykinin, substance P and serotonin (Perticone et al. 2001; Widlansky et al. 2003). Each agonist acts via a membrane receptor, with signal transduction functioning via G proteins (Perticone et al. 2001). In humans, endothelial function can be assessed in coronary arteries angiographically by Doppler flow measurements during the infusion of ACh or increased blood flow (Endemann & Schiffrin 2004; Widlansky et al. 2003). In healthy individuals, the response to these stimuli is by the endothelium releasing vasodilator substances, specifically
NO. It has been found that coronary and peripheral endothelial dysfunction predicts long term progression of atherosclerosis and CVD events in patients (Perticone et al. 2001). Endothelial function can be measured in the forearm resistance arteries via blood flow measurements using venous occlusion plethysmography. This method involves intra-arterial infusion of agonists such as ACh via a catheter in a dose response manner (Perticone et al. 2001; Widlansky et al. 2003). Endothelial function can also be assessed in vitro from isolated thoracic aortic rings. The endothelium-dependent and -independent vasodilating responses to ACh and sodium nitroprusside (SNP) respectively, are measured in a dose response manner in the presence of a submaximal contraction to norepinephrine (Martinez-Revelles et al. 2008).

2.5 Stroke-prone spontaneously hypertensive (SHRSP) rat model

The SHRSP rat was first established by Okamoto et al. in 1974. It is a model of human essential hypertension and stroke, and is derived from the SHR and WKY rat strains. In 1962 and 1963, the SHR colony was produced by selective inbreeding of WKY rats. Further breeding continued from this colony, and an inbred strain of SHR was obtained in 1969 (Okamoto et al. 1974). The occurrence of stroke in SHR was observed and mating was carried out amongst the substrains and generations. The offspring of which one or both parents developed stroke spontaneously were maintained. Stroke-prone rats were obtained after successive selective breeding in 1974 (Okamoto et al. 1974). The SHRSP rat strain showed an increase in blood pressure from a young age (around 5 weeks of age) and severe hypertension of around 240-250 mmHg (from 15 weeks of age) (Okamoto et al. 1974). The initial signs of stroke and hypertension encephalopathy were
observed in the rats, and included: hyperirritability, excitement (piloerection), behavioural and psychological depression (hyporesponsiveness, hypokinesia and hypotonia) and motion disturbances (Okamoto et al. 1974). After initial symptoms of stroke or hypertension were observed, the rats died from stroke within a few days to 24 weeks. Brain lesions were usually located in the cortical or subcortical areas of the optical, frontal and medial areas of the telencephalon. However, in some rats lesions were found in the basal ganglia (Okamoto et al. 1974). In addition, the incidence of stroke was greater in males than in females. Males showed rapid development of severe hypertension in contrast to females from a younger age (Okamoto et al. 1974).

The SHRSP rat model has been extensively used to investigate the effects of numerous antihypertensive agents as potential preventive effects against stroke and mortality (Richer et al. 1997). Agents such as angiotensin I-converting enzyme inhibitors and angiotensin II AT1-receptor blockers have shown to be very effective (Richer et al. 1997). The beneficial effects of these agents include the prevention of fibrinoid necrosis formation in cerebral arterioles and the limitation of blood pressure rise (Richer et al. 1997). Also, losartan, an angiotensin II AT1 receptor antagonist has shown to prevent stroke and improve endothelial cell and cerebral artery’s smooth muscle functions in SHRSP rats (Vacher et al. 1996). In addition, the SHRSP rat is a model of human insulin resistance (James et al. 2001). It is characterised by impaired fatty acid metabolism and decreased insulin-mediated glucose removal in isolated adipocytes (James et al. 2001). Amongst the common rat strains, the SHRSP rat strain develops the highest blood pressure. At 5 weeks of age SHRSP rats are
hypertensive, and in males the systolic blood pressure increases to at least 250 mmHg compared to the blood pressure in SHR which rises to 200 mmHg (Doggrell & Brown 1998), and in WKY rats it is under 150 mmHg (Yamori & Horie 1977). Low protein and high-salt intake have been suggested as high risk factors for this animal model, which are also suggested for human apoplexy (Huang et al. 1997). High-salt intake is associated with increased blood pressure (Flegel & Magner 2009). SHRSP rats have a deficiency of cholesterol in blood and in cell membranes. Thus, they have weak and more fragile cell membranes than other rat strains (Ratnayake et al. 2000b). Dietary salt loading accelerates the development of hypertension and stroke. The intake of fibre, calcium, fish oil or cholesterol reduces the adverse effects of salt loading (Ratnayake et al. 2000b). SHRSP rats live to around 52-64 weeks old (early to mid-adulthood) or around 14-20 weeks old following salt loading, compared to SHR and WKY which live to around 2 and 3 years, respectively (Doggrell & Brown 1998).

### 2.5.1 Canola oil intake and SHRSP rats

Evidence has shown that higher intakes of saturated and trans fats are associated with an increased risk of CVD, whilst higher intakes of monounsaturated and polyunsaturated fats, such as canola oil reduce the risk (Tanasescu et al. 2004). Previous studies using this animal model have investigated the effects of vegetable oils such as canola oil on lifespan and physiological changes. In 1974, through selective plant breeding a new rapeseed variety was released that contained low erucic acid and glucosinolate contents, and was renamed canola oil (also referred to as double low rapeseed oil) (Tatematsu et al. 2004). Oil from traditional rapeseed contained erucic acid and thyrotoxic sulphur compounds
derived from glucosinolates, which are two anti-nutritional factors. Euric acid is an omega-9 monounsaturated fatty acid and high exposure levels have been associated with myocardial lipidosis and heart lesions in Sprague-Dawley rats (FSANZ 2003). Glucosinolates are organic compounds containing sulphur and nitrogen, which affects the taste and quality of the oil, and is responsible for the sharp taste and the low palatability of the oil (Mailer & Wratten 1985). Canola oil has a high content of oleic acid (55-60%) and a low content of saturated fatty acids (SFA) (6-7%). In addition, it provides a low ratio of linoleic to linolenic acids (2:1) and is a good source of α-linolenic acid (7-10%) (Tatematsu et al. 2004). It contains a slightly higher level of sulphur compounds compared to other vegetable oils.

In SHRSP rats, ingestion of canola oil as the sole dietary fat source (added at 10 wt/wt% to standard rat chow) has been reported to shorten life span compared to soybean oil or perilla oil (Table 2.1) (Huang et al. 1996 & 1997; Natio et al. 2003; Ogawa et al. 2003; Ohara et al. 2006; Ratnayake et al. 2000a & 2000b). It has been found to shorten the lifespan of SHRSP rats by 15-20% under 1% NaCl loading (Ratnayake et al. 2000b). NaCl loading is used as it accelerates the progression of hypertension within these animals. Even without NaCl loading, canola oil was found to shorten the life span of SHRSP rats compared to soybean oil, 254 ± 12 and 416 ± 16 days, respectively (Huang et al. 1997). The life shortening effect of canola oil has only been observed in SHRSP rats and has not been carried out in any other rat strains such as the SHR and WKY rats, to determine whether the effects are the same. Possible contributing factors within canola oil have been examined, which include the fatty acid, the sulphur and the
phytosterol composition of canola oil. Studies have shown that the sulphur and fatty acid composition of canola oil does not appear to cause the shortened life span (Miyazaki et al. 1998; Ratnayake et al. 2000a). A study by Ohara et al. (2006) tried to fractionate the causative(s) in canola oil by super gas extraction technique. Three different fractions were obtained: 180-bar, 350-bar and residue fraction during the extraction of canola oil. SHRSP were fed 10 wt/wt% soybean oil, canola oil, 180-bar fraction, 350-bar fraction or residue fraction. The survival times were significantly shorter in the canola oil, 350-bar fraction and the residue fraction groups. In the kidney, chronic nephropathy was common in the canola oil and all the fraction groups, and cerebral necrosis was found in the residue group. However, the fraction containing the causative(s) was not found. This has been the only published study that has carried out fractionation work on canola oil to identify the possible causative(s) contributing to the shortened life span observed in SHRSP rats. On the other hand, a recent study by Ohara et al. (2009) found that the canola oil and interesterified canola oil mimic (consisting of safflower oil, flaxseed oil and erucic acid) diets elevated blood pressure, increased plasma lipids, increased hepatic activities of glucose-6-phosphate dehydrogenase (G6PD), reduced platelets and induced kidney abnormalities in WKY rats. The fatty acid composition unique to canola oil appears to affect the pathophysiology of the rat. However, the fatty acid composition is not asserted to be the direct causative of the life shortening effect of canola oil (Ohara et al. 2009).

Furthermore, the concentration of phytosterols in canola oil has also been suggested to be a contributing factor. A previous study carried out by Ratnayake et al. (2000b) found that the groups containing the added phytosterols and the
canola oil group had significantly reduced lifespan compared to the soybean oil group. They also found that the RBC membrane deformability index was significantly higher in the canola oil group and in the groups with the added phytosterols indicating an increase in membrane fragility. Another study found the addition of phytosterols to soybean oil caused an increased in systolic blood pressure and promoted the onset of stroke. However, a fivefold increase of phytosterols was needed to produce the same effects as canola oil (Ogawa et al. 2003). The same study also found that intestinal mRNA expression of Abcg5 and Abcg8, which are genes associated with the selective transport of dietary sterols in the intestine were decreased in SHRSP and WKY rats compared with Wistar rats. This may be partly responsible for the early onset of stroke in these animals (Ogawa et al. 2003). However, conflicting results have shown no clear correlation between the content of phytosterols in the diet and tissues and survival time observed (Ohara et al. 2006; Tatematsu et al. 2004). In addition, NaCl loading may mask the effects of dietary phytosterols and canola oil in the SHRSP rat. A recent study showed that dietary phytosterols and phytostanols increase blood pressure in WKY rats in the absence of NaCl loading (Chen et al. 2010).

Moreover, recent studies suggest that one of key mechanisms leading to the shortened life span in SHRSP rats is via the acceleration of hypertension-related deterioration of organs (Natio et al. 2003; Ohara et al. 2006). An increase in blood pressure and enhanced Na+, K+-ATPase activity have been reported as a result of canola oil ingestion, and may promote the deterioration of organs (Natio et al. 2000c & 2003). Other hypertension-related conditions induced by canola oil ingestion in SHRSP rats include tissue lesions in the heart and kidney, as well
enhanced tension development in isolated aortic rings after 4 weeks of ingestion (Natio et al. 2000c). Furthermore, as mentioned in section 4.0, evidence suggests that oxidative stress is involved in the pathogenesis of hypertension and is elevated in SHRSP rats. The damage and deterioration of organs may be exacerbated by oxidative stress due to canola oil intake, which has not been examined previously in SHRSP rats.

Evidence has shown that in WKY rats, the activity of hepatic G6PD was increased, along with an increase in blood pressure and plasma lipids, and the activities of catalase and SOD were reduced in the canola oil fed group compared to the control group (Natio et al. 2000d). It is well established that ROS interacts with endothelial NO, thus decreasing the vasodilating response of the blood vessel. The increase in blood pressure in the canola oil group may be due to the reduced endothelium-dependent vasodilation mechanism of the blood vessel, as the activities of catalase and SOD are decreased (Natio et al. 2000d). However, a study found that in SHR and WKY rats the endothelium-dependent and endothelium-independent vasodilating responses to ACh and SNP, respectively, were not different between the canola oil and soybean oil groups, despite an increase in blood pressure (Natio et al. 2000b). In addition, vascular lesions in the heart and kidney were observed in the canola oil fed SHR and WKY rats (Natio et al. 2000a; Ohara et al. 2008a). A recent study by Ohara et al. (2008a) found increased plasma lipids and increased G6PD activities in the liver and erythrocytes of canola fed SHR, as well as increased abundance of cyclooxygenase-2 (COX-2) through immunohistochemical staining. The results imply that canola oil ingestion brings about hyperlipidemia in these animals, and
the increased G6PD may serve as a NADPH provider. The study also found an increase RBC GSH and glutathione reductase, with a decrease in the activity of RBC GPx. Furthermore, in the hepatic cytosol, the activity of SOD and catalase were significantly reduced (Ohara et al. 2008a). Similar results were also found in a study by Ohara et al. (2009), in which the activities of catalase, GPx and glutathione reductase were decreased in the liver of WKY rats. However, a significant elevation in hepatic SOD levels was found. The study also found an increase in blood pressure in the canola oil fed WKY rats compared to the soybean oil group (Ohara et al. 2009). Furthermore, the enhanced activity of G6PD in the liver may increase NADPH oxidase production and facilitate lipogenesis, and possibly oxidative stress. Alternatively, the increased activity of G6PD may be a protective adaptation against enhanced oxidative stress, as increased G6PD can provide a source of electrons for the reduction of glutathione reductase which converts GSSG to GSH (O'Brien et al. 2000). However, the expression of NADPH oxidase has not been analysed, and this may provide evidence into whether the generation of ROS is increased. As noted above, the oxidative status is altered in canola oil fed SHR and WKY rats; therefore, the role of oxidative stress in canola oil fed SHRSP rats requires further investigation.

In addition, the unfavourable effects of canola oil ingestion seem to be confined and only seen in SHRSP rats and its related strains (SHR and WKY rats). This is evident from a study carried out by Ohara et al. (2008b), which found that canola oil ingestion did not induce any abnormalities in the Wistar rats, except increase plasma concentrations of Na⁺ and aldosterone compared to the soybean oil group. The results suggest that the unfavourable effects of canola oil ingestion appear to
be restricted to SHRSP, SHR and WKY rats. Furthermore, a study by Tatematsu et al. (2004) examined the effect of canola oil and soybean oil intake in SHRSP rats through 2 generations. The authors postulated that the factors responsible for the life shortening effect of canola oil may be transmissible to the next generation. The study found that the male pups from the canola oil fed dams had retarded growth. In addition, the pups from the canola oil dams and the soybean oil dams that were weaned on the canola oil diet till 4 weeks old had significantly reduced lifespan compared with the pups that were weaned with soybean oil (Tatematsu et al. 2004).
Table 2.1: Summary of the lifespan studies examining canola oil fed SHRSP rats compared to soybean oil

<table>
<thead>
<tr>
<th>Studies</th>
<th>Experiment</th>
<th>Diets</th>
<th>Findings</th>
<th>Mean survival time (days)</th>
</tr>
</thead>
</table>
| Huang et al. (1996)      | • Lifespan study                                                          | • SHRSP rats fed: 10 wt/wt% canola, soybean, perilla or microbial oil. | • Canola oil intake significantly reduced the lifespan compared to all other diets  
  • No difference in systolic and diastolic blood pressure in all dietary groups | Soybean Oil: 79.6        | Canola Oil: 50.1*                                                      |
|                          | • 1% NaCl loading                                                          |                                                                      |                                                                                                                                                                                                          |                          |
| Ratnayake et al. (2000a) | • Lifespan study                                                          | • SHRSP rats fed: 10 wt/wt% soybean oil, canola oil, high-palmitic canola oil, low-sulphur canola oil, high-oleic safflower oil, a fat blend mimicking fatty composition of canola oil, fat blend high in saturated fatty acids or normal rat chow. | • Survival times were not different between the canola oil and low-sulphur canola oil group  
  • Groups given the non-canola oil based diets lived longer than groups on canola oil and low-sulphur canola oil  
  • No differences in fatty acid composition of RBCs and liver from the animals in each group | Soybean Oil: 102 ± 15.4 | Canola Oil: 88 ± 10.4*                                                  |
|                          | • 37 day feeding trial for tissue analysis                                |                                                                      |                                                                                                                                                                                                          |                          |
| Ratnayake et al. (2000b) | • Lifespan study                                                          | • SHRSP rats fed: canola oil, soybean oil, canola oil + phytosterols, soybean oil + phytosterols, corn oil, olive oil or Canadian fat mimic. | • Lifespan of rats fed canola oil, canola oil + phytosterols, soybean oil + phytosterols and corn oil significantly shorter compared to soybean oil and Canadian fat mimic  
  • Groups fed high phytosterol diets had greater levels of phytosterols in plasma, RBC, liver and kidney | Soybean Oil: 95 ± 9.3     | Canola Oil: 87 ± 4*                                                     |
|                          | • 30-32 day feeding trial for blood and tissue analysis                   |                                                                      |                                                                                                                                                                                                          |                          |
| Ogawa et al. (2003)      | • Lifespan study                                                          | • SHRSP rats fed: 10 wt/wt% canola oil, soybean oil, soybean oil + 0.06% phytosterol or soybean oil + 4.5% phytosterol. | • Addition of phytosterols to soybean oil caused an increased in SBP and promoted the onset of stroke  
  • Intestinal mRNA expression of Abcg5 and Abcg8 were decreased in SHRSP and WKY rats compared with Wistar rats | Soybean Oil: 117.9 ± 7.1 | Canola Oil: 99.5 ± 3.5*                                                  |
<p>|                          | • Tissue analysis in SHRSP rats, WKY and Wistar rats                     |                                                                      |                                                                                                                                                                                                          |                          |</p>
<table>
<thead>
<tr>
<th>Studies</th>
<th>Experiment</th>
<th>Diets</th>
<th>Findings</th>
<th>Mean survival time (days)</th>
</tr>
</thead>
</table>
| Natio et al. (2003) | • Lifespan study  
• 4 week feeding trial for tissue analysis | • SHRSP rats fed: 10 wt/wt% canola oil or soybean oil.  
• 1% NaCl loading | • Mean systolic blood pressure was higher in canola oil compared to soybean oil group (233 ± 2 and 223 ± 3 mmHg, respectively)  
• ↑ phytosterol levels in plasma and RBC membranes in canola oil group  
• ↑ Na⁺, K⁺-ATPase activities in brain, heart and kidneys in canola oil group | Soybean Oil: 68 ± 3  
Canola Oil: 62 ± 2* |
| Ohara et al. (2006) | • Lifespan study  
• 8 week feeding trial for tissue analysis | • SHRSP rats fed: 10 wt/wt% soybean oil, canola oil, 180-bar fraction, 350-bar fraction or residue fraction.  
• 0.5% NaCl loading | • Lifespan was significantly shorter in the canola oil, 350-bar fraction and the residue fraction groups  
• In the kidney, chronic nephropathy was common in the canola oil and fraction groups.  
• Cerebral necrosis was found in the residue group | Soybean Oil: 63 ± 3  
Canola Oil: 53 ± 2* |
| Papazzo et al. (2011) | • Lifespan study | • SHRSP rats fed: 10 wt/wt% soybean oil or canola oil  
• 1% NaCl loading | • Canola oil intake significantly reduced the lifespan compared to soybean oil.  
• Canola oil intake lead to changes in oxidative status | Soybean Oil: 98.3 ± 3.4  
Canola Oil: 85.8 ± 1.1* |

*Statistically significant \( P < 0.05 \)
2.5.2 Canola oil and human health

Canola oil is considered to provide positive cardiovascular benefits due to its favourable fatty acid content (Ratnayake et al. 2000a). A study by Lichtenstein et al. (1993) assessed the effect of different vegetable oils which are high in monounsaturated or polyunsaturated fatty acids on plasma lipids as part of the National Cholesterol Education Program (NCEP) Step 2 diet in humans. The study consisted of middle-aged and elderly subjects (mean age 61 years), and were given diets containing canola, olive or corn oil. The results showed that the consumption of canola, corn and olive oil enriched diets significantly reduced plasma cholesterol concentrations, plasma low-density lipoprotein cholesterol (LDL-C) and apolipoprotein (apo)B levels. It is evident that canola oil is considered to provide positive effects in middle-aged and elderly hypercholesterolemic subjects. Saturated fatty acids in the diet can be substituted with oils rich in polyunsaturated or monounsaturated fatty acids as part of the NCEP Step 2 diet (Lichtenstein et al. 1993). In addition, a study by McDonald et al. (1989) evaluated the changes caused by dietary lipids to plasma lipids and lipoproteins and the production of thromboxane A2 and prostacyclin in vivo in healthy young men aged between 19 – 32 years. The young men were given 75% of fat added to their diet, and given either canola oil or sunflower oil for 18 days during the experimental period. Canola oil reduced total plasma cholesterol and LDL-C in the young men, and the results were similar to that of sunflower oil (McDonald et al. 1989). Evidence suggests that canola oil has antithrombotic effects as bleeding times were found to be longer and prostacyclin production increased (McDonald et al. 1989). A study by Sticker et al. (2008) found that in patients with peripheral arterial occlusive disease, 2 tablespoons/day of canola oil
for 8 weeks decreased total cholesterol and LDL-C levels, and improved endothelial function compared with sunflower oil (Sticker et al. 2008). However, there were a number of limitations to this study. At baseline, there was randomisation between the canola oil and sunflower oil groups, which included unmatched medications and significance in endothelial function and heart rate variability measures. Food diaries were not taken before the commencement of the trial, which would have provided information about the patient’s diet, as this could have influenced the results between the groups. Overall, the results obtained in this study may not be a true representative of what is occurring as a result of canola oil intake. Further research is required to investigate the effects of canola oil ingestion in patients with CVD. Furthermore, in 1981 toxic oil syndrome appeared in Spain due to the ingestion of canola oil which had been denaturated with aniline and marketed for human consumption. It affected more than 20,000 people and has caused 2500 deaths (Valades et al. 2003). Valades et al. (2003) carried out a cohort study with a total of 20,084 toxic oil syndrome subjects between the periods of 1981 to 1995. The shortest survival times were for women and subjects < 40 years of age. The major clinical manifestations observed include: liver disease, motor neuropathy, pulmonary infection, pulmonary hypertension and eosinophilia (Valades et al. 2003). In addition, experimental and epidemiological findings suggest that the use of unrefined canola oil in high temperature wok cooking has been associated with increased lung cancer in China (Shields et al. 1995). There has been no other evidence reported showing that canola oil ingestion has a negative impact on human health. It is possible that the observed effect of canola oil has been overlooked in human nutrition, as many people consume an abundance of saturated fatty acids from
different sources (Okuyama et al. 1997). If there is a subsection of the human population that responds in a similar manner as SHRSP rats to canola oil, then this could be an important clinical area that warrants investigation.
CHAPTER 3
Differential effects of dietary canola and soybean oil intake on oxidative stress in stroke-prone spontaneously hypertensive rats

Publication

3.1 Abstract

Background
Canola oil shortens the lifespan of SHRSP rats compared with rats fed soybean oil when given as the sole dietary lipid source. One possible mechanism leading to the damage and deterioration of organs due to canola oil ingestion is oxidative stress. This study investigated the effect of canola oil intake on oxidative stress in this animal model.

Method
Male SHRSP rats, were fed a defatted control diet containing 10 wt/wt% soybean oil or a defatted treatment diet containing 10 wt/wt% canola oil, and given water containing 1% NaCl. Blood pressure was measured weekly. Blood was collected prior to beginning the diets and at the end of completion of the study for analysis.
RBC antioxidant enzymes, RBC and plasma MDA, plasma 8-isoprostane and plasma lipids.

**Results**

Canola oil ingestion significantly decreased the lifespan of SHRSP rats compared with soybean oil, 85.8 ± 1.1 and 98.3 ± 3.4 days, respectively. Systolic blood pressure increased over time with a significant difference between the diets at the 6th week of feeding. Canola oil ingestion significantly reduced RBC SOD, GPx and catalase activities, total cholesterol and LDL-C compared with soybean oil. There were no significant differences in RBC MDA concentration between canola oil fed and soybean oil fed rats. In contrast, plasma MDA concentration and 8-isoprostane was significantly lower in the canola oil group compared to the soybean oil group.

**Conclusion**

In conclusion, canola oil ingestion shortens the lifespan of SHRSP rats and leads to changes in oxidative status, despite an improvement in the plasma lipids.

**3.2 Introduction**

The SHRSP rat is a model of human essential hypertension and stroke, and is derived from the SHR and the WKY rat strain (Huang et al. 1997; Ogawa et al. 2003; Okamoto et al. 1974; Tatematsu et al. 2004). In this model, ingestion of canola oil as the sole dietary fat source (added at 10 wt/wt% to standard rat chow) has been reported to shorten lifespan compared to the soybean oil or perilla oil (Huang et al. 1996 & 1997; Natio et al. 2003; Ogawa et al. 2003; Ohara et al. 2006; Ratnayake et al. 2000a & 2000b). The sulphur and fatty acid composition
of canola oil does not appear to cause the shortened life span (Ratnayake et al. 2000a). Furthermore, the concentration of phytosterols in canola oil has also been suggested to be a contributing factor (Natio et al. 2000a & 2003, Ogawa et al. 2003; Ratnayake et al. 2000b). However, conflicting results have shown no clear correlation between the content of phytosterols in the diet and tissues and survival time observed (Ohara et al. 2006; Tatematsu et al. 2004). Recent studies suggest that one of the key mechanisms leading to the shortened lifespan in SHRSP rats is via the acceleration of hypertension-related deterioration of organs (Natio et al. 2003; Ohara et al. 2006). An increase in blood pressure (Natio et al. 2000c) and enhanced Na+, K+-ATPase activities (Natio et al. 2003) have been reported as a result of canola oil ingestion, and may promote the deterioration of organs. Other hypertension-related conditions induced by canola oil ingestion include tissue lesions in the heart and kidney (Ohara et al. 2006).

There is growing evidence that oxidative stress leads to vascular damage and plays a critical role in the pathogenesis of cardiovascular diseases such as hypertension (Berry et al. 2001; Fearon & Faux 2009). It has been found that production of ROS is increased in both experimental and clinical hypertension, with a decrease in the antioxidant reserve in vascular tissues (de Champlain et al. 2004; Touyz & Schiffrin 2004). In both experimental and clinical hypertension, ROS and oxidative stress have been found to be associated with end-stage organ damage (Touyz & Schiffrin 2004). Hypertension is also a risk factor for stroke (Lo et al. 2003). It has been shown that in both ischemic and hemorrhagic stroke, ROS production is increased, and oxidative stress is a key mediator of tissue damage (Cherubini et al. 2005; Lo et al. 2003).
Cells are protected against ROS by a complex antioxidant system present within the cells, which includes SOD, catalase and GPx (Lakomkin et al. 2005). Within RBCs, protective mechanisms exist to detoxify and scavenge ROS, and protect endothelial cells against free radical damage (Richards et al. 1998). There is an inverse relationship between reduced activities of antioxidants (SOD, GPx and vitamin E) and increased lipid peroxidation products in blood and cardiovascular disease (Kalenikova et al. 2004). The activities of the antioxidant enzymes, SOD, catalase and GPx have been found to be reduced both in whole blood and peripheral mononuclear cells in hypertensive subjects (Redon et al. 2003). In addition, concentrations of MDA in whole blood and peripheral mononuclear cells have been shown to be increased in hypertensive subjects compared to control (Redon et al. 2003). MDA is an end product of lipid peroxidation, and therefore increased concentrations indicate an increase in ROS concentration and resulting oxidative damage (Redon et al. 2003). After an acute ischemic stroke the activities of SOD and GPx were reduced and the concentration of MDA was increased in RBCs of human subjects (Demirkaya et al. 2001). This study aimed to determine if enhanced oxidative stress and altered antioxidant capacity is associated with canola oil ingestion in SHRSP rats.
3.3 Materials and methods

3.3.1 Animal husbandry and study design

Experimental design was based on previous lifespan studies investigating canola oil intake in SHRSP rats (Natio et al. 2003) to allow direct comparisons to be made. Thirty-four male SHRSP rats (Deakin University, Australia) were used for this study. Ten of the SHRSP rats were sacrificed at 5 weeks of age and blood was collected for analysis (pre-treatment data). Twenty-four 4 week old SHRSP rats were randomly assigned to a control and treatment group and acclimatized for one week. During acclimatization they were given a standard pellet diet (Specialty Feeds, Western Australia) and water ad libitum. Male rats were used as previous studies investigating the canola oil-induced life shortening effect in SHRSP rats were carried out using male animals. Following this, the two groups were fed, respectively, a defatted control diet containing 10 wt/wt% soybean oil or a defatted treatment diet containing 10 wt/wt% canola oil (Speciality Feeds, Western Australia), and life span was determined. The fatty acid compositions and total antioxidant status of the diets are shown in Table 3.1. Each group was given water containing 1% NaCl to accelerate the development of hypertension. This has been used in previous lifespan studies investigating canola oil intake in SHRSP rats (Huang et al. 1996 & 1997; Natio et al. 2003; Ogawa et al. 2003; Ohara et al. 2006; Ratnayake et al. 2000a & 2000b). The rats were housed in pairs in plastic cages with saw dust/timber shavings as the bedding. The animals were maintained on a 12 hr light/dark photo-period with a room temperature of 21 ± 2°C. Animal body weights, food intake and water consumption were determined once a week, while the health of the animals was monitored daily. When a rat was found to suffer from stroke, paralysis, to be in pain or to have lost > 25% of their
body weight they were anaesthetised via intra-peritoneal injection with lethabarb (50 mg/kg), and blood was collected for analysis (post-treatment data). Approval for this project was granted by the Deakin University Animal Welfare Committee (Ethics approval no. A21/08).

Table 3.1: Fatty acid composition of canola oil and soybean oil diets.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Soybean oil (%)</th>
<th>Canola oil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0 Myristic acid</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>16:0 Palmitic acid</td>
<td>11.0</td>
<td>7.0</td>
</tr>
<tr>
<td>16:1 Palmitoleic acid</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>18:0 Stearic acid</td>
<td>4.0</td>
<td>2.0</td>
</tr>
<tr>
<td>18:1 Oleic acid</td>
<td>23.0</td>
<td>53.0</td>
</tr>
<tr>
<td>18:2 Linoleic acid</td>
<td>48.0</td>
<td>23.0</td>
</tr>
<tr>
<td>18:3 Linolenic acid</td>
<td>6.0</td>
<td>10.0</td>
</tr>
<tr>
<td>18:4 Stearidonic acid</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>20:1 Gadoleic acid</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>20:5 EPA</td>
<td>0.2*</td>
<td>0.2*</td>
</tr>
<tr>
<td>22:6 DHA</td>
<td>0.5*</td>
<td>0.5*</td>
</tr>
<tr>
<td>Total Antioxidant Status (TEAC mmol/L)</td>
<td>0.67 ± 0.01</td>
<td>0.66 ± 0.01</td>
</tr>
</tbody>
</table>

*Fatty acid source coming from fish meal found within the diet.
Information provided by Speciality Feeds (Western Australia), which produced both diets.
3.3.2 Measurement of blood pressure

Blood pressure was measured weekly in all the treatment groups using a tail cuff sphygmomanometer (Biopac Systems, USA). The animals were placed into a restraint and the tail was warmed briefly under a heat lamp. Then the latex cuff was placed around the base of the tail and the blood pressure was recorded. For each animal systolic blood pressure was obtained as an average of three readings.

3.3.3 Blood collection and processing

After the animal was anaesthetised, blood was collected via cardiac puncture into EDTA coated tubes. Immediately after blood collection, samples were centrifuged at 600 xg for 10 minutes at 4°C. The plasma was then removed and stored at -80°C until analysis of plasma lipids: triglycerides, total cholesterol, high density lipoprotein cholesterol (HDL-C) and LDL-C, and MDA. RBCs were then washed 3 times by adding an equal volume of 0.9% (w/v) NaCl, mixed carefully and centrifuged at 4°C at 600 xg for 10 minutes. The supernatant was removed and discarded. An equal volume of cold distilled water and RBCs were mixed well to lyse the cells. The hemolysate was stored at -80°C for subsequent analysis of antioxidant enzymes: SOD, catalase and GPx, and MDA.

3.3.4 Erythrocyte antioxidant enzymes

3.3.4.1 SOD assay

SOD activity was determined using a commercially available kit (Cayman Chemical Company, USA) following manufactures instructions. This assay utilizes xanthine oxidase and hypoxanthine to generate superoxide radicals
detected by tetrazolium salt. One unit of SOD is defined as the amount of enzyme required to inhibit the distmutation of the superoxide radical by 50%.

Firstly, all reagents within the kit were equilibrated to room temperature prior to beginning the assay. Then the following reagents were prepared. The assay buffer dilute was made up by adding 3 ml assay buffer concentrate provided to 27 ml milli-Q water. The sample buffer was made up by adding 2 ml sample buffer concentrate provided to 18 ml milli-Q water. The radical detector was made up prior to use, and 50 µl of radical detector was added to 19.95 ml assay buffer dilute. Xanthine oxidase was made up by adding 50 µl of the enzyme to 1.95 ml sample buffer dilute and stored on ice. The SOD stock standard (4.25 mM) was prepared by diluting 20 µl of SOD standard with 1.98 ml sample buffer dilute. Then the SOD stock was further diluted in separate eppendorf tubes with sample buffer dilute as shown in Table 3.2.

**Table 3.2: SOD standards**

<table>
<thead>
<tr>
<th>Tube</th>
<th>Formaldehyde Stock (µl)</th>
<th>Sample Buffer (µl)</th>
<th>Final Concentration (µM formaldehyde)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>1,000</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>980</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>40</td>
<td>960</td>
<td>15</td>
</tr>
<tr>
<td>D</td>
<td>80</td>
<td>920</td>
<td>30</td>
</tr>
<tr>
<td>E</td>
<td>120</td>
<td>880</td>
<td>45</td>
</tr>
<tr>
<td>F</td>
<td>160</td>
<td>840</td>
<td>60</td>
</tr>
<tr>
<td>G</td>
<td>200</td>
<td>800</td>
<td>75</td>
</tr>
</tbody>
</table>

The SOD reaction was set up in a 96 well plate. Firstly, 200 µl of diluted radical detector and 10 µl of standard were added to the designated wells on the plate.
Then, the positive control wells were prepared: 200 µl of diluted radical detector and 10 µl of diluted catalase were added to two wells. Next the sample wells were prepared: 200 µl of diluted radical detector and 10 µl of sample to two wells. Then the reaction was initiated by adding 20 µl of dilute xanthine oxidase to all the wells. The plate was covered and incubated at room temperature for 20 minutes. Next, the absorbance was read at 540 nm using a microplate analyser (Fusion-Alpha HT, PerkinElmer).

3.3.4.2 Catalase assay

Catalase activity was determined using a commercially available kit (Cayman Chemical Company, USA) following manufactures instructions. This method is based on the reaction of methanol with the enzyme in the presence of an optimal concentration of hydrogen peroxide.

Firstly, all the reagents within the kit were equilibrated to room temperature prior to beginning the assay. Then the following reagents were prepared. The assay buffer was made up by adding 2 ml assay buffer to 18 ml milli-Q water. The samples buffer was made up by adding 5 ml sample buffer to 45 ml milli-Q water. The catalase control was made up by reconstituting the catalase enzyme with 2 ml sample buffer dilute. Then 100 µl of the reconstituted enzyme was added to 1.9 ml sample buffer dilute, store on ice. Potassium hydroxide was made up by adding 4 ml cold milli-Q water. Hydrogen peroxide was made up by adding 40 µl hydrogen peroxide with 9.96 ml milli-Q water. The hemolysate was diluted to 3000/1 using sample buffer dilute. The formaldehyde stock standard (4.25 mM) was prepared by diluting 10 µl of formaldehyde standard with 9.99 ml
sample buffer. Then the formaldehyde stock was further diluted in separate eppendorf tubes (labelled A-G) with sample buffer as shown in Table 3.3.

### Table 3.3: Formaldehyde standards

<table>
<thead>
<tr>
<th>Tube</th>
<th>Formaldehyde Stock (µl)</th>
<th>Sample Buffer (µl)</th>
<th>Final Concentration (µM formaldehyde)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>1,000</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>990</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>30</td>
<td>970</td>
<td>15</td>
</tr>
<tr>
<td>D</td>
<td>60</td>
<td>940</td>
<td>30</td>
</tr>
<tr>
<td>E</td>
<td>90</td>
<td>910</td>
<td>45</td>
</tr>
<tr>
<td>F</td>
<td>120</td>
<td>880</td>
<td>60</td>
</tr>
<tr>
<td>G</td>
<td>150</td>
<td>850</td>
<td>75</td>
</tr>
</tbody>
</table>

The catalase reaction was set up in a 96 well plate. Firstly, 100 µl of assay buffer, 30 µl of methanol and 20 µl of standard was added to the designated wells on the plate. Then, the positive control wells were prepared: 100 µl of assay buffer, 30 µl methanol and 20 µl of diluted catalase were added to two wells. Next the sample wells were prepared: 100 µl of assay buffer, 30 µl methanol and 20 µl of sample to two wells. Then the reaction was initiated by adding 20 µl of dilute hydrogen peroxide to all the wells. The plate was covered and incubated at room temperature for 20 minutes. Then to each well 30 µl of potassium hydroxide was added followed by 30 µl of Purpald (chromogen). The plate was covered, placed on a platform shaker and incubated at room temperature for 10 minutes. Then, to each well 10 µl of potassium periodate was added and the plate was covered and further incubated at room temperature for five minutes on a platform shaker. Next, the plate was placed in a microplate analyser (Fusion-Alpha HT, PerkinElmer) and the absorbance read at 540 nm.
3.3.4.3 GPx assay

GPx activity was determined using a modification of the method of Wheeler et al. 1990. This assay is based on the oxidation of NADPH following the reduction of t-butyl hydroperoxide. A decrease in absorbance at 340 nm results from oxidation of NADPH to NADP+ and the rate of this decrease is proportional to the GPx activity in the sample.

Firstly the NADPH, glutathione reductase, glutathione, daily reagent and start reagent solutions were prepared. The NADPH solution was made up by mixing 4 mg of NADPH together with 2.133 ml of 0.1% NaHCO₃. The glutathione reductase solution was made up by adding 13 µl glutathione reductase to 250 µl phosphate buffer, pH 7.0. The glutathione solution was made up by mixing 35 mg of glutathione reductase together with 500 µl phosphate buffer, pH 7.0. The daily reagent was made up by adding 9.25 ml of phosphate buffer, pH 7.0, 1.25 ml NADPH solution, 0.250 µl glutathione reductase solution and 500 µl glutathione solution. The start reagent was made by adding 10 ml of distilled water along with 18.5 µl of t-butyl hydroperoxide. The hemolysate was diluted to 300/l with distilled water. Next, 24 µl of sample, 30 µl of distilled water and 135 µl of daily reagent was added to a cuvette and incubated at room temperature for 50 seconds. Then to the cuvette 20 µl of start reagent and 16 µl distilled water was added and the absorbance read every 25 seconds for 4 minutes and 10 seconds at 340 nm using a spectrophotometer (Biochrom, USA).
3.3.5 Haemoglobin assay

The antioxidant enzymes were all normalised to haemoglobin. Firstly, the hemolysate was diluted to 1:20 by adding 6 µl hemolysate to 54 µl distilled water. Then to a cuvette, 20 µl of diluted hemolysate was added to 480 µl Darbkin’s solution (1:500 dilution), mixed and left to stand for 5 minutes. Then the absorbance was read at 540 nm using a spectrophotometer (Biochrom, USA). The haemoglobin concentration for each sample was calculated using the following equation:

\[
\text{Hb (g/ml)} = \frac{\text{Abs}}{44} \times 64.458 \times \frac{401}{10}
\]

Where:
- \text{Abs} = \text{absorbance @ 540 nm of 500/1 dilution of sample in Drabkins}
- 64.458 = \text{molecular weight of Hb (g/mol)}
- 44 = \text{millimolar extinction coefficient of Hb at 540 nm}

The GPx samples were normalised to haemoglobin using the following equations:

Equation 1: \( \text{Hb sample} = \frac{\text{Hb (g/ml)}}{100} \times 24 \times 300 \)

Equation 2: \( \text{GPx U/ml Hb} = \frac{\text{GPx sample (U)}}{\text{equation 1}} \)

Where:
- 100 = conversion of g/dL to g/ml
- 24 = GPx sample quantity
- 300 = GPx sample dilution

The SOD samples were normalised to haemoglobin using the following equation:

\( \text{SOD U/gm Hb} = \frac{\text{SOD sample (U/ml)}}{\text{Hb (g/ml)}} \)

The catalase samples were normalised to haemoglobin using the following equation:

\( \text{Catalase mmol/min/gm Hb} = \frac{\text{Catalase sample (nmol/min/ml)}}{\text{Hb (g/ml)}} \times 1000 \)
3.3.6 Lipid peroxidation analysis

3.3.6.1 MDA

MDA was determined via high performance liquid chromatography (HPLC) in plasma and erythrocytes according to the method by Sim et al. 2003. The principle of this method is that 2,4-di-nitrophenylhydrazine (DNPH) is used to derivatise MDA contained in plasma or erythrocytes which forms stable hydrazones that can be easily separated by HPLC.

The MDA standard stock solution (10 mM) was prepared by adding 23.9 µl of tetraethoxypropane to 10 ml 1% sulphuric acid. This stock solution was further diluted to 100 µM by adding 50 µl standard stock solution to 4.95 ml milli-Q water. External standards of MDA aliquots of suitable concentrations were used by diluting the 100 µM standard solution as shown in Table 3.4. Standards were prepared for the analysis of plasma and hemolysate samples. For the analysis of the plasma samples, 50 µl of each standard along with 50 µl distilled water was added to separate eppendorf tubes. For the analysis of the hemolysate samples, 100 µl of each standard was added to separate eppendorf tubes. Then the standards were treated in the same manner as the samples, described below.

Table 3.4: MDA standards

<table>
<thead>
<tr>
<th>Final µM concentration of standard</th>
<th>Volume (µl) of 100 µm standard</th>
<th>Volume of milli-Q water (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>50</td>
<td>950</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>900</td>
</tr>
<tr>
<td>20</td>
<td>200</td>
<td>800</td>
</tr>
<tr>
<td>40</td>
<td>400</td>
<td>600</td>
</tr>
</tbody>
</table>
The hemolysate samples were diluted to 1 in 4 using distilled water. Then, 100 µl of the hemolysate samples or 50 µl of the plasma samples along with 50 µl of distilled water were added to separate eppendorf tubes. To the standards and samples 400 µl of 1.3 M sodium hydroxide (NaOH) was added to hydrolyse the standards and samples, then the eppendorf tubes were vortexed, incubated at 60°C for 60 minutes and cooled on ice for 5 minutes. To precipitate the proteins, 200 µl of 35% perchloric acid was added to the samples and standards, cooled on ice for 5 minutes and centrifuged at 8000 rpm for 10 minutes. Then, 650 µl of the supernant was removed and placed into an eppendorf tube. The standards and samples were protected from light from this step onwards. To the supernant, 30 µl of DNPH reagent was added and incubated for 10 minutes at room temperature. Next, the aqueous phase was extracted by adding 700 µl of hexane, and the eppendorfs were placed on a platform shaker for 10 minutes at room temperature. Then, 650 µl of the aqueous phase (hexane layer, supernant) was removed and transferred into eppendorf tubes. The extraction of the aqueous phase was repeated a further time, and to the pellet 700 µl of hexane was added, and the eppendorfs were placed on a platform shaker for 10 minutes at room temperature. The aqueous phase was removed and transferred and combined with the first extracted aqueous phase. The hexane was evaporated and the dry extract was reconstituted with a 100 µl mobile phase, which contains 450 ml acetonitrile, 550 ml distilled water and 2 ml glacial acetic acid (the mobile phase was filtered and degassed prior to use). Then, a volume of 45 µl was injected into the column for analysis. MDA concentrations were determined at 310 nm using HPLC (Agilent Technologies, Australia) with an Eclipse XDB-C18 column (150 x 4.6 mm, 5 µm, 1 ml/min flow rate, 9.8 MPa backpressure).
3.3.6.1 8-isoprostane

Total 8-isoprostane concentrations were analysed in plasma using an enzyme immunoassay (EIA) kit (Cayman Chemicals, USA) following manufactures instructions. This assay is based on the competition between 8-isoprostane and an 8-isoprostane acetylcholinesterase (AChE) conjugate for a limited number of 8-isoprostane -specific rabbit anti-serum binding sites.

The plasma samples were prepared by using the method from Cell Biolabs, INC. The plasma samples were hydrolysed by adding 25 µl 2M NaOH to 100 µl plasma sample. The samples were incubated at 45°C for 2 hours. Following this, 25 µl 10N HCl acid was added and the samples were centrifuged for 5 minutes at 12,000 RPM. The supernatant was removed and used for the determination of total 8-isoprostane using the EIA kit.

The standards were prepared by transferring 100 µl 8-isoprostane EIA standard into an eppendorf tube along with 900 µl milli-Q water (standard stock concentration 5 ng/ml). Then 8 eppendorf tubes were labelled from 1 to 8, and 900 µl EIA buffer was added to the eppendorf tube labelled 1 and 750 µl EIA buffer to the eppendorf tubes labelled 2-8. Then 100 µl of standard stock (5 ng/ml) was transferred to tube 1 and mixed thoroughly. The standards were serially diluted by removing 500 µl from tube 1 and placing in tube 2; mixed thoroughly. Next, 500 µl from tube 2 was removed and placed in tube 3; mixed thoroughly. This process was repeated for tubes 4-8.
The reactions were set up in a mouse anti-rabbit IgG coated plate. To the non-specific binding (NSB) wells and to the maximum binding (B₀) wells 10 µl EIA buffer or 50 µl EIA buffer were added, respectively. Next, 50 µl of standard or sample were added to the wells. Following this 50 µl 8-isoprostane AChE tracer was added to the well except to the total activity (TA) and blank (Blk) wells. Then, 50 µl 8-isoprostane EIA antiserum was added to each well except to the TA, NSA and Blk wells. The plate was incubated at 4°C for 18 hours. The wells were emptied and rinsed 5 times with wash buffer. Next, 200 µl Ellman’s reagent was added to each well and to the TA wells 5 µl of tracer was added. The plate was covered with foil and incubated at room temperature for 90 minutes. The plate cover was removed and the absorbance read between 405-420 nm using a microplate analyser (Fusion-Alpha HT, PerkinElmer).

3.3.7 Plasma lipids analysis

3.3.7.1 Triglycerides

Plasma triglycerides were determined using a commercially available kit (Thermo Scientific, USA). This procedure was set up in a 96 well plate, and 3 µl of calibrator or sample was added to 300 µl of triglyceride reagent and incubated at 37°C for 5 minutes. The absorbance was read at 500 nm using a microplate analyser (Fusion-Alpha HT, PerkinElmer).

3.3.7.2 Total cholesterol

Total cholesterol was determined using a commercially available kit (Thermo Scientific, USA). This procedure was set up in a 96 well plate, and 3 µl of calibrator or sample was added to 300 µl of cholesterol reagent and incubated at
37°C for 5 minutes. The absorbance was read at 500 nm using a microplate analyser (Fusion-Alpha HT, PerkinElmer).

### 3.3.7.3 HDL-C

HDL-C was determined using a commercially available kit (Thermo Scientific, USA). This procedure was set up in a 96 well plate, and 4 µl of calibrator or sample was added to 300 µl of HDL reagent 1 and incubated at 37°C for 5 minutes. Then, 100 µl of HDL reagent 2 was added and incubated at 37°C for 3 minutes. The absorbance was read at 600 nm using a microplate analyser (Fusion-Alpha HT, PerkinElmer).

### 3.3.7.4 LDL-C

LDL-C was determined using the Friedewald equation (Warnick et al. 1990):

\[
\text{LDL cholesterol} = \text{Total Cholesterol} - \text{HDL cholesterol} - \left(\frac{\text{TG}}{5}\right).
\]

### 3.3.8 Statistical analysis

Statistical analysis was performed using the SPSS statistical package (version 17.0, SPSS Inc.) for repeated measures ANOVA and one-way ANOVA. The results are represented as mean ± SEM. Comparisons between groups for animal body weight, food intake and water intake data were analysed using repeated measures ANOVA. A post hoc pair-wise comparison was also carried out. Statistical analysis of the survival time data was performed by Log-Rank and Wilcoxon’s nonparametric tests using GraphPad Prism 5 software. The differences between group means for the antioxidant enzymes, MDA and plasma...
lipids were made by one-way ANOVA. Significance was established at the 95% confidence level ($P < 0.05$).

3.4 Results

3.4.1 Establishment of canola-induced life shortening model

Canola oil ingestion significantly decreased the lifespan of SHRSP rats ($P < 0.001$). Mean lifespan of the two diets was 98.27 ± 3.35 days in the soybean oil group compared to 85.83 ± 1.13 days in the canola oil group (Figure 3.1), confirming the establishment of the model.

![Survival curves of SHRSP rats fed a diet containing 10% soybean oil or canola oil with 1% NaCl loading.](image)

The curves are significantly different ($P < 0.001$, Log-rank and Wilcoxon’s test). At the commencement of the study the number of animals in each group was 12.
3.4.2 Body weight, food intake and water intake

Body weight of the animals increased gradually until the 5\textsuperscript{th} week of administration in both diet groups (Figure 3.2). There were no significant differences ($P > 0.05$) between soybean oil and canola oil groups. There were also no significant differences ($P > 0.05$) in food consumption and water intake between the soybean oil and canola oil groups (Figure 3.3).

![Figure 3.2: Mean body weight of SHRSP rats fed canola oil compared with soybean oil diet.](image)

Values are means ± SEM. At the commencement of the study the number of animals in each group was 12. The numbers on the graph represent the n in each group.

* $P < 0.05$ represents a significant difference between canola and soybean oil groups.
Figure 3.3: Food and water intake of SHRSP rats fed canola oil compared with soybean oil diet.

Values are means ± SEM. At the commencement of the study the number of animals in each group was 12. The numbers on the graph represent the n in each group.
3.4.3 Blood pressure

Mean systolic blood pressure increased over time in the soybean oil and canola oil groups. At the 6th week of administration the mean systolic blood pressure was significantly higher in the canola oil group compared to soybean oil, 230.7 ± 3.52 and 213.61 ± 3.33 mmHg, respectively ($P < 0.05$) (Figure 3.4).

**Figure 3.4:** Mean systolic blood pressure of SHRSP rats fed canola oil compared with soybean oil diet.

Values are means ± SEM. At the commencement of the study the number of animals in each group was 12.

*$P < 0.05$ represents a significant difference between canola and soybean oil groups.
3.4.4 Antioxidant enzymes and oxidative damage

Markers of antioxidant status and oxidative damage are represented in Table 3.5. Canola oil ingestion significantly reduced \((P < 0.05)\) the activities of RBC SOD, GPx and catalase compared to soybean oil. The activities of RBC SOD and GPx were significantly lower \((P < 0.05)\) in the canola oil group compared to pre-treatment, however, there were no significant differences \((P > 0.05)\) between the soybean oil group and pre-treatment group.

Canola oil ingestion significantly decreased \((P < 0.05)\) plasma MDA and 8-isoprostane in SHRSP rats compared to the soybean oil group. Nevertheless, plasma 8-isoprostane in the soybean oil group and MDA in both canola oil and soybean oil groups was significantly higher \((P < 0.05)\) than the pre-treatment group. Lastly, RBC MDA in the soybean oil group was significantly lower \((P < 0.05)\) than the pre-treatment group.
Table 3.5: Antioxidant status and oxidative damage in SHRSP rats fed canola oil compared with soybean oil diet.

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Soybean oil</td>
</tr>
<tr>
<td>RBC SOD (U/gm Hb)</td>
<td>1608.7 ± 121.8</td>
<td>1454 ± 111.3</td>
</tr>
<tr>
<td>RBC GPx (U/gm Hb)</td>
<td>523.0 ± 25.9</td>
<td>512 ± 36.6</td>
</tr>
<tr>
<td>RBC Catalase (mmol/min/gm Hb)</td>
<td>155.6 ± 13.4</td>
<td>208.2 ± 35.1</td>
</tr>
<tr>
<td>RBC MDA (µM)</td>
<td>28.6 ± 1.6</td>
<td>16.6 ± 3.5#</td>
</tr>
<tr>
<td>Plasma MDA (µM)</td>
<td>11.8 ± 0.5</td>
<td>17.9 ± 0.5#</td>
</tr>
<tr>
<td>Plasma 8-isoprostane (pg/ml)</td>
<td>75.2 ± 12.3</td>
<td>96.2 ± 3.4#</td>
</tr>
</tbody>
</table>

Values are means ± SEM; Number of animals at pre-treatment was 10. Number of animals in each group at post-treatment for soybean oil and canola oil was 9 and 8, respectively. SOD, superoxide dismutase; GPx, glutathione peroxidase; MDA, malondialdehyde.

*P < 0.05 represents a significant difference between canola and soybean oil groups; #P < 0.05 represents a significant difference between pre-treatment vs post-treatment.
3.4.5 Plasma lipids

Canola oil ingestion significantly reduced ($P < 0.05$) the concentration of total cholesterol and LDL-C compared with soybean oil. Total cholesterol and LDL-C were significantly lower ($P < 0.05$) in the canola oil group compared with pre-treatment, however, there were no significant differences ($P > 0.05$) between the soybean oil group and pre-treatment group. HDL-C in both canola oil and soybean oil groups was significantly lower ($P < 0.05$) than the pre-treatment group. There were no significant differences ($P > 0.05$) in the concentration of triglycerides between groups (Table 3.6).

Table 3.6: Plasma lipids in SHRSP rats fed canola oil compared with soybean oil diet.

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment (mmol/L)</th>
<th>Post-treatment (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soybean oil</td>
<td>Canola oil</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>7.9 ± 0.2</td>
<td>7.9 ± 0.4</td>
</tr>
<tr>
<td>LDL-C</td>
<td>5.3 ± 0.2</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>HDL-C</td>
<td>2.4 ± 0.1</td>
<td>1.8 ± 0.1#</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SEM; Number of animals at pre-treatment was 10. Number of animals in each group at post-treatment for soybean oil and canola oil was 9 and 8, respectively.

$*P < 0.05$ represents a significant difference between canola and soybean oil groups; $^#P < 0.05$ represents a significant difference between pre-treatment vs post-treatment.
3.5 Discussion

Previous studies have shown that the lifespan of SHRSP rats is shortened when canola oil is the only dietary fat source (Huang et al. 1996 & 1997; Natio et al. 2003; Ogawa et al. 2003; Ohara et al. 2006; Ratnayake et al. 2000a & 2000b), which is confirmed by the present study, which showed shortened lifespan of about 13% with canola oil feeding compared with soybean oil under 1% NaCl loading. Our results are similar to the study published by Ratnayake et al. (2000b), which produced a 13% reduction in life span with canola oil feeding. Canola oil has been reported to shorten the life span of SHRSP rats compared with soybean oil, 254 ± 12 and 416 ± 16 days, respectively, even without NaCl loading (Huang et al. 1997).

Evidence indicates that canola oil intake has an effect on blood pressure in the SHRSP rat and its related strains. However, blood pressure in the present study is not a key contributing factor to the shortened life span, as there was only one time difference between soybean oil and canola oil. The results of the present study show an increase in systolic blood pressure over time in both treatment groups, with a difference between the groups at the 6th week of administration. A study by Huang et al. (1996) observed no significant change in systolic blood pressure in the canola oil group compared to the soybean oil group at 4 and 8 weeks of age (Huang et al. 1996). Another study by Ratnayake et al. (2000a) found no significant differences in the SBP among different dietary groups in SHRSP rats (Ratnayake et al. 2000a).
While previous literature has shown that decreased antioxidants and oxidative stress play a role in hypertension, to our knowledge this is the first study to investigate the effect of canola oil on measures of antioxidant status and oxidative damage in SHRSP rats. In the present study canola oil ingestion decreased the activities of SOD, GPx and catalase compared with the soybean oil group by 25, 18 and 31%, respectively. Previous studies have analysed several antioxidant enzymes in SHR and WKY rats. In the SHR, the activity of RBC GPx and the activities of SOD and catalase in the hepatic cytosol were found to be reduced (Ohara et al. 2008). In WKY rats, canola oil ingestion reduced the activity of catalase in the hepatic cytosol (Ohara et al. 2009), while an earlier study showed reduced activities of SOD and catalase in the hepatic cytosol of WKY rats (Natio et al. 2000d). Our data are largely consistent with these findings. Taken together these results indicate that canola oil ingestion affects antioxidant enzyme activity in different tissues. Indeed, previous research has shown an inverse relationship between erythrocyte GPx activity and the incidence of cardiovascular disease (Blankenberg et al 2003). Given the physiological significance of the decreased erythrocyte GPx activity, further research is required to determine the mechanism to explain the canola-oil induced changes to antioxidant enzyme activity.

In the present study, canola oil ingestion decreased the plasma 8-isoprostane and MDA concentration when compared to the soybean oil group. These results are unexpected given the canola oil-induced decrease in erythrocyte antioxidant enzyme activity. The mechanism to explain the decreased plasma 8-isoprostane and MDA concentration despite a decrease in RBC antioxidant enzyme activity is currently unknown. Plasma 8-isoprostane in the soybean oil and MDA in both
the canola oil and soybean oil groups was higher than the pre-treatment group. In a previous study in WKY rats, canola oil ingestion increased lipid peroxide levels in the hepatic cytosol (Ohara et al. 2009), while an earlier study showed no change in lipid peroxide levels in the hepatic cytosol of SHR (Ohara et al. 2008). The increased plasma 8-isoprostane in the soybean oil group and MDA concentrations in both dietary groups compared to pre-treatment indicates an increased amount of ROS induced lipid peroxidation over time independent from the consumption of either canola or soybean oil. Previous investigations that have determined the potential for cellular membranes to undergo oxidation based on their fatty acid composition (Tsalouhidou et al. 2006) suggest that the change in lipid peroxidation observed in the present study is in contrast to what may be expected based on the fatty acid composition of canola and soybean oil. Canola oil has relatively more n-9 monounsaturated fatty acids, more readily oxidised, compared to soybean oil that has proportionally more n-6 polyunsaturated fatty acids, less readily oxidised (Tatematsu et al. 2004). However, only focusing on the fatty acid composition alone of the two oils is too simplistic as the TAS for both diets was the same and the lower concentration of RBC MDA in the soybean oil group may have resulted from the higher RBC antioxidant activities compared to the canola oil group. Whichever the case the specific mechanism for these differences in RBC lipid peroxidation associated with the consumption of canola or soybean oil was not identified in the present study. Therefore, more research is clearly required to investigate the effect of canola oil intake on oxidative damage in SHRSP rats.
Canola oil ingestion reduced the concentration of total cholesterol and LDL-C compared with soybean oil and the pre-treatment group. HDL-C was lower in both dietary groups compared with pre-treatment, and there were no changes found in the triglyceride levels between the groups. However, previous studies in SHR and WKY rats have shown increases in total cholesterol, triglycerides and HDL-C with administration of canola oil compared to soybean oil (Natio et al. 2000b & 2000d; Ohara et al. 2008 & 2009). A study by Ohara et al. (2006) found no changes in the plasma lipids in the canola oil group after an eight week feeding trial in SHRSP rats (Ohara et al. 2006). Canola oil is considered to provide protective cardiovascular effects due to its favourable fatty acid composition (Ratnayake et al. 2000b). Canola oil has a high content of oleic acid (55-60%), a low content of SFA (6-7%), and provides a good source of omega-3 fats (Tatematsu et al. 2004). The present study supports the beneficial health effects of canola oil, as the plasma lipids were reduced in SHRSP rats. A previous study has shown that a canola oil rich diet reduced plasma cholesterol, LDL-C and HDL-C concentrations in middle-aged and elderly hypercholesterolemic subjects (Lichtenstein et al. 1993). Canola oil has also been shown to reduce total plasma cholesterol and LDL-C in the young men (McDonald et al. 1989). Whilst this present study supports the beneficial health effects of canola oil on blood lipids, it should also be pointed out that the metabolism of cholesterol in rats is different from cholesterol metabolism in humans (Dietschy & Wilson 1970). Therefore direct extrapolation of our circulating cholesterol results into humans must be done so with caution. However, the effects of canola oil ingestion in the SHRSP rat is still detrimental as this oil leads to a shorten lifespan in SHRSP rats.
There are a number of limitations associated with the present study in addition to the differences between cholesterol metabolism in rats and humans. The first is the question of whether the life shortening effect associated with canola oil consumption is specific to rats originally derived from the normotensive WKY rat strain. When canola oil was the only dietary fat fed to WKY rats, a significant increase in systolic blood pressure after five weeks of canola oil feeding was observed and persisted until the conclusion of the trial at week 13, compared to soybean oil fed rats (Natio et al. 2000). In addition when SHR rats were fed canola oil canola they developed vascular lesions in the kidney, had elevated plasma lipids and G6PD activation in the liver and erythrocytes compared to SHR fed soy oil (Ohara et al. 2008a). In human populations there is only one published incidence of canola oil being associated with the development of disease (Posada de la Paz et al. 2001) and this due was to manufacturing error when producing canola oil. The present study did not directly investigate the effect of canola oil on cell membrane fatty acid composition, fluidity, antioxidant capacity or oxidized membrane lipids. Although MDA was measured in plasma and lysed red blood cells, the membrane fraction of red blood cells or other organs was not isolated prior to measurement of MDA. A change to cell membrane composition has previously been investigated as a potential mechanism to explain the life shortening effects of canola oil in the SHRSP rat. These previous studies suggested that hyperabsorption and accumulation of plant sterols found in canola oil altered the fluidity of the membranes present in red blood cells, liver and kidney and contribute to the life shortening effect observed in SHRSP rats (Ratnayake et al. 2000b). Finally NaCl loading is used to accelerate the development of hypertension in the SHRSP rat (Huang et al. 1996 & 1997; Natio
et al. 2003; Ogawa et al. 2003; Ohara et al. 2006; Ratnayake et al. 2000a), however it may be masking the effects of canola oil in the SHRSP rat, thus further investigation on the life shortening effect of canola oil in the absence of NaCl are required.

In conclusion, we have shown that canola oil ingestion mediated lifespan shortening of SHRSP rats leads to changes in oxidative status. The plasma lipids were reduced after canola oil ingestion highlighting the health benefits of canola oil intake. Despite the improvement in the plasma lipids, canola oil is detrimental to the SHRSP rat as their lifespan is reduced. Further research is required to determine whether oxidative stress plays a role in SHRSP rats due to canola oil ingestion.
CHAPTER 4
The effect of short-term canola oil ingestion on oxidative stress in the vasculature of stroke-prone spontaneously hypertensive rats

Publication

4.1 Abstract

Background
This study aimed to determine if 25 days of canola oil intake in the absence of excess dietary salt or together with salt loading affects the antioxidant and oxidative stress markers in the circulation. A further aim was to determine the mRNA expression of NADPH oxidase subunits and SOD isoforms in the aorta of SHRSP rats.

Methods
Male SHRSP rats, were fed a defatted control diet containing 10 wt/wt% soybean oil or a defatted treatment diet containing 10 wt/wt% canola oil, and given tap water or water containing 1% NaCl. Blood was collected at the end of study for analysis of RBC antioxidant enzymes, RBC and plasma MDA, plasma 8-
isoprostane and plasma lipids. The aorta was removed and the mRNA expression of NOX2, p22<sup>phox</sup>, CuZn-SOD, Mn-SOD and EC-SOD were determined.

**Results**

In the absence of salt canola oil reduced RBC SOD and GPx, and increased total cholesterol and LDL-C compared with soybean oil. RBC GPx was significantly lower in both the salt loaded groups compared to the soybean oil only group. In addition, RBC MDA and plasma HDL-C were significantly higher in both the salt loaded groups compared to the non salt groups. Plasma MDA concentration was higher and LDL-C concentration lower in the canola oil group loaded with salt compared to the canola oil group without salt. The mRNA expression of NADPH oxidase subunits and SOD isoforms were significantly reduced in the canola oil group with salt compared to canola oil group without salt.

**Conclusion**

In conclusion, these results indicate that canola oil reduces antioxidant status and increases plasma lipids, which are risk factors for CVD. However, canola oil in combination with salt intake increases MDA, a marker of lipid peroxidation and decreases NAPDH oxidase subunits and SOD aortic expression.

4.2 Introduction

Evidence has shown that ingestion of canola oil as the sole dietary fat source (added at 10 wt/wt% to standard rat chow) shortens the life span of SHRSP rats compared to the soybean oil or perilla oil (Huang et al. 1996 & 1997; Natio et al. 2003; Ogawa et al. 2003; Ohara et al. 2006; Ratnayake et al. 2000a & 2000b). Our recent study strengthened this finding, and showed that canola oil ingestion
reduced the lifespan of SHRSP rats compared to soybean oil following 1% NaCl loading, 85.8 ± 1.1 and 98.3 ± 3.4 days, respectively (Papazzo et al. 2011).

The mechanism by which canola oil reduces lifespan is currently unknown; however, decreased antioxidant activity and heightened oxidative stress have been implicated. The results from our lifespan study showed that canola oil intake reduced the antioxidant activities of RBC SOD, GPx and catalase compared to soybean oil in SHRSP rats following NaCl loading at the end of their lifespan (Papazzo et al. 2011). Furthermore, canola oil intake increased plasma MDA compared to pre-treatment, suggesting an increase in lipid peroxidation overtime (Papazzo et al. 2011). RBCs can provide protective mechanisms against oxidative damage to endothelial cells by neutralising ROS in the circulation (Richards et al. 1998b). Previous research has shown an inverse relationship between reduced activities of antioxidants (SOD and GPx) and increased lipid peroxidation products in blood and cardiovascular disease (Kalenikova et al. 2004). Evidence has shown that in canola oil fed SHR there was an increase in RBC glutathione and glutathione reductase, with a decrease in the activity of RBC GPx. Furthermore, in the hepatic cytosol, the activity of SOD and catalase were significantly reduced (Ohara et al. 2008a). Similar results were also found in a study by Ohara et al. (2009), in which the activities of catalase, GPx and glutathione reductase were decreased in the liver of canola oil fed WKY rats. Taken together these results indicate that canola oil ingestion affects antioxidant enzyme activity in different tissues.
In vascular cells, NADPH oxidase is a major source of ROS, and is functionally active within all the layers of the vessel wall: in the endothelium, the media and the adventitia (Dworakowski et al. 2008; Touyz & Schiffrin 2004; Wassmann et al. 2004). In hypertensive patients, VSMCs from resistance arteries have increased ROS generation, and this increase is linked to NADPH oxidase (Paravicini & Touyz 2008). Evidence has shown that in SHR and SHRSP rats there was an enhanced production of $O_2^-$ derived by NADPH oxidase, and this was associated with the upregulation of $p22^{phox}$ mRNA expression in the aorta (Touyz & Schiffrin 2004; Zalba et al. 2001). Furthermore, NOX2 mRNA expression in the aorta was found to be greater in SHR compared to the normotensive WKY rats (Briones et al. 2011). In vascular cells, SOD is a major cellular antioxidant that provides defence against superoxide ($O_2^-$) (Wassmann et al. 2004). There are three isoforms of SOD which have been identified and include CuZn-SOD, Mn-SOD and Ec-SOD. The main vascular SOD is Ec-SOD, and is produced and secreted by VSMCs (Paravicini & Touyz 2008; Wassmann et al. 2004). Evidence has shown that in atherosclerotic vessels Ec-SOD expression is increased in apoE-deficient mice, a mouse model of atherosclerosis (Fukai et al. 1998).

Furthermore, the concentration of phytosterols in canola oil has also been suggested to be a contributing factor to the shortened lifespan. However, conflicting results have shown no clear correlation between the content of phytosterols in the diet and tissues and survival time observed (Ohara et al. 2006; Tatematsu et al. 2004). Moreover, NaCl loading may be masking the effects of dietary phytosterols and canola oil in the SHRSP rat. A recent study showed that
dietary phytosterols and phytostanols increase blood pressure in Wistar Kyoto rats in the absence of NaCl loading (Chen et al. 2010). Furthermore, research has shown that salt intake can induce oxidative stress, and leads to an increase in \(-O_2^-\) production in SHR and Sprague-Dawley rats (de Cavanagh et al. 2010; Kitiyakara et al. 2003). It has been shown that the increase in \(O_2^-\) production in Sprague-Dawley rats due to salt intake was accompanied by an increase in renal activity and mRNA expression of NADPH oxidase, and a decrease in CuZn-SOD and MnSOD mRNA expression (Kitiyakara et al. 2003). More research is required to investigate the effect of canola oil intake on oxidative damage and to examine the changes in the absence of excess dietary salt. This study aimed to determine if 25 days of canola oil intake in the absence or together with salt loading affects the antioxidant and oxidative stress markers in circulation and mRNA expression of NADPH oxidase subunits and SOD isoforms in the aorta of SHRSP rats.

4.3 Methods

4.3.1 Animal husbandry and study design

Approval for this project was granted by the Deakin University Animal Welfare Committee (Ethics approval no. A67/09). Forty male SHRSP rats (Deakin University, Australia) aged 4 weeks were randomly assigned to either group 1 (n = 20) or group 2 (n = 20). Within each group the rats were randomly assigned to a control and treatment group and acclimatized for one week. During acclimatization they were given a standard pellet diet (Specialty Feeds, Western Australia) and water ad libitum. The groups were then fed the following diets respectively, a defatted control diet containing 10 wt/wt% soybean oil or a
defatted treatment diet containing 10 wt/wt% canola oil (Speciality Feeds, Western Australia) for 25 days. Twenty five days was chosen based on our pervious study, which showed that the mean life span of the canola oil group was 85 ± 1.1 days (Papazzo et al. 2011). Given that the rats were 35 days old when they started the trial, they were on the diet for a mean of 50 days. We wanted to examine weather canola oil intake had an effect on oxidative stress earlier on in their life span and, thus, 25 days was chosen as a mid point. The fatty acid compositions of the diets are shown in Table 3.1 (Chapter 3). Group 1 was given water containing 1% NaCl and group 2 was given tap water throughout the trial. The reason for having the group without NaCl in the drinking water is to rule out any interfering factor the salt loading may have when analysing the tissue. The animals were maintained on a 12 hr light/dark photo-period with a room temperature of 21 ± 2°C. Animal body weights, food intake and water consumption were determined once a week, while the health of the animals was monitored daily. At the end of the 25 days the rats were anaesthetised via intra-peritoneal injection with lethabarb (50 mg/kg), and blood was collected for analysis. Following this, tissue collection was carried out and the aorta was removed, washed in saline solution and snap frozen in liquid nitrogen.

4.3.2 Measurement of blood pressure

Blood pressure was measured weekly and at day 25, and was preformed as outlined in Chapter 3, section 3.3.2.

4.3.3 Blood collection and processing

The blood was collected and processed as outlined in Chapter 3, section 3.3.3.
4.3.4 Erythrocyte antioxidant enzymes

4.3.4.1 SOD assay
SOD activity was measured as described in Chapter 3, section 3.3.4.1.

4.3.4.2 Catalase assay
Catalase activity was measured as described in Chapter 3, section 3.3.4.2.

4.3.4.3 GPx assay
GPx activity was determined using a commercially available kit (Cayman Chemical Company, USA) following manufacturers instructions. This assay is based on the oxidation of NADPH following the reduction of hydroperoxide. A decrease in absorbance at 340 nm results from oxidation of NADPH to NADP+ and the rate of this decrease is proportional to the GPx activity in the sample.

Firstly, the following reagents were prepared. The assay buffer dilute was made up by reconstituting the vial with 27 ml milli-Q water. The sample buffer dilute was made up by adding 2 ml sample buffer provided to 18 ml milli-Q water. The GPx control was made up by adding 10 µl of the enzyme to 490 µl sample buffer dilute, and stored on ice. The GPx co-substrate mixture was prepared by reconstituting the vial with 2 ml milli-Q water and stored at 25°C while carrying out the assay. The GPx reaction was set up in a 96 well plate. Firstly, the non-enzymatic wells were prepared: 120 µl of assay buffer dilute and 50 µl of co-substrate mixture were added to two wells. Then, the positive control wells were prepared: 100 µl of assay buffer dilute, 50 µl of co-substrate mixture and 20 µl of diluted GPx control were added to two wells. Next the sample wells were
prepared: 100 µl of assay buffer dilute, 50 µl of co-substrate mixture and 20 µl of sample were added to two wells. Then to each well 20 µl of cumene hydroperoxide was added to initiate the reaction. Next, the plate was placed in a microplate analyser (Fusion-Alpha HT, PerkinElmer) and the absorbance read at 540 nm once every minute for 10 minutes.

4.3.5 Haemoglobin assay

The antioxidant enzymes were all normalised to haemoglobin, and was measured as described in Chapter 3, section 3.3.5.

4.3.6 Lipid peroxidation analysis

4.3.6.1 MDA

MDA was determined via HPLC in plasma and erythrocytes and was measured as described in Chapter 3, section 3.3.6.1.

4.3.6.2 8-isoprostane

8-isoprostane was determined in plasma and was measured as described in Chapter 3, section 3.3.6.2

4.3.7 Plasma lipids analysis

4.3.7.1 Triglycerides

Plasma triglycerides were determined as described in Chapter 3, section 3.3.7.1.

4.3.7.2 Total cholesterol

Total cholesterol was determined as described in Chapter 3, section 3.3.7.2.
4.3.7.3 HDL-C

HDL-C was determined as described in Chapter 3, section 3.3.7.3.

4.3.7.4 LDL-C

LDL-C was determined as described in Chapter 3, section 3.3.7.4.

4.3.8 mRNA gene expression analysis

4.3.8.1 RNA extraction

RNA was extracted from the aorta using TRI reagent (Molecular Research Centre, USA) following the manufactures instructions. Firstly, all visible fat and connective tissue was removed from the aorta and 30 mg of tissue was crushed with liquid N₂ using a mortar and pestle. This crushed tissue was placed in a 2 ml screw top cryovials with 0.5 g homogenisation beads and 1ml of TRI Reagent. The cryovials were then placed in the fast-prep homogenizer (MP Biomedicals, Australia) for 20 seconds at speed 6 and samples were cooled on ice for 5 minutes. The homogenisation step in the fast prep for 20 seconds at speed 6 was repeated another two times, followed by centrifuging the samples at 12,000 xg for 10 minutes at 4°C. The supernant was removed and transferred to an eppendorf tube. Next, 100 µl of bromochloropropane was added and the samples were shaken vigorously for 15 seconds. The samples were stored for 5 minutes at room temperature, and then centrifuged at 12,000 xg for 15 minutes at 4°C. The supernatant (aqueous phase) was transferred to an eppendorf tube and the interphase and organic phase was kept and stored at -80°C for protein isolation. To the supernatant, 500 µl of cold isopropanol and 20 µl of 75% ethanol were added, and then the samples were gently shaken for 10 seconds. Next, the
samples were stored for 5 minutes at room temperature and then centrifuged at 12,000 xg for 8 minutes at 4°C. The supernatant was removed and discarded, and 1ml of cold 75% ethanol was added, and the samples were vortexed for 20 seconds. Then the samples were centrifuged at 7,500 xg for 5 minutes at 4°C. The supernatant was then removed and discarded and the RNA pellet was briefly air-dried for 5 minutes. The RNA was then dissolved in 30 µl of nuclease-free water and the solution was passed 5 times through a pipette tip. The RNA samples were then quantified using the NanoVue (GE Healthcare Biosciences, Australia).

4.3.8.2 Reverse transcription

First-strand cDNA was generated from 0.5 µg RNA using the Marligen first strand cDNA synthesis kit (Marligen, USA). Following RNA extraction, the samples were placed on ice. Next, a 10 µl reaction was set up in a micro-eppendorf tube for each sample by adding the following reagents: 2 µl of cDNA synthesis, 0.5 µl of reverse transcriptase, 1 µg (0-15 µl) of RNA sample and nuclease free water to make up the final volume of 10 µl. Next, the samples were placed in a MyCycler Thermal Cycler (Bio-Rad, USA) and the settings were as follows: 1 cycle at 22°C for 5 minutes, 1 cycle at 42°C for 30 minutes, 1 cycle at 85°C for 5 minutes and held at 4°C. The cDNA samples were then stored at -80°C until analysis of genes.

4.3.8.3 Reverse transcription-real-time PCR measurement of mRNA

Real-time PCR was performed using the iQ5 multicolour real-time PCR detection system (Bio-Rad, USA), with PCR reactions carried out using the iQ SYBR
Green Supermix (Bio-Rad, USA). The following targeted genes were analysed: NOX2, p22<sub>phox</sub>, CuZn-SOD, MnSOD and Ec-SOD, and the primers were all obtained from previous published articles (Table 4.1) (Reinehr et al. 2005; Cediel et al. 2003; Chabrashvili et al. 2003).

**Table 4.1: Real-time PCR primer sequences for genes of interest**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOX 2</td>
<td>TCAAGTGTCGCCAGGTATCC</td>
<td>CTTCACTGGCTGTACCAAGGTACCAAGGG</td>
</tr>
<tr>
<td>p22&lt;sub&gt;phox&lt;/sub&gt;</td>
<td>GCTCATCTGTCTGCTGGAGTA</td>
<td>ACGACCTCATCTGTCACTGGA</td>
</tr>
<tr>
<td>CuZn-SOD</td>
<td>TGTGTCCATTGAAGATCGTGTGA</td>
<td>TCTTGTGTCTCGTGGACCACC</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>TTAACGCAGCAGATCGATGCA</td>
<td>CCTCAGGTGACGTTCAGATTGT</td>
</tr>
<tr>
<td>Ec-SOD</td>
<td>GGCCCAGCTCCAGACTTGA</td>
<td>CTCAGGTCCCCGAACCTCAG</td>
</tr>
</tbody>
</table>

The primers were all obtained from previous published articles and were ordered through Geneworks (Australia): NOX2 (Reinehr et al. 2005), p22<sub>phox</sub> (Cediel et al. 2003), CuZn-SOD (Chabrashvili et al. 2003), MnSOD (Chabrashvili et al. 2003) and Ec-SOD (Chabrashvili et al. 2003).

Firstly, each primer was rehydrated to a final concentration of 100 µM using nuclease free water, and then further diluted to 2.4 µM. The cDNA samples prior to use were diluted to 1:10 using nuclease free water. RT-PCRs were set up in a total reaction of 25 µl that contained 12.5 µl iQ SYBR green supermix, 3.12 µl forward primer, 3.12 µl reverse primer, 3.12 µl nuclease free water and 3.12 µl cDNA sample. The PCR conditions were: 95°C for 4 minutes, and 40 cycles of 95°C for 10 seconds, 50°C for 30 seconds and 72°C for 20 seconds, followed by 95°C for 1 minute and then from 50-95°C read every 1°C, held every 3 seconds. Fluorescent emission data were captured and mRNA levels were analysed using the critical threshold (CT) value. The relative expression of the gene of interest
was calculated using the expression $2^{\Delta CT}$ and normalised to the cDNA concentration, and reported as arbitrary units (Sullivan-Gunn et al. 2011). Briefly, the cDNA concentration was quantified using the Quant-iT OliGreen ssDNA quantitation reagent kit (Invitrogen, Australia) according to the manufactures instructions. Firstly, the oligo standards were prepared as shown in table 4.2. Then, to a black 96-well fluoro plate 5 µl of standard or sample were loaded into the wells. Following this, 95 µl of 1x TE buffer and 100 µl of 1x ssDNA reagent were added to the wells. The plate was incubated at room temperature for 5 minutes protected from light, and the fluorescence read using a microplate analyser (Fusion-Alpha HT, PerkinElmer) set at excitation 485 nm and emission 538 nm.

### Table 4.2: Oligo standards

<table>
<thead>
<tr>
<th>Tube</th>
<th>Standard Concentration (ug/ml)</th>
<th>Volume of Standard</th>
<th>Volume of TE buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td>1 µl of stock</td>
<td>49 µl</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>10 µl of A</td>
<td>10 µl</td>
</tr>
<tr>
<td>C</td>
<td>0.5</td>
<td>5 µl of A</td>
<td>15 µl</td>
</tr>
<tr>
<td>D</td>
<td>0.25</td>
<td>5 µl of A</td>
<td>35 µl</td>
</tr>
<tr>
<td>E</td>
<td>0.1</td>
<td>16 µl of D</td>
<td>24 µl</td>
</tr>
<tr>
<td>F</td>
<td>0.05</td>
<td>20 µl of E</td>
<td>20 µl</td>
</tr>
<tr>
<td>G</td>
<td>0.025</td>
<td>10 µl of F</td>
<td>10 µl</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>N/A</td>
<td>20 µl</td>
</tr>
</tbody>
</table>
4.3.9 Statistical analysis

Statistical analysis was performed using the SPSS statistical package (version 17.0, SPSS Inc.) for repeated measures ANOVA and one-way ANOVA. The results are represented as mean ± SEM. Comparisons between groups for animal body weight, food intake and water intake data were analysed using repeated measures ANOVA. A post hoc pair-wise comparison was also carried out. Significance was established at the 95% confidence level ($P < 0.05$).
4.4 Results

4.4.1 Body weight, food intake and water intake

Body weight of the animals increased gradually over the course of the trial in all diet groups (Figure 4.1). There were no significant differences ($P > 0.05$) between soybean oil and canola oil groups. There were also no significant differences ($P > 0.05$) in food consumption between all dietary groups (Figure 4.2). The water intake in soybean oil and canola oil groups with salt were significantly increased ($P < 0.05$) between the soybean oil and canola oil groups without salt (Figure 4.2).

Figure 4.1: Mean body weight of SHRSP rats fed canola oil compared with soybean oil diet in the absence or presence of NaCl loading. Values are means ± SEM.
Figure 4.2: Food and water intake of SHRSP rats fed canola oil compared with soybean oil diet in the absence or presence of NaCl loading.

Values are means ± SEM. ‡P < 0.05 represents a significant difference from soybean oil and canola oil no salt groups.
4.4.2 Blood pressure

Blood pressure increased over time in all diet groups, with significant changes ($P < 0.05$) seen between different groups at days 14, 21 and 25 (Figure 4.3). At day 14, the blood pressure was significantly increased ($P < 0.05$) in the canola oil group without salt compared to the soybean oil group without salt. At day 21 and 25, the blood pressure was significantly increased ($P < 0.05$) in the soybean oil group with salt compared to the soybean oil group without salt. Also, at day 25 the blood pressure was significantly increased ($P < 0.05$) in the canola oil group with salt compared to the canola oil group without salt.
Figure 4.3: Mean systolic blood pressure of SHRSP rats fed 25 days of canola oil compared with soybean oil in the absence or presence of NaCl loading.

Values are means ± SEM. ◇P < 0.05 represents a significant difference between soybean oil no salt and soybean oil with salt groups; ◆P < 0.05 represents a significant difference between canola oil no salt and soybean oil no salt groups; ※P < 0.05 represents a significant difference between canola oil no salt and canola oil with salt groups.
4.4.3 Antioxidant enzymes and oxidative damage

Markers of antioxidant status and oxidative damage are represented in Table 4.3. In the absence of salt, canola oil ingestion significantly reduced ($P < 0.05$) the activities of RBC SOD and GPx compared with soybean oil alone. The activity of RBC GPx was significantly reduced ($P < 0.05$) in the soybean oil and canola oil groups in the presence of salt compared with soybean oil alone. There were no significantly differences ($P > 0.05$) in the activity of catalase between the groups.

Canola oil and soybean oil ingestion with salt loading significantly increased ($P < 0.05$) RBC MDA compared to both the canola oil and soybean oil groups without salt. Plasma MDA in the canola oil group in the presence of salt was significantly higher ($P < 0.05$) than the canola oil group without salt. There were no significantly differences ($P > 0.05$) in the concentration of 8-isoprostane between the groups.
Table 4.3: Antioxidant status and oxidative damage in SHRSP rats fed 25 days of canola oil compared with soybean oil diet in the absence and presence of NaCl loading.

<table>
<thead>
<tr>
<th></th>
<th>Soybean oil no salt</th>
<th>Canola oil no salt</th>
<th>Soybean oil salt</th>
<th>Canola oil Salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC SOD (U/gm Hb)</td>
<td>484.7 ± 76.7</td>
<td>277.6 ± 51.9*</td>
<td>337 ± 68.1</td>
<td>354.9 ± 59.9</td>
</tr>
<tr>
<td>RBC GPx (mmol/min/gm Hb)</td>
<td>85.4 ± 4.1</td>
<td>57.2 ± 9*</td>
<td>62.4 ± 8.7*</td>
<td>61.4 ± 7.8*</td>
</tr>
<tr>
<td>RBC Catalase (mmol/min/gm Hb)</td>
<td>377.6 ± 40.1</td>
<td>320.4 ± 36.6</td>
<td>303.2 ± 33.8</td>
<td>331.1 ± 42.1</td>
</tr>
<tr>
<td>RBC MDA (µM)</td>
<td>10.2 ± 0.3</td>
<td>10.2 ± 0.3</td>
<td>11.1 ± 0.2‡</td>
<td>11.1 ± 0.2‡</td>
</tr>
<tr>
<td>Plasma MDA (µM)</td>
<td>15.9 ± 0.6</td>
<td>14.5 ± 0.4</td>
<td>15.8 ± 1.1</td>
<td>16.4 ± 0.5‡</td>
</tr>
<tr>
<td>Plasma 8-isoprostane (pg/ml)</td>
<td>64.9 ± 8.5</td>
<td>83.2 ± 10.3</td>
<td>86.6 ± 8.7</td>
<td>105.1 ± 15.5</td>
</tr>
</tbody>
</table>

Values are means ± SEM; SOD, superoxide dismutase; GPx, glutathione peroxidase; MDA, malondialdehyde.

* *P < 0.05 represents a significant difference from soybean oil no salt group; ‡ *P<0.05 represents a significant difference from canola oil no salt group; † *P<0.05 represents a significant difference from soybean oil and canola oil no salt groups.
4.4.4 Plasma lipids

Canola oil ingestion alone significantly increased \((P < 0.05)\) the concentration of total cholesterol and LDL-C compared to soybean oil alone. LDL-C concentration was significantly lower \((P < 0.05)\) in the canola oil group loaded with salt compared to the canola oil group without salt. HDL-C concentration was significantly higher \((P < 0.05)\) in both the salt loaded groups compared to the no salt groups. There were no significant differences \((P > 0.05)\) in the concentration of triglycerides between groups (Table 4.4).

<table>
<thead>
<tr>
<th></th>
<th>Soybean oil no salt</th>
<th>Canola oil no salt</th>
<th>Soybean oil salt</th>
<th>Canola oil salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>3.1 ± 0.1</td>
<td>3.4 ± 0.1*</td>
<td>3.1 ± 0.1</td>
<td>3.3 ± 0.1*</td>
</tr>
<tr>
<td>LDL-C</td>
<td>1.4 ± 0.1</td>
<td>1.7 ± 0.1*</td>
<td>2.2 ± 1.3</td>
<td>1.1 ± 0.2#</td>
</tr>
<tr>
<td>HDL-C</td>
<td>1.3 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>2.3 ± 1.1†</td>
<td>2.3 ± 0.4‡</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.8 ± 0.1</td>
<td>2 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.9 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SEM; *\(P<0.05\) represents a significant difference from soybean oil and canola oil no salt groups; *\(P<0.05\) represents a significant difference from soybean oil no salt group; #\(P<0.05\) represents a significant difference from canola oil no salt group.
4.4.5 mRNA gene expression

In the absence of salt there were no changes seen in the mRNA expression of NOX2 and p22\textsubscript{phox} (Table 4.5). However, when salt loading was used canola oil intake significantly reduced \((P < 0.05)\) NOX2 mRNA expression compared to the soybean oil group with salt and the canola oil group without salt. Canola oil intake with salt significantly reduced \((P < 0.05)\) p22\textsubscript{phox} mRNA expression compared to the canola oil group without salt.

In the absence of salt, there were no changes seen in the mRNA expression of CuZn-SOD, Mn-SOD and EC-SOD (Table 4.5). However, when salt loading was used canola oil intake significantly decreased \((P < 0.05)\) CuZn-SOD and Mn-SOD mRNA expression compared with the soybean oil group with salt and both the canola oil and soybean oil groups without salt. EC-SOD mRNA expression was significantly reduced in the canola oil group with salt compared with the soybean oil group with salt and the canola oil group without salt.
Table 4.5: Effect of 25 days of canola oil intake compared to soybean oil intake on mRNA expression in the aorta of SHRSP rats in the absence and presence of NaCl loading.

<table>
<thead>
<tr>
<th>Gene</th>
<th>mRNA expression (Arbitrary units)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soybean oil</td>
<td>Canola oil</td>
<td>Soybean oil</td>
<td>Canola oil</td>
</tr>
<tr>
<td></td>
<td>no salt</td>
<td>no salt</td>
<td>salt</td>
<td>Salt</td>
</tr>
<tr>
<td>p22^phox</td>
<td>7.1 ± 2.4</td>
<td>7.6 ± 2</td>
<td>4.2 ± 1.4</td>
<td>2.6 ± 1#</td>
</tr>
<tr>
<td>NOX2</td>
<td>5.3 ± 1.5</td>
<td>9.9 ± 3</td>
<td>9.1 ± 3.6</td>
<td>1.9 ± 0.7†,#</td>
</tr>
<tr>
<td>CuZn-SOD</td>
<td>46.6 ± 19.4</td>
<td>29.7 ± 7.2</td>
<td>22.9 ± 4.5</td>
<td>8.9 ± 2.5†,‡</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>126.1 ± 41.2</td>
<td>94.6 ± 20.1</td>
<td>93.5 ± 20.9</td>
<td>37.3 ± 6.7†,‡</td>
</tr>
<tr>
<td>Ec-SOD</td>
<td>68.9 ± 17.4</td>
<td>82.7 ± 12.1</td>
<td>89.3 ± 17.2</td>
<td>25 ± 1.9†,#</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. †P < 0.05 represents a significant difference between canola oil and soybean oil with salt groups; #P < 0.05 represents a significant difference from canola oil no salt group; ‡P<0.05 represents a significant difference from soybean oil and canola oil no salt groups.
4.5 Discussion

The data from this study has shown that canola oil ingestion alone decreased the activities of SOD and GPx compared with soybean oil by 53 and 33%, respectively. These results indicate that canola oil ingestion alone for 25 days affects antioxidant enzyme activity. Previous studies have shown that the in the canola oil group (with no added salt) the activities of SOD and catalase were reduced in the hepatic cytosol of SHR and WKY rats (Natio et al. 2000d; Ohara et al. 2008). The present study has also shown that in the presence of salt, both canola oil and soybean oil ingestion reduced the activity of GPx compared with the soybean oil group without salt. The reduced activity of RBC GPx in the salt loaded canola oil group is consistent with our previous findings in chapter 3 (Papazzo et al. 2011). However, the data from our lifespan study showed that canola oil ingestion along with salt loading in SHRSP rats reduced RBC SOD, catalase as well as GPx at the end of their life span, mean lifespan of 85 days (Papazzo et al. 2011). Supporting evidence shows that there is an association between reduced antioxidants and CVD (Kalenikova et al. 2004). In addition, previous research has shown an inverse relationship between erythrocyte GPx activity and the incidence of CVD.

The present study has also shown that canola oil ingestion with salt loading increased the plasma MDA concentration when compared to the canola oil group without salt. In addition, RBC MDA concentration was increased in the canola oil and soybean oil groups in the presence of salt compared with the non salt groups. The increased plasma and RBC MDA concentration indicates an increased amount of ROS induced lipid peroxidation, which may be due to the salt loading
in combination with the diets. A previous study found an increase in urine MDA concentration in Sprague-Dawley rats as a result of salt intake (Kitiyakara et al. 2003). They found a difference between the low salt (0.03%) and normal salt (0.3) groups, and between the low salt and high salt (6%) groups. In addition, they also found an increase in urine 8-isoprostane levels as a result of salt intake. However, there were no changes found in the 8-isoprostane levels in the present study. In a previous study in WKY rats, canola oil ingestion in the absence of extra dietary salt decreased lipid peroxide levels in the hepatic cytosol (Ohara et al. 2009), while an earlier study showed no change in lipid peroxide levels in the hepatic cytosol of SHR with 1% NaCl loading (Ohara et al. 2008). The mechanism by which canola oil intake in combination with salt induces MDA is currently unknown.

The present study has shown that in the presence of salt, canola oil intake decreased the mRNA expression of CuZn-SOD, Mn-SOD and Ec-SOD. The decrease in the SOD isoforms indicates a reduced ability to eliminate O$_2^-$ in the presence of canola oil and salt. High salt intake (6%) has been shown to reduce renal expression of CuZn-SOD and Mn-SOD in Sprague-Dawley rats (Kitiyakara et al. 2003). The present study has also showed that p22$^{phox}$ and NOX2 mRNA expression was reduced in the canola oil group with the presence of salt, indicating that O$_2^-$ generated from NADPH oxidase may be decreased. Our current study shows for the first time that canola oil intake with salt reduces NADPH oxidase subunits and SOD isoforms in the aorta of SHRSP rats after 25 days of feeding. Evidence has shown that O$_2^-$ generated from NADPH oxidase is increased in hypertension (Paravicini & Touyz 2008). In addition, Kitiyakara et
al. (2003) found that salt intake in Sprague-Dawley rats lead to an increase in $O_2^-$ production. This was accompanied by an increase in renal activity and mRNA expression of NOX2 and p47$^{phox}$, and a decrease in CuZn-SOD and MnSOD mRNA expression (Kitiyakara et al. 2003). However, in the vasculature, there are several other sources of ROS, which include: xanthine oxidase, uncoupled nitric oxide synthase, lipoxygenase and the mitochondrial respiratory chain (Jiang et al. 2004; Schulz et al. 2011). In the present study, ROS generation may be coming from other sources within the vasculature, and requires further investigation. Furthermore, it would have been ideal to examine the protein levels. However, there was insufficient protein to carry out the western blotting analysis. A study found that plasma Ec-SOD activity was decreased in hypertensive patients, while there were no changes found in protein levels (Zhou et al. 2006). Therefore, the reduction in Ec-SOD activity is not due to the down regulation of Ec-SOD. In the present study, SOD activity was reduced in the canola oil group without salt, while there were no changes seen in the mRNA expression of the SOD isoforms.

Previous studies have reported an increase in plasma lipids due to canola oil ingestion (Natio et al. 2000b & 2000d; Ohara et al. 2008 & 2009). In the present study, canola oil ingestion alone increased the concentration of total cholesterol and LDL-C compared with soybean oil alone. When salt loading was used canola oil intake increased total cholesterol compared with canola oil without salt. HDL-C in both the canola oil and soybean oil groups with salt was higher compared with the non salt groups. Previous studies in SHR and WKY rats have shown increases in total cholesterol and HDL-C with administration of canola oil compared to soybean oil (Natio et al. 2000b & 2000d; Ohara et al. 2008 & 2009).
It is well established that increased LDL-C is a risk factor for atherosclerosis (Diaz et al. 1997). However, in the present study, the combination of canola oil and salt intake resulted in a decrease in LDL-C compared with the canola oil without salt group. This data is consistent with the results from our lifespan study in chapter 3, which showed a decrease in LDL-C, as well as HDL-C and total cholesterol in the canola oil group (Papazzo et al. 2011). There is substantial evidence suggesting that high salt intake increases the risk of CVD. However, some studies suggest that low salt intake and its adverse effects on blood lipids can have a detrimental effect on CVD risk (Harsha et al. 2004). The mechanisms by which salt intake affects the blood lipids are not clear. A meta-analysis on humans reported that a reduction in salt intake from 200 to 20 mmol/day resulted in a significant increase in total cholesterol and LDL-C (Graudal et al. 1998). A study by Harsha et al. (2004) found that within each diet (the typical American diet (control) and the Dietary Approaches to Stop Hypertension (DASH) diet), sodium intake (50, 100 or 150 mmol/d) did not significantly affect the serum levels of LDL-C, total cholesterol, HDL-C and triglycerides. However, at each sodium concentration, LDL-C, HDL-C and total cholesterol were lower in the DASH diet compared to the American control diet (Harsha et al. 2004). Taken together these results suggest that a diet low in salt leads to an increase in LDL-C.

The results of the present study show an increase in systolic blood pressure in both the canola oil and soybean oil groups with salt compared to the dietary groups without salt at the end of the feeding trial. The association between salt intake and hypertension is well known, which is evident in the present the study. Evidence indicates that canola oil intake has an effect on blood pressure in the
SHRSP rat and its related strains. However, the blood pressure in the canola oil groups was not consistently different from soybean oil. Our lifespan study showed that blood pressure in the canola oil group was not different from soybean oil. A study by Huang et al. (1996) observed no significant change in systolic blood pressure in the canola oil group compared to the soybean oil group at 4 and 8 weeks of age (Huang et al. 1996). Another study by Ratnayake et al. (2000a) found no significant differences in the SBP among different dietary groups in SHRSP rats (Ratnayake et al. 2000a). Taken together these results suggest that canola oil intake in the presence or absence of salt does not affect blood pressure. Therefore, the life shortening effect of canola oil may not be directly due to an increase in blood pressure.

In conclusion, canola oil ingestion in the absence of dietary salt decreased the activities of RBC SOD and GPx, and increased both total cholesterol and LDL-C, which are risk factors for CVD. However, the combination of canola oil and dietary salt intake resulted in an increase in plasma MDA, a decrease in LDL-C, and a decrease in NADPH oxidase subunits and SOD aortic expression when compared to canola oil intake alone. ROS generation may be coming from other sources within the vasculature. The increase in RBC and plasma MDA, with a decrease in RBC GPx and SOD mRNA expression may indicate an elevation in oxidative stress. More research is required to determine if canola oil intake in the absence or presence of salt leads to oxidative stress and altered vascular changes such as endothelial dysfunction in a longer duration study.
CHAPTER 5
The effect of 50 days of canola oil ingestion on oxidative stress in the vasculature and endothelial function in SHRSP rats

5.1 Abstract

Background
This study aimed to determine if 50 days of canola oil intake in the absence of excess dietary salt or together with salt loading effects: (1) the antioxidant and oxidative stress markers in the circulation, (2) aortic mRNA expression of NADPH oxidase subunits and SOD isoforms and (3) endothelial function in SHRSP rats.

Methods
Male SHRSP rats were fed a defatted control diet containing 10 wt/wt% soybean oil or a defatted treatment diet containing 10 wt/wt% canola oil, and given tap water or water containing 1% NaCl for 50 days. Blood was collected at the end of study for analysis of RBC antioxidant enzymes, RBC and plasma MDA, plasma 8-isoprostane and plasma lipids. The aorta was removed and the mRNA expression of NOX2, p22phox, CuZn-SOD, Mn-SOD and EC-SOD were determined. The contractile responses to norepinephrine, and endothelium-dependent and -independent vasodilating responses to ACh and SNP, respectively, were measured in thoracic aortic rings. Male WKY rats were used as a normotensive control to assess endothelial function and were fed 10 wt/wt% soybean oil for 50 days.
Results

In the absence of salt, canola oil increased RBC SOD, total cholesterol and triglycerides, \(p22^{phox}\), NOX2 and CuZn-SOD mRNA expression and decreased RBC GPx. In the presence of salt, canola oil reduced RBC SOD, catalase, LDL-C, and \(p22^{phox}\) mRNA expression compared with canola oil alone, whereas plasma MDA was reduced and LDL-C was higher compared with soybean oil with salt. In addition, RBC MDA was significantly increased in the canola oil group with salt compared to the soybean oil group with salt and to the non salt groups. In the presence of salt, the canola oil group had significantly reduced endothelium-dependent vasodilating responses to ACh and contractile responses to norepinephrine compared with the canola oil group without salt and to the WKY rats.

Conclusion

In conclusion, these results indicate that canola oil alone increases \(p22^{phox}\) and NOX2 mRNA expression suggesting an increase in \(O_2^-\) generation. However, canola oil ingestion in combination with salt leads to endothelial dysfunction.

5.2 Introduction

In the study described in the previous chapter, short-term ingestion (25 days) of canola oil in the absence of excess dietary salt resulted in a decrease in RBC SOD and GPx, and an increase in LDL-C and total cholesterol, which are risk factors for CVD. However, in the presence of salt, canola oil ingestion lead to an increase in MDA and a decrease in NAPDH oxidase and SOD aortic mRNA expression. Our lifespan study described in chapter 3, found that canola oil
ingestion decreased RBC antioxidant enzymes and increased RBC MDA (Papazzo et al. 2011). However, the effect of canola oil ingestion in the absence or presence of excess dietary salt for a longer period of time on oxidative stress has not been examined previously in SHRSP rats.

An increase in ROS activity contributes to the impaired regulation of physiological processes within the vascular wall, which leads to structural and functional changes observed in hypertension (Zalba et al. 2001). Recent research has shown that endothelial dysfunction is an early sign of CVD (Davies 2009). Increased ROS activity in the vasculature can lead to a decrease in NO bioavailability and impaired endothelium-dependent vasorelaxation, which results in endothelial dysfunction (Berry et al. 2001; Zalba et al. 2001; Datla & Griendling 2010). An excess of vascular ROS generation or reduced levels of antioxidants has been associated with the pathogenesis of endothelial dysfunction in SHRSP rats (Ulker et al. 2003).

This study aimed to determine if 50 days of canola oil intake in the absence of excess dietary salt or together with salt loading affects: (1) the antioxidant and oxidative stress markers in the circulation, (2) the mRNA expression of NADPH oxidase subunits and SOD isoforms in the aorta, and (3) endothelial function in SHRSP rats.
5.3 Materials and methods

5.3.1 Animal husbandry and study design

5.3.1.1 Fifty day treatment experiment

Forty male SHRSP rats (Deakin University, Australia) aged 4 weeks were randomly assigned to either group 1 (n = 20) or group 2 (n = 20). Within each group the rats were randomly assigned to a control and treatment group and acclimatized for one week. During acclimatization they were given a standard pellet diet (Specialty Feeds, Western Australia) and water ad libitum. The groups were the fed the following diets respectively, a defatted control diet containing 10 wt/wt% soybean oil or a defatted treatment diet containing 10 wt/wt% canola oil (Speciality Feeds) for 50 days. Our previous study examined the effect of 25 days of canola oil ingestion, therefore, 50 days was chosen to observe the use of canola oil ingestion for a longer period of time. In addition, 50 days was also chosen based on our pervious study, which showed that the mean lifespan of the canola oil group was 85 ± 1.1 days (Papazzo et al. 2011). Given that the rats were 35 days old when they started the trial, they were on the diet for a mean of 50 days. The fatty acid composition of the diets is shown in Table 3.1 (Chapter 3). Group 1 was given water containing 1% NaCl and group 2 was given tap water throughout the trial. The reason for having the group without NaCl in the drinking water is to rule out any interfering factor the salt loading may have when analysing the tissue. The animals were maintained on a 12 hr light/dark photo-period with a room temperature of 21 ± 2°C. Animal body weights, food intake and water consumption were determined once a week, while the health of the animals was monitored daily. At the end of the 50 days the rats were anaesthetised via intra-peritoneal injection with lethabarb (50 mg/kg), and blood
was collected for analysis. The aorta was then removed, washed in saline solution and snap frozen in liquid nitrogen.

5.3.1.2 Vascular function experiment

Forty male SHRSP rats and ten male WKY rats (Deakin University, Australia) aged 4 weeks were acclimatized for one week. During acclimatization they were given a standard pellet diet (Specialy Feeds, Western Australia) and water ad libitum. After the acclimatization period, the SHRSP rats were fed the following diets respectively, a defatted control diet containing 10 wt/wt% soybean oil or a defatted treatment diet containing 10 wt/wt% canola oil (Speciality Feeds) for 50 days. The WKY rats were fed 10 wt/wt% soybean oil control diet for 50 days and given tap water. The WKY rats were used as a normotensive control to assess endothelial function. The animals were maintained on a 12 hr light/dark photo-period with a room temperature of 21 ± 2°C. The health of the animals was monitored daily. At the end of the 50 days the rats were anaesthetised via intra-peritoneal injection with sodium pentobarbitone (50 mg/kg). The aorta was removed and used for the isolated aortic rings experiment.

5.3.2 Measurement of blood pressure

Blood pressure was measured weekly, and was preformed as outlined in Chapter 3, section 3.3.2.

5.3.3 Blood collection and processing

The blood was collected and processed as outlined in Chapter 3, section 3.3.3.
5.3.4 Erythrocyte antioxidant enzymes

5.3.4.1 SOD assay
SOD activity was measured as described in Chapter 3, section 3.3.4.1.

5.3.4.2 Catalase assay
Catalase activity was measured as described in Chapter 3, section 3.3.4.2.

5.3.4.3 GPx assay
GPx activity was measured as described in Chapter 4, section 4.3.4.3.

5.3.5 Haemoglobin assay
The antioxidant enzymes were all normalised to haemoglobin, and was measured as described in Chapter 3, section 3.3.5.

5.3.6 Lipid peroxidation analysis

5.3.6.1 MDA
MDA was determined via HPLC in plasma and erythrocytes and was measured as described in Chapter 3, section 3.3.6.1

5.3.6.2 8-isoprostane
8-isoprostane was determined in plasma and was measured as described in Chapter 3, section 3.3.6.2
5.3.7 Plasma lipids analysis

5.3.7.1 Triglycerides
Plasma triglycerides were determined as described in Chapter 3, section 3.3.7.1.

5.3.7.2 Total cholesterol
Total cholesterol was determined as described in Chapter 3, section 3.3.7.2.

5.3.7.3 HDL-C
HDL-C was determined as described in Chapter 3, section 3.3.7.3.

5.3.7.4 LDL-C
LDL-C was determined as described in Chapter 3, section 3.3.7.4.

5.3.8 mRNA gene expression analysis

5.3.8.1 RNA extraction
RNA extraction from the aorta was performed as previously described in Chapter 4, section 4.3.8.1.

5.3.8.2 Reverse transcription
Reverse transcription was carried out as previously described in Chapter 4, section 4.3.8.2.
5.3.8.3 Reverse transcription-real-time PCR measurement of mRNA

Real-time PCR was performed as previously described in Chapter 4, section 4.3.8.3.

5.3.9 Isolated aortic rings

After the aorta was removed, it was placed immediately into Tyrode solution (containing in mM: 136.9 NaCl, 5.4 KCl, 1.05 MgCl₂, 1.8 CaCl₂, 22.6 NaHCO₃, 0.42 NaH₂PO₄, 5.5 glucose, 0.28 ascorbic acid and 0.1 EDTA) bubbled with carbogen (95% oxygen and 5% carbon dioxide). All visible fat and connective tissue was removed. The aorta was then cut into three 4 mm rings and suspended in an organ bath chamber and bathed in Tyrode solution bubbled with carbogen. The organ baths are water jacketed and the water temperature was maintained at 35°C ± 0.5. Prior to the introduction of each of the vasoactive substances, the aortic rings were equilibrated for 1 hour in Tyrode solution with a resting tension of 10 mN. During the equilibration period the Tyrode solution was changed every 15 minutes. A cumulative concentration-response curve was performed for norepinephrine (final bath concentration 10⁻⁹ to 10⁻⁴ M) on one aortic ring to assess vasoconstriction. After a submaximal contraction to norepinephrine was reached in the other two aortic rings, endothelium-dependent and -independent vasodilation was assessed using ACh and SNP, respectively (final bath concentration 10⁻⁹ to 10⁻⁴ M). Force of contraction in the aorta was measured isometrically with force transducers (FT03C, Grass, USA) connected via amplifiers to a computer via a LabChart system (AD Instruments, Australia).
5.3.10 Statistical analysis

Statistical analysis was performed using the SPSS statistical package (version 17.0, SPSS Inc.) for repeated measures ANOVA and one-way ANOVA. The results are represented as mean ± SEM. Comparisons between groups for animal body weight, food intake and water intake data were analysed using repeated measures ANOVA. A post hoc pair-wise comparison was also carried out. Significance was established at the 95% confidence level (P < 0.05).
5.4 Results

5.4.1 Body weight, food intake and water intake

Body weight of the SHRSP rats increased gradually over the course of the trial in all diet groups. There were no significant differences ($P > 0.05$) between soybean oil and canola oil groups (Figure 5.1). There were also no significant differences ($P > 0.05$) in food consumption between all dietary groups. The water intake in soybean oil and canola oil groups with salt were significantly increased ($P < 0.05$) between the soybean oil and canola oil groups without salt (Figure 5.2).

![Figure 5.1: Mean body weight of SHRSP rats fed canola oil compared with soybean oil diet in the absence or presence of NaCl loading. Values are means ± SEM.](image-url)
Figure 5.2: Food and water intake of SHRSP rats fed canola oil compared with soybean oil diet in the absence or presence of NaCl loading.

Values are means ± SEM. ‡P < 0.05 represents a significant difference from soybean oil and canola oil no salt groups.
5.4.2 Blood pressure

Blood pressure increased over time in all dietary groups (Figure 5.3). At day 14, the blood pressure was significantly increased ($P < 0.05$) in the canola oil group without salt compared to the soybean oil group without salt. From day 21 to day 50, the blood pressure was significantly increased ($P < 0.05$) in the soybean oil group with salt compared to the soybean oil group without salt. Also, from day 28 to day 50, the blood pressure was significantly increased ($P < 0.05$) in the canola oil group with salt compared to the canola oil group without salt. At day 42, the blood pressure was significantly increased ($P < 0.05$) in the canola oil group with salt compared to the soybean oil group with salt.
Figure 5.3: Mean systolic blood pressure of SHRSP rats fed 50 days of canola oil compared with soybean oil in the absence or presence of salt.

Values are means ± SEM. †P <0.05 represents a significant difference between canola oil no salt and soybean oil no salt; ‡P <0.05 represents a significant difference between canola oil no salt and canola oil with salt; *P <0.05 represents a significant difference between soybean oil no salt and soybean oil with salt; ‡‡ P <0.05 represents a significant difference between canola oil with salt and soybean oil with salt.
5.4.3 Antioxidant enzymes and oxidative damage

Markers of antioxidant status and oxidative damage are represented in Table 5.1. In the absence of salt, canola oil ingestion significantly increased \( P < 0.05 \) the activity of RBC SOD and significantly reduced \( P < 0.05 \) the activity of RBC GPx compared with soybean oil alone. In the presence of salt, RBC SOD and catalase were significantly reduced \( P < 0.05 \) in the canola oil group compared with the canola oil group without salt. RBC GPx and catalase were significantly reduced \( P < 0.05 \) in the soybean oil group with salt compared with the soybean oil group without salt.

Canola oil ingestion with salt significantly increased \( P < 0.05 \) RBC MDA compared to the soybean oil group with salt and to the non salt groups. Plasma MDA in the canola oil group with salt was significantly lower \( P < 0.05 \) compared to the soybean oil group with salt. Soybean oil intake with salt significantly increased \( P < 0.05 \) plasma 8-isoprostane compared to the canola oil group with salt and to the non salt groups.
Table 5.1: Antioxidant status and oxidative damage in SHRSP rats fed 50 days of canola oil compared with soybean oil diets in the absence and presence of NaCl loading.

<table>
<thead>
<tr>
<th></th>
<th>Soybean oil no salt</th>
<th>Canola oil no salt</th>
<th>Soybean oil salt</th>
<th>Canola oil salt</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RBC SOD (U/gm Hb)</strong></td>
<td>281.6 ± 48.1</td>
<td>425.7 ± 52.7*</td>
<td>297.9 ± 45.1</td>
<td>290.7 ± 31.2#</td>
</tr>
<tr>
<td><strong>RBC GPx (mmol/min/gm Hb)</strong></td>
<td>140 ± 8.6</td>
<td>110 ± 8.6*</td>
<td>74.1 ± 12.5*</td>
<td>96.6 ± 17.1</td>
</tr>
<tr>
<td><strong>RBC Catalase (mmol/min/gm Hb)</strong></td>
<td>455.2 ± 54.1</td>
<td>381.5 ± 35.1</td>
<td>302 ± 26.8*</td>
<td>216.2 ± 36.2*.#</td>
</tr>
<tr>
<td><strong>RBC MDA (µM)</strong></td>
<td>14.7 ± 1.3</td>
<td>17.7 ± 2.2</td>
<td>17.6 ± 1.9</td>
<td>34.5 ± 5.8†.‡</td>
</tr>
<tr>
<td><strong>Plasma MDA (µM)</strong></td>
<td>18.8 ± 0.6</td>
<td>18.8 ± 1.6</td>
<td>19.6 ± 0.6</td>
<td>16.1 ± 0.8†</td>
</tr>
<tr>
<td><strong>Plasma 8-isoprostane (pg/ml)</strong></td>
<td>110.4 ± 8.9</td>
<td>97.7 ± 15.3</td>
<td>155.2 ± 17.9†.‡</td>
<td>87.6 ± 10.9</td>
</tr>
</tbody>
</table>

Values are means ± SEM; SOD, superoxide dismutase; GPx, glutathione peroxidase; MDA, malondialdehyde.

†P < 0.05 represents a significant difference between canola oil and soybean oil with salt groups; #P < 0.05 represents a significant difference from canola oil no salt group; ‡P < 0.05 represents a significant difference from soybean oil and canola oil no salt groups. *P < 0.05 represents a significant difference from soybean oil no salt group.
5.4.4 Plasma lipids

Canola oil ingestion alone significantly increased ($P < 0.05$) the concentration of total cholesterol and triglycerides compared to soybean oil alone. Total cholesterol was significantly higher ($P < 0.05$) in the canola oil loaded with salt compared to the canola oil group without salt. LDL-C was significantly increased ($P < 0.05$) in the canola oil group with salt compared to both the canola oil and soybean oil groups without salt. The concentration of triglycerides was significantly lower in the canola oil group with salt compared to the soybean oil group with salt. In the soybean oil group in the presence of salt, the concentration of total cholesterol, LDL-C, HDL-C and triglycerides were significantly higher ($P < 0.05$) compared to soybean oil alone (Table 5.2).

**Table 5.2: Plasma lipids in SHRSP rats fed 50 days of canola oil compared with soybean oil diets in the absence and presence of NaCl loading.**

<table>
<thead>
<tr>
<th></th>
<th>mmol/L</th>
<th>Soybean oil no salt</th>
<th>Canola oil no salt</th>
<th>Soybean oil salt</th>
<th>Canola oil salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td></td>
<td>2.8 ± 0.1</td>
<td>3.2 ± 0.1*</td>
<td>4.2 ± 0.3*</td>
<td>3.5 ± 0.2†</td>
</tr>
<tr>
<td>LDL-C</td>
<td></td>
<td>1 ± 0.04</td>
<td>1.2 ± 0.1</td>
<td>2.1 ± 0.2*</td>
<td>1.7 ± 0.2‡</td>
</tr>
<tr>
<td>HDL-C</td>
<td></td>
<td>1.4 ± 0.04</td>
<td>1.5 ± 0.04</td>
<td>1.6 ± 0.06*</td>
<td>1.6 ± 0.06</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td>1.5 ± 0.1</td>
<td>2.1 ± 0.1*</td>
<td>2 ± 0.2*</td>
<td>2 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SEM; *$P < 0.05$ represents a significantly difference from soybean oil no salt group; †$P < 0.05$ represents a significant difference between canola oil and soybean oil with salt groups; ‡$P < 0.05$ represents a significant difference from soybean oil and canola oil no salt groups.
5.4.5 mRNA gene expression

In the absence of salt, canola oil intake significantly increased \((P < 0.05)\) \(p22^{phox}\) and NOX2 mRNA expression compared to soybean oil alone (Table 5.3). In the presence of salt, canola oil and soybean oil intake significantly reduced \((P < 0.05)\) the mRNA expression of \(p22^{phox}\) compared to the canola oil group without salt. NOX2 mRNA expression was significantly increased \((P < 0.05)\) in the soybean oil group with salt compared to both the soybean oil and canola oil groups without salt.

CuZn-SOD mRNA expression was significantly increased in both the canola oil and soybean oil groups with salt \((P < 0.05)\) compared to the soybean oil group without salt. There were no changes seen in the mRNA expression of Mn-SOD and Ec-SOD between groups.

**Table 5.3: Effect of 50 days of canola oil intake compared to soybean oil intake on mRNA expression in the aorta of SHRSP rats in the absence and presence of NaCl loading.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Soybean oil no salt</th>
<th>Canola oil no salt</th>
<th>Soybean oil salt</th>
<th>Canola oil Salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>(p22^{phox})</td>
<td>7.6 ± 1.4</td>
<td>21.8 ± 3.5*</td>
<td>8.9 ± 2*</td>
<td>11.5 ± 4.1*</td>
</tr>
<tr>
<td>NOX2</td>
<td>1.4 ± 0.6</td>
<td>12.1 ± 5*</td>
<td>65 ± 22.6†</td>
<td>26.2 ± 6</td>
</tr>
<tr>
<td>CuZn-SOD</td>
<td>22.2 ± 5.1</td>
<td>39.2 ± 7.8*</td>
<td>60.5 ± 15.9*</td>
<td>54.9 ± 15*</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>76 ± 19.9</td>
<td>56.7 ± 9.1</td>
<td>100 ± 18.6</td>
<td>95.2 ± 22.2</td>
</tr>
<tr>
<td>Ec-SOD</td>
<td>7.1 ± 1.9</td>
<td>4.4 ± 0.9</td>
<td>8.1 ± 1.7</td>
<td>7.6 ± 1.8</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. ‡\(P < 0.05\) represents a significant difference from soybean oil and canola oil no salt groups; *\(P < 0.05\) represents a significant difference from soybean oil no salt group; †\(P < 0.05\) represents a significant difference from canola oil no salt group.
5.4.6 Vascular responses

Canola oil fed SHRSP rats with salt intake showed significantly reduced ($P < 0.05$) contractile responses to norepinephrine compared to SHRSP rats fed only canola oil (Figure 5.4A). The contractile responses to norepinephrine were significantly greater ($P < 0.05$) in the SHRSP rats fed only canola oil compared to the SHRSP rats fed only soybean oil. Contractile responses to norepinephrine were significantly greater ($P < 0.05$) in the WKY rats compared to the SHRSP rats fed canola oil and soybean oil in the presence of salt and to the soybean oil only group.

Relaxation responses to SNP were not significantly different between groups ($P > 0.05$) (Figure 5.4B). The ACh dilating responses were not significantly different ($P > 0.05$) between the SHRSP rats fed canola oil and soybean oil without salt (Figure 5.4C). Canola oil fed SHRSP rats with salt loading showed a significantly impaired ($P < 0.05$) endothelium-dependent relaxation response of the thoracic aorta compared to the canola oil only group and WKY rats.
Figure 5.4: Cumulative concentration-response curves for norepinephrine (A), SNP (B) and ACh (C) in aortic rings from SHRSP and WKY rats.

Values are mean ± SEM. $^* P < 0.05$ represents a significant difference from soybean oil no salt; $^\dagger P < 0.05$ represents a significant different from canola oil and soybean oil with salt; $^\# P < 0.05$ represents a significant difference from canola oil no salt; $^\ddagger P < 0.05$ represents a significant difference from canola oil with salt; $^\Omega P < 0.05$ represents a significant difference from all dietary groups; $^\alpha P < 0.05$ represents a significant difference from WKY rats.
5.5 Discussion

In the present study, canola oil ingestion alone increased the activity of RBC SOD and reduced RBC GPx compared with the soybean oil group. Similar results have also been found in a study by Ohara et al. 2009, in which the activities of GPx and catalase were reduced in the liver of WKY rats following canola oil ingestion. Furthermore, our previous study in chapter 4 found that 25 days of canola oil ingestion alone decreased the activities of RBC SOD and GPx. The results from the present study suggest that 50 days of canola oil ingestion alone leads to changes in antioxidant levels. The present study has also shown that in the presence of salt, canola oil ingestion reduced the activities of RBC SOD and catalase compared with the canola oil group without salt. The soybean oil group in the presence of salt also showed reduced activities of RBC SOD and catalase compared to the soybean oil group without salt. This data is consistent with our lifespan study data in chapter 3, which showed a decrease in RBC SOD and catalase, however, RBC GPx was also reduced. Furthermore, the results from the lifespan study did not find any changes in the activities of the RBC antioxidants in the soybean oil group. RBCs are under constant exposure to ROS and oxidative stress due to their role in transporting oxygen and carbon dioxide, and their increased content of heme iron (Cimen 2008). Therefore, the oxidative/antioxidant balance can become altered. Supporting evidence shows that there is an association between reduced antioxidants and CVD (Kalenikova et al. 2004). In addition, previous research has shown an inverse relationship between erythrocyte GPx activity and the incidence of CVD (Blankenberg et al. 2003).
The present study has also shown that canola oil ingestion in combination with salt leads to an increase in RBC MDA compared to all the other dietary groups. Whereas, plasma MDA was reduced in the canola oil group with salt compared to the soybean oil group with salt. This result is consistent with our lifespan study data in chapter 3, which showed a decrease in plasma MDA in the canola oil group compared to the soybean oil group. The mechanism by which canola oil along with salt intake leads to a decrease in plasma MDA is currently unknown. Our previous study found that short term intake (25 days) of canola oil resulted in an increase in both RBC and plasma MDA. The present study has also shown that soybean oil ingestion in combination with salt lead to an increase in plasma 8-isoprostane compared to all the other dietary groups. In the present study the increase in RBC MDA and plasma 8-isoprostane indicates an increase in lipid peroxidation. Salt intake may be a contributing factor to the changes seen, as there were no changes observed in the markers of oxidative stress in the diets without salt intake. A previous study observed an increase in urine MDA concentration in Sprague-Dawley rats as a result of salt intake (Kitiyakara et al. 2003). They found a difference between the low salt (0.03%) and normal salt (0.3%) groups, and between the low salt and high salt (6%) groups. In addition, they also found an increase in urine 8-isoprostane levels as a result of salt intake.

Evidence has shown that NADPH oxidase derived $\text{O}_2^-$ is enhanced in hypertension (de Cavanagh et al. 2010; Kitiyakara et al. 2003). In the present study, canola oil ingestion in the absence of salt increased $p22^{phox}$ and NOX2 mRNA expression compared to soybean oil alone. This data suggests that $\text{O}_2^-$ generated from NADPH oxidase is elevated in the presence of canola oil alone. In
addition, canola oil ingestion in the presence of salt reduced p22phox mRNA expression compared to the canola oil group without salt. However, NOX2 mRNA expression was increased in the soybean oil group with salt intake compared to the non-salt groups. The previous study in chapter 4 showed that 25 days of canola oil ingestion with salt reduced the mRNA expression of p22phox compared to the canola oil group without salt, which is consistent with the results from the present study. However, in the 25 day study, NOX2 mRNA expression was also found to be reduced in the canola oil group with salt, which was not evident in the present study. A study by Kitiyakara et al. (2003) found that salt intake in Sprague-Dawley rats lead to an increase in O$_2^-$ production. This was accompanied by an increase in renal activity and mRNA expression of NOX2 and p47phox, and a decrease in CuZn-SOD and Mn-SOD mRNA expression (Kitiyakara et al. 2003). However, the results from the present study show that canola oil ingestion without salt, and both the soybean oil and canola oil groups with salt increased the mRNA expression of CuZn-SOD compared to soybean oil alone. The increase in the mRNA expression of CuZn-SOD along with an increase in RBC SOD in the canola oil group without salt may be an adaptive response to an increase in O$_2^-$ generation. The previous study in chapter 4 showed that 25 days of canola oil ingestion with salt reduced the mRNA expression of CuZn-SOD, Mn-SOD and Ec-SOD, which is not evident in the present study. There were no changes found in Mn-SOD and Ec-SOD in the present study. Furthermore, in the present study, ROS generation in the canola oil group in the presence of salt may be coming from other sources within the vasculature, such as xanthine oxidase, uncoupled nitric oxide synthase, lipoxygenase and the mitochondrial respiratory chain (Jiang et al. 2004; Schulz et al. 2011).
The results from the present study show that contractile responses to norepinephrine were reduced in the canola oil group with salt compared to the canola oil only group, indicating smooth muscle cell contractile dysfunction. In addition, in the present study the contractile responses were greater in the SHRSP rats fed only canola oil compared to the soybean oil only group. Also, the WKY rats had greater contractile responses compared to the soybean oil only group and to both the canola oil and soybean oil groups with salt. These results show that SHRSP rats have reduced smooth muscle contractile function; however, the intake of canola oil alone may result in reducing contractile dysfunction. Furthermore, a study by Natio et al. (2000c) found that in SHRSP rats, in which salt loading was not used, the contractile responses in isolated mesenteric vascular bed to norepinephrine and to other agonists such as angiotensin II, arachidonic acid, ATP, endothelin-1 or serotonin were not different between the canola oil and soybean oil group after 4 weeks of feeding. The results from the present study also show that canola oil ingestion in combination with salt leads to a decrease in the endothelium-dependent vasodilating response to ACh compared to canola oil ingestion alone. The results suggest that salt intake in combination with canola oil is leading to endothelial dysfunction. A study found that in SHR and WKY rats the endothelium-dependent and endothelium-independent vasodilating responses to ACh and SNP, respectively, were not different between the canola oil and soybean oil groups in each strain following salt loading (Natio et al. 2000b). These results are consistent with the results found in the present study, as there were no changes found in the vasodilating responses to ACh and SNP between the canola oil and soybean oil groups with salt. However, the vasodilating responses to ACh were reduced in the canola oil group with salt...
compared with the WKY rats. In addition, studies have shown an association between high salt intake and endothelial dysfunction (Bragulat & de la Sierra 2002). A study has found that long term high salt intake caused a reduction in endothelium-dependent vasorelaxation in salt-sensitive rats (Li et al. 2009). Evidence has shown that in Sprague-Dawley rats, high salt intake leads to endothelial dysfunction by reducing the concentration of NO and eNOS activity (Datla & Griendling 2010). It is well established that ROS interacts with endothelial NO, thus decreasing the vasodilating response of blood vessels. However, p22phox mRNA expression was reduced in the canola oil group with salt, suggesting a decrease in NADPH oxidase derived $O_2^{-}$ generation. The interaction between salt intake and canola oil is not clearly understood, however, it seems that canola oil in combination with salt leads to reduced smooth muscle contractile function and impaired endothelium-dependent vasorelaxation.

Previous studies have reported an increase in plasma lipids due to canola oil ingestion (Natio et al. 2000b & 2000d; Ohara et al. 2008a & 2009). Canola oil is considered to provide protective cardiovascular effects due to its favourable fatty acid composition (Ratnayake et al. 2000b). However, in the present study, canola oil ingestion alone increased the concentration of total cholesterol and triglycerides compared with soybean oil alone. These results are consistent with the results from the previous study in chapter 4, which showed that short term ingestion of canola oil alone increased total cholesterol as well as LDL-C. Evidence has shown that dietary salt restriction from 10g to 2g per day for 5 days in patients with essential hypertension significantly increased total cholesterol and LDL-C (Sharma et al. 1990). Furthermore, there is an associated between
increased concentrations of plasma total cholesterol, triglycerides and LDL-C and the development of CVD such as atherosclerosis (Ballantyne et al. 2005). In addition, the present study has also shown that canola oil ingestion in the presence of salt increased total cholesterol and LDL-C, whereas the concentration of triglycerides was reduced. Our life span study showed that canola oil ingestion following salt loading reduced total cholesterol and LDL-C compared with soybean oil (Papazzo et al. 2011). In addition, the results from the present study also show that soybean oil in the presence of salt increased total cholesterol, LDL-C, HDL-C and triglycerides compared to soybean oil alone. In the present study, salt intake is having an interacting effect on the plasma lipids, as the increase in the total cholesterol and LDL-C is common in both the canola oil and soybean oil groups with salt loading. The mechanism to explain the salt interaction and the increase in plasma lipids is unknown, and warrant further investigation.

The association between salt intake and hypertension is well known (Taylor et al. 2011), which is evident in the present study. The results of the present study show an increase in systolic blood pressure in both the canola oil and soybean oil groups with salt compared to the dietary groups without salt at the end of the feeding trial. Evidence indicates that canola oil intake has an effect on blood pressure in the SHRSP rat and its related strains (Natio et al. 2000, 2000b & 2003). However, the blood pressure in the canola oil group was not consistently different from soybean oil. The results from the previous studies in chapter 3 and 4 also show that the blood pressure in the canola oil group is not different to the soybean oil group. Taken together these results suggest that canola oil intake in
the presence or absence of salt does not affect blood pressure. This is also supported by other studies, which have shown no significant changes in blood pressure between the canola oil and soybean oil groups in SHRSP rats (Huang et al. 1996; Ratnayake et al. 2000a).

In conclusion, canola oil intake alone increased p22phox and NOX2 mRNA expression, suggesting an increase in O$_2^-$ generation. In addition, canola oil ingestion alone increased RBC SOD, total cholesterol and triglycerides and decreased RBC GPx, which are risk factors for CVD. Canola oil in combination with salt intake increased RBC MDA, total cholesterol and LDL-C and decreased RBC SOD, catalase, plasma MDA and p22phox mRNA expression. Furthermore, canola oil intake with salt loading significantly impaired endothelial function in SHRSP rats when compared to canola oil intake alone.
CHAPTER 6
Overall conclusions

6.1 Summary of the major findings

The overall objectives of the thesis were to determine the effect of canola oil ingestion on oxidative stress in the vasculature and endothelial function in SHRSP rats. Evidence has shown that ingestion of canola oil as the sole dietary fat source (added at 10 wt/wt% to standard rat chow) shortens the lifespan of SHRSP rats. The mechanism by which canola oil reduces lifespan is currently unknown. However, recent studies suggest that one of key mechanisms leading to the shortened lifespan in SHRSP rats is via the acceleration of hypertension-related deterioration of organs (Natio et al. 2003; Ohara et al. 2006). The damage and deterioration of organs may be exacerbated by oxidative stress due to canola oil intake. Growing evidence has found that oxidative stress leads to vascular damage and plays a critical role in the pathogenesis of CVD (Berry et al. 2001).

There is an inverse relationship between reduced activities of antioxidants (SOD and GPx) and increased lipid peroxidation products in blood and CVD (Kalenikova et al. 2004).

The results from the first study strengthened the finding that canola oil as the only dietary fat source reduces the lifespan of SHRSP rats. This study found that canola oil ingestion reduced the lifespan of SHRSP rats compared to soybean oil following 1% NaCl loading, 85.8 ± 1.1 and 98.3 ± 3.4 days, respectively (Papazzo et al. 2011). The results also showed that blood pressure increased over
time in both the soybean oil and canola oil groups; however, there was no difference found between each diet. In addition, canola oil ingestion reduced RBC SOD, GPx and catalase activities, total cholesterol and LDL-C compared with soybean oil. There were no significant differences in RBC MDA concentration between canola oil fed and soybean oil fed rats. In contrast, plasma MDA concentration and 8-isoprostane were significantly lower in the canola oil group compared to the soybean oil group. Also, the plasma 8-isoprostane level in the soybean oil group and MDA level in both the canola oil and soybean oil groups were higher than the pre-treatment group. In addition, NaCl loading was used in this lifespan study to accelerate the development of hypertension. However, salt loading may have been masking the effects of canola oil ingestion in SHRSP rats. Therefore, the second study was carried out to determine the effect of short-term (25 days) ingestion of canola oil in the absence or presence of excess dietary salt on oxidative stress in the vasculature of SHRSP rats. The results from the 25 day study showed that canola oil ingestion in the absence of dietary salt decreased the activities of RBC SOD and GPx, and increased both total cholesterol and LDL-C compared with soybean oil. However, the combination of canola oil and dietary salt intake resulted in an increase in plasma MDA and a decrease in LDL-C concentration, NOX2, p22phox, CuZn-SOD, Mn-SOD and Ec-SOD aortic mRNA expression when compared to canola oil intake alone.

The final study was carried out to determine the effect of canola oil ingestion in the absence or presence of excess dietary salt for a longer period of time in SHRSP rats. Research has shown that endothelial dysfunction is an independent
predicator of CVD (Davies 2009). ROS leads to a decrease in NO, resulting in endothelial dysfunction (Fortuno et al. 2005). Therefore, the final study examined the effect of 50 days of canola oil ingestion on endothelial function and oxidative stress in the vasculature of SHRSP rats. The results showed that canola oil intake alone increased RBC SOD, total cholesterol, triglycerides, \( p22^{phox} \) and NOX2 mRNA expression, and decreased RBC GPx compared with soybean oil. However, canola oil in combination with salt intake increased RBC MDA compared to the soybean oil group with salt and to the canola oil only group. In addition, canola oil intake with salt lead to an increase in total cholesterol and a decrease in plasma MDA compared to the soybean oil group with salt. The results also showed that LDL-C was elevated and RBC SOD, catalase, plasma MDA and \( p22^{phox} \) mRNA expression were reduced in the canola oil group with salt compared to the canola oil only group. Furthermore, the canola oil group in combination with salt intake had reduced contractile responses to norepinephrine compared to the canola oil group without salt. Also, the contractile responses were greater in the SHRSP rats fed only canola oil compared to the soybean oil only group. The WKY rats had greater contractile responses compared to the soybean oil only group and to both the canola oil and soybean oil groups with salt. In addition, canola oil ingestion with salt loading showed impaired endothelium-dependent vasodilating responses to ACh compared to the canola oil group without salt and WKY rats.
6.2 Discussion of the major findings

Canola oil ingestion shortened the lifespan of SHSRP rats compared to soybean oil. These results strengthen and support the findings from previous studies. Evidence indicates that canola oil intake has an effect on blood pressure in the SHRSP rat and its related strains (Natio et al. 2000, 2000b & 2003). However, the blood pressure in the canola oil group was not consistently different from soybean oil in all three studies. Taken together these results suggest that canola oil intake in the presence or absence of salt does not affect blood pressure. Therefore, the results indicate that the life shortening effect of canola oil is not directly related to an increase in blood pressure over time. This is also supported by other research, which has shown no significant change in blood pressure between the canola oil and soybean oil groups in SHRSP rats (Huang et al. 1996; Ratnayake et al. 2000a). Also, the association between salt intake and hypertension is well known (Taylor et al. 2011), which is evident in both the 25 day and 50 day studies. Our results demonstrated an increase in systolic blood pressure in both the canola oil and soybean oil groups with salt compared to the dietary groups without salt at the end of the feeding trial.

To our knowledge this is the first study to investigate the effect of canola oil intake on measures of antioxidant status and oxidative damage in SHRSP rats. The results from the lifespan study in chapter 3 showed that canola oil ingestion reduced RBC SOD, GPx and catalase activities compared with soybean oil by 25, 18 and 31%, respectively. Evidence has shown that in the SHR, the activity of RBC GPx and the activity of catalase in the hepatic cytosol were found to be reduced (Ohara et al. 2008). In WKY rats, canola oil ingestion increased the
activity of SOD and reduced the activity of catalase in the hepatic cytosol (Ohara et al. 2009), while an earlier study showed reduced activities of SOD and catalase in the hepatic cytosol of WKY rats (Natio et al. 2000d). Our data are largely consistent with these findings. Taken together these results indicate that canola oil ingestion affects antioxidant enzyme activity in different tissues. The second study showed that the ingestion of canola oil for 25 days in the absence of excess dietary salt decreased RBC SOD and GPx compared with soybean oil alone. Whereas, 50 days of canola oil ingestion in the absence of salt loading increased RBC SOD and decreased GPx activity. The decrease in the activity of RBC GPx is consistent in both the 25 day and 50 day studies. In the presence of salt, both canola oil and soybean oil ingestion for 25 days decreased the activity of GPx compared with the soybean oil group without salt. Whereas, 50 days of canola oil ingestion in combination with salt intake resulted in a decrease in RBC SOD and catalase. The data is consistent with the lifespan study, which showed a decrease in RBC SOD and catalase, however, RBC GPx was also reduced. Supporting evidence shows that there is an association between reduced antioxidants and CVD (Kalenikova et al. 2004). RBCs are under constant exposure to ROS and oxidative stress due to their role in transporting oxygen and carbon dioxide, and their increased content of heme iron (Cimen 2008). Therefore, the oxidative/antioxidant balance can become altered. In addition, previous research has shown an inverse relationship between erythrocyte GPx activity and the incidence of CVD (Blankenberg et al. 2003).

In the first study, canola oil ingestion decreased the plasma 8-isoprostane and MDA concentration when compared to the soybean oil group. These results were
unexpected given the canola oil-induced decrease in erythrocyte antioxidant enzyme activity. The mechanism to explain the decreased plasma 8-isoprostane and MDA concentration despite a decrease in RBC antioxidant enzyme activity is currently unknown. However, the plasma 8-isoprostane in the soybean oil and MDA in both the canola oil and soybean oil groups was higher than the pre-treatment group. In a previous study in WKY rats, canola oil ingestion increased lipid peroxide levels in the hepatic cytosol (Ohara et al. 2009), while an earlier study showed no change in lipid peroxide levels in the hepatic cytosol of SHR (Ohara et al. 2008). The increased plasma 8-isoprostane in the soybean oil group and MDA concentrations in both dietary groups compared to pre-treatment indicates an increased amount of ROS induced lipid peroxidation over time independent from the consumption of either canola or soybean oil. In the presence of salt, 25 days of canola oil ingestion increased the plasma MDA concentration when compared to the canola oil group without salt. In addition, RBC MDA concentration was increased in the canola oil and soybean oil groups in the presence of salt compared with the non salt groups. The mechanism by which canola oil intake in combination with salt induces MDA is currently unknown. Whereas, 50 days of canola oil intake together with salt increased RBC MDA compared to all the other dietary groups. While plasma MDA was reduced in the canola oil group with salt compared to the soybean oil group with salt. This result is consistent with the life span study which showed a decrease in plasma MDA in the canola oil group compared to soybean oil. A previous study found an increase in urine MDA concentration in Sprague-Dawley rats as a result of salt intake (Kitiyakara et al. 2003). They found a difference between the low salt (0.03%) and normal salt (0.3%) groups, and between the low salt and high salt
(6%) groups. In addition, they also found an increase in urine 8-isoprostan levels as a result of salt intake. The results from the 25 day and 50 day studies suggest that salt intake is having an effect on the concentration of both plasma and RBC MDA when in combination with canola oil ingestion, as there were no changes observed in the dietary groups without salt intake.

The results from the 25 day study showed that in the presence of salt, canola oil ingestion resulted in a decrease in NOX2, p22phox, CuZn-SOD, Mn-SOD and Ec-SOD aortic mRNA expression when compared to canola oil intake alone. This study shows for the first time that 25 days of canola oil intake with salt reduces NADPH oxidase subunits and SOD isoforms in the aorta of SHRSP rats. The decrease in the SOD isoforms indicates a reduced ability to eliminate O$_2^-$ in the presence of canola oil and salt. High salt intake (6%) has been shown to reduce renal expression of CuZn-SOD and Mn-SOD in Sprague-Dawley rats (Kitiyakara et al. 2003). The results from the 50 day study showed that in the absence of salt, canola oil ingestion increased p22phox and NOX2 mRNA expression compared with soybean oil. Whereas, canola oil ingestion in the presence of salt decreased p22phox mRNA expression compared with the canola oil only group. It appears that canola oil ingestion in combination with salt decreases NADPH oxidase mRNA expression. The results also showed that canola oil ingestion without salt, and both the soybean oil and canola oil groups with salt increased the mRNA expression of CuZn-SOD compared to soybean oil alone. The increase in p22phox and NOX2 mRNA expression suggests an increase in O$_2^-$ generation as a result of canola oil ingestion alone. In addition, the increase in CuZn-SOD in the canola oil group without salt may be an adaptive response to an increase in O$_2^-$ derived
from NADPH oxidase. Evidence has shown that $O_2^\cdot$ generated from NADPH oxidase is increased in hypertension (Paravicini & Touyz 2008). In addition, Kitiyakara et al. (2003) found that salt intake in Sprague-Dawley rats lead to an increase in $O_2^\cdot$ production. This was accompanied by an increase in renal activity and mRNA expression of NOX2 and p47$^{phox}$, and a decrease in CuZn-SOD and MnSOD mRNA expression (Kitiyakara et al. 2003). However, in the vasculature, there are several other sources of ROS, which include: xanthine oxidase, uncoupled nitric oxide synthase, lipoxygenase and the mitochondrial respiratory chain (Jiang et al. 2004; Schulz et al. 2011). In the studies, ROS generation may be coming from other sources within the vasculature, and requires further investigation. Furthermore, it would have been ideal to examine the protein levels of the genes of interest. However, there was insufficient protein to carry out the western blot analysis.

The results from the lifespan study (Chapter 3) showed that canola oil ingestion reduced total cholesterol and LDL-C compared with soybean oil. This data supports the health benefits of canola oil intake. Canola oil is considered to provide protective cardiovascular effects due to its favourable fatty acid composition (Ratnayake et al. 2000b). However, in the absence of salt, 25 days of canola oil ingestion increased both total cholesterol and LDL-C compared with soybean oil. Whereas, canola oil in combination with dietary salt intake lead to a decrease in LDL-C compared with canola oil intake alone. This data is consistent with the results from our first study (Chapter 3), which showed that canola oil intake decreased LDL-C, as well as total cholesterol. However, the results from our 50 day study showed that in the absence of salt, canola oil ingestion increased
total cholesterol and triglycerides. These results are consistent with the results from the 25 day study, which showed that canola oil intake alone increased total cholesterol, as well as LDL-C. In the presence of salt, canola oil increased total cholesterol and LDL-C. In addition, the results from the 50 day study also showed that soybean oil in the presence of salt increased total cholesterol, LDL-C, HDL-C and triglycerides compared to soybean oil alone. Evidence has found an associated between increased concentrations of plasma total cholesterol, triglycerides and LDL-C and the development of CVD such as atherosclerosis (Ballantyne et al. 2005). In addition, there is substantial evidence suggesting that high salt intake increases the risk of CVD (Harsha et al. 2004). However, some studies suggest that low salt intake and its adverse effects on blood lipids can have a detrimental effect on CVD risk (Harsha et al. 2004). The mechanisms by which salt intake affects the blood lipids are not clear. A study has shown that dietary salt restriction from 10g to 2g per day for 5 days in patients with essential hypertension significantly increased total cholesterol and LDL-C (Sharma et al. 1990).

The results from the 50 day study showed that contractile responses to norepinephrine were reduced in the canola oil group with salt compared to the canola oil only group, indicating smooth muscle cell contractile dysfunction. Also, the contractile responses were greater in the SHRSP rats fed only canola oil compared to the soybean oil only group. In addition, the WKY rats had greater contractile responses compared to the soybean oil only group and to both the canola oil and soybean oil groups with salt. These results show that SHRSP rats have reduced smooth muscle contractile function; however, the intake of canola
oil alone may result in reducing contractile dysfunction. In addition, canola oil ingestion with salt loading showed impaired endothelium-dependent vasodilating responses to ACh compared to the canola oil group without salt and WKY rats. The results suggest that salt intake in combination with canola oil is leading to endothelial dysfunction. A study by Natio et al. (2000b) found that in SHR and WKY rats, the endothelium-dependent and endothelium-independent vasodilating responses to ACh and SNP, respectively, were not different between the canola oil and soybean oil groups in each strain following salt loading (Natio et al. 2000b). These results are consistent with the results found in the present study, as there were no changes found in the vasodilating responses to ACh and SNP between the canola oil and soybean oil groups with salt. In addition, evidence has shown an associated between high salt intake and endothelial dysfunction (Bragulat & de la Sierra 2002). The interaction between salt intake and canola oil is not clearly understood, however, it appears that canola oil ingestion in combination with salt leads to reduced smooth muscle contractile function and impaired endothelium-dependent vasorelaxation.

### 6.3 Study limitations

There are a number of limitations associated with the thesis studies. The first is the question of whether the life shortening effect associated with canola oil consumption is specific to SHRSP rats. Canola oil ingestion has been shown to increase blood pressure in WKY rats (Natio et al. 2000d), and cause vascular lesions in the kidney and elevate plasma lipids in SHR (Ohara et al. 2008a). In human populations there is only one published incidence of canola oil being associated with the development of disease (Posada de la Paz et al. 2001). Thus,
the consumption of canola oil on lifespan needs to be tested in other rat strains and even in humans. Also, our studies did not identify the potential toxic compound in canola oil that is causing the life shortening effect. Lastly, it would have been ideal to examine the aortic protein expression of NOX2, p22phox and the SOD isoforms. However, there was insufficient protein from the aortic samples to carry out western blot analysis. A maximum of 20 µg of protein was able to be used per sample and the protein bands were undetectable. However, studies have used 40 to 50 µg of protein to determine the aortic protein expression of the NADPH oxidase subunits and SOD isoforms (El-Awady et al. 2011; de Moraes et al. 2008; Wand et al. 2007).

6.4 Future directions

The research findings described in this thesis have raised questions that warrant further investigation. The results have shown that canola oil ingestion in the absence and presence of salt leads to changes in oxidative status and aortic mRNA expression of NADPH oxidase subunits. However, O$_2^-$ generation was not measured directly, and therefore, aortic sections should be stained with dihydroethidium (DHE) to determine whether O$_2^-$ production is enhanced. Furthermore, in the vasculature, there are several other sources of ROS, which include: xanthine oxidase, uncoupled nitric oxide synthase, lipoxygenase and the mitochondrial respiratory chain (Jiang et al. 2004; Schulz et al. 2011). Therefore, future studies should examine other sources of ROS. In addition, the results from this thesis have shown that canola oil ingestion in combination with salt leads to reduced smooth muscle contractile function and impaired endothelium-dependent vasorelaxation. It is well established that ROS interacts with endothelial NO, thus
decreasing the vasodilating response of blood vessels. Evidence has shown that in Sprague-Dawley rats, high salt intake leads to endothelial dysfunction by reducing the concentration of NO and eNOS activity (Datla & Griendling 2010). Therefore, a future study should measure NO and eNOS activity, as well as examine its aortic mRNA and protein expression. Furthermore, endothelial dysfunction is characterised by impaired vasodilatory responses, enhanced generation of vasoconstricting substances, platelet activation, increased expression of adhesion molecules and a proinflammatory and prothrombotic phenotype (Cai & Harrison 2000; Chhabra 2009; Savoia et al. 2011). Inflammation contributes to vascular remodelling, and in patients with CVD the expression and plasma concentrations of inflammatory markers and mediators such as interleukin-6, tumour necrosis factor-a and C-reactive protein are elevated (Savoia et al. 2011). The results from this thesis have shown that canola oil in combination with salt resulted in impaired vasodilatory responses; therefore, raising the question as to whether these animals are in a proinflammatory state. Hence, future studies should examine the expression and plasma concentrations of inflammatory markers.
References


Flegel, K. M. M. and Magner, P. M. D. (2009). "Get excess salt out of our diet." CMAJ 180(3): 263-


139


ammonia increase oxidative stress by NADPH oxidase in cultured astrocytes and vital brain slices.” Glia, 55:758-771.


