THE ROLE OF ANTIOXIDANTS IN ATHEROGENESIS AND SALT-SENSITIVE HYPERTENSION

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THE ROLE OF ANTIOXIDANTS IN ATHEROGENESIS AND SALT-SENSITIVE HYPERTENSION

by

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DECLARATION

I, Anand Nadar, Registration Number: 8014515 hereby declare that the dissertation/thesis entitled:

The Role of Antioxidants in Atherogenesis and Salt-Sensitive Hypertension,

is the result of my own investigation and research and that it has not been submitted in part or in full for any other degree or to any other university. Where use was made of the work of others, it is duly acknowledged in the text.

A. Nadar

Date

CONTENTS

LIST OF APPENDICES vii

ABBREVIATIONS viii

LIST OF TABLES x

LIST OF FIGURES xii

LIST OF GRAPHS xiii

ABSTRACT xiv

INTRODUCTION 1

1.0 LITERATURE SURVEY 4

1.1 **Hypertension** 4

1.2 **Salt Sensitivity** 10

1.3 **Free Radicals** 11

1.3.1 Sources of Free Radicals 12

1.4 **The Antioxidant System** 14

1.4.1 Glutathione Peroxidase 16

1.4.1.1 GPx and Selenium 17

1.4.2 Superoxide Dismutase 18

1.4.3 The Depth of the Antioxidant System 19

1.5 **Free Radicals and Hypertension** 21

1.6 Effects of Oxidative Stress 23

1.6.1 Endothelium 23

1.6.2 Peroxidation of Lipids 26

1.6.2.1 Effects of Aldehydes 27

1.6.3 Free Radicals in Vascular Remodeling 28

1.7 Independent Factors associated with Hypertension 29

1.7.1 Hyperglycemia 29

1.7.2 Hyperinsulinemia 31

1.8 Reactive Oxygen Species and Atherosclerosis 33

1.9 The Dahl Model and its Relevance 41

2.0 MATERIALS AND METHODS 44

2.1 Animals 44

2.2 Plasma Lipids 45

2.2.1 Triglycerides 46

2.2.2 Total Cholesterol 46

2.2.3 High Density Lipoprotein 47

2.3 RBC Glutathione Peroxidase 48

2.4 Superoxide Dismutase 49

2.5 Glutathione 51

2.6 Malonaldehyde 53

2.7 **Red Blood Cell Membrane Cholesterol** 55

2.8 **Catalase** 56

2.9 **H_2O_2 Standardisation** 57

2.10 **Tissue Analysis** 58

2.11 **Cytosolic Protein Concentration** 59

2.12 **Preparation for Chemical Hypoxia** 61

2.12.1 Isolation of Adult Rat Ventricular Myocytes 61

2.12.2 Preparation of Coverslips 63

2.12.3 Preparation of the Cell Tak[R] 63

2.12.4 Culture of Myocytes 63

2.12.5 Loading Myocytes with Fluorescent Probes 64

2.12.6 The Induction of Chemical Hypoxia and Reoxygenation 64

2.13 **Blood Pressure and Diet** 67

2.13.1 Blood Pressure Measurement 67

2.13.2 Diets 68

3.0 RESULTS 70

3.1 **Blood Pressure** 70

3.2 **Body and Organ Mass** 71

3.3 **The Antioxidant Status of the Heart** 73

3.4 **Growth and Food Parameters** 74

3.5 **Chemical Hypoxia of isolated Myocytes** 75

5 CONCLUSIONS AND RECOMMENDATIONS 142

6 ACKNOWLEDGEMENTS 147

7 REFERENCES 148

8 APPENDIX 165

LIST OF APPENDICES

Heart GSH **165**

Graphs 1 - 4 **166-169**

RBC Glutathione Peroxidase **170**

Graphs 5 - 9 **171–175**

RBC Superoxide Dismutase **176**

Graphs 10 - 13 **177–180**

Table 12a and 12b: Average Weekly Food Consumption **181**

LIST OF ABBREVIATIONS

DSS	:	Dahl salt sensitive
DSR	:	Dahl salt resistant
DSS 1% and 8%	:	Dahl salt sensitive rats on 1% and 8% salt diets respectively
DSR 1% and 8%	:	Dahl salt resistant rats on 1% and 8% salt diets respectively
NO	:	Nitric oxide
O_2.	:	Superoxide ion / radical
SOD	:	Superoxide dismutase
GPx	:	Glutathione peroxidase
GSH	:	Glutathione (reduced)
GSSH	:	Glutathione disulfide
ROS	:	Reactive oxygen species
CAD	:	Coronary artery diseases
RBC	:	Red blood cell/ corpuscle
LDL	:	Low density lipoprotein
HDL	:	High density lipoprotein
SHR	:	Spontaneously hypertensive rat

MDA	:	Malondialdehyde
H_2O_2	:	Hydrogen peroxide
$OH^{\bullet}$	:	Hydroxyl radical
Ox LDL	:	Oxidized low density lipoprotein fraction
PGI_2	:	Prostacyclin
TBA	:	Thiobarbituric acid
XO	:	Xanthine oxidase
ANOVA	:	Analysis of variance
PAF	:	Platelet activating factor
CAT	:	Catalase
W/L	:	Wall-to-lumen ratio
NADPH	:	Nicotinamide – Adenine Dinucleotide Phosphate
EDAM	:	Endothelium derived adhesion molecules
•	:	Radical
-	:	Anion
+	:	Cation

LIST OF TABLES

Table 1a:	Classification of hypertension	**4**
Table 2a:	Reactive oxygen species and their characteristic	**12**
Table 3a:	The major primary and secondary antioxidants in mammals	**15**
Table 4a:	AIN-76 modified sodium deficient diet	**69**
Table 1:	Changes in blood pressure and heart rate of Dahl Salt Resistant (DSR) and Dahl Salt Sensitive (DSS) rats treated with normal NaCl (1%) and high NaCl (8%) diet.	**83**
Table 2:	Changes in body weight, heart weight, left ventricle weight and the respective ratios of Dahl Salt Resistant (DSR) and Dahl Salt-Sensitive (DSS) rats on normal (1% NaCl) and high sodium (8% NaCl) diet.	**84**
Table 3:	Antioxidant enzymes' activities in LV heart tissue of Dahl Salt-Resistant (DSR) and Salt-Sensitive (DSS) rats on normal (1%) and high (8%) sodium chloride diet.	**85**
Table 4:	Changes in body weight, blood pressure and heart rate in Dahl Salt-Resistant (DSR) and Dahl Salt-Sensitive (DSS) rats on normal Na (1%) and high Na (8%) diet for six weeks.	**86**
Table 5:	The effect of chemical hypoxia without reoxygenation on the morphology and fluorescence of isolated myocytes of Dahl Salt-Resistant (DSR) and Salt-Sensitive (DSS) rats on normal (1%) and high (8%) NaCl diets.	**87**

Table 6:	Antioxidant enzymes' activities and glutathione in red blood cells of Dahl Salt-Resistant (DSR) and Salt-Sensitive (DSS) rats on normal (1%) and high (8%) sodium chloride diet.	**88**
Table 7:	Blood glucose and antioxidant enzyme activity in red blood cells of Dahl Salt-Resistant (DSR) and Dahl Salt-Sensitive (DSS) rats, on normal (1%) and high (8%) diet for six weeks estimated by Randox antioxidant kits.	**89**
Table 8:	Oxidative challenge test of red blood cells of Dahl Salt-Resistant (DSR) and Dahl Salt-Sensitive (DSS) rats, after exposure to different concentrations of H_2O_2.	**90**
Table 9:	Plasma lipid profile of Dahl Salt-Resistant (DSR) and Dahl Salt-Sensitive (DSS) rats on normal NaCl (1%) and high NaCl (8%) diets for six weeks.	**91**
Table 10:	Red blood cells membrane cholesterol of Dahl Salt-Resistant and Dahl Salt-Sensitive rats on normal (1%) and high (8%) NaCl diet for six weeks.	**92**
Table 11:	Haematological parameters of Dahl Salt Resistant (DSR) and Dahl Salt Sensitive (DSS) rats on normal diets	**93**

LIST OF FIGURES

Figure 1: Chemical interrelationship between ascorbic acid and tocopherol showing the recycling of the antioxidants. **20**

Figure 2: Hypothesised Role of ROS in Atherosclerosis **40**

LIST OF GRAPHS

Graph 1: Systolic blood pressures of DSR and DSS rats **94**

Graph 2: Diastolic blood pressures of DSR and DSS rats **95**

Graph 3: Oxidative challenge of RBC to H_2O_2 **96**

Graph 4: Percentage increase in mass/week **97**

Graph 5: Protein BSA Standard Curve **98**

Graph 6: Haemoglobin Cyanometh Standard Curve **99**

Graphs 7 to 10: **166 – 169**

Graphs 11 to 15: **171 – 175**

Graphs 16 to 19: **177 - 180**

ABSTRACT

Reactive oxygen species have been strongly associated with various cardiovascular diseases including atherosclerosis and hypertension. It remains unresolved whether the compromised antioxidant status develops before the elevation in blood pressure or develops as a consequence of it. This study was undertaken to evaluate the antioxidant status in both weanling and adult Dahl salt sensitive (DSS) rats and to monitor the progression of hypertension over a six week period. This animal model is ideal since they display hypertension and atherosclerotic changes in early life; so the role of free radicals in both these processes could be determined.

The antioxidant status of the red blood plasma cells (RBC), and cardiac tissue was evaluated on the basis of the superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione (GSH) levels. The levels of malonyl-dialdehyde, a biomarker for lipid peroxidation, was also determined after subjecting RBC to forced peroxidation. In addition isolated myocytes were subjected to chemical hypoxia using hydrogen peroxide.

The results have shown that weanling DSS rats which are normotensive have a compromised antioxidant status compared to their respective controls, the Dahl salt resistant strain. Both SOD and GPx levels were significantly lower in the RBC of DSS weanling rats compared to DSR animals. This indicates that the compromised antioxidant status precedes the elevation in blood pressure in DSS weanlings.

Adult DSS, hypertensive rats on normal diets displayed significantly lower levels of SOD and GPx levels in the RBC, generated higher levels of lipid peroxides, displayed a greater degree of dyslipidemia and had higher levels of serum CAT than normotensive DSR animals. Salt loading (dietary salt increased to 8%) did not alter the RBC status or lipid profile significantly.

Analysis of cardiac tissue has revealed that there was a non significant increase in SOD levels coupled with significant decrease in both GPx and GSH levels and evidence of elevated intracellular Ca^{+2} and H_2O_2 levels in DSS compared to DSR animals. Salt loading further exaggerated this trend in DSS animals only.

These results support the role of antioxidants as antihypertensive agents and highlight the need of early therapeutic intervention in salt sensitive hypertension and atherosclerosis, if extrapolated to humans.

INTRODUCTION

There is increasing evidence that many of the pathophysiological changes evident in hypertension are due to the associated increase in free radicals (Maxwell, 2000). Although these species (oxidant) have relatively short half lives, they pose a serious threat to cellular integrity by reacting with membrane lipids, various carbohydrates and proteins, and nuclei acid of cells (Jacob, 1995). Considering their diverse threat to cellular homeostasis it is therefore ironical that these species are generated by normal physiological metabolic mechanisms (Yu, 1994).

In order to maintain homeostasis and preserve the integrity of their cells, aerobic organisms have numerous molecules and enzymes that react with these species, effectively quenching them. These antioxidants are either of dietary origin or are synthesized endogenously in response to the levels of their respective pro-oxidants. Should the delicate balance that exists between the pro-oxidants and antioxidants shift towards the former, the resulting oxidative stress that would prevail has been implicated to have widespread effects. The major free radicals that have been consistently found to be elevated in hypertension are $O_2^{\cdot}$ and H_2O_2 (Lacy, *et al.*, 1998). These are quenched by SOD and GPx respectively. Catalase also plays a significant role in quenching H_2O_2 especially when levels are high. One of the complications with elevated levels of $O_2^{\cdot}$ is that after the dismutation reaction by SOD, H_2O_2 is formed. This species being less toxic but more diffusible, poses problems for the antioxidant system albeit at a less hazardous level. This illustrates

both the integrated nature of the antioxidant system and the depth at which it functions (Jacobs, 1995).

Oxidative stress could also arise when the antioxidant status is compromised. This has been attributed to factors ranging from inadequate dietary intake to genetic abnormalities. Genetic irregularities that alter expression of antioxidants, also have been reported to play a role (Pogan, *et al.*, 2001). If the alleles responsible for hypertensive mechanisms co-segregate with those responsible for expression of antioxidant enzymes, in individuals that are genetically predisposed to hypertension, this could therefore explain the elevated levels of free radicals in hypertensives. Therefore any factor/s that alter the stoichiometric balance between free radicals and antioxidants could result in an increase in oxidative stress.

Decades of research in cardiovascular diseases have identified many independent factors to play a causal role. Factors such as hyperglycemia, hyperinsulinemia with insulin resistance and dyslipidemia amongst others have been positively correlated with hypertension and cardiovascular diseases including atherosclerosis. Free radical research has added an additional dimension to the complications posed by these independent factors. In virtually all cases, free radicals have been linked to these independent risk factors. Therefore these radicals could potentially work directly and indirectly in the pathogenesis of various diseases including hypertension and atherosclerosis.

Salt and hypertension have been synonymous for decades since epidemiological and clinical studies have shown increased risk of developing hypertension with high dietary intake of NaCl. Salt-sensitive hypertension is of polygenic origin and develops primarily as a result of salt retention. Recent research has shown that in experimental salt sensitive models, such as the Dahl rat, there are critical periods called "developmental windows" during which environmental factors impact strongly on cardiovascular phenotype of adulthood. High salt diets and elevated levels of free radicals early in life had been shown to independently accelerate the onset and severity of hypertension (Zicha, *et al.*, 1999). Many studies have demonstrated that various cell types from hypertensive subjects are subjected to increase oxidative stress *in vivo* (Russo, *et al.*, 1998; Pogan, *et al.*, 2001). What has, however not been answered is whether the increase in free radicals precedes the elevation in blood pressure or arises as a consequence of the hypertension. In addition no direct causative link between free radicals and hypertension has been established. The present study was therefore undertaken to evaluate the antioxidant status of weanling and adult salt sensitive rats, monitor the onset of hypertension and determine the effect of salt loading on the antioxidant status of these rats.

Since the introduction of genetic models of hypertension such as the Dahl rat, evidence that has been gathered over the last three decades has, and will continue to help understand human hypertension more closely. Besides being able to develop effective therapeutic measures as has been the case up until now, significant preventive measures can also be developed from this research.

1.0 LITERATURE SURVEY

1.1 Hypertension

Extensive clinical trials have shown that arterial blood pressure has a normal distribution in the population. It is therefore difficult to differentiate between normotensive as opposed to hypertensive blood pressures, in addition this differentiation could be seen as arbitrary. Hypertension has traditionally been defined as arterial pressures that are positively correlated with severe cardiovascular pathophysiology (WHO guidelines, 1999). The World Health Organisation (WHO), based on extensive epidemiological studies and clinical trials presently identifies systolic and diastolic pressures greater than 140 and 90 mm Hg respectively as hypertensive (WHO Guidelines, 1999).

In addition, to facilitate the management of therapy, the presently accepted guidelines further classifies hypertension into three categories/ groups depending on severity (Table 1a).

TABLE 1a: Classification of Hypertension

Category	Systolic	Diastolic
Normal	< 130	< 85
High normal	130 – 139	85 – 89
Grade 1 hypertension (mild)	140 – 159	90 – 99
Subgroup: borderline	140 – 149	90 – 94
Grade 2 hypertension (moderate)	160 – 179	100 – 109
Grade 3 hypertension (severe)	≥ 180	≥ 110

In the case of individuals in which the causes of the hypertension can be identified (~10%) the condition is referred to as secondary hypertension. There are various forms of secondary hypertension, example, endocrine, drug induced, renovascular, pregnancy, etc. This type of hypertension can be normalized by therapy due to its identifiable causes.

In a large percentage of hypertensive individuals however (~90%) the reason for the increase in blood pressure is unknown, hence it has been referred to as essential or primary hypertension. Therapeutic intervention therefore relies largely on symptomatic treatment. The difficulty in identifying the aetiology of the hypertension is due to the fact that the hypertension appears to be of polygenic origin and environmental influences, multi-factorial. In addition the interaction between these two factors (nature and nurture) is also a variable that compounds the problem (Zicha, *et al.*,1999).

It is postulated that when the genetic and environmental factors convene repeatedly over a period of time, coupled with poor diets and a lack of exercise, this results in a gradual increase and subsequent fixing of arterial blood pressure at elevated levels (Zicha, *et al.*, 1999).

A good knowledge therefore of the ontogenic changes that occur in the cardiovascular apparatus and renal system would therefore facilitate research in the field. Functional changes that have been implicated to play a key role in hypertension (including the salt- sensitive type) are summarized below:

1. Structural vascular changes in humans and experimental animal models (including salt sensitive) are the hallmark of chronic hypertension. As yet however, no studies have demonstrated that these structural changes of the vasculature in hypertensives is linked to a genetic abnormality. This implies that no individuals have been shown to be genetically susceptible to the structural changes of vessels evident in hypertensives indicating that they could be induced by other factors or arise as a consequence of the hypertensive syndrome.

Simon *et al* (1998) have proposed that hypertension is preceded by a prehypertensive period in which the vasculature (especially the resistance vessels) is stimulated by trophic stimuli that potentiate the vasoconstrictor and pressor responses. The elevated levels of free radicals prior to the onset of hypertension adds strength to this hypothesis since free radicals have been identified as one of the many stimuli that could be implicated in vascular remodeling which subsequently lead to hypertension (Uehara, *et al.*, 1992).

The irreversible structural changes of the vasculature once established, dominate haemodynamics so significantly that the genetic and environmental factors that contributed to the syndrome are difficult to pin point (Folkow, *et al.*, 1992). The resultant increase in blood pressure therefore occurs at even relatively normal levels of cardiac and vascular function.

2. In genetic forms of hypertension, the kidneys tend to conserve Na^+ (sodium retention) due to amongst other factors, a shift of the pressure natriuresis curve to the right and a decrease in glomerular filtration capacity. Sodium loading in such cases exacerbates the hypertension (Rapp, 1982). In addition there is an abnormal response of the renal vasculature to prostaglandins which result in an increase in renal pressure (Tobian, 1997). The kidneys are however also a target organ because they are damaged as a consequence of the hypertension (Ruilope, *et al.*, 1989).

3. Besides the increase in sympathetic innervation in individuals that are genetically predisposed to hypertension, there is an increase in sympathetic activity. Hypertensives also display a characteristic non dipper pattern in sympathetic activity in which there is no decrease in nocturnal activity (Grassi, *et al.*, 1998; Zicha, *et al.*, 1999).

4. There has been a host of abnormalities associated with the renin-angiotensin system (RAS). Genetic studies have shown blood pressure was linked to blood renin thus enhanced RAS activity is associated with most forms of hypertension (Zicha, *et al.*, 1999). Exceptions however include the Dahl experimental model which is considered a low renin model of hypertension (Tobian, 1997).

5. The endothelium despite being a mono-layer, plays a very important role in various physiological processes such as serving as a selective filter to plasma

components and homeostasis (Henning, *et al.*,1987). In addition to these functions the endothelium is implicated in regulating smooth muscle tone and hence blood pressure through the synthesis and release of vasoactive substances such as nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor (Grunfeld *et al.*, 1995; Pogan *et al.*, 2001). Nitric oxide is perhaps the most potent of these vasoactive substances. Due to this secretory ability of the endothelium, it is often considered as an endocrine gland in itself by many physiologists (Anggard, 1994).

NO synthetase, the enzyme responsible for the synthesis of nitric oxide from the precursor L-arginine is expressed in all endothelial cells. NO being highly diffusible binds with receptors in the smooth muscle cells of the vasculature. This, after a cascade of intracellular reactions leads to the relaxation of the smooth muscle with resultant vasodilation (Bonnardeaux *et al.*, 1995).

Under normal physiological conditions the stimuli that trigger this response are shear stress, histamine, substance P and vasopressin. Inhibition of NO synthesis by many researchers has resulted in this vasodilatory controls being lost, followed by an increase in arterial blood pressure in both humans and animal models (Bonnardeaux *et al.*, 1995 and Grunfeld, *et al.*). This indicates that the resistance vessels are in a constant state of NO-mediated vasodilation (Anggard, 1994). This argument is strengthened by the fact that no study has demonstrated this vasodilatory effect on the venous system.

It would therefore appear that this mechanism (release of NO) plays a significant role in regulating arterial blood pressure *in vivo*.

The integrity of the endothelial layer is therefore very important in maintaining arterial blood pressure and damage to this layer would result in the blunting of the endothelium dependent vasodilation. Various mechanisms have been identified that could render the endothelium dysfunctional. These range from genetic abnormalities in metabolism to physical injury.

This theory therefore suggests that the endothelium could either play a causal role in hypertension only after being rendered dysfunctional, indicating that the hypertension is consequential or secondary phenomenon. Alternatively the hypertension *per se* could be responsible for endothelial damage in which case the dysfunctional endothelium could exacerbates the process.

Whilst many studies have shown that endothelial cells of spontaneously hypertensive animals produce less NO than their respective controls, Bonnardeaux, (1995) in a classic genetic study demonstrated that there is no linkage and association between the NO synthetase gene and essential hypertension in humans. This therefore indicates that the levels of NO produced by individuals that are genetically predisposed to hypertension would be similar to their "normotensive" counterparts. It would therefore appear that there are other mechanisms in individuals that genetically predispose them to develop hypertension.

1.2 Salt Sensitivity

The positive correlation between salt intake and the prevalence of hypertension in a population has been reported by many studies (Okuguchi *et al.*, 1999). But in populations that have been known to consume larger quantities of salt, up to 60% of that population does not develop hypertension. This occurrence indicates that a significant number of individuals in a population are genetically susceptible to the pressor effects of salt loading whereas the majority was not. The former sub group is referred to as being salt sensitive in contrast to the latter group that are called salt resistant. The salt sensitive sub group has been found in all populations but the prevalence amongst black Americans, that is, people of African descend is very high (Sullivan, 1991).

When describing salt sensitive and resistant sub groups in a population, the misconception that resistant individuals do not develop hypertension at all, often arises. In the resistant sub group blood pressure could increase but it is not triggered by salt intake neither does the blood pressure reach levels as high as that recorded in the sensitive sub group (Sullivan, 1991).

The hypertension displayed by salt sensitive individuals is more often associated with left ventricular hypertrophy, microalbuminuria, a lack of a circadian pattern of blood pressure than in hypertensive salt resistant individuals. In the Dahl strain of rats used in this study, the pathological changes mentioned above, occur in salt sensitive rats during hypertension but salt loading exacerbates it (Somova, *et al.*,

1999). It would therefore appear that salt loading abolishes the nocturnal drop in blood pressure, accelerates the onset of hypertension and leads to rapid progress of end organ damage in salt sensitive individuals.

The end organ damage that is evident in hypertension cannot be directly linked to the hypertension *per se* but to many other independent factors (Lacy, *et al.*, 1998).

1.3 Free Radicals

Free radicals are chemical species that possess an unpaired electron in the outermost valence shell. They are illustrated by a bold superscript dot, example $HO^{\bullet}$ (hydroxyl radical) and $O_2^{\bullet}$ (superoxide anion) (Jacobs, 1995). Due to this unpaired electron they readily extract electrons from other biomolecules that they come in contact with, oxidizing them, whilst being reduced in the process. In addition, many molecules after being oxidized become free radicals themselves. This indicates that initial levels of free radicals could be amplified in this manner (Yu, 1994) and the cycle could be perpetuated.

The damage caused by free radicals is widespread due to their ubiquitous presence and involves lipid peroxidation, DNA and RNA base modifications and alteration of protein structure and function. The damage wrought by these species has therefore been implicated in a wide range of diseases from diabetes and cardiovascular complications to neurological diseases (Jacobs, 1995).

The term “free” used in reference to these species indicates that they are capable of existing independently unlike reactive oxygen species (ROS) that are derivatives of oxygen. Despite this difference both free radicals and reactive oxygen species are classified as oxidants and many authors use the terms interchangeably.

1.3.1 Sources of Free Radicals

All aerobic organisms generate free radicals constantly as a byproduct of oxidative phosphorylation. These biological processes are redox reactions viz. oxidation and reduction are coupled in the same reaction. It is therefore ironical that the normal physiological processes that are essential to life, generate species that are potentially damaging.

TABLE: 2a Reactive oxygen species and their characteristic

Species	Symbol	Half life at 37 °C	Property
Hydroxy radical	HO•	1×10^{-9}	Considered to be the most reactive oxidant in the body
Superoxide anion	$O_2^{-\cdot}$	1×10^{-6}	Good reductant, poor oxidant
Hydrogen peroxide	H_2O_2	-	High diffusion capability, good oxidant
Peroxyl radical	$R00^{\cdot}$	1×10^{-2}	Lower oxidizing ability to hydroxy radical, HO•, High diffusion capability
Alkoxyl radical	$RO^{\cdot}$	1×10^{-6}	Oxidizing power between that of HO• and $R00^{\cdot}$

The greatest source of $O_2^{-\bullet}$ and H_2O_2 is perhaps from the respiratory chain within the mitochondrial compartment. These free radicals are formed during the sequential transfer of electrons when O_2 is reduced to molecular H_2O by the oxidases, specifically at the ubiquinone cytochrome b site. Due to the constant production of $O_2^{-\bullet}$ in this manner, intramitochondrial concentrations are in the order of 8×10^{-12} M. Virtually all of the $O_2^{-\bullet}$ produced within the mitochondria is converted to H_2O_2 by superoxide dismutase. The potentially harmful effects of the $O_2^{-\bullet}$ radical is therefore avoided in this manner.

It is argued that a small amount of H_2O_2 that is produced, due to its high diffusion capability, diffuses out of the mitochondria into the cytosol. Since H_2O_2 is not as serious a threat as $O_2^{-\bullet}$ to cellular homeostasis, it would therefore be advantageous to maximize the dismutation of the $O_2^{-\bullet}$ radical that is generated in the mitochondria. Virtually all intra-mitochondrial H_2O_2 is generated in this manner (Yu, 1994).

In steady states it would therefore appear that quantity of cytosolic H_2O_2 is minimal and hence does not pose a serious threat to cellular homeostasis. But it has been demonstrated that various factors could in fact increase the rate and quantity of H_2O_2 production within the mitochondria. It is perhaps under these abnormal circumstances that cytosolic H_2O_2 could increase significantly.

Mitochondrial production of the superoxide radical has been shown to be maximized when the carriers of the respiratory chain are highly reduced (Yu,1994). Conditions *in vivo* that result in such a status quo are limited substrate, oxygen and ADP levels.

In the case of oxygen, if concentrations fall well below the norm to about 1 to 3 mm Hg the resultant accumulation of reduced co-factors in the respiratory chain results in the production of $O_2^{-\bullet}$ instead of H_2O (Freeman, 1982). Superoxide radicals are also produced by the auto-oxidation of catecholamines. This is discussed further on.

1.4 The Antioxidant System

There exists an intricate network of defense systems in aerobic organisms that chemically neutralize free radicals and receive oxygen species to maintain the integrity of cellular homeostasis. This efficient biochemical network is collectively referred to as the antioxidant defense system. The term “system” is necessary due to the interactions that occur among the components and the high level of complexity that these components function at. In addition there are almost as many different antioxidants as free radicals themselves, this being necessary to maximize protection against the potentially deleterious effects of these species. Our knowledge of how this system functions in a holistic manner and the role of a dysfunctional/ compromised system in pathophysiology, is in many areas however very speculative (Freeman, 1982).

Due to the compartmentalized nature of eukaryotic cells, the antioxidants are strategically found in the various sub-cellular organelles in the cytosol and extracellularly, thereby quenching both the free radicals that are generated by the organelles and the species that make their way to the cytosol as well.

The compounds that constitute this defense system are basically biochemical compounds with antioxidant action such as urate, vitamin E and cysteine or enzymes with such action viz. superoxide dismutase, glutathione peroxidase and catalase. Irrespective of the type of compound, antioxidants have been classified as being either primary or secondary.

The primary defense system is largely responsible for the first line of defense against free radicals and comprises enzymes and compounds with chain breaking antioxidant potential. Despite being efficient the primary defense system is not able to quench all the free radicals produced by the body, the balance of which is neutralized by the secondary defenses. The secondary system is also responsible for repair to bio-molecules that have subjected to oxidative damage (Jacobs, 1995).

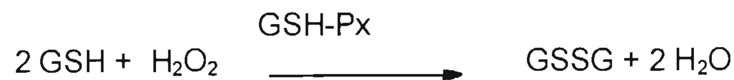
TABLE 3a: The major primary and secondary antioxidants in mammals

Primary antioxidants		
Endogenous Antioxidants	**Dietary Antioxidants**	**Metal Binding Proteins**
NADPH and NADH Glutathione and thiols (-SH) Ubiquinol (coenzyme Q) Uric acid Bilirubin Metalloenzymes	Vitamin C (Ascorbic acid) Vitamin E (Tocopherols) Carotenoids	Ceruloplasmin (copper) Metallothionein (copper) Albumin (copper) Transferrin (iron) Ferritin (iron) Myoglobin (iron)

Secondary/ Free Radical Scavenging Enzymes	
Enzymes	**Reaction**
Superoxide dismutase (SOD) Catalase Glutathione peroxidase (GPx) GSH = reduced glutathione GSSG = oxidized glutathione	$2O2\bullet- + 2H^+ \rightarrow H_2O_2 + O_2$ $2H_2O_2 \rightarrow 2H_2O + O_2$ $ROOH + 2GSH \rightarrow ROH \rightarrow H_2O + GSSG$

1.4.1 Glutathione Peroxidase (GPx) EC 1.11.1.9

This ubiquitous enzyme which is selenium dependant catalyses the reduction of organic hydro-peroxides and hydrogen peroxide (H_2O_2) (Lawrence, 1976). The H_2O_2 that is produced by the metabolic processes of the cells is reduced to water. In this reaction reduced glutathione (GSH) is oxidized as follows:



$$2\,GSH + H_2O_2 \xrightarrow{GSH\text{-}Px} GSSG + 2\,H_2O$$

The optimum reaction rate coincides with physiological pH and temperature (Paglia, 1967). GPx is highly specific for this reaction and its activity in tissue or plasma is therefore an excellent indication of the antioxidant capacity/potential of the system in question (Paglia, 1967). Glutathione (GSSG) on the otherhand can serve as a co-substrate in other enzymatic reactions as well, example, glutathione S-transferases which are involved in conjugated reactions with various pharmacologically active compounds such as alkylating agents. The glutathione status therefore is not as specific as that of GPx when assessing a systems capacity to neutralize peroxides (Moron, 1978).

Many studies have also looked at levels of oxidized glutathione (GSSG) in tissue and plasma as an indication of oxidative stress (Adams *et al.*, 1983 and Thom *et al.*, 1997). Plasma GSSG levels are a particularly good index because mammalian cells efficiently excrete GSSG, effectively keeping intracellular levels low thus favoring the equilibrium of this reaction to the right (Adam *et al.*, 1983). Therefore if plasma GSSG and GSH are expressed as a ratio, excretion of GSSG into the plasma compartment results in the change being amplified.

This enzyme and co-substrate therefore forms part of the essential antioxidant system that neutralizes/ quenches the peroxides that are generated *in vivo*. In addition the peroxides that are generated during the activation of arachidonic acid metabolism are also scavenged. This effectively results in an inhibitory modulation of the cascade of reactions that generate prostanoids such as thromboxane A_2. Thromboxane A_2 is a powerful vasoconstrictor and pro-aggregating agent for platelets (Girelli, *et al.*, 1993).

Therefore in addition to detoxifying the body of potentially harmful peroxides, the aggregation of platelets is also prevented thus indicating the widespread protective role that this enzyme has.

1.4.1.1 GPx and Selenium

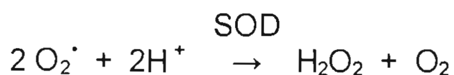
The molecular structure of both intra and extra-cellular glutathione peroxidases show that these tetramenic proteins have four gram-atoms of selenium per molecule enzyme, that is each a subunit of the structure has a selenium atom (Burk, *et al.*, 1994). It is due to this stoichiometric relationship that body selenium levels can be indirectly estimated using GPx levels. The determination of Se status using commercially available kits such as Randox kits are based on this. That GPx activity was dependant on Se levels was demonstrated by Smith *et al* in various rat tissues. GPx activity was shown to increase as a logarithmic function of dietary selenium (Reddy, 1974). A compromised Se status would therefore impact on the antioxidant ability of the body. This is perhaps clearly evident in chronic renal failure and Keshan

disease, which are characterized by low selenium levels. The accelerated atherosclerosis evident in such individuals strongly indicates the possible etiological role of a compromised antioxidant status in this disease (Girelli, *et al.*, 1993).

In addition glutathione peroxidase in platelets is responsible for neutralizing the peroxides generated during the metabolism of arachidonic acid. This effectively prevents the generation of pro aggregating agents such as thromboxane A_2 and thus reduces the risk of thrombosis.

1.4.2 Superoxide Dismutase (EC 1.15.1.1)

This enzyme is found in virtually all aerobic organism and is responsible for catalyzing the dismutation of the superoxide free radical as follows:



$$2\,O_2^{\cdot} + 2H^{+} \xrightarrow{SOD} H_2O_2 + O_2$$

In doing so, this enzyme is therefore responsible for the protection of organisms from the highly reactive superoxide radical. Although the production of H_2O_2 , a free radical itself might appear paradoxical, the potentially deleterious effect of $O_2^{-\cdot}$ is however greater than H_2O_2 (Halliwell, 1989). The dismutation of $O_2^{-\cdot}$ is a spontaneous reaction but this enzyme speeds up the reaction by a factor of 10^4 (Winterbourn, *et al.*, 1975). Therefore the $O_2^{-\cdot}$ that is generated either accidentally or functionally in the body is "neutralized" very efficiently in this manner.

There are three classes of dismutases depending on the metallic co-factor, they are Cu/Zn SOD, Mn SOD and Fe SOD. Despite being found in all cell types and in all compartments of the body, the majority (~70%) of its action is in the cytosolic compartment (Halliwell, 1989).

SOD in eukaryotes is a mitochondrial enzyme, the synthesis of which is controlled by nuclear genes. The expression of SOD is controlled by oxygen levels or one of it metabolites, especially $O_2^{-\bullet}$ (Yu, 1994). Induction of SOD has been shown to occur in bacteria, yeasts and rat hepatocytes after being subjected to high oxygen levels (Harris,1992). Regulation of SOD levels has been shown to occur independently and hence supporting evidence that a specific genetic locus, termed SOXR, exists (Harris, 1992). Under normal physiological conditions SOD levels in rats have been shown to be constant from birth to adulthood.

1.4.3 The Depth of the Antioxidant System

A large number of compounds with antioxidant abilities or potential have been identified in all compartments of aerobic organisms (Pezeshk, 1989). In addition their role in pathophysiology is well documented. The possible organization of all these antioxidants in each compartment, forming a system with "depth" to it, is however often overlooked in the literature (Jacobs, 1995). This theory therefore suggests that there is a synergist interrelationship amongst antioxidants and more importantly that antioxidants are organized into various levels of defense against free radicals. The merits of this organization are obvious in that two or more compounds with antioxidant properties can work together as a system; recycling or sparing each

other so that at any point in time there is an adequate amount of compound in the reduced form to neutralize free radicals (Jacobs, 1995).

A factor that complicates quenching of free radicals is that there are both lipid and aqueous phases in the body and both are subject to attack by free radicals. The interaction between ascorbic acid, which functions as an antioxidant in the aqueous phase and tocopherol in the lipid phase is well documented. This indicates that this "level" of antioxidants work as a pair to recycle each other to ensure protection of both aqueous and lipid components also demonstrating diversity of protection (Jacobs, 1995).

This chemical relationship between ascorbic acid and tocopherol occurs both spontaneously and enzymatically are shown below (Figure 1).

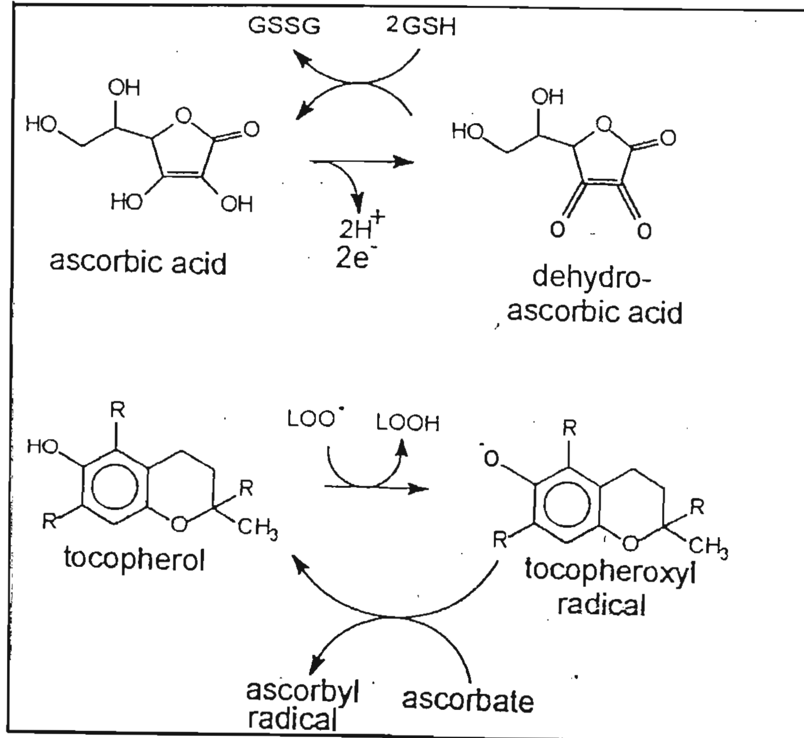


Figure 1: Chemical interrelationship between ascorbic acid and tocopherol showing the recycling of the antioxidants

Other interrelationships between antioxidants have been described, these include the ascorbic acid, glutathione system and ascorbic acid working in concert with glutathione to maintain tocopherol (Jacob, 1995).

Mammalian systems could have evolved this inter-relationship between antioxidants to ensure that radicals are continually being quenched highlighting the threat that free radicals pose to us.

1.5 Free Radicals and Hypertension

The increase in leucocyte count during hypertension could perhaps be one of many sources contributing to the increase in free radicals, observed during this condition. (Friedman *et al.*, 1990; Uehara, 1992). During phagocytosis, the respiratory burst displayed by leucocytes, releases reactive oxygen species that are thought to play a significant role in the inflammatory process. The production of $O_2^{-\bullet}$ and H_2O_2 by leucocytes is due to membrane bound oxidases that are stimulated during phagocytosis (Yu, 1994).

Xanthine oxidase (XO), a cytoplasmic enzyme catalyses the reaction in which oxygen is reduced to H_2O_2. $O_2^{-\bullet}$ is produced in varying proportions depending on pH and O_2 concentrations, etc. It has been suggested that significant concentrations $O_2^{-\bullet}$ and H_2O_2 are generated from this source (Lacy *et al.*, 1998 and Hoshikawa *et al.*, 2001).

Xanthine oxidase in humans also serves as an NAD^+ dependant dehydrogenase in the cascade of energy generating reactions. At this step however no free radicals are generated. It has nevertheless being shown that during ishemic conditions this enzyme is converted from the dehydrogenase to the oxidase form, thus generating free radicals viz. $O_2^{-\bullet}$ (Freeman, *et al.*, 1982). This therefore suggests that the increase in free radicals that is evident after a compromised oxygen status could be in part related to this mechanism (Freeman, *et al.*, 1982 and Hoshikawa *et al.*, 2001).

The positive correlation between hypertension and elevated levels of circulating catecholamines has been noted by many studies (Grassi, *et al.*, 1998, Modan, *et al.*, 1991). In addition individuals that are classified as being salt sensitive display hypertension that is referred to as a non dipper pattern, that is there is no nocturnal fall in blood pressure (Okuguchi, *et al.*, 1999). This lack of a circadian rhythm in blood pressure has been strongly implicated in organ damage that is evident in hypertension (Okuguchi *et al.*,1999). With a non dipper pattern of hypertension, circulating catecholamine levels are elevated and are maintained without a nocturnal drop. A relative increase in catecholamine levels has been shown to generate catechol-o-quinone, a reactive species, and electrons through an auto-oxidative pathway (Singal, *et al.*, 1967). This could result in a two fold problem, firstly the electrons that are generated would be captured by molecular oxygen to form superoxide radicals. Secondly the catechol-o-quinone is also classified as a reactive species due to its unpaired electrons. This compound as a result reacts strongly with other molecules, extracting electrons. Its reaction with sulphydryl groups which are present in numerous proteins, including cell and organelle membrane, is particularly strong (Yuan, *et al.*, 1998).

This possible dual action by the products of catecholamine induced autoxidation are similar to the damage caused by free radicals generated from other sources. Singal, (1967) demonstrated that many of the changes induced in rats by the administration of isoproterenol (a product of autoxidation of catecholamines *in vivo*) were similar to changes induced by free radicals during hypertension viz. high intracellular Ca^{+2} levels, cardio-myopathy, depletion of ATP stores, etc. In addition, these changes were reversed by the administration of an antioxidants viz. vitamin E. This strongly indicates that catecholamine induced autooxidation yield products with free radical properties (Singal *et al.*, 1967).

If this mechanism is indeed a significant source of free radicals in conditions such as hypertension and stress, then it would appear that this mechanism is secondary to the condition in question. Therefore this mechanism would not play an important role in the etiology of hypertension but could nevertheless be responsible for sustaining and perhaps ensuring that the hypertension reaches pathological proportions.

1.6 Effects of Oxidative Stress

1.6.1 Endothelium

The endothelial cells, other cells of blood vessels and cellular components of blood, especially the neutrophils produce free radicals as by products of metabolism (Grunfeld *et al.*, 1995). The antioxidant system, discussed elsewhere neutralizes/ quenches this species under normal physiological conditions. In many pathological

states however, including hypertension, studies have shown a significant increase in free radical concentration. Pogan, (2001) using direct measurements have shown a 250% increase in concentration of the superoxide anion ($O_2^{\bullet}$) in a suspension of endothelial cells of hypertensive rats. Besides the $O_2^{\bullet}$, there are reports in the literature of an increase in H_2O_2 and OH- ions as well (Swei *et al.*, 1997). In pre-eclampsia however, Chen *et al* have shown that there is depressed SOD activity in neutrophils and red blood cells indicating that the mechanism resulting in the hypertension during pregnancy is different from that seen in essential hypertension and spontaneously hypertensive rats (Grunfeld *et al.*, 1995). Irrespective of the free radical species or its origin (that is, intra or extra-cellular) the underlying problem is that the body in general and the endothelium in particular is subjected to an increase in oxidative stress during hypertension.

Whether the increase in free radical concentration precedes hypertension or occurs as a consequence of hypertension is a question that has not been addressed conclusively in the literature (Vehara *et al.*,1992; Pogan *et al.*, 2001). We, as part of this study attempted to address this question by evaluating the antioxidant status of weanling DSS and DSR red blood cells. Many studies have shown that the damage wrought by free radicals begins early in life and that the endothelium injury is inevitable. The hypothesized vicious cycle that leads to and maintains endothelial cell injury is illustrated in Figure 2.

Various morphological changes, attributed to free radicals that lead to a functional derangement of the endothelium have been reported (Henning, *et al.*, 1988; Pogan

et al., 2001; Simon *et al.*, 1998). These include damage to the endothelial junctions, cytocontractile and cytoskeletal elements which result in disarray of the closely arranged endothelial monolayer. The glycocalyx which maintains a charge barrier and effectively ensures that the endothelium is nonimmunogenic is also damaged by these species (Hennig, *et al.*,1988). These changes result in the loss of permeability and subsequent immunologic attack of the endothelium leading to dysfunction. This impacts on the functioning of layer altering its endocrine ability as well (Grunfeld *et al.*, 1996). Although Bonnardeaux (1995) have demonstrated that the expression of NO synthetase is similar in hyper and normotensive individuals, the loss of integrity of endothelial cells would definitely result in a net decrease in NO production (Henning, *et al.*, 1988).

The increase in free radicals (especially $O_2^{\bullet}$) has a possible dual role in the etiology of hypertension. Firstly the $O_2^{\bullet}$ reacts with NO to form the peroxynitrite which in contrast to NO is a very weak vasodilator (Pogan *et al.*, 2001). This means that the NO dependant vasodilation mechanism which is regarded as an important regulatory one would be blunted resulting in an increase in blood pressure. This could translate into a vicious cycle as arterial blood pressure increases, resulting in a parallel increase in the production of $O_2^{\bullet}$ (Swei *et al.*, 1997). In our model of hypertension, the Dahl salt sensitive rat, the increase in circulatory leucocytes could perhaps explain the very high levels of free radicals observed in the strain thus quenching NO levels to a greater extent than their respective control, the DSR (Swei *et al.*, 1997).

1.6.2 Peroxidation of Lipids

MDA is a short chain aldehyde that is formed when polyunsaturated fatty acids undergo spontaneous or enzymatic peroxidation. During the process there are, however many related aldehydes that are also formed these include 4-hydroxy-nonenal, 4-hydroxyhexanal and acrolien. For the purposes of this study however, we only investigated MDA levels since it is perhaps the most economic assay. The precursor for MDA formation in biological systems especially membranes is mainly arachidionic acid (20 : 4) and docosahexanoic acid (22:6) (Smith, *et al.*, 1975). There is increasing evidence that lipid peroxidation is induced by free radicals that are generated *in vivo* by the cells metabolic processes (Esterbauer *et al.*, 1991).

During oxidative stress and hypertension the increase in free radicals would therefore result in an increased rate of lipid peroxidation (Jacobs, 1995).

The pathophysiological role of aldehydes in biology is well documented and is due largely to two factors viz.

1. The half life of aldehydes is far greater than other reactive species such as free radicals. This results in these species migrating to many areas of the body, hence their effects are widespread, often, far away from the site of peroxidation.

2. The initial stimulus of peroxidation is amplified many fold by the reactive adehydes that are produced. These aldehydes due to their strong reactive ability often serve as second messengers to bring about widespread tissue damage. The effects of lipid peroxidation are therefore often described in terms of a complex chain of reactions (primary and secondary).

1.6.2.1 Effects of Aldehydes

Irrespective of how the aldehydes are formed, they react strongly with the amino acids in protein particularly the E-amino acids and lysine under physiological conditions. In addition, MDA reaction with other amino acids such as histidine, methionine and arginine have also being reported but to a less significant degree.

The resultant cross linkage that is formed between the amino groups and MDA results in a modified protein whose biological integrity is flawed. Evidence strongly supporting the role of MDA in pathophysiology is the presence of MDA conjugated proteins in atherosclerotic plaques of rabbits. In addition the presence of these modified proteins have also being detected in human plasma of heart patients, confirming that these conjugated proteins do play a role in the pathogenesis of diseases associated with the circulatory system (Esterbauer, 1991).

Besides altering the structural integrity of biological systems, aldehyde such as MDA have been demonstrated to react with the nucleosides in DNA especially guanine (Negishi, *et al.*, 2001). Although this occurs at high levels of MDA and is therefore not a major threat to genetic abnormalities, the long term effects of sub-minimal levels are yet to be demonstrated. MDA has nevertheless been shown to have a mutagenic effect on bacteria and isolated mammalian cells.

1.6.3 Free Radicals in Vascular Remodeling

There is increasing evidence that the apoptotic process that plays a focal role in vascular remodeling could in fact be linked to free radicals. *In vitro* studies have demonstrated that an increase in extra-cellular H_2O_2 concentration and oxidatively modified low density lipoprotein induce apoptosis in cultured smooth muscle cells whereas high intracellular H_2O_2 levels have been shown to stimulate the synthesis of various growth factors by vascular smooth muscle cells. Whilst these have been *in vitro* studies and hence the role of free radicals speculative, the fact that a large volume of studies have shown the correlation between elevated free radical levels and hypertension (with associated structural changes) is nevertheless an important consideration. In addition the process of remodeling is dependent on apoptosis and abnormal growth, both of which have been shown to be mediated by free radicals such as H_2O_2 (Li, *et al.*, 1997).

1.7 Independent Factors associated with Hypertension

1.7.1 Hyperglycemia

Hyperglycemia or elevated blood glucose levels occur when there is a functional or actual deficiency of insulin (Coffee,1998).

During sustained hyperglycemic states, as seen in conditions such as insulin resistance, diabetes and hypertension, the glucose in plasma undergoes a non-enzymatic chemical reaction with protein referred to as auto-oxidative glycosylation. The product, Schiffs base (unstable) is able to reduce molecular oxygen to oxidizing intermediates such as $O_2^{\bullet}$, OH- and H_2O_2. These free radicals, as discussed previously are very unstable and are responsible for widespread damage in the body. They, especially $O_2^{\bullet}$, contribute to the development of hypertension through the decomposition of NO. Decreased levels of NO can lead to vasoconstriction of the resistance vessels with a resultant elevation in arterial pressure as discussed previously (Paolisso, *et al.*, 1996).

In addition, in tissue that are not insulin dependent for glucose transport, for example, endothelial cells, hyperglycemia results in a relatively high intracellular glucose concentration (Coffee, 1998). In order to metabolise this glucose there is an increase in the aldose reductase and sorbitol-dehydrogenase reactions which are NADPH dependant.

Depending on the severity of the hyperglycemia, this could result in NADPH stores being depleted resulting in the activity of other NADPH dependant enzymes to be decreased or inhibited (Paolisso, *et al.*,1996).

Two reactions are worthy of consideration since they would impact on the hypertensive state. Firstly, the NO synthase reaction in which L-arginine is converted to NO, if lower levels of NO are generated this could result in an elevation of blood pressure as discussed previously. Secondly glutathione reductase which is responsible for the enzymatic conversion of glutathione disulfide (GSSG) to glutathione (GSH) would stop.

With lower levels of glutathione especially within the endothelial cells, the susceptibility of these cells to damage by H_2O_2 is increased, rendering them dysfunctional as pointed out earlier (Paolisso, *et al.*, 1996). This latter mechanism could also explain partly the damage associated with hyperglycemia in other tissues such as nerve, retina and the lens. It must be emphasized though that much of the damage in tissues that are non insulin dependent is due to the accumulation of sorbitol within them. This occurs after the sorbitol-dehydrogenase reaction ceases to function due to depletion of NADPH mentioned earlier.

Sustained hyperglycemia could therefore result in an increase in oxidative stress which could subsequently contribute to the pathogenesis of hypertension (Paolisso, *et al.*,1996). This is perhaps why many studies have consistently found a correlation between diabetes and hypertension.

1.7.2 Hyperinsulinemia

Ravens syndrome/ cluster is associated with hyperinsulinemia, insulin resistance and hypertension. In the Dahl model of hypertension, studies have also shown a positive correlation between hyperinsulinemia, insulin resistance, dyslipidemia and hypertension (Uehara *et al.*, 1992; Hayakawa, *et al.*, 1999). In addition the increase in free radicals in hypertension is well documented. The positive correlation therefore between free radicals, hyperinsulinemia, insulin resistance and hypertension is perhaps an indication that there is a causal interrelationship amongst them. Habib *et al.*, have demonstrated that increased insulin concentrations are associated with an increase in free radical production in rats. This was also observed in an *in vitro* study using weanling normotensive Dahl salt sensitive rats which have been shown to be hyperinsulinemic. The present study has also demonstrated that weanling DSS rats that are not yet hypertensive have a diminished antioxidant status of the red blood cells (Table 7) indicating a possible increase in free radicals. This strongly suggests that hyperinsulinemia and oxidative stress precede hypertension (Paolisso, *et al.*, 1996).

Whether these two factors work independently or in concert to produce hypertension is a hypothesis that has not been answered, further highlighting the multifunctional nature of the etiology of hypertension. It has been speculated nevertheless, that hyperinsulinemia precedes oxidative stress and could even be responsible for the increase in free radical production. Many mechanism have been suggested for the increase in free radicals by hyperinsulinemia (Paolisso, *et al.*, 1996).

Firstly, hyperinsulinemia has been known to be responsible for an increase in activity of the sympathetic nervous system which would therefore result in an increase in circulating catecholamines. High levels of these species have been shown to generate free radicals as discussed through auto oxidation. (Paolisso, *et al.*, 1996) It is therefore possible that this is the mechanism through which free radicals are increased in non insulin dependent diabetes (Modan, 1991). Higher sympathetic activity also would result in an increase in vascular tone, that is neurogenic hypertension.

One factor that adds immense strength to this theory is that in the early stages of hypertension there are no structural changes in the vasculature and hence the hypertension is reported as of the neurogenic type (Zicha, *et al.*, 1999).

Irrespective of whether the increase in free radicals is due to the hyperinsulinemia or not, these species have been shown to impair insulin action. The damaged caused by free radicals to the lipid bi-layer of cell membranes is well established (Macchia *et al.*, 1999). Once the chemical properties of the membranes are altered, this impacts on the physical properties. Many studies have shown that the membrane fluidity is altered significantly after membrane peroxidation by free radicals. This would therefore result in loss of its intrinsic properties of the membrane such as permeability, ion transport and altered receptor function (Macchia *et al.*, 1999). It has also been demonstrated using red blood cells of rat that glucose entry into cells is directly related to membrane fluidity, glucose entry being optimum with maximum membrane fluidity (Macchia *et al.*, 1999). In non insulin dependant diabetes mellitus

it has also been shown that insulin action is impaired with declined membrane fluidity. If this hypothesis of hyperinsulinemia leading to an increase in free radicals is correct then this link between free radicals and insulin action can be described as a vicious cycle, that ensures that the hypertension deteriorates to a severe state.

1.8 Reactive Oxygen Species and Atherosclerosis

Atherosclerosis is the most common coronary artery disease (CAD) and is a leading cause of death in western countries (Maxwell, 2000). The characteristic plaque that is formed in medium to large vessels consists of lipids (intra and extra-cellular origin), smooth muscle cell, connective tissue and glycosaminoglycans. Over a period of time, this abnormal deposition renders the vessels less compliant resulting in abnormal systolic wave propagation and elevation in blood pressure (Chan, 1998).

Although many factors have been implicated to play an aetiological role in the pathogenesis of atherosclerosis, ROS have been linked to two of the major hypotheses viz. the lipid and endothelial injury hypotheses respectively (Hennig, 1987; Maxwell, 2000). The underlying processes of these hypotheses are however not totally independent, in fact a certain level of synergism exists between them. This synergism has come to the fore as our understanding of the chemistry and biological role of free radicals expanded. It is generally accepted that atherogenesis commences after an insult occurs to the endothelium. The factor/s causing this insult have been diverse at the same time not very specific.

Elevated levels of free radicals in hypertension and other pathological states with a compromised antioxidant status have been discussed previously. The role of free radicals in oxidatively modifying the low density lipoprotein fraction which subsequently plays a role in the pathogenesis of atherosclerosis was initially proposed by Parthasarathy, *et al.*, (Yu, 1994). Besides being the source of the characteristic fatty streak in the walls of atherosclerotic vessels, oxidized LDL (Ox-LDL) has been more recently demonstrated to possess cytotoxic properties. It has therefore been suggested that oxidized LDL could in fact be responsible for both the initial insult and the subsequent deposition of lipids in the vessel walls (Chan,1998; Yu,1994).

I. The Lipid Theory

The plasma lipids in humans circulate as complexes with proteins called lipoproteins. Many cardiovascular and associated diseases have been linked to an abnormal proportion of the various lipoproteins, a condition that is increasingly being referred to as dyslipidemia (Channa, *et al.*). The alterations in LDL and HDL fractions, rather than total cholesterol concentrations have proved to be a more accurate predicator of cardiovascular diseases with LDL and HDL showing positive and negative risk associations respectively in humans (Chan, 1998; Morel, *et al.*, 1984). The analysis of these fractions therefore plays a central role in the diagnosis and management of lipid disorders and their associated pathologies. Once a lipid profile has been determined, it is therefore possible to therapeutically intervene by using the appropriate drug/s that is/are available.

For a number of years it was perceived that LDL *per se* was responsible for the pathogenesis of cardiovascular diseases such as atherosclerosis. Research over the last two decades has however, produced increasing evidence that the pathogenicity of LDL was contrary to this and that it was due to a modified fraction of LDL that was initially referred to as toxic LDL (Morel *et al.*, 1984).

This fraction has subsequently been called oxidized LDL due to the peroxidation of the lipid component of the lipoprotein. Despite variations in its density due to sterol loss, the characteristic feature is its increase in electro-phoretic mobility by 30%-70% (Morel *et al.*, 1984). The resultant lipid hydro-peroxides and endoperoxides that are formed by this peroxidation have the potential to decompose into aldehydes such as MDA, which have been demonstrated to have deleterious effects on cells (Berliner, 1993; Chan, 1998).

Extensive work has demonstrated that free radicals such as H_2O_2 and $O_2^{-\bullet}$ the levels of which are elevated in various pathological states, are capable of performing this per-oxidation both *in vitro* and *in vitro* (Chan, 1998, Russo *et al.*, 1998, Lacy *et al.*, 1998, Pogan *et al.*, 2000). Besides the well documented toxic effects of the free radicals themselves, oxidatively modified LDL has been demonstrated to be bioactive due to its strongly hydrophobic nature. The MDA adduct that has the potential of being formed can cause cross linking and polymerisation of cell membranes thus altering its properties such as deformability ion transport and micro-viscosity (Freeman, 1982). It therefore appears that much of potential damage that can be caused by Ox-LDL whether directly or indirectly mimics the actions of free radicals.

Although factors such as Ca^{+2} ions have been demonstrated to generate oxidized LDL both *in vitro* and *in vivo*, Solonen, (1992) have demonstrated that the malondialdehyde (MDA) generated species viz. MDA – LDL is found in significantly higher concentration than from other sources in Finnish heart patients. Besides being found in high concentration in the serum of these patients, immuno-globulins specific for oxidized LDL have also been identified in atherosclerotic plaques of man and rabbits (Salonen *et al.*, 1992).

Exposure of endothelial cells to Ox-LDL has been shown to increase expression of endothelium derived adhesion molecules which actively promotes the adhesion of macrophages and monocytes to the endothelial layer and their subsequent movement into the intimal layer of vessels. Once these activated cells are within the intimal layer they are converted into the characteristic lipid laden, foam cells which are the hallmarks of atherosclerosis. Consistent evidence over the last two decades has shown that the LDL fraction that is encorporated into the foam cells is not native LDL but Ox-LDL. (Chan, 1998; JA Berliner, 1993; Henning, 1987). This abnormal uptake is achieved through the scavenger receptor that lacks the feedback regulation of the receptor that binds native LDL. (Berliner, 1993). It has been reported by many human studies that the severity of the atherosclerosis is positively correlated with the levels of Ox-LDL both in plasma and vessel walls (Hayakawa, *et al.*,1999).

II The Endothelial Injury Hypothesis

Elevated levels of free radicals such as H_2O_2 and $O_2^{-\bullet}$ in hypertension and other disorders are well documented (Lacy, *et al.*, 1998, and Ceriello, *et al.*, 1991). These species could be generated either by endothelial cells, extra-cellular sources or from other cells (Hennig, 1987). Overwhelming of the antioxidant system in and around the endothelial cells would result in the ROS oxidizing molecules such as polyunsaturated fatty acids in membranes, etc. Once membrane integrity is lost in this fashion, this can lead to the endothelial layer becoming dysfunctional (Pogan *et al.*, 2000). With a compromised endothelial layer, the sub-endothelial layer is therefore exposed to blood borne elements such as leucocytes, ROS, Ox-LDL and platelets leading to a cascade of events that characterize the atherosclerotic process.

Besides the possible action of free radical many other factors have been implicated in endothelial cell injury such as mechanical sheer forces, viral attacks and auto-immune reactions (Hennig, 1987).

Besides exposing the sub-endothelial layer to remodeling, damaged endothelial cells would not sub-serve endothelial function itself, which is essential to the vascular apparatus. Since the endothelium has the full complement of organelles, it is responsible for the synthesis of collagen, glycosaminoglycons (key composition of the glycocalyx), various enzymes and extra-cellular proteins such as plasma proteins and fibronectin (Henning, 1987). Damage to the endothelium would thus result in a

compromised glycocalyx which would render the layer vulnerable to immunogenic attack and loss of the closely appositioned nature of the cells. In addition the inability to maintain the cytoskeleton and cytocontractile nature would effectively result in the complete disorganisational loss of this mono-layer (Henning, 1987).

In addition to the possibility that the endothelial layer is being compromised from external sources, dynamics with this cell layer itself could lead to destability. It has been demonstrated that endothelial cells passes xanthine oxidase activity, which by its action on hypoxanthine (generated within the endothelial cells) produces $O_2^{-\bullet}$. It has been therefore hypothesized that, the generation of $O_2^{-\bullet}$ from this source is responsible for endothelial cell injury. In addition both the spontaneous and enzymatic dismutation of $O_2^{-\bullet}$ would yield H_2O_2, a free radical of lower oxidizing potential than $O_2^{-\bullet}$. The generation of H_2O_2 in significant quantities from these sources to overwhelm the antioxidant system completely, is debatable. It has been nevertheless demonstrated, in cultured endothelial cells, that formation of lipid peroxides decreases significantly when SOD is introduced into the medium (Hennig, 1987). This strongly suggests that the quantity of $O_2^{-\bullet}$ generated *in vivo* is perhaps sufficient to cause lipid peroxidation that is associated with atherosclerotic changes in endothelial cells and that these changes are mediated by the $O_2^{-\bullet}$ radical.

In vitro studies using endothelial cell suspensions have also demonstrated that besides the possible alterations to the cells structural components, Ox-LDL is capable of altering the cells biochemical processes. The production of NO and PGI_2 is attenuated with PGI_2 by endothelial cells *in vitro* when incubated with Ox-LDL

(Chan, 1998). The anti-thrombotic role of PGI_2 is well documented and decreased levels of PGI_2 would alter the delicate balance that exists between PGI_2 and thromboxane A_2 to favour platelet aggregation. Platelet aggregation is identified as one of the many mechanisms that play an etiological role in atherosclerosis.

The link between free radicals, platelets and endothelial cells and their role in atherosclerosis is further strengthened by the observation that cultured endothelial cells synthesize platelet activating factor (PAF) when exposed to extra-cellular H_2O_2. This response was also a dose and time dependant one, indicating that the mechanism could respond in an amplified manner to increasing levels of H_2O_2 (Lewis *et al.*, 1988). High levels of H_2O_2 have been reported in hypertensives and individuals that are genetically predisposed to hypertension (Lacy *et al.*, 1998).

In addition it has been demonstrated recently that ROS (H_2O_2) can induce apoptosis of vascular smooth muscle cells *in vitro* by possibly altering the cellular redox state. Apoptotic cells have also been found in appreciable quantities in advanced atherosclerotic plaques (Li, *et al.*, 1997) indicating that apoptosis is part of the cascade of changes that take place in plaque formation. Vascular smooth muscle cell death due to apoptosis was also prevented by the administration of catalase to the medium, this protection strongly linking ROS to the process (Li, *et al.*,1997).

There is therefore a plethora of evidence indicating that many factors interact at vascular level leading to the pathogenesis of atherosclerosis. There is strong evidence that free radicals may be involved in initiating this process either directly

and/ or indirectly (Chan, 1998; Maxwell, 2000). Once this process commences however, it becomes a self-perpetuating cycle in which free radicals also play a role, these events are summarized in Figure 2.

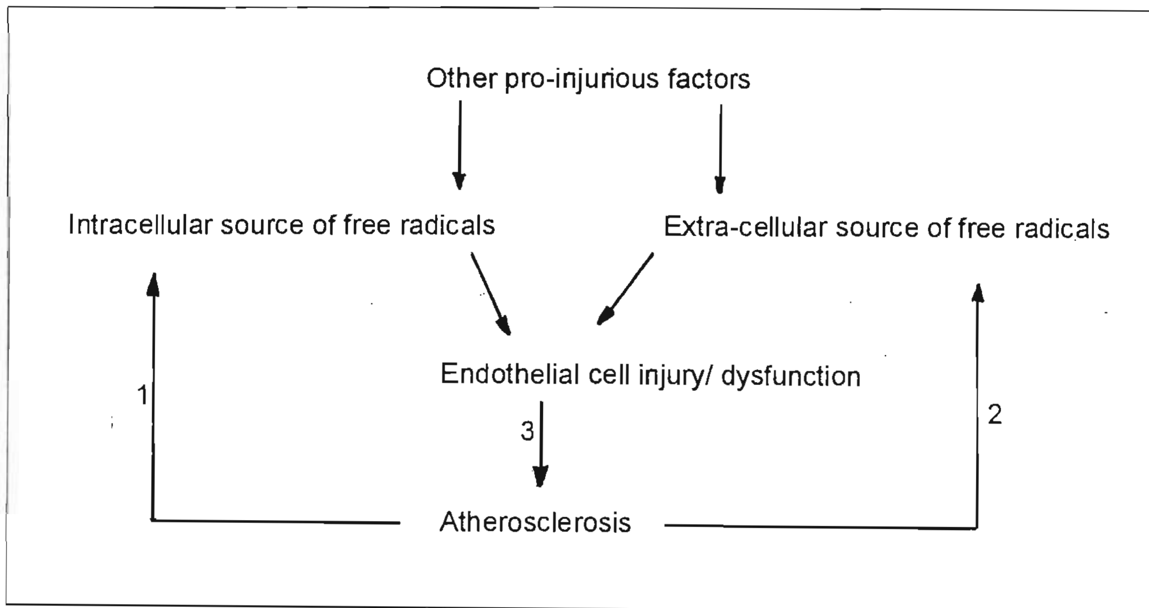


Figure 2: Hypothesised Role of ROS in Atherosclerosis

1. Intracellular source, ROS generated within endothelial cells
2. Extra-cellular source, ROS generated by sources other than endothelial cells
3. Damaged/ dysfunctional endothelium generating additional ROS, a vicious cycle

1.9 The Dahl Model and its Relevance

When using an animal model to study hypertension, it is imperative that the pathogenic mechanisms of the hypertension in that model is understood. This therefore means that the model has to be well characterized. In the case of salt sensitive hypertension, the Dahl rats, the salt sensitive (DSS) and salt resistant (DSR) strains, are considered excellent models (Sullivan, 1991). The salient differences in the DSS versus DSR strains that have been implicated in the pathogenesis of hypertension are discussed below.

Although Na^{+} exchange and Ca^{+2} uptake in various cell types of DSS rats have been shown to be altered compared to DSR rats, these changes only become evident during salt loading. It has been suggested that these changes may occur as a consequence of the hypertension (Ferrari *et al.*, 1995). Since these animals do develop hypertension on a normal salt diet, albeit at slower rate, there are therefore other significant mechanisms that occur in the DSS strain that play an etiological role in hypertension.

Major mechanisms have been identified in the kidneys of DSS rats that have been documented by many studies which are independent of neural control, and are considered major causative factors in hypertension (Rapp, 1982, Sullivan, 1991). Kidney function tests have shown that the glomerular filtration rates (GFR) and Na^{+} excretion are lower in DSS animals than DSR. In addition salt loading highlighted this difference significantly. This is due to pressure-natriuresis curve in DSS rats

being shifted to the right rather than changing its slope when animals are salt loaded. This effectively means that kidneys of DSS animals require a higher level of perfusion pressure for a given level of sodium excretion compared to DSR animals (Ferrari *et al.*, 1995).

This intrinsic defect in the renal handling of Na is also coupled with proteinuria in DSS rats. The accompanying expansion of the extra-cellular fluid volume results in an increase in blood pressure and heart rate. Characteristically however, the hypertension subsequently evolves into a condition marked largely by an increase in total peripheral resistance (Sullivan, 1991).

These renal abnormalities work in concert with neural, endocrine and metabolic changes to ensure that the hypertension becomes chronic, often leading to end organ injury. Peripheral sympathyectomy in weanling DSS rats ensures that they do not develop hypertension irrespective of salt intake yet renal denervation does not attenuate the hypertension. Incidentally DSS rats have a higher density of renal α_2 receptors than the DSR strain. This indicates that whilst the nervous system plays a role in the pathogenesis and maintenance of hypertension, its actions are complex (Ferrari *et al.*, 1995).

Many hormonal differences exist between DSS and DSR strains, which, are evident before the increase in blood pressure and are thought to be genetically determined. The significant increase in 18-OH deoxycorticosterone synthesis by the adrenal gland is in itself sufficient to elevate blood pressure (Ferrari *et al.*, 1995) (similar to

primary hyperaldosteronism). But this also effectively suppresses the renin-angiotensin system and induces hypokalemia. The Dahl rat is recognized as a model of hypertension with low renin activity. This altered pattern in steroidogenesis is controlled by a single gene locus that has been appropriately referred to as Hyp-1 (Rapp, 1982).

From this brief overview, it is apparent that the Dahl model of genetic salt sensitive hypertension is well characterized and that the hypertension is of a polygenic and multi-factorial origin. In addition, this model is a relevant one to study hypertension due to the many similarities that exist between it and hypertensive humans. For instance, in renal transplant patients, there is a correlation in blood pressure between the recipient and donor after transplant. This indicates that the kidney induces hypertension through a pressor mechanism that is under genetic control as well (Ferrari *et al.*,1995). The renal handling of Na^+ is also similar in the Dahl strain compared to individuals that are sensitive to salt. It therefore appears that the theoretical knowledge acquired with research in this model can in many instances be applied to human salt sensitive hypertension. This study was therefore undertaken to evaluate the antioxidant status of this model and the possible role that free radicals may have in supplementing existing, well established mechanisms in the pathogenesis of hypertension.

2.0 MATERIALS AND METHODS

2.1 Animals

Twenty-four male Dahl salt sensitive weanling rats and an equivalent number of Dahl salt esistant weanlers, which served as controls were used for the study. In each group the animals were randomly subdivided into two groups, one group fed a normal salt diet and the other a high salt diet. They were housed individually in metabolic cages with suspended floors (Techniplast, Italy). The diets were based on the AIN-76 diet the composition of which is described. The animals were fed these diets *ad libitum* for eight weeks, had free access to deionised water (Millipore) and were maintained on a twelve hour light-dark cycle at constant temperature.

Food consumption was monitored on a daily basis and body mass weekly. After the sixth week dietary regime the animals were fasted overnight and under anaesthesia (sodium thiopentone 40 mg/ kg – intraperitoneally) the left common carotid was cannulated. Blood was collected in chilled EDTA, heparinised and plain (no anticoagulant) glass tubes and stored on ice. The tubes with no anticoagulant were allowed to stand at room temperature for thirty minutes thereafter a glass Pasteur pipette with an annealed tip was used to gently remove any clotted material that adhered to the wall of the tube below the meniscus. The tubes were then centrifuged at 3000 x g for ten minutes at 4°C (Eppendorf 5043, Germany). The serum was used to determine the catalase concentration in the sample (50 nM Tris 0,1 mM EDTA buffer). The heart was excised and rinsed in cold pH 7.6, blotted dry and weighed. The right ventricle was dissected out, weighed and prepared for

histological work. The balance of the myocardium was immediately put into plastic storage bags and plunged into liquid nitrogen. It was thereafter stored in a biofreezer at -70°C. The aortic tree (the brachycephalic arteries to their bifurcations and the aorta to the iliac branching) was dissected, cut longitudinally and visually examined for any atherosclerotic lesions.

The blood that was collected in EDTA tubes was used for the analysis of red blood cell membrane cholesterol.

The heparinised blood was alloquoted and used for the following:

1. Haematological parameters: Red cell, white cell and platelet counts
2. Plasma lipids: Total cholesterol, high density lipoprotein and low density lipoprotein fractions and triglycerides
3. Super oxide dismutase
4. Glutathione
5. Glutathione peroxidase
6. Oxidative challenge test on RBC

2.2 Plasma Lipids

The blood was centrifuged at 3500 x g at 4°C for ten minutes. The plasma was aspirated using disposable liqui-pipettes and stored in polypropylene microfuge tubes at -20°C.

2.2.1 Triglycerides

The triglycerides concentration was obtained using the Periodochrom ^{R} method (Roche GmbH, Germany). This method is based on the principle in which the triglyceride fractionis involved in a multi-stepped enzymatic reaction to eventually yield 4(p-benzoquinone-mono-imino)-phenazone. This compound is detected spectrophotometrically and its concentration is directly proportional to intensity of colour. The absorbance was multiplied by the factor 8.66 as supplied by Roche.

The assay was performed in polypropylene tubes, twenty microlitre (20 *ul*) of sample was added to two millilitre of reagent. The reaction mixture was gently vortexed and incubated at 25°C for ten minutes. The optical density was obtained using glass cuvettes at 500 nm in a dual beam spectrophotometer (Varian, Cary IE).

2.2.2 Total Cholesterol

Total cholesterol was assayed enzymatically by the CHOD-PAP method (Roche Diagnostics GmbH, Germany). The assay is based on the principle in which the cholesterol ester of the plasma reacts in a multi stepped enzymatic reaction with reactants in the reagent to yield the compound, 4-(p-benzoquinone monoimino) phenazona, that is detected spectro-photometrically at 500 nm.

The assay was carried out in polypropylene tubes. To two millilitre (ml) of reagent 0,02 ml (20 microlitre) of plasma was added. The mixture was gently vortexed and incubated at 25°C for ten minutes. Two ml of reagent served as reagent blank. The optical densities were obtained using glass cuvettes (1 cm path length) at 500 nm in a dual beam spectrophotometer (Varian, Cary IE, Australia).

Precinorm L and Beckman decision level II were used as controls. The results obtained using these methods produced values that fell within the ranges recommended by the respective supplier. The results (in mmol/l) were obtained by multiplying the absorbance value by the factor 14.9. as supplied by the manufacturer.

2.2.3 High Density Lipoprotein

In order to determine the high density lipoprotein (HDL) concentration in the sample, the chylomicrons, very low density lipoproteins (VLDL) and low density lipoproteins (LDL) were precipitated using the HDL cholesterol precipitant (Roche Diagnostics GmbH, Germany). The HDL concentration was determined using the enzymatic method described earlier for total cholesterol. The reagent blank however, had 20 *ul* of deionised H_2O in 2 ml of reagent. The concentration in mmol/l was obtained by multiplying the absorbance by 5.67. The LDL concentration in mmol/l was obtained from the following formula:

$$\text{LDL Cholesterol} = \text{Total cholesterol} - \frac{\text{triglycerides}}{2.2} - \text{HDL cholesterol}$$

2.3 RBC Glutathione Peroxidase (GPx)

This biochemical assay was based on the established method of Paglia, (1967) which requires the sample RBC count to be approximately 3 x 10^6 per *ul*. After washing erthrocytes in cold saline thrice, the concentration was adjusted accordingly using a Coulter. The lysate was thereafter prepared by adding 0.1 ml cell suspension to 0.4 ml ultra-pure water and subjecting this to three freeze-thaw cycles for ten minutes using a freeze mixture that comprised dry ice and acetone. Equal volumes of the resultant lysate was added to double strength Drabkins reagent so that all the haemoglobin was converted to stable cyanomethaemoglobin. This was stored at -70°C until it had been assayed.

To 0.1 ml of hemolysate Drabkins mixture (well thawed and vortexed) the following reagents were added in a 5 ml polypropylene tube in this specific order

1. 2.58 and 0.05 M phosphate buffer pH 7.0 containing 0.005 M EDTA
2. 0.10 ml 0.0084 M NADPH
3. 0.01 ml GSSG-R
4. 0.01 ml 1.125 M NaN_3
5. 0.10 ml 0.15 M GSH

The reaction mixture was vortexed, dispensed into a glass cuvette and the enzymatic reaction was commenced by the addition of 0.1 ml, 0.0022 M H_2O_2 solution, after cuvette was put into the cell compartment of the spectrophotometer. The conversion of NADPH to NADP was observed at 340 nm between two to four

minutes. This step was initially monitored for eight minutes and the OD change per minute showed that optimum reaction rates were in fact between two to four minutes as described by the authors (Paglia, *et al.*, 1967).

Using the same batch of reagents, a non-enzymatic conversion of NADPH to NAPD was determined by replacing the sample with equal volume of H_2O. The true enzymatic activity of each sample was determined by subtracting the non-enzymatic from the total activity determined above.

2.4 Superoxide Dismutase (EC 1.15.1.1)

This method is based on the method of Winterbourn, (1974). The blood was centrifuged at 3000 x g at 4°C for ten minutes. After the plasma and buffy coat was removed the red blood cells were washed in four volumes of cold saline and centrifuged at 3000 x g for ten minutes.

This was repeated three times. After the final wash, 1 ml of cells were removed and added to 10 ml of cold ultra-pure water (Millipore, France). The sample was thereafter subjected to three, freeze-thaw cycles. Dry ice, acetone was used as the freeze mixture (-77°C).

After adjusting the haemoglobin concentration to about 10 g/dl, 0.5 ml of haemolysate was added to 3.5 ml cold water, followed by 1.0 ml ethanol. The mixture was vortexed thoroughly and a further 0.6 ml of chloroform was added. This final solution was vortexed for one minute and thereafter centrifuged for ten minutes at 3000 x g at 4°C. The clear uppermost layer which contains the enzyme was removed and the following volumes (micro-litres) were dispensed into six tubes : 10, 20, 40, 60, 80, 500. A further three tubes with no extract was included in the assay, these were used as blanks. These steps were conducted with as little light as possible. The following reagents were then added to the nine tubes as follows:

1. 0.2 ml EDTA / NaCN (0.1M EDTA containing 1.5 mg NaCN/ dl)
2. 0.1 ml NBT (1.5 mM Nitroblue tetrazolium)
3. 0.05 ml Riboflavin (0.12 mM)
4. Phosphate buffer (0.15 M pH 7.8) to a final volume of 3.00 ml. The tubes were thoroughly mixed.
5. The tubes were placed in a specially built light box for twelve minutes. The internal dimensions of the box is 560 x 305 x 200 mm (L,W,H). The entire inner surface of the box was painted white and a two feet fluorescent light (white light) was used as light source. To ensure that the tubes were evenly exposed to the light, mesh type test tube racks were used. The tubes were incubated for twelve minutes.

The optical densities were thereafter determined using glass cuvettes at 560 nm in a dual beam spectrophotometer (Varian, Cary, IE, Australia). A graph of % inhibition (V) vs volume lysate was blotted for each sample. The volume, V in ul of lysate that give 50% inhibition was determined from the graph. Since extract haemoglobin concentration was 1 gm/dl, the enzyme activity was obtained by the following formula:

$$\frac{100\ 000}{V}$$

Reagents:

1. EDTA/ NaCN: 0.1 M EDTA containing 1.5 mg NaCN/ dl (BDH, AR grade, Poole, England)
2. 1.5 mM Nitroblue tetrazolium (NBT) (Sigma chemicals, St.Louis, MO, USA)
3. 0.12 mM Riboflavin (Sigma chemicals, St Louis, MO, USA)
4. 0.15 M Phosphate buffer pH 7.8 : 0.12 M Potassium dihydrogen phosphate (7.4 ml) and 0.12 M disodium hydrogen phosphate (92.6 ml). pH was adjusted to 7.8 (BDH, AR grade, Poole, England).

2.5 Gluthathione

The heparinised blood was centrifuged at 1500 x g for ten minutes at 4°C. After removing the plasma and buffy coat the mass of the red blood cells were determined per millilitre. One ml of cells was then added to 10 ml of cold deionised water (4°C)

and allowed to stand at 4°C for ten minutes. One ml of this lysate was added to 5 ml of cold 5% Trichloroacetic acid (T.C.A.) in 1 mM EDTA. After mixing the sample was centrifuged at 12 000 x g at 4°C for five minutes. The deproteinised supernatant was alloquotted into polypropylene microfuge vessels and stored at -70°C.

The thawed sample was used in the assay as follows. In 5 ml polypropylene tubes, 2 ml of 0.6 mM 5'-5'-dithiobis-2-nitrobenzoic acid (DTNB) and 0.8 ml of phosphate buffer (0.1 M, pH 8.0) were added and mixed. 0.2 ml of sample was added to start the reaction.

The optical density was taken at 412 nm in a dual beam spectrophotometer (Varian, Cary 1E, Australia). In the reference cuvette, 0.2 ml of 5% TCA was substituted for the sample.

Reagents:

1. 5% Trichloroacetic acid ($^{w}/_{v}$) in 1 mM EDTA (BDH, AR grade, Poole, England)
2. 0.6 mM 5'-'5' dithiobis-2-nitrobenzoic acid (DTNB) (Sigma Chemicals, St Loius, MA, USA)
3. 0.1 M Phosphate buffer: 0. 1 M KH_2 PO_4 (3.7 ml) and Na_2HPO_4 2 H_2O (96.3 ml) pH adjusted 8.00 (BDH, AR grade Poole, England)

2.6 Malonaldehyde

The heparinised sample was centrifuged at 1500 x g at 4°C for ten minutes. After removing plasma and buffy coat the packed cells were washed in ten volumes cold saline (4°C) and centrifuged at 1500 x g at 4°C for ten minutes. This was repeated three times. After the last wash, the packed cells were weighed and the suspension adjusted to 5% by mass ($^{m}/_{v}$) using saline containing 2 mmol/l Sodium Azide (Na N_3).

After gently inverting the container many times so that an even suspension was obtained, the sample was used as follows. Five polypropylene tubes with the following H_2O_2 concentrations 0; 2.5; 5.0; 10.0 and 20 mM were put in a water bath at 37°C. Two ml of cell suspension was added to each tube and the mixture was incubated for one hour. The rack was gently shaken every ten minutes.

The reaction was terminated by adding 5 ml of 28% TCA with 0.1 mol/ l sodium arsenite. The sample was then centrifuged at 5000 x g at 4°C for fifteen minutes.

Two ml of supernatant was removed from each tube and transferred to an appropriately labeled glass, capital Pyrex tube. To each tube 0.5 ml of thio-barbituric acid (1% in 50 mM NaOH) was added. The mixture was thoroughly vortexed and the tubes were heated to 95°C for fifteen minutes.

The tubes were thereafter cooled rapidly in cool tap water. The optical density of each tube was taken at 532 nm and at 453 nm. In order to compensate for the effects of reduced glutathione and RBC hemolysate, and hence improve specificity of the test 20% of the absorbance at 453 nm was subtracted from the corresponding optical density at 532 nm the extinction co-efficient 1. 55 x 10^5 $M^{-1}.cm^{-1}$ was used.

Reagents:

1. Saline
2. Buffered Saline: 0.5 mmol/ l KH_2PO_4
   3.44 mmol/l K_2HPO_4
   150.0 mmol/l NaCl

   containing 2 mmol/l NaN_3 (Sigma, St Lious, MO, USA). The pH was adjusted to 7.4
3. 28% trichloroacetic acid with 0.1 mol/l Sodium arsenite (BDH, AR grade, Poole, England)
4. 1% thiobarbituric acid (Sigma chemical St Lious, MO, USA)
5. 2.5; 5.0; 10.0; 20.0 and 40.0 mM hydrogen peroxide solutions. A working stock solution of 40 mM/l was initially prepared and other concentrations were prepared with appropriate dilutions from this working standard.

2.7 Red Blood Cell Membrane Cholesterol

Blood used for this analysis was collected in EDTA tubes. The tubes were centrifuged in EDTA tubes. The tubes were centrifuged at 1500 x g for ten minutes at 10°C after which the plasma and buffy coats were removed. The cells were washed with three volumes of saline and centrifuged at 1000 x g for ten minutes at 10°C. The saline was renewed and, the wash was repeated three times.

After the final wash, the saline was partially removed to yield a 1:1 ratio of saline to cells. 250 *ul* of this cell suspension was removed and dispensed into a polypropylene tube. This was vortexed at high speed so that a thin film of cells was formed. 4.5 ml of isopropanol/ chloroform mixture with BHT was added dropwise along the walls of tubes. The tubes were capped and shaken vigorously for thirty seconds. They were thereafter allowed to stand in a dark cupboard overnight at room temperature. 300 *ul* of the extract was removed and evaporated in a fume cupboard at 37°C. The door of the fume cupboard was drawn down to leave a gap of 5 cm. This significantly increased the negative pressure within the fume cupboard and hence maximized the rate of evaporation of the extracting mixture. The residue was stored at -20°C in capped tubes. For analysis the residue was dissolved in 0.5 ml of cholesterol reagent solution (Roche) and optical densities determined as described for plasma cholesterol levels.

2.8 Catalase (E1, 11, 1, 6)

Catalase activity was determined in the serum sample. In order to establish catalase activity, the reactions were carried out in four polypropylene tubes as follows:

In tube one, 0.2 ml of sample was added to 1.0 ml of substrate. The mixture was gently vortexed and incubated for 60 seconds at 37°C in a water bath. The reaction was stopped by addition of 1.0 ml of Ammonium molybdate. The optical density was read in a glass cuvette at 405 nm against blank 3 (described below) in a dual beam spectrophotometer (Varian, Cary 1E, Australia). The spectrophotometer was initially zeroed against air.

In tube two, blank one was prepared with 0.2 ml serum, 1.0 ml substrate and 1.0 ml of ammonium molybdate. The mixture was gently vortexed and the O.D. read against air at 405 nm.

In tube three, blank two was prepared with the following reagents: 0.2 ml of buffer, 1.0 ml substrate, 1.0 ml ammonium molybdate and was gently vortexed. The O.D. was also read against air.

In tube four, blank three was prepared with 0.2 ml buffer, 1.0 ml buffer and 1.0 ml ammonium molybdate. After gently vortexing this blank was used as mentioned above.

Catalase activity was determined in batches of three to four samples. Blanks two and three therefore served as common blanks for the assay.

Reagents:

1. Sodium-potassium buffer 60 mmol, pH 7.4 60 mmol/l solutions of potassium dihydrogen orthophosphate and di-sodium hydrogen phosphate were prepared in deionised water (BDH, AR grade, Poole, England). For each 100 ml of buffer, 19.7 ml of KH_2PO_4 was added to 80.3 ml of Na_2HPO_4, $2H_2O$. pH was adjusted with either 0.1 N HCl or 0.1 N NaOH (BDH, AR grade, Poole, England).
2. Substrate containing 65 umol/ ml H_2O_2 in 60 mmol/l sodium-potassium buffer. The concentrations of the H_2O_2 was verified as described by Merck 32.4 mmol/l.
3. Ammonium molybdate (BDH, AR grade, Poole, England).

2.9 H_2O_2 Standardisation

Due to its highly reactive nature, the H_2O_2 concentration was determined each time that it was used. Dilution to the appropriate concentration was based on this value. The procedure was as follows:

1. 0.1 g H_2O_2 was weighed out and added to a 50 ml volumetric flask. This was taken up to mark using ultra pure water.
2. One ml of H_2SO_4 (concentration) was added gently to the solution with constant stirring.
3. This was titrated against 0.02 M K_2MnO_4. End point was the first permanent, faint pink colour that developed.
4. The calculation was based on the fact that 1 ml 0.02 M K_2MnO_4 = 0.0017007 g H_2O_2

   This method was recommended by Merck, (Darmstad, Germany).

2.10 Tissue Analysis

The left ventricle was thawed, blotted dry and weighed. A 1:10 ratio of heart (g) to homogenizing buffer (ml) was prepared (50 mM TRIS in 0.1 mM EDTA pH 7.6). This was always kept on ice. The homogenate was prepared using a homogeniser (Ultra-Turrax TP 18-10, Staufen, Germany). The tissue and buffer were subjected to two, fifteen seconds cycles with the homogeniser running at 25% of maximum speed.

Immediately thereafter 0.5 ml of homogenate was removed and 10 ml of cold 25 % TCA was added to it in a polypropylene tube. The sample was stoppered inverted three times and centrifuged at 12 000 x g at 4°C for five minutes in a Beckman ultracentrifuge using a SW 40 Ti rotor (Beckman, USA). The supernatant was removed and rapidly frozen in liquid nitrogen. The sample was then stored at -70°C until used for Glutathione analysis.

The remaining homogenate was centrifuged at 105 000 x g at 4°C for fifteen minutes in a Beckman ultracentrifuge (L5-50B) with a SW 40 Ti rotor (Beckman, USA). The resultant tissue cytosolic fraction was removed alloquoted for glutathione peroxidase, superoxide dismutase, protein concentration and haemoglobin levels. The samples were rapidly frozen in liquid nitrogen and thereafter stored at -70°C.

2.11 Cytosolic Protein Concentration

This assay was carried out since the levels of superoxide dismutase and glutathione peroxidase are expressed per gram protein of the cytosolic fraction.

The reagent used for this assay, Bio-rad (Bio-rad, USA) is based on the method of Bradford (Bradford, 1976) in which an acidic dye, Coomassie Blue binds to protein. The colour change is proportional to the product that is formed at 595 nm.

The corresponding protein concentration is extrapolated from a standard curve using bovine serum albumin as standard.

The standard curve was prepared as follows:

1. A standard protein of 1.4 mg/ ml was prepared using bovine serum albumin, fraction V (Merck, Darmstadt, Germany).

2. Six standards in duplicate were prepared in glass tubes using the following volumes (in *ul*) of the stock 0; 11; 16.5; 24.2; 27.5 and 33; these tubes corresponded to protein concentrations of 0; 0.28; 0.42; 0.56; 0.70; 0.84 mg/ml respectively.

3. To each tube, 2.75 ml of reagent was added (Bio-rad, USA). The tubes were vortexed and incubated at 25°C for 5 minutes.

4. The optical densities were read against the reagent at 595 nm in a dual beam spectrophotometer (Varian, Cary 1E, Australia). The standard curve was plotted using the optical densities (ordinate) against their respective concentrations (absisca).

   The assay of the samples were carried out as specified by Bio-Rad.

2.12 Preparation for Chemical hypoxia

2.12.1 Isolation of Adult Rat Ventricular Myocytes

Twenty four Dahl salt sensitive and salt resistant rats fed both high and low salt diets for six weeks were used for this study. Adult male rats (200 to 250 grams) were anesthetised with thiopentone sodium (30 mg; intraperitoneally). The heart was excised and mounted onto the aortic cannula of a modified Langendorff perfusion apparatus.

The heart was initially perfused for five minutes with a calcium free, HEPES bicarbonate buffer, called *buffer A* (500 ml) containing (in mmol/L) NaCl 109; NaH_2PO_4 1.15; KCl 4.95; glucose 30.0; 2,3-butane dione monoxime 19.98; HEPES 25.0; $NaHCO_3$ 28.0; carnitine 1; taurine 1; creatine 1; adenosine 0.249; insulin[1] 0.25 ml (0.025 iu/L) and pen/strep[2] 0.50 ml (pen = 10 iu/L and strep = 0.01 mg/L), pH 7.4. The buffer was gassed with 95 % O_2 and 5% CO_2 during the perfusion (temperature: 37°C).

This was followed by a ten minute perfusion with 50 ml *buffer A*, to which was added 25 uM/L $CaCl_2$, collagenase (30 mg/ 50 ml) (Boehringer Mannheim) and protease (3 mg/ 50 ml (Sigma).

Thereafter the $CaCl_2$ concentration was increased to 50 uM/L, and then to 100 uM/L at five minute intervals. The solution was gassed with 95% O_2 and 5% CO_2 throughout the isolation procedure.

The ventricles were cut off and the myocytes gently dispersed in 50 ml *buffer A*, containing 100 uM/L calcium chloride (post digestion buffer). The $CaCl_2$ concentration was increased by 100 uM/L to 1 mmol/L at ten minute intervals.

The cells were allowed to settle; half of the buffer was decanted and replaced with 25 ml nutrient medium containing M199[3] (2.455 grams); $NaHCO_3$ (23.0 mmol/L); creatine (1 mmol/L); taurine (1 mmol/L); caprylic acid (1 mmol/L); carnitine (1 mmol/L); pen/strep[2] 0.25 ml (pen = 10 iu/L and strep = 0.01 mg/L); insulin[1] 0.125 ml (0.025 iu/L).

The cells were again allowed to settle and the buffer (25 ml) nutrient medium (25 ml) mixture replaced by 50 ml nutrient medium. 3.5 x 4.7 x 10^6 rod-shaped, viable, calcium tolerant myocytes were obtained with each isolation from one heart. After gentle agitation, a fifth of this cell suspension was diluted to 50 ml with nutrient medium. The cells were now ready for attachment to coverslips and culturing.

1 (100 iu/ml)

2 (pen 10.000 iu/ml; strep 10 mg/ml)

3 (Gibco BRL)

2.12.2 Preparation of Coverslips

At a laminar flow bench, 10 to12 round coverslips (0.15 mm thick and 31 mm in diameter) were sterilized in 70% ethanol for one hour. The coverslips were then placed in 35 mm petri dishes, under UV light for one hour, to dry.

2.12.3 Preparation of the Cell Tak^R

30 µl of Cell tak (687 mg/L acetic acid) was diluted with 230 µl $NaHCO_3$ (100 mmol/L $NaHCO_3$).

20 µl portions of this mixture was aliquotted onto the centre of each coverslip. It was allowed to stand until dry and then rinsed with nutrient medium.

2.12.4 Culture of Myocytes

The coverslips were seeded with about 2 ml of diluted cell suspension and the density further adjusted with nutrient medium to ensure a sparse arrangement of cells in any, one microscopic field of view (15 to 20 rod-shaped myocytes in any one field using the x 20 microscope objective).

The cells were placed in a CO_2 (5%) incubator at 37°C for one hour, after which the medium was replaced with fresh nutrient medium. The cells were then incubated overnight to allow them to stabilize and to ensure proper attachment.

2.12.5 Loading Myocytes with Fluorescent Probes

The next morning the nutrient medium was removed and replaced with 2 ml *buffer B* which consisted of the following (in mmol/L) $CaCl_2$ 1.25; NaCl 114; KCl 4.98; KH_2PO_4 1.0; $MgSO_4$ 1.2; HEPES 25; glucose 5, pH 7.4, *Tetra-methyl-rhodamine* (TMRM) (1 μM/L) to which one of the following fluorophores were added and the myocytes pre-loaded for ten minutes at 37°C in a 5% CO_2 incubator:

- *2,7 dichlorodihydrofluorescein* (1 μmol/L), or
- *dihydrorhodamine 123* (1 1 μmol/L), or
- *fluo 3, AM* (2.5 μmol/L)

2.12.6 The Induction of Chemical Hypoxia and Reoxygenation

After loading the myocytes with the appropriate fluorophores, the coverslip was placed in an acetyl water jacketed chamber.

Chemical hypoxia was introduced by exposing the myocytes to 10 ml hypoxic buffer, pH 7.4 at 37°C. The chemical hypoxic buffer consisted of buffer B in which glucose was replaced by 20 mmol/L 2-deoxyglucose and 1.5 mmol/L KCN. The buffer was gassed with 100% nitrogen for one hour prior to the addition of KCN.

The cells were re-oxygenated for fifteen minutes by exposing the cells to buffer B, pH 7.4 containing 5 mmol/L glucose.

PI (5 mmol/L) and a low concentration of TMRM (200 nmol/L) were also included in the hypoxic and re-oxygenation buffers.

Reagents:

1. Stock Solutions:

Buffer A Stock [20 X Stock]

NaCl	64.284 g
KCl	3.727 g
NaH_2PO_4	1.6555 g
H_20	500 ml

$CaCl_2$ Stocks (147mw)

25mM:	183mg/50ml
100mM:	735mg/50ml

BUFFER A

KCl, NaCl, NaH_2PO_4	25 ml	50 ml
Carnitine	98.85 mg	0.1977
Taurine	62.55 mg	0.1251
Creatine	65.55 mg	0.1311
Octanoic acid	83.1 mg	0.1662
Adenosine	33.4 mg	0.0668
Glucose	2.705 g	5.41
BDM	1.01 g	2.02
Pen/ Strep	0.5 ml (units)	1 ml
Insulin	0.25 ml (units)	0.5 ml
HEPES	2.98 g	5.96
$NaHCO_3$	1.18 g	2.36
MEM	-	1000 ml
M199	qs to 500 ml, pH 7.3	

3. Digestion Buffer

Buffer A	50 ml
25mM $CaCl_2$	50 µl = 25 µM [Ca]
Collagenase (B)	30 mg
Protease	3 mg

4. Post Digestion Buffer

Buffer A	50 ml
25 mM $CaCl_2$	200 µl = 100 µM [Ca]

2.13 Blood Pressure and Diet

2.13.1 Blood Pressure Measurement

Blood pressure (systolic) was measured non-invasively using the tail cuff method. The computerised system (IITC, Model 31) uses a photoelectric sensor in the tail cuff that detects the pulse of the caudal artery. The system is automated in that it has a pump that inflates cuff pressure beyond a predetermined value. During deflation the amplitude of the pulse and the pulse rate are displayed as data plots. After the determination, a summary of the results is displayed on the screen. Using this method of detection, systolic pressure and heart rate can be calculated. Diastolic pressure, however is calculated by the software of the unit.

Optimum results were obtained when restrained animals were maintained at 28 to 29°C prior to and during the test in an oven with a circulating fan. Due to the possibility of inducing stress, animals were kept in the restrainer at this temperature for no longer than ten minutes. Three sizes of restrainers were used during the course of the study depending on the physical size of the animal. A common cuff was used to prevent any variation in results. To ensure acclimatization and to minimize stress, the animals were initially subjected to the entire procedure on at least three occasions before recording commenced. The results of recordings that appear in table 1 are an average of three measurements.

Recordings were carried out at approximately the same time each day and results of this study compared favourably with results obtained by cannulation in our laboratory (Somova, 1999). All blood pressure measurement was obtained with the artifact filter switched on.

2.13.2 Diets

Diets that were based on the AIN-76^{R}. The semi synthetic diets, was prepared in our laboratory (AIN 76). This diet was prepared using all precautions recommended for trace element studies this was necessary due to sodium, potassium and selenium being experimental variables of this study. The AIN 76 vitamin and mineral mixes were prepared in bulk and stored in dark airtight containers at 4°C. The final diet was prepared freshly on a weekly basis as described below under "composition." These diets were also stored in air tight, light resistant containers at 4°C until used. This diet has been shown to be ideal for promoting optimum growth and development in rodents (AIN 76). The details of the diet are as follows:

TABLE 4a: AIN-76 Modified Sodium Deficient Diet (Rats and Mice)

COMPOSITION	
Casein (Low Trace Element)	20.0 %
DL-Methionine	0.3 %
Corn Starch	15.0 %
Sucrose	50.0 %
Alphacel, Hydrolyzed	5.0 %
Corn oil	5.0 %
Choline Bitartrate	0.2 %
AIN 76 Mineral Mix	3.5 %
AIN 76 Vitamin mixture	1.0 %
AIN 76 MINERAL MIXTURE (gms/ kg of Mixture)	
Calcium Phosphate Dibasic	500.00 gm
Potassium Citrate Monohydrate	220.00 gm
Potassium Sulfate	52.00 gm
Magnesium Oxide	24.00 gm
Manganese carbonate (43-48% Mn)	3.50 gm
Ferric citrate (16-17% Fe)	6.00 gm
Zinc carbonate (70% Zn O)	1.60 gm
Cupric carbonate (53-55% Cu)	0.30 gm
Potassium iodate	0.01 gm
Sodium Selenite ($Na_2SeO_2{\cdot}5H_2O$)	0.01 gm
Chromium Potassium Sulfate	0.55 gm
Sucrose, finely powdered	192.00 gm
AIN 76a VITAMIN MIX (per kg of Mixture)	
Thiamine Hydrochloride	600.00 mg
Riboflavin	600.00 mg
Pyridoxine Hydrochloride	700.00 mg
Nicotinic acid	3.0 gm
D-Calcium Pantothenate	1.6 gm
Folic acid	200.0 mg
D-Biotin	20.0 mg
Cyanocobalamin (Vitamin B12)	1.0 mg
Retinyl Palmitate (Vit. A), Pre-Mix (250,000 iu/gm)	1.6 gm
DL-Tocopherol Acetate (250 iu/ gm)	20.0 gm
Cholecalciferol (Vit. D_3) (400,000 iu/gm)	250.0 mg
Menaquinone (Vit. K_2)	50.0 mg
Sucrose, finely powdered	972.9 gm

*N.B.
1. **Based on a modified AIN-76 formulation. Sodium selenite is included in this diet. The contribution to Na level is negligible.**
2. **All chemicals were either AR or food grade.**

RESULTS

All data, excluding those pertaining to chemical hypoxia and confocal microscopy (Table 5), are presented as a mean value ± standard error of the mean (SEM). For the analyses the INSTAT V2.04 program was used including the one-way ANOVA, t-test and chi-square test. A p value < 0.05 was considered statistically significant.

Difference in fluorescence intensity of all three fluorophores were evaluated using the Scion Image software package (Scion Corporation, MD, USA) to determine how they differed from baseline values. The mean fluorescence intensity of the myocytes was measured by object histoprogramming without background subtraction since the latter was negligible.

3.1 Blood Pressure

The results of both blood pressure and heart rates that were measured when the experiment commenced and subsequently at two week intervals, appear in Table 1. The higher baseline values of the DSR,1% group was due to the fact that they were not age matched (refer to mass in Table 1). The slight increase in blood pressure in both DSR, 1% and DSR, 8% animals during the six week experimental period is age related. Salt loading had no significant effect on blood pressures of DSR, 8% animals. The DSR animals irrespective of their diet therefore remained normotensive for the duration of the study.

The DSS, 1% group showed a significant increase in blood pressure and tachycardia compared to their controls in the fourth week whereas the DSS, 8% displayed this significant trend by the second week. In the sixth week both the DSS, 1% and DSS 8% groups remained hypertensive but the blood pressures of DSS 8% were significantly higher than the DSS 1% group.

3.2 Body and Organ Mass

Body Mass

The data in table 2 shows that DSS rats displayed a higher gain in mass per week than DSR animals. In the sixth week DSS animals had a significantly higher mass than DSR animals. Salt loading resulted in a lower body mass in both DSS and DSR animals.

Heart Mass

The masses of the whole heart and the left ventricle was determined. Both these parameters were expressed in terms of gram tissue per 100 grams body mass as well. These data appear in table 2.

The hearts of DSS 1% rats were significantly heavier than their respective controls (39.8%). Salt loading significantly decreased the heart mass in the DSR strain. In the

DSS strain however, salt loading resulted in a significant hypertrophy of the entire heart.

There was no significant difference in the left ventricular (LV) masses of both DSR 1% and DSR 8% animals. The left ventricular mass of DSS 1% rats was significantly higher than the DSR 1% group. Salt loading resulted in a decrease in left ventricular mass in DSS 8% group.

When heart mass was expressed in terms of body mass (g/100g), there were no differences in this ratio between DSR 1%, and DSR 8% groups, that is, they were independent of sodium intake. In the case of the DSS 1%, their heart/ body weight ratio was significantly higher than their respective controls the DSR 1%. Sodium loading resulted in a significant increase in this ratio compared to DSS 1%.

In the left ventricular weight/ body weight ratio there were no significant differences in the DSR groups, that is they were not affected by sodium loading. In the DSS groups, this ratio was identical in both DSS 1% and DSS 8%. In both cases they were significantly higher in their respective controls.

3.3 The Antioxidant Status of the Heart (Left Ventricle)

The results of all three antioxidant enzymes appear in table 3.

Glutathione peroxidase levels of left ventricle (LV) were significantly lower in DSS 1% compared to DSR 1% animals. Salt loading resulted in a significant decrease in GPx levels in both DSS and DSR animals.

Superoxide dismutase (SOD) levels of LV showed the opposite trend, there was a non-significant increase in SOD levels in DSS 1% compared to DSR 1% animals. Salt loading also produced a non-significant increase in SOD levels in both DSS and DSR animals. There was also a non-significant increase in SOD in the DSS 8% compared to DSR 8%.

Glutathione (GSH) levels of LV in DSS 1% animals were significantly lower than their respective controls. Salt loading resulted in a non-significant increase in GSH levels in the DSR group. In the case of DSS 8% group there was a significant increase in GSH levels compared to DSS 1% rats.

3.4 Growth and Food Parameters

Feed Efficiency Ratio

The feed efficiency ratio (FER) is a quotient of the body mass gain / food consumed during the six week experimental period. These data are available in other tables elsewhere. There was a significant increase in the FER in DSS 1% with respective to their controls (67%). Salt loading had no effect on the FER in the DSR strain. In the DSS strain however salt loading resulted in a significant decrease in the FER (14.9%) (Table 2).

Food Consumption

The average weekly food consumption was determined for each group. These appear in the appendix. This data was used to determine the feed efficiency ratio (Table 2) this, in view of the fact that this ratio also takes the increase in body mass into account.

Percentage increase in Mass

The mass of the animals on day one of the trial was used as baseline (100 %) all subsequent increases per week were expressed as a percentage increase. This data was not subject to statistical evaluation. The results appear in table 4 and in graph 4.

3.5 Chemical Hypoxia of Isolated Myocytes

The fluorescent probes and what they evaluated are listed below:

1. Dichlorodihydro – fluorescein diacetate (DCDHF) labels H_2O_2
2. Dihydrorhodamine 123 (DHR) is a general indicator of free radicals, viz, non specific oxidative stress
3. Fluo 3 is an indicator of intracellular calcium.

After subjecting oxygenated myocytes to 125 *u*M H_2O_2 (chemical hypoxia), the number of cells showing an increase in DCDHF, DHR and Fluo 3 fluorescence that exceeded baseline values were counted. These results, in table 5 are expressed as a percentage of the total number of cells evaluated.

This test revealed that under normoxemic conditions there was no significant difference between DSS and DSR myocytes with respect to H_2O_2 levels, reactive oxygen species (non-specific) and intra-cellular Ca^{+2} . After exposure to chemical hypoxia for thirty minutes, the myocytes of both strains showed an increase in H_2O_2 and ROS levels as well as increased intra-cellular Ca^{+2} accumulation. The number of damaged myocytes also increased. These changes were significant in the case of the DSS animals.

Due to an element of subjectivity in interpreting the results and the inconsistencies experienced with this method, the results of this test were not considered to be a significant contribution to this study.

3.6 The Antioxidant Status of Red Blood Cells

The antioxidant status of RBC was performed using established biochemical assays and using commercially available kits in the case of GPx and SOD (Randox). The results of these tests appear in tables 6 and 7 respectively. Included in table 7 is the antioxidant status of RBC of weanling rats on normal rat chow.

Results of Biochemical Assays, Glutathione perioxidase (GPx)

There was a significant decrease in RBC levels of this enzyme in DSS 1% rats compared to DSR 1%. Salt loading produced a non significant increase in the case of DSR animals but a significant increase in DSS animals.

Superoxide Dismutase (SOD)

The levels of this enzyme were not determined in salt loaded animals due to loss of sample. They were however determined in DSR 1% and DSS 1% using Randox kits (table 7). There was a significant decrease in SOD levels in DSS 1% compared to DSR 1% animals.

Serum Catalase

Serum catalase (CAT) was significantly elevated in DSR 1% compared to DSS 1% rats. This difference was very large and translated to a 152% increase.

3.7 Blood Glucose and Antioxidant Status of the RBC in both Weanlings and Adults

Blood Glucose

Whole blood glucose was determined using the Glucometer elite, the precision and accuracy of which was validated previously in our laboratory.

There was no significant difference in blood glucose levels between the two strains of weanling rats (Table 7). But the respective adult strain on normal diet showed an elevation in blood glucose levels. DSS adult rats on normal diets showed a marked increase in blood glucose levels compared to their respective controls. In addition salt loading of DSS rats resulted in a significant decrease in blood glucose levels compared to the group on normal salt intake.

Glutathione Peroxidase (GPx) and Superoxide Dismutase (SOD)

The glutathione peroxidase and superoxide dismutase levels of RBC that appear in Table 7 were assayed using Randox kits. The performance of these kits were initially validated against well established biochemical assays. Both the GPx and SOD kits are based on the principle of the respective biochemical assays.

In weanling rats which were not salt loaded both GPx and SOD levels in the RBC were significantly lower in DSS animals compared to DSR. Refer to table 1, the age of these weanlings corresponds to those at the beginning of the experiment period at which time they were normotensive.

In adult animals after the experimental period, GPx levels were significantly lower in RBC of DSS, animals than their respective controls. Salt loading had no significant effect on RBC GPx levels in this group.

The SOD levels in RBC of DSS 1% animals also showed a significant decrease compared to the DSR strain but salt loading further decreased SOD levels but the decrease was not significant.

The SOD: GPx ratio was calculated for discussion purposes since the antioxidant system functions in an integrated manner. This was not subjected to statistical evaluation.

3.8 The Oxidative Challenge Test

After exposing RBC of both strains to concentrations of H_2O_2 ranging from 2.5 to 20%, the results of Malondialdehyde (MDA) concentration, a product of lipid peroxidation appear in table 8 and graph 3.

The MDA concentrations in unchallenged RBC subjected to test conditions (that is 0% H_2O_2) were negligible with no significant differences between DSS and DSR animals.

Exposure of RBC to H_2O_2 concentrations of 2.5% and 5.0% did not result in significant differences in product formation between the two strains. There was however a > 500% and 700% increase in MDA formation in these concentrations respectively. This indicates that the test was successful in generating lipid peroxides.

At H_2O_2 concentrations of 10% and 20% there was a significant increase in MDA formed in the RBC of DSS animals compared to DSR. This indicates that the strain difference in response to the challenge only becomes evident when H_2O_2 concentration in the medium exceeds 10%.

3.9 Plasma Lipids

Plasma lipid levels were determined using commercially available kits (Roche diagnostics) and appear in table 9. All results are expressed in mmol/l .

Total Cholesterol

There was a significant increase in cholesterol concentration in DSS 1% compared to DSR 1%. This increase was large and translated to 108%.

In the DSR strain there was a non-significant decrease in total cholesterol levels in salt loaded animals whereas in the case of DSS 8% there was a significant decrease.

High Density Lipoprotein (HDL)

The levels of this fraction were significantly higher in DSS animals compared to the DSR group on normal diets. Although salt loading decreased the levels of HDL in both strains, this decrease was not significant.

Low Density Lipoprotein (LDL)

The DSS 1 % had a significantly higher level of the LDL fraction compared to the DSR 1% group. Salt loading produced opposite trends in these strains. In the case of the DSR strain there was a significant decrease in HDL levels. But salt loading produced a non-significant increase in LDL levels in the DSS strain.

Triglyceride Levels (TRIG)

The DSS strain had a significantly higher level of TRIG than DSR animals on normal diet. This difference was large, amounting to 230% increase.

Salt loading did not affect TRIG levels in DSR rats whereas the levels dropped significantly in DSS animals. There was no significant difference in both strains when they were salt loaded.

3.10 Red Cell Membrane Cholesterol and Haemoglobin

The red blood cell membrane cholesterol (ChoM) assays were performed as described by Macchia *et al.*, 1991. The results appear in table 10. The results appear in table 10. There was a significant decrease in ChoM in DSS animals compared to the DSR strain. Salt loading significantly increased ChoM in both DSS and DSR animals. Although there was this significant increase, the percentage was similar in both cases viz, a 33% in the DSR group and a 32% in the case of the DSS animals.

RBC Haemoglobin (Hb) concentration also appear in table 10. These levels are different from values that appear in table 11 which are from whole blood. The RBC Hb levels in table 10 are from blood that was washed with saline and re-suspended as described by Macchia *et al* for the RBC ChoM analysis. The haematocrit and therefore Hb levels would not correspond with those in table 11.

3.11 Blood Profile

The haematological parameters performed on a Coulter counter appear in table 11. There was a significant increase in white cell count in DSS 1% compared to DSR 1% animals. In the cases of RBC, haemoglobin, haematocrit (HCT), mean cell volume and mean cell haemoglobin concentrations, there was a significant difference in the DSS 1% animals compared to the DSR 1% group.

Although platelet levels were higher in the DSS 1% group compared to DSR 1%, they were not significant.

Due to lack of sample availability these parameters could not be investigated in the salt loaded animals.

TABLE 1

Changes in blood pressure (mm Hg) and heart rate (beats/min) of Dahl Salt Resistant (DSR) and Dahl Salt Sensitive (DSS) rats treated with normal NaCl (1%) and high NaCl (8%) diet.

Group/ Parameter	Control			2 weeks diet			4 weeks diet			6 weeks diet		
	SBP	DBP	HR	SBP	DBP	HR	SBP	DBP	HR	SBP	DBP	HR
DSR Normal NaCl (1%) (10)	128 ± 2.7	80 ± 1.7	459 ± 9.2	138 ± 4.1	88 ± 3.7	462 ± 9.0	128 ± 1.5	84 ± 3.5	474 ± 12.7	134* ± 3.5	78 ± 3.9	440 ± 10.2
DSR High NaCl (8%) (10)	115 ± 2.0	70 ± 3.9	477 ± 8.8	127 ± 4.3	74 ± 4.5	486 ± 11.5	133* ± 5.0	92* ± 7.1	423 ± 12.3	132* ± 5.9	82 ± 4.1	435 ± 7.3
DSS Normal NaCl (1%) (12)	116 ± 1.9	77 ± 1.3	445 ± 18.6	120* ± 2.1	81 ± 2.0	436 ± 14.4	162*+ ± 7.8	106*+± 7.1	496* ± 12.1	150*+ ± 7.3	106*+ ± 7.4	481*+ ± 14.0
DSS High NaCl (8%) (12)	116 ± 2.5	78 ± 1.2	478 ± 19.2	156*+ ± 3.4	93*+ ± 3.3	461 ± 19.8	162*+ ± 7.6	109* ± 8.0	531*+ ± 14.6	174*+ ± 3.3	124*+ ± 7.1	487 ± 18.9

Mean ± SEM

The number in brackets denotes the number of animals

*	Significant compared to control value of the same group
+	Significant compared to the respective DSR group
SBP	Systolic Blood Pressure
DBP	Diastolic Blood Pressure
HR	Heart Rate

TABLE 2

Changes in body weight, heart weight, left ventricle weight and the respective ratios of Dahl Salt Resistant (DSR) and Dahl Salt-Sensitive (DSS) rats on normal (1% NaCl) and high-sodium (8% NaCl) diet

Group/ Parameter	Body Weight (g)	Heart Weight (g)	Left Ventricle weight (g)	Heart Weight/ Body Weight (g/g x 100)	Left Ventricle Weight/ Body Weight (g/g x 100)	Feed Efficiency Ratio
DSR 1% NaCl diet	226 ± 6.0	0.827 ± 0.04	0.462 ± 0.01	0.36 ± 0.01	0.20 ± 0.01	0.189 ± 0.037
DSR 8% NaCl diet	198* ± 4.1	0.692* ± 0.01	0.410 ± 0.01	0.35 ± 0.01	0.21 ± 0.01	0.194 ± 0.042
DSS 1% NaCl diet	259+ ± 3.7	1.156+ ± 0.03	0.688 ± 0.02	0.43+ ± 0.03	0.27+ ± 0.01	0.316 ± 0.019*
DSS 8% NaCl diet	241+* ± 4.2	1.254+* ± 0.03	0.648+* ± 0.02	0.52+* ± 0.03	0.27+ ± 0.01	0.269 ± 0.02*+

Values are presented as Mean ± SEM.
+ Significant compared to the respective DSR group
* Significant compared to the respective group on normal diet

TABLE 3
Antioxidant enzymes' activities in LV heart tissue of Dahl Salt-Resistant (DSR) and Salt-Sensitive (DSS) rats on normal (1%) and high (8%) sodium chloride diet

Group/ Parameter	DSR 1% NaCl	DSR 8% NaCl	DSS 1% NaCl	DSS 8% NaCl
Glutathione peroxidase (nmol NADPH.min^{-1}.mg^{-1} protein)	131 ± 5.6	84* ± 8.6	114+ ± 4.5	93* ± 7.0
Superoxide dismutase (u/mg protein)	12.70 ± 2.8	19.47 ± 2.8	18.67 ± 2.4	22.78 ± 3.2
Glutathione (nmol/mg tissue)	2.91 ± 0.55	3.12 ± 0.42	1.72+ ± 0.14	2.52* ± 0.26

Values are means ± SEM, n = 10
\+ Significant strain difference (DSS vs DSR)
* Significant difference between normal (1%) and high (8%) sodium chloride diet in the same strain
All values are corrected for RBC contamination

TABLE 4

Changes in Body weight, blood pressure and heart rate in Dahl Salt-Resistant (DSR) and Dahl Salt-Sensitive (DSS) rats on normal Na (1%) and high Na (8%) diet for six weeks

	Control				6 weeks diet			
Group/ Parameter	Body weight (g)	Systolic BP (mm Hg)	Diastolic BP (mm Hg)	Heart Rate (beats/min)	Body weight (g)	Systolic BP (mm Hg)	Diastolic BP (mm Hg)	Heart rate (beats/min)
DSR Normal Na (1%)	45 ± 2.7	116 ± 2.3	66 ± 6.8	358 ± 15.0	150 ± 3.5	120 ± 7.9	81 ± 5.6	448* ± 4.4
DSR High Na (8%)	48 ± 2.6	124 ± 4.1	80 ± 2.7	458 ± 14.2	192 ± 4.7	128 ± 2.9	82 ± 2.6	443 ± 10.4
DSS Normal Na (1%)	45 ± 6.4	115 ± 8.3	68 ± 6.7	461 ± 2.5	187 ± 10.6	150*+ ± 7.5	109*+ ± 6.3	520*+ ± 6.3
DSS High Na (8%)	60 ± 8.6	117 ± 4.6	82 ± 6.2	438 ± 18.0	188 ± 7.5	170*+ ± 8.0	115*+ ± 8.0	500*+ ± 11.6

Mean ± SEM

* Significant to the control value

\+ Significant to the value of respective DSR group

TABLE 5
The effect of chemical hypoxia without reoxygenation on the morphology and fluorescence of isolated myocytes of Dahl Salt-Resistant (DSR) and Salt-Sensitive (DSS) rats on normal (1 %) and high (8 %) NaCl diets

	DSR				DSS			
Group/Parameter	Chemical hypoxia	Grade 0	Grade 1	Increased fluorescent Probe	Chemical Hypoxia (min)	Grade 0	Grade 1	Increased Fluorescent Probe
DCDHF Normal (1%) NaCl	10	10	0	10	10	13	0	13
	15	10	1	10	15	11	3	13
	20	5	5	9	20	7	10	10
DCDHF High (8%) NaCl	10	10	0	10	10	12	1	13
	15	10	0	10	15	11	3	13
	20	5	5	9	20	8	7	13
DHR Normal (1%) NaCl	10	10	0	0	10	13	0	0
	15	7	1	1	15	12	1	1
	20	4	5	5	20	7	7	7
DHR High (8%) NaCl	10	11	0	0	10	12	0	0
	15	11	0	0	15	11	1	1
	20	6	5	5	20	8	4	4
FLUO Normal (1%) NaCl	10	10	0	0	10	13	1	0
	15	8	1	1	15	11	2	2
	20	4	5	6	20	6	7	7
FLUO High (8 %) NaCl	10	10	0	0	10	11	0	0
	15	8	2	2	15	9	2	2
	20	5	5	5	20	7	5	6

Results are expressed as percentage of the total number of cells evaluated
n = 90 cells (15 cells/group) were evaluated from six separate myocyte preparations made on different days
Grade 0: % viable, rod-shaped myocytes
Grade 1: % viable, contracted cells with slight myofibrilar disarray and occasional membrane blebs

TABLE 6
Antioxidant enzymes' activities and glutathione in red blood cells of Dahl Salt-Resistant (DSR) and Salt-Sensitive (DSS) rats on normal (1 %) and high (8 %) sodium chloride diet

Group/Parameter	DSR 1% NaCl	DSR 8 % NaCl	DSS 1% NaCl	DSS 8 % NaCl
Glutathione peroxidase (nmol NADPH.min^{-1}.mg^{-1} Hb)	89.0 ± 6.03	94.0 ± 10.2	54.0+ ± 5.11	95* ± 5.05
Superoxide dismutase (u/mg Hb)	12.74 ± 0.42	-	9.42+ ± 0.58	-
Glutathione (nmol/mg RBC)	2.87 ± 0.46	2.70 ± 0.20	1 .59+ ± 0.19	1.28+ ± 0.19
Serum catalase (ku/l)	89.18 ± 16.00	-	224.88+ ± 33.99	-

Values are Means ± SEM, n = 10
+ Significant strain difference (DSS vs DSR)
* Significant difference between normal (1%) and high (8%) sodium chloride diet in the same strain
Note: The estimation of GSH-Px was performed by the method of Paglia and Valentine, and that of SOD by the method of Winterbourn et al.

TABLE 7

Blood Glucose and Antioxidant enzyme activity in red blood cells of Dahl Salt-Resistant (DSR) and Dahl Salt-Sensitive (DSS) Rats, on normal (1 %) and high (8 %) diet for six weeks estimated by Randox antioxidant kits

Group/Parameter	DSR-Weanling	DSS-Weanling	DSR on normal diet	DSS on normal diet	DSS-Sodium loaded
Blood glucose (mmol/l)	41 ± 0.05	3.9 ± 0.10	5.0 ± 0.28	6.8 ± 0.20+	6.2 ± 0.22*
Gluthathione peroxidase (units/ml)	100.17 ± 2.50	90.66 ± 2.98+	103.03 ± 2.30	82.47 ± 1.67+	85.79 ± 7.9
Superoxide dismutase (units/ml)	63.73 ± 1.53	52.70 ± 3.21+	278.07 ± 20.54	219.40 ± 15.25+	199.73 ± 7.75
SOD: GPx	0.63	0.58	2.70	2.68	2.32

Values are Mean ± SEM, n = 6

+ Significant strain difference (DSS vs DSR)

* Significant difference between normal (1 % NaCl) and high (8 % NaCl) in the sodium chloride diet

TABLE 8
Oxidative challenge test of red blood cells (Malondialdehyde, nmol/g RBC) of Dahl Salt-Resistant (DSR) and Dahl Salt-Sensitive (DSS) rats, after exposure to different concentrations of H_2O_2

Dahl Salt-Resistant Rats					Dahl Salt-Sensitive Rats				
H_2O_2 Concentrations (%)					H_2O_2 Concentrations (%)				
0	2.5	5	10	20	0	2.5	5	10	20
20.20 ± 1.08	124.49 ± 6.85	148.36 ± 4.27	158.24 ± 3.48	171.30 ± 5.19	18.71 ± 0.98	128.26 ± 5.67	157.30 ± 5.84	172.24 ± 3.28*	188.88 ± 4.09*

Mean ± SEM
*Significant compared to the respective concentration of the control DSR rats

TABLE 9
Plasma lipid profile of Dahl Salt-Resistant (DSR) and Dahl Salt-Sensitive (DSS) rats on normal NaCl (1%) and high NaCl (8%) diets for six weeks

Group/Parameter	Total cholesterol (mmol/l)	HDL (mmol/l)	LDL (mmol/l)	Triglycerides (mmol/l)
DSR 1 % NaCl	1.57 ± 0.09	0.92 ± 0.04	0.45 ± 0.07	0.44 ± 0.05
DSR 8 % NaCl	1.30 ± 0.09	0.81 ± 0.04	0.28 ± 0.04*	0.49 ± 0.16
DSS 1 % NaCl	3.27 ± 0.09+	1.76 ± 0.10+	0.88 ± 0.13+	1.45 ± 0.33+
DSS 8 % NaCl	2.34 ± 0.26*	1.25 ± 0.31	0.97 ± 0.14	0.64 ± 0.13*

Mean ± SEM
+ Significant strain difference (DSS vs DSR)
* Significant difference between normal (1%) and high (8%) NaCl in the same strain

TABLE 10
Red blood cells membrane cholesterol of Dahl Salt-Resistant and Dahl Salt-Sensitive rats on normal (1 %) and high (8 %) NaCl diet for six weeks

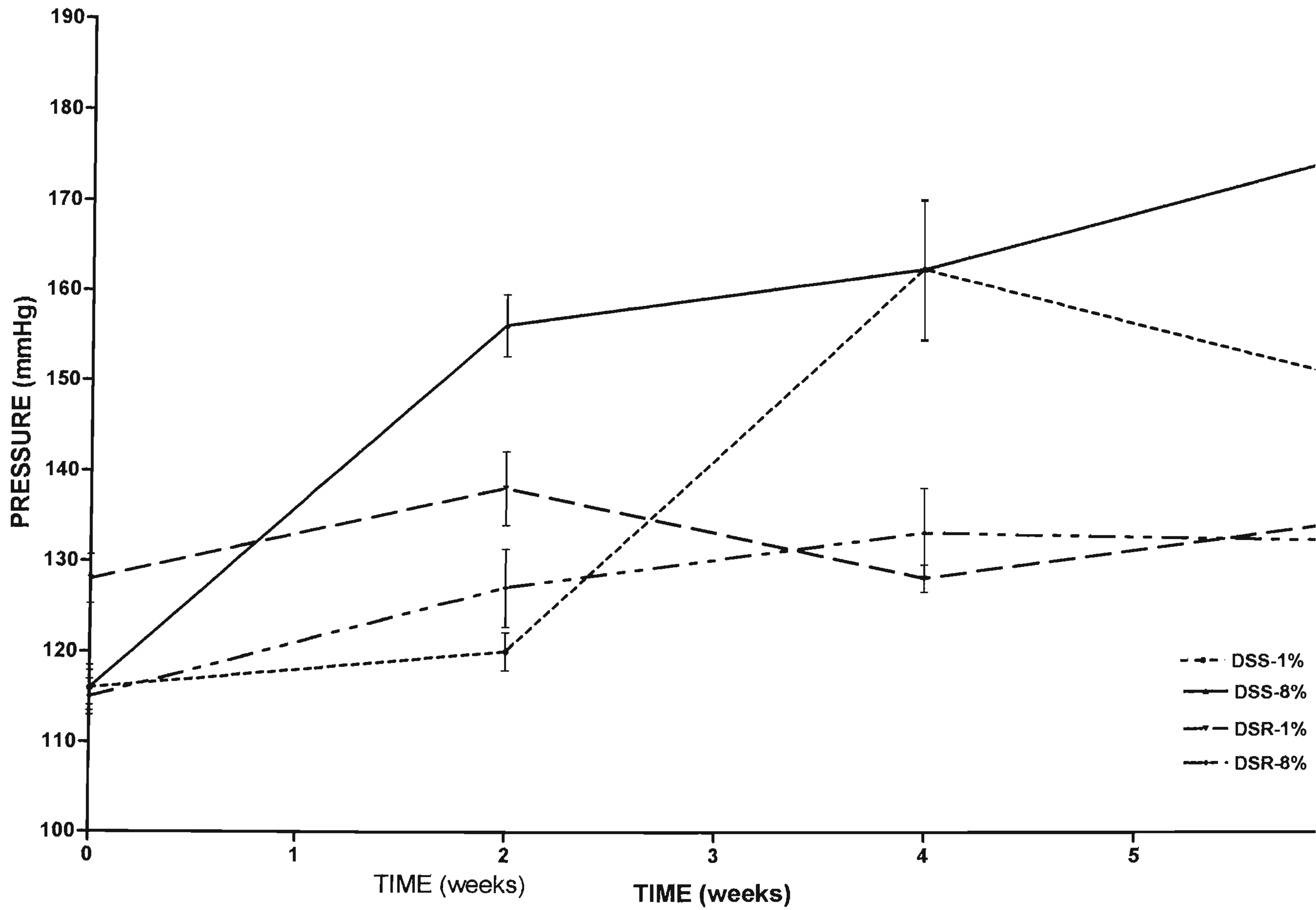
Group/ Parameter	DSR 1 % NaCl	DSR 8 % NaCl	DSS 1 % NaCl	DSS 8 % NaCl
RBC Haemoglobin (mg/dl)	0. 289 ± 0.019	0. 185 ± 0.008*	0. 272 ± 0.010	0. 234 ± 0.009*
RBC membrane cholesterol (mmol/ mg Hb)	19.50 ± 1.61	26.03 ± 1.85*	14.66 ± 1.81+	19.37 ± 1.50*
In mmol/l	6.75	14.01	5.39	8.28

Mean ± SEM
+ Significant strain difference (DSS vs DSR)
* Significant difference between normal (1 %) and high (8 %) NaCl in the same strain

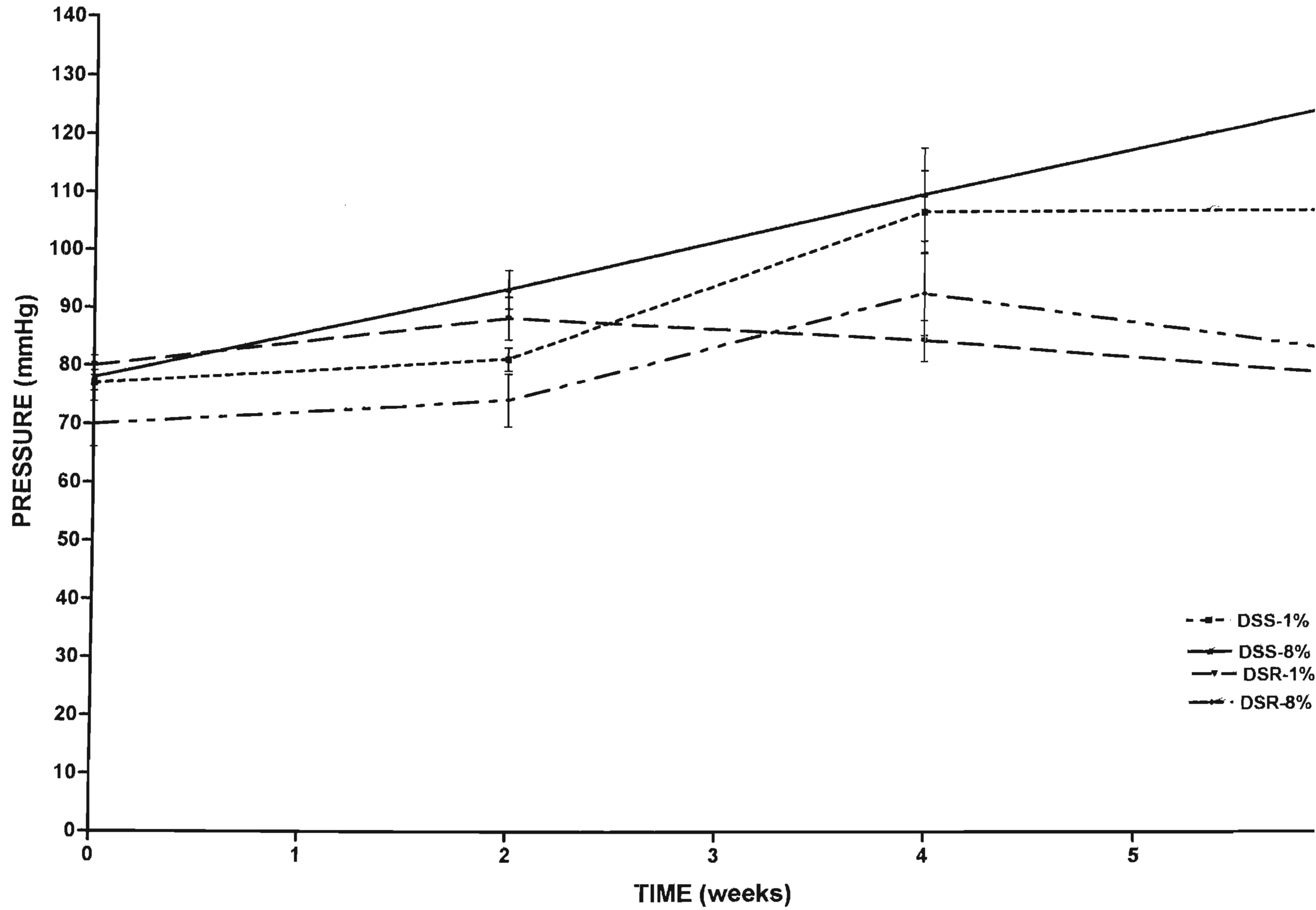
TABLE 11
Haematological Parameters of Dahl Salt Resistant (DSR) and Dahl Salt Sensitive (DSS) Rats on Normal Diets

Group/Parameter	White blood cells $(x\ 10^{3}/_{1})$	Red blood cells $(x\ 10^{12}/_{1})$	Haemoglobin (g/dl)	HCT (%)	MCV fl	MCHC g/dl	Platelets $(x\ 10_{-3})$
DSR on 1 % Na diet	2.90 ± 0.22	7.62 ± 0.16	12.8 ± 0.04	44.6 ± 0.52	69 ± 0.87	33 ± 0.20	710 ± 29
DSS on 1 % Na diet	3.90* ± 0.25	7.10* ± 0.10	12.2* ± 0.05	42.4* ± 1.0	63* ± 0.96	29* ± 0.10	735 ± 27

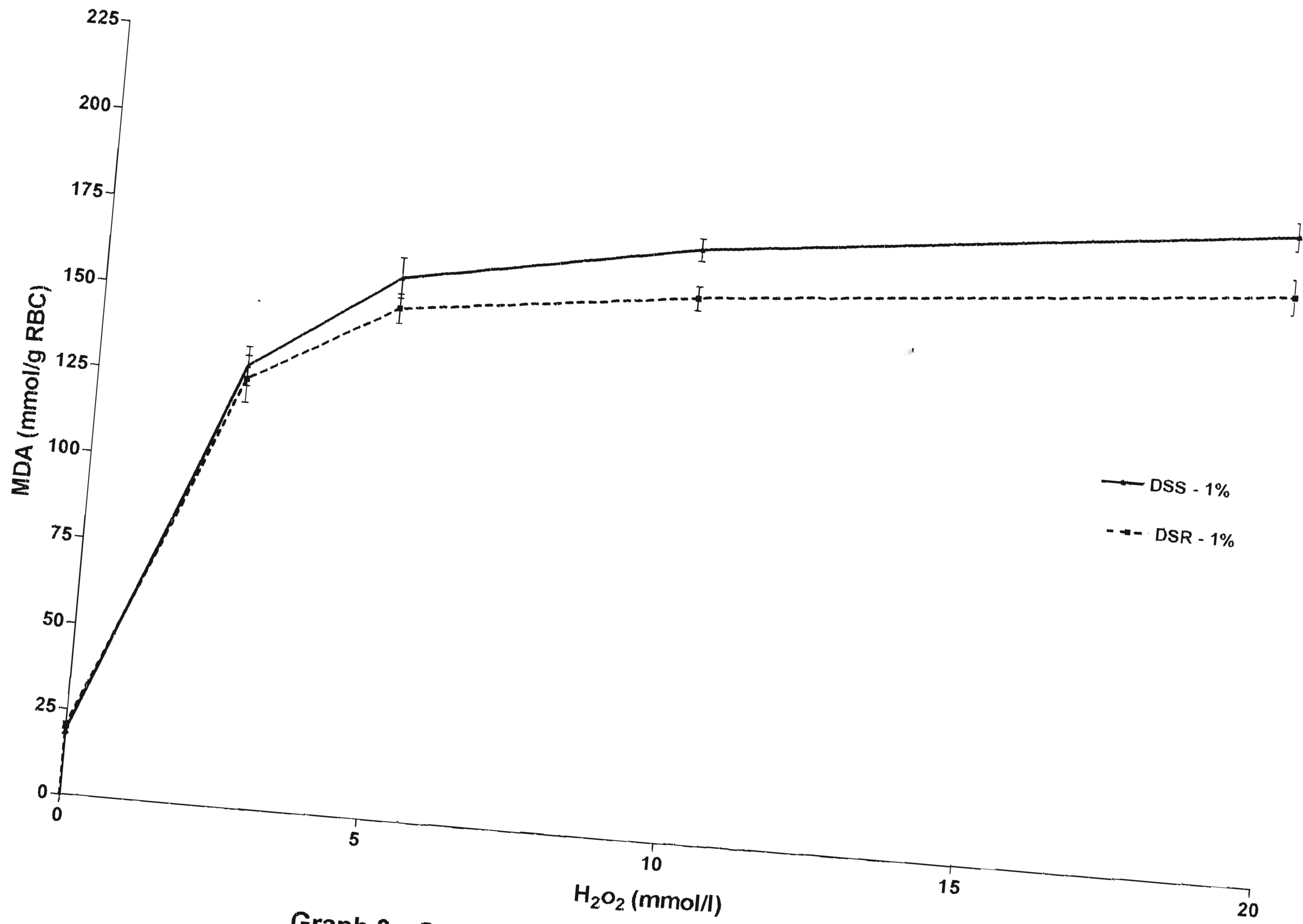
Mean ± SEM
* Significant compared to the value of the respective DSR group



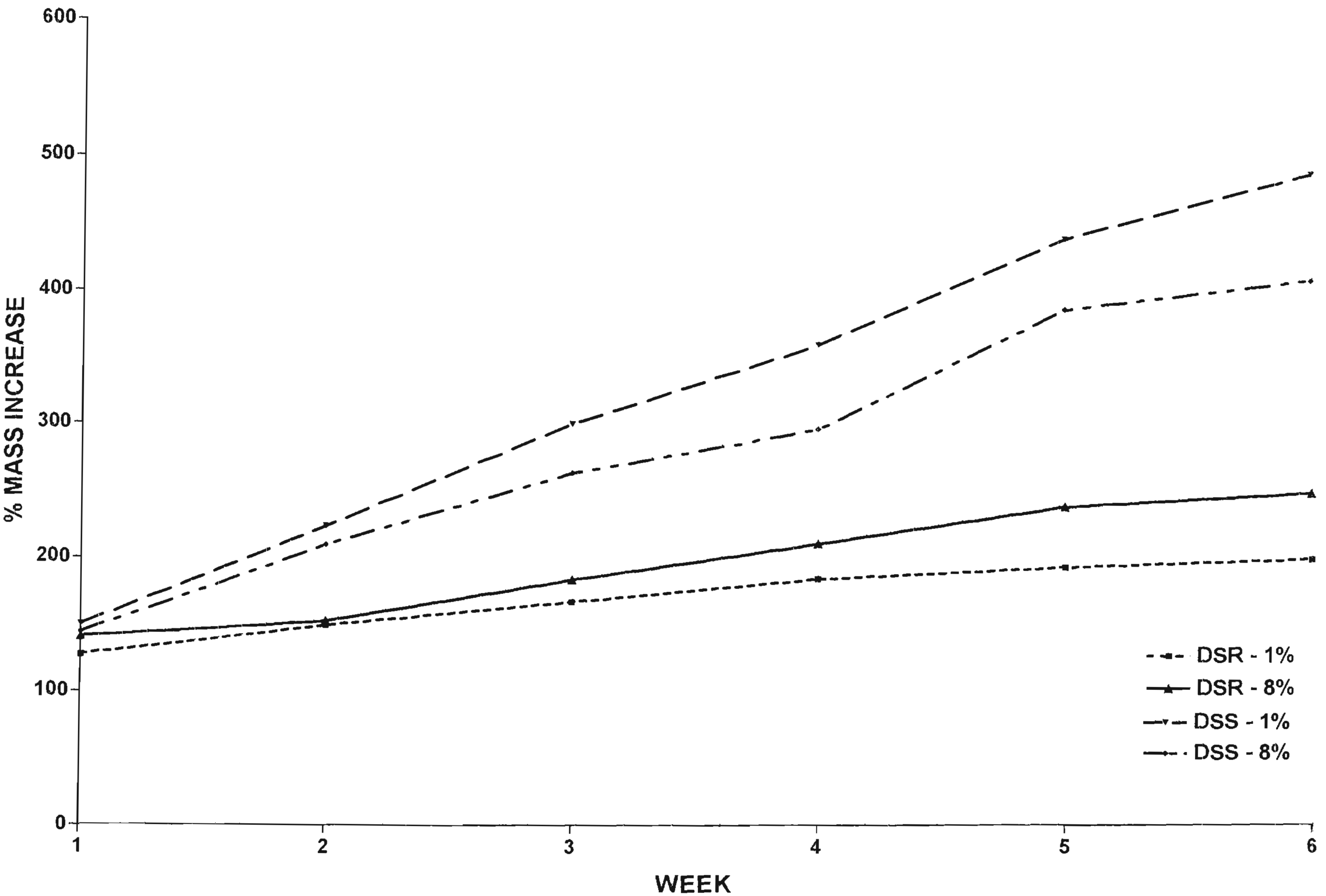
Graph 1 : Systolic blood pressures of DSR and DSS rats



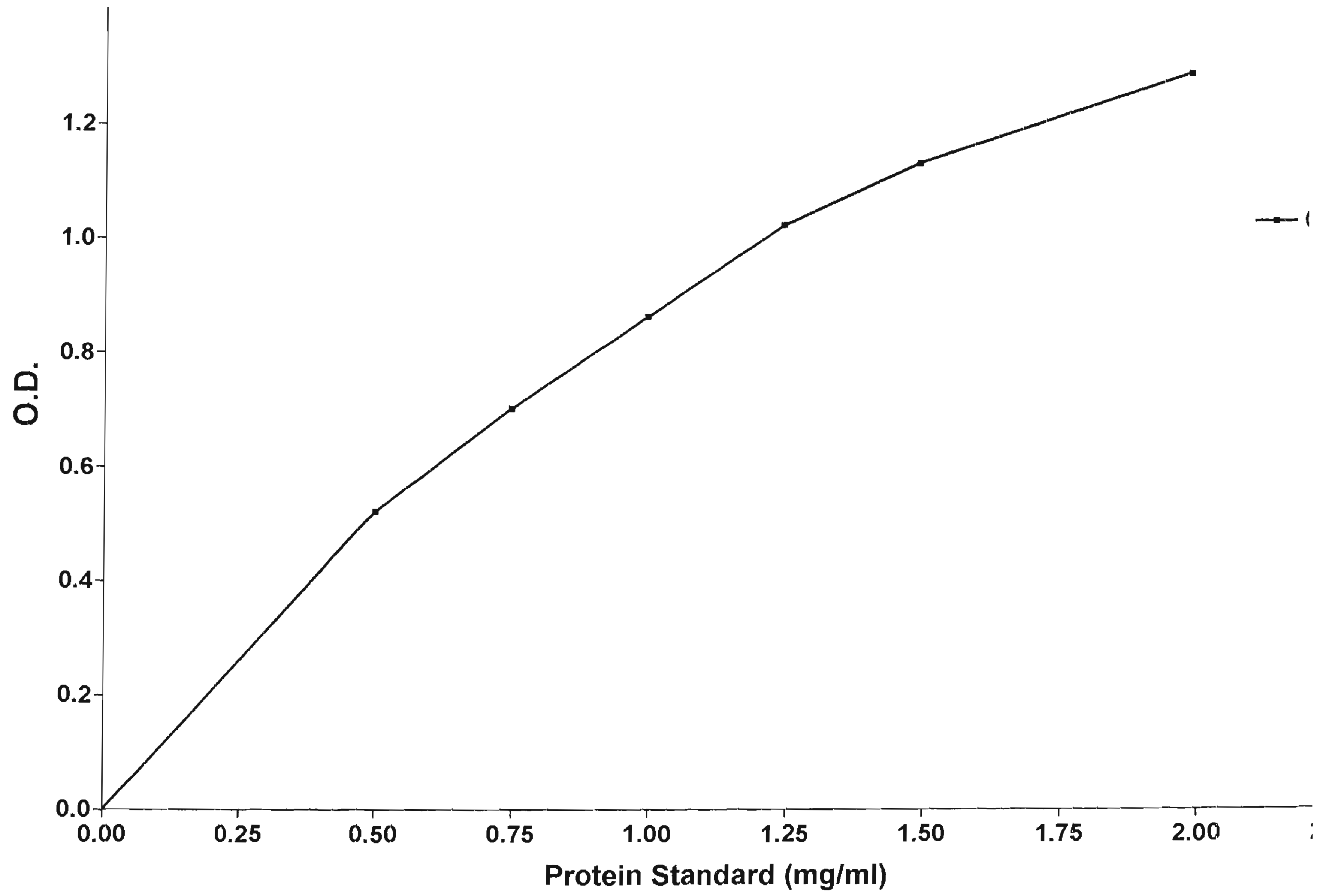
Graph 2 : Diastolic blood pressures of DSS and DSR rats



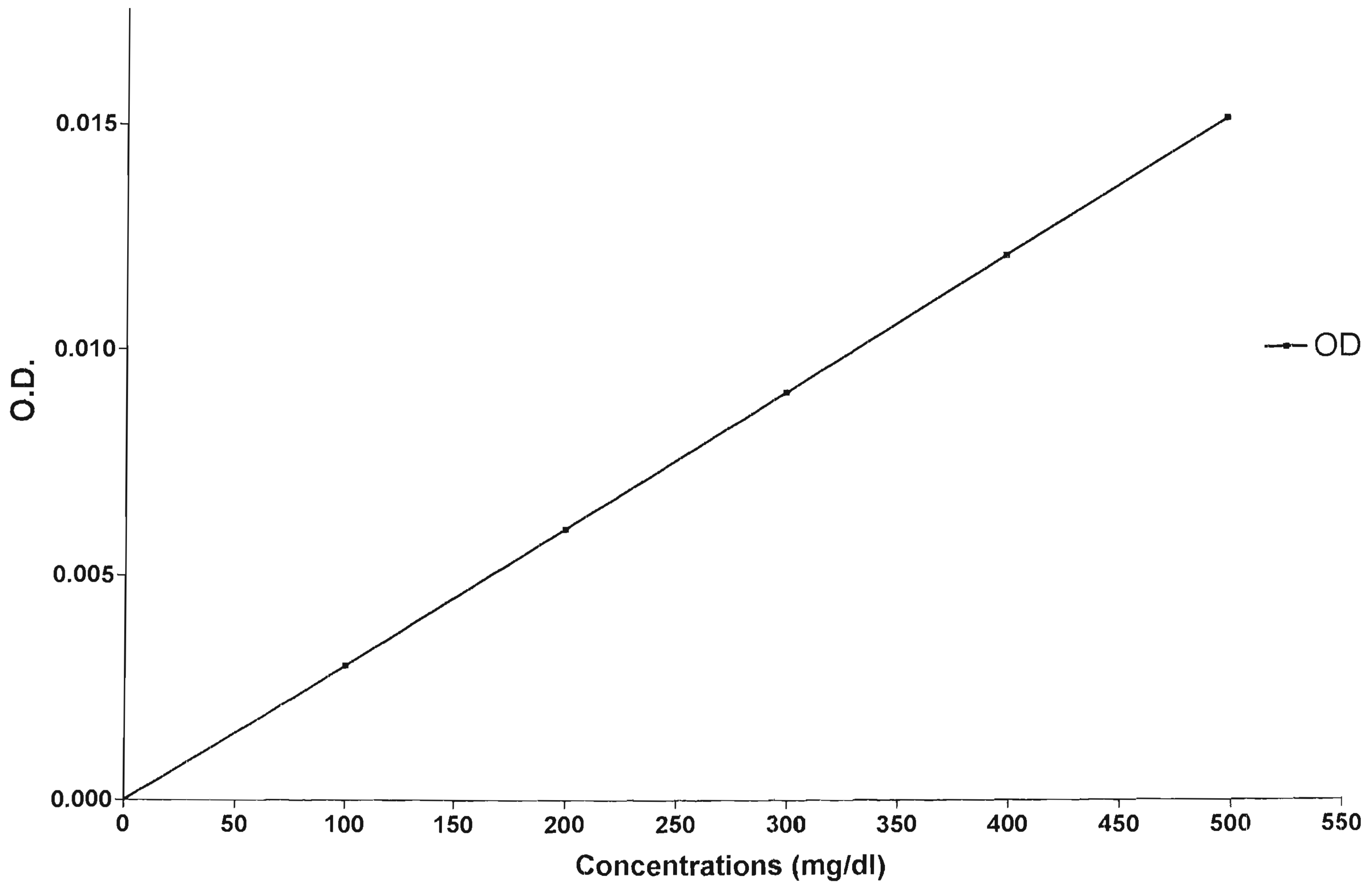
Graph 3 : Oxidative Challenge of RBC to H_2O_2



Graph 4 : Percentage increase in mass/week



Graph 5 : Protein BSA Standard Curve (Bradford)



Graph 6 : Haemoglobin Cyanometh Standard curve

4.0 DISCUSSION

This study has demonstrated the following in Dahl salt sensitive rats:

1. There is a compromised antioxidant status in the RBC, serum and heart as demonstrated by decreased levels of GPx, GSH, SOD and CAT levels.
2. The compromised antioxidant status was also coupled with elevated levels of serum CAT, dyslipidemia and hyperglycemia.
3. The compromised antioxidant status precedes the elevation in blood pressure indicating that the increase in free radicals do not occur as a consequence of the hypertension, as is currently being debated in the literature.
4. The coupling of the compromised antioxidant status with other risk factors highlights the multifactorial origins of hypertension.
5. The Dahl rat is a suitable model for the investigation of the etiological role of free radicals in hypertension.

The status of the antioxidant system that formed a pivotal part of this study is dependant largely on a balanced diet since many of the antioxidants are of dietary origin. Therefore in order to exclude this as a variable, comments on the diet are necessary. The AIN 76 diet has been shown to be a well balanced, isocaloretic diet for rodents since growth curves, reproductory rates and lactation are optimal (AIN,76). In particular the levels of trace elements such as selenium and vitamins A, C and E are adequate especially vitamin E levels which has been subsequently increased as a recommendation by the American Institute of Nutrition (AIN,1980). To

ensure that per-oxidation of fats and loss of vitamin activity was minimized, the diets were prepared in small batches (~2 kg) and stored in light resistant containers at 4°C.

Salt loading was achieved by supplementing the diet with sodium chloride and 8% was chosen as the high level since it is reported in the literature that sodium chloride at between 0.5 and 1.5 percent is considered normal whereas levels above 4% were considered to be high (Tobian, 1997).

The body weight of the control animals (DSR) on normal diet (1% NaCl) compared favorably with reports in the literature (Doi, *et al.*, 2000). Salt loading of DSR animals however resulted in a significant decrease in body weight compared to their respective controls (Table 2). Although the percentage increase in body mass during the experimental period showed a significant difference between the DSR 1% and DSR 8% respectively, there was no significant difference in the feed efficiency ratio (Table 3). This could be attributed to the fact that the average mass of the DSR, 1% group at week 0 (when study commenced) was significantly higher than the DSR, 8% group. It is well documented that rats experienced a growth spurt between 60 to 80 grams. This indicates that the feed efficiency ratio is a more accurate means of expressing this data since growth rate and food intake are related in normal physiological states.

The results in the DSS groups were however different. There was a significant decrease in the percentage growth in DSS, 8% group compared to the DSS, 1%

group, this is also evident in the feed efficiency ratio (Table 2). This indicates that salt loading resulted in a decrease in food intake and hence a reduced percentage increase in mass. This trend was apparent from the first week of the experiment and persisted until the sixth week.

The higher percentage growth and feed efficiency ratios in DSS groups compared with their respective controls indicates a possible lower metabolic rate in the DSS groups. It has also been hypothesized that antioxidant enzymes could work in concert to alter the metabolic activities of tissues (Yuan, *et al.*, 1998). The altered antioxidant status of both RBC and heart in DSS animals (discussed later) could therefore in some way alter the metabolic activity of this strain and therefore explain the increase in the feed efficiency ratio and the higher growth rates. The increase in mass can also be partly attributed to the expansion of the fluid volume in DSS rats, this however would only account for a few percentage points increase. During the dissections however, there were no visible signs of an increase in body fat.

4.1 Blood Pressure

A similar trend was observed in diastolic pressures as well, that is, DSS animals display higher diastolic pressures than DSR animals and salt loading significantly increased the diastolic pressure in the DSS animals whilst having no significant effect on the DSR group (Table 1, graph 2). These changes in blood pressures compares favourably with other studies in the literature and previous results of our laboratory (Yuan, *et al.*, 1995; Uehara *et al.*, 1992; Somova, *et al.*, 2003).

Although structural changes to the vascular apparatus is the hallmark of hypertension, it is debatable whether the initial increase in blood pressure as observed in this study can be attributed to significant structural changes. Many studies have shown that expression of integrins ($_v\beta_3$ and $_s\beta_1$) and collagen only commences at between six to twenty weeks of age in spontaneously hypertensive rats. This implies that major remodeling only commences after this period (Intengan, *et al.*, 2000).

Moreso, Simon (1998), has shown that there is a clear dissociation between blood pressure and wall: lumen ratio in the early stages of hypertension in rats. The role played by increased sympathetic tone, trophic and pressor stimuli are therefore highlighted in this period which is increasingly being referred to as an important "developmental window."

The resetting of the pressure natriuresis curve is regarded as one of the most significant primary factors that lead to hypertension in salt sensitive humans and experiment models such as the Dahl rat (Rapp, 1982). This results in salt retention with a corresponding expansion of the fluid volume resulting in an elevation in blood pressure. An increase in sympathetic activity, as evident by the increase in heart rate in hypertensive DSS animals, is also a significant factor that has been implicated in increasing arterial blood pressure (Grassi, *et al.*, 1998). In addition salt loading has been shown to eliminate nocturnal fall in blood pressure. (Okuguchi, *et al.*, 1999). The blood pressures of animals in the present study was done in the mornings so this pattern could not be verified in this model of salt sensitivity. This factor however

should not be excluded as a possible cause of the pathological changes that were observed (refer to paper in appendix).

An interesting pattern has however emerged in this study in which the increase in systolic blood pressure precedes the increase in heart rate (Table 1). This suggests that factors other than an increase in sympathetic activity could be involved in the early hypertensive process. The young DSS animals have been shown to be subjected to an increased oxidative stress (discussed later). Free radicals, especially $O_2^{\cdot}$ and H_2O_2 have been implicated in altering blood pressures in rats and humans by abolishing the NO dependant vasodialatory mechanism discussed in 4.3.1.

4.2 Free Radical and Hypertension

4.2.1 Weanling: Do free radical precede hypertension?

One of the significant stimuli that have been identified to play a key role in the "developmental window" are free radicals such as the superoxide radical. Nakazono, (1991) has even suggested that hypertension in spontaneously hypertensive rats be classified as a "free radical disease." The results of superoxide dismutase levels in weanling rats appear in Table 7. SOD levels in DSS weanlings are significantly lower than the DSR group. These rats were fed a standard pellet chow and were therefore not salt loaded. Despite a lack of salt loading these weanlings showed a decrease in

SOD levels in the RBC. This indicates that there is either an increase in free radical production, (specifically O_2^-) or a compromised SOD status in DSS weanling rats which were not even salt loaded. Measurement of $O_2^{-\cdot}$ was beyond the scope of this study for many reasons, however since the DSR animals are regarded as the appropriate controls, it can be assumed that the SOD status in Dahl rats is not compromised, but rather this observation is due to an increase in $O_2^{-\cdot}$ production in DSS weanlings. The quenching of increasing $O_2^{-\cdot}$ levels would therefore result in diminished SOD levels (Freeman, *et al.*, 1982). It must be emphasised however that this argument is restricted to the red blood cells. Since the antioxidant status varies in different tissue types. The RBC is, according to the literature, a good reflection of the antioxidant status of the body nevertheless (Yuan, *et al.*, 1995).

Elevated levels of free radicals have been reported in experimental hypertension (Yuan, *et al.*, 1995; Pezeshk, *et al.*, 2000). The superoxide radical in particular has been strongly linked to hypertension and diabetes. Since O_2^- had been shown to react spontaneously with NO to form the peroxynitrite. This abolishes the constant NO dependant vasodilation that is present in resistance vessels effectively resulting in an increase in arterial pressure. That this mechanism is significant in regulating arterial blood pressure has been demonstrated in studies in which administration of antioxidants have shown to decrease blood pressure significantly (Ceriello, *et al.*,1991).

The formation of peroxynitrite in significant quantities poses a serious threat due to its cytotoxic properties as well as explained in detail later. Peroxynitrite has been

shown to have a diverse range of actions ranging from its action on DNA, lipid and protein molecules. The reaction with protein is potentially deleterious due to the coupling with tyrosine residues to form the tyrosyl radicals which in itself has adverse biological effects (Vaziri, *et al.*, 2000).

Another factor that has been highlighted in weanling DSS rats is that they have been shown to have hyperinsulinemia, a state that is responsible for an increase in sympathetic drive (Channa, *et al.*, 2003). This would have a twofold effect in young DSS rats that have not developed full blown hypertension. Firstly, an increase in sympathetic activity would result in an increased neurogenic component to vascular resistance and hence an elevation in blood pressure. This mechanism has been shown to play a significant role in altering the blood pressure since blocking of β_1 adrenergic receptors and neonatal sympathectomy attenuated the development of hypertension in young Dahl sensitive rats (Zicha, *et al.*,1999). Secondly, sustained high levels of catecholamines yield free radicals that play a pivotal role in the etiology of hypertension (Singal *et al.*, 1983).

An interesting pattern that has been observed in many studies is that young salt sensitive rats, as opposed to adults, display an expansion of the plasma and total body fluid volume and that this is exacerbated with salt loading. This is therefore perhaps another mechanism that contributes to the increase in blood pressure before maturity which has been attributed to the fact that the immature kidney is damaged significantly by various factors that come to the fore during the early hypertensive process (Zicha *et al.*, 1999).

Evidence is therefore very strong that the initial increase in blood pressure in DSS rats as observed by the second and third week and salt sensitive individuals is biochemically mediated and that it precedes the structural changes of the vascular apparatus. Therefore an imbalance between pro and antioxidants could play a significant role early in the hypertensive process in this model of hypertension and salt sensitive individuals.

Although there has been some debate as to when structural changes to the vascular apparatus commence, most studies agree that these changes are evident at an age of about ten weeks (Simon, *et al.*, 1998). This corresponds to the fourth week of this experiment during which average systolic and diastolic pressures of DSS rats (1% and 8%) were significantly higher than their respective controls DSR 1% and 8% respectively (Graphs 1 and 2). Salt loading of DSS animals exacerbates the process to such an extent, that animals on a high salt diet develop elevated systolic blood pressures by the third week that are similar in magnitude to that of DSS rats on a 1% diet for six weeks (extrapolated from Graph 1).

There is sufficient evidence in the literature indicating that the hypertension that coincides with this period (roughly age ten week) is due largely to changes in the property of the arterial walls. It is perhaps the most important change amongst a series of changes that ensure that the hypertension is "self sustaining" (Simon, 1998).

The changes to the vasculature in salt sensitive rats which are both dose and time dependant is first evident in the small resistance arterioles, marked by an increased wall/lumen ratio. This occurs largely as a result of remodeling of the vasculature during which there is either an increase in vessel wall area due to hypertrophy or hyperplasia of the vascular wall. Alternatively there could be a decrease in lumen diameter due to restructuring of the vessel wall. (Simon,1998). This type of change to resistance vessels is called hypertrophic remodeling and is characteristic of Dahl salt sensitive hypertension and in human renovascular hypertension (Intengan, *et al.*, 2000).

Studies have also shown that salt loading of young DSS rats result in a decrease in arterial compliance. This effectively dampens pulse pressure which further elevates systolic blood pressure (Zicha, *et al.*,1999).

It is therefore evident from the data of this study and others in the literature that by increasing the salt intake in young salt sensitive rats, permanent changes in blood pressure can be induced, this has prompted Simon (1998) **to suggest a new definition of salt sensitivity namely, rats that respond to salt loading by an increase in wall/lumen ratio of the small resistance vessels as opposed to those that do not, should be termed salt sensitive**. (Simon *et al.*, 1998).

An independent factor that could play a significant role in ensuring that the hypertension, irrespective of its aetiology, progresses to a chronic state, is the mechanical strain induced by elevated blood pressure *per se* on the vascular smooth

muscle (USM) layer. It has recently been shown that VSM cells express a growth factor, TGF β_1 m RNA in response to increasing mechanical strain. This results in an increase matrix synthesis and deposition which would render vessels less compliant.

Levels of TGF β_1 m RNA have also been shown to be elevated in hypertensive rats and humans. This qualitative change to the vascular composition in response to hypertension could therefore initiate a vicious cycle in which vessel compliance could progressively decrease. This independent variable could work in concert with other factors to accelerate and sustain the hypertension (O' Callaghan, *et al.*, 2000).

4.2.2 Free Radicals and Hypertension in Adult Animals

It is therefore apparent from the foregoing discussion that many factors have been identified in the pathogenesis of hypertension and that these factors often work in concert or synergistically to produce the hypertension. Antioxidants have nevertheless been shown to have a significant anti-hypertensive effect thus implicating free radicals as potential hypertensive agents (Somova, *et al.*, 2003; Simon, 1998).

Of the antioxidant assays performed in this study, SOD levels in RBC, is perhaps the "compartment" that would exert the greatest effect on the vascular apparatus. SOD levels of the RBC of DSS rats were significantly lower than DSR controls in both weanling (discussed previously) and adults. This could perhaps indicate that salt

sensitive rats are genetically predisposed to a compromised SOD status or produce a larger quantity of free radicals (specifically O_2^-). Many studies have however reported an increase in production of free radicals in experimental and essential hypertension (Russo *et al.*, 1988, Grunfeld, *et al.*, 1995).

Using adult rats, Grunfeld *et al.*, have shown that O_2^- levels are significantly higher in the aortas of these rats compared to Wistar controls. This could explain the lower SOD status in the RBC adult DSS rats since SOD levels would decrease stoichiometrically as a result of the quenching action (Grunfeld *et al.*,1995). The reaction of O_2^- and NO to form the peroxynitrate is very rapid and results in the attenuation of the NO dependent vasodilatory effect thus effectively increasing blood pressure (Nakazono *et al.*, 1991). Intravenous administration of SOD, Hb, an artificially synthesized form of SOD, decreases the blood pressure of SHR but not of Wistar controls indicating that this mechanism could play a significant role in salt sensitive rats as well (Nakazono, et al., 1991). It thus appears that an increase in O_2^- levels in the vicinity of the vascular apparatus could play a significant role in hypertension in DSS rats. Salt loading however did not alter the status of SOD levels in the RBC yet blood pressures increased greatly by salt loading (Table 1, Graph 1 and 2). This clearly highlights the fact that whilst this mechanism could contribute to the hypertensive process, there are other mechanisms working in parallel with it that perhaps become more prominent during salt loading. This of course does not exclude the fact that additional independent mechanisms come to the fore during salt loading.

From the previous discussion of hypertension in weanling rats and as is the case in essential hypertension, it is apparent that the hypertensive process commences early in life, but the circumstances in adulthood that affect this process is different from that in the young (Simon 1998, Intengan, *et al.*, 2000). The elevation in blood pressure *per se* has also been identified as a stimulus to excascerbate the process since the shear forces imposed by the elevated pressure induce changes in the vascular wall (Simon, 1998).

The major factors that come to the fore in adulthood which ensure that the hypertension remains chronic syndrome are:

4.2.3 Changes in the Vasculature

A. Structural Change

In SHR changes to the vasculature are first evident after age ten weeks and in humans between the second and third decade of life (Zicha, *et al.*, 1999). These changes to the vasculature are regarded as the hallmark of hypertension basically involved an increase in wall to lumen ratio of the major resistance vessels (Simon, 1998). This is achieved by remodeling of the vessels, the nature and development of which has been studied extensively (Simon, 1998, Intengan, *et al.*, 2000, O'Callaghan, *et al.*, 2000). Various factors have been implicated in inducing vascular changes, they are therefore beyond the scope of this discussion. Two factors

however are worthy of mention here. Firstly, Simon (1998) has demonstrated that salt fed rats displayed a trend in the resistance vessels in which there was an increase in wall to lumen. They have even suggested a new definition of salt sensitivity as well, as a result of their observations, that is, rats that display an increase in wall to lumen of resistance vessels after salt supplementation should be termed salt sensitive as opposed to those that do not. (Simon,1998). This is perhaps one of the many reasons as to why salt loading excascerbates the hypertension in salt sensitive individuals and in the Dahl rat, which is considered an excellent model for studying salt sensitivity and hypertension (Somova, *et al.*, 2001). Although no morphological work was performed on any of the resistance vessels of animals in this study, analysis of the myocardium has revealed significant changes to the ultra-structure that is in agreement with changes associated with hypertension in general.

Secondly it has been hypothesised by many researchers in this field of study that chronic hypertension in adulthood develops as a result of prolonged exposure to a pressor stimulus of sub-minimal intensity (Simon, 1998). Free radicals have been identified as one of these stimuli that could be directly involved in inducing structural changes to the vascular apparatus (Maxwell, 2000, Hayakawa *et al.*, 1999). In this study the antioxidant status of the RBC of adult animals was determined and appear in tables 6 and 7. As mentioned previously, the results of table 6 were obtained using accepted chemical assays whereas the data in table 7 were obtained using commercially available kits. The discussion will be restricted to table 7. The antioxidant status of the RBC is an accepted indication of the balance between pro-antioxidants in the vascular apparatus (Yuan, *et al.*, 1996, Winterbourne, *et al.*,

1974). There was a significant drop in both GPx and SOD levels in DSS animals compared to their normotensive counterparts. Salt loading had no significant effect on the antioxidant levels. Although the units used to express the results were as recommended by Randox, the results (after conversion) compared to the values obtained by Yuan *et al* using SHR. The SOD values were however 40 to 50 % lower in this study but the trends were similar when comparing hypertensive to normotensive animals (Yuan *et al.*, 1998). The significant drop in SOD in hypertensive rats has been postulated to be due to an increase in production of O_2^- rather than a decrease in expression of SOD (Pogan, *et al.*, 2000, Grunfeld, *et al.*, 1995). *In vitro* studies have demonstrated that cultured endothelial cells and aortic rings from SHR produce significantly larger concentrations of O_2^- than cells of normotensive controls. In addition, immunohistochemical studies have shown that the endothelial cells of resistance vessels in SHR have much higher levels of xanthine oxidase than larger vessels (Nakazono *et al.*, 1991). Since this enzyme has been identified as a significant source of O_2^- *in vivo*, once generated by the endothelial cells, this radical could then make its way into the lumen of vessel thereby being quenched by SOD even within the RBC.

Further evidence strengthening the aetiological role of free radicals in vascular remodeling has been demonstrated in DSS rats that were administered with indapamide, a diuretic with known antioxidant action *in vivo* (Uehara *et al.*, 1992). Despite salt loading the hypertension was ameliorated and vascular remodeling failed to occur as long as the animals were kept on the drug. In addition the organ damage (mainly kidneys) that is associated with hypertension and has been thought

to develop as a result of the action of free radicals failed to develop. These observations therefore strongly suggest that free radicals are directly involved in alterations of the cardiovascular system and play a significant role in organ damage (Uehara *et al.*, 1992).

The O_2^- radical has been shown to react spontaneously and rapidly with NO forming peroxynitrite (Galley, *et al.*, 1996; Rossi, *et al.*, 2001). This action effectively scavenges the NO resulting in lower levels of NO despite normal levels of synthesis. Prolonged blocking (~3 weeks) of NO synthesis has resulted in remodeling of the vasculature largely by altering the endothelium and increasing the thickness of the medial layer in rats. This effectively alter the wall to lumen ratio and hence increases the blood pressure, a pattern that is synonymous with experimental (including salt sensitive) and essential hypertension (Rossi, *et al.*, 2001).

The compromised SOD status of DSS animals in this study, therefore strongly suggests that there is an increase in $O_2^{\bullet}$ production which could play a role in vascular remodeling. This could in turn have an independent pressor response in this model of hypertension.

B. Alteration in Arteriolar Tone

Despite remodeling of the vasculature in hypertension (salt sensitive and essential), both humans and animals respond to antihypertensive therapy. Response times to antihypertensive therapy are however, slower after vascular modeling has occurred.

This implies that there are other significant mechanisms that work in concert with structural changes to elevate blood pressure. Of the many mechanisms identified, arteriolar tone, is thought to be significant. An increase in arteriolar tone effectively decreases vessel lumen diameter increasing the peripheral resistance, which effectively increases blood pressure.

Arteriolar tone is generally controlled neurohumorally in normotensive individuals. Two mechanisms, linking free radicals to an alteration in arteriolar tone have been described. The first mechanism involves H_2O_2, proposed by Swei *et al.*, in which a correlation between arteriolar tone and plasma H_2O_2 was demonstrated in Dahl rats. This has been attributed to the direct action of H_2O_2 on the vascular smooth muscle (Swei, *et al.*, 1997). In addition a significant observation in salt sensitive rats has been an increase in H_2O_2 production during salt loading (Lacy *et al.*, 1998). Plasma H_2O_2 is derived from many sources, the major mechanisms being the dismutation of O_2^- by SOD and from the action of xanthine oxidase both in plasma and endothelium. In the present study there was a significant decrease in SOD levels in DSS compared to DSR animals indicating that this enzyme system was being stressed/ overwhelmed to a greater degree by elevated levels of O_2^- which would in turn produce increasing levels of H_2O_2. Elevated levels of H_2O_2 have been reported by other studies on hypertension as mentioned above. Although a direct effect of H_2O_2 on arteriolar tone has been demonstrated by Swei (1997), suggesting that this radical could contribute directly to the elevation in blood pressure it must be borne in mind that H_2O_2 is highly diffusible and has reducing powers as well, thus contributing to oxidative stress.

The second mechanism involves the link between the O_2^- and the NO dependant vasodilatory mechanism. The present study has shown that the decrease in SOD levels in DSS animals as discussed previously, is possibly due to an increase in O_2^- a trend observed in other studies as well (Yuan *et al.*, 1996). Direct measurement of O_2^- levels, using chemiluminescene, in endothelial cells and aortic rings have shown that cells/ tissues of SHR produce significantly higher levels of O_2^- than normotensive controls (Grunfeld, *et al.*, 1995). This radical, under normal physiological conditions is neutralized/ quenched efficiently by SOD (Hamilton, 2002). However in many pathological states including hypertension elevated levels of O_2^- have been shown to react with NO that is synthesized by endothelial cells. Blood pressure is controlled in part by the vasodilatory action of NO therefore O_2^- has the potential to inactivate this control resulting in an elevation in blood pressure (Hamilton, 2002).

Variation in NO levels, synthesized by the endothelium, would also result in altered blood pressures. A defective NO synthesis pathway has been implicated in salt sensitive Dahl/ Rapp rats (Bonnardeaux *et al.*, 1995). There is a strong probability that this could be a possible mechanism to induce hypertension in the salt sensitive animals as opposed to their resistant counterparts. In addition salt loading of Dahl/ Rapp salt resistant rats has been shown to increase NO synthesis thus resisting hypertension (Bonnardeaux, 1995). It would therefore appear that in addition to being a "free radical" animal which directly attenuates the vasodilatory mechanism, the DSS rats is further hindered by a defect in NO synthesis. This could explain the

rapid onset of hypertension observed in this study and supports the genetic link to hypertension in this model and perhaps in essential hypertension as well.

4.2.4 Free Radical and Calcium Homeostasis

Proper Ca^{+2} homeostasis is essential to cell functioning and has been reported by many studies (Kayama *et al.*, 1999; Pogan *et al.*, 2001). Free radicals such as O_2^- and H_2O_2 have been implicated in altering Ca^{+2} homeostasis in vascular smooth muscle cells and endothelial cells, this effectively alters vascular tone and impacts on blood pressure (Kayama *et al.*, 1999). In addition, the pressor effect of high dietary intake of salt becomes more evident when cytosolic calcium ion concentrations are altered. In SHR the cytosolic Ca^{+2} concentration of vascular smooth muscle cells have been reported to be higher than their normotensive controls, indicating that the Ca^{+2} homeostatic balance is upset in salt sensitive and genetic models of hypertension (Pogan, *et al.*, 2001).

The present study has demonstrated that in salt sensitive hypertension there is a compromised antioxidant status in the RBC of Dahl rats. Evidence in the literature is strong that this compromised status is due to an increase in free radical production rather than a decrease in antioxidant levels. Both O_2^- and H_2O_2 have been implicated in altering Ca^{+2} homeostasis in hypertension (Lacy, *et al.,* 1998; Pogan *et al.*, 2001).

Basically Ca^{+2} signaling involves firstly the intracellular release of Ca^{+2} from the endoplasmic reticulum that is mediated through inositol 1,4,5, triphosphate (IP_3) action. This is subsequently followed by entry of Ca^{+2} from the extra-cellular medium through voltage-gated channels into the cell (Ceolotto *et al.*, 1998; Pogan *et al.*, 2001).

In a recent *in vitro* study using endothelial cells, it was demonstrated that cells from SHR as opposed to normotensive animals display a lower quantity of releasable Ca^{+2} from their internal stores. Evidence in the literature indicates that this is due to the action of ROS on the IP_3 receptor when oxidized by these species thus rendering these dysfunctional (Pogan, *et al.*, 2001). It has also been suggested that depleted intracellular stores of Ca^{+2} could be due to the inhibition of the Ca^{+2}, ATPase pumps and an increase in permeability of the endoplasmic reticulum to Ca^{+2} ions. Both these changes have been linked to the action of ROS (Pogan, *et al*). The abnormal Ca^{+2} signaling that is evident in various models of hypertension can be attributed directly to the action of ROS on various areas of the cell. Confirmation of this role of ROS is evident when SOD, catalase and various other antioxidants were added in physiological doses to incubated cells. Ca^{+} flux increased significantly under these conditions in cells of SHR compared to cells of normotensive animals (Pogan *et al.*, 2001).

Evidence is therefore strong that free radicals such as O_2^- and H_2O_2 alter endothelial cell function through their Ca^{+2} signaling mechanisms. A dysfunctional endothelium has been reported by many studies in hypertension (Bonnardeaux *et al.*,1994;

Henning *et al.*,1988) and has been shown to be responsible in part, to elevating blood pressure. This is due to the attenuation of the NO dependant vasodilatory mechanism (Pogan *et al.*, 2001).

An interesting observation made by Pogan, (2001) that parallels the results of this study was the impaired Ca^{+2} signaling in weanling SHR. The inhibited IP_3 activity in weanling rats preceded the increase in blood pressure (Pogan *et al.*, 2001). The antioxidant status of the RBC of DSS was compromised relative to their DSR controls and this was achieved without salt loading (Table 6). This was presumably due to an increase in free radicals, O_2^- and H_2O_2 since western blot analysis has also shown recently that the expression of antioxidants is not decreased in SHR compared to normotensive controls (Pogan, *et al.*, 2001). This means that abnormal Ca^{+2} signaling commences early in life in the SHR leading to dysfunctionality of both the endothelium and the vascular smooth muscle layer. Since these components of the vasculature play an integral role in regulating blood pressure in part, this free radical mediated mechanism could help usher in the hypertension from an early age in DSS and SHR animals.

4.3 Independent Factors associated with Hypertension

4.3.1 Hypoxanthine and Superoxide

One of the sources of O_2^- *in vivo* is from the action of xanthine oxidase when acting on the substrate, hypoxanthine. O_2^- is generated from molecular O_2 as a by-product in this reaction (Freeman, *et al.*, 1982). The generation of O_2^- from this source is regarded as significant since xanthine oxidase inhibitors, administered to hypertensives, result in a significant decrease in blood pressure and many studies have reported higher levels of xanthine oxidase in hypertension than in normotensives (Ohtahara, *et al.*, 2001; Yu, 1994). Since this study has shown that SOD levels in RBC of DSS rats are significantly lower than their DSR controls (Table 7), this suggests a possible increase in the O_2^- radical. Many studies have demonstrated using direct measurements that there is a significant increase in O_2^- levels in hypertension (Pogan *et al.*, 2001). Elevated O_2^- levels exert strong biological effects as discussed previously often working synergistically with other factors to excascerbate the hypertensive process.

Serum uric acid has also being regarded as an important risk factor of cardiovascular disease. Uric acid, besides being produced after metabolism is also produced during the action of xanthine oxidase on hypoxanthine.

It is a well documented fact that the substrate hypoxanthine, is released from skeletal muscles under normal physiological conditions. It has been demonstrated

recently however that the skeletal muscles of hypertensives release larger quantities of hypoxanthine than that of normotensives (Ohtahara, *et al.*, 2001). Since xanthine oxidase is expressed in large concentrations by endothelial cells, the generation of O_2^- from this source has been shown to be significant. Xia *et al* have shown that by elevating circulating levels of hypoxanthine, the levels of oxygen free radicals could be increased *in vivo*. In addition tissue injury evident during ischemia could be attributed to the ROS that are produced by the enhanced activity of XO (Ohtahara *et al.*, 2001). This is perhaps also the mechanism that results in elevated uric acid levels in hypertension, an observation that has been reported by many studies (Yu, 1994).

It therefore appears that even the skeletal muscles of hypertensives contribute to the hypertensive process through an abnormal expression of hypoxanthine which is thereafter a basis of O_2^- generation. The present study has also revealed that the RBC of DSS weanling rats had a compromised SOD status and that the elevation in blood pressure preceded major structural changes of the vasculature. As discussed previously, this status quo strongly suggests that the hypertension is in part, biochemically mediated. Since the hypoxanthine levels in skeletal muscles is genetically controlled, there is a possibility therefore that hypoxanthine levels would be elevated in weanling DSS rats as well. Elevated levels of O_2^- that overwhelm SOD and result in a lower SOD status, could therefore arise from this mechanism in DSS weanlings.

Further evidence strengthening this argument is evident in the results of antihypertensive drugs. Angiotensin converting enzyme inhibitors (ACE) improve endothelial function and restore NO synthesis. The relatively higher efficiency of this drug compared to calcium channel antagonists and α_1, blockers is due to the restoration of NO release as well. NO has been shown to react with O_2^- forming peroxynitrate with a resultant attenuation of the vasodilatory action of NO (Nakazono *et al.*, 1991).

Since O_2^- levels have been shown by many studies to be constantly high in hypertension ACE inhibitors negate this by increasing NO synthesis and thus restoring the vasodilatory action of this mechanism. (This excludes its primary action of angiotensin on angiotensinogen).

4.3.2 Hyperglycemia and Insulin Resistance

This study has shown that fasted blood glucose concentrations are significantly higher in adult DSS rats compared to DSR controls (Table 7). There was also a significant increase in blood glucose between weanlings and their respective adult groups. These results concur with many studies that have reported an association between hyperglycemia, insulin resistance, hypertension, cardiovasculature diseases and obesity (Zemel, *et al.*, 1995; Landsberg, 2001; Paolisso, *et al.*, 1996). A possible decrease in blood glucose levels in DSS 8% animals was possibly due to the fact that in the sixth week of this study food intake in this group had decreased

significantly. The AIN-76 diet being semi synthetic has a high sucrose content (50%), a decrease in food consumption would perhaps result in lower blood glucose concentrations. In addition the significant decrease in the food efficiency ratio (Table 2) in DSS, 8% compared to DSS, 1% could be due to an increase in insulin resistance as reported by other studies (Yuan *et al.*, 1998).

Insulin resistance of salt loaded, hypertensive Dahl rats was reported by our laboratory (In press) and hyperinsulinemia is frequently associated with insulin resistance (Paolisso, *et al.*, 1996). Elevated levels of insulin on the other hand have been shown to be implicated in altering Na^{+} transport across membranes of various tissues. The increase in intra-cellular Na^{+} concentration in tissues of hypertensives have also been well documented (Modan,1991). Insulin has also been shown to directly attenuate Ca^{+2} influx through voltage gated channels in vascular smooth muscle cells. This results in an alteration in both Ca^{+2} homeostasis and signaling with a resultant vascoconstriction (Zemel, *et al.*, 1995). There is also a growing body of evidence that alteration in Ca^{+2} homeostasis in cells results in the down regulation of insulin mediated uptake of glucose. Although this has been demonstrated in many cell types, the mechanism remains unknown (Paolisso, *et al.*, 1996). The interplay between these two factors could result in a vicious cycle in the smooth muscle cells of the vasculature with a significant vasoconstriction.

Hypertension has often being described as a chronic syndrome that could be ushered in over a long period of time by stimuli that are regarded as subminimal (Simon,1998). Elevated blood glucose levels have been identified as such a factor

since glucose, when found in high concentrations in the blood has been shown to undergo a spontaneous oxidative reaction called enolisation. This chemical process also reduces molecular O_2, generating oxidizing intermediates such as O_2^- (Halliwell,1989, Paolisso, *et al.*, 1996). The oxidative stress and resultant injury to many tissues evident in diabetes has to been postulated to be in part, due to this mechanism (Halliwell, 1989). High levels of free radicals have also been implicated in the pathogenesis of hypertension by many studies (Yuan *et al.*,1998; Somova *et al.*, 2001). It therefore appears that in salt sensitive hypertension, the hyperglycemia and hyperinsulinemia that are often associated with these conditions, could play a role in the hypertensive process *per se* by acting directly on the vascular apparatus.

In addition there are other cell types such as the endothelial cells in which glucose uptake is insulin independent (Coffee, 1998). Persistently elevated levels of blood glucose would obviously result in higher than normal levels of intracellular glucose. This would result in higher levels of aldose reductase and sorbitol dehydrogenase in order to metabolise the "excess" glucose. These enzymes are NADPH dependant and this is one possible mechanism that explains why NADPH levels are depleted/ lowered in endothelial cells of diabetics as well (Paolisso, *et al.*,1996).

The depletion of NADPH due to hyperglycemia has potentially far reaching implication for the generation of free radicals and the hypertensive process. NO synthetase, the enzyme responsible for NO synthesis and glutathione reductase, that regenerates glutathione (GSH) from its oxidized form, glutathione disulfide is both NADPH enzymes. Depleted NADPH levels would therefore impair the action of

these enzymes. Glutathione disulfide is generated by GSH after being oxidized by ROS. If the activity of glutathione reductase is decreased this would imply that the antioxidant potential would be diminished because of lower GSH levels.

Since NO has been shown by many studies to be involved in vasodilation in the resistance vessels, decreased levels of NO would result in this mechanism being partly abolished hence increasing peripheral resistance and elevating blood pressure (Grunfeld *et al.*, 1995). This particular mechanism could become accentuated during hyperglycemia since high glucose levels have also been shown to generate O_2^- which reacts spontaneously with NO, forming peroxynitrate which has no vasoactive properties but is nevertheless cytotoxic (Vaziri, *et al.*, 2000). Therefore in addition to a possible decrease in enzyme activity, there could be an increase in the substrate that alters the nature of this mechanism.

It therefore appears that persistent hyperglycemia and hyperinsulinemia as observed in this model of hypertension further supplement existing mechanisms that have implicated to play a role in the pathogenesis of hypertension in salt sensitivity.

4.4 Plasma Lipids, Hypertension and Atherosclerosis

Analysis of the lipid profiles appear in Table 9. Total cholesterol concentration in DSS rats fed both diets (1% and 8% NaCl) were significantly higher than their respective controls.

Salt loading however decreased total cholesterol concentrations in both DSS and DSR groups. The results of animals fed normal diets were in contrast to other studies (Yuan *et al*) but were similar to previous results of our laboratory using Dahl rats (Somova, *et al.*, 2001). The decrease in blood cholesterol concentration in salt loaded animals could be due to the fact that dietary intake in salt loaded animals decreased after the fourth week of the experiment. The correlation between dietary intake and plasma lipids is well documented.

There is a positive correlation between dietary and plasma lipids especially when animals are fed *ad libitum* (Yuan, *et al.*, 1996).

The decrease in dietary intake of salt loaded animals has introduced an additional variable in this study especially with regards to the lipid profile. Discussion will therefore involve mainly animals on normal diets.

It has also been demonstrated using the Dahl rat that hypercholestrolemia coupled with a compromised antioxidant status, is involved in inducing endothelial dysfunction in the kidney and mesenteric vessels (Hayakawa, *et al.*, 1999). It is well documented by both experimental and clinical studies that the kidney is involved in the pathogenesis of hypertension. Hypercholestrolemic DSS rats have also displayed inhibition or attenuation of the NO synthesis in the renal medulla. Besides causing marked impairment of the endothelium dependant vasodilatory mechanism, compromised NO synthesis also results in a shift of the pressure natriuresis curve to the right. This effectively increases salt sensitivity and results in an increase in blood

pressure (Hayakawa, *et al.*, 1999). It therefore appears that hypercholestrolemia in DSS animals further excascerbates the hypertensive process by inducing changes to endothelial cells of kidney.

Although the total blood cholesterol concentration is not a reliable risk factor for coronary artery diseases (CAD), it is nevertheless used routinely when screening patients (Parks *et al.*, 1998). The significant increase in total cholesterol concentrations in DSS, on normal diet compared to their DSR counterparts does reveal that DSS animals are dyslipidemic compared to their controls. Both the high and low density lipoprotein fractions in DSS animals increased proportionately compared to DSR. In addition, the ratio between HDL: LDL fractions remained unchanged in DSS, 1% (2.00) compared to DSR, 1% (2.04). This basically means that the entire lipid profile in DSS, 1% animals was proportionately higher than their respective controls.

The disproportionate increase in the triglyceride concentration in DSS,1% animals could indicate impaired lipoprotein lipase activity since this enzyme is responsible for lipolysis of this fraction (Assman, *et al.*, 1991). Other studies have shown that fasting levels of triglycerides are very low and generally consistent in rats (Oschy Eisenberg,1982). Abnormalities with lipoprotein lipase in humans have been linked to genetic abnormalities (Assman, *et al.*, 1991). In such cases however triglyceride concentrations as high as ≥ 11,0 mmol/ l have been reported in the literature (Assman, *et al.*,1991). Elevated triglycerides are nevertheless considered a risk factor for the etiology of cardiovascular disease and is often considered symptomatic

of genetic abnormalities of lipid metabolism (Somova, *et al.*, 2003, Assman, *et al.*, 1991).

Although the rat is used extensively as a model in experimental work, lipoprotein metabolism in the rat is different in many respects from other mammals. This obviously make extra-polation of data on plasma lipids to humans difficult especially when one tries to compare the HDL fraction. Besides lacking the HDL_3 subfraction, rats accumulate cholesteryl esters from various origins in the HDL, subfraction due to the lack of the cholesteryl ester transferase enzyme (Oschry, *et al.*, 1982). This is the underlying reason why the HDL:LDL ratio and its beneficial effects should be used with caution in the rat (Oschry, *et al.*,1982).

Significant increases in the total cholesterol and especially the LDL fraction are considered significant independent risk factors in the etiology of atherosclerosis and other associated cardiovascular diseases (Palinski *et al.*, 1990; Somova *et al.*, 2003). This study has shown that in DSS,1% rats the LDL fraction had increased significantly compared to respective controls. Although the electrophoretic mobility of LDL was not performed, oxidized LDL (ox-LDL) has been implicated more strongly in CAD than native LDL (unmodified) (Palinski *et al.*, 1990; Steinbrecher *et al.*, 1990). The LDL fraction has been shown by many studies to be oxidatively modified by factors such as Cu^{+} ions and free radicals (Hayakawa *et al.*, 1999; Maxwell, 2000). The macrophages, which are the foam cell precursors, cannot take up large enough quantities of native LDL through LDL receptors to transform them into foam cells, ox-LDL on the other hand has been shown to be taken up by special scavenger

receptors that lacks the sensitive feedback control that involves down regulation to receptors (Maxwell, 2000; Chan, 1998). There is overwhelming evidence that foam cells play a central role in endothelial cell dysfunction and plaque formation, events that are synonymous with atherosclerosis (Chan, 1998; Steinbrecher, *et al.*,1990).

The results of this study has shown that the conditions in the vascular compartment of DSS rats are ideal for generating ox-LDL. Firstly there is a significant increase in the LDL fraction. Secondly the antioxidant status of the RBC has suggested that there is an increase in free radical production. Many recent studies have used direct means to demonstrate that there is a high concentration of ROS in hypertensives (Pogan *et al.*, 2001). The generation of lipid peroxides, as revealed by the MDA test (table 8) in this study has also revealed greater lipid peroxidation in the RBC of DSS animals compared to DSR controls. Since oxidative modification of LDL involves peroxidation of its polysaturated fatty acids, there is a strong possibility, that the LDL fraction would be subjected to oxidative modification by ROS in DSS animals. This substantiates the results of human studies that have demonstrated a direct correlation between the severity of the atherosclerosis and the levels of ox-LDL in plasma (Palinski *et al.*, 1990, Steinbrecher, *et al.*,1990).

4.4.1 Oxidatively Modified LDL and Atherosclerosis

The bioactivity of ox-LDL is not only restricted to the kidney since it has been shown by experimental and clinical studies that endothelial cells within the entire vasculature can be affected in varying degrees (Chan, 1998). The endothelium is regarded as a distinct metabolic and endocrine organ and is therefore involved in many physiological processes. A dysfunctional endothelium has therefore being implicated in many pathophysiological states (Maxwell, 2000). Atherosclerosis is a complex process in which many factors interact resulting in the characteristic plaque formation of largely the medium sized vessels. Discussion will however concentrate on the role of free radicals and ox-LDL in the pathogenesis of atherosclerosis.

It is generally accepted that atherosclerosis commences with damage to the endothelium, this often being referred to as an "insult." Besides the damage wrought directly by ROS on all cell membranes, there is increasing evidence that ox-LDL also has a deleterious effect on the endothelium (Steinbrecher *et al.*, 1990). For instance, when endothelial cells are incubated with ox-LDL, their production of PGI_2 decreases significantly. This has been shown to be due to the decrease in expression of $cPLA_2$, the rate limiting enzyme that is responsible for the release of arachidonic acid, the precursor of PGI_2 (Chan,1998). The resultant lower levels of PGI_2 would decrease the anti-thrombotic effect exerted by this compound and hence increase platelet aggregation, an activity associated with atherosclerosis (Buczynski *et al.*, 1993).

One of the early observations in atherosclerosis is the increased adherence displayed by monocytes to the endothelial layer. This is a significant step because many of these monocytes subsequently migrate into the intima where they are transformed into macrophages and subsequently into foam cells (Chan 1998; Maxwell, 2000). This adherence phenomenon displayed by monocytes has been shown to be mediated by endothelium derived adhesion molecules (EDAM), a protein synthesized by endothelial cells.

Endothelial cells when exposed to ox-LDL but not native LDL (Chan, 1998) display an increased expression of these EDAM. Evidence strongly implicating ROS in initiating these series of events, is the decrease in EDAM expression when antioxidants such as vitamin E are administered to cultured endothelial cells (Chan, 1998).

Besides the significant involvement of ROS ox-LDL in the early stages of atherosclerosis, many *in vitro* and *in vivo* studies have demonstrated that these factors also play a role, as the atherosclerosis progresses towards a pathological state. Using monoclonal antibodies specific for MDA-lysine, Palinski (1990) have demonstrated that a significant part of the "material" of plaque in rabbits comprise MDA modified LDL (MDA-LDL). Two important facts emerge from this study. Firstly the adduct of LDL used is significant because when ROS react with lipids, one of the products that is formed is MDA (Yu, 1994). This strongly indicates that peroxidation of the LDL fraction *in vivo* is accomplished by ROS at least in part, if not totally. Secondly MDA-LDL is the major lipid peroxide that is deposited in plaques of

atherosclerotic lesions. These observations therefore strongly implicate ROS in the pathogenesis of atherosclerosis. This study has also shown that since the antioxidant status of DSS rats was compromised, the probality that there was an increase in free radical production is strong. In addition histological studies of blood vessels of hypercholesterolemic Dahl rats has shown that functional changes that resemble atherosclerosis become evident after an experimental period of eighteen weeks (Hayakawa *et al.*, 1999).

The cardioprotective role of antioxidants is well documented (Hayakawa, *et al.*,1999, Somova, *et al.*, 2003). Whilst their mechanisms of action might vary, in many cases they have demonstrated that the role of free radicals in hypertension and atherosclerosis is a causal one (Somova *et al.*, 2003; Haya kawa, *et al.*, 1999).

An example to illustrate this point is vitamin E, which has been shown to demonstrate anti-hypertensive and anti-atherosclerotic properties (Chan, 1998). Vitamin E is carried in significant quantities in the LDL fraction and prevents peroxidation of the lipid component of the LDL fraction. Enrichment of LDL fraction therefore minimizes peroxide formation that is due to amongst other factors, free radicals within the vascular compartment. There is therefore a subtle difference between the action of antioxidant enzymes investigated in this study (SOD and GPx) and that of vitamin E. The former group quenches the free radicals that cause oxidative modification of the LDL fraction, whereas the latter affords direct protection by preventing peroxidation. The vitamin E content of the diet used in this study was

to the integrated nature of the antioxidant system *in vivo*, results of SOD levels can explain this pattern. In the case of each group in this study, a decrease in GPx levels is coupled with an increase in SOD levels. Since it is estimated that SOD is responsible for the generation of 70% of H_2O_2 from O_2^- generation in cardiac tissue. Superoxide ions are generated from xanthine oxidase (XO) amongst other sources. Although xanthine oxidase levels were not assayed in this study, many studies have reported elevated levels of uric acid in hypertension. Uric acid is a major metabolite of xanthine and therefore its levels are an indication of xanthine oxidase levels. Our laboratory in a previous study has also reported elevated levels of uric acid levels in hypertensive DSS rats compared to normotensive DSR (Channa, *et al.*, 2003).

Using western blot analysis, however, Pogan, (2001) has shown that there was no difference in the expression of SOD in the endothelial cells of SHR compared to those of normotensive Sprague-Dawley rats. In addition chemical assays of enzyme levels confirmed these results (Pogan *et al.*, 2001). In the neonate lung of rat, however, an enhanced expression of SOD has been demonstrated and the superior resistance of the neonate to hypoxia has been attributed to this. In bacteria, the gene locus that regulates SOD, called the *soxyR* regulon, is well characterized. In and bacteria, SOD levels have been shown to be finely regulated in response to O_2^- levels (Harris, 1992). The issues above point to the following:

1. There is a possibility that the expression of SOD in DSS and DSR animals are very different from the SHR.

2. There could be a difference in expression of SOD in different tissues/ organs within a particular organism.

3. There is perhaps a difference in SOD expression in the neonate compared to the adult in a species and lastly the antioxidant system in multi-cellular organism is far more complex than in the bacterial model that is often used in studies. The complexity/ diversity and depth of the mammalian antioxidant system has been described in the literature and this is what makes its study complex (Jacob, 1995).

In an intact antioxidant system, GPx converts H_2O_2 to water by oxidizing reduced glutathione (GSH) to oxidized glutathione (GSSG). This is a good example to illustrate the integrated nature of the anti-oxidant system (Harris, 1992). Although GSH is synthesized by most tissues, there is a constant efflux of GSH from tissue/ organs into the plasma compartment and plasma GSH is cleared rapidly by the kidneys (Adams *et al.*, 1983). In this study there was a significant decrease in cardiac GSH in DSS animals compared to DSR controls (Table 3). This trend alludes to the fact that levels of H_2O_2 are elevated in hypertension. Since the levels of GPx was also significantly lower in cardiac tissue of DSS animals, the paralleled decrease in GSH concentration is DSS animals strongly suggests that the levels of H_2O_2 had increased in this group. Therefore the decrease in both the antioxidant, GPx and its cofactor, GSH indicate that cardiac tissue of hypertensive salt-sensitive rats are subjected to an increase in oxidative stress. The increase in H_2O_2 in

hypertension have also been reported by other studies (Lacy *et al.*, 1998, Pogan *et al.*, 2001).

A compromised GPx status either due to abnormality in GPx expression, Se deficiency or an increase in H_2O_2 has serious clinical implications. This is evident in Keshan's disease in which low levels of GPx have been reported in cardiac tissue and RBC due to low dietary intake of Se. This is due to low bioavailability of Se in an area in China. The pathological changes seen in these individuals is therefore an ideal case to observe, the effects of low GPx levels. Consistent symptoms in acute Keshans disease and Se deficient animals are cardiac hypertrophy and varying degrees of myocardial necrosis followed by diastolic dysfunction (Konz *et al.*, 1991). *In vitro* experiments using perfused hearts of Se deficient rats with H_2O_2 have also displayed diastolic dysfunction before systolic dysfunction compared to rats that were on a balanced diet (Konz *et al.*, 1989). This indicates the GPx affords the myocardium protection against H_2O_2 and that processes that govern diastole are more sensitive to oxidative attack than those that do with systole.

Salt loading in both DSS and DSR group however produced an increase in GSH concentration in cardiac tissue. In the case of DSR animals there was a 7.2% increase compared to a 46.5% increase in DSS rats. Since 70% of plasma GSH is cleared by the kidneys, our observations during salt loading are perhaps due to the alteration in clearance rates. Kidney function, including clearance capacity are altered during renovascular and salt sensitive hypertension (Rapp, 1982).

The kidney function of DSS animals are altered to a greater extent than DSR animals, a condition that would explain the greater increase in cardiac GSH levels in salt loaded DSS rats compared to the respective control. Studies using Sprague Dawley rats have shown that plasma and hepatic GSH levels are unaltered even if these animals are subjected to huge oxidative stress (Adams *et al.*, 1983) whether this also happens in hypertensive Dahl rats is as yet unanswered.

Morphological studies have confirmed numerous changes to the myocardium of DSS hypertensive rats that are associated with hypertension and CVD, including edematous areas within the myocardium. Under such conditions there would be an increase in leucocytes, a fact confirmed by our haematological analysis (Table 11). Leucocytes with their characteristic "burst reaction" release significant amounts of H_2O_2 as part of their mechanism to annhilate bacteria (Friedman *et al.*, 1990). This would no doubt subject the myocardium to an increase in oxidative stress as evident by lower GPx levels hypertensive rats. This source of H_2O_2 is thus an independent one that is generated as a consequence of the hypertension and its associated pathological changes.

From the data of this study, there is therefore a strong probability that there is an increase in free radical production by the myocardium of salt sensitive hypertensive rats. The compromised antioxidant status could therefore be attributed to this increase. The major ROS that stresses the myocardium is probably O_2^- since there was a non-significant increase in expression of SOD and GPx levels indicate greater quenching by the H_2O_2 that is generated by SOD. Are Dahl rats therefore similar to

patients with Bloom's syndrome, a rare genetic disease in which there are elevated levels of SOD and O_2^-? The resultant increase in H_2O_2 levels and the corresponding overwhelming of the GPx and catalase antioxidant enzymes in these patients is considered a major factor in the disorder (Harris, 1992). This question can only be answered if the nature of expression of the various antioxidant enzymes are determined and if free radical levels can be measured directly in different tissues or compartments of DSS and DSR animals.

Besides Keshans disease, an excellent model that provides evidence of the roles of antioxidants and free radicals is the occlusion/ reperfusion model. The brief occlusion of a coronary artery followed by reperfusion results in the previously ischemic area displaying a transient alterations in both contractile function and biochemical properties. This post ischemic "stunning" has been attributed in part at least to the effects of free radicals. (Przyklenk *et al.*,1986). The reperfusion period has great relevance to this study because the rate of free radical production is very high at this point and this is coupled with a washout of local antioxidants such as SOD, GPx and catalase. The cardiac tissue is therefore in a compromised situation and would thus be susceptible to free radial mediated attack. Many studies have demonstrated the deleterious effects of free radicals such as O_2^- and H_2O_2 on the myocardium under these conditions, these range from decreased contractility to ultrastructural changes (Kok *et al.*, 1989; Konz *et al.*, 1990; Przyklenk *et al.*, 1986). In a parallel study we have also shown in a histological evaluation of the myocardium that in DSS animals there were abnormalities of the sarcoplasmic reticulum, T tubules, mitochondria were swollen and besides the separation of intercalated discs

from fibres there was an increase in interfibre space (Somova *et al.*, 2001). Most of these observations are in agreement with other studies and perhaps demonstrate the etiological role, at least in part, of free radicals on myocardial pathology.

Therefore the changes evident in the occlusion/ reperfusion model which demonstrates the possible short term effects of ROS parallels the changes that were evident in this study which can be regarded as a relatively long term one. Many of the histological changes that were evident in the cardiac tissue could in all probability be due, in part, to the elevated levels of ROS in this model of salt sensitive hypertension.

In addition Przyklenk, (1986) have demonstrated using canine hearts that there is considerable improvement in the post ischaemic myocardium when antioxidants, SOD and catalase were administered. This therefore strongly suggests that free radicals are at least in part, responsible for the "stunning" and its associated changes that is displayed by cardiac tissue during reperfusion (Przyklenk *et al.*, 1986).

4.6 Chemical Hypoxia, Myocytes

The results of this procedure confirmed many of the observations noted in the other assays discussed earlier and in a parallel study that investigated morphological changes to the heart (Somova *et al.*, 2001). In myocytes that were unchallenged, that is normoxemic, florescence activity of the DCDHF, DHR and Fluo-3 probes revealed that there were no significant differences in H_2O_2 and intra-cellular Ca^{+2} accumulation between the DSS and DSR strains respectively. The DCDHF flourescence did however suggest that H_2O_2 levels were high in both strains. This radical is generated largely from the action of SOD and is removed by GPx and catalase. Biochemical assays of SOD levels in cardiac tissue has however (discussed previously) revealed that SOD activity was more pronounced in DSS compared to DSR rats and salt loading elevated these levels even further. The non-significant difference in H_2O_2 levels between the strains despite it being elevated as shown in this procedure, therefore these observations cannot be explained in terms of the antioxidant status that was biochemically determined.

After subjecting myocytes to oxidative stress by chemical hypoxia (125 µM H_2O_2), there were morphological changes, increase in both H_2O_2 and ROS and increased intra-cellular Ca^{+2} accumulation (Table 5) all of which were significantly higher in DSS versus DSR rats. This data suggests that cardiac tissue of DSS rats are subjected to an increase in oxidative stress. Many of the morphological changes that were induced by chemical hypoxia are synonymous with changes mediated by free

radicals. Disturbances in Ca^{+2} homeostasis due to free radical mediated alteration in cell membranes and the scarcoplasmic reticulum is well documented (Pogan *et al.*, 2001; Somova *et al.*, 2001).

One of the hypothesis of hypertrophied hearts is that it could be subjected to periods of relative hypoxia. Exhaustive studies using the occlusion/ reperfusion model has demonstrated that after a period of brief hypoxia (after occlusion) followed by reperfusion, there is a significant increase in free radical production (Przyklenk *et al.*,1986). The increase in free radicals have been implicated in various structural and functional alterations to the myocardium. These changes to the myocardium could therefore be considered an independent risk factor for injury to the myocardium in hypertension (Somova *et al.*, 2001).

4.7 CONCLUSIONS AND RECOMMENDATIONS

The Dahl rat is an excellent experimental model for salt sensitivity and its associated patho-physiology (Ferrari, *et al.*, 1995). The present study has shown that in addition to the well established changes that are evident in this model, there is a compromised antioxidant status in two critical areas (RBC and myocardium) that impact on cardiovascular diseases. Whether this compromised status quo is due to an increase in production of free radicals or a decrease in expression of antioxidants, needs further investigation. Most mammalian systems, however alter their expression of antioxidant enzymes in response to levels of free radicals, that is, they adapted to oxidative stress.

The only evidence of an increase in expression of the antioxidant enzymes in this study was the increase in serum catalase and the non significant increase in cardiac SOD levels. This trend also suggests that the major oxidative stressors against the RBC and myocardium are different, since SOD is responsible for dismutation of $O2^{\bullet}$ and catalase for H_2O_2 respectively. Due to the highly integrated nature and depth of the antioxidant system, an increase in antioxidant activity at one level could have an overwhelming effect at another with resultant deleterious effects. This has been demonstrated when SOD expression in brain is increased significantly, thus resulting in accumulation of H_2O_2. The myocardium is therefore potentially at great risk in DSS hypertensive animals since both biochemical assays and florenscence activity of the

probes have suggested elevated levels of H_2O_2. Direct measurement of cytosolic H_2O_2 levels however could only confirm this observation.

Both GPx and catalase are responsible for neutralizing/ quenching H_2O_2. Catalase, rather than GPx has been shown to react when H_2O_2 levels are high (Yu, 1994). The high levels of serum catalase therefore strongly suggests that H_2O_2 is the major free radical in the vascular compartment of DSS animals. Many pathophysiological changes associated with hypertension and cardiovascular diseases, including atherosclerosis, have been attributed to the action of free radicals. This hypertensive model has therefore provided indirect evidence that free radical levels are high in DSS animals. Genetic studies on the expression of the anti-oxidant enzymes coupled with direct measurements of free radical levels will only confirm this.

There has been much debate whether the hypertension *per se* is responsible for the pathophysiological changes associated with hypertension and cardiovascular diseases. This study has demonstrated that many independent factors that are strongly linked to these diseases are present in the hypertensive DSS animals. These include hyperglycemia, hyperinsulinemia and dyslipidemia. The interplay amongst these factors and free radicals are considered by many in the field to be ideal for the pathogenesis of end organ damage and conditions such as atherosclerosis (Maxwell, 2000). Although the rat is not considered an ideal model for atherosclerosis, recent studies have shown that with an extended experimental period (> 18 weeks), changes in the vasculature that are synonomous with early atherosclerotic changes were evident in DSS rats (Hayakawa *et al.*, 1999). In a

parallel study we have also shown numerous morphological changes to the myocardium that are associated with cardiovascular complications (Somova, *et al.*, 2001).

If, the LDL fraction can be critically evaluated in the future its pathophysiological role in hypertension and atherosclerosis would be identified with certainty. This would involve the identification and quantification of the Ox-LDL fraction by electrophoresis and using immunological techniques identify the major lipid adduct of oxidized LDL. Most studies suggest that it could be MDA derived LDL. Using the oxidative challenge test we have shown that higher levels of MDA are formed in the RBC of DSS rats. These analyses would also confirm the role of free radicals in oxidatively modifying the LDL fraction.

By assessing the antioxidant status of RBC in both weanlings and adults sourced from the same colony this study has shown that the compromised antioxidant status in DSS animals (either due to an increase in free radical production or a decrease in antioxidant synthesis) precedes the hypertension. This therefore clarifies the "anomalies" of other studies that have shown a dissociation between the wall: lumen ratio and blood pressure in the early stages of hypertension (Simon, *et al.*, 1998). Studies to date have shown that there exists a clear dissociation between the wall: lumen ratio of resistance vessels in the early stages of hypertension (Simon *et al.*, 1998). This implies that the initial increase in blood pressure is due to factors other than vascular remodeling. Since free radicals have been implicated directly as hypertensive agents, the initial increase in blood pressure in young DSS rats could

be, in part, biochemically mediated (Ceriello *et al.*, 1991). In order to assess the possible aetiological of free radicals in the pathogenesis of hypertension *per se*, it would advantageous to administer antioxidants that have antihypertensive properties from weanling stage and monitor both the antioxidant status of the animals and the parallel changes in blood pressure in a longitudinal study.

Salt loading did exacerbate the hypertensive process in DSS animals, confirming their salt sensitivity, which can be explained in terms of salt retention and other well documented mechanisms (Rapp, 1982; Simon, 1998). Salt loading did however also result in lower GPx levels in both the RBC and myocardium. This suggests the possibility of a correlation between blood and H_2O_2 levels. Elevated levels of H_2O_2 have been reported in many hypertensive studies (Lacy, *et al.*, 1998). This can perhaps be evaluated in a future study by measuring H_2O_2 levels directly using methods such as the Clark electrode. A statistical relationship between H_2O_2 concentration and blood pressure can thereafter be obtained, if any. Alternatively, *in vitro* experiments using segments of vasculature can be used by varying H_2O_2 levels in media and recording contractility through a transducer.

This study has therefore shown that adult Dahl salt sensitive rats irrespective of dietary salt have a compromised antioxidant status based on lower levels of SOD and GPx in both the RBC and myocardium. This was coupled with hypertension, dyslipidemia, hyperglycemia and elevated levels of lipid peroxides. In addition, the compromised antioxidant status preceded the increase in blood pressure as evident

by lower levels of SOD and GPx in the RBC of weanling DSS animals. These results therefore suggests a strong genetic involvement in either the compromised antioxidant status and/or an increase in free radical production.

Therefore, evidence provided in the present study and others in the literature, overwhelmingly indicate that free radicals have an etiological role in hypertension of salt sensitive rats. This study further confirmed the multifactorial and polygenic nature of hypertension, in particular salt sensitive hypertension and highlights the therapeutic role of antioxidants as antihypertensive agents in salt sensitive hypertension. Besides the reduction in blood pressure, the cardiovascular apparatus and target organs would be afforded protection against the deleterious effects of oxidative stress.

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APPENDIX

HEART GSH

The reaction was monitored for a total of five minutes as indicated in the following four graphs. It is recommended that the average O.D. of three consecutive readings be used for the calculation from a linear part of the curve. Prior to the actual test runs, O.D. change of a few reactions were monitored for eight minutes to obtain the period at which this occurs. In all cases linearity was obtained between three to five minutes. This is evident in the four graphs following this text.

The average O.D. was divided by the extinction coefficient which yielded the quantity of GSH formed in nmol. This was then divided by the mass of tissue in milligram and the results were expressed as nmol/mg.

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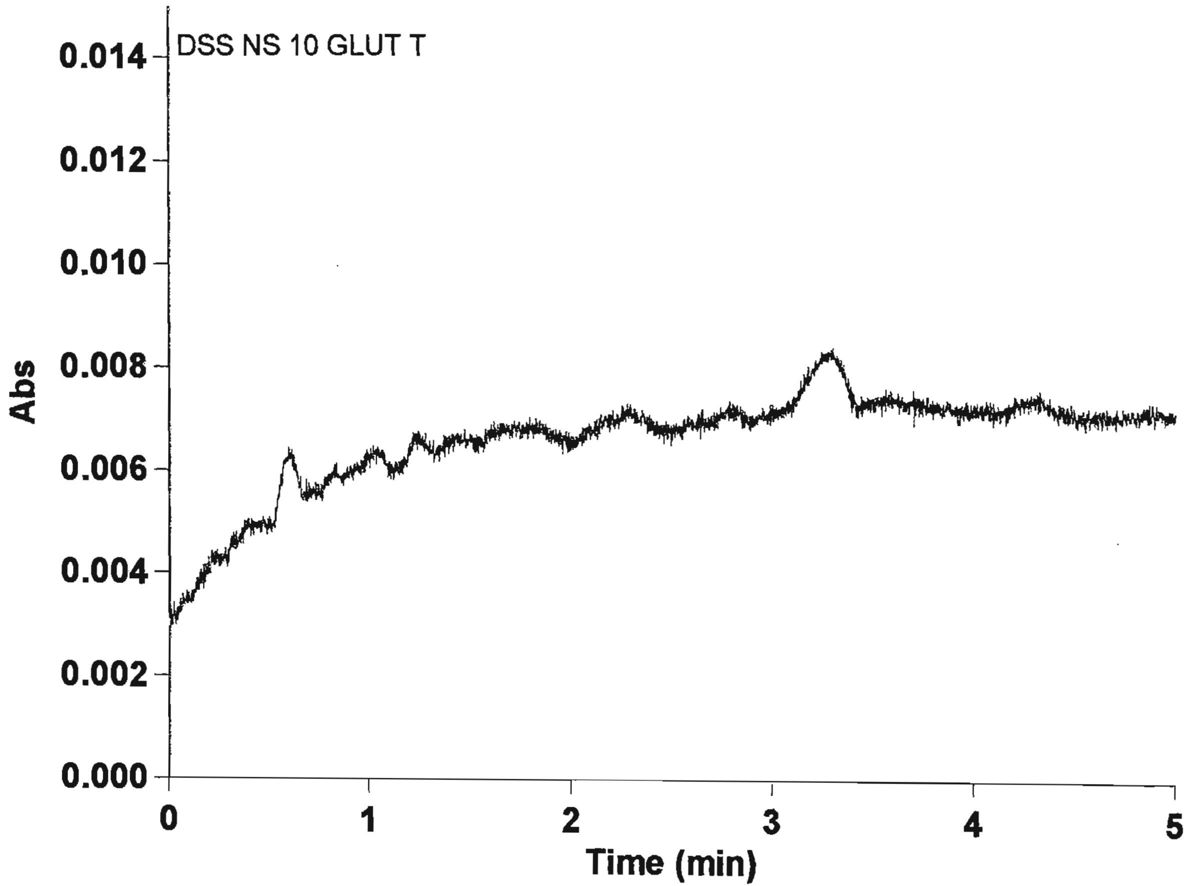

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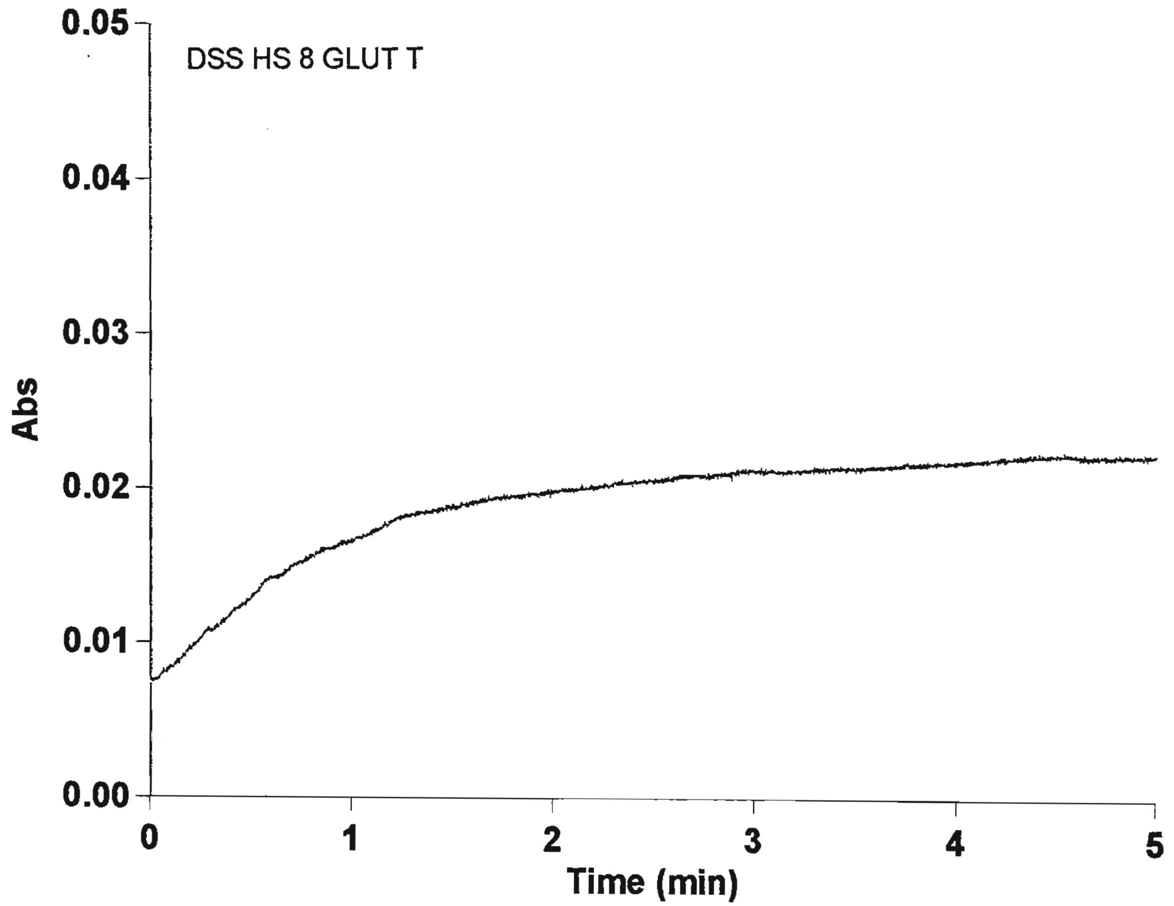

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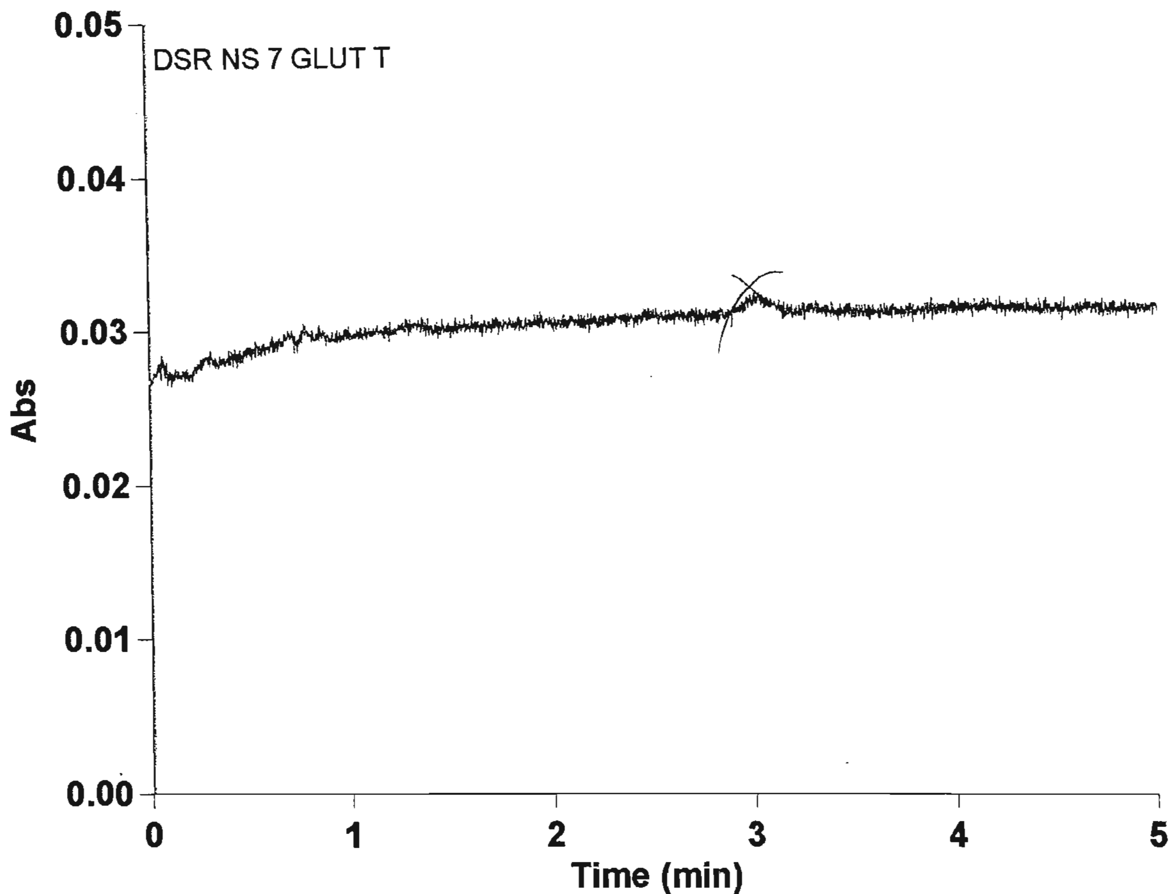

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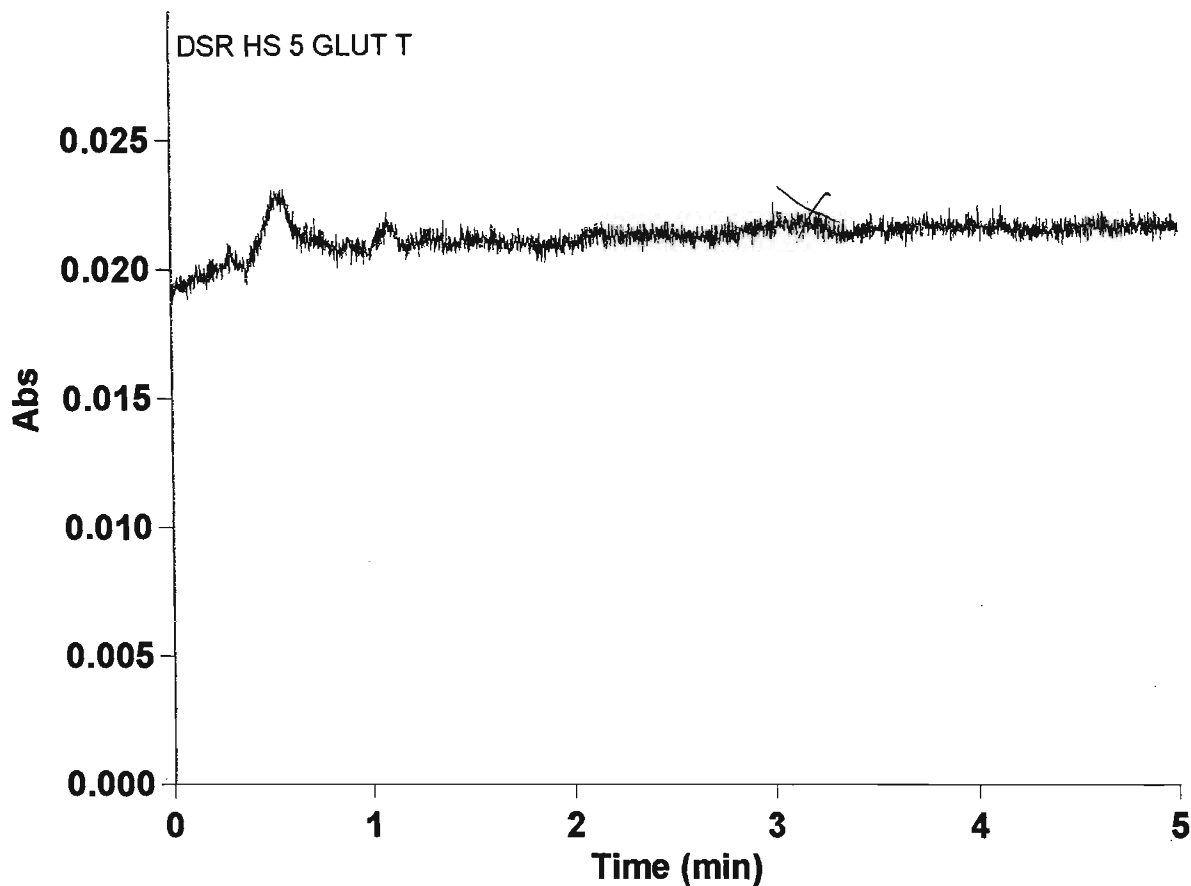

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RBC GLUTATHIONE PEROXIDASE

The five graphs following this text are examples of data generated in this assay. The first graph represents a typical result obtained in a non enzymatic reaction in which the RBC was replaced with equal volume of H_2O, that is non enzymatic conversion of NADPH to NADP.

The reactions (that is change in O.D.) were monitored for a period of four minutes commencing immediately after the addition of the sample. The change in O.D. between two and four minutes were obtained from the recordings. This was divided by two to give the change in O.D. per minute. Since the monitored change in O.D. is a combination of both enzymatic and non enzymatic reactions, the change in O.D. of the latter was subtracted from each sample recording to yield true enzymatic conversion of NADPH to NADP.

The O.D. was divided by the extinction coefficient to yield results in nmol. This was then divided by the corresponding protein concentration of each sample to yield the final result in nmol/mg.

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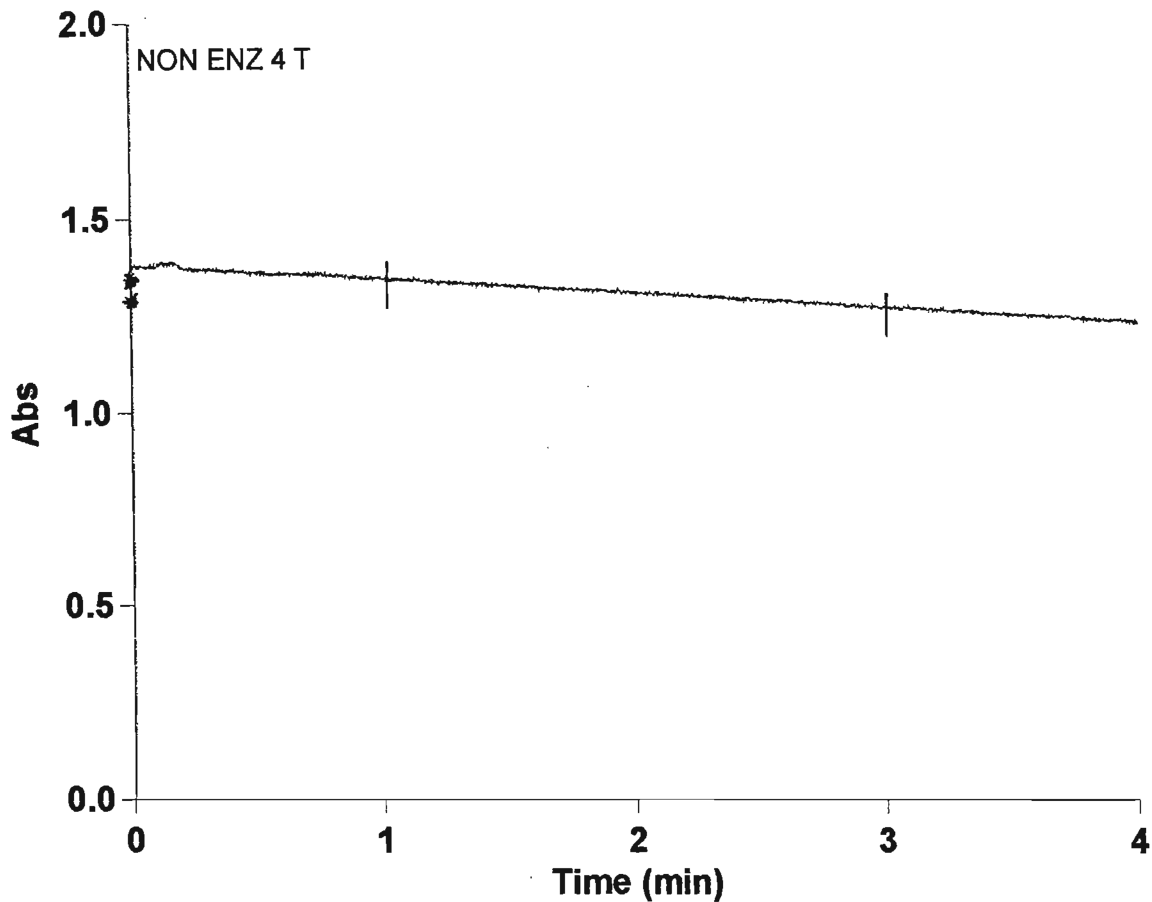

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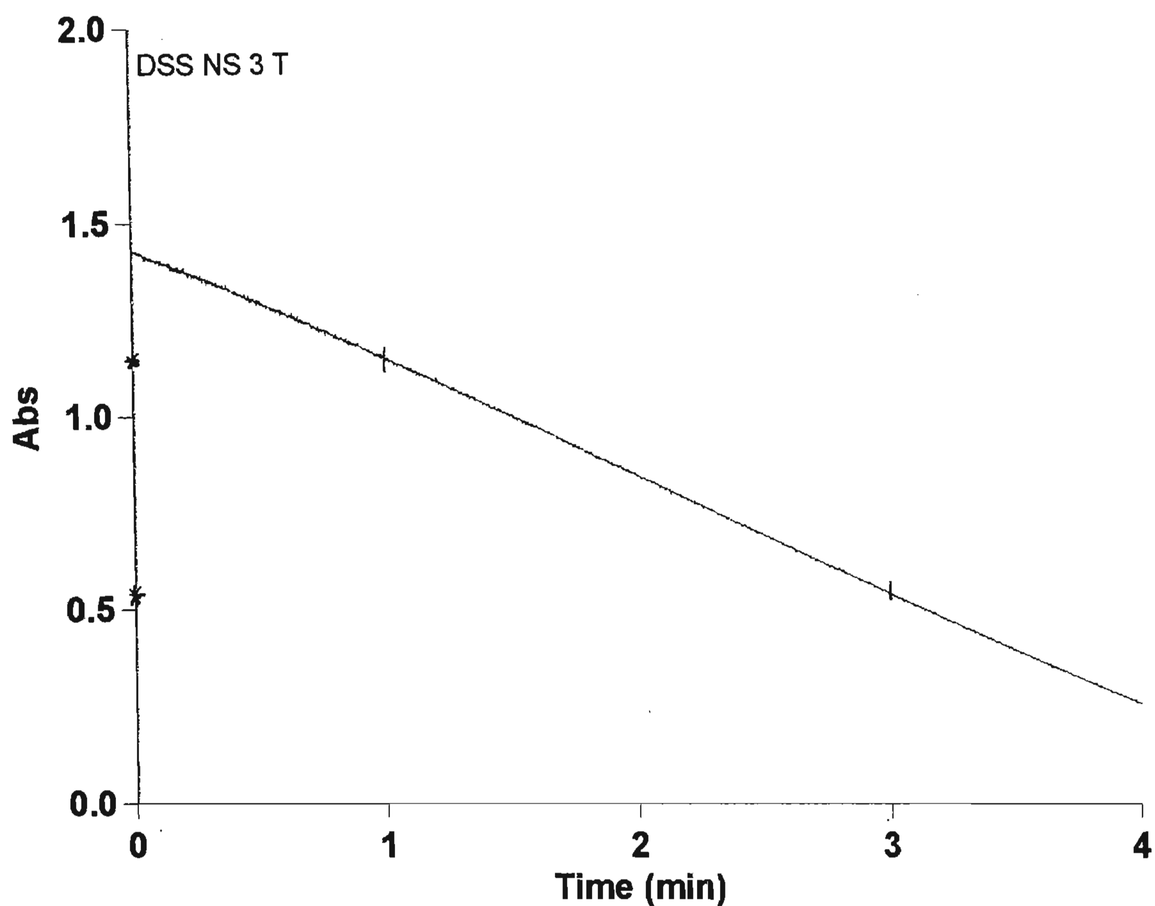

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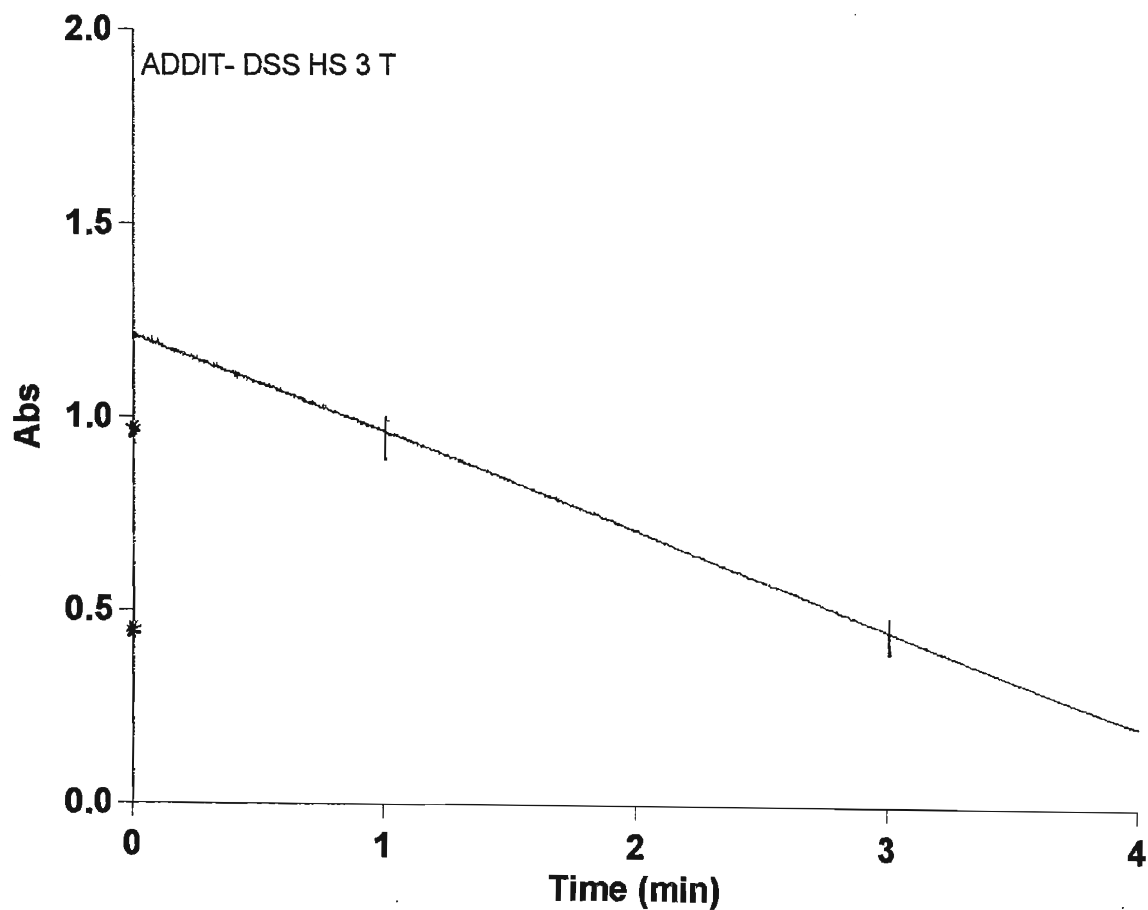

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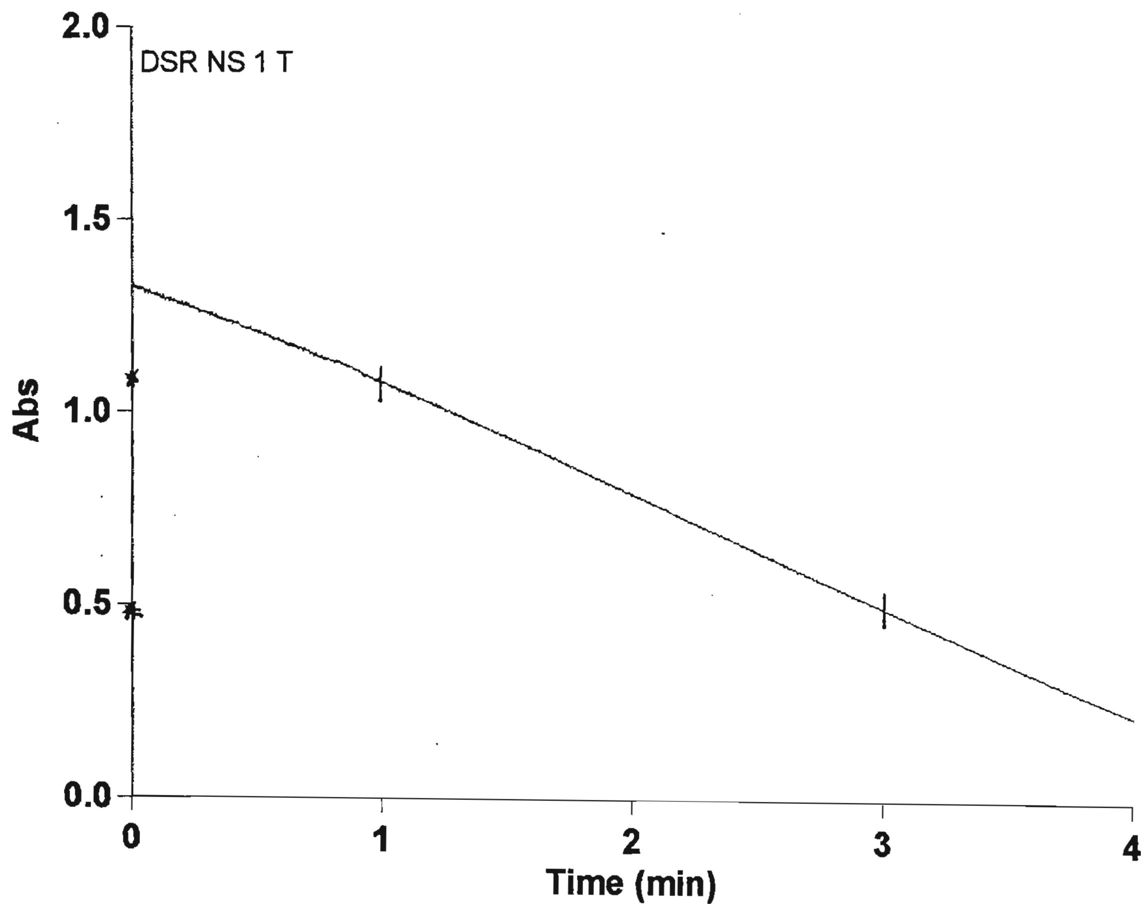

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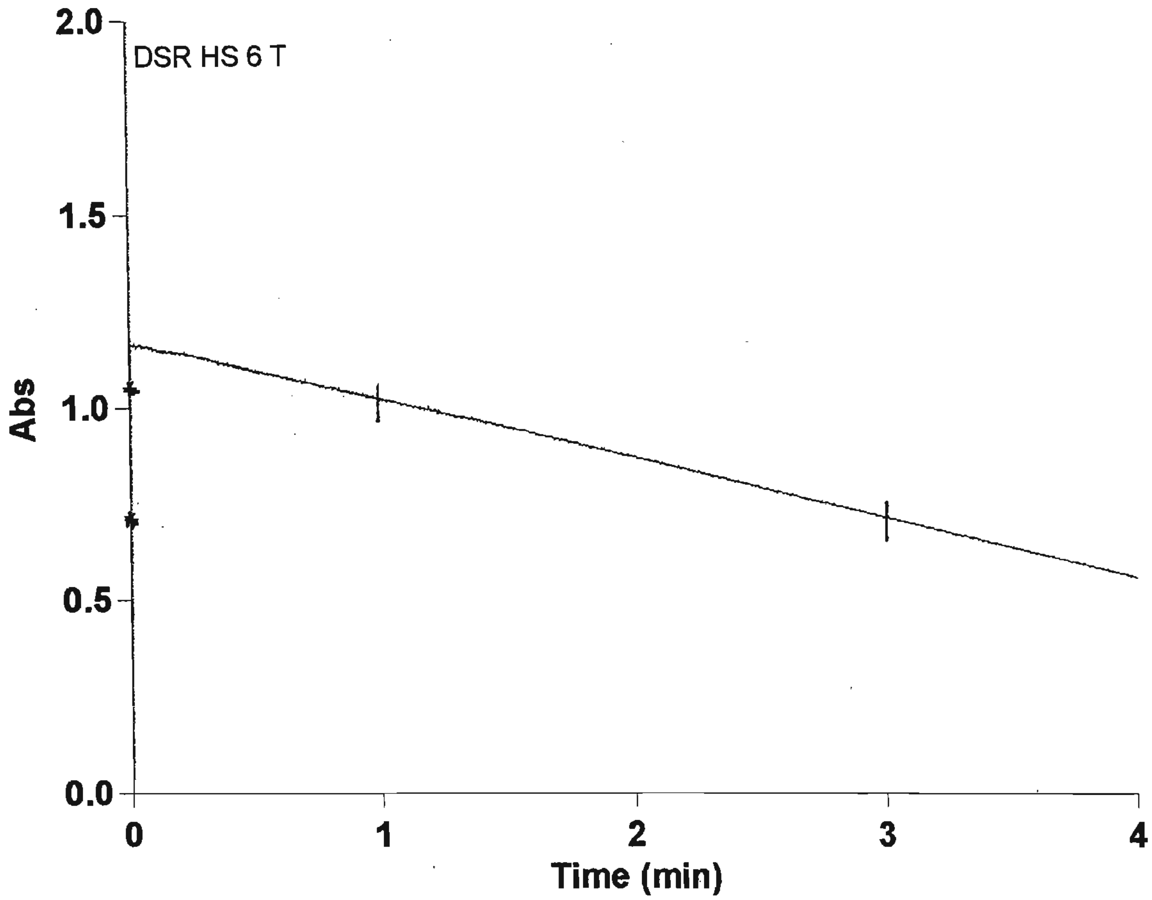

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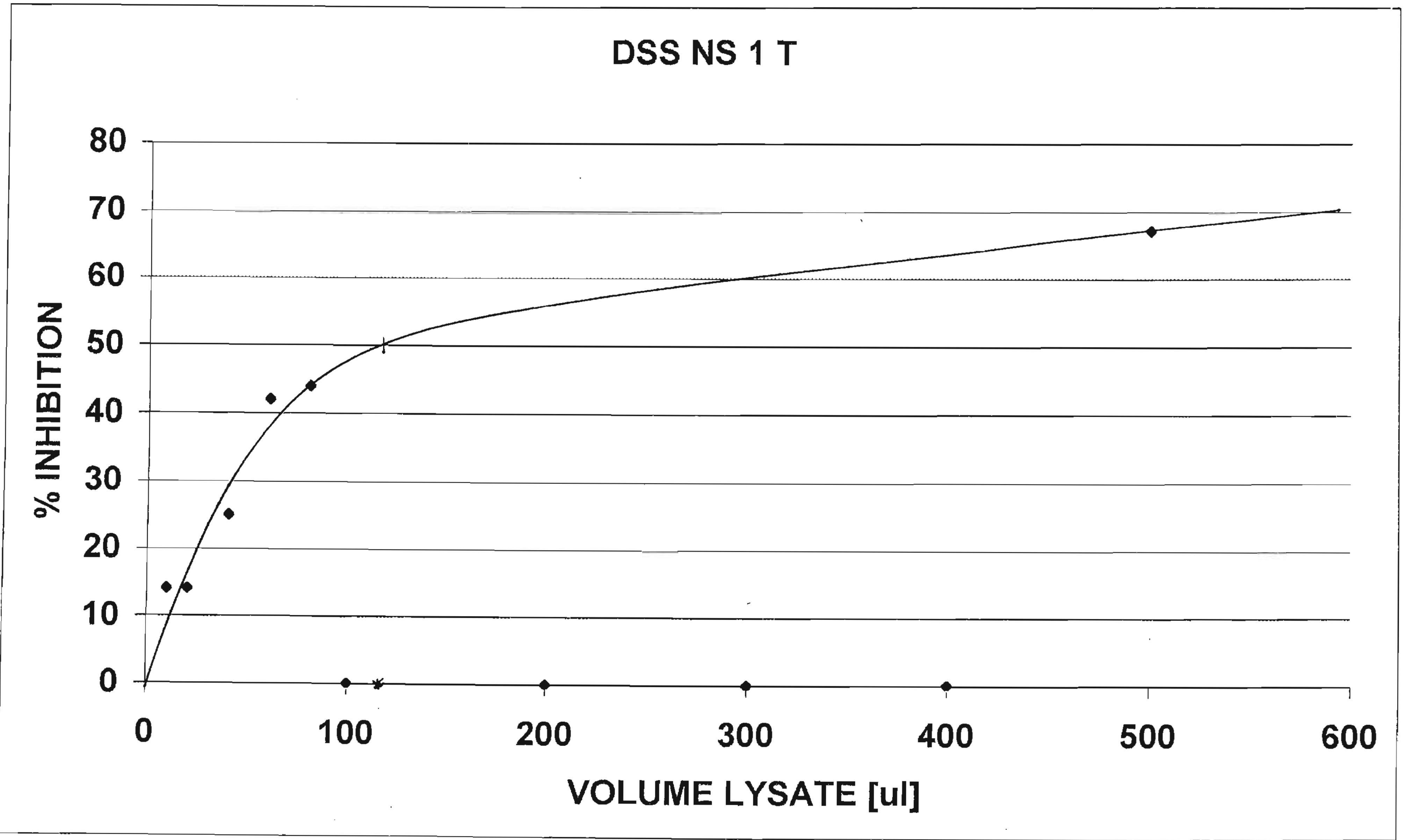
RBC – SUPEROXIDE DISMUTASE

In this assay, for each sample there were six tubes in duplicate corresponding to the following volumes of lysate (ul) 10, 20, 40, 60, 80 and 500. After incubation for twelve minutes in a specially constructed light box, the optical density of each tube was determined against a blank that comprised all reagents besides the lysate. The average O.D. per volume was determined. In parallel with the sample assay, a non enzymatic reaction was also carried out in triplicate.

The test is based on the ability of superoxide dismutase to inhibit the reduction of nitroblue tetrazolium (NBT). The percentage inhibition of each tube with varying volumes of lysate was determined by dividing the average O.D. by the O.D. of the non enzymatic reaction. The four graphs that follow this text represent one from each group. The volume that corresponds to 50% inhibition was used in the calculation. This point is marked with an asterisk on the x-axis.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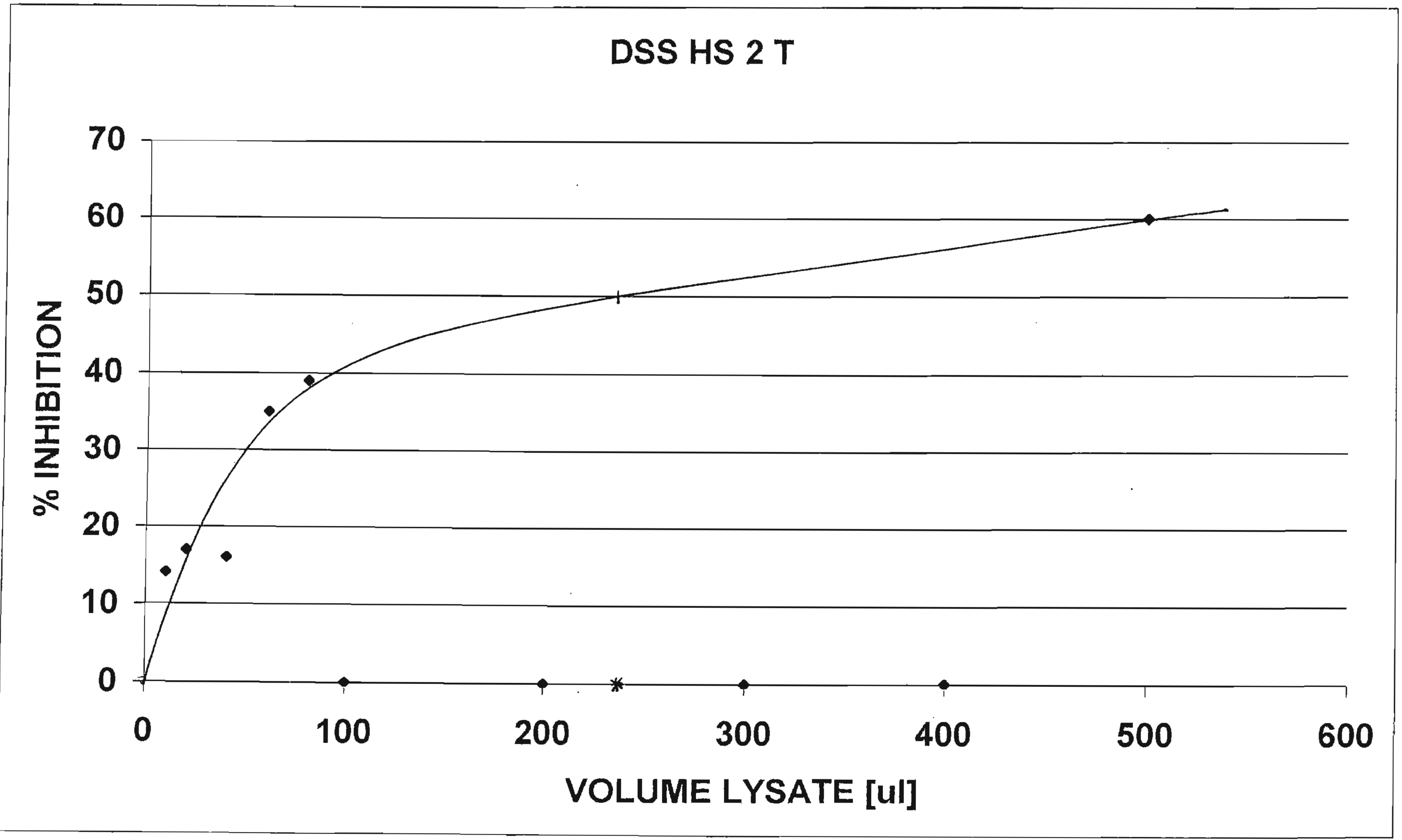
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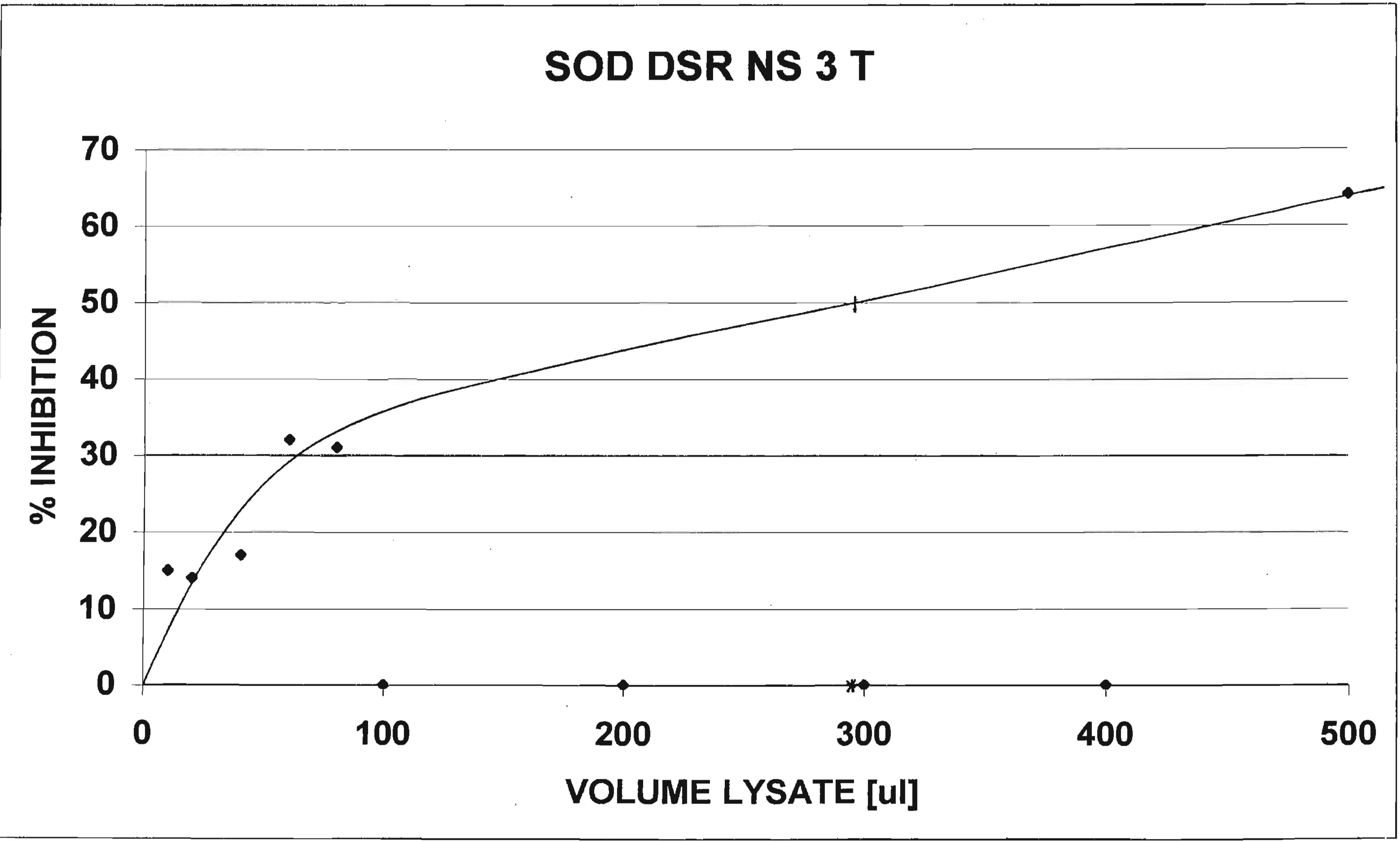
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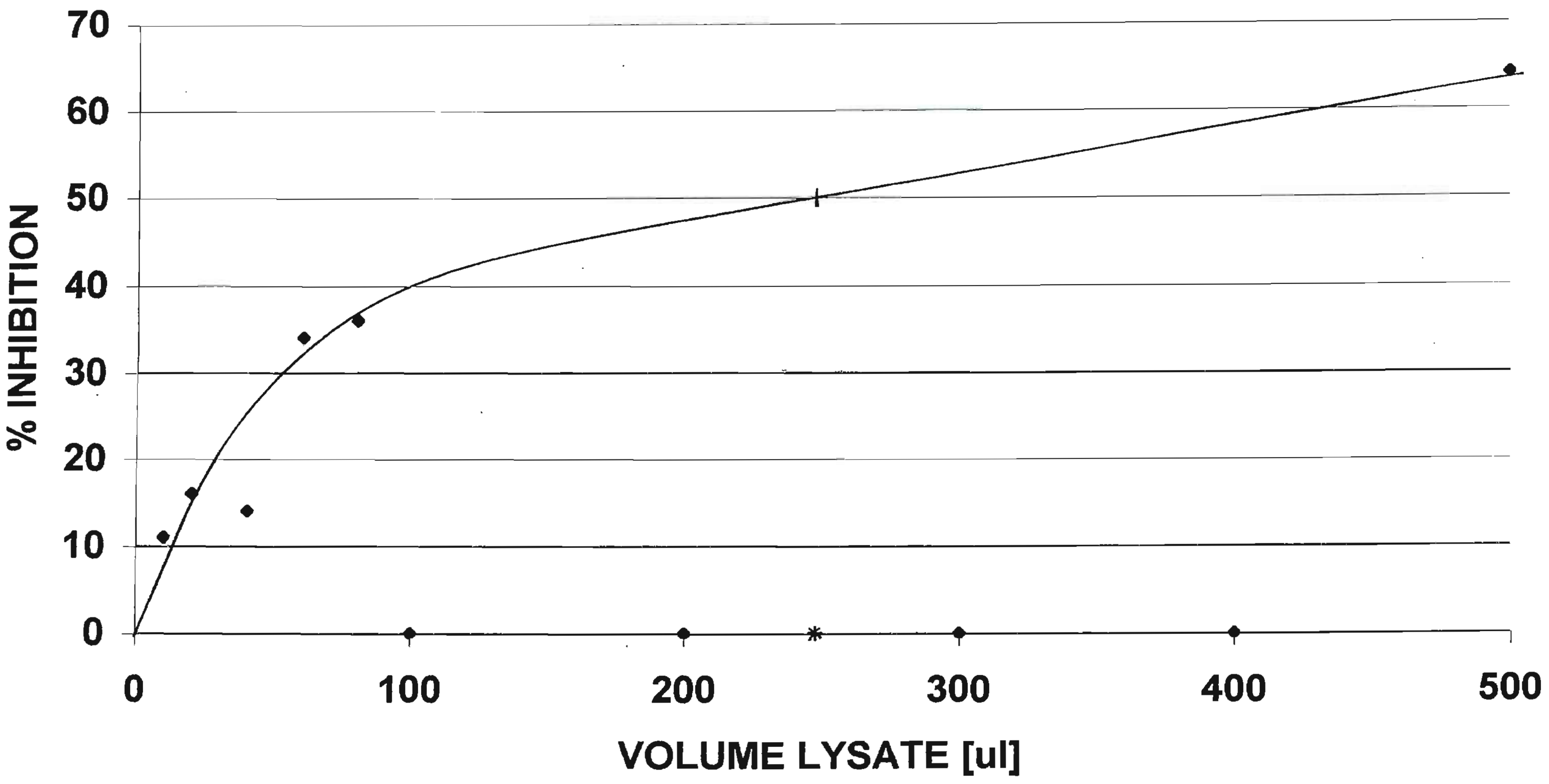

SOD DSR HS 5 T
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TABLE 12a: Average Weekly Food Consumption (grams)

Group/Week	DSR-Normal Na (1%)	DSR-High Na (8%)	DSS-Normal Na (1%)	DSS-High Na (8%)
1	97.0 ± 18.6	89.5 ± 14.9	106.6 ± 9.0	100.6 ± 8.5
2	103.9 ± 11.9	86.3 ± 7.0	112.9 ± 6.0	103.0 ± 8.0
3	102.1 ± 13.1	99.2 ± 6.4	129.9 ± 7.4	118.8 ± 5.8
4	102.3 ± 11.9	103.5 ± 7.7	135.5 ± 11.9	130.4 ± 4.7
5	98.3 ± 10.5	98.3 ± 7.5	140.8 ± 10.2	135.0 ± 12.5
6	98.6 ± 12.8	104.0 ± 8.7	136.8 ± 8.3	100.1 ± 41.4

TABLE 12b: Average Weekly Food Consumption (grams)

Group/Week	DSR-Normal Na (1%)	DSR-High Na (8%)	DSS-Normal Na (1%)	DSS-High Na (8%)
0	120.7 ± 28.6	78.2 ± 20.2	63.3 ± 11.5	60.9 ± 6.0
1	153.5 ± 30.7	110.2 ± 21.2	95.0 ± 13.1	87.8 ± 9.2
2	179.8 ± 34.5	118.8 ± 16.0	140.7 ± 15.1	127.0 ± 10.2
3	200.5 ± 37.1	142.7 ± 15.3	188.6 ± 15.1	159.0 ± 10.6
4	220.5 ± 39.3	163.2 ± 12.6	225.1 ± 14.8	178.5 ± 11.8
5	230.2 ± 39.2	184.0 ± 13.4	274.3 ± 13.9	231.7 ± 19.4
6	236.3 ± 41.2	191.0 ± 12.5	304.4 ± 13.8	244.3 ± 19.4

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Antioxidant Status of the Hypertrophic Heart of Dahl Hypertensive Rat as a Model for Evaluation of Antioxidants

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SUMMARY

Development of hypertension, myocardial hypertrophy and the cardiac antioxidant status of male Dahl salt-sensitive (DSS) genetically hypertensive rats was evaluated and compared to that of normotensive Dahl salt-resistant (DSR) controls. In order to obtain exaggerated and more severe hypertension, half of the animals (10 per group) were Na loaded (8% NaCl diet) for 6 weeks. The myocardial antioxidant status was estimated in tissue homogenates on the basis of tissue glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). The results showed that 6 weeks of hypertension resulted in left ventricle myocardial hypertrophy, documented by weight, morphometry and morphological changes. The compromised myocardial antioxidant status of the DSS rats was defined by significantly decreased GSH-Px and glutathione activity (13% and 41%, respectively) as compared to DSR rats. SOD in DSS myocardium was increased by 47% compared to that in DSR myocardium, an effect that is considered a compensatory mechanism to the oxidative stress. All of the above changes were exaggerated by NaCl loading. It was concluded that DSS rats, on either a normal or high NaCl diet, displayed decreased antioxidant capacity, which is most likely genetically determined. Before the Dahl rat can be considered as a suitable model for testing new cardiac antioxidants, a full characterization of the level of cardiac oxygen free radicals is required. © 2001 Prous Science. All rights reserved.

Key words: Antioxidant status - Myocardial hypertrophy - Salt-sensitive hypertension

INTRODUCTION

There is increasing clinical and epidemiological evidence to suggest that the association between hypertension and atherosclerosis may be related to the *in vivo* oxidative status of plasma and tissue lipid components (1, 2). Several studies that have investigated the role of oxygen-derived free radicals in cardiovascular disease (CVD), such as ischemic heart disease and stroke, report enhanced lipid peroxidation and reduced endogenous antioxidant capacities from patients exhibiting various CVD risk factors. Elevated levels of lipid peroxides, decreased activities of tissue antioxidant enzymes, such as catalase (CAT) and glutathione peroxidase (GSH-Px), and compensatory increase of superoxide dismutase (SOD) have been observed in patients with coronary heart disease (3, 4). Hypertension has also been reported to coincide with increased production of reactive oxygen species, as well as decreased levels of antioxidant glutathione and reduced GSH-Px activity (5). Thus, these reports suggest a role for oxidative processes in the pathogenesis of both hypertension and atherosclerosis.

Animal models, including various strains of rats, have been used to determine whether CVD risk factors, such as hypertension and hyperlipidemia, influence the development of atherosclerosis, and the data has been used to provide models for better therapeutic strategies (6-9). The antioxidant status of the Dahl salt-sensitive rodent model of hypertension has not been investigated so far, despite it being considered the best combination of hypertension and atherosclerosis development due to the animals' salt sensitivity and the fact that sodium loading exacerbates hypertension and facilitates the development of atherosclerosis.

The objective of the present study was to establish, enzymatically, the antioxidant status of the heart of Dahl salt-sensitive, salt-loaded hypertensive rats and correlate this finding with any pathomorphological changes that may occur in the myocardium. It was hoped that these data could be used to evaluate the potential therapeutic value of antioxidants in the prevention of hypertension-mediated cardiac complications in man.

MATERIALS AND METHODS

Experimental animals

All procedures employed during the course of the study were approved by the ethics committee of the University of Durban-Westville. Twenty 1-month-old male weanling Dahl salt-sensitive (DSS) rats (Group 1)

were matched with 20 male Dahl salt-resistant (DSR) animals (Group 2) as controls. All animals were housed in individual metabolic cages at the Biomedical Resource Center of the University and were exposed to a 12-h light/dark cycle at constant humidity. Each animal received, *ad libitum*, deionized water and a semi-synthetic diet (AIN76), recommended for rodents by the American Institute of Nutrition and South African Research Programme for Nutritional Intervention. Half of the animals from each group were fed a diet containing 1% NaCl (Groups 1A and 2A), while the other half were fed a higher salt diet (8% NaCl) (Groups 1B and 2B) for 6 weeks. Blood pressure and heart rate were measured by using a tail-cuff, computerized blood pressure monitor (IITC Life Sciences 31, USA). The details regarding preparation of diet, feeding and blood pressure measurement are described elsewhere (10). At the conclusion of the experiment (6 weeks) the animals were fasted overnight, anesthetized (40 mg/kg body weight i.p. sodium thiopentone) and exsanguinated by cannulating the left carotid artery.

Biochemical determinations

Tissue sample preparation

The aortic tree (the brachycephalic arteries to their bifurcations and the aorta to the iliac branching) was dissected, opened longitudinally and examined for atherosclerotic lesions. The heart was excised and placed in chilled 50 mM Tris 0.1 mM EDTA, pH 7.6, homogenizing buffer. The heart was blotted dry and weighed; the left ventricle was separated, weighed and kept in nitrogen. The homogenate was prepared in fresh, chilled homogenizing buffer using an Ultra-Turrax TP 18-10 homogenizer (Staufen, Germany). Tissue cytosolic fractions used in enzymatic assays were prepared by ultracentrifugation at 105,000 x g, 15 min at 4 °C, using a Beckman L5-50B ultracentrifuge (USA) with a SW50Ti rotor (Beckman, USA). The cytosolic fractions were assayed for protein content according to Bradford (11). Blood contamination was assayed for Hb (12) and corrections for the red blood cells enzyme activity in the tissue cytosolic fractions were made. Enzyme activity and Hb determinations were carried out using a computerized Cary IE UV-vis spectrophotometer (Varian, Australia).

Glutathione peroxidase activity

Heart GSH-Px (EC 1.11.1.9) activity was assayed according to Paglia and Valentine (13) and expressed on the basis of nanomoles of nicotinamide adenine dinucleotide phosphate (NADPH) oxidized to NADP per minute per milligram protein, using the extinction coefficient of NADPH at 340 nm of 6.22 x 10^6.

Superoxide dismutase activity

Heart SOD (EC 1.15.1.1) activity was assayed according to the method of Winterbourn *et al.* (14) and expressed as units of SOD per milligram protein. One unit of SOD activity is defined as the amount of enzyme activity that causes 50% inhibition of nitroblue tetrazolium (NBT) reduction. The rate of inhibition of NBT reduction by superoxide generated by photoreduction of riboflavin was determined by measuring the absorbance at 560 nm.

Tissue sulfhydryl group content as an indirect measure of glutathione

Cardiac GSH was measured according to the method of Moron *et al.* (15), with minor modifications (16). Tissue homogenate treated with ice-cold 25% trichloroacetic acid (TCA) (Sigma, USA), followed by centrifugation (Eppendorf 5403, Germany) at 12,000 x g, 4 °C, for 5 min, was assayed for acid-soluble sulfhydryl groups at 412 mm using 0.6 mM 5′5′-dithiobis-2-nitrobenzoic acid (DTNB, Sigma) in 0.1 M phosphate buffer, pH 8.0.

Morphological studies

Light microscopy and morphometry

Two hearts from each subgroup were prepared for microscopy. Myocardial tissue from the left ventricle of each heart was immediately immersed in Karnovsky's fixative (17). After being diced into 1-mm cubes and reimmersed in fresh fixative, the tissue was washed in 0.1 M sodium cacodylate buffer (pH 7.2) prior to post-fixation in 1% osmium tetroxide. Thereafter the specimens were dehydrated through graded ethanols, cleared in propylene oxide, perfused with Spurr epoxy resin, embedded in Beem polythene capsules and polymerized in a vacuum embedding oven at 60 °C for 36 h. Thick (1 um) sections for light microscopy and morphometry were cut with glass knives using an Ultracut ultramicrotome and stained with 1% alkaline, toluidine blue. The sections were visualized using a Nikon light microscope with an x40 objective. Morphometric measurements of myofiber diameters were made from oblique/transversely sectioned fibers as per the method of Mars and Gregory (18). In brief, images were displayed on the CRT of a Noran Voyager 2100 image analyzer by means of a video camera interfaced with the microscope. The diameters of approximately 120 fibers were measured from 2 or more sections from each specimen.

Electron microscopy

Having identified areas of interest by light microscopy, expendable areas were removed and ultrathin sections, approximately 60 nm in thickness, were cut using a diamond knife. Sections were stretched with chloroform vapor, picked up on uncoated, 200 mesh copper grids and double stained with Reynold's lead citrate (19) and ethanolic uranyl acetate. The sections were

TABLE 1. Changes in blood pressure (mmHg) and heart rate (beats/min) of Dahl salt-resistant (DSR) and Dahl salt-sensitive (DSS) rats treated with normal NaCl (1%) and high NaCl (8%) diets.

Group/parameter	Control			2-week diet			4-week diet			6-week diet		
	SBP	DBP	HR	SBP	DBP	HR	SBP	DBP	HR	SBP	DBP	HR
DSR normal NaCl (1%) [10]	128 ± 2.7	80 ± 1.7	459 ± 9.2	138 ± 4.1	88 ± 3.7	462 ± 9.0	128 ± 1.5	84 ± 3.5	474 ± 12.7	134 ± 3.5*	78 ± 3.9	440 ± 10.2
DSR high NaCl (8%) [10]	115 ± 2.0	70 ± 3.9	477 ± 8.8	127 ± 4.3	74 ± 4.5	486 ± 11.5	133 ± 5.0*	92 ± 7.1*	423 ± 12.3	132 ± 5.9*	82 ± 4.1	435 ± 7.3
DSS normal NaCl (1%) [12]	116 ± 1.9	77 ± 1.3	445 ± 18.6	120 ± 2.1*	81 ± 2.0	436 ± 14.4	162 ± 7.8*+	106 ± 7.1*+	496 ± 12.1*	150 ± 7.3*+	106 ± 7.5*+	481 ± 14.0*+
DSS high NaCl (8%) [12]	116 ± 2.5	78 ± 1.2	478 ± 19.2	156 ± 3.4*+	93 ± 3.3*+	461 ± 19.8	162 ± 7.6*+	109 ± 8.0*	531 ± 14.6*+	174 ±3.3*+	124 ± 7.1*+	487 ± 18.9

Mean ± SEM. The number in brackets denotes the number of animals. *Significant compared to control value of the same group; +significant compared to the respective DSR group. SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate.

TABLE 2. Changes in body weight, heart weight, left ventricle weight and the respective ratios of Dahl salt-resistant (DSR) and Dahl salt-sensitive (DSS) rats on normal (1% NaCl) and high sodium (8% NaCl) diets.

Group/parameter	Body weight (g)	Heart weight (g)	Left ventricle weight (g)	Heart weight/body weight (g/g x 100)	Left ventricle weight/ body weight (g/g x 100)	Myofiber diameters (um)
DSR 1% NaCl diet	226 ± 6.0	0.827 ± 0.04	0.462 ± 0.01	0.36 ± 0.01	0.20 ± 0.01	15.51 ± 0.21 [363]
DSR 8% NaCl diet	198 ± 4.1*	0.692 ± 0.01*	0.410 ± 0.01	0.35 ± 0.01	0.21 ± 0.01	17.96 ± 0.23* [428]
DSS 1% NaCl diet	259 ± 3.7+	1.156 ± 0.03+	0.688 ± 0.02+	0.43 ± 0.03+	0.27 ± 0.01+	16.40 ± 0.27+ [390]
DSS 8% NaCl diet	241 ± 4.2*+	1.254 ± 0.03+*	0.648 ± 0.02*+	0.2 ± 0.03+*	0.27 ± 0.01+	19.09 ± 0.27++* [414]

Values are presented as mean ± SEM. The number in brackets shows the number of myofibers measured. +Significant compared to the respective DSR group. *Significant compared to the respective group on normal diet.

examined with a Jeol 1010 transmission electron microscope at 60 Kv.

Statistical analysis

The results are presented as a mean value ± standard error of the mean (SEM). For all analyses the INSTAT V2.04 program was used, including the one-way ANOVA, *t*-test and chi-square test. A p value < 0.05 was considered statistically significant.

RESULTS

The results of blood pressure and heart rate measurement are presented in Table 1. They indicate that DSS rats developed hypertension with tachycardia at the end of the fourth week (2 months old), irrespective of the diet. The DSR rats remained normotensive with a slight increase of blood pressure, corresponding to their age. Sodium chloride loading (8% sodium NaCl) exacerbated the hypertensive state of DSS rats. The results of body weight, heart weight, left ventricle weight and the respective ratios are presented in Table 2. They showed that DSS rats gained weight more rapidly than DSR rats. The hypertension in DSS rats was accompanied by significant cardiac hypertrophy, more specifically left ventricle hypertrophy.

Morphometry

The morphometric data are presented in Table 2. Figures 1 and 2 show the marginal distribution of fiber diameters in Groups 1A and 1B and 2A and 2B. The chi-square test showed a significant difference in the marginal distribution of fiber diameters in Group 1A and 1B and 2A and 2B data. The data showed that in both cases, myofiber diameters were larger after exposure to 8% NaCl in the diet.

Tissue antioxidant analysis

The results are presented in Table 3 and can be summarized as follows: a nonsignificant increase in SOD activity coupled with a significantly decreased GSH-Px and glutathione activity in the heart of both hypertensive DSS groups on normal and high Na diets as compared to the enzymes of DSR groups was found. Sodium chloride loading influenced *per se* the enzymes by further increasing SOD activity and decreasing GSH-Px and glutathione in both DSR and DSS groups.

Heart morphology

Myofibers in Group 2A myocardium contained normal nuclei, mitochondria and other sarcoplasmic organelles. The fibers were closely packed together and interspersed by morphologically normal capillaries (Fig. 3). Group 1A myocardium was also populated with myofibers containing typically normal nuclei, myofibrils and mitochondria (Fig. 4). Intercalated disks appeared morphologically normal but there was evidence of swelling of sarcoplasmic, reticulum and T-tubes (Fig. 5). Although measurements were not made, there appeared to be a subjective increase in the space between fibers in

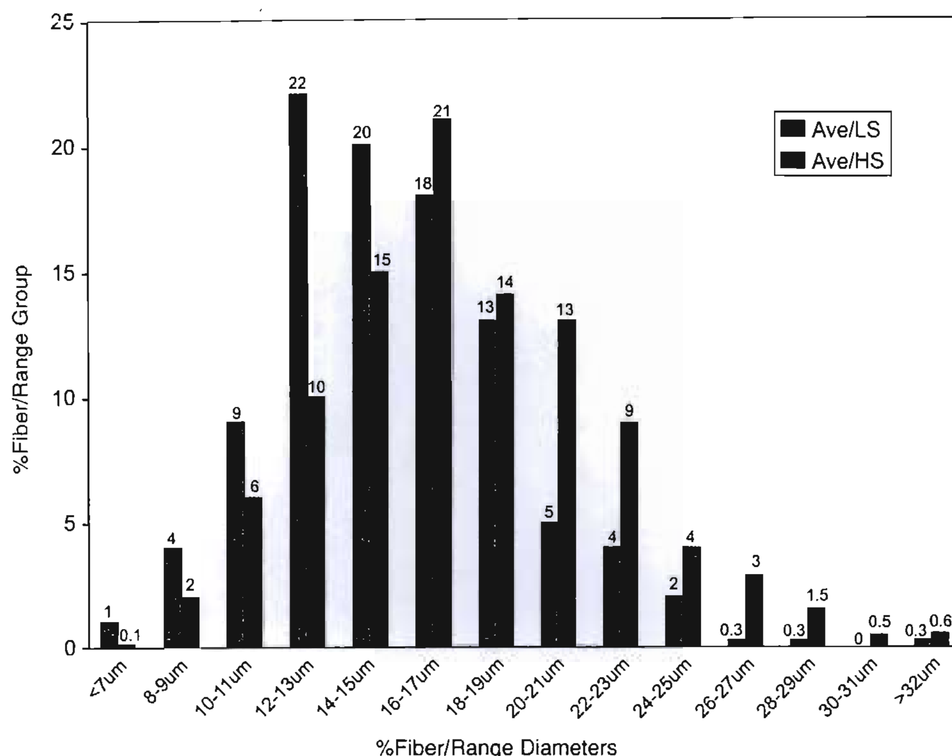

FIG. 1. The marginal distribution of fiber diameters in DSR rats (Group 2). Note there is a significant skewing of the distribution to larger diameters in animals fed with a high salt diet (Group 2B: Ave/HS). LS: low salt; HS: high salt.

TABLE 3. Activities of antioxidant enzymes in left ventricle heart tissue of Dahl salt-resistant (DSR) and salt-sensitive (DSS) rats on normal (1%) and high (8%) sodium chloride diets.

Group/parameter	DSR 1% NaCl	DSR 8% NaCl	DSS 1% NaCl	DSS 8% NaCl
Glutathione peroxidase (nmol NADPH.min^{-1}·mg^{-1} protein)	131 ± 5.6	84 ± 8.6*	114 ± 4.5^{+}	93 ± 7.0*
Superoxide dismutase (u/mg protein)	12.70 ± 2.8	19.47 ± 2.8	18.67 ± 2.4	22.78 ± 3.2
Glutathione (nmol/mg tissue)	2.91 ± 0.55	3.12 ± 0.42	1.72 ± 0.14^{+}	2.52 ± 0.26*

Values are means ± SEM, n = 10; $^{+}$Significant strain difference (DSS *vs.* DSR); *Significant difference between normal (1%) and high (8%) sodium chloride diet in the same strain. All values are corrected for RBC contamination.

some specimens. Many myofibers in group 2B myocardium exhibited pathomorphological alterations. These included crenation of nuclei intermyofibrillar vacuoles, especially in the vicinity of sometimes mildly disassociating intercalated disks, and mitochondrial swelling and occasional crystolysis (Fig. 6). While interfiber spaces appeared to have increased, endothelial cells and capillaries within these spaces appeared normal. There were considerable pathomorphological alterations in most myofibers in the myocardium of Group 1B specimens. In some fibers, while mitochondria appeared normal, nuclei were crenated and the central core of fibers appeared to have become edematous (Fig. 7). Such areas contained numerous membrane bound vacuoles, myofibrils appeared to be lysing into the edematous pool and no obvious sarcoplasmic organelles were present. Mitochondria were often swollen and in various phases of crystolysis and degeneration. These were often observed in close proximity to intercalated disks (Fig. 8). Intercalated disks appeared to be separating (Fig. 9) and in one instance, an endothelial cell had infiltrated into the space between the two lamina of an intercalated disk suggesting that such separation had been proceeding for a considerable time before sacrifice of the animal (Fig. 10). While there was considerable interfiber edema, endothelial cells and capillaries appeared normal.

DISCUSSION

It is still unresolved whether elevated blood pressure alone represents a risk for cardiovascular complications in hypertensive subjects (20). Few studies have reported a direct link between high blood pressure *per se* and the

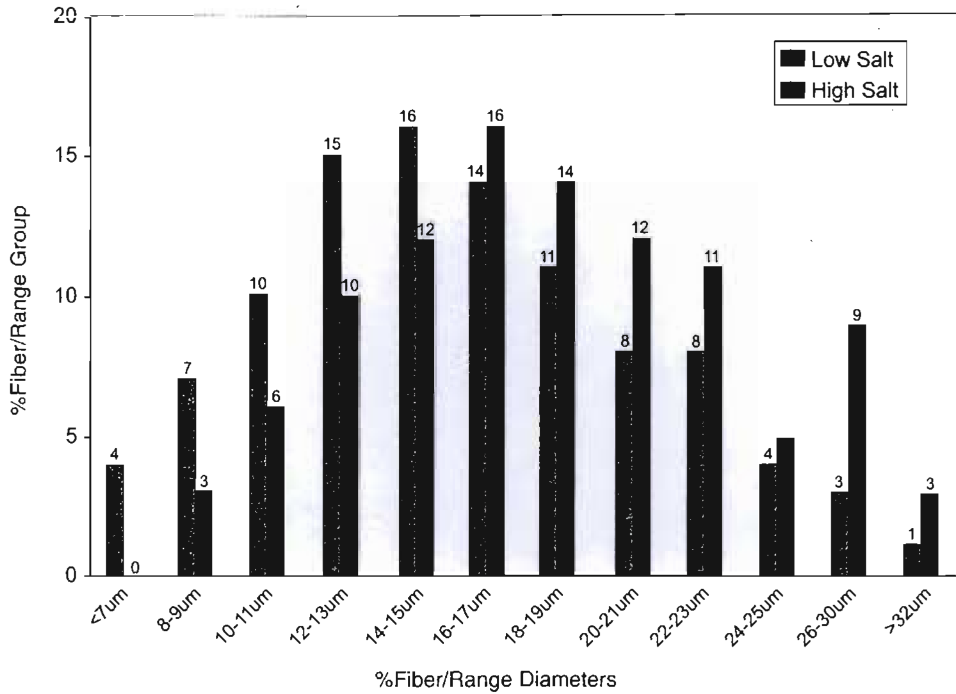

FIG. 2. The marginal distribution of fiber diameters in DSS rats (Group 1). Note there is a significant skewing of the distribution to larger diameters in animals fed with a high salt diet (Group 1B: high salt).

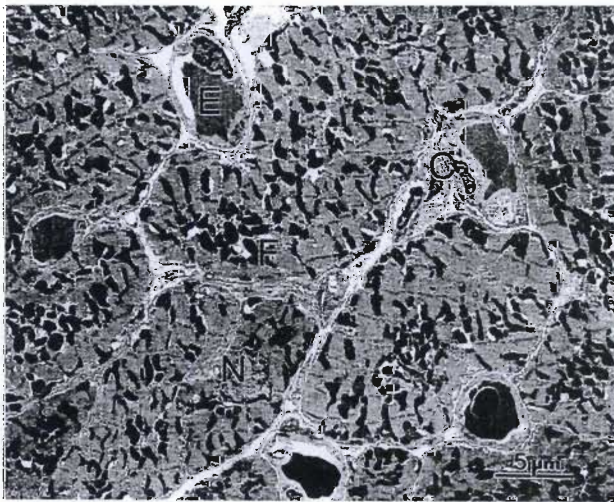

FIG. 3. A transmission electron micrograph (TEM) showing oblique/cross-sectioned myofibers in Group 2A (DSR: control) animals. Note tightly packed fibers (F) interspersed with morphologically normal capillaries (C). E = erythrocytes; N = nucleus.

hypertensive complications, without indications of the mechanisms that predispose hypertensive patients to organ injury, more specifically the heart. Recent studies suggest that the progression of the hypertensive state in Dahl salt-sensitive rats was accompanied by overproduction of oxygen free radicals in the circulation (20) and myofibers (21).

Left ventricle cardiac hypertrophy is one of the early signs of development of hypertension, developing as a result of the increased cardiac afterload due to high blood pressure. Cardiac hypertrophy is considered to be an independent risk factor for coronary heart disease (22), along with other factors such as smoking, dyslipidemia, diabetes and obesity. In many cases, these risk factors tend to coexist, acting synergistically to increase the risk of atherogenesis (16).

It was reported that the *in vivo* antioxidant status of spontaneously hypertensive rats (SHR) was associated with a greater susceptibility of myocardial tissue to lipid

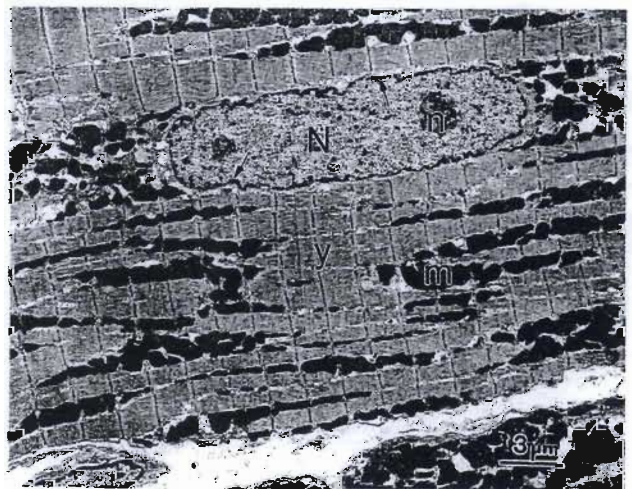

FIG. 4. TEM: Longitudinal section of myofiber from Group 1A (DSS) animals. Note normal arrangement of myofibrils (y) interspersed with healthy mitochondria (m). n = nucleolus; peripheral chromatin (arrows).

FIG. 5. TEM: Group 1A: Intercalated disks appeared normal (Id), but some mitochondria were swollen as were some T-tubes and elements of the sarcoplasmic reticulum (arrows).

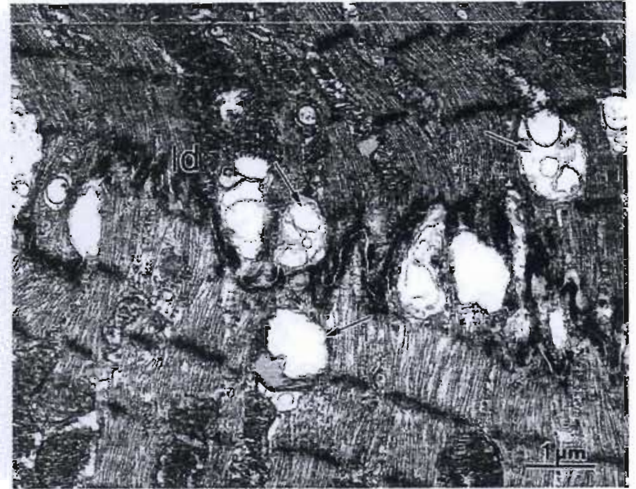

FIG. 8. TEM: Group 1B: Edematous and necrotic mitochondria (arrows) in the vicinity of an intercalated disk. Note myelin figures within vacuolated mitochondria.

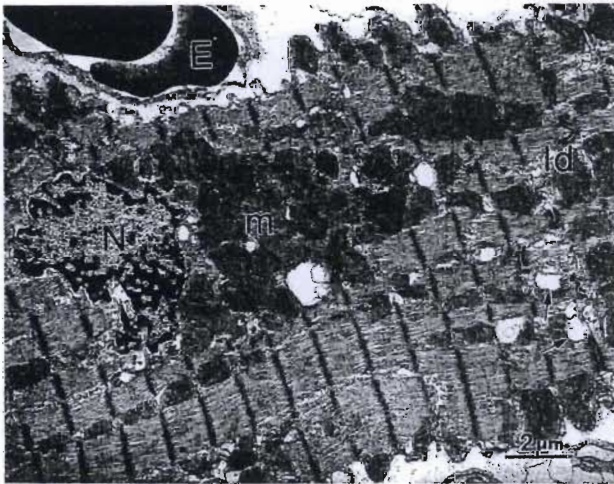

FIG. 6. TEM: Group 2B: Myofiber with crenated nucleus. Note vacuoles (arrows) in the vicinity of a normal intercalated disk.

FIG. 9. TEM Group 1B: Separation of intercalated disk. Note numerous necrotic mitochondria undergoing crystolysis near the Id.

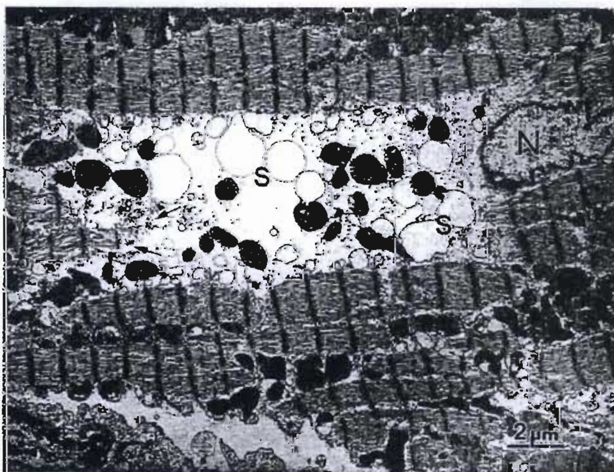

FIG. 7. TEM: Group 1B: Myofibrilysis in central core of a myofiber. Note vacuolated sarcoplasmic reticulum(s) and myofilaments dispersing into the central space (arrows).

FIG. 10: TEM: Group 1B: Separated intercalated disk. Note many mitochondria exhibit crystolysis and a portion of a morphologically healthy endothelial cell (e) has penetrated the interfiber space.

peroxidation (16, 23), but the factors behind myocardial vulnerability in SHR hypertrophied heart are still unknown, since SHR as any rodents, are resistant to atherosclerosis (16). Since the hypertrophied myocardium may undergo relative hypoxia, membrane lipids could be peroxidated by free radicals and thus may be, independent of atherosclerosis, a causative factor in hypertensive myocardial cell vulnerability. In our experiments on young Dahl rats there was no macroscopic evidence of the development of aortic atherosclerotic plaque or lesions and no electron microscopic changes in myocardial arterioles. The development of early (at 2 months of age) hypertension was evident and despite the short duration of the hypertensive state (4 weeks), left ventricle cardiac hypertrophy was prominent, as documented by weight and morphometry.

The electron microscopic results showed a difference between the hypertrophic hearts of DSS rats on normal and high NaCl diets. The former group, despite being hypertensive, showed morphologically less myocardial changes. The sodium-loaded DSS rats showed edema, necrosis and loss of myofibrils. The morphological changes in both DSS groups were paralleled with milder antioxidant vulnerability in DSS rats on normal diet, and distinctly decreased antioxidant status in sodium-loaded DSS rats. The changes in the latter group could be explained by a direct effect of Na^+. It is believed that the hormonal or neurotransmitter-induced inhibition of adenylate cyclase in various membrane systems is regulated by Na^+ (24) and the regulatory effect of Na^+ on the receptor-adenylate cyclase complex is exerted throughout binding to the inner aspect of the plasma membrane, an effect similar to that of the sympathetic nervous system (25, 26). These membrane changes may induce alterations in membrane-associated enzyme activities such as Na^+/K^+- ATPase (27), and even in intracellular Ca^{2+} concentrations (28). Whatever the mechanism of Na^+ that produced damage is, it may lead to free oxygen radical generation causing peroxidation of the plasma membrane phospholipids (29, 30).

Recently we reported (31) that the Dahl salt-sensitive rat is a model of nondiabetic, nonobese insulin resistance with secondary hyperinsulinemia. In studies investigating the effect of insulin on *in vivo* antioxidant enzyme activity (32), it was demonstrated that insulin was inhibitory to glutathione reductase activity, meaning that this mechanism might additionally contribute to suppression of antioxidants in DSS rats.

The compromised antioxidant status in the myocardium of DSS rats was defined by the significantly decreased GSH-Px and glutathione activity (13% and 41 %, respectively) compared to the activity in DSR rats. It could be speculated that suppressed antioxidant activity in DSS rats as compared to their DSR controls is genetically determined as a result of the salt sensitivity and NaCl retention and insulin resistance. SOD activity, in the DSS myocardium, was increased by 47% compared to that in the DSR myocardium. This late result may indicate a compensatory antioxidant enzyme response to enhanced levels of reactive oxygen species (ROS), more specifically H_2O_2, either in the heart or in the circulation of the DSS rat (20). Studies that have investigated the role of oxygen-derived free radicals in CVD reported that ROS production was increased in hypertensive state (33). Regarding DSS hypertensive rats, the compensatory increased myocardial SOD activity showed that the rats still have the ability to respond to cardiac oxidative stress.

In summary, in our animal model of the Dahl salt-sensitive hypertensive rat we found decreased myocardial antioxidant activity, which is most likely genetically determined. The cardiac hypertrophy consequent to development of hypertension *per se* could further reduce the antioxidant status of the hypertensive animals, most likely due to relative hypoxia. In sodium chloride loaded hypertensive animals, the damaging effect of Na^+ could explain the exaggerated development of hypertension, the disintegration and necrosis of hypertrophied myofibrils, leading to distinct alterations in antioxidant enzyme profiles with an increased susceptibility to *in vitro* lipid oxidation. The insulin-resistant state of Dahl hypertensive rats might contribute to the above changes.

Dahl salt-sensitive hypertensive rats, on either a normal or high NaCl diet, displaying increased susceptibility to lipid oxidation and decreased antioxidant capacity of cardiac myofibrils, could be a suitable model for testing new cardiac antioxidant drugs.

ACKNOWLEDGMENTS

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