OXIDATIVE STRESS OF TISSUE IN HYPERTENSIVE DAHL RATS

by

Melvin M. Govender

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Submitted in partial fulfilment of the requirements for the degree of Masters Medical Science (M.Med.Sc.) in the Department of Human Physiology and Physiological Chemistry, in the School of Basic and Applied Medical Sciences, in the Faculty of Health Science at the University of Kwa-Zulu Natal – Westville Campus.

Supervisor: Dr A. Nadar

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DEDICATION

The following study is dedicated to both my parents, for without their undying love, guidance, moral and financial support, my attempt at academic success would be a futile one. This study is a physical manifestation of the strong educational philosophy that they have instilled in me, and therefore duly dedicated to them.

DECLARATION

I, Melvin Megandran Govender, Student Number: 200000375, hereby declare that the dissertation/thesis entitled:

Oxidative Stress of Tissue in Hypertensive Dahl Rats,

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 or Tertiary Institution. Where use was made of the work of others, it is duly acknowledged in the text. The research done in this study was carried out under the supervision of Dr A. Nadar.

M.M. Govender

11/04/06

Date

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LIST OF ABBREVIATIONS

Ang II	:	Angiotensin II
ANOVA	:	Analysis of Variance
BSA	:	Bovine Serum Albumin
CAT	:	Catalase
cGPx	:	"Classic" Glutathione Peroxidase
DNA	:	Deoxyribonucleic Acid
DSS	:	Dahl Salt Sensitive
DSR	:	Dahl Salt Resistant
EC	:	Endothelial Cells
FER	:	Food Efficiency Ratio
GPx	:	Glutathione Peroxidase
GSH	:	Reduced Glutathione
GSSG	:	Oxidized Glutathione
H_2O	:	Water
H_2O_2	:	Hydrogen Peroxide
HAE	:	4-Hydroxyalkenals
HIV	:	Human Immunodeficiency Virus
HRPO	:	Horseradish Peroxidase
HS	:	High Salt Diet (2% NaCl)
MDA	:	Malonyldialdehyde
NaCl	:	Sodium Chloride
NADPH	:	Nicotinamide Adenine Dinucleotide Phosphate
NO	:	Nitric Oxide

NS	:	Normal Salt Diet
O_2	:	Molecular Oxygen
O_2^-	:	Superoxide Radical
OH^-	:	Hydroxyl Radical
$ONOO^-$	:	Peroxynitrite
PDGF	:	Platelet Derived Growth Factor
PHGPx	:	Phospholipid Hydroperoxide Glutathione Peroxidase
PRS	:	Phenol Red Solution
RNS	:	Reactive Nitrogen Species
ROS	:	Reactive Oxygen Species
RPM	:	Revolutions per Minute
SEM	:	Standard Error of Mean
SHR	:	Spontaneously Hypertensive Rat
SOD	:	Superoxide Dismutase
SOD-1	:	Cytosolic Copper/Zinc - containing Superoxide Dismutase
SOD-2	:	Mitochondrial Manganese - containing Superoxide Dismutase
SOD-3	:	Extracellular Superoxide Dismutase
TBA	:	Thiobarbituric Acid
UV	:	Ultraviolet
VSMC	:	Vascular Smooth Muscle Cells
W/L	:	Wall to Lumen Ratio
WHO	:	World Health Organisation

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ABSTRACT

Oxidative stress, resulting from an antioxidant/free radical imbalance, is considered to be an important etiologic factor in the patho-physiological changes associated with salt sensitive hypertension. An important unresolved issue in hypertension research is the mechanism for organ damage during the development of the syndrome. Reactive oxygen species (ROS) such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-), may play a critical role in the pathogenesis of hypertension by targeting the very tissue that is responsible for regulating blood pressure, during the hypertensive state.

Thus, this study was undertaken to evaluate the antioxidant and free radical status in the DSS rat strain, which has been shown to be an excellent model of salt sensitive hypertension. The antioxidant status was evaluated on the basis of the vascular superoxide dismutase (SOD) and glutathione peroxidase (GPx) levels, and the free radical status was evaluated on the basis of the plasma H_2O_2 concentration. The levels of malonyldialdehyde (MDA), which is a bio-marker for lipid peroxidation was used to determine the level of oxidative stress in the kidney, liver and brain. The kidney and liver were also subjected to an induced free radical mediated lipid peroxidation, by exposing the tissue to increasing known concentrations of H_2O_2 (2.5mM – 15mM). The level of lipid peroxidation was used to assess the tissues antioxidant buffering capacity to an induced free radical "attack".

The results have shown that the DSS strain may have a compensatory increase in vascular SOD levels, to counter an increase in O_2^-. SOD levels were significantly lower during salt loading. The GPx levels were significantly lower in the DSS strain,

and showed a slight increase during salt loading. The results demonstrate that the DSS strain has a compromised antioxidant status compared to the DSR strain. The plasma H_2O_2 concentration displayed non-significant changes in the DSS strain, however salt loading did result in a non-significant increase in the plasma H_2O_2 concentration in the DSS strain. The GPx : H_2O_2 ratio, demonstrated an inadequate increase in GPx levels during salt loading to neutralise this non-significant increase in H_2O_2 concentration.

The kidney showed an increased level of *in vivo* lipid peroxidation, which could implicate increased tissue damage, and thus confirm the kidney as being a target organ during the hypertensive state. The liver and brain showed non-significant differences in the level of *in vivo* lipid peroxidation and are therefore thought not to be target tissue in the hypertensive state. The kidney displayed a decreased antioxidant buffering capacity to the induced free radical "attack", thereby demonstrating the tissue's decreased ability to neutralise an increased free radical level. Although the liver displayed a "normal" level of *in vivo* lipid peroxidation, it also displayed a decreased antioxidant buffering capacity to an induced free radical "attack", showing that the liver is able to cope with *in vivo* free radical levels, but at higher free radical levels, its loses its ability to quench a free radical "attack" and thereby minimise lipid peroxidation. The *in vivo* lipid peroxidation levels of the kidney, liver and brain have shown that tissues have varying abilities to cope with tissue oxidative stress, and behave differently, in their free radical quenching abilities.

These results have shown that a compromised free radical and antioxidant status results in oxidative damage to the tissue responsible for regulating blood pressure.

1. INTRODUCTION

Free radicals are constantly being generated in the body, as a result of normal metabolic processes. Under physiological conditions, damage due to free radicals is countered by antioxidants (Halliwell and Gutteridge., 1999). When excessive free radical formation occurs in the body, and/or there are compromised levels of antioxidants, the body cannot cope, i.e. the pro-oxidants overwhelm the antioxidants, and the resulting situation is referred to as oxidative stress. Thus oxidative stress is a general term used to describe a state of potential damage caused by free radicals (Halliwell and Gutteridge., 1999).

Compelling experimental evidence indicates that reactive oxygen species (ROS) play an important patho-physiological role in the development of hypertension. This is due, in large part to a relative increase in superoxide (O_2^-) and hydrogen peroxide (H_2O_2) levels and the resultant decreased nitric oxide bioavailability in the vasculature and kidneys (Touyz., 2004).

Oxidative damage to tissue has also been implicated in a wide variety of human diseases such as, atherosclerosis, diabetes and cancer. An important unresolved issue in hypertension research is the mechanism of organ damage during the development of the syndrome. Reactive oxygen species such as O_2^-, H_2O_2 and the hydroxyl radical (OH^-), may play a critical role in the pathogenesis of hypertension, and its associated organ damage (Swei *et al.*, 1997).

Another issue that remains unresolved is whether the increased oxidative stress is due to an increase oxygen free radical concentration or due to a decrease in antioxidant

production. Although the deleterious effects of free radicals in biological systems have been previously demonstrated, their role in kidney and liver damage in salt sensitive hypertension has not been evaluated (Jacob., 1995, Yuan., 1998). Since these organs play an important role in metabolism and blood pressure regulation, attack by free radicals could well contribute to organ damage that is evident in hypertension.

The Dahl rat has been established as an excellent model of salt sensitive hypertension. Therefore this study determined the antioxidant status of this model by assessing the levels of superoxide dismutase (SOD) in the red blood cell and the level of glutathione peroxidase (GPx) in whole blood. The free radical status was determined by using the plasma H_2O_2 concentration as an indicator of the relative free radical concentration *in vivo*. This, being used because H_2O_2, is thought to be one of the few ROS, that is intimately involved with the hypertensive state. The status of both these parameters will be able to show whether a compromised antioxidant and/or free radical status results in the increased level of oxidative stress associated with hypertension.

The level of lipid peroxidation of the kidney, liver and brain would be used as an indicator of tissue oxidative stress with potential organ damage. This would enable the study to determine the role that tissue oxidative stress with resultant organ damage plays during the hypertensive state. Thus, with these parameters it would be possible to identify the role that free radicals and antioxidants play in either affecting or contributing to the hypertensive state displayed by this model of salt sensitive hypertension.

Thus this study would provide a means of assessing the role that antioxidant therapy may play in minimising the level of tissue damage associated with hypertension. Along with this, the research undertaken in this study would provide further depth into the putative role that increased free radicals and decreased antioxidant levels have, with regard to the deleterious effects of hypertension related organ damage. Thus the depth provided by this research would allow further preventative research to be developed.

2. LITERATURE REVIEW

2.1. Hypertension

Blood pressure is a normally distributed variable with no distinction between normal, high and low levels. There is a positive correlation between high blood pressure and the risk of associated organ damage. This risk increases with increasing levels of blood pressure (Hickey and Graham.,1988). Shapiro *et al.*, (1991) defined hypertension as the level of blood pressure associated with an increased morbidity and mortality. Although a more general clinical definition states that hypertension is that level of blood pressure at which detection and treatment does more good than harm and this offers a new challenge to define hypertension as a risk factor rather than a pathological condition (Evans and Rose., 1971).

Hypertension, or a persistent high blood pressure, is defined by The World Health Organisation (WHO Guidelines., 1999) as a systolic pressure of 140mm Hg or greater and diastolic blood pressure of 90mm Hg or greater *(Table 1.)*.

Table 1. Classification system to rank blood pressure values for adults - (WHO Guidelines., 1999).

	Systolic (mmHg)	**Diastolic (mmHg)**
NORMAL	Less than 130 mmHg	Less than 85 mmHg
HIGH-NORMAL	130 – 139 mmHg	85 – 89 mmHg
HYPERTENSION	140 mmHg or greater	90 mmHg or greater
Stage 1 (Mild)	140 – 159 mmHg	90 – 99 mmHg
Stage 2 (Moderate)	160 – 179 mmHg	100 – 109 mmHg
Stage 3 (Severe)	180 – 209 mmHg	110 – 119 mmHg
Stage 4 (Very Severe)	210 mmHg or greater	140 mmHg or greater

2.1.1. Types and causes of Hypertension

A persistently elevated blood pressure that cannot be attributed to any identifiable cause is termed primary (essential) hypertension (Tortora and Grabowski., 1996). In the region of 90-95% of all hypertension cases fit this definition. It is suspected that several factors combine to predispose a person to hypertension, including diet, lack of exercise, metabolic effects, stress, and heredity, thereby manifesting itself as a multi-factorial disease. The remaining 5-10% of cases is secondary hypertension which results as a consequence of other disease states such as kidney disease, endocrine disorders and complications to some drug treatments.

2.1.2. Effect of Dietary Sodium in Hypertension

The evidence that salt has a definitive link with hypertension is not difficult to marshal. The relationship between dietary salt intake and the development of hypertension has been the subject of continuing debate for decades. There is abundant epidemiological and experimental observations demonstrating a link between salt and blood pressure (Weinberger., 1996). Although many controversies still exist regarding the role of dietary sodium intake in the pathogenesis of hypertension, it is accepted that there is a strong positive correlation between sodium intake and hypertension, and even modest dietary sodium excess induces hypertension in salt-sensitive individuals who are genetically susceptible (Laragh and Brenner., 1990).

2.2. Salt Sensitivity

Salt sensitivity is defined as the interindividual difference in the blood pressure response to changes in dietary sodium chloride intake; it results in an alteration in the slope of the pressure-natriuresis relationship (Strazzullo., 2003). Salt sensitivity is a common trait in patients with essential hypertension and seems to have both an inherited and an acquired component (e.g. is influenced by aging and renal insufficiency). Studies show that for a given load of dietary salt, the blood pressure response is variable amongst hypertensives and normatensives. Salt-Sensitive hypertensives experience a significant rise in blood pressure when switching from a low salt to a high-salt diet (Weinberger., 1996).

Salt-sensitive hypertension, which is due to alterations in dietary sodium, is apparent from studies in both humans and certain animal models. The relevance of salt-sensitive hypertension lies in demographical studies that have found people of African

heritage to consistently have a greater frequency of salt sensitivity, when compared to other demographic groups (Weinberger., 1996). The development of animal models reinforced the concept that varying levels of salt intake result in varying levels of blood pressure. Dahl *et al.*, (1962) developed the Dahl Sensitive and Resistant rat strains, these strains demonstrated a marked sensitivity and resistance, respectively, with respect to the ability of a high-salt intake to raise blood pressure.

Although salt sensitivity and sodium sensitivity of blood pressure are often thought of interchangeably, considerable evidence suggests that both sodium and chloride must be provided to fully express salt sensitivity (Laragh and Brenner., 1990). Thus, in animal models, feeding sodium without chloride or chloride without sodium does not raise the blood pressure to the extent that NaCl feeding does (Boeghold and Kotchen., 1991).

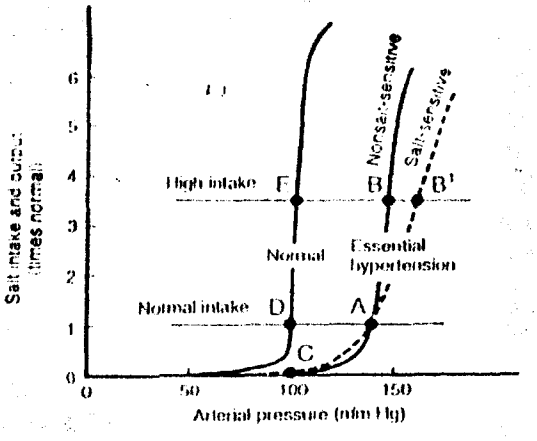


Figure 1. Analysis of arterial pressure regulation in salt-sensitive and non-salt-sensitive essential hypertension

In all forms of chronic hypertension, the renal pressure-natriuresis mechanism is abnormal because sodium (chloride) excretion is the same as in the normotensive individual, despite an increase in blood pressure. A resetting of the pressure natriuresis relationship necessitates an increased blood pressure to maintain sodium balance. By analysing the characteristics of the pressure natriuresis relationship in hypertensive animals and by making comparisons with normotensive controls, it is possible to gain insight into blood pressure-elevating mechanisms in experimental animal models. The underlying abnormality in sodium excretion may involve the kidneys or other hormonal factors. A parallel, rightward shift of the pressure-natriuresis relationship *(Fig. 1)* is characteristic for a non–salt-sensitive (resistant) form of hypertension. A decreased slope in the pressure-natriuresis relationship indicates the presence of a salt-sensitive form of hypertension (Gross *et al.,* 1997).

2.2.1. Salt Sensitivity and Hypertension

The underlying etiology of salt-sensitive hypertension is multi-factorial and complex. This is partly because it represents a syndrome rather than a specific disease entity and also because of the difficulty in defining the characteristics of the disease (Sanders., 1996). The importance of sodium as a determinant of blood pressure has been substantiated by the numerous epidemiological, clinical and experimental evidence, which supports a positive correlation between sodium and hypertension. Thus the role for excess salt ingestion in the genesis and development of hypertension is well established. It is the exact mechanism by which it engenders an increased blood pressure that is under investigation by current studies.

It has been reported that for a given dietary salt load, the blood pressure response varies amongst hypertensives and normotensives (Sullivan *et al.,* 1988). It is this variable response that gives rise to the concept of salt sensitivity and blood pressure (Santello *et al.,* 1997).

Ferri *et al.,* (1998) characterized salt-sensitive hypertension as a cluster of renal, hormonal, and metabolic derangements that might favour the development of cardiovascular and renal complications. Although there is strong evidence that the kidney is the final common pathway in the long-term control of blood pressure, the initial elevation in blood pressure need not be attributed necessarily to the kidney. This elevation could be attributed to other factors such as metabolic defects, genetic defects, and diet (Cowley *et al.,* 1995). A variety of factors can lead to a reduction of renal excretory function and result in hypertension. These include circulating hormones such as angiotensin II, aldosterone, atrial natriuretic peptide and renal sympathetic nerve activity, which all have an important influence on the pressure-natriuresis relation and lead to various forms of hypertension.

2.3. Free Radicals

Atoms are most stable in the ground state. An atom is considered to be "ground" when every electron in the outermost shell has a complimentary electron that spins in the opposite direction. By definition a free radical is any atom (e.g. oxygen, nitrogen) with at least one unpaired electron in the outermost shell, and is capable of independent existence. A free radical is easily formed when a covalent bond between entities is broken and one electron remains with each newly formed atom. Free radicals are highly reactive due to the presence of these unpaired electron(s) (Karlsson., 1997). When free radicals donate an electron to, or accept an electron from a surrounding compound or molecule, that compound or molecule becomes a free radical itself. This process initiates a self-perpetuating chain reaction that ultimately results in tissue damage.

2.3.1. Reactive Oxygen Species (ROS)

Any free radical derived from oxygen can be referred to as reactive oxygen species (ROS) (Goldfarb, A. H., 1999). Molecular oxygen in the ground state is a bi-radical, containing two unpaired electrons in the outer shell (also known as a triplet state). Since the two single electrons have the same spin, oxygen can only react with one electron at a time and therefore it is not very reactive with the two electrons in a chemical bond. On the other hand, if one of the two unpaired electrons is excited and changes its spin, the resulting species (known as singlet oxygen) becomes a powerful oxidant as the two electrons with opposing spins can quickly react with other pairs of electrons, especially double bonds. The reduction of oxygen by one electron at a time produces relatively stable intermediates. The superoxide anion ($O_2^{-}\bullet$), which is a product of one-electron reduction of oxygen, is the precursor of most ROS and a

mediator in oxidative chain reactions. Dismutation of $O_2^{-}\bullet$ (either spontaneously or through a reaction catalysed by superoxide dismutase) produces hydrogen peroxide (H_2O_2), which in turn may be fully reduced to water or partially reduced to hydroxyl radical (OH•), one of the strongest oxidants in nature. (Reaction series shown below) (Turrens., 2003).

$O_2 + e^- \rightarrow O_2^{-}\bullet$ - Superoxide Radical *(1)*

$O_2 + H_2O \rightarrow HO_2\bullet + OH^-$ - Hydroperoxyl radical *(2)*

$HO_2 + e^- + H \rightarrow H_2O_2$ - Hydrogen Peroxide *(3)*

Superoxide ($O_2^{-\bullet}$) and hydroxyl ($OH^{-\bullet}$) are examples of reactive oxygen radicals. However, the term reactive oxygen species can also refer to oxygen-derived non-radicals such as hydrogen peroxide (H_2O_2), ozone (O_3), hypochlorus acid (HOCl) and singlet oxygen ($^1O_2$). Nitric oxide ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) are nitrogen radicals, but the term ROS also encompasses certain non-radicals such as nitrous acid (HNO_2) and peroxynitrate ($ONOO^{-\bullet}$) Table 2. (Halliwell *et al.*, 1992).

Table 2. Reactive Oxygen Species

Species	Common Name	Half-Life (37^0C)
$HO^{\bullet}$	Hydroxyl radical	1 nanosecond
$HO_2^{\bullet}$	Hydroperoxyl radical	unstable
$O_2^{\bullet}$	Superoxide anion radical	enzymatic
$^1O_2^{\bullet}$	Singlet oxygen	1 microsecond
$RO^{\bullet}$	Alkoxyl radical	1 microsecond
$ROO^{\bullet}$	Peroxlyl radical	7 seconds
$NO^{\bullet}$	Nitric oxide radical	1-10 seconds
H_2O_2	Hydrogen peroxide	stable
HOCl	Hypochlorus acid	stable
R = lipid		

Reactive oxygen species are found in several cells including macrophages and vascular smooth muscle cells. At low concentrations reactive oxygen species can act as physiological mediators of cellular responses such as signal transduction, cell growth and inflammation. At higher concentrations ROS may cause cell damage and death (Irani., 2000). The major sources of reactive oxygen species are "leakages" from the electron transport chains of mitochondria and endoplasmic reticulum. Only 1–2% of these electrons are "leaked" to generate superoxide radicals in reactions mediated by coenzyme Q and ubiquinone and its complexes. Neutrophils and macrophages produce reactive oxygen species during phagocytosis ('oxygen burst') or stimulation with several agents through the activation of nicotinamide adenine dinucleotide phosphate reduced [NAD(P)H] oxidase that is assembled at the plasma membrane from resident plasma membrane components and cytosolic protein components (Forman and Torres., 2002). NAD(P)H oxidase is also, a major source of vascular superoxide production. It should be noted that the activation of vascular NAD(P)H oxidase by angiotensin II stimulates both O_2^- production and NO production (Pueyo *et al.*, 1998, Tepel., 2003).

It has become clear that the renin-angiotensin system plays a major role in hypertension. The mechanism of renin-angiotensin system–induced hypertension has generally been attributed to the vasoconstrictor effects of angiotensin II and the mineralocorticoid effects of aldosterone. However, recent work has revealed an additional potential mechanism in which Ang II increases blood pressure during the hypertensive state. Angiotensin II has been shown to stimulate O_2^- generation by increasing the activity of the enzyme NAD(P)H cytochrome P-450 oxidoreductase, more commonly termed NAD(P)H oxidase (McIntyre *et al.*, 1999).

The production of ROS by nonphagocytic NAD(P)H oxidase isoforms plays a role in the regulation of intracellular signalling cascades in various types of nonphagocytic cells including fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac myocytes, and thyroid tissue (Droge., 2002).

Problems occur when production of ROS exceeds their neutralisation by the natural antioxidant defence system, or when the latter is compromised. This imbalance between production of ROS and the diminished ability of cells to efficiently defend against them, is called oxidative stress (Ebadi *et al.,* 1996).

2.3.2. Implications of Free radicals in the Disease State

Pathological conditions may develop in cases of persistently elevated reactive oxygen species (ROS) levels. These conditions result in a change in homeostatic balance. Accordingly, pathological symptoms may result from both the damaging effects of ROS in tissue and from ROS-mediated changes in gene expression (Droge., 2002).

Free radicals are very reactive species that damage biologically critical molecules (Halliwell *et al.,* 1992). ROS react directly with cellular lipids, proteins, and DNA, causing cell damage, leading to cell death. Damage to DNA can occur directly by free radicals in close proximity to the DNA or indirectly, for example, by impairing production of proteins needed to repair DNA (Dhalla *et al.,* 2000). Alteration in DNA is a major factor in the development of cancer. Free radicals can attack fatty acid side chains of intracellular membranes and lipoproteins and a chain reaction known as lipid peroxidation ensues. The products of lipid peroxidation can further damage membrane proteins, making the cell membrane " leaky" and eventually leading to loss

of membrane integrity. The last structures damaged by oxidative stress are cellular proteins. Oxidized proteins may trigger antibody formation, autoimmune processes and can cause inactivation of critical enzymes that induce denaturation thereby rendering proteins non-functional (Halliwell and Gutteridge., 1999).

2.4. The Antioxidant System

All organisms that respire aerobically have evolved defence mechanisms against free radicals, which are known collectively as antioxidants. An antioxidant is a substance that prevents oxidation. In biological systems antioxidants can work in various ways, including catalytic removal of free radicals, as scavengers of free radicals or in the form of proteins that minimize the availability of pro-oxidants (molecules that promote free radical formation) such as metal ions. However, there are circumstances in which certain antioxidants can actually behave as pro-oxidants (Halliwell., 1996).

The major endogenous antioxidants are:

1) Superoxide dismutase (SOD) which neutralises $O_2^{-\bullet}$
2) Catalase (CAT) which converts H_2O_2 to water (H_2O) and O_2, and
3) Glutathione Peroxidase (GPx) which aids with H_2O_2 neutralisation and thereby prevents hydroxyl radical ($OH^{\bullet}$) formation.

Many other substances such as uric acid, iron-binding proteins, selenium, ceruloplasmin, bilirubin and estrogen can also function as antioxidants in specific compartments. Lipoic acid and coenzyme Q10 are other antioxidants under investigation. Lipoic acid has the unique ability to regenerate several other antioxidants such as vitamin E, vitamin C, coenzyme Q10 and glutathione. Coenzyme Q10 can also regenerate vitamin E from its radical form and is capable of scavenging oxygen radicals and preventing disruption of lipid cell membranes (Table. 3) (Halliwell., 1996, Packer and Coleman., 1999).

Table 3. 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 (Cu) Metallothionein (Cu) Albumin (Cu) Transferrin (Fe) Ferritin (Fe) Myoglobin (Fe)
Secondary / Free radical Scavenging Enzymes		
Enzymes	**Reaction**	
Superoxide dismutase (SOD) Catalase (CAT) Gluthione peroxidase (GPx)	$2O_2^{-}\cdot + 2H^{+} \rightarrow H_2O_2 + O_2$ $2H_2O_2 \rightarrow 2H_2O + O_2$ $ROOH + 2GSH \rightarrow ROH \rightarrow H_2O + GSSG$ GSH = reduced glutathione GSSG = oxidized glutathione	

2.4.1. Superoxide Dismutase (SOD)

The superoxide dismutases (SOD) are a major cellular defence system against superoxide. These enzymes contain redox metals in the catalytic center and convert superoxide radicals to hydrogen peroxide and oxygen (Table. 3). Three different isoforms of SOD have been identified: the mitochondrial manganese-containing SOD (MnSOD, SOD-2), the cytosolic copper/zinc-containing SOD (CuZnSOD, SOD-1), and the extracellular SOD (ecSOD, SOD-3), which is also a copper/zinc-containing enzyme that is mainly produced and secreted by vascular smooth muscle cells (VSMC) (Wassmann *et al.*, 2004).

SOD-1 is located in the cytosol and nucleus of all cell types. Whereas the SOD isoenzymes are normally thought to be protective, it is postulated that increased SOD-1 activity produces increased amounts of H_2O_2, which become toxic in the presence of normal glutathione and catalase activity (Yarom *et al.*, 1988).

SOD-2 is synthesized in the cytoplasm and directed to the mitochondria by a signal peptide, where it is involved in dismutating the O_2^- generated by the respiratory chain of enzymes. The essential role of SOD-2 is to maintain mitochondrial function (Li *et al.*, 1995).

SOD-3 is produced in fibroblasts and glial cells and secreted into the extracellular fluid, where it is the principal SOD. SOD-3 exists in the vasculature mainly bound to the surface of the endothelial cells and the extracellular matrix. Because of its location, SOD-3 has been identified as the principal regulator of endothelium-derived nitric oxide (NO) bioavailability, although this does not exclude cytosolic SOD-1 which is also thought to be important regulator (Marklund *et al.*, 1982, Oury *et al.*, 1996, McIntyre *et al.*, 1999).

2.4.2. Glutathione Peroxidase (GPx)

Glutathione peroxidases are widely distributed in animal tissues. Reduced glutathione (GSH) plays a major role in the regulation of the intracellular redox state of vascular cells by providing reducing equivalents for many biochemical pathways. Glutathione peroxidase (GPx) is a selenium-containing antioxidant enzyme that effectively reduces hydrogen peroxide and lipid peroxides to water and lipid alcohols, respectively, and gluthione is in turn is oxidized to glutathione disulfide *(Table. 3)* (Wassmann *et al.*, 2004).

Glutathione peroxidases are a family of tetrameric enzymes that require selenium for activity. Dietary selenium actively participates in the catalytic reaction, and this is often the basis for antioxidant protection offered by supplemental selenium. Selenium-containing peroxidases comprise a family of enzymes of at least four types. The

"classic" gluthione peroxidase (cGPx) acts on H_2O_2 and hydroperoxides of fatty acids and cholesterol, but not esterified lipids such as those present in lipoproteins. Phospholipid hydroperoxide gluthione peroxidase (PHGPx) is the only enzyme known to reduce complex lipid hydroperoxides in lipoproteins (Stocker and Keaney., 2004).

In the absence of GPX activity or inadequate glutathione levels, hydrogen peroxide and lipid peroxides are not detoxified and may be converted to hydroxyl radicals and lipid peroxyl radicals, respectively, by transition metals (e.g., Fe^{2+}). The GPx/gluthione system is thought to be a major defense in low-level oxidative stress (Wassmann *et al.*, 2004).

Reduced gluthione (GSH) is recognized as one of the most important non-enzymatic oxidant defenses within the body. It exists in very large quantities (mM levels) within cells where it acts to detoxify peroxides as well as maintain other physiologically important antioxidants in their reduced form (Tarpey *et al.*, 2004). It has been suggested by Jones., 2002 that, 'the balance of GSH and GSSG provides a dynamic indicator of oxidative stress *in vivo*". Therefore gluthione levels *in vivo*, is an important indicator of oxidative stress.

2.4.3. The Antioxidant System as a Functional Unit

The antioxidant system which is responsible for cellular protection against oxidative stress is as diversified as the free radicals themselves. These scavengers are strategically compartmentalized in subcellular organelles within the cell to provide maximum protection (Ji *et al.,* 1988, Yu., 1994).

The complexity of the intracellular network of various antioxidants has impeded understanding of the overall protective efficiency of the antioxidant defense system (Wassmann *et al.,* 2004). It has been proposed by Davies., 1988, that the following scheme is a comprehensive classification of antioxidant defense systems. Primary defenses include; 1) antioxidant compounds such as Vitamins E, A and C, gluthione, and uric acid and 2) antioxidant scavenging enzymes such as superoxide dismutase, catalase and peroxidases. For Secondary defenses, he suggested lipolytic enzymes, phospholipases, proteolytic enzymes, proteases, peptidases, DNA repair enzymes, endonuclease, exonuclease and ligase. This scheme offers the greatest versatility and utility to compartmentalize the antioxidant system (Yu., 1994).

In addition to the integration of intracellular cytosolic defenses, the cooperative interaction between the various antioxidants is crucial for maximum suppression of free radical reactions in extracellular compartments (Yu., 1994).

Although antioxidants are specific to the respective free radicals, the antioxidants display a "concert effect" pertaining to their actions. These actions are well coordinated and provide a maximal protection against free radicals. An example to demonstrate the cooperative actions of different antioxidants is in the breakdown of the superoxide radical. As shown previously *(Table 3.)*, SOD neutralizes the

superoxide radical by converting it to hydrogen peroxide. The resultant hydrogen peroxide, which is in itself a free radical, is then broken down by GPx and catalase, into H_2O and O_2, which are stable end products. If not for the coordinated actions of these antioxidants, then the self-perpetuating actions of the superoxide radical and hydrogen peroxide, would be deleterious to the organism. Thus, this one example alone demonstrates the coordinated depth of the antioxidant system as a functional unit.

2.5. Oxidative Stress

2.5.1. The Concept of Oxidative Stress

Oxidative stress can be defined as the disruption of the equilibrium between the factors that promote free-radical formation and antioxidant defense mechanisms (Halliwell *et al.*, 1992). According to Turrens., 2003, 'oxidative stress' is an expression used to describe various deleterious processes resulting from an imbalance between the excessive formation of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) and limited antioxidant defences.

Whilst small fluctuations in the steady-state concentration of these oxidants may play a role in intracellular signalling, uncontrolled increases in the steady-state concentrations of these oxidants lead to free radical-mediated chain reactions which indiscriminately target proteins, lipids, polysaccharides and DNA (Droge., 2002).

2.5.2 Implications of Oxidative Stress

Oxidative stress occurs in most human diseases. However this does not imply that oxidative stress is the cause of most diseases. The increase in free radicals may be secondary to the disease process (Halliwell and Gutteridge., 1999).

There is a growing awareness that oxidative stress plays a role in various clinical conditions (Droge., 2002). Malignant diseases, diabetes, atherosclerosis, hypertension, chronic inflammation, human immunodeficiency virus (HIV) infection, neurodegenerative diseases and sleep apnea are important examples. These diseases fall into two major categories. In the first category, diabetes mellitus and cancer show commonly a pro-oxidative shift, suggesting that skeletal muscle mitochondria may be

the major site of elevated ROS production. These conditions may be referred to as "mitochondrial oxidative stress". The second category may be referred to as "inflammatory oxidative conditions" because it is typically associated with an excessive stimulation of NAD(P)H oxidase activity by cytokines or other agents. In this case increased ROS levels or changes in intracellular glutathione levels are often associated with pathological changes. These pathological changes are indicative of a dysregulation of signal cascades and/or gene expression, exemplified by altered expression of cell adhesion molecules (Cutolo *et al.*, 1993, Dosquet *et al.*, 1992).

In malignant diseases ROS are potential carcinogens because they facilitate mutagenesis, tumour promotion, and progression. Certain types of cancer cells produce substantial amounts of ROS. The growth-promoting effects of ROS are related to redox-responsive signalling cascades. Even normal cells often show increased proliferation and expression of growth-related genes if exposed to hydrogen peroxide or superoxide. The apparent inconsistency between the uncontrolled cell growth in ROS-producing malignant cells and the ROS-induced senescence in normal cells suggests, however, that ROS production may be necessary but not sufficient to induce malignant cell growth (Dreher *et al.*, 1996, Ha *et al.*, 2000, Droge., 2002).

Elevated ROS levels have also been implicated in diabetes mellitus. In this case oxidative stress is associated with a pro-oxidative shift of the glutathione redox state in the blood. Elevated glucose levels are associated with increased production of ROS by several different mechanisms. In addition the process of glucose auto-oxidation generates superoxide. Glucose auto-oxidation results in plasma glucose undergoing a non-enzymatic chemical reaction with proteins, the products of which are able to

reduce molecular O_2 to ROS such as O_2^-, H_2O_2 and OH^-. The increase in ROS production contributes to the development of complications associated with diabetes such as atherosclerosis and other vascular complications (Nishikawa *et al.,* 2000, Baynes., 1991, Droge., 2002).

Atherosclerosis is a multifactorial disease characterized by hardening and thickening of the arterial wall. The vascular areas affected by this disease contain mononuclear cells, proliferating smooth muscle cells, and extracellular matrix components. Atherosclerosis is commonly viewed as a chronic inflammatory disease and is associated with certain risk factors such as hyperlipidemia, diabetes, and hypertension. Excessive ROS production has been implicated in the pathogenesis of atherosclerosis and hypertension. Oxidative stress induces the expression of protein kinases such as focal adhesion kinase and intercellular adhesion molecules (Alexander., 1999, Auch-Schwelk *et al.,* 1992, Droge., 2002).

Oxidative stress has also been implicated by numerous studies in neurodegenerative diseases such as Downs Syndrome and Alzheimer's Disease (Droge., 2002). The available literature on these topics is specific, vast and well investigated. This shows that local uncontrolled production of ROS/RNS occurs in several diseases which indicates how damaging these species can be (Evans and Halliwell., 2001).

2.5.3. Lipid peroxidation as an Indicator of Oxidative Stress

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malonyldialdehyde (MDA) and 4-hydroxyalkenals (HAE) upon decomposition. Measurement of MDA and HAE has been used successfully as an indicator of lipid peroxidation (Esterbauer *et al.,* 1991).

Many of the methods used to detect lipid peroxidation in urine, blood plasma, or tissue are non-specific, relying on the detection of thiobarbituric acid (TBA)- reactive substances such as MDA or other reactive aldehydes generated *in vivo* or *in vitro* by the decomposition of lipid peroxidation products (Tarpey *et al* 2004). The literature shows that non-enzymatic, free radical-induced lipid peroxidation produces F_2-like prostanoid derivatives of arachidonic acid called F_2-isoprotanes. These F_2-isoprotanes have proven to be a more reliable indicator of oxidative stress, because of their stable nature, and that they can be measured in extra cellular fluids such as plasma and urine thereby making it a relatively non-invasive approach in assessing oxidative stress (Roberts and Morrow., 2000, Tarpey *et al.,* 2004).

Other analytical tests to assess lipid peroxidation include the following techniques: fluorometry of lipofuscin-like substances in serum, spectrophotometry of conjugated dienes in lipid extracts of plasma and microsomes, gas chromatography of ethane or pentane in exhaled breath, hydroperoxide determination, fluorometry, high-performance liquid or gas–liquid chromatography, measurements of other saturated

and unsaturated aldehydes and oxygen uptake during lipid peroxidation (Wilson *et al.*, 1997). The above-mentioned methods are used for the determination of generalised oxidative stress *in vivo* and *in vitro*.

Lipid peroxidation has been linked to a variety of disorders, including atherogenesis, diabetes and UV-induced carcinogenesis (Girotti., 1998). There are a variety of methods available in the literature, with respect to the direct measurement of ROS, this provides a specific measurement of the related ROS inducing the oxidative stress.

2.6. Free Radicals, Oxidative stress and the link to Hypertension

Reactive oxygen species such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), play a critical role in the pathogenesis of hypertension, as well as in other conditions such as atherosclerosis, stroke, and myocardial infarction (Gryglewski *et al.,* 1986) Oxygen free radicals and the resulting oxidative stress play a dual role in hypertension. On one hand, they affect vascular resistance by inactivating NO, thereby causing arteriolar vasoconstriction and elevation of peripheral hemodynamic resistance; on the other hand, they may serve as trigger mechanisms for lesion formation and organ damage (Haliwell and Gutteridge., 1999, Swei *et al.,* 1997).

2.6.1. Nitric Oxide (NO)

Essential hypertension in several animal models of hypertension, including salt-sensitive models are associated with increased peripheral vascular resistance. Due to nitric oxide (NO) being an endogenous vasodilator, there are theoretical reasons to why reduced NO production or bioavailability would lead to vasoconstriction and hence, increased peripheral vascular resistance. NO has been found to regulate the tone of normal vessels, including resistance vessels. In addition, NO causes renal vasodilatation with consequent diuresis and natriuresis. These actions would tend to lower blood pressure; therefore, an attenuation in this mechanism could theoretically contribute to hypertension (McIntyre *et al.,* 1999).

Several studies suggest the superoxide radical interacts with NO and thus limits its bioavailability. The affinity of NO for superoxide is so high that its reaction rate is limited only by diffusion. Because superoxide effectively degrades NO, the biological

activity of NO may be determined by the availability of superoxide (Schnackenberg., 2002).

During oxidative stress, NO depletion occurs by the reaction of NO with the superoxide anion to form peroxynitrite ($ONOO^-$). The presence of peroxynitrite can lead to a number of adverse effects, including protein nitration, lipid peroxidation, DNA degradation and enhanced tubuloglomerular feedback responses (Unlap *et al.,* 2003, Hemnani and Parihar., 1998).

Studies have shown that increased oxidative stress affects hypertension, in part by reducing the levels of NO. Intravenous administration of SOD-Hb, an artificially synthesised form of SOD, significantly decreases the blood pressure of SHR but not of the Wistar controls, demonstrating the significant role played by the decreased bioavailability of NO due to superoxide (Nakazono *et al.,* 1991). Reduction in NO can lead to augmented vasoconstrictive responses, increased blood viscosity, resistance to blood flow, and hypertension (Unlap *et al.,* 2003, Welch and Wilcox., 2001).

2.6.2. Vascular Changes

Reactive oxygen Species (ROS) production is intimately involved in many of the processes leading to both hypertrophic and proliferative vascular smooth muscle cells (VSMC) growth. It has been known for many years that the vasoactive peptide Angiotensin II (Ang II) can induce VSMC hypertrophy. Ang II increases NAD(P)H-driven superoxide production in cultured vascular smooth muscle cells and fibroblasts (Taniyama and Griendling., 2003). ROS also mediate the full proliferative response to agonists such as platelet-derived growth factor (PDGF) and thrombin. H_2O_2 has been

shown to induce VSMC proliferation, although this effect is critically dependent on the concentration of H_2O_2 to which cells are exposed. Furthermore, VSMC proliferation by PDGF or thrombin requires H_2O_2 generation. (Brown *et al.,* 1999). Endogenously produced H_2O_2 may also be important in modulating survival and proliferation of VSMCs. High physiological concentrations of H_2O_2 have been found to induce apoptosis (programmed cell death) (Taniyama and Griendling., 2003).

Endothelial injury or exposure to $O_2^{\cdot-}$ and H_2O_2 induces apoptosis of endothelial cells (EC), which leads to EC loss and results in atherogenesis and a procoagulative state (Dimmeler and Zeiher., 2000). ROS regulate apoptotic mechanisms induced by a variety of stimuli other that the ROS themselves. Another type of programmed cell death, anoikis, results from detachment of ECs from the extracellular matrix. This process is also associated with increased intracellular ROS, probably from mitochondria (Li *et al.,* 1999). EC migration, proliferation, and tube formation are essential events that lead to apoptosis which results in angiogenesis. ROS may be directly involved in all these mechanisms, as H_2O_2 has been shown to induce proliferation and migration of ECs and to mediate lymphocyte-activated tubulogenesis (Maulik and Das., 2002, Taniyama and Griendling., 2003).

Intracellular H_2O_2 may also act as a second messenger for a variety of growth factors. For example, stimulation of VSMC's by platelet-derived growth factor, epidermal growth factor, fibroblast growth factor and angiotensin II all lead to a rise in intracellular H_2O_2 concentration. H_2O_2 does not function as a mitogen for VSMC, instead it is a stimulus to trigger VSMC apoptosis (Li *et al.,* 1997).

Structural vascular changes are the hallmark of chronic hypertension, and increased wall-to-lumen ratio (W/L), of resistance arteries is the prominent lesion. The increase in relative "thickness" of resistance arteries is responsible for the "amplifier" property of the arterial circulation in hypertension, which functionally manifests itself as a pressor or vasoconstrictor hyper responsiveness (Simon *et al.*, 1998).

2.6.3. Organ Damage due to Lipid Peroxidation

Peroxidation of membrane lipids plays an important role in cell physiology and pathology (Roders *et al.*, 1978). The peroxidative degradation of polyunsaturated fatty acids has been found to produce changes in the fluidity of membranes as well as other membrane parameters. The resultant disruption of the biological membranes result in alterations in the activity of a number of membrane bound enzymes (Pradhan *et al.*, 1990). Decomposition of the polyunsaturated fatty acids of organelle membrane phospholipids by peroxides, would result in specific abnormalities of organelle function leading to cell injury or cell death (Popova and Popov., 2002).

Hypertension induces important functional and structural alterations in the kidney, resulting in proteinuria, glomerular sclerosis, and other morphological changes, eventually leading to end-stage renal disease. Reducing blood pressure in hypertensive patients retards the progression of renal failure and reduces the morbidity and mortality rates, but the mechanisms by which hypertension causes renal damage are not clear. Recent experimental data have shown that renal damage in Dahl Sensitive rats occurs concomitantly with the long-term increases in arterial pressure. Increased oxidative stress resulting in lipid peroxidation contributes to this renal damage (Meng *et al.*, 2003).

The aldehydes released during lipid peroxidation have been implicated as causative agents in cytotoxic processes, and it has been postulated that, when released from cell membranes they may diffuse, interact, and induce oxidative modifications in other cells (Redon *et al.* 2003). Oxidative stress-induced cell signalling, may originate with lipid peroxidation and culminate with apoptotic cell death, which results in cell shrinkage, loss of plasma membrane asymmetry, protease and endonuclease activation, and internucleosomal fragmentation of nuclear DNA. The apoptosis thereby damaging the integrity of the cells and tissue (Girotti., 1998).

Regulation of cell survival or death by oxidative stress is a complex process. Depending on the severity and duration of stress, cells exhibit proliferative or apoptotic responses that are mediated by a variety of different, complex and often-interacting pathways.

An increased level of lipid peroxidation is the evidence most frequently cited in support of the involvement of oxidative stress and damage in tissues (Liu *et al.*, 2000). There is increasing evidence that oxidative stress contributes to organ damage by apoptosis and necrosis, in a multitude of disease states. The resulting organ damage is due to an increase in the peroxidation of membrane lipids in the presence of increased oxidative stress. Oxidative stress is an important aetiological factor in hypertension, which is accompanied by architectural changes in the kidney, heart and vessels that are often deleterious and can eventually contribute to end-organ disease such as renal failure, heart failure and coronary disease (Raij., 1998).

2.7. Animal Models in Hypertension

Various animal models of hypertension have been developed over the past fifty years. A number of animal strains, that include several strains of rats, at least one strain of rabbit and one strain of dog readily display spontaneous hereditary hypertension. Experimental hypertensive rats are widely employed in investigative studies, on the pathogenesis of human hypertension. The most widely employed rat strain, is by far the spontaneously hypertensive rat (SHR).

Characteristics of an ideal Animal Model

For investigative purposes an ideal animal model should have five characteristics viz.

1. Mimic the Human disease.
2. Allow studies in chronic, stable disease.
3. Produce symptoms, which are predictable and controllable.
4. Satisfy economical, technical and animal welfare considerations.
5. Allow measurement of relevant cardiac, biochemical and hemodynamic parameters (Doggrell *et al.,* 1998).

Genetic susceptibility plays a definitive role with various genetic strains of rats, since there is a marked difference in susceptibility to NaCl-induced hypertension (Laragh and Brenner., 1990). The Dahl Salt-sensitive rat is a classic example of a strain of rat with a strong genetic susceptibility to NaCl-induced hypertension.

2.7.1. Dahl Salt-sensitive Rats

2.7.1.1. History

The effects of a high salt diet on the blood pressure response were studied, in the 1950's by Meneely and Bail. This study remarked, "there was a marked degree of individual variation" in the blood pressure response to the given salt ingestion (Meneely and Bail., 1958).

The Dahl Salt-sensitive rats were developed by Dahl *et al.,* (1962), as a genetic model for salt induced hypertension. Two strains of rats were developed by them, which were either susceptible or resistant to the hypertensive effects of a high salt (8%NaCl) intake, from the Sprague Dawley line. For the first few generations, inbreeding was done to produce a sensitive and resistant strain of rat. An inbred strain is one for which brother-sister mating have been made for 20 generations. These strains were homozygous at almost 100% of genetic loci, thus fixing the characteristics of the strain (Rapp, 1982).

The rats produced were termed the Dahl Salt-sensitive (DSS/Jr) and Dahl Salt Resistant (DSR/Jr). Since receiving the above-mentioned strains from Dr John Rapp in 1986, the Harlem Sprague Dawley, Inc (Indianapolis, Ind) has maintained the Sensitive and Resistant rats with a strict program of inbreeding. The inbreeding of both these strains for more that 50 generations has resulted in a very reliable physiological response *(Figure 1)* with respect to salt-sensitive hypertension, due to a very high level of genetic homogeneity within each strain.

2.7.1.2. Characteristics of the Dahl Salt-sensitive strain.

When fed high salt diets, the Dahl Salt-sensitive (DSS) rats develop severe hypertension with often fatal consequences, whereas the Dahl Salt Resistant rats do not develop hypertension with salt loading. The DSS rats also become hypertensive when fed normal salt diets, clearly demonstrating that this is a model of genetic hypertension, with the added feature of salt sensitivity (Rapp, 1982).

The magnitude of the blood pressure response in Dahl Salt-sensitive rats is partly determined by the age at which a high salt diet is initiated. When Dahl Salt-sensitive rats were placed on a high salt diet (8% NaCl) at weaning (21-23 days of age), they rapidly developed hypertension and all the rats died by the 16^{th} week of salt loading. If the high salt loading was delayed until 3 months of age, the hypertension developed less rapidly and systolic blood pressure increased to about 185mmHg by 16-20 weeks of age (Dahl *et al.,* 1962).

After 2-3 weeks of a high salt diet, renal injuries appear in Dahl Salt-sensitive rats; the lesions are of a focal nature and comparable to malignant hypertensive renal disease encountered in humans (Karlsen *et al.,* 1997). The Dahl Salt-sensitive rats when placed on a high salt diet early in life, they typically die after 4-8 weeks (Rapp and Dene., 1985). The reason for the rapid development of end-stage renal disease in the Dahl Salt-sensitive rat is unknown, however it has been suggested that the glomeruli are exposed to the damaging effect of an elevated pressure caused by a decrease in afferent arteriolar resistance (Azar *et al.,* 1979).

A widespread misconception that exists is that Dahl Salt-sensitive rats only become hypertensive when placed on a high NaCl diet. The fact remains that, when fed a normal diet that contains 1% NaCl the Dahl Salt-sensitive rats become markedly hypertensive, but it does take a longer time for this to occur (months instead of weeks). The salt merely accelerates and exacerbates the onset of hypertension in this strain (Sustarsie *et al.*, 1981).

3. MATERIALS AND METHODS

3.1. Animal Protocol

3.1.1. Housing and Standard Protocol

Forty-seven male Dahl rats were used for the purposes of this study. The animals were acquired from the Biomedical Resource Centre (University of Kwa-Zulu Natal – Westville Campus). The animals were of 2 strains viz. 24 Dahl Salt Sensitive (DSS) rats and 23 control animals - Dahl Salt Resistant (DSR) rats. These two groups were broken down further with no prejudice into the following groups:

Table 4. Animal Grouping

Strain	Normal Salt	High Salt (2%NaCl)
DSS	n = 12	n = 12
DSR	n = 12	n = 11

Animals were distinguished from each other by ear notching. All animals were individually housed in the Biomedical Resource Centre (University of Kwa-Zulu Natal – Westville Campus) in metabolic cages (Techniplast – Italy). These cages allowed for the separation and collection of urine and faeces via a funnel collection system, to prevent contamination of samples. Cages were also washed daily, in order to prevent contamination of samples.

Animals were fed standard Epol rat chow and deionised water (Millipore). The high salt groups had their water supplemented with 2% $^{m}/_{v}$ NaCl. It should be noted food and water consumption was monitored and recorded daily, and rounded off to the closest gram and millilitre respectively for all groups.

Body mass was monitored once a week for the adult groups on a weigh balance (Mettler) and recorded to the closest gram. The room that the animals were housed in was maintained on a 12hour light/dark cycle and maintained at a constant ambient temperature and pressure for the duration of the study for all groups.

3.1.2.Duration

The experiment commenced with weanlings (~21 days old). All groups were acclimatised for a period of 1 week, and thereafter, the necessary data (food consumption, water intake, urine output, mass and blood pressure) was monitored and recorded accordingly.

The study was done over a period of 8 weeks including the acclimatisation week. The high salt groups were maintained on a normal salt diet, till the third week, at this point salt loading commenced with supplementation of drinking water with 2% NaCl, and lasted for a period of 4 weeks.

3.1.3. Blood Pressure

Blood pressure was monitored using the non-invasive tail-cuff method. Blood pressure was monitored weekly for the duration of the study on all groups. Blood pressure was recorded using the II TC Model 31 NIBP, blood pressure recording equipment in the Department of Physiology (Westville Campus).

3.1.3.1. Blood Pressure Training Protocol

All animals were trained during the acclimatisation week. Animals were first exposed to the restrainers as a group, by allowing them to explore the restrainers in a communal cage. When they gained confidence in moving in and out of the restrainers, they were individually held and allowed to walk into the restrainers. This was done over ~2days.

The animals were then exposed to the warming chamber and all animals were placed a minimum of 3 times in restrainers and placed into the warming chamber for periods not exceeding 10 minutes. The animals were then exposed to the tail-cuff, with repetitive inflation and deflation cycles. All animals were well trained for blood pressure measurements within the acclimatisation week.

3.1.3.2. Blood Pressure Recording Protocol

Systolic blood pressure, diastolic blood pressure and heart rate measurements were obtained by the use of a non-invasive computerized tail-cuff system. The system is comprised of an automatic scanner and pump, a tail cuff with a photoelectric sensor and amplifier to measure and count the pulse rate in the animals tail (II TC Model 31

NIBP). The principle of operation is related to the Riva-Rocca method used in humans.

Small, medium and large sizes of restraining devices were used. This was to compensate for the increase in mass, size and diameter of the tail, of the animals during the 8-week duration of the study. The restrainers used were hollow Perspex cylinders, which fitted the rats snugly so as to minimize voluntary movement of the experimental animal, which could have an affect on the blood pressure values. The restrainers allowed for the protrusion of the tail through the tail cuff at one end and ventilation of the head at the other end. The tail cuff was attached to the restrainer by a studded end plate.

Animals were allowed to pre-heat in the restrainer for ~20minutes in a warming chamber maintained at ~31°C. This was found to be the optimum temperature at which consistent pulses could be detected. The warming chamber consisted of a circulating heated fan and a mounted, ventilated Perspex walls and cover. The warming chamber allowed for 3 animals to be pre-heated and monitored simultaneously. The optimum temperature in the warming chamber was maintained by the heated circulating fan and a temperature control.

After preheating the animals, the tail cuff was automatically inflated by the pump, which resulted in the arterial blood supply to the tail to be occluded. The tail cuff was then slowly deflated and at the reappearance of a pulsation, which was detected by the photoelectric sensor, this was taken as the systolic blood pressure. As the pressure continued to fall, the computer automatically stored the detected high pulse point,

which was accepted as the mean pressure if there was no subsequent higher pulse pressure within the next two seconds. The diastolic pressure for recording was computed using the equation Diastolic= (3mean – Systolic) / 2, this feature was part of the software used (BPMON Version 2.1.).

The results were displayed as data plots and summary data of systolic, diastolic, mean blood pressure and heart rate on the computer screen, this information being available in printable form. The blood pressure measurement information was displayed in 2 forms viz. plots of analog waveforms and digital values. The same occluding tail cuff was used for all animals, to minimise any variables in blood pressure monitoring. All results were done with the artefact filter switched on. The validation of the method and equipment was previously carried out in the same laboratory (Somova *et al.*, 1998).

3.1.4. Sacrifice

Animals were sacrificed at the end of the specified duration, viz. 8 weeks for both salt loaded and non-salt loaded groups, in the Department of Physiology (Westville Campus). Animals were starved overnight and animals were sacrificed the next morning. Animals were anaesthetised using sodium thiopentane (40mg/kg) administered intraperitoneally.

The abdominal cavity was then exposed and blood was collected in heparinised syringes via the bifurcation of the abdominal aorta. The heparinised blood was then aliquoted and stored in heparinised vials for the following analysis:

1. Haematological parameters (Whole Blood)
2. Superoxide Dismutase (Whole Blood)
3. Gluthione Peroxidase (Whole Blood)
4. Hydrogen peroxide (Plasma)

Required tissue (kidney, liver, brain) was then harvested and weighed. These tissues were then placed in separate plastic bags and quenched in liquid nitrogen and stored for later analysis in a bio freezer (-70° C). It should be noted that all harvesting was done immediately, with the time delay kept to a minimum, to maintain the integrity of the tissue.

3.2. Glutathione Peroxidase

Glutathione Peroxidase (GPx) was quantified using a commercially available kit from Randox Chemicals (RANSEL - Cat No. RS 504). The method is based on that of (Paglia and Valentine., 1967). GPx catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH the oxidised glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340nm was measured.

$$2GSH + ROOH \xrightarrow{GPx} ROH + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} NADP^+ + 2GSH$$

The method allowed for GPx to be quantified in whole blood. The sample, had to be diluted using the diluting agent supplied, until the absorbance change per minute was below the threshold of 0.1000, set by the supplier, this is done to ensure that there was linearity in the equation supplied. The dilution of all groups fell into the following range: (80 – 100 x). GPx was quantified using the following equation that was supplied by the agent:

Units of GPx / Litre of Haemolysate = 8412 x ΔAbsorbance (340nm) / Minute

The reaction was carried out in matched glass cuvettes and readings were obtained from a dual beam spectrophotometer (Varian, Cary IE).

3.3. Superoxide Dismutase (SOD)

Superoxide Dismutase (SOD) was quantified using a commercially available kit from Randox Chemicals (RANSOD – Cat No. SD125). The assay principle is based on the fact that the role of SOD is to accelerate the dismutation of the toxic superoxide radical ($O_2^{-}\cdot$), produced during oxidative energy processes, to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). The method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The SOD activity is then measured by the degree of inhibition of this reaction. A standard curve was plotted using the standards provided by the kit. During successive assays, a standard was also assayed, to ensure that the standard fell into the same range on the standard curve.

The assay was carried out on the lysate of the whole blood. The lysate was prepared by centrifuging 0.5ml the aliquoted whole blood for 10minutes at 3000rpm at 4^0C. The buffy coat and plasma was aspirated off. The remaining erythrocytes were thoroughly washed with cold saline solution (0.9% NaCl). The suspension was then centrifuged for 10minutes at 3000rpm at 4^oC. This washing process was repeated four times to ensure thorough washing of cells.

The washed and centrifuged erythrocytes were then made up to 2.0ml with cold redistilled water (Millipore), they were then mixed and left to stand on ice (4^oC) for 15minutes. The resulting lysate was then diluted with a 0.01 mmol/l phosphate buffer (pH 7.0), the dilution was done to ensure that the %inhibition fell between 30 – 60%, as required by the supplier. The dilution of all groups fell into the following range:

(60 – 100 x). The reaction was carried out in matched glass cuvettes and readings were obtained from a dual beam spectrophotometer (Varian, Cary IE).

The %inhibition was calculated from the equation supplied by the manufacturer and the concentration of SOD was then extrapolated from the %inhibition on the standard curve.

$$\% \text{ inhibition} = 100 - (\Delta A_{\text{sample/min}} \times 100) / (\Delta_{\text{sample diluent/min}})$$

3.4. Lipid Peroxidation

3.4.1. Total Malonyldialdehyde (MDA)

MDA concentration was quantified in the following tissues kidney, liver and brain. The modified method based on that of Buegue and Aust., 1976, was adopted for the analysis of tissue. The method is based on the principle that MDA is formed from the breakdown of polyunsaturated fatty acids, that serves as a convenient index for determining the extent of the peroxidation reaction. MDA has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid (TBA), to give a red species absorbing at 535nm.

Tissue for MDA analysis was stored in a bio freezer at –70°C, to prevent extrinsic lipid peroxidation. Tissue was collected from the bio freezer and immediately placed on ice (`4°C), to thaw, the reason for using the ice was to lower the temperature gradient that the tissue was exposed to during the thawing process. The tissue was slow thawed and excess tissue was discarded, and no tissue was exposed to more than one freeze-thaw cycle.

The thawed tissue was then weighed (~0.2 – 0.4g) and washed in cold buffered saline (pH 7.4), to remove any excess blood on the tissue, the tissue was then blotched dry on filter paper. The weighed tissue was then cut with a dissection blade into smaller portions. These portions were suspended in the cold-buffered saline (pH 7.4) and the volume adjusted to 5% mass ($^{m}/_{v}$). The suspension was placed on ice. The buffered saline (pH 7.4), contained 2mmol/l Sodium Azide (NaN_3). The NaN_3 was found to minimise the level of lipid peroxidation *in vitro* as the NaN_3 inhibits lipid peroxidation.

The suspension was then transferred into a glass homogenising tube and homogenised using a tissue homogeniser. The homogenising step was standardised for all samples, all samples were subjected to the same number of homogenising pulses (50) and the revolutions per minute (rpm), was kept at a constant 5000rpm. The homogenising tube was held in a beaker containing ice during the entire homogenising process, to minimise a drastic temperature increase of the suspension. The homogenate was then transferred to a polypropylene tube and stored on ice.

In a glass test tube (Pyrex),1,5ml of the homogenate was added to 1,5ml of the TBA cocktail. This reaction mixture was then lightly vortexed, and immediately transferred to a boiling water bath (~95°C) and incubated for 15 minutes.

The test tubes were then removed and immediately placed into an in an ice slurry, for rapid cooling, to stop the reaction and inhibit further formation of the red species. The cooled tubes were then centrifuged at 3000rpm for 10 minutes at room temperature (~21°C).

The supernatant was decantered into glass cuvettes and the absorbance was read at 535nm and 453nm against a blank containing all the reagents except the homogenate. To improve the specificity of the test, 20% of the absorbance at 453nm was subtracted from the corresponding optical density at 535nm. The concentration of MDA in the sample was calculated by using an extinction coefficient of $1.56 \times 10^5 M^{-1}cm^{-1}$. MDA concentration was represented as per milligram tissue protein; therefore, homogenates

were subjected to protein estimation by the Folin-Lowry Method described in Section 3.5.

3.4.1.1. Reagents

1. Buffered Saline:

- Saline (0.9%)
- Sodium Azide (NaN_3) – 2mmol/l

2. TBA Cocktail Stock:

- Trichloroacetic acid (TCA) - 15% $^{w}/_{v}$
- Thiobarbituric acid (TBA) - 0.375% $^{w}/_{v}$
- Hydrochloric acid (conc.) - 0.25 *N*

A stock of 100ml was made up, with distilled water being used to bring the solution up to 100ml. To assist with the dissolution of the TBA, the solution was mildly heated and a magnetic bead stirrer was used. This stock solution was stored at room temperature in a dark cupboard. A fresh stock was made on the morning of each assay. The cocktail was found to be highly corrosive and latex gloves were used when handling all reagents in the cocktail.

3.4.2. Oxidative Challenge Test

The oxidative challenge test provided an index of assessing the ability of tissue (kidney and liver), to buffer an oxidative onslaught, and assess the tissues antioxidant buffering to limit lipid peroxidation. This was achieved by exposing the tissue homogenate to known concentrations of a stable free radical species viz. hydrogen peroxide (H_2O_2) and then quantifying the concentration of MDA produced by the method of Buegue and Aust., 1976.

The tissue homogenate was prepared following the same protocol as for the Total MDA quantification. The same meticulous precautions were also observed for the challenge test, to minimise an increase in homogenate temperature, and thereby limit extrinsic lipid peroxidation. (Section 3.4.1.)

A fresh working stock of H_2O_2 (40 mM), verified at 30% $^{w}/_{v}$ (Section3.6.), was prepared on the morning of the assay. Serial dilutions of the working stock with distilled water, were made to achieve the following H_2O_2 concentrations: 15Mm, 10mM, 5mM and 2.5mM. A control concentration of 0mM was achieved by using distilled water.

The reactions were carried out in sterilised plastic tablet holders (20ml). To each of the five reaction vessels, 1,5ml of the known concentration of H_2O_2 was added. To each of these, 1.5ml of tissue homogenate was added. The reaction vessels were placed into a modified test tube holder and the holder was placed into a heated oscillating water bath. The samples were incubated at 37°C, for 60minutes and subjected to 100 ossiscillations per minute.

The test tube holder was then removed and 1,5ml of the incubated sample was then immediately added to 1,5ml of the TBA cocktail. This reaction was carried out in glass test tubes. The same assay protocol was followed as for the Total MDA analysis. Optical densities were read and recorded as per the Total MDA protocol (Section 3.4.1.). The same calculations were made as in the Total MDA analysis. MDA concentration was represented as per milligram tissue protein, therefore, homogenates were subjected to protein estimation by the Folin-Lowry Method described in Section **3.5.**

3.4.2.1. Reagents:

1. Hydrogen Peroxide (H_2O_2) – Working Standard (40mM)

The working standard was made up from a ~30% H_2O_2 stock (Sigma. Co.). The concentration of the stock was verified as described in Section 3.6. A 40mM working stock was made up with distilled water, and successive serial dilutions were made with distilled water to produce the experimental concentrations. The working stock was wrapped in foil, placed on ice and stored in a dark cupboard. This was done to prevent any break down of the free radical species. The serial dilutions were done just before the addition of the homogenate.

2. Buffered Saline (pH 7.4):

- Saline (0.9%)
- Sodium Azide (NaN_3) – 2mmol/l

3. TBA Cocktail Stock:

- Trichloroacetic acid (TCA) - 15% $^{w}/_{v}$
- Thiobarbituric acid (TBA) - 0.375% $^{w}/_{v}$
- Hydrochloric acid (conc.) - 0.25 *N*

The same protocol was followed as described in Section 3.4.1.1., for making up the TBA cocktail.

3.5. Hydrogen Peroxide (H_2O_2) Estimation

Hydrogen Peroxide (H_2O_2) concentration was estimated in the blood plasma of all groups. The estimation was based on a modified method employed by Pick and Keisari., 1980. The principle of the assay is based on the horseradish peroxidase (HRPO) mediated oxidation of phenol red by H_2O_2, which results in the formation of a compound demonstrating increased absorbance at 610nm.

The aliquoted heparinised blood drawn from the animal was centrifuged at 3000rpm for 10minutes at 4°C. The plasma was then aspirated off with sterile disposable pipettes and placed into labelled capped tubes (2ml). The tube was then immediately plunged into liquid nitrogen, and stored in a bio freezer (-70°C).

The plasma samples were removed from the bio freezer at the time of the assay and immediately placed on ice and allowed to slow thaw. When fully thawed, the tube was inverted a few times. The reaction was carried out in 10ml polypropylene tubes. To 2ml of Phenol Red Solution (PRS), 20*u*l of the plasma sample was added. This reaction mixture was lightly vortexed, and allowed to incubate at room temperature for 5minutes. Immediately after the incubation, 20*u*l of NaOH (1*N*) was added to the test tube. The tube was then vigorously vortexed for ~3 – 4 seconds.

The samples were then transferred to matched glass cuvettes, and the absorbance was read at 610nm against a blank containing all reagents except the plasma, which was replaced with distilled water. The colour was found to be stable for ~15 – 20minutes. The concentration of H_2O_2 was calculated from an extrapolation of the optical density on the standard curve. (Section 3.5.1.)

3.5.1. Standard Curve for H_2O_2

A standard curve of absorbance versus known concentration of H_2O_2 was plotted according to the method described by Pick and Keisari., 1980. A working stock solution of H_2O_2, was made up by diluting 1.02ml of ~30% H_2O_2 in 100ml of distilled water. This resulted in a working stock solution concentration of 100 *u*M.

The working stock was then serial diluted with distilled water to give the following concentrations of H_2O_2: 10*u*m, 5*u*m, 2.5*u*m, 1.25*u*m, 1*u*m, 0.5*u*m, 0.25*u*m, 0.125*u*m and 0.075*u*m. For a H_2O_2 concentration of 0*u*m distilled water was used. These standards were then subjected to the same protocol as described in Section 3.5. All standards were assayed in triplicate and the average optical densities were used to plot the standard curve, which displayed a linear relationship.

3.5.2. Reagents

Working Stock Solutions:

1. Phenol Red (Sigma Chem.) - (0.028 M)
   - Dissolved in distilled water

2. Horseradish Peroxidase (Sigma Chem.) - (5mg/ml) (5000 units)
   - Dissolved in 0.05 M Potassium Phosphate Buffer (pH 7.0)
   - Stock was made up and aliquoted and plunged in liquid nitrogen, and stored in a bio freezer (-70°C), until required.

3. NaCl (BDH Chem. - AR grade) - 1.40 M
   - Dissolved in distilled water.

4. Dextrose (BDH Chem.) - 0.055 M

- Dissolved in distilled water.

5. NaOH (BDH Chem.) - 1*N*

- Dissolved in distilled water.

Buffers:

1. 0.05 M Potassium Phosphate Buffer (pH 7.0)
2. 0.001 M Potassium Phosphate Buffer (pH 7.0)

Phenol Red Solution (PRS)

A stock solution of 100ml of PRS was made up as follows and consisted of:

1. NaCl - (140mM)

- 10ml of NaCl working stock was used to get the required concentration in 100ml of PRS

2. Dextrose - (5.5mM)

- 10ml of Dextrose working stock was used to get the required concentration in 100ml of PRS.

3. Phenol Red - (0.28mM)

- 1ml of Phenol Red working stock was used to get the required concentration in 100ml of PRS.

4. Horseradish Peroxidase - (50*u*g/ml)

- The working stock was first slow thawed on ice, inverted and then dispensed into the solution. The horseradish peroxidase was added just before the commencement of the assay.

- 1ml of Horseradish peroxidase working stock was used to get the required concentration in 100ml of PRS.

5. Potassium Phosphate Buffer (pH 7.0) - (0.001M)

- The solution was brought up to 100ml with the cold buffer.

The PRS solution was made up just prior to the assay, to prevent degradation of the solution. The solution was wrapped in foil and stored on ice. Fresh PRS was made up for successive assays and was never stored.

3.6. Hydrogen Peroxide (H_2O_2) – Verification

The concentration of the stock H_2O_2 – 30% (stabilised) (Sigma Chem.) was verified using the following method. 0,1008g of stock was weighed out, this was diluted in 50ml of distilled water. 1ml of H_2SO_4 (conc.) was then added to the solution. The solution was titrated against 0.02M $KMnO_4$. The end point was taken as the first stable pink colour change.

It is known that 1ml 0.02M $KMnO_4$ is equivalent to 0.0017007g H_2O_2. The following equation was used to verify % concentration of H_2O_2:

$$\% H_2O_2 \ {}^{w}/_{w} = \text{Reading of } KMnO_4 \times 0.0017007 \times 100 / \text{Mass of } H_2O_2$$

The verification was done in triplicate, to ensure stability of the H_2O_2 Stock.

3.7. Protein Estimation

All tissue homogenates (kidney, liver and brain) that were analysed for MDA concentration, were also subjected to a tissue protein estimation by the Folin-Lowry Method. This was done, so as to represent the MDA concentration as per milligram tissue protein.

The homogenate was diluted with saline solution 0.9% NaCl (10 –15x). The dilutions differed amongst the range of tissue analysed. The dilution was done, so that the concentration fell in the range of 1 – 2.5 mg protein/ml of substrate, as required by the method used.

The reaction was carried out in 10ml polypropylene test tubes. 200*ul* of the diluted sample was added to 800*ul* of saline. 5ml of Alkaline Copper Reagent was then added to the test tube and the solution was lightly vortexed and left to stand at room temperature for 10minutes. 0.5ml of diluted Folin-Ciocalteu reagent was then added to the solution, this was then vigorously vortexed for ~4-5 seconds, and left to stand at room temperature for 30minutes.

The spectrophotometer was zeroed with distilled water and the absorbance of the samples was read at 660nm. A blank was made up with saline and subtracted from the sample absorbance, the blank represented the background absorbance. The final absorbance was extrapolated from a standard curve. The extrapolated values were subjected to the dilution factors, with the result being the protein concentration of the tissue.

3.7.1. Protein Estimation – Standard Curve

A standard curve of known protein concentration versus absorbance was plotted according to the method described by Lowry. A working stock solution of bovine serum albumin (BSA) (200*u*g/ml) was used as the known concentration standard. A subsequent serial dilution of the working standard was made with saline. These standards were subjected to the same protocol, that was described for the unknown samples. All standards were done in triplicate and the average absorbances were used to plot the standard curve.

3.7.2. Reagents

1. Saline - 0.9% NaCl

2. Alkaline Copper Reagent

- 0.5ml of 1% $^{w}/_{v}$ Copper Sulphate ($CuSO_4.5H_2O$)
- 0.5ml of 2% $^{w}/_{v}$ Sodium Potassium Tartrate ($NaKC_4H_4O_6$)
- 50ml of 2% $^{w}/_{v}$ Na_2CO_3 diluted in 0.1M NaOH

Stock solutions that made up the reagent were stored in a fridge (4°C). Reagent was made up prior to the assay and stored at room temperature.

3. Folin-Ciocalteu Reagent

- A stock reagent was obtained from BDH Chem.
- The stock was diluted 1:1 with distilled water.
- The stock was stored in a fridge (4°C).

4. Bovine Serum Albumin (BSA) Stock Protein

- BSA stock was obtained from Merck Ltd.

- 0.02g of stock was diluted with 100ml of saline to give a protein concentration of (200*u*g/ml)

- A serial dilution of the stock was made with saline .

4. RESULTS

All data is represented as the Mean ± Standard Error of the Mean (SEM). The statistical analysis of all results was performed using Graphpad Instat Ver. 3.00. The analysis performed used one-way Analysis of variance (ANOVA) and t-test. A **p** value < 0.05 was considered to be statistically significant.

4.1. Food Consumption

The average weekly food consumption for each group was determined and is shown in *Table 5.*, and represented in *Figure 2*. The results show that there is a significant increase in the food consumption of the DSS groups when compared to the control DSR groups for the entire 7 weeks of the study.

Salt loading was initiated, in the 4th week of the study in the High Salt (HS) groups, by supplementing the drinking water with 2% NaCl. Salt loading had a significant decrease in food consumption after the 2nd week of salt loading in the DSS group and after the 1st week of salt loading in the DSR group when compared to the respective non-salt loaded controls. Salt loading also had a significant decrease in food consumption in the DSR (HS) group when compared to the DSS (HS) group.

The ratio of mass gain to food intake (Food Efficiency Ratio – FER) is shown in *Table 6*. This is a quotient of the Mass Gain per week and the food consumption per week. Salt loading had a significant decrease in this ratio in both of the salt loaded groups when compared to their respective controls. The DSR (HS) group however showed a "normal" FER, relative to the DSR (NS) group, during the final week of salt loading.

Table 5. **Average Weekly Food Consumption**

Food Consumption (grams/week)							
Strain / Week	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
DSS NS (12)	140 ± 2.96	169 ± 4.08	175 ± 2.85	197 ± 3.08	191 ± 3.39	188 ± 2.26	198 ± 4.46
DSR NS (12)	118 ± 3.09	145 ± 2.30	150 ± 2.79	158 ± 4.64	161 ± 3.18	157 ± 2.96	161 ± 2.98
DSS HS (12)	153 ± 3.73	173 ± 4.27	193 ± 3.76	189 ± 3.68	184 ± 4.33	145 ± 7.41	157 ± 8.72
DSR HS (11)	131 ± 6.21	141 ± 14.10	154 ± 4.39	109 ± 5.86	103 ± 8.13	108 ± 5.56	113 ± 4.95

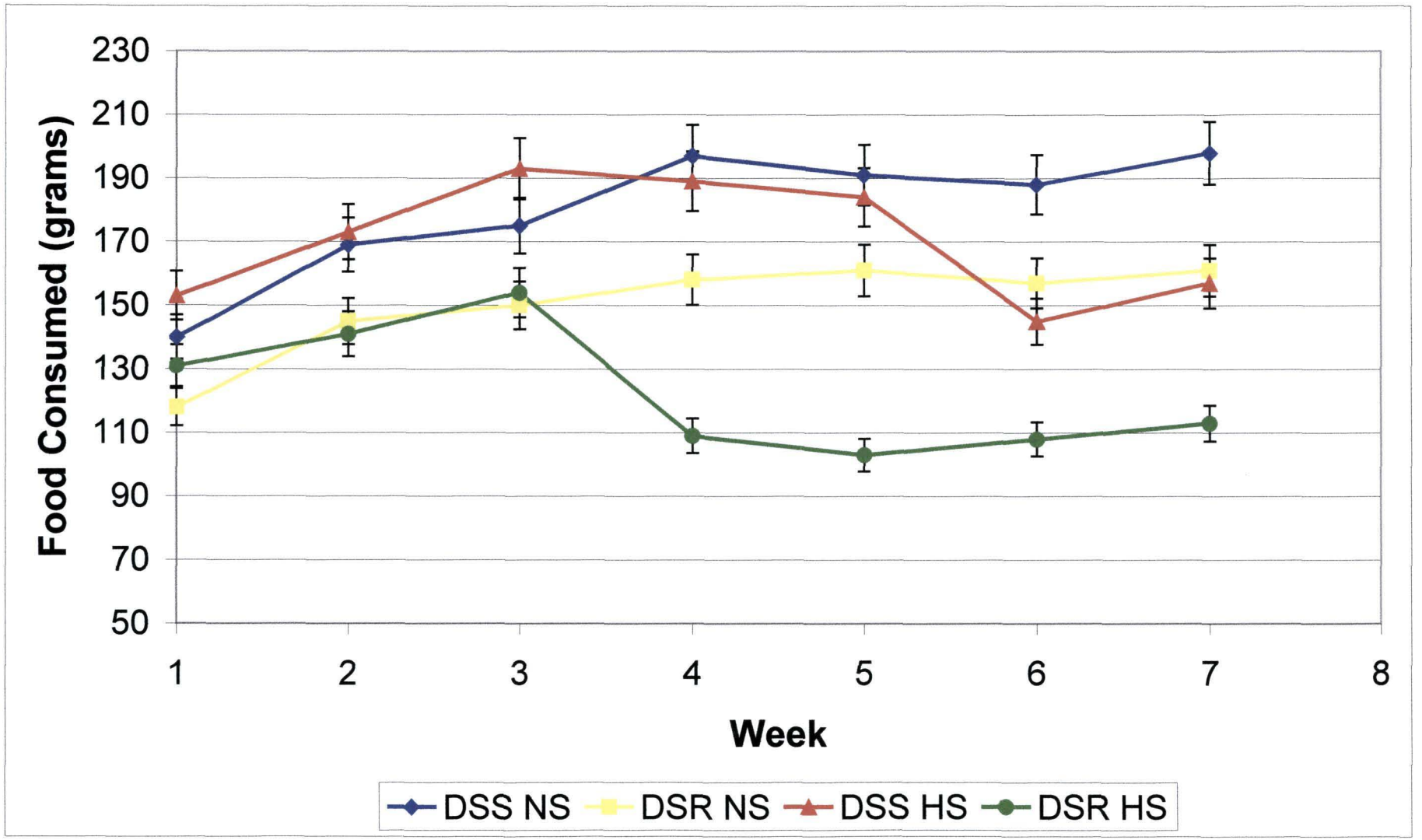
Values shown as Mean ± SEM
Food Consumption expressed in grams
Numbers in brackets indicates n – value (sample number)
☐ - Salt loading with 2% NaCl

Table 6. **Food Efficiency Ratio**

Food Efficiency Ratio (FER) (Mass gain / Food Consumption)							
Strain / Week	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
DSS NS (12)	-	0.357 ± 0.010	0.247 ± 0.010	0.213 ± 0.008	0.209 ± 0.004	0.142 ± 0.010	0.141 ± 0.012
DSR NS (12)	-	0.373 ± 0.018	0.200 ± 0.008	0.203 ± 0.011	0.189 ± 0.009	0.140 ± 0.007	0.115 ± 0.009
DSS HS (12)	-	0.339 ± 0.006	0.272 ± 0.007	0.236 ± 0.006	0.189 ± 0.006	0.122 ± 0.011	0.073 ± 0.029
DSR HS (11)	-	0.373 ± 0.026	0.394 ± 0.020	0.053 ± 0.017	0.084 ± 0.032	0.088 ± 0.030	0.122 ± 0.041

Numbers in brackets indicates n – value (sample number)
Ratio is a quotient of Mass Gain / Food consumption
☐ - Salt loading with 2% NaCl

Figure 2. **Average Weekly Food Consumption**



4.2. Water Intake

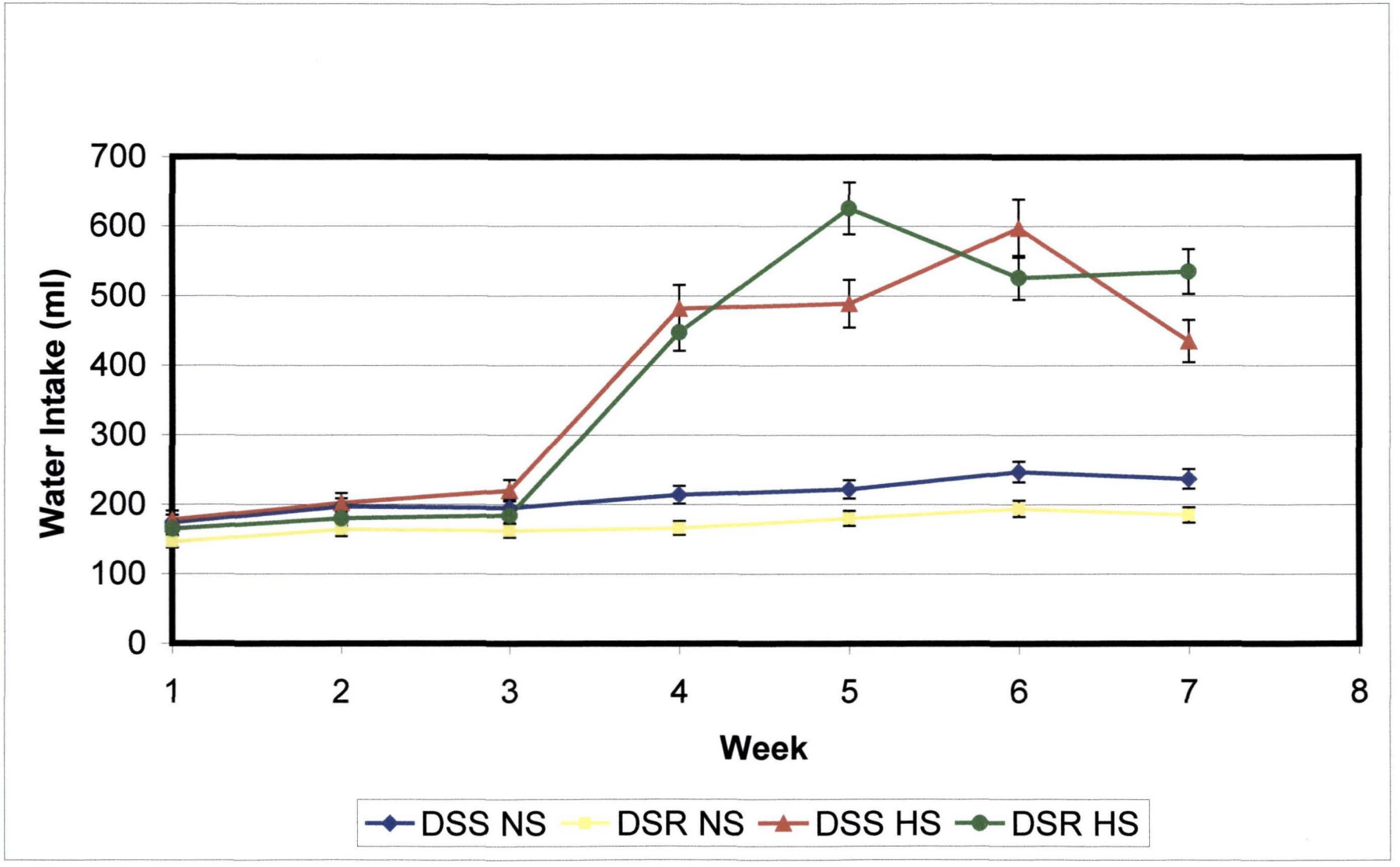
The average weekly water intake for each group was recorded and is shown in *Table 7.*, and represented in *Figure 3*. The water was supplemented with 2% NaCl in the 4th week of the study for the designated high salt (HS) groups. There was a significant increase in water intake of the DSS group when compared to the control DSR group during the 7-week study. Salt loading had a significant increase in water intake of the HS groups when compared to the NS group, with the water intake being ~3-4 times that of the non-salt loaded controls. Salt loading also had a significant effect on the water intake of both the salt loaded groups when compared to each other, after the 2nd week of salt loading.

Table 7. **Average Weekly Water Consumption**

Strain / Week	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
DSS NS (12)	174 ± 5.32	197 ± 4.69	195 ± 5.74	214 ± 7.64	222 ± 8.79	247 ± 12.84	237 ± 10.10
DSR NS (12)	146 ± 2.95	164 ± 2.77	162 ± 4.99	166 ± 3.60	180 ± 4.51	194 ± 8.87	185 ± 6.12
DSS HS (12)	178 ± 4.32	202 ± 4.03	220 ± 3.35	482 ± 34.43	489 ± 29.24	597 ± 43.69	435 ± 36.75
DSR HS (11)	165 ± 4.41	180 ± 7.31	184 ± 4.53	448 ± 34.70	626 ± 70.53	526 ± 83.03	535 ± 85.87

Values shown Mean ± SEM
Numbers in brackets indicates n – value (sample number)
Water Intake represented in millilitres
▭ - Salt loading with 2% NaCl

Figure 3. **Average Weekly Water Consumption**



4.3. Urine Output

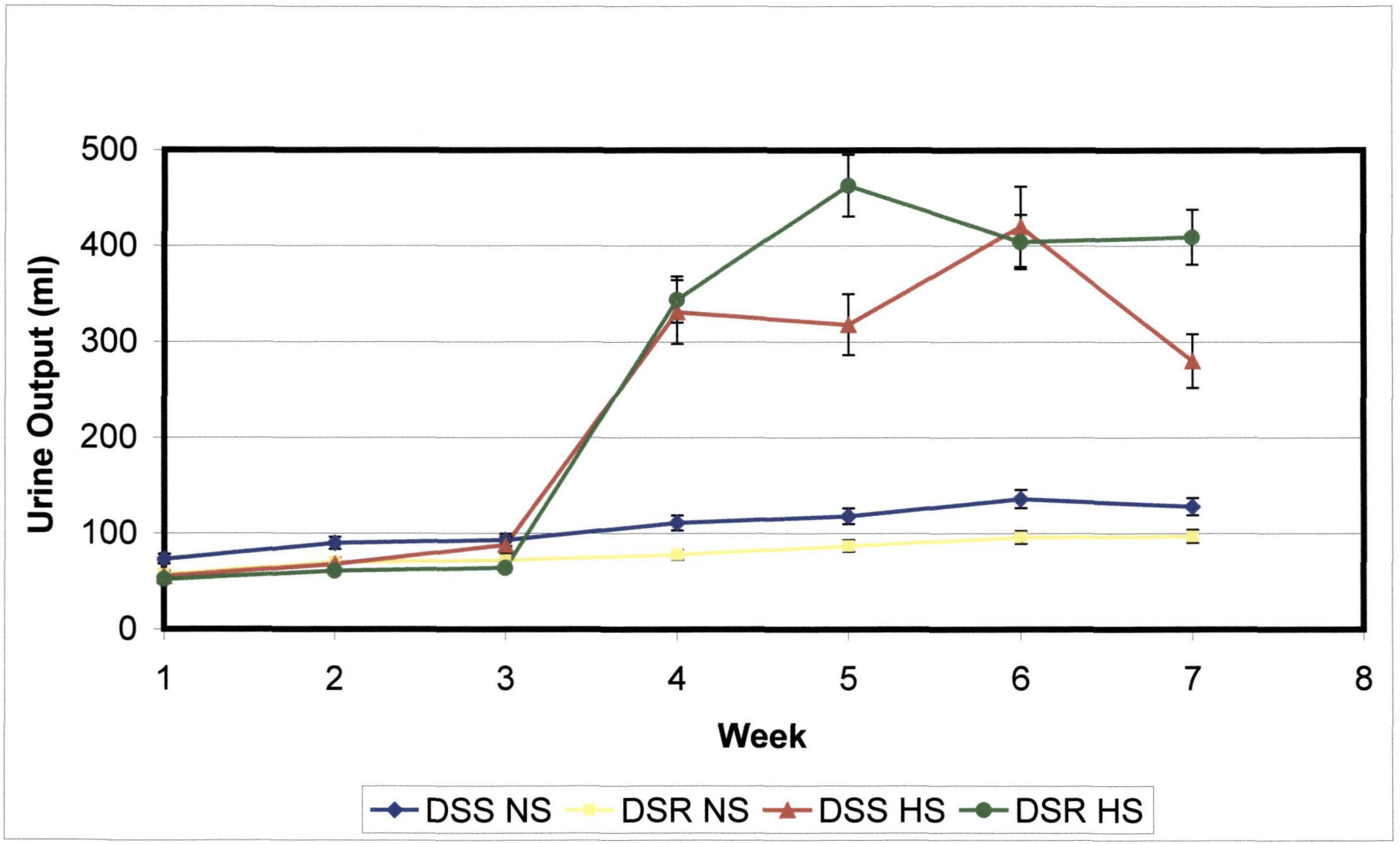
The average weekly urine output for each group is shown in *Table 8.*, and represented in *Figure 4.* There was a significant increase in the urine output of the DSS group when compared to the control DSR group, during the entire 7-week study. Salt loading had a significant increase in the urine output of both the salt loaded groups when compared to the respective non-salt loaded controls. There was a significant difference in urine output of the salt loaded groups when compared to each other in the 2^{nd} and 4^{th} week of salt loading. The salt loaded DSS group had a significant decrease in urine output in the final week of the study.

Table 8. **Average Weekly Urine Output**

Strain / Week	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
DSS NS (12)	73 ± 4.97	90 ± 5.91	93 ± 5.28	111 ± 5.99	118 ± 6.46	136 ± 10.35	128 ± 9.40
DSR NS (12)	57 ± 2.42	70 ± 2.38	72 ± 2.63	78 ± 4.06	87 ± 3.12	96 ± 4.60	97 ± 4.72
DSS HS (12)	55 ± 3.60	68 ± 1.88	88 ± 2.77	331 ± 18.95	318 ± 23.16	420 ± 31.96	280 ± 23.55
DSR HS (11)	52 ± 1.83	61 ± 3.30	64 ± 4.55	344 ± 26.16	463 ± 43.85	404 ± 58.27	409 ± 60.25

Values shown Mean ± SEM
Numbers in brackets indicates n – value (sample number)
Urine output represented in millilitres
▭ - Salt loading with 2% NaCl

Figure 4. **Average Weekly Urine Output**



4.4. Body Mass

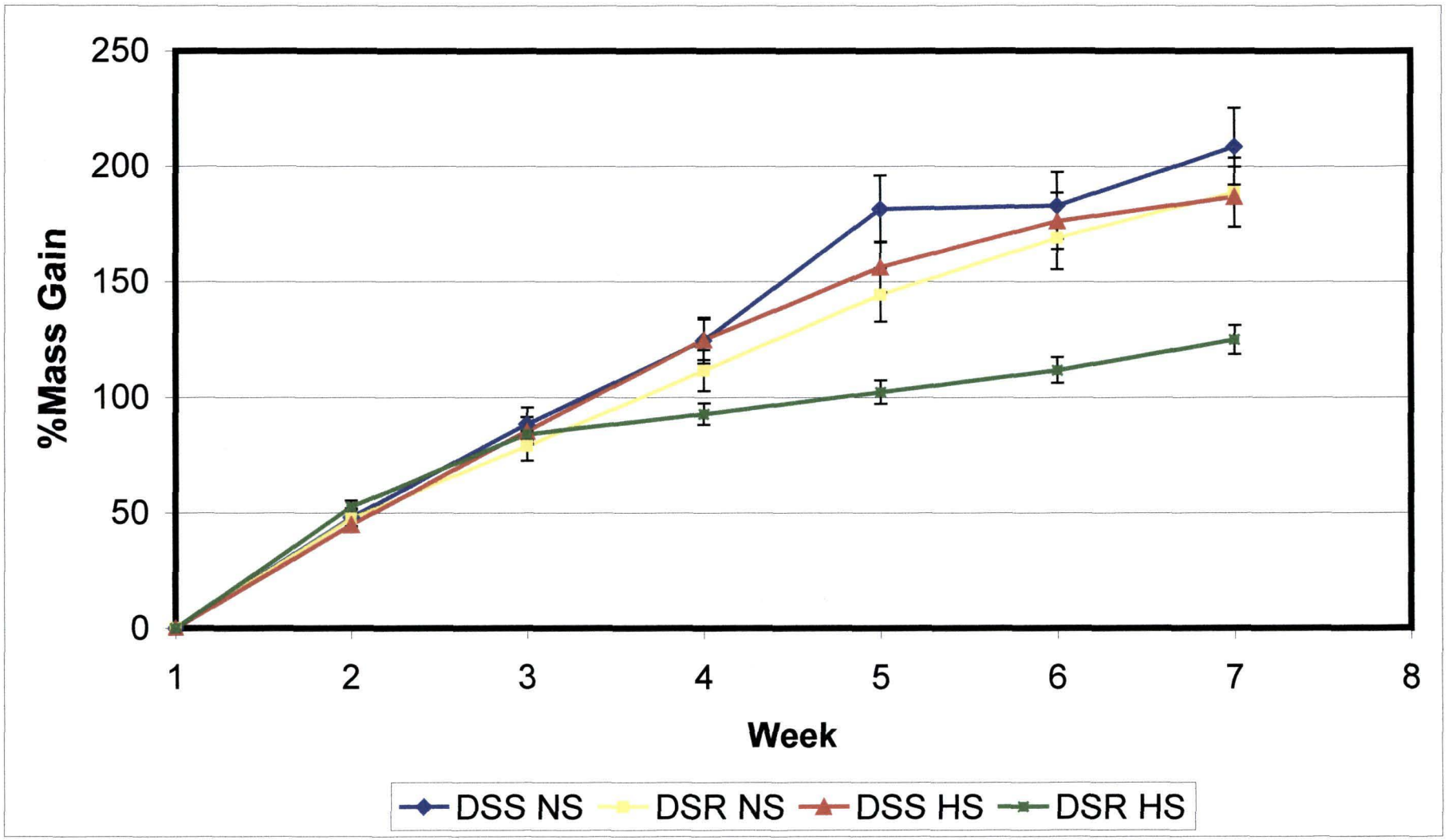
Body mass is expressed as a percentage mass gain per week, with the mass in the 1st week being taken as the baseline (100%), with all subsequent increases in mass per week being expressed as the percentage increase. This data is shown in *Table* 9., and represented in *Figure 5*. There was a significant increase in percentage (%) mass gain of the DSS group when compared to the control DSR group from the 4th week of the study to the end of the study in the 7th week. Salt loading had a significant decrease in % mass gain from the 2nd week of salt loading of both the HS groups when compared to the respective NS groups. The DSS groups, both salt loaded and non-salt loaded had a higher end of study body mass than their respective controls.

Table 9. **Weekly Percentage Mass Gain**

Strain / Week	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
DSS NS (12)	0	48.0 ± 1.62	88.5 ± 3.66	124.6 ± 4.16	181.5 ± 11.18	183.0 ± 6.36	208.7 ± 6.48
DSR NS (12)	0	47.5 ± 2.97	78.9 ± 4.63	111.6 ± 5.16	144.4 ± 6.86	168.9 ± 8.29	188.6 ± 9.19
DSS HS (12)	0	44.8± 1.38	85.5 ± 2.70	125.0 ± 4.10	156.3 ± 5.56	176.3 ± 7.70	186.7 ± 7.15
DSR HS (11)	0	52.6 ± 4.63	84.1 ± 5.49	92.8 ± 6.24	102.3 ± 7.42	111.8 ± 7.86	124.9 ± 8.09

Values shown ± SEM
Numbers in brackets indicates n – value (sample number)
Body Mass represented as % Mass gain per week, Week 1 taken as Baseline
▭ - Salt loading with 2% NaCl

Figure 5. **Weekly Percentage Mass Gain**



4.5. Blood Pressure and Heart Rate

The blood pressure and heart rate was monitored weekly. The systolic and diastolic blood pressures are shown in *Table 10* and *Table 11* respectively and represented diagrammatically in *Figure 6* and *Figure 7* respectively. There was a significant increase in both systolic and diastolic blood pressures in the DSS group when compared to the control DSR group from the 3rd week to the end of the study in the 7th week. Salt loading had a significant increase in systolic blood pressure in the 2nd and 3rd week of salt loading, in the DSS (HS) group when compared to the DSS (NS) group. There was no significant difference in the diastolic blood pressure during salt loading.

The heart rate recorded in beats per minute (bpm) is shown in *Table 12.*, and represented in *Figure 8.* There was no significant difference in heart rate during the first 4 weeks of the study. There was a significant increase in heart rate of the DSS group when compared to the control DSR group from Week 5 to the end of the study in the 7th week. Salt loading had a significant decrease in the heart rate of the DSS group during the 6th week of the study. There was no significant difference in the DSR groups during salt loading.

Table 10. **Average Weekly Systolic Blood Pressure**

Strain / Week	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
DSS NS (12)	135.5 ± 2.49	138.3 ± 2.38	154.4 ± 3.83	154.9 ± 3.25	164.1 ± 2.76	178.6 ± 4.38	194.4 ± 4.77
DSR NS (12)	112.6 ± 5.60	134.0 ± 2.82	132.8 ± 2.75	134.3 ± 1.86	133.3 ± 2.93	136.0 ± 3.30	137.3 ± 3.10
DSS HS (12)	104.8 ± 9.51	155.3 ± 3.50	156.2 ± 5.09	168.1 ± 1.70	182.2 ± 6.24	193.3 ± 4.96	205.8 ± 5.81
DSR HS (11)	116.5 ± 4.08	124.2 ± 2.37	120.0 ± 3.53	138.4 ± 3.34	113.4 ± 6.08	128.6 ± 5.50	132.2 ± 3.90

Values shown as Mean ± SEM
Numbers in brackets indicates n – value (sample number)
Blood pressure represented in millimetres Mercury (mmHg)
▭ - Salt loading with 2% NaCl

Table 11. **Average Weekly Diastolic Blood Pressure**

Strain / Week	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
DSS NS (12)	72.9 ± 1.92	89.1 ± 1.99	97.8 ± 2.15	111.3 ± 1.68	129.7 ± 0.91	139.7 ± 1.50	136.9 ± 11.50
DSR NS (12)	72.8 ± 1.32	79.3 ± 0.92	83.3 ± 1.13	80.9 ± 1.40	84.7 ± 1.20	89.8 ± 1.93	90.2 ± 1.91
DSS HS (12)	64.1 ± 7.96	108.2 ± 2.08	106.8 ± 3.23	116.3 ± 3.08	126.8 ± 4.25	140.8 ± 6.47	143.2 ± 6.44
DSR HS (11)	74.8 ± 2.18	78.4 ± 1.07	67.4 ± 1.64	80.9 ± 2.53	68.5 ± 2.06	78.9 ± 1.50	89.8 ± 1.17

Values shown as Mean ± SEM
Blood pressure represented in (mmHg)
Numbers in brackets indicates n – value (sample number)
▭ - Salt loading with 2% NaCl

Figure 6. **Average Weekly Systolic Blood Pressure**

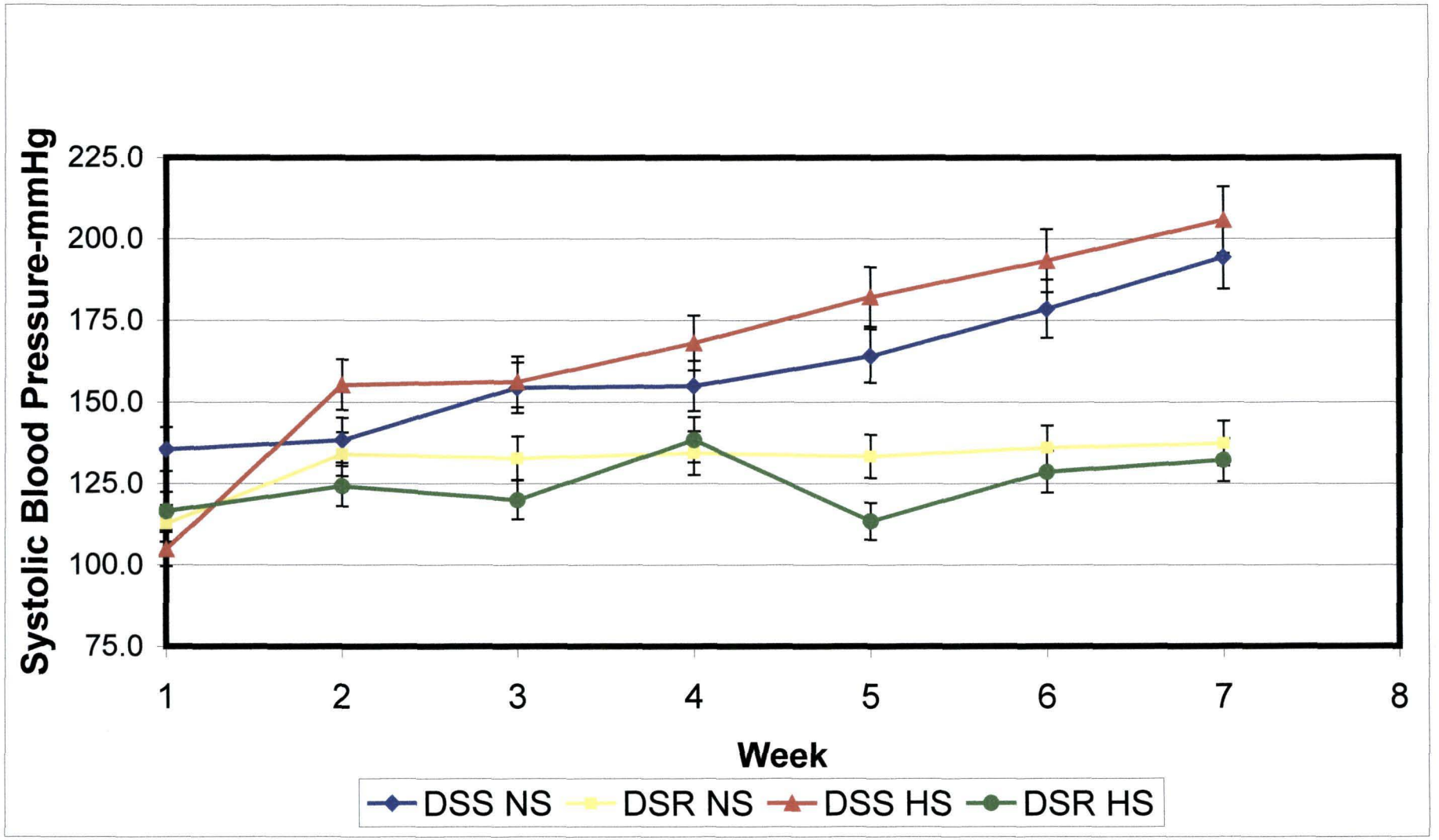


Figure 7. **Average Weekly Diastolic Blood Pressure**

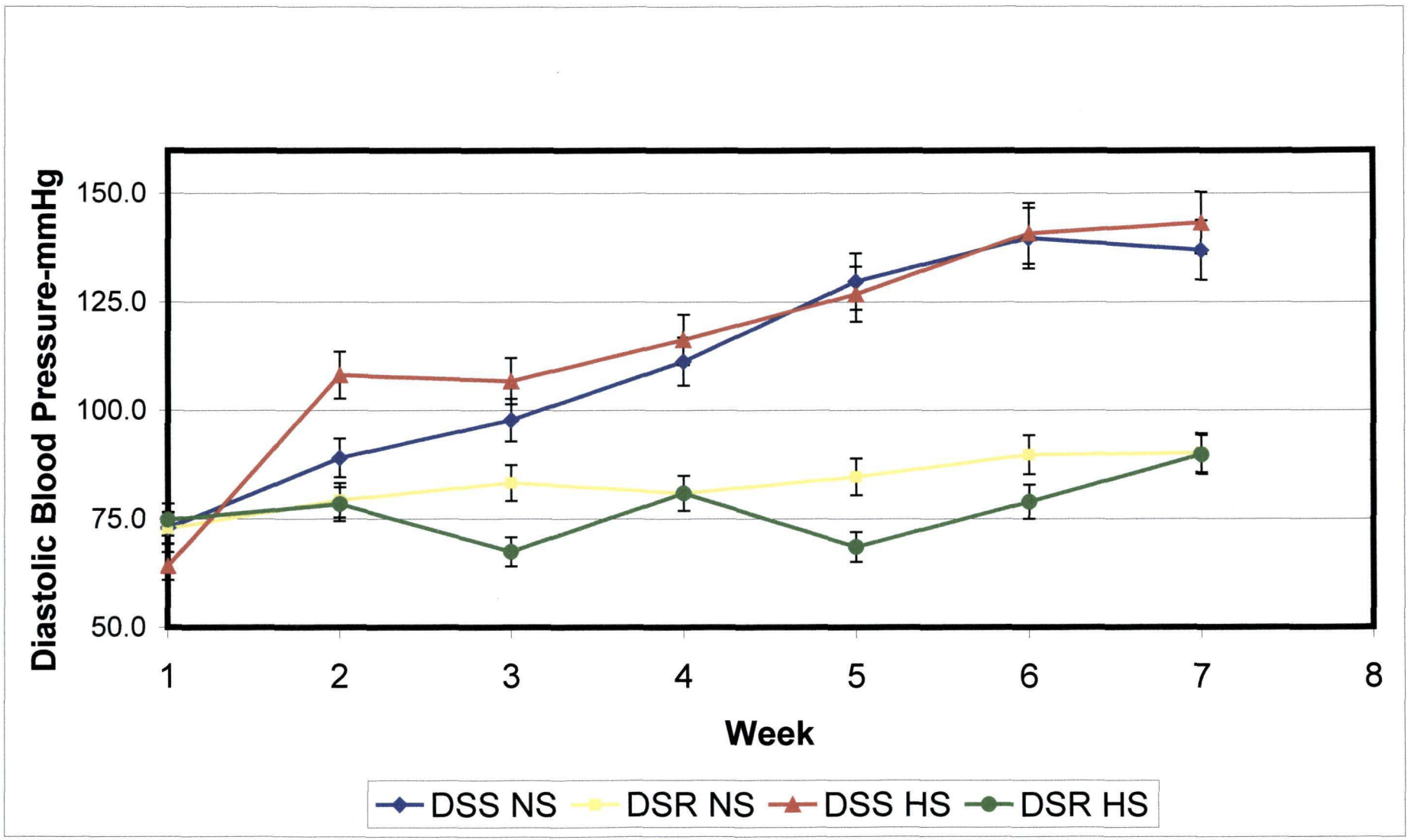
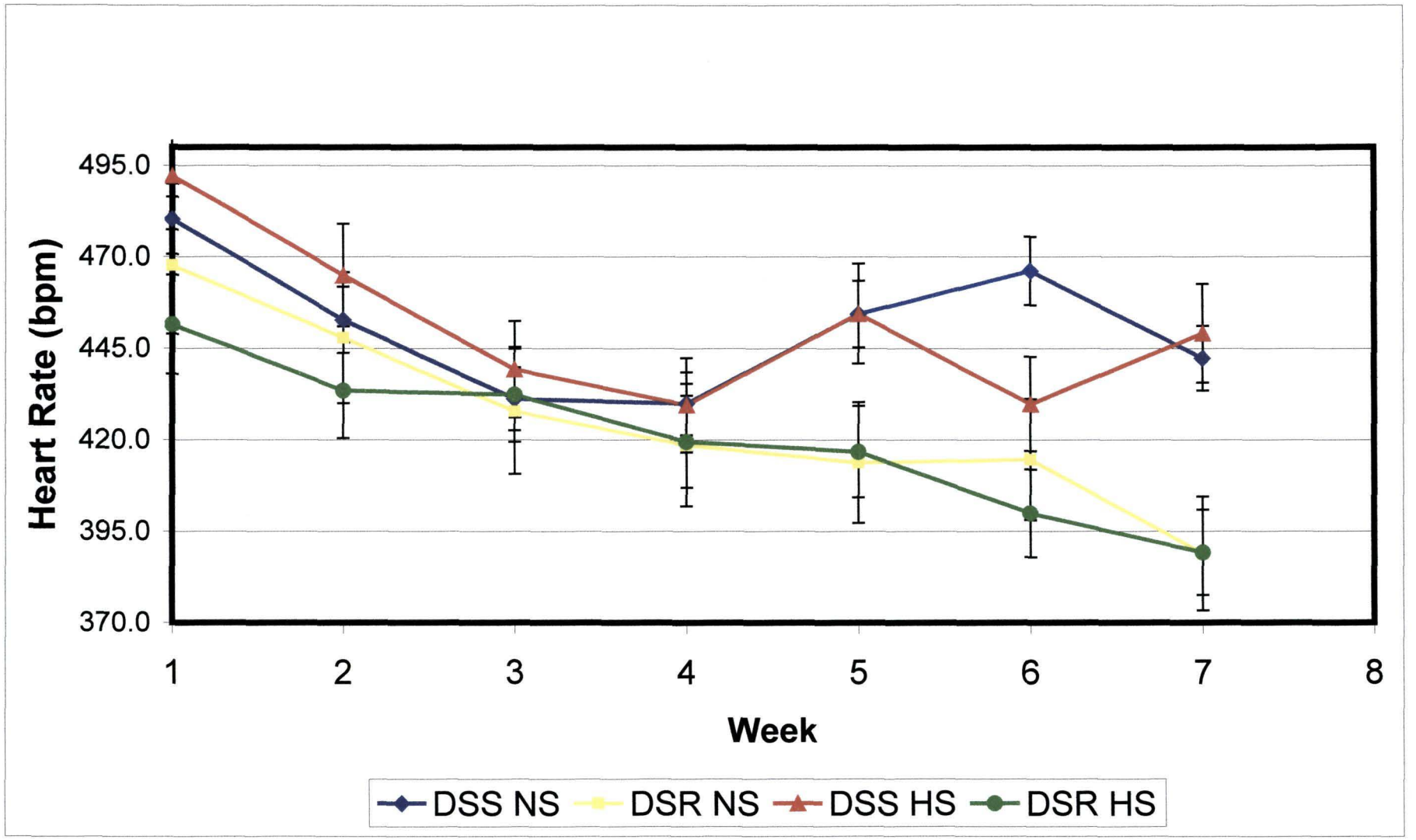


Table 12. **Average Weekly Heart Rate**

Strain / Week	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
DSS NS (12)	480.3 ± 13.52	452.8 ± 13.50	431.2 ± 7.91	429.9 ± 7.82	454.4 ± 4.65	466.1 ± 7.41	442.3 ± 13.03
DSR NS (12)	467.6 ± 6.65	447.9 ± 8.36	427.8 ± 6.11	418.6 ± 5.68	413.8 ± 5.52	414.6 ± 7.45	388.9 ± 4.97
DSS HS (12)	492.1 ± 8.49	465.0 ± 11.91	439.3 ± 11.08	429.5 ± 10.04	454.5 ± 16.97	429.8 ± 11.90	449.1 ± 13.57
DSR HS (11)	451.5 ± 11.61	433.5 ± 8.74	432.4 ± 8.42	419.5 ± 8.53	416.8 ± 6.62	399.9 ± 5.63	389.2 ± 10.31

Values shown as Mean ± SEM
Heart Rate represented in Beats per Minute (bpm)
Numbers in brackets indicates n – value (sample number)
▭ - Salt loading with 2% NaCl

Figure 8. **Average Weekly Heart Rate**



4.6. Organ Weight

Organ weights of the right kidney, liver and brain are shown in *Table* 13., and represented in *Figure 9.* There was a significant increase in the mass of the right kidney of the DSS group when compared to the control DSR group. Salt loading significantly increased the right kidney mass of the DSS HS group. There was no significant difference in the right kidney mass of the salt loaded DSR group.

There was no significant difference in the mass of the liver in the DSS group when compared to the control DSR group, however during salt loading there was a significant increase in the liver mass in both the DSS and DSR salt loaded groups.

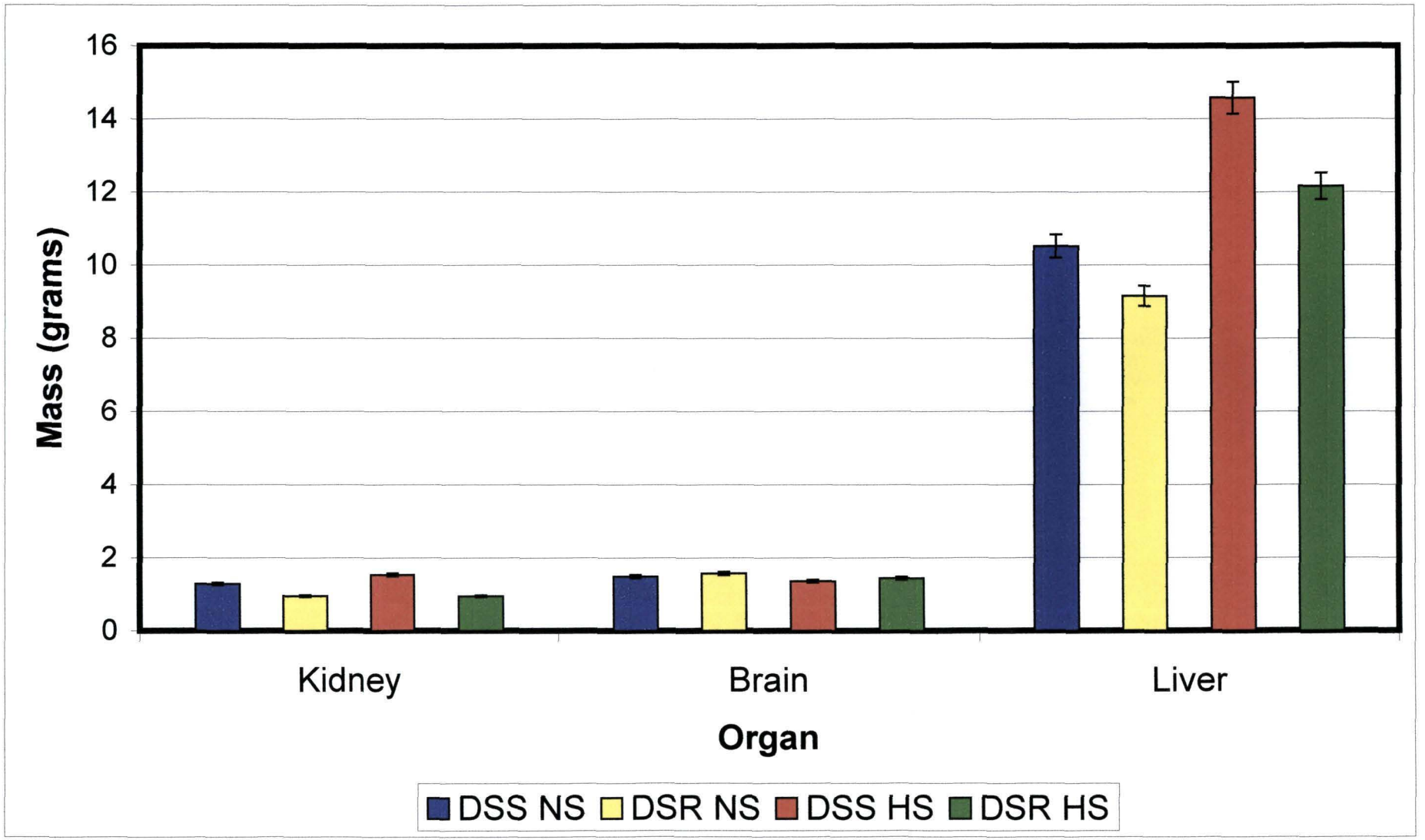
There was no significant difference in the brain mass of the DSS group when compared to the control DSR group. Salt loading showed no significant difference in the mass of the brain in both the DSS and DSR groups.

Table 13. **Average Right kidney, liver and brain weight**

Organ / Strain	DSS NS (12)	DSR NS (12)	DSS HS (12)	DSR HS (11)
Right Kidney	1.28 ± 0.04	0.95 ± 0.03	1.53 ± 0.05	0.94 ± 0.02
Liver	10.51 ± 0.28	9.15 ± 0.45	14.58 ± 0.50	12.16 ± 0.65
Brain	1.48 ± 0.05	1.57 ± 0.04	1.35 ± 0.03	1.43 ± 0.04

Values shown as Mean ± SEM
Numbers in brackets indicates n – value (sample number)
Organ mass represented in grams

Figure 9. **Average Right kidney, liver and brain weight**



4.7. Antioxidant Enzymes

4.7.1. Superoxide Dismutase (SOD)

The SOD levels evaluated in the red blood cell is shown in *Table 14.*, and represented in *Figure 10.* There is a significant increase in SOD levels in the DSS group when compared to the control DSR group. Salt loading resulted in a significant decrease in the SOD levels of the salt loaded groups when compared to the respective non-salt loaded groups.

4.7.2. Gluthione Peroxidase (GPx)

The GPx levels evaluated in whole blood are shown in *Table 14.*, and represented in *Figure 11.* There was a significant decrease in GPx levels in the DSS group when compared to the control DSR group. Salt loading resulted in no significant difference in GPx levels in both the DSS and DSR salt loaded groups.

4.8. Hydrogen Peroxide (H_2O_2)

The hydrogen peroxide levels determined in the blood plasma are shown in *Table 14.*, and represented in *Figure 12.* Statistically there was no significant difference ($p > 0.05$) in the hydrogen peroxide levels in all groups including the salt loaded groups when compared to their respective control groups. The GPx : H_2O_2 ratio which is shown in *Table 14.*, is a quotient of the GPx and H_2O_2 concentration and is expressed as GPx units per nanomol H_2O_2, and was used to determine the bioavailability of GPx to neutralise H_2O_2.

Table 14. **SOD, GPx, Hydrogen Peroxide Concentration, GPx : H_2O_2 Ratio and Total MDA concentration in the brain**

Parameter / Strain	DSS NS (12)	DSR NS (12)	DSS HS (12)	DSR HS (11)
SOD	104.2 ± 2.7	71.2 ± 4.1	57.9 ± 3.2	34.4 ± 0.9
GPx	60975.8 ± 1297.7	77738.8 ± 1557.0	65547.0 ± 2510.1	77190 ± 1585.3
H_2O_2	0.0489 ± 0.0070	0.0541 ± 0.0058	0.0694 ± 0.0057	0.0697 ± 0.0101
GPx : H_2O_2 Ratio	1523.67 ± 257.83	1855.70 ± 450.27	1025.54 ± 106.30	1916.96 ± 629.54
Total MDA Brain	2837.6 ± 109.8	3183.5 ± 127.0	3217.7 ± 113.5	3165.1 ± 138.4

Values shown as Mean ± SEM
Total MDA expressed as nmol MDA / mg Tissue Protein
GPx expressed as GPx units / ml of Blood
SOD expressed as SOD units / ml of Blood
H_2O_2 expressed as uM H_2O_2 / ml Plasma
GPx : H_2O_2 Ratio is expressed as GPx units per nanomol(nm) H_2O_2

Figure 10. **Superoxide Dismutase**

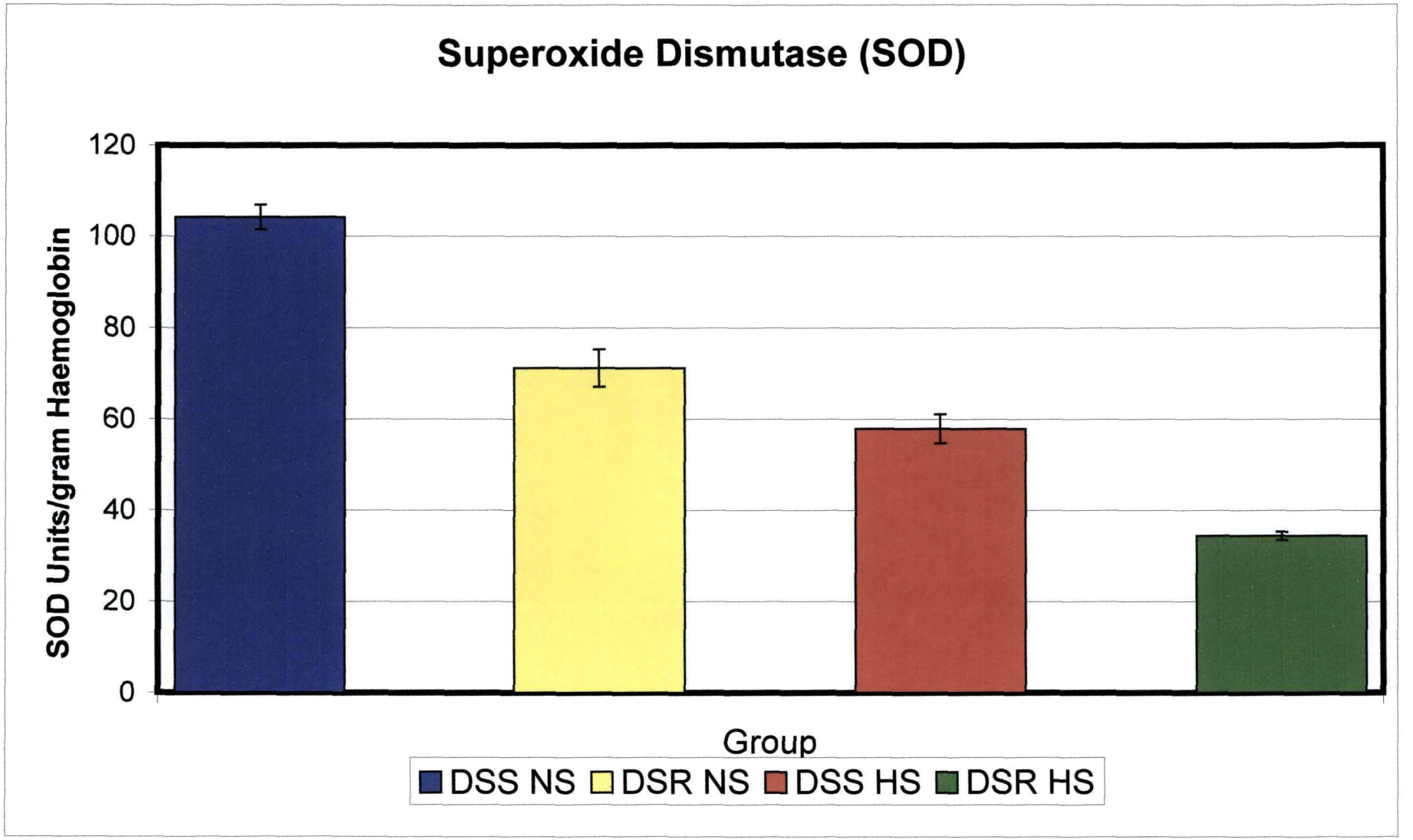


Figure 11. **Glutathione Peroxidase**

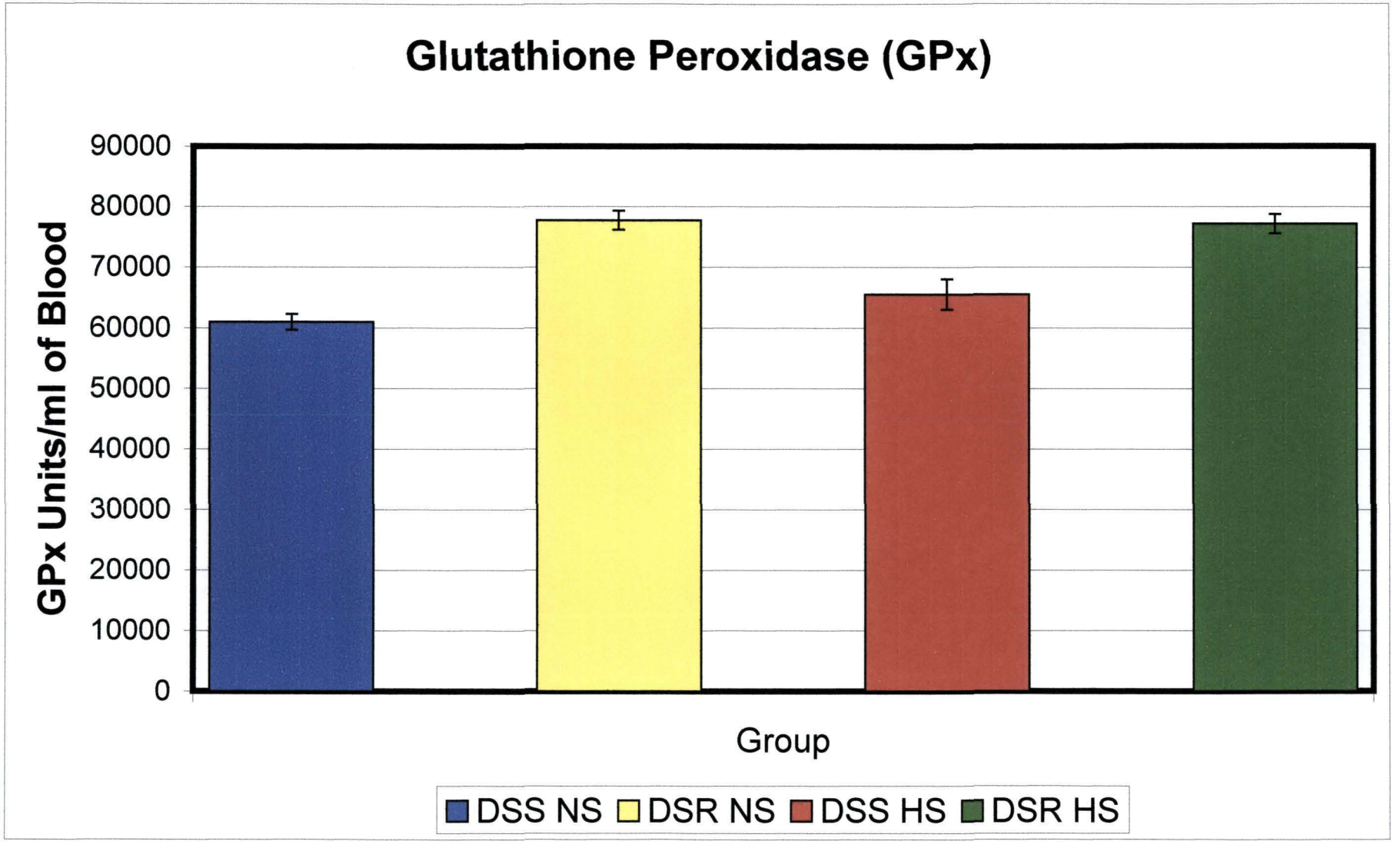
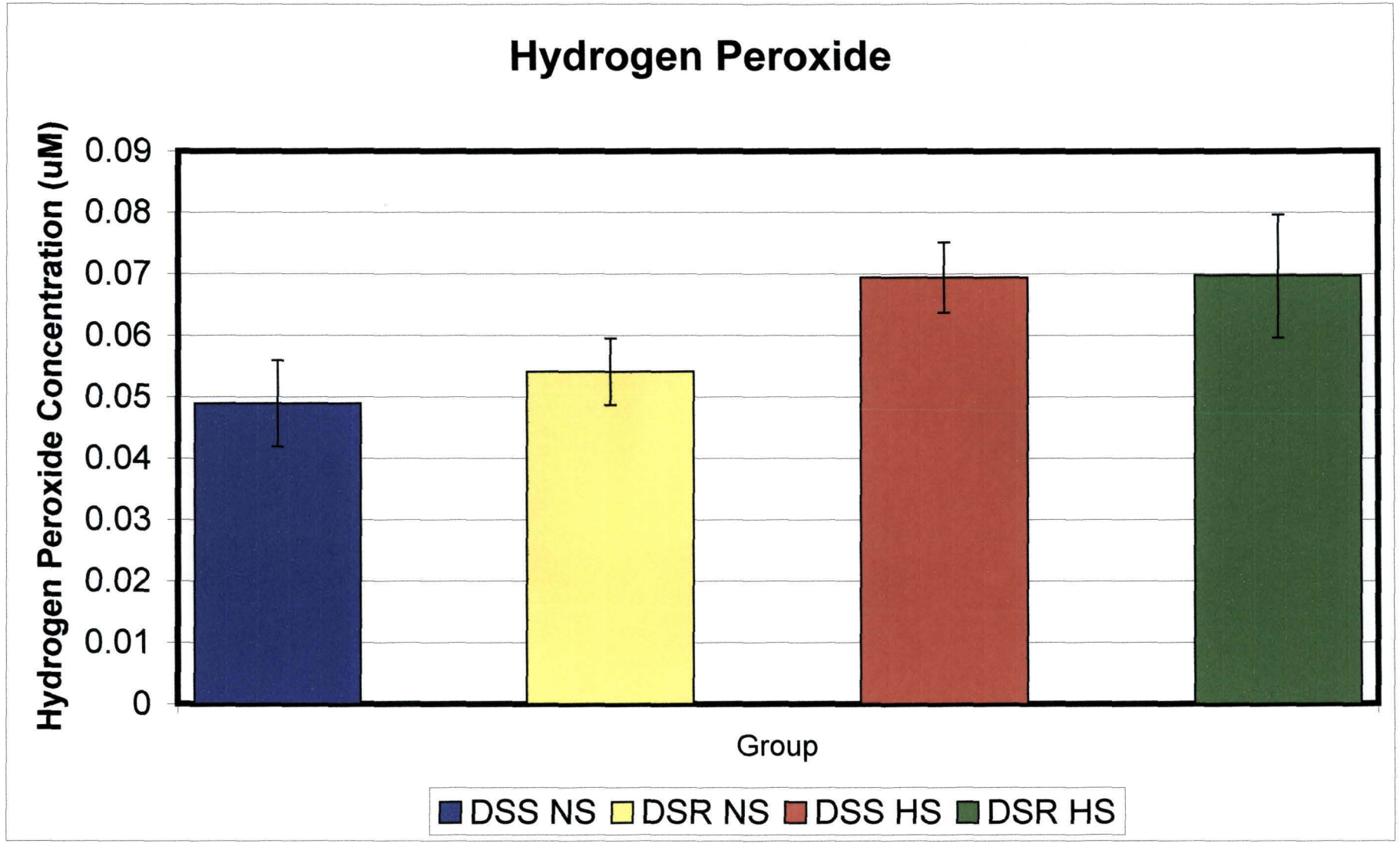


Figure 12. **Hydrogen Peroxide per ml of Plasma**



4.9. Lipid Peroxidation

4.9.1. Kidney

The kidney homogenates of all groups were exposed to increasing concentrations of hydrogen peroxide (H_2O_2) viz. (2.5 mM, 5 mM, 10mM and 15 mM). The homogenate was then subjected to the TBA test, and the concentrations of Malonyldialdehyde (MDA), which is a product of lipid peroxidation are shown in *Table* 15., and represented in *Figure 13.*

The level of lipid peroxidation *in vivo* (Total MDA) of the kidney was taken as the concentration of MDA generated after being exposed to 0 mM H_2O_2. The Total MDA concentration was significantly higher in the DSS group when compared to the control DSR group. Salt loading resulted in no significant difference in the Total MDA concentration when compared to the respective non-salt loaded controls.

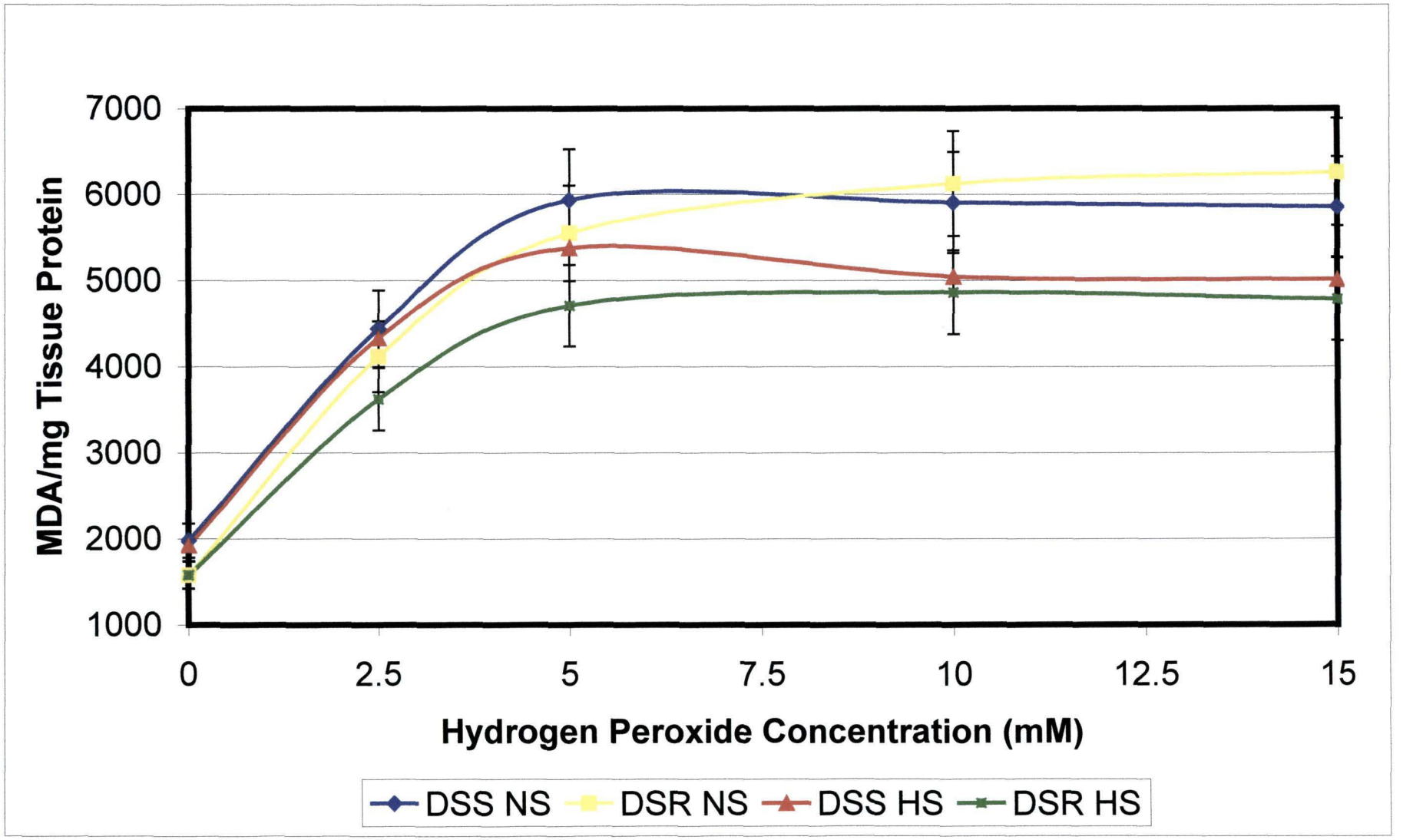
There was no significant difference in MDA concentration at the increasing concentrations of H_2O_2, of each group when compared to their respective controls. In all groups however, there was a significant increase in MDA concentration at the increasing levels of H_2O_2 when compared to the MDA concentration at 0mM H_2O_2. The maximal MDA concentration, being reached at differing concentrations of H_2O_2 for each group.

Table 15. **Oxidative Challenge Test – Kidney**

Strain / [ ] H_2O_2	0 mM	2.5 mM	5 mM	10 mM	15 mM
DSS NS (11)	1978.6 ± 113.9	4442.4 ± 278.3	5934.2 ± 362.8	5906.5 ± 338.7	5855.3 ± 282.0
DSR NS (12)	1576.3 ± 78.2	4116.8 ± 301.9	5548.9 ± 391.7	6127.0 ± 296.3	6261.1 ± 327.7
DSS HS (10)	1925.2 ± 99.3	4334.6 ± 356.2	5376.8 ± 498.2	5044.9 ± 488.2	5012.6 ± 559.7
DSR HS (10)	1581.4 ± 68.5	3623.6 ± 169.2	4708.6 ± 232.1	4863.1 ± 230.2	4781.9 ± 220.8

Numbers in brackets indicate n – value (Sample Size)
Values expressed as MDA(nmol)/mg Tissue Protein
Values shown as Mean ± SEM

Figure 13. **Oxidative Challenge Test – Kidney**



4.9.2. Liver

The liver homogenates of all groups were exposed to increasing concentrations of hydrogen peroxide (H_2O_2) viz. (2.5 mM, 5 mM, 10mM and 15 mM). The homogenate was then subjected to the TBA test, and the concentrations of Malonyldialdehyde (MDA), which is a product of lipid peroxidation are shown in *Table* 16., and represented in *Figure 14.*

The level of lipid peroxidation *in vivo* (Total MDA) of the liver was taken as the concentration of MDA generated after being exposed to 0 mM H_2O_2. There was no significant difference in the concentration of MDA of the DSS group when compared to the control DSR group. Salt loading resulted in no significant difference in MDA production at 0 mM H_2O_2, when compared to the non-salt loaded controls.

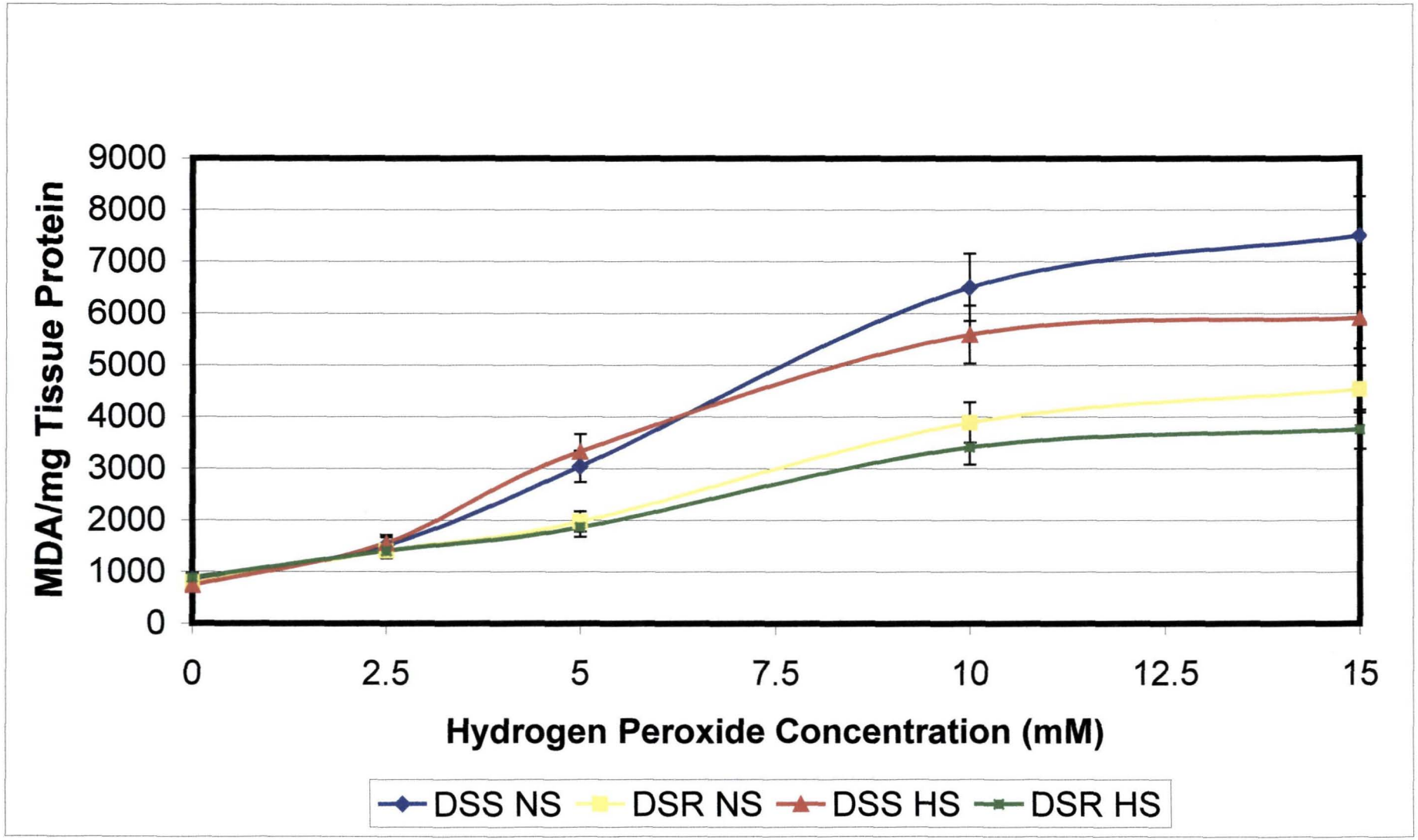
A significant increase in MDA production was evident at 5mM, 10mM and 15mM in both the DSS groups when compared to respective control DSR groups. At a H_2O_2 concentration of 5mM, 10mM and 15mM, there was a significant difference in MDA concentration of the salt loaded DSS group when compared to the non-salt loaded DSS group.

Table 16. **Oxidative Challenge Test – Liver**

Strain / [ ] H_2O_2	0 mM	2.5 mM	5 mM	10 mM	15 mM
DSS NS (11)	846.4 ± 25.4	1514.4 ± 49.7	3042.6 ± 240.9	6505.1 ± 369.6	7506.5 ± 365.5
DSR NS (10)	789.4 ± 33.1	1402.2 ± 35.1	1973.4 ± 166.5	3893.7 ± 520.8	4538.6 ± 518.8
DSS HS (10)	740.4 ± 47.7	1556.0 ± 158.4	3331.2 ± 208.5	5591.8 ± 215.0	5918.5 ± 223.6
DSR HS (11)	884.7 ± 26.4	1403.3 ± 84.7	1865.4 ± 59.9	3417.7 ± 214.9	3759.8 ± 230.3

Numbers in brackets indicate n – value (Sample Size)
Values expressed as MDA(nmol)/mg Tissue Protein
Values shown as Mean ± SEM

Figure 14. **Oxidative Challenge Test – Liver**



4.9.3. Brain

The brain homogenates were subjected to a direct TBA test, which resulted in the concentration of MDA present *in vivo* for the brain. This MDA concentration was taken as the total MDA of the brain, the results are shown in *Table 14*. There was no significant difference in MDA concentration in both the non-salt loaded and salt loaded DSS groups when compared to their respective DSR controls.

4.10. Standard Curves

The standard curves used in the study are shown in *Figure 15.*, (Superoxide Dismutase), *Figure 16.*, (Hydrogen peroxide) and *Figure 17.*, (Protein Estimation). All plotted values on the standard curves, were done in triplicate, and the average of these values were used to plot the standard curves. A best-fit line was plotted, using these values. The equations used to extrapolate the unknown values are shown on the respective plots.

Figure 15. **Standard Curve - Superoxide Dismutase (SOD)**

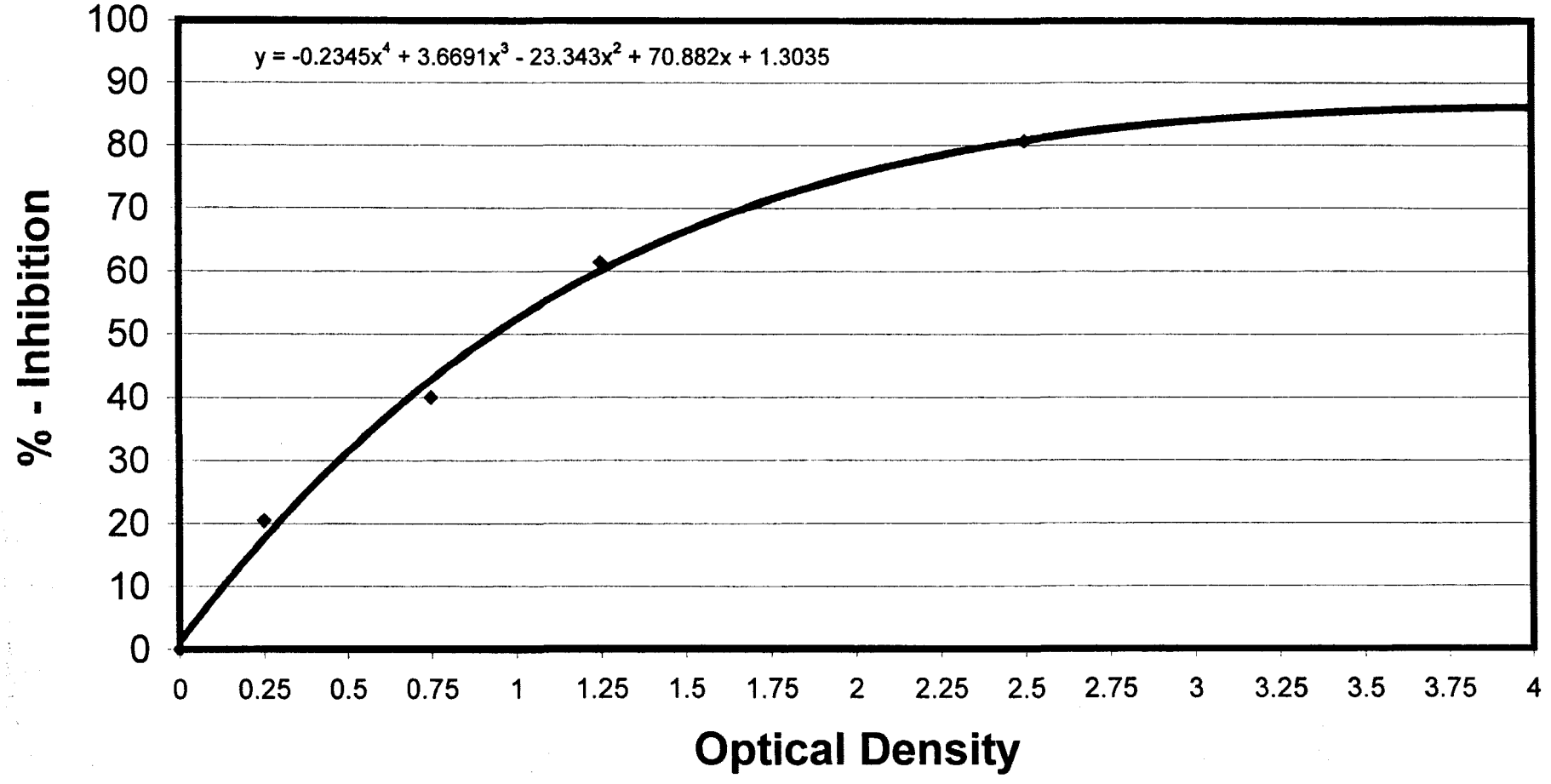


Figure 16. **Standard Curve - Hydrogen Peroxide**

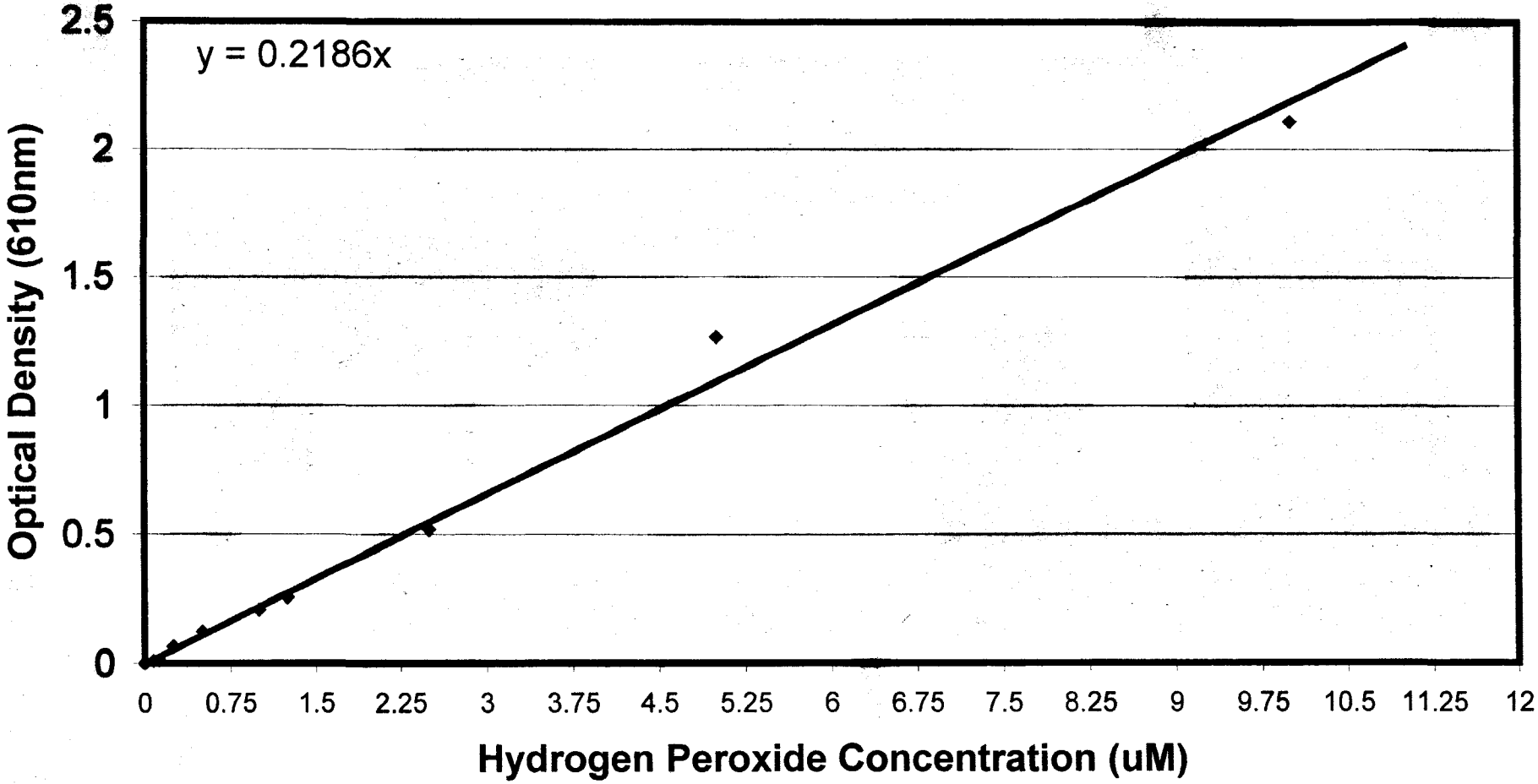
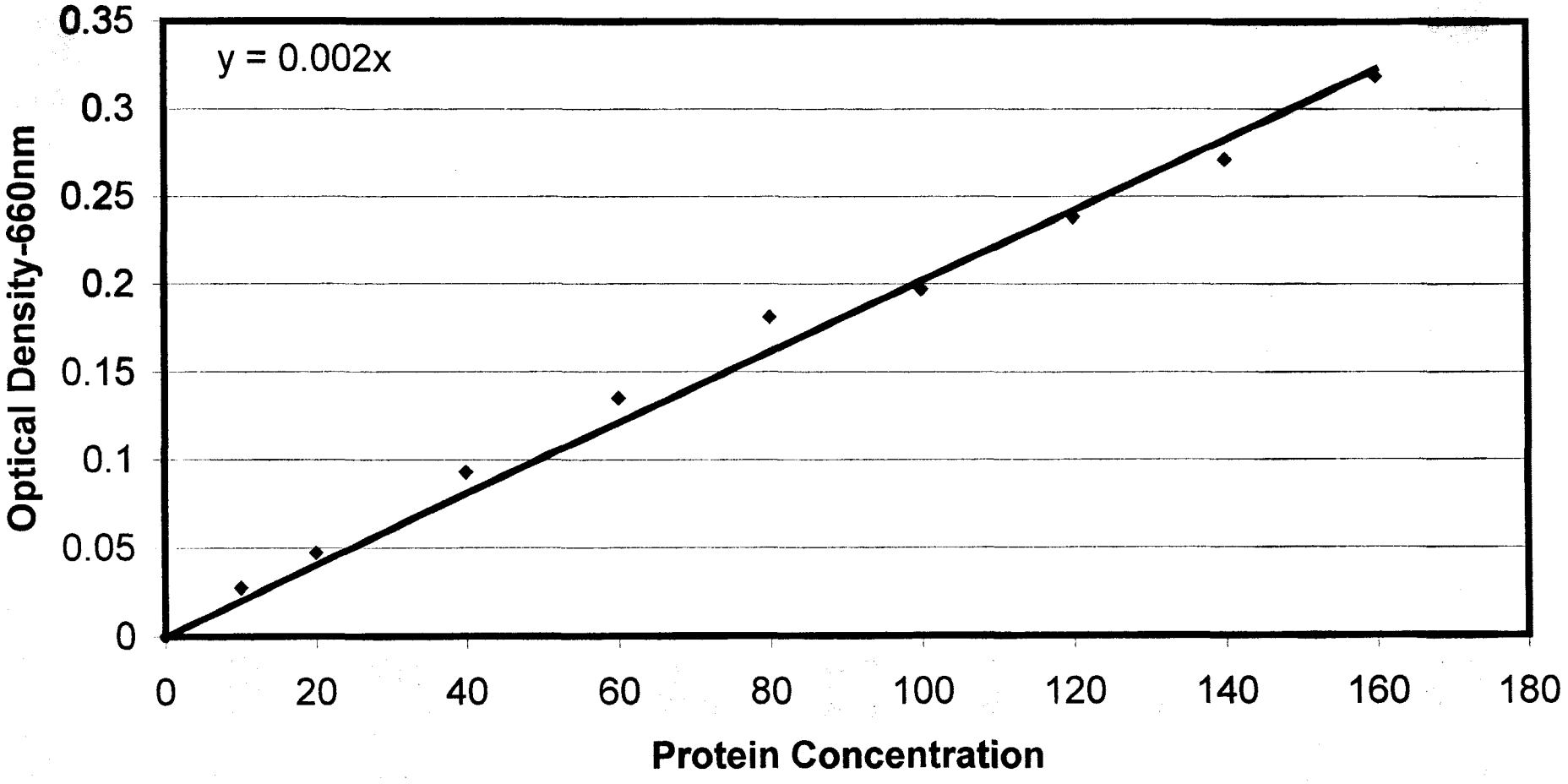


Figure 17. **Standard Curve - Protein Estimation (Lowry)**



5. DISCUSSION

This study has shown that the Dahl Salt Sensitive (DSS) strain, which is a well-established salt sensitive model of hypertension, demonstrates an evident link between salt sensitive hypertension and both the free radical and antioxidant status with resulting increase in tissue oxidative stress.

This has been demonstrated by the following patho-physiological changes in the Dahl Salt Sensitive (DSS) rat:

1. This strain has an increased vascular superoxide dismutase (SOD) concentration, which could be a compensatory mechanism to counter an increased *in vivo* level of free radicals.

2. Salt loading resulted in a decrease in vascular SOD concentration, indicating that salt loading decreases the bioavailability of SOD, due to an increase in the superoxide radical (O_2^-) found during salt loading.

3. The vascular glutathione peroxidase (GPx) levels are significantly decreased in the both the salt-loaded and non-salt loaded DSS group, thereby reducing its ability to neutralise an increased H_2O_2 concentration.

4. Salt loading increased the vascular GPx levels, but not sufficiently to counter the increase in plasma H_2O_2 concentration evaluated, as assessed by the GPx:H_2O_2 ratio.

5. Salt loading resulted in an increased plasma H_2O_2 concentration.

6. This strain showed an increase in the, *in vivo* levels of lipid peroxidation in specific target tissues viz. kidney, as a result of a suspected increase in tissue oxidative stress.

7. This strain has shown that tissue viz. kidney and liver has a reduced antioxidant buffering capacity in countering an induced free radical challenge, resulting in increased *in vitro* tissue oxidative stress due to increased lipid peroxidation.

8. It has also shown that tissue types adapt and react to increased free radicals, in different ways, suggesting that, different tissue types undergo differing levels of oxidative stress relative to their antioxidant buffering capacity.

From the results obtained thus far, it has been established that the DSS strain, has both a compromised antioxidant and an elevated free radical status due to changes in antioxidant enzyme concentrations and the related free radical concentrations. This compromised status results in oxidative stress. The DSS strain has also demonstrated that target tissue viz. kidney, has a higher level of *in vivo* tissue oxidative stress and it has a lower tissue antioxidant buffering capacity to induced oxidative stress. The study has thus shown that these parameters are linked to the genetic hypertensive state that the DSS model displays via the mechanisms discussed below.

5.1. Nutritional Parameters

The Dahl Salt Sensitive (DSS) strain on the normal salt (NS) diet showed an increasing trend in food consumption during the first 4 weeks of the study, with a plateau phase, in the successive weeks. The increasing trend corresponds with a similar trend apparent in the percentage (%) mass gain, during the first 4 weeks. The DSS strain had a higher food consumption per week than the control DSR animals, and a proportionally higher % mass gain per week. This increase in food consumption and subsequent increase in % mass gain could be due to a lower metabolism in the DSS strain. It has been hypothesised that antioxidant enzymes and free radicals could work in concert to alter metabolic activities of tissues (Yuan *et al.*, 1998). The altered status of both free radical and antioxidants found in the metabolically important tissue such as the kidney and liver (discussed later), could affect the metabolic rate and explain the increase in food consumption and %mass gain, apparent in the DSS strain.

Salt loading showed a significant drop in food consumption from the first week of salt loading in the 4^{th} week of the study in both the salt loaded DSS and DSR strains. The decrease in food consumption was manifested as a decrease in the %mass gain and seemed to stunt growth with respect to the % mass gain, in both the DSS and DSR control animals. The salt loaded strains had a significantly lower, end of study body mass when compared to their non-salt loaded controls, thus showing that metabolic activities are disrupted in salt sensitive hypertension.

The water intake of the DSS animals was significantly higher in the successive weeks of the study. The urine output of both the salt-loaded and non-salt loaded groups showed a corresponding trend to the water intake. Salt loading showed a significant

increase in water intake, in both the salt loaded groups when compared to the non-salt loaded groups. The water intake was ~ 3-4 times higher in the salt loaded groups than in the non-salt loaded controls. The increased water intake manifesting itself with a corresponding increase in urine output. The increased water intake in both the DSS salt loaded and non-salt loaded groups, is characteristic of salt sensitive models which display a downward shift in the pressure-natriuresis curve (Rapp., 1982). This shift results in salt retention with a corresponding expansion of fluid volume, resulting in the elevation of blood pressure (Gross *et al.*, 1997). This reported sodium retention would result in an increase in water intake to maintain homeostatic balance. This being evident in the DSS model which displays an increased water intake.

Salt loading was achieved by supplementing the water with 2% NaCl. The literature suggests that 0.5 – 1% is considered normal and levels above 4% are considered high (Tobian., 1997). The reason that 2% NaCl was chosen as a high salt intake was due to results obtained in a pilot study performed on the DSS and DSR strains. The pilot study used a 4% NaCl salt load, as a high salt load. This study showed abnormal changes in the nutritional and metabolic parameters of both strains. To eliminate the abnormal variations in these parameters, a 2% NaCl salt load was employed. This 2% NaCl salt load showed no abnormal variations in nutritional and metabolic parameters, but showed an incremental increase in blood pressure consistent with salt loading and was therefore employed as a high salt load.

The DSS strain in the last week of the study had a significant drop in urine output, when compared to the previous 3 weeks of salt loading, with a similar water intake. This significant drop in urine output could be due a decreased renal efficiency evident

with gross renal damage. This renal damage could be caused by the increase in blood pressure and increased lipid peroxidation in the kidney (discussed later) of this strain. The decreased urine output is consistent with decreased natriuretic and diuretic properties reported in the kidney during hypertension, which could result from renal damage (Meng *et al.*, 2003). This decrease in renal efficiency may result in water retention with a resultant expansion in fluid volume and thereby contribute to the increase in blood pressure.

5.2. Blood Pressure and Heart Rate

Both the systolic and diastolic blood pressures of the DSS strain showed an increasing trend through the successive weeks of the study. In contrast the DSR control strains showed a steady systolic and diastolic blood pressure through the successive weeks of the study. The slight increase in the systolic and diastolic blood pressure's when comparing the 1st week of the study to the 7th week in the DSR strain can be attributed to an age related increase in blood pressure. The steady state blood pressure of the salt loaded DSR group further confirms the resistance of this strain to an increase in blood pressure due to salt loading and emphasising the strains salt resistance. The changes in blood pressure obtained in this study are consistent with previous results obtained from this model in our laboratory (Somova *et al.*, 2001).

Salt loading had a significant increase in the blood pressure of the DSS strain, further emphasising the strains susceptibility to salt loading. The salt loading exacerbated, the increase in blood pressure, this being evident when comparing the salt loaded DSS strain to the non-salt loaded controls from the 2nd week of salt loading. Thus reinforcing the strains genetic susceptibility to an increase in salt intake. The resulting increase in blood pressure which was due to an increase in salt intake, thus demonstrates the salt sensitivity of this model as reported in other studies (Weinberger., 1996).

One of the most significant primary factors that lead to hypertension in salt sensitive humans and experimental models such as the Dahl rat is the shift seen in the pressure natriuresis curve (Rapp., 1982) (Fig 1.). This shift results in salt retention with a corresponding expansion of the fluid volume, resulting in an elevation in blood

pressure. The increase in heart rate that is displayed by the DSS strain, could be attributed to an increase in sympathetic activity that has been previously reported in this genetic model of salt sensitive hypertension (Grassi *et al.,* 1998).

The steady increase in blood pressure evident in the DSS strain, even without salt loading further emphasises this models genetic susceptibility to an increase in blood pressure associated with the hypertensive state. The salt loading merely exacerbates the increase in both the systolic and diastolic blood pressure evident in this study (Sustarsie *et al.,* 1981).

5.3. Antioxidants, Free radicals, Tissue Oxidative Stress and the link with Hypertension

The antioxidant status of all groups were evaluated by assessing the levels of the major antioxidants in the vascular compartment viz. Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx). The results show that the DSS strain on a normal salt diet had a significantly higher SOD concentration than the control Dahl Salt Resistant (DSR) strain on the same diet. SOD is the antioxidant enzyme that neutralises the deleterious reactive oxygen species (ROS), superoxide (O_2^-), to a more stable and relatively less deleterious free radical, hydrogen peroxide (H_2O_2) (Halliwell., 1996).

The increased SOD concentration observed in the DSS strain, is consistent with studies done in Angiotensin II (Ang II)-induced hypertension in rats, that shows that an increase in SOD concentration is probably an adaptive, negative feedback mechanism, where Ang II increased the vascular expression of extracellular SOD (SOD-3 / ecSOD) *in vitro* and *in vivo* (Fukai *et al.,* 1999, Wassmann *et al.,* 2004). The hypertensive state displayed by the DSS strain has been linked to an increase in Ang II by studies done on this model previously (Meng *et al.,* 2002, Rajagopalan., 1996). The DSS strain may have an adaptive mechanism with respect to the levels of SOD, that is due to an increase in O_2^- levels *in vivo*, this compensatory adaptation aiding in the neutralisation of a free radical "onslaught" from the O_2^- species.

The mechanism of renin-angiotensin system–induced hypertension has generally been attributed to the vasoconstrictor effects of Ang II and the mineralocorticoid effects of aldosterone. Recent work has however revealed an additional potential mechanism.

Ang II has been shown to stimulate O_2^- generation by increasing the activity of the enzyme NAD(P)H cytochrome P-450 oxidoreductase, more commonly termed NAD(P)H oxidase, in cultured rat vascular smooth muscle cells and in intact aortas of rats made hypertensive by angiotensin II infusion. This appears to be a fairly specific effect, as rats made hypertensive to a similar degree by infusion of noradrenalin showed no increase in NAD(P)H oxidase activity. Blood pressure and vascular reactivity could be restored by exogenous liposome-encapsulated SOD in the angiotensin II hypertensive rats, but not the noradrenalin hypertensive rats. This further implicates O_2^- in hypertension associated with high angiotensin II states. They concluded that this was further evidence that angiotensin II–induced hypertension activates the NAD(P)H oxidase system and that this system is directly involved with the pathology of hypertension (Rajagopalan *et al.*, 1996, McIntyre *et al.*, 1999).

This study has shown that salt loading had a significant decrease in the vascular levels of SOD *in vivo*, when compared to the non-salt loaded controls. This demonstrating that in salt sensitive hypertension, there is a decrease in the ability to counter a free radical "onslaught" by O_2^-. The decrease in SOD levels in the salt-loaded group may also indicate an increase in O_2^- levels associated with salt loading, and this increase in O_2^-, thereby limiting the bioavailability of SOD. The H_2O_2 levels evaluated in the study showed that the salt-loaded DSS strain, had a non-significant increase in the plasma concentration of H_2O_2. These increased levels are consistent with the decreased levels of vascular SOD. The decreased SOD, which may be due to the increased neutralisation of O_2^-, would result in increased H_2O_2 as an end product, this increase has been shown in the plasma levels of H_2O_2 established in this study. This study therefore demonstrated results that are similar to a study, that showed the

hypertensive high salt DSS strain has a significantly higher plasma H_2O_2 than the non-salt loaded controls, which was determined via the Clarke electrode method (Swei *et al.,* 1997). The disparity in significance of the H_2O_2 levels that were found in this study when compared to the above mentioned study could have resulted from the reported non-specificity of the method employed in this study (Tarpey *et al.,* 2004).

It has been established that SOD is the antioxidant enzyme that neutralises the superoxide (O_2^-) radical. The role that O_2^- plays in hypertension per se is a well-established and putative one. The most significant role is the effect that O_2^- has on nitric oxide (NO). NO is a potent endogenous vasodilator (Palmer *et al.,* 1987), it regulates vascular tone in normal vessels, which includes resistance vessels, and it causes renal vasodilatation with consequent diuresis and natriuresis. The actions of NO would tend to lower blood pressure and therefore a reduction in this mechanism is a means in which O_2^- contributes to hypertension (McIntyre *et al.,* 1999).

O_2^- reduces the bioavailability of NO, due to its high affinity to NO and thereby forming peroxynitrite ($ONOO^-$) (Rubanyi and Vanhoutte., 1986). Therefore circumstances that result in increased O_2^-, such as hypertension can be harmful in several ways, firstly by halting the beneficial effects of NO by reducing its bioavailability and secondly by the damaging effects of $ONOO^-$, which can be protonated to peroxynitrous acid, the cleavage products of which are among the most reactive oxygen species, in the biological system (Beckman *et al.,* 1994, McIntyre *et al.,* 1999). In addition to this, studies have demonstrated that O_2^- can act directly as a vasoconstrictor (Stocker and Keaney., 2004).

The putative role that $O_2^{\cdot-}$ plays during hypertension can be successfully assessed with reference to studies that have administered synthetic SOD, to neutralise increased $O_2^{\cdot-}$ levels, in hypertensive rats and were successful in lowering the blood pressure (Nakazono *et al.,* 1991). Thus the mechanism by which $O_2^{\cdot-}$ acts on NO to increase blood pressure during hypertension is an important one. It can be further demonstrated how the deleterious effects of $O_2^{\cdot-}$ are successfully neutralised by SOD and thereby lowering blood pressure, in studies linked with Down syndrome. The human gene for Cu/Zn SOD (SOD-1) has been localized to the 21q22.1 region of chromosome 21. Therefore, patients with Down syndrome (trisomy 21) have an extra copy of the gene and have been shown to have SOD-1 activity 50% greater than the normal diploid population, in keeping with the gene-dosage effect (De la Torre *et al.,* 1996). Transgenic rats containing an extra copy of the human SOD-1 gene display some of the neurological defects characteristic of Down syndrome, including premature aging, suggesting that this gene is involved in the pathogenesis of Down syndrome (Yarom *et al.,* 1988). The increased SOD-1 activity in Down syndrome may further indicate a role for $O_2^{\cdot-}$ in hypertension. With a higher SOD-1 activity, Down syndrome patients will have reduced $O_2^{\cdot-}$ levels. If $O_2^{\cdot-}$ excess is involved in the pathogenesis of hypertension, then one would expect Down syndrome patients to have lower blood pressure. This was recently found to be the case in a well-controlled study by Morrison *et al.,* 1996 (McIntyre *et al.,* 1999). This thereby confirming the positive role that $O_2^{\cdot-}$ plays in increasing blood pressure during hypertension by the effects discussed earlier.

There are many factors both established and some still under investigation that seem to be involved in increasing the production of $O_2^{\cdot-}$ and thereby decreasing the

bioavailability of NO, resulting in an increased blood pressure during the hypertensive state. A major implicated source of O_2^- is the oxidant enzyme NADPH oxidase. It is known that phagocytes, adventitial fibroblasts, vascular smooth muscle cells (VSMC) and endothelial cells (EC) contain a plasma-membrane bound multi-component oxidase that utilises electrons derived from NADPH to reduce molecular O_2 to O_2^- (Stocker and Keaney., 2004). An over expression of this enzyme due to Angiotensin II (Ang II) infusion was found to result in an increase in the O_2^- production, during the hypertensive state (McIntyre *et al.*, 1999). It has been previously reported that the DSS strain has increased levels of Ang II, this would result in an increase in the expression of NADPH oxidase and may therefore result in the increase in O_2^-, apparent in this study (Rajagopalan *et al.*, 1996).

The GPx levels in the vascular compartment showed a significant decrease in the DSS strain when compared to the DSR control group. GPx is the antioxidant enzyme that with catalase neutralises H_2O_2 to H_2O and molecular O_2. The DSS strain showed a significantly decreased level of GPx with no significant difference in plasma H_2O_2 levels. A similar study on the DSS strain by Swei *et al.*, 1997, found a significant increase in plasma H_2O_2 levels, which was measured using the Clarke electrode. The evident disparity in results could be due to the reported, relative inaccuracy of the method employed in this study (Tarpey *et al.*, 2004). The GPx levels demonstrate that the DSS animal has a compromised ability to neutralise H_2O_2 *in vivo*, due to the lower vascular GPx levels. This is consistent with other studies that found a decrease in the *in vivo* GPx levels in the DSS strain (Turi *et al.*, 2003). Salt loading had a non-significant increase in vascular GPx concentration in the DSS strain. This increase may be due to the non-significant increase in H_2O_2 levels during salt loading, and may

be an adaptive mechanism to this increased H_2O_2 concentration *in vivo*. The GPx : H_2O_2 ratio which illustrates the relative "number" of GPx units available to neutralise 1nanomol H_2O_2, was used in this study as a theoretical measure of the bioavailability of GPx to neutralise H_2O_2. This ratio clearly demonstrates, that although the salt-loaded DSS strain demonstrated an increase in GPx concentration during salt loading, the decrease in this ratio, illustrates a decrease in the ability of GPx to neutralise the H_2O_2 free radical during salt loading.

It has been thus far demonstrated that SOD is a major defence against an increase in blood pressure associated with hypertension, via the dismutation of O_2^-. However, although the dismutation of O_2^-, prevents the deleterious effects of this free radical as discussed previously, hydrogen peroxide (H_2O_2) is an end product of this reaction. GPx is the antioxidant enzyme that with catalase neutralises H_2O_2 to oxygen (O_2) and water (H_2O). An increase in the dismutation of O_2^- by SOD would result in a relative increase in H_2O_2 formation, with a resulting decrease in SOD, as found in this study. If the GPx levels are compromised as shown in this study, then this would result in an accumulation of H_2O_2. An increase in the *in vivo* H_2O_2 concentration in the DSS model has been shown in similar studies (Swei *et al.,* 1997) and has been linked to the hypertensive state by the mechanisms discussed below.

Virtually all types of vascular cells produce O_2^- and H_2O_2. The endothelium plays a crucial role in the regulation of vascular tone and vascular remodelling. Endothelial injury or exposure to O_2^- and H_2O_2 induces apoptosis (programmed cell death) of endothelial cells (EC), which leads to EC loss and results in atherogenesis and a procoagulative state (McIntyre *et al.,* 1999). These reactive oxygen species (ROS)

regulate apoptotic mechanisms induced by a variety of stimuli. Another type of programmed cell death, termed anoikis, results from the detachment of EC's from the extracellular matrix and this process is associated with increased intracellular ROS. EC migration, proliferation and tube formation are essential events in the process of apoptosis resulting in angiogenesis. ROS maybe directly involved in all these mechanisms, as H_2O_2 has been shown to induce proliferation and migration of EC's and to mediate lymphocyte-activated tubulogenesis. These ROS also act as mediators of angiogenic growth factors, such as vascular endothelial growth factor (VEGF) (Taniyama and Griendling., 2003).

The production of ROS O_2^- and H_2O_2 is involved in many of the processes leading to both hypertrophic and proliferative vascular smooth muscle cell (VSMC) growth. These ROS also mediate the full proliferative response to agonists such as platelet derived growth factor (PDGF) and thrombin. H_2O_2 itself induces VSMC proliferation, although this effect appears to be critically dependant on the concentration of H_2O_2, which cells are exposed to. It has been found that endogenously produced H_2O_2 may also be important in modulating survival and proliferation of VSMC's (Taniyama and Griendling., 2003).

It has been shown previously, that relatively high concentrations of H_2O_2 induce apoptosis, whereas a moderate concentration causes cell cycle arrest. This demonstrating that concentrations of H_2O_2 from moderate to high, have deleterious effects in the vasculature (Taniyama and Griendling., 2003). H_2O_2 does not function as a mitogen for VSMC's, instead, it serves as a stimulus to trigger VSMC apoptosis (Li *et al.,* 1997) These factors suggest that H_2O_2 contributes to hypertension by

altering vascular remodelling. Structural vascular remodelling is a hallmark of chronic hypertension, and an increased wall to lumen ratio of resistance arteries is the predominant lesion. The increase in relative "thickness" of resistance arteries is responsible for the "amplifier" property of the arterial circulation in hypertension, which functionally manifests itself as a pressor or vasoconstrictor hyperresponsiveness (Simon *et al.,* 1998).

An accumulation of H_2O_2 *in vivo* as reported, could have the following deleterious effect if not neutralised by GPx and catalase. In the presence of the transition metals Iron (Fe) and Copper (Cu) and O_2^-, H_2O_2 is broken down to the hydroxyl radical (OH^-), which is considered to be potentially the most potent oxidant encountered in biological systems (Yu., 1994). The OH^- radical causes strand breaks and base modification in DNA leading to changes in gene expression, mutation and apoptosis. Protein side chains are oxidised which can result in enzyme, receptor and carrier dysfunction. The resulting lipid peroxidation due to OH^- alters functional properties of membranes and delivery of lipids to tissues (Halliwell., 1996).

Thus an accumulation of H_2O_2 results in the formation of OH^-, which has been reported to cause an increase in lipid peroxidation resulting in tissue damage associated with the hypertensive state. Thus increased levels of H_2O_2 contribute to the hypertensive state via two main mechanisms, firstly via vascular remodelling and secondly by the formation of OH^-, leading to lipid peroxidation and eventual tissue damage.

Oxidative stress leading to tissue damage, appears to be a focal point in hypertension research. The tissue damage associated with lipid peroxidation due to free radicals could be a major factor in the pathogenesis of hypertension, since it is accompanied by architectural changes in the kidney, heart and vessels that are often deleterious and can eventually contribute to end organ diseases such as renal failure, heart failure and coronary disease (Raij., 1998).

The level of oxidative stress was assessed in the kidney, liver and brain of the DSS strain, by quantifying the *in vivo* concentration of malonyldialdehyde (MDA) in these tissues. MDA is a byproduct of lipid peroxidation by free radicals and is regarded as a reasonable indicator of oxidative stress *in vivo* (Buczynski *et al.,* 1993). The above-mentioned tissues were also subjected to an oxidative challenge, by exposing them to increasing, known concentrations of H_2O_2. This was done to assess the antioxidant buffering capacity of the tissue to an induced free radical challenge.

The oxidative challenge test in the kidney of the DSS strain showed a significantly higher MDA concentration, and therefore a higher level of lipid peroxidation. Total MDA concentration *in vivo*, was assessed as the MDA concentration, with no induced free radical challenge i.e. (0mM H_2O_2). The total MDA concentration showed that the kidney of the DSS strain had a significantly higher level of lipid peroxidation than the DSR control group. This demonstrates that lipid peroxidation in the kidney of the DSS strain could be due to a higher concentration of free radical in the kidney and/or a lower tissue antioxidant concentration. The kidney of the DSS strain could therefore be undergoing an increased level of oxidative stress relative to the DSR control strain.

This therefore supports the hypothesis/theory that hypertension is a "free radical" disease with a resultant increase in tissue oxidative stress.

Salt loading had a non-significant increase in the level of lipid peroxidation in the kidney of the DSS strain, this could indicate that salt loading does not manifest itself as an increased level of lipid peroxidation in the kidney and/or the kidney could have a compensatory antioxidant buffering capacity to an increased free radical concentration, established during salt loading. When the kidney was subjected to the oxidative challenge test with increasing concentrations of H_2O_2, the level of lipid peroxidation increased, demonstrating a curvilinear relationship with the increasing levels of free radical. A H_2O_2 concentration of 5mM, proved to be the concentration at which a maximal level of lipid peroxidation was reached. The curve was consistent up to this concentration. The concentrations above 5mM H_2O_2 could have introduced variables that, induced the inconsistency apparent at these concentrations. Analysing these variables was beyond the scope of this study and therefore for the discussion of the oxidative challenge, these higher H_2O_2 concentrations would be ignored.

Salt loading showed a non-significant decrease in the level of lipid peroxidation in the kidney of the salt-loaded DSS strain. This could suggest a compensatory increase in the antioxidant buffering capacity of the kidney to an increased free radical concentration in the kidney during salt loading. This analysis being purely speculative and a confirmation would lie in assessing the free radical and antioxidant levels in the kidney. Unfortunately these parameters where beyond the scope of this study. The study thus shows that during salt sensitive hypertension, the kidney of the DSS strain demonstrates an increased level of lipid peroxidation and therefore an associated

increase in tissue damage. This shows that the kidney has a relatively higher level of oxidative stress and that it is a target organ for free radical induced oxidative stress during the hypertensive state. These findings are consistent with other studies that have been done to assess the level of damage in the kidney during salt sensitive hypertension (Meng *at al.,* 2003).

This study has therefore shown that the kidney appears to be a target organ during the hypertensive state, due to an increased level of lipid peroxidation products (MDA) ***in vivo***. MDA, the most abundant among the reactive aldehydes derived from lipid peroxidation, has been found to be significantly increased in blood as well as in peripheral mononuclear cells. These aldehydes are implicated causative agents in cytotoxic processes, and when released from cell membranes they may diffuse, interact and induce oxidative modifications in other cells and thus increasing the risk of both cardiovascular and renal damage in hypertension (Steinberg *et al.,* 1989, Redon *et al.,* 2003).

Hypertension induces important functional and structural alterations in the kidney, resulting in proteinuria, glomerular sclerosis, and other morphological changes, eventually leading to end-stage renal disease. Reducing blood pressure in hypertensive patients retards the progression of renal failure and reduces the morbidity and mortality rates (Meng *et al.,* 2003). The kidney appears to be both cause and victim in the hypertensive process. Renal hemodynamics are abnormal even in the early stages of primary hypertension, and the changes become pronounced with the severity of the disease (Ruilope *at al.,* 1990).

A variety of studies in humans and animals suggest a renal abnormality or multiple alterations in renal function due to salt-sensitive hypertension (Weinberger., 1996). ROS can act as signal transduction messengers for several transcription factors, which play a critical role in the activation of multiple genes that contribute to the inflammatory response and end organ damage (Schnackenberg., 2002).

It can thus be shown that the increased tissue oxidative stress seen in the kidney of the DSS model, represented by a high level of *in vivo* lipid peroxidation would contribute to the hypertensive state by impacting on the renal efficiency, and thereby contribute to the hypertensive state, by decreasing the natriuretic and diuretic properties of the kidney, which tends to lowers blood pressure (Meng *et al.,* 2003).

The decreased antioxidant buffering capacity of both the kidney and liver demonstrated in this study, shows that increased levels of free radical would result in an increase in tissue oxidative stress. This would result in tissue damage due to lipid peroxidation, and thereby decreasing the homeostatic roles of these organs. The decreased homeostatic capacity of these organs would contribute to a number of disease states associated with damage to these organs. This study has shown that the kidney appears to be a target organ for increased oxidative stress and is consistent with other studies demonstrating tissue oxidative stress in the hypertensive state (Meng *et al.,* 2003, Raij., 1998, Zhou *et al.,* 2000).

The total MDA concentration which was taken as the level of lipid peroxidation of the liver *in vivo*, showed a non-significant increase in the level of lipid peroxidation in the DSS strain. This demonstrates the liver of the DSS strain has a "normal" level of lipid

peroxidation relative to the control strain. However when the liver of the DSS strain was exposed to the oxidative challenge test, with increasing concentrations of H_2O_2, a significant increase in the level of lipid peroxidation was apparent from 5mM H_2O_2 to 15mM H_2O_2. This clearly demonstrating that the liver of the DSS strain has a significant decrease in the antioxidant buffering capacity to counter a higher concentration of free radical, and thus is susceptible to tissue damage due to lipid peroxidation at a higher concentration of free radical than is present *in vivo*.

When comparing the total MDA concentration to the MDA concentration at the increasing concentrations of induced free radical (2.5mM – 15mM H_2O_2), a clear picture arises that the liver of the DSS strain copes extremely efficiently in neutralising an increased free radical concentration associated with the hypertensive state. In contrast when the liver is exposed to higher levels of free radical (> 5mM), the tissue is overcome by this free radical onslaught and loses the efficient antioxidant buffering capacity evident at a lower *in vivo* free radical concentration.

Salt loading had a non-significant increase in the level of lipid peroxidation in the liver, in the salt-loaded DSS group. The salt-loaded group displayed the same curvilinear relationship as the non-salt loaded controls, when exposed to the oxidative challenge test. Thus confirming that the liver has an efficient *in vivo* antioxidant buffering capacity to an increased free radical concentration, that has been established in the DSS strain, and that the liver of the DSS strain copes efficiently with the increased free radical levels associated with salt loading and resulting salt sensitivity. The liver of the DSS strain shows a limited, non-significant level of tissue oxidative stress during the hypertensive state. This suggests that the liver may not be a target

organ for free radicals in salt sensitive hypertension. Thus when comparing the level of *in vivo* lipid peroxidation in the kidney and in the liver, it is apparent that the kidney has a higher level of *in vivo* tissue oxidative stress than the liver during salt sensitive hypertension. This difference in the level of lipid peroxidation, therefore strongly suggests that the kidney is targeted by oxidative stress, with resultant free radical mediated lipid peroxidation, during salt sensitive hypertension.

The total MDA concentration in the brain of the DSS strain showed no significant difference in the MDA concentration. It can thus be established that the brain has a normal level of lipid peroxidation relative to the control. Salt loading had a non-significant increase in the MDA concentration *in vivo*, of the salt-loaded DSS group. This non-significant increase may indicate that the brain is not isolated from the increased free radical concentration, established during salt loading, but the brain appears to have an efficient antioxidant buffering capacity during increased levels of free radicals. The whole brain was analysed for this study, in retrospect, the isolated brain stem would have been a more accurate indicator of increased oxidative stress during the hypertensive state. The brain stem contains the major "control centres" for cardiovascular regulation, and is suspected to be a target for free radicals during the hypertensive state, and thus would have proved to be a more accurate measure of tissue damage associated with tissue oxidative stress and the hypertensive state.

It has been shown that the kidney is a target organ during the hypertensive state due to an increased level of lipid peroxidation. This increased level of *in vivo* lipid peroxidation could be due to a compromised antioxidant and free radical status. The

study has also shown that these compromised states seem to have a non-significant bearing on the level of lipid peroxidation due to free radicals, in the liver and brain.

This can be explained by a recent study that shows that lipid peroxidative damage occurs in differing levels that are determined by organ type, age and subcellular structure-linked differences (Popova and Popov., 2002). It has also been reported that various tissues contain different levels and activities of antioxidants and enzymes such as vitamin E, C, glutathione, GPx, SOD and catalase (Lew *et al.,* 1985, Gohil *et al.,* 1986; Di Meo *et al.,* 1996, Popova and Popov., 2002). This may also be so because it has been shown that O_2^- is not membrane permeable and is therefore restricted to reacting in the compartment in which it is generated (McIntyre *et al.,* 1999). This would therefore explain the "normal" levels of lipid peroxidation shown in the liver and brain and that these tissues may not be target tissue, by oxidative stress in the hypertensive state or that they are able to buffer the free radical "attack" sufficiently to minimise lipid peroxidation. This being purely speculative and direct measurements of *in vivo* tissue free radical and antioxidant levels would provide a reliable definition of these tissues, antioxidant and free radical status during the hypertensive state.

6. CONCLUSION AND RECOMMENDATIONS

This study has provided further confirmation that the Dahl rat is an excellent model for assessing the patho-physiological changes associated with salt sensitive hypertension. It has shown that the Dahl Salt Sensitive strain (DSS), has a compromised antioxidant status when both on normal and high salt diet in the vascular compartment. This compromised status is due to the decreased levels of superoxide dismutase (SOD) and the non-sufficient increase in the glutathione peroxidase (GPx) concentrations during salt loading. This model also showed that it may have developed a compensatory increase in SOD, due to an increase in the formation of the superoxide (O_2^-) free radical species. This being demonstrated by the increase in SOD concentration, in the vascular compartment, which was independent of salt loading.

The plasma hydrogen peroxide (H_2O_2) concentration, which was used as a measure of the vascular free radical status, demonstrated that the DSS strain has a non-significant increase in plasma H_2O_2 concentration during salt loading, which could be caused by an increase in the dismutation of O_2^- by SOD. Thereby confirming that the model has a compromised free radical status, with resultant oxidative stress. It has been established that H_2O_2 induces vascular remodelling, and thereby contributing in part to the hypertensive state. To substantiate these findings, a more specific method to assess the H_2O_2 concentration would need to be employed, such as the Clarke electrode, because this study found non-significant changes, which may be due to the reported non-specificity of the method employed (Tarpey *et al.*, 2004).

With regard to H_2O_2, it is known that both GPx and catalase are responsible in neutralising the species. This study only assessed the vascular GPx levels, it would be more reliable to also assess whether catalase levels are also compromised. A measure of both these enzymes, would provide a more accurate means of assessing the level of the compromised antioxidant status. This would a provide a means of also assessing the integrated nature of the antioxidant system with respect to neutralising an increased level of H_2O_2, found during the hypertensive state.

The important role that O_2^- plays during the hypertensive state with respect to increasing blood pressure by decreasing the bioavailability of nitric oxide (NO), is an important, established one. Many studies have shown that synthetic SOD infusion, resulted in a significant decrease in blood pressure (Nakazono., 1991). Although the decrease in blood pressure is an important factor in reducing the level of hypertensive related damage, a coupled investigation with respect to the effect that SOD infusion has on buffering the level of tissue oxidative stress, would prove a means of assessing the exact role that O_2^- plays in lipid peroxidation resulting in end organ disease.

In this coupled study the H_2O_2 levels should also be assessed, as increased dismutation of O_2^- would result in an increase in the production of H_2O_2. The role that H_2O_2 plays in vascular remodelling is a putative one. Therefore, although SOD infusion appears to be a short-term measure in decreasing blood pressure, a long-term histological study on the vasculature would prove a means of assessing the deleterious effects of increased levels of H_2O_2 in the vasculature during long term SOD infusion. This being due to vascular remodelling, and increased lipid peroxidation by the

formation of the hydroxyl radical (OH^-), both of which are suspected to occur during H_2O_2 accumulation.

Angiotensin II (Ang II), has been found to contribute to the hypertensive state, not only by the vasoconstrictor effect that has been established. Ang II infusion has been found to increase the vascular expression of NADPH oxidase. NADPH oxidase is known to increase O_2^- production via the conversion of molecular O_2. Although there are other sources of O_2^-, a study done to show an over expression of NADPH oxidase, would be a reliable indicator of an increased production of O_2^-. This would be a more accurate means of assessing vascular O_2^- levels, since measuring O_2^- levels *in vivo*, could be inaccurate due to the short half life and resultant high reactivity of this free radical.

The Dahl rat has been established as an excellent model of genetic salt sensitive hypertension. It should be further investigated the exact effects, if any, that free radicals have in changing the genetic characteristics of this model, because this study showed an increase in SOD production independent of salt loading. Free radicals have been found to damage genetically critical material. These genetic changes could work in concert with the established genetic mechanisms of this model to exacerbate the hypertensive state that this model displays. A genetic vascular and tissue profile would be an excellent measure of this models relevance to free radical and antioxidant research. This profile will also expose the deleterious effect that free radicals have in up regulation and/or down regulation of critical enzymes and factors, such as SOD, NADPH and H_2O_2. This profile should include the effect, if any that salt loading has on the expression of these enzymes and factors.

This study showed that during the hypertensive state, organs critical in buffering the hypertensive state viz. the kidney have an increased level of *in vivo* lipid peroxidation. Increased lipid peroxidation would result in tissue damage and eventual end organ disease. It has been shown in this study that the hypertensive state contributes to the increased lipid peroxidation seen in the kidney. This tissue damage in the kidney would in turn contribute to the hypertensive state by decreasing the natriuretic and diuretic properties of the kidney, and thereby resulting in an increase in blood pressure. Thus by controlling the free radical levels *in vivo*, one would be able to minimise the deleterious effects that free radicals have on the kidney by decreasing lipid peroxidation and the resultant tissue damage. In this study the role of antioxidant therapy should be investigated, so as to determine the buffering capacity of these antioxidant supplements and therapies to reduce tissue damage and may provide a means of therapeutic management in hypertension.

The kidney and liver also displayed a compromised antioxidant buffering capacity during induced free radical mediated lipid peroxidation. This showed that both the kidney and liver have a limited tolerance to free radicals. A sudden increase in free radical levels would result in increased lipid peroxidation due to a decreased antioxidant buffering capacity of the specific tissue. The lipid peroxidation estimation showed that tissues have differing capacities to counter a free radical "attack". Although this being evident from the level of induced lipid peroxidation from the oxidative challenge test, this could be further substantiated, if the level of free radicals and antioxidant were assessed in the specific tissue. It is known that O_2^- is not membrane permeable and thus acts in the compartment where it is produced. A direct

measure of the free radical and antioxidant levels in the tissue would demonstrate the differing abilities of a tissues antioxidant buffering capacity. It would be interesting to note the levels of free radicals and antioxidants in different tissues to assess 2 important parameters, firstly the levels of free radicals and antioxidants to assess which one if not, both are compromised, leading to increased oxidative stress and secondly to assess exactly which tissues are affected the most during the hypertensive state. This would provide a means for targeted antioxidant therapy, which could minimise the level of lipid peroxidation in tissue identified with a compromised antioxidant status.

Although this study showed that the kidney has an increased level of *in vivo* lipid peroxidation, through an increased level of oxidative stress, it would be interesting to note the exact damage that lipid peroxidation induces. This would answer if lipid peroxidatic damage is specific to certain areas of the kidney, or it targets the entire organ. A histological study coupled with a free radical and antioxidant status determination in specific organs such as the kidney would show the exact damage that free radicals induce in the tissue. This study would assess the level of glomerulosclerosis, nephrosclerosis and other histological parameters in the kidney. This would provide a means of assessing the exact level of damage induced in the kidney by a compromised free radical and antioxidant status, with resultant tissue oxidative stress.

From the evidence in this study, it can be seen that a compromised antioxidant and free radical status contributes to the hypertensive state in a multitude of ways. Free radicals seem to be indiscriminate in their "attack" during the hypertensive state. This

indiscriminate action "attacks" enzymes, expression factors, cells and a host of other biologically critical molecules. These indiscriminate actions tend to lead to the detriment of the effected organism. With this it would be appropriate to end by stating that free radicals and oxidative stress may not be the primary cause of hypertension but rather a mechanism by which the hypertensive state contributes to its own deleterious cycle.

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8. APPENDIX

Appendix 1	-	Body Mass – Raw Data
Appendix 2	-	Superoxide Dismutase - % Inhibition
Appendix 3	-	Sample of Blood Pressure Recordings

Appendix 1 - Body Mass - Raw data

Body Mass - DSS NS							
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
DSS 1	101	149	191	230	273	299	322
DSS 2	127	175	202	242	281	308	333
DSS 3	118	166	203	235	276	303	318
DSS 4	96	148	191	224	266	294	318
DSS 5	103	155	198	236	279	306	330
DSS 6	94	140	179	214	256	285	304
DSS 7	111	161	217	260	305	324	345
DSS 8	103	162	215	240	281	319	345
DSS 9	109	162	212	253	293	317	347
DSS 10	98	141	184	231	341	269	305
DSS 11	95	146	179	218	322	249	287
DSS 12	100	148	185	223	329	265	302

Body Mass - DSR NS							
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
DSR 1	94	139	176	219	252	283	308
DSR 2	99	141	166	196	226	248	266
DSR 3	96	138	164	195	230	251	272
DSR 4	96	142	170	206	246	263	282
DSR 5	79	120	151	183	225	253	277
DSR 6	90	133	156	183	214	236	263
DSR 7	96	140	164	196	229	251	270
DSR 8	117	145	173	203	226	246	266
DSR 9	105	146	174	209	233	252	265
DSR 10	86	140	170	193	213	238	249
DSR 11	80	128	162	182	206	224	236
DSR 12	85	133	166	193	220	246	256

Body Mass - DSS HS							
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
DSS 1	121	183	230	276	302	313	335
DSS 2	133	186	235	280	312	323	343
DSS 3	119	171	223	270	307	336	362
DSS 4	135	186	236	273	308	327	343
DSS 5	109	159	213	264	309	341	319
DSS 6	127	183	230	278	312	330	348
DSS 7	104	156	193	240	282	313	322
DSS 8	101	153	204	250	287	320	335
DSS 9	109	162	214	254	293	313	320
DSS 10	114	163	211	268	307	338	356
DSS 11	130	180	222	266	303	325	334
DSS 12	97	139	175	214	242	257	268

Body Mass - DSR HS							
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
DSR 1	-	-	-	-	-	-	-
DSR 2	95	144	178	194	215	227	244
DSR 3	97	146	175	164	167	183	194
DSR 4	109	145	172	179	192	209	225
DSR 5	97	156	185	190	210	221	229
DSR 6	106	161	195	192	179	178	189
DSR 7	106	142	169	180	193	202	208
DSR 8	81	112	138	151	164	174	187
DSR 9	91	170	200	209	220	219	205
DSR 10	89	132	159	177	194	208	219
DSR 11	81	131	156	167	179	195	214
DSR 12	81	131	165	174	162	157	189

Appendix 2 - Superoxide Dismutase (SOD) - % Inhibition

Normal Salt			
DSS 1	56.61	DSR 1	40.07
DSS 2	51.20	DSR 2	45.33
DSS 3	56.62	DSR 3	38.26
DSS 4	55.33	DSR 4	42.34
DSS 5	51.93	DSR 5	43.34
DSS 6	55.79	DSR 6	44.06
DSS 7	57.49	DSR 7	43.52
DSS 8	57.31	DSR 8	46.61
DSS 9	57.40	DSR 9	40.71
DSS 10	59.55	DSR 10	56.00
DSS 11	59.00	DSR 11	50.37
DSS 12	60.96	DSR 12	52.42

High Salt			
DSS 1	48.42	DSR 1	-
DSS 2	38.42	DSR 2	32.00
DSS 3	35.79	DSR 3	31.66
DSS 4	36.84	DSR 4	38.12
DSS 5	34.21	DSR 5	43.23
DSS 6	38.68	DSR 6	35.36
DSS 7	40.00	DSR 7	41.14
DSS 8	44.25	DSR 8	40.16
DSS 9	37.89	DSR 9	39.17
DSS 10	43.42	DSR 10	38.19
DSS 11	46.05	DSR 11	42.43
DSS 12	31.32	DSR 12	42.26

Appendix 3 – Blood Pressure Recordings (Sample)