THE ROLE OF ANGIOTENSIN II AND OXIDATIVE STRESS IN THE SPONTANEOUSLY HYPERTENSIVE RAT

by

MELVIN M. GOVENDER

2011

THE ROLE OF ANGIOTENSIN II

AND OXIDATIVE STRESS IN

THE SPONTANEOUSLY HYPERTENSIVE RAT

by

MELVIN M. GOVENDER

2011

Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Health Sciences (PhD Health Sciences) in the Department of Physiology, in the School of Basic and Applied Medical Sciences, in the Faculty of Health Sciences at the University of Kwa-Zulu Natal – Westville Campus.

Supervisor: Dr A. Nadar

Date Submitted: June 2011

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

DEDICATION

The following study is duly dedicated to my parents, for without whom this study would have been but a near impossible task. If not for their love, support, guidance and dedication, my attempt at academic success would be but a futile exercise. 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:

THE ROLE OF ANGIOTENSIN II AND OXIDATIVE STRESS IN THE SPONTANEOUSLY HYPERTENSIVE RAT

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.

_____ 14th June 2011

MM Govender Date

ACKNOWLEDGEMENTS

The following people are thanked for their assistance and support in this study:

1. **The National Research Foundation,** for their financial assistance for the study.
2. **Dr A. Nadar,** for his invaluable expertise, technical assistance, encouragement and supervision through the entirety of the study.
3. **The Staff of the Department of Physiology (University of Kwa-Zulu Natal-Westville Campus),** for their assistance and support.
4. **The Staff of the Department of Physiology (University of Limpopo – Medunsa Campus),** for their assistance and support.
5. **Mr R. Singh,** for his technical expertise and support.
6. **Mr S. Rambharose**, for his technical assistance and support.
7. **Mr S.V. Ramesar,** for this academic input and technical assistance.
8. **Prof M.L. Channa,** for his academic mentorship, advice and assistance.
9. **Dr S. Singh and the Staff of the Biomedical Resource Unit – Westville Campus,** for their assistance, support and expertise with regards to the animal component of the study.
10. **The Electron Microscope Unit – Westville Campus,** for their assistance and expertise with regards to the histological analysis.
11. **The Staff at the Pfizer Molecular Laboratory,** for their assistance with and use of their equipment.
12. **Department of Microbiology-Westville Campus,** for the use of equipment.
13. **Department of Biology-Westville Campus,** for the use of equipment.
14. **Mrs P. Reddy,** for proof reading the draft.
15. **Mr S. Reddy and Mr V. Govender,** the Health Science Principle Faculty officers, for their administrative expertise, assistance and support.
16. **My Family and Friends,** for their unwavering love, moral support and encouragement.
17. **The Divine Grace that is God,** for his divine inspiration, love, knowledge, spiritual guidance, and for his faith and belief in me.

TABLE OF CONTENTS

NO	TITLE	PAGE
1.	**INTRODUCTION**	1
2.	**LITERATURE REVIEW**	4
2.1.	HYPERTENSION	4
2.1.1.	Definition	4
2.1.2.	Types and Causes	6
2.1.2.1.	Essential hypertension	6
2.1.2.2.	Secondary hypertension	6
2.1.3.	Role of heredity in essential hypertension	7
2.2.	FREE RADICALS	8
2.2.1.	Reactive oxygen species	8
2.2.2.	Implications of free radicals in the disease state	11
2.3.	ANTIOXIDANTS	12
2.3.1.	Definition	12
2.3.2.	Types/Classes	12
2.3.2.1.	Enzymatic antioxidants	12
2.3.2.1.1.	The Superoxide dismutases	13
2.3.2.1.1.1.	Cu/Zn SOD (SOD-1) expression	14
2.3.2.1.2.	The Glutathione peroxidases	15
2.3.2.1.3.	The Catalases	17
2.3.2.2.	Non-enzymatic antioxidants	19
2.3.3.	The antioxidant system as a unit of defence	20

2.4.	THE RENIN-ANGIOTENSIN SYSTEM	21
2.4.1.	Angiotensin II	21
2.4.2.	The Angiotensin II receptors	24
2.5	NADPH OXIDASE	25
2.6	OXIDATIVE STRESS	27
2.6.1.	Physiological implications of oxidative stress	27
2.6.2.	Free radicals, oxidative stress and the link to hypertension	29
2.6.2.1.	Nitric oxide	29
2.6.2.2.	Vascular remodelling	31
2.6.2.3.	Lipid peroxidation as an indicator of oxidative stress	34
2.6.2.3.1.	Organ damage due lipid peroxidation	35
2.7.	THE SPONTANEOUSLY HYPERTENSIVE RAT	37
3.	**MATERIALS AND METHODS**	40
3.1.	ANIMAL PROTOCOL	40
3.1.1.	Ethical approval and standard animal protocol	40
3.1.2.	DURATION	41
3.1.3.	ALZET^{TM} OSMOTIC PUMP	42
3.1.3.1.	Background of the ALZET^{TM} osmotic pump	42
3.1.3.2.	Surgical procedure for the insertion of the ALZET^{TM} osmotic pump	43
3.1.3.3.	Infusion dosage	44
3.1.4.	BLOOD PRESSURE	45
3.1.4.1.	Blood pressure training protocol	45
3.1.4.2.	Blood pressure recording protocol	45
3.1.5.	SACRIFICE	48

3.2.	SAMPLE ANALYSIS	50
3.2.1.	GLUTATHIONE PEROXIDASE	50
3.2.2.	SUPEROXIDE DISMUTASE	51
3.2.3.	ANGIOTENSIN II	53
3.2.4.	NITRIC OXIDE	55
3.2.5.	F_2-ISOPROSTANES	56
3.2.6.	HYDROGEN PEROXIDE	60
3.2.7.	CATALASE	61
3.2.8.	HISTOPATHOLOGY	63
3.2.8.1.	TISSUE PROCESSING	63
3.2.8.2.	EMBEDDING	64
3.2.8.2.1.	Embedding equipment	64
3.2.8.2.2.	Embedding procedure	64
3.2.8.3.	SECTIONING	65
3.2.8.3.1.	Trimming the wax block	65
3.2.8.3.2.	Cutting sections	65
3.2.8.3.3.	Floating out sections	65
3.2.8.3.4.	Temporary mounting	66
3.2.8.4.	SLIDE SELECTION	67
3.2.8.5.	STAINING	67
3.2.8.5.1.	Principle	67
3.2.8.5.2.	Reagents	67
3.2.8.5.3.	Procedure	68
3.2.8.5.4.	Permanent mounting	70
3.2.8.6.	MORPHOMETRIC ANALYSIS	70

3.2.9.	GENE EXPRESSION AND ANALYSIS	71
3.2.9.1.	Trizol purifiaction of RNA	71
3.2.9.2.	Realtime PCR quantification	73
3.2.9.2.1.	p22phox	73
3.2.9.2.2.	gp91phox	74
3.2.9.2.3.	Cu/Zn SOD	75
3.2.9.2.4.	AT-1a	76
4.	**RESULTS**	77
4.1.	BLOOD PRESSURE	78
4.1.1.	Systolic blood pressure	78
4.1.2.	Diastolic blood pressure	80
4.2.	HEART RATE	82
4.3.	PLASMA ANGIOTENSIN II	85
4.4.	PLASMA NITRIC OXIDE	87
4.5.	SUPEROXIDE DISMUTASE	90
4.6.	GLUTATHIONE PEROXIDASE	92
4.7.	SERUM CATALASE	94
4.8.	PLASMA HYDROGEN PEROXIDE	98
4.9.	KIDNEY F_2-ISOPROSTANES	100
4.10.	VASCULAR REMODELLING	102
4.10.1.	Aorta media wall thickness	102
4.10.2.	Aorta wall to lumen ratio	104
4.10.3.	Iliac artery media wall thickness	106
4.10.4.	Iliac artery wall to lumen ratio	108

4.11.	GENE EXPRESSION	111
4.11.1.	p22phox mRNA expression	111
4.11.2.	gp91phox mRNA expression	114
4.11.3.	Cu/Zn SOD mRNA expression	116
4.11.4.	AT-1a mRNA expression	119
5.	**DISCUSSION**	123
5.1.	BLOOD PRESSURE AND HEART RATE	128
5.2.	ANGIOTENSIN II, DOES IT UNDERLIE THE PATHOGENESIS OF HYPERTENSION?	131
5.3	NADPH OXIDASE, IS IT ANOTHER MECHANISM BY WHICH ANGIOTENSIN II ELEVATES BLOOD PRESSURE?	137
5.4.	A COMPROMISED ANTIOXIDANT STATUS : IS IT THE CAUSE OR CONSEQUENCE OF AN ELEVATED BLOOD PRESSURE IN HYPERTENSION?	141
5.4.1.	SOD THE PRIMARY DEFENCE AGAINST THE DELETERIOUS O_2^-	141
5.4.2.	NITRIC OXIDE A VITAL “COG IN THE WHEEL” TO REDUCE BLOOD PRESSURE	147

5.4.3.	GPx, CATALASE AND HYDROGEN PEROXIDE, DO THEY PLAY A ROLE IN THE PATHOGENESIS OF HYPERTENSION?	149
5.4.4.	IS THE ANTIOXIDANT STATUS OF THE EXPERIMENTAL GROUPS COMPROMISED?	155
5.5.	TISSUE OXIDATIVE STRESS: IS THE KIDNEY A TARGET FOR REACTIVE OXYGEN SPECIES IN HYPERTENSION?	156
5.6.	VASCULAR REMODELLING AN AMPLIFIER AGENT IN HYPERTENSION.	158
6.	**CONCLUSION AND RECOMMENDATIONS**	161
7.	**REFERENCES**	167
8.	**APPENDIX**	189

LIST OF ABBREVIATIONS

ACC	:	Associated Clinical Conditions
ACE	:	Angiotensin Converting Enzyme
Ang (1-7)	:	Angiotensin 1-7
Ang II	:	Angiotensin II
Ang III	:	Angiotensin III
Ang IV	:	Angiotensin IV
ANOVA	:	Analysis of Variance
AT_1	:	Angiotensin Type 1 Receptor
AT_2	:	Angiotensin Type 2 Receptor
BHT	:	Butalyated Hydroxytoulene
BNF	:	Buffered Neutral Formalin
BP	:	Blood Pressure
BPM	:	Beats Per Minute
CAT	:	Catalase
cDNA	:	Complementary Deoxyribonucleic Acid
CNS	:	Central Nervous System
Cu	:	Copper
Cu/Zn SOD	:	Copper/Zinc – containing Superoxide Dismutase
CVD	:	Cardiovascular Disease
DBP	:	Diastolic Blood Pressure
DNA	:	Deoxyribonucleic Acid
EC	:	Endothelial Cells
EIA	:	Enzyme Immunoassay
ecSOD	:	Extracellular Superoxide Dismutase

GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
GPx	:	Glutathione Peroxidase
GSH	:	Reduced Glutathione
GSSG	:	Oxidized Glutathione
H_2O	:	Water
H_2O_2	:	Hydrogen Peroxide
HIV	:	Human Immunodeficiency Virus
MDA	:	Malonyldialdehyde
MeOH	:	Methanol
Mn	:	Manganese
mRNA	:	Messenger Ribonucleic Acid
NADPH	:	Nicotinamide Adenine Dinucleotide Phosphate
NO	:	Nitric Oxide
O_2^-	:	Superoxide Radical
O_2	:	Oxygen
OH^-	:	Hydroxyl Radical
$ONOO^-$	:	Peroxynitrate
PCR	:	Polymerase Chain Reaction
PDGF	:	Platelet Derived Growth Factor
RAS	:	Renin Angiotensin System
RBC	:	Red Blood Cell
RNS	:	Reactive Nitrogen Species
ROS	:	Reactive Oxygen Species
SBP	:	Systolic Blood Pressure
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
SPE	:	Solid Phase Extraction
TBARS	:	Thiobarbituric Acid Reactive Substances
TOD	:	Target Organ Damage
TPR	:	Total Peripheral Resistance
VSMC	:	Vascular Smooth Muscle Cells
W/L Ratio	:	Wall To Lumen Ratio
WHO	:	World Health Organisation
XOD	:	Xanthine oxidase
Zn	:	Zinc

LIST OF TABLES

Table 1	:	Classification and Stratification of risk to quantify prognosis of Hypertension (WHO Guidelines., 2003)	5
Table 2	:	Reactive Oxygen Species	9
Table 3	:	Classification of Antioxidants	19
Table 4	:	Target Organs and Effects of Angiotensin II	22
Table 5	:	Animal Grouping	40
Table 6	:	Systolic Blood Pressure, Diastolic Blood Pressure and Heart Rate	84
Table 7	:	Plasma Angiotensin II, Nitric Oxide and Hydrogen Peroxide levels and Kidney F_2-Isoprostane levels	89
Table 8	:	Antioxidant Enzyme levels	97
Table 9	:	Morphometric Measurements of the Aorta and Iliac Artery	110
Table 10	:	Aorta mRNA Expression	122
Table 11	:	Effect of angiotensin II on oxidative stress related parameters in the experimental groups	126

LIST OF FIGURES

Figure 1	:	Renin Angiotensin System	21
Figure 2	:	Classification of Vascular Remodelling	32
Figure 3	:	Summary of Angiotensin II and Oxidative Stress	39
Figure 4	:	Reaction Series for Ang II ELISA	53
Figure 5	:	Graph of Systolic Blood Pressure	79
Figure 6	:	Graph of Diastolic Blood Pressure	81
Figure 7	:	Graph of Heart Rate	83
Figure 8	:	Graph of Plasma Angiotensin II	86
Figure 9	:	Graph of Plasma Nitric Oxide	88
Figure 10	:	Graph of Superoxide Dismutase	91
Figure 11	:	Graph of Glutathione Peroxidase	93
Figure 12	:	Graph of Serum Catalase	96
Figure 13	:	Graph of Plasma Hydrogen Peroxide	99
Figure 14	:	Graph of Kidney F_2-Isoprostanes	101
Figure 15	:	Graph of Aorta Media Wall Thickness	103
Figure 16	:	Graph of Aorta – Wall to Lumen Ratio	105
Figure 17	:	Graph of Iliac Artery Media Wall Thickness	107
Figure 18	:	Graph of Iliac Artery – Wall to Lumen Ratio	109
Figure 19	:	Graph of p22phox mRNA Expression	113
Figure 20	:	Graph of gp91phox mRNA Expression	115
Figure 21	:	Graph of Cu/Zn SOD mRNA Expression	117
Figure 22	:	Graph of AT-1a mRNA Expression	121
Figure 23	:	Summary of the link between Ang II and Oxidative Stress and its Role in Elevating Blood Pressure	127

ABSTRACT

Oxidative stress resulting from an imbalance between free radicals and antioxidants is considered to be an important etiological factor in the development and maintenance of hypertension. Angiotensin II (Ang II) has been shown to be an important regulator of blood pressure and acts to elevate blood pressure by its pressor effects. The pressor effects of Ang II are well documented but recent evidence has suggested another possible role of Ang II in elevating blood pressure, whereby it acts via an independent mechanism that is directly linked with oxidative stress.

The spontaneously hypertensive rat (SHR) is a widely used model in the investigation of the pathophysiological mechanisms involved in hypertension. This study was therefore undertaken to determine whether Ang II acts as a causative factor via oxidative stress in the development and maintenance of hypertension in the SHR. This was elucidated by evaluating the role of both the endogenous *in vivo* levels of Ang II as well as an exogenous sub-pressor dose of Ang II, on oxidative stress and its associated parameters. The parameters evaluated included, the antioxidant status of the model on the basis of the levels of the major antioxidant enzymes viz. SOD, GPx and catalase; the free radical generating capacity, by assessing the activity of the membrane bound enzyme NADPH oxidase and the levels of H_2O_2. The study also evaluated the levels of the endogenous vasodilator nitric oxide (NO), remodelling of the vasculature and the level of tissue oxidative stress in the kidney.

The results show that the SHR has an elevated plasma Ang II level and an elevated level of oxidative stress, thus showing that in this model there is an intimate link between oxidative stress and Ang II. The SHR also shows depleted levels of NO and thus a decreased vasodilatory capacity and increased remodelling of the vasculature. The kidney showed an increased level of lipid peroxidation, which was due to the elevated levels of oxidative stress.

All of these pathophysiological changes contribute to the elevation in blood pressure in this model.

The long term infusion of the sub-pressor dose of Ang II affected the SHR to a greater extent than the Wistar. Although the dose of Ang II elevated the blood pressure in both models, the degree of the pathophysiological changes associated with the elevation in blood pressure was greater in the SHR. The Ang II infusion in both these models demonstrated that in the SHR which is genetically predisposed to hypertension, adjustments are made to the antioxidant system, that result in an elevated level of protection against oxidative stress.

These results show that Ang II acts as a causative factor in the pathogenesis of hypertension in the SHR via its well documented pressor effects, as well as via a multitude of independent mechanisms that are linked to oxidative stress. This is substantiated by the significant decrease in NO that is caused by the elevated oxidative stress, as well as the previously described pathophysiological changes. This study has therefore shown that Ang II has an intimate causative link with oxidative stress that results in parallel mechanisms that work concomitantly with each other in hypertension in this model.

1. INTRODUCTION

Free radical generation in an organism occurs as a result of the organisms normal metabolic processes. Under physiological conditions, free radical mediated derangements are prevented by antioxidants. When excessive free radical formation occurs in the body and/or there are compromised levels of antioxidants i.e. the pro-oxidants overwhelm the antioxidants, 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. This state of potential free radical mediated damage has been intimately linked with various disease states including hypertension (Sies., 1997; Halliwell and Gutteridge., 1999).

It is well known that Angiotensin II (Ang II) plays an important role in blood pressure regulation. The actions of Ang II are fairly widespread, and Ang II acts on the blood pressure regulating organs/tissues to bring about an elevation in blood pressure.

There also exists substantial experimental evidence indicating that reactive oxygen species (ROS) play an important pathophysiological role in the development and maintenance of hypertension, this being due to a relative increase in the production of the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) in hypertension. The O_2^- radical has been shown to scavenge nitric oxide and reduce its bioavailability, thus reducing its vasodilatory role on the vasculature (McIntyre *et al.,* 1999). The relative increase of both O_2^- and H_2O_2 with resultant tissue oxidative stress has also been shown to decrease the natriuretic and diuretic properties of the kidney, thus reducing the organs ability to lower blood pressure (Meng *et al.,* 2003). Both these species have also been shown to promote vascular smooth muscle cell (VSMC) growth, and contribute to the blood pressure amplifier consequence of vascular remodelling (Touyz., 2003; Taniyama and Griendling., 2003; Touyz., 2004; Paravicini and Touyz., 2008).

Compelling experimental evidence suggests that both the Ang II and the oxidative stress mechanisms are intimately linked. Ang II has been shown to stimulate the production of free radicals in the vasculature of rats, via the activation of the membrane bound enzyme NADPH oxidase (Griendling *et al.*, 1994; Rajagopalan *et al.*, 1996). Thus this study will attempt to investigate whether the activation of this membrane bound enzyme (NADPH oxidase) by Ang II initiates the production of free radicals and results in a state of elevated oxidative stress. A state of elevated oxidative stress and its deleterious derangements have been previously shown in part, to be responsible for an elevation of blood pressure in hypertension (de Champlain *et al.*, 2004).

The spontaneously hypertensive rat (SHR) has proven to be an excellent model to investigate the pathophysiological changes that occur in hypertension. Thus this study will investigate the role of Ang II in this model of genetic hypertension, and elucidate whether Ang II elevates the blood pressure by stimulating the production of free radicals and thus inducing a state of oxidative stress. The study will also aim to answer whether the elevation in blood pressure evident in this model arises as a consequence of oxidative stress, or whether the oxidative stress is a consequence of the elevation in blood pressure.

Furthermore, the study will aim to investigate the role of oxidative stress as a possible mechanism by which a long term sub-pressor dose of Ang II (i.e. a dose of Ang II that has been shown to not elicit immediate pressor effects in the kidney and in the vasculature, but elevates blood pressure over the long term) is able to elevate the blood pressure of normotensive rats (Simon *et al.*, 1995). This would allow the study to show whether there exists other mechanisms by which Ang II is capable of increasing the blood pressure, and investigate whether oxidative stress is one of these mechanisms.

The study will also subject the SHR to a long term sub-pressor dose of Ang II, and investigate whether this dose will exacerbate the onset and level of hypertension in this model, and determine the role, if any that is played by oxidative stress. This would allow the study to compare and contrast the response of the free radical and antioxidant systems of both the genetically normotensive model (i.e. Wistar), and the genetically hypertensive model (i.e. SHR) to the sub-pressor dose of Ang II.

The evidence that this study will provide, will allow for the development of strategies to strengthen the use of antioxidant therapies that are co-administered with Ang II converting enzyme (ACE) inhibitors and Ang II antagonists (AT-1 blockers) in the treatment and management of hypertension. This would also allow for the use of these therapies as both preventative and management treatments for hypertension. It is also hoped that this research would provide a basis for further hypertension preventative and management research to be developed.

2. LITERATURE REVIEW

2.1 HYPERTENSION

2.1.1. Definition

Blood pressure has previously been described as a quantitative trait that is highly variable (Parati *et al*., 1998). However, with a persistent elevation in the level of blood pressure, there is a positive correlation with an increased risk for associated organ damage, especially cardiovascular diseases such as stroke, myocardial infarction and heart failure as well as renal disease (Carretero and Oparil., 2000). Hypertension can therefore be stated to be the elevated level of blood pressure that is associated with an increase in morbidity and mortality.

Since, more general clinical definitions show that hypertension, is that level of blood pressure at which detection and treatment will do more good than harm to an individual's health, it can be defined as both a risk factor as well as a pathological condition, and therefore offers a new challenge in the management of this condition (WHO Guidelines., 2003).

Hypertension, is characterized by the World Health Organization (WHO) as a systolic blood pressure (SBP) of 140 mmHg or greater and a diastolic blood pressure of 90 mmHg or greater. Table 1 shows a classification system by WHO to rank blood pressure into different grades, linked with patient history and predisposing risk factors, and classifies patient risk (WHO Guidelines., 2003). This classification allows for the individual patient dependant clinical management of the condition.

Table 1: Classification and Stratification of risk to quantify prognosis of Hypertension (WHO Guidelines., 2003)

Other risk Factors and Disease History	**BLOOD PRESSURE (mmHg)**		
	GRADE 1 (SBP 140-159 or DBP 90-99)	**GRADE 2** (SBP 160-179 or DBP 100-109)	**GRADE 3** (SBP ≥ 180 or DBP ≥ 110)
No other risk factors	LOW RISK	MEDIUM RISK	HIGH RISK
1 – 2 Risk Factors	MEDIUM RISK	MEDIUM RISK	HIGH RISK
3 or more risk factors, or TOD, or ACC	HIGH RISK	HIGH RISK	HIGH RISK

SBP – Systolic blood pressure

DBP – Diastolic blood pressure

TOD – Target organ damage

ACC – Associated clinical conditions e.g. left ventricular hypertrophy, chronic kidney disease, diabetic nephropathy, heart failure and cerebrovascular disease

Risk Factors include, unhealthy diet, physical inactivity, tobacco use, glucose intolerance and hyperlipidemia

2.1.2. Types and Causes

Classically hypertension has been divided into 2 types/classes viz:

i) Essential/Primary/Idiopathic Hypertension and

ii) Secondary Hypertension

2.1.2.1. Essential Hypertension

Essential hypertension is also referred to as primary or idiopathic hypertension and according to the many studies on the classification of hypertension this class/type of hypertension accounts for 90-95% of all hypertensive cases. Messerli *et al.*, (2007), defined essential hypertension as, "a rise in blood pressure of unknown cause that increases the risk for cerebral, cardiac and renal events". It is postulated that this type of hypertension does not have a single cause, but rather, in most cases, it is a combination of several etiological factors such as diet, lack of exercise, stress, metabolic effects, obesity and heredity thus manifesting as a disease condition with multifactorial origins.

2.1.2.2. Secondary hypertension

The remaining 5-10% of hypertension cases is termed secondary hypertension, which results as a consequence of other known or identifiable diseases/medical states such as renal disease, endocrine disorders, drug treatment complications and pregnancy (Onusko., 2003).

2.1.3. The Role of Heredity in Essential Hypertension

The two phenotypes that determine blood pressure viz. total peripheral resistance and cardiac output are further controlled by intermediate phenotypes which include vasopressor/depressor hormones, the structure of the cardiovascular system, the autonomic nervous system, body fluid volume and renal function amongst others. This fact thus complicates the identification of variant genes that contribute to the development of hypertension (Carretero and Oparil., 2000).

Although it is not known exactly which genes cause a variation in genetically determined blood pressure, from the many familial studies, it has been shown that genetically determined blood pressure can range from a low normal to severe hypertension (Carretero and Oparil., 2000).

Recent studies have shown that blood pressure can be either raised (from a normal blood pressure) or lowered (from high blood pressure) through a common pathway that involves increasing or decreasing salt and water absorption respectively, by the nephron, and this can arise due to mutations in at least 10 variant genes (Lifton., 1995; Lifton., 1996; Carretero and Oparil., 2000).

The literature shows that the causative role that heredity plays via genetic variations (this includes most blood pressure regulating factors) in essential hypertension is a very important one (Luft., 1998). When this genetic susceptibility is combined with the environmental or metabolic factors stated previously, this leads to essential hypertension becoming a deleterious syndrome that affects a fairly large percentage of the population.

2.2. FREE RADICALS

An atom is most stable in the ground state. In order for the atom to be in this ground state every electron in the outermost shell must have a complimentary electron that spins in the opposite direction, and thus the atom is considered to be "ground". If an atom has at least one unpaired electron in the outermost shell, this species is referred to as a free radical; importantly this resultant species is capable of independent existence. Free radicals thus become highly reactive due to the presence of these unpaired electron(s) (Karlsson., 1997). In addition 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. Biologically this process initiates a self-perpetuating chain reaction that ultimately results in a disturbance in both intracellular and extracellular homeostatic balance and eventual cellular damage (Halliwell., 1994).

2.2.1. Reactive Oxygen Species

A free radical that is derived from oxygen is referred to as a reactive oxygen species (ROS). Molecular oxygen in the ground state is a bi-radical, which means that it contains two unpaired electrons in the outer shell, and this is also known as the triplet state. The two single (i.e. the unpaired) electrons have the same spin, resulting in the oxygen molecule only being able to react with one electron at a time. Therefore the oxygen molecule is not very reactive when the two electrons are in a chemical bond. However, if one of the two unpaired electrons reaches an excited state and changes the direction of its spin, the resulting species which is now called a singlet oxygen, becomes a powerful oxidant. This is due to the two electrons having an opposing spin, and can thus quickly react with other electron pairs. This is especially so for double bonds. The one electron reduction of molecular oxygen produces relatively stable intermediates. The superoxide anion (O_2^-), is one such product, and is the

precursor of most ROS and is also a mediator in oxidative chain reactions. Dismutation of O_2^- (either spontaneously or through a reaction catalysed by superoxide dismutase - SOD) produces hydrogen peroxide (H_2O_2), which in turn may be fully reduced to water or partially reduced to the hydroxyl radical (OH^-), one of the strongest oxidants in nature (Reaction series shown below) (Turrens., 2003).

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

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

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

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

Table 2. Reactive Oxygen Species

Species	**Common Name**	**Half-Life (37^0C)**
HO^-	Hydroxyl radical	1 nanosecond
O_2^-	Superoxide anion radical	1 x 10^{-6} seconds
$^1O_2^-$	Singlet oxygen	1 microsecond
RO^-	Alkoxyl radical	1 microsecond

ROO^-	Peroxyl radical	7 seconds
NO^-	Nitric oxide radical	1-10 seconds
H_2O_2	Hydrogen peroxide	stable
HOCl	Hypochlorus acid	stable

R = lipid

Biologically ROS are found in several cells including macrophages, vascular smooth muscle cells (VSMC) and endothelial cells (EC). At low concentrations these ROS can act as physiological mediators of cellular responses such as signal transduction, cell growth and inflammation. However at higher concentrations ROS may cause cell damage and death due to its indiscriminate targeting of proteins, lipids, polysaccharides and DNA (Irani., 2000). Biologically the "leakages" from the electron transport chains of mitochondria and endoplasmic reticulum are the major sources of ROS. 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 [NAD(P)H] oxidase that is assembled at the plasma membrane from resident plasma membrane components and cytosolic protein components (Forman and Torres., 2002). NADPH oxidase is also, a major source of vascular superoxide production (Pueyo *et al*., 1998; Tepel., 2003; Shimokawa and Matabo., 2004).

2.2.2. Implications of Free radicals in the Disease State

Pathological conditions such as cancer, inflammation and hypertension may develop in cases of persistently elevated ROS levels. Pathological conditions result in a change in homeostatic balance. Accordingly, pathological symptoms may result from both ROS-mediated changes in gene expression and/or the damaging effects of ROS on the integrity of the tissue (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. Direct DNA damage is usually caused by free radicals in close proximity, whereas indirect damage for example, can be caused by free radicals impairing production of the proteins that are needed to repair DNA (Dhalla *et al.*, 2000; McGee *et al.*, 2003). Alteration in DNA has been shown to be a major factor in the development of cancer. Free radicals have been shown to attack fatty acid side chains of intracellular membranes and lipoproteins, this self-perpetuating attack causes a chain reaction known as lipid peroxidation. The by-products of lipid peroxidation cause further damage to membrane proteins, by making the cell membrane " leaky" and eventually leading to loss of membrane integrity. The last structures damaged by free radicals 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).

The literature thus shows that, damage due to free radicals is intimately linked with many disease states. The causes of the increase in free radical production in the different disease states may be diverse and disease dependant, but the implication of the free radical damage is similarly deleterious.

2.3. ANTIOXIDANTS

2.3.1. Definition

All organisms that respire aerobically have evolved defense mechanisms against free radicals and these agents are collectively known as antioxidants. A concise definition of an antioxidant was provided by Halliwell and Gutteridge, (1989), who defined an antioxidant as "any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate." This definition would include antioxidants of both an enzymatic and non-enzymatic origin (Sies., 1997).

2.3.2. Types/Classes

Thus due to their origin, antioxidants can be broken up broadly into 2 types/classes viz.;

i) Enzymatic antioxidants and

ii) Non-enzymatic antioxidants

2.3.2.1. Enzymatic antioxidants (Intracellular/Endogenous Antioxidants)

There are 3 major classes/types of enzymatic antioxidants (antioxidants enzymes) viz;

1. The Superoxide Dismutases (EC 1.15.1.1)

2. The Glutathione Peroxidases (EC 1.11.1.9)

3. The Catalases (EC 1.11.1.6)

2.3.2.1.1. The Superoxide Dismutases

The superoxide dismutases (SOD) are the major cellular defense against the superoxide radical (O_2^-). They convert (dismutate) O_2^- to hydrogen peroxide (H_2O_2) via the following reaction:

$$2O_2^{\cdot-} + 2H^- \rightarrow H_2O_2 + O_2$$

These enzymes contain redox metals such as copper (Cu), zinc (Zn) and manganese (Mn) in their catalytic centre and are found in the mitochondria and the cytosol (Halliwell., 1996).

There exists 3 different isoforms of SOD, firstly the mitochondrial Mn-containing SOD (MnSOD, SOD-2), then the cytosolic Cu/Zn containing SOD (Cu/Zn-SOD, SOD-1) and the extracellular SOD (ecSOD, SOD-3), which is also a Cu/Zn containing enzyme that is produced and secreted mainly by vascular smooth muscle cells (VSMC's) (Wassmann *et al.*, 2004).

SOD-1 is located in the cytosol and nucleus of all cell types. Whereas the SOD iso-enzymes 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 and prevent an accumulation of O_2^- in the organelle (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 an important regulator (Marklund *et al.,* 1982; Oury *et al.,* 1996; McIntyre *et al.,* 1999).

2.3.2.1.1.1. Cu/Zn SOD (SOD-1) Expression

Cu/Zn SOD has been shown to have a fairly widespread distribution, in many different cell types (Crapo *et al.,* 1992). The expression of the cytoplasmic Cu/Zn SOD has been shown to be stable and its relative activity is often adopted as an internal control for Cu/Zn SOD gene expression (Zelko *et al.,* 2002).

The Cu/Zn SOD mRNA levels have been shown to be regulated by various physiological and pathological conditions. An elevation of Cu/Zn SOD mRNA levels has been shown in response to many different conditions such as elevated hydrogen peroxide (Yoo *et al*., 1999a), arachidonic acid (Yoo *et al*., 1999b), and depressed nitric oxide levels amongst others (Frank *et al*., 2000; Zelko *et al.,* 2002).

A down regulation of Cu/Zn SOD has been shown in experimental animals exposed to hypoxic environments (Jackson *et al*., 1996).

As can be seen, the changes in expression of Cu/Zn SOD in many pathological conditions are adaptive to conditions that promote free radical formation. These adaptive changes act to minimise the deleterious effects of O_2^-, and thus allow the organism a means to maintain homeostatic balance.

2.3.2.1.2 The Glutathione Peroxidases

The glutathione peroxidases (GPx) are widely distributed in animal tissues. They are essentially a selenium-containing antioxidant enzyme that efficiently reduces hydrogen peroxide and lipid peroxides to water and lipid alcohols, respectively. Within this process the glutathione is in turn oxidized to glutathione disulfide (Wassmann *et al.,* 2004).

Selenium is vital for the activity of these tetrameric enzymes. 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" glutathione peroxidase acts on H_2O_2 and hydroperoxides of fatty acids and cholesterol, but not esterified lipids such as those present in lipoproteins. Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is the only enzyme known to reduce complex lipid hydroperoxides in lipoproteins (Stocker and Keaney., 2004).

Inadequate glutathione levels, or the absence of GPx activity, would result in hydrogen peroxide and lipid peroxide accumulation, and these substances may then be converted to hydroxyl radicals and lipid peroxyl radicals, respectively, by transition metals (e.g., $Fe^{2+}$and Cu^{2+}). By detoxifying and thus preventing the accumulation of H_2O_2 and the lipid peroxyl radicals, the GPx/glutathione system is thought to be a major defense in low-level oxidative stress (Wassmann *et al.,* 2004).

Reduced glutathione (GSH) is said to be one of the most important enzymatic oxidant defenses within the body. It is found 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). Jones., (2002), has suggested that, “the balance of

GSH and GSSG provides a dynamic indicator of oxidative stress *in vivo*". *In vivo* levels of glutathione are thus an important indicator of the level/extent of oxidative stress.

2.3.2.1.3. The Catalases

The catalases are responsible with glutathione for decomposing H_2O_2 to H_2O and O_2. The catalases are located in peroxisomes and to some extent in the cytosol. The catalases are widely distributed in the human body, with high concentrations been shown in the liver, kidney and erythrocytes (Reimer *et al.*, 1994; Nishikawa *et al.*, 2009).

A single molecule of catalase (CAT) has been shown to have the potential to breakdown 40 million molecules of H_2O_2 per second. The process of CAT decomposing H_2O_2 indirectly detoxifies O_2^- radicals, because the dismutation of the superoxide radical by SOD produces H_2O_2 as an end product (Wassmann *et al.*, 2004).

The enzyme has also been shown to have peroxidase activity and thus reacts with organic peroxides and H^+ donors, and breaks them down to H_2O and organic alcohols. These peroxidatic reactions have been shown to be present at low levels of H_2O_2, but the catalatic reaction predominates at higher H_2O_2 concentrations. The main difference in the catalatic and peroxidatic reactions, can be attributed to their reaction rates, where the catalatic reaction rate has been shown to be one of the fastest known enzymatic reaction rates, and the peroxidatic rate is relatively slow (Kirkman and Gaetani., 2006). Thus this shows that catalase is more efficient during states where H_2O_2 levels are higher than when there are lower H_2O_2 levels. Therefore during states of high-level oxidative stress (when H_2O_2 levels are high), catalase will be the primary defense to protect cells from the endogenously produced H_2O_2. Thus catalase would be very important during cases of limited glutathione levels/decreased GPx activity and would play an important role in the development of tolerance to oxidative stress (Cai., 2005; Kirkman and Gaetani., 2006; Paravicini and Touyz., 2008).

CAT deficiency has been categorized into 2 types, firstly when there is less than 10% of normal CAT activity, this is known as acatalasemia, and when there is ~50% of normal catalase activity this is categorized as hypocatalasemia (Goth and Eaton., 2000). These conditions usually result in a dysregulation of H_2O_2, and H_2O_2 accumulation , which has been shown to result in hydrogen peroxide-induced lipid peroxidation and it has also been shown to induce vascular remodeling, amongst other pathologies (Hyoudou *et al.,* 2006; Nishikawa *et al.,* 2009).

2.3.2.2. Non-enzymatic Antioxidants

As shown in Table 3, many substances of non-enzymatic origin can act as antioxidants, of these, the main non-enzymatic antioxidants are α-tocopherol, Vitamin C, the cartenoids and the flavenoids.

Table 3: Classification of Antioxidants

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

Vitamin E is the most important and researched α-tocopherol and is found in membranes and lipoproteins. It has been shown to inhibit the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals, and has been shown to be extremely important for protection in cardiovascular diseases (Halliwell., 1994).

2.3.3. The Antioxidant System as a Unit of Defence

The antioxidant system is essentially responsible for the protection of cellular components against oxidative damage. This system has been shown to be as diversified as the free radicals themselves. Although in previous descriptions the role of antioxidants has been discussed in isolation, this is however not how they function *in vivo* (Yu., 1994; Arora *et al*., 2003).

The antioxidant system, although having individual components which at times have specific targets, the system and its components actually work together in a synergistic manner as a functional unit to quench the free radical threat and thus prevent/minimize the deleterious effects of oxidative stress. Yu., (1994), best described the integrated nature of the antioxidant system with the following statement; “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.”

Although antioxidants are specific to the respective free radicals, the antioxidants display a “concert effect” pertaining to their free radical quenching actions. These actions are well coordinated and provide maximal protection against free radicals. An example to demonstrate the cooperative actions of the antioxidants is in the neutralizing of the superoxide radical. As discussed previously 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 example demonstrates the coordinated depth of the antioxidant system as a functional unit and shows how the antioxidants work in a synergistic manner with each other, to act against the diverse free radicals and thus manifest as a single functional unit of defense and protection.

2.4. THE RENIN ANGIOTENSIN SYSTEM

The Renin Angiotensin System (RAS) has typically been seen as a hormonal circulating system that is solely involved in blood pressure regulation and Na^+, K^+ and fluid balance/homeostasis. The system responds to a drop in blood pressure, or changes to ionic concentrations of Na^+/K^+, and brings about its actions via Angiotensin II. The effects/mechanism of action of this system is best described by the following flow diagram:

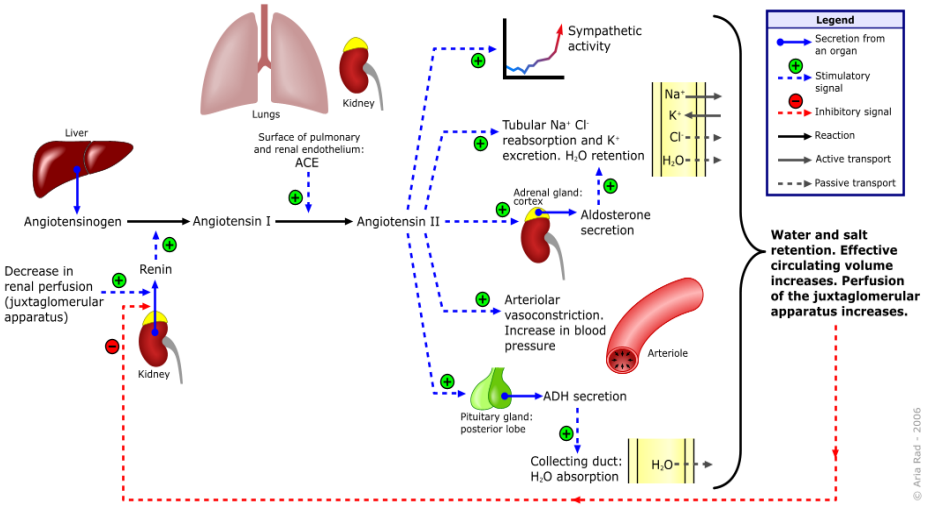


Figure 1: Renin Angiotensin System

http://www.ourmed.org/images/a/a2/Renin-angiotensin-aldosterone_system.png

Thus, it can be seen that the active component of the system is Angiotensin II, and it acts broadly to increase blood pressure, Na^+ and H_2O retention and K^+ excretion.

2.4.1. Angiotensin II

Ang II is an octapeptide (8 amino acids) whose effects are fairly wide acting. It brings about its effects on the CNS, kidneys, heart, blood vessels and adrenal glands. (Table 4)

Table 4: Target Organs and Effects of Angiotensin II

TARGET ORGAN	EFFECT
Central Nervous System (CNS)	Increases Vasopressin/ADH secretion
Kidneys	i. Decreases renal blood flow by vasoconstriction of the renal afferent arteriole or by augmentation of tubuloglomerular feedback ii. Increases tubular Na^+ reabsorption by increased Na^+-H^+ exchange at the S_1 segment of the proximal tubule iii. Increases K^+ excretion
Heart	i. Causes pathological cardiac hypertrophy by stimulating myocyte hypertrophy, myocyte gene reprogramming, fibroblast proliferation and extracellular matrix protein accumulation.* ii. Increased sympathetic nerve activity induced heart rate and contractility.
Blood Vessels	i. Induces VSMC proliferation, with resultant vascular remodelling, characterised by increasing vascular media width, increasing media cross sectional area, and increasing media/lumen ratios. ii. Increased sympathetic nerve activity induced vasoconstriction.
Adrenal Glands	Stimulation of aldosterone biosynthesis in the adrenal glomerulosa.*

*Sealy and Laragh., 1995

Thus the localized role of Ang II and its systemic actions act to increase the blood pressure over both the short-term and the long term. The final product Ang II, can be further broken down to Ang III, Ang IV and Ang (1-7). Ang III has been shown to be centrally involved in the regulation of blood pressure. Ang IV has been shown to have vasodilatory,

antiproliferative and cognitive actions and Ang (1-7) has been shown to have vasorelaxant properties (Sernia., 2001).

A study by Griendling *et al.,* 1994, has revealed an additional probable mechanism in which Ang II acts to increase blood pressure. Ang II infusion has been shown to increase the activity of the enzyme NADPH oxidase and this enzyme is responsible for the generation of O_2^- by VSMC's.

The O_2^- produced by this process, as stated previously, will increase blood pressure by acting as a potent localised vasoconstrictor, and also, due to its high affinity for NO, will readily combine with NO and thus limits/prevents NO's vasodilatory actions. Thus these actions of O_2^- will bring about an increase in blood pressure, by increasing peripheral resistance.

Other studies that have used Ang II infusion to induce hypertension in normotensive rats, have shown that the vasculature of these animals (viz. the aorta), responds to the increased levels of circulating Ang II, by increasing its activity of NADPH oxidase. This seems to be a fairly specific effect of Ang II, as normotensive rats made to be hypertensive to a similar degree with an infusion of noradrenalin, showed no change in the activity of the same enzyme (McIntyre *et al.*, 1999).

2.4.2. The Angiotensin II receptors

There are 2 types of Angiotensin II receptors viz. Angiotensin II Type 1 (AT_1) and Angiotensin II Type 2 (AT_2). The distribution of these receptors vary in different tissues with the AT_1 receptor being extensively distributed in adult tissues that include the vasculature, heart, kidney, adrenal gland, liver, brain and lung, and the AT_2 receptor is distributed mainly in the developing foetal tissues and in adult tissues that include the uterus, ovary, heart and adrenal medulla (Suzuki *et al*., 1993; Kim and Iwao., 2000).

In rats and mice there are further subtypes of the AT_1 receptor viz. AT_{1a} and AT_{1b} subtypes. They have been shown to have a 94% homology with respect to their amino acid sequence and have similar tissue distribution and pharmacological properties (Kim and Iwao., 2000).

The AT_1 receptor mediates all the known functions of Ang II to elevate blood pressure, such as vasoconstriction, increased cardiac contractility, aldosterone release and, renal Na^+ and H_2O absorption (Timmermans *et al*., 1993; Kim and Iwao., 2000), as well as the previously mentioned increase in NADPH oxidase activity (Seshiah *et al.*, 2002; Paravicini and Touyz., 2008).

This as well as the accumulating literature shows that Angiotensin II is mediated almost exclusively by the AT_1 receptor in many cardiovascular and renal diseases. This evidence has been supported by the development of the many AT_1 receptor antagonists (commonly known at AT_1 blockers) in the successful clinical treatment of patients diagnosed with hypertension (Kim and Iwao., 2000). Thus the regulation of the activity of the AT_1 receptors is an important tool in mediating the effects of Ang II.

2.5. NADPH OXIDASE

NADPH oxidase, as mentioned previously, is a common name given to a membrane bound enzyme NADPH cytrochrome P-450 oxidoreductase. It is assembled at the plasma membrane from resident plasma membrane components and cytosolic protein components (Forman and Torres., 2002). This multi-subunit enzyme catalyzes O_2^- production via the 1-electron reduction of O_2 using NADPH/NADH (Paravicini and Touyz., 2008).

NADPH oxidase is thought to be one of the main enzymatic sources of ROS in the vascular wall that have been found to play a putative role in hypertension. Other enzymes include nitric oxide synthase, xanthine oxidase and cyclooxygenase (Mohazzab *et al.,* 1994; Griendling *et al.,* 1994; Pagano *et al.,* 1995; Jones *et al.,* 1996; Zalba., 2001).

The vascular NADPH oxidase enzyme consists of a cytochrome b558 membrane complex, which is composed of membrane bound p22phox and gp91phox (Nox 2) sub-units, both these sub-units have been shown to be expressed in EC's and fibroblasts, and the p22phox sub-unit is expressed in VSMC's. The enzyme also consists of 3 cytosolic components viz., p47phox, p67phox and rac. The p47 and rac are expressed in EC's, fibroblasts and VSMC's while the p67phox being only expressed in EC's and fibroblasts (Zalba., 2001).

Studies have shown that vascular NADPH oxidase can also be responsive to numerous factors amongst them are growth factors, cytokines, mechanical forces (e.g. shear stress) and G protein-coupled receptor agonists (Bayraktutan *et al.,* 2000; Li and Shah., 2002; Lassegue and Clempus., 2003; Miller *et al.,* 2006; Takeya and Sumimoto., 2006; Paravicini and Touyz., 2008). Ang II has been shown to be one of the most important and potent regulators of NADPH oxidase. Ang II carries this out via its AT-1 receptors (Seshiah *et al.,* 2002; Paravicini and Touyz., 2008).

Thus any disturbance in the activity of this membrane bound enzyme caused by the factors mentioned previously would result in an increase in production of the deleterious ROS O_2^-, and increase the potential for ROS related pathology.

2.6. OXIDATIVE STRESS

Oxidative stress can be defined as the disruption of the equilibrium between the factors that promote free-radical formation and the antioxidant defense mechanisms that attempt to inhibit them (Halliwell *et al*., 1992). Turrens., (2003) stated that, "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".

It is known that minute changes in the steady-state concentration of these oxidants may play an integral role in intracellular signalling. However, situations that cause an uncontrolled increase in the steady-state concentrations of these oxidants leads to **free** radical-mediated chain reactions which indiscriminately target cellular components (Droge., 2002; Turrens., 2003).

2.6.1. Physiological Implications of Oxidative Stress

Oxidative stress occurs in most human diseases, however this does not imply that oxidative stress is the primary cause of, or is responsible for the pathogenesis of these diseases. The increase in free radicals may actually be secondary to the disease process (Halliwell and Gutteridge., 1999).

The clinical conditions that oxidative stress was found to play a role in were listed in an excellent review by Droge., (2002). This list included malignant diseases, diabetes, atherosclerosis, hypertension, chronic inflammation, human immunodeficiency virus (HIV) infection, neurodegenerative diseases and sleep apnoea. These diseases have also been shown to fall into two major categories. The first category is made up of, diabetes mellitus and cancer, and these conditions commonly show a pro-oxidative shift, suggesting that skeletal

muscle mitochondria may be the major site of the elevated production of ROS. These conditions may therefore be referred to as "mitochondrial oxidative stress". The second category may be referred to as "inflammatory oxidative stress conditions" because it is typically associated with an excessive stimulation of NADPH oxidase activity by cytokines or other factors/agents. In these cases, increased ROS levels or changes in intracellular glutathione levels are often associated with the pathological changes (Droge., 2002).

Oxidative stress has also been implicated by numerous studies in neurodegenerative diseases such as Down 's 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 indicate how deleterious these species can be (Evans and Halliwell., 2001).

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

The reactive oxygen species such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-), play a critical role in the pathogenesis of hypertension, as well as in other CVD's such as atherosclerosis, stroke, myocardial infarction, diabetes and ischemia-reperfusion injury (Paravicini and Touyz., 2008).

In hypertension ROS and the resulting oxidative stress play a dual role. On the one hand, they affect vascular resistance by inactivating NO, thereby promoting vasoconstriction and an elevation of peripheral hemodynamic resistance. On the other hand, they may serve as trigger mechanisms for lesion formation and organ damage (Swei *et al.*, 1997; Haliwell and Gutteridge., 1999; Touyz., 2003).

2.6.2.1. Nitric Oxide (NO)

Essential hypertension in several animal models of hypertension, including genetic models such as the SHR, is associated with an increased peripheral vascular resistance (Schnackenberg *et al.*, 1998). de Champlain *et al.*, (2004), stated that "the homeostatic modulation of blood pressure depends on the harmonious balance between the vasoconstrictive and vasodilatory mechanisms."

Nitric oxide (NO) is known to be one of the most powerful endogenous vasodilators. Due to this, there are theoretical reasons as to why reduced levels of NO production or diminished bioavailability would lead to vasoconstriction and thus an increased peripheral vascular resistance. NO has been found to regulate the tone of normal vessels and this includes resistance vessels. NO also causes renal vasodilatation which results in diuresis and natriuresis. These are actions that would tend to lower blood pressure. Therefore, an

attenuation of this mechanism could theoretically cause an elevation in blood pressure (McIntyre *et al.,* 1999).

Several studies suggest that 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 (Zalba *et al.,* 2001; Schnackenberg., 2002; Shimokawa and Matabo., 2004).

During oxidative stress, NO depletion occurs due to the reaction of NO with the superoxide anion to form peroxynitrite ($ONOO^-$). The presence of peroxynitrite in cells can lead to a number of adverse effects, including protein nitration, lipid peroxidation, DNA degradation and enhanced tubuloglomerular feedback responses (Hemnani and Parihar., 1998; Unlap *et al.,* 2003). The reaction of O_2^- and NO is biologically significant because it reduces the vasodilatory capacity of the vessel (Rojas *et al*., 2006). O_2^- and $OONO^-$ have been shown to stimulate vascular smooth muscle cell growth and thus vascular remodelling, and these products also favour inflammation of the vasculature and platelet aggregation (Cai and Harrison., 2000; Zalba *et al.,* 2001; de Champlain *et al.,* 2004).

The above mentioned studies provides convincing evidence that oxidative stress plays an important role in hypertension, in part by reducing the levels of NO. The most compelling evidence is provided by studies that have intravenously administrated SOD-Hb, an artificially synthesised form of SOD (a SOD mimetic), the SOD-Hb significantly decreased the blood pressure of the SHR (which had been shown to have elevated levels of O_2^-), but not of the Wistar controls (which had been shown to have lower levels of O_2^-), demonstrating the significant role played by the decreased bioavailability of NO due to O_2^- (Nakazono *et al.,* 1991). More recent studies using the SOD mimetic tempol have shown similar results, and

have thus provided evidence of the putative role played by NO, O_2^-, $OONO^-$ and the resultant oxidative stress in hypertension (Simonsen *et al.,* 2009).

2.6.2.2. Vascular Remodelling

ROS production is closely involved with many of the processes that lead to both hypertrophic and proliferative vascular smooth muscle cell (VSMC) growth (Taniyama and Griendling., 2003). ROS act as mediators in the proliferative response to agonists such as platelet-derived growth factor (PDGF) and thrombin (Berk., 2001). 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 (Rao and Berk., 1992). 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 also been found to induce apoptosis (programmed cell death), while moderate concentrations cause cell cycle arrest (Deshpande *et al.,* 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 PDGF, epidermal growth factor, fibroblast growth factor and Ang 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).

It has been shown previously that Ang II can induce VSMC hypertrophy. NADPH-driven superoxide production in cultured vascular smooth muscle cells and fibroblasts, is increased in the presence of Ang II, and this causes VSMC hypertrophy (Ushio-Fukai *et al.,* 1999; Touyz *et al*., 2003).

Dimmeler and Zeiher., (2000), have shown that exposure to O_2^- or endothelial injury and H_2O_2 induces apoptosis of EC's, which leads to EC loss and results in atherogenesis and thus

a procoagulative state. ROS regulate apoptotic mechanisms induced by a variety of stimuli other that the ROS themselves (Taniyama and Griendling., 2003). Another type of programmed cell death, anoikis, results from detachment of ECs from the extracellular matrix. This process is associated with increased intracellular ROS, probably originating from mitochondria (Li *et al.*, 1999). EC proliferation, migration and tube formation are crucial events that lead to apoptosis which essentially 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).

Structural vascular changes are the hallmark of chronic hypertension, and increased wall-to-lumen ratio (W/L), of resistance vessels is the prominent lesion. Alterations in the structure of resistance vessels account for many of the features associated with essential hypertension (Mulvany., 1999). The increase in the relative "thickness" of resistance vessels 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).

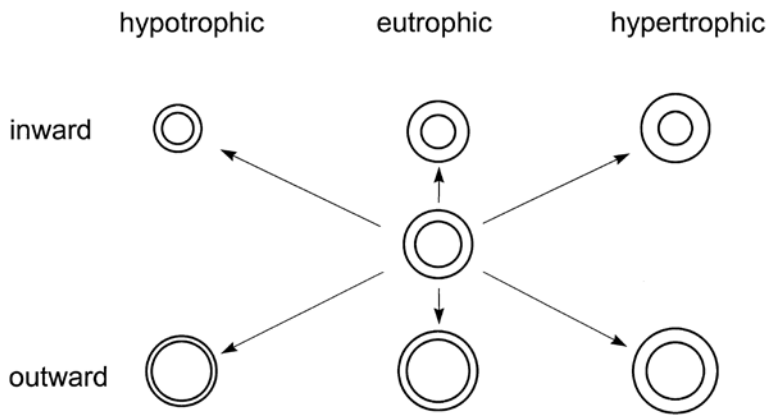


Figure 2: Classification of Vascular Remodelling (Mulvany., 1999)

The term “vascular remodelling” has been said to be used indiscriminately in the past to describe changes in the vasculature. The following proposal was put forward by Mulvany, (1999) in a review, it states: “... the term remodelling is to be used where there is a structurally determined change in lumen diameter, and that it be classified into the six changes indicated in Figure 2. It was suggested that remodelling should be termed inward or outward remodelling, depending on whether the process has resulted in a decrease or increase in diameter respectively. Furthermore, since remodelling can result in an increase, no change, or a decrease in the amount of material, that there should be a sub-classification into hypertrophic, eutrophic and hypotrophic remodelling, respectively”, as demonstrated in Figure 2.

The author thus suggests that by adopting this uniform framework/classification for defining the various modes of vascular remodelling, it would become easier (for researchers) to discuss the mechanisms involved in this process.

2.6.2.3. Lipid peroxidation as an Indicator of Oxidative Stress

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, that is mediated by free radicals, and is used as an indicator of oxidative stress in cells and tissues. It is a degenerative process that affects mainly cell membranes and other lipid-containing structures, during oxidative stress (Girroti., 1998; Arora *et al.,* 2003). Lipid peroxides are unstable by nature and decompose to form a complex series of compounds including reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate malonyldialdehyde (MDA), 4-hydroxynonenal (4-HNE) and isoprostanes upon decomposition. Measurement of these decomposition end-products (MDA, 4-HNE and isoprostanes) has been used successfully as an indicator/measure of the level of *in vivo* lipid peroxidation (Esterbauer *et al.,* 1991; Janika *et al.,* 2010).

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 reactive substances (TBARS), 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, and these substances are called F_2-isoprostanes. These F_2-isoprostanes have been demonstrated to be a more reliable indicator of *in vivo* oxidative stress, because of their stable nature, and also because they can be measured in extracellular fluids such as urine and plasma. Because of this, it is now possible for a relatively non-invasive approach in assessing levels of *in vivo* oxidative stress (Roberts and Morrow., 2000; Tarpey *et al.,* 2004).

Lipid peroxidation has been linked to a variety of disorders, including atherogenesis, diabetes, hypertension and UV-induced carcinogenesis (Girotti., 1998), and has been shown to be a deleterious consequence of the reported oxidative stress in these disorders.

2.6.2.3.1. Organ Damage due to Lipid Peroxidation

The consequent peroxidation of membrane lipids caused by free radicals plays an important role in cell physiology and pathology (Wang and Salahudheen., 1995). The peroxidative degradation of polyunsaturated fatty acids has been found to produce changes in most of the membrane parameters, and the resultant disruption of the biological membranes results in alterations to the activity of the 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 has been shown to encourage significant structural and functional modifications in the kidney, that result in proteinuria, glomerular sclerosis, and other morphological changes, and it is these modifications that eventually lead to end-stage renal disease. A reduction in the blood pressure of hypertensive patients appears to hinder the progression of renal failure and also reduces the morbidity and mortality rates (Meng *et al.*, 2003).

Increased oxidative stress resulting in lipid peroxidation has been shown to contribute to both renal damage and impaired renal functioning. Studies that have infused isoprostanes into the kidney have been shown to induce renal vasoconstriction, thus decreasing glomerular filtration rate and thus natriuresis and diuresis (Morrow *et al.,* 1992; Morrow *et al.,* 1994; Reckelhoff *et al*., 2000). The aldehydes released during lipid peroxidation have been implicated as causative agents **in** cytotoxic processes, due to their self-perpetuating characteristics, in that when released from cell membranes they may diffuse, **in**teract, and **in**duce oxidative modifications **in** other cells (Redón *et al.* 2003). Apoptotic cell death has been shown to result in cell shrinkage, loss of plasma membrane asymmetry, protease and

endonuclease activation, and internucleosomal fragmentation of nuclear DNA. The apoptosis thereby resulting in an almost total loss to the integrity of the cells and tissue (Girotti., 1998).

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. THE SPONTANEOUSLY HYPERTENSIVE RAT

The spontaneously hypertensive rat (SHR) is one of the most widely used animal models for the study of essential hypertension. This model originated from the selective inbreeding of Wistar rats with an elevated blood pressure, which resulted in 100% of the progeny having naturally occurring hypertensive disease (Okamoto., 1963).

The model develops a high blood pressure from the age of 5-6 weeks and the systolic blood pressure may reach values between 180-200 mmHg in the adult. There is a steady state increase in blood pressure as the animal ages. The SHR also has been shown to develop characteristics associated with cardiovascular disease, such as hypertrophy of the heart and blood vessels (Conrad., 1995).

Studies have shown that the genetic basis for hypertension, is not yet fully understood. However this model has demonstrated a polygenic trait, with studies showing at least 3 major genes to be involved in early development, and an additional gene identified on chromosome 10 has been shown to play a role in the development and maintenance of hypertension during aging in the SHR (Ely and Turner., 1990; Yamori *et al*., 1999). More recent evidence suggests that the genetic mechanism of hypertension in the SHR, can be attributed to both vascular and neural alterations (Pinto *et al*., 1998; Lerman *et al*., 2005).

Thus the characteristics of this widely used model, has proven over the years to be a reliable model for the study of the pathophysiological changes that occur during genetic hypertension.

SUMMARY

The flow diagram that follows, summarises the literature on the link between Ang II and oxidative stress, and the role of this link in contributing to an elevation in blood pressure.

The factors/agents that are highlighted in red, are some of the parameters that were quantified by this study, and provide motivation and justification of the objectives of this study in light of the available literature.

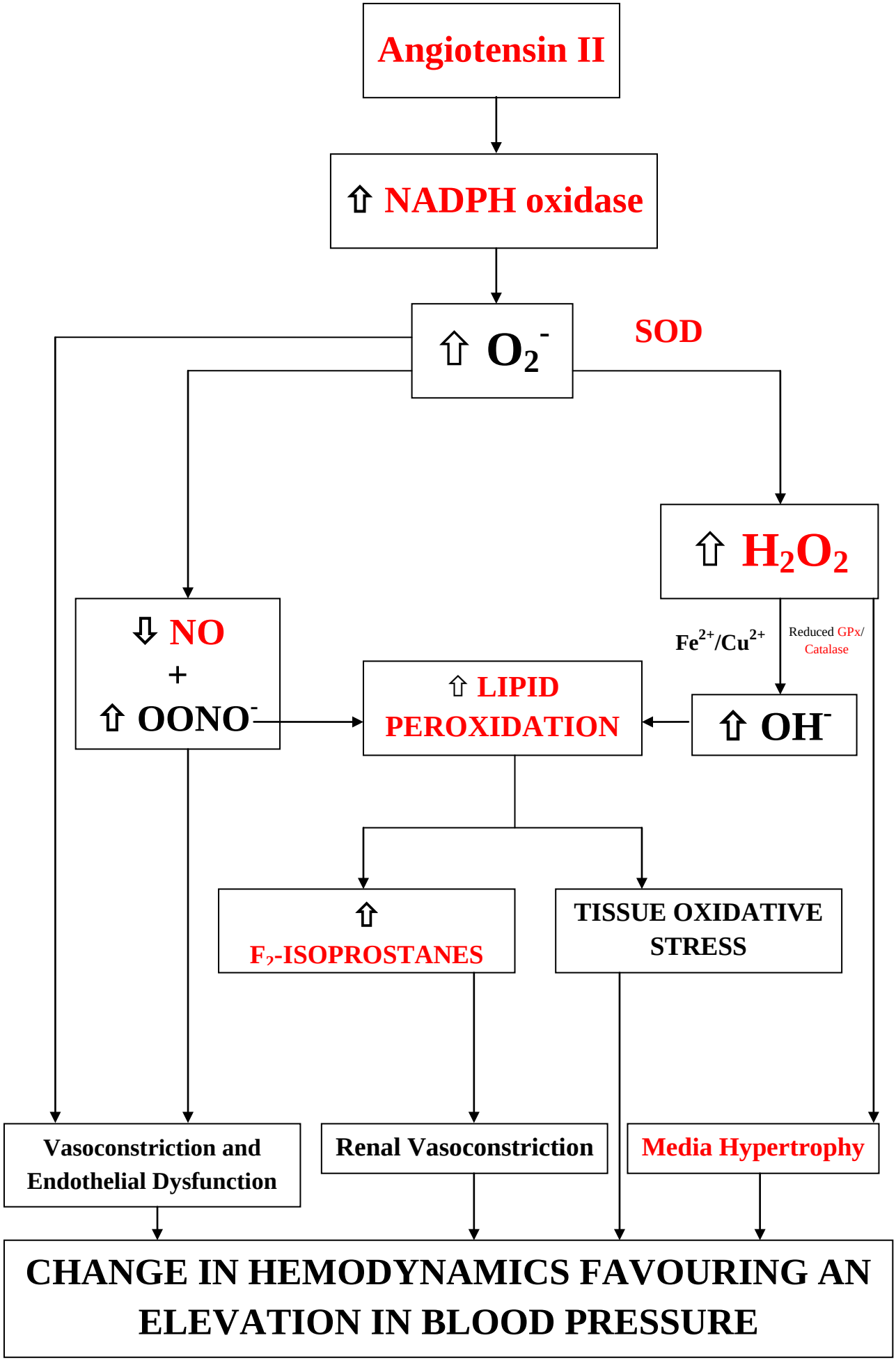


Figure 3: Summary of the link between Ang II and Oxidative Stress

3. MATERIALS AND METHODS

3.1. ANIMAL PROTOCOL

3.1.1. Ethical Approval and Standard Animal Protocol

Ethical approval was obtained from the Animal Ethics Committee of the University of Kwa-Zulu Natal (Reference Number: 015/08/Animal).

Sixty two male rats were used in this study. The animals were acquired from the Biomedical Resource Unit at the University of Kwa-Zulu Natal – Westville Campus. The animals were of 2 strains i.e. 31 Spontaneously Hypertensive Rats (SHR) rats and 31 normotensive Wistar control rats. These two groups were broken down further with no prejudice into the following groups (Table 5):

Table 5. Animal Grouping

Strain	Normal	Weanling	Angiotensin II Infused	Saline Infused
SHR	n = 8	n = 8	n = 7	n = 8
Wistar	n = 7	n = 8	n = 8	n = 8

Animals were distinguished from each other by ear notching. All animals were housed in the Biomedical Resource Unit at the University of Kwa-Zulu Natal – Westville Campus, in standard rodent cages. Animals were fed standard Epol rat chow and deionised water (Millipore) was provided *ad libitum*.

The room that the animals were housed in was maintained on a 12 hour light/dark cycle and maintained at a constant ambient temperature (23^0C) and pressure (101.3 kPa) for the duration of the study for all groups.

3.1.2. DURATION

The experiment commenced with all animals at weanlings (21 days old). Each of the groups in the table above (Table 5) had a different experimental protocol and duration.

SHR–Normal and Wistar–Normal groups:

Maintained for 28 days from weanling, at which point they were sacrificed.

The SHR–Weanling and Wistar–Weanling groups:

Sacrificed at 21 days old.

The SHR–Angiotensin II Infused and Wistar–Angiotensin II Infused groups:

Underwent minor surgery at weanling (21 days old) where they had a mini osmotic pump containing Angiotensin II subcutaneously inserted (as described in Section 3.2.1). These animals were then maintained for 28 days post surgery, after which they were sacrificed. It should be noted that the SHR – Ang II infused group, was sacrificed after 21 days of infusion (7 days earlier), due to the group's health status, which was assessed by the in-house veterinarian. He found their health status to be unstable. Based on this assessment, the study was shortened, for this group.

The SHR–Saline Infused and Wistar–Saline Infused groups:

These groups also underwent the previously mentioned minor surgical procedure at weanling (21 days old), where they also had a mini osmotic pump inserted subcutaneously, however these pumps contained saline (0.9% NaCl), and were the sham operated control groups. These animals were also maintained for 28 days post surgery, after which they were sacrificed.

3.1.3. ALZET™ OSMOTIC PUMP

Angiotensin II delivery was achieved with the aid of an Alzet mini-osmotic pump (Durect Corporation) – Model 2004. This pump once surgically inserted into the animal delivers a set dose at a constant rate for 28 days. The 2004 model pump used for this experiment delivers the pre-determined dose at a rate of 0.25*ul*/hr for the 28 days.

3.1.3.1. Background of ALZET™ Osmotic Pump

ALZET™ osmotic pumps are miniature, implantable pumps that are widely used for research in mice, rats and other laboratory animals. These pumps can deliver pre-determined doses of drugs, hormones, and other test agents at a controlled rate.

ALZET™ pumps operate because of an osmotic pressure difference between a compartment within the pump, called the salt sleeve, and the tissue environment in which the pump is implanted. The high osmolality of the salt sleeve causes water to flux into the pump through a semi-permeable membrane which forms the outer surface of the pump. As the water enters the salt sleeve, it compresses the flexible reservoir, displacing the test solution from the pump at a controlled, predetermined rate. Because the compressed reservoir cannot be refilled, the pumps are for single use only. The rate of delivery by an ALZET™ pump is controlled by the water permeability of the pump's outer membrane. Thus, the delivery profile of the pump is independent of the drug formulation dispensed. Drugs of various molecular configurations, including ionized drugs and macromolecules, can be dispensed continuously in a variety of compatible vehicles at controlled rates. The molecular weight of a compound, or its physical and chemical properties, has no bearing on the rate of delivery by the ALZET™ pumps (Information quoted as from package insert provided by the Durect Corporation).

3.1.3.2. Surgical Procedure for the Insertion of the ALZET™ Osmotic Pump

The pump was inserted subcutaneously between the scapula of the animal. The animal was anaesthetized using a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg) with or without inhalant gaseous anaesthesia of 0.5-2% halothane to maintain anaesthesia. The animal was then placed on a heating pad to maintain body temperature and the surgery was performed on the heating pad. The animal was shaven (mid scapular region) and the area was cleaned with a standard solution of 5% hepataine (5ml standard solution of chlorohexadine in 95ml water). The area was dried with sterile gauze, and betadine (a commercially available antiseptic spray) was applied to the area. Contact time of a few minutes was permitted before the area was cleaned with sterile swabs. The animal was surgically draped and prepared for surgery. A mid-scapular incision of ~1cm was then made with a #10 scalpel blade. Blunt dissection was performed with a small forceps and a haemostat to separate skin from fascia in order to make a pocket for the pump. When a large enough pocket was made, the filled pump was then inserted into this pocket. The incision was closed with nylon sutures (size 2-0 or 3-0). The surgical area was swabbed and sterilised with betadine. The operated animal was left to recover with regular observation for 24 hours. The wound/incision was monitored and disinfected twice daily with betadine to prevent infection until the wound/incision had healed completely and during this period the animals health status was checked for any adverse reactions, by both the researcher and the in-house veterinarian.

3.1.3.3. Infusion Dosage

Angiotensin II, was obtained as a lysate from Sigma (Catalogue No. : A9525) and dissolved in Saline (0.9% NaCl) and delivered using the 2004 model ALZET™ mini osmotic pump, at a dosage equivalent to 10ng/kg/min^{-1}. This dosage was adopted from previous studies, where it was shown to be an optimum sub-pressor dose in rats (Reckelhoff *et al.*, 2000).

3.1.4. 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 Biomedical Resource Unit (UKZN-Westville Campus).

3.1.4.1. 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 ~2 days.

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.

3.1.4.2. 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 28 day 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 effect on the blood pressure readings. 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^{o}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 surrounded by 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 which had 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 being occluded. The tail cuff was then slowly deflated. The reappearance of a pulsation, which was detected by the photoelectric sensor, was taken as the systolic blood pressure. As the pressure continued to drop, 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 analogue 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).

3.1.5. SACRIFICE

Animals were sacrificed by exsanguination, at the end of the specified duration, viz. 28 days after weanling for both the infused and non-infused groups and at 21 days old for the weanling groups. The sacrifice was carried out in the Animal Operating Theatre in the Biomedical Resource Unit (UKZN - Westville Campus). Animals were fasted overnight and were sacrificed the next morning. Animals were sedated with halothane (5mg/kg) with the use of a gas chamber.

Once fully sedated, blood was collected using a 16 gauge sterilised needle and syringe, via cardiac puncture. This blood was aliquoted for the different assays in pre-cooled collection tubes as follows:

1. Superoxide Dismutase (Whole Blood)
2. Glutathione Peroxidase (Whole Blood)
3. Catalase (Serum)
4. Hydrogen peroxide (Plasma)
5. Angiotensin II (Plasma)
6. Nitric Oxide (Plasma)

After the blood collection, the animals abdominal and thoracic cavity was surgically exposed and the required tissue viz. arch of aorta, kidney and the required vasculature was dissected out. The kidney and aorta were placed in mircocentrifuge tubes and were snap frozen in liquid nitrogen and stored in a biofreezer (-70^0C) for later analysis. The harvested vasculature was stored in 10% buffered neutral formalin (BNF) for histological analysis. 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. SAMPLE ANALYSIS

3.2.1. 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 340 nm was measured.

Reaction series:

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

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

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. 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).

3.2.2. 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 role of SOD which is to accelerate the dismutation of the toxic superoxide radical (O_2^-), which is 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 an erythrocyte lysate. The lysate was prepared by centrifuging 0.5ml the aliquoted whole blood for 10 minutes at 3000 rpm 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 10 minutes at 3000 rpm 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 ml with cold redistilled water (Millipore), they were then mixed and left to stand on ice (4^oC) for 15 minutes. The resulting lysate was then diluted with a 0.01 mmol/l phosphate buffer (pH 7), the dilution was done to ensure that the percentage inhibition fell between 30 – 60%, as required by the supplier. The reaction was carried out in matched glass cuvettes and readings were obtained from a dual beam spectrophotometer (Varian, Cary IE).

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

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

3.2.3. ANGIOTENSIN II

Ang II was quantified using a commercially available kit from BACHEM (Kit No: S1133). The kit was a competitive peptide enzyme immunoassay. The working principle as explained in the kit insert is as follows:

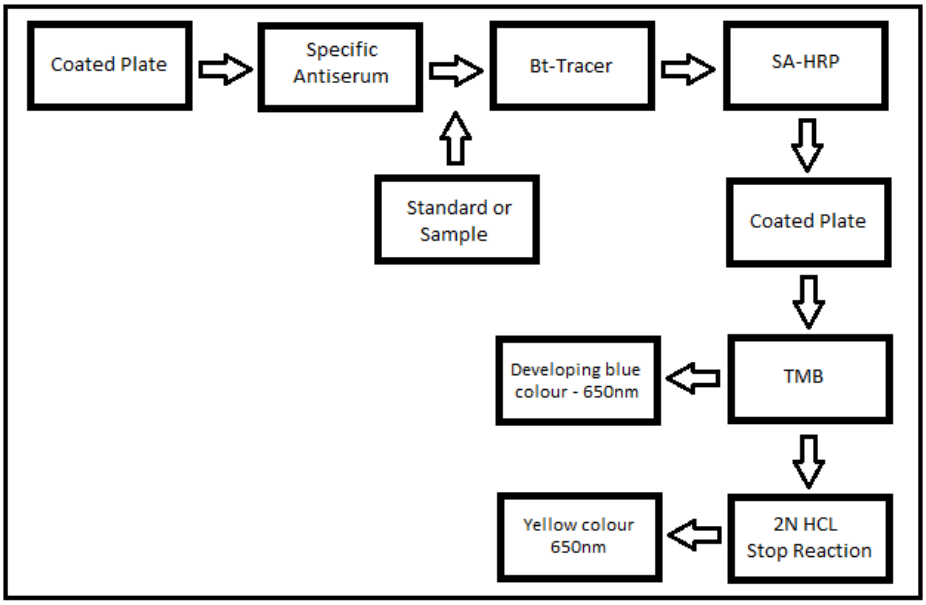


Figure 3: Reaction Series for Ang II EIA.

The antiserum is immobilized on a 96-well plate. A constant concentration of Bt-tracer (biotinylated tracer) and varying concentrations of standard/sample compete for binding specifically to the anti-serum. Captured Bt-tracer is subsequently bound to SA-HRP (streptavidin-conjugated horseradish peroxidase), which produces a soluble coloured product after TMB (3,3`,5,5`-tetramethyl benzidine dihydrochloride) is added. This reaction is then stopped with 2N hydrochloric acid, and the yellow end point colour (optical density) is read on a plate reader at a wavelength of 450 nm.

The plasma samples had to be first prepared/purified by sample extraction. The following solid phase extraction (SPE) was performed on all plasma samples before Ang II quantification.

3.2.3.1. Reagents

Buffer A – 1% triflouroacetic acid (TFA)

Buffer B – 60% acetonitrile, 1% TFA and 39% distilled water

3.2.3.2. Procedure

1. 200 ul of Buffer A was added to an equal volume of sample (plasma)
2. This was centrifuged at 5000 rpm for 20 minutes at 4^0C
3. The supernatant was transferred to a new tubes discarding any pellet that was present
4. A SEP-COLUMN (containing 200 mg of C18 – Cat No: Y-1000 – BACHEM) was equilibrated by washing with 1 ml Buffer B, followed by 3 washes of 3 ml Buffer A
5. The plasma sample was loaded into the column
6. The column was slowly washed with Buffer A (3 ml, twice)
7. The peptide was eluted slowly with Buffer B into a microcentrifuge tube
8. The eluant was freeze-dried by immersing the tube into liquid nitrogen
9. The freeze-dried sample was evaporated in a centrifugal evaporator.
10. The residue was dissolved in a suitable volume of EIA buffer such that the concentration of the peptide fell close to the IC_{50}. This dissolved peptide was now ready for analysis using the EIA.

3.2.4. NITRIC OXIDE

Nitric oxide was quantified in plasma with the aid of a commercially available kit from BioAssay Systems (Quantichrom^{TM} Nitric oxide assay kit – Cat No: DINO 250).

The final products of NO *in vivo* are nitrite and nitrate. The relative proportion of nitrite and nitrate is variable and cannot be predicted with certainty. Thus the method uses the sum of both these species as total NO production. The principle is based on the improved Griess method, in two steps, where the first step involves the conversion of nitrate to nitrite utilizing a nitrate reductase. The second step involves adding the Griess reagents (i.e. Sulphanilamide and Ethylenediamine) which convert nitrite into a deep purple azo compound that can be read using a spectrophotometer at 540nm.

Results were extrapolated of an 8 point standard curve with the standards provided as part of the kit.

3.2.5. F_2-ISOPROSTANES

Kidney F_2-isoprostanes were evaluated using a commercially available kit from Northwest Life Science Specialties (Product Number: NWK-IS001)

Isoprostanes are prostaglandin-like compounds that are produced by free radical mediated peroxidation of lipoproteins. Levels of 15-isoprostane F_{2t} a representative isoprostane in biological fluids, has been shown to be useful for assessment of oxidant stress *in vivo*.

This assay according to the supplier utilizes a competitive enzyme-linked immunoassay format. Briefly, 15-isoprostane F_{2t} in the samples or standards is allowed to compete with 15-isoprostane F_{2t} conjugated to horseradish peroxidise (HRP) for binding to a polyclonal antibody specific for 15-isoprostane F_{2t} coated on the microplate. Subsequent TMB (3,3`,5,5`-tetramethyl benzidine dihydrochloride) substrate addition results in a blue colour development that is inversely proportional to the quantity of 15-isoprostane F_{2t} in the original samples or standards. Addition of an acid stop solution causes a colour change to yellow where absorbance is read at 450nm on a microplate reader.

The following solid phase extraction (SPE) protocol was used to extract the 15-isoprostane F_{2t} fraction from the kidney.

3.6.1. Reagents

1. Folch Solution:

   2:1 Chloroform:Methanol + 5 mg Butalyated Hydroxytoulene (BHT)/100 ml solution

2. Methanol Solution:

   Methanol (MeOH) + 5 mg BHT/100 ml MeOH

3. 15% Potassium Hydroxide (KOH)

4. pH 3.0 water

5. Ethyl acetate Heptane solution:

   Ethyl acetate : Heptane (1:1)

6. Ethyl acetate Methanol solution

   Ethyl acetate : Methanol (1:1)

3.6.2. Procedure

1. 20 ml of Folch solution was added to a 40 ml flat bottom centrifuge tube, and placed on ice.

2. The kidney was weighed and between 0.5 - 1 g of tissue was added to the Folch Solution on ice.

3. The tube was vortexed vigorously for 1 min.

4. The solution was homogenised with a blade homogeniser for 30 seconds.

5. The homogenate solution was placed under nitrogen (N_2) for 1 hour at room temperature, and was vortexed occasionally.

6. 4 ml of saline (0.9% NaCl) was added to the homogenate solution and vortexed vigorously.

7. The homogenate solution was centrifuged at 5000 rpm for 3 minutes.

8. The upper aqueous layer (MeOH/saline) was discarded.

9. The lower phase was transferred to a glass 50 ml conical test tube (much care was taken to avoid the middle protein layer).

10. This solution was evaporated under N_2.

11. When the solution had fully evaporated, 2 ml of MeOH+BHT, as well as an equal volume of 15% KOH were added to the test tube.

12. The solution was incubated in a water-bath maintained at $37^{O}C$ for 30 minutes.

13. The solution was diluted with 40 ml of pH 3.0 water so that the MeOH was less than 5% of the total volume. This sample was then ready for the solid phase extraction.

14. 3 ml of pH 3.0 water was added to 10 ml of the diluted sample.

15. The pH of this solution was adjusted to 3.

16. C-18 Sep Pak:

    i. The column was washed with 5 ml ethanol

    ii. This was followed with 5 ml pH 3.0 water

    iii. The sample was loaded

    iv. The column was washed with 10 ml pH 3.0 water

    v. The column was washed with 10 ml Heptane

    vi. The peptide was eluted with 10 ml Ethyl acetate: Heptane (1:1) solution into a plastic tube.

    vii. 1 pea-sized scoop of sodium sulphate was added to the eluant.

    viii. The eluant was aspirated into a new test tube with a disposable pipette.

    ix. The sample was ready for the next column.

17. Silica Sep-Pak

i. The column was washed with 5 ml MeOH

ii. This was followed with 5 ml ethyl acetate

iii. The sample was loaded (i.e. the Ethyl acetate:Heptane eluant)

iv. The column was washed with 5 ml ethyl acetate

v. The peptide was eluted with 5 ml of an Ethyl acetate:Methanol (1:1) solution into a plastic tube.

vi. This solution was evaporated under N_2

18. The sample was ready to be reconstituted and analysed.

3.2.6. HYDROGEN PEROXIDE

Hydrogen Peroxide (H_2O_2) was quantified in plasma using a commercially available kit from Assay Designs – Stressgen (Catalogue Number: 907-015). The kit was a simple colorimetric assay based on the following working principle as explained in the kit insert.

The kit is designed to measure low concentrations of H_2O_2 in biological matrices. The principal is based on a colour reagent that contains a dye, called xylenlol orange, in an acidic solution with sorbitol and ammonium iron sulphate that reacts to produce a purple colour that is proportional to the concentration of H_2O_2, in a sample. This colour change was then read at 450 nm.

3.2.7. CATALASE

Catalase levels were quantified in serum using a biochemical assay, based on a modified method adapted from Goth., (1991). The serum was obtained by allowing blood to stand at room temperature for 30 minutes in a glass serum tube upon collection, the clot was then removed via centrifugation at 3000 rpm for 10 minutes and the serum was aspirated and stored at -70^{o}C.

3.2.7.1. Reagents

1. Substrate – 65 umol/ml H_2O_2 in 60 mmol/l Na-K phosphate buffer (pH 7.4)

   30% H_2O_2 (0.05525 g/25 ml)

2. 32.4mmol/l Ammonium molybdate – $Na_2HPO_4.2H_2O$ (40.05 g/l)

3. Buffer (phosphate) for dilution of sample (If > 100 kU/l) – 2-10 fold

4. Na-K buffer – 60 mmol/l (pH 7.4)

   a) KH_2PO_4 - 8.165 g/l

   b) $Na_2HPO_4.2H_2O$ - 10.679 g/l

   For 100ml buffer a (19.7 ml) + b (80.3 ml) – Mix, adjust pH to 7.4

3.2.7.2. Procedure

Sample :

i) 0.2 ml serum was added to 1 ml of substrate

ii) This was incubated at 37^{0}C for 1 minute

iii) The reaction was stopped with 1 ml $(NH_4)_6Mo_7O_{24}$)

iv) The optical density was read at 405 nm against Blank 3

The reaction series was prepared as follows:

Blank 1: 0.2 ml serum + 1 ml substrate + 1 ml $(NH_4)_6Mo_7O_{24}$

Blank 2: 0.2 ml buffer + 1 ml substrate + 1 ml $(NH_4)_6Mo_7O_{24}$

Blank 3: 0.2 ml buffer + 1 ml buffer + 1 ml $(NH_4)_6Mo_7O_{24}$

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

Serum catalase activity was calculated using the following formula:

Serum catalase activity = [(Abs blank 1- Abs sample) / (Abs blank 2- Abs blank 3)] × 271

3.2.8. HISTOPATHOLOGY

3.2.8.1. TISSUE PROCESSING

The vasculature was removed from the fixative i.e. 10% BNF. The need for fixation was to ensure that the integrity of the cellular components of the tissue was maintained, as is standard procedure for histological study.

Histoprocessing was conducted with the use of the H2500 microwave processor. The tissue was placed in appropriately labelled cassettes that were immersed in a plastic beaker containing ethyl alcohol (100%). The beaker was thereafter placed in the microwave processor, with the temperature probe manoeuvred centrally into the bath. The microwave processor power level was turned to 450W (80%) and the temperature set at 67^0C for 15 minutes.

After the set time had elapsed, the cassettes were removed and placed in a bath containing isopropanol. This bath was placed in the microwave processor with the temperature probe being centered again. The microwave processor was then set at 450 W (80%) and 75^0C for 15 minutes. The cassettes were then placed into a paraffin rack, and immersed into a plastic beaker containing melted paraffin, the paraffin's melted state being maintained on a stove at 67^0C.

The plastic beaker containing the paraffin and cassettes was then placed into the microwave processor. Microwave processing in paraffin was carried out in two steps at different temperature settings viz. 70^0C for 10 minutes and 82^0C for 10 minutes.

At the completion of these two steps the tissue was ready for embedding.

3.2.8.2. EMBEDDING

3.2.8.2.1. Embedding Equipment

The embedding procedure was carried out with the use of the Shandon Histocentre 2. The equipment essentially consists of a paraffin tank, cold plate, hot plate, base moulds, a pair of warmed forceps and a warming tank.

3.2.8.2.2. Embedding Procedure

The processed vasculature was placed in a stainless steel holding tank containing molten paraffin. These cassettes were then removed and placed in the warming tank.

An appropriate base mould was then selected from the prewarmer which housed various base moulds of differing sizes. The mould was placed on the hot plate and paraffin wax was dispensed from the paraffin tank into the mould, filling it to about quarter of the level of the base mould.

The tissue from the warming tank was orientated in the base mould so as to have the exposed portions of the vessel facing down. The tissue was then held in position with a pair of heated forceps and placed on the cold plate. The molten paraffin then changed from translucent to opaque and it hardened. Thereafter an appropriately labelled cassette was placed on top of the base mould and filled with the molten wax. The base mould was then placed back on the cold plate for rapid cooling and hardening of the molten wax. After the right amount of cooling was achieved, the specimen attached to the cassette was carefully removed from the base mould. The base mould was then returned to the warming tank.

3.2.8.3. SECTIONING

3.2.8.3.1. Trimming the wax block

A rotary microtome was used for the cutting of paraffin embedded serial sections. The coarse feed mechanism of the microtome was advanced by hand before commencement of each stroke. This was done in order to trim away the surplus wax and to expose an appropriate area of tissue for sectioning. On exposing a suitable area a thickness of 2μm was chosen for sectioning.

3.2.8.3.2. Cutting Sections

The paraffin blocks were placed on ice before cutting. This is done because the integrity of the paraffin block is dependent on atmospheric temperature, melting point of wax and humidity. However, if contact is limited on the block face this is not detrimental and this cooling effect on the block face by the ice promotes ease in production of flat sections.

It was found that blowing lightly on the section the moment the section commenced up the cutting edge of the knife was beneficial in obtaining a consistent ribbon of wax. Warm breath had an expansion effect on the wax, thereby flattening the cut sections. The selected sections were then immediately floated out on a constant temperature water bath ($60^{o}C$).

3.2.8.3.3. Floating out of Sections

The floating out actions had to be smooth, with the trailing end of the ribbon making contact with the hot water bath first. The slight drag produced when the ribbon touches the water surface is sufficient to produce tension in the ribbon and to thereby remove most of the folds from these sections.

The remaining wrinkles and folds were removed by carefully teasing them apart with a pair of forceps, as the ribbon came to rest on the water surface. After several minutes on the water surface, complete flattening and expansion of sections were achieved. Prolonged floating was avoided since this may cause the tissue to expand beyond their normal size and become distorted or irregular.

The hot water bath was maintained at a temperature of ~60^{o}C. A higher temperature would cause the paraffin to melt and dissolve sections, and a lower temperature would not have the desired effect of flattening out the sections.

3.2.8.3.4. <u>Temporary Mounting</u>

Once the sections were evenly flattened on the water bath, they were separated into individual sections or groups of sections. The desired sections were picked up on a clean, appropriately labelled slide, which was previously coated with egg albumen. The slide was placed approximately three quarters into the water bath and was manoeuvred so as to position the tissue in a desired position on the coated side. The slide was then slowly picked up with the section safely flattened onto the slide. The egg albumen used is a general purpose section-adhesive. It is an adhesive which results in a minimal production of background staining.

The slides were then tapped vertically on a paper towel to remove excess water and placed on a warming plate. The warming plate was maintained at a temperature of ~40^{0}C, and the slides were placed on it for ~8 minutes to provide maximal adhesion of the tissue section to the slide and to also evaporate excess paraffin wax from the section. The slides were then ready for staining.

3.2.8.4. SLIDE SELECTION

Twelve slides for each animal, in each group were chosen for staining. These slides were chosen on the context of well sectioned slides.

3.2.8.5. STAINING

3.2.8.5.1. Principle

Staining was done using a basic Haematoxylin-Eosin stain. Haematoxylin stains the nuclear component and eosin is responsible for cytoplasmic staining. In the first step, the nuclei are stained with a haematoxylin solution. The nuclei stain blue, dark violet to black. The second step is counterstaining with eosin, where the cytoplasmic portions stain pink. This stain therefore allows for the identification of individual cells, as well as components.

3.2.8.5.2. Reagents

The following reagents were made up in the Physiological Chemistry Laboratory in the Department of Human Physiology:

1. Harris Haematoxylin

   Haematoxylin : 10 g

   Absolute Alcohol : 100 ml

   Potassium Alum : 200 g

   Distilled Water : 2000 ml

   Mercuric Oxide :5g

The haematoxylin was dissolved in the absolute alcohol in a small beaker. The potassium alum was then dissolved in warm distilled water in 5 litre flask and brought up to boiling

point. The mixture was then removed from the heat and the mercuric oxide was added and then cooled rapidly in a sink of iced water. The reagent was now ready for use and stored in a light safe holding vessel.

2. Eosin

Eosin Y	: 1 g
Distilled Water	: 20 ml
Ethanol (100%)	: 80 ml

The Eosin Y was dissolved in distilled water and then added to the ethanol. A working solution was then made up in a 1:2 ratio with 70% ethanol. 0.5 ml of glacial acetic acid was added to each 100 ml of stain just before use.

3. Acid Alcohol

70% Alcohol	: 70 ml
Concentrated Hydrochloric Acid	: 2 ml
Distilled Water	: 28 ml

The hydrochloric acid was added to the alcohol in a fume cupboard, and the distilled water was added to this mixture to bring the solution up to a 100 ml.

3.2.8.5.3. <u>Procedure</u>

Eight slides were stained on each staining run, in appropriate staining glassware. The time for each step was monitored using a stop watch.

1. The slides were first de-paraffinised by immersing them into a solution of xylene for 2 minutes. This step was repeated 3 times.

2. The tissue was hydrated to water. This was achieved by using decreasing concentrations of alcohol. The tissue was first placed into a 100% solution of alcohol for 1 minute. This step was repeated two more times. The tissue was then placed into a 95% solution of alcohol for 1 minute and this was also repeated two more times. The tissue was thereafter placed into 90%, 80% and 70% alcohol solutions respectively for a period of 1 minute each. Finally the tissue was washed twice in distilled water, with each slide set being dipped 10 times. This step completed the hydration of the tissue.

3. The hydrated tissue was immersed into the Haematoxylin solution for 1 minute.

4. The slides were dipped into distilled water to remove the excess stain.

5. The slides were washed under running tap water for 10 minutes. This was done to remove the excess Haematoxylin and to prevent over-staining.

6. The slides were immersed in the Acid Alcohol solution for 1 minute.

7. The slides were washed twice in distilled water, for 2 minutes for each wash.

8. The slides were immersed in the eosin solution for 1 minute.

9. The tissue was dehydrated. This was achieved by placing the tissue in increasing concentrations of alcohol. The tissue was first placed in a 70% solution of alcohol for 1 minute. The tissue was thereafter immersed into 80%, 90% and 95% alcohol solutions for 1 minute each. The tissue was then placed into absolute alcohol (100%) for 1 minute. This step was repeated 3 times.

10. The slides were cleared by placing them in Xylene, for a period of 2 minutes. This was repeated 3 times.

The slides were then ready to be permanently mounted.

3.2.8.5.4. Permanent Mounting

The stained sections were immediately mounted by placing the slide on a coverslip, which had a drop of DPX™ mountant on them. Pressure was applied to the slide so as to spread the DPX™ evenly and prevent the formation of air bubbles. The mounted sections were allowed to dry for 24 hours.

The slides were then ready for histological analysis.

3.2.8.6. MORPHOMETRIC ANALYSIS

Slides were analysed in the Electron Microscope Unit (EMU) at the University of Kwa-Zulu Natal - Westville Campus.

Slides were viewed under a Nikon Compound Microscope, at 10x magnification. Images were captured using a Nikon DS U2/L2 camera, with the aid of NIS Elements D 3.0 histology software. Morphometric measurements on captured images were then made, with the use of the iTEM software. Each image was first calibrated at a 10x magnification scale, and then measurements were made. The software allowed for measurement of area and perimeter in um to be made.

3.2.9. GENE EXPRESSION AND ANALYSIS

3.2.9.1. Trizol purification of RNA

(Perou *et al.* 1999)

1. The frozen tissue specimen was removed from the bio-freezer and using a scalpel, the tissue was cut into small pieces. 1 ml Trizol was added per 50-100 mg tissue processed. This tissue was then homogenized in the Trizol until it could easily pass through a 21 gauge needle.

2. The sample was incubated at room temperature for 5 to 10 minutes after homogenization.

3. The sample was centrifuged at 12000 rpm for 20 minutes at 4^0C.

4. The upper fat layer was removed using a Pasteur pipette (Upper fat layer, if present has a yellowish appearance while the Trizol homogenate, remains red.)

5. 200 ul chloroform per 1 ml Trizol reagent used in step 2 was added to the sample. The solution was shaken vigorously for 15-30 seconds and incubated at room temperature for 20 minutes.

6. The sample was centrifuged at 12000 rpm for 20 minutes at 4^0C.

7. The upper aqueous phase which contains the total RNA was carefully removed, and placed into a new micro-centrifuge tube.

8. The RNA was precipitated by adding 500 ul isopropanol and 1 ul glycogen per 1 ml Trizol. This was thereafter incubated at room temperature for 20 minutes.

9. The solution was centrifuged at 12000 rpm for 20 minutes at 4^0C.

10. The supernatant was carefully discarded and the remaining pellet was washed once with 1ml 75% ethanol per 1 ml Trizol.

11. This solution was centrifuged at 12000 rpm for 10 minutes at 4^0C.

12. The resulting supernatant was discarded and the sample was centrifuged for 1 minute to remove residual ethanol.

13. The resulting pellet was immediately redissolved in 35 ul of RnaSecure.

14. The solution was placed in an oven at 60^oC for 10 minutes.

15. The sample was stored in a biofreezer at -70^oC.

16. The samples thereafter underwent a DNAse treatment to remove residual genomic DNA from the extracted sample.

17. This sample thereafter underwent cDNA synthesis using a commercially available kit by BioRad (iScript cDNA synthesis kit)

18. The sample was then ready to undergo Realtime PCR quantification.

3.2.9.2. Realtime PCR quantification

3.2.9.2.1. p22phox

p22phox gene expression was examined by using SYBR Green 1 (Roche Diagnostics) chemistry with primers obtained from Inqaba Biotech to uniquely amplify the p22phox sequence. The primers for p22phox were 5'-GCTCATCTGTCTGCTGGAGTA-3' (forward) and 5'-ACGACCTCATCTGTCACTGGA-3' (reverse). The housekeeping gene used was GAPDH. The primers for GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) were 5'-TCCATGACAACTTTGGCATC-3' (forward) and 5'-CATGTCAGATCCACCACGGA-3' (reverse), (Moritz *et al.*, 2003). Each real-time PCR reaction was performed in a total volume of 10 ul that was made up of 5.05 ul water, 1.2 ul $MgCl_2$ (3 mmol/ul), 0.5 ul reverse primer, 0.25 ul of forward primer, 1 ul Fast Start SYBR Green I and 2 ul sample cDNA. All reactions were run in a Roche Lightcycler Ver. 1.5 with 1 cycle of 95^{o}C (10 minutes), followed by 45 cycles of 95^{o}C (6 seconds), 58^{o}C (10 seconds) and 72^{o}C (6 seconds). Each sample was run in duplicate. Relative mRNA copy numbers were calculated by generating standard curves using serial dilutions of a known concentration of cDNA. p22phox and GAPDH mRNA levels were calculated as number of molecules per ug of cDNA. Results that follow are depicted as a ratio of p22phox to GAPDH molecules per ug of cDNA.

3.2.9.2.2. gp91phox

gp91phox gene expression was examined by using SYBR Green 1 (Roche Diagnostics) chemistry with primers obtained from Inqaba Biotech to uniquely amplify the gp91phox sequence. The primers for gp91phox were 5'-CCAGTCTGAGACTCAAAAAGATCTACT TC-3' (forward) and 5'-GTCTGCGAACCACTCAAAAGC-3' (reverse). The housekeeping gene used was GAPDH. The primers for GAPDH were 5'-TCCATGACAACTTTGGCATC-3' (forward) and 5'-CATGTCAGATCCACCACGGA-3' (reverse), (Moritz *et al.*, 2003). Each real-time PCR reaction was performed in a total volume of 10 ul that was made up of 4.8 ul water, 1.2 ul $MgCl_2$ (3 mmol/ul), 0.5 ul reverse primer, 0.5 ul of forward primer, 1 ul Fast Start SYBR Green I and 2ul sample cDNA. All reactions were run in a Roche Lightcycler Ver. 1.5 with 1 cycle of 95^{o}C (10 minutes), followed by 42 cycles of 95^{o}C (6 seconds), 60^{o}C (6 seconds) and 72^{o}C (6 seconds). Each sample was run in duplicate. Relative mRNA copy numbers were calculated by generating standard curves using serial dilutions of a known concentration of cDNA. gp91phox and GAPDH mRNA levels were calculated as number of molecules per ug of cDNA. Results that follow are depicted as a ratio of gp91phox to GAPDH molecules per ug of cDNA.

3.2.9.2.3. Cu/Zn-SOD

Cu/Zn-SOD gene expression was examined by using SYBR Green 1 (Roche Diagnostics) chemistry with primers obtained from Inqaba Biotech to uniquely amplify the Cu/Zn-SOD sequence. The primers for Cu/Zn-SOD were 5'-CGGATGAAGAGAGGCATGTTG-3' (forward) and 5'-TTGGCCACACCGTCCTTT-3' (reverse) (van Lindhout *et al.,* 2008). The housekeeping gene used was GAPDH. The primers for GAPDH were 5'-TCCATGACAACTTTGGCATC-3' (forward) and 5'-CATGTCAGATCCACCACGGA-3' (reverse) (Moritz *et al.,* 2003). Each real-time PCR reaction was performed in a total volume of 10 ul that was made up of 4.8 ul water, 1.2 ul $MgCl_2$ (3 mmol/ul), 0.5 ul reverse primer, 0.5 ul of forward primer, 1 ul Fast Start SYBR Green I and 2 ul sample cDNA. All reactions were run in a Roche Lightcycler Ver. 1.5 with 1 cycle of 95^{o}C (10 minutes), followed by 40 cycles of 95^{o}C (6 seconds), 58^{o}C (10 seconds) and 72^{o}C (6 seconds). Each sample was run in duplicate. Relative mRNA copy numbers were calculated by generating standard curves using serial dilutions of a known concentration of cDNA. Cu/Zn-SOD and GAPDH mRNA levels were calculated as number of molecules per ug of cDNA. Results that follow are depicted as a ratio of Cu/Zn-SOD to GAPDH molecules per ug of cDNA.

3.2.9.2.4. AT-1a

AT-1a gene expression was examined by using SYBR Green 1 (Roche Diagnostics) chemistry with primers obtained from Inqaba Biotech to uniquely amplify the AT-1a sequence. The primers for AT-1a were 5'-CGGATGAAGAGAGGCATGTTG-3' (forward) and 5'-TTGGCCACACCGTCCTTT-3' (reverse) (Naito *et al.,* 2002). The housekeeping gene used was GAPDH. The primers for GAPDH were 5'-TCCATGACAACTTTGGCATC-3' (forward) and 5'-CATGTCAGATCCACCACGGA-3' (reverse) (Moritz *et al.,* 2003). Each real-time PCR reaction was performed in a total volume of 10 ul that was made up of 4.8 ul water, 1.2 ul $MgCl_2$ (3 mmol/ul), 0.5 ul reverse primer, 0.5 ul of forward primer, 1 ul Fast Start SYBR Green I and 2 ul sample cDNA. All reactions were run in a Roche Lightcycler Ver. 1.5 with 1 cycle of 95^{o}C (10 minutes), followed by 45 cycles of 95^{o}C (6 seconds), 65^{o}C (6 seconds) and 72^{o}C (6 seconds). Each sample was run in duplicate. Relative mRNA copy numbers were calculated by generating standard curves using serial dilutions of a known concentration of cDNA. AT-1a and GAPDH mRNA levels were calculated as number of molecules per ug of cDNA. Results that follow are depicted as a ratio of AT-1a to GAPDH molecules per ug of cDNA.

4. RESULTS

All data is represented as the Mean ± Standard Error of Mean (SEM). The statistical analysis of all the results presented was performed using Graphpad Instat Ver. 5.0. The statistical analysis performed was a one-way analysis of variance (ANOVA) and an unpaired t-test.

A p-value < 0.05 was considered to be statistically significant.

4.1. BLOOD PRESSURE

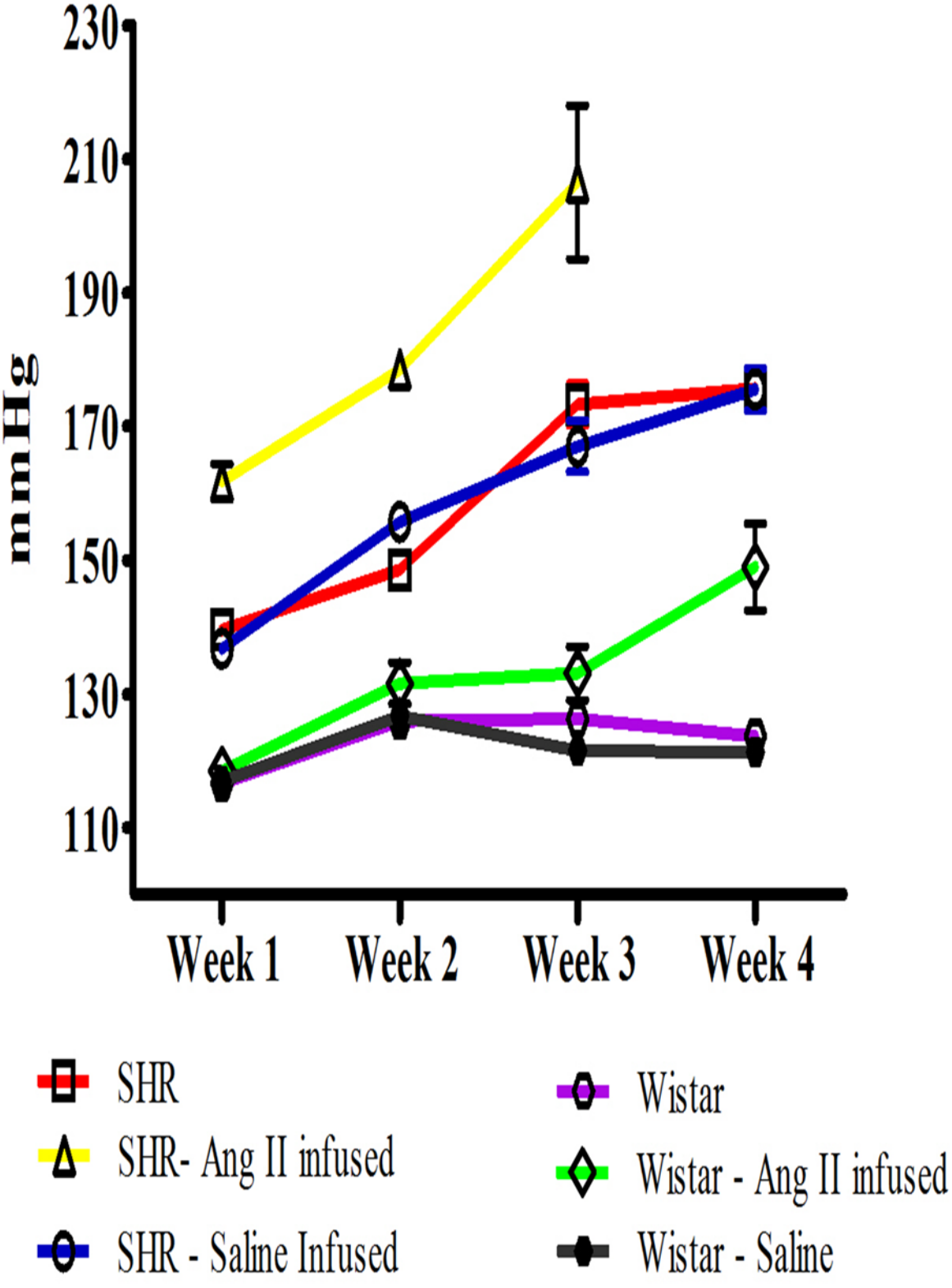
The blood pressure was monitored weekly using the non-invasive tail-cuff method. The systolic and diastolic blood pressures are shown in Table 6 and are represented in Figure 5 and Figure 6 respectively.

4.1.1. Systolic Blood Pressure (SBP)

The results show that there was a gradual increase in the SBP of the SHR group over the 4 successive weeks of the study when compared to the normotensive Wistar control, which had a steady SBP over the successive weeks (Week 1 – 140 ± 2 mmHg vs 117 ± 1 mmHg; Week 2 - 149 ± 2 mmHg vs 126 ± 1 mmHg; Week 3 – 173 ± 3 mmHg vs 126 ± 2 mmHg and Week 4 – 176 ± 1 mmHg vs 124 ± 1 mmHg)

The Angiotensin II infusion had a significant increase on the SBP of both the SHR and the Wistar groups when compared to their respective saline infused controls. The increase in the SHR-Ang II infused groups SBP was observed from the first week of infusion until the termination of the group in the third week. (Week 1 - 161 ± 3 mmHg vs 143 ± 4 mmHg; Week 2 – 179 ± 2 mmHg vs 157 ± 2 mmHg and Week 3 – 207 ± 11 mmHg vs 168 ± 2 mmHg). The Wistar-Ang II infused group had a significant increase in SBP in the third and fourth week of the study when compared to the saline infused control. (Week 1 – 132 ± 3 mmHg vs 116 ± 2 mmHg; Week 2 - 134 ± 4 mmHg vs 126 ± 1 mmHg; Week 3 – 149 ± 7 mmHg vs 123 ± 1 mmHg and Week 4 – 152 ± 6 mmHg vs 121 ± 2 mmHg)

Figure 5: Systolic Blood Pressure



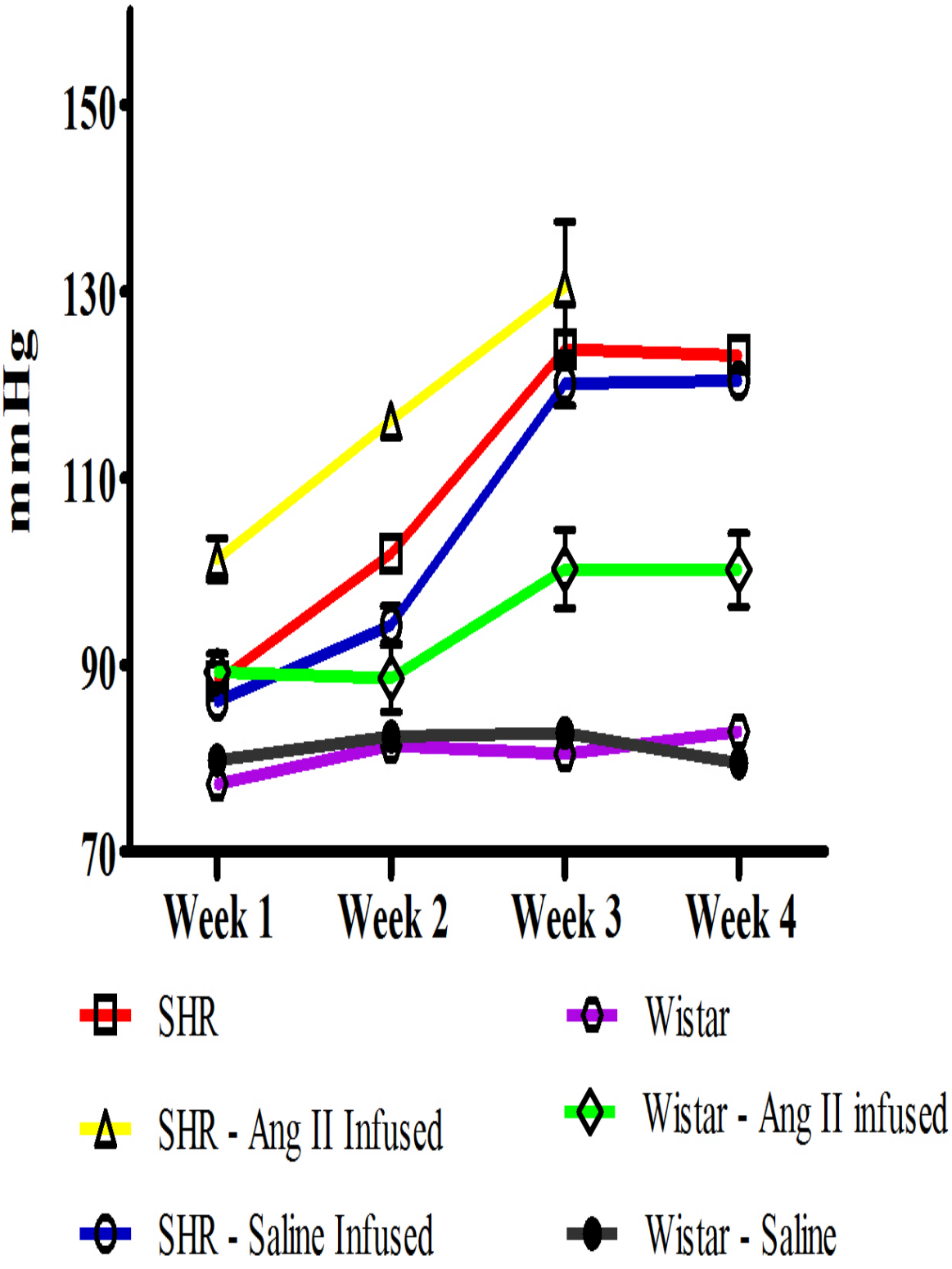
4.1.2. Diastolic Blood Pressure (DBP)

The results show that there was an increase in the DBP of the SHR group between Week 2 and Week 3, with the DBP stabilizing from Week 3 till the end of study. This increase in DBP was statistically significant when compared to the normotensive Wistar control, which showed a stable normotensive DBP, through the entire duration of the study. (Week 1 – 88 ± 1 mmHg vs 77 ± 1 mmHg; Week 2 - 102 ± 1 mmHg vs 81 ± 1 mmHg; Week 3 – 124 ± 1 mmHg vs 80 ± 2 mmHg and Week 4 – 123 ± 1 mmHg vs 83 ± 1 mmHg)

The Ang II infusion showed a significant weekly increase in the DBP of both the SHR and Wistar infused groups, when compared to their respective saline-infused control groups. The SHR-Ang II infused group had a significant increase in DBP from Week 2 of the infusion till the termination of the group at the end of the third week. (Week 1 – 100 ± 3 mmHg vs 94 ± 2 mmHg; Week 2 - 116 ± 1 mmHg vs 105 ± 2 mmHg; Week 3 – 131 ± 7 mmHg vs 121 ± 2 mmHg)

The Wistar-Ang II infused group had a significant increase in DBP from Week 3 till the end of study at 4 weeks, this when compared to its respective saline infused control (Week 1 – 89 ± 2 mmHg vs 80 ± 1 mmHg; Week 2 - 89 ± 4 mmHg vs 82 ± 1 mmHg; Week 3 – 100 ± 4 mmHg vs 83 ± 1 mmHg and Week 4 – 99 ± 4 mmHg vs 79 ± 1 mmHg).

Figure 6: Diastolic Blood Pressure



4.2. HEART RATE

The heart rate (HR) was monitored weekly, using the non-invasive tail cuff method for measuring blood pressure. The heart rates are shown in Table 6 and represented as beats per minute (bpm) in Figure 7.

The results show that the SHR had a steady decrease in HR over the successive weeks (Week 1 –407 ± 5 bpm; Week 2 - 380 ± 5 bpm; Week 3 – 378 ± 7 bpm; Week 4 - 372 ± 9 bpm). The normotensive Wistar control showed a similar trend with a steady weekly decrease in HR (Week 1 – 450 ± 10 bpm; Week 2 - 399 ± 8 bpm; Week 3 – 368 ± 9 bpm; Week 4 - 357 ± 18 bpm). The SHR group had a higher HR than the normotensive Wistar control at the end of study (372 ± 9 bpm vs 357 ± 18 bpm), but this higher HR was not statistically significant ($p>0.05$).

The Ang II infusion caused an increase in the HR of the SHR group at the termination of the group at the end of 3 weeks when compared to its saline infused control, at the same time (392 ± 5 bpm vs 372 ± 8 bpm), this increase in HR was however not statistically significant ($p>0.05$).

The Wistar-Ang II infused group, showed a decrease in the HR in the first 2 weeks of infusion and then subsequently the HR returned to the saline-infused value at the end of study (Week 1 – 398 ± 6 bpm vs 420 ± 8 bpm; Week 2 – 366 ± 7 bpm vs 399 ± 9 bpm; Week 3 – 374 ± 11 bpm; vs 372 ± 11 bpm; Week 4 – 361 ± 8 bpm vs 357 ± 12 bpm)

Figure 7: Heart Rate

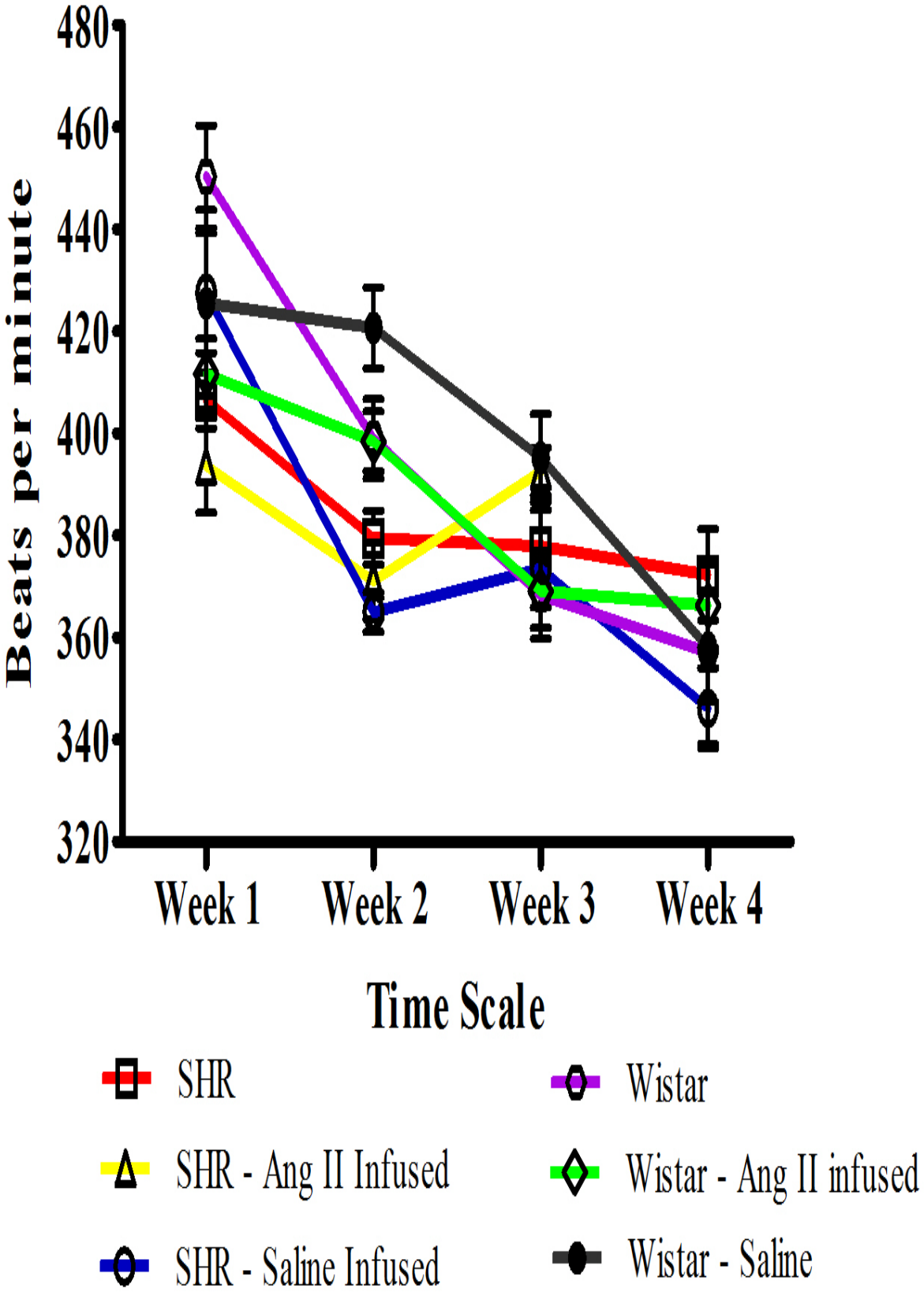


Table 6. Systolic Blood Pressure, Diastolic Blood Pressure and Heart Rate

SYSTOLIC BLOOD PRESSURE (mmHg)				
	Week 1	**Week 2**	**Week 3**	**Week 4**
SHR	140 ± 2*	149 ± 2*	173 ± 3*	176 ± 1*
Wistar	117 ± 1	126 ± 1	126 ± 2	124 ± 1
SHR - Weanling	138 ± 3*	-	-	-
Wistar - Weanling	120 ± 1	-	-	-
Wistar - Saline	116 ± 2	126 ± 1	123 ± 1	121 ± 2
Wistar - Ang II	132 ± 3*	134 ± 4	149 ± 7*	152 ± 6*
SHR - Saline	143 ± 4	157 ± 2	168 ± 4	176 ± 3
SHR - Ang II	161 ± 3*	179 ± 2*	207 ± 11*	-

DIASTOLIC BLOOD PRESSURE (mmHg)				
	Week 1	**Week 2**	**Week 3**	**Week 4**
SHR	88 ± 1*	102 ± 1*	124 ± 1*	123 ± 1*
Wistar	77 ± 1	81 ± 1	80 ± 2	83 ± 1
SHR - Weanling	89 ± 1*	-	-	-
Wistar - Weanling	80 ±1	-	-	-
Wistar - Saline	80 ± 1	82 ± 1	83 ± 1	79 ± 1
Wistar - Ang II	89 ± 2*	89 ± 4	100 ± 4*	99 ± 4*
SHR - Saline	94 ± 2	105 ± 2	121 ± 2	120 ± 1
SHR - Ang II	100 ± 3	116 ± 1*	131 ± 7*	-

HEART RATE (beats per minute)				
	Week 1	**Week 2**	**Week 3**	**Week 4**
SHR	407 ± 5*	380 ± 5	378 ± 7	372 ± 9
Wistar	450 ± 10	399 ± 8	368 ± 9	357 ± 18
SHR - Weanling	421 ± 6	-	-	-
Wistar - Weanling	409 ± 17	-	-	-
Wistar - Saline	420 ± 8	399 ± 9	372 ± 11	357 ± 12
Wistar - Ang II	398 ± 6*	366 ± 7*	374 ± 11	361 ± 8
SHR - Saline	364 ± 4	353 ± 7	372 ± 8	346 ± 8
SHR - Ang II	394 ± 10*	374 ± 9	392 ± 5*	-

*- Denotes statistically significant ($p<0.05$) when compared to the relevant control

4.3. PLASMA ANGIOTENSIN II

Angiotensin II levels were quantified in the plasma of all groups. The levels are shown in Table 7 and represented in Figure 8. The results show that the SHR-normal group had a 5.6 fold higher plasma Ang II concentration than the normotensive Wistar control (8.99 ± 1.22 vs 1.61 ± 0.43 pg/ml), and this higher concentration was statistically significant ($p<0.0001$).

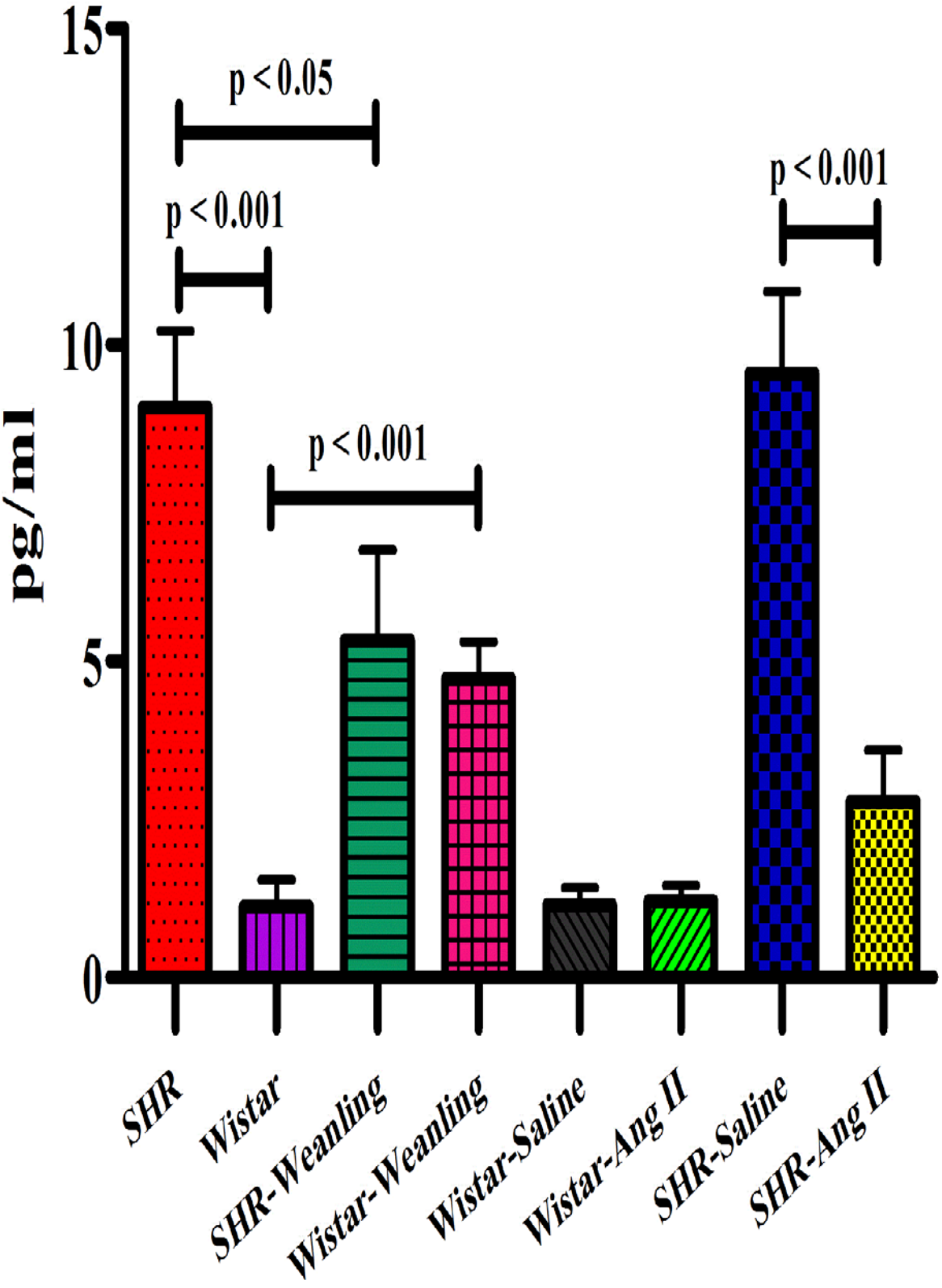
The SHR-weanling group showed no significant difference in plasma Ang II levels when compared to the Wistar-weanling (5.33 ± 1.42 vs 4.72 ± 0.59 pg/ml). The SHR-adult group showed a 1.7 fold increase in Ang II levels when compared to the SHR-weanling group (8.99 ± 1.22 vs 5.33 ± 1.42 pg/ml), and this difference in Ang II levels was statistically significant. ($p>0.05$). This trend was the opposite when the Wistar-adult group was compared to the Wistar-weanling group, with the Wistar-adult group having a 2.9 fold lower plasma Ang II level than the Wistar-weanling group (1.61 ± 0.43 vs 4.72 ± 0.59 pg/ml), and this difference was statistically significant ($p<0.001$).

The SHR-Ang II infused group showed a 3.4 fold decrease in plasma Ang II levels when compared to the SHR-saline infused control group (2.79 ± 0.81 vs 9.54 ± 1.30 pg/ml), and this decrease was statistically significant ($p<0.001$).

The Wistar-Ang II infused group did not demonstrate the same trend and showed no significant change in plasma Ang II levels when compared to its saline infused control (1.21 ± 0.25 vs 1.13 ± 0.28 pg/ml).

The degree of change due to the infusion of Ang II with regards to the plasma Ang II levels in the SHR and Wistar was compared and these results show that the sub-pressor dose of Ang II decreased plasma Ang II levels in the SHR by 71%, and did not change the plasma Ang II levels in the Wistar.

Figure 8: Plasma Angiotensin II



4.4. PLASMA NITRIC OXIDE

Nitric oxide (NO) levels were quantified in the plasma. The levels are shown in Table 7 and represented in Figure 9. The results show that the SHR had a 1.4 fold lower plasma NO level when compared to its normotensive Wistar control (38.4 ± 1.5 vs 53.7 ± 2.9 ug/ml). This difference in plasma NO levels was statistically significant ($p<0.05$).

The SHR-weanling showed no difference in the plasma NO level when compared to the Wistar-weanling control (62.6 ± 6.0 vs 64.1 ± 5.1 ug/ml). When the SHR-adult group was compared to SHR-weanling group, the SHR-adult group showed a 1.6 fold decrease in the plasma NO level (38.4 ± 1.5 vs 62.6 ± 6.0 ug/ml), and this difference was statistically significant ($p<0.05$). The Wistar-adult showed a similar trend with a 1.2 fold decrease when compared to the Wistar-weanling (53.7 ± 2.9 vs 64.1 ± 5.1 ug/ml), however this decrease was not statistically significant ($p>0.05$).

The SHR-Ang II infused group had a 1.6 fold lower plasma NO level than the saline infused control (24.2 ± 2.9 vs 38.0 ± 1.5 ug/ml), and this decrease was statistically significant ($p<0.05$).

The Wistar-Ang II infused group showed a similar trend with a 1.1 fold decrease in plasma NO levels (47.9 ± 1.0 vs 54.2 ± 6.2 ug/ml), this decrease was however not statistically significant ($p>0.05$).

The degree to which the infusion of the sub-pressor dose of Ang II decreased the plasma NO levels in the SHR and Wistar was compared, and the results show that in the Wistar infused with Ang II, the NO levels were decreased by 13%, but the SHR infused with Ang II showed a greater degree of decrease, where the plasma NO levels were decreased by 36%.

Figure 9: Plasma Nitric Oxide

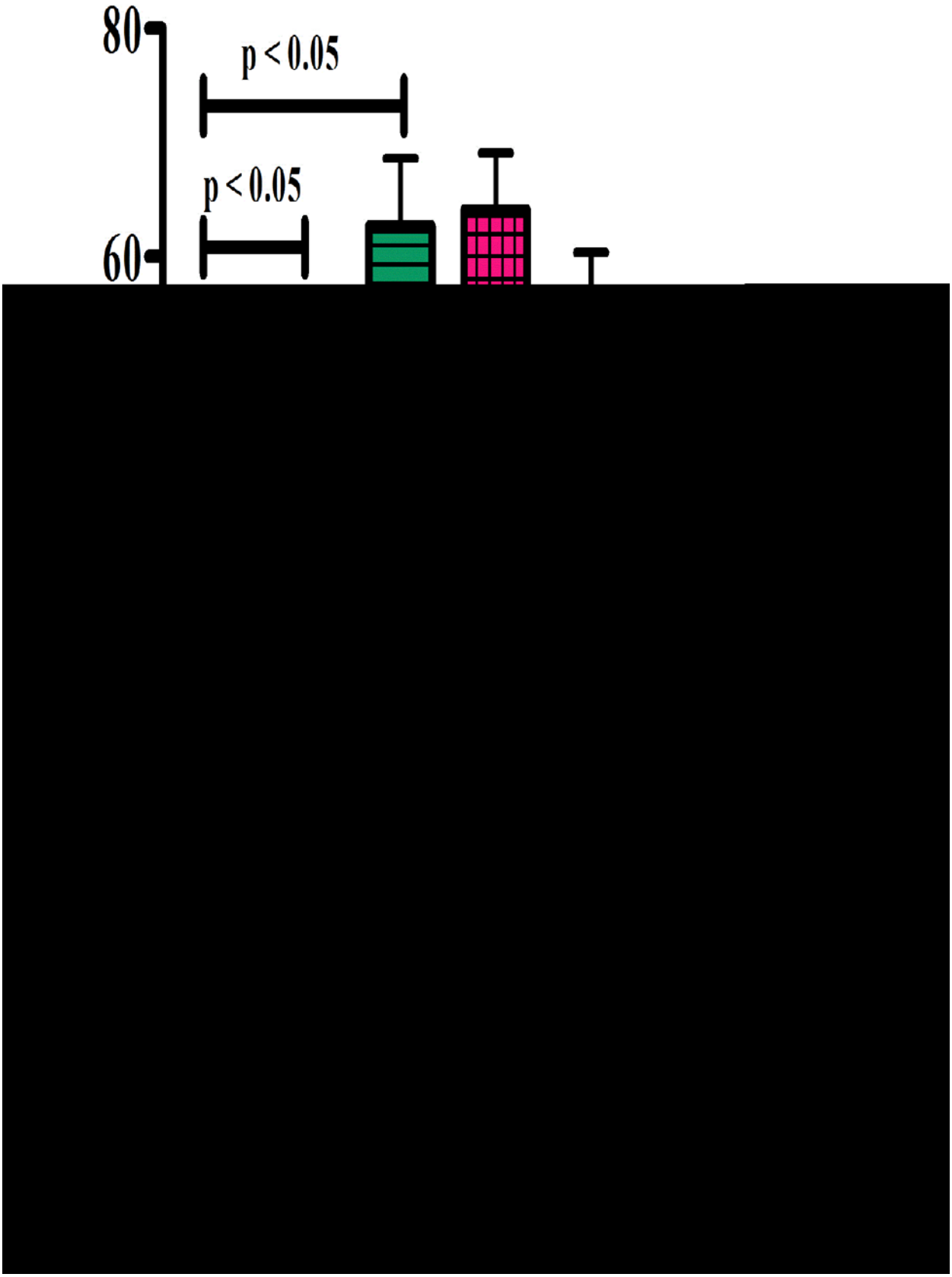


Table 7. Plasma Angiotensin II, Nitric Oxide, Hydrogen Peroxide and Kidney F_2-Isoprostane levels

	SHR (n=8)	**Wistar (n=7)**	**SHR Weanling (n=8)**	**Wistar Weanling (n=8)**	**Wistar Saline (n=8)**	**Wistar Ang II (n=8)**	**SHR Saline (n=8)**	**SHR Ang II (n=7)**
Plasma Angiotensin II (pg/ml)	8.99 ± 1.22*	1.61 ± 0.43*	5.33 ± 1.42*	4.72 ± 0.59	1.13 ± 0.28	1.21 ± 0.25 (0%)	9.54 ± 1.30	2.79 ± 0.81* (71%⇩)
Plasma Nitric Oxide (ug/ml)	38.4 ± 1.5*	53.7 ± 2.9	62.6 ± 6.0*	64.1 ± 5.1	54.2 ± 6.2	47.9 ± 1.0 (12%⇩)	38.0 ± 1.5	24.2 ± 2.9* (36%⇩)
Plasma Hydrogen Peroxide (ng/ml)	671.2 ± 52.7*	490.0 ± 46.0	635.4 ± 41.5	376.5 ± 20.6*	499.0 ± 49.6	425.0 ± 58.6 (15%⇩)	640.8 ± 21.6	456.5 ± 38.1* (29%⇩)
Kidney F_2-isoprostanes (ng/mg)	1.079 ± 0.047*	0.847 ± 0.041	1.114 ± 0.039	0.873 ± 0.043*	0.805 ± 0.082	0.796 ± 0.037 (0%)	1.003 ± 0.068	0.998 ± 0.037 (0%)

Statistical Analysis Comparison Key:

* SHR :Statistically significant difference when compared to the Wistar group

* Wistar :Statistically significant difference when compared to the Wistar-Weanling group

*SHR-Weanling :Statistically significant difference when compared to the SHR group

* Wistar-Weanling :Statistically significant difference when compared to the SHR-Weanling group

*Wistar-Ang II :Statistically significant difference when compared to the Wistar-Saline infused group

*SHR-Ang II :Statistically significant difference when compared to the SHR-Saline infused group

N.B. Red % – below the Wistar and SHR Ang II group values, means % increase (⇧)/decrease(⇩) compared to the respective Saline infused control

4.5. SUPEROXIDE DISMUTASE

SOD was quantified in the vascular compartment (red blood cell). The results are shown in Table 8 and represented in Figure 10.

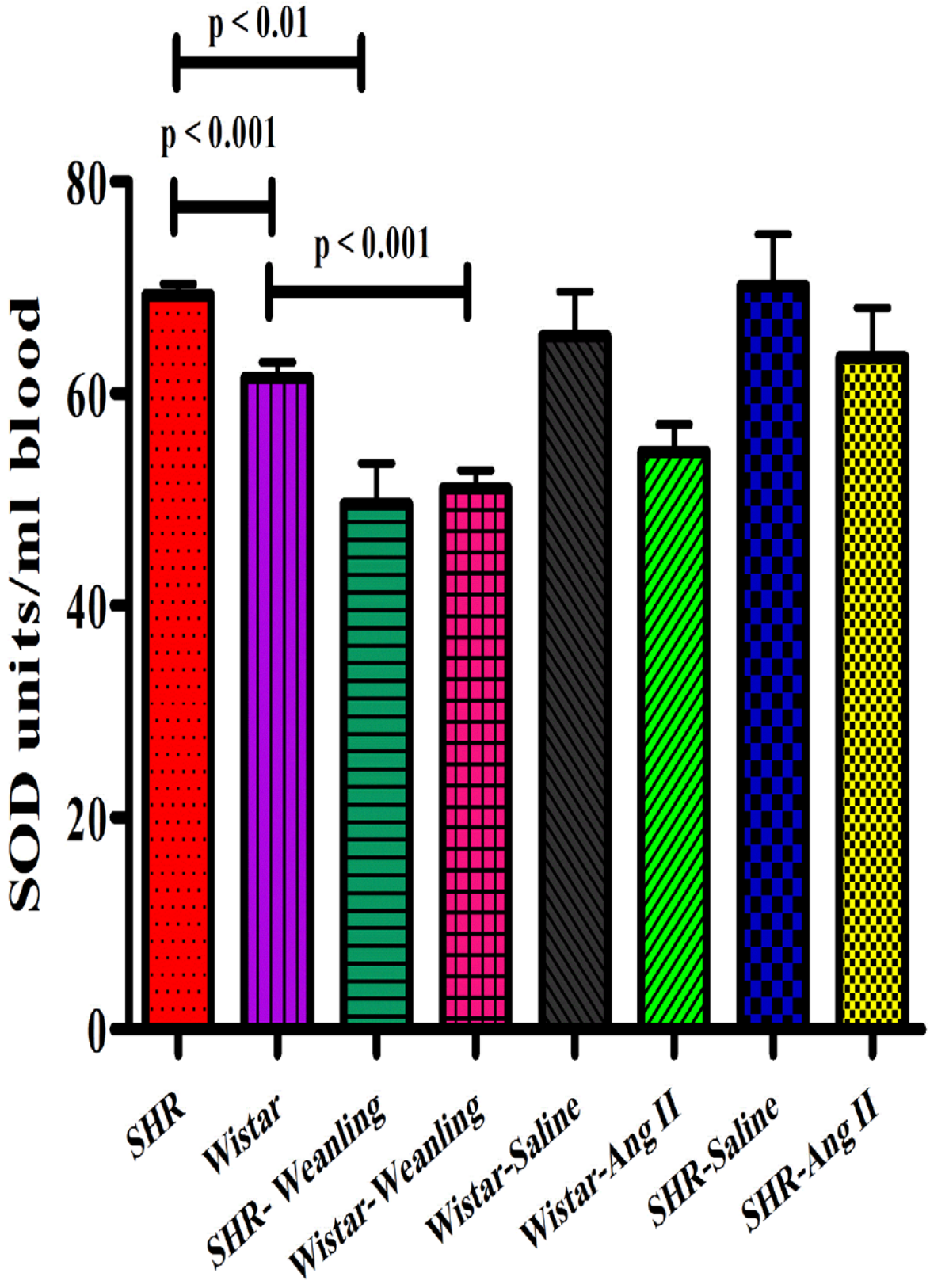
The SHR group had a 1.1 fold higher SOD level than the normotensive Wistar control (69.3 ± 1.1 vs 61.5 ± 1.5 SOD units/ml), and this increase was statistically significant ($p>0.05$). The SHR-weanling group showed no difference in SOD levels when compared to the Wistar-weanling (49.6 ± 3.9 vs 51.0 ± 1.8 SOD units/ml), however when the SHR-adult was compared to the SHR-weanling, there was a statistically significant ($p<0.05$) 1.4 fold increase in SOD levels (69.3 ± 1.1 vs 49.6 ± 3.9 SOD units/ml) in the SHR adult group. This trend was the same when the Wistar-adult group was compared to the Wistar-weanling group, with the Wistar-adult group having a 1.2 fold increase in SOD levels (61.5 ± 1.5 vs 51.0 ± 1.8 SOD units/ml), and this difference was also statistically significant ($p<0.001$).

The SHR-Ang II infused group demonstrated a 1.1 fold decrease in SOD concentration when compared to the saline infused control group (63.4 ± 4.6 vs 70.2 ± 4.9 SOD units/ml), but this difference was not statistically significant ($p>0.05$).

The Wistar-Ang II infused group also showed a similar trend with a 1.2 fold decrease in SOD levels (54.5 ± 2.6 vs 65.4 ± 4.2 SOD units/ml), and this difference was also not statistically significant ($p>0.05$).

The degree to which the infusion of the sub-pressor dose of Ang II decreased the SOD levels in the SHR and Wistar was compared, and the results show that in the Wistar infused with Ang II, the SOD levels were decreased by 17%, and the SHR infused with Ang II, showed a smaller degree of decrease, where the SOD levels were decreased by 10%.

Figure 10: Superoxide Dismutase



4.6. GLUTATHIONE PEROXIDASE

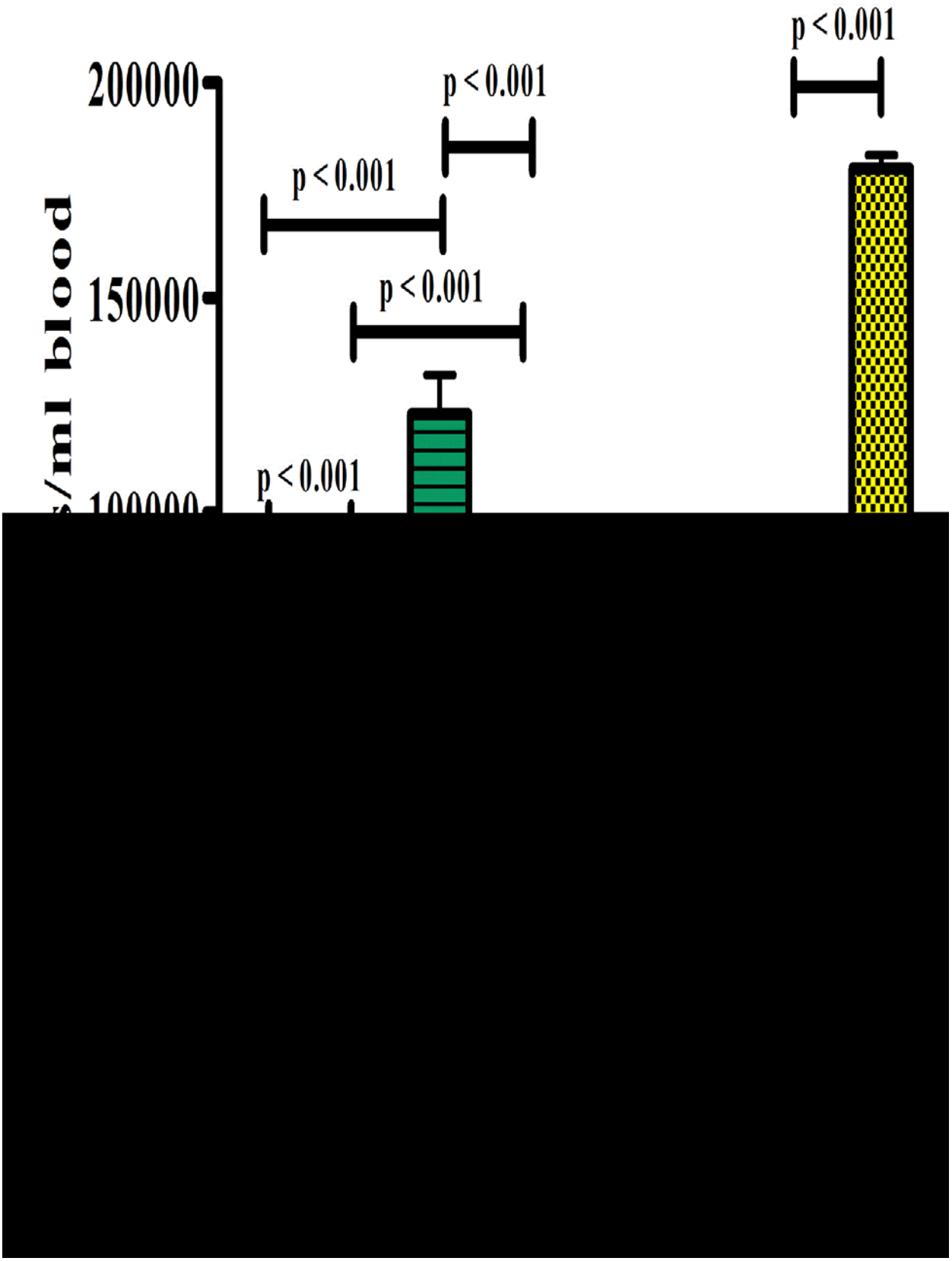
GPx was quantified in whole blood and the results are shown in Table 8 and represented in Figure 11.

The SHR group showed a 1.8 fold lower GPx level, when compared to its normotensive Wistar control (46975 ± 5242 vs 82567 ± 2537 GPx units/ml), and this difference was statistically significant ($p<0.001$). This trend was the opposite when the SHR-weanling was compared to the Wistar-weanling group, with the SHR-weanling group having a 2 fold higher GPx level than the Wistar-weanling (122839 ± 9225 vs 62189 ± 2476 GPx units/ml), and this difference was also statistically significant ($p<0.001$). When the SHR-adult was compared to the SHR-weanling group, the SHR-adult group had a 2.6 fold decrease in GPx levels, and this decrease was statistically significant (46975 ± 5242 vs 122839 ± 9225 GPx units/ml) ($p<0.001$). The Wistar-adult displayed an opposite trend with a 1.3 fold increase in GPx levels when compared to the Wistar-weanling (82567 ± 2537 vs 62189 ± 2476 GPx units/ml), and this increase was also statistically significant ($p<0.05$).

The SHR-Ang II infused group had a 4.1 fold increase in GPx levels when compared to the saline infused control (180023 ± 3241 vs 43858 ± 3447 GPx units/ml), and this difference was statistically significant ($p<0.001$). The Wistar-Ang II infused group showed a 1.1 fold increase in GPx levels (87553 ± 3881 vs 81922 ± 3163 GPx units/ml), but this increase was not statistically significant ($p>0.05$).

The degree to which the infusion of the sub-pressor dose of Ang II increased the GPx levels in the SHR and Wistar was compared, and the results show that in the Wistar infused with Ang II, the GPx levels were increased by 7%, but the SHR infused with Ang II showed a greater degree of increase, where the GPx levels were increased by 310%.

Figure 11: Glutathione Peroxidase



4.7. SERUM CATALASE

Catalase was quantified in serum and the results are shown in Table 8 and represented in Figure 12.

The results show that SHR group had a 1.6 fold higher level of serum catalase than the normotensive Wistar control (689.4 ± 48.3 vs 423.0 ± 45.6 Catalase units/ml), and this higher serum catalase level was statistically significant ($p<0.01$). The SHR-weanling had a 1.8 fold lower serum catalase than the Wistar-weanling (352.2 ± 26.3 vs 644.3±36.1 Catalase units/ml), and this difference in serum catalase levels was also statistically significant ($p<0.001$). When the serum catalase levels of the SHR-adult is compared to the SHR-weanling, the SHR-adult had a 2 fold increase in serum catalase levels (689.4 ± 48.3 vs 352.2 ± 26.3 Catalase units/ml), and this difference was also statistically significant ($p<0.001$). The Wistar-adult showed an opposite trend with a 1.5 fold decrease in serum catalase levels (423.0 ± 45.6 vs 644.3 ± 36.1 Catalase units/ml), and this decrease in catalase levels was statistically significant ($p<0.001$).

The SHR-Ang II infused group had a 1.3 fold decrease in the level of serum catalase when compared to the saline infused control (462.3 ± 47.8 vs 612.8 ± 36.3 Catalase units/ml), and this decrease was statistically significant ($p<0.05$).

The Wistar-Ang II infused group showed an opposite trend in serum catalase levels when compared to the saline infused control, with the Wistar-Ang II infused group having a 1.8 fold increase in serum catalase levels (987.3 ± 42.6 vs 556.3 ± 27.2 Catalase units/ml), and this difference in serum catalase was statistically significant ($p<0.05$).

The degree of change caused by the infusion of Ang II with regards to the serum catalase levels in the SHR and Wistar was compared, and the results show that the sub-pressor dose of

Ang II decreased serum catalase levels in the SHR by 25%, and increased the serum catalase levels by 77% in the Wistar.

Figure 12: Serum Catalase

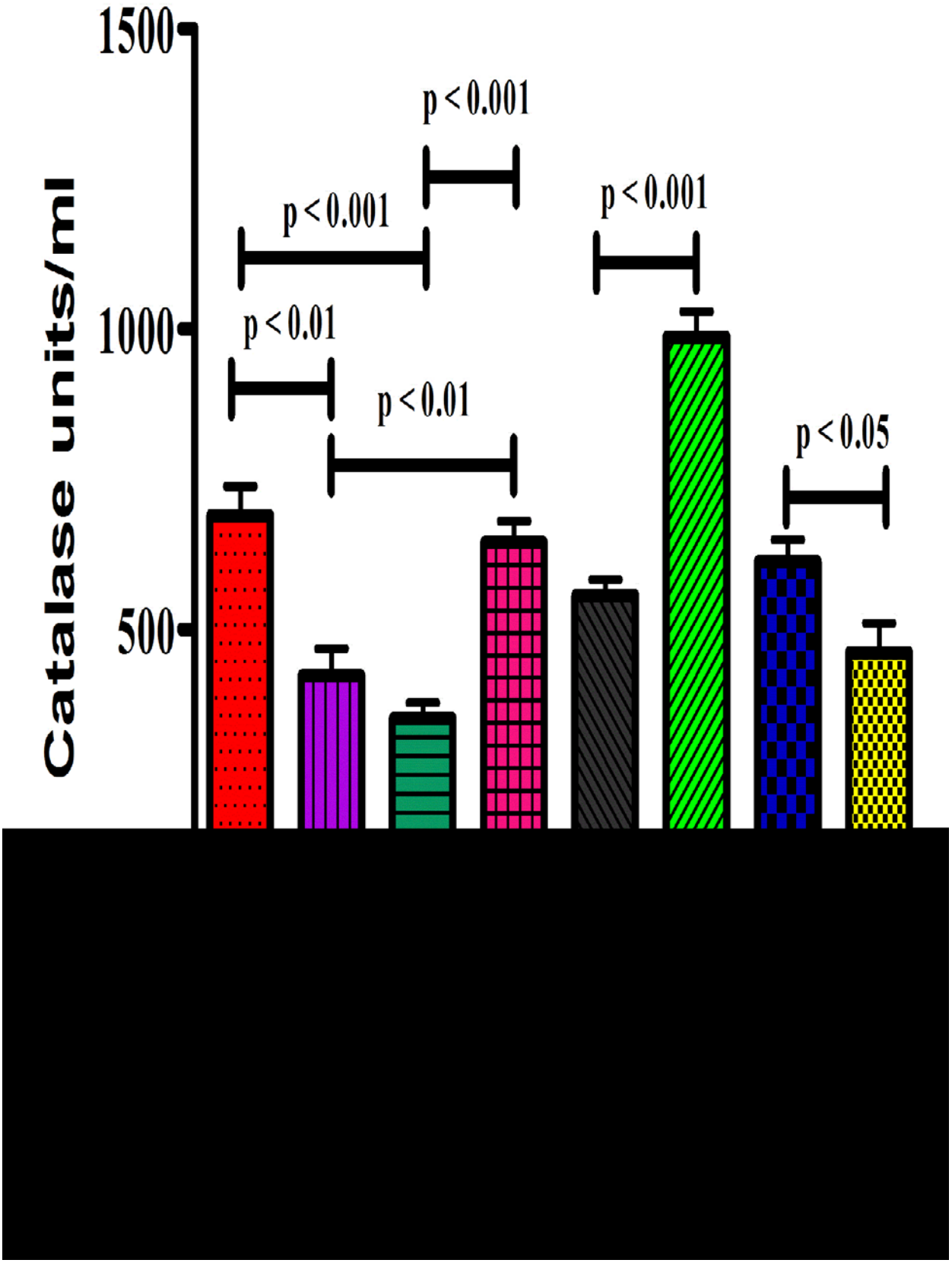


Table 8. Antioxidant Enzyme levels

	SHR (n=8)	Wistar (n=7)	SHR Weanling (n=8)	Wistar Weanling (n=8)	Wistar Saline (n=8)	Wistar Ang II (n=8)	SHR Saline (n=8)	SHR Ang II (n=7)
SOD (SOD units/ml)	69.3 ± 1.1*	61.5 ± 1.5*	49.6 ± 3.9*	51.0 ± 1.8	65.4 ± 4.2	54.5 ± 2.6 (17%⇩)	70.2 ± 4.9	63.4 ± 4.6 (10%⇩)
GPx (GPx Units/ml)	46975 ± 5242*	82567 ± 2537*	122839 ± 9225*	62189 ± 2476*	81922 ± 3163	87553 ± 3881 (7%⇧)	43858 ± 3447	180023 ± 3241* (310%⇧)
Catalase (Catalase units/ml)	689.4 ± 48.3*	423.0 ± 45.6*	352.2 ± 26.3*	644.3 ± 36.1*	556.3 ± 27.2	987.3 ± 42.6* (77%⇧)	612.8 ± 36.3	462.3 ± 47.8* (25%⇩)

Statistical Analysis Comparison Key:

* SHR :Statistically significant difference when compared to the Wistar group

* Wistar :Statistically significant difference when compared to the Wistar-Weanling group

*SHR-Weanling :Statistically significant difference when compared to the SHR group

* Wistar-Weanling :Statistically significant difference when compared to the SHR-Weanling group

*Wistar-Ang II :Statistically significant difference when compared to the Wistar-Saline infused group

*SHR-Ang II :Statistically significant difference when compared to the SHR-Saline infused group

N.B. Red % – below the Wistar and SHR Ang II group values, means % increase (⇧)/decrease(⇩) compared to the respective Saline infused control

4.8. PLASMA HYDROGEN PEROXIDE

Hydrogen peroxide (H_2O_2) levels were quantified in the plasma and the results are shown in Table 7 and represented in Figure 13.

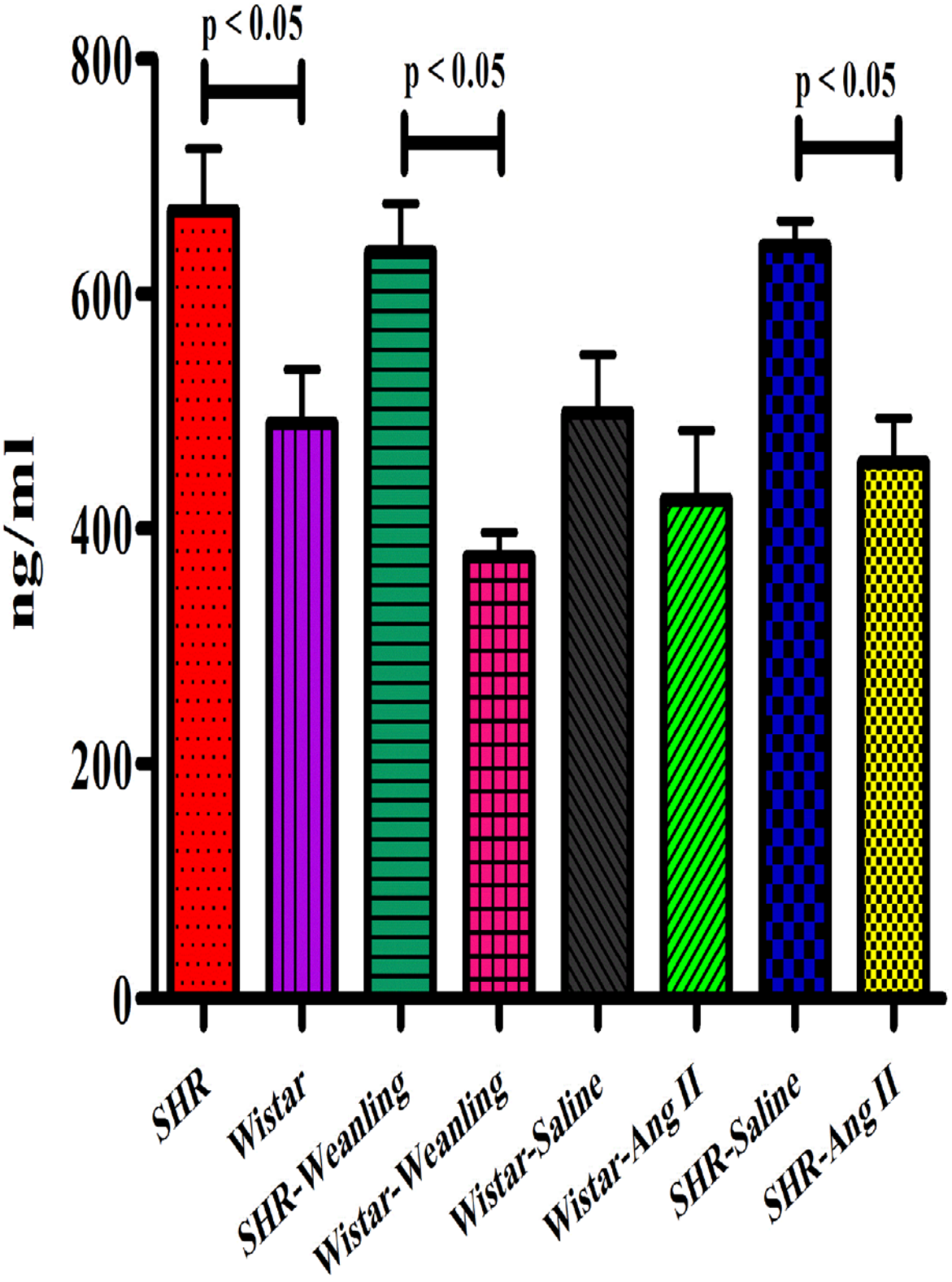
The results show that SHR group had a 1.4 fold higher plasma H_2O_2 level than the normotensive Wistar control (671.2 ± 52.7 vs 490.0 ± 46.0 ng/ml), and this higher plasma H_2O_2 level was statistically significant ($p>0.05$). The SHR-weanling had a 1.7 fold higher plasma H_2O_2 level than the Wistar-weanling (635.4 ± 41.5 vs 376.5 ± 20.6 ng/ml), and this difference in plasma H_2O_2 levels was also statistically significant ($p<0.05$). When the SHR-adult group was compared to the SHR-weanling and the Wistar-adult to the Wistar-weanling group, there was no significant differences in the plasma H_2O_2 levels of these groups (671.2 ± 52.7 vs 635.4 ± 41.5 ng/ml) and (490.0 ± 46.0 vs 376.5 ± 20.6 ng/ml) respectively.

The SHR-Ang II infused group had a 1.4 fold decrease in plasma H_2O_2 when compared to the saline infused control (456.5 ± 38.1 vs 640.8 ± 21.6 ng/ml), and this decrease in plasma H_2O_2 levels was statistically significant ($p<0.05$).

The Wistar-Ang II infused group showed a similar trend with a 1.2 fold decrease in plasma H_2O_2 levels (425.0 ± 58.6 vs 499.0 ± 49.6 ng/ml), however this decrease was not statistically significant ($p>0.05$).

The degree to which the infusion of the sub-pressor dose of Ang II decreased the plasma H_2O_2 levels in the SHR and Wistar was compared, and the results show that in the Wistar infused with Ang II, the plasma H_2O_2 levels were decreased by 15%, but the SHR infused with Ang II showed a greater degree of decrease, where the plasma H_2O_2 levels were decreased by 29%.

Figure 13: Plasma Hydrogen Peroxide



4.9. KIDNEY F_2-ISOPROSTANES

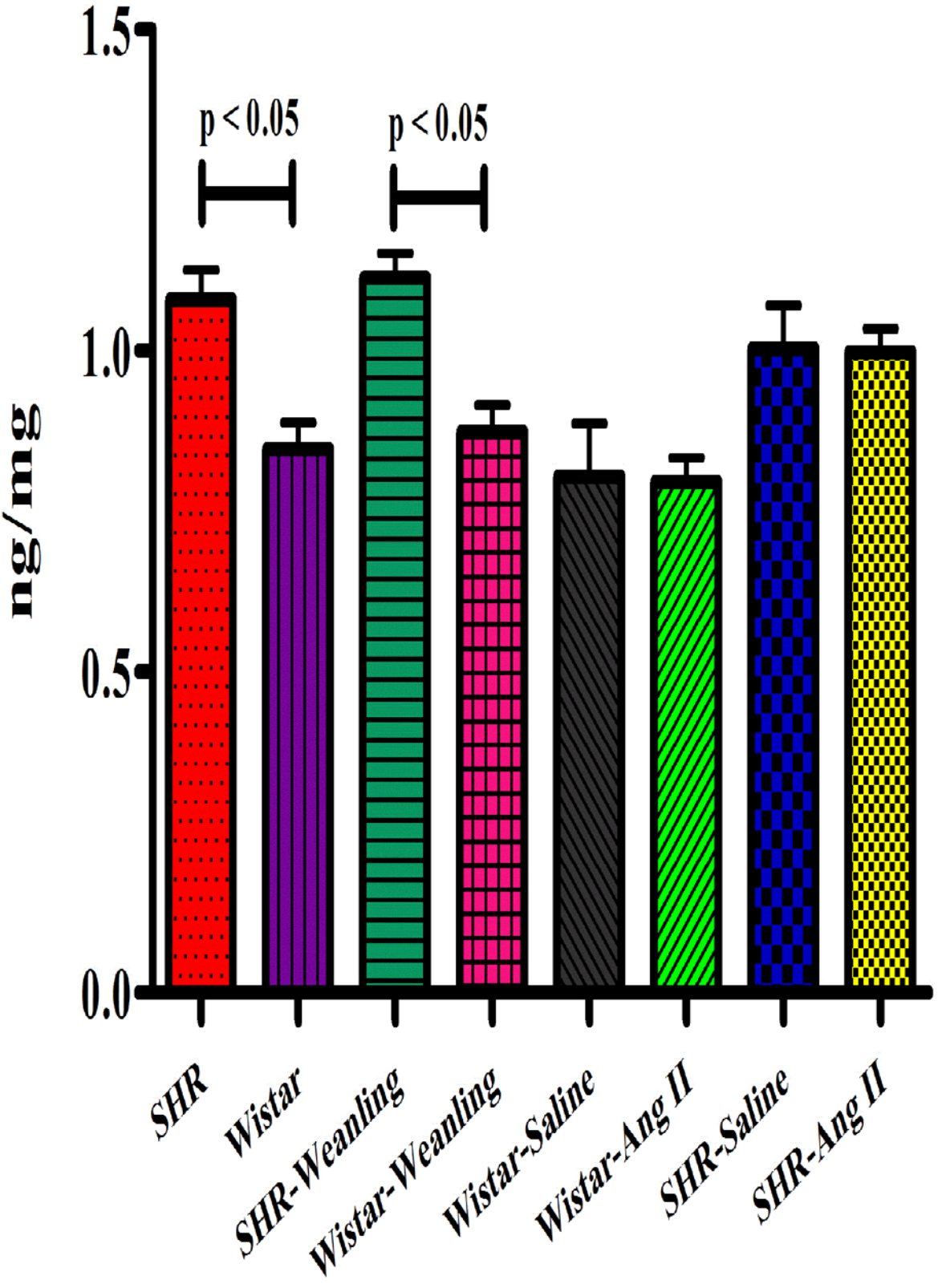
F_2-isoprostanes which is regarded as an indicator of *in vivo* lipid peroxidation was quantified in kidney tissue. The results are shown in Table 7 and represented in Figure 14.

The results show that SHR group had a 1.3 fold higher level of F_2-isoprostanes than the normotensive Wistar control (1.079 ± 0.047 vs 0.847 ± 0.041 ng/mg), and this increase was statistically significant ($p<0.05$). The SHR-weanling had a 1.3 fold higher F_2-isoprostanes level than the Wistar-weanling (1.114 ± 0.039 vs 0.873 ± 0.043 ng/mg), this difference was also statistically significant ($p>0.05$). When the SHR-adult group was compared to the SHR-weanling group and the Wistar-adult to the Wistar-weanling group, there was no significant change in the plasma F_2-isoprostane levels (1.079 ± 0.074 vs 1.114 ± 0.039 ng/mg) and (0.847 ± 0.041 vs 0.873 ± 0.043 ng/mg) respectively.

The SHR-Ang II infused group showed no change in F_2-isoprostane levels when compared to the saline infused control (0.998 ± 0.037 vs 1.003 ± 0.068 ng/mg).

The Wistar-Ang II infused group showed a similar trend with no change in F_2-isoprostane levels when compared to the saline infused control (0.796 ± 0.037 vs 0.805 ± 0.082 ng/mg).

Figure 14: Kidney - F_2-Isoprostanes



4.10. VASCULAR REMODELLING

4.10.1. Aorta Media Wall Thickness

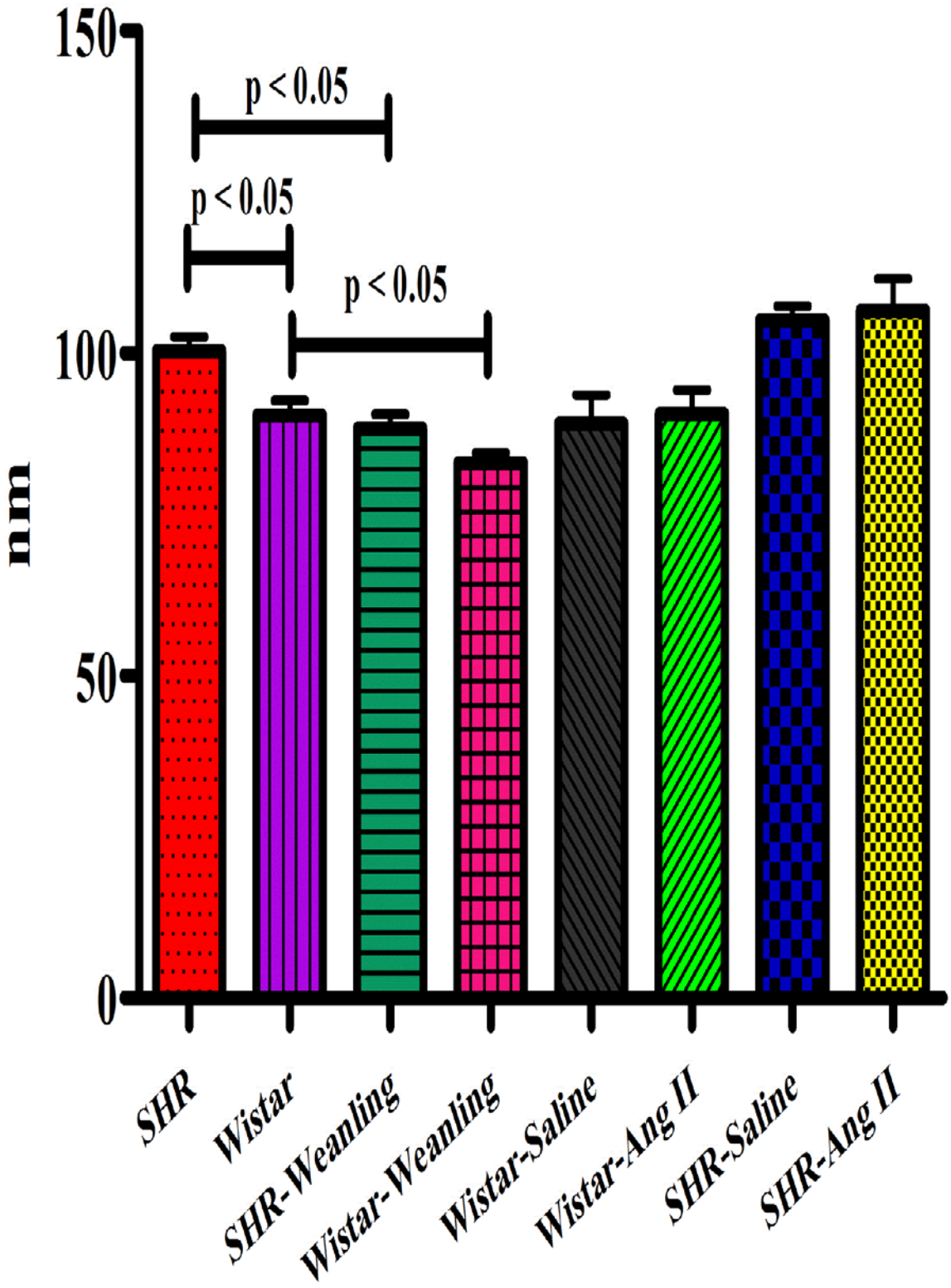
The media wall thickness was measured in the aorta and the results are shown in Table 9 and represented in Figure 15.

The results show that the SHR group had a 11.1% thicker tunica media than the normotensive Wistar control (100.3 ± 2.3 vs 90.3 ± 2.3 nm). This increase in thickness was statistically significant ($p<0.05$). The SHR-weanling showed a 6.5% thicker tunica media when compared to the Wistar-weanling (88.3 ± 2.3 vs 82.9 ± 1.7 nm), however this difference was not statistically significant ($p>0.05$). When the SHR-adult was compared to the SHR-weanling, the SHR- adult group showed a 13.6% increase in the tunica media thickness (100.3 ± 2.3 vs 88.3 ± 2.3 nm), and this increase was statistically significant ($p<0.05$). The Wistar-adult group showed a 8.9% increase in media wall thickness, when compared to the Wistar-weanling (90.3 ± 2.3 vs 82.9 ± 1.7 nm), and this difference was also statistically significant. ($p<0.05$)

The SHR-Ang II infused group, showed no significant difference in the tunica media thickness of the aorta (106.6 ± 5.0 vs 105.0 ± 2.3 nm).

The Wistar-Ang II infused group also showed no significant difference in the tunica media thickness when compared to the Wistar-saline infused group (90.5 ± 3.9 vs 89.0 ± 4.6 nm).

Figure 15: Aorta - Media Wall Thickness



4.10.2. Aorta Wall to Lumen Ratio

The wall to lumen ratio was assessed in the aorta and the results are shown in Table 9 and represented in Figure 16.

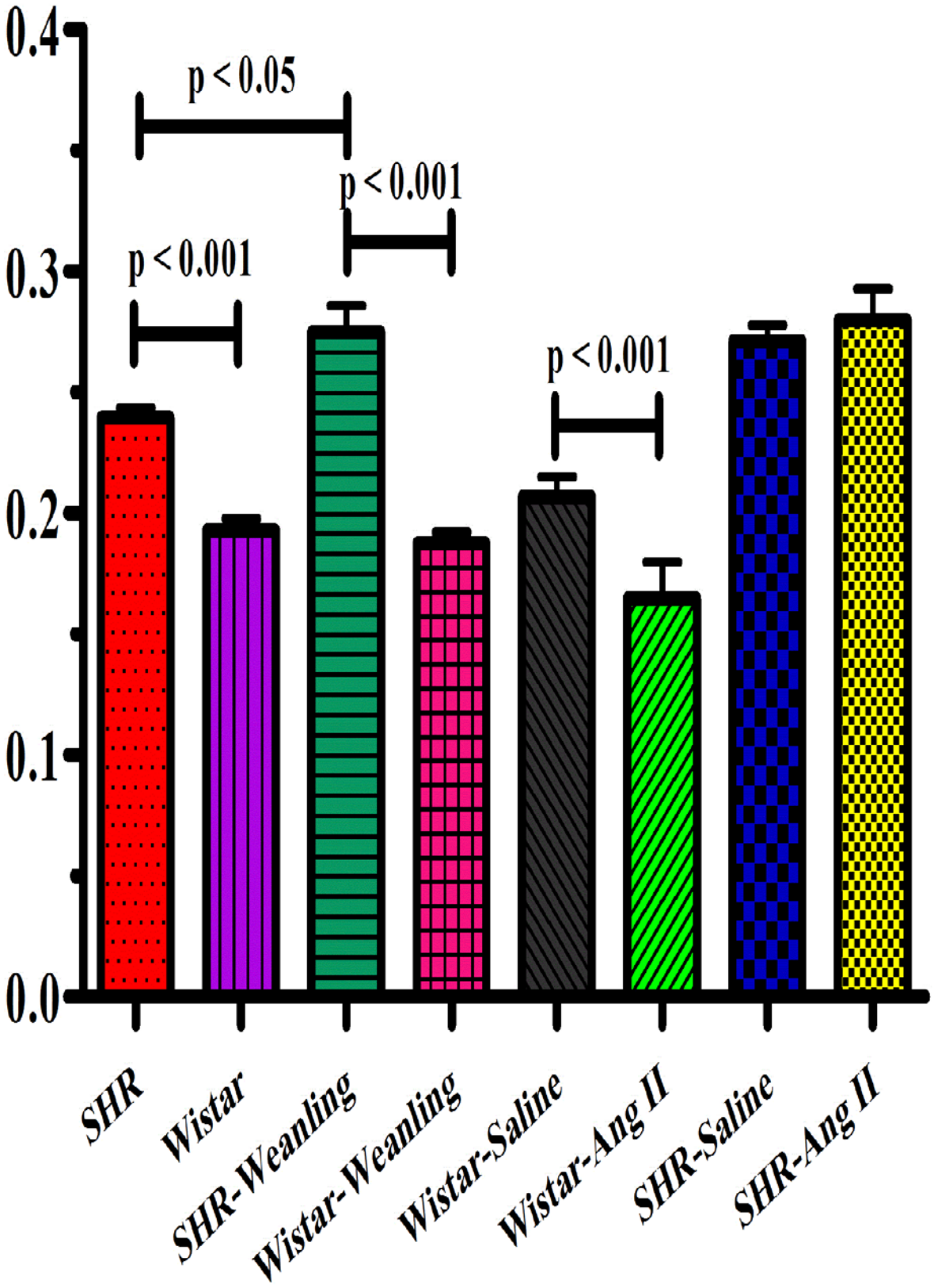
The results show that the SHR group had a 24.3% greater W/L ratio than the normotensive Wistar control group (0.2396 ± 0.004 vs 0.1927 ± 0.005), and this difference in W/L ratio was statistically significant ($p<0.05$). The SHR-weanling had 46.7% greater W/L ratio than the Wistar-weanling (0.2749 ± 0.011 vs 0.1874 ± 0.005), this difference in W/L ratio was also statistically significant ($p<0.05$). When the SHR-adult was compared to the SHR-weanling, the SHR-adult showed a 14.7% decrease in the W/L ratio, and this decrease was statistically significant (0.2396 ± 0.004 vs 0.2749 ± 0.011). The Wistar adult showed no significant change to the W/L ratio of the aorta when compared to the Wistar-weanling group (0.1927 ± 0.005 vs 0.1874 ± 0.005).

The SHR-Ang II infused group, showed no significant difference in the W/L ratio of the aorta when compared to the saline infused control (0.2797 ± 0.013 vs 0.2713 ± 0.007).

When the Wistar-Ang II infused group was compared to the Wistar-saline infused group, there was a 20.2% decrease in the W/L ratio (0.1649 ± 0.013 vs 0.2068 ± 0.008), and this decrease was statistically significant ($p>0.05$).

The degree of change caused by the infusion of Ang II with regards to the W/L ratio of the aorta in the SHR and Wistar was compared, and the results show that the sub-pressor dose of Ang II did not change the W/L ratio of the aorta in the SHR, but decreased the W/L ratio of the aorta by 20% in the Wistar.

Figure 16: Aorta - Wall to Lumen Ratio



4.10.3. Iliac Artery Media Wall Thickness

The media wall thickness was measured in the iliac artery and the results are shown in Table 9 and represented in Figure 17.

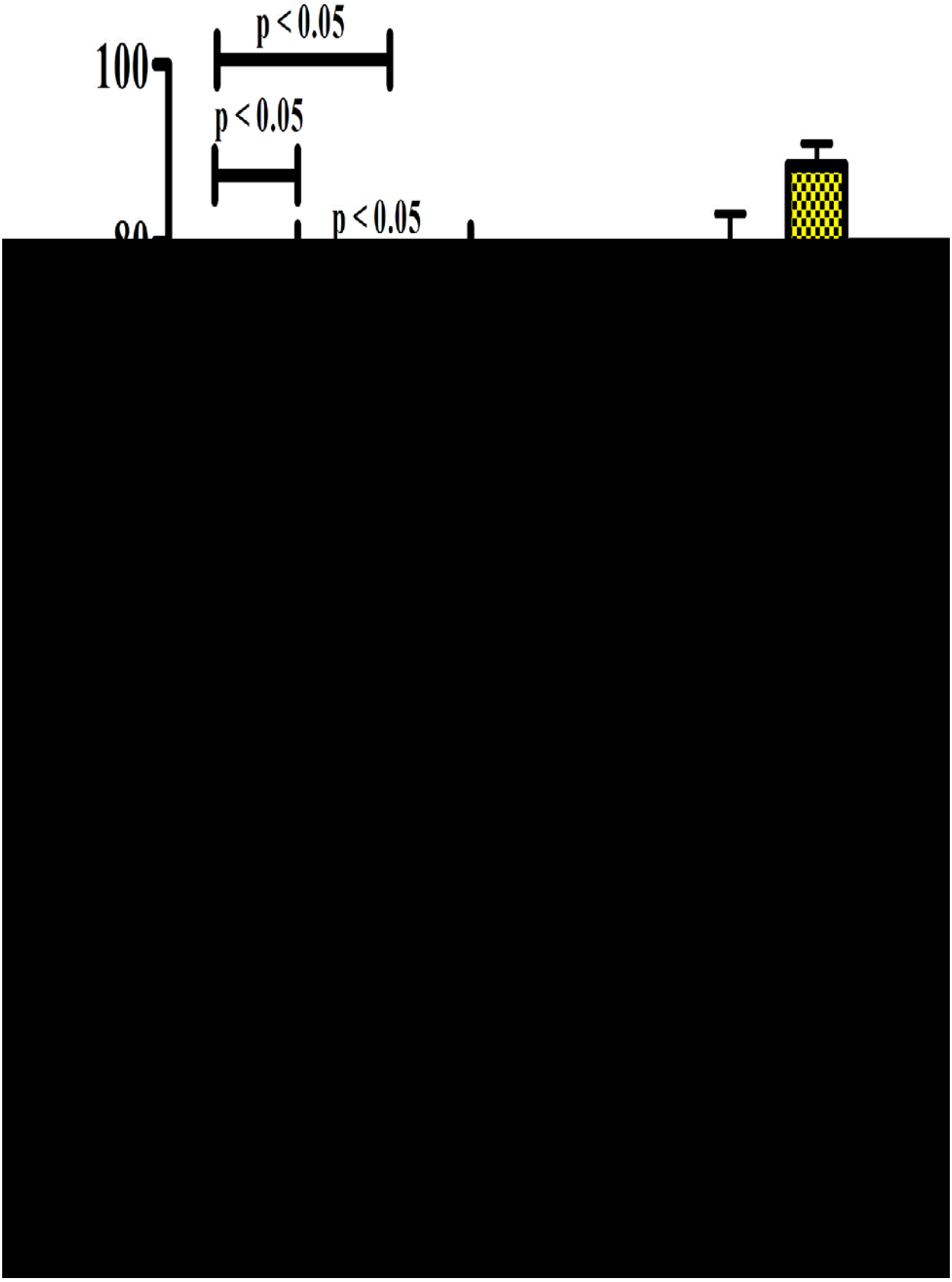
The results show that the SHR group had a 7.8% thicker tunica media than the normotensive Wistar control (77.8 ± 1.4 vs 72.2 ± 1.1 nm) and this greater thickness was statistically significant ($p<0.05$). The SHR-weanling showed a similar trend and had a 12.3% thicker tunica media than the Wistar-weanling (67.4 ± 4.4 vs 60.0 ± 2.8 nm), however this greater thickness was not statistically significant ($p>0.05$). When the SHR-adult was compared to the SHR-weanling, there was a 15.4% increase in the media wall thickness of the SHR-adult (77.8 ± 1.4 vs 67.4 ± 4.0 nm), and this increase was statistically significant ($p<0.05$). The Wistar-adult showed a 20.3% increase in wall thickness when compared to the Wistar-weanling and this difference was statistically significant (72.2 ± 1.1 vs 60.0 ± 2.8 nm) ($p<0.05$).

The SHR-Ang II infused group, showed an 8.8% increase in the tunica media thickness when compared to the SHR-saline infused group (86.6 ± 2.5 vs 79.3 ± 3.9 nm), however this increase was not statistically significant ($p>0.05$).

The Wistar-Ang II infused group showed no significant change in the tunica media thickness when compared to the Wistar-saline infused group (65.4 ± 3.2 vs 67.0 ± 1.6 nm).

The degree of change caused by the infusion of Ang II with regards to the media wall thickness of the iliac artery in the SHR and Wistar was compared, and the results show that the sub-pressor dose of Ang II increased the media wall thickness of the iliac artery in the SHR by 12%, but did not change the media wall thickness of the iliac artery in the Wistar.

Figure 17: Iliac Artery - Media Wall Thickness



4.10.4. Iliac Artery Wall to Lumen Ratio

The wall to lumen ratio was assessed in the iliac artery and the results are shown in Table 9 and represented in Figure 18.

The results show that the SHR group had a 7.6% greater W/L ratio than the normotensive Wistar control group (0.2685 ± 0.0142 vs 0.2496 ± 0.0145), however this difference in W/L ratio was not statistically significant ($p>0.05$). The SHR-weanling had a 21.6% greater W/L ratio than the Wistar-weanling (0.2984 ± 0.0159 vs 0.2453 ± 0.0100), however this difference was also not statistically significant ($p>0.05$). When the SHR-adult was compared to the SHR-weanling, the SHR-adult had a 10.0% decrease in W/L ratio (0.2685 ± 0.0142 vs 0.2984 ± 0.159), this decrease was however also not statistically significant ($p>0.05$). When the Wistar adult was compared to the Wistar-weanling group there was no significant change in the W/L ratio of the iliac artery (0.2496 ± 0.0145 vs 0.2453 ± 0.100).

The SHR-Ang II infused group, showed a 21% increase in W/L ratio when compared to the SHR-saline infused group (0.3194 ± 0.0153 vs 0.2641 ± 0.0137), and this increase was statistically significant ($p<0.05$).

The Wistar-Ang II infused group was compared to the Wistar-saline infused group, and there was an opposite trend, with a 10% decrease in the W/L ratio (0.2191 ± 0.0122 vs 0.2436 ± 0.0135), however this decrease was not statistically significant ($p>0.05$).

The degree of change caused by the infusion of Ang II with regards to the W/L ratio of the iliac artery in the SHR and Wistar was compared, and the results show that the sub-pressor dose of Ang II increased the W/L ratio of the aorta in the SHR by 21%, but decreased the W/L ratio of the iliac artery by 10% in the Wistar.

Figure 18: Iliac Artery - Wall to Lumen Ratio

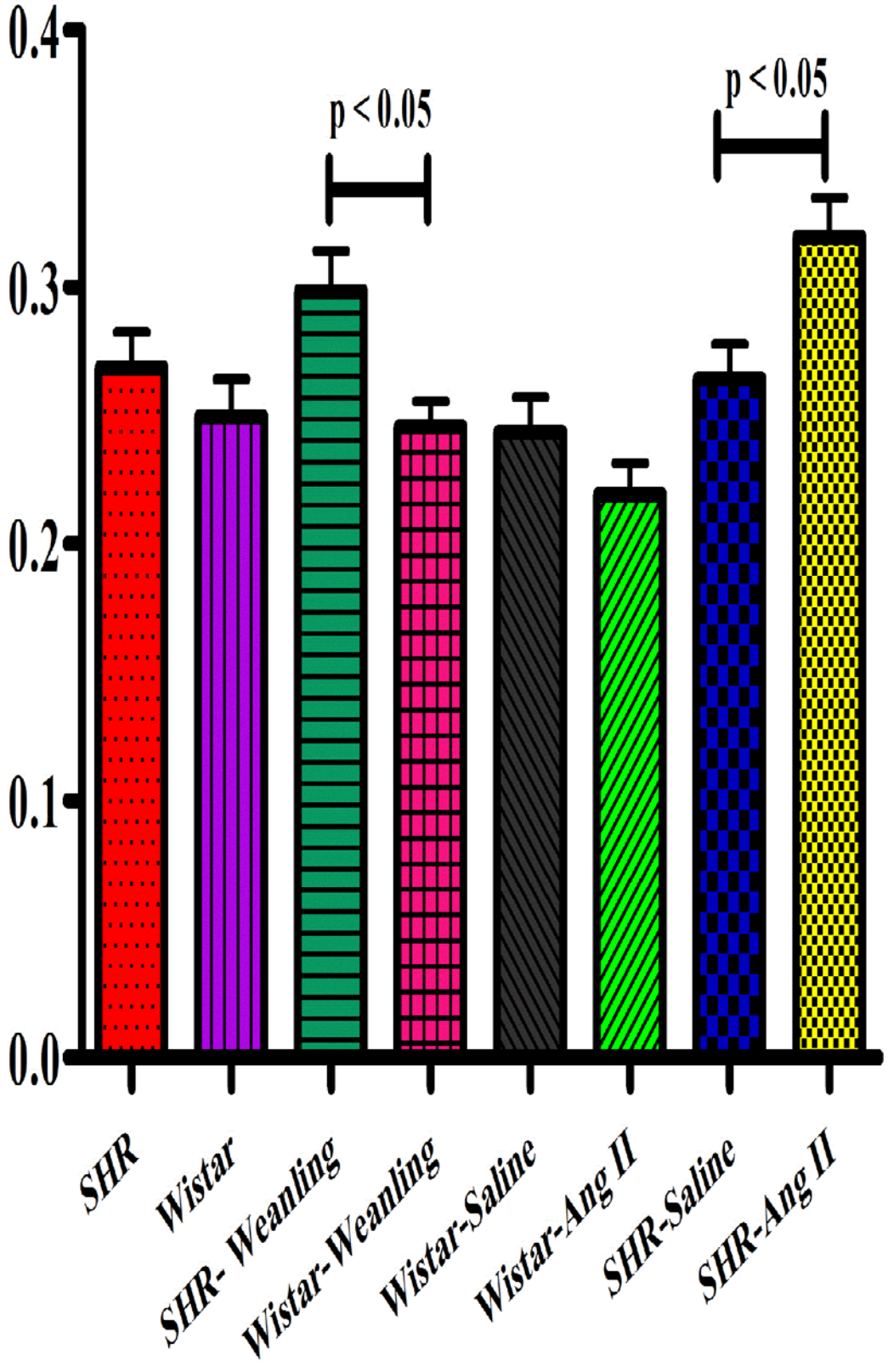


Table 9. Morphometric measurements of the Aorta and Iliac Artery

	SHR (n=8)	Wistar (n=7)	SHR Weanling (n=8)	Wistar Weanling (n=8)	Wistar Saline (n=8)	Wistar Ang II (n=8)	SHR Saline (n=8)	SHR Ang II (n=7)
Aorta: Tunica Media Thickness (nm)	100.3 ± 2.3*	90.3 ± 2.3*	88.3 ± 2.3*	82.9 ± 1.7	89.0 ± 4.6	90.5 ± 3.9 (0%)	105.0 ± 2.3	106.6 ± 5.0 (0%)
Aorta: Wall to Lumen Ratio	0.2396 ± 0.004*	0.1927 ± 0.005	0.2749 ± 0.011*	0.1874 ± 0.005*	0.2068 ± 0.008	0.1649 ± 0.015* (20%⇩)	0.2713 ± 0.007	0.2797 ± 0.013 (0%)
Aorta: Total Cross Sectional Area (nm^2)	166200 ± 17144	148374 ± 11050	94131 ± 4175	132328 ± 2617	141420 ± 14335	152216 ± 9311 (8%⇧)	162826 ± 10517	150876 ± 6846* (7%⇩)
Iliac Artery: Tunica Media Thickness (nm)	77.8 ± 1.4*	72.2 ± 1.1*	67.4 ± 4.0*	60.0 ± 2.8	67.0 ± 1.6	65.4 ± 3.2 (0%)	79.3 ± 3.9	88.6 ± 2.5 (12%⇧)
Iliac Artery: Wall to Lumen Ratio	0.2685 ± 0.0142	0.2496 ± 0.0145	0.2984 ± 0.0159	0.2453 ± 0.0100*	0.2436 ± 0.0135	0.2191 ± 0.0122 (10%⇩)	0.2641 ± 0.0137	0.3194 ± 0.0153* (21%⇧)
Iliac Artery: Total Cross Sectional Area (nm^2)	67596 ± 3862	58770 ± 4205	54557 ± 3655	42886 ± 3864	60263 ± 4886	66344 ± 5095 (10%⇧)	68296 ± 6693	77199 ± 3421 (13%⇧)

Statistical Analysis Comparison Key:

* SHR :Statistically significant difference when compared to the Wistar group

* Wistar :Statistically significant difference when compared to the Wistar-Weanling group

*SHR-Weanling :Statistically significant difference when compared to the SHR group

* Wistar-Weanling :Statistically significant difference when compared to the SHR-Weanling group

*Wistar-Ang II :Statistically significant difference when compared to the Wistar-Saline infused group

*SHR-Ang II :Statistically significant difference when compared to the SHR-Saline infused group

N.B. Red % – below the Wistar and SHR Ang II group values, means % increase (⇧)/decrease(⇩) compared to the respective Saline infused control

4.11. GENE EXPRESSION

4.11.1. p22phox mRNA Expression

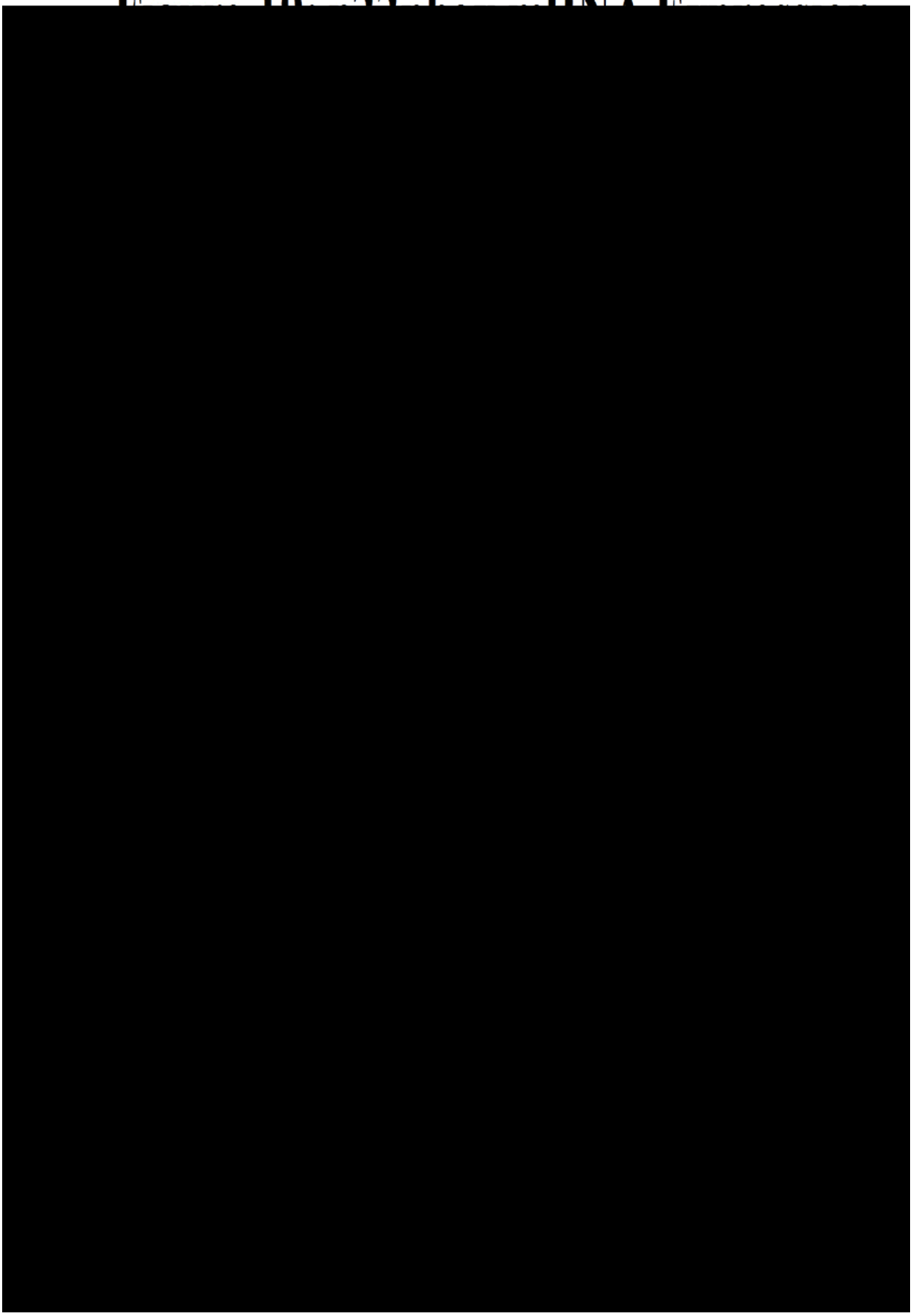
Real-time PCR was used to quantify the p22phox mRNA in aorta tissue for all groups. p22phox gene expression data for each of the groups mRNA was normalized with GAPDH, by expressing data as a ratio of p22phox to GAPDH mRNA expression. The results are shown in Table 10 and represented in Figure 19.

The SHR group showed a 3.24 fold lower p22phox mRNA expression when compared to the normotensive Wistar control (1.91 ± 0.28 vs 6.18 ± 0.58), and this difference was statistically significant ($p>0.05$). The SHR-adult showed a 4.73 fold decrease in p22phox expression when compared to the SHR-weanling (1.91 ± 0.28 vs 9.03 ± 0.58), and this decrease was also statistically significant ($p<0.05$). The SHR-weanling showed a 3.24 fold higher p22phox expression when compared to the Wistar-weanling group (9.03 ± 0.58 vs 2.79 ± 0.78), and this difference in p22phox expression was statistically significant ($p<0.05$). The Wistar-adult showed a 2.22 fold increase in p22phox mRNA expression when compared to the Wistar-weanling (6.18 ± 0.58 vs 2.79 ± 0.78), and this increase was also statistically significant. ($p<0.05$)

The SHR-Ang II infused group showed a 2.92 fold increase in p22phox expression when compared to the saline infused control (6.22 ± 0.90 vs 1.42 ± 0.10), and this increase was statistically significant ($p>0.05$).

The Wistar-Ang II infused group showed a similar trend with a 2.27 fold increase in p22phox expression when compared to its saline infused control (14.61 ± 1.25 vs 5.75 ± 1.13), and this increase in expression was statistically significant ($p<0.05$).

The degree to which the infusion of the sub-pressor dose of Ang II increased the p22phox expression in the SHR and Wistar was compared, and the results show that in the Wistar infused with Ang II, the p22phox expression levels were increased by 154%, but the SHR infused with Ang II showed a greater degree of increase, where the p22phox expression levels were increased by 338%.



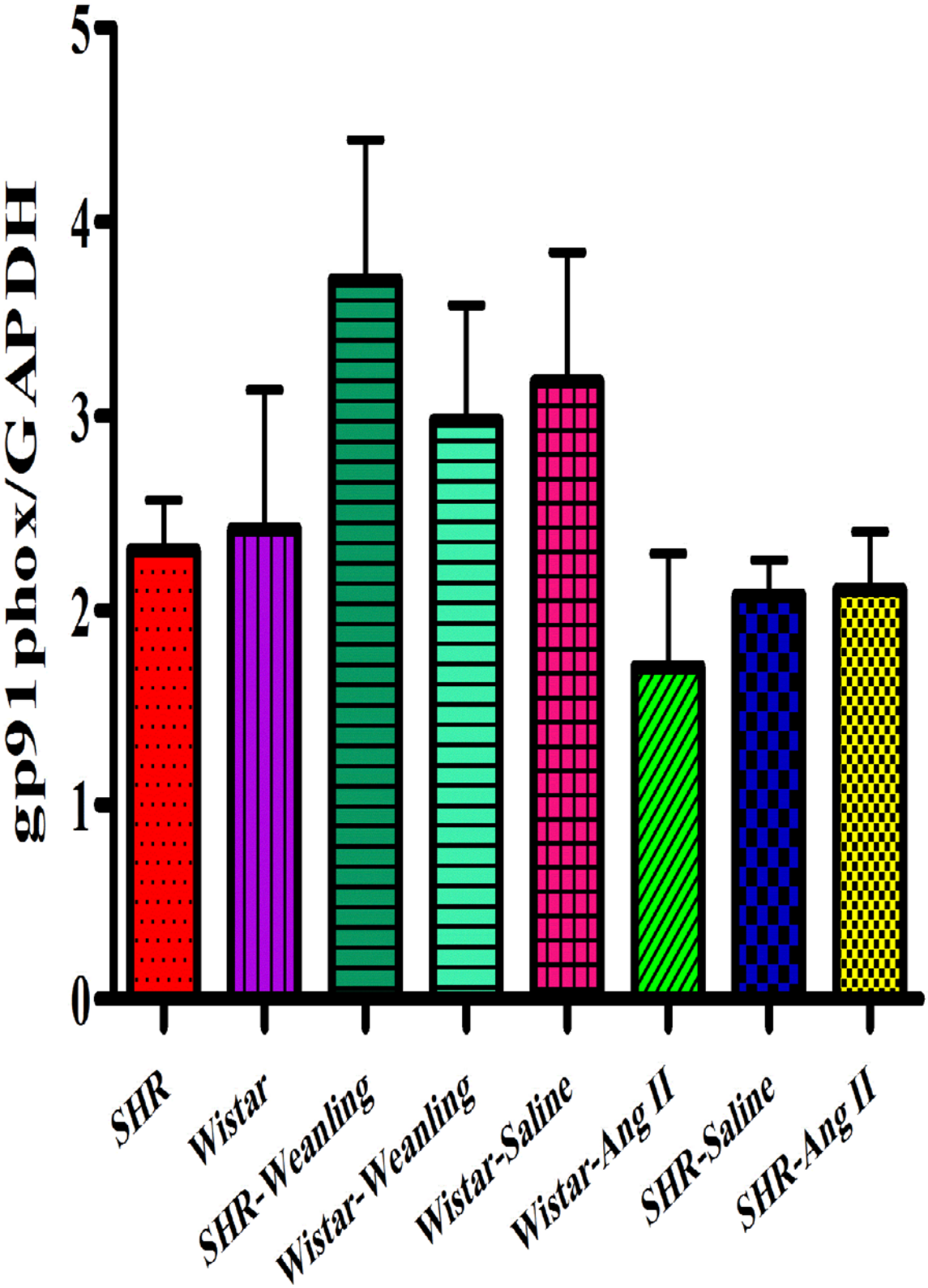
4.11.2. gp91phox mRNA Expression

Real-time PCR was used to quantify gp91phox mRNA in aorta tissue for all groups. gp91phox gene expression data for each of the groups mRNA was normalized with GAPDH, by expressing data as a ratio of gp91phox to GAPDH mRNA expression. The results are shown in Table 10 and represented in Figure 20.

The SHR group showed no difference in gp91phox mRNA expression when compared to the normotensive Wistar control (2.31 ± 0.26 vs 2.42 ± 0.72). This trend was repeated when the SHR-weanling was compared to the Wistar-weanling groups (3.70 ± 0.72 vs 2.98 ± 0.60). The SHR adult showed a 1.60 fold decrease in gp91phox expression when compared to the SHR-weanling (2.31 ± 0.26 vs 3.70 ± 0.72), however this decrease in expression was not statistically significant ($p>0.05$). The Wistar adult showed no significant difference in the expression of gp91phox when compared to the Wistar-weanling group (2.42 ± 0.72 vs 2.98 ± 0.60).

The SHR-Ang II infused group showed no difference in gp91phox expression when compared to the saline infused control (2.11 ± 0.30 vs 2.08 ± 0.19). The Wistar-Ang II infused group however showed a 1.86 fold decrease in gp91phox expression when compared to its saline infused control (1.71 ± 0.58 vs 3.18 ± 0.67), however this decrease in expression was not statistically significant ($p>0.05$).

Figure 20: gp91phox mRNA Expression



4.11.3. Cu/Zn SOD mRNA Expression

Real-time PCR was used to quantify Cu/Zn-SOD mRNA in aorta tissue for all groups. Cu/Zn-SOD gene expression data for each of the groups mRNA was normalized with GAPDH, by expressing data as a ratio of Cu/Zn-SOD to GAPDH mRNA expression. The results are shown in Table 10 and represented in Figure 21.

The SHR group showed no significant difference in Cu/Zn-SOD mRNA expression when compared to the normotensive Wistar control (2.93 ± 0.67 vs 2.38 ± 0.36). When the SHR-weanling was compared to the Wistar-weanling the SHR-weanling showed a 6.60 fold higher Cu/Zn-SOD mRNA expression (45.04 ± 6.77 vs 6.82 ± 0.82), this difference in expression was statistically significant ($p<0.001$).The SHR-adult showed a 15.37 fold decrease in Cu/Zn-SOD expression when compared to the SHR-weanling (2.93 ± 0.67 vs 45.04 ± 6.77), and this difference in expression was statistically significant ($p<0.001$). The Wistar-adult showed a similar trend when compared to the Wistar-weanling group, with a 2.87 fold decrease in Cu/Zn-SOD expression (2.38 ± 0.36 vs 6.82 ± 0.82), and this decrease was also statistically significant. ($p<0.05$)

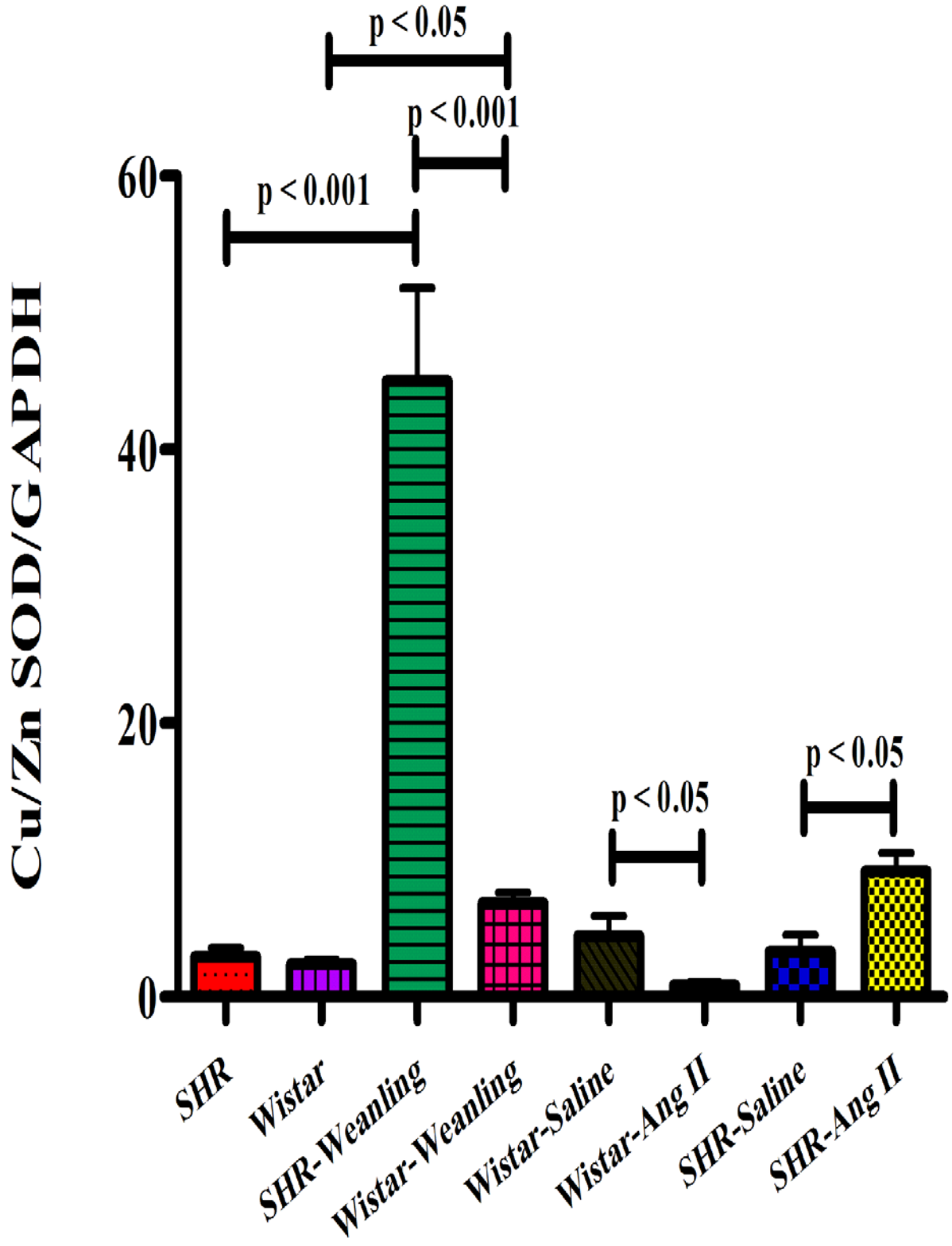
The SHR-Ang II infused group showed a 2.80 fold increase in Cu/Zn-SOD expression when compared to the saline infused control (9.12 ± 1.33 vs 3.26 ± 1.27), and this increase in expression was statistically significant ($p<0.05$).

The Wistar-Ang II infused group showed an opposite trend with a 5.35 fold decrease in Cu/Zn-SOD expression when compared to its saline infused control (0.83 ± 0.25 vs 4.44 ± 1.49), and this difference in expression was also statistically significant ($p<0.05$).

The degree of change caused by the infusion of Ang II with regards to the Cu/Zn-SOD expression in the SHR and Wistar was compared, and the results show that the sub-pressor

dose of Ang II increased Cu/Zn-SOD expression in the SHR by 180%, but decreased the Cu/Zn-SOD expression by 81% in the Wistar.

Figure 21: Cu/Zn SOD Expression



4.11.4. AT-1a mRNA Expression

Real-time PCR was used to quantify AT-1a mRNA in aorta tissue for all groups. AT-1a gene expression data for each of the groups mRNA was normalized with GAPDH, by expressing data as a ratio of AT-1a to GAPDH mRNA expression. The results are shown in Table 10 and represented in Figure 22.

The SHR group showed no significant difference in AT-1a mRNA expression when compared to the normotensive Wistar control (1.28 ± 0.14 vs 1.61 ± 0.29). When the SHR-weanling was compared to the Wistar-weanling, the SHR-weanling showed a 4.42 fold higher AT-1a mRNA expression (15.15 ± 1.78 vs 3.36 ± 0.84), and this difference in AT-1a expression was statistically significant ($p<0.001$). The SHR-adult showed a 11.8 fold decrease in AT-1a mRNA expression when compared to the SHR-weanling (1.28 ± 0.14 vs 15.15 ± 1.78), and this difference in expression was statistically significant ($p<0.001$). The Wistar-adult showed a 2.08 fold decrease in AT-1a mRNA expression when compared to the Wistar-weanling group, and this difference in expression was also statistically significant (1.61 ± 0.29 vs 3.36 ± 0.84) ($p<0.05$).

The SHR-Ang II infused group showed a 3.98 fold increase in AT-1a expression when compared to the saline infused control (4.97 ± 0.84 vs 1.25 ± 0.54), and this increase in AT-1a mRNA expression was statistically significant ($p<0.05$).

The Wistar-Ang II infused group showed a similar trend with a 2.26 fold increase in AT-1a expression when compared to its saline infused control (9.77 ± 0.52 vs 4.33 ± 2.89), and this difference in expression was also statistically significant ($p<0.05$).

The degree to which the infusion of the sub-pressor dose of Ang II increased the AT-1a expression in the SHR and Wistar was compared, and the results show that in the Wistar

infused with Ang II, the AT-1a mRNA expression levels were increased by 126%, but the SHR infused with Ang II showed a greater degree of increase, where the AT-1a mRNA expression levels were increased by 298%.

Figure 22: AT-1a Expression

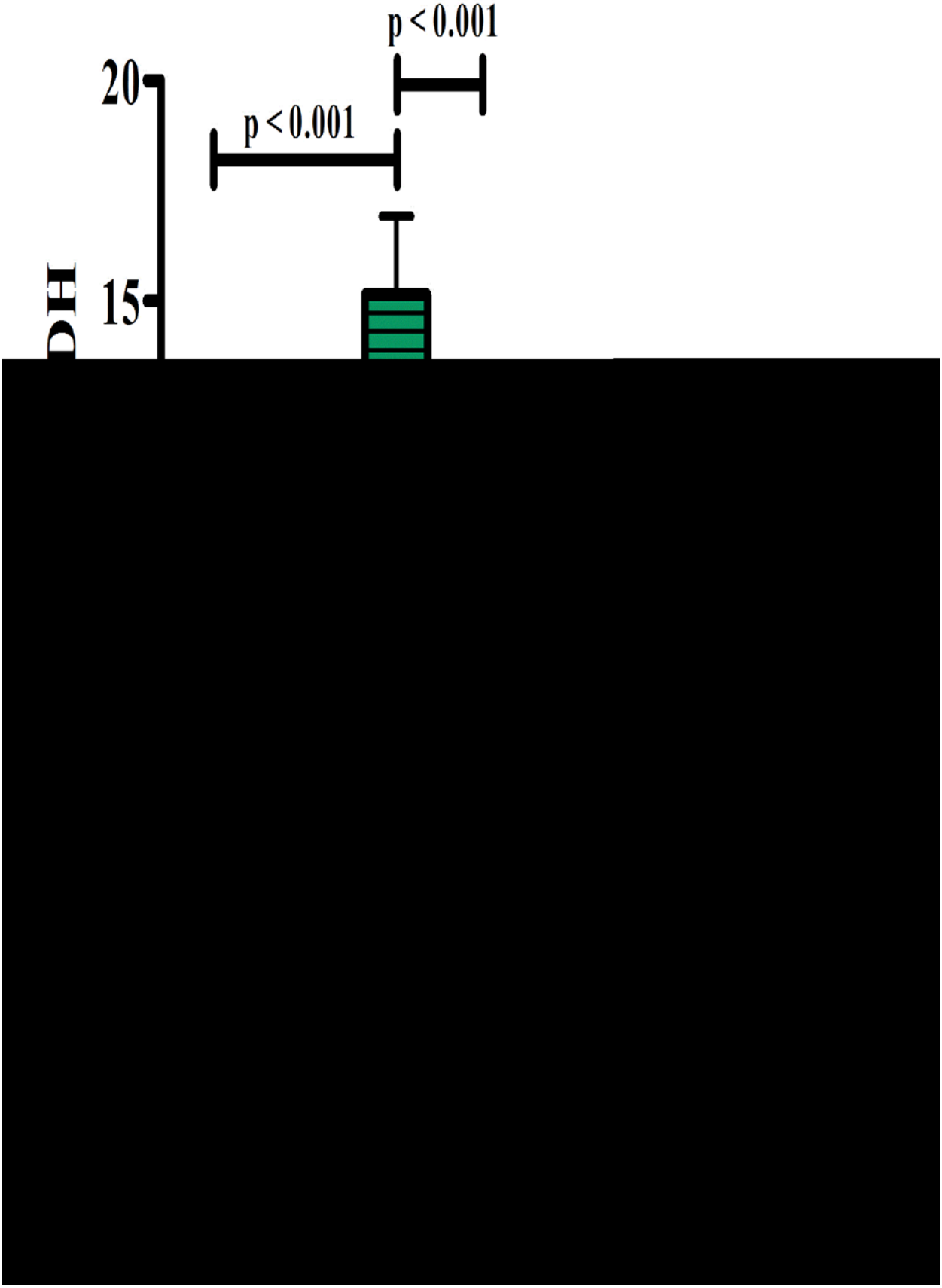


Table 10: Aorta mRNA Expression

	SHR (n=8)	Wistar (n=7)	SHR Weanling (n=8)	Wistar Weanling (n=8)	Wistar Saline (n=8)	Wistar Ang II (n=8)	SHR Saline (n=8)	SHR Ang II (n=7)
AT-1a mRNA Expression	1.28 ± 0.14	1.61 ± 0.29*	15.15 ± 1.78*	3.36 ± 0.84*	4.33 ± 2.89	9.77 ± 0.52* (126%⇧)	1.25 ± 0.54	4.97 ± 0.84* (298%⇧)
Cu/Zn SOD mRNA Expression	2.93 ± 0.67	2.38 ± 0.36*	45.04 ± 6.77*	6.82 ± 0.82*	4.44 ± 1.49	0.83 ± 0.25* (81%⇩)	3.26 ± 1.27	9.12 ± 1.33* (180%⇧)
gp91phox mRNA Expression	2.31 ± 0.26	2.42 ± 0.72	3.70 ± 0.72	2.98 ± 0.60	3.18 ± 0.67	1.71 ± 0.58 (46%⇩)	2.08 ± 0.19	2.11 ± 0.30 (0%)
p22phox mRNA Expression	1.91 ± 0.28*	6.18 ± 0.58*	9.03 ± 0.58*	2.79 ± 0.78*	5.75 ± 1.13	14.61 ± 1.25* (154%⇧)	1.42 ± 0.10	6.22 ± 0.90* (338%⇧)

Statistical Analysis Comparison Key:

* SHR :Statistically significant difference when compared to the Wistar group

* Wistar :Statistically significant difference when compared to the Wistar-Weanling group

*SHR-Weanling :Statistically significant difference when compared to the SHR group

* Wistar-Weanling :Statistically significant difference when compared to the SHR-Weanling group

*Wistar-Ang II :Statistically significant difference when compared to the Wistar-Saline infused group

*SHR-Ang II :Statistically significant difference when compared to the SHR-Saline infused group

- Red % – below the Wistar and SHR Ang II group values, means % increase (⇧)/decrease(⇩) compared to the respective Saline infused control
- Expression ratios are a ratio of the gene sequence of interest, and the housekeeping gene (GAPDH)

5. DISCUSSION

This study has shown that the SHR, a well-established model of essential hypertension, demonstrates an evident link between Ang II and hypertension. Ang II has been shown to act as a causative factor in the pathogenesis of hypertension not only by its well established pressor effects, but also by its influence on the antioxidant and free radical systems via NADPH oxidase, resulting in a state of elevated oxidative stress in the model. This recently established mechanism although independent of other blood pressure elevating mechanisms in this model, works in parallel with them, resulting in the vicious cycle that is evident in hypertension.

This has been demonstrated by the following pathophysiological changes in the SHR and the other experimental models used in this study (viz. the SHR - Ang II infused group and the Wistar - Ang II infused group):

1. The SHR has an elevated circulating plasma Ang II level, and this increased level of Ang II plays a major role in elevating the blood pressure of the model via its previously reported pressor effects.

2. The SHR has a compromised antioxidant status (on the basis of the levels of the major antioxidant enzymes viz. SOD, GPx, and catalase), as well as an increased level of free radical production. This results in the model being in a state of high level oxidative stress. This high level oxidative stress appears to be intimately linked with the hypertension in this model.

3. A sub-pressor dose of Ang II administered to a normotensive rat model (i.e. the Wistar - Ang II infused group) over a four week period, elevated its blood pressure and also increased the activity of vascular NADPH oxidase. This increases the free

radical production in the vasculature and subsequently results in an overwhelmed antioxidant system. This results in the experimental model being in a state of high level oxidative stress. It is thus evident that there is a strong link between the increased oxidative stress and the elevation in blood pressure, with the Ang II being the causative factor in this link.

4. A sub-pressor dose of Ang II administered to the SHR (i.e. the SHR - Ang II infused group) over a three week period, exacerbated the onset and level of the hypertension in this model and also increased the activity of vascular NADPH oxidase. Interestingly this infused model makes adjustments to the antioxidant system in response to an increase in NADPH oxidase activity that is not mirrored by the normotensive Ang II infused model. This demonstrates that in the SHR which is genetically predisposed to hypertension, the antioxidant system reacts differently to an added stressor (i.e. Ang II), than in the normotensive Wistar model.

5. All experimental groups (i.e. the SHR, the SHR - Ang II infused group and the Wistar - Ang II infused group) showed changes to the vasculature, i.e. vascular remodelling, and these changes contribute to an increased peripheral resistance and/or decreased vascular compliance, and thus acts to elevate blood pressure. It is important to note that the vascular remodelling appears to precede the elevation in blood pressure in the SHR, and plays an important initiator and amplifier role in hypertension. It can thus be said to be a parallel blood pressure elevating mechanism, working in concert with the other established mechanisms to initiate, exacerbate and maintain the hypertension.

6. The SHR showed an elevated level of kidney F_2-isoprostanes, which is an *in vivo* marker for lipid peroxidation as well as a potent renal vasoconstrictor, thus these

elevated levels demonstrate an elevated level of tissue oxidative stress. The renal damage due to tissue oxidative stress has been reported to result in a reduction in the natriuretic and diuretic properties of the kidney which acts to decrease blood pressure, thus contributing to the elevation in blood pressure in this model. Furthermore the elevated F_2-isoprostanes would result in an increase in renal vasoconstriction and this would further decrease the natriuretic and diuretic properties of the kidney, and thus elevate blood pressure

Thus from the results it has been established that the SHR, has both a compromised antioxidant status and elevated free radical levels, resulting in the model being exposed to high level oxidative stress. Ang II has been shown to be a causative factor in this model via mechanisms other than that of its established pressor role, and these mechanisms are intimately linked with the antioxidant and free radical systems (and the consequent oxidative stress). This study has thus shown that these pathophysiological changes are linked to the hypertension that the SHR is predisposed to, as well as in the SHR – Ang II infused and the Wistar – Ang II infused groups, and that these parallel mechanisms will work in concert, leading to the vicious cycle that is evident in hypertension. These are the mechanisms that will be the focus of the discussion that follows.

N.B. The table and flow diagram that follows is a summary of the link between Ang II and oxidative stress and their role in hypertension.

Table 11: Effect of Angiotensin II on Oxidative Stress related parameters in the Experimental groups

Group/ Parameter	SHR	SHR Ang II Infused	Wistar Ang II Infused
SOD	⇧	⇩ (10%)	⇩(17%)
CuZn SOD (Aorta)	-	⇧(180%)	⇩(81%)
GPx	⇩	⇧(7%)	⇧(310%)
Catalase	⇧	⇧(77%)	⇩(25%)
p22phox (Aorta)	⇩	⇧(338%)	⇧(154%)

All values are shown as increase (⇧), decrease (⇩) or no change (-) when compared to their respective controls.

The magnitude of these changes are shown within brackets.

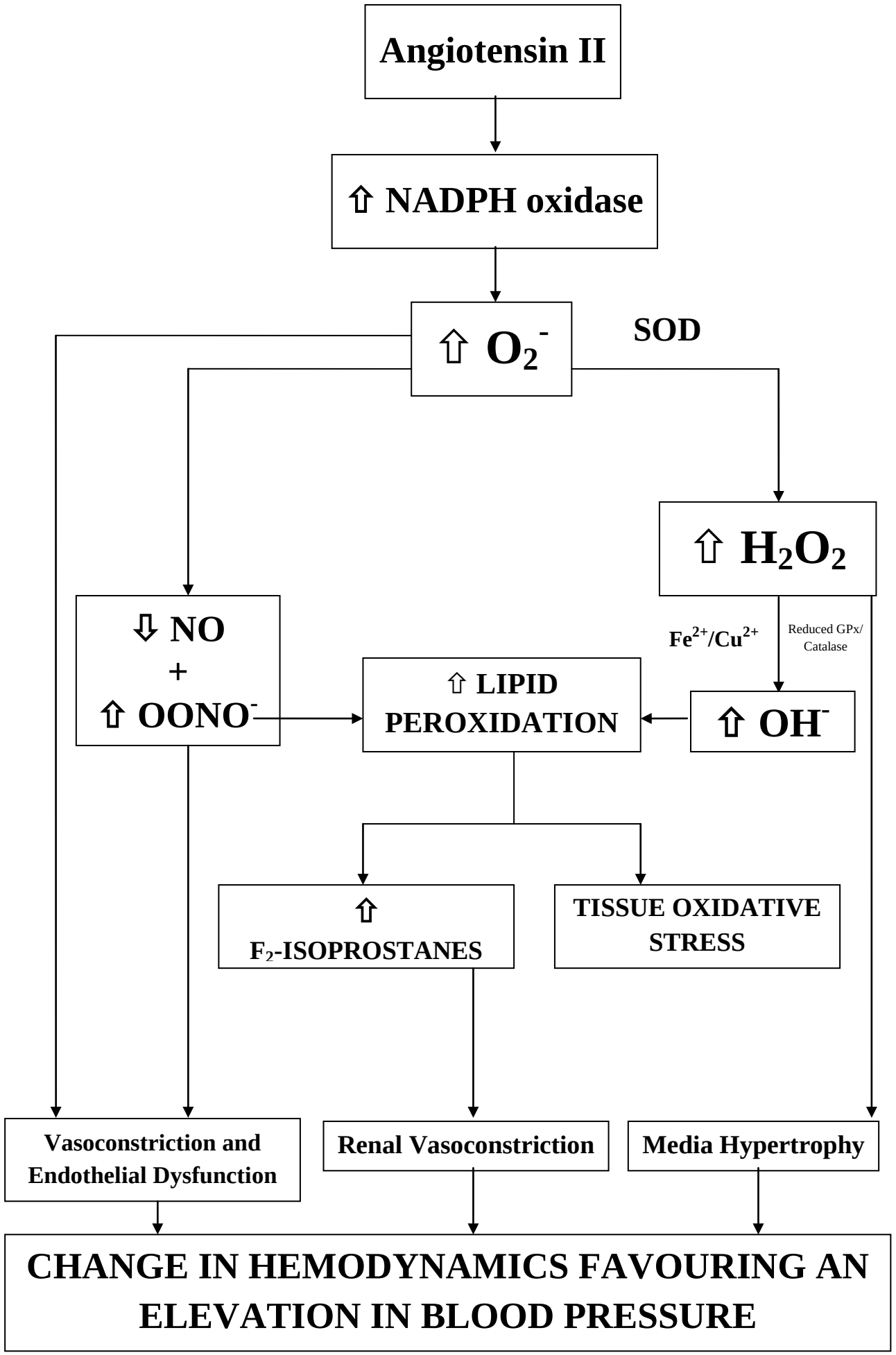


Fig 23. Summary of the link between Ang II and oxidative stress and its role in elevating blood pressure

5.1. BLOOD PRESSURE AND HEART RATE

The SHR had a significantly higher systolic and diastolic blood pressure over the four successive weeks of the study when compared to the normotensive Wistar control. The SHR showed a steady increase in SBP from the first week till the end of study at four weeks. If one adopts the hypertension classification system provided by the WHO in the 2003 report as a means of comparison to evaluate the "extent" of the hypertension, then the SHR progresses from Grade 1 to the highest Grade 3, by the end of the final week of the study (WHO., 2003). The Wistar control on the other hand displayed a steady state normotensive systolic and diastolic blood pressure over the same time period. These results confirm the suitability of the genetically predisposed SHR and the normotensive Wistar controls to be excellent models for assessing and comparing the pathophysiological changes that occur in hypertension.

The elevated blood pressure of the SHR becomes significant when one looks at the vascular remodelling (to be discussed in detail in Section 5.6), which is prevalent in this model. The SHR showed significant changes to the vasculature with a significantly thicker tunica media in both the aorta and the iliac artery. This increased thickness of the tunica media results in a greater W/L ratio in the aorta. The increase in W/L ratio, and the subsequent decrease in lumen diameter, will cause an increase in vascular resistance, and manifest as the elevated blood pressure that is shown in the SHR.

The elevated blood pressure of the SHR also becomes significant when one looks at the plasma NO levels (to be discussed in detail in Section 5.4.2). This model had significantly lower plasma NO levels than the Wistar control. It is this compromised NO level, and the models subsequent reduced vasodilatory capacity, that contribute to and manifest as the elevated blood pressure in this model.

The SHR had a significantly higher heart rate than the Wistar control at the beginning of the study. These results are consistent with other studies that have shown the SHR to have a higher heart rate due to an increased level of sympathetic activity, which acts to increase the chronotrophic and inotrophic actions of the heart (Kirby *et al.*, 1989). However, while the systolic and diastolic blood pressures increased over the successive weeks the heart rate showed a steady decrease. The Wistar model also showed a steady decrease in heart rate as the animal aged, with no change (i.e. increase or decrease) in blood pressure, thus the steady decrease in heart rate in the SHR and Wistar is age related. The SHR did however have a higher heart rate at the end of the study, and this is due to the increased sympathetic activity of this model.

The SHR - Ang II infused group, showed significantly higher systolic and diastolic blood pressures from the beginning of the infusion till they were sacrificed at the end of the 3rd week, thus the infusion of the sub-pressor dose of Ang II over the 3 week period, exacerbated both the onset and the level of hypertension. The model reached high systolic and diastolic levels viz. 179 ± 2 mmHg and 116 ± 2 mmHg respectively by the end of the second week, as compared to the saline infused control only reaching this level in the fourth week (these levels are comparable with Grade 3 hypertension according to the WHO classification of 2003). This therefore suggests that a long term infusion of a sub-pressor dose of Ang II is able to exacerbate the onset and level of hypertension in the SHR. Therefore, this experimental model would be useful in investigating the mechanisms by which the sub-pressor dose of Ang II (viz. an increase in oxidative stress and its related blood pressure elevating mechanism) act to exacerbate the blood pressure in this model.

The SHR - Ang II infused group showed no significant changes in the heart rate over the successive weeks of the study, however it did show a significantly higher heart rate at the end of the third week when compared to the saline infused control, and thus these results suggest

that Ang II has a positive chronotrophic effect. This is consistent with other studies that have shown that Ang II infusion increases the intrinsic heart rate, by affecting the baroreceptor reflex sensitivity, which inevitably causes an increase in heart rate (Machado and Salgado., 1990).

The Wistar Ang II infused model showed significant increases in blood pressure from the third week of infusion, and by the end of study, after four weeks these high levels in the systolic and diastolic blood pressure are comparable with the Grade 2 hypertension according to the WHO classification of 2003. This therefore shows that the sub-pressor dose of Ang II in the normotensive Wistar increases blood pressure slowly over a long period. This is consistent with other studies that have shown a similar increase in blood pressure due to the infusion of sub-pressor doses of Ang II (Simon *et al.,* 1995). This therefore confirms the suitability of this model to investigate the long-term non-pressor role of Ang II that acts to elevate blood pressure.

The heart rate of the Wistar - Ang II infused group was significantly lower in the second week of the infusion, demonstrating a possible compensatory change by the cardiovascular system to the increase in blood pressure caused by the Ang II. The heart rate then mirrored that of the saline infused control, suggesting a long term effect of Ang II to sustain the increased heart rate and provided further evidence that Ang II is a positive chronotrophic factor, which acts to increase the intrinsic heart rate, as mentioned previously by affecting the baroreceptor sensitivity, which inevitably causes an increase in heart rate (Machado and Salgado., 1990).

5.2. ANGIOTENSIN II, DOES IT UNDERLIE THE PATHOGENESIS OF HYPERTENSION?

Angiotensin II levels were assessed in the plasma of all groups and the results show that the SHR had a significantly higher plasma Ang II level when compared to the Wistar control. The pathophysiological role that Ang II plays in hypertension is well established, and is usually attributed to its pressor effects i.e. its vasoconstrictor properties which act to increase total peripheral resistance, its stimulation of the thirst centre in the hypothalamus to increase fluid intake, its stimulatory role on aldosterone (which increases Na^+ and H_2O retention), and its stimulatory role on vasopressin (to prevent fluid loss in the urine). All of these factors act to essentially expand the plasma volume and thus increase blood pressure (Sealy and Laragh., 1995). Thus an increase in circulating Ang II would result in an increase in blood pressure, by the above mentioned pressor effects, as well as the NADPH oxidase enzyme mechanism that is in question by this study. This therefore means that in the SHR the increase in blood pressure, is due in large part to the increase in circulating Ang II, as has been shown in previous studies (Bolterman *et al.*, 2005).

Although the SHR had higher plasma Ang II levels, the AT-1a mRNA expression in the aorta showed no difference in expression when compared to the normotensive control and this suggests no difference in AT-1a receptor expression/density (Suzuki *et al.,* 1993), but the SHR still had a significantly higher blood pressure. The AT-1 receptor has been shown to mediate all the known functions of Ang II that act to elevate blood pressure, such as vasoconstriction, increased cardiac contractility, aldosterone release and, renal Na^+ and H_2O reabsorption (Timmermans *et al*., 1993; Kim and Iwao., 2000). Thus these results show that the vasculature where the AT-1a mRNA expression was quantified viz. the aorta, is not a tissue that is a target for the increased circulating Ang II, to act to increase the blood pressure. These results are consistent with previous studies that have shown other target tissues which

include the kidney, heart, brain and smaller resistance vessels that show an increase in AT-1a mRNA expression, in the SHR and other models exposed to high circulating Ang II levels (Suzuki *et al.,* 1993; Ito *et al.,* 1995; Lenkei *et al.,* 1997; Kim and Iwao *et al.,* 2000). Thus without a change in the density of this receptor in the aorta, it confirms that the aorta, is not a target for Ang II in the SHR, but rather the other previously reported tissues, which play a major role via AT-1 in elevating the blood pressure.

The link between Ang II and oxidative stress in hypertension, is an area that has received much attention in recent research, this being due to a mechanism by which Ang II increases blood pressure indirectly via the NADPH oxidase enzyme system. Ang II has been shown to stimulate O_2^- generation by increasing the activity of the enzyme; NADPH cytochrome P-450 oxidoreductase, more commonly termed NADPH oxidase. This was demonstrated in cultured rat vascular smooth muscle cells and in intact aortas of rats made hypertensive by Angiotensin II infusion (Griendling *et al.,* 1994; Zalba *et al.,* 2001). However when rats were made hypertensive to a similar degree, by an infusion of noradrenalin, this model showed no increase in NADPH oxidase activity (Laursen., 1997). Interestingly, the blood pressure and vascular reactivity could be brought back to normal by exposure to an exogenous liposome-encapsulated SOD in the Angiotensin II hypertensive rats, but not in the noradrenalin hypertensive rats (Griendling *et al.,* 1994; Laursen., 1997; Zalba *et al.,* 2001). The combined findings of these studies fortifies the causative role of O_2^- in hypertension that is associated with high Ang II states, and thus proves that elevated Ang II results in a state of increased *in vivo* oxidative stress. These studies concluded that Ang II – induced hypertension activates the NADPH oxidase system and increases O_2^- production and the increase in O_2^- is directly involved in the pathology of hypertension (Rajagopalan *et al.,* 1996; McIntyre *et al.,* 1999). The results of this study show that although the plasma Ang II levels of the SHR are elevated, the p22phox (a sub-unit of NADPH oxidase) mRNA expression in the aorta is not. This

finding is not consistent with the results of the previously mentioned studies where Ang II infusion and exposure increases p22phox mRNA expression. This is thought to be due to two main factors in this study, firstly although the SHR has elevated *in vivo* levels of Ang II, it is thought that the threshold for Ang II to increase/stimulate p22phox mRNA expression in the aorta, is greater than that which this model is exposed to at this age, thus there is no elevation of p22phox mRNA in the aorta. Secondly, the results indicate again that the aorta is not a target by Ang II in the SHR at this age as shown in previous studies and by the AT-1a mRNA expression results in this study. It is rather the smaller resistance vasculature that experience a higher shear stress. These vessels have been shown in this model to have an increased expression of the p22phox subunit of NADPH oxidase as well as an elevated level of O_2^- (Zalba *et al*., 2001), whereas the aorta shows elevated levels of p22phox expression, at a much later age (~30 weeks old), and this is thought to be linked to the elevated shear stress levels that the aorta is exposed to at that age (Zalba *et al.*, 2000).

When the SHR-adult is compared to the SHR-weanling, there was a significant age-related increase in plasma Ang II levels in the adult. This demonstrates that in the adult, Ang II production is increased. This increase is due to the reported increased activity of the renin-angiotensin system (RAS) in this model as it ages (Matsushima *et al.*, 1988). The AT-1a mRNA expression results show that the SHR-adult had a significant decrease in AT-1a mRNA expression in the aorta. This therefore suggests that this model has an age related decrease in AT-1a receptor density in the aorta and again suggests that the aorta is not a target tissue for Ang II to elevate the blood pressure in this model at this age. It is rather the previously reported tissues such as the kidney, heart, brain and smaller resistance vasculature (Suzuki *et al.*, 1993; Ito *et al.*, 1995; Lenkei *et al.*, 1997; Kim and Iwao *et al.*, 2000). The age related decrease is confirmed by the changes to the AT-1a mRNA expression and plasma Ang II results when the Wistar-adult is compared to the Wistar-weanling. The Wistar-adult

showed a significant decrease in plasma Ang II levels and a significant decrease in AT-1a mRNA expression, with no change in blood pressure. This confirms that the decrease in AT-1a mRNA expression of the aorta in the SHR is age related, rather than pathological. These results therefore show that although the vasculature of the hypertensive and normotensive adults show an age related decrease in the receptor density of the AT-1a receptor in the aorta, the SHR, has a genetic predisposition to increased levels of circulating Ang II, via increased activity of the RAS system and the Wistar does not. Thus the elevated levels of Ang II are a contributing factor to the age related increase in blood pressure demonstrated by the SHR.

The sub-pressor dose of Ang II (i.e. a dose of Ang II that has been shown to not elicit immediate pressor effects in the kidney and in the vasculature) infused into the SHR over three weeks, significantly decreased plasma Ang II levels (Simon *et al.,* 1995). The infusion however exacerbated the onset and the level of hypertension displayed by this model, as demonstrated by the blood pressure results. The infusion of Ang II also significantly increased the AT-1a mRNA expression in the aorta, suggesting an increase in AT-1a receptor density. These changes show that an exogenous increase of Ang II via a sub-pressor dose in the SHR, results in a negative feedback inhibition of Ang II formation via RAS, and thus acts to reduce circulating plasma Ang II levels. Interestingly the AT-1a mRNA expression in the aorta is increased. This has also been demonstrated in other studies where an infusion of Ang II, resulted in a decrease in circulating Ang II levels, and it is this decreased circulating Ang II levels that is shown to cause an upregulation of AT-1 (Schiffrin *et al.,* 1984). The upregulation of AT-1 will thus result in an increase in the Ang II mediated blood pressure elevating effects, even though the circulating levels are decreased. These results are consistent with other studies that have shown this negative feedback regulation of Ang II when circulating levels reach a threshold, and that the decreased Ang II levels subsequently enhance AT-1 receptor activation and activity (Douglas and Brown., 1982; Schiffrin *et al.,*

1984; Lassegue *et al.,* 1995; Nickenig and Harrison., 2002). Thus although the circulating Ang II levels decrease in the SHR by 71%, the AT-1a receptor density increases by 338%, and this will potentiate the effects of Ang II. The potentiation of Ang II effects as illustrated by the blood pressure results, manifests as an 'accelerated' onset and level of hypertension.

The Wistar – Ang II infused group did not display the same trend and showed no significant change to the plasma Ang II levels. The group did however show a 126% increase in AT-1a mRNA expression. These results thus confirm that a sub-pressor dose of Ang II would inhibit the RAS system, and prevent an increase in Ang II levels, but target cells (in this case the vasculature) become more sensitive by increasing their AT1-a receptor density and thus potentiating the effects of Ang II (Diep *et al.,* 2002; Le *et al.,* 2003).

The results also show that a three week infusion of a sub-pressor dose of Ang II in the SHR decreases its Ang II levels to a greater extent than that in the Wistar. The greater inhibition of the RAS in the SHR, causes a greater degree of AT-1a upregulation, and thus the effect of this upregulation of AT-1a on the SHR will be more exaggerated. The results also indicate that the SHR, has a much higher threshold level for Ang II feedback inhibition, than the normotensive Wistar, and provides a reason for the higher "normal" circulating Ang II levels in the SHR.

Thus these results show that an increased long term exposure to sub-pressor doses of Ang II, increases AT-1a receptor density in the vasculature and thus increasing Ang II's blood pressure elevating properties. Other studies have shown that sub-pressor doses of Ang II, do not immediately increase blood pressure, but in the long term has slow pressor effects that work independently of the classic pressor mechanisms and significantly increase blood pressure (Romero and Reckelhoff., 1999). These mechanisms are still under investigation, but from this study it can be seen that Ang II increases AT-1a mRNA expression over the

long term, and thus the increase in receptor density would potentiate the blood pressure elevating properties of Ang II.

5.3. NADPH OXIDASE, IS IT ANOTHER MECHANISM BY WHICH ANG II ELEVATES BLOOD PRESSURE?

NADPH oxidase has been shown to be a major source of ROS in vascular cells. It consists of 2 membrane bound subunits viz. p22phox and gp91phox, and 3 cytosolic subunits p67phox, p47phox and GTPase rac 1. Phagocytes, adventitial fibroblasts, vascular smooth muscle cells (VSMC) and endothelial cells (EC) contain this plasma-membrane bound multi-component oxidase that utilises electrons derived from NADPH to reduce molecular O_2 to O_2^- (Stocker and Keaney., 2004).

This study assessed the p22phox and gp91phox mRNA expression in the aorta and used the mRNA expression as a measure of the activity of the NADPH oxidase enzyme. The activity of NADPH oxidase was then used as an indirect measure of O_2^- production (Fukui *et al.*, 1997; Zalba *et al*., 2000; Laude *et al.*, 2005).

The gp91phox mRNA expression showed no significant changes amongst all groups in the study. The reason for this is similar to the findings of other studies that have shown that gp91phox is expressed as very low levels in VSMC's, and at higher levels in EC's and adventitial tissue (Zalba *et al*., 2001). The tissue where the mRNA expression was assessed viz. the intact aorta showed non-significant changes to gp91phox mRNA expression across all groups. The reason for this is due to the previously mentioned selective expression of gp91phox in EC's. It is thought that in the handling and storage of the intact aorta segments, there was probable damage and loss of the endothelium and thus the EC's. The loss of the endothelium thus resulted in the lack of gp91phox expression, and thus, gp91phox mRNA expression results will be excluded from the discussion.

The p22phox subunit (which is largely expressed by VSMC's) mRNA expression results show that the SHR had a significantly lower p22phox mRNA expression than the

normotensive Wistar control. These results would therefore suggest that the SHR had a lower O_2^- production. However previous studies have shown the SHR to have a higher O_2^- production/level (Boulemie *et al*., 1997). These results therefore suggest that the NADPH oxidase specifically in the aorta is not the main contributor to the increased O_2^- production in the SHR. This is especially so when taking into consideration the age of the group i.e. 8 weeks old as discussed previously in Section 5.2. The reason as to why age needs to be considered, is that, these results are similar to results in a study by Zalba *et al.,* (2001). The study showed that the SHR at 16 weeks of age, showed no difference in p22phox mRNA expression in the aorta, when compared to the normotensive controls but they did have higher O_2^- levels. However when 30 week old SHR's in the same study were compared to 30 week old normotensive controls, there was a significantly greater p22phox mRNA expression in the aorta. The results from that study therefore suggest that p22phox and thus NADPH oxidase in the aorta play a more pertinent role with respect to O_2^- production as the SHR ages and has long term exposure to an elevated blood pressure. The elevated blood pressure in this model is thought to be caused and maintained in part by the pressor effects of the increased Ang II viz. vasoconstriction of the smaller resistance vasculature and Na^+ and H_2O retention. Other studies have found that the SHR has a significantly higher level of O_2^- production than the normotensive control, and this is independent of age (Boulemie *et al*., 1997). Thus the results of this study combined with the reports of the other studies suggest that the source of the O_2^- is other than that of NADPH oxidase in the aorta, and is from smaller resistance vasculature, that experience higher/sustained shear stress, and this increased shear stress has been shown to increase the activity of NADPH oxidase (Zalba *et al.,* 2001). Thus the age at which this group is at, the aorta is not exposed to a high enough levels of shear stress as compared to the older animals to increase the activity of NADPH oxidase. However when the shear stress is elevated, as demonstrated in the SHR - Ang II infused group, the p22phox

mRNA expression increases significantly. The Ang II results combined with the findings here, and of those in the previously mentioned studies, show that the elevation in blood pressure in this model, is not caused by a single factor or agent, but rather a multitude of mechanisms that are working in concert to initiate and maintain an elevated blood pressure. This was demonstrated in studies whose findings show that the elevated ROS production occurs before the development of hypertension in the SHR, thus highlighting that the ROS, play a role in both the development and maintenance of hypertension in this model (Kitiyakara and Wilcox., 1998; Houston., 2005; Paravicini and Touyz., 2008).

The Ang II infusion caused a significant increase in p22phox mRNA expression of both the SHR - Ang II infused group and the Wistar - Ang II infused group when compared to their respective saline infused controls. These results therefore suggest that the long term sub-pressor dose of Ang II enhances specifically the p22phox subunit and therefore increases local O_2^- production i.e. by the vasculature, via the increased activity of NADPH oxidase. These results are consistent with other studies that have shown an increase NADPH oxidase activity in the vasculature due to stimulation by Ang II (Rajgopalan *et al*., 1996; Ushio-Fukai *et al.,* 1996; Fukui *et al.,* 1997). These results also demonstrate that the exacerbated increase in blood pressure (i.e. in the SHR – Ang II infused group), could be due to an increase in O_2^- production (mechanism to be discussed later in the role of O_2^- in hypertension – Section 5.4.1) as well as the effects of the increased circulating Ang II (discussed in Section 5.2) and vascular remodelling (to be discussed in Section 5.6). This again demonstrates the multitude of mechanisms that are working in concert to initiate and maintain an elevation in blood pressure, in the SHR.

Interestingly when the degree to which the infusion of the sub-pressor dose of Ang II increases p22phox expression in the SHR and Wistar is compared, it can be seen that in the Wistar, the p22phox expression increased by 154%, but in the SHR, the same sub-pressor

dose of Ang II increased p22phox expression by 338%. This therefore shows that the SHR has a greater sensitivity to Ang II stimulation and thus a greater capacity for ROS production and the resultant oxidative stress.

These results demonstrate that Ang II has a significant localised effect on the vasculature to increase the production of O_2^- via its stimulation of NADPH oxidase. This brings the study to the point of now discussing how the organism attempts to neutralise O_2^- and the pathophysiological consequences of an increased O_2^- in hypertension.

5.4. A COMPROMISED ANTIOXIDANT STATUS: IS IT THE CAUSE OR CONSEQUENCE OF AN ELEVATED BLOOD PRESSURE IN HYPERTENSION?

The antioxidant status of all groups was evaluated by assessing the levels of the major antioxidant enzymes in the vascular compartment viz. SOD, GPx and Catalase.

5.4.1. SOD THE PRIMARY DEFENCE AGAINST THE DELETERIOUS O_2^- RADICAL

The results show that the SHR had a significantly higher SOD level than the normotensive control. SOD is the antioxidant enzyme that neutralises the deleterious reactive oxygen species, O_2^-, to a more stable and relatively less deleterious ROS H_2O_2.

This greater SOD buffering capacity of the SHR is an adaptive mechanism that this genetically hypertensive rat model has developed to counter an increase in O_2^- production and minimise oxidative stress. The SHR has been shown in previous studies to have higher O_2^- levels than the normotensive control (Bouloumie *et al.,* 1997). This adaptive trait is further reinforced by the H_2O_2 results, which show that the SHR had a significantly higher plasma H_2O_2 level than the normotensive control. Since SOD dismutates O_2^- to H_2O_2, an increase in SOD and O_2^- would result in an increase in H_2O_2 levels, which has been found in the SHR in this study.

An increase in the SOD enzyme levels, should be indicative of a systemic increase in the mRNA expression of that enzyme as shown in previous studies viz. an increase in Cu/Zn SOD (Zelko *et al*., 2002), however the results of this study do not show this relationship. The reason for this is due to the Cu/Zn SOD mRNA expression being assessed in the vasculature (i.e. the aorta) and is indicative of cytosolic SOD (SOD-1) levels of the vascular cells that make up the aorta (mainly VSMC's) and the SOD enzyme levels on the other hand were

assessed in the RBC and is indicative of RBC cytosolic SOD (SOD-1). The results thus show that differing cell types, within the same compartment express this enzyme at different levels. These levels of expression appear to be dependent on the cells production of and exposure to O_2^-, thus in the cells of the aorta, the O_2^- production is not elevated (this is confirmed by the p22phox mRNA expression results, which show this), and thus the cytosolic SOD, is therefore not elevated. Thus in the aorta of the SHR, there is normal O_2^- and SOD levels, and this is not indicative of the reported elevated levels of O_2^- that this model is exposed to (Bouloumie *et al.*, 1997). Previous studies in this model, at this age, as discussed previously, have shown that the aorta, does not have an elevated level of O_2^- production via NADPH oxidase, and it is rather the smaller resistance vessels, exposed to a higher shear stress, that have elevated O_2^- levels via increased activity of NADPH oxidase (Fukui *et al.,* 1999; Zalba *et al.,* 2001; Faraci and Dideon., 2004). Thus it can be seen that in the SHR, it is these smaller resistance vessels that play a major role in contributing to the elevated levels of ROS and its related derangements that result in an elevation in blood pressure.

When the SOD enzyme levels of the SHR-adult is compared to the SHR-weanling group, there is a significant increase in SOD levels in the SHR-adult, and this suggests that there is an age related increase in SOD, which is due to the reported age related increase in O_2^- production (Bouloumie *et al.,* 1997). This previous study has shown that this increase in SOD in the adult is an adaptive mechanism to deal with higher levels of O_2^-. Interestingly the weanling had a significantly higher Cu/Zn SOD expression than the adult. This is also age related, and shows that as the model ages the vasculature decreases its antioxidant buffering capacity and production, and is thus more prone to oxidative stress, as demonstrated in the adult. This also shows that the adaptation of this major antioxidant (increase in SOD) is a response to the hypertension, and demonstrates an adaptive trait to deal with an elevated level/production of O_2^-. The Wistar showed a similar trend with regards to SOD, when

compared to the weanling and confirms that the changes in the SOD levels are age related. However the degree of decrease in Cu/Zn SOD is greater in the SHR than in the Wistar and suggests that this decrease in SOD buffering capacity would result in an increase in O_2^- in the vasculature and contribute to the elevation in blood pressure in this model. This also shows that the age related compensations are inadequate in the SHR which is exposed to higher levels of ROS than the Wistar, and thus the accumulation of these ROS play an important role in this model of genetic hypertension.

The infusion of the sub-pressor dose of Ang II resulted in no significant change to the RBC (cytosolic) SOD levels in both the SHR and Wistar models, suggesting no change to the antioxidant buffering capacity of the RBC. However when looking at the Cu/Zn SOD mRNA expression i.e. the cytosolic SOD levels of the aorta the results show that the Ang II infusion resulted in a significant increase in the expression of this enzyme in the SHR. This suggests that there is an adaptive response to the significant increase in p22phox expression in the vasculature. Thus the increase in SOD would attempt to scavenge the increased O_2^- derived from NADPH oxidase and attempt to prevent the deleterious effects of O_2^- accumulation, and delay this process and its subsequent pathology.

In the Wistar infused with the sub-pressor dose of Ang II there was an opposite trend with regards to the Cu/Zn SOD mRNA expression where the infusion resulted in a significant decrease in the expression of this important enzyme. This therefore suggests a decrease in the SOD antioxidant buffering capacity of the vasculature. The infusion also resulted in a significant increase in p22phox expression, and thus an increase in O_2^- production in the vasculature via NADPH oxidase. When considering both these results it suggests that in the Wistar – Ang II infused group there would be an accumulation of O_2^- in the vasculature, and this being due to both a decreased SOD antioxidant buffering capacity and an increased production of O_2^- via NADPH oxidase. These findings also suggest that Ang II inhibits

Cu/Zn SOD expression and stimulates NADPH oxidase activity and thus promotes the deleterious blood pressure elevating effects of O_2^- in the Wistar – Ang II infused model, and thus contributes to the increase in blood pressure demonstrated by this model.

When the degree to which the infusion of the sub-pressor dose of Ang II changed the SOD antioxidant buffering capacity in the SHR and the Wistar is compared, the results show that the infusion had no significant effect on the RBC SOD i.e. the cytosolic SOD. However the infusion increased the expression of the Cu/Zn SOD in the vasculature of the SHR by 180%, but decreased the Cu/Zn SOD expression in the Wistar by 81%. This is significant when taking into consideration the changes to the p22phox expression, where the SHR showed a 338% increase in p22phox expression and the Wistar showed a 154% increase in p22phox expression. This therefore suggests that although the SHR makes adjustments to the SOD antioxidant buffering capacity in response to the increase in O_2^- production via NADPH oxidase, the degree of O_2^- production is greater than the increase in SOD antioxidant buffering capacity and this would result in O_2^- accumulation. These results also suggest that the genetically hypertensive SHR has developed “protective” mechanisms with regards to the antioxidant system, that are not apparent in the normotensive model. These compensatory/protective/adaptive adjustments appear to stem from the SHR being genetically susceptible to elevated Ang II levels, and the Wistar not having this susceptibility. These findings are novel and more in-depth work on the exact mechanisms involved in this differential expression of this important antioxidant enzyme needs to be done to conclusively comment on these findings.

The role that O_2^- plays in hypertension per se is a well-established and putative one. The most significant role is the interaction between O_2^- and nitric oxide (NO). NO is a potent endogenous vasodilator, it regulates vascular tone in normal vessels, which includes resistance vessels. It also causes renal vasodilatation with consequent diuresis and natriuresis.

The actions of NO would act to lower blood pressure and therefore a reduction in NO would result in a decrease in the vasodilatory capacity with a consequent elevation in blood pressure (McIntyre *et al.,* 1999).

O_2^- reduces the bioavailability of NO, due to its high affinity for NO. The combination of O_2^- and NO, results in the formation of peroxynitrite ($ONOO^-$). Therefore conditions that promote O_2^- formation, such as in hypertension can be harmful in several ways. This being firstly by halting the beneficial effects of NO by reducing its bioavailability and secondly by the damaging effects of $ONOO^-$. If $ONOO^-$ is protonated to peroxynitrous acid, the cleavage products are among the most reactive oxygen species, this includes the breakdown of arachidonic acid which results in F_2-isoprostanes. The F_2-isoprostanes have been shown to cause renal arteriolar vasoconstriction, 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 , as well as induce hypertrophy and proliferation of VSMC's, resulting in irreversible changes to the vasculature i.e. vascular remodelling (Section 5.6) (Stocker and Keaney., 2004).

The putative role that O_2^- plays during hypertension can be successfully assessed with reference to studies that have administered synthetic SOD to neutralise the O_2^- threat in hypertensive rats. The results of these studies were successful in lowering the blood pressure of these hypertensive rats (Nakazono *et al.,* 1991). Thus the mechanism by which O_2^- 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^- are successfully neutralised by SOD and thereby lowering blood pressure, in studies linked with Down's 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's 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's syndrome, including premature aging, suggesting that this gene is involved in the pathogenesis of Down's syndrome (Yarom *et al.*, 1988). The increased SOD-1 activity in Down's syndrome may further indicate a role for O_2^- in hypertension. With a higher SOD-1 activity, Down's syndrome patients will have reduced O_2^- levels (McIntyre *et al*., 1999). If O_2^- excess is involved in the pathogenesis of hypertension, then one would expect Down's syndrome patients to have a lower blood pressure, and this trend was shown to be the case in a study by Morrison *et al.*, (1996). This thereby confirms the positive role that O_2^- plays in increasing blood pressure during hypertension by causing the effects discussed earlier. This adds further evidence to hypertension being classified as a disease that is intimately linked with an increased level of oxidative stress.

As can be seen from these results, SOD depletion and increased O_2^- production, play an integral role in elevating the blood pressure in the SHR and in the experimental models, mainly due to the interaction between O_2^- and NO, and the next section will discuss the *in vivo* changes to NO in these models of hypertension.

5.4.2. NITRIC OXIDE: A VITAL "COG IN THE WHEEL" TO REDUCE BLOOD PRESSURE

Nitric oxide levels were assessed in the plasma and the results show that the SHR had significantly lower plasma NO levels than the Wistar control. As discussed previously NO is a potent vasodilator and regulator of vascular tone, and thus plays an important role in blood vessels to decrease total peripheral resistance (TPR) and thus blood pressure. It is also known to increase renal vasodilation which increases natriuresis and diuresis, the result of which will tend to lower blood pressure. So the results indicate that the SHR has a decreased ability to reduce TPR and increase renal natriuresis and diuresis and ultimately decrease blood pressure (McIntyre *et al.,* 1999). The decreased availability of NO in the SHR, manifests as the significant elevation in blood pressure demonstrated by this model. This elevation in blood pressure may be due to the models decreased vasodilatory capacity, and thus the subsequent increased vascular resistance.

The reason for the decrease in NO, is due to the reported increase in O_2^- production by the SHR (Bouloumie *et al*., 1997; Zalba *et al.,* 2001; Wassmann *et al.,* 2004). O_2^- has a very high affinity for NO as discussed earlier, and thus with an increase in O_2^-, in the face of depleted SOD, this would result in an increase scavenging of NO by O_2^-, thus decreasing NO's vasodilatory activity/capacity and contributing to an increase in blood pressure.

When the SHR-adult is compared to the SHR-weanling, it can be seen that there is a significant age related decrease in NO levels. This trend was not apparent in the normotensive Wistar groups (i.e. adult vs weanling). Thus the decrease in NO can be attributed to the reported age related increase in O_2^- production of the SHR. This therefore contributes to the adult SHR's decreased vasodilatory capacity and resultant elevated blood pressure. Studies have shown that the biological activity of NO is determined by the

availability of O_2^- (Zalba *et al.*, 2001; Schnackenberg., 2002; Shimokawa and Matabo., 2004). This therefore presents further evidence on the putative role played by NO depletion due to O_2^- in elevating blood pressure in hypertension.

The Ang II infusion significantly decreased the NO levels in the SHR - Ang II infused group, and again this decrease can be attributed to the increase in O_2^- production via NADPH oxidase in the vasculature as demonstrated by the increase in p22phox mRNA expression results. The Wistar - Ang II infused group showed a similar trend with a decrease in NO levels. This decrease could also be attributed to an increased p22phox mRNA expression also seen in this model, which means an increase in O_2^- production by the vasculature. The decrease in NO in both these experimental models manifests as the significant increase in blood pressure noted in these models, which is due to the increased vascular resistance caused by the reduced vasodilatory capacity and subsequent vasoconstriction.

When the degree to which the infusion of the sub-pressor dose of Ang II decreased NO levels in the SHR and Wistar are compared, the results show that in the SHR, the NO levels decreased by 36%, but in the Wistar the NO levels decreased by 12%. This can be attributed to the greater degree of O_2^- production in the SHR as compared to the Wistar. Thus the Ang II depletes NO indirectly via O_2^- to a greater extent in the SHR than in the Wistar, and thus decreases the vasodilatory capacity of the SHR more than that of the Wistar.

The results of both these groups suggest that Ang II plays an important indirect role in modulating vascular tone. This is due to Ang II diminishing the vasodilatory capacity of the vessel wall by stimulating O_2^- production via the NADPH oxidase mechanism, and ultimately decreasing NO activity. This would result in an increase in vasoconstriction and subsequently vascular resistance and thus increases blood pressure.

5.4.3. GPX, CATALASE AND HYDROGEN PEROXIDE, DO THEY PLAY A ROLE IN THE PATHOGENESIS IF HYPERTENSION?

GPx and Catalase are the antioxidant enzymes that neutralise H_2O_2 to H_2O and O_2. The SHR had a significantly lower GPx level than the normotensive control. This difference therefore suggests a decrease in the GPx antioxidant capacity of the SHR, to neutralise H_2O_2. This decrease in GPx antioxidant capacity will lead to subsequent H_2O_2 accumulation, evidence of this is provided by the plasma H_2O_2 results that show the SHR to have a significantly higher plasma H_2O_2 levels than the normotensive control.

GPx levels however, cannot be viewed independently of catalase levels, as both these enzymes work in unison to neutralise H_2O_2 and prevent its accumulation. The catalase results show that the SHR had a significantly higher serum catalase level than the normotensive control. This is biologically significant and previous studies have shown that catalase becomes elevated and plays the chief role at high concentrations of H_2O_2, during high levels of oxidative stress and also during states of reduced GPx activity (Cai., 2005; Kirkman and Gaetani., 2006; Paravicini and Touyz., 2008). These results i.e. the catalase and H_2O_2 levels indicate that in the SHR the catalase levels are high due to the high levels of H_2O_2 and the reduced GPx activity, and this therefore suggests that the SHR is in a state of high level oxidative stress. This state of high level oxidative stress is due primarily to elevated levels of O_2^- and its subsequent dismutation by SOD discussed earlier and the elevated levels of H_2O_2 discussed here. The same trend did not occur in the normotensive control, which had higher GPx levels, but lower catalase levels, which is due to the lower levels of H_2O_2, and thus would indicate that this model is not in a state of high level oxidative stress. The difference in trends of the genetically hypertensive and normotensive models, adds further evidence to link the hypertension demonstrated by the SHR to high level oxidative stress.

The source of the H_2O_2 can be attributed to the previously discussed results i.e. SOD levels and NADPH oxidase activity (p22phox mRNA expression). These results show that the increase in SOD levels in the SHR was due to an increase in O_2^- production, and this would lead to an increased dismutation of O_2^- to H_2O_2 and result in an increase in H_2O_2 levels and thus H_2O_2 accumulation, as the results show. This therefore demonstrates the integrated nature of the antioxidant system where an increase in SOD due to O_2^-, must also cause an increase in GPx and CAT to prevent oxidative stress via H_2O_2 accumulation. It is imperative that the antioxidant system functions in an integrated manner, thus minimizing oxidative stress and its associated pathological changes.

When the SHR-adult is compared to the SHR-weanling, there was a significant decrease in the GPx levels in the adult, demonstrating that this model develops a reduced GPx activity as it ages. This characteristic exposes the SHR to increased levels of ROS and thus the SHR is unable to provide a resistance to oxidative stress via H_2O_2 accumulation. The Wistar adult does not mimic the same trait when compared to the Wistar-weanling, and instead shows a significant increase in GPx levels. This results in an increased GPx antioxidant buffering capacity, and is able to provide resistance to oxidative stress via H_2O_2. The Wistar adult also shows a significant decrease in CAT levels and thus demonstrates that the GPx system is able to cope with any increase in ROS that the Wistar might encounter as it ages. This importantly indicates that the model is not in any form of elevated oxidative stress. The SHR on the other hand is in a state of high level oxidative stress, this being due to the significant increase in CAT levels. Previous studies have shown that elevated levels of CAT are an indicator of high level oxidative stress (Kirkman and Gaetani., 2006). It can also be seen that although the CAT levels increase significantly, the H_2O_2 levels remain high. This would result in H_2O_2 accumulation in the SHR and also indicates an increased H_2O_2 production as described earlier, this being due to the dismutation of O_2^- by SOD.

The Ang II infusion results show that there was a significant increase in GPx levels in the SHR - Ang II infused group when compared to the saline infused control. This therefore suggests that Ang II increases GPx levels. This is thought to be a compensatory response in an attempt to deal with the increase in H_2O_2 resulting from the increased dismutation of O_2^- by SOD discussed earlier. When analysing the H_2O_2 results, these results do not directly show this, as there was a significant decrease in H_2O_2 levels in the SHR - Ang II infused group. This therefore means that the significant increase in GPx levels is coping with the increased H_2O_2 production and actually decreasing H_2O_2 levels, and thus preventing the deleterious consequences of H_2O_2 accumulation. This is further confirmed by the significant decrease in CAT levels, which appear to decrease in response to the lower H_2O_2 levels.

There is a contrast when looking at the Wistar - Ang II infused group, where there was no significant change in GPx levels, but a significant increase in catalase levels. These results firstly suggest that the Ang II infusion increases the activity of the catalase enzyme and secondly that the Wistar - Ang II infused group is being exposed to high levels of H_2O_2. However the increased CAT compensation is sufficient to neutralise the H_2O_2 levels and bring them down to below normal and prevent H_2O_2 accumulation. Although the H_2O_2 levels in the Wistar - Ang II infused group show no significant change to the control, this could be due to the increase in catalase that lowers H_2O_2 levels and attempts to prevent H_2O_2 accumulation. As discussed previously the p22phox mRNA expression results suggests a significant increase in O_2^- production via the NADPH oxidase mechanism and thus an increase H_2O_2 production via O_2^- dismutation by SOD, but the increase in CAT in response to Ang II appears to be preventing an accumulation of H_2O_2.

When the degree to which the infusion of the sub-pressor dose of Ang II affected the GPx, CAT and H_2O_2 levels in the SHR and the Wistar are compared, the results show that in the SHR, the infusion increased GPx levels by 310%, but had no significant effect on the Wistar.

This therefore suggests that the genetic susceptibility of the SHR to high Ang II levels, allows the SHR and not the Wistar to make compensatory adjustments to the GPx system. This adjustment minimises a further increase in the high level of oxidative stress experienced by this model, and also attempts to prevent H_2O_2 accumulation.

When looking at the CAT results, the infusion of the sub-pressor dose of Ang II significantly decreased the CAT levels in the SHR by 25%, but in the Wistar, the CAT levels were increased by 77%. This therefore suggests that the Wistar is in a state of high level oxidative stress, while the SHR is maintaining and actually attempting to reduce its "normal" high level oxidative stress. Thus the genetic susceptibility of the SHR to high levels of Ang II and oxidative stress appear to have equipped this model with mechanisms to prevent a further increase in H_2O_2 accumulation. Interestingly the comparative GPx and CAT results show that in the SHR which has a genetic susceptibility to high Ang II levels, the GPx is the first line of resistance to an increased oxidative stress by H_2O_2 accumulation. However in the Wistar which lacks this genetic susceptibility to high Ang II levels, the CAT system is the first line of resistance to the increased oxidative stress by H_2O_2 accumulation.

The consequences of exposure to increased levels of H_2O_2 are deleterious, and the prevention of H_2O_2 accumulation is imperative in maintaining homeostatic balance. Virtually all types of vascular cells produce O_2^- and H_2O_2. The endothelium plays a fundamental role in the regulation of vascular tone and vascular remodelling. Exposure of the EC's (endothelial cells) to O_2^- and H_2O_2 has been shown to induce the deleterious process of apoptosis (programmed cell death), this process essentially leads to EC loss and results in atherogenesis and a procoagulative state (McIntyre *et al.,* 1999; Dimmeler and Zeiher., 2000; Taniyama and Griendling., 2003). 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 also

associated with increased intracellular ROS (Li *et al.,* 1999; Taniyama and Griendling., 2003).

The production of ROS O_2^- and H_2O_2 are 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 therefore demonstrates 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 hyper-responsiveness (Simon *et al.,* 1998).

An accumulation of H_2O_2 *in vivo*, could have the following deleterious effect if not neutralised by GPx and CAT. In the presence of the transition metals Iron (Fe^{2+}) and Copper (Cu^{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 and ultimately decreases cellular integrity and functioning (Halliwell., 1996).

Thus an elevated level of H_2O_2 in the face of depressed GPx and CAT will cause an accumulation of H_2O_2 and thus would result in the formation of OH^-. OH^- 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 hypertension via two main mechanisms, firstly via vascular remodelling and secondly by the formation of OH^-, leading to lipid peroxidation and eventual tissue/end-organ damage.

Oxidative stress leading to tissue damage, appears to be a focal point in hypertension research. The resultant tissue damage associated with lipid peroxidation is considered to be a major factor in the pathogenesis of hypertension. Empirical evidence has also shown that the architectural changes that are induced during oxidative stress in the kidney, heart and blood vessels contribute to a vicious cycle, that leads to end organ damage (Raij., 1998).

5.4.4. IS THE ANTIOXIDANT STATUS OF THE EXPERIMENTAL GROUPS COMPROMISED?

From the results discussed above, it can be seen that the experimental groups viz. the SHR, the SHR - Ang II infused group and the Wistar - Ang II infused groups have a compromised antioxidant status. This is primarily due to the dysfunctional levels of the primary antioxidant enzymes. Although the system attempts to make compensatory adjustments to some of these enzymes, these adjustments appear to be insufficient to deal with the increased levels of the ROS. This leads to these experimental models/groups being in a state of high level oxidative stress. The antioxidant status of these groups appear to be both the cause and the consequence of the oxidative stress. This being due to either the reduced antioxidant capacity, or to the deleterious increase in free radical generation during hypertension. It can thus be seen from the results of this study that in the hypertension in these models, there is an evident link with high level oxidative stress, where the consequences of a dysfunctional antioxidant system are highlighted. The dysfunctional nature of the antioxidant system shown here that is due to and/or resulting in oxidative stress, has also been shown in previous studies in humans, and thus highlights the importance of this system in hypertension (Lacy *et al.*, 2000).

5.5. TISSUE OXIDATIVE STRESS: IS THE KIDNEY A TARGET FOR REACTIVE OXYGEN SPECIES IN HYPERTENSION?

Tissue oxidative stress with resultant lipid peroxidation was assessed in the kidney, using a reliable indicator of *in vivo* lipid peroxidation viz. F_2-isoprostanes. The reason for the kidney being used to assess tissue damage via lipid peroxidation, was due to previous studies that have shown the kidney to be a target organ for tissue oxidative stress, and also because of the important homeostatic role played by the kidney in blood pressure regulation (Badr and Abi-Antoun., 2005).

The results show that the kidney of the SHR had a significantly higher level of F_2-isoprostanes than the normotensive control. This therefore suggests an increase in the level of *in vivo* lipid peroxidation and thus an increased threat to the structural and functional integrity of the organ. The increased level of lipid peroxidation in the SHR could be due to the previously discussed results that suggest the SHR to have a higher level of ROS that cause lipid peroxidation and also a compromised level of antioxidant enzymes that would act to prevent lipid peroxidation (Chabrashvili *et al.*, 2002). The accumulation of the ROS's, especially the elevated levels of H_2O_2 and the increase in $OONO^-$, could be the causative factors for the increased lipid peroxidation and the F_2-isoprostane formation (Paravicini and Touyz., 2008).

The lipid peroxidative damage will result in impaired renal functioning and contribute to an increase in blood pressure by reducing the natriuretic and diuretic properties of the kidney. Also the F_2-isoprostanes, which as previously stated are products of the lipid peroxidative breakdown of arachidonic acid by OH^- and $OONO^-$, have been shown to be potent renal vasoconstrictors. Renal vasoconstriction would result in a decrease in glomerular filtration and would thus further decrease the natriuretic and diuretic properties of the kidney. Thus

both of these mechanisms would contribute to an elevation in blood pressure. Interestingly studies have shown that the increased renal expression of NADPH oxidase subunits (a source of ROS in the kidney) precedes the development of hypertension. Thus, increased expression of NADPH oxidase and the consequent increase in ROS production resulting in increased tissue oxidative stress appears to be one of the causative factors in, rather than a consequence of hypertension (Chabrashvili *et al.,* 2002; Wilcox., 2002).

The Ang II infusion models i.e. the SHR - Ang II infused group and the Wistar - Ang II infused group, showed no significant difference in their kidney F_2-isoprostane levels when compared to their relative controls, and thus did not show an increased level of lipid peroxidation. Although these groups showed an increased level of oxidative stress, due to increased ROS production, they did not show significant increases in lipid peroxidation in the kidney. This could perhaps be due to the fact that the experimental period of four weeks was not sufficiently long enough for changes in this parameter. Also from the results the compensatory increases in GPx and CAT, suggest that the changes to these important antioxidant enzymes, successfully prevented H_2O_2 accumulation by neutralising this threat, and thus preventing an increase in lipid peroxidative damage to the kidney.

This adds further evidence that the increase in blood pressure in these models are independent of the Ang II pressor effects on the kidney, and thus shows that the O_2^- role via NADPH oxidase on the vasculature is one of the primary short-term mechanisms of blood pressure elevation in both of these experimental models.

5.6. VASCULAR REMODELLING: AN AMPLIFIER AGENT IN HYPERTENSION

Vascular remodelling refers to the architectural changes that occur in the blood vessel. It is characterised by an increase or decrease in the thickness of the tunica media (i.e. due to proliferation and hypertrophy of the VSMC's), with a resultant change in the wall to lumen ratio (W/L ratio). An increase in the W/L ratio signifies an increase in the thickness of the media and a consequent decrease in lumen diameter. A decrease in the W/L ratio signifies a decrease in media thickness and a consequent increase in lumen diameter. Both these characteristic changes to the blood vessel have profound effects on the patency of the blood vessel by adversely affecting vascular tone. They either increase vascular resistance (by decreasing the lumen diameter, and thus increasing resistance to blood flow, resulting in an increase in blood pressure), or decreasing vascular elasticity/compliance contributing to 'stiff vessels' or less compliant vessels (these vessels are unable to adjust to or accommodate an elevation in blood pressure, thus acting as an amplifier to the elevated blood pressure) (Mulvany., 1999).

Vascular remodelling was assessed in 2 major blood vessels viz. a portion of the aorta and a portion of the iliac artery. The results show that the SHR had a significantly thicker tunica media (in both the aorta and the iliac artery), than the normotensive control, this therefore suggests increased hypertrophy of the VSMC's. This is due to the higher plasma H_2O_2 levels displayed by the SHR as H_2O_2 has been shown by previous studies to increase VSMC's hypertrophy/proliferation (Li *et al.*, 1997; Brown *et al.*, 1999; Laurent *et al.*, 2001; Taniyama and Griendling., 2003). The source of the the H_2O_2 has been shown by previous studies and demonstrated in this study to be derived from O_2^- produced by the NADPH oxidase system, and the subsequent dismutation of this O_2^- by SOD. This therefore supports the concept that both O_2^- and H_2O_2 whose production is stimulated by Ang II are growth promoting and hypertrophic factors and that their existence is mutually dependent (Zafari *et al.*, 1998).

The increase in the thickness of the tunica media of the SHR manifests as a significantly greater W/L ratio in the aorta with no significant difference in total cross sectional area of the vessel, suggesting a smaller lumen diameter. According to the model provided by Mulvany, (1999) which has been adopted by this study, this can be classed as eutrophic inward remodelling. This type of remodelling would therefore result in an increase in vascular resistance and manifests as the significant increase in blood pressure that is shown by this model, as discussed previously. The significantly greater W/L ratio in the genetically hypertensive strain as compared to the normotensive strain is consistent with previous studies in these models. This suggests that the differences are due to the genetic predisposition of the model to an increased blood pressure and shear stress exposure, and are thus one of the initial contributing factors to the elevation in blood pressure of the SHR (Auch-Schwelk., 1989; Mulvany *et al*., 1996; Taniyama and Griendling., 2003). This is further supported by the SHR-weanling which had a significantly greater W/L ratio than the normotensive-weanling, with no significant difference in the thickness of the tunica media and a significantly smaller total cross sectional area (meaning a smaller vessel size at the same age). This demonstrates the genetically predisposed nature of this model to an increased peripheral resistance. This shows that the vascular remodelling is an early etiological factor that even precedes the elevation in blood pressure in the SHR and is thus an important initiatory contributor to the hypertension in the SHR.

The Wistar - Ang II infused group showed no significant changes in the tunica media thickness of the aorta, but a significant decrease in the W/L ratio with no significant change to the total cross sectional area. Interestingly this was not mirrored by the smaller iliac artery. This type of remodelling can be classed as eutrophic outward remodelling, and suggests an adjustment by this portion of the vasculature to compensate for the increased blood pressure and shear stress. It would be expected, that the vessel wall would increase in thickness, due to

the hypertrophic and inflammatory stimulation of the VSMC's, by Ang II and H_2O_2, as shown in previous studies **(**Griendling and Ushio-Fukai., 2000), but this is not evident here, and this finding requires further investigation.

The SHR - Ang II infused group, showed no significant change in the thickness of the media wall of both the aorta and the iliac artery, however there was a significant increase in the W/L ratio of the iliac artery. This increase in W/L ratio is due to the increase in the media wall thickness of the iliac artery. This change in the iliac artery can be classed as eutrophic inward remodelling due to there being no significant change in the total cross sectional area of the vessel. This suggests hypertrophy of the VSMC's due to Ang II and H_2O_2, resulting in a decrease in lumen diameter (Griendling and Ushio-Fukai., 2000). This decrease would result in an increased vascular resistance, and an increase in blood pressure. This decrease in lumen diameter is supported by the significant elevation in blood pressure shown in this model.

Vascular remodelling is the hallmark of essential hypertension, and acts to increase vascular resistance and thus exacerbates the elevation of blood pressure in the condition. It can thus be seen that the vasculature, plays an important hemodynamic role in hypertension, in the SHR and that these changes precedes the elevation in blood pressure. Over time, more changes to the vasculature dictated by factors such as Ang II and ROS amongst others, compounds and becomes maladaptive, and contributes via a mechanism that works in concert (i.e. increased vascular resistance) with the other established causative mechanisms in hypertension.

6. CONCLUSION AND RECOMMENDATIONS

This study has provided further evidence that the SHR is an excellent model for assessing the pathophysiological changes that are associated with essential hypertension. It has shown that the SHR has an increased level of oxidative stress, due to the reported O_2^- and H_2O_2 accumulation. Although the SHR responds to the oxidative stress by making compensatory adjustments to the major antioxidant enzymes viz. SOD, GPx, and catalase, these adjustments appear to be inadequate to deal with the increased free radical generating capacity of the model, resulting in a state of high level oxidative stress. This is confirmed by the H_2O_2 accumulation displayed in the model. This suggests that H_2O_2 accumulation and its resultant deleterious effects such as increased lipid peroxidation and vascular remodelling are some of the many independent mechanisms that are working together to contribute to the vicious cycle that is the hallmark of essential hypertension. This is further evidence to the accepted norm that essential hypertension is a multi-factorial disease condition.

Future studies should consider quantifying the plasma O_2^- levels directly. There are two reasons as to why this study did not do this, the first was primarily due to the protocol and equipment required for the analysis, and this was beyond the infrastructural capabilities of our laboratory and the second reason was due to the cost of the analysis. A direct measure of O_2^- combined with the NO and $OONO^-$ levels would be an excellent measure of the exact *in vivo* relationship of these important causative factors in essential hypertension in these models.

Another recommendation would be to assess the plasma SOD (ecSOD/SOD-3) levels rather than the RBC SOD (cytosolic Cu/Zn SOD – SOD-1) levels, as the antioxidant and free radical system is cell and compartment specific, and this would have been a better indicator of the level of this essential antioxidant enzyme. It will also be interesting to compare the

different compartments and how they react in this model to counter the established increased activity of the ROS, in specific compartments. This would enable one to demonstrate whether oxidative stress is compartment specific, and it can thus be shown whether; during oxidative stress in hypertension, specific compartments are targeted and affected.

Apart from the free radical related changes, NO appears to be a main target for the O_2^-. The deficiency of this important vasodilator, is thought to initially contribute to and thus essentially responsible for the elevation in blood pressure/decreased ability to reduce blood pressure in this model. The diminished NO levels compounded with the vascular remodelling noted in the model, due to the effects of the free radicals (especially O_2^- and H_2O_2 which causes VSMC's hypertrophy and proliferation respectively), add strong evidence of the link between oxidative stress and hypertension in the SHR. The free radicals are shown to bring about slow pressor changes, especially an increase in vascular resistance, and thus the ROS can be classed as long term pressor agents/factors.

The role of Ang II in hypertension has been actively researched and is hence well documented. This study has added further evidence to strengthen the hypothesis that Ang II derangement is deleterious with regards to its blood pressure elevating properties. The statement/idea that essential hypertension is intimately linked with oxidative stress is further strengthened by the role established in other studies and fortified in this study i.e. Ang II directly increases the production of free radicals, by stimulating the activity of the NADPH oxidase enzyme. The resultant oxidative stress potentiates other mechanisms that thereafter work in parallel with the classic pressor mechanisms of Ang II, to elevate blood pressure.

This study has also concluded that the SHR has a genetic predisposition to an increase in blood pressure due to an increased capacity to produce free radicals, and that this free radical production precedes the increase in blood pressure. This was deduced by comparing the adult

model to the weanling model, thus implicating the increase in the free radical generating capacity as being one of the possible causative factors in the elevation of blood pressure in this model. This study has also shown that vascular remodelling precedes the elevation in blood pressure in the SHR. These results fortifies the notion of the multifactorial nature of essential hypertension and provides justification for the call of therapeutic intervention in those who are predisposed to hypertension.

This study has also shown that sub-pressor doses of Ang II administered to a normotensive model (i.e. Wistar - Ang II infused group), causes an elevation in blood pressure, via the activation of the NADPH oxidase enzyme. The activation of this system has been shown to increase O_2^- production in the vasculature. This occurs concomitantly with a decrease in VSMC production of SOD. This results in specific derangements viz. decrease in NO bioavailability, increase in VSMC hypertrophy (vascular remodelling) and vasoconstriction, thus resulting in an elevation of blood pressure. Thus, by using a sub-pressor dose this study has provided evidence of an independent mechanism that acts to elevate blood pressure.

The SHR - Ang II infused group, further strengthened the hypothesis that the hypertension displayed in this model is intimately linked with oxidative stress. Interestingly with the added hypertensive stress of the sub-pressor doses of Ang II, the antioxidant system makes significant adjustments to counter the increase in free radical production; however these adjustments seem to be overwhelmed by the concomitant increase in free radical production. The Ang II infusion has shown that specific tissues become more receptive to the actions of Ang II when exposed to increased concentrations of the peptide, this is apparent from the AT-1a receptor expression, which showed significant increases in the infused groups. This suggests that Ang II displays a positive feedback with regards to the receptors, and thus potentiating its blood pressure elevating effect.

A recommendation would be to remove the exogenous source of Ang II after a period of infusion and then analyse the model after the Ang II levels have returned to normal *in vivo* levels. This is to determine whether the AT-1a receptor expression recovers to normal or whether they are maintained at the infused level. This would demonstrate whether exposure to increased levels of Ang II sensitises target tissue for a longer period than is necessary, thus increasing the blood pressure for longer than is required for homeostatic regulation. This is a very promising aspect of the study that needs to be further investigated, as a previous study demonstrated that models who have been exposed to exogenous sources of Ang II, to induce hypertension, become salt sensitive when the exogenous source has been removed and Ang II levels return to normal (Lombardie *et al.,* 1999). This thereby confirms that chronic/acute exposure to increased levels of Ang II *in vivo* causes long term derangements to the organs/systems responsible for regulating blood pressure. Thus the use of selective doses of Angiotensin II antagonists (AT 1 blockers) may play an important role to counter the sensitisation of target tissues.

This study has also concluded that the kidney of the SHR is a target organ for oxidative stress in essential hypertension. Although it is not necessarily the primary cause of the elevated blood pressure, the oxidative stress related damage and derangement contribute to decrease the kidneys natriuretic and diuretic role which would act to lower blood pressure. It can thus be said that the kidney is both a causative factor and a consequent victim of this free radical related disease.

A recommendation for future studies would be to compare different tissues involved in blood pressure control and regulation, and the tissue oxidative stress levels of these tissues to assess whether the free radical ‘attack’ is specific or indiscriminate. This would provide important information to help protect specific tissues/organs to decrease the derangement caused by the free radicals.

Another recommendation would be to assess the level of oxidative stress continuously over the entire study period by assessing the F_2-isoprostane levels in the urine. This would determine whether the lipid peroxidation increases with increasing blood pressure or whether the level remains constantly high during essential hypertension and thus attempt to correlate the rise in blood pressure and the level of *in vivo* oxidative stress.

The study has also concluded that vascular remodelling plays an important amplifier role in hypertension. It has also shown that the architectural changes in the vasculature precedes the development of hypertension, and is therefore an early etiological factor in the elevation of blood pressure. These vascular changes are also subsequently stimulated and thus amplified by Ang II and the ROS, and also play a pertinent role in maintaining the hypertension. This confirms the notion that vascular remodelling is the hallmark of hypertension and has both an early and an amplifier role to play in the process.

With these conclusions and recommendations for future studies this study has fortified the call for the use of antioxidant therapies in conjunction with currently administered medication in the treatment and management of essential hypertension. It has also provided justification for the call for the use of these therapies as a preventative measure in genetically susceptible individuals, due to the fact that the antioxidant intervention, targets more than just a single mechanism, and would thus aim to treat and attempt to address the multi-factorial nature of essential hypertension. The study has also provided evidence to the causative nature of Ang II in hypertension, and thus provided further reason for the use of Ang II antagonists and ACE inhibitors in the treatment and management of hypertension.

The study has also demonstrated the significant role played by Ang II in the SHR, and shown Ang II to be a causative factor in this animal model of essential hypertension. The study has also highlighted the fact that there are many blood pressure elevating mechanisms that work

in parallel in the SHR and thus strengthens the validity of the use of this model in investigating the pathophysiology of essential hypertension. It has also demonstrated the intimate link between Ang II and oxidative stress in this model, and shown that the pathology of essential hypertension in this model is due in large part to the so called “Unholy Alliance”, as it was referred to by Ritz and Haxsen, (2003), between Ang II and oxidative stress.

7. REFERENCES

Arora S., Chopra S.C., Gupta C. (2003). Free radicals and Antioxidants. *J Anaesth Clin Pharmacol.* 19(1): 15-32.

Auch-Achwelk A., Katusic Z.S., Vanhoutte P.M. (1989). Contractions of oxygen-derived free radicals are augmented in aorta of the SHR. *Hypertension.* 13: 859-864.

Badr K.F. and Abi-antoun T.E. (2005). Isoprostanes and the kidney. *Antioxidants and Redox Signalling.* 7: 236-243.

Bayraktutan U., Blayney L., Shah A.M. (2000). Molecular characterization and localization of the NAD(P)H oxidase components gp91-phox and p22-phox in endothelial cells. *Arterioscler Thromb Vasc Biol.* 20:1903–1911.

Beckman J.S., Chen J., Ischiropoulos H., Crow J.P. (1994). Oxidative chemistry of peroxynitrite. *Methods of Enzymology.* 229-240.

Berk B.C. (2001). Vascular smooth muscle growth: Autocrine growth mechanisms. *Physiological Reviews*. 81(3): 999-1030.

Bolterman R.J., Manriquez M.C., Ortix Ruiz M.C., Juncos L.A., Romero J.C., (2005). Effects of Captropril on the rennin-angiotensin system, oxidative stress and endothelin in normal and hypertensive rats. *Hypertension*. 46: 943.

Bouloumie A., Bauersachs J., Linz W., Schalkens B.W., Wiemer G., Fleming I., Busse R., (1997). Endothelial dysfunction coincides with an enhanced Nitric oxide synthase expression and superoxide anion production. *Hypertension*. 30: 934-941.

Brown M.R., Miller F.J. Jr., Li W.G., Ellingson A.N., Mozena J.D., Chatterjee P., Engelhardt J.F., Zwacka R.M., Oberley L.W., Fang X., Spector A.A., Weintraub N.L., (1999). Overexpression of human catalase inhibits proliferation and promotes apoptosis in vascular smooth muscle cells. *Circ Res.* 85: 524–533.

Cai H., (2005). Hydrogen peroxide regulation of endothelial function: origins, mechanisms, and consequences. *Cardiovasc Res.* 68: 26-36.

Cai H., Harrison D.G. (2000). Endothelial dysfunction in cardiovascular diseases. The rold of oxidant stress. *Circ Res.* 87: 840-844.

Carretero O.A and Oparil S., (2000). Essential Hypertension: Part I: Definition and Etiology. *Circulation.* 101: 329-335.

Conrad CH., (1995). Myocardial fibrosis and stiffness with hypertrophy and heart failure in the spontaneously hypertensive rat. *Circulation*. 9: 161–70.

Chabrashvili T., Tojo A., Onozato ML., Kitiyakara C., Quinn MT., Fujita T., Welch W.J., Wilcox C.S., (2002). Expression and cellular localization of classic NADPH oxidase subunits in the spontaneously hypertensive rat kidney. *Hypertension*. 39(2):269-74.

Crapo J.D., Dury T., Rabouille C., Slot J.W., Chang L.Y. (1992). Cu-Zn SOD is primarily a cytosolic protein in human cells. *Proc. Natl. Acad. Sci.* 89: 10405-10409.

De Champlain J., Wu R., Girouord H., Karas M., El Midaou A., Laplante M., Wu L. (2004). Oxidative stress in Hypertension. *Clinical and Experimental Hypertension.* 26(7-8): 593-601.

De la Torre R., Casado A., Lopez-Fernandez E., Carrascosa D., Ramirez V., Saez J., (1996). Overexpression of copper-zinc superoxide dismutase in trisomy 21. *Experientia.* **52:** 871–873.

Deshpande N.N., Sorescu D., Seshiah P., Ushio-Fukai M., Akers M., Yin Q., Griendling K.K. (2002). Mechanism of Hydrogen Peroxide-Induced Cell Cycle Arrest in Vascular Smooth Muscle. *Antioxidants & Redox Signaling.* 4(5): 845-854.

Dhalla N.S., Temsah R.M., Netticadan T., (2000). Role of oxidative stress in cardiovascular diseases. *J Hypertens.* 18: 655.

Diep Q.N., El Mabarouk M., Cohn J.S., Endemann D., Amiri F., Virdis A., Neves M.F., Schriffin E.L. (2002). Structure, endothelial function, cell growth and information in blood vessels of Ang II infused rats. *Circulation.* 105: 2296-2302.

Dimmeler S., Zeiher A.M., (2000). Reactive oxygen species and vascular cell apoptosis in response to angiotensin II and pro-atherosclerotic factors. *Regul Pept.* 90: 19–25.

Douglas J.G., Brown G.P., (1982). Effect of prolonged low dose infusion of Angiotensin II and aldosterone on rat smooth muscle and adrenal Angiotensin II receptors. *Endocrinology.* 111: 988-992.

Droge W., (2002). Free radicals in the Physiological Control of Cell Function. *Physiological Reviews.* 82: 47-95.

Ely D.L. and Turner M.E., (1990). Hypertension in the spontaneously hypertensive rat is linked to the Y chromosome. *Hypertension.*16: 277-281

Esterbauer H., Schaur R.J., Zollner H., (1991). Chemistry and Biochemistry of 4-Hydroxynonenal, Malonaldehyde and Related Aldehydes. *Free Rad. Biol. Med.* 11: 81-128.

Evans P., Halliwell B., (2001). Micronutrients: oxidant/antioxidant status. *British Journal of Nutrition.* 85: Suppl. 2, S67-S74.

Faraci F.M. and Didion S.P. (2004). Vascular protection: Superoxide dismutase isoforms in the vessel wall. *Arteriolar Thromb Vasc Biol.* 24: 1367-1373.

Forman H.J., Torres M., (2002). Reactive oxygen species and cell signaling: respiratory burst in macrophage signaling. *Am J Respir Crit Care Med.* 166: S4–S8.

Frank S., Kampfer H., Podda M., Kaufmann R., Pfeilschifter J. (2000). Identification of copper/zinc superoxide dismutase as a nitric oxide-regulated gene in human (HaCaT) keratinocytes: implications for keratinocyte proliferation. *Biochem. J.* 346(3):719–728.

Fukui T., Ishizaka N., Rajagopalan S., Laursen S.B., Copers Q IV., Taylor W.R., Harrison D.G., de Leon H., Wilcox J.N., Griendling K.K. (1997). p22phox mRNA expression and NADPH oxidase activity are increased in aorta's from hypertensive rats. *Circ Res*. 80: 45-51.

Fukui T., Siegfried M.R., Ushio-Fukai M., Griendling K.K., Harrison D.G. (1999). Modulation of extracellular superoxide dismutase expression by Ang II and Hypertension. *Circ Res.* 85: 23-28.

Girotti A.W., (1998). Lipid hydroperoxide generation, turnover, and effector action in biological systems. *J Lipid Research.* 39: 1529-1542.

Goth L. (1991). A simple method for determination of serum catalase activity and revision of reference range. *Clinica Chimica Acta.* 196: 143-152.

Goth L., Eaton J.W., (2000). Hereditary catalase deficiencies and increased risk of diabetes. *Lancet.* 356: 1820-1821.

Griendling K.K., Minieri C.A., Ollerenshaw J.D., Alexander R.W., (1994). Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res.* 74: 1141-1148.

Griendling K.K., Ushio-Fukai M., (2000). Reactive oxygen species as mediators of angiotensin II signaling. *Reg Peptides*. 91: 21–27.

Halliwell B, Gutteridge J.C., Cross C.E., (1992). Free radicals, antioxidants, and human disease: Where are we now?. *J Lab Clin Med.* 119: 598.

Halliwell B. (1994). Free radicals, Antioxidants and human disease: Curosity, Cause or Consequence. *The Lancet.* 344: 721-724.

Halliwell B., (1996). Antioxidants in human health and disease. *Annu Rev Nutr.* 16: 33.

Halliwell B., Gutteridge J.C., (1989). Free Radicals in Biology and Medicine, 2nd ed. Oxford, UK. Clarendon Press.

Halliwell B., Gutteridge J.C., (1999). Free Radicals in Biology and Medicine, 3rd ed. London, England. Oxford University Press.

Hemnani T., Parihar M.S., (1998). Reactive oxygen species and oxidative DNA damage. *Indian J Physiol Pharmacol.* 42: 440-452.

Houston M.C., (2005). Nutraceuticals, vitamins, antioxidants, and minerals in prevention and treatment of hypertension. *Prog Cardiovasc Dis.* 47: 396-449.

Hyoudou K., Nishikawa M., Kobayashi Y., Umeyama Y., Yamashita F., Hashida M., (2006). PEGylated catalase prevents metastatic tumor growth aggravated by tumor removal. *Free Rad Biol Med.* 41: 1449-1458.

Irani K., (2000). Oxidant signaling in vascular cell growth, death, and survival: a review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. *Circ Res*. 87: 179–183.

Ito M., Oliverio M.I., Mannon P.J., Best C.F., Maeda N., Smithies O., Coffman T.M. (1995). Regulation of bloop pressure by the type 1a Angiotensin II receptor gene. *Proc. Natl. Acad. Sci. USA.* 92: 3521-3525.

Jackson R.M., Parish G., Ho Y.S. (1996). Effects of hypoxia on expression of superoxide dismutases in cultured ATII cells and lung fibroblasts. *Am. J. Physiol.* 271:L955–962.

Janicka M., Kot-Wasik A., Kot J., Namieśnik J. (2010). Isoprostanes-biomarkers of lipid peroxidation: Their utility in evaluating oxidative stress and analysis. *Int J Mol Sci*. 11: 4631-4659.

Jones D.P. (2002). Redox potential of GSH/GSSG couple: assay and biological significance. *Methods Enzymol.* 348: 98-112.

Jones S.A., O'Donnell V.B., Wood J.D., Broughton J.P., Hugher E.J., Jones O.T., (1996). Expression of phagocyte NADPH oxidase components in human endothelial cells. *Am J Physiol.* 271: H1626-H1634.

Karlsson, J. (1997). Introduction to Nutraology and Radical Formation. In: Antioxidants and Exercise. Human Kinetics Press., Illinois. P: 1-143.

Kim S. and Iwao H. (2000). Molecular and cellular mechanisms of Angiotensin II mediated cardiovascular and renal diseases. *Pharmacological Reviews*. 52: 11-34.

Kirby R.F., Callahan M.F., McCarty R., Johnson A.K. (1989). Cardiovascular and sympathetic nervous system responses to an acute stressor in borderline hypertensive rats (BHR). *Physio and Behavior*. 46(2): 309-313.

Kirkman H.N., Gaetani G.F. (2006). Mammalian catalase: A venerable enzyme with new mysteries. *Trends in Biochem Sc.* 32(1): 44-50.

Kitiyakara C., Wilcox C.S., (1998). Antioxidants for Hypertension. *Curr Opin Nephrol Hypertension.* 7: 531-538.

Lacy F., Kailasam M.T., O'Connor D.T., Schmid-Schönbein G.W., Parmer R.J., (2000). Plasma hydrogen peroxide in human essential hypertension. *Hypertension.* 36: 878.

Lassegue B., Alexander R.W., Nickenig G., Clark M., Murphy T.J., Griendling K.K., (1995). Angiotensin II down regulates the vascular smooth muscle AT1 receptor by transcriptional and posttranscriptional mechanisms: evidence for homologous and heterologous regulation. *Mol Pharmacol.* 48: 601-609.

Lassegue B., Clempus R.E. (2003). Vascular NAD(P)H oxidases: specific features, expression, and regulation. *Am J Physiol Regul Integr Comp Physiol.* 285:R277–R297.

Laude K., Cai H., Fink B., Hoch N., Weber D.S., McCann L., Kojda G., Fukai T., Schmidt H.H., Dikalov S., Ramasamy S., Gamez G., Griendling K.K., Harrison D.G., (2005). Hemodynamic and biochemical adaptations to vascular smooth muscle overexpression of p22phox in mice. *Am J Physiol. Heart Circ Physiol:* . 288: H7-H12.

Laurent S., Boutouyrie P., Lacolly P. (2001). Structural and genetic basis of arterial stiffness. *Hypertension*. 45: 1050-1055.

Laursen J.B., Rajagopalan S., Galis Z., Tarpey M., Freeman B.A., Harrison D.G. (1997). Role of superoxide in Angiotensin II-induced but not catecholamine-induced hypertension. *Circulation*. 95: 588-593.

Le T.H., Kim H.S., Allen A.M., Spurney R.F., Smithies O., Coffmann T.M. (2003). Physiological impact of increased expression of AT 1 angiotensin II receptors. *Hypertension*. 42: 507-514.

Lenkei Z., Palkovits M., Carvol P., Llorens-Cutés C. (1997). Expression of Angiotensin type-1 (AT1) and type-2 (AT2) receptor mRNA's in the adult rat brain: A functional neuroanatomical Review. *Frontiers in Neuroendocrinology*. 1: 383-439.

Lerman L.O., Chade A.R., Sica V., Napoli C., (2005). Review Article: Animal Models of hypertension: An overview. *J Lab Clin Med.* 146: 160-173.

Li A.E., Ito H., Rovira I.I., Kim K.S., Takeda K., Yu Z.Y., Ferrans V.J., Finkel T., (1999). A role for reactive oxygen species in endothelial cell anoikis. *Circ Res.* 85: 304–310.

Li J.M., Shah A.M. (2002). Intracellular localization and preassembly of the NAD(P)H oxidase complex in cultured endothelial cells. *J Biol Chem* 277:19952–19960.

Li P.F., Dietz R., von Harsdorf R., (1997). Reactive oxygen species induce apoptosis of vascular smooth muscle cell. *Fed. Euro. Biochem. Soc. (FEBS).* 404: 249-252.

Li Y., Huang T.T., Carlson E.J., Melov S., Ursell P.C., Olson J.L., Noble L.J., Yoshimura M.P., Berger C., Chan P.H., Wallace D.C., Epstein C.J., (1995). Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet.* 11: 376–381.

Lifton R.P., (1995). Genetic determinants of human hypertension. *Proc Natl Acad Sci USA.* 92: 8545-8551.

Lifton R.P., (1996). Molecular genetics of human blood pressure variation. *Science.* 272: 676-680.

Liu J., Yeo H.C., Overvik-Douki E., Hagen., Doniger S.J., Chu D.W., Brooks G.A., Ames B.N., (2000). Chronically and acutely excercised rats: biomarkers of oxidative stress and endogenous antioxidants. *J Appl Physiol.* 89: 21-28.

Lombardie D., Gordan K.L., Polinsky P., Suga S., Schwartz S.M., Johnson R.J., (1999). Salt-sensitive hypertension develops after short-term exposure to Angiotensin II. *Hypertension.* 33(4):1013-9.

Luft F.C. (1998). Molecular genetics of human hypertension. *J. Hypertension*. 16: 1871-1878.

Machado B.H., Salgado H.C. (1990). Intrinsic heart rate after infusion of Angiotensin II in rats with sino-aortic deafferentation. *Braz J Med Biol Res.* 23(3-4): 337-341.

Marklund S.L., Holme E., Hellner L., (1982). Superoxide dismutase in extracellular fluids. *Clin Chim Acta.* 126: 41–51.

Matsushima Y., Kwamura M., Akabane S., Imanishi M., Kuramochi M., Itoi K., Omar T. (1988). Increases in renal Angiotensin II content and tubular Angiotensin II receptors in prehypertensive SHR's. *J Hypertension*. 6(10): 791-796.

Maulik N., Das D.K., (2002). Redox signaling in vascular angiogenesis. *Free Radic Biol Med.* 33: 1047–1060.

McGee S. A., Wiggins S. A., Pierce J.D. (2003). What Advanced Practice Nurses Need To Know About Free Radicals . *The Internet Journal of Advanced Nursing Practice.* 6: 1.

McIntyre M., Bohr D.F., Dominiczak., (1999). Endothelial Function in Hypertension: The Role of Superoxide Anion. Brief Review. *Hypertension.* 34: 539-545.

Meng S., Cason G.W., Gannon A.W., Racusen L.C., Manning R.D. Jr., (2003). Oxidative Stress in Dahl Salt-Sensitive Hypertension. *Hypertension.* 41:1346.

Messerli F.H and Williams B., (2007). Essential Hypertension. Lancet. 370: 591-603.

Miller A.A., Drummond G.R., Sobey C.G. (2006). Novel isoforms of NADPH-oxidase in cerebral vascular control. *Pharmacol Ther.* 111:928–948.

Mohazzab K.M., Kaminski P.M., Wolin M.S., (1994). NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. *Am J Physiol*, 266: H2568-H2572.

Moritz F., Montiel C., Isabelle M., Bauer F., Renet S., Mulder P., Richard V., Thuillez C., (2003). Role of reactive oxygen species in cocaine-induced cardiac dysfunction. *Cardiovascular Research.* 29: 834-843.

Morrison R.A., McGrath A., Davidson G., Brown T.J., Murray G.D., Lever A.F. (1996). Low blood pressure in Downs Syndrome: A link with Alzheimers disease? *Hypertension.* 28: 569-575.

Morrow J.D., Awad J.A., Boss H.J., Blair J.A., and Roberts L.J. II. (1992). Non-cyclooxygenase derived prostanoids (F_2-isoprostanes) are formed in situ on phospholipids. *Proc. Natl. Acad. Sci. USA.* 89: 10721–10725

Morrow J.D., Minton T.A., Mukundan C.R., Campbell M.D., Zackert W.E., Daniel V.C., Badr K.F., Blair I.A., Roberts L.J. II. (1994). Free radical-induced generation of isoprostanes in vivo: evidence for the formation of D-ring and E-ring isoprostanes. *J. Biol. Chem*. 269: 4317–4326.

Mulvany M.J., Baumbach G.L., Aalkjaer C., Heagerty A.M., Korsgaard N., Schiffrin E.L., Heistad D.D., (1996). Vascular remodeling. *Hypertension*. 28: 505–506.

Mulvany M.J., (1999). Review: Vascular remodelling of resistance vessels: can we define this? *Cardiov Res.* 41: 9-13.

Naito Y., Tsujino T., Fujioka Y., Ohyanagi M., Iwasaki T. (2002). Augmented diurnal variations of cardiac rennin-angiotensin system in hypertensive rats. *Hypertension.* 40: 827-833.

Nakazono K., Watanabe N., Matsuno K., Sasaki J., Sato T., Inoue M., (1991). Does superoxide underlie the pathogenesis of hypertension? *Proc. Natl. Acad. Sci. USA.* 88: 10045-10048.

Nickenig G., Harrison D.G., (2002). The AT1-Type Angiotensin receptor in oxidative stress and Atherogenesis: Part II: AT 1 Receptor regulation. *Circulation.* 105: 530-536.

Nishikawa M., Hashida M., Takakura Y., (2009). Catalase delivery for inhibiting ROS-mediated tissue injury and tumor metastasis. *Adv Drug Delivery Rev.* 61: 319-326.

Okamoto AK., (1963). Development of a strain of spontaneously hypertensive rat. *Jap Circ J*. 27: 282–293.

Onusko E. (2003). Diagnosing secondary hypertension. *Am Fam Physician.* 67(1): 67-74.

Oury T.D , Day B.J., Crapo J.D., (1996). Extracellular superoxide dismutase: a regulator of nitric oxide bioavailability. *Lab Invest.* 75: 617–636.

Pagano P., Ito Y., Tornheim K., Gallop P., Tauber A., Cohen R., (1995). An NADPH oxidase superoxide-generating system in the rabbit aorta. *Am J Physiol.* 268: H2274-H2280.

Paglia D.E., Valentine W.N., (1967). Studies on the quantitative and qualitative characterisation of erythrocyte glutathione peroxidise. *J Lab Clin Med.* 70: 158.

Parati G., Di Rienzo M., Ulian L., Santucciu C., Girard A., Elghozi J.L., Mancia G. (1998). Clinical relevance of blood pressure variability. *J Hypertension.* 16(S3): 525-533.

Paravicini T.M., Touyz R.M. (2008). NADPH oxidases, reactive oxygen species and hypertension. *Diabetes Care*. 31(52): 5170-5180.

Perou C.M., Jeffery S.S., van de Rijn M., Rees C.A., Eisen M.B., Ross D.T., Pergamenschikov A., Williams C.F., Zhu S.X., Lee J.C.F., Lashkari D., Shalon D., Brown P.O., Botstein D. (1999). Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. *PNAS.* 16: 9212-9217.

Pinto Y.M., Paul M., Ganten D., (1998). Lessons from rat models of hypertension: from Goldblatt to genetic engineering. *Cardiovasc Res*. 39(1):77– 88.

Popova M.P., Popov C.S., (2002). Damage to Subcellular structures evoked by lipid Peroxidation. *Z. Naturforsch.* 57c: 361-365.

Pradhan D., Weiser M., Lunley-Sapanski K., Frazier D., Kemper S., Williamson P., Schlegel R.A., (1990). Peroxidation-induced perturbations of erythrocyte lipid organisation. *Biochem. Biophys. Acta. Biomembranes.* 1023: 398-404.

Pueyo M.E., Arnal J.F., Rami J., Michel J.B., (1998). Angiotensin II stimulates the production of NO and peroxynitrite in endothelial cells. *Am J Physiol.* 274: C214–C220.

Raij L., (1998). Nitric Oxide in Hypertension: Relationship with renal injury and left ventricular hypertrophy. *Hypertension.* 31: 189.

Rajagopalan S., Kurz S., Műnzel T., Tarpey M., Freeman B.A., Griendling K.K., Harrison D.G. (1996). Angiotensin II – mediated hypertension in the rat increase vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations in vascular tone. *J. Clin. Invest.* 97(8): 1916-1923.

Rao G.N., Berk B.C. (1992). Active oxygen species stimulate vascular smooth muscle growth and proto-oncogene expression. *Circ Res.* 70: 593-599.

Reckelhoff J.F., Zhang H., Srivastava K., Roberts L.J. II., Morrow J.D., Romero J.C. (2000). Sub-pressor doses of Ang II increase plasma F_2-isoprostanes in rats. *Hypertension.* 35: 476.

Redón J., Oliva M.R., Tormos C., Giner V., Chaves J., Iradi A., Sáez G.T., (2003). Antioxidant Activities and Oxidative Stress Byproducts in Human Hypertension. *Hypertension.* 41:1096-1101.

Reimer D.L., Bailley J., Singh S.M., (1994). Complete cDNA and 5' genomic sequences and multilevel regulation of the mouse catalase gene. *Genomics.* 21: 325-336.

Ritz E., Haxsen V., (2003). Angiotensin II and Oxidative Stress: An Unholy Alliance. *J Am Soc Nephrol*. 14: 2985-2987.

Roberts I.J., Morrow J.D., (2000). Measurement of F(2)-isoprostanes as an index of oxidative stress in vivo. *Free Rad. Biol. Med.* 28: 505-513.

Rojas A., Figueroa H., Re L., Maraui M.A. (2006). Oxidative stress at the vascular wall: Mechanistic and Pharmacological Aspects. *Archives of Med Res.* 32: 436-448.

Romero J.C., Reckelhoff J.F. (1999). Role of Angiotensin II and oxidative stress in essential hypertension. *Hypertension.* 34: 943-949.

Schnackenberg C.G., (2002). Physiological and pathophysiological role of oxygen radicals in the renal microvasculature. *Am J Physiol Rugul Integr Comp Physiol.* 282: 335-342.

Schnackenberg C.G., Welch W.J., Wilcox C.S. (1998). Normalization of blood pressure and renal vascular resistance in spontaneously hypertensive rats with a membrane-permeable SOD mimetic: Role of nitric oxide. *Hypertension.* 32(1): 59-64.

Schiffrin E.L., Gutkowska J., Genest J., (1984). Effect of Angiotensin II and deoxycorticosterone infusion on vascular Angiotensin II receptors in rats. *Am J Physiol.* 246: 608-614.

Sealey J,E., Laragh J.H., (1995). The Renin-Angiotensin-Aldosterone system for normal regulation of blood pressure and sodium and potassium homeostasis. *Hypertension: Pathophysio, Diagnosis and Management.* 2nd Ed. Chapter 105. Raven Press Ltd. New York.

Schiffrin E.L., Gutkowska J., Geneset J., (1984). Effect of Angiotensin II and deoxycorticosterone infusion on vascular Angiotensin II receptors in rats. *Am J Physiology.* 246: 608-614.

Sernia C., (2001). A critical appraisal of the intrinsic pancreatic Angiotensin-generating system. *J.Pancreas (Online).* 2(1): 50-55.

Seshiah P.N., Weber D.S., Rocic P., Valppu L., Taniyama Y., Griendling K.K. (2002). Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res.* 91:406–413.

Shimokawa H., Matoba T. (2004). Hydrogen peroxide as an endothelium-derived hyperpolarizing factor. *Pharmacol. Res.* 49: 543-549.

Sies H. (1997). Oxidative stress: Oxidants and Antioxidants. *Experimental Physiology.* 82: 291-295.

Simon G., Abraham G., Cserep G. (1995). Pressor and sub-pressor Angiotensin II administration. Two experimental models of hypertension. *Am J Hypertension*. 8: 645-650.

Simon G., Illyes G., Csiky B., (1998). Structural Vascular Changes in Hypertension – Role of Angiotensin II, Dietary Sodium supplementation, Blood pressure and Time. *Hypertension.* 32: 654-660.

Simonsen U., Christensen F.H., Buus N.H. (2009). The effect of tempol on endothelium-dependant vasodialation and blood pressure. *Pharmacology and Therapeutics.* 122: 109-124.

Somova L.I., Khan M.S., Chetty S., (1998). Stress and salt intake, experimental data on Dahl Salt Resistant and Dahl Salt Sensitive rats. *Stress Medicine*. 14: 125-134.

Stocker R., Keaney J.F. Jr., (2004). Role of Oxidative Modifications in Atherosclerosis. *Physiol. Rev.* 84: 1381-1478.

Suzuki J., Matsubaru H., Urakami M., Inada M., (1993). Rat Angiotensin II (type 1a) receptor mRNA regulation and subtype expression in myocardial growth and hypertrophy. *Circ. Res*. 73: 439-447.

Swei A., Lacy F., DeLano F.A., Schmid-Schonbein G.W., (1997). Oxidative stress in the Dahl Hypertensive Rat. *Hypertension.* 30: 1628-1633.

Takeya R., Sumimoto H. (2006). Regulation of novel superoxide-producing NAD(P)H oxidases. *Antioxid Redox Signal.* 8:1523– 1532.

Taniyama Y., Griendling K.K., (2003). Reactive Oxygen Species in the Vasculature: Molecular and Cellular Mechanisms. *Hypertension.* 42: 1075.

Tarpey M.M., Wink D.A., Grisham M.B., (2004). Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. *Am J Physiol Regul Integr Comp Physiol.* 286: R431-R444.

Tepel M., (2003). Oxidative stress: does it play a role in the genesis of essential hypertension and hypertension of uraemia. *Nephrol Dial Transplant.* 18: 1439-1442.

Timmermans P.B.M.W.M., Wong P.C., Chiu A.T., Herblin W.F., Benfield P., Carini D.J., Lee R.J., Wexter R., Saye J., Smith R. (1993). Angiotensin II receptors and Angiotensin II receptor agonists. *Pharmacol Rev.* 45:205-251.

Touyz R.M. (2003). Reactive oxygen species in vascular biology: Role in arterial hypertension. *Expert Rev. Cardiovasc. Ther.* 1: 91-106.

Touyz R.M. (2004). Vascular oxidative stress and Redox Signaling in hypertension: What is the clinical significance. *Hypertension*. 44: 248.

Touyz R.M., Cruzado M., Tabet F., Yao G., Saloman S., Schiffrin E.L. (2003). Redox-dependant MAP kinase signalling by Angiotensin II in vascular smooth muscle cells: Role of receptor tyrosine kinase transactivation. *Can J Physiol Pharmacol.* 81: 159-167.

Turrens J. F., (2003). Mitochondrial formation of reactive oxygen species. *J Physiol.* 552.2: 335-344.

Unlap M.T., Bates E., Williams C., Komlosi P., Williams I., Kovacs G., Siroky., Bell P.D., (2003). Na^{+}/Ca^{2+} Exchanger – Target for oxidative stress in salt-sensitive hypertension. *Hypertension.* 42: 363-368.

Ushio-Fukai M., Zafari A.M., Fukui T., Ishizaka N., Griendling K.K. (1996). $p22^{phox}$ is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates Angiotensin II induced hypertrophy in vascular smooth muscle cells. *J Biol Chem*. 271: 23317-23321.

Ushio-Fukai M., Alexander R.W., Akers M., Yin Q., Fijio Y., Walsk., Griendling K.K. (1999). Reactive oxygen species mediate the activation of Akt/protein kinase B by Angiotensin II in vascular smooth muscle cells. *J Biol Chem.* 274: 22699-22704.

van Linthout S., Spillman F., Riad A., Trimpert C., Lievens J., Meloni M., Escher F., Filenberg G., Demir O., Li J., Shakibaei M., Schimke I., Stoudt A., Felix S.B., Schultheiss H-P., de Geest B., Tschope C. (2008). Human Apolipoprotein A-I gene transfer reduces the development of experimental diabetic cardiomyopathy. *Circulation.* 117: 1563-1573.

Wang C., Salahudeen A.K. (1995). Lipid peroxidation accompanies cyclosporine nephrotoxicity: effects of vitamin E. *Kidney Int*. 49: 927–934.

Wassmann S., Wassmann K., Nickenig G. (2004). Modulation of oxidant and antioxidant enzyme expression and function in vascular cells. *Hypertension.* 44: 381.

Wilcox C.S. (2002). Reactive Oxygen Species: Roles in blood pressure and kidney function. *Hypertension.* 4:160–166.

World Health Organisation, International Society of Hypertension Writing Group. (2003). WHO/International Society of Hypertension (ISH) statement on hypertension. *J Hypertension*. 21: 1983-1992.

Yamori Y., (1999). Implication of hypertensive rat models for primordial nutritional prevention of cardiovascular diseases. *Clin. Exp. Pharmacol. Physiol.* 26: 568-572.

Yarom R., Sapoznikov D., Havivi Y., Avraham K.B., Schickler M., Groner Y., (1988). Premature aging changes in neuromuscular junctions of transgenic mice with an extra human CuZnSOD gene: a model for tongue pathology in Down's syndrome. *J Neurol Sci.* 88: 41–53.

Yoo H.Y., Chang M.S., Rho H.M. (1999a). The activation of the rat copper/zinc superoxide dismutase gene by hydrogen peroxide through the hydrogen peroxide-responsive element and by paraquat and heat shock through the same heat shock element. *J. Biol. Chem.* 274: 23887–23892.

Yoo H.Y., Chang M.S., Rho H.M. (1999b). Induction of the rat Cu/Zn superoxide dismutase gene through the peroxisome proliferatorresponsive element by arachidonic acid. *Gene.* 234:87–91.

Yu B.P., (1994). Cellular Defenses against Damage from Reactive Oxygen Species. *Physiol Rev.* 74: 139-160.

Zafari A.M., Ushio-Fukai M., Akers M., Yin Q., Shah A., Harrison D.G., Taylor R.W., Griendling K.K., (1998). Role of NADH/NADPH Oxidase–Derived H2O2 in Angiotensin II–Induced Vascular Hypertrophy. *Hypertension.* 32: 488-495.

Zalba G., Beaumont F.J., San José G., Fortuńo A., Fortuńo M.A., Etayo J.C., Diez J. (2000). Vascular NADH/NADPH oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats. *Hypertension.* 35: 1055.

Zalba G., San José G., Moreno M.U., Fortuńo M.A., Fortuńo A., Beaumont F.J., Diez J. (2001). Oxidative stress in arterial hypertension: Role of NADPH oxidase. *Hypertension.* 38: 1395-1399.

Zelko I.N., Mariani T.J., Folz R.J. (2002). SOD multigene family: A comparison of the CuZn-SOD (SOD-1), Mn-SOD (SOD-2) and EC-SOD (SOD-3) gene structures, evolution and expression. *Free Radical Biology and Medicine*. 33: 337-349.

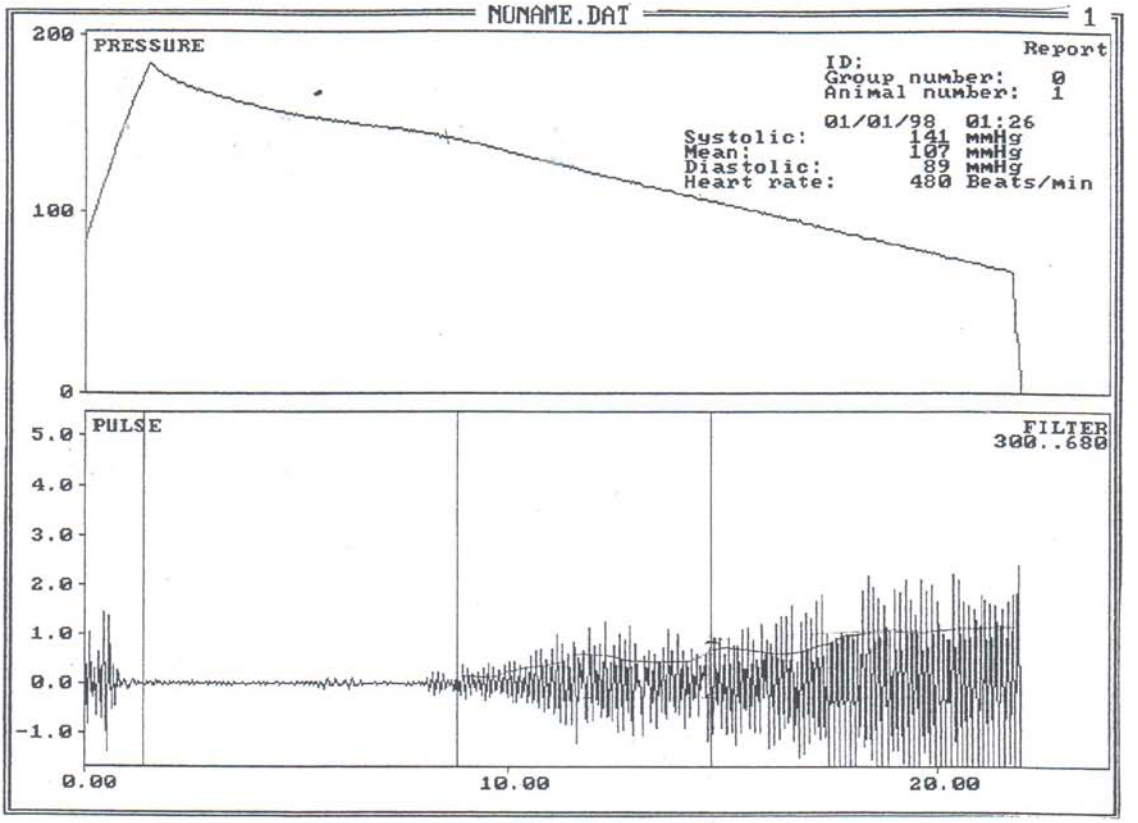
8. APPENDIX

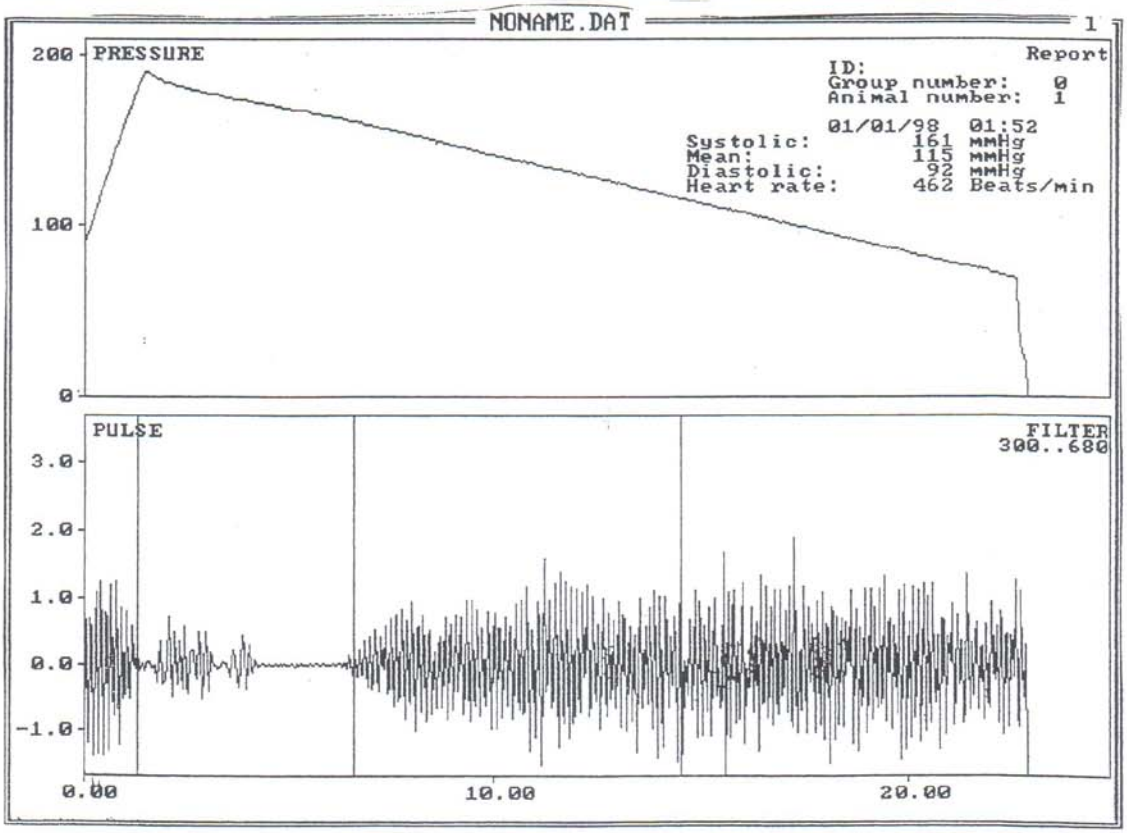
Appendix 1 - Sample of Blood Pressure Recordings

Appendix 2 - Standard Curves

Appendix 1

Sample of Blood Pressure Recordings





Appendix 2

Standard Curves

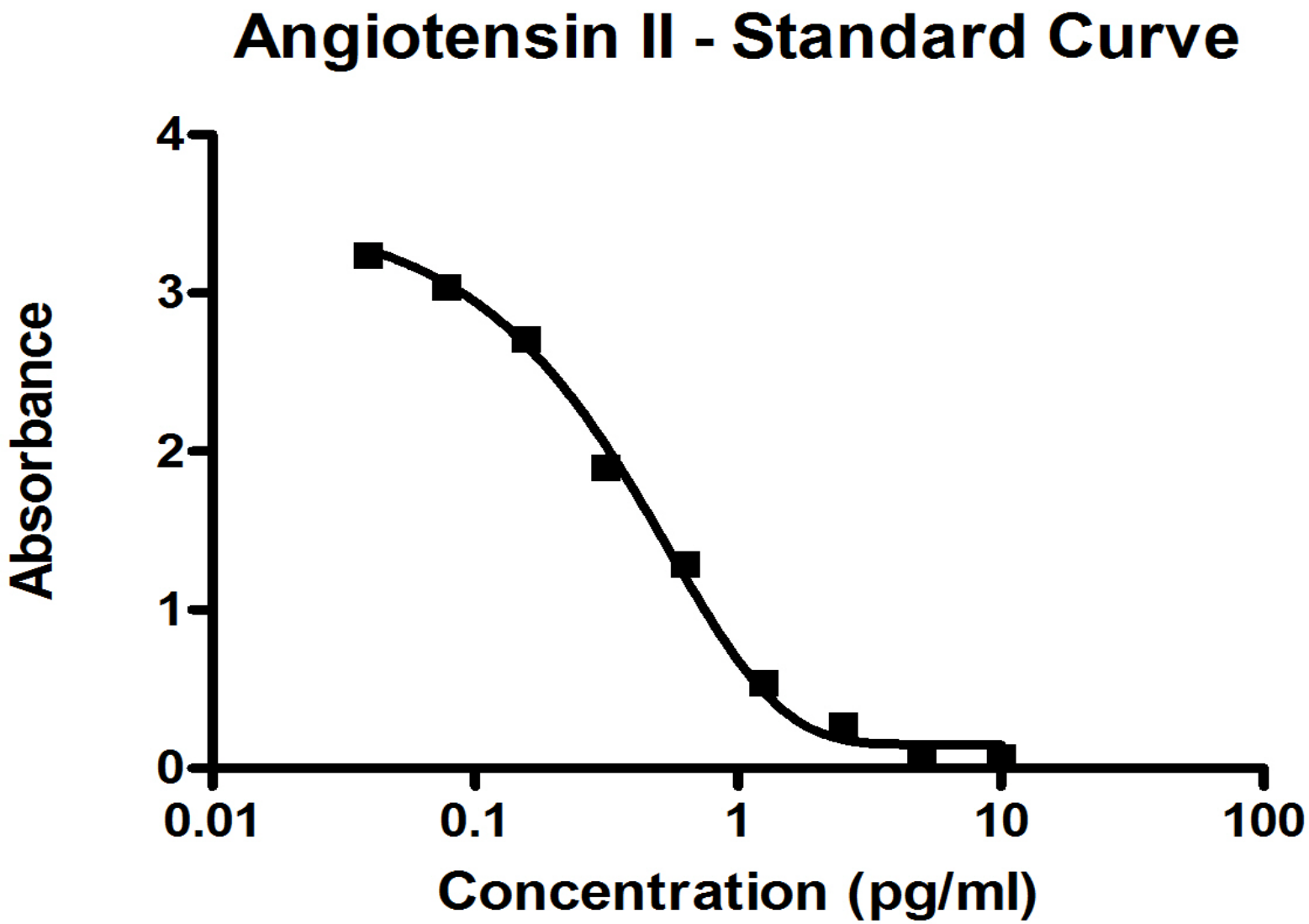
Angiotensin II

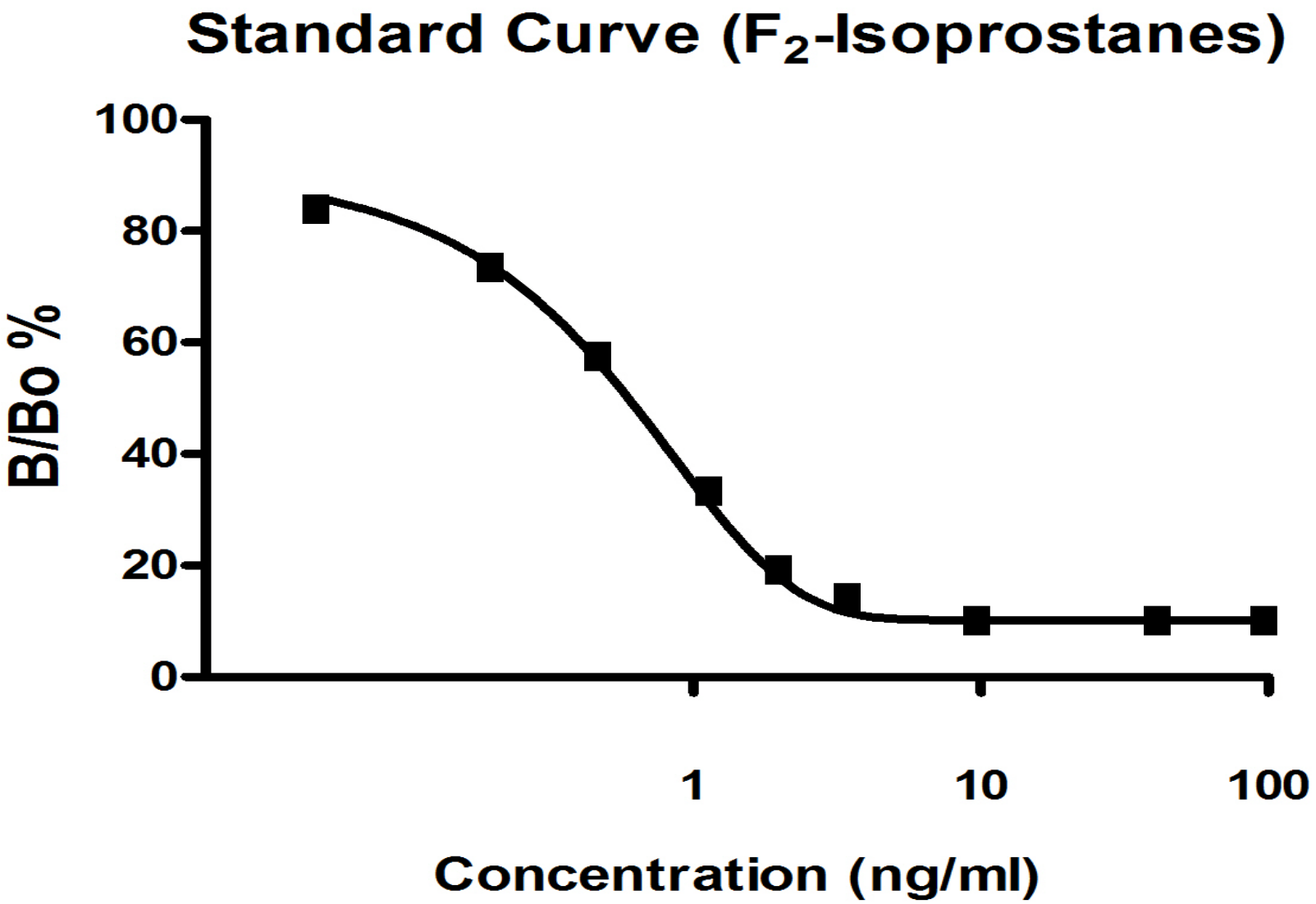
F_2-isoprostanes

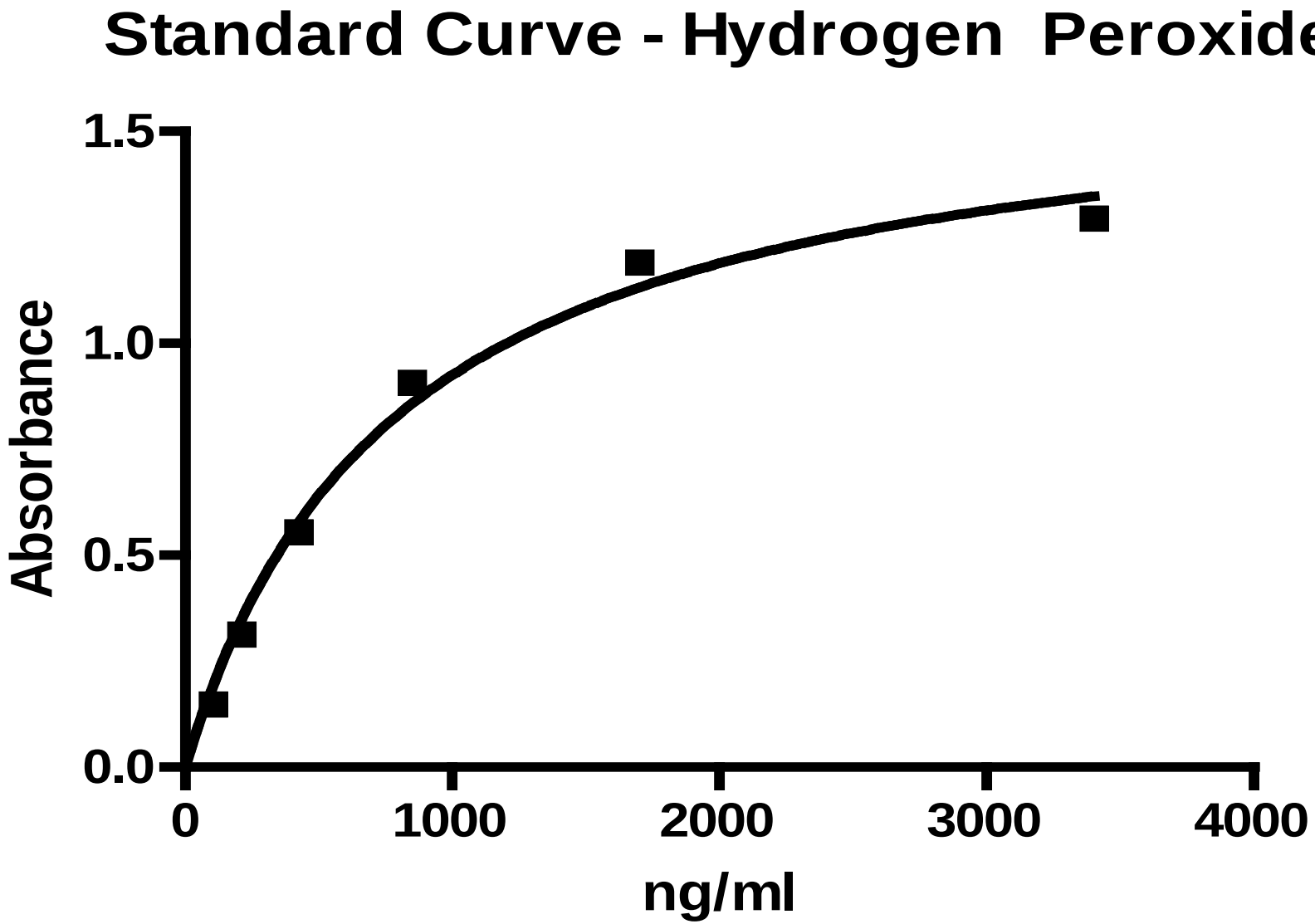
Hydrogen Peroxide

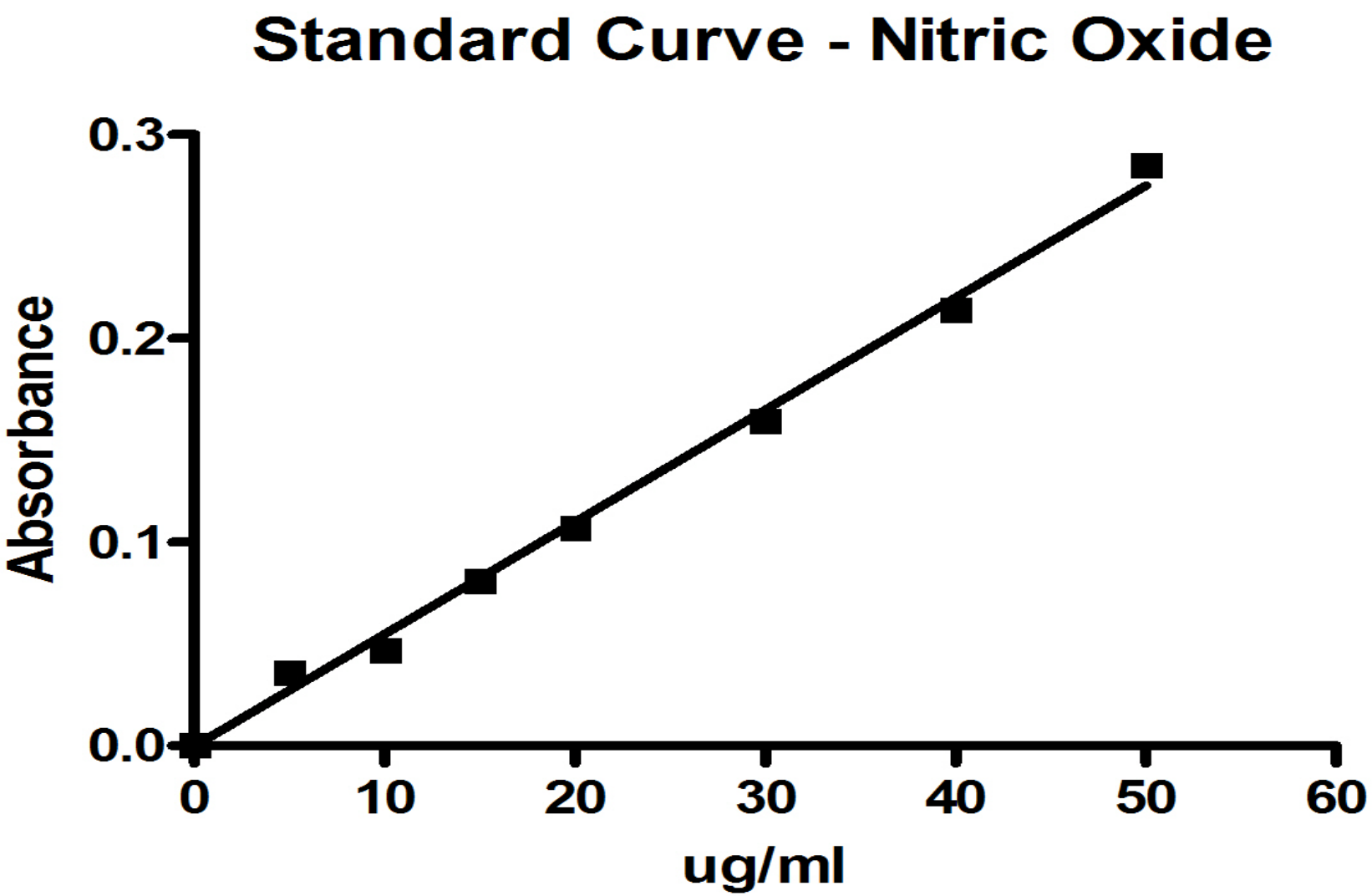
Nitric Oxide

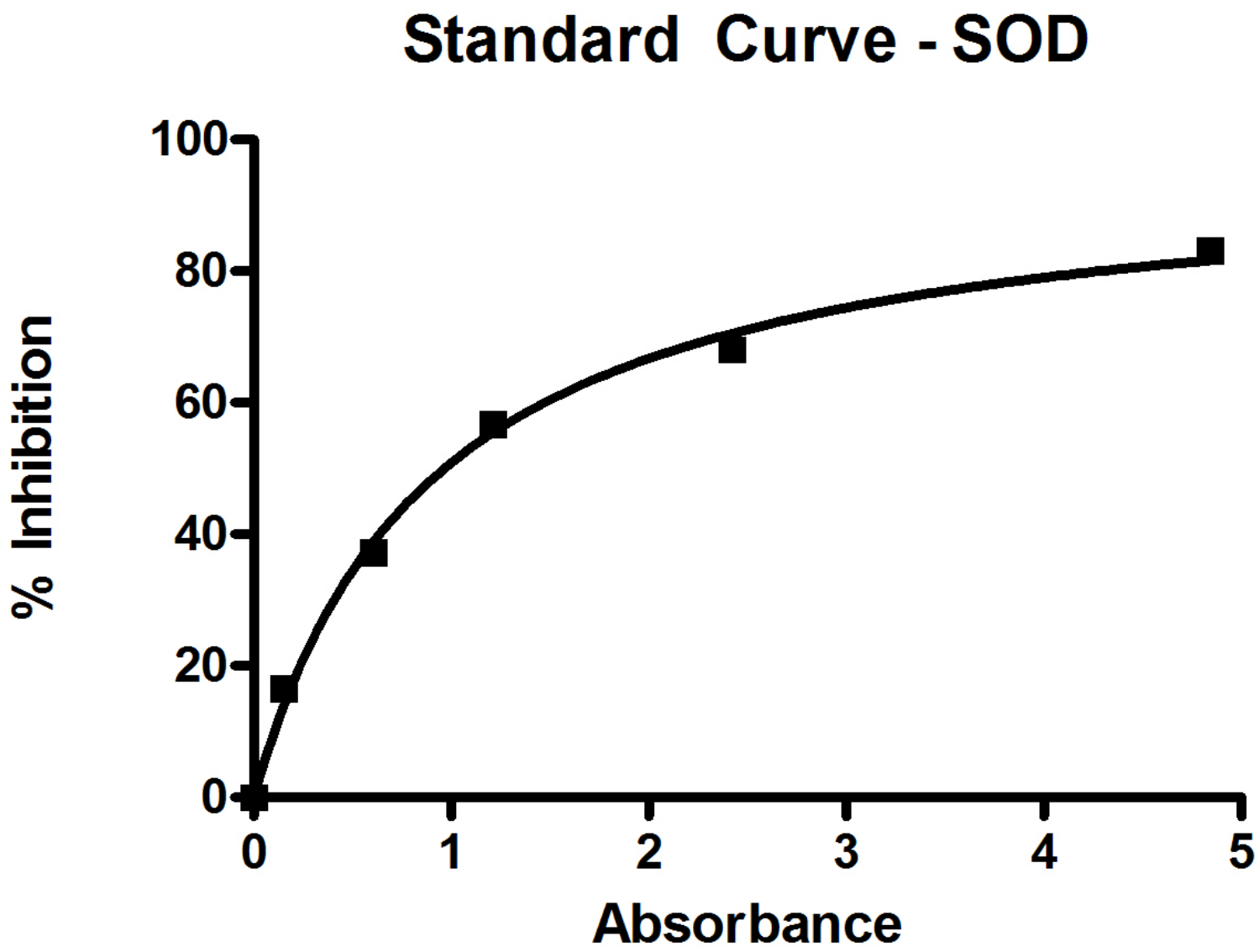
Superoxide Dismutase


Angiotensin II - Standard Curve
Absorbance
4
3
2
1
0
0.01
0.1
1
10
100
Concentration (pg/ml)


Standard Curve (F_2-Isoprostanes)
B/Bo %
100
80
60
40
20
0
1
10
100
Concentration (ng/ml)


Standard Curve - Hydrogen Peroxide
Absorbance
1.5
1.0
0.5
0.0
0
1000
2000
3000
4000
ng/ml


Standard Curve - Nitric Oxide
Absorbance
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
ug/ml


Standard Curve - SOD
% Inhibition
100
80
60
40
20
0
0
1
2
3
4
5
Absorbance