EFFECTS OF ANDROGENS IN THE ONSET AND MAGNITUDE OF SALT – INDUCED HYPERTENSION IN MALE SPRAGUE – DAWLEY RATS

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, UNIVERSITY OF LAGOS, LAGOS NIGERIA

IN FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY IN PHYSIOLOGY BY

OLOYO, AHMED KOLADE

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DECLARATION

This work titled “Effects of androgens in the onset and magnitude of salt-induced hypertension in male Sprague-Dawley rats” submitted to the School of Postgraduate Studies, University of Lagos, Lagos, Nigeria for the award of Doctor of Philosophy in Physiology is an original research carried out by OLOYO, Ahmed Kolade in the Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, under the supervision of Professor O.A. Sofola and Dr C.N. Anigbogu.

This work has not been submitted previously, in whole or in part, to qualify for any other academic award.

PROF. O.A SOFOFA
Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Idi-Araba, P.M.B. 12003, Lagos, Nigeria.

DR. C.N. ANIGBOGU
Associate Professor, Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, Idi-Araba, P.M.B. 12003, Lagos, Nigeria.

OLOYO, AHMED KOLADE (Candidate)
DEDICATION

This work is dedicated

to

The loving memory of my father

Alhaji Subair Olaitan Ajetunmobi Alabi OLOYO

who died on the 12th of February 2008

For the loving and caring father he was

For the principles that guided his life

For the culture of fairness, honesty and self-confidence he instilled in his children

I will always love you "Papa"

May Almighty ALLAH (SWT) grant him Aljanat Firdaus
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ABSTRACT

Blood pressure is consistently higher in males compared with females from puberty onwards and men show an increased risk for hypertension compared to women. The gender disparity in cardiovascular functions and diseases has been linked to the effect of sex hormones on vascular reactivity. Although previous studies have suggested that the effect of chronic exposure to testosterone is an increase in vascular tone, it therefore implies that lack of testosterone should elicit vasorelaxation. Salt-sensitive hypertension in humans is associated with higher morbidity and mortality which may also be gender related. The role of gender in salt-sensitivity is not clear and the long-term effects of androgens on vascular reactivity especially in salt-induced hypertension have not been studied experimentally.

Therefore experiments were designed to assess the effect of androgens on the development of salt-induced hypertension in male Sprague-Dawley rats by assessing vascular relaxation response to agonist as well as vascular smooth muscle histomorphology in orchidectomy-induced androgen deficient rats placed on a normal or high salt diet with or without testosterone supplementation.

Weanling male Sprague-Dawley rats aged 8 weeks were divided into 8 groups of 16 rats each. They were either bilaterally orchidectomised or sham-operated under anaesthesia, with or without testosterone replacement (10mg/kg sustanon 250® i.m once in 3 weeks). They were placed on normal (0.3%) or high (8%) NaCl diet for 6 weeks. Arterial blood pressure was determined in conscious rats before and weekly throughout the experimental period using non-invasive tail cuff method. Terminal arterial blood pressure was also determined via femoral artery cannulation. Serum concentration of testosterone was determined using enzyme
immunoassay (EIA) technique at the end of the feeding period. Thoracic aorta was isolated and 3mm aortic rings were suspended in organ baths and relaxation responses to acetylcholine (ACh), sodium nitroprusside (SNP) forskolin, diazoxide, testosterone and DHEA in the presence or absence of L-nitro-Arginine methyl ester (L-NAME), indomethacin, barium chloride, flutamide and Aminoglutethetemide in noradrenaline pre-contracted rings were studied. Lipid peroxidation was studied in the heart and kidney homogenates using the TBARs method. The serum activity of super oxide dismutase (SOD) was also determined. Histological examination of thoracic aorta and mesenteric artery were carried out with specific dyes using haematoxylin and eosin stain for the cytoplasm and nucleus and Verhoeff – Van Geison and Picro-sirius red stains for elastin and collagen content estimation respectively. Histomorphometric analysis was determined using a programmed software IMAGEREADER 6.1.

The results indicate a significant increase (P < 0.001) in the mean arterial blood pressure (MABP) of rats placed on high salt diet (HSD), when compared with control or orchidectomised rats. Orchidectomy elicited a reduction in MABP while testosterone replacement normalized MABP to values seen in intact rats placed on high salt diet. High salt diet reduced relaxation response to ACh both in the presence and absence of eNOS inhibition with L-NAME. There was a significant decrease (P < 0.05) in the relaxation response to forskolin in rats placed on high salt diet when compared with controls. High salt diet reduced the relaxation response to diazoxide but not in orchidectomised rats while testosterone supplementation re-established the blunted diazoxide relaxation. The results also show a significant increase (P < 0.001) in lipid peroxidation of HSD groups compared with controls. Orchidectomy elicited a reduction in lipid peroxidation while testosterone supplementation returned it to the level observed in the intact groups. On the other hand there was a significant decrease (P < 0.01) in the serum level of SOD
of the HSD groups when compared with controls, while orchidectomy increased the SOD activity, testosterone supplementation restored it to the level observed in the intact groups.

Tunica media thickness and cross sectional area, elastin and collagen contents were all significantly elevated in the rats placed on high salt diet while orchidectomy significantly reduced the values of the parameters in high salt group but concomitant administration of testosterone restored them to the levels observed in intact rats. Both aromatase inhibition and androgen receptor blockade did not prevent the relaxing effect of testosterone on rings from rat aorta. There was also no significant difference between testosterone relaxation response in the presence or absence of L-NAME and indomethacin. However, 3µM BaCl₂ almost completely abolished the aortic ring relaxation response to testosterone while 1 µM nifedipine potentiated the vasorelaxing effect of testosterone.

The results indicate that in male Sprague-Dawley rats, endogenous testosterone promotes blood pressure-elevating effect of a HSD such that bilateral orchidectomy reduced the blood pressure and attenuated the impaired endothelial function induced by HSD. However, this was reversed by concomitant administration of testosterone, suggesting a role for androgens in enhancing long term vascular smooth muscle tone and hence the maintenance of arterial blood pressure. Endogenous testosterone enhanced ROS-generating and promoted vascular hypertrophic effect of a HSD in the rats. On the other hand, exogenous testosterone relaxes rat aorta directly via a non-genomic pathway which is independent of endothelial derived vasoactive substances.
CHAPTER ONE

1.0 INTRODUCTION

There is a consistent gender gap in life span of approximately 6 years in most populations of the world (The World Health Report, 2002; World Population Data Sheet, 2002). This gender gap favours women, meaning that women live longer than men or men die younger compared to
women in these populations. The gender gap in life span derives from gender differences to a large extent in age-specific cardiovascular death rates, which rise steeply in parallel for both gender, but 5-10 years earlier in men (Liu et al., 2003). From the viewpoint that cardiovascular disease is the leading cause of human deaths (Murray and Lopez, 1996; Chockalingam et al., 2006), gender differences in its onset and severity must make an important contribution to the gender gap in life span.

Gender differences in cardiovascular diseases may be due to genetic, hormonal or lifestyle factors or a combination of mechanisms. Genetic contributions due to either Y-linkage or X-dosage remain largely speculative. The single example is Y-chromosome linkage due to SRY gene, which induces testis formation and the male phenotype including testosterone secretion. This is an interesting circumstance in which a potentially modifiable hormonal pathway is the effector mechanism for a genetic risk usually considered to be unmodifiable. However, a probable candidate gene on the rat Y-chromosome which could be responsible for genetic hypertension in spontaneously hypertensive rat (SHR) has not been characterized (Ely et al., 2000). There is also no evidence for protective dosage effects of any X-Chromosomal gene, which might hypothetically suppress cardiovascular disease in women, an unlikely mechanism, considering random X-inactivation (Iyonization) in female. Gender differences in lifestyle risk factors of cardiovascular disease, notably diet, physical activity, smoking and other behavioral characteristics have been the focus of much attention (Kromhout et al., 2002) although they contribute to, they do not explain the gender gap in cardiovascular diseases (Barett-Connor, 1997).
Hormonal effects are the most tractable for practical therapeutics, considering the various reproductive steroids that are available. Reinforcing the hormonal effect as the most important factor in gender differences in cardiovascular diseases is the fact that receptors for sex steroids are expressed in varying numbers in both the endothelium and vascular smooth muscle of multiple vascular systems (Thompson and Khalil, 2003). These sex hormone receptors appear to have different subtypes, tissue distribution and sub-cellular location and can be modified by various agonists and antagonists. The interaction of sex steroids with cytosolic / nuclear receptors triggers long-term genomic effects that could stimulate endothelial cell growth while inhibiting smooth muscle proliferation. Activation of plasmalemal sex steroids receptors may trigger acute non-genomic responses that could stimulate endothelium-dependent mechanism of vascular relaxation such as the nitric oxide – eNOS / prostacyclin – cGMP and hyperpolarisation pathways (Heinlein and Chang, 2002). The effects of sex steroids on the blood vessels may contribute to the gender differences observed in vascular tone and subsequently onset and severity of cardiovascular diseases.

Hypertension and coronary artery disease are two of the most common cardiovascular diseases. As at 1984 using the cut-off of 160/95mmHg, the prevalence of hypertension in adults all over the world was estimated at 10% to 16% (World Health Organization, 1984). With the adoption of 140/90mmHg cut-off, the prevalence of hypertension in the adult worldwide is about 26.4% (Kearny et al., 2005), and 36.6% in adult Nigerians (Adedoyin et al., 2008). Hypertension is a complex disease that may arise from dysfunction of blood vessels, central nervous system regulation, hormonal homeostasis, or kidney functions. Hypertension and the accompanying complications; stroke, heart disease, and kidney disease, arise from interactions between genetic risk factors, environmental risk factors and sex-specific hormonal effectors (Gerhold et al.,
Genetic risk factors in humans include a mutation in the preangiotensionogen gene which leads to a blunted response to angiotensin II under high salt conditions which is observed mostly in males (Williams et al., 2000). Environmental risk factors include high salt and high protein diets. Blood pressure has been reported to be consistently higher in males compared with females from puberty onwards and men show an increased risk for hypertension and renal disease compared to women, a risk that interacts with genes and with diet (Gerhold et al., 2007). Likewise epidemiological reports have shown that there is a greater incidence of hypertension and coronary artery disease in men and post-menopausal women compared with pre-menopausal women (Barrett – Connor, 1997; Reckelhoff et al., 2000; Orshal and Khalil, 2004), suggesting sexual dimorphism in incidence and prevalence of hypertension in humans.

High salt diet has been implicated in the pathogenesis of human hypertension particularly in salt sensitive individuals, as high salt in the diet was correlated with the level of blood pressure (Sanders, 2009). An individual that shows a change in blood pressure in response to a change in dietary salt loading or salt restriction is termed salt sensitive. About 73% of hypertensive black, 29% – 51% of hypertensive whites and 36% of normotensive blacks are salt sensitive (Weinberger et al., 2001). Experimental studies have also shown that high salt diet results in elevated blood pressure in animals such as; Dahl salt sensitive (DSS) rats (Meng et al., 2003; Zheng et al., 2008), Spontaneously hypertensive rats (SHR) (Ahn et al., 2004; Matavelli et al., 2007), Sabra rats (Khalid et al., 2002) Sprague Dawley rats (Liu et al., 1999; Sofola et al 2003) and dogs (Hainsworth et al., 2003). Bibbins–Domingo et al., (2010) suggested that a modest reduction of 1g of salt per day would have significant benefits on cardiovascular health, a view that is strongly supported by Appel and Anderson, (2010).
Salt sensitivity in human exhibits gender differences and this has been the subject of many studies, but at best, results from such studies have been inconsistent. Some studies reported that girls are more likely than boys to show blood pressure (BP) reduction in response to low sodium diet (Wilson et al., 1999), while boys showed more increase in BP compared with girls in response to high sodium diet (Wilson et al., 1996); this finding is consistent with that of Dietary Approach to stop Hypertension DASH – Sodium trial (Vollmer et al., 2001). On the contrary, a more recent study by the Genetic Epidemiology Network of Salt Sensitivity (GenSalt) group reported that BP responses to dietary sodium intake were greater in females compared to males, thus female have higher sensitivity to salt diet (He et al., 2008). Sexual dimorphism has also been reported in salt sensitivity in experimental animals. For instance, when fed a high salt diet, blood pressure elevation is higher in male rats compared with female rats (Hinojosa-Larbode et al., 2004; Chappel et al., 2008).

Suggested mechanisms by which salt sensitivity causes hypertension include; left ventricular hypertrophy (Ahn et al., 2004), increased sympathetic activity (Ehmke, 2005), sodium excretion impairment as well as glomerular dysfunction (Calstrom et al., 2007). Schmidlin et al., (2007) suggested that systemic vascular dysfunction that is expressed as an impaired vasodilatory response to dietary NaCl loading is the initiating pressor effect in salt sensitive individual. Studies have also shown that elevated dietary salt intake leads to an impaired relaxation of blood vessels to vasodilator stimuli in animals (Nurkiewicz and Boegehold, 2007; Zhu et al., 2007). The effect of sex hormones on vascular reactivity is considered as one of the underlying factors contributing to gender differences in cardiovascular functions and diseases (Sader and
Celermajer, 2002; Thompson and Khalil, 2003). Gender difference in vascular tone has been described in many vascular beds in both human and experimental animals (Geary et al., 2000). However, most studies have focused on the influence of oestrogen on the blood vessel wall (Geary et al., 2000; Golding and Keplar, 2001), less attention is being paid to the vascular effect of testosterone. Most available data are on acute vasodilatory effect of testosterone and these effects were observed at supraphysiological concentrations (Chou et al., 1996; Ding and Stallone, 2001). Few studies available on the effect of endogenous androgens on the blood vessel were carried out in normal non hypertensive human and rat cerebral arteries (Penotti et al., 2001; Gonzales et al., 2004). The role of gender in salt sensitivity is not conclusive neither is the long-term effects of androgens on vascular reactivity especially in salt-dependent hypertension studied.

Cells living under aerobic conditions constantly face the oxygen paradox. Oxygen is required to support life, but its metabolites such as reactive oxygen species (ROS) can modify cell functions, endanger cell survival or both (Agarwal, 2005). Generation of ROS has been implicated as one of the mechanisms by which high salt diet induces hypertension (Zhuo et al., 2003; Tian et al., 2007). High salt diet has been reported to prevent normal dilatory response to acetylcholine (ACh) and increased shear stress in many vascular beds in rats such as skeletal muscle arterioles, cerebral and mesenteric arteries (Zhu et al., 2007). Widespread inactivation of NO could occur with high salt intake if this diet leads to the generation of reactive oxygen species (ROS) in or near the microvascular endothelium. These highly reactive molecules can be formed in the microvasculature via the enzymatic activity of cyclooxygenase, xanthine oxidase, NAD(P)H oxidase, or NO synthase (Wolin, 2009). Superoxide anions generated by one or more of these
pathways can rapidly oxidize endothelium-derived NO and so interfere with its normal influence on vascular tone (Berry et al., 2001). Increased ROS generation and the consequent inactivation of endothelium-derived NO have been implicated in the depressed endothelium-dependent vascular responses associated with hypertension (Touyz et al., 2004), diabetes (Mohanty et al., 2000; Whiteside, 2005), and ischemia-reperfusion (Raat et al., 2009).

Some clinical studies have shown that oxidative stress is higher in men than women (Ide et al., 2002), a finding that is consistent with reported higher blood pressure in males compared with females (Barrett-Connor, 1997; Reckelhoff et al., 2000). More recently studies have shown that there are gender differences in oxidative stress in laboratory animal models of experimental hypertension (Lopez-Ruiz et al., 2010; Venegas-Pont et al., 2010). However, the role of androgens on ROS generating, and antioxidant enzyme depleting effects of high salt diet is yet to be studied experimentally.

1.1.0 Statement of problem

Women live longer than men in most populations of the world. This fact is not unconnected with sexual differences in cardiovascular related deaths which favour women more than men. Cardiovascular disease accounts for the highest percentage of human death all over the world and hypertension is one of the most common cardiovascular diseases. There is a greater incidence and prevalence of hypertension in males compared with their age-matched females.
This gender disparity has been interpreted primarily as reflecting estrogen-mediated protection against cardiovascular diseases. Whereas previous studies have demonstrated acute relaxant responses to testosterone in various vascular beds, this observation is not consistent with the greater predisposition of males to cardiovascular diseases. Even though oestrogen mediated protection of females from cardiovascular diseases remains unproven by prospective clinical trials, this dominant belief shapes the direction of much mechanistic research leaving to be less studied the plausible alternative, that androgens promotes cardiovascular diseases. The need to study the plausible alternative is further sharpened by the need for careful scrutiny of the safety of wider androgen use in the community. This is because even minor deleterious effects of androgens on cardiovascular disease as the most frequent cause of death, are likely to outweigh any substantial perceived benefits from androgens in any medical, lay or abusive context. Salt sensitivity increases the risk of death whether or not a person has high blood pressure. Salt-sensitive hypertension in humans is associated with higher morbidity and mortality due to cardiovascular diseases than hypertension of other aetiologies. Therefore, considering the role of high salt content in the diet as a major lifestyle factor, especially in salt-sensitive populations, the need to study the interactions of sex steroid hormones (the major factor responsible for gender difference in cardiovascular disease) with salt becomes more imperative.

### 1.2.0 Aim of the study

This work is designed to study the possible effects of androgens, and their mechanism of action in the onset and magnitude of salt-induced hypertension in non-genetically modified Sprague-Dawley rats.

### 1.2.1 Objectives
1) To evaluate the role of androgens in the onset and magnitude of blood pressure elevating effect of a high salt diet in male Sprague-Dawley rats.

2) To assess the effect of bilateral orchidectomy and testosterone supplementation as well as the effect of a high salt diet on serum level of androgens in intact and orchidectomized Sprague-Dawley rats.

3) To study the effects of androgens on vascular responses to agonists in Sprague-Dawley rats fed a high salt diet.

4) To study the mechanisms by which androgens affect salt-induced hypertension by:
   i. Assessing the effects of androgens on oxidative stress in Sprague-Dawley rats fed a high salt diet.
   ii. Assessing the effects of androgens on vascular smooth muscle cells of Sprague-Dawley rats fed a high salt diet.

5) To assess the non-genomic effects of androgens on vascular responses in intact and orchidectomized Sprague-Dawley rats fed a high salt diet.

1.3.0 Significance of the study

The gender gap in life span has not narrowed over the last century, and it seems unlikely to diminish without effective targeting. If the sex differential in mortality is to be reduced, the preferable route is by decreasing male mortality, not increasing female mortality. The outcome of this study may add to the available knowledge on how to improve male cardiovascular health.
thereby reducing male mortality due to cardiovascular diseases, hence reducing the gender gap in life span.

This study will give an insight into:

- The role that androgens may play in the onset and magnitude of salt-induced hypertension.
- The mechanisms that may compound vascular changes in salt-induced hypertension.
- The mechanism of actions of androgens on blood vessels and the basis for gender differences in vascular tone and cardiovascular diseases such as salt-sensitive hypertension.
- The potential beneficial vascular effect of hormone replacement therapy (HRT) during natural and surgically-induced deficiencies of androgens.

1.4.0 Operational Definition of Terms

**Genomic:** refers to the effect of androgens elicited through the activation of the classical cytosolic or nuclear androgen receptors (AR) with the ensuing cascade of reactions involving nuclear activation, gene transcription and translation and protein synthesis. Effects elicited through this pathway are slow and last longer.
**Hormone replacement therapy (HRT):** is a medical treatment with a medication containing one or more hormones for surgically menopausal, perimenopausal, post menopausal female and hypogonadal male.

**Intact rats:** these were groups of rats that surgery was not performed on either for actual bilateral removal of their testicles or false removal.

**Non-genomic:** refers to the effect of androgens that do not involve nuclear activation and, gene transcription, translation and protein synthesis. Effects mediated through this mechanism are fast and short-lived.

**Orchidectomy:** refers to the surgical removal of the rats testicles under aseptic condition.

**Oxidative stress:** refers to an imbalance in the production of reactive oxygen species and ability to detoxify the reactive intermediates or repair the resulting damage in the body.

**Salt-induced hypertension:** refers to experimental induction of blood pressure elevation following high salt loading in experimental animals for six weeks.

**Salt-sensitive hypertension:** refers to an elevation of blood pressure above the normal values following high dietary salt loading in humans, it is commonly used interchangeably with salt-dependent hypertension.

**Sexual dimorphism:** refers to the systematic differences in form between individuals of different sex in the same species.

**Sham orchidectomy:** refers to false orchidectomy whereby the rats underwent anaesthesia and the scrotal sac was opened, but the testicles were not actually removed.

**Testosterone supplementation:** refers to parenteral administration of testosterone to the rats following orchidectomy. It is synonymous to hormone replacement therapy in humans.
CHAPTER TWO

2.0 REVIEW OF LITERATURE

2.1 Gender Gap in Life Span
A strikingly consistent feature of human populations is the gender gap in life span (The World Health Report, 2002; World Population Data Sheet, 2002). At birth, life expectancy is consistently shorter for men than women across virtually all population. In 186 out of 191 (97%) United Nation Member countries, men have shorter life expectancy by an average of 5.6 years in those countries above a minimal level (GDP in US dollar, of 3,000 per capita) economic development. If women that died while giving birth or in pregnancy is not considered, female human life expectancy is considerably higher than those of men (The World Health Report, 2002). The reasons for this are not entirely certain.

In developed countries, females outlive men by seven years: life expectancy at birth for females is 79 and for males it is 72. However, there is variation across the developed world in the magnitude of the sex difference in life expectancy—ranging from a low of five years in many of the countries that made up the former Yugoslavia (averaging 75 years for females and 70 years for males) to a high of twelve years in Russia (73 years for females; 61 years for males) (United Nation, 2000; Trovato, 2005). Trends in mortality favored women in the more developed countries from at least the beginning of the twentieth century to the early 1980s. Mortality trends in different age groups contributed differentially to this overall trend of widening the sex gap in mortality. Nearly two-thirds of the widening can be attributed to mortality among people aged sixty-five and above. In other words, death rates for older women declined more quickly than death rates for older men (Lopez et al., 1995; Trovato, 2005). One-quarter of the increase resulted from mortality trends among people aged fifty-five to sixty-four, for whom, as well, female death rates declined more than male death rates. Very little of the increase was due to mortality among children aged one to fourteen, (less than 3 percent). This is unlike the case in the third world, where death rates among children figure prominently in mortality trends and
differentials. In contrast, trends in infant mortality operated in opposite fashion, to narrow the sex difference in mortality. High male infant mortality was overcome to a considerable degree so that eventually, for the most part, only the genetically caused higher susceptibility of male infants to death remained (United Nation, 1998).

According to the Population Reference Bureau circa, (2001), in less developed countries the life expectancy at birth of females exceeded that of males by three years (66 versus 63 years). In the 1950s the differential was approximately two years (42 versus 40 years). While considerable progress in life expectancy at birth has been made, and approximately equivalently for males and females, there is much concern that women in third world countries are disadvantaged in terms of mortality (Women’s International Network News, 1990). In part, this concern stems from the gender mortality gap favoring females in developed countries that is interpreted to mean that, given more equal treatment, females will have lower mortality than males. The clearest evidence of female disadvantage comes from demographic research on the third world, which estimates that between 60 million and 100 million girls and women are "missing." They are missing as a combined result of female infanticide, sex-selective abortion and health care neglect, nutritional deficiency, and mistreatment that lead to death (Sen, 1992). Maternal mortality also plays a role; the World Health Organization (WHO) and UNICEF estimate that more than half a million women die per year due to pregnancy-related problems, most in developing countries. Worldwide, women face a 1 in 75 lifetime risk of dying due to maternity-related causes. This risk varies from 1 in 4,085 in industrialized countries to 1 in 16 in the least developed parts of the world (World Health Organization, UNISEF, and UNFPA, 2001).
Traditional arguments tend to favour socio-environmental factors: men, on average, consume more tobacco, alcohol and drugs than females in most societies, and are more likely to die from some associated diseases such as lung cancer, tuberculosis and cirrhosis of the liver (World Health Organization, 2004). Men are more likely to die from injuries, whether unintentional (automotive accidents, etc.), or intentional (suicide, violence, war) (Boyd, 2000; World Health Organization, 2004).

Women tend to have a lower mortality rate at every age. In the womb, male fetuses have a higher mortality rate (males are conceived at a ratio of about 124 males/100 females, but by birth, the ratio is only 105 males/100 females) (Waldron, 1998). Females have a higher survival rate among the smallest premature babies. It has universally been found that more male than female babies are born. While the magnitude of the sex ratio at birth (the number of male births per 100 female births) varies somewhat, it is almost always in the range of 103 to 107. There is some debate about why male births exceed female births, in particular on the issue of sex differences in the number of conceptions. It has been argued that considerably more males are conceived, but that the male fetus is biologically weaker (Waldron, 1998). The data available suggest that males do have higher mortality from around the third to the fifth month of gestation age. While it is extremely difficult to know about mortality risk during the first few months of pregnancy, the limited evidence suggests that there are more male embryos by the second month after conception. This means either that more males are conceived or that female embryos have higher mortality risk in the weeks after conception, or both (Waldron, 1998).

While there is uncertainty about sex mortality differentials in the early gestational period, data for the first year of life are very clear (United Nation, 1998). In virtually all places and times
infant mortality rates are higher for males than for females. Most evidence points to biological factors playing an important role in the higher mortality of infant males (Waldron, 1998; United Nation, 2000). Despite their higher birth-weights (a factor associated with infant survival), male babies are more likely to suffer from congenital abnormalities that lead to death and to have immune deficiencies associated with X chromosome-linked genetic defects and with exposure to testosterone prenatally and in early infancy (Retherford, 1975; Waldron, 1998). This latter factor may also contribute to greater activity levels associated with higher accident mortality (Boyd, 2000).

The mortality gap varies during other stages of life. Between ages 15 and 24 years, men are four to five times more likely to die than women. This timeframe coincides with the onset of puberty and an increase in reckless and violent behavior in males. Researchers refer to it as a “testosterone storm.” Most deaths in this male group come from motor vehicle accidents, followed by homicide, suicide, cancer, and drowning (Chase, 1998). After age 24, the difference between male and female mortality narrows until late middle age. In the 55- to 64-year-old range, more men than women die, due mainly to heart disease, suicide, car accidents, and illnesses related to smoking and alcohol use. Heart disease kills five of every 1,000 men in this age group (United Nation Secretariat, 1988; Perl and Fretts, 1998).

Other biologically based factors has been suggested to contribute to gender differences in mortality, such include the protective effect of women’s XX chromosome structure against heart disease, especially at ages under fifty-five (Waldron, 2000), and a propensity to violence among men that can have lethal consequences (Boyd, 2000). The degree to which men are more violence-prone than women and the reasons for it are, however, hotly debated and it cannot be
stated if and how biology may be implicated (Boyd, 2000). While biological factors can explain part of gender differences in mortality, these differences vary too much by time and place to be accounted for by biology to any great extent.

Differential trends in various causes of death contributed to the widening of the sex mortality difference. By far the most important cause of death in the widening is diseases of the circulatory system, which include ischemic heart disease and strokes (Murray and Lopez, 1996). While there is variation from country to country, the overall fact that male deaths due to circulatory disease declined less than female deaths is responsible for approximately three years of the widening gap. Of the different kinds of circulatory diseases, trends in ischemic heart disease played the biggest role in this three-year widening, with men's death rates increasing over most of the twentieth century while women's death rates were stable. Rheumatic heart disease and strokes (for which women and men have approximately equal risks of death) have decreased in importance as causes of death. Thus, the composition of the circulatory disease category with an increasing prevalence of ischemic heart disease played a role in widening the sex mortality differential (World health Organization, 2004).

The second most important cause of death in explaining the widening sex differential in mortality is malignant neoplasm (cancer). At the turn of the twentieth century, female mortality from cancer (especially due to breast cancer and cancers of the female genital organs) tended to be higher than male cancer mortality. However, over the course of the twentieth century increasing rates of male mortality due to respiratory (e.g., lung) cancers served to widen the male-female mortality difference (Valkonen and Van Poppel, 1997). In the United States for the period from 1900 to the early 1980s shifts in the trends and pattern of cancer mortality accounted
for more than one-third of the widening sex mortality differential; in other Western countries such as England and Australia, the contribution made by malignant neoplasm to widening the sex mortality ratio was even greater differential (World health Organization, 2004).

Other causes of death are much less important contributors to the widening sex mortality differential. For example, declines in maternal mortality, although very substantial, have had only a small effect. Trends in accident mortality and suicide—two causes of death that are higher for males—have not played a big role either. In contrast, declines in infectious and parasitic diseases, for which males in the West tended to have higher mortality than females, had an opposite effect, that is, to narrow the sex gap in mortality differential (World health Organization, 2004).

The increase in respiratory cancer among men and the slower decreases in circulatory system mortality among men have been attributed to smoking differences, in large part. Over the earlier years of the twentieth century, men (much more so than women) took up cigarette smoking, the effects of which show up in mortality statistics among older age groups (Lopez, et al., 1995; Valkonen and Van Poppel, 1997).

2.2.0 Gender Differences in Cardiovascular Diseases

Globally, cardiovascular disease will remain the major causes of human death well into the 21st century (Murray and Lopez, 1996). Because of this, gender difference in its onset and severity must make an important contribution to the gender gap in life span. Cardiovascular diseases are some of the most common and costly diseases in the industrialized world. Several reports have shown that there is greater incidence of cardiovascular diseases in males compared to age–matched females (Liu et al., 2003; Khalil, 2005). Figure 1 shows the age-adjusted mortality rates
for coronary artery diseases (CAD) across some countries. It can be inferred from this diagram that mortality rates for (CAD) is higher in males compare with females across all the countries studied.
Fig. 1: Age-adjusted mortality rates for Coronary Artery Disease (CAD) by country and sex. (age 35–74 yr). (National Heart, Lung, and Blood Institute, 2000).
Higher male susceptibility to cardiovascular disease may be due to various factors ranging from genetic, hormonal or lifestyle factors or a combination of mechanisms. Genetic contributions to gender differences in cardiovascular diseases due to either Y-linkage or X- dosage have remained speculative. The single example is Y-chromosome linkage due to SRY gene, which induces testis formation and the male phenotype including testosterone secretion. This is an interesting circumstance in which a potentially modifiable hormonal pathway is the effector mechanism for a genetic risk usually considered to be unmodifiable. A gene on the Y-chromosome of spontaneously hypertensive rat (SHR) which is responsible for genetic hypertension, has been suggested to involve testosterone, the androgen receptor (Ely et al., 1991) and an unidentified extrarenal substance (Harrap et al., 1992) and endothelium-derived pressor substances (Honda et al., 1999), but it has not yet been characterized (Ely et al., 2000). Likewise there is no evidence for protective dosage effects in any X-Chromosomal gene. Hypothetically, X-chromosome may suppress cardiovascular disease in women, but this is an unlikely mechanism, considering random X-inactivation (Iyonization) in females.

Gender differences in lifestyle risk factors of cardiovascular disease, notably diet, physical activity, smoking and other behavioral characteristics have been the focus of much attention (Kromhont et al., 2002). These factors contribute to, but do not explain the gender gap in cardiovascular diseases (Barrett-Connor, 1997). From the beginning, diet played a prominent role in research on the origin of coronary heart disease (Connor, 1999; Menotti, 1999). The original diet-heart hypothesis was very simple. Cholesterol is a constituent of the atherosclerotic plaque. Therefore, it was thought that there was a direct relation between cholesterol in the diet (i.e., eggs), cholesterol in the blood, cholesterol in the plaque, and its clinical complications, such
as myocardial infarction (Connor, 1999). In the second part of the past century, it became clear that dietary cholesterol played a minor role in regulating serum cholesterol levels. It was also shown that dietary fatty acids are the major determinants of serum cholesterol (Menotti, 1999). The study of lipoprotein metabolism showed that the cholesterol-rich LDL fraction, not total cholesterol, was most strongly related to the development of atherosclerosis and its sequel (Gofman, 1950). Experimental research was essential to understand the mechanisms by which genes, hormones, and diet interact to regulate the serum cholesterol level (Brown et al., 1981). Low density lipoprotein (LDL) cholesterol levels can be increased by saturated fatty acids, especially those with 12 to 16 carbon atoms, and by trans-fatty acids (Clarke et al., 1997).

Several hypotheses have been proposed to explain the initiating events in atherogenesis, e.g. the response-to-injury, response-to-retention, and oxidation hypotheses (Steinberg et al., 1989; Boren et al., 2000). These hypotheses are not mutually exclusive and may even be compatible with each other. The oxidation hypothesis emphasizes the importance of oxidative modification in the atherosclerotic process, because compared with native LDL, oxidized LDL is preferentially taken up in the arterial wall (Steinberg et al., 1989). This hypothesis makes a role of diet and lifestyle in atherogenesis likely, because LDL can be oxidized by smoking, for example, and oxidation can be prevented by dietary antioxidants, e.g. vitamins and polyphenols. There is overwhelming evidence that smoking, alcohol, and physical activity are important determinants of coronary heart disease. Prospective cohort studies showed a strong, graded relationship between cigarette smoking and coronary heart disease (Doll and Peto, 1976). A moderate alcohol intake of 1 or 2 drinks per day is associated with a 30% to 40% lower risk of coronary heart disease (Thun et al., 1997). Prospective cohort studies have also shown that the relative risk of coronary heart disease for inactive subjects compared with active persons is about
2 times higher (Powell, 1987). Complex interactions between diet, lifestyle, and lipoprotein metabolism determine the development of atherosclerosis and its complications (Kromhout et al., 2002).

A study carried out on the effects of smoking on sex differences in life expectancy in Demark, Finland, Norway, Sweden and the Netherlands during 1970–1989 demonstrated that on average, 2.4 years or more than 40% of the total sex difference in life expectancy in 1970–1974 was estimated to be attributable to smoking in the 5 countries (Valkonen and Van Poppel, 1997). By 1985–1989 the contribution of smoking dropped to 1.8 years or approximately 30% of the total difference. The contribution of smoking to the sex difference was greatest in the Netherland, smallest in Sweden. As a result of the decline in smoking-attributable male mortality, the sex difference in life expectancy diminished in Finland in by 1.6 years (Valkonen and Van Poppel, 1997). Since 1980 in the United States, men's rates of lung cancer mortality, although still increasing, have slowed down in pace; in contrast, women's rates of lung cancer mortality have skyrocketed. This trend reflects, in large part, the later adoption of smoking by women (Kromhout et al., 2002).

Out of all the factors that could account for sexual differences in cardiovascular disease, hormonal effects are the most culpable, considering the plethora of reproductive steroids that are available. Furthermore, sex hormone receptors have been found in the cytosol and nuclear compartments of various cell types including endothelium and vascular smooth muscle (Orshal and Khalil, 2004), alteration in vascular tone, which are inadvertently dependent on the activities of these cells play a major role in the control of blood pressure and the coronary circulation and thereby the incidence of hypertension and coronary artery disease. Gender difference in vascular
tone has been described in many vascular beds in both human and experimental animals (Xia and Khalil, 2010). For example Noradrenalin elicits less forearm vasoconstriction in women than in men (Kneale et al., 2000), contraction to noradrenalin and phenylephrine is greater in the aorta of intact male than intact female rats (Kneale et al., 2000), while oxidized low-density lipoprotein enhances 5-hydroxytryptamine-induced contraction to a greater extent in coronary arteries from male than female pigs (Cox and Cohen, 1997). Xia and Khalil, (2010) reported a higher contractile response in the inferior vena cava of male rats than the females and that, $[\text{Ca}^{2+}]_i$, Ca$^{2+}$-dependent contraction, and the myofilament contraction sensitivity to $[\text{Ca}^{2+}]_i$ are reduced in female compared with male rat inferior vena cava. Likewise several studies have suggested significant effect of sex steroids on the renal control mechanisms of the blood pressure, particularly the rennin angiotensin system (Reckelhoff et al., 2000; Reckelhoff, 2001). For example estradiol has been reported to inhibit rennin release and angiotensin converting enzyme (Takeda-Matsubura, 2002), whereas testosterone may increase the blood pressure by activating the rennin – angiotensin system (Reckelhoff, 2001).

2.3.0 Hypertension

The World Health Report (2002) identified hypertension, or high blood pressure, as the third ranked factor for disability-adjusted life years. Hypertension is one of the primary risk factors for heart disease and stroke, the leading causes of death worldwide (Ezzati et al., 2002). Analyses have shown that as of the year 2000, there were 972 million people living with hypertension worldwide, and it is estimated that this number will escalate to more than 1.56 billion by the year 2025 (Kearny et al., 2005). As at 1972, Akinkugbe had put the prevalence in the adult African population as 10 to 15% (Akinkugbe, 1972). A survey by the expert committee on non
Communicable diseases in Nigeria revealed the prevalence of hypertension in adult Nigerians aged 15 years and above, to be 11.2%, using 160/95 mmHg as the cut off, with rates in the rural and urban communities of 9.8% and 14.6% respectively (Akinkugbe, 1997). Adjusting these, with the cut-off point of 140/90 mmHg, these results would lead to a current prevalence rate of 36.6% (Adedoyin et al., 2008).

The risk of developing not only cardiac disease but also neurologic, renal and vascular disease is significantly increased among hypertensive individual. The higher the blood pressure the greater the risk (Price and Wilson, 1986). Hypertension is defined as an abnormal elevation of systolic and / or diastolic blood pressure. The precise delineation of abnormality is somewhat arbitrary, acceptable values vary with age and sex. In general, systolic values ranging from 140 to 160 mmHg and diastolic values from 90 to 95 mmHg are considered indicative of borderline hypertension. The diagnosis of hypertension is unequivocal with values excess of 160 mmHg systolic and 95 mmHg diastolic pressure (Price and Wilson, 1986). These values are consistent with a conceptual definition of hypertension as a pressure elevation associated with an increase in cardiovascular mortality of over 50 percent.

The cause of hypertensive disease is particularly insidious; hypertensive individuals can remain asymptomatic for many years. This latent period masks disease progression until significant organ damage occurs. Symptoms, if present, are typically non-specific, such as headache or dizziness. If hypertension remains undetected and untreated, death results from heart failure, myocardial infarction, stroke, or renal failure. However early detection and effective treatment of hypertension can significantly decrease associated morbidity and mortality. Consequently routine
blood pressure screening is of paramount importance in hypertension control. The cause of hypertension is unknown in approximately 90% of the cases. This idiopathic form of hypertension is referred to as primary or essential hypertension. The precise pathogenesis appears to be extremely complex with interaction of multiple variables. Proposed mechanisms include alteration in the following: renal excretion of sodium and water, baroreceptor sensitivity, vascular responsiveness and renin secretion.

The remaining 10 percent of hypertensive disease is secondary to some other underlying disease processes such as renal parenchymal disease or primary aldosteronism. The mechanisms by which hypertension produce disability and death relate directly to its effects upon the heart and blood vessels. The elevation in systemic blood pressure increase the resistance to left ventricular ejection; consequently, cardiac work load is increased. In response, the ventricle hypertrophies to increase the force of contraction. However the ability of the ventricle to sustain cardiac output via compensatory hypertrophy is eventually exceeded and cardiac dilation and failure result. The heart is further compromised by an associated acceleration of coronary arteriosclerosis (Price and Wilson, 1986). As coronary arteriosclerosis progresses, myocardial oxygen supply is reduced, Angina or myocardial infarction can result since a concurrent rise in myocardial oxygen demand occurs secondary to the ventricular hypertrophy and increased cardiac work. Approximately one-half of hypertensive deaths are due to myocardial infarction or failure (Price and Wilson, 1986).

Accelerated arteriosclerosis and medial necrosis of the aorta predispose to the formation of aneurysms and dissection. Structural changes in the small arteries and arterioles cause
progressive occlusive vascular disease (Price and Wilson, 1986). As the vascular lumen narrows, arterial flow is compromised and tissue microinfarction can result. The consequences of these vascular changes are most striking in the brain and kidneys. Cerebral vascular occlusion or rupture accounts for approximately one-third of hypertensive deaths. Progressive sclerosis of the renal vasculature with resultant organ dysfunction and renal failure can also be fatal. The therapy of mild to moderate essential hypertension remains controversial in that despite the obvious benefits of hypertension control, antihypertensive therapy may involve some disagreeable side effect and long term patient compliance is difficult to achieve. Usually therapy initially consists of restriction of dietary sodium intake and weight reduction to ideal body weight. If this approach is unsuccessful, pharmacologic therapy with diuretics, sympatholytic agents, vasodilators, or a combination of these may be indicated. Hypertension secondary to another cause is treated by reversing the underlying process. Price and Wilson, 1986.

2.3.1.0 High salt diet and blood pressure

There is an increasing interest in the influence of high dietary salt intake on the incidence of high blood pressure. Epidemiological reports have shown that communities that ingest high salt in their diet (e.g. Northern Japan) do have a high incidence of hypertension, which may be as high as 40% of the population (SACN, 2003; Meneton et al., 2005). Whereas communities that consume little or no salt in their diet (e.g. Eskimos, Kalahari tribesmen of Southern Africa, and Yanomamo Indians in Brazil) may have an incidence of hypertension that is virtually zero (Meneton et al., 2005). Likewise it was reported recently that low sodium diet intervention reduced blood pressure and improved vascular function in hypertensive individual (Al-Soulaiman et al., 2009; Jablonski et al., 2009). The recommended average daily intake of salt is
about 5g (about 86 mmol) per day (equivalent to about 1 teaspoon), but the actual consumption ranges from as little as 1g/day to as high as >10 – 12g/day in some populations (Meyer, 2010).

Salt sensitivity which is usually taken as a change in BP in response to change in salt and water homeostasis was reported to be about 73% in hypertensive black, 29% – 51% in hypertensive whites and 36% in normotensive blacks (Weinberger et al., 2001). Salt-sensitive hypertension is defined as an increase in blood pressure (BP) with high salt (HS) intake or a decrease in BP caused by salt restriction (Weinberger et al., 2001). Salt-sensitive hypertension in humans is associated with higher morbidity and mortality due to cardiovascular disease than hypertension of other etiologies (Morimoto et al., 1997; Weinberger et al., 2001). A large body of evidence suggests that salt sensitivity leads to hypertension through mechanisms such as left ventricular hypertrophy (Ahn et al., 2004), increased sympathetic activity (Ehmke, 2005), impaired sodium excretion and glomerular dysfunction (Carlström et al., 2007; Matavelli et al., 2007). Pressor effect of dietary salt in hypertension may be in part, due to increase in plasma factor that inhibits Na⁺ - K⁺ - ATPase (Gawoski et al., 2005; Wang et al., 2003). Acute volume expansion in dogs increases the capacity of plasma to inhibit Na⁺ - K⁺ - ATPase, and this increase is also observed in essential hypertension, spontaneously hypertensive rats SHR, and Milan hypertensive rat (Meneton et al., 2005). However the nature of the substance responsible for Na⁺ - K⁺ - ATPase inhibition in hypertension is not clear. Schmidlin et al., (2007) suggested that systemic vascular dysfunction that is expressed as an impaired vasodilatory response to dietary NaCl loading is the initiating pressor effect in salt sensitive individual.
2.3.1.1 Sexual differences in salt sensitivity

Reports on sex differences in salt sensitivity in human have been inconsistent. For instance, Wilson et al., (1999) reported that during salt restriction, girls are more likely than boys to show BP reduction, while boys showed more increase compared with girls in BP in response to salt loading (Wilson et al., 1996), this finding is consistent with that of Dietary Approach to stop Hypertension DASH – sodium trial (Vollmer et al., 2001). On the contrary, a more recent study by the Genetic Epidemiology Network of Salt Sensitivity (GenSalt) group reported that BP responses to dietary sodium intake was greater in females compared with males suggesting that females have higher sensitivity to salt diet (He et al., 2008). Experimental studies have also shown that sexual dimorphism exists in salt sensitivity in laboratory animals. Several studies have suggested that male rats are more sensitive to dietary NaCl exposure than female rats (Chappel et al., 2008). When male and female DS rats are fed high salt diet, males become more hypertensive (Hinojosa – Larbode et al., 2004).

Endothelin and expression of its 2 receptor subtypes have been implicated in salt dependent hypertension (Pollock, 2000; Pollock and Schneider, 2006). Recently, Nakano and Pollock, (2009) reported that, differences in ET$_A$ and ET$_B$ receptors expression determine unique physiological actions of the endothelin system to control sodium excretion in salt-dependent hypertension. They further adduced less susceptibility of ET$_A$ receptors to reduced blood flow in the kidneys, and their consequent effect of increasing sodium excretion without increasing BP in the female rat compared to male as a possible mechanism for gender differences in salt sensitivity. Yane et al., (2009) suggested up-regulation of the intrarenal RAAS as a possible mechanism by which testosterone influences the development of hypertension and renal injury in
male DS rats fed a high salt diet. Recent observation in animal models of genetically determined salt sensitive hypertension suggest that dietary NaCl loading can induce a pressor effect that depends on dysfunctional vascular response to NaCl (Schmidlin et al., 2005).

Studies have shown that elevated dietary salt intake leads to an impaired relaxation of blood vessels to vasodilator stimuli (Lombard et al., 2003; Nurkiewicz and Boegehold, 2007; Zhu et al., 2007). The effect of sex hormones on vascular reactivity is considered as one of the underlying factors contributing to gender differences in cardiovascular functions and diseases (Sader and Celermajer, 2002; Thompson and Khalil, 2003). Gender differences in vascular tone have also been described in many vascular beds in both human and experimental animals (Geary et al., 2000). However most studies have focused on the influence of hormones on the blood vessel wall (Geary et al., 2000; Golding and Kaplan, 2001), with less attention being paid to the effects of testosterone on the vasculature, particularly in the longer term and in induced-pathologies.

2.3.2.0 Oxidative stress and hypertension

The role that oxidative stress plays in mediating hypertension has been the subject of many researches in recent years. Many clinical trials have been carried out to know whether treatment of hypertensive individuals with antioxidant will reduce their blood pressure. Data from these clinical trials have been in the least inconsistent, with antioxidants having no effect, decreasing, or even increasing blood pressure in hypertensive individuals (Czernichow et al., 2005; Ward et al., 2007).
Reactive oxygen species (ROS) are now known to have many important roles in the control of vascular functions. A major source of the variability in vascular regulation seen in arteries and veins, and the microcirculation of different organ systems during the progression of aging and disease processes originates from changes in ROS and their interactions with systems controlling endothelial mediator release and vascular function (Wolin, 2009). One of the most important ROS in the vasculature is superoxide (O$_2^-$), which is formed by the univalent reduction of oxygen (Droge, 2002). This reaction is mediated by several enzyme systems including NAD(P)H oxidases and xanthine oxidase (XO). Although O$_2^-$ can itself exert effects on vascular function, it is also pivotal in generating other reactive species. Reaction of O$_2^-$ with NO generates peroxynitrite (NOO$^-$), a potentially deleterious ROS. Dismutation of O$_2^-$ by superoxide dismutase (SOD) produces the more stable ROS, hydrogen peroxide (H$_2$O$_2$), which is then converted enzymatically into H$_2$O by catalase and glutathione peroxidase (GPx). Hydrogen peroxide (H$_2$O$_2$) can also react with reduced transition metals to be converted to the highly reactive hydroxyl radical (·OH), or it can be metabolized by myeloperoxidase (MPO) to form hypochlorous acid (HOCl). Virtually all types of vascular cells produce O$_2^-$ and H$_2$O$_2$ (Greindling et al., 2000). Multiple enzymatic systems produce O$_2^-$ and its derivatives in the vasculature, including NAD(P)H oxidases, XO, nitric oxide synthases (NOS), and MPO. The relative importance of each of these proteins appears to vary with the physiological state of the vasculature.

ROS such as superoxide and the hydroxyl radical (OH) are characterized by having one or more unpaired electrons and are chemically unstable and highly reactive (Berry et al., 2001). The established enzymatic sources of superoxide production within the vascular wall include
nicotinamide adenine dinucleotide phosphate, reduced form (NAD(P)H) oxidase (Mohazzab et al., 1994) xanthine oxidase (Ohara et al., 1993), and nitric oxide synthase (NOS) enzyme (McIntyre et al., 1997). The cellular sources of vascular superoxides are the endothelium (Mohazzab et al., 1994), vascular smooth muscle cells (VSMC) (Miller et al., 1998), and fibroblasts within the adventitia (Wang et al., 1999). Studies have demonstrated that ROS bioavailability and consequently vascular tone, is modulated by endogenous scavenging systems, such as superoxide dismutase (SOD) (Gupte et al., 1999).

Increased ROS has been demonstrated in the SHR (Suzuki et al., 1995), DOCA-salt hypertensive (Somers et al., 2000), and Dahl salt-sensitive rats (Fujii et al., 2003), as well as in hypertension induced by angiotensin II (Rajagopalan et al., 1995). In humans, enhanced production of superoxide and hydrogen peroxide has been demonstrated in untreated subjects with mild to moderate hypertension. Increased rates of vascular production of O$_2^-$ and H$_2$O$_2$ contribute to initiation of proinflammatory events, with transcriptional regulation of the gene expression of vascular cellular adhesion molecule – 1 and monocyte chemotactic protein – 1 sensitive to changes in cellular oxidant production (Tsao et al., 1996), as well as to modulation of cell signaling events (Ushio – Fukai et al., 1999). Peroxynitrite (ONOO$^-$), the diffusion – limited product of O$_2^-$ and NO, although limiting the bioavailability of NO, has been proposed to mediate many of the cytotoxic effects associated with NO because of the multiplicity of its reactions with cellular thiols, proteins, and DNA (Patel et al., 1999).

The antioxidant property of bilirubin, the end product of haem catabolism in mammals, was first demonstrated by Stocker et al., (1987). Heme oxygenase (HO) is the rate limiting enzyme for the
breakdown of haem to generate carbon monoxide, iron, and biliverdin. Biliverdin is rapidly converted to bilirubin by biliverdin reductase (Kirkby and Adin, 2006). Experimental evidence suggests that the induction of HO-1, the inducible isoform of HO, is an important endogenous mechanism for cytoprotection and the downstream products of haem degradation may mediate the beneficial effects, such as antioxidant, anti-inflammatory properties, etc (Kirkby and Adin, 2006).

Induction of HO-1 has also been demonstrated to lower blood pressure in several animal models, including the spontaneous hypertensive rat (SHR) and experimental renovascular hypertension, as well as in angiotensin II-dependent hypertension (Botros et al., 2005; Pradhan et al., 2006; Vera et al., 2007). In vitro studies have demonstrated that bilirubin exerts an antioxidant effect in either free or albumin-bound form (Nagel and coworkers, 1996). Several studies have shown a relationship between serum bilirubin and oxidative stress-mediated diseases, including coronary artery disease (Wiesel et al., 2001), angiotensin II-mediated hypertension (Kittyakara and Wilcox, 1998), and renal ischemia-reperfusion injury in vivo (Adin et al., 2005; Kirkby et al., 2007).

Several laboratory investigations have provided a biological background to explain the antioxidant and anti-inflammatory effects of bilirubin. Although bilirubin was known to scavenge peroxyl radicals in an in vitro study (Stocker et al., 1987), the antioxidant effect of bilirubin is usually mediated by inhibition of NADPH oxidase (Lanone et al., 2005) and protein kinase C activity (Chin et al., 2009). Increased endothelial NADPH oxidase activity is a key mediator of atherosclerosis and hypertension (Berry et al., 2001; Kitada et al., 2003), and activation of
NADPH oxidase appears to play a key role in TGF-beta signaling and in the responsiveness of collagen synthesis, which is a common pathway to vascular smooth muscle proliferation (Sanders, 2009; Arribas et al., 2010).

In humans, the effects of mildly increased serum bilirubin levels have been reported to be a decreased risk for the development of coronary artery disease and atherosclerosis (Novotny and Vitek, 2003). In an animal hypertensive model using hyperbilirubinemic Gunn rats infused with angiotensin II, the rise in systolic blood pressure was markedly blunted compared to that in control rats (Pflueger et al., 2005). Oxidative stress was attenuated by bilirubin in the Gunn rat and in the aortic rings from angiotensin II-infused rats (Pflueger et al., 2005). Thus, mild hyperbilirubinemia may have, at least in part, an anti-hypertensive role by the quenching of oxidative stress (Pflueger et al., 2005). However, few human studies have demonstrated the relationship between serum bilirubin level and the development of hypertension, but there is paucity of information on the level of bilirubin in salt dependent hypertension. Likewise there is little or no data on the effect of androgen on level of bilirubin in salt sensitive hypertension.

### 2.3.2.1 High salt diet and Oxidative stress

One of the mechanisms by which high salt diet induces hypertension includes generation of ROS and the consequent elevation of oxidative stress (Zhuo et al., 2003; Tian et al., 2007). It has been reported that in skeletal muscle of normotensive rats fed a high-salt diet, endothelium-dependent arteriolar responses to acetylcholine (ACh) and increased shear stress are reduced because of a suppression of local nitric oxide (NO) activity (Zhu et al., 2007). Reduced endothelium-dependent responses associated with high salt intake have also been documented in rat cerebral...
and cremaster muscle arterioles (Liu et al., 1999) and in small feed arteries of rat gracilis muscle (Liu et al., 1999).

The reduced influence of NO on arteriolar tone in rats fed a high salt diet is apparently not caused by a decrease in vascular smooth muscle responsiveness to NO, as judged by normal arteriolar responses to the NO donor sodium nitroprusside (SNP) in those animals (Liu et al., 1999; Frisbee et al., 2001). It is more likely that high salt intake leads to either a decrease in endothelial release of NO or the premature inactivation of released NO. Widespread inactivation of NO could occur with high salt intake if this diet leads to the generation of reactive oxygen species (ROS) in or near the microvascular endothelium. ROS is implicated in endothelial – dependent vascular dysfunction (Somers et al., 2000; Sofola et al., 2002), and the impaired sensitivity of VSMC to cGMP (Leite et al., 2000) in DOCA salt induced hypertension. Likewise in Dahl salt sensitive (DSS) rats, xanthine oxidase mediated ROS production has been implicated in the pathogenesis of vascular damage. Vascular hyperplasia and hypertrophy may be stimulated by increased ROS activity (Griendling et al., 2000), likewise ROS are involved in modulating a variety of intracellular signaling pathways involved in VSMC growth regulation (Irani, 2000).

2.3.2.2 Sex differences in oxidative stress

Clinical findings have shown that there is sexual difference in oxidative stress in humans. Oxidative stress is higher in men when compared with women (Ide et al., 2002), a finding that is consistent with higher blood pressure observed in males when compared with females (Barrett-Connor, 1997; Reckelhoff, 2001). Several experimental studies have also shown oxidative stress
to be higher in males animals compared to females, for example angiotensin II infusion in male rats resulted in an increase in blood pressure with a corresponding increase in superoxide production in an isolated aorta (Rajagopalan et al., 1996), while angiotensin II infusion in female rats, though elevates blood pressure, failed to change the activity of NADPH oxidase (Ebrahimian et al., 2007), or the expression of p67phox, a subunit of NADPH oxidase (Tatchum-Talom et al., 2005). Likewise tempol a superoxide dismutase mimetic reduced blood pressure in male SHR while it had no effect on female SHR (Yanes et al., 2005; Sartori-Valinotti et al., 2007) and female mREN2 Lewis rats (Chappel et al., 2008) blood pressures.

Sex differences in hem oxygenase (HO) activity have been suggested. The effect of bilirubin on the development of hypertension was reported to be more evident in females than male human subjects (Chin et al., 2009). Likewise trauma and hemorrhage was reported to double the hepatic HO-1 expression in female rats compared with male rats (Toth et al., 2003), implying sexual differences in the activity of bilirubin. With all these findings it becomes imperative to evaluate experimentally the role of androgens on ROS generating, and antioxidant enzyme depleting effects of a high salt diet.

2.3.3.0 The kidney and blood pressure regulation

A major hypothesis for the development of hypertension is that abnormal renal excretory function is critical for the initiation, development, and maintenance of primary hypertension.

The renal body fluid feedback mechanism couples the long-term regulation of arterial pressure to extracellular volume (sodium and water) homeostasis via pressure-natriuresis, whereby the kidneys respond to changes in arterial pressure by altering urinary sodium and water excretion.
The obligatory requirement for maintenance of sodium and water balance by the kidneys is believed to be primary in the long-term control of arterial pressure. An increase in arterial pressure (via increases in total peripheral resistance or cardiac output or both) leads to an increased urinary sodium and water excretion via the pressure-natriuresis mechanism, with consequent reduction in blood volume until arterial pressure is returned to normal. Thus, factors that decrease renal excretory function and disrupt the maintenance of sodium and water balance by the kidneys lead to an increase in arterial pressure, which is required to reestablish and maintain sodium and water balance. Based on computer modeling studies, a long-term increase in arterial pressure can only occur if there is a chronic and sustained decrease in renal excretory function (Coffman and Crowley, 2008).

The kidney plays a major role in the chronic regulation of blood pressure via modulation of sodium and water excretion. The central role for the kidney in BP control and the relationship between alterations in systemic blood pressure and changes in renal sodium excretion is well documented (Aperia et al., 1971, Guyton et al., 1991). For example, an elevation in perfusion pressure in the renal artery results in a rapid increase in sodium and water excretion by the kidney, so-called “pressure-natriuresis” (Aperia et al., 1971). Based on such observations, Guyton et al., (1972) suggested that whenever arterial pressure is elevated, activation of this pressure-natriuresis mechanism will cause sufficient excretion of sodium and water to return systemic pressures to normal. Many of the genes identified in humans and animals as controllers of BP regulate renal sodium handling, thus further establishing renal sodium handling, which changes with dietary salt, as central mechanism in long-term regulation of BP (Lifton et al., 2001). Furthermore mutations in these genes that decrease the ability of the kidney to excrete
sodium would cause salt and water retention and thereby increase plasma volume, cardiac output, and BP (Khalil, 2006).

The renin-angiotensin system (RAS) is an important participant in the development and maintenance of hypertension. The RAS is an essential regulator of blood pressure and fluid balance. This biological system is a multienzymatic cascade in which angiotensinogen, its major substrate, is processed in a 2-step reaction by renin and Angiotensin-converting enzyme (ACE), resulting in the sequential generation of angiotensin I and angiotensin II. Along with its importance in maintaining normal circulatory homeostasis, abnormal activation of the RAS can contribute to the development of hypertension and target organ damage (Coffman and Crowley, 2008). The RAS has potent actions to modulate pressure-natriuresis relationships in the kidney (Hall, 1999) and these actions shape the characteristics of RAS-dependent blood pressure regulation in normal physiology and in disease states. For example, chronic infusion of angiotensin II causes a shift of the pressure-natriuresis curve to the right, suggesting that when the RAS is activated, higher pressures are required to excrete an equivalent sodium load (Hall, 1999). Conversely, administration of ACE inhibitors or angiotensin receptor blockers shifts the curve to the left, meaning that natriuresis is facilitated at lower levels of blood pressure. The basic features of endogenous control of the RAS are consistent with these homeostatic functions. The system is activated at low levels of salt intake stimulating renal sodium reabsorption and conservation of body fluid volumes and blood pressure. In contrast, with high sodium intake, the system is suppressed, facilitating natriuresis.
2.3.3.1 Sexual dimorphism in renal regulation of blood pressure

Several studies have suggested significant effects of sex steroids on the renal control mechanisms of the blood pressure, particularly the rennin-angiotensin system (Reckelhoff et al., 2000, Reckelhoff, 2001; Venegas-Pont et al., 2010). There is sexual dimorphism in rennin-angiotensin in human system. Plasma renin activity was reported to be higher in men than in aged-matched women (Liu et al., 2003). It has also been shown that plasma renin activity is higher in postmenopausal women than in premenopausal women or postmenopausal women receiving hormone replacement therapy (Liu et al., 2003). In animals, studies have shown that renal angiotensinogen mRNA levels in normotensive rats are affected by gender, with angiotensinogen mRNA being higher in kidneys from males than from females (Liu et al., 2003). Castration decreases while testosterone treatment of ovariectomised females increases angiotensinogen mRNA (Liu et al., 2003). Estrogen treatment of ovariectomised female rats has also been shown to reduce angiotensin converting enzyme activity and mRNA (Reckelhoff, 2001). Takeda-Matsubara et al. (2002) suggested, estradiol inhibits renin release and the angiotensin converting enzyme (ACE), whereas testosterone may increase the blood pressure by activating the rennin-angiotensin system (Reckelhoff et al., 2000). But the interactions of sex steroids with the RAS are complex and appear to be influenced by age and genetic background (Ely et al., 2000). Moreover, the role of sexual dimorphism on renal handling of salt loading is unclear.

2.4.0 Vascular Biology

The vessel wall is the final common pathway for the impact of cardiovascular risk factors and genetic predisposition to pathological conditions such as hypertension, arteriosclerosis and
atherothrombotic events (Minson and Green, 2008). Therefore its health and function may therefore reflect aggregate cardiovascular risk. Figure 2 shows the histomorphological representation of the blood vessel.

Fig. 2: Histomorphological representation of the blood vessel (McGeachie, 2006)
2.4.1.0 Vascular endothelium

Endothelial cells are very flat, have a central nucleus, and are about 1-2 µm thick and some 10-20 µm in diameter. They form flat, pavement-like patterns on the inside of the vessels and at the junctions between cells there are overlapping regions which help to seal the vessel. These extraordinary cells were once considered to be simple lining cells with very few functional roles, other than to keep cells within the blood from leaking out of the vessels. However, for some years research on endothelial cells has revealed that they have an amazing array of functional and adaptive qualities. Moreover, they are the key determinants of health and disease in blood vessels and play a major role in arterial disease.

The vascular endothelium, a single cell layer separating blood and vascular smooth muscle, regulates vessel tone through release of vasoactive factors such as nitric oxide (NO) and prostanoids (Sader and Calermajer, 2002). As a cellular plane, it forms the surface on which blood cells, vessel wall growth, and adhesive factors interact to form the nidus of atherogenic lesion. The endothelial cell behaves as a receptor-effector structure that senses different physical or chemical stimuli that occur inside the vessel, and therefore modify the vessel shape or releases the necessary products to counteract the effect of the stimulus and maintain homeostasis. The endothelium is capable of producing a large variety of different molecules, as agonists as antagonists, therefore balancing the effects in both directions. Endothelium produces vasodilators and vasoconstrictors, procoagulants and anticoagulants, inflammatory and anti-inflammatory, fibrinolytics and antifibrinolytics, oxidizing and antioxidizing, and many others (Vanhoutte, 1999). When endothelial cell lose their ability to maintain this delicate balance, the conditions are right for the endothelium to be invaded by lipids and leukocytes (monocytes and T-
lymphocytes). The inflammatory response is incited and fatty streaks appear the first step in the formation of the atheromatous plaque. If the situations persist, fatty streaks progress and the plaque are exposed to rupture and set the condition for thrombogenesis and the vascular occlusion (Esper et al., 2006).

2.4.1.1 Nitric oxide (NO)

Nearly all stimuli that produce vasodilatation do it through nitric oxide (NO), a volatile gas, biologically active, present practically in all tissue and thanks to its low molecular weight and its lipophilic properties it diffuses easily across cell membranes. The NO crosses the endothelial intima and reaches the smooth muscular tissue of the arterial wall, and, through nitrosilation of the heam from the guanylate-cyclase degrades GTP releasing cGMP, which in turn regulates the cytosolic Ca\(^{2+}\) and causes smooth muscle fiber relaxation and therefore vasodilatation (Loscalzo and Welch, 1995).

NO is produced by the action of nitric oxide synthases (NOS) on L-arginine amino acid, producing NO and L-citruline, requiring O\(_2\) and Nicotinamide Adenine Dinucleotid Phosphate (NADP) coenzyme, essential in Redox process. Tetra-hydro-biopterine accelerates this process, which is favored by other cofactors like flavin-adenine dinucleotide, and thiol groups like cysteine and reduced glutathione. Three NOS isoenzimes are known, two constitutive and low-production, NOS-I from neurological tissue (nNOS) and NOS-III from endothelial cells (eNOS), both respond to agonist that increase intracellular Ca\(^{2+}\). The other one, inducible NOS-II (iNOS), is specially expressed in macrophage and endothelial cells due to the effect of pro-inflammatory cytokines and can release several times more NO than the constitutives NOS. Both constitutive
and inducible NOS are in the endothelial cells. Constitutive NOS produces NO for short periods when it is induced by vasodilators like acetylcholine or bradykinin. Inducible NOS synthesize NO for longer periods in a constant manner when the stimulus comes from pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) (Loscalzo and Welch, 1995).

The most important stimulation for NO release comes from shear stress, that is caused by the increase in blood velocity and leads to a vasodilatation proportional to the amount of NO released by the endothelium (Cooke and Tsao, 2001). This vasodilatation is called endothelium-dependent. The endothelial cell membranes contains specialized ion channels, such as Ca\(^{2+}\)-activated K\(^+\) channels, that open in response to shear stress (Miura et al., 2001). The effect is to hyperpolarize the endothelial cell, increasing the driving force for Ca\(^{2+}\) entry and activates the enzyme NOS-III and the subsequent generation of NO (Miura et al., 2001). Nitrates given in any way are NO donors, unconfined NO into the circulation directly releasing cGMP in the smooth muscle cell and causing a vasodilatation that is not dependent of the endothelial response, for this reason it is called endothelium-independent vasodilatation. Shear stress induces a persistent production of NO that maintains a constant vasodilatation (Ludmer et al., 1986). Shear stress explains the importance of the haemodynamic factor in the formation, localization and plaque fissure. This usually develops in areas were the shear stress is low (<6 din/cm\(^2\)), oscillating or retrograde, where NO release is diminished and adhesion molecules are increased and chemical and growth factors create a proinflammatory atmosphere. On the other hand, a high shear stress (>70 din/cm\(^2\)) can cause endothelial erosion and provoke platelet aggregation, or cause plaque rupture or damage. Thus, a low or retrograde shear stress allows plaque formation. Physiological
levels of shear stress that protect the endothelium are between these two values (6 to 70 din/cm²) (Feldman and Stone, 2001).

Nitric oxide apart of being a vasodilator also reduces vascular permeability and the monocyte and lymphocyte adhesion molecules synthesis. NO also reduces platelet aggregation, tissue oxidation, tissue inflammation, activation of thrombogenic factors, cell growth, proliferation and migration, also it inhibits proatherogenic and pro-inflammatory cytokines expression and it favors fibrinolysis. Nuclear factor kappa-B (NFκB) inhibitor (I-kB) is also expressed by NO. All these factors reduce atherogenesis and its complication. For this reason NO is considered the antiatherogenic molecule (Baldwin, 2001; Libby et al., 2001).

2.4.1.2 Prostanoids

The prostanoids are part of a family of biologically active lipids derived from the action of cyclooxygenases or prostaglandin synthases upon the twenty-carbon essential fatty acids or eicosanoids. They can be further subdivided into three main groups, the prostaglandins, prostacyclins and thromboxanes, each of which is involved in some aspect of the inflammatory response (Buczynki et al., 2009). It is useful to consider the other great source of vasodilatation, via arachidonic acid cascade that ends in prostacyclines, which release cAMP from ATP that regulates cytosolic Ca²⁺, therefore producing relaxation and vasodilatation (Iniguez et al., 2008).

Two prostanoids are especially important and have essential but opposing functions in the maintenance of vascular homeostasis, i.e. thromboxane TXA₂ and prostacyclin PGI₂ (Fetalvero et al., 2007). TXA₂ is synthesized mainly in platelets which express only COX-1, production
being enhanced during platelet activation, and it promotes platelet aggregation, vasoconstriction, and smooth muscle proliferation, even though it has a half-life of only 20-30 seconds (Flower, 2006). In contrast, prostacyclin is the main product of macro-vascular endothelial cells. It is a potent vasodilator, and it inhibits platelet aggregation and smooth muscle cell proliferation. Thus, it contributes substantially to myocardial protection (Fulton and Stallone, 2002; Fetelvero et al., 2007). COX-2 is the enzyme that provides the main source of prostacyclin. Both TXA₂ and PGI₂ are therefore important mediators of pathological vascular events including thrombosis and atherogenesis, and it is evident that the correct balance between the two prostanoids is essential to good cardiovascular health (Fetelvero et al., 2007). The ratio of TxA₂:PGI₂ seems to be more important than the absolute amounts of these mediators that are produced in vivo. Further relevant factors are increased expression and activation of the TP receptor (for TXA₂) in atherosclerotic lesions, which can directly accelerate atherogenesis and plaque growth. The cardio-protective effect of aspirin, established by clinical trials (Fulton and Stallone, 2002), is exerted by the irreversible long-term inhibition of platelet COX-1 and thence of TXA₂ biosynthesis for the lifetime of a platelet in the circulation (aspirin has little effect on PGI synthesis) (Tilley et al., 2001). Indeed, aspirin appears to be the only COX inhibitor with proven cardioprotective activity. In contrast, there is some concern that specific COX-2 inhibitors may have pro-thrombotic effects by inhibiting prostacyclin synthesis relative to that of thromboxanes (Parente and Peretti, 2003).

2.4.1.3 Endothelium Derived Hyperpolarizing Factor (EDHF)

Combined blockade of endogenous production of NO and prostacyclin, do not completely abolish endothelium dependent relaxation in some vascular beds, especially those with smaller
diameters (Feletou et al., 2003). This suggests there is a third autacoid that contributes to endothelium dependent vasodilatation mechanisms in these vascular beds. This autacoid is called endothelium dependent hyperpolarizing factor because its vasodilator effects are strongly associated with smooth muscle hyperpolarization, and because the nature of EDHF was unknown (Feletou et al., 2003) and remains controversial (McGuire et al., 2001; Busse et al., 2002).

There are currently three main suggestions as to the nature of EDHF, which are not mutually exclusive but may represent differences between species, between vascular beds and between different endothelial stimulants. One suggestion is that EDHF represents endothelial hyperpolarization generated by the activation of Ca\(^{2+}\)-activated K\(^+\) channels (K\(_{\text{Ca}}\)) that spread passively via myoendothelial gap junctions to result in hyperpolarization of the smooth muscle cells (Coleman et al., 2004). According to this idea, endothelial K\(_{\text{Ca}}\) channels would influence smooth muscle contractile activity by reducing Ca\(^{2+}\) influx via voltage-operated Ca\(^{2+}\) channels and by suppression of key enzymes involved in agonist-induced transduction pathways (Feletou et al., 2003). Another suggestion is that EDHF is a product of the cytochrome P450 pathway, such as an epoxyeicosatrienoic acid (EET), and since EETs can activate large-conductance, Ca\(^{2+}\)-activated K\(^+\) channels (BKCa), it has been inferred that EDHF evokes hyperpolarization via the activation of BKCa channels on the smooth muscle cells (Coleman et al., 2004). The third suggestion is that K\(^+\) efflux from endothelial cells via intermediate- and small-conductance Ca\(^{2+}\)-activated K\(^+\) channels (IKCa and SKCa, respectively), activates inward rectifier K\(^+\) channels (KIR) and the Na\(^+\)/K\(^+\)ATPase on the smooth muscle cells (Coleman et al., 2004).
The mechanism of endothelium-dependent hyperpolarization, once attributed to an elusive endothelium derived hyperpolarizing factor (EDHF), is now better understood. EDHF-mediated responses are triggered by an increase in the endothelial intracellular calcium concentration that is followed by the opening of two populations of potassium channels, the calcium-activated potassium channels of small and intermediate conductance ($SK_{Ca}$ and $IK_{Ca}$), which results in the hyperpolarization of the endothelial cells. This response is transmitted to the smooth muscle cells by direct electrical coupling through myoendothelial junctions and/or by the accumulation of potassium ions in the intercellular myoendothelial space (Busse et al., 2002).

EDHF plays an increasingly prominent role in vasodilatation as arterial diameter decreases, and is thus likely to be important in tissue perfusion. EDHF has been suggested to be a compensatory mechanism for maintenance of blood flow in pathological conditions such as hypertension and diabetes where the availability of NO may be reduced (Ledoux et al., 2006). This implies that the synthesis and mechanism of action of EDHF must be insensitive to reactive oxygen species (Hecker, 2000). EDHF has been reported to be targeted in diseases such as hypertension and diabetes, and oxidative stress is involved either in the pathogenesis or as a consequent of these disease conditions. Oxidative stress in the pathogenesis of salt sensitive hypertension has been reported to exhibit sexual dimorphism, but it is not clear whether EDHF activity is affected by sex and/or sex steroids in the vasculature. The knowledge of the role of androgen on mechanisms underlying EDHF activity would give an improved understanding of the nature of EDHF and impact on our understanding of the sexual differences that exist in the regulation of vascular tone in health and in disease.
2.4.2 **Vascular smooth muscle**

There is a wealth of evidence to support the contention that regulation of vascular smooth muscle tone and ultimately vascular resistance is dependent on a tightly coupled balance between vasoconstrictor and vasodilator influences. Factors tending to shift the balance towards constriction increase vascular resistance, while factors that shift the balance toward dilation decrease vascular resistance. Studies in recent years have more clearly defined the cellular pathways associated with constriction and relaxation of vascular (Taylor et al., 1999).

2.4.2.1 **Contraction**

Contractile stimuli produce an increase in intracellular calcium, phosphorylation of myosin and cross bridge formation. The dependence of smooth muscle contraction on calcium (Ca$^{2+}$) was first determined by Filo et al., (1965), who demonstrated that a critical concentration range of Ca$^{2+}$ (180–1000 nM) was needed to evoke contraction of permeabilized vascular smooth muscle. Myosin phosphorylation, a necessary event for smooth muscle contraction, was later shown to be a Ca$^{2+}$ dependent process (Sobieszek, 1977). Dabrowska et al., (1978) consequently identified myosin light chain kinase (MLCK) in vascular smooth muscle. The calcium dependent modulator protein, calmodulin, has been shown to activate MLCK, which phosphorylates the regulatory light chains of myosin. This phosphorylation increases the affinity of myosin filaments for binding sites on the actin filaments. Based upon these findings, a general model of smooth muscle contraction has been proposed.

Stimulation of smooth muscle cells causes an increase in cytosolic Ca$^{2+}$ concentration [Ca$^{2+}$], (van Breemen, 1977). Calcium ions form complexes with calmodulin (four Ca$^{2+}$ ions, one
calmodulin molecule) which binds to and activates MLCK (Kemp et al., 1987). Active MLCK transfers the terminal phosphates of ATP to regulatory light chains of myosin, thereby altering the affinity of myosin for actin. The terminal phosphates are transferred to serine-19 on each of the two 20 kDa myosin light chain subunits (also called LC20). Binding of actin then stimulates myosin ATP-ase, resulting in myosin cross-bridge cycling along actin filaments and muscle contraction (Adelstein and Klee, 1981).

In order to appreciate the cellular mechanism whereby pathological states can lead to altered vasoregulatory function, it is important to consider first, the means by which vascular smooth muscle generates force. Figure 3 shows the two primary mechanisms of vascular smooth muscle contraction, electromechanical and pharmacomechanical that was described Taylor et al. (1999). Electromechanical coupling begins with membrane depolarization via the gating of ion channels (e.g. K+, Cl-, and Ca2+). As a result of the altered electrochemical gradient, voltage gated Ca2+ channels open (L- and T-channels), allowing the diffusion of Ca2+ down its concentration gradient and into the cell (Nilius and Droogmans, 2001). Under these conditions, the primary trigger for force development appears to be calcium availability. Pharmacomechanical coupling, does not require cell depolarization, but involves the binding of a ligand (e.g. norepinephrine, vasopressin, angiotensin II) to a G-protein coupled membrane receptor (Meldrum et al., 1991). Membrane associated G-proteins are heterotrimers comprised of α, β, and γ subunits. Upon activation, the α-subunit dissociates from the βγ-subunits to trigger a cascade of events. Differences in Gα-subtypes confer some degree of specificity to the G-protein response. Three key α-subtypes are involved in G-protein coupled responses of vascular smooth muscle. The Gαs and Gαi subtypes activate, and inhibit adenylyl cyclase, respectively. The αq-subunit of G-
proteins induces vasoconstriction primarily through the activation of cellular phospholipases. For constrictors such as α-adrenergic agonists, the β-isoform of phospholipase-C (PLC-β) is stimulated. This enzyme hydrolyzes membrane-associated phosphatidylinositol 4,5-bisphosphate (PIP$_2$), yielding inositol 1,4,5-trisphosphate (IP$_3$) and 1,2-diacylglycerol (DAG) (Meldrum et al., 1991). The targets of each of these cleavage products differ. DAG activates protein kinase-C (PKC) which is known to increase ionic conductance through transmembrane channels (L-type Ca$^{2+}$ and K$^+$ channels) (Nilius and Droogman, 2001). PKC is also believed to modulate the calcium sensitivity of the contractile apparatus (Taylor et al., 1999). IP$_3$ binds receptors on the SR membrane causing the release of Ca$^{2+}$ into the cytoplasm (Berridge, 2005). Furthermore, muscarinic or α$_1$-adrenergic agonists can bind to G-protein coupled receptors and activate phospholipase 2 (PLA$_2$) causing generation of arachidonic acid. Arachidonic acid inhibits MLCP thereby increasing calcium sensitivity (Taylor et al., 1999). In addition to the well-defined L-type channels, the smooth muscle cell membrane also contains other types of Ca$^{2+}$ channels. Release of Ca$^{2+}$ from the SR has been shown to induce Ca$^{2+}$ influx through store-operated channels (SOC) on the plasma membrane. This mechanism allows for the refilling of depleted internal Ca$^{2+}$ stores (hence the term capacitative Ca$^{2+}$ entry) and allows for additional increases in cytosolic Ca$^{2+}$ concentration (Berridge, 2005; Putney, 2007). Another source of calcium influx is the receptor operated Ca$^{2+}$ channel (ROC). Binding of a vasoconstrictor to its receptor may elicit Ca$^{2+}$ influx or cause depolarization and subsequent activation of L-type Ca$^{2+}$ channels via mechanisms that do not involve G-proteins. T-type channels are voltage-gated channels on cell membranes which open transiently in response to cell depolarization. However, unlike the L-type channels, T-type channels do not appear to contribute substantially to the regulation of intracellular calcium levels in vascular smooth muscle (Nilius and Droogman, 2001). The route
of Ca\(^{2+}\) entry and its relative contribution to constriction appear to be agonist-dependent. Diacylglycerol activates PKC, which may enhance contraction in various ways. PKC may phosphorylate and open L-type Ca\(^{2+}\) channels, eliciting an increase in Ca\(^{2+}\) influx. PKC may also increase Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels indirectly by phosphorylating and inhibiting membrane potassium channels, allowing for cell depolarization (Domeier et al., 2008). Additionally, PKC has been suggested to enhance Ca\(^{2+}\) sensitivity in smooth muscle, possibly through inhibition of MLCP or phosphorylation of microfilament regulatory proteins such as caldesmon and calponin (Cobine et al., 2007). In both electromechanical and pharmacomechanical coupling, the resultant increase in [Ca\(^{2+}\)], may cause further Ca\(^{2+}\) release through stimulation of ryanodine sensitive receptors located on SR membranes (Bootman and Roderick, 2008). This Ca\(^{2+}\)-induced Ca\(^{2+}\) release (ICR) has been implicated in a number of complex intracellular Ca\(^{2+}\) signaling events such as Ca\(^{2+}\) oscillations and waves (Berridge, 2005). Stretch-activated Ca\(^{2+}\) channels that are mechanosensitive cation channels believed to be important for normal myogenic control of vascular tone have also been implicated in mechanism of smooth muscle contraction.

2.4.2.2 Relaxation

Removal of the contractile stimulus is the first step towards smooth muscle relaxation. The primary mechanism of smooth muscle relaxation is reduction of free cytosolic calcium concentration through sarcoplasmic reticulum (SR) sequestration and plasmalemmal pump activity. Reduction of calcium leads to the dissociation of calmodulin from MLCK. Myosin is then dephosphorylated by MLCP which minimizes actin: myosin interactions, thereby disengaging the contractile mechanism. However, certain ligands (e.g. β-adrenergic agonists,
glucagon, and prostacyclin) can cause receptor-mediated active relaxation of vascular smooth muscle. Other substances such as nitric oxide cause active vasorelaxation by receptor-independent pathways. Receptor-mediated relaxation is most often associated with elevations in cyclic nucleotides (Taylor et al., 1999). Most often, vasodilation is associated with the elevation of cyclic nucleotides, cAMP and cGMP, in vascular smooth muscle cells (Taylor et al., 1999). Cyclic AMP is generated through the binding of certain ligands (e.g. β-agonists, prostaglandins, and glucagon) to membrane receptors which triggers the dissociation of the heterotrimeric Gs protein (Hofer and Lefkimmiatis, 2007). The Ga subunit activates the membrane-bound adenylyl cyclase, which catalyzes formation of cAMP from ATP (Brunton et al., 2008). Cellular functions of cAMP are manifested through the activation of cAMP-dependent protein kinase (PKA) (Taylor et al., 1999). Saturation of the receptor or binding with cAMP causes dissociation and activation of the two catalytic subunits of this tetrameric enzyme. The catalytic subunits of PKA catalyze the phosphorylation of various cellular targets, altering their activity. Cyclic AMP is degraded by cellular phosphodiesterase (PDE) enzymes, producing the inactive metabolite 5′-AMP. In vascular smooth muscle, these PDEs are typically comprised of the cAMP-specific isoform PDE4 and the cGMP-inhibitable isoform PDE3 (Brunton et al., 2008). Cyclic AMP is postulated to inhibit vasoconstriction by two distinct mechanisms, which are, decreasing VSM cell Ca\(^{2+}\) concentrations and by reducing Ca\(^{2+}\) sensitivity of the contractile apparatus. McDaniel et al., (1991) found that elevating cAMP with the adenylyl cyclase activator forskolin reduced Ca\(^{2+}\) concentrations in swine arterial smooth muscle as measured with aequorin. A number of investigators have reported similar effects on myoplasmic Ca\(^{2+}\) concentration during periods of cAMP elevation (Taylor et al., 1999). Since smooth muscle contraction is calcium-dependent, one may conclude that cAMP relaxes smooth muscle by lowering calcium availability. However,
evidence indicating that cAMP substantially alters the association between $[\text{Ca}^{2+}]_i$ and tension also exists. A number of investigators have reported cAMP-mediated alteration of $\text{Ca}^{2+}$ influx from the extracellular compartment. Unlike skeletal and cardiac muscle, where cAMP elevation increases $\text{Ca}^{2+}$ entry through PKA phosphorylation of voltage sensitive L-type $\text{Ca}^{2+}$ channels (Taylor et al., 1999), cAMP inhibits inward $\text{Ca}^{2+}$ current through membrane $\text{Ca}^{2+}$ channels in vascular smooth muscle. It remains unclear how PKA inhibits activity of L-type $\text{Ca}^{2+}$ channels in vascular smooth muscle and stimulates L-type $\text{Ca}^{2+}$ channels in skeletal and cardiac muscle although the existence of cell-specific regulatory proteins seems most likely. It has been proposed that cyclic nucleotide reduction of voltage dependent $\text{Ca}^{2+}$ influx and associated vascular smooth muscle relaxation may result from regulation of membrane $\text{K}^+$ channels. Under such conditions, cyclic AMP would increase $\text{K}^+$ efflux through these channels, thereby hyperpolarizing the cell and limiting $\text{Ca}^{2+}$ influx through the voltage sensitive L-type channels. Four types of $\text{K}^+$ channels have been identified in vascular smooth muscle plasma membranes (Nelson and Quayle, 1995). The voltage-gated $\text{K}^+$ channel ($\text{Kv}$), calcium-activated $\text{K}^+$ channels ($\text{K}_{\text{Ca}}$), ATP-sensitive $\text{K}^+$ channels ($\text{K}_{\text{ATP}}$) and the inward rectifying potassium channel ($\text{K}_{\text{IR}}$). Evidence indicates that some of these channels are regulated by vasodilator stimuli (Huang et al., 2000), hence implicating the activities of various potassium channels in smooth muscle relaxation. Studies have shown that when smooth muscle preparations were exposed to inorganic nitrogen compounds or classic vasodilators (e.g. sodium nitroprusside or nitroglycerine), vasorelaxation was accompanied by increased cGMP levels (Tang et al., 2005). The production of cGMP is stimulated by the enzymatic activity of guanylyl cyclase on guanosine triphosphate (GTP). There are two types of guanylyl cyclase in vascular smooth muscle cells. One is the particulate enzyme, which is actually a transmembrane spanning receptor that contains guanylyl
cyclase within the intracellular domain (Brunton et al., 2008). The other form is free in the cytoplasm and referred to as soluble guanylyl cyclase. Particulate guanylyl cyclase binds ANPs, whereas soluble guanylyl cyclase is stimulated by NO. The physiological effects of cGMP in cells are achieved by its interactions with intracellular proteins (Brunton et al., 2008).

There are three main types of target proteins for cGMP, namely PKGs, cGMP-regulated ion channels, and cGMP-phosphodiesterases (Taylor et al., 1999). Cyclic guanosine monophosphate phosphodiesterases degrade cGMP. Thus, cyclic GMP can alter cell function through protein phosphorylation or through mechanisms not directly related to protein phosphorylation. The mechanisms of action of cGMP are analogous in some respects to cAMP in that cGMP has been found to act predominantly through the activation of PKG. Unlike PKA, PKG is made up of two identical subunits with regulatory and catalytic domains within the same peptide (Taylor et al., 1999). Binding of cGMP induces a conformational change in the kinase, exposing the catalytic sites. The activated catalytic subunits of the kinase serve to phosphorylate cellular proteins, enzymes and ion channels which may lead to reduced free calcium in the sarcoplasm (Taylor et al., 1999).
Fig. 3: Key cellular events associated with pharmacomechanical and electromechanical coupling.

L-type Ca\(^{2+}\) channels (L) can be activated by either pathway. Dotted lines indicate inhibitory effects. (Abbreviations: SOC, store-operated Ca\(^{2+}\) channels; ROC, receptor-operated Ca\(^{2+}\) channels; R, G-protein coupled receptor; PLA\(_2\), phospholipase A\(_2\); PLC, phospholipase C; PC, phosphatidylcholine; AA, arachidonic acid; PIP\(_2\), phosphatidylinositol 4,5-bisphosphate; DAG, 1,2-diacylglycerol; PKC, protein kinase C; IP\(_3\), inositol 1,4,5-trisphosphate; IP\(_3\)R, IP\(_3\) receptor; RyR, ryanodine receptor; Cam, calmodulin; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; SERCA, sarcoplasmic endoplasmic reticulum calcium-ATPase; CaP, calponin; CaD, caldesmon; A, actin; M, myosin) (Taylor et al., 1999).
2.4.3 Ions and their channels in the vasculature

2.4.3.1 Calcium

Vascular tone and hence blood pressure are determined by the contractile state of vascular smooth muscle cells (VSMCs) within the blood vessel wall, which is regulated by intracellular calcium concentration \([\text{Ca}^{2+}]_i\). Vasoconstrictors act through increasing \([\text{Ca}^{2+}]_i\) as well as on the apparent calcium sensitivity of the contractile process in VSMCs, whereas relaxing factors have the opposite effect. In contrast, an elevation in endothelial \([\text{Ca}^{2+}]_i\) induces relaxation of the adjacent VSMCs. Therefore, fine tuning of \([\text{Ca}^{2+}]_i\) in both cell types is imperative for precise regulation of organ and tissue perfusion (Ledoux et al., 2006).

The major pathways for an increase in myocytes \([\text{Ca}^{2+}]_i\) are voltage-dependent Ca\(^{2+}\) channels (VDCC) and nonselective cation channels in the plasmalemmal membrane and internal store release channels, i.e., the ryanodine (RyR) and the inositol trisphosphate \((\mathrm{IP}_3)\) receptors found in the sarcoplasmic reticulum (SR) membrane. Global \([\text{Ca}^{2+}]_i\) is mainly dictated by the open state probability of L-type VDCC, which are finely controlled by the membrane potential (Ledoux et al., 2006). Activity of different ion channels present at the plasma membrane such as \(\text{K}^+\), \(\text{Cl}^-\), and cation channels govern the membrane potential and therefore affect the VDCC activity and calcium entry. Opening of \(\text{K}^+\) channels such as the large-conductance \(\text{Ca}^{2+}\)-activated \(\text{K}^+\) channel (BK) hyperpolarizes the membrane, promoting closure of VDCC and thus opposing vasoconstriction. In contrast to the VSMCs, an increase in endothelial \([\text{Ca}^{2+}]_i\) results in vascular relaxation through endothelium-derived relaxing factors such as nitric oxide (NO), prostacyclin (PGI\(_2\)), and endothelium-derived hyperpolarizing factor (EDHF). Furthermore, due to the absence of VDCC, hyperpolarization of the endothelial membrane by activation of \(\text{Ca}^{2+}\)-sensitive
K⁺ channels does not reduce calcium influx. In fact, membrane potential hyperpolarization would promote calcium entry possibly involving transient receptor potential channels (TRPC1, TRPC3, TRPC4, TRPC6, TRPV4, etc.) through an elevation of the electrochemical driving force for Ca²⁺ (Ahmed and Malik, 2005). An increase in [Ca²⁺]ᵢ in endothelium or in VSMCs exerts opposing influences on blood vessel diameter. The nature of calcium signals in endothelium and smooth muscle are also fundamentally different. Endothelium-dependent vasodilators such as acetylcholine appear to act in part through the elevation of endothelial [Ca²⁺]ᵢ, followed by the generation of endothelium-derived relaxing factors. However, the nature of the intracellular calcium dynamics at rest in the endothelium remains unclear (Ledoux et al., 2006).

Three different forms of calcium signaling have been identified in smooth muscle. First, global cytosolic [Ca²⁺]ᵢ represents averaged calcium levels throughout the entire cytoplasm and controls smooth muscle contractility through the activation of the myosin light chain kinase (Hai and Murphy, 1989). Global calcium can also influence gene expression via a calmodulin (CaM) kinase activation of the transcription factor cAMP response element binding protein (Wellman et al., 2001) or via the nuclear factor of activated T-cells (Hill-Eubanks et al., 2003).

A second form of calcium signaling reported in vascular smooth muscle consists of propagating waves (“calcium waves”). Calcium waves can be induced by extracellular alkalization or exposure to caffeine (Hepner et al., 2002) as well as by endogenous vasoconstrictors such as UTP (Jaggar et al., 2000) or norepinephrine (Boittin et al., 1999). These calcium waves or oscillations result from intracellular calcium release through IP₃R and/or RyR (Bootman and Roderick, 2008). Whereas calcium waves are thought to be responsible for smooth muscle...
contraction (Boittin et al., 1999), they can also paradoxically activate BK channels, promoting membrane hyperpolarization and thereby opposing smooth muscle contraction (Young et al., 2001). Although information regarding calcium waves in vascular smooth muscle is rapidly emerging, their physiological function remains only partly resolved (Ledoux et al., 2006).

The general view of a necessary homogeneous elevation of calcium throughout the entire cell for effective calcium signaling has been altered by the discovery of highly spatially and temporally localized calcium transients (“calcium sparks”) in skeletal (Klein et al., 1996), cardiac (Cheng et al., 1993), and smooth (Nelson et al., 1995) muscle cells. Calcium sparks result from the opening of RyRs in the SR, and a single calcium spark generates a highly restricted (~1% of the cell volume) and large (10–100µM) increase in \([Ca^{2+}]_i\), whereas the global calcium is raised by only 2 nM. A calcium spark, by virtue of its massive local calcium elevation, has the potential to modulate \(Ca^{2+}\)-dependent processes that are not responsive to global increases in \([Ca^{2+}]_i\) (Berridge, 2005).

### 2.4.3.2 Potassium

Potassium is the most abundant intracellular ion, a feat that is achieved and maintained by \(Na^+\)-\(K^+\)-ATPase in the plasma membrane. The sodium pump is the active transport system that is responsible for maintenance of the transmembrane gradients of \(Na^+\) and \(K^+\). Because these gradients provide energy for several essential cellular functions (e.g., control of membrane potential, cell volume, and pH), it is not surprising that this transport protein is present in all animal cells (Hardy et al., 2006). Potassium channels help determine the resting membrane potential and regulate cell volume. Because cells maintain a much higher intracellular
concentration of potassium than that present in the extracellular medium, the opening of potassium channels induces a change in membrane potential toward more negative values (repolarization or hyperpolarization). They play a key role in many cellular signaling events, including the regulation of smooth muscle tone and blood flow (Shieh et al., 2000).

Potassium channels are classified into four subgroups (according to Nomenclature Compendium—International Union of Pharmacology subcommittee on potassium channels): the voltage-gated (KV), the calcium-activated (KCa), the two-pore-domain (K2P), and the inward rectifier (Kir) potassium channel families (International Union of Pharmacology, 2002). Four types of K+ channels have been identified in vascular smooth muscle plasma membranes (Nelson and Quayle, 1995). The voltage-gated K+ channel (Kv) regulates smooth muscle membrane potential in response to depolarizing stimuli. Calcium-activated K+ channels (KCa) are activated by cytosolic Ca2+ as well as changes in membrane potential. ATP-sensitive K+ channels (KATP) are sensitive to cell metabolic status (i.e. open in response to low ATP) and a number of other intracellular signals. The inward rectifying potassium channel (Kir) is known to open under hyperpolarizing conditions (Nelson and Quayle, 1995).

Calcium activated potassium channels (KCa) are classified into three groups, the large conductance (BKCa), intermediate conductance (IKCa) and the small conductance (SKCa) calcium activated potassium channels. The native BKCa channel is composed of four α- and four β-subunits (Tanaka et al., 1997). The α-subunit, encoded by the Slo gene, is the pore-forming portion of the channel and consists of 11 hydrophobic domains (S0–S10). Current opinion suggests that the transmembrane-spanning domains (S0–S6) constitute the so-called “core”
region with an extracellular NH2 terminus. The four remaining domains (S7–S10) are thought to be located in the cytoplasm and form the COOH-terminal “tail” of the protein (Ledoux et al., 2006). Recent studies suggest that the α-subunit also contains an intrinsic sensitivity to [Ca\(^{2+}\)]. The predominant β-subunit in VSMCs is the β1 isoform (Tanaka et al., 1997). The β1-subunit interacts with the S0 domain and the extracellular NH2 terminus of the α-subunit, thereby increasing the apparent voltage and calcium sensitivity of the channel (Brenner et al., 2000; Nishimaru et al., 2004). Although the α-subunit determines the basic calcium sensitivity of the BK channel, the β1-subunit appears to be responsible for the translation of physiological calcium signals to regulate vascular function. Indeed, genetic deletion of the β1-subunit leads to a decreased calcium sensitivity of the BK\(_{\text{Ca}}\) channel, resulting in an uncoupling of BK\(_{\text{Ca}}\) channels from calcium sparks. Furthermore, deletion of the β1-subunit leads to a more depolarized membrane potential, an elevation of [Ca\(^{2+}\)], followed by vasoconstriction and ultimately, an elevation of arterial blood pressure (Brenner et al., 2000). Consistent with these results, a gain-of-function mutation of the β1-subunit was reported to protect against human diastolic hypertension by increasing the apparent calcium and voltage sensitivity of the BK channel (Fernandez-Fernandez et al., 2004).

Another class of Ca\(^{2+}\)-activated K\(^+\) channels with a smaller unitary conductance (10–40 pS) was first identified in the brain. It is referred to as small-conductance Ca\(^{2+}\)-activated K\(^+\) channels (SK\(_{\text{Ca}}\) channels). The expression pattern of SK channels is quite different from the BK channel. SK\(_{\text{Ca}}\) channels are expressed in neurons, endothelium, epithelium, and several types of smooth muscles (e.g., urinary bladder) but not in VSMCs. However, IK\(_{\text{Ca}}\) was shown to be expressed in VSMCs, but only when the myocytes were in a proliferative state (Neylon et al., 1999). Opening
of the SK pore is independent of the membrane potential but strictly depends on $[\text{Ca}^{2+}]_i$. The calcium sensitivity of SK channels depends on CaM. The binding of calcium ions to the coupled CaM induces a conformational change of the complex involving an interlacing of cytoplasmic loops that leads to the channel pore opening.

The critical influence of $\text{Ca}^{2+}$-activated $\text{K}^+$ channels in both endothelium and smooth muscle cells suggests an involvement in cardiovascular diseases. Indeed, the prevalence of hypertension increases with age, and this is correlated with a reduction of $\text{BK}_{\text{Ca}}$ channel expression (Marijic et al., 2001; Nishimaru et al., 2004). A number of recent findings support the importance of the smooth muscle $\text{BK}_{\text{Ca}}$ channel in the regulation of vascular tone and blood pressure. Ablation of the gene for the smooth muscle specific $\beta_1$-subunit of the $\text{BK}_{\text{Ca}}$ channel leads to an increase in vascular tone and hypertension, accompanied by left ventricular hypertrophy in mice (Lohn et al., 2001). Fernández-Fernández et al. (2004) reported a mutation of the human $\beta_1$ gene leading to a gain of function of the $\text{BK}_{\text{Ca}}$ channel associated with a low incidence of human diastolic hypertension. Moreover, $\beta_1$ relative to the $\alpha$-subunit expression is down-regulated in chronically hypertensive rats (Amberg and Santana, 2003). $\text{SK}_{\text{Ca}}$ and $\text{IK}_{\text{Ca}}$ channels are expressed in vascular endothelium, and suppression of expression of SK3 channels leads to an elevation of blood pressure (Taylor et al., 2003). Attenuated vasoconstrictor responses in rats with cirrhosis are associated with elevated endothelial $\text{SK}_{\text{Ca}}$ channel expression (Kohler et al., 2003), and blunted endothelium-dependent vasodilatation of carotid arteries after balloon catheter injury appear to be linked to decreased $\text{SK}_{\text{Ca}}$ and $\text{IK}_{\text{Ca}}$ expression in regenerated endothelial cells (Barriere et al., 2001).
Inwardly rectifying K\(^+\) channels (K\(_{\text{IR}}\)) may be crucial for the maintenance of membrane potential in some small resistance arteries (coronary, cerebral and mesenteric (Schubert et al., 1996). The inward rectification means that the channel conducts potassium current more readily in the cells than out of the cells over a wide range of potentials. When the membrane potential is negative compared with the equilibrium potential for K\(^+\) (E\(_{\text{K}}\)), the driving force for the flux of K\(^+\) is in the inward direction. However, for positive membrane potentials (compared to E\(_{\text{K}}\)), the outward flow of K\(^+\) through K\(_{\text{IR}}\) is smaller. Under physiological conditions, the membrane potential of vascular cells is always positive compared with E\(_{\text{K}}\), so it is the relatively small efflux of K\(^+\) that plays a role (Nelson and Quayle, 1995; Ford et al., 2002). In the vascular wall, K\(_{\text{IR}}\) channels are expressed in both the endothelial and the smooth muscle cells (Droogman and Nilius, 2001).

The expression of the K\(_{\text{IR}}\) channel is more abundant in the smooth muscle of autoregulatory vascular beds such as the coronary and cerebral circulations (Robertson et al., 1996). In the general circulation, the expression of the K\(_{\text{IR}}\) channel appears to increase as the diameter of the artery decreases (Kim et al., 2005). K\(_{\text{IR}}\) channels are blocked by micromolar concentrations of barium and certain imidazoline compounds (Quignard et al., 2003). A unique feature of K\(_{\text{IR}}\) channels is the effect of extracellular potassium on its gating. A moderate increase in potassium concentration, in the range of 1 to 15 mM, enhances potassium efflux through K\(_{\text{IR}}\) at physiologically relevant potentials (Quignard et al., 2003). In some arterial smooth muscle cells, this moderate increase in extracellular potassium concentration leads to hyperpolarization and relaxation (Nelson and Quayle, 1995). The K\(_{\text{IR}}\) channel most likely involved in potassium-induced relaxation is composed of the K\(_{\text{IR}}\) \(\alpha\)-subunits, as these relaxations disappear in mice with deletion of K\(_{\text{IR}}\) (Zaritsky et al., 2000). In some arteries, potassium-induced relaxation involves the contribution of endothelial K\(_{\text{IR}}\) (Crane et al., 2003).
2.5.0 Androgens

Androgen is the name given to the male sex steroids. Cholesterol is the precursor of the five major classes of steroid hormone: progestogens, glucocorticoids, mineralocorticoids, estrogens and androgens. The term ‘androgen’ means any steroid hormone that has masculinizing effects. Therefore it includes male sex hormones produced elsewhere in the body besides the testis (the major site of production). The testis secretes several male sex hormones, which are collectively called androgens: testosterone, dihydrotestosterone and androstenodione. Testosterone is formed by the interstitial cells of Leydig which lie in the interstices between the seminiferous tubules and constitute about 20 percent of the mass of the adult testes (Guyton and Hall, 1996). Figure 4 shows the step by step synthesis of androgens. The synthesis of androgens starts with hydroxylation of progesterone at carbon-17 (C-17). The side chain consisting of carbon-20 and carbon-21 is then cleaved to yield androstenedione, an androgen. Testosterone is formed by reduction of the 17-keto group of androstenodione. Androgens contain nineteen carbon atoms. Estrogens are synthesized from androgens by the loss of a C-19 angular methyl group and the formation of an aromatic A ring. This reaction requires NADPH and oxygen. Estrone, an estrogen is derived from androstenodione, whereas estradiol, another estrogen is formed from testosterone (Stryers, 1988).

Figure 5 shows the structure of testosterone contains the notable features of a 17-β-OH (hydroxyl) and a 3-keto group which are necessary for activity. Oxidation of the 17-β-OH to a 17-keto group, or conversion to a 17-α-OH, results in steroids with significantly decreased androgen activity. Likewise, the 3-OH instead of 3-keto group is much less active partly due to increased metabolism. The primary site of testosterone synthesis is the testes (Leydig cells) with
low level synthesis occurring in the adrenal cortex. Low levels of testosterone are also synthesized in the ovary. These compounds are under tight biosynthetic control, with short and long negative feedback loops that regulate the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary and gonadotropin releasing hormone (GnRH) by the hypothalamus. Low levels of circulating sex hormone reduce feedback inhibition on GnRH synthesis (the long loop), leading to elevated FSH and LH. The latter peptide hormones bind to gonadal tissue and stimulate P450ssc activity, resulting in sex hormone production via cAMP and PKA mediated pathways. By a long feed-back loop, testosterone inhibits its own synthesis by acting upon the hypothalamus to turn off luteinizing hormone releasing hormone (LHRH) secretion. Classic androgenic effects include the development of male sex characteristics, both fetal and at puberty, ongoing spermatogenesis, and maintenance of male characteristics. Anabolic effects include increased muscle mass and protein synthesis. Testosterone may act by blocking protein breakdown induced by cortisol, which is secreted from the adrenal cortex in response to stress (Guyton and Hall, 1996).
Fig. 4: Synthesis of the male sex hormones in Leydig cells of the testis.

P450SSC is P450-linked side chain cleaving enzyme, 3β-DH is 3β-dehydrogenase, P450c11 is 11β-hydroxylase, P450c17 is 17α-hydroxylase and P450c21 is 21β-hydroxylase (Foye et al., 1995).
2.5.1 Testosterone analog, structure and activity relationships

Testosterone analogs show increased activity if the double bond between carbons 4 and 5 is reduced. While this reduction occurs normally during activation in some tissues by the action of 5-α reductase, the enzyme is not present in muscle tissue. Introduction of fluorine at C9, as for glucocorticoids, increases activity. Addition of an alkyl group to position 17 increases stability by preventing oxidation to an inactive 17-keto form. Oxidation to the 17-keto form normally occurs in the liver and is a significant obstacle to oral administration. The 17-α methyl testosterone has twice the activity of native testosterone and can enter through the buccal mucosa.
(mouth membranes). Removal of the 19-CH₃ enhances selectivity for anabolic activity by decreasing selectively the androgenic effect. Creation of an ester at the 17-β position leads to longer acting analogs, presumably by creation of prodrugs (Foye et al., 1995). Figure 6 shows the structures of testosterone propionate and cypionate, typical testosterone esters.

Fig. 6: Chemical structure of Testosterone propionate and testosterone Cypionate (Foye et al., 1995)

2.5.2 Side effects of testosterone analogs

Use of androgen-like steroids by women sometimes (but not always) leads to irreversible virilization including hair loss and male pattern baldness. Use by men can lead to testicular atrophy and decreased spermatogenesis due to negative feedback to the hypothalamus (decreased
LHRH). Men can obtain the anabolic effect of administered testosterone or its derivatives systemically, but the high concentrations inhibit LH (leutinizing hormone) release from the anterior pituitary with decreased maintenance of testes and spermatogenesis. Human chorionic gonadotropin (hCG) has been used in the past by some male athletes to maintain testicular function, presumably due to its perceived structural similarity to LH, but with unverified results. High doses of anabolic steroids can slow the rate of liver metabolism and lead to increased liver toxicity. Anabolic steroids can cause premature bone fusion in children leading to stunted growth (Foye et al., 1995). Anabolic steroids can aggravate cardiovascular disease by lowering levels of HDL's (high density lipoproteins). Anabolic steroids are banned in high-level sporting competitions. Athletes in such competitions are routinely screened for natural and synthetic steroids and metabolites. Use of testosterone itself is the most difficult to detect, but the ratio of the concentrations of testosterone (17-β OH) to epitestosterone (17-α OH) in the urine is tested. The normal ratio for the epimers in the urine is 1:1, so it is illegal to have a ratio of >6:1 β: α OH testosterone in sporting competitions. Taking testosterone in the absence of exercise does not build muscle mass, steroids only enhance the extent of increased muscle mass in those who train rigorously and strenuously, such as competitive weightlifters (Foye et al., 1995).

2.5.3 Testosterone inhibitors

Antiandrogens may have beneficial effects as potential treatments for prostate cancer or acne (testosterone affects the sebaceous glands beginning at puberty). No steroidal antiandrogen drugs have been approved for general use. An example of an experimental antiandrogen steroid drug is cyproterone acetate. Flutamide is an example of a non-steroidal inhibitor which binds to the androgen receptor and prevents testosterone action. Flutamide is indicated for use in conjunction
with LHRH agonists such as leuprolide, for treatment of metastatic prostate carcinoma. Flutamide and leuprolide treatment when started simultaneously increased median survival rates from 27.9 to 34.9 months in one study. Flutamide inhibits both androgen uptake and binding in target tissue. Finasteride is a synthetic 4-azasteroid compound that acts as a specific inhibitor of steroid 5-a reductase, the intracellular enzyme that converts testosterone into the potent androgen 5-α dihydrotestosterone (DHT). It is given to reverse the enlargement of the prostate gland which is often associated with urinary symptoms and decreased urine flow. Finasteride is a competitive and specific inhibitor of 5-α-reductase and has no affinity for the androgen receptor (Foye et al., 1995).

Doxazosin mesylate has an unlabelled use for treatment of benign prostatic hypertrophy, and is indicated for treatment of hypertension and severe congestive heart failure (in conjunction with diuretics and cardiac glycosides). It acts by inhibiting post-synaptic alpha-adrenergic receptors with resulting vasodilation and decrease in total peripheral resistance and blood pressure (Foye et al., 1995). Extracts of saw palmetto contain phyosterols that reportedly inhibit 5 a-reductase activities. Saw palmetto extracts may be beneficial in the treatment of benign prostatic hyperplasia (Goepel et al., 1999).

### 2.5.4 Metabolism of Androgens

After secretion by testis about 97% of the testosterone becomes either loosely bound with plasma albumin or more tightly bound with a beta globulin called sex hormone binding globulin and circulates in the blood in these states for about 30 minutes to 1 hour. By this time testosterone either becomes fixed to the tissues or is degraded into inactive products that are subsequently
excreted. Much of the testosterone that becomes fixed to the tissue is converted within the cells to Dihydrotestosterone, especially in certain target organs such as the prostate gland in the adult male and the external genitalia of the fetal male. Some actions of testosterones are dependent on this conversion, whereas other actions are not. Testosterone that does not become fixed to the tissue is rapidly converted mainly by the liver, into androsterone and dehydroepiandrosterone and simultaneously conjugated as either glucuronides or sulfates (glucuronides particularly). These are excreted either into the gut in the liver bile or into the urine through the kidney (Guyton and Hall, 1996).

2.5.5 Mode of Actions of Androgens

The classical pathway of androgen action involves steroid binding to the androgen receptor (AR), a ligand activated transcription factor and single copy member of the nuclear receptor superfamily, acting on the genome (Quigley et al., 1995). Androgens mediate their effects in target cells predominantly by interacting with the androgen receptor (AR). Like other steroid hormone receptors, the AR consists of an N-terminal hypervariable domain, a DNA-binding domain (DBD) and a ligand-binding domain (LBD) (MacLean et al., 1997). Inside target cells, androgens bind to the LBD, resulting in dissociation of inhibitory receptor-associated proteins. Once activated, the AR undergoes dimerisation, phosphorylation, nuclear translocation and DNA binding to androgen response elements located within target genes to activate or repress transcription (Brinkmann et al., 1999). This is referred to as genomic action of androgen (Notini et al., 2005). The genomic action of AR is modulated by a large variety of co-regulators, which are proteins that fine-tune target gene expression by enhancing (co-activator) or restraining (co-repressor) transcription (Heinlein and Chang, 2002). The development of the first non-steroidal
androgens (Edward et al., 1998) has promise in exploiting tissue-specific differences in androgen sensitivity (Yin et al., 2002). Whether such tissue-specific partial androgen agonists (selective AR modulators) which are structurally non-aromatizable and functionally dihydrotestosterone (DHT) analogs, would have useful roles in vascular therapeutic mediated via AR remain to be clarified. Pharmacological targeting of non-genomic and androgenic vasodilator mechanisms in vascular smooth muscle seems promising, in contrast to endothelial and vessel wall mechanism that involve aromatization.

There is now considerable evidence for rapid, non-genomic effect of steroids, including androgens (Losel and Wehling, 2003). Non-genomic steroid action is distinguished from genomic effect by, rapid onset (seconds to minutes) that is faster than genomic mechanisms. Insensitivity to inhibition of RNA and protein synthesis, effects produced by steroids unable to access the nucleus (either by covalently-linked to membrane impermeable macromolecules or in cells devoid of nucleus) and, not usually blocked by classical antagonists due to different steroid specificity from classical cognate nuclear receptor (Heinlein and Chang, 2002). A full understanding of the nature of these mechanisms is still far from being accomplished, only descriptive information on different non-transcriptional effects of steroid hormones has been accumulated (Moggs and Orphanides, 2001).

One of the most compelling arguments in favor of the presence of non-nuclear actions of steroid hormones is represented by the localization of a pool of steroid receptors (SRs) at the cell membrane level. Pioneering evidence for the existence of such a distinct subpopulation was provided in the late 1970s by the work of Pietras & Szego, who described the presence of
cytoplasmic membrane binding sites for estradiol in endometrial cells (Pietras and Szego, 1977). Since then, several reports on these receptors have been contributed using imaging techniques, and evidence that these molecules mediate important cellular actions has accumulated (Levin, 1999). Indeed, plasma membrane estrogen receptors (ERs) have been advocated to be involved in the regulation of cell membrane ion channels (Valverde et al., 1999), G-protein-coupled receptors (GPCRs) (Kelly and Wagner, 1999), tyrosine kinases and mitogen-activated protein kinases (MAPKs) (Watters et al., 1997). Moreover, they have also been shown to activate adenylate cyclase production (Aronika et al., 1994), as well as to trigger phospholipase C (PLC) activation (Le Mellay et al., 1997).

Non-genomic androgen effects characteristic involve the rapid induction of conventional second-messenger signal transduction cascades, such as increases in cytoplasmic calcium and activation of protein kinase A, protein kinase C, and MAPK, leading to diverse cellular effects including smooth muscle relaxation, neuromuscular and junctional signal transmission and neuronal plasticity (Heinlein and Chang, 2002). No membrane AR has been characterized, but preliminary evidence of a low affinity microsomal membrane binding site for alkylated androgens (Luzardo et al., 2000) and an endothelial cell plasma membrane dehydroepiandrosterone (DHEA) binding site (Liu and Dillion, 2002) still require functional proof of specific receptor status. Also a plasma membrane SHBG membranes and initiating intracellular cAMP signaling has been described in humans (Kahn et al., 2002). The sex hormone binding globulin (SHBG) receptor remains to be fully characterized, and it is not clear whether it has any physiological role in species like rodents that lack circulating SHBG.
The earliest suggestion that testosterone may have an effect on vascular function, emanated from the findings of Hamn and Walker both of them working separately but coincidentally at the same time reported that testosterone injection has a relieving effect on patient with angina pectoris (Liu et al., 2003). This opens the gate to flood of researches into how testosterone could be beneficial to men suffering from cardiovascular diseases an assertion that negates the higher prevalence of cardiovascular diseases in males. More recently testosterone was shown to be beneficial to men with coronary artery disease (English et al., 2000) or congestive heart failure (Malkin et al., 2009), and it elicits relaxation response in the rabbit airway smooth muscle (Koloumenta et al., 2006), on human radial artery, (Seyrek et al., 2007) and pulmonary artery (Smith et al., 2008). Most of the in-vitro non genomic effects of androgen on the vascular tone have been described in normal experimental animals (Yue et al., 1995; Ding and Stallone 2001). To the best of our knowledge there is paucity of literature on the effect of testosterone on vascular reactivity in experimental animals with induced-pathologies such as hypertension or hypogonadism.

2.5.6 Metabolic activation of testosterone

A key issue in the biological effects of testosterone is its conversion to bioactive metabolites. Although only a small fraction (< 5%) of testosterone output undergoes such transformation usually in local tissues, conversion both amplifies and diversifies testosterone action. Conversion to its 5α-reduced metabolite, DHT by either type 1 or type 2 5α-reductase amplifies testosterone action because DHT has higher molar potency due to its more avid binding affinity and slower dissociation rate from the AR (Grino et al., 1990). Type 1 and type 2 5α-reductase has been identified in vascular tissue based on immunoreactivity (Eicheler et al., 1995), and
enzymatic activity (Martel et al., 1994), but the biological consequences of androgen amplification in vessel walls remain to be clarified (Higashiura et al., 1996). Conversion of testosterone to estradiol by the enzyme aromatase (CYP19) diversifies androgen action by activating estrogen receptors (ER). Aromatase gene expression (Murakami et al., 2001; Nathan et al., 2001), protein (Murakami et al., 2001; Nathan et al., 2001), and enzymatic activity (Bayard et al., 1995) have been detected in vascular tissues, including human coronary arteries (Diano et al., 1999) particularly in endothelium and smooth muscle.

2.6.0 Dehydroepiandrosterone (DHEA)

Dehydroepiandrosterone (DHEA) is a controversial hormone. There is debate about its physiological roles, its mechanisms of action, its relationship to health and disease, indeed, whether it is a hormone at all (Labrie et al., 2005). Despite uncertainty about its status, it is widely promoted and sold as a complementary medicine (Komesaroff, 2008). DHEA and its sulfated prohormone, DHEA sulfate (DHEAS) are quantitatively the most abundant circulating adrenal steroid hormones in humans. Circulating DHEAS serves as a reservoir for DHEA, with conversion by sulfotransferases occurring in a wide range of tissues. There is also extensive metabolism to estrogens and androgens, giving rise to the view that many of its effects are mediated by these hormones or other metabolites (Hanning et al., 1991). Plasma DHEAS levels decline with age and vary with sex, ethnicity, and environmental factors (Orentreich et al., 1984). DHEA has been linked, usually controversially, to many diseases, including malignancies (Rao et al., 1992), neurological dysfunction (Maurice et al., 2006), and systemic lupus erythematosus and other immune disorders (Crosbie et al., 2007), and claims have been made that DHEA deficiency contributes to the symptoms associated with adrenal insufficiency (Allolio
et al., 2007), ageing (Grimley et al., 2006), menopause (Raven and Hinson, 2007), and disorders of sexual function (Panjari and Davis, 2007). However, the major interest in the hormone stems from epidemiological studies that have been said to show an inverse relationship between cardiovascular mortality and plasma DHEA(S) levels in men (La croix et al., 1992), but these data have been seriously contested (Tchermof and Labrie, 2004).

Over the last few years, evidence has steadily mounted in support of a physiological role for DHEA in cardiovascular tissues. Animal studies have shown its anti-atherogenic actions in several models of vascular dysfunction (Hayashi et al., 2000). In vitro data have shown actions on vascular endothelium and smooth muscle (Williams et al., 2002) and on key mediators of atherogenesis. DHEA(S) influences proliferation of vascular endothelial and smooth muscle cells independently of androgen and estrogen receptors (Williams et al., 2004). In endothelial cells, it increases expression of nitric oxide synthase (NOS) and thereby secretion of NO, an important regulator of vascular functions, and protects these cells against apoptosis (Simoncini et al., 2003).

Dehydroepiandrosterone (DHEA) administration to humans improves vascular endothelial function (Williams et al., 2004), reduces known cardiovascular risk markers (Beer et al., 1996), and appears to inhibit atherosclerosis (Hayashi et al., 2000). The effects on endothelial cells have been reported to be mediated, at least in part, through the activation of the MAPK ERK1/2 (Williams et al., 2004). Progress has been made in elucidating the receptors through which DHEA acts. Although the best characterized steroid receptors are nuclear transcription factors, it is now recognized that steroids can in some cases also activate plasma membrane receptors and
thereby initiate cytosolic kinase cascades (Simoncini et al., 2003). Liu and Dillion, (2002) reported a membrane-bound, G protein coupled receptor for DHEA identified in bovine vascular endothelial cells. The binding of ligand was of high affinity ($K_d = 48.7 \text{ pM}$) and saturable. These authors further showed that the receptor is maximally activated by 1–10 nm DHEA to stimulate endothelial NOS and enhance NO production (Liu and Dilion, 2004), and more recently they reported that the effect of DHEA on endothelium occurs via activation of extracellular signal-regulated kinase (ERK1/2) (Liu et al., 2008). They provide evidence linking DHEA effects at the endothelial plasma membrane with cellular proliferation and angiogenesis by a process mediated by pertussis toxin-sensitive G proteins and ERK1/2 (Liu et al., 2008).

Yet, uncertainty remains about the control of the biologic events of DHEA, given the high circulating concentrations of DHEA. It is possible that the specificity of DHEA action is established through control of hormone delivery to tissues by yet to be characterized binding proteins, tissue-specific DHEA receptor expression, regulation of the expression and activity of the receptor, expression of coreceptors, or other mechanisms (Komesaroff, 2008).

### 2.7.0 Androgens and Blood Vessels

Until recently, researchers assumed that the targets for sex hormones were primarily the reproductive organs: the breast, female reproductive tract (uterus and ovary), and male reproductive tract (testes and epididymis) (Wierman, 2007). Bone was known to be a target of sex hormones based on the data that gonadectomy of either sex resulted in osteoporosis and sex-specific hormonal replacement restored bone structure and function (Lindsay, 2004). An expanded list of sex hormone targets became apparent when investigators examined the
phenotypes of naturally occurring mutations in humans and genetically altered mouse models (Wierman, 2007). Deficiency of aromatase (the enzyme that converts testosterone to estradiol) or knockout of the ER, progesterone receptor (PR), or androgen receptor (AR) in mice showed tissue-specific deficits (Matsumoto et al., 2005). Together, this research suggested that sex steroid hormones function in an expanded list of target tissues (Wierman, 2007). These include the vascular system, central nervous system, gastrointestinal tract, immune system, skin, kidney, and lung. An understanding of the tissue-specific roles of gonadal hormones is important when predicting the benefits or risks of replacing natural ligands or use of steroid hormone antagonists in humans.

Recent investigation has shown the importance of sex hormone action in the vasculature in both sexes (Mendelsohn and Karas, 2005). Research has documented the presence of ERs, PRs, and ARs in vascular endothelial cells, smooth muscle cells, and cardiomyocytes as targets of sex hormone ligands. Estrogen administration has been shown to improve vascular reactivity, increase nitric oxide production, decrease free radical production, and prevent programmed cell death in normal vasculature. In contrast, in the diseased vessel, a different gene program may be activated in response to estrogen administration that promotes plaque destabilization and thrombosis through the activation of metalloproteinases. Importantly, studies have confirmed that there is a dose-response relationship to various sex steroid ligands in different tissues. These basic studies may give insight into the unexpected toxicities observed when combined conjugated equine estrogen and daily progestins were given broadly to postmenopausal women in Women’s Health Initiative trials (Turgeon et al., 2006).
Gender differences are characteristic of animal models of atherosclerosis. Males develop earlier and more extensive atherosclerotic plaques independent of lipid levels in diet-induced models in several laboratory animals (Liu et al., 2003). For instance, testosterone treatment consistently inhibited atherosclerosis in castrate, cholesterol-fed male rabbits. The most detailed study showed that castration of male rabbits increased, whereas both testosterone and DHEA treatment inhibited aortic atherosclerosis. Androgens also enlarged coronary diameter (Obasanjo et al., 1996) and enhanced endothelium-dependent acetylcholine vasodilator responses (Adams et al., 1995), consistent with vasodilator effect of androgens. AR is expressed in all cells of the vasculature, including endothelial cells smooth muscle cells, myocardial fibers, macrophages and platelets (Liu et al., 2003). In earlier studies myocardial (Lin et al., 1990) and aortic (Tamaya et al., 1993; Knauthe et al., 1996), AR content was similar in male and female rats (Knauthe et al., 1996) rabbit (Tamaya et al., 1993) and non human primates (Lin et al., 1990). However males showed more nuclear localization consistent with greater AR activation by endogenous testosterone (Lin et al., 1990). Studies using more sensitive detection methods find consistent gender differences in vascular tissue AR content. Higher AR expression in males is reported for rat vascular smooth muscle (Higashiura et al., 1997) human macrophages from peripheral blood (McCrohon et al., 2000) or synovium (Cutolo et al., 1996) and mesenteric artery and endothelial cells (Death et al., 2003). Hormonal regulation of AR protein levels in non-reproductive tissue including the vasculature is not well defined.
2.8.0 Androgens and Hypertension

Blood pressure is higher in men than in women from puberty onward (Barrett-Connor, 1997; Reckelhoff, 2001) so that the prevalence and complications of hypertension exhibit consistent gender differences. Animal models suggest that this is primarily due to testosterone increasing blood pressure, but the mechanism remains only partly understood. The nature of the testosterone effect remains elusive, although kidney transplantation demonstrates the involvement of an extra-renal factor (Harrap et al., 1992) and endothelium-derived substance causing higher polarization of vascular smooth muscle involving potassium channels (Honda et al., 1999).

Evidence for key involvement of the Renin-Angiotensin System (RAS) as an effect of an angiotensin-I-converting enzyme (ACE) inhibitor (Reckelhoff et al., 2000) is less convincing given the non-specific hypotensive effect of ACE blockade. Further studies using transgenic models would be more definitive. The hypothesis that the role of testosterone in hypertension-prone rats may reflect variations in tissue androgen sensitivity (Reckelhoff, 2001) warrants further analysis, including the association of the CAG triplet repeat functional polymorphism in the human AR that influences tissue androgen sensitivity (Jin et al., 2000). Similar findings are reported for the salt-sensitive hypertensive male rat model with testosterone regulating renal α-2β adrenoreceptors (Khalid et al., 2002). There is also evidence for a rat Y-chromosome locus producing gender selective hypertension mediated by testosterone and the AR (Ely et al., 2000) and involving sympathetic nervous system activation (Ely et al., 1991). The responsible rat locus, its human homologues and the specific mechanisms remain to be identified.
Neonatal androgens imprinting determines the sexually dimorphic nature of blood pressure patterns of spontaneously hypertensive rat (SHR) (Plut et al., 2002), and susceptibility to diet-induced hypertension (Plut et al., 2002). Likewise a single neonatal testosterone dose administered to new born female increased their blood pressure to male levels at maturity (Cambotti, 2000). This may be an important clue, when considered in conjunction with the natural history of gender differences in cardiovascular mortality that points to atherogenesis having similar progression in men and women but with men having a head start at some undefined early stage in pathogenesis. A key event in early male life is the perinatal androgen surge when blood testosterone concentrations reach adult levels for months. This epoch is critical for hormonal imprinting of brain (Arnold and Gorski, 1984), prostate (Prins, 2001), and probably other androgen sensitive tissues, perhaps including vascular tissues. More specific examination of gender in relation to fetal environmental programming and related perinatal events, such as the androgen surge and hormonal imprinting may be informative. Therefore studying the effect of withdrawing androgens using bilateral orchidectomy at weaning, would aid in the understanding of the sexually dimorphic nature of salt-sensitivity, oxidative stress and renin-angiotensin system and their role in the maintenance of normal and elevated blood pressure in a non-genetically hypertensive rat model such as Sprague-Dawley rats. Outcome of this study is likely to be highly informative on how gender and androgens modulate the cardiovascular system.
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1.0 Animals

One hundred and thirty eight weanling male Sprague-Dawley rats were used for this study. Inbred weanling male Sprague-Dawley rats from College of Medicine of the University of Lagos Laboratory Animal Center, aged 8 weeks old with a weight range of 90 - 100 g were housed in steel cages and maintained under standard lighting conditions 12 hours light, 12 hours dark period. Food and water were provided *ad libitum*. The rats were divided into eight groups of 16 rats each. In addition ten inbred male Sprague Dawley rats weighing between 250 – 300g were used for the non-genomic study at the Department of Cellular and Molecular Cardiology (DCMC), Clinical Wing, Sree Chitra Tirunal Institute of Medical Sciences and Technology (SCTIMST), Kerala, India. The rats were identified as Sprague-Dawley strains by Dr. I.A. Taiwo of the Department of Cell Biology and Genetics, Faculty of Science, University of Lagos, Lagos. Rats used for the non-genomic study were identified by Dr A.C.E. Fernandez, The Head and Scientist-in-Charge, Department of Laboratory Animal Sciences (DLAS), Bio Medical Technology Wing (BMT), SCTIMST, Kerala, India.

3.1.1 Surgery

Sixty four weanling male rats were anesthetized with a mixture of ketamine (91mg / kg, i.m) and xylazine (9mg / kg, i.m) (Gonzales et al., 2004) for bilateral removal of the testes under aseptic surgical conditions. As soon as the animals were at the deep surgical state, they were placed on their back on a surgical table. The perineal and the scrotal area were cleared of fur, wiped with
methylated spirit, and a small incision was made at the lower end of the scrotal sac. A neat midline dissection was carried out and each layer of the skin and the fascia was carefully separated until the testes were exposed. The scrotal arteries were tied and the two testes were removed. The scrotal sac was carefully sutured back using size 3/0 silk braided suture. Thirty two sham-orchidectomised animals underwent all these procedures except that when the scrotal sac was opened the testes were not removed, and the scrotal sac was also sutured back. All operated rats received an injection of penicillin (300,000 i.u/kg/body weight of benzylpenicillin sodium B.P/ procaine penicillin B.P i.m) each at the time of surgery to prevent infections (Zhu et al., 2005) and after recovery from anaesthesia, all animals were returned to their cages. The animals were allowed a 3-day recovery period (Zhu et al., 2005), before the beginning of the experiment.

3.1.2 Salt–Loading

Hypertension was induced by feeding male Sprague-Dawley rats with a diet containing 8% NaCl for 6 weeks. Estimates indicate that 8% salt diet to rats is equivalent to 40g/day in humans, an intake that is significantly higher than what is obtainable in the normal human diet (Luft, 1989). This method of induction of hypertension is very widely used and consistently produces a sustained elevation in blood pressure in many rat strains, such as Sprague Dawley (Adegunloye and Sofola, 1997; Ebuehi et al., 2003; Porter et al., 2007), Wistar Kyoto (Vasdev et al., 2007), Sabra (Khalid et al., 2002), Dahl Salt sensitive (Dahl-SS) (Tian et al., 2007; Yane et al., 2009) and spontaneously hypertensive (SHR) (Varagic et al., 2008; Susic et al., 2010) rats. Salt-loading was carried out by feeding the rats with high salt diet. High salt diet was prepared by adding
7.7% (w/w) NaCl to the rats’ chow which already contained 0.3 % NaCl to give 8% (Sofola et al., 1993, Adegunloye and Sofola, 1997).

3.1.3 Testosterone supplementation

Testosterone supplementation which is synonymous with hormone replacement therapy was carried out through intramuscular injection of Sustanon 250® (10mg/Kg body weight) once in three weeks. The rats were injected in the thigh region. Sustanon 250® is a trade name for an oil-based injectable blend of four esterised testosterone compounds: 30mg testosterone propionate; 60mg testosterone phenylpropionate; 60mg testosterone isocaproate and 100mg testosterone decanoate. The different testosterone esters have different half-lives (Foye et al, 1995). Esterization of the testosterone molecules provides a sustained (but non-linear) release of testosterone from the injection depot into the blood plasma. The intention of the mixed testosterone esters in Sustanon is to provide a more stable serum testosterone levels (Schubert, 2006). Sustanon 250® is a product of N.V. Organon Oss Holland. A stock of 10mg/ml was made in a 10% ethanol solution and stored in a refrigerator from where the subsequent doses were drawn.

3.1.4 Animal Groups

Group I: Intact rats that were given normal rat chow and water only.

Group II: Intact rats that were given high salt diet and water.

Group III: Orchidectomized rats that were given normal rat chow and water only.

Group IV: Orchidectomized rats that were given high salt diet and water only.
Group V: Orchidectomized rats that were supplemented with parenteral testosterone. They were fed with normal rat chow and water only.

Group VI: Orchidectomized rats that were supplemented with parenteral testosterone. They were fed with high salt diet and water only.

Group VII: Sham-operated rats that were given normal rat chow and water only.

Group VIII: Sham-operated rats that were given high-salt diet and water only.

3.2.0 Determination of body weight

The animals were weighed before and weekly throughout the period of the experiment, using a Duet top loading scale (Salter, England). After the experimental period, the percentage weight gain was calculated by subtracting the initial weight from the final weight and dividing the difference by the initial weight and then multiplied by 100. The final weights of the animals were compared across the groups.

3.3.0 Collection of blood and separation of serum

The animals were sacrificed by cervical dislocation, and blood was collected immediately. The thoracic cavity was opened and blood was collected via cardiac puncture using a 5ml syringe and 21g needle. Blood was collected into a 5ml sterile sample bottles without any anticoagulant and allowed to clot. The blood was then spun in a centrifuge at 3000 rpm for ten minutes. The supernatant was carefully withdrawn into a 3ml Eppendorff’s tube, using a Pasteur pipette. Serum samples were stored at -80°C at the Department of Molecular Medicine, Bio Medical
3.4.0 Determination of Heart, Kidney and Liver weights

After sacrifice the, heart, kidneys and liver were removed, carefully cleared of connective tissues, dried between filter paper and weighed. The weight index of each organ was taken as the division of such organ by the total body weight multiplied by 100. And the weight index of each organ was compared across the groups.

3.5.0 Polygraph and transducers

The blood pressure measurements were determined after arterial cannulation was carried out using a blood pressure transducer (Statham Gould, Model P23D, Hato Rey, Puerto Rico). Some of the vascular reactivity studies were carried out using an isometric force transducer (Model FT03C, Grass Instruments, Mass, USA). The transducers were coupled to a grasm polygraph (Model 7D, Grass Instruments) via a preamplifier and a driver amplifier to an ink writing stylus. The transducers were calibrated daily before the commencement of measurement. Calibration of the driver amplifier of the polygraph involved selecting a polarity switch position either for blood pressure or isometric force recording. A convenient baseline and driver sensitivity were also chosen before switching the driver amplifier to “USE”. The preamplifier was then calibrated. An appropriate balance voltage and a sensitivity level (usually 1mV / cm) were selected for use with the blood pressure transducer or isometric force transducer. After calibration of the polygraph, the blood pressure transducer was connected to a preamplifier and then calibrated. The transducer was filled with 1% heparinised saline, made free of air bubbles
and then connected to aneroid barometer. The blood pressure transducer was calibrated by taking consistent recordings of 40mmHg increments in pressure using mercury manometer with a cuff. This was carried out before the commencement of actual blood pressure recordings. The isometric force transducers were calibrated after coupling to the polygraph, using a standard 1g weight. The pen sensitivity was adjusted such that 1g weight gives 20mm deflection (Ettarh, 2004).

3.6.0 Blood pressure studies

3.6.1 Invasive blood pressure measurement: Anaesthesia and cannulation

Five randomly selected animals from each group were used for invasive blood pressure measurement. Invasive blood pressure measurement was carried out with arterial cannulation (Dick et al., 2008). The rats were anaesthetized with a solution of 25% w/v urethane and 1% w/v α-chloralose injected intraperitoneally at a dose of 5ml/kg body weight of rats (Ettarh, 2004). The anaesthetized rat was placed on its back on the operating table and the limbs were fastened to the table. A small incision was made on the anterior part of the neck exposing the anterior longitudinal muscles of the neck in front of the trachea. The muscles, fascia and connective tissues were carefully teased apart by blunt dissection with a curved forceps and the trachea exposed. Two loops of thread were then placed around the trachea, to hold in place a plastic cannula of similar diameter that was inserted into the trachea. The blood pressure measurements were obtained by cannulation of either of the femoral arteries. The artery was exposed by blunt dissection in the inguinal area of the lower limb. It was carefully separated from the vein and nerve situated on the either side. Two ligatures were placed around a free length of the artery using fine silk threads. The distal thread was tied and a bulldog clip was placed at the proximal
end. An oblique cut was made in the artery between the tied ligature and the bulldog clip, and a polyethylene cannula filled with 1% heparinised saline was inserted and tied place. The cannula was gently pushed just past the bulldog clip and the ligature firmly tied. The cannula was connected to the pressure transducer by a 3-way tap. The tap was closed to artery and transducer, and then the air bubbles were flushed out at the point of connection. This was to ensure a continuous column of fluid from blood vessel to transducer membrane during measurement (Ettarh, 2004). For the invasive blood pressure determination, mean arterial blood pressure (MABP) was calculated using the formula: diastolic pressure + 1/3 pulse pressure (Guyton and Hall, 1996).

3.6.2 Non invasive blood pressure monitoring
The conscious rats were placed in a restrainer on a heated pad (37°C) and allowed to adapt/rest inside for 15 minutes before blood pressure was measured weekly (Dominiczak et al., 1991; Pojoga et al., 2008). The rat tail was placed inside 9mm or 11mm tail cuff, and the cuff was inflated and released several times to allow the animal become conditioned to the procedure. Five consecutive blood pressure and heart rate measurements were obtained using the non-invasive blood pressure monitor MP35 (BIOPAC System Inc USA), which was connected to a computer. Blood pressure tracings were obtained through preinstalled software for BSL Pro.3.7. A picture of the non-invasive blood pressure measurement set-up and a typical blood pressure tracing from the measurement are attached in appendix 1a and 1b respectively.
3.7.0 Testosterone assay

Serum testosterone levels were measured by enzyme-linked immunoassay (EIA) (Marcus and Durnford, 1986) using a commercial kit from Biotech Laboratories (Suffolk, UK) according to the protocol of the manufacturer. The kit uses the principle of competitive microplate enzyme immunoassay, whereby testosterone present in the sample competes with enzyme-testosterone conjugate for binding with anti-testosterone coated microplate to form an antigen-antibody complex. Briefly, the serum samples stored at -80°C were allowed to thaw at room temperature. 25µl of each of the calibrator, control serum and the samples were added to the pre-labelled microplate well and thereafter 100µl of the enzyme-testosterone conjugate was added. The whole set-up was incubated on a plate shaker at 800 rpm for 30 minutes at 37°C. After the incubation period the unbound conjugate was removed by washing each of the well was washed 5 times with 300µl of diluted wash buffer and the washed plates were dried by firmly tapping them against an absorbent paper. The enzyme activity was determined by a color change in the tetramethylbenzidine (TMB)-substrate solution. 100µl of TMB-solution was added to each of the wells at timed-intervals and incubated for 30 minutes at room temperature in the dark. After incubation 150µl of the stop reagent was added into each well at the same timed-interval and gently mixed for about 10 seconds. Thereafter, absorbance of each of the samples was read 3 times each on the microplate reader at 450nm.

3.7.1 Calculation of testosterone assay result

The enzyme activity in the antibody-bound fraction is inversely proportional to the testosterone concentration in the serum sample (Erkins, 1990). The mean absorbance value of each of the calibrator duplicate was calculated and a calibrator curve was drawn on a graph paper with mean absorbance on Y-axis and the calibrator concentration on the X-axis. The mean absorbance
values for each of the test samples were calculated from the calibration curve. The value of testosterone concentration (nmol/l) in the samples was read directly from the calibrator curve. The kit has an assay dynamic range of 0-40nmol/l and an analytical sensitivity of 0.2nmol/l. Its intra and inter assay variation were 5.6% and 7.1% respectively. The calibration curve form is attached in appendix 2.

3.8.0 Isometric Tension Studies in Isolated Aortic Rings

3.8.1.0 Grass Polygraph 7D Model and paper recorder

Rats for aortic ring study were sacrificed by cervical dislocation, thereafter the thoracic cage was opened. Heparin was injected into the ventricle to prevent blood clotting. The aorta was cut at the visible ends and quickly placed in a Petri dish containing cold physiological salt solution (PSS) (modified Krebs solution). The aorta was freed of connective tissue and cut into 2-3mm ring segments. The rings were gently rinsed to remove blood left in the lumen. Special care was taken to avoid contact with the endothelial surface during the removal and mounting of the rings. The ring was then mounted horizontally between two fine stainless steel rods. The lower rod was connected to the base of the organ bath. The upper rod was attached to the isometric force transducer (Model FT03C, calibrated with a 2gm weight, Grass instruments, Mass, USA) which was coupled to the Grass polygraph (Model 7D, Grass instruments) via a preamplifier and a driver amplifier to an ink writing stylus. This was used in recording the force displacement by the tissue (Dominiczak et al., 1991; Ettarh, 2004). The rings were superfused in 20ml double jacketed organ bath with PSS gravity-fed from a 1 liter reservoir. The PSS temperature in the organ bath was maintained at 37°C and gassed with 95% O₂: 5%CO₂ mixture. The gentle bubbling with the gas mixture ensured constant stirring of the bath solution. The pH of the PSS
was usually between 7.35-7.40. The baths used had a simultaneous parallel connection to the source of PSS. The composition of the modified Krebs Physiological Salt Solution (PSS) in mmol/L was: NaCl, 119.0; KCl, 4.7; KH$_2$PO$_4$, 1.2; MgSO$_4$, 1.2; NaHCO$_3$, 24.9; CaCl$_2$, 1.6 and glucose, 11.5 (Sofola et al., 2003). A schematic diagram of the in-vitro vascular reactivity study using the double-jacketed organ bath is attached in appendix 3.

3.8.1.1 Experimental protocol

After mounting, a passive tension of 2g was applied to each ring and then allowed to equilibrate in the PSS for 90min, during which each ring was subjected to administration of a sub-maximal dose (0.1µM) of noradrenaline (Sigma-Aldrich, USA) at 30 min interval. Each of the stabilizing stimulations lasted for 5 minutes after which the ring was rinsed with PSS. The PSS was replaced after every 15 minutes during the duration of the experiment. Stabilization was required to ensure consistent responses of the aortic rings throughout the experiment. At the end of the 90 minutes stabilization period the various protocols described below were carried out (Ettarh, 2004).

3.8.1.2 Vascular relaxation response to acetylcholine

Relaxation response to acetylcholine (ACh) (Sigma-Aldrich, USA) an endothelial dependent vasodilator was assessed in endothelial intact aortic rings. Aortic rings were precontracted with 0.1µM noradrenaline, after the contraction has reached a plateau, cumulative doses of ACh (0.01 – 10µM) were added to the organ bath (Sofola et al., 2002). Noradrenaline and acetylcholine were dissolved and diluted in distilled water.
3.8.1.3 Vascular relaxation response to acetylcholine in the presence of L–Nitro Arginine Methyl Ester (L–NAME)

Involvement of endogenous NO production on the relaxation response of aorta to ACh across the groups was studied by blocking endogenous production of NO using eNOS inhibitor L–NAME. Aortic rings were incubated with L–Nitro Arginine Methyl Ester (L-NAME) (Sigma-Aldrich, USA) (100µM), for 30 minutes. After the 30 minutes incubation period the rings were precontracted with 0.1µM noradrenaline, after which cumulative doses of ACh (0.01 – 10µM) were added to the organ bath (Sofola et al., 2002). L-NAME was dissolved and diluted in distilled water.

3.8.1.4 Vascular relaxation response to sodium nitroprusside (SNP) in the presence of L–NAME

To assess the vascular relaxation response to exogenous nitric oxide, vascular relaxation response to SNP (Sigma-Aldrich, USA) (an NO donor) was studied across the groups. Aortic rings were incubated with L-NAME (100µM), for 30 minutes. After the 30 minutes incubation period the rings were precontracted with 0.1µM noradrenaline, after which cumulative doses of SNP (0.1nM – 0.1µM) were added to the organ bath (Tang et al., 2005). SNP was dissolved and diluted in distilled water.

3.8.2.0 ADInstrument Top loader Transducer and software

The concluding parts of the vascular reactivity studies were carried out using a top force transducer MLT 050/D (ADInstruments Australia) connecting a Panlab LETICA series 01 organ baths to a Powerlab 2/25 hardware and a chart 5 software recorder (ADInstruments, Australia).
Aortic ring was mounted between a fine stainless steel rod, and a small S – shaped steel hook attached to a thread. The upper part of the rod was attached to the clamp of the micropositioner, while the thread was attached to the isometric force transducer (MLT 050/D ADInstruments, Australia). The rings were superfused in 20ml organ bath (Panlab LETICA series 01), with Hepes buffer solution at 37°C and gassed with 100% Oxygen. The pH of the Hepes buffer was between 7.35-7.40. The baths used simultaneously had a parallel connection to the source of Hepes buffer physiological solution. The composition of the solution in mmol/L was: NaCl 133, KCl 3.6, CaCl₂ 1.8, MgCl₂·6H₂O 1.2, Glucose 16, Hepes 3, and KH₂PO₄ 1.18.

After mounting the ring, a passive tension of 2g was applied to each ring and then allowed to equilibrate for 90 minutes, during which each ring was subjected to a sub-maximal dose (0.1µM) of noradrenaline at 30 min interval. The sensitivity of the transducer was set at 1g/mV. At the end of the 90 minutes stabilization period, the following studies were carried out. A picture of the ADInstruments toploader transducer setup is attached in appendix 3.

3.8.2.1.0 Experimental protocol

3.8.2.1.1 Vascular relaxation response to forskolin

To assess the role of cyclic adenosine monophosphate (cAMP) pathway on vascular reactivity, cumulative relaxation response curves to forskolin (Sigma-Aldrich Banglore, India); an adenylyl cyclase activator were obtained. Aortic rings were precontracted with 0.1µM noradrenaline following which the rings were exposed to cumulative doses (0.01µM to 10µM) of forskolin (Sofola et al., 2003). Forskolin was dissolved and diluted in dimethylsulphuroxide (DMSO).
3.8.2.1.2 Vascular relaxation response to diazoxide

The possible involvement of potassium ion channels in the effect of androgens on vascular reactivity was assessed by studying the aortic relaxation response to diazoxide (Sigma-Aldrich Banglore, India); a potassium channel activator. This was carried out across all the groups. Aortic rings were precontracted with 0.1µM noradrenaline, after which a cumulative dose (0.01µM to 10µM) of diazoxide was added to the organ bath (Newgreen et al., 1990). Diazoxide was dissolved and diluted in dimethylsulphuroxide (DMSO).

3.9.0 Oxidative Stress and Reactive Oxygen Species (ROS) study

3.9.1 Lipid peroxidation

Lipid peroxidation was measured by determining malondialdehyde (MDA) production, using thiobarbituric acid reactive substances (TBARS) assay. The sensitivity of TBARS has made this assay the method for screening and monitoring lipid peroxidation, a major indicator of oxidative stress (Armstrong and Browne, 1994; Yagi, 1998). A biological marker that indicates oxidative stress with respect to lipid peroxidation in body fluids or cells is malondialdehyde (MDA). MDA is a by-product of the arachidonate cycle, as well as lipid peroxidation (Jain, 1988; Wasowicz et al., 1993), and it is detectable in quantifiable amounts employing the TBA assay. The methods of Niehaus and Samuelson, (1968), modified by Buege and Aust, (1978) and Yagi, (1998) were used. The heart and the kidney were washed in normal saline, and immediately placed in different containers. Each was minced and homogenized separately in 2ml 0.1N Tris – HCl solution. Trichloroacetic acid (TCA) – Thiobarbituric acid (TBA) reagent was prepared as follows; 3g of TCA was weighed in brown bottle and was dissolved in 9.58ml of distilled water to which 0.42ml of concentrated HCl was added. TBA (75mg) was weighed and dissolved in
10ml distilled water. The two separate solutions were then added together to form the TCA – TBA reagent. Lipid peroxides were measured by adding 2ml of TCA - TBA reagent to 1ml of the homogenates. The mixture was then heated in boiling water bath for 15 minutes. TBA and MDA react to form a Schiff base adduct under high temperature and acidic condition to produce a chromogenic product that can be easily measured using analytical techniques such as spectrophotometry (Hoving et al., 1992; Lapenna et al., 2001). After cooling, the suspension was centrifuged at 1000g for 10 minutes. The supernatant was then separated and absorbance was measured at 532nm in a UV I Spectrophotometer (Thermo Electron Corporation). The MDA concentration of the sample was calculated using the formula below

MDA/mol/L = OD × V × DF

Σ v

OD = Absorbance (Optical Density) of sample
Σ = Molar extinction coefficient = 1.56 x 10⁵
V = Total volume of the reacting sample
V = Volume of the sample
DF = Degree of Freedom.

3.9.2 Super Oxide Dismutase (SOD) activity

Misra and Fridovich, (1972) reported that the observation that SOD acted as a potent inhibitor of the spontaneous oxidation of epinephrine at elevated pH provided a convenient and sensitive assay for this enzyme. Super oxide dismutase (SOD) level in the serum was assayed at 30°C according to the method described by Misra and Fridovich, (1972). The assay was performed in 3ml of 50mM Na₂CO₃ buffer (pH 10.2), 0.02ml of the sample was added to 3ml of buffer and 0.03ml of the substrate which is epinephrine. The oxidation of epinephrine was followed in terms
of the production of adrenochrome, which exhibits an absorption maximum at 480nm, with an extinction coefficient of 4020 M⁻¹ cm⁻¹. A blank was prepared as follows; 0.02ml of distilled water was added to 3ml of the buffer and 0.03ml of the substrate. Absorbance of the sample was taken at 480nm over 3 to 5 minutes. One unit of SOD was defined as the amount of enzyme, which causes 50% inhibition of epinephrine auto-oxidation. SOD concentration in the sample was calculated as follows:

\[
\text{SOD (µg/ml)} = \frac{\Delta A_{480} \times V \times DF}{\Sigma \Delta A_{480}}
\]

\[
\Delta A_{480} = \text{Difference in absorbance between the sample and the blank}
\]

\[
\Sigma = \text{Extinction coefficient (4020M}^{-1} \text{cm}^{-1})
\]

\[
V = \text{Total volume of the reacting sample}
\]

\[
V = \text{Volume of the sample}
\]

\[
DF = \text{Degree of Freedom}
\]

3.10.0 Biochemical assays to determine the serum level of bilirubin

The serum level of total bilirubin and direct bilirubin was determined using the Erba Chem – 7 automated machine with specific Erba reagents and/or assay kits for each parameters that were measured. The machine was calibrated with serum based XL multical calibrator and the XL results were calculated automatically by the machine. To ensure adequate quality control normal and abnormal controls with assayed values were ran as unknown samples.

3.10.1 Serum level of total bilirubin

Bilirubin is a breakdown product of haemoglobin, which is usually formed in the reticuloendothelial system. It is transported bound by albumin to the liver. This bilirubin is water
insoluble and is known as indirect or unconjugated bilirubin. In the liver, bilirubin is conjugated onto glucoronic acid to form direct bilirubin. Both the total and direct bilirubin were measured. Total bilirubin = indirect bilirubin + Direct bilirubin. Total bilirubin level was measured in the serum using an automated Erba chem. – 7 machine and total bilirubin assay kit. The determination procedure follows the Walters and Gerarde, (1970) method. Bilirubin is coupled with diazotized sulfanilic acid in the presence of ethylene glycol and dimethylsulfoxide (DMSO) as solvents to produce an intensely coloured diazo compound. The intensity of colour of this solution is proportional to the concentration of the bilirubin total in the sample. Optical Density (OD) for total bilirubin reagents and standard were 0.0020 and 0.2224 respectively. The machine presents the value/concentration of total bilirubin in mg/dl. Total bilirubin assay reagent composition is as follows; Sulphalinic acid (25.6 mmol/L), HCl (40 mmol/L) and sodium nitrite (1mmol/L).

3.10.2 Serum level of direct bilirubin

The azobilirubin produced by the reaction between bilirubin and the diazonium salt of sulfanilic acid shows maximum absorption at 550nm in an acidic medium. The intensity of the colour produced is directly proportional to the quantity of bilirubin which has reacted. In the absence of an accelerator, only conjugated (direct) bilirubin reacts. In the presence of an accelerator, DMSO, the non – conjugated bilirubin also participate in the reaction, thus determining the level of total bilirubin. . Optical Density (OD) for direct bilirubin reagents and standard were 0.0010 and 0.1640 respectively. The machine presents the value/concentration of direct bilirubin in mg/dl. Direct bilirubin assay kit’s components are sulphanilic acid (27.74 mmol/L), HCl (40 mmol/L), and sodium nitrite (1.38 mmol/L).
3.11.0 Histological Studies

Thoracic aorta and mesenteric arteries from two rats from each group were taken immediately after the animals were opened up. The arteries were fixed in 10% formalin, so as to preserve the various constituents in their normal micro-anatomical position and prevent them from any degenerative or autolytic changes. The histological section was carried out adapting the methods described by Bancroft and Cook (1984). Briefly, the tissues were cut into bits with a scalpel blade (1cmx5mmx2mm) size and placed in the tissue cassettes individually and were labeled. The cassettes with the tissue bits were then replaced into the 10% formalin. Automatic LEICA TP1020 tissue processor was used for the processing of the tissues. The machine was programmed for 17 hours.

The tissues were dehydrated because water is not miscible with paraffin. This was achieved by putting the tissues in ascending grades of alcohol (automatically in the tissue processor). Ascending concentration of alcohol was used so as to prevent sudden rushing out of water from the tissues, so that the cell will not be distorted or damaged. After dehydration, alcohol was also removed from the tissues because it was not miscible with paraffin. The tissues were infiltrated with xylene, which replaced alcohol and was also miscible with paraffin. Xylene also made the opaque tissues to be transparent, therefore the name clearing stage. After the clearing in xylene, the tissue bits were placed in a melted paraffin wax 1 hour with VAC, and for another 90 minutes with VAC. After processing the tissue cassettes were transferred in melted paraffin containers of SLEE MPS/P1 dispenser. The tissue paraffin blocks were made with SLEE MPS/P1 paraffin dispenser. The blocks were allowed to cool by placing them on SLEE MPS/C cooler plate. The tissue blocks were trimmed using trim program on the tissue processor.
(microtome Leica RM 2255). The tissue blocks were fixed in tissue block holder of the microtome Leica RM 2255, and tissue sections were cut with Microtome Leica RM 2255 into a size of 5µM. The cut ribbons were transferred into a LEICA water bath set to 49°C. One surface of a slide was made sticky by rubbing it with a drop of egg albumen. Afterward the thin sections were held onto microslides and labeled. The labeled slides were then incubated at 25°C overnight and then stained.

3.11.1 Morphometric Analysis

Morphometric analyses (nucleus count, elastin and collagen estimate as well as thickness and area measurement) were performed using IMAGE-PRO 3DS 6.1 software. Three sagittal sections, 5µm thick were cut in Leica microtome RM 2255, and were specifically stained to obtain a monochromatic colour associated with the various structures of interest in the thoracic aorta and mesenteric artery media. The histological sections were stained with hematoxylin and eosin (H & E) for the general histology. Special staining was done for collagen and elastin estimations. Elastin took up blue black colour on staining with Verhoff - Vangeison staining and collagen took up red colour on staining with Picro-sirius red staining. Nuclear counting was done on H & E stained sections. Air bubbles were prevented from getting in, and a photomicrograph of the slide preparation was then taken using the pre-installed camera in the light microscope (Olympus BX 51, Japan). The images were opened in the IMAGE-PRO software and measurements were taken randomly from four different areas of the image at magnification of 50X. To correct for shrinkage during the fixation and staining procedure, the shrinkage index (X 1.25 for length and X1.56 for area) were used (Sho et al., 2004). (The images of the morphometric analysis are attached in appendix 5 a - e).
3.12.0 Non – genomic effect of testosterone study

Non genomic effect of testosterone on vascular reactivity was studied by obtaining cumulative concentration – response curves to testosterone propionate and dehydroepiandrosterone in the presence or absence of different pharmacological inhibitors and blockers. To remove the possible effect of ethanol on the aortic rings relaxation to testosterone, 100µL of ethanol (0.5%) was added to each 20ml organ bath before studying the relaxation response to testosterone. Testosterone propionate was dissolved in absolute ethanol.

3.12.1 Vascular relaxation response to exogenous testosterone

Aortic rings were precontracted with 0.1µM noradrenaline, after a plateau was achieved, cumulative doses of testosterone propionate (Sigma-Aldrich, Bangalore, India) (0.1µM to 100µM) was added to the organ baths (Ding and Stallone, 2001). This study was carried out across the groups. Testosterone was dissolved and diluted in ethanol.

3.12.2 Vascular relaxation response to dehydroepiandrosterone (DHEA)

Direct relaxation effect of DHEA (Sigma-Aldrich Bangalore, India) on the aorta was also assessed. Aortic rings were precontracted with 0.1µM noradrenaline, after which cumulative doses of DHEA (0.1µM to 100µM), were added to the organ baths (Ding and Stallone, 2001). DHEA was dissolved and diluted in ethanol.
3.12.3 Experiments on possible mechanism by which exogenous testosterone elicit vascular relaxation.

The following experiments described below were carried out only on aortic rings of the control group, to elucidate the possible mechanism of action of direct effect of testosterone on aorta relaxation.

3.12.3.1 Role of androgen receptor

The role of the conventional androgen receptor (AR) was assessed by incubating aortic rings in flutamide for 30 minutes (Ding and Stallone, 2001). Flutamide (Sigma-Aldrich Bangalore, India) is a classical androgen receptor blocker. After the incubation period, the rings were precontracted with 0.1µM noradrenaline, and which cumulative doses (0.1µM to 100µM), of testosterone propionate was added to the organ baths. Flutamide was dissolved and diluted in ethanol.

3.12.3.2 Role of aromatase enzyme

The role of aromatase (CPY19), an enzyme that converts testosterone to estradiol in the peripheral tissues, was assessed by incubating some aortic rings in 5µM aminogluthetemide (Sigma-Aldrich Bangalore, India) an inhibitor of aromatase enzyme for 30 minutes (Ding and Stallone, 2001). After the incubation period, the rings were precontracted with 0.1µM noradrenaline, and cumulative doses (0.1µM to 100µM), of testosterone propionate was added to the organ baths. Aminogluthetemide was dissolved and diluted in ethanol.
3.12.3.3 Role of endothelial vasoactive substances

The involvement of endothelial vasoactive substances on the aortic ring relaxation response to testosterone was assessed by incubating some aortic rings with L-NAME (Sigma-Aldrich Bangalore, India) (100µM) an endothelial nitric oxide synthase (eNOS) inhibitor, and indomethacin (Sigma-Aldrich Bangalore, India) (10µM) a cyclooxygenase -2 (Cox-2) inhibitor for 30 minutes (Yue et al., 1996). After the incubation period, the rings were precontracted with 0.1µM noradrenaline, and cumulative doses (0.1µM to 100µM), of testosterone propionate were added to the organ baths. L-NAME was dissolved and diluted in distilled water, while indomethacin was dissolved in 2.5% sodium carbonate.

3.12.3.4 Role of potassium channel activation

The involvement of different potassium channels in the relaxation effect of testosterone on the aorta was assessed. Concentration – response curves to testosterone propionate were obtained after incubation of aortic rings for 30 minutes in some potassium channel blockers; barium chloride 3µM, a non specific K\(^+\) channel blocker (Yue et al., 1996), tertiapin – Q (100nM), a selective inward rectifier K\(^+\) channel blocker (Brindeiro et al., 2008), apamin (1µM), charybdotoxin (1µM), and iberiotoxin (25nM) which are, selective small, intermediate and large conductance calcium activated K\(^+\) channel blockers respectively (Ledoux et al., 2006). The effect of each compound was tested on fresh rings. After the incubation period, the rings were precontracted with 0.1µM noradrenaline, and cumulative concentrations (0.1µM to 100µM), of testosterone propionate were added to the organ baths. Barium chloride, tertiapin-Q, charybdotoxin, apamin and iberiotoxin were purchased from Sigma-Aldrich Bangalore, India. Barium chloride and tertiapin-Q were dissolved and diluted in distilled water. Charybdotoxin and
Iberiotoxin were dissolved in 100mM of sodium chloride while apamin was dissolved in 5% acetic acid and diluted in distilled water.

3.12.3.5 **Role of voltage gated calcium channel**

The role of voltage dependent calcium channel (VDCC) on the effect of testosterone was assessed by incubating some aortic rings in nifedipine (Sigma-Aldrich Bangalore, India) (1µM), an L – type calcium channel blocker for 30 minutes. After the incubation period, the rings were precontracted with 0.1µM noradrenalin, and cumulative doses (0.1µM to 100µM), of testosterone propionate were added to the organ baths. Finally, in order to evaluate if calcium channel blockade augments the relaxing effect of testosterone on the aortic rings, its calcium channel blocking potential was compared with nifedipine, some aortic rings were incubated in 1µM testosterone for 30 minutes, after the incubation period the rings were precontracted with 0.1µM noradrenaline and cumulative doses (0.1µM to 100µM) of nifedipine was added to the organ baths. Nifedipine was dissolved in 50% ethanol.

3.13.0 **Statistical Analysis**

The collected data were expressed as mean ± S.E.M. The data were analyzed using one way analysis of variance (ANOVA). Student-Newman-keuls post hoc test was used to identify differences between individual means. Confidence interval was placed at 95%, so that in all cases a value of P < 0.05 was considered significant. Differences in the body weights of rats in each of the groups before and after the experiment were presented as percentage difference. In the non – invasive blood pressure study, the difference in the blood pressure before and after the experiment was also presented as percentage difference. In the vascular reactivity studies,
statistical analysis was performed on the values of –Log EC$_x$ ($x$ = concentration causing $x\%$ of maximal response), these values were obtained by programmed statistical software (Graphpad Prism 5, USA). Percentage maximum relaxation responses to each agonist in each of the groups were also compared.
CHAPTER FOUR

4.0 RESULTS

4.1 Body weight

At the end of the six weeks experimental period, the final body weights of the rats were determined across all the groups. Table 1 shows the body weights of rats in each group before and after the experimental period and the percentage weight gain in each group. In all groups, there were increases in body weight after the six weeks. However the percentage increase in weight in the high salt group was significantly less (P < 0.001) when compared with that of the intact and normal salt group (control). Also the percentage weight gain in the testosterone supplemented and high salt diet group and sham orchidectomised and high salt diet group was significantly less (P < 0.001) when compared with their corresponding normal diet (control) groups, but there was no significant difference between the percentage weight gain of normal diet and high salt diet of the orchidectomised groups. However, the percentage weight gain and the final body weight of the orchidectomy and high salt diet group was significantly higher (P < 0.01) when compared with that of intact and high salt diet group. There were no significant changes in the percentage weight gain and final body weight of both the normal and high salt testosterone supplemented groups when compared with their corresponding intact groups. Likewise there were no significant differences in the percentage weight gain and final body weight of the sham orchidectomised groups when compared with that of their corresponding intact groups. Figure 7 shows the weekly changes in the mean body weights of the rats.
Table 1: Initial, final and percentage body weight gain of the rats across the groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Before Treatment (g)</th>
<th>After Treatment (g)</th>
<th>% Weight Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 10)</td>
<td>Week 0</td>
<td>Week 6</td>
<td></td>
</tr>
<tr>
<td>INT + NS</td>
<td>96 ± 2.21</td>
<td>192.0 ± 3.27</td>
<td>50.10 ± 0.80</td>
</tr>
<tr>
<td>INT + HS</td>
<td>99.0 ± 2.33</td>
<td>160.0 ± 2.58</td>
<td>38.03 ± 1.65***</td>
</tr>
<tr>
<td>ORCH + NS</td>
<td>97.0 ± 2.13</td>
<td>183.0 ± 3.96</td>
<td>47.41 ± 1.29</td>
</tr>
<tr>
<td>ORCH + HS</td>
<td>99.0 ± 2.33</td>
<td>183.0 ± 3.00</td>
<td>45.87 ± 0.95††</td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>100.0 ± 2.36</td>
<td>187.8 ± 4.01</td>
<td>46.10 ± 1.25</td>
</tr>
<tr>
<td>ORCH + TES + HS</td>
<td>98.0 ± 3.07</td>
<td>189.0 ± 3.42</td>
<td>42.40 ± 1.80***</td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>100.0 ± 4.22</td>
<td>200.0 ± 2.75</td>
<td>47.74 ± 0.87</td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>97.5 ± 3.66</td>
<td>163.8 ± 4.20</td>
<td>38.46 ± 1.28</td>
</tr>
</tbody>
</table>

**Keys:** INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are presented as Means ± S.E.M (n = 10). **Weight gain is significantly less (P < 0.001)** when compared with corresponding control groups, ††weight gain is significantly high (P < 0.01) when compared with intact and high salt diet group.
Fig. 7: Mean body weights of intact and orchidectomised Sprague Dawley rats fed a normal or high salt diet with or without testosterone supplementation.

Keys: INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone.

Data are presented as Means ± S.E.M. (n = 10). Final body weight significantly less (⁎P < 0.05, ⁎⁎⁎P < 0.001) when compared with control. ^^Final body weight significantly high (P < 0.001) when compared with that of the intact and high salt group. †††Final body weights significantly less when compared with that of orchidectomy and high salt diet group.
4.2 Organ weights

The relative weights of the heart, kidney and the liver to the body weights were obtained and recorded as weight index. Table 2 shows the mean heart, kidney and liver indices of the rats across the groups. There was a significant increase ($p < 0.05$) in the mean heart index of the intact high salt diet group when compared with the intact normal salt diet group (control). However there was a significant decrease ($P < 0.05$) in the heart index of orchidectomy and high salt group when compared with intact and high salt diet group, while there was no significant difference between the orchidectomised and high salt group when compared with the intact and normal salt group (control). There was a significant increase ($p < 0.001$) in the kidney index of high salt diet group when compared with their corresponding control groups, except in the orchidectomised groups where there was no significant difference between the kidney index of the normal diet group and high salt group. There was a significant decrease ($P < 0.01$) in the kidney index of orchidectomy and high salt group when compared with intact and high salt diet group, while orchidectomy almost restored the kidney index in the high salt diet group back to the value observed in the intact and normal salt diet group (control).
Table. 2: Mean heart, kidney and liver index of rats across the groups.

<table>
<thead>
<tr>
<th>Groups (n = 5)</th>
<th>Heart Index</th>
<th>Kidney Index</th>
<th>Liver index</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT + NS</td>
<td>0.29 ± 0.006</td>
<td>0.57 ± 0.03</td>
<td>3.07 ± 0.14</td>
</tr>
<tr>
<td>INT + HS</td>
<td>0.31 ± 0.007*</td>
<td>0.65 ± 0.04**</td>
<td>2.99 ± 0.11</td>
</tr>
<tr>
<td>ORCH + NS</td>
<td>0.26 ± 0.012</td>
<td>0.55 ± 0.01</td>
<td>2.72 ± 0.39</td>
</tr>
<tr>
<td>ORCH + HS</td>
<td>0.28 ± 0.009†</td>
<td>0.57 ± 0.02††</td>
<td>3.02 ± 0.16</td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>0.28 ± 0.009</td>
<td>0.55 ± 0.02</td>
<td>3.08 ± 0.07</td>
</tr>
<tr>
<td>ORCH + TES + HS</td>
<td>0.28 ± 0.008</td>
<td>0.60 ± 0.01**</td>
<td>2.88 ± 0.07</td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>0.28 ± 0.002</td>
<td>0.52 ± 0.01</td>
<td>3.16 ± 0.07</td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>0.30 ± 0.004</td>
<td>0.59 ± 0.00†</td>
<td>2.80 ± 0.18</td>
</tr>
</tbody>
</table>

**Keys:** INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are presented as Means ± S.E.M (n = 5). Significant increase, (*P < 0.05, **P < 0.01, ***P < 0.001) when compared with corresponding control, significant decrease (†P < 0.05, ††P < 0.001) when compared with intact and high salt group.
4.3 Blood pressure

4.3.1 Invasive Blood Pressure Measurement

4.3.1.1 Systolic blood Pressure

Terminal blood pressure parameters were determined using invasive method via cannulation of the femoral artery. Table 3 shows the mean systolic, diastolic, mean arterial and pulse pressures of the rats across the groups. Systolic blood pressure (SBP) was elevated in all the high salt diet groups, and these elevations were significant ($P < 0.001$) when compared with those of their corresponding control (normal salt diet) groups. However the rise in SBP observed in the orchidectomised high salt group was significantly less ($P < 0.01$) when compared with that of the intact high salt diet group. On the other hand SBP were significantly higher ($P < 0.01$) in the testosterone supplemented groups when compared with their corresponding orchidectomy only (without testosterone replacement) groups. Also SBP was significantly lower ($P < 0.01$) in the orchidectomy and normal diet groups when compared with that of the control.

4.3.1.2 Diastolic blood Pressure

Table 3 shows the mean diastolic pressure of the rats in all the groups. Diastolic blood pressure (DBP) was significantly higher ($P < 0.001$) in the high salt diet groups when compared with their corresponding controls. However the increase in DBP in the orchidectomised and high salt diet group was of lower magnitude when compared with the intact high salt diet group and this was significant ($P < 0.01$). Diastolic blood pressure values were also significantly higher ($P < 0.01$) in the testosterone supplemented groups when compared with their corresponding orchidectomy only (without testosterone replacement) groups. Also DBP was significantly lower ($P < 0.01$) in
the orchidectomy and normal diet group when compared with the intact and normal diet (control) group.

4.3.1.3 Mean Arterial Blood Pressure (MABP)

Table 3 shows the MABP of the rats in all the groups. Mean arterial blood pressure (MABP) was significantly elevated (P < 0.001) in all the high salt diet groups when compared with their corresponding control groups. However the increase in the MABP of the orchidectomised and high salt diet group was lower in magnitude when compared with the intact high salt diet group and this was significant (P < 0.01). On the other hand MABP was significantly higher (P < 0.01) in the testosterone supplemented groups when compared with their corresponding orchidectomy only (without testosterone replacement) groups. MABP was significantly lower (P < 0.05) in the orchidectomy and normal salt diet when compared with the intact and normal salt diet group.

4.3.2 Heart Rates

Figure 8 shows the heart rates of the rats across the groups. The heart rate in orchidectomy and high salt rats were significantly lower (P < 0.05) when compared with the orchidectomy and normal salt and the control groups.
Table 3: Systolic, diastolic, mean arterial and pulse pressure of the rats across the groups

<table>
<thead>
<tr>
<th>Groups (n = 5)</th>
<th>Systolic Pressure (mmHg)</th>
<th>Diastolic Pressure (mmHg)</th>
<th>Mean Arterial Pressure (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT + NS</td>
<td>133.5 ± 3.02</td>
<td>109.50 ± 2.16</td>
<td>117.9 ± 2.39</td>
</tr>
<tr>
<td>INT + HS</td>
<td>156.4 ± 2.21***</td>
<td>132.0 ± 3.04***</td>
<td>138.8 ± 2.68***</td>
</tr>
<tr>
<td>ORCH + NS</td>
<td>120.3 ± 4.33##</td>
<td>97.67 ± 2.33##</td>
<td>105.2 ± 2.87##</td>
</tr>
<tr>
<td>ORCH + HS</td>
<td>††136.3 ± 4.59***</td>
<td>††119.3 ± 2.81***</td>
<td>††125.3 ± 2.25***</td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>132.0 ± 3.27</td>
<td>114.4 ± 3.25</td>
<td>123.0 ± 3.21</td>
</tr>
<tr>
<td>ORCH + TES + HS</td>
<td>144.0 ± 2.88***</td>
<td>121.6 ± 2.60***</td>
<td>130.0 ± 1.79***</td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>129.4 ± 2.71</td>
<td>107.2 ± 2.50</td>
<td>116.8 ± 2.97</td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>149.4 ± 3.79***</td>
<td>129.2 ± 2.65***</td>
<td>133.9 ± 3.13***</td>
</tr>
</tbody>
</table>

**Keys:** INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are presented as means ± S.E.M n = 5. **Significantly higher (P < 0.001) when compared with corresponding control, ††significantly lower (P < 0.01), when compared with intact and high salt group. ##Significantly lower (P < 0.01) when compared with control.**
Fig. 8: Mean heart rates of the rats across the groups.

**Keys:** INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are presented as means ± S.E.M (n = 5). *significantly lower (P < 0.05), when compared with orchidectomised and normal salt and control groups.*
4.3.3 Non-invasive blood pressure monitoring

Table 4 shows the initial, final and percentage difference between initial and final mean arterial blood pressure (MABP) of the rats in all the groups. Figure 9 shows the weekly mean MABP of the rats.

Non invasive blood pressure monitoring via tail plethysmography showed that the blood pressure elevating effect of a high salt diet set in around the second week of salt-loading, and by the third week the intact and high salt group had developed hypertension. Orchidectomy delayed the onset of hypertension following a high salt diet till about the fifth week of salt-loading. In rats that were given testosterone supplementation following orchidectomy and concurrently fed a high salt diet, blood pressure elevation also set in around the second week, and reached the hypertensive level about the third week.

After the six week experimental period, there was an increase in the mean arterial blood pressure of rats in all the groups. However, the increase in MABP in the high salt diet groups was significantly higher (P < 0.001) from the third week upward to the sixth and the final week of the experiment, when compared with their corresponding control (normal salt diet) groups. The elevation in the MABP in the orchidectomy and high salt group was significantly less (P < 0.05) when compared with the intact and high salt diet group (Figure 9).

On the other hand the increase in the MABP of rats in the testosterone supplemented groups was significantly higher (P < 0.01) when compared with their corresponding orchidectomised groups without testosterone supplementation. The increase in MABP of the testosterone supplemented
groups is almost comparable to that of the intact groups. Also elevation of MABP after the six week experimental period in the orchidectomy and normal salt group was significantly less (P < 0.01) when compared with the intact and normal salt diet group. Sham orchidectomy has no significant effect (P > 0.05) on the MABP both in the normal diet and high salt diet groups (Figure 9).

The percentage difference between the final and initial MABP of the rats follows the same trend as above, i.e. after the six week experimental period, there were percentage increases in the final MABP when compared with the initial MABP across all the groups. However the percentage increase in the MABP of all the high salt fed groups were significantly higher (P < 0.001) when compared with the groups fed a normal salt diet, but the percentage increase recorded in the orchidectomy and high salt group was significantly less (P < 0.01) when compared with intact and high salt diet group. Also the percentage increase in the MABP of the orchidectomy and normal diet group was significantly less (P < 0.01) when compared with the intact and normal diet group (Table 4).

Conversely, the increase in the percentage difference in the testosterone supplemented groups was significantly higher (P < 0.01) when compared with their corresponding orchidectomised groups i.e. groups without testosterone supplementation (Table 4).
Table 4: Initial, final, and percentage difference of mean arterial blood pressure of the rats across the groups.

<table>
<thead>
<tr>
<th>Groups (n = 6)</th>
<th>Initial MABP (mmHg)</th>
<th>Final MABP (mmHg)</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT + NS</td>
<td>99.5 ± 2.43</td>
<td>112.2 ± 3.71</td>
<td>15.23 ± 0.78</td>
</tr>
<tr>
<td>INT + HS</td>
<td>100.3 ± 2.33</td>
<td>134.3 ± 4.28***</td>
<td>28.57 ± 1.23***</td>
</tr>
<tr>
<td>ORCH + NS</td>
<td>100.0 ± 1.68</td>
<td>106.0 ± 3.02##</td>
<td>7.55 ± 0.65##</td>
</tr>
<tr>
<td>ORCH + HS</td>
<td>100.3 ± 1.95</td>
<td>††124.2 ± 2.96***</td>
<td>††19.08 ± 1.01***</td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>100.5 ± 1.56</td>
<td>112.5 ± 2.85</td>
<td>10.67 ± 0.74</td>
</tr>
<tr>
<td>ORCH + TES + HS</td>
<td>101.0 ± 1.68</td>
<td>132.7 ± 3.52***</td>
<td>23.71 ± 1.65***</td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>99.67 ± 1.84</td>
<td>116.6 ± 3.11</td>
<td>17.23 ± 0.61</td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>101.7 ± 2.49</td>
<td>134.7 ± 3.54***</td>
<td>32.3 ± 1.62***</td>
</tr>
</tbody>
</table>

Keys: INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are presented as means ± S.E.M (n = 6). *** significant increase (P < 0.05) when compared with corresponding control, †† significant decrease (P < 0.01), when compared with intact and high salt group. ### significant decrease (P < 0.001) when compared with control.
Fig. 9: Mean arterial blood pressure of intact and orchidectomised male Sprague Dawley rats fed a normal or high salt diet with or without testosterone supplementation

Keys: ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone.

Data are presented as means ± S.E.M (n = 6). Significantly high (**P < 0.01, ***P <0.001) when compared with Control. ^^^Significantly high (P < 0.01) when compared with orchidectomy and high salt diet group. ##significantly less (P < 0.01) when compared with control and testosterone supplemented and normal salt diet group. †††significantly less (P < 0.001) when compared with intact high salt group.
4.4.0 Testosterone assay

Figure 10 shows the serum level of testosterone across the groups. There was a significant decrease ($P < 0.001$) in the serum level of testosterone in the orchidectomised groups when compared with the intact groups. Serum level of testosterone was also significantly reduced ($P < 0.001$) in the orchidectomised groups when compared with that of the groups that received testosterone supplementation following orchidectomy and the sham-orchidectomised groups. Testosterone supplementation restored the serum testosterone level to that observed in the intact groups as there was no significant difference ($P > 0.05$) between the testosterone supplemented group and the intact group. Likewise there was no significant difference ($P > 0.05$) in the serum testosterone level of the sham-orchidectomised groups when compared with that of the intact groups. High salt diet did not affect the serum level of testosterone because there was no significant change ($P > 0.05$) in the serum level of testosterone in the groups fed a normal salt diet when compared with that of the high-salt fed groups.
Fig 10: Serum level of testosterone across the groups.

Keys: INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone supplementation, SHAM = sham orchidectomy.

Data are presented as mean ± S.E.M, (n = 5). Significant decrease (***P < 0.001) when compared with the intact, testosterone supplemented and sham-orchidectomised groups.
4.5  *In-vitro* vascular studies

4.5.1 Vascular relaxation response to Acetylcholine (Ach)

Cumulative relaxation response curves of the noradrenaline precontracted aortic rings to acetylcholine were obtained across the groups. Table 5, shows the EC$_{50}$, EC$_{75}$ and percentage maximum relaxation response of aortic rings from each group to acetylcholine. There was a significant reduction (P < 0.001) in the percentage maximum relaxation response of aortic rings to acetylcholine from high salt diet groups, when compared with the corresponding normal salt diet groups. However, there was a significant increase (P < 0.01) in the percentage maximum relaxation response to acetylcholine of aortic rings from orchidectomised and high salt group when compared with that from intact and high salt diet group. There was also a significant decrease (P < 0.01) in the relaxation response to acetylcholine in the testosterone supplemented groups when compared with the orchidectomy alone groups. Figure 11 shows the effects of a high salt diet, orchidectomy, testosterone supplementation and sham orchidectomy respectively on cumulative concentration relaxation response curves to acetylcholine of aortic rings from male Sprague Dawley rats.
Table. 5: EC$_{50}$, EC$_{75}$ and percentage maximum relaxation response of aortic rings to acetylcholine across the groups.

<table>
<thead>
<tr>
<th>Groups (n=12)</th>
<th>EC$_{50}$ (-Log mol/L)</th>
<th>EC$_{75}$ (-Log mol/L)</th>
<th>Maximum Relaxation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT + NS</td>
<td>6.91 ± 0.14</td>
<td>7.90 ± 0.29</td>
<td>81.97 ± 5.70</td>
</tr>
<tr>
<td>INT + HS</td>
<td>6.70 ± 0.06</td>
<td>7.50 ± 0.13</td>
<td>58.12 ± 4.12***</td>
</tr>
<tr>
<td>ORCH +NS</td>
<td>6.97 ± 0.07</td>
<td>7.84 ± 0.14</td>
<td>83.81 ± 5.20</td>
</tr>
<tr>
<td>ORCH + HS</td>
<td>6.39 ± 0.19</td>
<td>7.25 ± 0.33</td>
<td>#76.31 ± 4.57**</td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>6.82 ± 0.21</td>
<td>7.71 ± 0.42</td>
<td>78.56 ± 5.77</td>
</tr>
<tr>
<td>ORCH + TES + HS</td>
<td>6.51 ± 0.12</td>
<td>7.56 ± 0.34</td>
<td>††68.63 ± 4.30***</td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>6.36 ± 0.01</td>
<td>7.65 ± 0.11</td>
<td>63.4 ± 4.7</td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>6.21 ± 0.22</td>
<td>7.42 ± 0.19</td>
<td>62.46 ± 3.61***</td>
</tr>
</tbody>
</table>

Keys: INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are presented as means ± S.E.M (n=12). Significant decrease (***P < 0.001, **P < 0.01) when compared with corresponding control groups. ##significant increase (P < 0.01) when compared with intact and high salt diet group. ††significant decrease (P < 0.01) when compared with orchidectomy and high salt group.
Fig. 11: Relaxation response to acetylcholine in aortic rings from intact and orchidectomised Sprague Dawley rats fed a normal or high salt diet with or without testosterone supplementation.

Keys: ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone.

Data are presented as means ± S.E.M (n = 12). Significant Decrease (⁺⁺⁺P < 0.001) when compared with control. ⁺⁺significant decrease (P < 0.01) when compared with orchidectomy and high salt diet group. #⁹significant increase (P < 0.01) when compared with intact and high salt group.
4.5.2 Vascular relaxation response to Acetylcholine in the presence of L – Nitroarginine Methyl Ester (L-NAME)

The role of the endogenous nitric oxide (NO) production in the relaxation response of aortic rings from orchidectomised Sprague Dawley rats fed a high salt diet to acetylcholine was determined by obtaining cumulative concentration response curves to acetylcholine after 30 minutes incubation in L – NAME, an endothelial nitric oxide synthase (eNOS) inhibitor.

Table 6, shows the EC$_{15}$, EC$_{50}$, and maximum relaxation response of aortic rings from rats in all the groups to acetylcholine in the presence of L – NAME. Comparison across the groups show that there was a significant increase ($P < 0.01$) in the EC$_{15}$ value of the orchidectomy and high salt group when compared with the orchidectomy and normal salt diet group. Also there was a significant increase ($P < 0.01$) in the EC$_{50}$ values of the high salt diet groups when compared with their corresponding normal diet groups. Likewise there was a significant decrease ($P < 0.01$) in the percentage maximum relaxation response of all the high salt diet groups when compared with their corresponding control. However orchidectomy failed to improve relaxation response to ACh in the presence of L – NAME, as there was no significant difference in maximum relaxation response of the aortic rings between orchidectomy and high salt group and intact and high salt group.

Table 7, shows the percentage inhibition of aortic rings relaxation response to ACh by L – NAME. Percentage inhibition was calculated as the difference between relaxation response before and after incubation in L – NAME divided by relaxation before inhibition multiplied by 100. There was no significant difference in the percentage inhibition of the relaxation to
acetylcholine in the presence of L – NAME across the groups. Figure 12 shows the effects of a high salt diet, orchidectomy, testosterone supplementation and sham orchidectomy on aortic rings relaxation response to ACh in the presence of L – NAME.
Table. 6: EC$_{15}$, EC$_{50}$ and percentage maximum relaxation response of aortic rings from rats across the groups to acetylcholine in the presence of L – NAME.

<table>
<thead>
<tr>
<th>Groups (n = 9)</th>
<th>EC$_{15}$ (-Log mol/L)</th>
<th>EC$_{50}$ (-Log mol/L)</th>
<th>Maximum Relaxation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT + NS</td>
<td>4.76 ± 0.55</td>
<td>6.04 ± 0.21</td>
<td>26.26 ± 3.79</td>
</tr>
<tr>
<td>INT + HS</td>
<td>4.47 ± 0.25</td>
<td>5.26 ± 0.12*</td>
<td>18.83 ± 2.40††</td>
</tr>
<tr>
<td>ORCH +NS</td>
<td>4.19 ± 0.31</td>
<td>6.15 ± 0.30</td>
<td>28.22 ± 4.14</td>
</tr>
<tr>
<td>ORCH + HS</td>
<td>3.12 ± 0.23**</td>
<td>4.61 ± 0.23**</td>
<td>21.67 ± 3.67††</td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>5.54 ± 0.31</td>
<td>6.44 ± 0.13</td>
<td>23.25 ± 3.59</td>
</tr>
<tr>
<td>ORCH + TES + HS</td>
<td>5.19 ± 0.64</td>
<td>6.32 ± 0.23</td>
<td>20.25 ± 3.73††</td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>4.80 ± 0.45</td>
<td>5.51 ± 1.18</td>
<td>21.1 ± 4.17†</td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>4.88 ± 0.34</td>
<td>5.39 ± 0.17**</td>
<td>19.21 ± 3.15††</td>
</tr>
</tbody>
</table>

Keys: INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are presented as means ± S.E.M (n = 9). Significant decrease (††P < 0.01) when compared with corresponding control groups. Significant increase (**P < 0.01, *P < 0.05) when compared with corresponding control.
Table 7: Percentage maximum relaxation to acetylcholine before and after incubation in L-NAME and percentage inhibition (differences) in aortic rings from rats in the groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Maximum Relaxation (%) Before LNNA n =12</th>
<th>Maximum Relaxation (%) After LNNA n = 9</th>
<th>% Inhibition (Differences)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT + NS</td>
<td>81.97 ± 5.70</td>
<td>26.26 ± 3.79</td>
<td>67.96 ± 4.23</td>
</tr>
<tr>
<td>INT + HS</td>
<td>58.12 ± 4.12</td>
<td>18.83 ± 2.40</td>
<td>67.60 ± 3.13</td>
</tr>
<tr>
<td>ORCH + NS</td>
<td>83.81 ± 5.20</td>
<td>28.22 ± 4.14</td>
<td>68.33 ± 4.17</td>
</tr>
<tr>
<td>ORCH + HS</td>
<td>76.31 ± 4.57</td>
<td>21.67 ± 3.67</td>
<td>72.91 ± 5.21</td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>78.56 ± 5.77</td>
<td>23.25 ± 3.59</td>
<td>70.40 ± 4.23</td>
</tr>
<tr>
<td>ORCH + TES + HS</td>
<td>68.63 ± 4.30</td>
<td>21.25 ± 3.13</td>
<td>77.49 ± 3.81</td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>83.76 ± 4.71</td>
<td>24.40 ± 4.67</td>
<td>70.06 ± 5.03</td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>62.46 ± 3.61</td>
<td>19.1 ± 2.7</td>
<td>91.34 ± 3.92</td>
</tr>
</tbody>
</table>

Keys: **INT** = intact, **ORCH** = orchidectomy, **NS** = normal salt, **HS** = high salt, **TES** = testosterone, **SHAM** = sham orchidectomy.

Data are presented as means ± S.E.M (n = 9 or 12). There was no significant difference (P > 0.05) in the percentage inhibition across the groups.
Fig. 12: Relaxation response to acetylcholine in the presence of L-NAME in aortic rings from intact and orchidectomised Sprague Dawley rats fed a normal or high salt diet with or without testosterone supplementation.

Keys: **INT** = intact, **ORCH** = orchidectomy, **NS** = normal salt, **HS** = high salt, **TES** = testosterone.

Data are presented as means ± S.E.M (n = 9). *Significant decrease (P < 0.01) when compared with control.* ††Significant increase (P < 0.01) when compared with orchidectomy and high salt group.
4.5.3 Vascular relaxation response to Sodium Nitroprusside (SNP) in the presence of L – Nitroarginine Methyl Ester (L-NAME)

To assess the role of exogenous nitric oxide on the relaxation responses of the aortic rings across the groups, cumulative concentration response curves to administration of sodium nitroprusside (an exogenous NO donor), after 30 minutes incubation in L - NAME were obtained. Table 8 shows the EC$_{50}$, EC$_{75}$ and percentage maximum relaxation response to SNP after inhibition of eNOS by L – NAME. There was no significant difference in the EC$_{50}$, and EC$_{75}$ values of the relaxation response to SNP across the groups. Figure 13 shows the cumulative relaxation curves of aortic rings in all the groups to SNP in the presence of L-NAME. There was no significant difference in the percentage maximum relaxation response of aortic rings to SNP across all the groups.
Table 8. EC$_{50}$, EC$_{75}$, and percentage maximum relaxation response of aortic rings from rats across the groups to sodium nitropruside (SNP).

<table>
<thead>
<tr>
<th>Groups n = 9</th>
<th>EC$_{50}$ (-Log mol/L)</th>
<th>EC$_{75}$ (-Log mol/L)</th>
<th>Maximum Relaxation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT + NS</td>
<td>8.89 ± 0.06</td>
<td>9.30 ± 0.09</td>
<td>108.40 ± 2.82</td>
</tr>
<tr>
<td>INT + HS</td>
<td>9.18 ± 0.04</td>
<td>9.72 ± 0.08</td>
<td>102.30 ± 5.73</td>
</tr>
<tr>
<td>ORCH + NS</td>
<td>9.16 ± 0.01</td>
<td>9.54 ± 0.01</td>
<td>110.20 ± 2.26</td>
</tr>
<tr>
<td>ORCH + HS</td>
<td>9.11 ± 0.01</td>
<td>9.51 ± 0.01</td>
<td>105.30 ± 2.79</td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>8.68 ± 0.17</td>
<td>9.35 ± 0.30</td>
<td>113.80 ± 2.34</td>
</tr>
<tr>
<td>ORCH + TES + HS</td>
<td>8.94 ± 0.12</td>
<td>9.38 ± 0.05</td>
<td>109.90 ± 2.22</td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>8.72 ± 0.03</td>
<td>9.19 ± 0.05</td>
<td>110.60 ± 2.16</td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>9.16 ± 0.03</td>
<td>9.65 ± 0.05</td>
<td>107.70 ± 3.08</td>
</tr>
</tbody>
</table>

Keys: **INT** = intact, **ORCH** = orchidectomy, **NS** = normal salt, **HS** = high salt, **TES** = testosterone. **SHAM** = sham orchidectomy.

Data are presented as means ± S.E.M (n = 9). There was no significant difference (P > 0.05) in relaxation response to SNP across the groups.
Fig. 13: Relaxation response to SNP in the presence of L – NAME in aortic rings from intact and orchidectomised Sprague Dawley rats fed a normal or high salt diet, with or without testosterone supplementation.

**Keys:** ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone.

Data are presented as means ± S.E.M (n = 9). No significant difference (P > 0.05) in relaxation response to SNP across the groups.
4.5.4 Vascular relaxation response to Forskolin

The role of cyclic adenosine monophosphate pathway in the vascular reactivity of the aortic rings from the rats in all the groups was assessed. Cumulative concentration response of aortic ring to forskolin, an adenylyl cyclase activator was obtained. Table 9 shows the EC$_{50}$, EC$_{75}$ and percentage maximum relaxation response of aortic rings to forskolin. There was a significant increase (P < 0.01) in the EC$_{50}$ values of the high salt diet groups when compared with the normal salt diet groups. However orchidectomy significantly reduced (P < 0.05) the EC$_{50}$ value when compared with intact high salt group. There was a significant decrease (P < 0.01) in the percentage maximum relaxation response of the high salt fed groups when compared with the normal diet groups. However there was a significant increase (P < 0.01) in the percentage maximum relaxation response of the orchidectomy high salt group when compared with that of the intact and high salt group. Also there was a significant decrease (P < 0.01) in the percentage maximum relaxation response of the testosterone supplemented groups when compared with their corresponding orchidectomy alone groups. Figure 14 shows the cumulative relaxation curves of aortic rings in all the groups to forskolin.
Table. 9: EC$_{50}$, EC$_{75}$ and percentage maximum relaxation response of aortic rings from the rats across the groups to forskolin.

<table>
<thead>
<tr>
<th>Groups (n =8)</th>
<th>EC$_{50}$ (-Log mol/L)</th>
<th>EC$_{75}$ (-Log mol/L)</th>
<th>Maximum Relaxation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT + NS</td>
<td>7.50 ± 0.06</td>
<td>7.29 ± 0.27</td>
<td>136.9 ± 5.36</td>
</tr>
<tr>
<td>INT + HS</td>
<td>6.74 ± 0.07*</td>
<td>7.13 ± 0.07</td>
<td>122.6 ± 3.70††</td>
</tr>
<tr>
<td>ORCH + NS</td>
<td>7.15 ± 0.05</td>
<td>7.16 ± 0.23</td>
<td>141.5 ± 3.57</td>
</tr>
<tr>
<td>ORCH + HS</td>
<td>6.93 ± 0.04*</td>
<td>7.31 ± 0.07</td>
<td>127.1 ± 2.97††</td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>7.03 ± 0.05</td>
<td>7.04 ± 0.83</td>
<td>^124.4 ± 4.51</td>
</tr>
<tr>
<td>ORCH + TES + HS</td>
<td>6.73 ± 0.05*</td>
<td>7.11 ± 0.06</td>
<td>^119.9 ± 2.42</td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>7.31 ± 0.05</td>
<td>7.22 ± 0.71</td>
<td>132.96 ± 3.87</td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>6.83 ± 0.06*</td>
<td>7.4 ± 0.74</td>
<td>119.5 ± 1.41</td>
</tr>
</tbody>
</table>

Keys: INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are presented as means ± S.E.M (n = 8). *significant increase (P < 0.05) when compared with corresponding control groups. Significant decrease (#P < 0.01, #P < 0.05) when compared with intact high salt group. ††significant decrease when compared with corresponding controls. ^^significant decrease (P < 0.01) when compared with orchidectomised groups.
Fig. 14: Relaxation response to forskolin in aortic rings from intact and orchidectomised Sprague Dawley rats fed a normal or high salt diet with or without testosterone supplementation.

**Keys:** ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone.

Data are presented as means ± S.E.M (n = 8.). Significant decrease (**P < 0.01, ***P < 0.001) #significant increase in relaxation response when compared with intact and high salt group.
4.5.5  Vascular relaxation response to Diazoxide

Cumulative concentration response curves of aortic rings from rats across the groups to diazoxide, an ATP-dependent Potassium channel activator was determined. Table 10 shows the \( EC_{50} \), \( EC_{75} \) percentage maximum relaxation response of aortic rings from rats across the groups to diazoxide. There was a significant increase (\( P < 0.05 \)) in the \( EC_{50} \) and \( EC_{75} \) values of the high salt diet groups when compared with their normal diet counterpart (control) groups. Likewise there was a significant decrease (\( P < 0.001 \), \( P < 0.01 \)) in the percentage maximum relaxation response in all the high salt diet groups when compared with their corresponding normal salt groups. However there was a significant increase (\( P < 0.05 \)) in the percentage maximum relaxation response of orchidectomy and high salt group when compared to intact high salt group. Figure 15 shows the cumulative relaxation response curves of aortic rings from all the groups to diazoxide.
Table. 10: EC\textsubscript{50}, EC\textsubscript{75} and percentage maximum relaxation response of aortic rings across the groups to diazoxide.

<table>
<thead>
<tr>
<th>Groups (n = 8)</th>
<th>EC\textsubscript{50} (-Log mol/L)</th>
<th>EC\textsubscript{75} (-Log mol/L)</th>
<th>Maximum Relaxation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT + NS</td>
<td>6.96 ± 0.14</td>
<td>7.73 ± 0.08</td>
<td>91.03 ± 2.47</td>
</tr>
<tr>
<td>INT + HS</td>
<td>6.79 ± 0.13\textsuperscript{*}</td>
<td>7.41 ± 0.10\textsuperscript{*}</td>
<td>65.56 ± 2.46\textsuperscript{†††}</td>
</tr>
<tr>
<td>ORCH + NS</td>
<td>6.87 ± 0.11</td>
<td>7.96 ± 0.16</td>
<td>84.12 ± 2.64</td>
</tr>
<tr>
<td>ORCH + HS</td>
<td>6.64 ± 0.15\textsuperscript{*}</td>
<td>7.62 ± 0.43</td>
<td>#70.44 ± 3.31\textsuperscript{†††}</td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>6.27 ± 0.14</td>
<td>7.43 ± 0.29</td>
<td>#70.90 ± 2.61</td>
</tr>
<tr>
<td>ORCH + TES + HS</td>
<td>6.24 ± 0.10</td>
<td>7.43 ± 0.16</td>
<td>67.56 ± 2.73</td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>6.99 ± 0.14</td>
<td>7.97 ± 0.14</td>
<td>86.03 ± 3.97</td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>6.85 ± 0.15</td>
<td>7.88 ± 0.17</td>
<td>82.66 ± 2.25</td>
</tr>
</tbody>
</table>

Keys: INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are expressed as means ± S.E.M (n = 8). \textsuperscript{*}Significant increase (P < 0.05) when compared with corresponding control groups (normal diet), significant decrease (\textsuperscript{††P} < 0.01, \textsuperscript{†††P} < 0.001) when compared with corresponding controls, \#significant increase (P < 0.05) when compared with intact high salt group.
Fig. 15: Relaxation response to diazoxide in aortic rings from intact and orchidectomised Sprague Dawley rats fed a normal or high salt diet with or without testosterone supplementation.

Keys: ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone.

Data were presented as means ± S.E.M (n = 8). Significant decrease (**P < 0.01, ***P < 0.001) in relaxation when compared with control. ††Significant increase (P < 0.01) in relaxation response when compared with orchidectomy and high salt group.
4.6 Oxidative Stress and Reactive Oxygen Species (ROS) Study

4.6.1 Lipid Peroxidation

4.6.1.1 Lipid Peroxidation in the Heart

The level of lipid peroxidation in the heart was taken as an indication of the oxidative stress in it, following six weeks salt loading and orchidectomy with or without testosterone supplementation.

Figure 16 shows the level of lipid peroxidation in the heart across the groups. There was a significant increase (P < 0.01) in the lipid peroxidation level in the heart of the high salt diet groups when compared with their corresponding normal salt groups. However, there was a significant decrease (P < 0.05) in the lipid peroxidation level in the heart of the orchidectomy and high salt group when compared with the intact intact and salt diet group. On the other hand, testosterone supplementation significantly increased (P < 0.05) the lipid peroxidation level in the heart toward that in the intact groups, when compared with the orchidectomised groups.
Fig. 16: Lipid peroxidation level in the heart of the rats across the groups.

Keys: INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone supplementation, SHAM = sham orchidectomy.

Data are expressed as means ± S.E.M (n = 6). **significant increase (P < 0.01) when compared with the control group. †significant decrease (P < 0.05) when compared with intact and high salt group. #significant increase (P < 0.05) when compared with the orchidectomised groups.
4.6.1.2 Lipid Peroxidation in the Kidney

The level of lipid peroxidation in the kidney was taken as an indication of the oxidative stress in it, following six weeks salt loading and orchidectomy with or without testosterone supplementation.

Figure 17 shows the level of lipid peroxidation in the kidney across the groups. There was a significant increase (P < 0.01) in the lipid peroxidation level in the kidney of the high salt diet groups when compared with their corresponding normal salt groups. However, there was a significant decrease (P < 0.01) in the lipid peroxidation level in the kidney of the orchidectomy and high salt group when compared with the intact and high salt diet group. Testosterone supplementation significantly increased (P < 0.05) the lipid peroxidation level in the kidney toward that in the intact groups, when compared with the orchidectomized groups.
Fig. 17: Lipid peroxidation level in the kidney of the rats across the groups.

**Keys:** INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone supplementation, SHAM = sham orchidectomy.

Data are expressed as means ± S.E.M (n = 6). **significant increase (P < 0.01) when compared with the control group. †† significant decrease (P < 0.01) when compared with intact and high salt group. # significant increase (P < 0.05) when compared with the orchidectomised groups.**
4.6.2 Super Oxide Dismutase activity in the Blood

Super oxide dismutase (SOD), is an important antioxidant enzyme system in the body.

Figure 18 shows the activity of SOD in the serum of the rats across the groups. There was a significant decrease (P < 0.05) in the SOD activity in the serum of the high salt diet groups when compared with their corresponding normal salt groups. The only exception to this is the testosterone-supplemented groups where there was no significant difference between the high salt group and the normal salt group. However, there was a significant increase (P < 0.05) in the serum activity of SOD in the orchidectomy and high salt group when compared with the intact high and salt diet group, but testosterone supplementation had no significant effect on SOD activity when compared with the orchidectomy groups.
Fig. 18: Serum level of super oxide dismutase (SOD) in the rats across the groups.

**Keys:** INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone SHAM = sham orchidectomy.

Data are expressed as means ± S.E.M (n = 6). Significant decrease (††P < 0.01, †P < 0.05) when compared with the corresponding control groups. *significant increase (P < 0.05) when compared with intact and high salt diet group.
4.7.1 Serum level of total Bilirubin

Figure 19 shows the level of total bilirubin in the serum across the groups. There was a significant decrease (P < 0.01) in the serum level of total bilirubin in the high salt diet groups when compared with their corresponding control groups. However, there was a significant increase (P < 0.05) in the serum level of direct bilirubin in orchidectomy and high salt diet group when compared with the intact and high salt diet group. There was no significant difference in the serum level of total bilirubin of the testosterone supplemented groups when compared with the orchidectomised groups.

4.7.2 Serum level of direct Bilirubin

Figure 20 shows the level of direct bilirubin in the serum across the groups. There was a significant decrease (P < 0.01) in the serum level of direct bilirubin in the high salt diet groups when compared with their corresponding control groups. However, there was a significant increase (P < 0.05) in the serum level of direct bilirubin in orchidectomy and high salt diet group when compared with the intact and high salt diet group. There was no significant difference in the serum level of direct bilirubin of the testosterone supplemented groups when compared with the orchidectomised groups.
Fig. 19: Serum level of bilirubin total in the rats across the groups.

Keys: INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are expressed as means ± S.E.M. (n = 6). †† significant decrease (P < 0.01) when compared with the corresponding control groups. * significant increase (P < 0.05) when compared with intact and high salt group.
Fig. 20: Serum level of bilirubin direct in the rats across the groups.

Keys: INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are expressed as means ± S.E.M. (n = 6). Significant decrease (‡‡P < 0.01, †P < 0.05) when compared with the control group. *significant increase (P < 0.05) when compared with intact and high salt group.
4.8.0 Histomorphometric study

Tables 11 and 12 show the histomorphometric data of the thoracic aorta and the mesenteric arteries across the groups.

4.8.1 Tunica Media thickness

There was a significant increase (P < 0.01, P < 0.001) in the thickness of the tunica media of the thoracic aorta and mesenteric arteries of the high salt fed groups when compared with that of their corresponding normal diet fed groups (controls). However there was a significant decrease (P < 0.01) in the tunica media thickness of the orchidectomised groups when compared with that of the intact groups. On the other hand, there was a significant increase (P < 0.01) in the thickness of the aorta and mesenteric artery tunica media of the groups of rats given testosterone supplementation following orchidectomy when compared with the orchidectomy alone groups (Table 11).

4.8.2 Media Cross Sectional Area (MCSA)

There was a significant increase (P < 0.001) in the medial cross sectional area (MCSA) of the thoracic aortas and mesenteric arteries of the high salt fed groups when compared with that of their corresponding normal diet fed groups (controls). But the MCSA of the orchidectomised groups were significantly less (P < 0.01) when compared with that of the intact groups. On the other hand, there was a significant increase (P < 0.01) in the MCSA of the groups of rats given testosterone supplementation following orchidectomy when compared with the groups that were orchidectomised but not given testosterone supplementation (Table 11).
4.8.3 Luminal Cross Sectional Area (LCSA)

There was a significant decrease (P < 0.001) in the luminal cross sectional area (LCSA) of the thoracic aortas and mesenteric arteries from the high salt fed rats when compared with that of their corresponding normal diet fed groups (controls). However there was a significant increase (P < 0.01) in the LCSA of the orchidectomised groups when compared with that of the intact groups. On the other hand, there was a significant decrease (P < 0.01) in the LCSA of the thoracic aorta from the groups of rats given testosterone supplementation following orchidectomy when compared with the orchidectomy alone groups (Table 11).

4.8.4 Elastin Content

There was a significant increase (P < 0.01, P < 0.001) in the elastin content of the thoracic aortas and mesenteric arteries of the high salt fed groups when compared with that of their corresponding normal diet fed groups (controls). But there was a significant reduction (P < 0.01) in the elastin content area of the orchidectomised groups when compared with that of the intact groups while testosterone supplementation significantly increase (P < 0.01) the elastin content in the thoracic aortas and the mesenteric arteries when compared with the orchidectomy alone groups (Table 11).

4.8.5 Collagen Content

There was a significant increase (P < 0.01, P < 0.001) in the collagen content of the thoracic aortas and mesenteric arteries of the high salt fed groups when compared with that of their corresponding normal diet fed groups (controls). But there was a significant reduction (P < 0.01) in the collagen content of the orchidectomised groups when compared with that of the intact
groups while testosterone supplementation significantly increase \( P < 0.01 \) the collagen content in the thoracic aortas and the mesenteric arteries when compared with the orchidectomy alone groups (Table 11).

### 4.8.6 Nucleus Count

Nucleus count was significantly higher \( P < 0.05, P < 0.01 \) in the thoracic aorta of the high salt fed groups when compared with that of their corresponding normal diet fed groups (controls). However, there was a significant decrease \( P < 0.01 \) in the nucleus count of the orchidectomised groups when compared with that of the intact groups. On the other hand, nucleus count was significant higher \( P < 0.01 \) in the thoracic aortas of the groups of rats given testosterone supplementation following orchidectomy when compared with the orchidectomy alone groups (Table 11).

The internal elastic laminae were intact in all aorta and the mesenteric arteries studied. However, an observation worthy of note, is the tortuosity and thickness or otherwise of the internal elastic lamina of aorta and mesenteric in each group. The internal elastic laminae of the high salt diet groups appear to be straightened out and thicker in comparison to that of their corresponding normal salt diet groups. Figures 21 –28 show the photomicrograph sections of the aorta and the mesenteric artery across the groups.
Table. 11: Histomorphometric measurement of the thoracic aorta of the rats across the groups.

<table>
<thead>
<tr>
<th>Groupings n = 10</th>
<th>Intima medial thickness (µm)</th>
<th>Media cross sectional area (µm²)</th>
<th>Luminal cross sectional area (µm²)</th>
<th>Elastin content (µm²/mm)</th>
<th>Collagen content (µm²/mm)</th>
<th>Nucleus count</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT + NS</td>
<td>76.16 ± 2.36</td>
<td>315786 ± 432</td>
<td>928459 ± 653</td>
<td>6347 ± 43</td>
<td>1994 ± 93</td>
<td>63 ± 2</td>
</tr>
<tr>
<td>INT + HS</td>
<td>99.94 ± 5.18***</td>
<td>407803 ± 672***</td>
<td>566767 ± 702***†††</td>
<td>8461 ± 97***</td>
<td>3193 ± 112**</td>
<td>79 ± 5**</td>
</tr>
<tr>
<td>ORCH + NS</td>
<td>††66.20 ± 3.62</td>
<td>††276812 ± 312</td>
<td>993876 ± 815</td>
<td>††4758 ± 67</td>
<td>1561 ± 69</td>
<td>††57 ± 3</td>
</tr>
<tr>
<td>ORCH + HS</td>
<td><strong>88.18 ± 5.96</strong>*</td>
<td><em><strong>375358 ± 491</strong></em></td>
<td><strong>74964 ± 245</strong>*†††</td>
<td><strong>7977 ± 71</strong>*</td>
<td>2509 ± 61</td>
<td><strong>72 ± 4</strong></td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>**81.88 ± 4.39</td>
<td>***354892 ± 198</td>
<td>**865974 ± 372</td>
<td>**6406 ± 54</td>
<td>2076 ± 56</td>
<td>**73 ± 3</td>
</tr>
<tr>
<td>ORCH + TES + HS</td>
<td><strong>94.75 ± 4.80</strong>*</td>
<td><em><strong>495652 ± 613</strong></em></td>
<td>*<em>119690 ± 297</em>†††</td>
<td>**8478 ± 82</td>
<td>2731 ± 87</td>
<td>*<em>77 ± 2</em></td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>78.57 ± 3.28</td>
<td>309624 ± 301</td>
<td>83438 ± 57.9</td>
<td>602 ± 0</td>
<td>1891 ± 49</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>98.22 ± 4.10***</td>
<td>400345 ± 265***</td>
<td>6691 ± 428***</td>
<td>8745 ± 12***</td>
<td>908 ± 63</td>
<td>77 ± 3**</td>
</tr>
</tbody>
</table>

Keys: INT = Intact, ORCH = Orchidectomy, NS = Normal salt, HS = High salt, TES = Testosterone, SHAM = Sham orchidectomy.

Data are expressed as mean ± S.E.M. (n = 10). ***Significant increase (P < 0.001) when compared with the corresponding controls (normal diet groups). †††Significant decrease (P < 0.001) when compared with corresponding controls. ††Significant decrease (P < 0.01) when compared with intact and normal diet (Control). ##Significant decrease when compared with intact and high salt group. ^Significant increase (P < 0.01) when compared with intact and high salt group. ^Significant increase (P < 0.01) when compared with corresponding orchidectomised groups. †††Significant increase (P < 0.01) when compared with intact and high salt group. ¶¶Significant decrease (P < 0.01) when compared with corresponding orchidectomised groups. ððSignificant increase (P < 0.01) when compared with intact and normal diet group.
Table 12: Histomorphometric measurement of the mesenteric artery of the rats across the groups.

<table>
<thead>
<tr>
<th>Groupings n = 10</th>
<th>Tunica medial thickness (µm)</th>
<th>Media cross sectional area (µm²)</th>
<th>Luminal cross sectional area (µm²)</th>
<th>Elastin content (µm²/mm)</th>
<th>Collagen content (µm²/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT + NS</td>
<td>28.43 ± 3.78</td>
<td>17424 ± 432</td>
<td>72017 ± 797</td>
<td>2771 ± 64</td>
<td>1790 ± 57</td>
</tr>
<tr>
<td>INT + HS</td>
<td>39.40 ± 3.91***</td>
<td>36651 ± 672***</td>
<td>46676 ± 837†††</td>
<td>3604 ± 89***</td>
<td>2413 ± 92***</td>
</tr>
<tr>
<td>ORCH + NS</td>
<td>126.82 ± 3.15††</td>
<td>15028 ± 312</td>
<td>81938 ± 536</td>
<td>2329 ± 71</td>
<td>1536 ± 54</td>
</tr>
<tr>
<td>ORCH + HS</td>
<td>32.34 ± 4.90*</td>
<td>23667 ± 491***</td>
<td>58050 ± 689†††</td>
<td>3086 ± 92***</td>
<td>1829 ± 77***</td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>29.84 ± 2.3</td>
<td>20265 ± 198</td>
<td>59773 ± 971</td>
<td>2433 ± 54</td>
<td>1714 ± 68</td>
</tr>
<tr>
<td>ORCH + TES +HS</td>
<td>37.90 ± 4.91***</td>
<td>26178 ± 613***</td>
<td>47097 ± 483</td>
<td>31027 ± 78</td>
<td>1947 ± 80</td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>28.07 ± 2.78</td>
<td>18648 ± 302</td>
<td>70252 ± 602</td>
<td>137 ± 70</td>
<td>175 ± 60</td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>38.01 ± 3.88***</td>
<td>33883 ± 265</td>
<td>44335 ± 513</td>
<td>1394 ± 88***</td>
<td>2333 ± 83***</td>
</tr>
</tbody>
</table>

Keys: INT = Intact, ORCH = Orchidectomy, NS = Normal salt, HS = High salt, TES = Testosterone, SHAM = Sham orchidectomy.

Data are expressed as mean ± S.E.M. (n = 10). Significant increase (***P < 0.001, *P < 0.05) when compared with the corresponding controls (normal diet groups). †††Significant decrease (P < 0.001) when compared with corresponding controls. Significant decrease (††P < 0.01, †P < 0.05) when compared with intact and normal diet (Control). #Significant decrease when compared with intact and high salt group. **Significant increase (P < 0.01) when compared with corresponding orchidectomised groups. §§Significant increase (P < 0.01) when compared with intact and high salt group. ¶¶Significant decrease (P < 0.01) when compared with corresponding orchidectomised groups. §§§Significant increase (P < 0.01) when compared with intact and normal diet group.
Fig. 21: Photomicrograph of aorta from the intact and normal salt diet group (Control).

Verhoeff’s haematoxylin and Van Gieson’s stain. 50µm
**Fig. 22:** Photomicrograph of aorta from the intact and high salt diet group: Harris’s Haematoxylin and Eosin stain. 50µm
Fig. 23: Photomicrograph of aorta from the orchidectomy and normal salt diet group:

Verhoeff’s haematoxylin and Van Gieson’s stain 50µm
Fig. 24: Photomicrograph of aorta from the orchidectomised testosterone supplemented and normal diet group: Verhoeff’s haematoxylin and Van Gieson’s stain. 50µm
Fig. 25: Photomicrograph of mesenteric artery from the intact and normal salt diet group:

Harris’s Haematoxylin and Eosin stain. 50µm
Fig. 26: Photomicrograph of mesenteric artery from the intact and high salt diet group:

Verhoeff’s haematoxylin and Van Gieson’s stain. 50µm
Fig. 27: Photomicrograph of aorta from the orchidectomised testosterone supplemented and normal salt diet group: Verhoeff’s haematoxylin and Van Gieson’s stain. 50μm
Fig. 28: Photomicrograph of aorta from the orchidectomised testosterone supplemented and high salt diet group: Harris’s Haematoxylin and Eosin stain. 50μm
4.9.1 Vascular relaxation response to exogenous testosterone

To assess the non genomic effect of testosterone on vascular relaxation response of aortic rings across the groups, cumulative concentration response to exogenous testosterone was obtained. Testosterone was dissolved and serially diluted in absolute ethanol.

Figure 29 shows the comparison of graded concentration of testosterone to time–matched ethanol (control). There was a significant dose dependent increase (P < 0.05, P < 0.001) in the relaxation response to 1, 10 and 100µM of testosterone when compared with time–matched (5 minutes) absolute ethanol solvent which serves as control.

Table 13 shows the EC_{50}, EC_{75} and percentage maximum relaxation response of aortic rings to testosterone propionate. There was a significant increase (P < 0.01) in the EC_{50} and EC_{75} values of the intact high salt groups when compared with intact normal salt diet group (control). There was a significant decrease in the percentage maximum relaxation response to all the high salt groups when compared with their corresponding normal salt group. Interestingly, there was a significant decrease (P < 0.001) in the maximum relaxation response of the orchidectomised groups when compared with the intact groups, while on the other hand testosterone supplementation significantly increased (P < 0.001) the maximum relaxation response to testosterone even more than the intact groups.

Figure 30 shows the percentage relaxation response of the aortic rings to increasing concentrations of testosterone propionate. There was a significant decrease (P < 0.01) in the percentage relaxation response at each concentration used, in all the high salt groups when
compared with their corresponding control groups. At each concentration used there was a significant decrease (P < 0.001) in the relaxation response of the orchidectomy groups when compared with the intact groups. On the other hand, at each concentration used, there was a significant increase (P < 0.001) in the percentage relaxation response of the testosterone supplemented groups when compared with both the orchidectomised and the intact groups.
Fig. 29: Relaxation response of aortic rings to testosterone and time–matched (5 minutes) ethanol (control).

Data are presented as means ± S.E.M. Significant increase (*P < 0.05, ***P < 0.001) when compared with time – matched control.
Table. 13: EC$_{50}$, EC$_{75}$ and percentage maximum relaxation response of aortic rings from rats across the groups to exogenous testosterone.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n = 10</th>
<th>EC$_{50}$ (-Log mol/L)</th>
<th>EC$_{75}$ (-Log mol/L)</th>
<th>Maximum Relaxation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT + NS</td>
<td>4.92 ± 0.12</td>
<td>5.45 ± 0.09</td>
<td>78.27 ± 2.55</td>
<td></td>
</tr>
<tr>
<td>INT + HS</td>
<td>3.96 ± 0.04 *</td>
<td>2.99 ± 0.24**</td>
<td>63.65 ± 4.36 ††</td>
<td></td>
</tr>
<tr>
<td>ORCH + NS</td>
<td>4.90 ± 0.17</td>
<td>3.90 ± 0.20</td>
<td>58.66 ± 5.08 ††</td>
<td></td>
</tr>
<tr>
<td>ORCH + HS</td>
<td>4.94 ± 0.06</td>
<td>4.37 ± 0.17</td>
<td>39.39 ± 2.86 †††</td>
<td></td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>5.45 ± 0.23</td>
<td>4.64 ± 0.09</td>
<td>92.8 ± 9.34 ***</td>
<td></td>
</tr>
<tr>
<td>ORCH + TES + HS</td>
<td>5.60 ± 0.12</td>
<td>5.08 ± 0.27</td>
<td>110.1 ± 6.23 ***</td>
<td></td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>4.39 ± 0.13</td>
<td>5.43 ± 0.13</td>
<td>58.13 ± 3.14</td>
<td></td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>3.29 ± 0.42 *</td>
<td>3.09 ± 0.11**</td>
<td>110.1 ± 6.23 ††</td>
<td></td>
</tr>
</tbody>
</table>

**Keys:** INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are presented as means ± S.E.M. ††significant decrease (P < 0.01) when compared with corresponding control groups. ***significant increase (P < 0.001) when compared with both orchidectomised and the control group.
Fig. 30: Percentage relaxation response of aortic rings across the groups to increasing concentrations of testosterone propionate.

**Keys:** INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are expressed as means ± S.E.M. (n = 10). #significant decrease when compared with their corresponding normal diet group (controls), *significant increase when compared with both orchidectomised and intact groups of rats.
4.9.2 Vascular relaxation response to Dehydroepiandrosterone (DHEA)

The direct effect of DHEA, a precursor/metabolite of testosterone, on vascular relaxation response of aortic rings across the groups was studied. Table 14 shows the EC\textsubscript{50}, EC\textsubscript{75} and percentage maximum relaxation response of the aortic rings to DHEA.

There was no significant difference in the maximum relaxation response of the high salt groups when compared with the normal salt diet groups. However there was a significant decrease (P < 0.001) in the percentage maximum relaxation response of the orchidectomised groups when compared with the intact groups. On the other hand there was a significant increase (P < 0.05) in the percentage maximum relaxation response of the testosterone supplemented groups when compared with both orchidectomised groups and the intact groups.

Figure 31 shows the percentage relaxation response of the aortic rings to increasing concentrations of DHEA. At each concentration used, there was a significant decrease (P < 0.01) in the percentage relaxation response of the orchidectomy groups when compared with the intact groups. On the other hand testosterone supplementation significantly increased (P < 0.001) the percentage relaxation response of the aortic rings to DHEA at each concentration used when compared with both the orchidectomy and the intact groups. However, testosterone supplementation seems to re-establish the vascular relaxation impairing effect of a high salt diet. At each concentration used, there was a significant decrease (P < 0.05) in the percentage relaxation response of the testosterone supplemented and high salt diet group when compared with the corresponding normal salt diet group.
Table. 14. EC$_{50}$, EC$_{75}$ and percentage maximum relaxation response of aortic rings from rats across the groups to dehydroepiandrosterone (DHEA).

<table>
<thead>
<tr>
<th>Groups n = 10</th>
<th>EC$_{50}$ (-Log mol/L)</th>
<th>EC$_{75}$ (-Log mol/L)</th>
<th>Maximum Relaxation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT + NS</td>
<td>4.37 ± 0.30</td>
<td>5.88 ± 0.68</td>
<td>62.44 ± 2.17</td>
</tr>
<tr>
<td>INT + HS</td>
<td>4.97 ± 0.21</td>
<td>5.45 ± 0.51</td>
<td>61.09 ± 3.03</td>
</tr>
<tr>
<td>ORCH + NS</td>
<td>2.14 ± 0.19</td>
<td>4.10 ± 0.23</td>
<td>35.89 ± 3.43†††</td>
</tr>
<tr>
<td>ORCH + HS</td>
<td>2.69 ± 0.27</td>
<td>3.86 ± 0.46</td>
<td>35.66 ± 4.11†††</td>
</tr>
<tr>
<td>ORCH + TES + NS</td>
<td>5.82 ± 0.19</td>
<td>5.19 ± 0.16</td>
<td>113.3 ± 3.02***</td>
</tr>
<tr>
<td>ORCH + TES + HS</td>
<td>6.01 ± 0.30</td>
<td>5.74 ± 0.18</td>
<td>113.3 ± 6.12</td>
</tr>
<tr>
<td>SHAM + NS</td>
<td>4.63 ± 0.46</td>
<td>5.30 ± 0.09</td>
<td>66.77 ± 3.17</td>
</tr>
<tr>
<td>SHAM + HS</td>
<td>4.89 ± 0.05</td>
<td>5.85 ± 0.17</td>
<td>62.9 ± 3.05</td>
</tr>
</tbody>
</table>

**Keys:** INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data are presented as means ± S.E.M. (n = 10). ††† significant decrease (P < 0.001) when compared with control groups. *** significant increase (P < 0.001) when compared with both the orchidectomy and the intact groups. # significant decrease (0.05) when compared with testosterone supplemented and normal salt diet.
Fig. 31: Percentage relaxation response of aortic rings across the groups to increasing concentrations of DHEA.

Keys: INT = intact, ORCH = orchidectomy, NS = normal salt, HS = high salt, TES = testosterone, SHAM = sham orchidectomy.

Data expressed as means ± S.E.M. (n = 10). †† significant decrease (P < 0.01) when compared with the control group. *** significant increase (P < 0.001) when compared with both orchidectomy and intact groups. # significant decrease (P < 0.05) when compared with testosterone supplemented and normal salt diet group.
4.9.3 Relaxation responses of aortic rings to exogenous testosterone and DHEA in the presence or absence of different inhibitors and activators

The probable mechanism by which testosterone elicits vascular relaxation was assessed by obtaining cumulative relaxation response curves of aortic rings to testosterone propionate and DHEA in the presence or absence of pharmacological inhibitors and activators i.e. flutamide, an androgen receptor blocker; aminogluthethemide, an aromatase enzyme inhibitor; L-NAME, an eNOS inhibitor; indomethacin, a COX-2 inhibitor; barium chloride, a non-specific potassium channel blocker, tertiapin –Q, an inward rectifier potassium channel blocker, apamin, charybdotoxin and iberiotoxin, small, intermediate and large calcium activated potassium channel blockers respectively.

Table 15 shows the EC$_{50}$, EC$_{75}$, and percentage maximum relaxation response of aortic rings to exogenous testosterone and DHEA in the presence or absence of different inhibitors and/or activators. There was a significant decrease (P < 0.05) in the percentage maximum relaxation response of aortic rings to DHEA when compared with that of testosterone (control). There was no significant difference in the percentage maximum relaxation response to testosterone after endothelial nitric oxide synthase (eNOS) and cyclooxygenase – 2 (COX - 2) inhibition with L-NNA and indomethacin respectively, when compared with the control. Also blockade of the three Ca$^{2+}$ dependent K$^+$ channels namely small, intermediate and large, and the inward rectifier K$^+$ channel by apamin, charybdotoxin iberiotoxin and tertiapin – Q respectively has no significant effect on the relaxation responses of the aortic rings to exogenous testosterone.
Blockade of conventional androgen receptor (AR) and aromatase enzyme inhibition with flutamide and aminogluthethemide respectively significantly increased (P < 0.05) the percentage relaxation response of aortic rings to testosterone when compared with the control. Also there was a significant increase (P < 0.01) in the percentage maximum relaxation response of the aortic rings to testosterone after incubation in 1µM nifedipine when compared with control (table 14).

However incubation of the aortic rings in 3µM barium chloride (BaCl₂) almost abolished the aortic rings relaxation response to testosterone. There was a significant decrease (P < 0.001) in the percentage maximum relaxation response of the aortic rings to testosterone after BaCl₂ incubation when compared with the control. There was a significant decrease (P < 0.001) in the percentage relaxation response of the aortic rings to nifedipine after incubation in testosterone, when compared with relaxation response of testosterone after incubation in nifedipine (table 14).

Figures 32 and 33 show cumulative relaxation response curves to testosterone and DHEA in the presence or absence of some of these pharmacological agents.
Table 15: EC<sub>50</sub>, EC<sub>75</sub>, and percentage maximum relaxation response of aortic rings to exogenous testosterone and DHEA in the presence or absence of different inhibitors and/or activators.

<table>
<thead>
<tr>
<th>Groupings</th>
<th>n = 10 or 6</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (-Log mol/L)</th>
<th>EC&lt;sub&gt;75&lt;/sub&gt; (-Log mol/L)</th>
<th>Maximum Relaxation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone (control)</td>
<td>4.92 ± 0.12</td>
<td>5.45 ± 0.09</td>
<td>78.27 ± 2.55</td>
<td></td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>4.37 ± 0.30</td>
<td>5.88 ± 0.68</td>
<td>62.44 ± 2.17†</td>
<td></td>
</tr>
<tr>
<td>Testosterone + Aminoglut + Flutamide</td>
<td>5.03 ± 0.09</td>
<td>5.54 ± 0.11</td>
<td>86.15 ± 3.46*</td>
<td></td>
</tr>
<tr>
<td>Testosterone + L-NAME + Indomethacin</td>
<td>4.75 ± 0.13</td>
<td>5.24 ± 0.05</td>
<td>79.09 ± 2.18</td>
<td></td>
</tr>
<tr>
<td>Testosterone + Barium Chloride</td>
<td>5.10 ± 0.34</td>
<td>5.05 ± 0.32</td>
<td>25.38 ± 1.18†††</td>
<td></td>
</tr>
<tr>
<td>Testosterone + Nifedipine</td>
<td>5.93 ± 0.26</td>
<td>6.71 ± 0.53</td>
<td>96.74 ± 1.70**</td>
<td></td>
</tr>
<tr>
<td>Nifedipine + Testosterone</td>
<td>5.26 ± 0.46</td>
<td>6.19 ± 0.64</td>
<td>43.80 ± 3.99###</td>
<td></td>
</tr>
</tbody>
</table>

**Keys:** AMINOGLUT = aminogluthetemide, L-NAME = L – nitroarginine methyl ester.

Data are expressed as means ± S.E.M. (n = 10 or 6). Significant decrease (†P < 0.05, †††P < 0.001) when compared with the control group. Significant increase (⋆P < 0.05, ⋆⋆P < 0.01) when compared with control group. ###significant decrease (P < 0.001) when compared with relaxation response to testosterone after incubation in nifedipine.
Fig. 32: Relaxation response of aortic rings to testosterone propionate and DHEA in the presence and absence of flutamide, aminogluthetemide, L-NAME and indomethacin.

**Keys:** AMINOGLUT = aminogluthetemide, FLUTA = flutamide, L-NAME = L-Nitro Arginine Methyl Ester, INDO = indomethacin, DHEA = dehydroepiandrosterone.

Data are expressed as means ± S.E.M. *significant decrease (P < 0.05) when compared with the control group.
Fig. 33: Relaxation response of aortic rings to testosterone propionate in the presence and absence of barium chloride and nifedipine

**Keys:** TEST = testosterone, BaCl$_2$ = barium chloride, NIFE = nifedipine.

Data are expressed as means ± S.E.M. (n = 10 or 6). †significant decrease when compared with the control group. *significant increase when compared with control group. #significant decrease (P < 0.05) when compared with relaxation response of aortic rings to testosterone after nifedipine incubation.
DISCUSSION

5.1 Discussion of Results

5.1.1 Body weight

Results from this study show that high salt diet reduced the rats’ body weight. Higher magnitude in the percentage increase in the final mean body weight of rats in the orchidectomy and high salt group when compared with the intact and high salt group (control) indicates that orchidectomy attenuated body weight reducing effect of a high salt diet. As there was no difference in the feeding habit of the rats in all the groups, the weight loss could be due to an increase in body water loss through excessive urination in the intact high salt diet group. An important observation in the intact high salt and sham orchidectomy high salt groups is the frequency of the need to change these rats’ beddings due to excessive soiling. Previous reports on high salt diet and body weight have in the least been controversial. Coelho et al. (2006) reported that high salt diet reduced body weights, while low salt diet reduced food intake yet increased body weight in rats. They ascribed the weight increase in the low salt diet group to positive energy balance in this group of rats, this view was shared by Heinman et al., 2006. But Cappuccio and Miller (2006), surmised that the findings of Coelho et al., 2006, on bodyweight increasing effect of low salt diet, which simultaneously decrease food intake negates the law of thermodynamics as energy can neither be created nor destroyed. This particular study did not include low salt diet in its design, but our findings on high salt diet and bodyweight corroborates that of Coelho et al., 2006 and Partovian et al., (1998) that high salt diet reduces body weight. Also body weight was reported to be reduced in rat model of angiotensin II induced hypertension (Brink et al., 1996). Conversely, 1% increase in body weight was reported in hypertensive patients on high compared to low salt diet (Dichtchekenian et al., 1992). The disparity in these findings could be due to the
fact that Dichtchekenian et al., (1992) studied the effect of a high salt diet on human subjects with established hypertension, while the present study and that of Coelho and colleagues were carried out on normotensive weanling rats on high salt diet.

5.1.2 Organ weights

Results from this study show that high salt diet increased the heart and kidney weight index in rats. The heart and kidney indices of the orchidectomy and high salt group were comparable to the control group, unlike that of the intact and high salt group that was higher than the control. More recently, it has been recognized that excess salt is strongly associated with cardiac hypertrophy, a structural pattern observed in both hypertensive men and rats independently of the level of blood pressure (Ahn et al., 2004; Matavelli et al., 2007). A temporal link between increased NaCl intake and aortic hypertrophy has also been noted (Lima et al., 1980; Partovian et al., 1998) in spontaneously hypertensive rats (SHR) in the absence of a significant change in blood pressure. This finding implicates testosterone in the increase in the weight indices of the heart and the kidney of rats fed a high salt diet.

5.1.3 Arterial blood pressures

Normal non-genetically modified Sprague-Dawley rats develop high blood pressure when fed a high salt diet from weaning (Adegunloye and Sofola, 1997; Ebuehi et al., 2003). In this study, non-genetically modified Sprague-Dawley rats were used as a model of salt-sensitivity in normotensive males. This allow for the question of how males develop high blood pressure in response to a high salt diet and what the role of androgens in this process is. This model was also
used to examine how in males the blood vessel responds to salt-stress in the presence or absence of androgens.

Blood pressure is timely regulated by many mechanisms such as, neural, hormonal and renal mechanisms. The neural and hormonal mechanisms react within seconds or minutes to change the diameter of the blood vessels and vascular resistance, while the renal mechanisms react within hours or days to restore blood pressure mainly by controlling the plasma volume. Hypertension involves abnormal and persistent changes in the blood pressure mechanisms, and high salt diet has been associated with hypertension (Khalil, 2006; Sanders, 2009).

Feeding the rats with a high salt diet for six weeks elevated all their blood pressure parameters (systolic, diastolic and the mean arterial blood pressure). Mean arterial blood pressure (MABP) of 115mmHg was used as the cut-off point for hypertension in this study. This was achieved by adding 10% increase to 105mmHg, the mean value of MABP in non-hypertensive rats (Rapp and Dene, 1985). In this study, the blood pressure elevating effect of a high salt diet set in around the second week of salt-loading. By the third week, the intact as well as the orchidectomised rats that were given testosterone supplementation and fed a high salt diet had developed hypertension. But the onset of hypertension was delayed to about the fifth week of salt-loading in the orchidectomised rats fed a high salt diet. These findings implicate testosterone in the modulation of mechanisms that are responsible for regulation of blood pressure during salt-stress and suggest that orchidectomy delayed while testosterone promoted the development of salt-induced hypertension in male Sprague-Dawley rats. Although blood pressure was elevated in the rats that were fed a high salt diet, the higher magnitude of blood pressure elevation in the intact and
orchidectomised rats that were given testosterone supplementation showed that testosterone is involved in the blood pressure elevating and consequently hypertension inducing effect of a high salt diet. The reduction in the magnitude of blood pressure elevation in orchidectomised rats fed a high salt diet shows that orchidectomy countered or prevented the blood pressure elevating effect of a high salt diet, therefore showing that testosterone plays a role in or complements the blood pressure elevating effect of a high salt diet and consequently salt-induced hypertension in male Sprague-Dawley rats. This present result is similar to the findings of other researchers, using different strains of rats, (Reckelhoff et al., 1999; Reckelhoff, 2001), reported that castration of young male SHR and DSS attenuates the development of hypertension when they were fed a high-salt diet. The study by Reckelhoff and Colleagues implicated renin angiotensin system in the gender difference in the development of hypertension in SHR as it partly involved pressure between male and female was abolished by chronic treatment with angiotensin-converting enzyme inhibitor. In addition, in their study, androgens failed to increase blood pressure in the presence of RAS blockade (Reckelhoff et al., 2001), suggesting that testosterone elicit blood pressure elevation in these rats by activation of the RAS. Khalid et al., (2002) also reported that in male Sabra rats (SBH), a genetically-modified salt-sensitive strain, gonadectomy prevented the full development of hypertension, however they suggested the effect of testosterone on the role of α2A-adrenoceptor as the basis for the blood pressure reducing effect of orchidectomy in this rat strain. Conversely, Yagil and Yagil, (2000) reported that both in male and female newly selected Sabra rats (SBHs) strain, gonadectomy had no effect on salt-induced hypertension. Discrepancy in their findings could be due to the different strains used, but this also shows that differences occur in physiological functions of laboratory animals from species to species and even within the same species. Findings from this present study shows that even
in the absence of genetic risk factor for onset of hypertension such as in SHR, DSS and SBH (as exemplified in Sprague-Dawley rats), high salt diet could induce high blood pressure and that development of high blood pressure is delayed and attenuated by orchidectomy, but promoted by testosterone in Sprague Dawley rats.

5.1.4. Serum Level of Testosterone

The testis is the primary source of androgens in males and testosterone is the major androgen in males (Guyton and Hall, 1996). The significant decrease in the mean serum concentration of testosterone in the orchidectomised rats when compared with that of the intact rats showed that the bilateral orchidectomy procedure was successful. The significant increase in the serum level of testosterone in the rats given testosterone supplementation following orchidectomy when compared with orchidectomised rats showed that intramuscular administration of Sustanon-250® was successful as a form of testosterone replacement in orchidectomised rats. This finding is further supported by absence of significant difference in the serum level of testosterone in rats given testosterone supplementation when compared with the intact rats. Intramuscular injection of Sustanon is a usual method of initiating testosterone treatment in hypogonadal males (Malkin et al., 2004). As expected sham orchidectomy had no effect on the serum level of testosterone as there was no significant difference in the mean serum testosterone concentration between the sham-orchidectomised groups and the intact groups.

Results from this present study shows that high salt diet had no effect of its own on the serum concentration of testosterone in male rats as there were no significant differences in the mean serum testosterone in all the groups placed on high salt diet when compared with groups of rats
placed on a normal diet. This finding is similar to that of Khalid et al., (2002), that reported that a high salt diet had no effect on the plasma testosterone concentration in salt-sensitive Sabra rats.

5.1.5.0 In-vitro vascular studies

Because the kidney is the primary site for the regulation of salt and water homeostasis, most of the search for mechanisms leading to salt-sensitivity has focused on functional and genetic differences in renal sodium handling. This is quite understandable, because many genes that are involved in salt-sensitivity both in humans and mouse have been linked to renal salt-handling (Meneton et al., 2005), and the usual saying is that “hypertension follows the kidney” suggesting that the kidney is often a source of the hypertensive phenotype. For instance, after kidney transplantation in humans, recipients show significantly higher blood pressure if the donor was hypertensive or had hypertensive relations. Conversely blood pressure is usually normalized in hypertensive recipients when the donor are normotensive (Gerhold et al., 2007). Likewise cross-transplantation experiments emphasized the role of kidney in hypertension. Insertion of a kidney from young hypertensive rats into nephrectomised normotensive rats is associated with a rise in blood pressure, and when a kidney from normotensive rat is inserted into young nephrectomised hypertensive rat, blood pressure of the hypertensive rat does not rise (Meneton, 2005). But interestingly and surprisingly, sexually dimorphic risk factors do not follow this rule. In SHR which are genetically hypertensive, male rats maintain higher blood pressure than females. In this strain, female rats transplanted with male kidneys are normotensive whereas male rats with female kidneys show elevated blood pressures, which are characteristics of the normal male rats (Reckelhoff and Granger, 1999). This suggests that sex differences in blood
pressure arise from the male / female hormonal regimens, which may hypothetically, masked the genetic influence that resides in the kidney or better still may manifest its effect more in the vascular functions, another platform on which factors that regulate blood pressure in hypertension can act. Gender differences have been reported of vascular tone in several arterial beds (Orshal and Khalil, 2004), and the effect of sex steroids on vascular reactivity has been suggested as one of the underlying factors that contribute to gender differences in cardiovascular functions and disease (Sader and Celermajer, 2002).

5.1.5.1 Vascular relaxation response to acetylcholine

Endothelium is known to play a significant role in the regulation of vascular tone in physiological and pathological conditions through the release of different vasoactive agents (Moncada et al., 1991; Moncada, 1999). Principal among these endothelial factors is nitric oxide (NO). In the vasculature NO is an important mediator of vasodilation, it is released by various vasoactive agents and provides protection for the endothelium (Moncada et al., 1991). NO is synthesized by endothelial cells from L-arginine by a Calcium dependent enzyme NO synthase (eNOS) (Moncada et al., 1991; Moncada, 1999). Acetylcholine (Ach) is an endothelium dependent vasodilator. Ach elicits vascular relaxation through a mechanism involving endothelial vasoactive substances such as NO, prostacyclin and endothelial dependent hyperpolarizing factor (EDHF).

Results from this study suggest that high salt diet impaired vascular relaxation response to ACh and orchidectomy attenuated the impairing effect of a high salt diet on vascular relaxation response to ACh, while testosterone supplementation re-established it. The increase in the EC$_{50}$
value of the cumulative relaxation response curve (CRRC) to acetylcholine of the intact and high salt diet group shows that higher concentration of ACh was required to elicit half maximal relaxation response of aortic rings from this group. This is further corroborated by the simultaneous decrease in the maximum percentage relaxation response to Ach in the intact high salt group. This indicates that high salt diet impairs endothelial capacity to respond to acetylcholine in male Sprague Dawley rats. The decrease in the EC$_{50}$ value, and increase in the maximum percentage relaxation response in the orchidectomy and high salt group suggests that orchidectomy attenuates the endothelial impairing effect of a high salt diet. The significant increase in the EC$_{50}$ and decrease in the maximum percentage relaxation response to Ach in the testosterone supplemented and high salt diet group when compared with the orchidectomy and high salt diet group indicates that testosterone supplementation restored impairment of endothelial function in high salt diet group. Panza et al. (1993), reported that hypertensive patients have less vasodilatation response and an increase in vascular resistance in comparison with normotensive patients. As endothelium maintains a constant vasodilatation level produced by NO, it was considered that in hypertension there is an endothelial dysfunction that reduced NO release, and with that a reduced basal vasodilatation. This led them to postulate that endothelial dysfunction could be one of the causes of hypertension. One of the suggested mechanisms by which a high salt diet elevates high blood pressure is inhibition of vascular relaxation response to agonist (Sofola et al., 2002). The result of the present study supports this suggestion, and it could be inferred that orchidectomy attenuated blood pressure elevating effect of a high salt diet by ameliorating its endothelial function impairing effect. Reestablishment of vascular impairment in form of reduced vascular relaxation response to Ach in high salt diet group by testosterone supplementation implicates testosterone in the impairment of endothelial...
function by a high salt diet. Absence of any significant differences between the sham-orchiectomy groups and the intact groups shows that observed differences in the orchidectomy and testosterone supplemented groups when compared with the intact groups is definitely not due to the surgical trauma the rats underwent during the surgical procedure.

5.1.5.2 Vascular relaxation response to acetylcholine in the presence of L – NAME

The role of endogenous NO production in the relaxation response to acetylcholine in the groups was studied by determining the concentration that produces 15% (EC_{15}), and 50% (EC_{50}) relaxation response from the cumulative relaxation response curves to ACh, as well as the maximum percentage relaxation response. The decrease in the maximum percentage relaxation response to ACh after endogenous NO production blockade with L – NAME in the high salt diet groups suggests high salt diet reduces bioavailability of NO in the blood vessel. This could either be due to reduction in the endogenous production of NO or an increase in inactivation of NO by high salt diet induced increase in generation of ROS in the vasculature. It could be that high salt diet had earlier reduced the available NO, either by down-regulating the endothelial nitric oxide synthase (eNOS) or by increasing the generation of O_2^- in the vasculature, which inadvertently use up the available NO, so that the remaining enzyme would be more inhibited in the presence of L-NAME, hence reducing relaxation response to Ach greatly. Although orchidectomy moderately improves relaxation response to ACh even in the presence of eNOS inhibition in the orchidectomy high salt group, yet there was a significant increase in the relaxation response in the orchidectomy normal salt group in comparison to the orchidectomy high salt group. Though the reason for this is currently not clear, but it is not impossible that androgens have other mechanisms of increasing vascular tone aside NO pathway for inhibiting vascular relaxation. If
such other mechanisms exist, thus removal of such effect of androgens on the vasculature via orchidectomy, would then account for the increase in relaxation response to ACh observed in orchidectomy normal salt group when compared with other groups, even in the presence of L – NAME an eNOS inhibitor.

5.1.5.3 Vascular relaxation response to sodium nitroprusside (SNP) in the presence of L-NAME

Sodium nitroprusside is an exogenous NO donor. A normal relaxation response of aorta from the high salt groups to SNP in the presence of L - NAME suggests that the vascular smooth muscle cells (VSMCs) internal mechanism to respond to NO is not impaired. Rather it could be the endothelial production of NO that is altered by high salt diet and androgens as observed earlier from the results of relaxation response to acetylcholine. Neither orchidectomy nor testosterone supplementation had any significant effect on the aortic rings relaxation response to exogenous NO donor.

5.1.5.4 Vascular relaxation response to forskolin

Activation of adenylyl cyclase and consequently cyclic Adenosine Mono Phosphate (cAMP) production causes vascular smooth muscle relaxation via several downstream mechanisms involving among others, PKA, intracellular calcium ion concentration modulation as well as opening of potassium channel. In this present study, high salt diet impairs relaxation response to forskolin, an adenylyl cyclase activator. This finding is consistent with that of Sofola et al., (2003) that reported attenuation of relaxation response of aorta from rats fed a high salt diet to adenylyl cyclase activator and a cAMP analog. Present experiment shows that orchidectomy
prevented the attenuating effect of a high salt diet on aortic rings relaxation response to forskolin while this effect of orchidectomy was reversed by testosterone supplementation. An alternative second-messenger pathway that plays a key role in eliciting relaxation of vascular muscle involves activation of adenylate cyclase, formation of cAMP, and activation of protein kinase A and myosin light-chain kinase within smooth muscle cells (Lamping, 2001). In general, cAMP-mediated relaxation of vascular smooth muscle does not involve the endothelium (Lamping et al., 2000). Likewise forskolin is an endothelium-independent vasodilator. Several studies have suggested that some “typical endothelium-independent” vasodilators may also release NO and activate guanylate cyclase (Cardillo et al., 1997; Ito et al., 1997), because relaxation to some classic endothelium-independent agents, including adenosine, prostacyclin, forskolin, and β-receptor agonists, is reduced by inhibitors of NO synthase (NOS) (Zanninger et al., 1993; Cardillo et al., 1997). This suggests that relaxation of vascular smooth muscle to these agents is mediated by an interaction between cGMP and cAMP pathways. Some studies suggest that cGMP can exert important functional effects by inhibiting cAMP phosphodiesterase and increasing levels of cAMP (Taylor et al., 1999). Cyclic adenosine monophosphate phosphodiesterase is present in a variety of cell types, including vascular tissue (Lamping, 2001). Alternatively, agents that increase both cAMP and cGMP attenuate phospholipase C activation and mobilization of Ca^{2+} from intracellular stores (Taylor et al., 1999), possible interactions may also occur at myosin light-chain kinase or Ca^{2+} adenosine triphosphatases (Walter, 1989). The mechanism(s) of NO/cGMP-mediated alterations in cAMP-dependent vascular responses is unclear.
Studies demonstrating that vascular responses to forskolin and isoproterenol are attenuated with inhibitors of NOS suggest that these endothelium-independent agents may also release NO (Taylor et al., 1999). The study by Zhang and coworkers suggests that the cAMP/NO interaction do not involve an increase in levels of mRNA or protein for NOS but rather an increase in NOS activity (Zhang andHintze, 2001). They suggest that cAMP increases NO through activation of protein kinase A and subsequent phosphorylation of endothelial NOS by protein kinase B through a phosphatidylinositol 3-kinase–mediated effect.

Attenuation of vascular relaxation response to forskolin by a high salt diet suggests that vascular function impairing effect of a high salt diet is both endothelial-dependent and independent. NO production seems to be a common pathway for both forskolin and acetylcholine in eliciting their vasorelaxation, which is attenuated by a high salt diet. This finding further reinforces the earlier suggestion that it is the production or bioavailability of NO that is affected by a high salt diet rather than the vascular internal mechanism to respond to NO. This is further corroborated by normal relaxation response to SNP, an exogenous NO donor, by aorta from rats fed a high salt diet. Counteraction of the effect of a high salt diet on relaxation response to forskolin and Ach by orchidectomy, and reestablishment of such effects by testosterone supplementation suggests testosterone modulates the cGMP/cAMP and NO pathways. The various signal-transduction pathways in vascular tissue that modulate vascular reactivity involve complex interactions, and these require further consideration.
5.1.5.5 Vascular relaxation response to diazoxide

Activation of potassium channel causes vascular smooth muscle relaxation via hyperpolarization (Ledoux et al., 2006). Diazoxide is a selective arteriolar vasodilator (Newgreen et al., 1990; Pasdois et al., 2008), it causes vasodilation by hyperpolarizing vascular smooth muscle by opening ATP-dependent K\(^+\) channel (Nilius and Droogmans, 2001). In the present study high salt diet attenuates the relaxation response of aorta to diazoxide an ATP – dependent potassium channel activator. Vascular relaxation response to diazoxide is restored back in orchidectomy and high salt diet group while testosterone supplementation reestablishes the impairment of vascular relaxation response to diazoxide by a high salt diet. Opening of K\(^+\) dependent channel by diazoxide is not solely dependent on ATP. Harding et al., (1993), reported the involvement of other cytosolic nucleotides including guanosine triphosphate (GTP), cAMP dependent protein kinase was reported to stimulate K\(_{ATP}\) in vascular smooth muscle (Nelson and Quayle, 1995; Zhang and Hintze, 2001). High salt diet has been reported to attenuate cAMP (Sofola et al., 2003), as well as NO / soluble guanylyl cyclase (sGC) / cGMP pathways-mediated vasorelaxation (Kagota et al., 2001).

On one hand, attenuation of aorta relaxation response to diazoxide by a high salt diet in this present study could be due to the effect of such diet on the vascular smooth muscle nucleotides and their respective pathways of eliciting vasorelaxation. This finding is further corroborated by the earlier finding that high salt diet impairs vascular relaxation response to forskolin, an adenylyl cyclase activator, which elicits its vasorelaxing effect via the cAMP pathway. On the other hand, attenuation of relaxation response to diazoxide in rat aorta by high salt diet could possibly be due to effect of the high salt diet on K\(_{ATP}\) sensitive channels. Potassium channels are
found on the endothelial and vascular smooth muscle cells (Nelson and Quayle, 1995), both of which, findings from this study have shown to be affected by a high salt diet. Data from this study implicate testosterone in the increase in vascular tone observed in male Sprague Dawley rats and suggest that part of such effect is mediated through cAMP pathway and K⁺ channel activation.

5.1.6.0 Oxidative stress study

5.1.6.1 Level of lipid peroxidation in the heart and kidney

Oxidative stress, a state of excessive ROS activity is associated with cardiovascular diseases such as hypertension, arteriosclerosis and diabetes (Berry et al., 2001). Lipid peroxidation is a major indicator of oxidative stress (Armstrong and Brown, 1994; Lefer et al., 1998) and corresponds with the concentration of superoxide radicals in a tissue. The level of lipid peroxidation in the heart and kidney, two of the most important target organs of hypertension was measured as an indication of the level of oxidative stress in the rats. Significant increase in lipid peroxidation in the heart and kidney of the high salt diet groups suggests an increase in oxidative stress in rats in these groups. This finding is consistent with other studies that report increasing oxidative stress effect of a high salt diet (Zhuo et al., 2003; Tian et al., 2007). ROS activities in other organ systems, such as the heart, nervous system, and kidneys, have also been implicated in the pathophysiology of hypertension (Wilcox, 2002; Zimmerman and Davisson, 2002). In particular, increased renal O₂⁻ production is associated with NO bio-inactivation, which influences afferent arteriolar tone, tubuloglomerular feedback responses, and sodium reabsorption, which are important in long-term BP regulation (Wilcox, 2002). High salt diet has been reported to affect both cardiac and renal functions negatively (Ahn et al., 2004; Matavelli et
The negative impact of high salt diet on the heart and kidney could be mediated through its ROS generating effect. Orchidectomy attenuated the increase in lipid peroxidation (LP), as observed in the orchidectomised and high salt diet group, while testosterone supplementation following orchidectomy increased the lipid peroxidation level almost back to what is obtained in the intact group. This result implicates testosterone in the oxidative stress promoting effect of a high salt diet. This current result is consistent with that obtained from the cardiac and renal indices experiment. The significant increase in the cardiac and renal indices of the high salt fed rats could be due to the increased oxidative stress observed in the heart and kidney of rat from this group because increased ROS generation has been implicated in cardiac and renal hyperthrophy (Ahn et al., 2004; Calstrom et al., 2007). Likewise the finding that orchidectomy reduced the cardiac and renal hyperthrophic effect of a high salt diet is also consistent with the finding that orchidectomy attenuated the ROS generating effect of a high salt diet in this study.

5.1.6.2 Serum activity of Super Oxide Dismutase (SOD)

Superoxide dismutase (SOD) is one of the most important antioxidant enzymes in the body (Berry et al., 2001). Maintaining a balance between ROS generation and antioxidant system in the body is necessary so as to prevent oxidative stress and its consequential negative effect. The serum level of SOD was measured as an indicator of the antioxidant system in the body. A decrease in SOD level signifies an increase in its activity, as it is usually used up when it mops up excess superoxide ions (O$_2^-$). The significant decrease in the SOD level in the high salt diet groups indicates that there is an increase in its activity in these groups. This is consistent with the data on lipid peroxidation discussed above. An increase in the level of lipid peroxidation in the
body indicates an increase in ROS generation e.g. O$_2^-$ which is a substrate for SOD. SOD reacts with O$_2^-$ converting it to hydrogen peroxide (H$_2$O$_2$) and H$_2$O$_2$ in another step reaction is converted to water and molecular oxygen. The significant increase in SOD level of the orchidectomy and HSD group compared to intact and HSD group suggests orchidectomy counteracted ROS generating effect of HSD therefore reducing the usage of antioxidant system (SOD) or it upregulates the production of SOD in the body as a way of preventing oxidative stress. Interestingly testosterone supplementation following orchidectomy has no significant effect on the SOD level when compared with both the intact and the orchidectomy groups.

It may be that the effect of testosterone on oxidative stress is on ROS generation rather than direct antioxidant system inactivation. ROS generated within the blood vessel wall may contribute to endothelial dysfunction and vascular hypertrophy in hypertension (Berry et al., 2001). ROS is implicated in endothelial–dependent vascular dysfunction (Conyers et al., 2000), and the impaired sensitivity of VSMCs to cGMP (Leite et al., 2000) in DOCA salt–induced hypertension. Likewise in Dahl salt sensitive (DSS) rats, xanthine oxidase mediated ROS production has been implicated in the pathogenesis of vascular damage. Vascular hyperplasia and hypertrophy may be stimulated by increased ROS activity (Griendling et al., 2000), likewise ROS are involved in modulating a variety of intracellular signaling pathways involved in VSMC growth regulation (Irani et al., 2000). Increased vascular ROS activity can lead to reduced bioavailable NO and impaired endothelium – dependent relaxation. In fact, alterations in both the rates of formation and extent of scavenging of O$_2^-$ have been implicated in vascular dysfunction seen in atherosclerosis, hypertension, diabetes and chronic nitrate tolerance as well as in postischemic myocardium (Berry et al., 2001; Iliescu et al., 2007).
The finding that orchidectomy improves oxidative balance by reducing ROS generating effect of a high salt diet and improving antioxidant enzyme activity is consistent with the earlier finding from this same study that orchidectomy improves impaired vascular function in the high salt fed group consequently the improved vascular relaxation response observed in the orchidectomised group is consistent with the low blood pressure observed in this group when compared with the intact group of rats fed a high salt diet.

5.1.6.3 Total and direct bilirubin

The result of the present experiment indicates that high salt diet decreased both the total and direct serum bilirubin levels. Bilirubin is not merely an end product of heme degradation but a potent antioxidant (Stocker et al., 1987) which usually acts by inhibiting NADPH oxidase (Lanone et al., 2005) and of protein kinase C activity (Sano et al., 1985). The reduction in the serum bilirubin level in the high salt diet group could be a result of an increase in the ROS level in these groups of rats. An increase in the ROS level will consequently lead to a decrease in the level of antioxidant system such as bilirubin, as the latter is used to mop up the excess ROS. Some studies have reported a relationship between serum bilirubin and oxidative stress-mediated diseases, including coronary artery disease (Endler et al., 2003; Novotny and Vitek, 2003), angiotensin II-mediated hypertension (Pflueger et al., 2005), and renal ischemia-reperfusion injury (Adin et al., 2005; Kirkby et al., 2007). Likewise laboratory investigations have provided a biological background to explain the anti-oxidant and anti-inflammatory effects of bilirubin.
Bilirubin scavenged peroxyl radicals in an in vitro study (Stocker et al., 1987), and its antioxidant effect was suggested to be mediated by inhibition of NADPH oxidase (Lanone et al., 2005) and protein kinase C activity (Sano et al., 1985). Increased endothelial NADPH oxidase activity is a key mediator of atherosclerosis and hypertension (Berry et al., 2001; Kitada et al., 2003), suggesting that increased NADPH oxidase is involved in mechanism responsible for endothelial dysfunction in these pathologies. Bilirubin is an endogenous antioxidant and is destroyed by ROS (Stocker et al., 1987). High salt diet generates ROS which might consume bilirubin, which possibly might be the reason for the reduced serum level of bilirubin observed in rats fed a high salt diet in this study. The result of this present study suggests that reduction of bioavailable bilirubin and hence its consequential effect on vascular functions is part of the mechanisms by which high salt diet induces hypertension.

Less serum bilirubin level observed in rats fed a high salt diet was attenuated by orchidectomy, while testosterone supplementation re-established it. This finding implicates testosterone in the antioxidant activities of bilirubin. Although is not imminently clear how testosterone reduces concentration of serum bilirubin, the gender effect on the haem oxygenase inducibility to stress, which is the rate-limiting enzyme to produce bilirubin, would be considerable. For instance Toth et al., (2003) reported that trauma and haemorrhage doubled the hepatic HO-1 expression, in female rats compared with male rats. Likewise Chin et al., (2009) reported that subjects with higher bilirubin level showed a lower incidence of hypertension than did the subjects with lower bilirubin level, especially in females.
Novotny and Vitek, (2003) reported that in humans, mildly increased serum bilirubin levels is a decreased risk for the development of coronary artery disease and atherosclerosis. Likewise in hyperbiliruminaemic Gunn rats infused with angiotensin II when compared with control, the rise in systolic blood pressure was markedly blunted, and oxidative stress was attenuated (Pflueger et al., 2005). The finding of this present study agrees with the above reports. In this study, an observation worthy of note is the lower blood pressure parameters in groups with higher serum level of bilirubin. Blood pressure reducing effect of orchidectomy is consistent with its serum bilirubin elevating effect in rats fed a high salt diet. Therefore increasing serum bilirubin level, either by promoting its production or preventing excess ROS generation which could have reduce the bilirubin bioavailability could be one of the mechanisms by which orchidectomy prevents or attenuates endothelial dysfunction and consequently blood pressure elevation in rats fed a high salt diet. On the other hand, blood pressure elevating effect of testosterone could be partly mediated by decreased serum bilirubin, which increases oxidative stress and consequently promotes endothelial dysfunction.

5.1.7.0 Histomorphometric study

Haemodynamic factors are very important in the regulation of the structure of the artery. Intraluminal pressure regulates the thickness of the artery wall through its effects on wall tension, and blood flow regulates arterial lumen diameter through changes in wall shear stress (Sho et al., 2005).
5.1.7.1 Tunica Medial Thickness

Vascular smooth muscle plays an important role in the development and maintenance of vascular tone. Therefore its structure and functions will be affected in cardiovascular diseases. For instance tunica media thickening is a fundamental morphologic feature of arteriosclerosis and restenosis (Nanjo et al., 2006). Smooth muscle cell proliferation and migration are thought to play an important role in the process of tunica media thickening (Nanjo et al., 2006). The result of this present experiment shows that high salt diet increased the thickness of the tunica media, while orchidectomy reduced but testosterone supplementation following orchidectomy promoted such effect of a high salt diet on the tunica media in the thoracic aorta and mesenteric artery, a conduit and resistant artery respectively. The increase in the medial thickness indicates hypertrophy of the vascular smooth muscle. Reduction of the thoracic aorta and mesenteric artery thickness by orchidectomy and the increase in the thickness of the media in the group of rats given testosterone supplementation following orchidectomy suggest testosterone induces vascular smooth muscle proliferation and consequently its hypertrophy.

5.1.7.2 Medial Cross Sectional Area (MCSA)

The increase in the MCSA indicates hypertrophy of the blood vessel and it is consistent with the increase in the medial thickness which is the major determinant of the MCSA. The finding that lowering blood pressure reverses arterial hypertrophy in some arterial beds (Owens et al., 1988) suggests that an increase in pressure plays an important role in the development of vascular hypertrophy during chronic hypertension. Therefore the increase in the intima media thickness and the MCSA of the high salt fed groups is consistent with blood pressure elevating effect of a high salt diet observed in this present study. Reduction of intima media thickness and MCSA by
orchidectomy and the subsequent increment in these parameters after testosterone supplementation following orchidectomy, implicates testosterone in the vascular hypertrophic effect of high salt diet. More so, when testosterone have been implicated in increased DNA synthesis in vascular smooth muscle cells (Somjen et al., 1998) as well as increasing proteoglycan synthesis via increasing the glycosaminoglycans (GAG) chain length (Hashimura et al., 2005).

5.1.7.3 Luminal Cross sectional Area (LCSA)

Sodium overload, which is present in high salt diet, has been reported to change the shear stress and geometric modifications of the blood vessels (Bevan, 1993; Davies 1995). The decrease in the LCSA in the high salt diet groups could be as a result of the increased media thickness which tends to narrow the vascular lumen. This finding opposes that of Partovian et al., (1998) that reported an increase in the luminal cross sectional area regardless of an increase in the intima media of spontaneously hypertensive rats fed a high salt diet for 16 weeks. They anchored this on a supposed compensatory mechanism in the blood vessel and proposed that it could be responsible for non elevation of blood pressure in the SHR after 16 weeks of salt loading. The reason for the difference in our findings and theirs could either be due to genetical differences in the experimental animal and hypertension model or due to the differences in the experimental period used. In this present study male Sprague-Dawley rats, a non genetic model of hypertensive rats were salt-loaded for six weeks, while they salt-loaded SHR a genetic model of hypertension for 16 weeks.
5.1.7.4 Elastin and Collagen Content

Extracellular matrix (ECM) of a blood vessel contributes substantially to the diverse functions of the blood vessel. First, the ECM constitutes the scaffold which keeps the histological structure of the vessel wall in shape but also bears the enormous and permanent mechanical forces levied on the vessel by the pulsatile blood flow in the arteries and by vasoconstriction, which regulates blood flow and pressure. The complex network of elastic fibers and tensile forces-bearing networks are well adapted to accomplish these mechanical tasks. Second, the ECM provides informational cues to the vascular cells, thus regulating their proliferation and differentiation. Third, ECM molecules can store, mask, present or sequester growth factors, thereby modulating their effects remarkably (Eble and Niland, 2009).

Elastin and collagen are two major extracellular matrix proteins found in the blood vessel. The increase in the elastin and collagen content is consistent with the findings from this study that a high salt diet increases the media thickness and the MCSA in the thoracic aorta. Hypertension is associated with hypertrophy of the heart and large conduit arteries and accumulation of extracellular matrix. Mechanisms that may contribute to arterial hypertrophy include elevated blood pressure, genetic factors, neural influences, and humoral factors (Bevan, 1993; Owens et al., 1988). In the literature, findings indicate strong interactions between a high salt diet and extracellular matrix accumulation in conduit arteries of rats with genetic hypertension, such as Dahl salt-sensitive rats (Benetos et al., 1995), SHR (Tobian, 1991) and stroke prone SHR (Contard et al., 1993). This present study shows that in a non-genetic hypertension model, such as in Sprague Dawley rat, both increased thickening of the tunica media and MCSA in the high salt fed groups is associated with a highly significant accumulation of elastin and collagen during
the study period. The reduction and increment in the elastin and collagen content observed in the orchidectomised and the testosterone-supplementation following orchidectomy groups respectively, implicates testosterone in the vascular hypertrophy recorded in the study. This further suggests that testosterone may induce and / or promotes vascular hypertrophic effect of a high salt diet by accentuating extracellular matrix protein accumulation or deposition in the aorta.

5.1.7.5 Nucleus Count

The increase in the nucleus count in the thoracic aorta of the rats fed a high salt diet is consistent with the earlier result on the tunica medial thickness and the media cross sectional area (MCSA). This finding suggests that high salt diet induces hypertrophy in the blood vessel by inducing and/or increasing vascular smooth muscle proliferation. The increase in the number of vascular smooth muscle nucleus connotes hyperplasia of the vascular smooth muscle, which is indicative of vascular smooth muscle cell proliferation. The increase in the nucleus count of the testosterone supplemented as well as the intact groups when compared with the groups that were orchidectomised without testosterone supplementation suggests testosterone enhances vascular smooth muscles proliferation. Somjen et al., (1998) reported that dihydrotestosterone regulates DNA synthesis in human vascular cells. It could be that hypertrophic effect of testosterone in the blood vessel is mediated by the peripheral conversion of testosterone to DHT in these cells. Vascular smooth muscle has been reported to express 5α-reductase (Liu et al., 2003; Orshal and Khalil, 2004), an enzyme required to convert testosterone to DHT in peripheral tissues.
Observation of the histological photomicrograph shows that the internal elastic laminae of the high salt diet groups appear to be straightened out and are thicker in comparison with their corresponding normal salt diet groups. These differences may either account for the increased blood pressure observed in the groups fed a high salt diet or the observed increase in the thickness of the inner elastic laminae of the high salt diet groups with higher MABP could be a compensatory or adaptive mechanism by the blood vessel to prevent the damage to the delicate inner elastic lamina by the elevated blood pressure. Although what these morphological differences portend is not clear, the preponderancy of supporting proofs from other set of experiments in this present study tempts one to suggest that these changes were caused by several factors affected by high salt diet. For instance, in one of the experiments, high salt diet increased ROS generation and also depotes the antioxidant level. Increased ROS activity has been reported to stimulate vascular hyperplasia and hypertrophy (Griendling et al., 2000), likewise ROS was reported to be involved in modulating a variety of intracellular signaling pathways involved in VSMCs growth regulation (Irani et al., 2000). Antioxidant effect of bilirubin was reported to be mediated by inhibition of NADPH oxidase (Lanone et al., 2005) and increased endothelial NADPH oxidase activity has been implicated in arteriosclerosis and hypertension (Kitada et al., 2003). Activation of NADPH oxidase plays a role in beta-tissue growth factor (TGF-β) signaling (Sanders, 2009), and in the responsiveness of collagen synthesis; a common pathway to vascular smooth muscle proliferation and hypertrophy (Arribas et al., 2010). Therefore the reduction in serum level of bilirubin in rats placed on high salt diet and the consequential elevation of serum bilirubin level in orchidectomised rats observed in this study is consistent with the increase and decrease vascular hypertrophy in the high salt and orchidectomised groups respectively.
One thing that is not imminently clear is that, are these various findings (endothelial dysfunction, ROS generation, endogenous antioxidant enzyme depletion and vascular hypertrophy) the cause of the elevated blood pressure and hence consequently hypertension, or are they as a result of the established hypertension by the high salt diet. A very important perspective would be to look at changes in these parameters continuously and consistently over a period of time. This will probably reveal the consequential factor.

5.1.8.0 Non-genomic pathway for vascular effect of androgens

The classical pathway of androgen action involves steroid binding to the androgen receptor (AR), a ligand activated transcription factor and single copy member of the nuclear receptor super family, acting on the genome (Quigley et al., 1995). There is now considerable evidence for a rapid, non-genomic effect of steroids, including androgens (Losel and Wehling, 2003). Non-genomic steroid action is distinguished from genomic effect by the rapid onset of its effect (seconds to minutes) that is faster than genomic mechanisms. Also such effects are insensitive to inhibition of RNA and protein synthesis and can be produced by steroids that have been rendered impermeable to the plasma membrane, thus unable to access the nucleus. This is usually achieved by either covalently-linking the androgens to membrane impermeable macromolecules or observing the free androgens effect in cells that are devoid of nucleus. Non transcriptional effects of androgens are also not usually blocked by classical antagonists due to their different steroid specificity from classical cognate nuclear receptor (Heinlein and Chang, 2002; Losel and Wehling, 2003; Foradori et al., 2008).
5.1.8.1 Vascular relaxation response to exogenous testosterone

Although it has been reported that testosterone injection has a relieving effect on patient with angina pectoris (Liu et al., 2003) and it is beneficial to men with coronary artery disease (English et al., 2000) or congestive heart failure (Malkin et al., 2009), suggesting that testosterone may have an effect on vascular function. Most of the non genomic effects of androgen on the vascular tone were described in normal experimental animals with no underlying or induced pathologies. For instance, testosterone relaxes rabbit coronary arteries and aorta, (Jiang et al., 1991; Yue et al., 1995), as well as thoracic aorta (Ding and Stallone, 2001). Recently Koloumenta et al., (2006) reported an in vitro relaxing effect of testosterone on rabbit airway smooth muscle while the vasorelaxing effect of testosterone on human radial artery (Seyrek et al., 2007) and pulmonary artery (Smith et al., 2008) have also been reported. All these animal data were in response of different arterial beds from normal animals, in humans to exogenous androgen. In spite of these emerging data supporting the role of testosterone and other androgens in different normal arterial beds, less is known of the role that exogenous androgens may play in vascular reactivity of these arterial beds in pathologic states.

The results of this present study indicate that high salt diet reduced relaxation response to testosterone propionate in the rat aorta at every concentration studied, suggesting that a high salt diet impairs vascular relaxation response to exogenous testosterone. Exogenous testosterone causes vascular relaxation by both endothelium-dependent and endothelium-independent mechanisms (Costarella et al., 1996; Deenadeyalu et al., 2001; Ding and Stallone, 2001). Likewise high salt diet has been reported to affect vascular tone by impairing endothelial and vascular smooth muscle functions (Adegunloye and Sofola, 1997; Kagota et al., 2001; Lombard
et al., 2003; Zhu et al., 2007). The finding from this study suggests that a high salt diet may have affected the pathways involved in both the endothelial and the vascular smooth muscle response to exogenous androgens, hence reducing relaxation response to testosterone propionate in the rat aorta. Orchidectomy almost abolished relaxation response to testosterone propionate in the aorta. Testosterone propionate at the lower concentrations even elicits contraction in the aortic rings of the orchidectomy groups studied. On the other hand testosterone supplementation restores relaxation response to testosterone propionate in the aorta to a higher degree compared to that observed even in the intact groups. This finding is interesting, considering the fact that, the direct acute vasorelaxing effect of testosterone is considered to be a non-genomic response, hence it should be independent of the classical nuclear androgen receptors which could have been affected by orchidectomy or testosterone supplementation. There is no literature on the direct effect of androgens on blood vessels from male animals fed a high salt diet and/or orchidectomised. Nonetheless, it could be that a minimal or optimum concentration of testosterone in the body is required for exogenous source to exert its vasorelaxing effect. It could be possible that there are other intermediate substances or chemicals required for eliciting the direct effect of androgens, as non-transcriptional actions of androgens have been reported to be exhibited through activation of different second messenger systems. It is therefore possible that such systems are affected by orchidectomy and / or testosterone supplementation.

The above finding is very important, it brings to the front the question of when does non-genomic effect of androgens turn to genomic effect. Much more important is the observation that, non-genomic effects of androgens on the vasculature are beneficial, while on the other hand, the genomic effects are considered to be deleterious. For instance, orchidectomy reduces
blood pressure in experimental animals to a level that is comparable to that of age-matched intact females (Reckelhoff et al., 2000; Reckelhoff, 2001). This implicates testosterone in the consistent higher blood pressure observed in males when compared with their age-matched females. Ironically exogenous testosterone and oestrogen acutely elicit relaxation in several arterial beds in both human and experimental animals. For example, testosterone relaxes rabbit coronary arteries and aorta, (Yue et al., 1995), as well as rat thoracic aorta (Ding and Stallone, 2001) and mesenteric artery (Tep-areenan et al., 2003), and oestrogen elicits relaxation in different arterial beds such as cerebral (Salam et al., 2001), mesenteric (Naderali et al., 2001) and saphenous (Kackus et al., 2001). These findings respectively, contrast the observed higher susceptibility of males to hypertension but, it is consistent with lower incidence and prevalence of hypertension in premenopausal women.

Be that as it may, the biological evidence that both testosterone and oestrogen are beneficial to vascular functions (from their acute vasorelaxing effects) prompted the idea of hormone replacement therapy (HRT) in hypogonadal males and postmenopausal women respectively. But a lot of confusion surrounds the benefits and risks of HRT, and this confusion continues unabated especially in the cardiovascular field. Several clinical trials have been carried out on the effect of HRT on cardiovascular health in both males and females. The outcomes of most of these trials are very controversial. For instance, in 2002, the oestrogen-progesterone wing of the Women Health Initiative (WHI), reported that HRT caused an increase in coronary heart events, stroke and venous embolism, as well as breast cancer. This view is supported by the Million Women Study (MWS), (2003), Women’s health initiative (2004), while some studies reported that HRT has no effect on coronary heart diseases and cardiovascular events (Manson et al.,
On the other hand, other studies reported a beneficial effect of HRT on cardiovascular functions (Gebara et al., 2009; Stevenson, 2009). The important issue of timing could be the basis for the controversial outcomes of the prospective clinical trials on hormone replacement therapy as a means of preventing or reducing cardiovascular diseases in post-menopausal women and hypogonadal men.

The results of this present experiment implicate timing as a probable cause of varying results of the different clinical trials on HRT. This result also provides biological evidence for the epidemiological findings that early commencement of HRT at about perimenopausal period could remove its negative effect and even be beneficial in preventing coronary heart diseases and cardiovascular events (Hsia et al., 2006; Roussow et al., 2007). This present study acute relaxing effect of testosterone on the aorta was almost abolished in orchidectomised rats after six weeks, while this effect is retained in rats given testosterone supplementation following orchidectomy, suggesting that the inability of exogenous testosterone to elicit adequate relaxation in the aorta of the orchidectomised animal may be due to the effect of the withdrawn androgen on the vasculature. The presence of exaggerated relaxation in the aorta of rats given testosterone supplementation following orchidectomy confirms this finding. Probably if in those clinical trials, hormone replacement therapy commences at perimenopause or immediately after menopause when some level of the circulating sex steroids functions are still retained, the outcome could be less controversial. A very important perspective would be to inculcate a well defined timing into the study of direct effect of androgens on the vasculature in both intact, androgen withdrawn and androgen supplemented animals.
5.1.8.2 Vascular relaxation response to dehydroepiandrosterone (DHEA)

Dehydroepiandrosterone (DHEA) is both a precursor and metabolite of testosterone. The results of this present study suggest that high salt diet have no effect on aorta relaxation response to DHEA. On the other hand orchidectomy almost abolished aorta relaxation response to exogenous DHEA, while testosterone supplementation re-established vascular relaxation response to DHEA even to a larger extent than observed in the intact groups. Numerous studies in recent years have provided evidence that exogenous androgens elicit relaxation in several arterial beds (Costarella et al., 1996; Deenadeyalu et al., 2001; Ding and Stallone, 2001). In spite of emerging data supporting this role of testosterone and others in different normal arterial beds, less is known of the potential role that exogenous androgens play in vascular reactivity of these arterial beds in pathologic states. The results of the present experiment on the effect of testosterone in the normal relaxation response of aorta to exogenous DHEA. Although the best characterized steroids receptors are nuclear transcriptor factors, it is now recognized that steroids, can in some cases also activate plasma membrane receptors and thereby initiate cytosolic kinase cascades (Simoncinni et al., 2003; Norman et al., 2004). DHEA is not known to act on the classical androgen receptor (AR), but recently Liu et al., (2008) reported that DHEA activates a plasma membrane receptor on vascular endothelial cells and phosphorylates ERK ½. It is possible that the expression of this plasma membrane receptors are modulated by circulating testosterone level, hence the attenuation of DHEA relaxation effect on aorta in orchidectomised rats and reestablishment of relaxation response to exogenous DHEA in rats supplemented with testosterone following orchidectomy.
5.2.8.3.0 Probable mechanism of relaxing effect of exogenous testosterone on the aorta

5.2.8.3.1 Role of peripheral conversion of testosterone in acute vasorelaxing effect of exogenous testosterone

A key issue in the biological effects of testosterone is its conversion to bioactive metabolites. Although only a small fraction (< 5%) of testosterone output undergoes such transformation usually in local tissues, conversion both amplifies and diversifies testosterone action. Conversion to its 5α-reduced metabolite, Dihydrotestosterone (DHT) by type 1 or type 2, 5α-reductase amplifies testosterone action because DHT has higher molar potency due to its more avid binding affinity and slower dissociation rate from the androgen receptor (AR) (Grino et al., 1990). Conversion of testosterone to estradiol by the enzyme aromatase (CYPI9) diversifies androgen action by activating estrogens receptors (ERs) (Coleman et al., 2004; Nathan et al., 2001).

The result from this study shows that neither flutamide, a classical androgen receptor blocker nor aminogluthetemide, an aromatase inhibitor blocked the relaxation response of the aortic rings to testosterone. This suggests that testosterone propionate causes relaxation in the aorta via a pathway that is independent of the nuclear receptors activation, nor through peripheral conversion to estradiol.

5.1.8.3.2 Role of endothelial vasoactive substances and potassium channel in acute vasorelaxing effect of exogenous testosterone

Non-genomic androgen effects characteristically involve the rapid induction of conventional second-messenger signal transduction cascades, such as increases in cytosolic calcium and
activation of protein kinase A, protein kinase C, and MAPK, leading to diverse cellular effects including smooth muscle relaxation, neuromuscular and junctional signal transmission and neuronal plasticity (Heilen and Chang, 2002). Modulations of ion channels activities are part of the mechanisms through which the effects of second messenger activation are observed. Activation of potassium channels both in the endothelium and vascular smooth muscle causes hyperpolarization which leads to vascular smooth muscle relaxation (Adams et al., 1989; Ledoux et al., 2006). Result from this study indicates that barium chloride, a non specific K⁺ channel blocker almost abolished aorta relaxation response to testosterone. Attenuation of vascular relaxation response to testosterone propionate in the presence of barium chloride, a non-specific potassium channel blocker, implicates potassium channel activation in the vasorelaxing effect of testosterone propionate as obtained in these present experiments.

In this present experiment, it was observed that the endothelium plays little or no role in the relaxing effect of testosterone propionate in the vascular smooth muscle. This is so because inhibition of endothelial nitric oxide synthase (eNOS) and prostacyclin by L-NAME and indomethacin respectively had no effect on the relaxation response of the aorta to testosterone. Endothelial K⁺ channels have been widely implicated in endothelium-dependent vasodilatation. It was thought that endothelial cell hyperpolarization, via the opening of K⁺ channels, would facilitate Ca²⁺ influx in these cells by increasing the driving force for Ca²⁺ (Adam et al., 1989; Hardy et al., 2006), and in this way enhance production of the “classical” endothelium-dependent vasorelaxants NO and PGI₂, which rely on increase in cytoplasmic free Ca²⁺.
However, the discovery of additional vasodilator phenomenon of endothelium-derived hyperpolarizing factor (EDHF) showed that in addition to causing increased cytoplasmic Ca\(^{2+}\), endothelial K\(^+\) channels inadvertently cause adjacent smooth muscle hyperpolarization. This arises from activation of small and intermediate calcium activated K\(^+\) channels in endothelial cells, resulting in endothelial hyperpolarization which spreads through myoendothelial junctions to result in the EDHF-attributed hyperpolarization and relaxation of the smooth muscle (Coleman, 2004). Small, intermediate and large conductance Ca\(^{2+}\)-activated K\(^+\) channels have been implicated in EDHF activity, while Inward rectifier K\(^+\) channels (K\(_{IR}\)) and Na\(^+\)/K\(^+\) ATPase have been suggested by some studies (Coleman et al., 2004). IK\(_{Ca}\) and SK\(_{Ca}\) channels occur in abundance in endothelial cells and their activation results in EDHF-like hyperpolarization of these cells. K\(^+\) efflux from endothelial cells via small and intermediate calcium activated K\(^+\) channels (SK\(_{Ca}\) and IK\(_{Ca}\) respectively), activates inward rectifier K\(^+\) channels (K\(_{IR}\)) and Na\(^+\)/K\(^+\) ATPase on the smooth muscle cells (Coleman et al., 2004). In this present study blockade by apamin, charybdotoxin and iberiotoxin of small, intermediate and large conductance calcium activated potassium channel respectively, which are found mainly on the endothelium had no effect on the vasorelaxing effect of testosterone. This is consistent with the earlier findings that reported that endothelium may not be involved in the relaxation effect of testosterone (Deendayalu et al., 2001; Ding and Stallone, 2001). In the vascular wall, inward rectifier potassium channels (K\(_{IR}\)) channels are expressed in both the endothelial and smooth muscle cells (Droogmans and Nilius, 2001). K\(_{IR}\) is expressed more in the smooth muscle of autoregulatory vascular bed such as cerebral and coronary artery (Robertson et al., 1996), while its expression in the general circulation increases with decreasing arterial diameter (Robertson et al., 1996). K\(_{IR}\) channel conducts potassium current more readily in the cells than out of the cells over a wide
range of potentials. Activation of $K_{IR}$ results in hyperpolarization and thus relaxation of smooth muscle (Haddy et al., 2006). In this present experiment, tertiapin – Q a specific inward rectifier of potassium channel failed to prevent the relaxing effect of testosterone on the aorta. As explained above, it could be that there is no significant expression of $K_{IR}$ in the aorta cells, or $K_{IR}$ activation is not involved in the pathway through which testosterone induces relaxation in the male Sprague Dawley rats aorta.

5.1.8.3.3  Role of L–type calcium channel in acute vasorelaxing effect of testosterone

Unlike potassium channels, calcium channel activation has opposing effect in the endothelium and the vascular smooth muscle cell (VSMc). In the endothelial cells calcium channel activation eventually leads to vascular smooth muscle relaxation while in the VSMc, activation of calcium channel causes contraction. In this present study, nifedipine, a conventional L-type calcium channel blocker, augments the relaxation response of testosterone on the aorta suggesting that calcium channel blockade could be another mechanism by which testosterone relaxes vascular smooth muscle in the aorta. This finding is consistent with that of (Scragg et al., 2007; Montano et al., 2008) that testosterone is a selective and potent inhibitor of L-type Ca$^{2+}$ channels. Also the molecular requirement for testosterone to act as a potent L-type Ca$^{2+}$ channels inhibitor was demonstrated by the same group of researchers (Scragg et al., 2007).

Calcium channel blocking effect of testosterone could be another reason why blockade of the three types of calcium activated potassium channel failed to have any effect on the vasorelaxing activities of testosterone. It could be that calcium channel blocking effect of testosterone prevented the intracellular / cytosolic concentration of calcium ions from reaching the required
level to activate these calcium ion dependent potassium channels; an important step in their activation (Coleman et al., 2004). Results from this present study suggest that testosterone is a more potent L-Ca\(^{2+}\) channel blocker compared to nifedipine a conventional L-Type calcium channel blocker. The fact that testosterone causes more relaxation even after incubation with nifedipine while nifedipine cannot elicit further relaxation after incubation in testosterone suggests that at the same concentration, testosterone blocks more L-Ca\(^{2+}\) channels than nifedipine.

The results of this present experiment therefore suggest that testosterone propionate relaxes aorta from male Sprague Dawley rats directly via a non-genomic pathway which is not dependent on the endothelial vasoactive substances. Potassium channel activation and L-type calcium channel blockade appear to be involved in the pathway by which testosterone induces its vasorelaxing activity in the aorta of male Sprague Dawley rats.
5.2.0 SUMMARY OF FINDINGS

1. Orchidectomy delayed the onset and magnitude of blood pressure elevation, while testosterone accentuated the development and severity of high blood pressure in a non-genetically modified Sprague-Dawley rat fed a high salt diet.

2. Bilateral orchidectomy drastically reduced the serum concentration of testosterone but parenteral supplementation restored the serum level back to those observed in the intact rats. High salt diet has no effects on the serum level of testosterone.

3. Orchidectomy improved while testosterone exacerbated both endothelial-dependent and endothelial-independent vascular relaxation response impaired by a high salt diet in a non-genetically modified Sprague Dawley rat.

4. Studies on mechanisms by which androgens affect salt-dependent hypertension show that
   i. Orchidectomy counteracted ROS level elevating and antioxidant level depleting-effect of a high salt diet in the rats. But testosterone supplementation following orchidectomy restored ROS generating effect of a high salt diet, but had no marked effect on production of antioxidant enzymes.
   ii. Orchidectomy ameliorated vascular hypertrophic effect of a high salt diet by reducing vascular smooth muscle proliferation and decreasing extracellular matrix protein deposition induced by high salt diet. Testosterone supplementation following orchidectomy accentuated vascular hypertrophic effect of a high salt diet.

5. High salt diet reduced vascular relaxation response to exogenous testosterone and orchidectomy almost abolished acute vasorelaxing effect of the exogenous testosterone.
High salt diet had no effect on vascular relaxation response to DHEA, but orchidectomy drastically reduced acute relaxing effect of exogenous DHEA on the aorta.

i. Exogenous testosterone relaxed aorta via a non genomic pathway that is mediated by potassium channel opening and L – type voltage gated calcium channel blockade.

5.3.0 CONCLUSION

In this study, testosterone promoted the development of salt-induced hypertension in non-genetically modified Sprague-Dawley rats. Testosterone did this by accentuating vascular function impairment effect of a high salt diet through exacerbation of oxidative stress as well as increasing the proliferation of vascular smooth muscle cell and deposition of matrix proteins leading to vascular hyperthrophy.

The implication of the overall findings from this present study is that in non-genetically predisposed salt-sensitivity, vascular dysfunction could be the underlying cause of blood pressure elevation. Accentuation the vascular dysfunction by testosterone could be one of the bases for sexual dimorphism in salt-induced hypertension.

Finally, risk factors for cardiovascular disease interact negatively in a synergistic manner; thus, blood pressure elevation, endothelial dysfunction, oxidative stress accentuation, cardiovascular and renal hypertrophy induced by the genomic effect of endogenous androgens in salt-induced hypertension may render males more susceptible to cardiovascular diseases, particularly hypertension than females.
5.4.0 CONTRIBUTIONS TO KNOWLEDGE

- Testosterone promotes early onset and increases the magnitude of blood pressure elevation in a non-genetically modified animal model of salt-induced hypertension.
- Testosterone promotes salt-induced hypertension in non-genetically modified Sprague-Dawley rats by accentuating impaired endothelial and vascular smooth muscle function effect of a high salt diet.
- Testosterone accentuates vascular function impairment effect of a high salt diet by promoting oxidative stress (which impairs endothelial functions) and vascular hypertrophy (which increases the vascular tone) through increasing ROS generation and deposition of matrix protein as well as increasing smooth muscle cell proliferation.
- Acute vasorelaxant effect of androgens on arteries involve potassium channel activation and calcium channel blockade, and this effect is impaired in salt-induced hypertension and surgically-induced hypogonadism.


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Appendix 1a: Non–invasive blood monitoring using the tail cuff recorder
Appendix 1b: A typical blood pressure tracing for the non invasive blood pressure measurement
Appendix 2: The calibrator curve for the testosterone enzyme immunoassay
Appendix 3: Schematic Figure of the double-jacketed organ bath used in the in-vitro vascular study
Appendix 4: Set up of the ADInstrument top loader transducer for the isometric tension study
Appendix 5a: Image of nucleus count
Appendix 5b: Image of elastin content area estimation
Appendix 5c: Image of medial cross sectional area measurement
Appendix 5d: Image of tunica media thickness measurement
Appendix 5e: Image of collagen content estimate in Picro-sirius red stain.