

Research Article

## Orchidectomy Ameliorates the Vascular Hypertrophic Effect of A High Salt Diet in Sprague-Dawley Rats

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**Keywords:**

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Testosterone,  
Vascular hypertrophy,  
Extracellular matrix protein,  
media thickness,  
Histomorphometry

**ABSTRACT**

Sodium overload, which is present in a high salt diet (HSD), induces hypertension by mechanisms that include change in the shear stress and geometric modifications of the blood vessels. Salt sensitivity exhibits sex difference, which is higher in males when compared with females. This has been attributed to the effect of androgens on blood vessels in males. Therefore, experiments were designed to study the vascular smooth muscle histomorphometry in weanling male rats that were either sham-operated or orchidectomised under (90mg/kg bodyweight ketamine and 10mg/kg bodyweight xylazine.i.p) anesthesia, with or without testosterone replacement (TR) (10mg/kg sustanon 250® i.m) once in 3 weeks. They were either placed on a diet with normal 0.3% or high 8% NaCl content for 6 weeks. Histological examination of thoracic aorta and mesenteric artery were carried out with specific dyes; hematoxylin 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 carried out using a programmed software IMAGE-PRO 3DS 6.1. Tunica media thickness, cross sectional area, elastin and collagen contents of the blood vessels were all significantly elevated ( $p < 0.05$ ) in the rats placed on HSD, while orchidectomy prevented the increase in these parameters but concomitant administration of testosterone restored them to the levels observed in intact rats. Orchidectomy ameliorated vascular hypertrophic effect of a HSD by reducing vascular smooth muscle proliferation and extracellular matrix protein deposition in the blood vessels.

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### INTRODUCTION

High salt diet has been shown to cause hypertension both in humans (Corruziet *et al.*, 2005; Weinberger *et al.*, 2001) and several species of laboratory animals (Matavelli *et al.*, 2007; Ahnet *et al.*, 2004; Khalid *et al.*, 2002; Sofola *et al.*, 2002). Part of the mechanisms by which a high salt diet induces hypertension includes

impairment of vascular function (Zhu *et al.*, 2007; Oloyo *et al.*, 2011). Haemodynamic factors are very important in the regulation of the structure of the artery (Shoet *et al.*, 2004) and sodium overload which is present in a high salt diet has been reported to change the shear stress and geometric modifications of the blood vessels (Bevan 1993). The basic haemodynamic abnormality in salt-dependent hypertension is an elevated peripheral resistance consequent of vascular dysfunction (Schmidlinet *et al.*, 2007). The increase in peripheral resistance in hypertension can be due to both functional and structural factors (Groset *et al.*, 2000). For example, impaired endothelial and vascular smooth muscle responses to agonists have been reported in salt-induced hypertension in rats (Sofola *et al.*, 2002; Oloyo *et al.*, 2011; Nurkiewicz and Boegehold, 2007)

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Likewise, in spontaneous hypertensive rats, a high salt diet induced structural changes such as increased arterial area and tunica media thickness (Partovian *et al.*, 1998).

However, the effect of a high salt diet exhibits sexual differences, with its adverse effect being usually higher in males when compared with females. For instance, when fed a high salt diet, males develop a higher BP compared with female rats (Hinojosa-Laborde *et al.*, 2004). Likewise, when adolescent humans were salt-loaded, boys responded with a greater pressure change compared with girls (Wilson *et al.*, 1996).

The sexual difference in cardiovascular diseases has been linked to the effect of sex steroids on the blood vessels (Sader and Celermajer, 2002). Estrogen is believed to possess vascular protective effect, while the vascular effect of testosterone especially in induced pathologies is not well studied. Although recently we reported the testosterone dependence of endothelial function impairing effect of a high salt diet (Oloyo *et al.*, 2011), but the role of androgens on vascular hypertrophic effects of a high salt diet is not clear. Therefore, considering the sexual differences in response to a high salt diet, and the implication of sex steroids in this effect, we chose to investigate the effect of testosterone on the blood vessel histomorphometry in male Sprague-Dawley rats fed a high salt diet.

## MATERIALS AND METHODS

Forty eight weanling male Sprague Dawley rats aged 8 weeks and weighing between 90 – 110g were obtained from the Department of Laboratory Animal Sciences, (DLAS), BioMedical and Technology (BMT) wing SreeChitra Tirunal Institute for Medical Sciences and Technology (SCTIMST) Kerala, India. They were housed in steel cages and maintained under standard lighting conditions 12 hours light, 12hours dark period. Food and water were provided ad libitum. The rats were divided into eight groups of six rats each. Groups 1 and II were intact rats, groups III and IV were orchidectomised rats, groups V and VI were rats that were given Sustanon<sup>®</sup> injection as testosterone replacement following orchidectomy and groups VII and VIII were sham orchidectomised rats. For orchidectomy, rats were anesthetized with ketamine and xylazine (90mg and 10mg/Kg/body weight i.m) (Oloyo *et al.*, 2011), respectively, for bilateral removal of the testes under aseptic surgical conditions while in groups VII and VIII rats, the scrotal sacs were opened and sutured back as a model of sham-orchidectomy. All operated rats received an injection of penicillin 300,000 i.u/Kg body weight at the time of surgery to prevent infections and were allowed a 3-day recovery period before the beginning of the experiments (Oloyo *et al.*,

2011). After recovery from anesthesia, all animals were returned to their cages. Rats in groups I, III, V, and VII were fed with rat diet containing normal salt concentration (0.3% NaCl) and tap water for 6 weeks. Rats in groups II, IV, VI and VIII were fed with a high salt diet (8% NaCl), and tap water ad libitum, for 6 weeks. Group V and VI rats received 10mg/Kg body weight of testosterone suspension (Sustanon 250<sup>®</sup>i.m) once in 3 weeks during the study, for testosterone supplementation. Sustanon 250<sup>®</sup> is a trade name for an oil-based injectable blend of four esterized testosterone compounds viz: 30mg Testosterone Propionate, 60mg Testosterone Phenylpropionate, 60mg Testosterone Isocaproate, and 100mg Testosterone Decanoate. The composition is a blend of fast, short acting and slow but long acting testosterone esters.

**Blood pressure monitoring:** Every week, conscious rats were placed in a restrainer on a heated pad and allowed to adapt / rest inside for 15 minutes before blood pressure was measured. The procedure was performed in a room with an ambient temperature of 25°C. 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 systolic blood pressure and heart rates 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.

**Histological Staining and histomorphometric analyses:** 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 17hours. 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 trimmed using trim program on the microtome (LEICA RM 2255). The tissue embedded paraffin blocks were placed in cassette holder of the microtome, and tissue sections were cut into size of 5µm. The cut ribbons were transferred into LEICA water bath set at 49°C. The tissue sections were held onto poly-l-lysine coated glass slides and labelled. The labeled slides were incubated at 25°C overnight and then specifically stained to obtain a monochromatic colour associated with the various structures of interest in the thoracic aorta and mesenteric artery. Haematoxylin and eosin stain was used for the cytoplasm and nucleus while Verhoeff – Van Geison and Picro-sirius red stains were used for elastin and collagen content estimation respectively. 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. Photomicrograph of the slide preparation was then taken using the pre-installed camera in the light microscope (Olympus BX 51, Japan). Histomorphometric analyses comprising nucleus count, elastin and collagen estimate as well as thickness and area measurement were performed using a programmed software IMAGE-PRO 3DS 6.1. 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 (Shoet *al.*, 2004).

### Statistical Analysis:

The collected data were expressed as mean  $\pm$  S.E.M, and 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.

## RESULTS

### Arterial blood pressure monitoring

After the six weeks experimental period, there was an increase in the systolic blood pressure (SBP) of rats in the high salt group when compared with that of the normal diet group (154 $\pm$ 4.0vs.130 $\pm$ 3.0). The SBP of the orchidectomy plus high salt group was significantly less ( $P < 0.05$ ) when compared with the intact plus high salt diet group (132 $\pm$ 4.0vs.154 $\pm$ 3.0). The SBP of rats in the testosterone replacement groups was significantly higher ( $P < 0.05$ ) when compared with their corresponding orchidectomised groups without testosterone replacement (132 $\pm$ 3.0vs.120 $\pm$ 3.0; 146 $\pm$ 3.0vs.132 $\pm$ 4.0), i.e. normal diet and high salt diet respectively. The SBP of the orchidectomy plus high salt diet group was similar to that of the intact plus normal diet group (control) (132 $\pm$ 4.0vs.130 $\pm$ 3.0), likewise the SBP of the testosterone replaced groups were also similar to that of the intact groups (132 $\pm$ 4.0vs.130 $\pm$ 3.0; 146 $\pm$ 3.0vs.154 $\pm$ 4.0), i.e. normal diet and high salt diet respectively.

### Histomorphometry of the thoracic and mesenteric artery

**Tunica Media thickness:** There was a significant increase ( $P < 0.01$ ) 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.

**Table. 1.**

Histomorphometric measurement of the thoracic aorta of the rats across the groups.

Groupings n = 10	Intima medial thickness	Media cross sectional area ( $\mu\text{m}^2$ )	Luminal cross sectional area ( $\mu\text{m}^2$ )
INT + NS	76.16 $\pm$ 2.36	315786 $\pm$ 432	928459 $\pm$ 653
INT + HS	99.94 $\pm$ 5.18 <sup>***</sup>	407803 $\pm$ 672 <sup>***</sup>	566767 $\pm$ 702 <sup>***</sup>
ORCH + NS	<sup>††</sup> 66.20 $\pm$ 3.62	<sup>††</sup> 276812 $\pm$ 312	<sup>††</sup> 993876 $\pm$ 815
ORCH + HS	<sup>##</sup> 88.18 $\pm$ 5.96 <sup>***</sup>	<sup>##</sup> 375358 $\pm$ 491 <sup>***</sup>	<sup>##</sup> 734964 $\pm$ 245 <sup>***</sup>
ORCH + TES + NS	<sup>^^</sup> 81.88 $\pm$ 4.39	<sup>^^</sup> 354892 $\pm$ 198	<sup>^^</sup> 865974 $\pm$ 372
ORCH + TES +HS	<sup>^^</sup> 94.75 $\pm$ 4.80 <sup>***</sup>	<sup>^^</sup> 495652 $\pm$ 613 <sup>***</sup>	<sup>^^</sup> 792700 $\pm$ 291 <sup>***</sup>
SHAM + NS	78.57 $\pm$ 3.28	309624 $\pm$ 301	934938 $\pm$ 549
SHAM + HS	98.22 $\pm$ 4.10 <sup>***</sup>	400345 $\pm$ 265 <sup>***</sup>	646521 $\pm$ 428 <sup>***</sup>

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

Data expressed as mean  $\pm$  S.E.M. (n = 10). <sup>\*\*\*</sup>Significant increase ( $P < 0.001$ ) when compared with the corresponding controls (normal diet groups). <sup>†††</sup>Significant decrease ( $P < 0.001$ ) when compared with corresponding controls. <sup>††</sup>Significant decrease ( $P < 0.01$ ) when compared with intact and normal diet (Control). <sup>##</sup>Significant decrease when compared with intact and high salt group. <sup>^^</sup>Significant increase when compared with corresponding orchidectomised groups. <sup>^^</sup>Significant increase ( $P < 0.01$ ) when compared with intact and high salt group. <sup>^^</sup>Significant decrease ( $P < 0.01$ ) when compared with corresponding orchidectomised groups. <sup>††</sup>Significant increase ( $P < 0.01$ ) when compared with intact and normal diet group.

**Table. 2.**

Extracellular matrix protein area measurement and nucleus count of the thoracic aorta of the rats across the groups.

Groupings n = 10	Elastin content area ( $\mu\text{m}^2/\text{mm}$ )	Collagen content area ( $\mu\text{m}^2/\text{mm}$ )	Nucleus count
INT + NS	6347 ± 43	1994±93	63 ± 2
INT + HS	11461 ± 97 <sup>***</sup>	3193±112 <sup>***</sup>	79 ± 5 <sup>**</sup>
ORCH + NS	<sup>††</sup> 4758 ± 67	<sup>††</sup> 1561±69	<sup>††</sup> 57 ± 3
ORCH + HS	<sup>###</sup> 8977 ± 71 <sup>***</sup>	<sup>###</sup> 2509±61 <sup>***</sup>	<sup>###</sup> 72 ± 4 <sup>**</sup>
ORCH + TES + NS	8406± 54	2076±56	<sup>~</sup> 73 ± 3
ORCH + TES +HS	10678 ± 83 <sup>***</sup>	2732±87 <sup>***</sup>	<sup>^</sup> 77 ± 2 <sup>*</sup>
SHAM + NS	6827 ± 67	1897±49	71 ± 4
SHAM + HS	12745 ± 88 <sup>***</sup>	2902±63 <sup>***</sup>	77 ± 3 <sup>**</sup>

Data expressed as mean ± S.E.M. (n = 10). <sup>\*\*\*</sup>Significant increase (P < 0.001) when compared with the corresponding controls (normal diet groups). <sup>††</sup>Significant decrease (P < 0.01) when compared with intact plus normal diet (Control). <sup>###</sup>Significant decrease when compared with intact plus high salt group.

**Table. 3:**

Histomorphometric measurement (in  $\mu\text{m}^2$ ) of the mesenteric artery of the rats across the groups.

Groupings n = 10	Tunica medial thickness	Media cross sectional area	Luminal cross sectional area
INT + NS	28.43 ± 3.78	17424 ± 432	72017 ± 797
INT + HS	39.40 ± 3.91 <sup>***</sup>	36651 ± 672 <sup>***</sup>	46676 ± 837 <sup>***</sup>
ORCH + NS	<sup>†</sup> 26.82 ± 3.15	<sup>††</sup> 15028 ± 312	<sup>00</sup> 81938 ± 536
ORCH + HS	<sup>###</sup> 32.34 ± 4.90 <sup>*</sup>	<sup>###</sup> 23667 ± 491 <sup>***</sup>	<sup>^^</sup> 58050 ± 689 <sup>***</sup>
ORCH + TES + NS	<sup>^^</sup> 29.84 ± 2.3	<sup>^^</sup> 20265 ± 198	<sup>††</sup> 69773 ± 971
ORCH + TES +HS	<sup>^^</sup> 37.90 ± 4.91 <sup>***</sup>	<sup>^^</sup> 26178 ± 613 <sup>***</sup>	<sup>††</sup> 47097 ± 4831 <sup>***</sup>
SHAM + NS	28.07 ± 2.78	18648 ± 301	70252 ± 602
SHAM + HS	38.01 ± 3.88 <sup>***</sup>	33883 ± 265 <sup>***</sup>	44335± 513 <sup>***</sup>

**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 (<sup>\*\*\*</sup>P < 0.001, <sup>\*</sup>P < 0.05) when compared with the corresponding controls (normal diet groups). <sup>\*\*\*</sup>Significant decrease (P < 0.001) when compared with corresponding controls. Significant decrease (<sup>††</sup>P < 0.01, <sup>†</sup>P < 0.05) when compared with intact and normal diet (Control). <sup>###</sup>Significant decrease when compared with intact and high salt group. <sup>~</sup>Significant increase (P < 0.01) when compared with corresponding orchidectomised groups. <sup>^^</sup>Significant increase (P < 0.01) when compared with intact and high salt group. <sup>††</sup>Significant decrease (P < 0.01) when compared with corresponding orchidectomised groups. <sup>00</sup>Significant increase (P < 0.01) when compared with intact and normal diet group.

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 replacement following orchidectomy when compared with the orchidectomy alone groups (Tables 1 and 3).

**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 testosterone replacement groups of rats when compared with the groups that were orchidectomised but not given testosterone supplementation (Tables 1 and 3).

**Luminal Cross Sectional Area (LCSA):** There was a significant decrease (P < 0.01) 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 (Tables 1 and 3).

**Elastin Content:** There was a significant increase (P < 0.01) 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 replacement significantly increase (P < 0.01) the elastin content in the thoracic aortas and the mesenteric arteries when compared with the orchidectomy alone groups (Tables 2 and 4).

**Table. 4:**

Extracellular matrix protein area measurement of the mesenteric artery of the rats across the groups.

Groupings n = 10	Elastin content ( $\mu\text{m}^2/\text{mm}$ )	Collagen content ( $\mu\text{m}^2/\text{mm}$ )
INT + NS	2771 $\pm$ 64	1790 $\pm$ 57
INT + HS	3604 $\pm$ 89 <sup>***</sup>	2413 $\pm$ 92 <sup>***</sup>
ORCH + NS	<sup>††</sup> 2329 $\pm$ 71	<sup>††</sup> 1536 $\pm$ 54
ORCH + HS	<sup>###</sup> 3086 $\pm$ 92 <sup>***</sup>	<sup>###</sup> 1829 $\pm$ 77 <sup>***</sup>
ORCH + TES + NS	2433 $\pm$ 54	1714 $\pm$ 68
ORCH + TES + HS	31027 $\pm$ 78 <sup>***</sup>	1947 $\pm$ 80 <sup>***</sup>
SHAM + NS	2607 $\pm$ 76	1731 $\pm$ 69
SHAM + HS	3294 $\pm$ 98 <sup>***</sup>	2333 $\pm$ 83 <sup>***</sup>

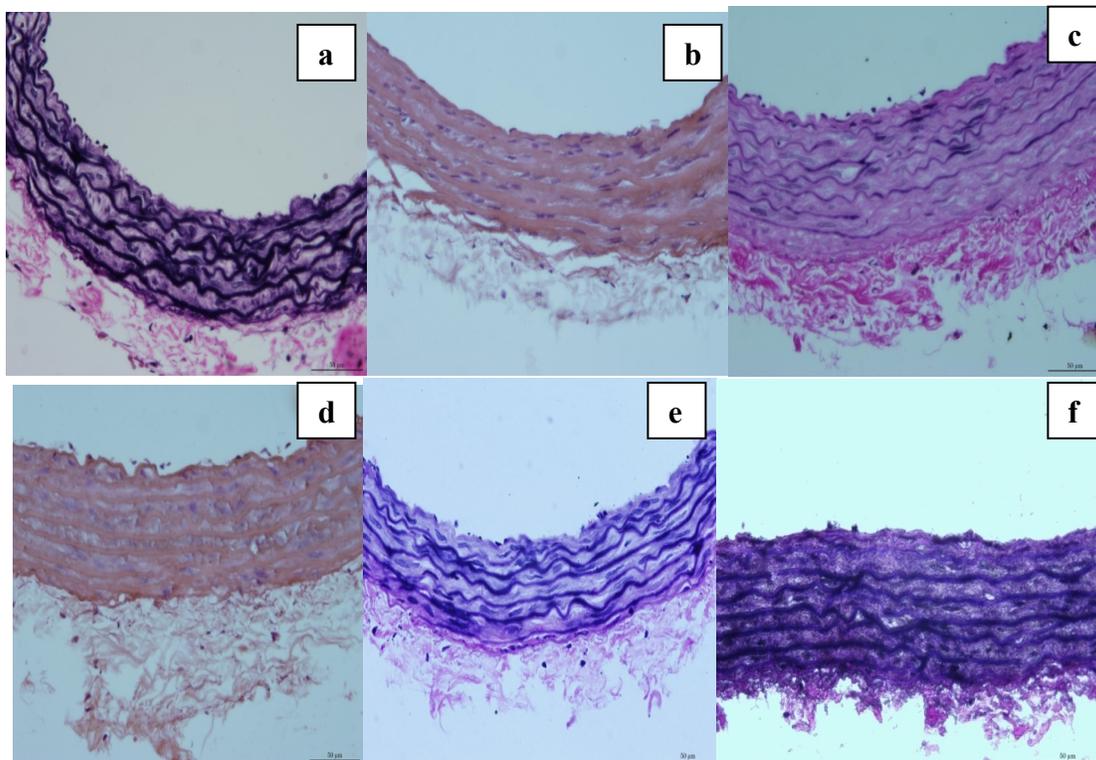
Data are expressed as mean  $\pm$  S.E.M. (n = 10). Significant increase (<sup>\*\*\*</sup>P < 0.001, \*P < 0.05) when compared with the corresponding controls (normal diet groups). Significant decrease (<sup>††</sup>P < 0.01, <sup>†</sup>P < 0.05) when compared with intact and normal diet (Control). <sup>###</sup>Significant decrease when compared with intact plus high salt group.

**Collagen Content:** There was a significant increase (P < 0.01) 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

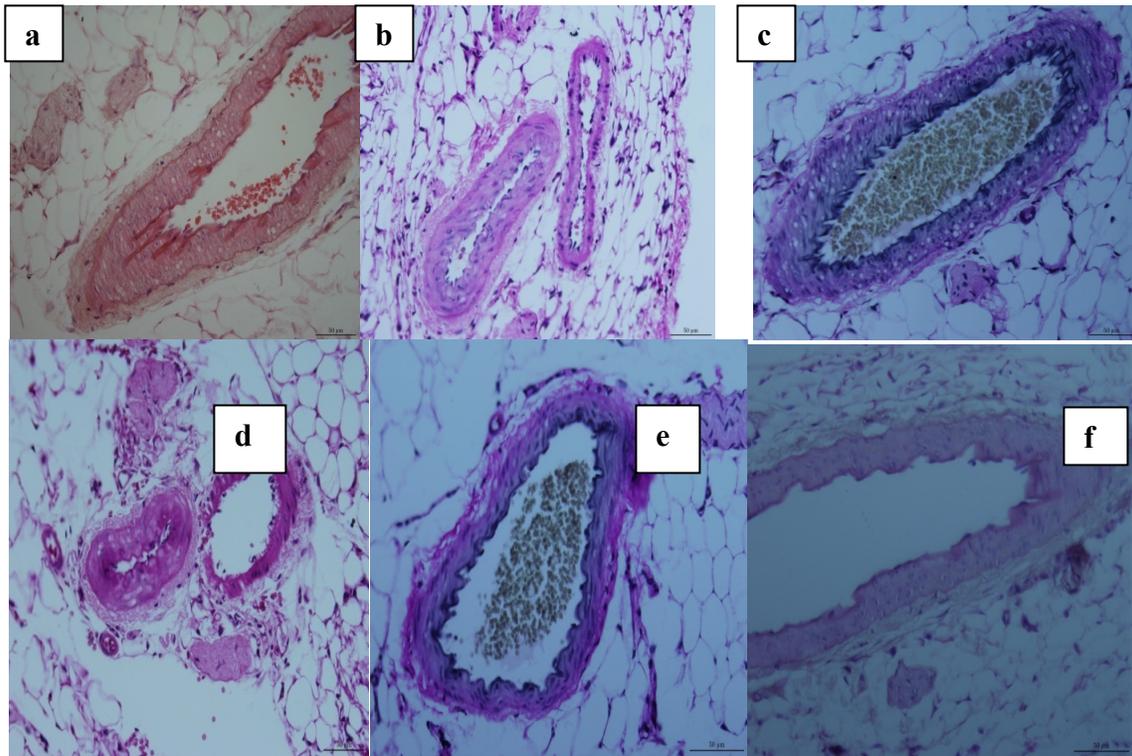
significantly increase (P < 0.01) the collagen content in the thoracic aortas and the mesenteric arteries when compared with the orchidectomy alone groups (Table 2 and 4).

**Nucleus Count:** Nucleus count was significantly higher (P < 0.05) 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 significantly higher (P < 0.01) in the thoracic aortas of the groups of rats given testosterone replacement following orchidectomy when compared with the orchidectomy alone groups (Table 2).

Figures 1a - f and 2a - f show the photomicrograph sections of the aortas and the mesenteric arteries of the rats across the groups. 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 artery 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.

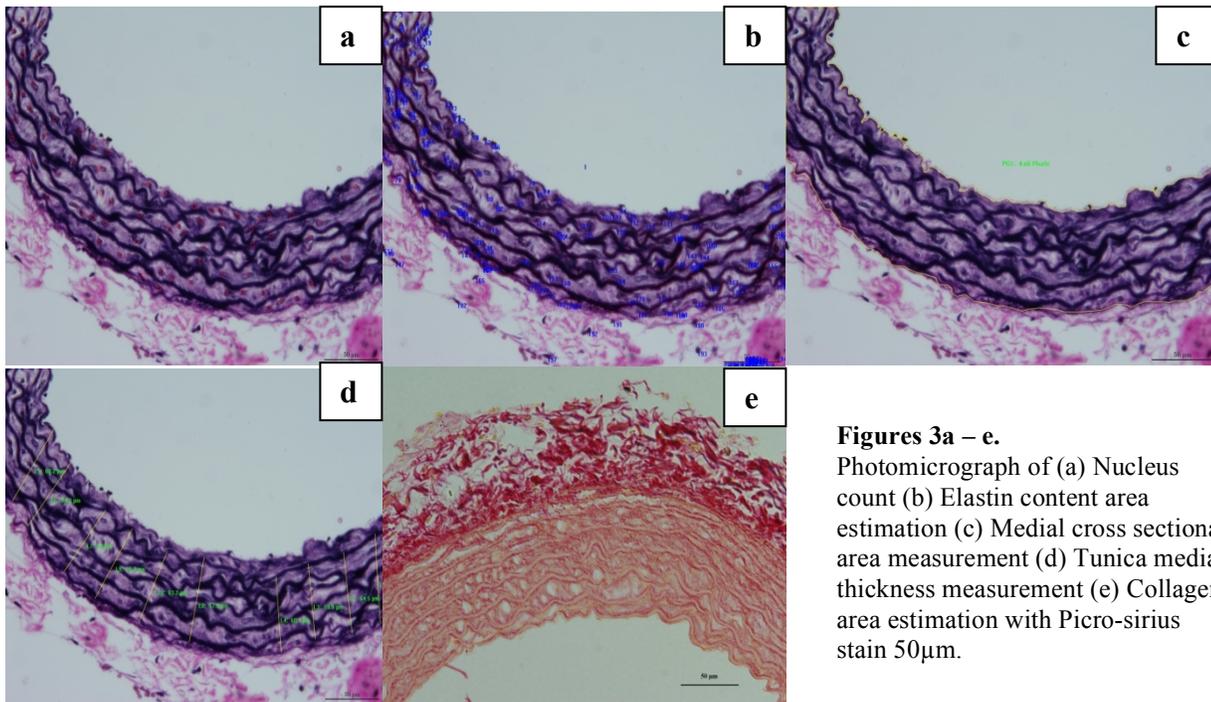


Figures. 1a – f: Photomicrograph of aorta from (a) Intact plus normal salt diet (Control). (b) Intact plus high salt diet (c) Orchidectomy plus normal salt diet (d) Orchidectomy plus high salt diet (e) orchidectomised plus testosterone plus normal diet (f) orchidectomised plus testosterone plus high salt diet groups. H&E and Verhoeff’s haematoxylin and Van Gieson’s stain. 50 $\mu\text{m}$ .



**Figures 2a – f:**

Photomicrograph of mesenteric artery from (a) Intact plus normal salt diet (Control). (b) Intact plus high salt diet (c) Orchidectomy plus normal salt diet (d) Orchidectomy plus high salt diet (e) orchidectomised plus testosterone plus normal diet (f) orchidectomised plus testosterone plus high salt diet groups. H&E, Verhoeff's haematoxylin and Van Gieson's stain. 50µm.



**Figures 3a – e.**

Photomicrograph of (a) Nucleus count (b) Elastin content area estimation (c) Medial cross sectional area measurement (d) Tunica media thickness measurement (e) Collagen area estimation with Picro-sirius stain 50µm.

Figures 3a – e show the illustration of the methods for nucleus count, elastin content area estimation, media cross sectional area measurement, media thickness measurement and collagen content area estimation respectively.

## DISCUSSION

Results from this study show that orchidectomy reduced but testosterone replacement reestablished the vascular hypertrophic effect of a high salt diet in male Sprague-Dawley rats. Studies have shown 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). Likewise, a temporal link between increased NaCl intake and aortic hypertrophy has also been noted (Limas *et al.*, 1980; Partovian *et al.*, 1998) in spontaneously hypertensive rats (SHR) in the absence of a significant change in blood pressure. However, these studies did not consider the effect of androgen on the hypertrophic effect of a high salt diet. Findings from the present study implicate a role for testosterone in the hypertrophic effect of a high salt diet in male rats.

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.*, 2004). 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 re-stenosis (Nanjo *et al.*, 2006) and smooth muscle cell proliferation and migration are suggested to play an important role in the process of tunica media thickening (Nanjo *et al.*, 2006). The result of the present experiment shows that a high salt diet increased the thickness of the tunica media, while orchidectomy reduced but testosterone replacement 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 groups of rats given testosterone replacement suggests testosterone enhanced vascular

smooth muscle proliferation and subsequently its hypertrophy.

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 tunica 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 tunica media thickness and MCSA by orchidectomy and the subsequent increment in these parameters after testosterone replacement 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).

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 is opposite to 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. Partovian and colleagues based their findings on a probable 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 genetic differences in the experimental animal and hypertension model or due to the differences in the experimental period used. In the present study male Sprague-Dawley rats, a non-genetic model of hypertensive rats were salt-loaded for six weeks, while Partovian *et al.*, salt-loaded SHR a genetic model of hypertension for 16 weeks.

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). Previous reports 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). The 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.

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 replacement as well as the intact groups when compared with the groups that were orchidectomised without testosterone replacement suggests testosterone enhances vascular smooth muscles proliferation. Somjenet *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 5 $\alpha$ - 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. On one hand, these differences may either account for the increased blood pressure observed in the groups fed a high salt diet, as the consistent elevated vascular resistance observed in hypertension has been linked to structural changes in the blood vessel (Gros *et al.*, 2000). On the other hand, the observed increase in the thickness of the inner

elastic laminae of the high salt diet groups with higher SBP 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 in this study, we did not determine the level of oxidative stress in the vasculature, however, findings in our laboratory have shown elevated ROS generation in the heart and kidney as well as reduced antioxidant enzymes (superoxide dismutase and bilirubin) activities in the serum of high salt fed male Sprague-Dawley rats, and these effects were attenuated by orchidectomy (unpublished data). 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- $\beta$ ) signaling (Sanders, 2009), and in the responsiveness of collagen synthesis; a common pathway to vascular smooth muscle proliferation and hypertrophy (Arribas *et al.*, 2010). In conclusion, testosterone potentiates the vascular hypertrophic effect of a high salt diet both in conduit and resistance vessels, and these mechanisms appear to be involved in part of the mechanisms by which testosterone enhances blood pressure elevating effect of a high salt diet.

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