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Castration induces progressive increase in the carotid intima-medial thickness of the male rat

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Abstract

Objectives: The role of androgens in the development of cardiovascular diseases remains controversial. The current study therefore aimed to determine the changes in the carotid intima-medial thickness of the male rat in surgically-induced hypogonadism.

Methods: Twenty-two *Rattus norvegicus* male rats aged two months were used. The rats were randomly assigned into baseline (4), experimental (9) and control (9) groups. Hypogonadism was surgically induced in the experimental group by bilateral orchiectomy under local anesthesia. At experiment weeks 3, 6 and 9, three rats from each group (experimental and control) were euthanized, their common carotid artery harvested, and routine processing was done for paraffin embedding, sectioning and staining. The photomicrographs were taken using a digital photomicroscope for morphometric analysis.

Results: The mean carotid intima-median thickness was 321.97 μm at baseline. There was a progressive increase in the carotid intima-median thickness by 17.6%, 37% and 67.1% of the baseline values in the castrated group at the end of the third, sixth and ninth week respectively ($p<0.001$). Although the carotid intima-median thickness increased in the non-castrated group with increasing duration of the study, these increases were not statistically significant ($p=0.110$). The increase in carotid intima-median thickness was associated with hyperplasia of the intimal layer as well as increased deposition of collagen fibers in the medial layer.

Conclusion: Androgen deprivation by surgical castration induces a progressive increase in the carotid intima-median thickness. This may constitute an anatomical basis for the higher predisposition of hypogonadal males to cardiovascular diseases.

Keywords: androgens; cardiovascular disease; carotid intima-medial thickness; castration; hypogonadism

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Introduction

Carotid intima-medial thickness (c-IMT), defined as the combined thickness of the intimal and medial layers of the common carotid artery (CCA),[1] is an important biomarker of subclinical atherosclerosis.[2,3] It is an established indicator of the presence, extent and severity of coronary artery disease (CAD).[4] It is also independent predictor of future cardiovascular events, with an absolute 0.1 mm increase in the c-IMT correlating to a 10–15% and 13–18% increase in the risk of future myocardial infarction (MI) and stroke respectively.[5] It is therefore utilized as a surrogate end point for cardiovascular events in interventional studies.[6–8]

The role of androgens in the development of cardiovascular diseases (CVDs) remains controversial.[9] Traditionally, androgenic hormones have been associated with an increased risk of CVDs. This has largely been based on the gender disparity in the incidences of CVDs, with a higher male preponderance[10] as well the abuse of anabolic steroids by athletes which has been linked to various adverse effects on the cardiovascular system including thrombosis, hypertension and heart failure.[11] Similarly, hyper-androgenic conditions such as congenital adrenal hyperplasia (CAH) and polycystic ovarian syndrome (PCOS) has been associated with an increase in C-IMT.[12–14] However, evidence from various population studies and animal studies demonstrate beneficial effects of physiological levels of androgens, reporting an inverse correlation between androgen levels and c-IMT & incidences of CVDs.[15–18] The relationship between androgens and CVDs/c-IMT therefore remains unclear.

Of note, previous studies mainly focused c-IMT measurements in the presence of physiological or supra-physi-

ological levels of androgens. Changes in the c-IMT in hypogonadal states remains relatively underexplored. The current study therefore sought to determine the changes in the c-IMT in orchiectomy-induced hypogonadism. This may be important in predicting the risk of cardiovascular events in patients with hypogonadism secondary to age-related decline in endogenous androgen levels,[19,20] androgen deprivation therapies[21] or disorders that either damage the testis or reduce gonadotropin stimulation.[22,23]

Materials and Methods

A total of 22 male rats aged 2 months were used in this study. Rats were used as the study model because of their ease of handling, low maintenance cost and close physiological resemblance to man. Further, the structure of the rat common carotid artery (CCA) is similar to that in man. Two-month old rats were used because this is the age at which they attain sexual maturity. Rats with visible neck or scrotal pathology were excluded. The animals were housed in cages floored with wood shavings that were changed regularly. They were kept in their cages for 2 weeks prior to commencement of the study for acclimatization. The animals were placed under a normal 12 hours' light/dark diurnal cycle and provided with standard rat pellets and water ad libitum. Ethical approval to conduct the study was granted by the Biosafety, Animal Care and Use committee of Nairobi University, Nairobi, Kenya (Ethical approval number: FVMBAUEC/2016/96).

Four rats were chosen using simple random sampling technique to demonstrate the baseline (day 0) histomorphology of the CCA. The remaining rats were divided randomly into 2 groups (11 experimental, 11 controls). Hypogonadism was induced in the experimental via bilateral orchiectomy under local anesthesia. The animals were placed in the dorsal recumbent position and under physical restrain. The scrotal skin disinfected using iodine solution. Two ml of 1% lignocaine was injected in the around the scrotal sac to provide local anesthesia. A 1.5 cm incision was made at the base of the hemi-scrotal sacs. Subcutaneous tissue was bluntly dissected to reveal the vaginal processes. This was then excised to access the testis and the spermatic cord which were then gently exteriorized. The spermatic cord was then clamped, ligated and removed together with the testis and epididymis. A 4-0 suture was then used to close the processus vaginalis. This procedure was repeated on the contralateral hemi-scrotal sac. The skin was thereafter closed with a 2-0 non-absorbable interrupted sutures. The wound was then covered with a cotton wool soaked in iodine and secured with a bandage. Post procedural pain was detected through observation based on the animals' behavior and attitude changes. These include reluctance to move, abnormal posturing, decreased appetite and vocalization. This post procedural pain was managed using ibuprofen 5 mg/kg mixed with water.

On experimental week 3, 6 and 9, three animals from both groups were picked randomly, euthanized and perfused with normal and formal saline solutions. Their CCA were harvested and processed for paraffin embedding and sectioning. The rats' CCA were fixed in 10% formalin for twelve hours. This was followed by dehydration in increasing grades of alcohol (70% up to absolute alcohol) at one hour intervals, and clearing in toluene. Thereafter, the vessels were placed in the memmert oven for wax infiltration. The CCA were embedded in paraffin wax and oriented for transverse sectioning. After cooling, the embedded tissues were blocked using wooden blocks and then serially cut into 7 µm sections using a microtome. Fifteen 7 µm sections were randomly obtained from the ten ribbons, floated on a 60 °C water bath and picked on a glass slide, then dried in an oven for 12 hours. Masson's Trichrome was used to display smooth muscle cells and collagen fibers while Wiegerts stain was used to display elastic fiber profile.

Photomicrographs of the sections were taken using a digital camera (Canon Powershot A640, 12 mp, Beijing, China) mounted on a photomicroscope (Carl Zeiss, Axiostar Plus Microimaging, Jena, Germany) for morphometric analysis using the Fiji-ImageJ. This is an open source software developed by the United States National Institute of Health for processing and analyzing images. The variable obtained was the carotid intima-medial thickness (c-IMT). The c-IMT measurements were obtained from four different parts of the vessel wall and an arithmetic mean of the four values used for analysis.

The collected were entered into the using the Statistical Package for Social Sciences (SPSS for Windows, version 21.0, Chicago, IL, USA) for coding, tabulation and statistical analysis. The c-IMT was expressed in micrometers. The data were grouped into two: Control group (non-castrated) and experimental (castrated) group. After confirming that the data was not normally distributed (using box plots and histograms), non-parametric tests were used for univariate analysis. Kruskal-Wallis H test was used to compare the medians of the c-IMTs along the various harvesting periods within each group. Mann-Whitney U test was used to compare the medians in c-IMTs between control and exper-

imental groups. A p-value <0.05 was considered significant at 95% confidence interval. Data is presented in tables and photomicrographs.

Results

The mean carotid intima-median thickness (c-IMT) was 321.97 μm at baseline. There was a progressive increase in the c-IMT by 17.6%, 37% and 67.1% of the baseline values in the castrated group at the end of the third, sixth and ninth week, respectively (p<0.001) (**Table 1**). These increases in c-IMT were more marked in rats exposed to hypogonadism over a longer period of time (**Figures 1a-f**). The increase in c-IMT was associated with hyperplasia of the intimal layer as well as deposition of collagen fibers in the medial layer (**Figures 1c** and **d**). Although the c-IMT increased in the non-castrated group with increasing duration of the study, these increases were not statistically significant (p=0.110) (**Table 1**).

Discussion

The present study demonstrates that endogenous androgen deprivation by bilateral orchiectomy results in a significant increase in the carotid intima-medial thickness (c-IMT) of the male rat. To the best of our knowledge, this is the first study to describe changes in the c-IMT in induced hypogonadism. Previous studies focused mainly on the correlation between the levels of endogenous androgens and c-IMT. Nonetheless, the findings of the current study are in concordance with studies by Muller et al.,[24] Farias et al.,[25] and Chan et al.,[26] who reported an inverse correlation between androgen levels and c-IMT. The findings of the current study are however at variance with those by Allameh et al.,[13] and Kim et al.,[14] who reported increased c-IMT measurements in hyperandrogenic conditions such as congenital adrenal hyperplasia (CAH) and polycystic ovarian disease (PCOS). Pooled together, the above findings suggest that physiological levels of androgens are beneficial, whereas extreme levels (hypogonadism and hypergonadism) are detrimental to the cardiovascular system.

The increase in c-IMT could be attributed to the hyperplasia of the tunica intima as well as increased deposition of collagen fibers in the tunica media that was observed in the current study. The development of intimal hyperplasia in induced hypogonadism, hitherto undescribed to the best of our knowledge, may be attributed to the increased production of pro-inflammatory cytokines[27] and reactive oxygen species (ROS)[28] that normally accompany androgen deprivation. These are known to cause endothelial injury, a crucial initial component in the pathogenesis of intimal hyperplasia.[29] Similarly, endogenous androgens are known to have inhibitory effects on vascular smooth muscle proliferation and migration. It is therefore plausible to postulate the loss of these inhibitory effects, hence proliferation of smooth muscle cells in the tunica intima, resulting in intimal-hyperplasia. Although the current study did not investigate the changes in lipid profiles of the study animals, previous studies have reported dysregulations in lipid metabolism in hypogonadal states, with decreased levels of high density lipoproteins (HDL) as well as increased levels of low density lipoproteins (LDL) and triglycerides (TGs).[9] This may plausibly constitute another basis for the development of intimal hyperplasia in the current study.

Increased carotid intima-medial thickness (c-IMT) is an established marker of atherosclerosis[8] and is an independent predictor of the presence and severity of coronary and peripheral artery disease.[4,30] Increased c-IMT seen in this study and those from previous studies therefore suggest that low androgen levels are associated with increased risk of atherosclerotic coronary and peripheral

Table 1
Changes in the carotid intima-medial thickness in control and experimental animals.

Group	Baseline	Week 3	Week 6	Week 9	p
Control (medians)	B1 - 322.64 B2 - 323.36 B3 - 320.39 B4 - 321.49	C1 - 327.31 C2 - 324.22 C3 - 326.44	C4 - 329.98 C5 - 331.28 C6 - 329.51	C7 - 344.43 C8 - 342.05 C9 - 345.36	0.11
Experimental groups (medians)	_	E1 - 379.31 E2 - 375.76 E3 - 378.01	E4 - 442.42 E5 - 438.04 E6 - 441.38	E7 - 538.89 E8 - 540.17 E9 - 537.36	<0.001*
p	_	0.002*	0.001*	<0.001*	

*p<0.05. **B:** baseline animals; **C:** control animals; **E:** experimental animals.

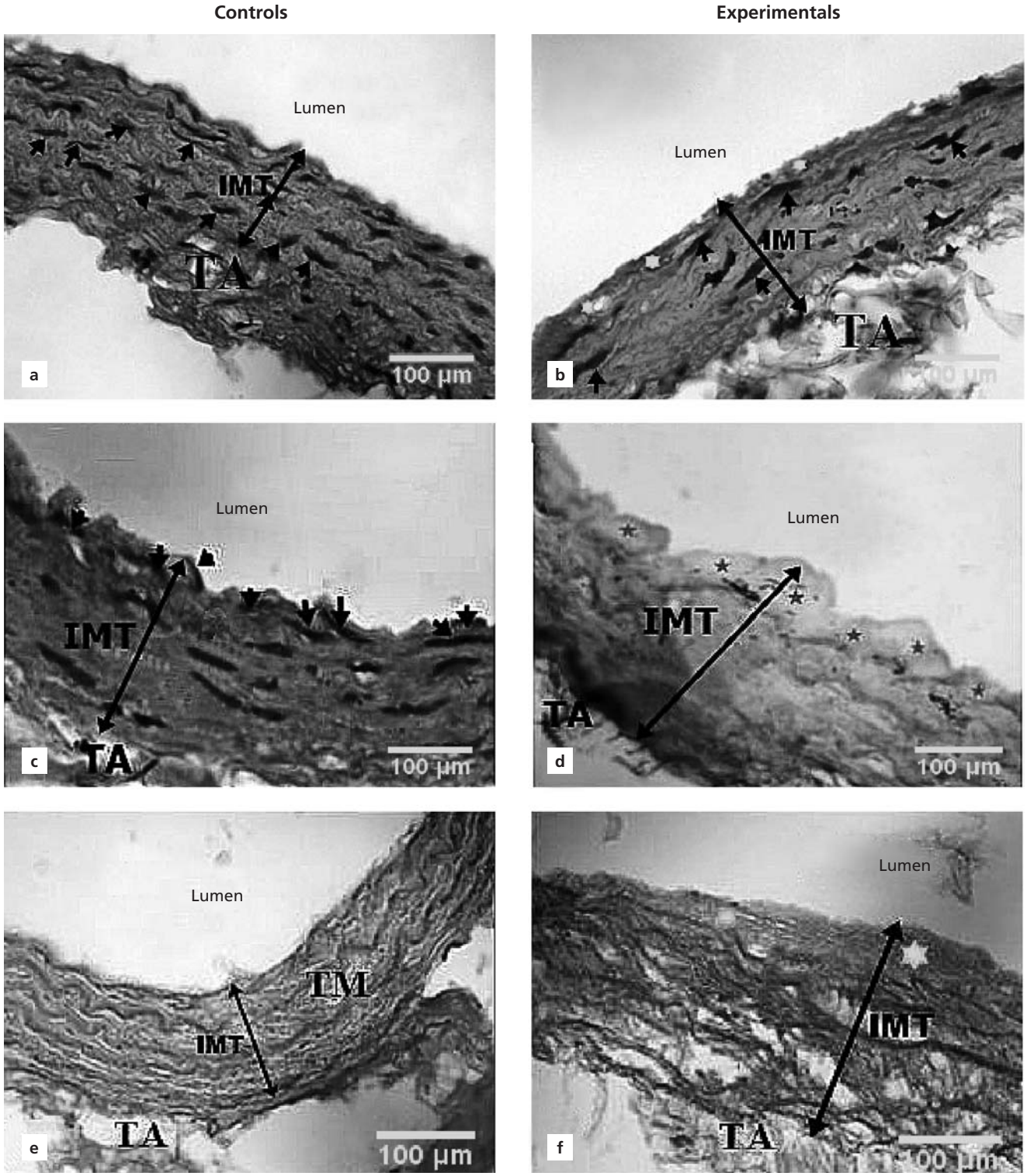

Figure 1. Photomicrograph showing the structure of the CCA of (**a**) a normogonadic rat at week 3, (**b**) three weeks after castration (note the increase in intima-medial thickness compared to the control in Figure 1a), (**c**) 9 weeks after castration (note the intima hyperplasia with smooth muscle cell nuclei), (**d**) hypogonadic rat seen 9 weeks after castration (note the intimal hyperplasia that's thicker than that in the control animal in Figure 1c) (**green stars**). Hematoxylin and Eosin stain. Photomicrograph showing the organization of tunica media of common carotid artery of (**e**) a normogonadic rat at week at week 6 of the study, (**f**) a hypogonadic rat seen 6 weeks after castration (note the increased intima-medial thickness as well as the intimal hyperplasia). **IMT:** intima-medial thickness; **TA:** tunica adventitia; **TM:** tunica media. Wiegerts' elastic stain. Scale bars=100 µm. [Color figure can be viewed in the online issue, which is available at www.anatomy.org.tr]

artery disease. This is in agreement with various observational studies that have reported higher incidences of these diseases in hypogonadal men compared to those with normal androgen levels.[15,16,18] Progressive hyperplasia and hypertrophy of the tunica intima is known to result in vascular stenosis. This may compromise vascular supply to important organs such as brain and heart affected by atherosclerosis making them more vulnerable to ischemic insults.[31] This may explain the higher incidences of stroke,[32] myocardial infarction[33] as well as ischemic lower limb amputations[34] among hypogonadal men.

The current study was limited by the fact that castration is a surgical procedure that causes tissue injury. Therefore, some of the changes observed in this study may have been due to reactive processes to tissue injury. This was delimited by the fact that the surgical procedure was carried out in the scrotum and did not tamper with the common carotid arteries located in the neck. Also, based on the fact that the changes observed in this study were proportional to the duration of androgen deficiency, they are most likely to be due to gonadal hormone deficiency rather than surgical trauma.

Conclusion

Androgen deprivation by surgical castration induces a progressive increase in the c-IMT. This may constitute an anatomical basis for the higher predisposition of hypogonadal males to cardiovascular diseases.

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