REVERSIBLE EFFECT OF TESTOSTERONE ON THE MORPHOLOGY OF THE CORONARY ARTERY IN ADULT MALE RABBITS;

AN INTERVENTIONAL STUDY

By

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(H56/76172/2014)

A dissertation in partial fulfilment of the requirements of the Masters of Science Degree in Human Anatomy in the University of Nairobi

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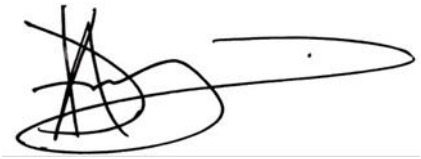
NOVEMBER 2021

DECLARATION

I hereby confirm that this dissertation is my original work and has not been presented elsewhere for approval or examination.



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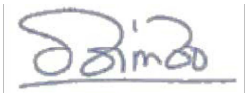

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LIST OF ABBREVIATIONS

IMT – Intimal-medial thickness

ANOVA – Analysis of Variance

TNFα – Tumor Necrosis Factor alpha

IL-1β –Interleukin 1 beta

ABSTRACT

Background; Male hypogonadism, marked by low serum levels of testosterone, is a relatively common endocrine disorder and its prevalence differs among populations. Declining quantities of serum testosterone have been linked with increased risk for cardiovascular diseases. On the contrary, a physiological rise in testosterone level is protective against cardiovascular diseases and ameliorates ongoing cardiovascular events. Androgens influence the cardiovascular system through multipronged mechanisms, one of them being the induction of histomorphological changes in the vascular wall. Varying levels of androgens have been associated with structural modifications in vessels such as the carotid and the aorta in various studies. Of note, studies show a correlation of reduced testosterone levels with an increase in intimal-medial thickness, connective tissue density of the vascular wall, and reduced luminal diameter in key vessels. Physiological studies on the efficacy of testosterone on various vascular beds illustrate non-uniformity, suggesting that its effect on the vascular structure may also not be homogenous.

Objective; This study sought to describe histomorphologic changes that occur in the coronary artery of the adult male rabbit following surgical castration and subsequent testosterone administration.

Design; Interventional study design

Materials and Methods; Twenty-eight (28) one year old male rabbits were randomly divided into an experimental/interventional group (14) and a control group (14). Two in each group were selected for baseline data. Animals in the interventional group underwent surgical orchidectomy while the controls underwent sham surgery (scrotal opening and closing without orchidectomy). Serum testosterone levels were recorded fortnightly. After the first six weeks, half the animals in each group were randomly picked, sacrificed and their coronary vessels harvested and processed for routine histology. From this point henceforth, the remaining

rabbits of the experimental group underwent weekly intramuscular injections of testosterone enanthate. At the end of the study (after another six weeks), the rest of the rabbits were sacrificed and their coronary vessels were harvested and processed for routine histology. Hematoxylin and eosin stain was used to demonstrate the intima-media span and smooth muscle cell nuclei. Masson's Trichrome was used to demonstrate collagen fibers. A ZeissTM digital photomicroscope was used to take photomicrographs for stereological analysis.

Data Management; Quantitative data on mean intimal-medial thicknesses, smooth muscle cell count, and adventitial collagen fiber density was entered into Statistical Package for Social Sciences (SPSS) software for analysis. After assessment for normality, a parametric test (Analysis of Variance, ANOVA) was used to compare the mean between groups. A p-value $\leq$ 0.05 was considered statistically significant at a 95% confidence level. Data was presented in tables, graphs and boxplots.

Results; Mean serum testosterone levels were 27.5 nmol/l, 0.9 nmol/l, and 15.4 nmol/l in controls, castrated rabbits and testosterone injected rabbits respectively. Intimal medial thickness was significantly increased in the castrated group (0.488mm) compared to controls (0.388mm) and subsequently declined to 0.440mm in the testosterone injected group. Adventitial collagen fiber density of the left coronary artery rose in the castrated group (66.63%) compared to controls (36.11%) but stayed elevated in testosterone injected group (65.19%). Medial smooth muscle cell count of the castrated rabbits was 26.96%, significantly lower than the count in the testosterone injected group (47.53%) and the controls (47.80%).

Conclusion; Varying testosterone levels are associated with reversible changes in some morphometric parameters of the coronary artery. These findings suggest that the testosterone hormone may have a role in modifying the structure of the left coronary artery, hence modifying the risk for cardiovascular disease.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Male hypogonadism, a condition that presents with reduced amounts of testosterone in circulation, is widely observed in elderly males (Fraietta et al., 2013). It is a relatively common endocrine disorder but its exact prevalence among different populations is not clear. The decline in serum testosterone concentrations with advancing age in men is actually with a function of age-related illnesses rather than chronological age per se (Harman et al., 2001). Of interest, chronically depleted serum testosterone is linked to a substantially higher risk of cardiovascular disease (Saad et al., 2016). Many biological factors utilized in the calculation of the Framingham risk score, a formula that estimates a patient's ten-year risk of acquiring cardiovascular illness, are influenced by circulating testosterone levels (Jahangiry et al., 2017).

According to epidemiological analyses, men in the reproductive age bracket have a higher risk of developing cardiovascular disease than age-matched females(Liu et al., 2003; Wu and von Eckardstein, 2003). This gender disparity had previously been ascribed to a cardiovascular protective effect of estrogen in women, versus a deleterious effect of androgens in men(Bernini et al., 2001). This hypothesis has since been disapproved by larger prospective studies. A growing body of evidence is now suggesting the converse; showing a protective effect of androgens in cardiovascular disease (Cai et al., 2016; Nettleship et al., 2009). Several randomized clinical trials and meta-analyses demonstrate that serum levels of circulating androgens are inversely correlated with risk factors and mortality in cardiovascular illness (Smith, 2007; Kintzel et al., 2008; Tivesten et al., 2009). In support of this, for instance, some studies show that patients having coronary heart disease and heart failure elicit better cardiovascular function after receiving testosterone treatment (Rosano et al., 2007;Morgentaler et al., 2015a; Gencer et al., 2021)

Androgens impart their beneficial effects on cardiovascular disease either by directly acting on the cardiovascular system or by modifying other risk factors. Experimental evidence suggests that physiologically high testosterone levels favorably affect the lipid profile, glycometabolism, hemostatic parameters, and vascular inflammation (Gyllenborg et al., 2001; Hak et al., 2002; Ng et al., 2002; Svartberg et al., 2006). Testosterone levels also have immune-modulating effects that significantly influence the incidence and advancement of atherosclerosis(Malkin et al., 2004, 2003) (Malkin et al 2003, Malkin et al 2004). This can be attributed to a reduction in proinflammatory cytokines such as TNFα, IL-1β, and a concomitant rise in anti-inflammatory cytokines such as IL 10 following testosterone injections (Malkin et al., 2004).

Most investigations on the effect of testosterone on cardiovascular disease have largely concentrated on acute physiological alterations resulting from endothelium and non-endothelium mediated vasodilation (Perusquía et al., 2012; Perusquía and Stallone, 2010). Fewer have described the influence of chronic hypogonadism on the structure of vessels, for instance by highlighting morphological markers of atherosclerosis. Tsujimura et al., 2012 characterized an association of decreased serum free testosterone with increased thickness of carotid intima-media in middle-age Japanese males. Geary et al., 2000 described the effect of low gonadal hormones on the decreased luminal diameter of cerebral arteries. Alexandersen et al., 1999 had earlier illustrated the reversibility of the effect of testosterone on structural markers of aortic atherosclerosis in rabbits. Studies on testosterone replacement therapy report evidence of deceleration in the progression of atherosclerosis, with one particular study showing decreased carotid intimal-medial thickness (IMT) in stable angina patients (Mathur et al., 2009).

However, there is a scarcity of literature that describes the relationship between low serum testosterone and structural markers of coronary artery atherosclerosis, and whether or not this

relationship is reversible. The vulnerability of the coronary artery to arterial disease has been documented, though the focus has been on physiological and metabolic derangements, most of which are affected by testosterone levels (Wu and von Eckardstein, 2003). Perusquía et al., 2012 assert that androgens have varying efficacy on different vascular beds and thus the effect of testosterone on morphological alterations is not necessarily a uniform pattern across all vessels. For instance, increase in intima-medial thickness is a prominent structural change in the carotid artery in both hypogonadic animal models (Nakashima et al., 2008) as well as studies done with human subjects (Bernini et al., 2001) while increase in collagen fibre density is reported in the aorta in similar study designs (Jenkins et al., 2007; Ogeng'o, 2017). Changes in structural markers like intima-media thickness are important subclinical markers of atherosclerosis and are useful in evaluation of risk of cardiovascular dieases (Lorenz et al., 2007; Uthoff et al., 2008). Thus, the elucidation of the effect of testosterone on the histomorphology of the coronary arteries in castrated rabbits may add to the growing body of knowledge on how androgens influence structural risk for cardiovascular disease.

1.2. LITERATURE REVIEW

The implication of hypogonadism on the reproductive function of males has traditionally been extensively documented (Basaria and Dobs, 2001). Subsequent studies have described a connection linking low serum levels of testosterone to a risk of cardiovascular diseases (Maggio and Basaria, 2009). Of interest, low testosterone level has been associated with atherosclerosis of most large vessels, with some experimental studies showing inhibition of plaque development by androgens in animal experiments (Hanke et al., 2001). The coronary arteries, carotids, and aorta are known to be particularly vulnerable to atherosclerosis (Hayashi et al., 2010). Some studies describe a link between low serum testosterone and morphological markers of atherosclerosis such as IMT and luminal diameter in the aorta and carotid arteries (Hak et al., 2002; Muller et al., 2004). The association between vascular connective tissue and testosterone levels has attracted relatively less attention, although Jenkins et al., 2007 showed increased collagen synthesis by adventitial fibroblasts in the coronary artery of rats treated with testosterone.

1.2.1. RELEVANT ANATOMY OF THE CORONARY ARTERY OF THE RABBIT

The number and branching patterns of coronary arteries in rabbits bear both similarities and differences with the human pattern. They both have a right and left coronary artery but the left is always dominant in the rabbit (Podesser et al., 1997). The right coronary in the rabbit is considerably smaller. The left coronary artery bifurcates or trifurcates (as commonly seen in humans) but with even prevalence (Figure 1). Studies have preferred the left coronary artery due to its size and accessibility (Podesser et al., 1997).

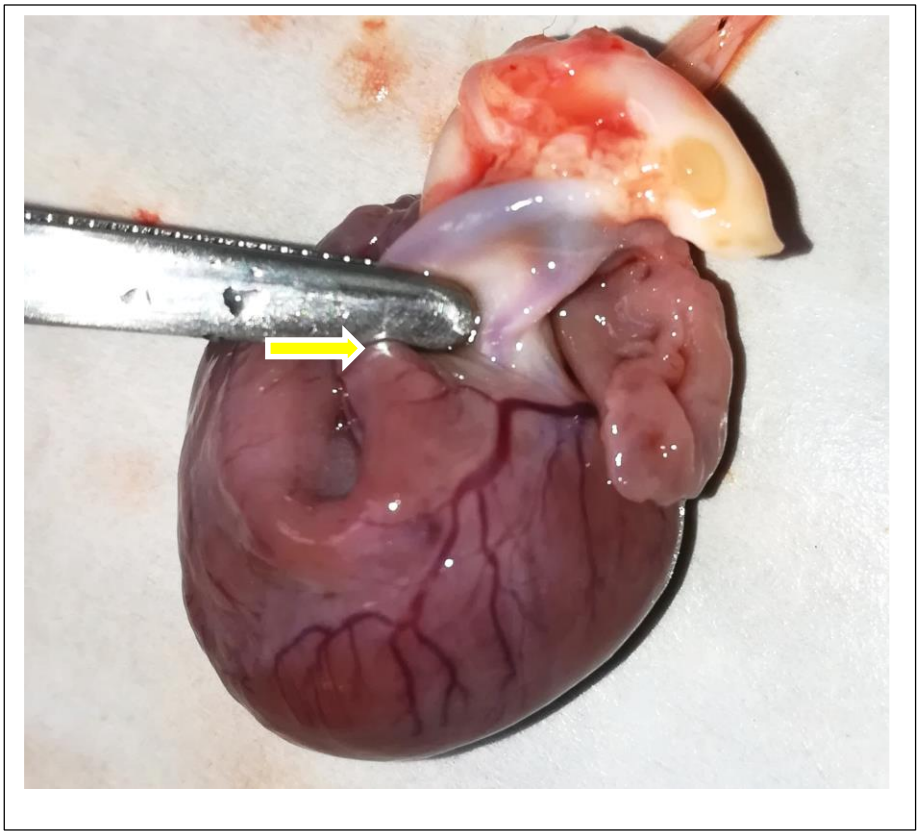


Figure 1; Left coronary artery (arrow) of an adult male rabbit used in our study. Histology slides were processed and stained from sections of proximal, middle and distal portions of the artery.

1.2.2. INTIMA-MEDIAL THICKNESS

The thickness of intima-media is a consistent and sensitive marker in cases of asymptomatic atherosclerosis, while also independently predicting the risk of adverse cardiovascular sequelae (Lorenz et al., 2007). It varies widely among different populations, and also varies with morphological parameters of the respective vessel (Ogeng'o, 2015). It proves valuable in evaluation, stratification of risk, prognostication, and monitoring of cardiovascular disease (Uthoff et al., 2008). Changes in intima-medial thickness may be considered as an adaptive response to luminal blood imparting lateral pressure and stress on the vessel wall (Stary et al., 1992; Deopujari and Dixit, 2010). It is not clear how its further progression leads to atherosclerosis.

Several theories have been put forward to account for how changes in IMT develop, and most of these admit difficulty in determining exactly when the atherosclerotic lesion initiates. The "response-to-retention" hypothesis, proposed by Williams and Tabas affirms that retention of atherogenic lipoproteins within the intima-medial layer of the artery marks the first stage in atherogenesis (Tabas et al., 2007). The process is induced stimuli such as inflammatory cytokines and mechanical stress increase the local synthesis of proteoglycans that bind lipoproteins within these layers(Camejo et al., 1998; Chait and Wight, 2000; Lee et al., 2001; Williams, 2001; Little et al., 2002). This interaction, which is ionic in nature, occurs between anions in the glycosaminoglycan component of proteoglycans and cationic residues of lipoproteins (Camejo et al., 1998; Chait and Wight, 2000). The hypothesis additionally factors in the increased susceptibility of lipoprotein–proteoglycan complexes to modifications such as oxidation and accumulation which eventually results in phagocytosis by macrophages that transform into foam cells (Hurt-Camejo et al., 1992; Tabas, 1999; Kaplan and Aviram, 2001).

Nakashima et al., 2008 classify intimal-medial thickening into two: diffuse intimal thickening and eccentric intimal thickening. Diffuse intimal thickening, otherwise known as musculoelastic intimal thickening, occurs at the non-branching sections of arteries and extends both longitudinally and circumferentially. Eccentric intimal thickening, or intimal cushion, involves a focal proliferation of the intima, especially at orifices and branching points. The occurrence of these classes of intimal thickenings is often simultaneous and may not always be distinguishable (Nakashima et al., 2008).

Diffuse intimal thickening is the process of intimal-medial thickening that most likely occurs in atherosclerosis (Stary et al., 1992; Schwartz et al., 1995; Virmani et al., 2000). The histological picture consistent with diffuse intimal thickening is consistently found within the walls of arteries prone to atherosclerosis, including the carotid arteries, coronary arteries, aorta and iliac arteries (Schwartz et al., 1995; Virmani et al., 2000; Nakashima et al., 2008, 2002).

Such findings have pointed to the fact that diffuse intimal thickening is an important mechanism of atherogenesis.

Smooth muscle cells become abundant during the process of diffuse intimal thickening and are principle cell types implicated in the initial steps of atheroma formation (Aikawa et al., 1993). Intimal smooth muscle cells produce various paracrine factors that promote cell proliferation, migration, and extracellular matrix transformation during atherogenesis (Nakata et al., 1996). Furthermore, the production of proteoglycans by smooth muscle cells increases during diffuse intimal thickening, hence potentiating lipid entrapment when they invade the tunica intima (Nakashima et al., 2008).

1.2.3. CONNECTIVE TISSUE FIBRES IN THE TUNICA ADVENTITIA

Previously, the tunica adventitia was assumed to play a passive role in the nutritional and physical integrity of the wall of an artery. Recently, evidence suggests that it plays an active role in the function, structure, and development of pathological processes in the arterial wall (Stenmark et al., 2013). Traditional descriptions of tunica adventitia describe it as being almost entirely composed of macrophages and fibroblasts. Ogeng'o et al., 2014 have additionally described immunoregulatory cells, progenitor cells, endothelial cells, and pericytes within the adventitia of carotid and coronary arteries.

The tunica adventitia is richly fibroelastic, having collagen fibers that confer tensile strength that enable it to withstand external forces and elastic fibers that enable stretching for vasodilation and constriction (Ogeng'o, 2017). Disproportionate amounts of collagen increase vessel stiffness and is correlated with multiple vascular pathologies including atherosclerosis and hypertension. The collagen-elastin ratio is known to be influenced by sex hormones, partly contributing to the gender disparity in cardiovascular illnesses. Fischer and Swain, 1977 demonstrated a correlation of low testosterone levels with a high collagen-elastin ratio in the

aorta of male rats after castration. More recently, Jenkins et al., 2007 showed increased collagen synthesis by adventitial fibroblasts in the coronary artery of rats treated with testosterone. We will attempt to illustrate how collagen fiber densities are affected by hypogonadism in a rabbit model.

1.2.4. VASCULAR SMOOTH MUSCLE DENSITY

Testosterone is known to exert acute vasodilatory effects in blood vessels through non-genomic pathways, including the modulation of calcium channels (Deenadayalu et al., 2001; English et al., 2002; Lorigo et al., 2020; Wynne and Khalil, 2003). There is however a scarcity of literature regarding the effect of androgens on the structure of the tunica media and density of vascular smooth muscles. Available data reveal a pattern of reduced vascular smooth muscle density of internal carotid arteries and penile erectile tissues in hypo-androgenic states (Cheruiyot et al., 2018). Changes that are described in the smooth muscle of erectile tissues include the disorganization of smooth muscle cells (Traish and Kim, 2005) and decreased myofilament quantity (Traish et al., 2007).

Supraphysiological levels of testosterone affect the vascular structure negatively by inducing hypertension and other pathological changes, through mitochondrial reactive oxygen species generation and NLRP3 inflammasome activation (Alves et al., 2020; Lopes et al., 2014). High levels of testosterone may eventually induce smooth muscle cell apoptosis leading to a reduction of smooth muscle density in the tunica media (Lopes et al., 2014). Interventions that replace testosterone levels in hypo-androgenic states should therefore be strictly limited to the restoration of physiological levels of serum testosterone to avoid adverse vascular effects. An investigation of the chronic effects of restoring the normal physiological levels of serum testosterone on the density of coronary arterial smooth muscle after hypo-androgenic state is further warranted. This study may elaborate on any potential benefit of such replacement therapy to the histologic structure of the coronary artery.

1.3. STUDY JUSTIFICATION

Male hypogonadism is a fairly common endocrine disorder. Even though its exact prevalence among different populations is not known, with reports suggesting that it is underdiagnosed (Fraietta et al., 2013). Reports from a study of male aging in Massachusetts indicated that androgen deficiency had an incidence rate of 12.3 per 1000 person-years (Araujo et al., 2004). This was noted to rise significantly with aging and could be inferred as about 481,000 cases per year acquired in men between forty and sixty-nine years of age. Varying levels of circulating androgens induce structural modifications in key vessels like the carotid and the aorta, and partly predispose these vessels to cardiovascular disease (Cheruiyot et al., 2018; Kintzel et al., 2008; Smith, 2007; Tivesten et al., 2009b). The consistent pattern observed is that reduced serum androgen levels correlate with structural changes that predispose to cardiovascular diseases (Tsujimura et al., 2012). With increasing evidence that androgens may have a nonuniform effect on the structural changes across the cardiovascular system (Perusquía et al., 2012), data on the effect of testosterone on the structure of all vulnerable vessels need to be documented. This information may contribute to the growing body of knowledge regarding the role of androgens in modifying structural risk factors for cardiovascular illnesses.

1.4. STUDY SIGNIFICANCE

Emerging research shows that lower serum androgen levels consistently correlate with the worsening of cardiovascular risk factors (Saad et al., 2016; Bashyal et al., 2019) and on the contrary, physiologically high levels of testosterone are demonstrably protective against cardiovascular diseases (Cai et al., 2016). Administration of testosterone for therapy in hypogonadal men facing cardiovascular illnesses appears promising as shown by accumulating data from clinical trials (Morgentaler et al., 2015a; Gagliano-Jucá and Basaria, 2019). Demonstration of the reversible effect of testosterone on the structure of a key vessel like the

coronary artery will bolster the evidence that androgens are beneficial to the vascular health of hypogonadal men.

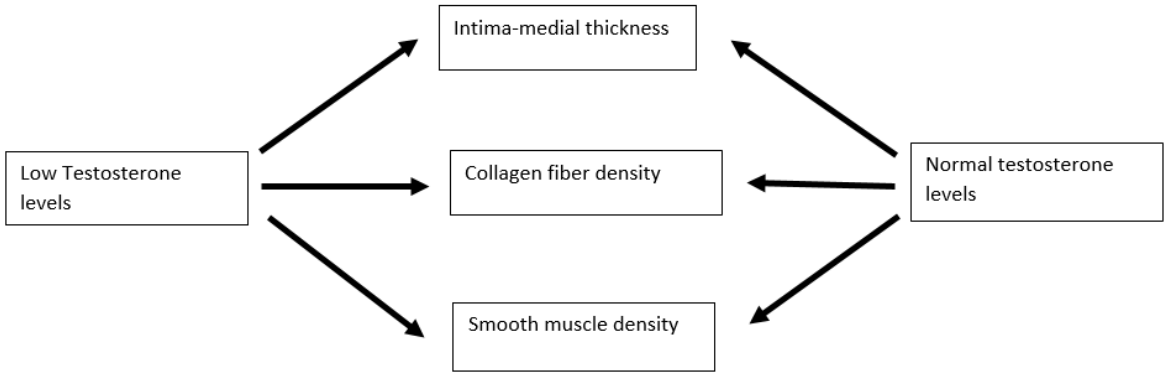
1.5. NULL HYPOTHESIS

Short term morphological changes in coronary arteries induced by hypogonadism via castration are not reversible with exogenous testosterone administration.

1.6. STUDY QUESTION

Are the morphological changes in coronary arteries induced by surgical castration reversible with exogenous testosterone administration?

1.7. CONCEPTUAL FRAMEWORK



1.8. OBJECTIVES

1.8.1. Broad Objective

To demonstrate the morphological changes in the coronary arteries of adult male rabbits associated with changes in testosterone levels induced through surgical castration and subsequent testosterone administration

1.8.2 Specific Objectives

1. To compare intimal-medial thickness of coronary arteries between adult male rabbits that were surgically castrated and not treated with testosterone and those that were surgically castrated and subsequently injected with testosterone.
2. To compare the density of collagen fibers in the tunica adventitia of the coronary arteries between adult male rabbits that were surgically castrated and not treated with testosterone and those that were surgically castrated and subsequently injected with testosterone.
3. To compare smooth muscle cell density of coronary arteries between adult male rabbits that were surgically castrated and not treated with testosterone and those that were surgically castrated and subsequently injected with testosterone.

CHAPTER 2: MATERIALS AND METHODS

2.1. STUDY DESIGN

The study followed an interventional design.

2.2. MATERIALS

2.2.1. Rabbits as a study model

Rabbits remain a popular animal model in biomedical research for the study of the cardiovascular system due to their ease of maintenance, affordability, and similar cardio physiology with humans (Fan et al., 2015). Several studies have used the rabbit model to investigate the cardiovascular system. The White New Zealand species have been used in descriptive studies due to close gross morphological and histological similarities to humans (Podesser et al., 1997). Therefore, rabbits are a suitable model for this study.

2.2.2. Study setting

The rabbits were purchased from the Department of Veterinary Anatomy, at the University of Nairobi. The study was conducted at the Department of Veterinary Anatomy animal house and later processing of the specimen was done at the Department of Human Anatomy of the University of Nairobi.

2.3. SAMPLING

2.3.1. Sample Size

A formula for interventional type study designs was suggested by Charan and Biswas, 2013, useful when mean values of two groups are compared. It is used to calculate sample size per group.

$$Sample\ size = 2SD^2 (Z_{\alpha/2} + Z_{\beta})^2 \div d^2$$

SD = Standard deviation, derived from published or pilot studies (0.6125 from our literature)

$Z_{\alpha/2}$ = 1.96, derived from Z tables, at type 1 error rate of 0.05

Z_{β} = 0.421, derived from Z tables. at a power of 80%

d = the effect size, derived from the difference between mean values (0.48 mm in past literature)

Therefore; $2 \times 0.6125^2 (1.96 + 0.421)^2 \div 0.48^2 =$ **13**

The sample size calculated is **13** per group.

For the convenience of grouping, **14 adult male rabbits per group** were selected making it a total sample size of **28 adult male rabbits**.

2.3.2. Selection Criteria

Twenty-eight (28) adult male rabbits of similar age and almost similar weight were used for this study. Animals selected for this study were about 1 year old since the male rabbit attains sexual maturity at that age (Steinberg, 2004).

There were no rabbits with variant cardiac/coronary arterial system and visible pathology in the scrotal regions, which would have been excluded from the study.

2.4. ETHICAL CONSIDERATIONS

The study topic and proposal was endorsed and passed at the department of Human Anatomy (University of Nairobi). Ethical approval for animal use was obtained from the Biosafety, Animal Use and Ethics Committee (BAUEC), University of Nairobi (Appendix 1). The rabbits were handled in strict adherence to the guidelines provided by the ethical committee. Sacrificing of the study subjects followed internationally accepted standards i.e use of inhaled halothane for quick painless death.

2.5. METHODS

All 28 animals were bought from the department of Veterinary Anatomy, University of Nairobi. Half (14 animals) were randomly assigned to the interventional or experimental group and 14 were controls. At the beginning of the study, 2 rabbits from each group were randomly selected and sacrificed and their coronary arteries were harvested for routine histology. Data obtained from these vessels were used as a baseline. All animals were tagged with coded numbers for randomization using a digital number generator.

At the start of the study, all twelve (12) of the remaining rabbits in the intervention group underwent surgical orchidectomy, as the other 12 of the control group underwent a sham surgery, where the scrotum was opened and closed without actual orchidectomy. Invasiveness of surgical opening of the scrotum is traumatic and likely to influence the outcomes of the study thus a sham procedure was performed in the controls to standardize conditions and minimize confounders. The surgeries were performed under sterile conditions using a combination of ketamine (20mg/kg) and xylazine (3mg/kg) for effective general anesthesia. Phenylbutazone 8mg/kg was also used as an analgesic. Amoxyl syrup (125mg/ml) was added to feeds after the operations to control infections. The animals were then allowed to heal as other conditions of both groups remained constant.

Measurements of serum testosterone levels were taken fortnightly in rabbits of all groups for the duration of the study (12 weeks). This was done by collecting blood samples via venipuncture of the ear vein after which they were sent to the veterinary laboratory at Pathologists Lancet Kenya for assays of total testosterone. After the first 6 weeks, 6 rabbits of the intervention group and 6 controls were randomly selected by employing a digital random number generator, sacrificed and their coronary vessels harvested and processed for routine histology. The 6-week duration was informed by studies that demonstrate vascular

morphological changes occur within 4 to 6 weeks in adult rabbits which corresponds to one year of human life (Hayashi et al., 2010; Fan et al., 2015).

From this point henceforth, the 6 remaining rabbits of the intervention group underwent weekly intramuscular injections of 25 mg testosterone enanthate (procured from a private veterinary pharmacy); this was similar to the method used by Alexandersen et al., 1999 with fortnightly measurements of serum testosterone level for all animals. Concurrently, the controls were given normal saline intramuscular injections. At the end of the study (after another 6 weeks) all remaining rabbits of both groups were sacrificed and their coronary vessels were harvested and processed for routine histology (Figure 2). The flow chart below illustrates the steps described.

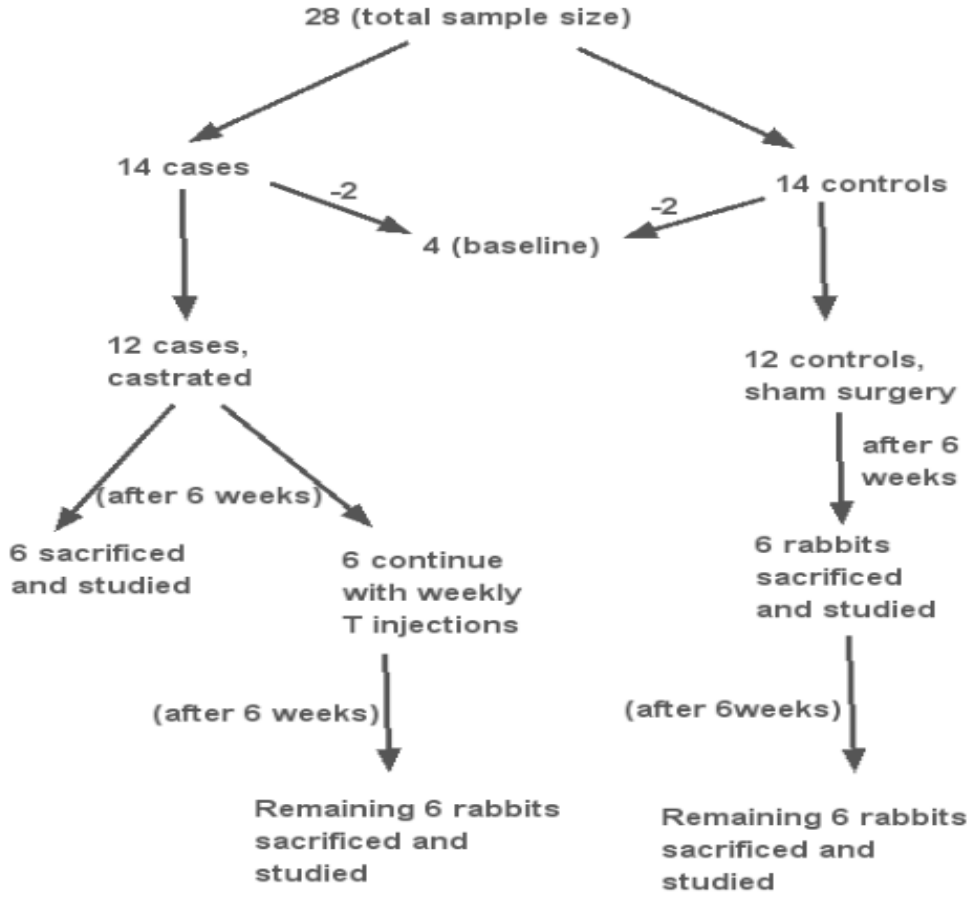


Figure 2; Flowchart illustrating the sequence of events during the study

2.5.1. Surgical induction of hypogonadism

Hypogonadism was induced by surgical castration under local anaesthesia at the beginning of the study in the intervention group using the prescrotal approach. With the animal lying in the dorsal recumbency position under physical restrain, perineal region was identified and the prescrotal area shaved. The skin was then cleaned with iodine solution then local anaesthesia (2 ml of 1% Lignocaine) was injected at the prescrotal area and around both scrotal sacs. A 2 cm incision was made on the midline just cranial to the base of the hemiscrotal sacs to access the testicle and spermatic cord, which were then gently grasped and exteriorized through the incision (figure 3). The ligament between the hemiscrotal sac and the tail of the epididymis was gently dissected, and the spermatic cord was clamped, ligated and removed en bloc with the testes and epididymis. The preplaced stay suture was then tied to close the vaginal process. The procedure was repeated on the contralateral side and the skin incision closed using Vicryl 2.0 stitch. The wound was covered by an Elastoplast which all the animals removed at varied times, from immediately to within 24 hours.

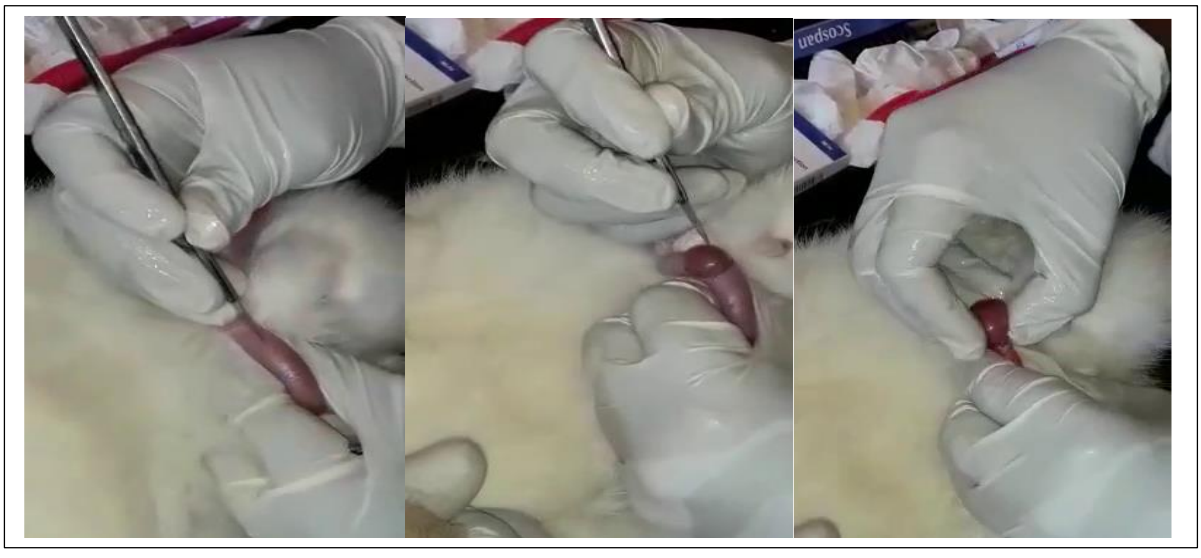


Figure 3; Midline incision of the hemiscrotal sacs to access the testicle and spermatic cord in a male rabbit.

2.5.2. Handling of study animals

The rabbits were fed and handled with close observation by attendants at the animal house. They were housed in standard rabbit-sized cages measuring 91 × 61 × 91 cms. Each cage contained two animals. The animals were coded and labeled for identification to prevent mixup during cage clean-ups. The floors of the rabbit cages were paved with wood shavings. The shavings were replaced every two days while cleaning the cages. The animals were fed standard rabbit pellets with water ad libitum and subjected to weekly inspection for good health.

2.5.3. Occupational health

The rabbits were held in a way to prevent any biting or scratching injuries to the handlers. To ensure proper restraining, the rabbits were ejected from cages by taking hold of both ears with one hand and their base with the other hand. The handlers wore bite-resistant leather gloves. Due to careful handling, there were no cases of defensive biting by the rabbit. All sharps, once used, were put in the safe disposal containers. Safety gloves were donned when handling caustic chemicals such as formalin and xylene in the histology lab.

2.6. TISSUE HARVESTING AND PROCESSING

The rabbits were euthanized by placing halothane-soaked cotton wool over their mouth and nostrils. The death of the rabbits was verified by the cessation of the heartbeat and diminished ocular reflexes. Subsequently, an incision was made through the midline chest and abdomen. Formal saline was then infused through an intra-cardiac injection. After perfusion, the heart was removed from the body by severing major vessels and removing it from the mediastinum. The coronary arteries were then carefully dissected and extracted from the heart. They were afterward cut into short segments.

These tissue segments were fixed by immersing in 10% formalin saline for approximately twenty-four hours. After proper fixation, the heart underwent dehydration in ascending

concentrations of alcohol, starting from 70% up to 100% alcohol between hourly intervals. The heart tissue was cleared using toluene and later infiltrated by immersing in a wax container in a memmert oven at 60 degrees for twenty-four hours. The tissue was then embedded in paraffin wax before cooling. The tissues, after embedding, were blocked using plastic cassettes. They were then sectioned transversely into seven micrometer thick slices using a rotary microtome (Leica® Model SM2400, Leica Microsystems, Nussloch GmbH, Germany). The sectioned ribbons were put on a warm water bath and picked using a glass slide, then dried at 35 degrees in an oven for twelve hours. Thereafter, the slides were stained using Hematoxylin and Eosin and Masson's Trichrome.

2.6.1. Hematoxylin and Eosin staining

The slides were dewaxed by passing through three changes of xylene, for five minutes periods each. The tissue in the slides was subsequently rehydrated by immersing in a series of solutions, beginning with 50:50 concentrated xylol followed by descending concentrations of alcohol from 100% to 70% with a three-minute interval per change. Thereafter, the slides were immersed in Iron Hematoxylin solution for a fifteen-minute interval then passed through running water for two minutes to clear excess stain. The slides were then stained in a 1% eosin solution for three minutes. This was followed by dehydration in increasing ethanol concentrations from 70% to 100%. The slides were finally cleared by passing in two xylene changes for five minutes before observing under a microscope.

2.6.2. Masson's Trichrome staining

The slides were dewaxed by passing through three changes of xylene, for five minutes per change. The tissues in the slides were subsequently rehydrated by immersing in a series of solutions, beginning with 50:50 concentrated xylol followed by descending concentrations of alcohol from 100% to 70% with a three-minute interval per change. Thereafter, the slides were

immersed in Iron Hematoxylin solution for a 15-minute interval. The stained slides were then dipped once in acid alcohol for differentiation then immersed in a container filled with running tap water for an hour for blueing. The slides were thereafter immersed in Ponceau stain for six minutes, followed by clearing in a container with distilled water. The slides were then placed in a mordant for four minutes, cleared in distilled water, then immersed in the light green stain for two minutes. This was followed by dehydration in increasing ethanol concentrations from 70% to 100%. The slides were finally cleared by passing in two xylene changes for five minutes before observing under a microscope.

The histology slides were read and interpreted, at the department of Human Anatomy histology laboratory, by two technologists independent of each other and blinded to the grouping of the study subjects.

2.7. MORPHOMETRIC ANALYSIS

A photomicroscope (ZeissTM digital photomicroscope, Carl Zeiss AG, Oborkochen, Germany) with a 12-megapixel digital camera was used to take photomicrographs of the sections. Magnifications of X40, X100, and X400 were used to capture the vascular wall tunics with higher magnifications showing collagen fibers in the adventitia. These photographs were entered into with Fiji Image J software (National Institutes of Health image program) whereby morphometric and stereological analysis was done.

2.7.1. Intimal-medial thickness measurement

Four arbitrary points of the arterial wall were measured using a digital imaging software (Image J v1.53) to obtain the IMT and an average recorded for the proximal, middle, and distal segments of all the arteries (figure 4).

IMT= (IMTa + IMTb + IMTc + IMTd) / 4

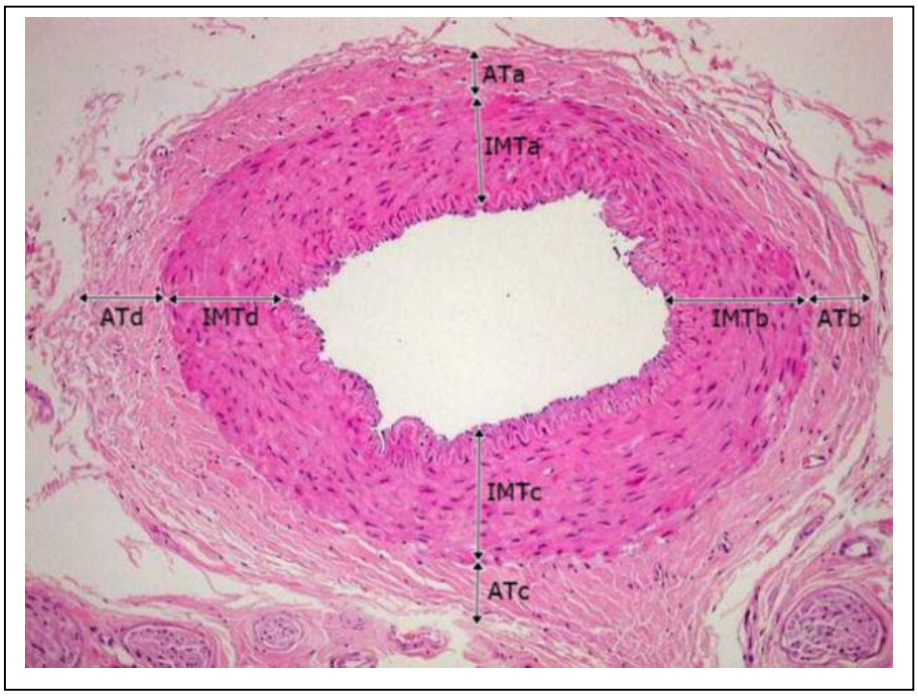


Figure 4; Transverse section of a left coronary artery of an adult male rabbit showing measurements of the IMT. *Hematoxylin and eosin stain X 40 magnification*.

2.7.2. Collagen fibre density estimation

Connective tissue volumetric density estimation was done using the Cavalieri principle of point counting (Mandarim-de-Lacerda, 2003) and data expressed as volume densities (%). The chosen fields of view were examined using an overlaid 42-point grid that was selected from the Image J app. The grid system has orthogonally arranged lines that cross at regular points, which enable estimation of tissue densities (Mandarim-de-Lacerda, 2003). The volumetric densities of the histological structures were evaluated while blinded on the animal group that tissue samples were from.

By stereological principles, the distribution area of a histological component of isotropic tissue, when established from a two-dimensional section, is directly proportional to its volume distribution (Pinheiro et al., 2000; Mandarim-de-Lacerda, 2003). The volume densities of the

vascular components were derived by the formula: Vv = Pp/Pt, where Vv was the volume density, p was the vascular component under consideration (smooth muscle, elastic or collagen fibers), Pp was the number of test points that cross on p, and Pt was the total number of points in the grid.

2.7.3. Smooth muscle cell count

Histological slides stained with hematoxylin and eosin were analyzed using a superimposed 42-point grid on the digital images on the monitor screen as described in the previous section (Figure 5). Smooth muscle cell nuclei were counted per unit area and expressed as percentages. The volume density of the muscle cells was calculated by the formula Vv = Pp/Pt, where Vv was the volume density, p was the tissue component under consideration (smooth muscle nuclei), Pp was the number of test points associated with p, and Pt was the total number of points of the test system.

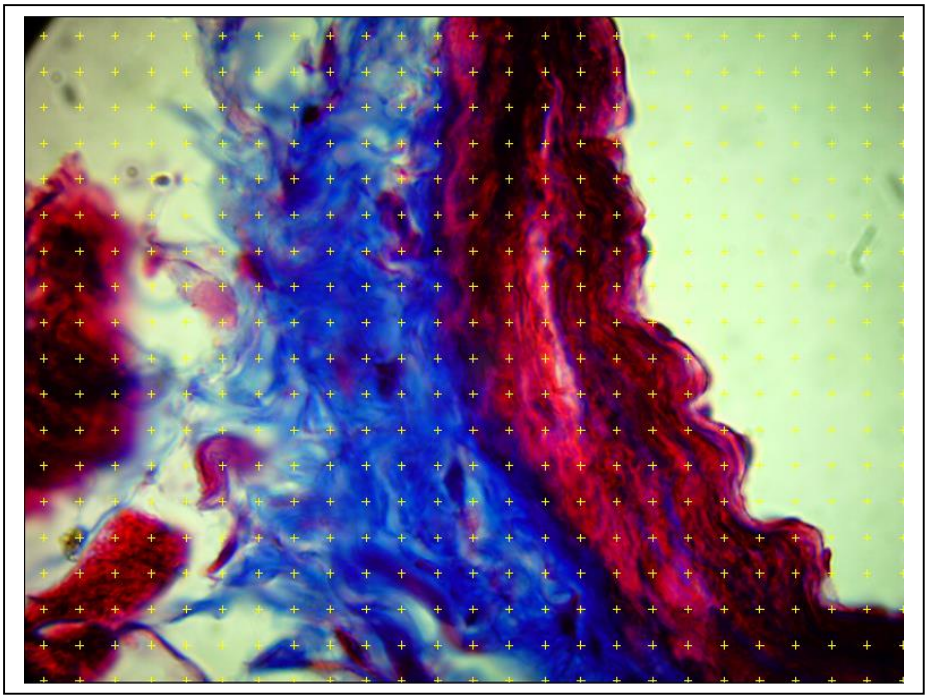


Figure 5: Point counting method to estimate volume densities of connective fibres in tunica adventitia of rabbit's left coronary artery. Masson's trichrome X400 magnification.

2.8. STATISTICAL ANALYSIS AND DATA MANAGEMENT

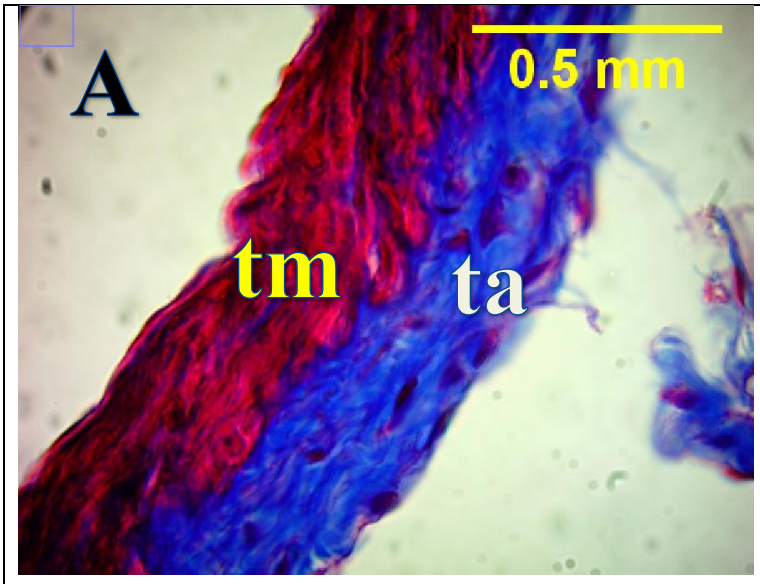
Morphometric data on the coronary artery thickness was entered by coding in the Statistical Package for Social Sciences software (SPSS, version 21.0, Chicago, Illinois) for further statistical analysis. Wall thickness was expressed in millimeters, collagen density, and smooth muscle densities were expressed as percentages. The data was split into the control group and two experimental groups datasets ('castrated' and 'testosterone injected' groups) for comparison. Data normality was tested using Wilk-Shapiro tests and normality graphs. The Analysis of Variance (ANOVA) test was used to compare means among the groups. A p-value less than or equal to 0.05 was interpreted as statistically significant (95% confidence level).

CHAPTER 3: RESULTS

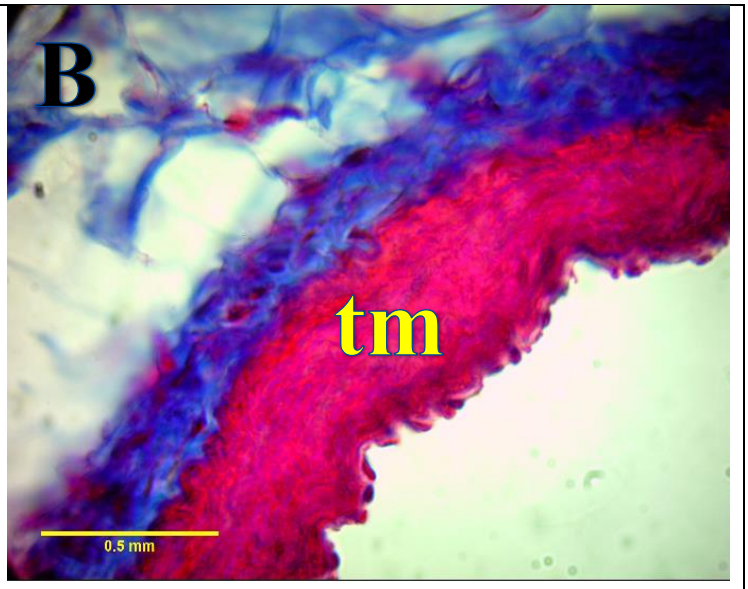
The rabbit coronary artery showed features of a muscular artery with three conventional tunics; tunica intima, tunica media, and tunica adventitia. Mean testosterone levels for separate groups were as follows; Baseline (30.1nmol/l); Castrated group; 0.9 nmol/l; Testosterone injected group; 15.4 nmol/l; and Controls; 27.5 nmol/l. The normal range of serum testosterone levels in adult male rabbits is 1.59 – 32.80 nmol/l.

3.1. INTIMAL-MEDIAL THICKNESS

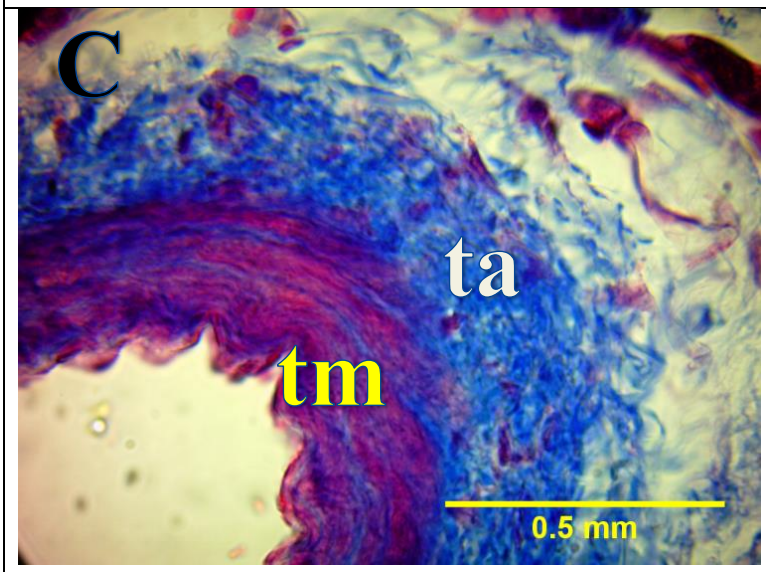
From a visual analysis of the photomicrographs, the thickness of the intimal-medial layer of the coronary arteries appeared to be greater in the castrated group compared to the controls (Figure 6A-B). The thickness of the intimal-medial layer was reduced when the castrated rabbits were administered with testosterone, with the thickness being comparable to the controls (Figure 6C).



Control group



Castrated group



Testosterone injected group

Figure 6; Representative slides of left coronary artery wall from controls (A), castrated (B), and testosterone injected rabbits (C). The vascular tunics are visible. The IMT is greater in the castrated group compared to the controls. The IMT is reduced in the testosterone administered group compared to the castrated group (C). *Masson's trichrome X400*, **tm**- tunica media; **ta**- tunica adventitia

After a morphometric analysis, a mean IMT of 0.488 mm was recorded in the castrated group, 0.440 mm in the testosterone injected group, and 0.388 mm in the control group (table 1). Standard deviation was 0.005 in all groups.

Group (mean Testosterone levels in nmol/l)	Mean IMT (mm)	Std. Deviation	95% Confidence Interval for Mean (mm)		Minimum (mm)	Maximum (mm)
			Lower Bound	Upper Bound		
Castrated (0.9)	0.488	.005	.487	.489	.478	.499
Control (27.5)	0.388	.005	.386	.389	.375	.399
Testosterone Injected (15.4)	0.440	.005	.439	.441	.427	.453

Table 1; Measurements for intimal-medial thickness (IMT) for each group as well as their means and standard deviations.

The data on IMT were normally distributed as assessed by the Shapiro-Wilk test ($p < 0.05$), and means between groups were compared by Analysis of Variance (ANOVA) (Table 2). All differences were statistically significant ($p = 0.000$). The greatest difference in IMT was between the castrated gropu and the controls.

		Mean IMT Difference (mm)	Std. Error	P-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Castrated	Control	.101*	.0008	.000	.099	.103
	Testosterone injected	.048*	.0008	.000	.046	.051
Control	Castrated	-.101*	.0008	.000	-.103	-.099
	Testosterone injected	-.052*	.0008	.000	-.055	-.050
Testosterone injected	Castrated	-.048*	.0008	.000	-.051	-.046
	Control	.052*	.0008	.000	.050	.055

Table 2; Results of the test of the differences in the mean intimal medial thickness (IMT) of the groups using ANOVA. Statistically significant differences among all groups ($p<0.05$).

The difference in IMT between the castrated group and the testosterone injected group was significant (0.048mm; $p<0.05$). The difference in means between the castrated groups and the controls (0.101mm), as well as between the testosterone injected group and the controls (0.052mm) were also statistically significant ($p<0.05$). A boxplot further demonstrated a reduction in IMT in the testosterone administered group (Figure 7).

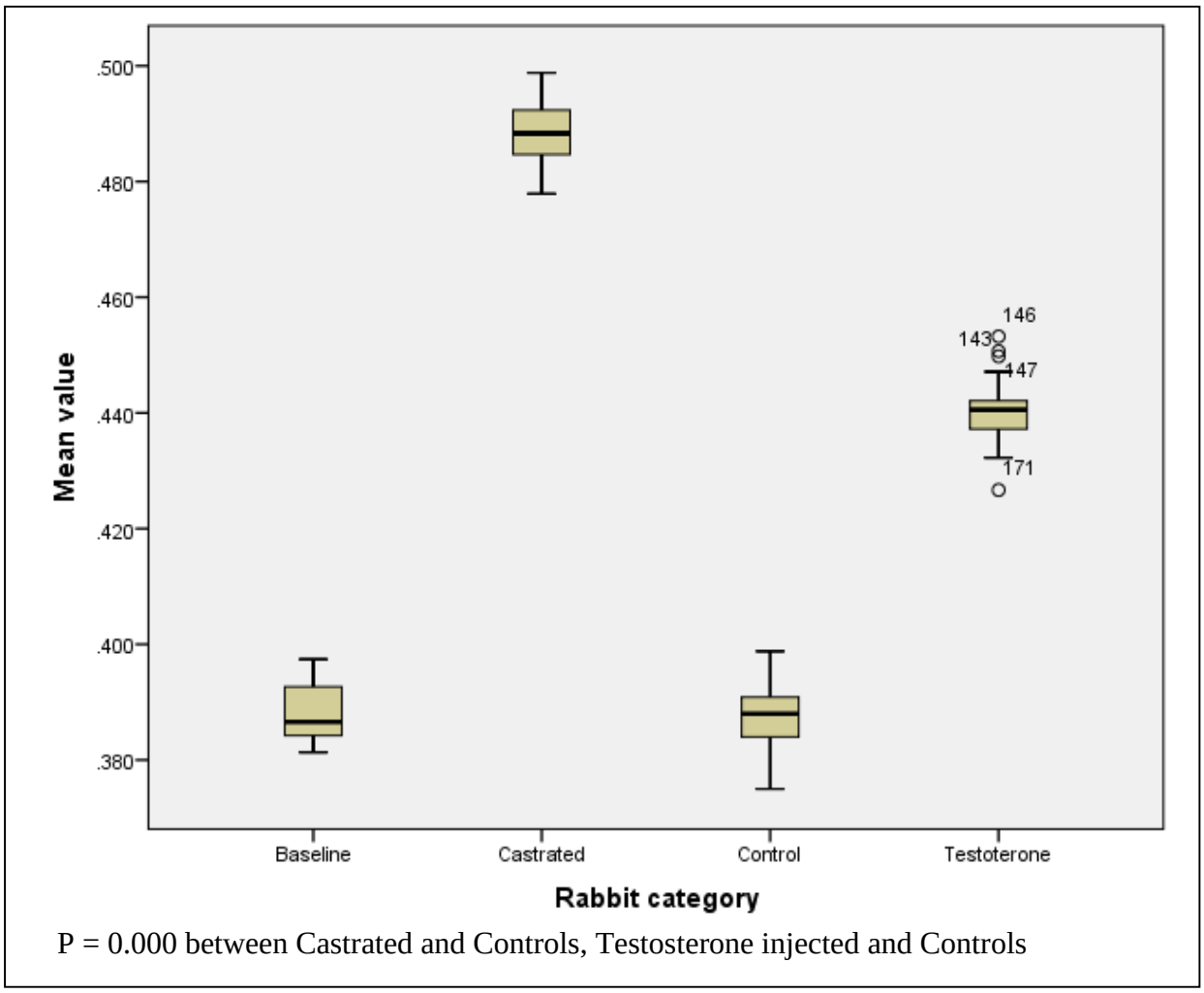
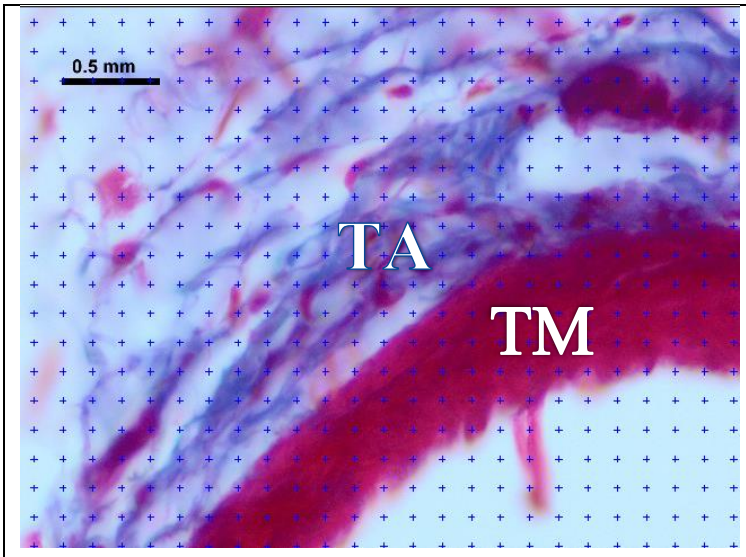


Figure 7; Box plot comparing means of the intimal medial thickness of the left coronary artery between the baseline, control, castrated and testosterone injected groups.

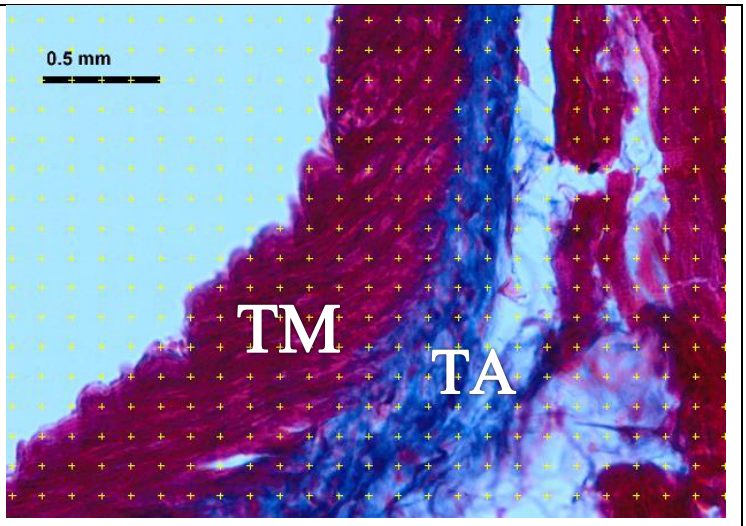
In summary, IMT was significantly increased in the castrated group compared to the control group and subsequently reduced in the testosterone injected group. However, IMT in both interventional groups (castrated and testosterone injected) was significantly higher compared to the controls.

3.2. ADVENTITIAL COLLAGEN FIBRE DENSITY

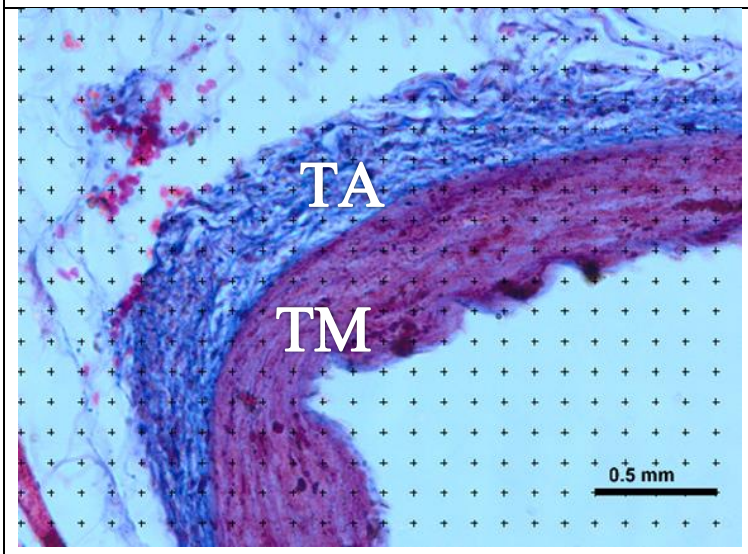
From a visual impression of the histology of the slides, the tunica adventitia of the coronary artery appeared to be denser in the castrated and testosterone injected groups compared to control. (Figure 8A-C). The density of collagen in the castrated and testosterone injected groups was not visually distinguishable (Figure 8B & C).



A. Control



B. Castrated



C. Testosterone Injected

Figure 8; Representative slides of left coronary artery wall from the control group (A), castrated group (B), and testosterone injected groups (C). The tunica adventitia of the coronary artery is denser in the castrated and testosterone injected groups compared to control. The collagen density is not visually distinguishable between the castrated and testosterone injected groups. *Masson's trichrome X400*, **TA**; - Tunica adventitia **TM**; - Tunica media

Collagen fiber density of the tunica adventitia of the coronary artery of the adult male rabbit ranged from 29.2% to 74.7%. A mean of 66.6% was recorded in the castrated group, 65.2% in the testosterone injected group and 36.1% in the control group (table 3).

Group (mean Testosterone levels in **nmol/l**)	**Mean (%)**	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum (%)	Maximum (%)
				Lower Bound	Upper Bound		
Baseline (**30.1**)	**34.53**	1.84	.41	33.67	35.39	30.43	38.58
Castrated (**0.9**)	**66.63**	3.32	.43	65.77	67.49	59.92	74.66
Control (**27.5**)	**36.11**	1.93	.25	35.62	36.61	29.92	40.34
Testosterone injected (**15.4**)	**65.19**	2.39	.31	64.57	65.80	61.05	71.46

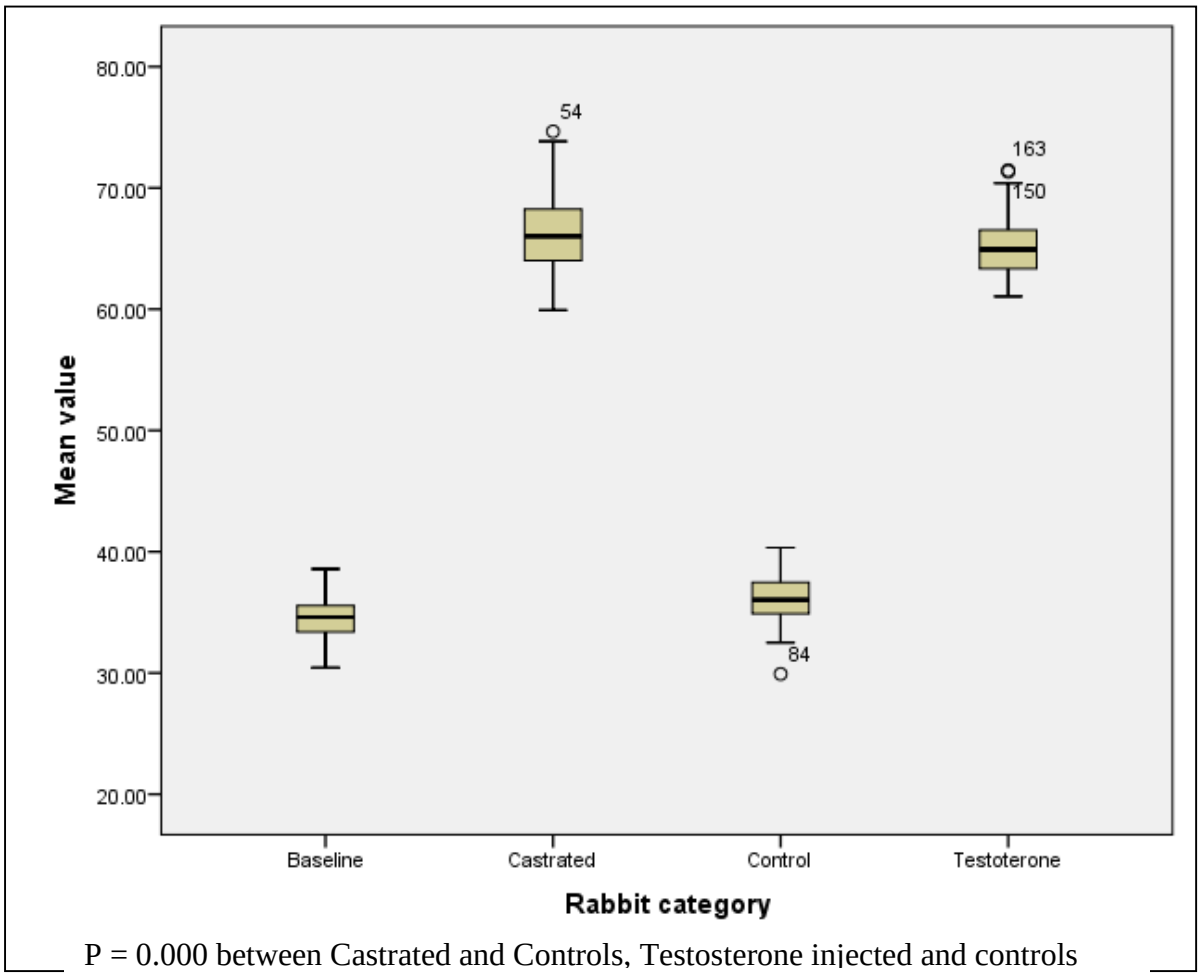
Table 3; Measurements for adventitial collagen fiber densities for each group as well as their means and standard deviations. The means of the baseline and control rabbits were almost 50% less as compared to those of the castrated and testosterone injected rabbits.

The means of collagen fibre density were compared by ANOVA and a significant rise in fiber density among the castrated (30.5%) as well as the testosterone injected groups (29.1%) compared to controls were recorded (Table 4). The greatest difference in mean density was between the castrated group and the controls. The differences in castrated and testosterone injected groups was not significant ($p<0.05$).

		Mean Difference	Std. Error	P-value	95% Confidence Interval	
					Lower Bound	Upper Bound
Castrated	Control	30.5*	.50	.000	29.22	31.81
	Testosterone	1.4	.53	.036	0.07	2.82
Control	Castrated	-30.5*	.50	.000	-31.81	-29.22
	Testosterone	-29.1*	.40	.000	-30.10	-28.04
Testosterone	Castrated	-1.4	.53	.036	-2.82	-0.07
	Control	29.1*	.40	.000	28.04	30.10

Table 4; Results of test of differences in the mean collagen fiber densities of the groups using ANOVA. Notably, differences in castrated and testosterone injected groups was not significant ($p<0.05$).

The difference in means collagen fiber density between the two interventional groups (castrated rabbits and the testosterone injected rabbits) was not significant. A boxplot demonstrated a higher density of collagen fibers in the adventitia of coronary arteries of castrated and testosterone administered rabbits compared to controls, with density in testosterone administered rabbits being slightly lower (Figure 9).



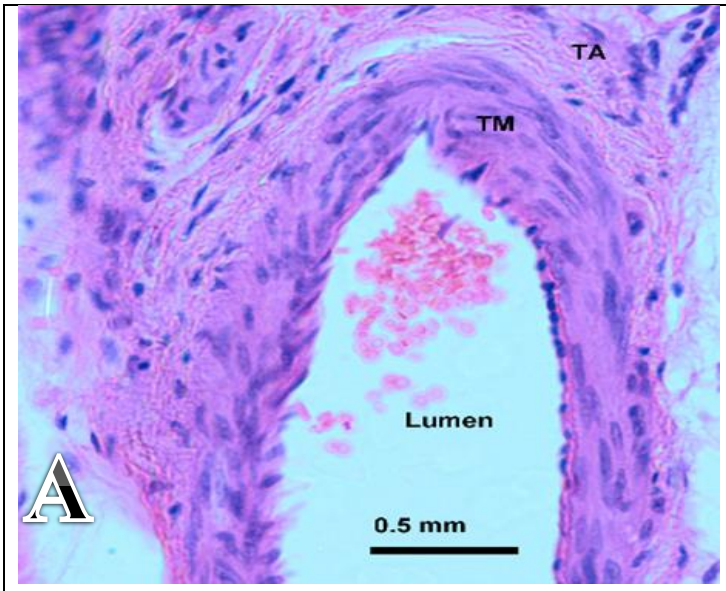
P = 0.000 between Castrated and Controls, Testosterone injected and controls

Figure 9; Boxplot comparison of mean collagen fiber densities among baseline, castrated, testosterone injected, and control groups.

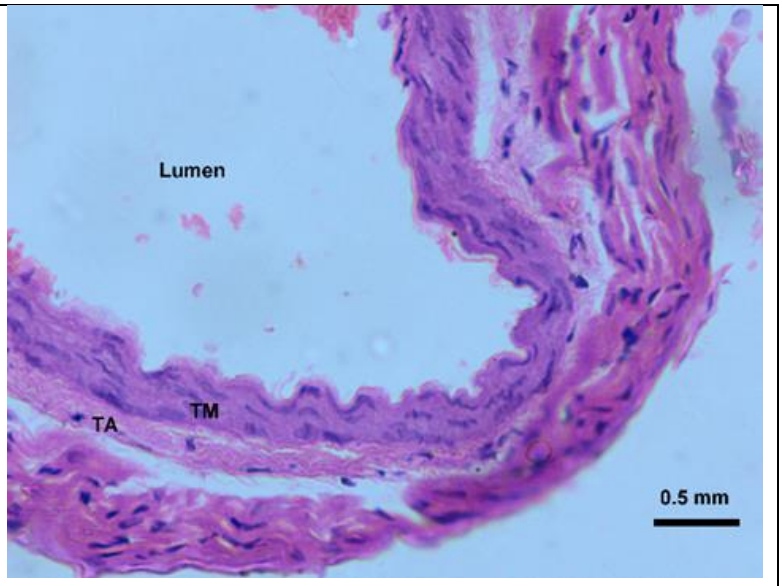
In summary, mean collagen fiber density in the adventitia of the left coronary artery of adult male rabbits rose after castration but did not decline with subsequent testosterone injections as compared to controls.

3.3. SMOOTH MUSCLE CELL COUNT

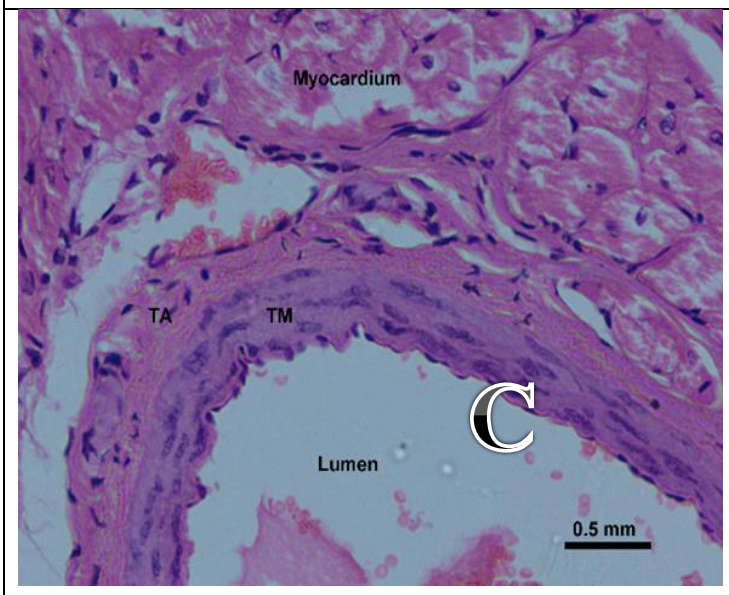
From a histological analysis, the density of smooth muscle cell nuclei appeared to be lower in the tunica media of coronary arteries of the castrated group compared to the controls. (Figure 10A-B). The density of smooth muscle cells was not visually distinguishable between the castrated and testosterone injected groups in the photomicrographs (Figure 10 B & C).



control group



castrated group



testosterone injected group

Figure 10; Sample slides of left coronary artery wall from the **control group (A), castrated group (B)** and **testosterone injected groups (C).** The smooth muscle density is lower in the castrated group compared to the controls and comparable between the castrated and testosterone injected groups in the photomicrographs. *Hematoxylin and eosin X100* TA; - Tunica adventitia TM; - Tunica media

Smooth muscle cell count of the tunica media of the coronary artery of the adult male rabbit was measured in percentages of cell nuclei numbers over the unit area and ranged from 23.43% to 50.69%. A mean of 26.96% was recorded in the castrated group, 47.53% in the testosterone injected group, and 47.80% in the control group (table 5).

Group (mean Testosterone levels in **nmol/l**)	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
Baseline (30.1)	**43.98**	1.53	0.34	43.26	44.69	39.29	46.10
Castrated (0.9)	**26.96**	1.57	0.20	26.56	27.37	23.43	30.68
Control (27.5)	**47.80**	1.18	0.15	47.50	48.11	43.58	50.69
Testosterone (15.4)	**47.53**	0.94	0.12	47.28	47.77	45.26	50.19
Total	41.08	9.42	0.67	39.77	42.40	23.43	50.69

Table 5; Recordings of smooth muscle cell counts for each group as well as their means and standard deviations. The mean density of castrated rabbits was almost 50% less as compared to those of the other groups.

The means were compared by ANOVA and a significant reduction in smooth muscle cell density among the castrated was (20.84% compared to controls and 20.56% compared to testosterone injected group) was recorded (Table 6).

Rabbit category		Mean Difference	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Castrated	Control	-20.84*	.25	.000	-21.49	-20.18
	Testosterone	-20.56*	.24	.000	-21.18	-19.95
Control	Castrated	20.84*	.25	.000	20.18	21.50
	Testosterone	.27	.19	.495	-.2327	.7810
Testosterone	Castrated	20.56*	.24	.000	19.95	21.18
	Control	-0.27	.19	.495	-0.78	.23

Table 6; Results of test of differences in the mean smooth muscle cell count of the groups using ANOVA. Notably, differences in means between testosterone injected and control groups was not significant ($p<0.05$).

The difference in means smooth muscle cell density between the testosterone injected group and the controls was not significant. A boxplot further corroborated these findings (Figure 11).

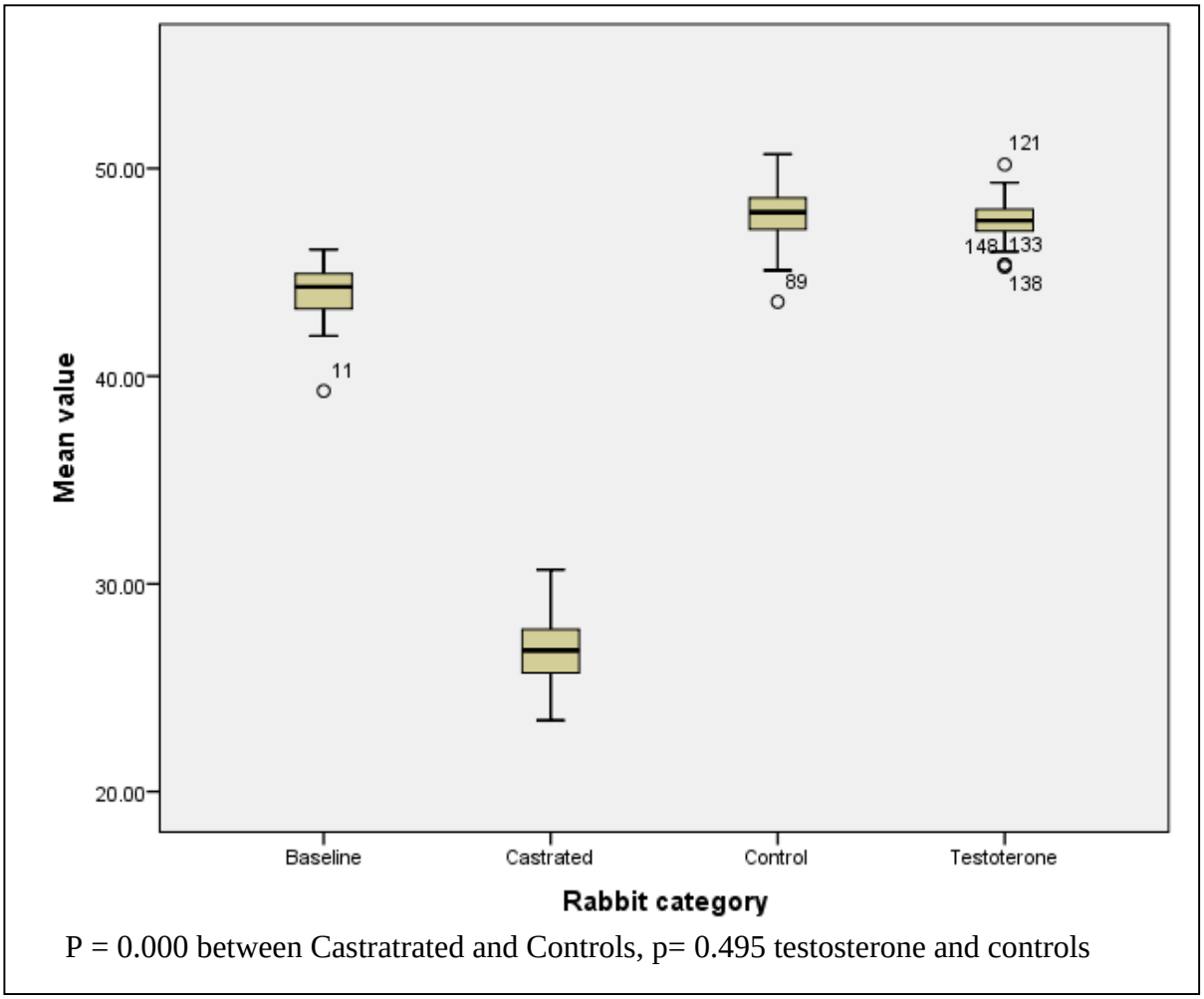


Figure 11; Boxplot comparison of mean smooth muscle cell counts among baseline, castrated, testosterone injected, and control groups. There was a significant drop in smooth muscle density in the castrated rabbits while the mean values of the other groups were almost similar ($p<0.05$).

In summary, smooth muscle cell density of the tunica media of the coronary artery significantly dropped in castrated adult male rabbits but rose to near control levels in adult male rabbits that were castrated and subsequently injected with testosterone.

CHAPTER 4: DISCUSSION

The association of low testosterone levels with morphological markers of atherosclerosis such as IMT has been observed in the aorta (Hak et al., 2002) and the carotid (Muller et al., 2004). Jenkins et al., 2007 have also demonstrated increased collagen synthesis by adventitial fibroblasts in the coronary artery of rats treated with testosterone. It remains largely unexplored as to whether these structural changes induced by hypogonadism can be reversed with the administration of testosterone within a physiological range, an effect that this study tries to describe.

4.1. INTIMAL-MEDIAL THICKNESS

This study found an increase in IMT in hypoandrogenic castrated rabbits and a comparative reduction of the same in castrated rabbits that had received testosterone injections. Cheruiyot et al., 2018 similarly demonstrated increased IMT in common carotid arteries of hypogonadic rats. This study additionally shows a significant reversal of IMT in castrated animals that had later received testosterone injections, though their mean values were still higher than baseline values. It is possible that if we extended the study period, then interventional findings closer to baseline values could have been reached. Multiple studies demonstrate an inverse relationship between IMT and serum testosterone levels (Malkin et al., 2003; Tsujimura et al., 2012), but an indication of whether testosterone administration would reverse the effects of an induced hypoandrogenic vascular structure is hardly reported. Our findings suggest a possible role for exogenous testosterone in reversing structural markers for cardiovascular diseases.

IMT is a reliable and sensitive marker of subclinical atherosclerosis and an independent predictor of cardiovascular events and target organ damage (Lorenz et al., 2007). It is valuable in the evaluation and stratification of cardiovascular disease risk, prediction of long-term outcomes, and monitoring ongoing disease progression and regression (Uthoff et al., 2008).

Intimal thickness can be regarded as an adaptive mechanism for increasing blood flow volume imparting lateral pressure and stress on the vessel wall (Stary et al., 1992; Deopujari and Dixit, 2010). It is not clear how its further progression leads to atherosclerosis.

The immunomodulatory effect of testosterone and its effect on programmed cell death of vascular smooth muscle cells may explain the association of increased IMT with hypoandrogenic states and vice versa. Experimental studies indicate that testosterone suppresses the activity of pro-inflammatory cytokines and enhances that of anti-inflammatory factors (Ng et al., 2002; Malkin et al., 2004). Testosterone also regulates apoptosis of vascular smooth muscle cells, an event that contributes to the progression of intimal hyperplasia and atherosclerosis (Bennett et al., 2010).

Although testosterone therapy has been used for many years to manage male hypogonadism, there is evidence to suggest that testosterone therapy would also be beneficial to hypoandrogenic men at risk of cardiovascular disease (Morgentaler et al., 2015a). Routine use of testosterone in clinical settings of hypoandrogenic patients who have cardiovascular disease has not been adopted due to the absence of large, prospective, placebo-controlled studies. Nevertheless, our observation of reversal of structural changes of the coronary artery after testosterone administration in hypoandrogenic models supports the mounting evidence that testosterone therapy should be considered where appropriate (Corona et al., 2014; Morgentaler et al., 2015b; Shabsigh et al., 2005).

4.2. ADVENTITIAL COLLAGEN FIBRE DENSITY

The present study demonstrates an increase in collagen fiber density in the tunica adventitia of the coronary artery in hypoandrogenic rabbits. Other studies have reported similar changes in the common carotid artery (Cheruiyot et al., 2018) and the penis (Olabu, 2014), under the setting of induced androgen deficiency. We also found that vascular collagen fiber density

remained elevated after the administration of testosterone to castrated rabbits. It is plausible that an increase in collagen deposition is a long-term phenomenon, not easily reversible with readjustment of hormone levels.

Multiple mechanisms have been proposed to explain how androgens influence vascular collagen deposition, one of which is the regulation of production of transforming growth factor β (TGFβ) (Chipuk et al., 2002). Testosterone suppresses expression of TGFβ, thus in settings of low androgen levels, upregulation of TGFβ results in fibroblast activation and deposition of collagen fiber. TGF-β also induces the differentiation of fibroblasts into the more synthetic myofibroblast phenotype. Hypoandrogenic states also upregulate angiotensin 2 receptors on smooth muscle leading to myofibroblast differentiation and increased collagen deposition (Kang et al., 2012).

The tunica adventitia is now known to play an active role in the structural integrity of the vascular wall and is not a passive participant as earlier thought (Stenmark et al., 2013; Ogeng'o, 2017). Tunica adventitia is richly fibroelastic with collagen fibers responsible for high tensile strength to enable it to withstand high pressures and elastic fibers to allow reversible stretchability. Changes in collagen increase vessel stiffness and have been associated with multiple cardiovascular diseases such as hypertension and atherosclerosis. This may be worsened in hypo-androgenic states. The presence of immunoregulatory cells, progenitor cells, endothelial cells, and pericytes within the adventitia of the aorta has additionally described, further elaborating the role of this layer as an active vascular component (Ogeng'o et al., 2015). Further studies may be necessary to investigate if androgens have an influence on these other cellular components of the tunica adventitia in the coronary arteries and other vessels.

4.3. SMOOTH MUSCLE CELL COUNT

This study reports a reduction of smooth muscle cell counts of the tunica media of the coronary artery in castrated adult male rabbits. Similar findings have been reported in the common carotid artery (Cheruiyot et al., 2018) and the penis (Olabu, 2014) under settings of induced hypogonadism. Smooth muscle cell counts increased to near control levels in adult male rabbits that were castrated and subsequently injected with testosterone, suggesting a reversible effect of testosterone on numbers of vascular smooth muscle cells.

A reduction in myofilament quantity is one of the described mechanisms through which reduction muscle volume is achieved in hypogonadal states (Traish et al., 2007). Further studies pinpoint programmed cell death as a key response of smooth muscle cells to the reduction of androgen levels (Ikeda et al., 2009; Kang et al., 2012). This has been demonstrated to be effected by caspases activated through angiotensin 2 receptors, which are upregulated in hypogonadic states. Others have touted an 'androgen deficiency- associated atrophy' of smooth muscle cells as seen in the penile corpus cavernosum (Traish and Kim, 2005; Olabu, 2014) similar to atrophy seen in skeletal muscle in men with low testosterone levels (Dandona and Rosenberg, 2010).

Experimental studies that performed blockade of 5α reductase also describe the dedifferentiation of smooth muscle cells into other phenotypes such as fibroblasts and adipocytes and partially explain the reduction of muscle mass in hypogonadic states (Arnold and Isaacs, 2002; Corradi et al., 2004). Loss of medial smooth muscle density in the coronary artery may increase the chances of aneurysms, calcification, and rupture of atherosclerotic plaques in arteries. Our observation that there is a reversal of muscle cell count with a return to normo-androgenic state further supports the notion that testosterone could have a therapeutic role in the management of cardiovascular diseases in hypogonadic states.

4.4. CONCLUSIONS AND NOVEL FINDINGS

In agreement with other studies, this study has shown that reduced testosterone levels are associated with alteration of the vascular structure. We further demonstrated that changes in IMT and vascular smooth muscle density can be reversed with the administration of testosterone within physiological ranges. Thus the null hypothesis was negated.

4.5. RECOMMENDATIONS

Further molecular studies regarding the mechanisms of the reversible effect of androgens on vascular structure may explain our observations. Further pharmacological studies are also required in human subjects to elucidate if testosterone administration may have some benefit in the reduction of risk of cardiovascular diseases.

4.6. STUDY LIMITATIONS AND DELIMITATIONS

Tissue injury caused by surgical castration may have caused reactive changes in the coronary vessels. We were also unable to determine whether the decrease in smooth muscle composition as a result of atrophy, apoptosis, or both. To delimit these, all experimental groups underwent scrotal opening with the actual removal of the testes done for the cases. The standardization of conditions for all subjects allowed for a direct comparison of the outcomes.

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APPENDIX 1

ETHICAL APPROVAL LETTER

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

P.O. Box 30197,
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Dr. Duncan Anangwe

University of Nairobi

Dept of Human Anatomy

REF: FVM BAUEC/2019/195

27/02/2019

Dear Dr. Anangwe,

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

Morphology of the coronary artery following surgical castration and testosterone administration in male rabbits; a quasi- experimental study.

By Dr. Anangwe (H56/76172/2014).

We refer to your revised MSc. proposal submitted to our committee for review and your application letter dated 31/01/2019.

We have reviewed your proposal, particularly section 8.6 that involves use of laboratory animals for surgical castration and testosterone administration in male rabbits.

We are satisfied that the proposed treatment and care of the animals meets acceptable standards for animal welfare. Furthermore, the numbers proposed are reasonable.

We have also noted that a registered veterinary surgeon Dr Ann Ndeke (**KVB 2574**) will supervise the animal surgery and humane end points.

We hereby give approval for you to proceed with the experiments as outlined in the submitted proposal.

Yours sincerely

Dr. Catherine Kaluwa, BVM, MSc, Ph.D
Chairperson,
Biosafety, Animal Use and Ethics Committee
Faculty of Veterinary Medicine.

APPENDIX 2

DATA COLLECTION TOOL

Group; - …….

Rabbit No; - ……

Histology Slide no.; ……

Testosterone level (nmol/l); - …….

Left Coronary artery (Masons Trichrome)			
	IMT	**Adventitial Collagen Density**	**Smooth muscle cell density**
A1			
A2			
A3			

Left Coronary artery (Hematoxylin and Eosin)			
	IMT	**Adventitial Collagen Density**	**Smooth muscle cell density**
A1			
A2			
A3			