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Reversible effect of castration induced hypogonadism on the morphology of the left coronary arteries in adult male rabbits

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Abstract: Hypogonadism is associated with an increased risk of coronary artery disease. This study sought to describe the histomorphology of the left coronary arteries of the adult male rabbit following orchiectomy and subsequent testosterone administration. We included 20 adult male rabbits, divided into a baseline group (n=2), an interventional group subjected to castration only (n=6), an intervention group subjected to castration followed by testosterone injection (n=6), and a control group (n=6). Key variables under investigation were serum testosterone levels, the intima-media thickness of coronary arteries, smooth muscle cell density, and adventitial collagen fiber density. The mean coronary arteries' intimal medial thickness was significantly higher in the castrated group than in controls (0.488 mm and 0.388 mm, respectively), while the testosterone-injected group had a mean of 0.440 mm. Mean smooth muscle cell density was significantly lower in the castrated rabbits vs. controls (26.96% and 47.80%, respectively), this observation being reversed with testosterone injection (47.53%). Mean adventitial collagen fiber density was significantly higher in the castrated group than in controls (66.6% and 36.1%, respectively), with a marginal difference after testosterone injection (65.2%). This study demonstrates that castration-induced hypogonadism causes morphological changes in the coronary arteries that are partly reversible using testosterone injections. These findings provide a morphological basis for understanding the role of testosterone in coronary arteries.

Key words: Testosterone, Hypogonadism, Coronary vessels, Cardiovascular diseases, Coronary artery disease

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Introduction

Male hypogonadism, characterized by low circulating testosterone levels, is one of the problems affecting males with advancing age [1, 2]. It is a relatively common endocrine disorder, the exact prevalence varying between populations. Age-related decline of serum testosterone levels in males can result from age-related illnesses or senescence-related decreases in secretion [3]. Androgens confer a protective influence on the cardiovascular system [4, 5].

Prospective clinical trials and meta-analyses indicate that circulating levels of endogenous androgens inversely correlate with risk factors and mortality from cardiovascular diseases [6-8]. Notably, chronically low testosterone levels are associated with a substantially higher risk of cardiovascular disease [9, 10], while patients with coronary artery disease or heart failure may elicit improved cardiovascular function after receiving testosterone treatment [11, 12].

Androgens benefit cardiovascular disease by directly acting on the cardiovascular system or modifying other risk factors. Experimental evidence suggests that physiologically

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high testosterone levels favourably influence the lipid profile, glycogen metabolism, hemostatic parameters, and vascular inflammation [13-16]. The immune-modulating effects of testosterone partly underpin its preventative influence on the development and progression of cardiovascular diseases like atherosclerosis [17, 18].

Most experimental studies have focused on changes in large-sized arteries induced by altering testosterone levels. Reported vascular structural changes under the setting of hypogonadism include increased carotid intima-media thickness (IMT) [19], increased collagen fiber density in the aorta [20], and decreased muscle density of carotid arteries [21]. Some evidence highlights that androgens have varying effects on different vascular beds [22]. Thus, the effect of testosterone on morphological alterations may not necessarily be a uniform pattern across all arteries. Further, little evidence indicates that structural vascular changes induced by varying testosterone levels are reversible.

Hypogonadism, characterized by diminished testosterone levels, has been linked to an elevated susceptibility to coronary artery disease. Given that coronary artery disease remains a significant cause of morbidity and mortality among middle-aged and older males [23], it is imperative to understand the role of testosterone and its deficiency in the structure of the coronary arteries. To elucidate the underlying mechanisms, our study delved into the alterations in histomorphology occurring within the left coronary arteries (LCAs) of adult male rabbits following orchiectomy and subsequent testosterone administration. Our primary aim was to ascertain the effects of castration-induced hypogonadism on coronary artery morphology and evaluate the potential reversibility of these changes through testosterone supplementation.

Materials and Methods

Study design

This study was a non-randomized quasi-experimental trial using the rabbit model. A total of 20 adult male rabbits aged 12 months were used in this study. We sought ethical approval to conduct the study from the Biosafety, Animal Use and Ethics Committee of the University of Nairobi in Kenya (Ethical approval number: FVMBAUEC/2019/98). Rules for the care and use of laboratory animals, as outlined by the Biosafety, Animal Use and Ethics Committee of the University of Nairobi, were followed. We adhered to the international guiding principles for biomedical research involving animals.

Animals and study setting

Rabbits of the white New Zealand species used as the study model are easy to handle, have low maintenance costs, and have a close cardio-physiological resemblance to humans. We obtained the animals from the Department of Veterinary Anatomy, University of Nairobi. All animals were selected at approximately 12 months because this is the age at which they attain sexual maturity. We excluded rabbits with visible pathology. The animals were housed in standard cages floored with wood shavings that were changed regularly. The animals were placed under a regular 12-hour light/dark diurnal cycle at a room temperature of 25°C±3°C and provided with standard rabbit pellets (Unga Farmcare) and water *ad libitum*.

Methods

We initiated the study by sacrificing two rabbits to establish baseline features of the LCAs. We then randomly divided the remaining 18 animals into interventional (12 rabbits) and control groups (6 rabbits). The interventional group underwent bilateral orchidectomy (surgical castration) under anaesthesia to induce hypogonadism, while the control group underwent sham surgery without actual orchidectomy. The surgeries were performed under sterile conditions using a combination of ketamine (30 mg/kg) and xylazine (2 mg/kg) intramuscularly for effective anaesthesia. Aspirin and amoxicillin were administered post-surgery for pain relief and infection prevention. Measurements of serum testosterone levels were taken in rabbits from all study groups. 5 ml of blood was collected from the jugular vein using heparinized bottles, and plasma samples were separated by centrifugation. A testosterone Enzyme Immuno-Assay kit (Immunometrics) was used to determine testosterone levels.

After the initial 6 weeks, six rabbits from the interventional group (castrated group) were euthanized, and their LCAs were harvested and processed for routine histology. This timeframe aligns with previous research indicating vascular morphological changes within 4 to 6 weeks in adult rabbits [24, 25]. After six weeks, the remaining six rabbits in the interventional group (testosterone-injected group) received weekly intramuscular injections of 25 mg of testosterone enanthate, a dosage used in past studies [26]. After 12 weeks, we sacrificed the remaining rabbits in both the intervention-

al and control groups, harvested their LCAs, and processed the tissues for histological analysis. The sections of LCAs for histology were consistently obtained at the midpoint of the artery's extent, equidistant from the aorta in all animals.

Tissue preparation and histological staining

The rabbits' LCAs were fixed in 10% formalin for twelve hours. This process was followed by dehydration in increasing grades of alcohol (70% up to absolute alcohol) at one-hour intervals, then clearing in toluene. After that, the tissues were placed in an oven for wax infiltration. The LCAs 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, picked on a glass slide, and dried in an oven for 12 hours. Masson's Trichrome staining displayed smooth muscle cells and adventitial collagen fibers, while hematoxylin and eosin (H&E) staining demonstrated the smooth muscle cell nuclei in the LCAs.

Microscopic analysis and morphometry

Photomicrographs of the sections were taken using a digital camera (Canon Powershot A640, 12 MP; Canon) mounted on a photomicroscope (Axiostar Plus Microimaging; Carl Zeiss). We noted and described the morphological changes of the LCAs. We conducted Stereological analysis using the Fiji Image J software (National Institute of Health), an open-source software for processing and analyzing images.

Statistical analysis

The variables we obtained included IMT, volumetric densities of adventitial collagen, and volumetric densities of smooth muscle. These measurements were obtained using standard morphometric methods in a past study on muscular arteries [21]. The collected data were then entered into the SPSS version 21 (IBM Co.) for coding, tabulation, and statistical analysis. Volumetric densities were expressed in frequencies. The data were grouped into the control group, castrated and testosterone-injected group. Mean differences in the parameters were determined as the differences from the controls. After confirming that the data were normally distributed using the Shapiro–Wilk test, analysis of variance (ANOVA) was used to compare means between groups. A *P*-value of ≤0.05 was considered significant at a 95% confidence interval. Data were visualized in tables, graphs, and photomicrographs.

Results

Baseline findings and serum testosterone

From the baseline, the LCAs showed features of a muscular artery with three conventional tunics: tunica intima, tunica media, and tunica adventitia. The tunica intima comprised a solitary layer of endothelial cells positioned atop a delicate sub-endothelial connective tissue. The most prominent layer, tunica media, featured unbroken elastic lamellae intermingled with smooth muscle cells and collagen fibers. As for the tunica adventitia, it consisted of dense collagen fiber bundles encircling the vessel wall. Mean serum testosterone levels for the separate study groups were as follows: baseline had serum testosterone of 27.9 nmol/L, controls had 27.5 nmol/L, the castrated group had 0.9 nmol/L, and the testosterone-injected group had 15.4 nmol/L.

Morphometric parameters of the coronary arteries

From a visual analysis of the photomicrographs, the thickness of the intimal-medial layer of the LCAs appeared to be more prominent in the castrated group than in the controls (Fig. 1A, B). The thickness of the intimal-medial layer seemed smaller when the castrated rabbits were ad-

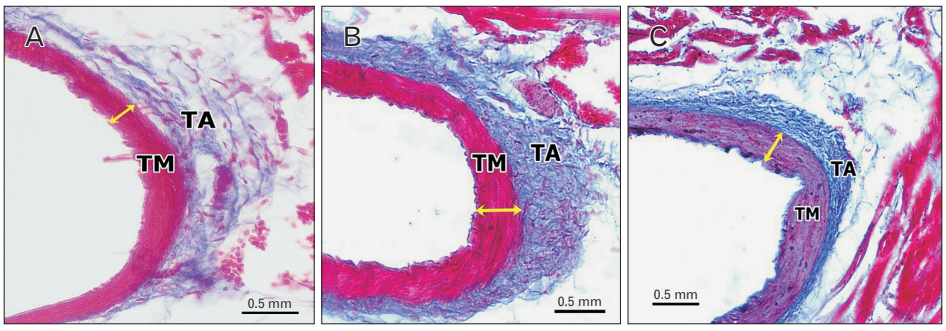

Fig. 1. Representative slides of the left coronary artery wall from controls (A), castrated (B), and testosterone-injected rabbits (C). The double-ended arrow is IMT. Mean IMT was greater in the castrated group compared to the controls. Masson's trichrome, ×100. TM, tunica media; TA, tunica adventitia; IMT, intima-media thickness.

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ministered with testosterone, with a thickness comparable to the controls (Fig. 1A, C). A mean IMT of 0.388 mm was recorded in the control group, 0.488 mm in the castrated group, and 0.440 mm in the testosterone-injected group (Fig. 2A). The difference in IMT between all three experimental groups was statistically significant (P<0.05) (Table 1).

From a histological analysis, the density of smooth muscle cell nuclei appeared lower in the tunica media of LCAs of the castrated group compared to the controls (Fig. 3A, B). The density of smooth muscle cells was visually indistinguishable between the control and testosterone-injected groups in the photomicrographs (Fig. 3A, C). A mean of smooth muscle density of 47.80%, 26.96%, and 47.53% was determined in the tunica media of the LCA in control, castrated, and testosterone-injected groups, respectively (Fig. 2B). A significantly lower smooth muscle cell density was noted among the castrated group than controls and testosterone injected group (P<0.001). The smooth muscle cell density difference between controls and testosterone-injected animals was not statistically significant (P=0.495) (Table 2).

From a visual impression of the histology of the slides, the tunica adventitia of the coronary LCAs appeared to be thicker in the castrated and testosterone-injected groups compared to the control (Fig. 1). The thickness of adventitial

Table 1. Differences in the mean intimal medial thickness of the control, castrated, and testosterone-injected groups using ANOVA

Study groups	Mean difference (mm)	*P*-value	95% confidence interval
Castrated vs. control	0.101*	<0.001	0.099–0.103
Castrated vs. testosterone injected	0.048*	<0.001	0.046–0.051
Testosterone injected vs. control	0.052*	<0.001	0.050–0.055

*Indicates statistically significant.

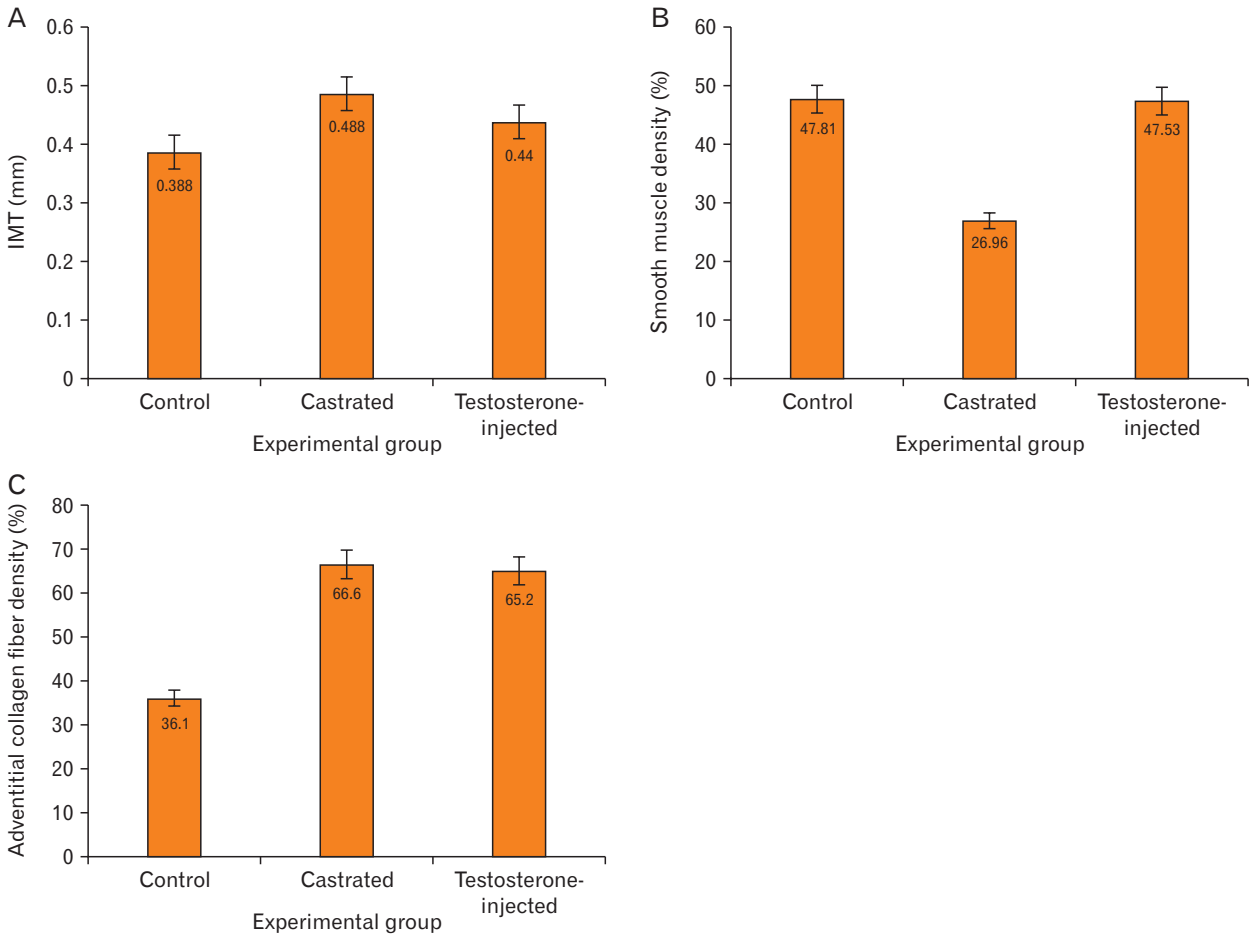

Fig. 2. Bar graphs showing means of the IMT (A), mean smooth muscle cell densities (B), and mean adventitial collagen fiber densities (C) of the left coronary artery in control, castrated, and testosterone-injected groups. IMT, intima-media thickness.

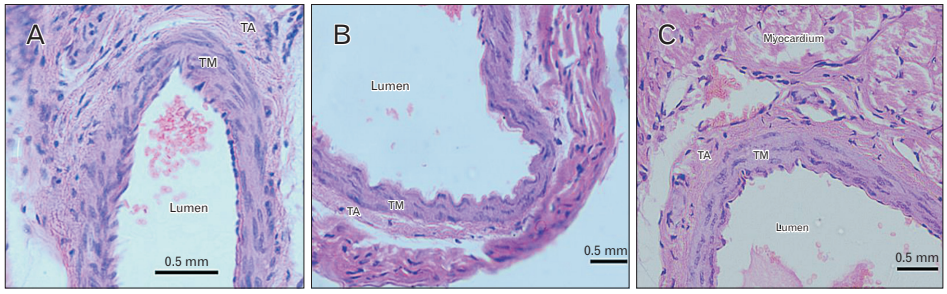

Fig. 3. Representative slides of the left coronary artery wall from the control group (A), castrated group (B), and testosterone-injected groups (C). There were fewer smooth muscle cells per unit area in the tunica media of the castrated group. Hematoxylin and eosin, ×100. TM, tunica media; TA, tunica adventitia.

Table 2. Differences in the mean smooth muscle cell densities of the control, castrated, and testosterone-injected groups using ANOVA

Study groups	Mean difference (mm)	*P*-value	95% confidence interval
Castrated vs. control	–20.84*	<0.001	–21.49 to –20.18
Castrated vs. testosterone injected	–20.56*	<0.001	–21.18 to –19.95
Testosterone injected vs. control	–0.27	0.495	–0.78 to 0.23

*Indicates statistically significant.

Table 3. Differences in the mean adventitial collagen fiber densities of the control, castrated, and testosterone-injected groups using ANOVA

Study groups	Mean difference (mm)	*P*-value	95% confidence interval
Castrated vs. control	30.5*	<0.001	29.22–31.81
Castrated vs. testosterone injected	1.4	0.036	0.07–2.82
Testosterone injected vs. control	29.1*	<0.001	28.04–30.10

*Indicates statistically significant.

collagen in the castrated and testosterone-injected groups was not visually distinguishable (Fig. 1B, C). A mean adventitial collagen fiber density of 36.1% was recorded in the control group, 66.6% in the castrated group, and 65.2% in the testosterone-injected group (Fig. 2C). Compared to controls, a significantly higher fiber density was recorded among the castrated and the testosterone injected groups (P<0.001) (Table 3).

Discussion

A link between low serum testosterone levels and various cardiovascular risk factors and diseases has been described in previous studies [27]. Low testosterone level has been associated with atherosclerosis of most large arteries, while endogenous and exogenous androgens confer a protective effect [26, 28]. The coronary arteries, carotids, and aorta are known to be particularly vulnerable to atherosclerosis [24]. 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 [14, 29]. We were interested in the association of low testosterone levels with morphological markers of atherosclerosis, such as IMT, adventitial collagen fiber density, and smooth muscle density in the LCAs.

The typical serum testosterone levels in adult male rabbits vary seasonally and may range from 1.59–32.80 nmol/L [30, 31]. Our study's serum testosterone levels were within range for the control rabbits and below the reference range for the castrated rabbits. The rabbits injected intramuscularly with testosterone regained testosterone levels within the reference range.

We found a larger IMT in the hypo-androgenic castrated rabbits than in controls. We also noted a significantly lower IMT in castrated rabbits that subsequently received testosterone injections. Cheruiyot et al. [21] similarly demonstrated increased IMT in common carotid arteries of hypogonadal rats. Few other studies indicate an inverse relationship between IMT and serum testosterone levels [17, 19], but an indication of whether testosterone administration would reverse the effects of hypogonadism on the vascular structure is barely reported.

Clinically, measuring IMT by ultrasonography is considered an early indicator of cardiovascular disease risk in individuals with hypogonadism. IMT is a reliable and sensitive marker of subclinical atherosclerosis and an independent predictor of cardiovascular events and target organ damage [32]. It is valuable in evaluating and stratifying cardiovascular disease risk, predicting long-term outcomes, and monitoring ongoing disease progression and regression [33]. The intimal thickness can be considered an adaptive mechanism to the blood flow, imparting lateral pressure and stress on the artery walls [34, 35].

The immunomodulatory effect of testosterone and its in-

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fluence on programmed cell death in vascular smooth muscle cells may explain the association of increased IMT with hypo-androgenic states. Experimental studies indicate that testosterone suppresses pro-inflammatory cytokine activity and enhances anti-inflammatory factors [36]. Testosterone also regulates the apoptosis of vascular smooth muscle cells, which contributes to the progression of intimal hyperplasia and atherosclerosis [37].

Anecdotal evidence suggests that testosterone therapy would benefit hypoandrogenic males at risk of cardiovascular disease [12, 38]. However, routine use of testosterone in clinical settings of hypo-androgenic patients with cardiovascular disease has not been adopted due to the absence of larger, prospective, placebo-controlled studies. Our observation of reversing structural changes of the LCAs after testosterone administration in hypo-androgenic models supports the mounting evidence that testosterone therapy should be considered where appropriate.

We reported a reduction of smooth muscle cell density of the tunica media of the LCAs in castrated adult male rabbits. Similar findings have been noted in the smooth muscle of the common carotid artery [21] and the penis [39] under settings of induced hypogonadism. Smooth muscle density increased to control levels in adult male castrated rabbits subsequently injected with testosterone, suggesting a reversible effect.

A reduction in myofilament quantity is described in hypogonadal states [40]. Proposed mechanisms that may explain the decrease in smooth muscle in hypo-androgenic animals include programmed cell death, atrophy, and de-differentiation of smooth muscle cells into other phenotypes [41, 42]. Kang et al. [43] observed an upregulation of angiotensin II receptors within the vascular wall in hypo-androgenic states. Angiotensin II receptors are potential activators of caspases, the primary mediators of apoptosis. Their activation also causes the downregulation of antiapoptotic molecules such as B cell lymphoma 2 (BCL2) in smooth muscle cells [43].

Certain studies have touted an 'androgen deficiency-associated atrophy' of smooth muscle cells as seen in the penile corpus cavernosum [39, 44]. This atrophy is similar to skeletal muscle atrophy in males with low testosterone levels [45]. Experimental studies that performed blockade of 5α reductase also describe the de-differentiation of smooth muscle cells into other phenotypes, such as fibroblasts and adipocytes, which partially explain the reduction of smooth muscle density in hypogonadal states [41, 46].

An *in vitro* experiment further demonstrated that testosterone administration delayed the senescence of vascular smooth muscle while inhibiting collagen synthesis in ageing vascular tissue via growth arrest-specific protein 6/Axl-8 and Akt/FoxO1a-dependent pathways [47]. These mechanisms may explain the protective effect of testosterone against vascular pathology. Our observation of a smooth muscle cell density reversal with an injection of testosterone further supports the notion that testosterone could be therapeutic in managing cardiovascular diseases in hypogonadal states.

The present study demonstrates increased collagen fiber density in the tunica adventitia of the LCAs in hypo-androgenic rabbits. Other studies have reported similar changes in collagen fibers in the common carotid artery [21] and the penis [39] under induced androgen deficiency. We also found that vascular adventitial collagen fiber density remained elevated after administering testosterone to castrated rabbits. It is plausible that an increase in collagen deposition is a long-term phenomenon that is not easily reversible with the restoration of hormone levels.

Multiple mechanisms have been proposed to explain how androgens influence vascular collagen deposition, including regulating transforming growth factor β (TGF-β) production. Testosterone suppresses the expression of TGF-β; thus, in settings of low androgen levels, upregulation of TGF-β results in fibroblast activation and deposition of collagen fibers [48]. TGF-β also induces the differentiation of fibroblasts into the more synthetic myofibroblast phenotype. Hypo-androgenic states additionally upregulate angiotensin II receptors on smooth muscle, leading to myofibroblast differentiation and increased collagen deposition [43].

Previously, the tunica adventitia was assumed to play a passive role in the nutritional and physical integrity of the wall of an artery. Fresh evidence suggests that it plays an active role in the function, structure, and development of pathological processes in the arterial wall [49]. Traditional descriptions of tunica adventitia refer to it as almost entirely composed of macrophages and fibroblasts. Ogeng'o et al. [50] have additionally demonstrated immunoregulatory cells, progenitor cells, endothelial cells, and pericytes within the adventitia of carotid and coronary arteries.

The tunica adventitia is richly fibroelastic, with collagen fibers that confer tensile strength to withstand external forces and elastic fibers that enable stretching for vasodilation and constriction [51]. A disproportionate amount of collagen

increases arterial wall stiffness and correlates with multiple vascular pathologies, including atherosclerosis and hypertension [49]. The collagen-elastin ratio is influenced by sex hormones, partly contributing to the gender disparity in cardiovascular illnesses. Fischer and Swain [52] 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. [20] showed increased collagen synthesis by adventitial fibroblasts in the coronary arteries of rats treated with testosterone. Further studies should clarify these disparate results and determine whether adventitial collagen changes in hypoandrogenism are amenable to testosterone therapy.

The partial reversal of adverse histologic changes with testosterone injection suggests that testosterone replacement therapy (TRT) might offer cardiovascular benefits [18, 30]. Clinicians managing hypogonadal patients, especially those at risk of or already diagnosed with coronary artery disease, may consider TRT as a potential therapeutic approach to mitigate the impact of hypogonadism on coronary artery morphology. However, the cautious administration of TRT and close cardiovascular monitoring would be imperative.

We acknowledge the limitations of our study. We could not account for any other morphological changes in the LCAs that may have been induced by the systemic reaction to tissue injury caused by surgical castration. We, therefore, also did sham surgeries on the scrotum in control animals to try and reproduce similar stress responses for a more precise comparison. We could not determine whether the decrease in smooth muscle composition resulted from atrophy, apoptosis, or both, and we recommend further immunohistology studies to assess the same.

In conclusion, we have shown that reduced testosterone levels are associated with the alteration of vascular structure in the LCAs in the castrated rabbit model. We have further demonstrated that some changes may be reversed with testosterone injection and serum testosterone level restoration. These findings may serve as a morphological basis to explain coronary artery disease associated with hypogonadism in males and the role of testosterone therapy in prevention. Further studies are required in human subjects to elucidate if testosterone administration may reduce the risk of coronary artery disease in hypogonadal males.

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Conceptualization: DA. Data acquisition: DA. Data analysis or interpretation: IHO. Drafting of the manuscript: DA, IHO. Critical revision of the manuscript: MMO, PBG. Approval of the final version of the manuscript: all authors.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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