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DEPARTMENT OF HUMAN ANATOMY AND PHYSIOLOGY

HISTOMORPHOMETRIC FEATURES OF THE COMMON CAROTID ARTERY OF ALBINO RATS (*Rattus norvegicus domesticus*) FOLLOWING LETROZOLE ADMINISTRATION

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A dissertation submitted in partial fulfillment of the requirements of the Master of Science Degree in Human Anatomy at the Department of Human Anatomy and Physiology, University of Nairobi.

DECLARATION

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

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DEDICATION

My daughter Grace,

How beauteous are the paths that God has destined for you!

Dream! The reality of your creative mind is in your grasp.

My dearest Emily,

For illuminating our journey to forever.

The crystal of happiness and joy.

Prof Hassan Saidi,

A true mentor, teacher and friend.

You continue to bridge mortality to inspire!

All things bright and beautiful,
All creatures great and small,
All things wise and wonderful,
The Lord God made them all.

-Cecil F. Alexander, 1848.

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LIST OF ABBREVIATIONS

AI- Aromatase inhibitor

ANOVA- Analysis of variance

AT- Adventitial thickness

bFGF- Basic fibroblast growth factor

CCA- Common carotid artery

CFD- Collagen fiber density

CIMT- Carotid intima-medial thickness

EFD- Elastic fiber density

EGF- Epidermal growth factor

MAPK- Mitogen-activated protein kinase

NO- Nitric oxide

PDGF- Platelet-derived growth factor

SEZ- Sub-endothelial zone

SMD- Smooth muscle cell density

TA- Tunica adventitia

TI- Tunica intima

TM- Tunica media

VSMC- Vascular smooth muscle cell

DEFINITION OF TERMS

Aromatase inhibitors (AIs): A class of drugs that block the enzyme aromatase, which converts androgens into estrogens in the body.

Letrozole: A type of aromatase inhibitor that is commonly used to treat hormone-responsive breast cancer in postmenopausal women.

Common carotid artery: A major blood vessel that supplies blood to the head and neck region.

Histomorphometric: Relating to the measurement and analysis of the microscopic structure and organization of tissues.

Stereological: Relating to the study of the three-dimensional structure of objects from two-dimensional images.

Intima-medial thickness (IMT): A measure of the thickness of the innermost two layers of the arterial wall, which reflects the degree of atherosclerosis.

Collagen fiber: A type of protein fiber that provides strength and flexibility to the connective tissue.

Vascular smooth muscle cell: A type of muscle cell that contracts and relaxes to regulate the diameter of blood vessels.

Adventitial: Relating to the outermost layer of the arterial wall, which contains nerves and blood vessels that supply the vessel wall itself.

Elastic fiber: A type of protein fiber that provides elasticity and resilience to the connective tissue.

Elastic lamellae: Thin sheets of elastic fibers that are arranged in parallel layers in the arterial wall, forming part of the tunic that forms the vessel wall.

SUMMARY

Background

Aromatase inhibitors (AIs) such as letrozole are used in treatment of hormone-responsive breast cancer by reducing levels of circulating estrogen. Their use has been associated with adverse cardiovascular events including myocardial infarction and stroke. Although their effect on the structure of blood vessels remains unexplored, their atherogenic potential has been demonstrated sonographically by presence of atheromatous plaques in the common carotid arteries of patients on treatment with AIs. The structural changes that underlie this are yet to be demonstrated. This study therefore sought to describe the structural effect of letrozole on the common carotid arteries.

Broad objective: To describe the histomorphometric features of the common carotid artery following letrozole administration.

Research question: What are the histomorphometric features of the common carotid artery following letrozole administration?

Study type: Quasi-experimental study

Methods: Twenty-seven male rats (3 baseline, 15 experimental, 9 control) aged three months were used for the study. The experimental group received 0.5 mg/kg of letrozole dissolved in distilled water daily by oral gavage while the control group received 2 ml of normal saline daily by oral gavage as placebo. The rats were randomly selected, euthanized, perfused and their carotid arteries harvested for histological processing on days 20, 35 and 50. Stereological techniques were used to determine the densities of different histological components. Data were analyzed using the statistical package for social sciences (SPSS V25, Chicago, Illinois). Results are presented in photomicrographs, tables and graphs.

Results: In response to letrozole administration, statistically significant temporal increases in carotid intima-medial thickness ($p=0.002$), collagen fiber density ($p<0.0001$), and vascular smooth muscle cell density ($p <0.001$) were noted. Adventitial thickness also increased albeit not statistically significant ($p=0.09$). Elastic fiber organization also displayed disruption with branching of elastic lamellae and fragmentation, and a reduction in elastic fiber density ($p=0.009$)

Conclusion: Letrozole administration is associated with increased carotid intima-medial thickness, vascular smooth muscle density and collagen fiber density and reduced elastic fiber density. These changes could affect the function of the vessel wall and may explain the greater incidence of cardiovascular disease in patients taking aromatase inhibitors.

1 INTRODUCTION

The common carotid artery (CCA) is the primary arterial supply to the head and neck (Standring, 2015). Arising from the aorta and brachiocephalic trunk on the right and left, respectively, it ascends to the neck to bifurcate into the external and internal carotid arteries (Sinnatamby, 2011; Standring, 2015). It is a large elastic artery that serves to dampen fluxes in blood flow in systole and diastole (Barrett et al., 2012). The CCA is commonly affected by atherosclerosis (Morbiducci et al., 2016). Indeed, the development of CCA atherosclerosis has been shown to correspond to the progression of atherosclerosis in other vascular beds (Ogeng'o et al., 2013). As such, morphometric measurements of the CCA, such as carotid intima-medial thickness and adventitial thickness, have found clinical utility in cardiovascular risk profiling (Ogeng'o et al., 2013).

Estrogens are steroid sex hormones responsible for the development of secondary sexual characteristics in females (Simpson, 2003). In the cardiovascular system, they have important regulatory effects on the various components of blood vessel walls. They have been found to reduce endothelial dysfunction and reduce proinflammatory thickening in all layers of blood vessel walls (Novella et al., 2012). As a result, estrogens are known to reduce the rate of atherogenesis (Miller et al., 2016; Novella et al., 2012). These modulatory benefits of estrogen on blood vessel walls are posited to be responsible for the overall reduction in cardiovascular risk in premenopausal women (Novella et al., 2012). Conversely, it is known that the incidence of cardiovascular diseases and their complications, including end-organ damage and cardiovascular events, increases in postmenopausal women (Moreau and Hildreth, 2014).

Breast cancer is a leading cause of morbidity and mortality among women. The incidence of breast cancer increases with age and is highest among post-menopausal women (Momenimovahed and Salehiniya, 2019). Aromatase inhibitors (AIs) are the preferred adjuvant treatment for estrogen-receptor (ER) positive breast cancer in post-menopausal women (Schneider et al., 2011). Though considered relatively safe, AIs have been associated with an increased risk of cardiovascular disease (Blondeaux et al., 2016). This includes the presence of atherosclerotic plaques in the CCA and changes in carotid intima-medial thickness (Skilton et al., 2011). Among the AIs commonly prescribed, letrozole has been found to have a higher association with cardiovascular disease (Zou et al., 2015). Letrozole is a medication that belongs to the class of aromatase inhibitors. It is used to treat breast cancer in postmenopausal women by

lowering the levels of estrogen (Simpson, Curran and Perry, 2004; Bhatnagar, 2007). Letrozole can cause some side effects, such as hair thinning, bone mass loss, increased risk of cardiovascular disease, and menopausal symptoms, such as hot flashes, vaginal dryness, weight gain, increased sweating, or increased cholesterol (Peters and Tadi, 2023).

While the risk for cardiovascular disease in women on treatment with letrozole is well documented, the structural changes in the vasculature that underlie these observations remain scantily explored. Moreover, while sonographic changes in the dimension of the CCA wall have been found following letrozole use (Blondeaux et al., 2016), the structural basis for these changes is yet to be described. This study therefore seeks to describe the structure of the CCA following letrozole administration.

2 LITERATURE REVIEW

2.1.1 TOPOGRAPHY AND DEVELOPMENT OF THE COMMON CAROTID ARTERY

The common carotid artery (CCA) and its branches constitute the main arterial supply to the head and neck (Sinnatamby, 2011; Standring, 2015). Its topography is similar in man and rodents (Aydin et al., 2013; Sinnatamby, 2011). It originates from the arch of the aorta and the brachiocephalic trunk on the left and right, respectively (Sinnatamby, 2011). In man, it is arbitrarily divided into three segments viz: the first from its origin to the sternoclavicular joint, the second from this point to the sixth cervical vertebra and the third distal segment from this point to its bifurcation usually at the junction of the third and fourth cervical vertebrae (Skandalakis et al., 2004).

The development of the CCA is closely related to the development of the aortic arches. The aortic arches are derived from the mesoderm and neural crest cells that contribute to the formation of the connective tissue and smooth muscle of the arterial walls (Nguyen and Duong, 2023). The CCA is derived from the proximal part of the third aortic arch, which is the first to form and the last to regress (Menshawi, Mohr and Gutierrez, 2015; Nguyen and Duong, 2023). The development of the CCA is influenced by several factors, such as the migration and differentiation of neural crest cells, the expression of various genes and signalling molecules, and the hemodynamic forces exerted by the blood flow (Hoefer, den Adel and Daemen, 2013; Garoffolo and Pesce, 2019). Some of the genes and molecules that are involved in the development of the CCA and other large vessels include bone morphogenetic proteins (BMPs), Notch, Gata family proteins, T-box transcription factors, myocardin, semaphorin family proteins and especially Hoxa3 (Khalid and Bordoni, 2023).

2.1.2 HISTOMORPHOMETRY OF THE COMMON CAROTID ARTERY

The common carotid arteries are large elastic arteries whose wall, like other vessels, is layered into tunica intima, tunica media and tunica adventitia (Ross and Pawlina, 2016). Histomorphometric dimensions of importance in the structure of the carotid artery include carotid intima-medial thickness (CIMT) and carotid adventitial thickness (AT) (Ross and Pawlina, 2016).

2.1.2.1 Carotid intima-medial thickness

The common carotid tunica intima is the innermost histological layer of the common carotid artery and consists of a single layer of endothelial cells supported by sub-endothelial connective tissue in the sub-endothelial zone (SEZ) (Ross and Pawlina, 2016). It is this zone that sometimes contains smooth muscle cells. The tunica intima is delineated from the tunica media by the internal elastic lamina (IEL) made of elastin (Ross and Pawlina, 2016). The endothelium has protean functions in vascular biology, among which are the maintenance of vascular tone and vascular homeostasis(Ganz and Vita, 2003). It is a key participant in vascular wall inflammation. Endothelial cells also elaborate mediators such as nitric oxide (NO), prostacyclin and other cyclooxygenase derivatives that are anti-spasmodic and anti-aggregating (Deanfield et al., 2007). This is known to stay the progression of atherogenesis. Indeed, it is this property that has earned the endothelium the moniker “the guardian of arterial integrity” (Arnal et al., 2010).

The CCA tunica media, on the other hand, consists primarily of multiple layers of regular, concentric elastic laminae with interspersed vascular smooth muscle cells (VSMCs) and collagen fibres (Ross and Pawlina, 2016). Each layer of elastic fibres and its associated VSMCs and collagen fibres make up the lamellar unit, also called the tension unit (Wagenseil and Mecham, 2012). These units remain throughout life, and their number is genetically determined (Wagenseil and Mecham, 2012). They confer recoil capacity to the vessel, maintaining luminal capacitance through diastole and systole (Barrett et al., 2012).

Carotid intima-medial thickness is the combined thickness of tunica intima and tunica media (Liviakis et al., 2010). This measurement is useful as a surrogate marker of atherosclerosis and is a widely accepted biomarker of cardiovascular risk (Ogeng’o et al., 2013). It is associated with the risk of cardiovascular events and mortality and constitutes a valuable tool for cardiovascular disease risk stratification (Cardoso et al., 2019; Roumeliotis et al., 2019; Sun et

al., 2020). High values of CIMT are often associated with high rates and degrees of histopathological atherosclerosis (Sun et al., 2020). In the clinical setting, it is measured sonographically. Increased CIMT occurs with and is evident in atherosclerosis.

Estrogens exert protective effects on both the tunica intima and media (Somani et al., 2019). Estrogen receptors within the endothelium and estrogen signalling modulate endothelial function and prevent endothelial activation in response to injury (Arnal et al., 2010; Simpson, 2003). Further, estrogens potentiate endothelium-dependent vasodilation (Freay et al., 1997). Within the media, estrogens are known to reduce vascular smooth muscle proliferation as well as reduce synthesis of collagen (Fardon et al., 2020; Fleenor, 2013). It is through these effects that estrogens protect against endothelial injury and reduce carotid intima-medial thickening and the attendant arterial stiffening. It is plausible that this protective effect may be attenuated by treatments that inhibit estrogen production.

2.1.2.2 Adventitial thickness

The outermost layer of the vessel, the tunica adventitia, consists predominantly of collagen fibres. Within these connective tissue fibres are interspersed fibroblasts, elastic fibres and vasa vasora (Ross and Pawlina, 2016). The structural integrity of the adventitia is maintained by the collagen fibres while the vasa vasora convey blood supply to the vessel wall. The adventitia also contains lymphatic channels, autonomic nerves and resident macrophages, T-cells, B-cells, mast cells and dendritic cells that mediate immune responses. Long thought to be inert, recent evidence has revealed that the adventitial layer in blood vessels is metabolically active, containing a stem cell niche capable of recruitment in vascular injury (Ogeng'o et al., 2017; Witter et al., 2017). Indeed, experimental removal of the tunica adventitia results in degeneration of the tunica media (Fagundes et al., 2012). The cellular components of the tunica adventitia, the fibroblasts, elaborate various paracrine and autocrine mediators key in adventitial response to inflammation and vascular injury (Majesky et al., 2011; Milutinović et al., 2019). These are growth factors and cytokines which result in fibroblast proliferation and differentiation that, in turn, lead to the synthesis of extracellular matrix components, including collagen fibres (Lacolley et al., 2012). Adventitial thickness refers to the breadth of the tunica adventitia. Due to the active contribution of the adventitia to the metabolism of the vascular wall, alterations in its dimensions may be due to its response to vascular mechanobiology (Wagenseil and Mecham, 2009). Thickening of the adventitia due to the deposition of collagen results in an overall increase in vascular wall thickness

and may result in vascular stiffening (Cecelja and Chowienczyk, 2012). This has been postulated as a possible mechanism for the development of essential hypertension. Adventitial thickening has also been demonstrated in atherosclerosis (Ogeng'o *et al.*, 2014). It is in this manner that the adventitia contributes to atherogenesis in an "outside-in" manner (Ogeng'o et al., 2017).

Within the adventitia, estrogens have been found to reduce the progression of inflammation following vascular injury (Oparil et al., 1999). Following endoluminal injury to blood vessels, the adventitia musters an inflammatory response that includes the proliferation and differentiation of adventitial fibroblasts to myofibroblasts (Milutinović et al., 2019). These myofibroblasts express α-smooth muscle actin and are involved in adventitial thickening (Ogeng'o et al., 2014). Estrogens have been shown to attenuate this response (Dehaini et al., 2018). They are posited to act in a similar manner to reduce vascular inflammation and limit vascular damage in atherogenesis. These protective effects of estrogens on the endothelium are believed to underlie the relatively low incidence of cardiovascular disease in premenopausal women (Moreau and Hildreth, 2014; Novella et al., 2012).

2.1.2.3 The role of vascular smooth muscle cells in mechanobiology of the common carotid artery

The mechanical properties of the CCA wall, as well as its luminal diameter, are a result of the interaction between the connective tissue fibres, VSMCs and other extracellular components synthesized by the VSMCs (Wagenseil and Mecham, 2012). VSMCs, therefore, play an important role in vascular contraction and relaxation. In normal blood vessels, VSMCs have a non-proliferative phenotype characterised by an abundance of contractile proteins that include α-actin as well as SM-1 and SM-2 myosin heavy chains (Bacakova et al., 2018; Lacolley et al., 2017). In atherogenesis and vascular inflammation, VSMCs change to a synthetic phenotype in which they attain migratory characteristics. In this case, VSMCs invade the tunica intima and result in intimal thickening (Bacakova et al., 2018; Lacolley et al., 2012, 2017). Further, synthetic VSMCs lay down collagen within the vessel wall, altering the connective tissue fibre composition and, hence, the mechanical properties of the vascular wall (Lacolley et al., 2017).

Estrogens are known to inhibit neointimal formation by reducing VSMC proliferation (Krom et al., 2007). This has been demonstrated in carotid arteries as well as the aorta and iliac arteries of experimental animals (Oparil et al., 1999). This action on VSMCs results from the inhibition of the switch from contractile to synthetic phenotypes through its receptor Erα (Dehaini et al.,

2018). Further, estrogens increase the rate of apoptosis of VSMCs by activating a MAPK (Mitogen-activated protein kinase) pathway via phosphorylation of p38 (Dehaini et al., 2018). As a result of this pro-apoptotic effect, the proportion of proliferative VSMCs is reduced and overall results in the prevention of intimal and medial thickening. In states of estrogen deprivation, therefore, it is likely that this pro-apoptotic effect of estrogens is reduced, leading to VSMC proliferation, a precursor to atherosclerosis (Vinay et al., 2015).

2.1.2.4 Connective Tissue Composition of the Common Carotid Artery

The connective tissue composition of the common carotid vessel wall comprises mainly elastic and collagen fibers (Ross and Pawlina, 2016).

Collagen Fibres

Collagen fibres confer structural integrity to the vessel wall. They are interspersed within the tunica intima's elastic lamellae and VSMCs, as well as the predominantly collagenous tunica adventitia. Collagen fibres prevent vessel overstretch in systole (Chow et al., 2014). The predominance of collagen, however, as occurs in oestrogen-deficient states such as menopause (Brincat et al., 2005; Mozos et al., 2017), results in arterial stiffening. This stiffening has been implicated in the pathogenesis of cardiovascular diseases such as hypertension and atherosclerosis (Fleenor, 2013). Estrogen deficiency results in the phenotypic conversion of VSMCs from contractile to synthetic. As such, more collagen is synthesized in the vessel wall, resulting in progressive stiffening (Fardon et al., 2020).

Elastic fibres

Elastic fibres maintain compliance with elastic arteries such as the common carotid artery. This role is achieved by storing recoil energy during arterial deformation that occurs in systole. The energy thus stored allows the artery to recoil to its original state in diastole (Ross and Pawlina, 2016). It is this mechanism that dampens blood flow in large conduit arteries such as the aorta and common carotid artery, allowing for smooth flow in systole and diastole (Wagenseil and Mecham, 2009).

In states of estrogen deficiency, such as menopause, there is a reduction in elastic fibre content and an increase in the synthesis of collagen (Moreau and Hildreth, 2014). Consequently, the load within the vessel wall is transferred to the less compliant collagen fibres. As a result, there is arterial stiffening, which results in systolic hypertension and may result in further downstream cardiovascular disease progression, including atherosclerosis and heart failure(Moreau and Hildreth, 2014).

2.1.3 AROMATASE INHIBITORS

2.1.3.1 Pharmacokinetics and pharmacodynamics of letrozole

Letrozole is an oral non-steroidal aromatase inhibitor that is used to treat breast cancer in postmenopausal women. Aromatase is an enzyme that catalyzes the conversion of androgens to estrogens, which are hormones that stimulate the growth of some types of breast tumors (Simpson, Curran and Perry, 2004). Letrozole inhibits the activity of aromatase and reduces the production of estrogens in peripheral tissues (Bhatnagar, 2007). Letrozole is a third-generation aromatase inhibitor that has greater potency and selectivity than other aromatase inhibitors, such as anastrozole, exemestane, formestane, and aminoglutethimide (Peters and Tadi, 2023). Letrozole does not affect the synthesis of other steroids, such as cortisol, aldosterone, and thyroxine and also has no significant estrogenic, progestogenic, or androgenic effects (Bhatnagar, 2007).

Letrozole is well absorbed after oral administration, with a bioavailability of about 100% and peak plasma concentration within 1 to 2 hours (Mukherjee et al., 2022). Letrozole is extensively metabolized in the liver by cytochrome P450 enzymes, mainly CYP2A6 and CYP3A4, with the major metabolites being inactive and excreted in the urine and feces (Haynes et al., 2003; Mukherjee et al., 2022). The elimination half-life of letrozole is about 2 days. The plasma protein binding of letrozole is about 60%. Letrozole is generally well-tolerated, but can cause some side effects, such as hot flashes, headache, dizziness, weakness, bone pain, muscle or joint pain, swelling, weight gain, increased sweating, or increased cholesterol, mostly due to the decrease in estrogen levels (Peters and Tadi, 2023). Letrozole can also affect the bone mineral density and increase the risk of osteoporosis and fractures. Therefore, it is important to have regular medical tests and check-ups while taking letrozole

2.1.3.2 Cardiovascular disease with aromatase inhibitor use

There is scientific consensus implicating estrogens and their metabolites in the development of breast cancer (Henderson and Feigelson, 2000). Binding to their receptors in estrogen-sensitive tissues, estrogens result in tissue proliferation that can result in carcinogenesis and tumor progression (Schneider et al., 2011). This has provided the basis for the use of hormonal treatment in breast cancer (Dixon et al., 2011). Estrogen deprivation of hormone-responsive-ER-positive tumours is indeed the preferred adjuvant chemotherapeutic option (Khosrow-Khavar et al., 2020). Letrozole, a third-generation aromatase inhibitor, is a non-steroidal aromatase

inhibitor available in oral formulation. It demonstrates 99% inhibition of endogenous aromatase (Fabian, 2007). Together with other AIs, letrozole has become the first-line treatment of estrogen-responsive breast cancer in postmenopausal women. Its use has resulted in high 5-year survival rates in these patients (Schneider et al., 2011). Moreover, due to good oral bioavailability and a relatively good safety profile, it has gained wide acceptance amongst both patients and clinicians (Geisler, 2011). It is available in oral formulations in once-a-day dosage forms (Miller, 1999). It is therefore convenient and its prescription results in better patient adherence.

The use of AIs in treatment of breast cancer has been associated with cardiovascular events such as stroke and myocardial infarction (Khosrow-Khavar et al., 2020). This is postulated to be due to the inhibition of the presumed cardiovascular protection afforded by estrogens against cardiovascular disease (Miller and Duckles, 2008). Indeed, the prevalence of cardiovascular disease in premenopausal women is significantly less than in postmenopausal women (Khosrow-Khavar et al., 2020). The use of AIs in postmenopausal women, which further reduces estrogen levels in these patients expectedly results in an increase in the risk of cardiovascular disease. There is, however, a potential for redesigning new AIs to be more precisely targeted to breast tissue and have fewer systemic negative effects. Some of the possible strategies for achieving this goal are developing selective AIs that can inhibit aromatase only in the breast tissue and developing tissue-specific delivery systems involving nanoparticles, liposomes, micelles, or other carriers (Eccles et al., 2023).

Several mechanisms have been postulated to explain the cardiovascular risk associated with AI use. Their use has been associated with dyslipidemia with resultant serum hypercholesterolemia (Blondeaux et al., 2016). This increase in serum cholesterol and low-density lipoproteins has been suggested as the reason for the elevated cardiovascular risk in these patients. Moreover, there is an overall increased rate of atherosclerosis with a higher presence of carotid plaques reported (Seo et al., 2019). Such structural changes in blood vessels especially in the carotid vasculature are a known biomarker of cardiovascular risk and disease (Centurión, 2016). Further, changes in surrogate markers of atherosclerosis such as CIMT which are associated with risk of cardiovascular events and mortality have been reported in patients taking AIs (Blondeaux et al., 2015; Seo et al., 2019).

The evidence for increased cardiovascular risk in patients taking AIs is well documented (Khosrow-Khavar et al., 2020). However, there is no consensus in literature on structural

changes that underpin this risk (Blondeaux et al., 2015; Matthews et al., 2018; Seo et al., 2019). While some studies have documented structural changes in the carotid arteries in patients taking AIs, others have reported no significant differences in CIMT and stenosis between patients treated with and without AIs (Blondeaux et al., 2016; Shin et al., 2006).

2.1.4 STUDY JUSTIFICATION AND SIGNIFICANCE

Cancer is a leading cause of morbidity and mortality worldwide (World Health Organization (WHO), 2020). In Africa, cancer accounts for more than half a million deaths annually (World Health Organization (WHO), 2020). In Kenya, cancer is the third leading cause of death after infectious disease and cardiovascular disease (Macharia et al., 2019; Wambalaba et al., 2019). Among these cancer deaths in Kenya, breast cancer is second to cervical cancer as a cause of cancer-related deaths among women accounting for 23% of all cases of cancer among women (Macharia et al., 2019). The age-adjusted incidence rate for breast cancer among Kenyan women is 40.3 per 100000 with a mortality rate of 7.1 per 100000 women (Ekpe et al., 2019).

The rate of late-stage diagnosis of breast cancer (diagnosis at stage III or IV) ranges from 30% to 98% in sub-Saharan Africa (Jedy-Agba et al., 2016) . In Kenya, late-stage diagnosis accounts for around 42% of the case load (Ekpe et al., 2019). Most women diagnosed with breast cancer in Kenya are post-menopausal. Of these patients, a majority have tumors that express estrogen receptors (ER) and are termed ER-positive (Ekpe et al., 2019). Treatment of these hormone-receptor-positive tumors employs adjuvant chemotherapy of which aromatase inhibitors are the preferred treatment of choice (Schneider et al., 2011).

Aromatase inhibitors are increasingly the treatment of choice for breast cancer, which is a leading cause of cancer-related deaths in Kenya and, indeed, worldwide(Schneider et al., 2011). The use of AIs is indicated in most of these patients since a majority are postmenopausal and have hormone receptor-positive tumors(Miller, 1999). Like other AIs, Letrozole, which is commonly prescribed in Kenya has been associated with cardiovascular events such as stroke and myocardial infarction (Mouridsen et al., 2007). This is especially poignant for Kenya as there is a structural predisposition to cardiovascular disease in the Kenyan population due to the relatively high rate of anatomical variations (Ogeng'o et al., 2013). While the cardiovascular risks with use of AIs are well documented, data are lacking on the structural changes that underlie this observation.

2.1.4.1 Study Significance

The findings of this study may contribute to the understanding of the risk profile of AIs, which are known to increase the incidence of cardiovascular events. This may inform caution in long-term follow-up of patients on treatment for breast cancer with AIs who, due to longer survival, may suffer morbidity and mortality due to cardiovascular disease. The study also contributes to

the existing knowledge on the mechanisms and consequences of aromatase inhibition on the vascular system therefore opening up new avenues for further research on the effects of letrozole and other aromatase inhibitors on different blood vessels and organs, as well as the possible ways to mitigate or reverse these effects.

2.1.5 CONCEPTUAL FRAMEWORK

Estrogens are known to exert vasoprotective effects that are the reason for less incidence of cardiovascular disease in premenopausal women. Estrogen deficiency results in changes in blood vessels as is seen in menopause. Letrozole, an aromatase inhibitor commonly prescribed to postmenopausal women for adjuvant treatment of breast cancer, is associated with an increased risk of cardiovascular events due to estrogen deprivation. The structural underpinning of this observation in the carotid arteries remains undescribed.

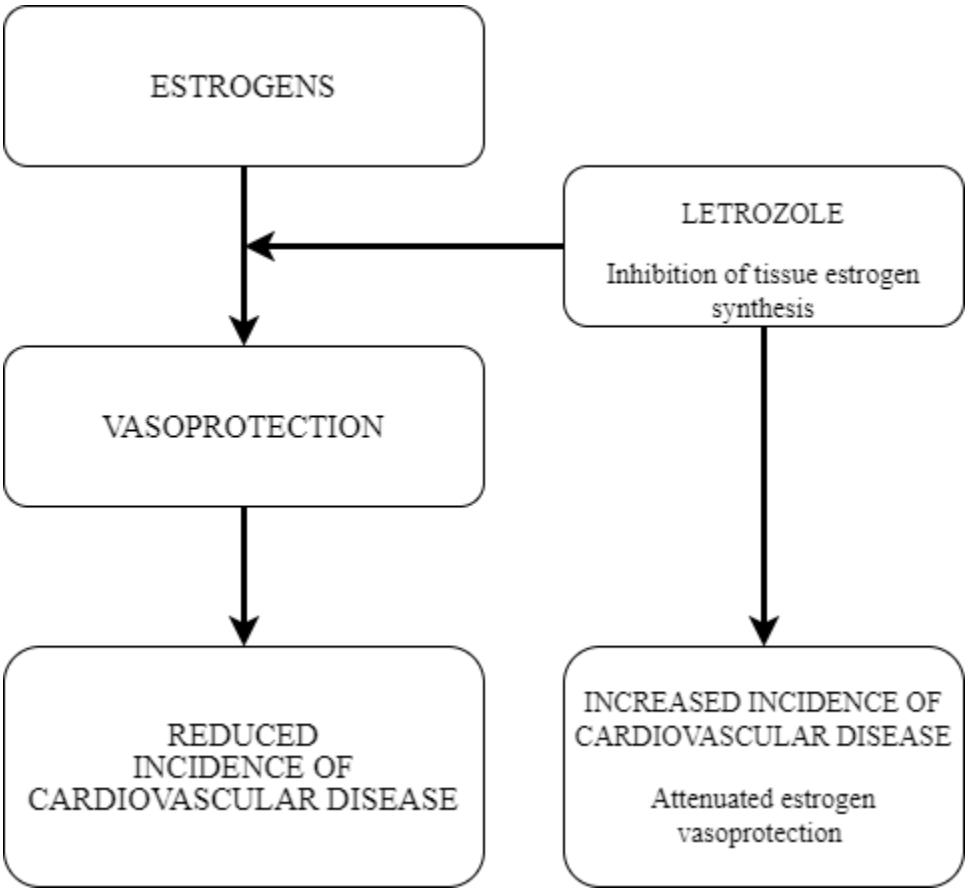

Figure 1: Conceptual framework

2.2 HYPOTHESIS

H_0 - There are no differences in the histological features of the Albino rat's common carotid artery following letrozole administration.

2.3 STUDY OBJECTIVES

Broad Objective

To describe the histological features of the common carotid artery of Albino rats *(Rattus norvegicus domesticus)* following administration of letrozole.

Specific Objectives

To determine the differences in the common carotid arteries of Albino rats *(Rattus norvegicus domesticus)* with and without administration of letrozole by comparing the:

1. Carotid intima-medial thickness
2. Adventitial thickness
3. Composition and distribution of vascular smooth muscle cells.
4. Connective tissue composition and organization.

3 MATERIALS AND METHODS

Study Design

This was a quasi-experimental study. The type of quasi-experimental design used in the study is the nonequivalent groups design. This is because the researcher compares two or more groups that are not randomly assigned, but instead are formed by selecting subjects that are in-bred, which is then a non-random criterion.

Study Setting

To reduce the number of animals used in experimentation, the study was nested in a quasi-experimental study investigating the effect of Letrozole on the cerebellar cortex (*APPENDICES II and III*) .

Rats for the study were obtained from the Department of Zoology Animal House, University of Nairobi. Rats were housed in the Department of Veterinary Anatomy and Physiology Animal House where in the study intervention was carried out. The harvested specimens were processed at the Department of Human Anatomy, University of Nairobi.

3.1 MATERIALS

Rats as the study model

Rodents are the preferred animal models for biomedical research due to their genetic, physiological, and anatomical similarity to humans, ease of maintenance, short life cycle and their small size (Homberg et al., 2017). Male rats were chosen as ovarian aromatase is relatively insensitive to AIs. Ovarian production of estrogens would have acted as a confounder in this study that focuses on the effects of letrozole which is commonly prescribed in postmenopausal women. It has also been noted that men display a higher level of tissue aromatase activity and expression compared to females (Biegon, 2016).

The rats used in the above study are albino rats (*Rattus norvegicus domesticus*), which are a type of laboratory rat with a genetic mutation that causes a lack of pigmentation in their skin, fur, and eyes. They are a direct descendant of the Norway rat (*Rattus norvegicus*), which is a common wild rat species that originated in northern China and spread to other continents through human activities. Albino rats were first domesticated in the 18th century by ratcatchers who used them for rat-baiting, a sport that involved pitting dogs against rats in a pit. Later, they were bred for scientific research, especially in the fields of physiology, pharmacology, and genetics. Albino

rats are one of the most widely used animal models in biomedical research, as they have many advantages such as easy breeding, low cost, high similarity to humans, and availability of genomic tools. This strain is also known for its docile temperament, large body size, long lifespan, and susceptibility to various diseases and environmental factors.

The albino rat is an appropriate model for cardiovascular research because it has a similar cardiovascular system to humans, and it can develop atherosclerosis and hypertension under certain conditions (Cheruiyot *et al.*, 2018b). The albino rat model has been successfully used in other related studies, such as those investigating the effects of different drugs on the coronary artery, the aorta, and the heart.

However, some limitations of this model are that it may not fully reflect the human response to letrozole, as there are differences in metabolism, pharmacokinetics, and gene expression between rats and humans (Homberg *et al.*, 2017). Therefore, caution should be exercised when extrapolating the findings from this model to human patients.

3.2 SAMPLE SIZE CALCULATION

Sample size estimation was calculated using the method described by Arifin and Zahirrudin, 2017. The between-subject error degrees of freedom (that is, the within-subject degrees of freedom) is calculated as:

DF = $N - k = kn - k = k(n - 1)$.

where N = total number of subjects

k = number of groups, and n = number of subjects per group.

By rearranging the formula, n is given as:

$n = DF/(k + 1)$

Based on the acceptable range of the DF, the DF in the formulas are replaced with the minimum (10) and maximum (20) DFs to obtain the minimum and maximum numbers of animals per group:

Minimum $n = 10/(k + 1)$

Maximum $n = 20/(k + 1)$

Substituting into the equation for the three groups:

Minimum n= 10/(3+1)= 4.3 which rounded off is four animals per group (Arifin and Zahiruddin, 2017).

To account for attrition, a group size of 5 rats was selected. However, only 3 rats were used in the control group as per ethical requirements. An additional 3 rats were used to demonstrate the baseline histological features of the common carotid artery.

N = (5*3) + (3*3) +3 = 27 rats

Hence, a total of 27 rats were used for the study.

3.3 ETHICAL CONSIDERATIONS

Approval to conduct the parent study was sought from the Biosafety, Animal Use and Ethics Committee, Faculty of Veterinary Medicine, University of Nairobi (Reference: *FVM BAUEC/2020/249, Appendix II*). Approval to nest this study to reduce the use of additional animals for experimentation was sought and obtained from the Chairman, Department of Human Anatomy.

The study was conducted and the animals handled according to the guidelines provided by the committee.

3.4 SELECTION CRITERIA

Inclusion criteria

Adult male rats aged three months were used in the study. This is because at this age they are considered to be sexually mature and physiologically stable. Younger rats may not have fully developed their reproductive and cardiovascular systems, while older rats may have age-related changes or diseases that could affect the results of the study. The rats were also pure in-bred rats.

Exclusion criteria

Rats with any obvious demonstrable pathology. Presence of pathology was established by observation and assistance from the animal house attendants.

Female rats were also excluded due to the ovarian aromatase that is relatively insensitive to AIs alone.

Sampling technique

All the rats were assigned a number randomly between 1 and 27. The rats were labelled using a non-toxic permanent marker on the base of their tails. The rats were split into 2 groups, the control group that was administered normal saline by oral gavage while the experimental group that was administered letrozole. Three animals were used to demonstrate the baseline histology of the carotid artery. Microsoft Excel Software was used to obtain random non-repeating numbers for grouping the rats. The first three numbers obtained served as the baseline. The next nine numbers obtained were assigned to the control group. The remaining 15 rats were assigned to the experimental group. The numbers assigned to the rats and the group to which they belong were then entered into Microsoft Excel 2013 for record-keeping purposes.

3.5 EXPERIMENT PROTOCOL

Timings for Perfusion

3 rats were perfused on Day 0 to demonstrate baseline histological features of the carotid arteries. 7 rats (3 control; 5 experimental) were perfused on day 20, 35 and 50 of the study (**Table 1**).

Table 1:Perfusion timings and animal grouping

DAY	CONTROL (normal saline)	EXPERIMENTAL (0.5 mg/kg letrozole)	Total
Day 0 (Baseline)	3		3
Day 20	3	5	8
Day 35	3	5	8
Day 50	3	5	8
Total	12	15	27

It has been hypothesized that 10 rat days are approximately equal to a human year (Sengupta, 2013). This was used as a basis for determining the perfusion timings in the current study.

On day 20: Equivalent to 2 human years. Time period over which AI use is considered safe (Gangadhara and Bertelli, 2009).

On day 35: Equivalent to about 3.5 human years.

On day 50: Equivalent to 5 human years. This time period corresponds to the mean duration for which AIs are taken as adjuvant therapy (Dellapasqua and Colleoni, 2010).

Handling of Animals

The rats were housed in standard cages measuring 109cm by 69 cm by 77.5cm. The rats were housed in clean and comfortable cages that were appropriate for their size, number, and behavior. The cages were made of durable and non-toxic materials, such as plastic and stainless steel. The cages were floored with bedding and nesting materials such as wood shavings. The bedding and The cages were floored with wooden shavings which were replaced every two days, or more often if they are wet or soiled, after cleaning the cages. The cages are also sanitized periodically using appropriate disinfectants and methods. Each cage had about 5 animals. The cages were placed in a room with controlled temperature and humidity in a normal 12hour light/dark diurnal cycle. The rats were kept in their cages for 7 days for acclimatization. The rats were fed with a standard rodent diet that met their nutritional requirements. The diet was provided in the form of rodent pellets, which were placed in a food hopper attached to the cage. The food hopper was checked daily and refilled as needed. The rats also had access to fresh water from a water bottle *ad libitum*. The water bottle or the water line was also checked daily for leaks and blockages, and the water quality was monitored regularly during the study period. The rats were handled under supervision of the animal house attendants. The rats were observed daily for any signs of disease, illness, or injury, such as changes in body weight, appetite, behavior, activity, coat condition, respiration, posture, or vocalization. Any abnormal findings were reported to the attending veterinarian assistant and the principal investigator.

Occupational Safety while handling the rats

The animals were handled carefully with the aid of bite-resistant gloves in order to prevent injury by biting or scratching. During transfer, feeding or cleaning, the animals were held at the base of the tails by using the index finger and thumb of the non-dominant hand. Protective equipment including a clean laboratory coat, sterile latex gloves and a gas mask was worn during fixing and tissue processing. This was done to reduce chances of getting into direct contact with skin and also inhalation of carcinogenic reagents such as formaldehyde. Sharps were disposed of in safety disposal containers.

Treatment Protocol

Each rat in the experimental group received a daily dose of 0.5 mg/kg letrozole dissolved in distilled water by oral gavage. Letrozole was administered orally as this is the preferred route in humans (Dellapasqua and Colleoni, 2010). This dosage was derived from the FDA-approved dose of 2.5 mg daily by calculation *a la* (Nair and Jacob, 2016). One letrozole tablet (2.5mg) was dissolved in 10 ml of distilled water and divided into five equal aliquots measuring 2 ml each. These were then administered to each rat in the experimental group by oral gavage. Due to the slightly lower solubility of letrozole in aqueous solutions (Hossain et al., 2014; Letrozole | C17H11N5 - PubChem, n.d.), the solution was shaken before each delivery in order to ensure uniform dosage delivery to each rat. The control group received 2 ml of normal saline by oral gavage daily as a placebo (**Table 2**).

GROUP	**TREATMENT PROTOCOL**
Experimental	2 ml of 0.5 mg/kg letrozole dissolved in distilled water by oral gavage
Control	2 ml of normal saline by oral gavage

Table 2:Treatment protocol used in the study

3.6 TISSUE HARVESTING AND PROCESSING

Tissue harvesting

The rats were weighed and then euthanized by placing them in sealed containers with 1% halothane soaked in cotton wool. Death was confirmed by the absence of heartbeat and ocular reflexes. Following death, a longitudinal incision in the midline of the body was made and normal saline was used via the trans-cardiac method to flush out all the blood. Thereafter, 10% formal saline was infused to start tissue fixation. The carotid arteries were dissected out using a standard protocol that involves exposing the neck region, isolating the common carotid artery from the surrounding tissues, releasing the branches of the artery, and cutting the artery at the proximal and distal ends. The artery would then be stored in a fixative solution until further processing. Both the right and the left carotid arteries were harvested from each rat.

Tissue processing

The study used a representative segment of the artery, the middle and the distal two-thirds of the carotid artery, which may have more pronounced changes than the proximal part. The carotid artery samples were processed for light microscopic examination by paraffin wax embedding. The samples were placed in specimen bottles containing 10% formalin for at least 24 hours in order to preserve the tissues in their living state. Following fixation, they were dehydrated in ascending grades of ethyl alcohol, starting from 70% alcohol to absolute alcohol at one-hour intervals. The tissue was cleared in toluene for 2 hours and then infiltrated with paraffin wax in the oven at 58°C overnight. Within the wax blocks, each tissue slice was oriented along its sagittal plane. After cooling, the embedded tissues were blocked using wooden blocks to facilitate sectioning by the rotary microtome. They were serially cut into 7 micrometer thick sections using a microtome (Leica® Model SM2400, Leica Microsystems, Nussloch GmbH, Germany). The sections were floated in a warm water bath to enhance spreading. The sections were fished from the water bath, onto a glass slide smeared with egg albumin adhesive. It was dried in an oven at 38°C for 12 hours.

Selection of sections

During sectioning of a carotid artery section, two ribbons of 16 successive 'cuts' were obtained. The third section was identified and picked after which every third section was floated. This gave a total of 5 sections for every ribbon. Hence, a total of 10 sections per block were selected. This process was repeated for each block. These sections were then stained as outlined below.

3.7 STAINING

Hematoxylin and Eosin staining

Dewaxing was done in three changes of xylene, each change taking 8 minutes. This was followed by rehydration starting from 50:50 xylol then descending grades of alcohol (100%, 95% ad 70%) for three minutes in each change. The slides were dipped in Ehrlich's Hematoxylin for 15 minutes then washed under running water for 30 minutes to remove excess stain. The slides were dipped in 1% acid alcohol solution to further remove excess stain. They were placed in running water for another 30 minutes in order to allow bluing of the sections. This was followed by staining the sections in 1% Eosin solution for 3 minutes, followed by dehydration in ascending grades of ethanol from 70% to absolute alcohol. The sections were cleared in two changes of xylene, each 8 minutes, before mounting using DPX mountant and observing under a light microscope.

Masson's Trichrome Staining

Dewaxing was done in three changes of xylene, each change taking 5 minutes. This was followed by rehydration starting from 50:50 xylol after which the tissues were dipped in descending concentration of ethanol for 3 minutes in each change. The slides were then kept in Iron Hematoxylin for 15 minutes. They were then differentiated in acid alcohol before being blued in running water for one hour. They were then dipped in Ponceau stain for 6 minutes followed by distilled water after which they were placed in mordant (Bouin's solution) for 4 minutes, dipped in distilled water and then kept in light green stain for 2 minutes. This was followed by dehydration in ascending grades of ethanol. Afterwards, the specimen was cleared in two changes of xylene before being mounted onto glass slides for light microscopy.

Weigert's elastin stain

Dewaxing was done in three changes of xylene, each change taking 5 minutes. This was followed by rehydration starting from 50:50 xylol after which the tissues were dipped in descending concentration of ethanol for 3 minutes in each change. The slides were then kept in Weigert's Hematoxylin for 15 minutes. They were then blued in running water for one hour. After this, the slides were kept in resorcin-fuchsin solution for 90 minutes. They were rinsed in acid alcohol and kept in Van-Gieson's solution for 1 minute. This was then followed by dehydrating them in ascending grades of ethanol. Afterwards, the specimen was cleared in two changes of xylene before being mounted for light microscopy.

3.8 HISTOMORPHOMETRIC ANALYSIS

Out of the 10 stained sections, the odd sections were chosen for histomorphometric analysis. Photomicrographs were taken using a Zeiss™ digital photomicroscope (Carl Zeiss AG Oborkochem, Germany). These photographs were entered into Fiji Image J Software Version 1.53 (National Institutes of Health Image Program).

3.8.1 Determination of carotid intima-medial thickness and Adventitial thickness

For measurements of the CIMT and AT four random points of the arterial wall were measured and their average calculated to obtain the CIMT and adventitial thickness (AT) **(Figure 2)**

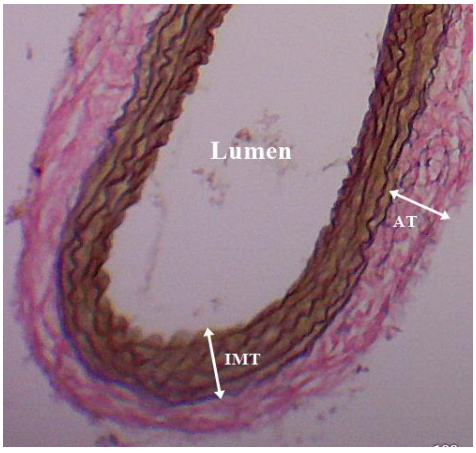

Figure 2: Photomicrograph of the common carotid artery showing the measurement of the carotid intima-medial thickness and the adventitial thickness. (Weigert Elastin, Magnification=X100)

$$CIMT = (CIMT_1 + CIMT_2 + CIMT_3 + CIMT_4) / 4$$

$$AT = (AT_1 + AT_2 + AT_3 + AT_4) / 4$$

3.9 VOLUME DENSITIES OF THE COMPONENTS OF THE COMMON CAROTID ARTERY

3.9.1 Estimation of vascular smooth muscle cells density

Vascular smooth muscle area density estimation was also done using the Delesse principle of point counting (Mandarim-de-Lacerda, 2003). The selected histological area was analyzed using a superimposed grid on the digital image using Fiji ImageJ Software (**Figure 3**). The volumetric densities (Vv) of the histological structures were evaluated while unaware of the source of the tissue samples.

3.9.2 Estimation of connective tissue fiber density

Connective tissue volumetric density estimation was done using the Cavalieri principle of point counting (Mandarim-de-Lacerda, 2003) and data expressed as volumetric densities (%). From each animal, 5 different 7 µm sections were sampled by simple random sampling. Three random fields from these sections were then sampled and the images captured at X1000 were loaded into Fiji ImageJ software. Following the technique described by Gundersen et al., (1988) (Gundersen et al., 1988), the selected histological areas were analyzed using a superimposed 100-point grid on the digital images on the monitor screen. Such a grid system produces point probes regularly arranged, which are used to facilitate estimation of the specific tissue densities (Mandarim-de-Lacerda, 2003).

In isotropic tissue, the distribution area of a structure, as determined on a two-dimensional section of a structure, is proportional to the volume distribution of the structure (Mandarim-de-Lacerda, 2003). The volume densities of the histological components were calculated by the formula Vv = P*p*/P*t*, where Vv is the volume density, *p* is the tissue component under consideration (smooth muscle, collagen or elastic fibers), P*p* is the number of test points associated with *p*, and P*t* is the total number of points of the test system.

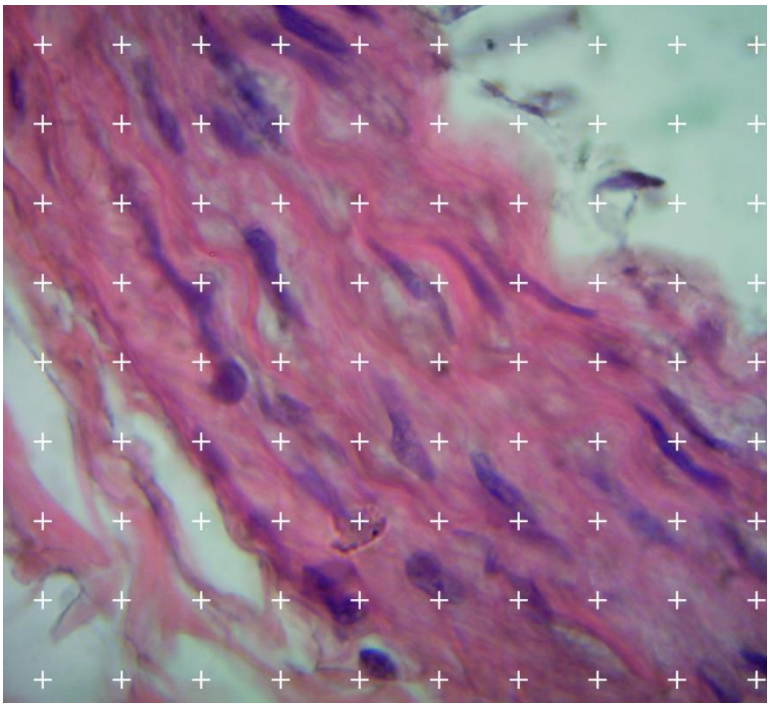

Figure 3:Image showing the point counting grid superimposed onto the photomicrograph of the common carotid artery. (Haematoxylin and Eosin, Magnification=X1000)

3.10 DATA ANALYSIS AND PRESENTATION

The data was sorted, cleaned, coded and entered into the statistical package for social sciences (SPSS V25, Chicago, Illinois). Normality of the data was assessed using visual inspection of histograms and Q-Q plots. Continuous variables were summarized as means ± standard deviation. One-way analysis of variance with Scheffe's *post hoc* test was used to check for statistically significant differences over time in both the control and experimental group over the study period. p value less than 0.05 was considered statistically significant.

Photomicrographs were used to demonstrate the histological findings. Data are presented in tables, line graphs and box and whisker plots.

4 RESULTS

4.1 GENERAL CHARACTERISTICS OF THE STUDY ANIMALS

All the animals recruited for this study had normal weight gain (Figure 4 below). They displayed normal social behaviour throughout the course of the experiment. None of the animals was lost to either disease or death. No obvious gross differences were evident between the intervention and control groups.

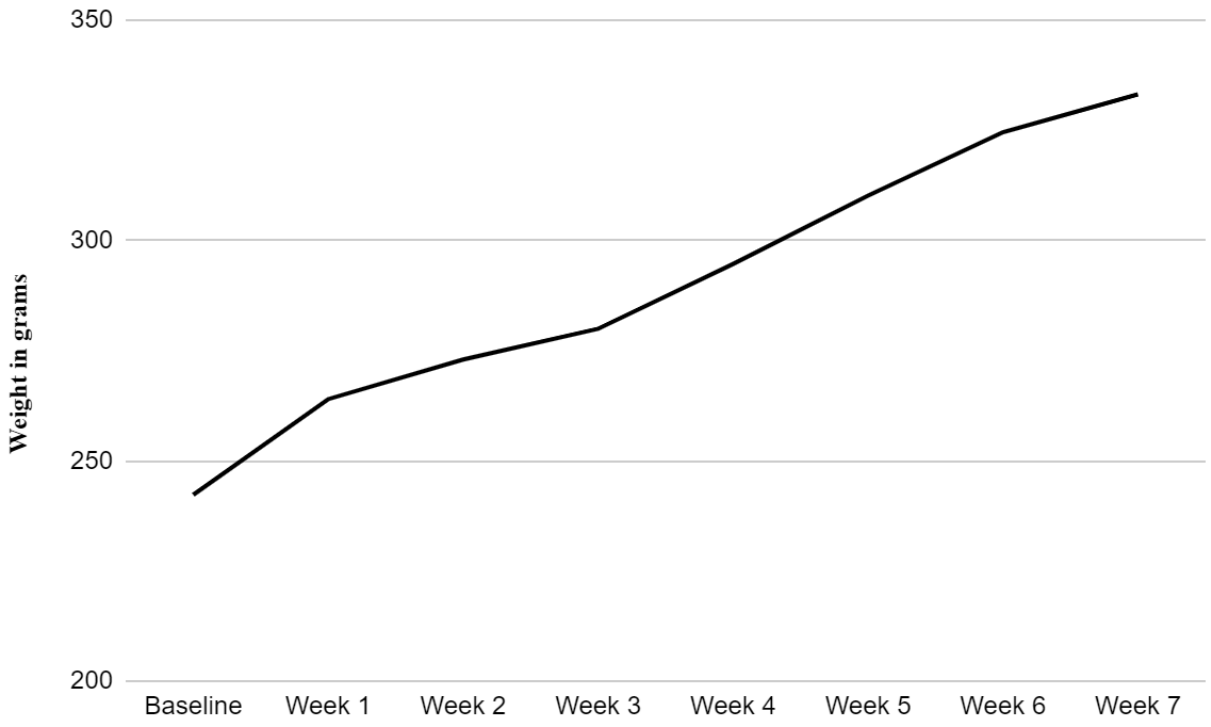

Figure 4: Trend of the average weight of the rats during the duration of the experiment

4.2 HISTOLOGICAL ORGANIZATION OF THE COMMON CAROTID ARTERY

The CCA studied showed features of an elastic artery. The vessels were organized into three histological tunics: the tunica intima, media and adventitia with the media being most prominent (**Figure 5**).

The tunica intima comprised a single layer of squamous endothelial cells which lay on a subendothelial zone of connective tissue **(Figure 5B)**. Tunica intima was separated from tunica media by the innermost elastic lamella, the internal elastic lamina (**Figure 5B)**. Tunica media itself was made of circumferential lamellae of connective tissue, predominantly elastic fibers **(Figure 5D)**. Interspersed amongst connective tissue lamellae were fusiform vascular smooth muscle cells (**Figure 5B)**. The outermost elastic lamella separated the tunica media from the tunica adventitia. The tunica adventitia was itself predominantly collagenous with collagen fibers arranged in a circumferential feltwork around the vessel (**Figure 5C)**.

LEGEND: HISTOLOGICAL ORGANIZATION OF THE COMMON CAROTID ARTERY

Figure 5A: Photomicrograph showing the histological organization of the common carotid artery. Note that the vessel is surrounded by three tunics. Shown in this section is the tunica media (TM) and tunica adventitia (TA). (Haematoxylin and Eosin, Magnification=X100).

Figure 5B: Photomicrograph showing the histological organization of the common carotid artery. Note the fusiform vascular smooth muscle cells (black arrows) in the tunica media (TM). Note also the tunica intima (white arrow) (Haematoxylin and Eosin, Magnification=X1000).

Figure 5C: Photomicrograph showing the histological organization of the common carotid artery. Note the squamous endothelial cells (black arrows). The tunica media (TM) is interposed between the tunica intima and the collagenous fibrous tunica adventitia (TA) (Masson's Trichrome, Magnification=X1000).

Figure 5D: Photomicrograph showing the histological organization of the common carotid artery. Note the tunica intima, separated from the tunica media (TM) by the internal elastic lamella (white arrows). Note also the continuous bands of the elastic fibers (black arrows) in the tunica media (TM) and fewer elastic fibers interspersed within the tunica adventitia (TA) (Weigert's Elastin, Magnification=X1000).

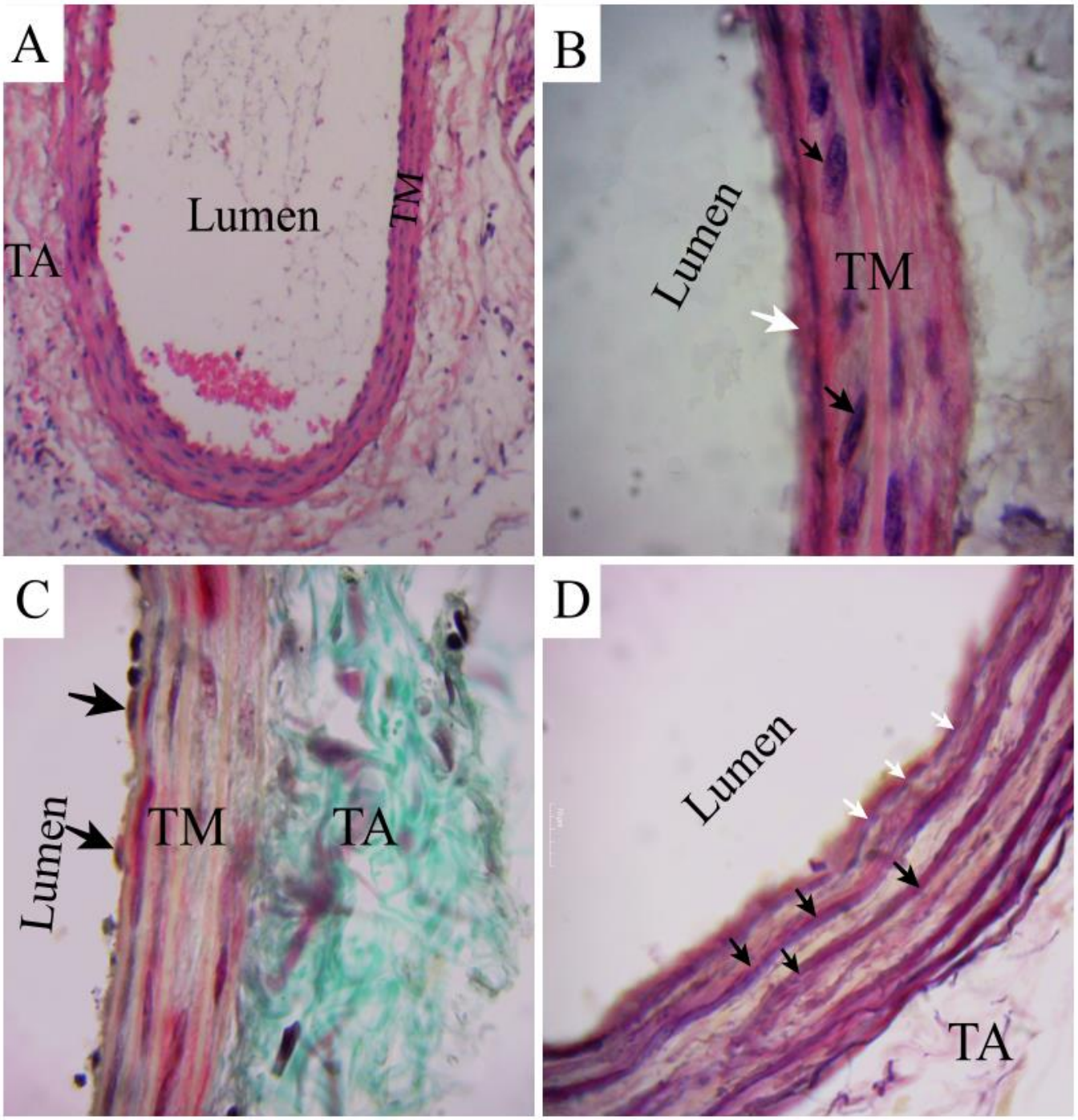

Figure 5: Photomicrographs showing the histological organization of the common carotid artery

4.3 HISTOMORPHOMETRIC CHANGES IN THE COMMON CAROTID ARTERY

Salient observations among the experimental animals revealed intimal thickening with resultant increase in carotid intima-media thickness and increased adventitial thickness.

4.3.1 CAROTID INTIMA-MEDIAL THICKNESS

Consequent to administration of letrozole, the CIMT of experimental animals increased significantly with the most significant difference being between the animals in the third sacrifice and the controls ($p<0.001$) (**Table 3)**. The average CIMT in control animals was 266.3µm ±20.57. In experimental animals, the CIMT was 307.9 ±29.8 µm, 374.8 ±55.1 µm and 490.9 ± 61.8 µm (**Figure 6).**

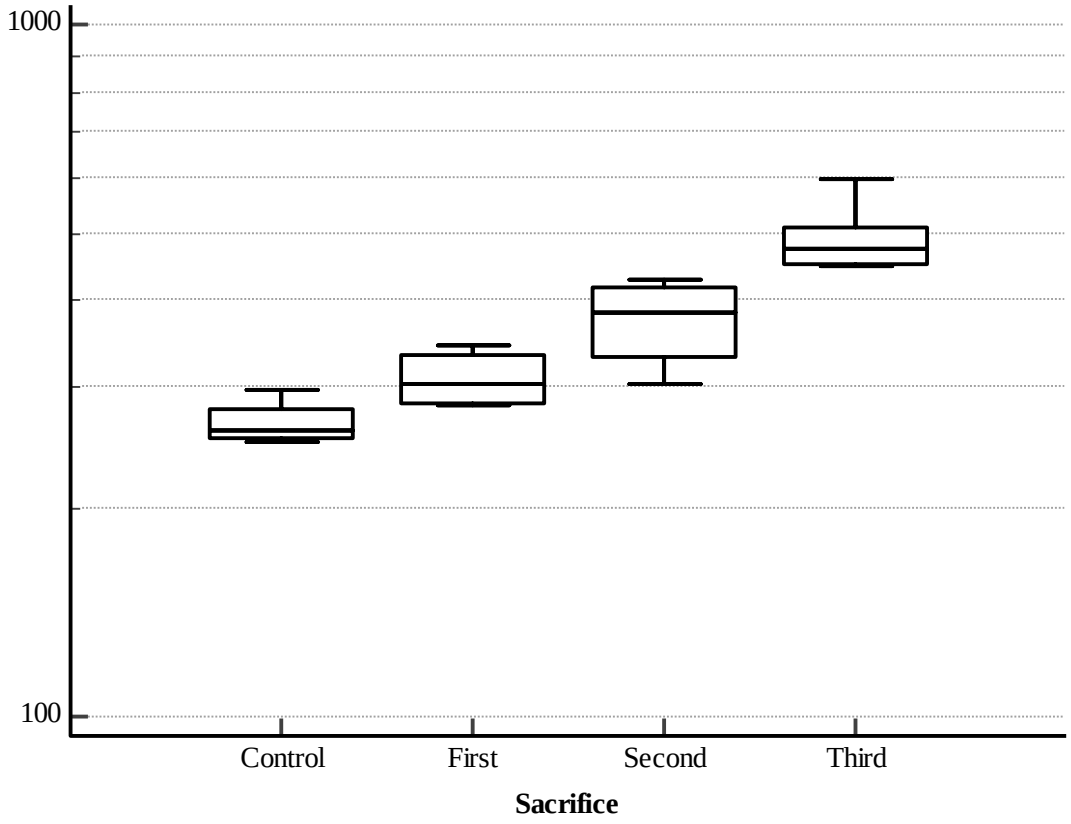

Figure 6: Box and whisker plot showing carotid intima-medial thickness in different animal groups

Table 3: Carotid intima-medial thickness (CIMT) in different animal groups

CIMT by Sacrifice	N	Mean (µm)	Mean SE*	SD	
Control	9	266.28438	23.341405	20.56829	
First	5	307.92500	23.341405	29.85715	
Second	5	374.75000	23.341405	55.07586	
Third	5	490.90000	20.877187	61.82059	
Pooled	24			46.68281	
Scheffé all comparisons					
Contrast	**Mean difference**	**Simultaneous 95% CI**		**SE**	**p-value**
Third - Control	224.61563	124.44623 to 324.78502		31.315781	**<0.001**
Third - First	182.97500	82.80561 to 283.14439		31.315781	**0.0006**
Third - Second	116.15000	15.98061 to 216.31939		31.315781	**0.0212**
Second - Control	108.46563	2.87781 to 214.05344		33.009731	**0.0432**
Second - First	66.82500	-38.76281 to 172.41281		33.009731	**0.2967**
First - Control	41.64063	-63.94719 to 147.22844		33.009731	**0.6693**

4.3.2 ADVENTITIAL THICKNESS

Average adventitial thickness in the control group was 722.4 ± 62.9 µm. The AT in experimental animals was 765.8 ± 71.0 µm, 741.1 ± 82.1 µm and 865.6 ± 72.3 µm in the first second and third sacrifice respectively (**Figure 7)**. This was a statistically significant increase in AT (p=0.03) in experimental animals with the most significant difference being between the animals in the third sacrifice and the controls (**Table 4)**. However, the differences between the animals in the other sacrifice groups and the controls were not statistically significant.

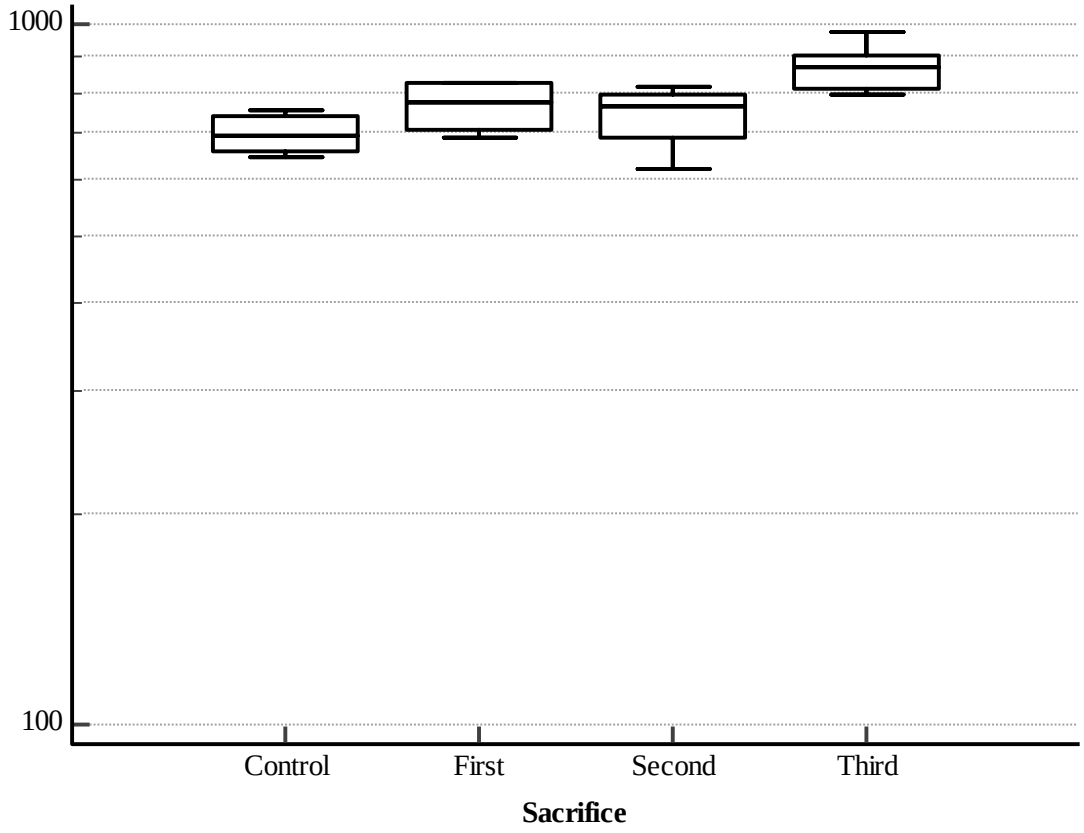

Figure 7: Box and whisker plot showing adventitial thickness in different animal groups

Table 4: Adventitial thickness in different animal groups

AT by Sacrifice	N	Mean	Mean SE*	SD	
Control	9	697.4125	34.97888	49.4601	
First	5	765.8000	34.97888	71.0235	
Second	5	741.0500	34.97888	82.0981	
Third	5	865.6400	31.28606	72.3377	
Pooled	24			69.9578	
Scheffé all comparisons					
Contrast	**Mean difference**	**Simultaneous 95% CI**		**SE**	**p-value**
Third - Control	168.2275	18.1160 to 318.3390		46.92909	**0.0262**
Third - Second	124.5900	-25.5215 to 274.7015		46.92909	**0.1200**
Third – First	99.8400	-50.2715 to 249.9515		46.92909	**0.2588**
First – Control	68.3875	-89.8439 to 226.6189		49.46761	**0.6044**
Second - Control	43.6375	-114.5939 to 201.8689		49.46761	**0.8533**
First – Second	24.7500	-133.4814 to 182.9814		49.46761	**0.9679**

4.4 COMPOSITION AND DISTRIBUTION OF VASCULAR SMOOTH MUSCLE CELLS

The average smooth muscle cell density (SMD) in the control group was 15.4%. In experimental animals, the average VSMC density was 19.5%, 25.4% and 40.2% in the first, second and third sacrifice respectively (**Figure 8).** This was a statistically significant ($p<0.001$) increase compared to control animals. Analysis of variance with Scheffe's post-hoc test revealed the difference to be greatest between animals sacrificed on day 50 (third sacrifice) and controls. A statistically significant change was also noted between the animals in the third sacrifice group and those in the second sacrifice group. The change in VSMC density was thus most evident in animals that had received letrozole for a longer period of time **(Table 5)**.

Compared to control animals, the CCA in experimental animals showed a progressive change in structure. CCA sections from experimental animals showed abundant smooth muscle cells localized in the tunica media and organized circumferentially around the vessel (**Figure 9**). Further, there was noted intimal thickening in the experimental animals (**Figure 9).**

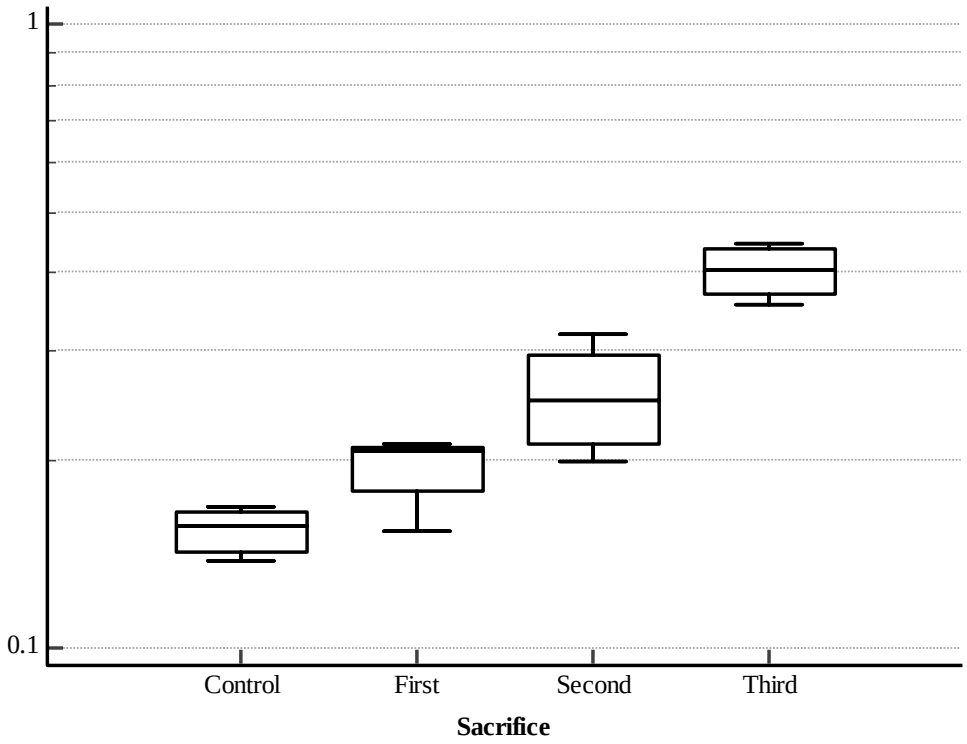

Figure 8: Box and whisker plot of vascular smooth muscle density in different animal groups

Table 5: Vascular smooth muscle density (SMD) in different animal groups

SMD by Sacrifice	N	Mean	Mean SE*	SD	
Control	9	0.154433333	0.021598598	0.015303359	
First	5	0.195130303	0.018704934	0.027422103	
Second	5	0.254641396	0.018704934	0.052514513	
Third	5	0.401907546	0.016730202	0.038066903	
Pooled	24			0.037409869	
Scheffé all comparisons					
Contrast	**Mean difference**	**Simultaneous 95% CI**		**SE**	**p-value**
Third - Control	0.247474214	0.159069131 to 0.335879296		0.027320305	**<0.0001**
Third - First	0.206777243	0.125571989 to 0.287982496		0.025095303	**<0.0001**
Third - Second	0.147266150	0.066060896 to 0.228471404		0.025095303	**0.0008**
Second - Control	0.100208064	0.007751815 to 0.192664312		0.028572259	**0.0323**
Second - First	0.059511093	-0.026086760 to 0.145108946		0.026452772	**0.2225**
First - Control	0.040696971	-0.051759278 to 0.133153219		0.028572259	**0.5831**

LEGEND: VASCULAR SMOOTH MUSCLE CELLS IN CONTROL AND EXPERIMENTAL RATS

FIGURE 9A: Photomicrograph showing the histological organization of the common carotid artery of a rat in the control group. Note the squamous endothelial cells (black arrows) in the tunica intima. Note the fusiform vascular smooth muscle cells (white arrowheads) in the tunica media (TM). Note the regular arrangement of these cells. Note also fibrous tunica adventitia (TA) (Haematoxylin and Eosin, Magnification=X1000).

FIGURE 9B: Photomicrograph showing the histological organization of the common carotid artery of a rat in the experimental first sacrifice group. Note the increased vascular smooth muscle cells (white arrowheads) in the tunica media (TM (Haematoxylin and Eosin, Magnification=X1000).

FIGURE 9C: Photomicrograph showing the histological organization of the common carotid artery of a rat in the experimental second sacrifice group. Note the increased vascular smooth muscle cells (white arrowheads) in the tunica media (TM) compared to the control group and the first sacrifice group. (Haematoxylin and Eosin, Magnification=X1000).

FIGURE 9D: Photomicrograph showing the histological organization of the common carotid artery of a rat in the experimental second sacrifice group. Note the increased vascular smooth muscle cells (white arrowheads) in the tunica media (TM) compared to the other animal groups. Note also the more prominent tunica intima (TI) (Haematoxylin and Eosin, Magnification=X1000).

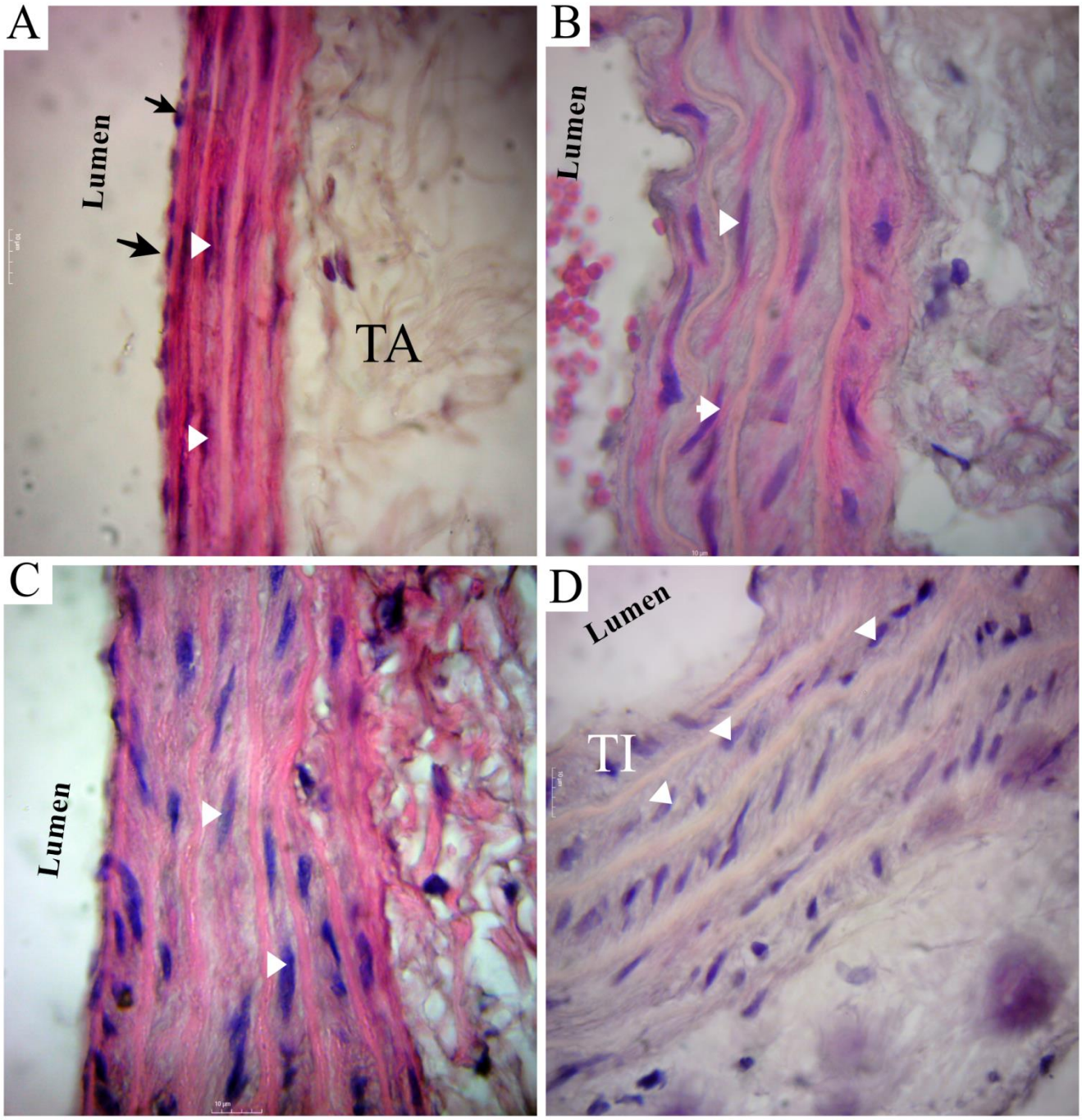

Figure 9: Photomicrographs showing the changes in vascular smooth muscles in response to administration of Letrozole

4.5 ORGANIZATION AND COMPOSITION OF CONNECTIVE TISSUE FIBERS

4.5.1 ORGANIZATION AND DENSITY OF COLLAGEN FIBERS

The average collagen fiber density (CFD) in the control group was 18.7%. There was a statistically significant increase in CFD in the experimental group, with average CFD in the first, second and third sacrifice being 40.3%, 45.3% and 53.3% respectively (**Figure 10)**.

The tunica media of experimental animals displayed progressive increase in collagen fiber quantity. This increase was most evident in animals that had received letrozole for longer (**Figure 10 and 11**). This increase was also seen in the tunica adventitia.

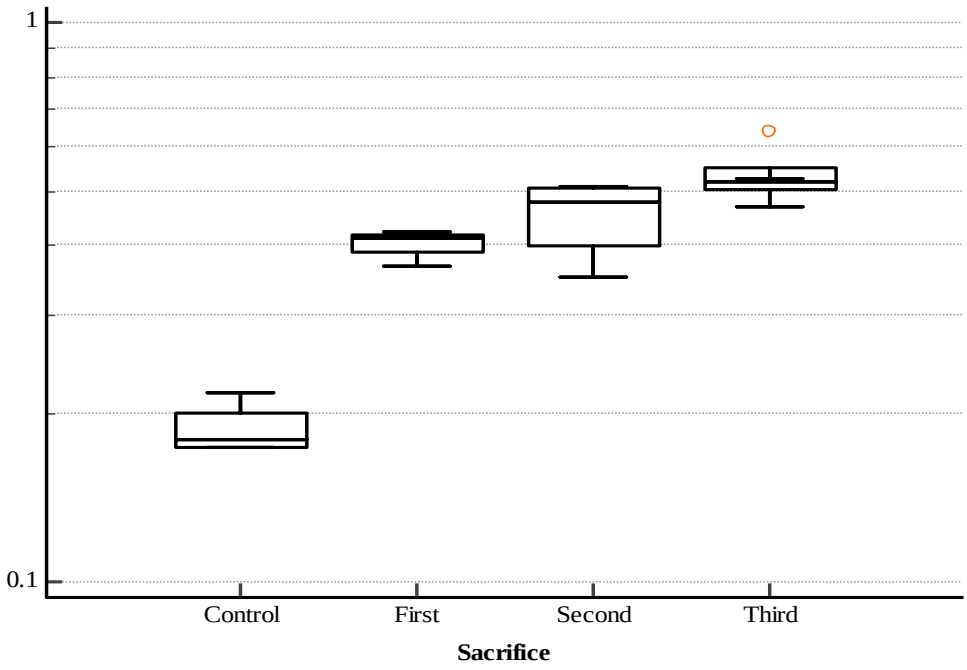

Figure 10: Box and whisker plot showing collagen fiber density in different animal groups

Scheffe's *post hoc* analysis revealed the change was greatest between the third sacrifice and the control group. A statistically significant change was also noted between the animals in the third sacrifice group and those in the first sacrifice (**Table 6).**

Table 6: Collagen fiber density (CFD) in different animal groups

CFD by Sacrifice	**N**	**Mean**	**Mean SE***	**SD**	
Control	9	0.187491702	0.025889433	0.020562703	
First	5	0.403348463	0.025889433	0.024518008	
Second	5	0.453190951	0.025889433	0.072754426	
Third	5	0.533533510	0.023156213	0.063051903	
Pooled	24			0.051778866	
Scheffé all comparisons					
Contrast	**Mean difference**	**Simultaneous 95% CI**		**SE**	**p-value**
Third - Control	0.346041809	0.234937581 to 0.457146036		0.034734319	**<0.0001**
Second - Control	0.265699249	0.148585110 to 0.382813388		0.036613187	**<0.0001**
First - Control	0.215856761	0.098742622 to 0.332970900		0.036613187	**0.006**
Third - First	0.130185047	0.019080820 to 0.241289275		0.034734319	**0.0198**
Third - Second	0.080342560	-0.030761668 to 0.191446787		0.034734319	**0.1999**
Second - First	0.049842488	-0.067271651 to 0.166956627		0.036613187	**0.6157**

LEGEND: ORGANIZATION OF COLLAGEN FIBERS

FIGURE 11A: Photomicrograph showing the organization of collagen fibers in the common carotid artery of a rat in the control group. Note that the collagen fibers within the tunica media (black arrows) are comparably less than the fibers in the tunica adventitia (TA (Masson's Trichrome, Magnification=X1000).

FIGURE 11B: Photomicrograph showing the organization of collagen fibers in the common carotid artery of a rat in the experimental first sacrifice group. Note the increased collagen fibers (arrowheads) within the Tunica Media (TM) (Masson's Trichrome, Magnification=X1000).

FIGURE 11C: Photomicrograph showing the organization of collagen fibers in the common carotid artery of a rat in the experimental second sacrifice group. Note the increased collagen fibers (arrowheads) within the Tunica Media (TM) (Masson's Trichrome, Magnification=X1000).

FIGURE 11D: Photomicrograph showing the organization of collagen fibers in the common carotid artery of a rat in the experimental third sacrifice group. Note the collagen fibers (arrowheads) within the Tunica Media (TM) (Masson's Trichrome, Magnification=X1000).

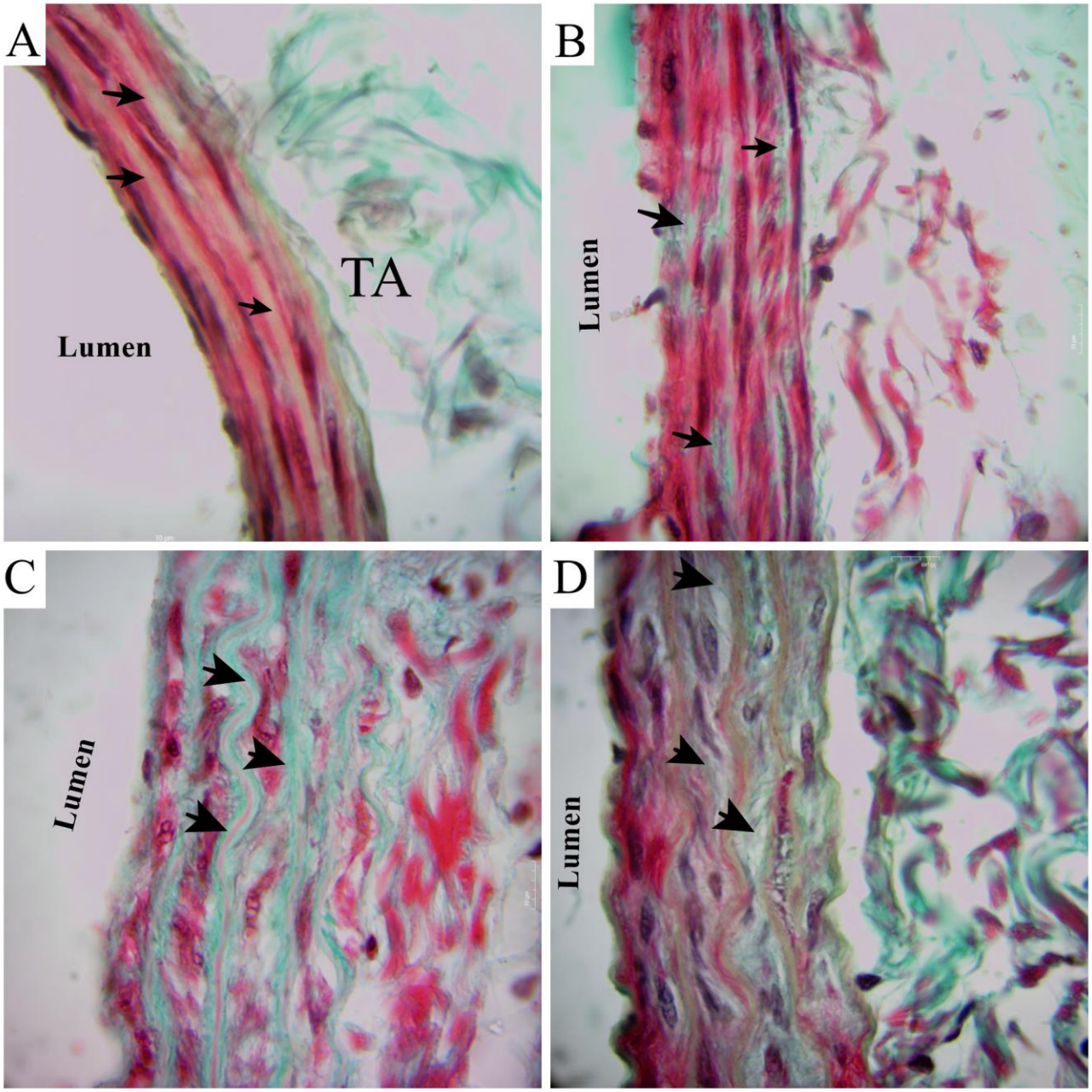

Figure 11:Photomicrographs showing the changes in collagen fiber composition and arrangement in response to administration of letrozole

4.5.2 ORGANIZATION AND DENSITY OF ELASTIC FIBERS

Elastic fiber density (EFD) in the CCA was 63.4% on average in the control group. There was a statistically significant (p=0.003) reduction in EFD in the experimental animals compared to the control group. Average EFD was 46.9%, 42.0% and 44.8% in animals in the first, second and third sacrifice respectively (**Figure 12**). ANOVA with Scheffe's *post hoc* analysis showed that while the differences between the control group and all experimental groups were statistically significant, the difference was most significant between the control group and animals in the second sacrifice (**Table 7)**.

Elastic fibers appeared further interspersed in experimental animals compared to controls. The regular lamellar organization of the elastic fibers was disrupted in experimental animals. While elastic fibers were arranged in regular circumferential lamellae in controls, they had fragmentation and branching coupled with reduced thickness in experimental animals (**Figure 13)**.

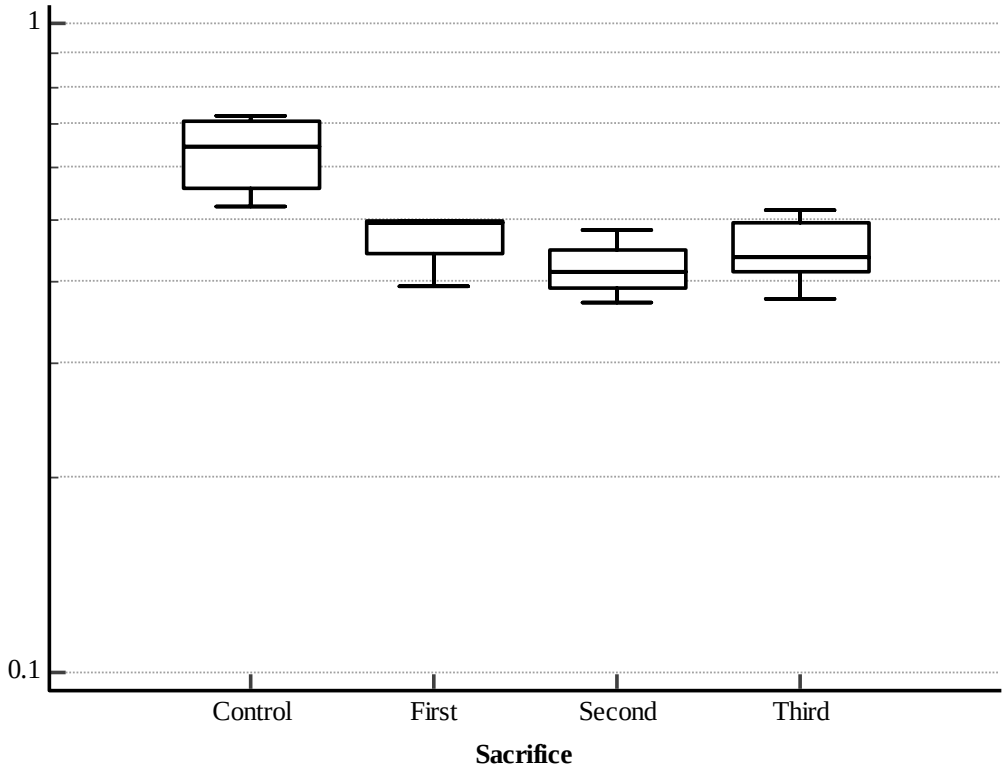

Figure 11:Box and whisker plot showing elastic fiber density in different animal groups

Table 7: Elastic fiber density (EFD) in different animal groups

EFD by Sacrifice	N	Mean	Mean SE*	SD	
Control	9	0.633603192	0.031175821	0.091521244	
First	5	0.469519956	0.031175821	0.050946329	
Second	5	0.420555978	0.031175821	0.045033318	
Third	5	0.448111675	0.027884502	0.053715584	
Pooled	24			0.062351642	
Scheffé all comparisons					
Contrast	**Mean difference**	**Simultaneous 95% CI**		**SE**	**p-value**
Control - Second	0.213047215	0.072019426 to 0.354075003		0.044089269	**0.0032**
Control - Third	0.185491518	0.051700810 to 0.319282225		0.041826753	**0.0062**
Control - First	0.164083237	0.023055449 to 0.305111025		0.044089269	**0.0207**
First - Second	0.048963978	-0.092063811 to 0.189991766		0.044089269	**0.7478**
Third - Second	0.027555697	-0.106235011 to 0.161346404		0.041826753	**0.9313**
First - Third	0.021408281	-0.112382427 to 0.155198988		0.041826753	**0.9657**

LEGEND: ORGANIZATION OF ELASTIC FIBERS

FIGURE 13A: Photomicrograph showing the organization of elastic fibers in the common carotid artery of a rat in the control group. Note the continuous regularly arranged, thick, dark-staining elastic bands. (Weigert's Elastin Stain, Magnification=X1000)

FIGURE 13B: Photomicrograph showing the organization of elastic fibers in the common carotid artery of a rat in the experimental first sacrifice group. Note the wider spacing in between the elastic bands. (Weigert's Elastin Stain, Magnification=X1000)

FIGURE 13C: Photomicrograph showing the organization of elastic fibers in the common carotid artery of a rat in the experimental second sacrifice group. Note the wide spacing in between the elastic bands. Note also the areas where the bands approximate (white arrow). Also note the frayed appearance of the elastic bands. The tunica intima (TI) is more prominent in this section compared to the sections from animals in the control group (Weigert's Elastin Stain, Magnification=X1000).

FIGURE 13D: Photomicrograph showing the organization of elastic fibers in the common carotid artery of a rat in the experimental third sacrifice group. Note the wide spacing in between the elastic bands. Note also the areas where the elastic fibers branch (white arrow). Also note the more frayed appearance of the elastic with irregular kinks (short black arrow) and fragmentation (long black arrow). Note also the tunica intima (TI), more prominent compared to animals in the control group. (Weigert's Elastin Stain, Magnification=X1000)

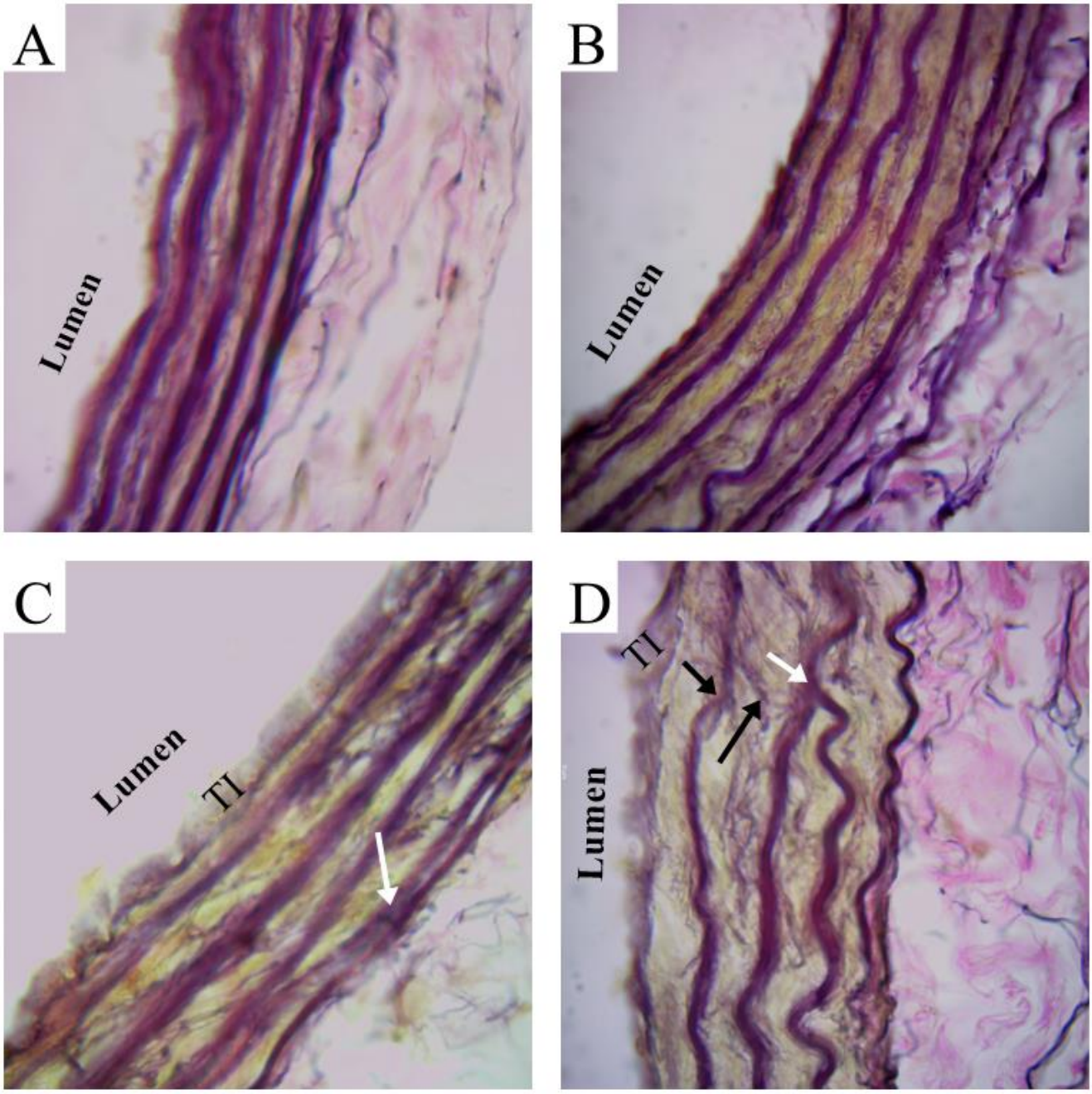

Figure 12: Photomicrographs showing the changes in elastic fibers in the common carotid artery following administration of letrozole

5 DISCUSSION

The findings of the current study demonstrate the effects of letrozole on the wall of the common carotid artery (CCA). In response to letrozole administration, carotid intima-medial thickness and adventitial thickness were increased. There was also increased vascular smooth muscle cell density, collagen fiber density and a reduction of elastic fiber density which was associated with disruption of elastic lamellae.

5.1 HISTOMORPHOMETRY OF THE COMMON CAROTID ARTERY

5.1.1 CAROTID INTIMA-MEDIAL THICKNESS

In the current study, there was an observed thickening of the tunica intima and a quantified increase in the carotid intima-medial thickness (Figure 6, Table 3).

Similar observations of CIMT increase in response to hormonal deprivation have been seen in androgen deprivation (Cheruiyot et al., 2018a). Conversely, estrogen replacement therapy is reported to reduce CIMT (Shufelt et al., 2016). Increased CIMT is thought to be due to intimal hyperplasia, as seen in the current study, as well as in increase in vascular smooth muscle density (Cheruiyot et al., 2018a). Increased CIMT is associated with atherosclerosis in the CCA and predicts the progression of atherosclerosis in other vessels, including the coronary arteries (Liviakis et al., 2010). CIMT is also an independent predictor of the severity of coronary and peripheral artery disease (Kotsis et al., 2005; Sadasivam et al., 2015). The increased CIMT in response to letrozole observed in the current study therefore, suggests an increased cardiovascular risk in women on treatment with AIs. Indeed, this predilection has been observed with a greater incidence of cardiovascular events amongst women on treatment with AIs compared to those on treatment with tamoxifen (Khosrow-Khavar et al., 2020).

As a result of increased CIMT, carotid luminal diameters are reduced. This affects luminal blood flow resulting in a vicious cycle of hypertension, endothelial injury and further intimal thickening (Milutinović et al., 2019; Vinay et al., 2015). As a result, there is a greater risk of ischemic injury to organs that are sensitive to ischemia such as the brain. This may explain the higher incidences of stroke among women on treatment with AIs(Khosrow-Khavar et al., 2020). A similar mechanism may also underlie the greater incidence of myocardial infarction amongst these patients.

5.1.2 ADVENTITIAL THICKNESS

The adventitia in experimental rats was noted to be thickened (Figure 7, Table 4). Adventitial thickening has been reported in vascular ageing (Fleenor, 2013) and at the onset and progression of atherosclerosis (Ogeng'o et al., 2014; Skilton et al., 2011). This increase in AT could be attributed to increased extracellular matrix (ECM) synthesis and deposition by adventitial fibroblasts (Majesky et al., 2011). Activation of adventitial fibroblasts is mediated by various pro-fibrotic cytokines such as TGF-β and TNF-α (Fleenor et al., 2010), which are normally released by inflammatory cells such as macrophages and monocytes (Vinay et al., 2015). Estrogens have an inhibitory effect on the production of these cytokines (Pfeilschifter et al., 2002). Upregulation of TGF-β and TNF-α in estrogen deprivation stimulates the conversion of adventitial fibroblasts to their synthetic phenotype (Fleenor et al., 2010). These synthetic fibroblasts are active in laying down extracellular matrix that results in adventitial thickening.

The tunica adventitia plays an important role in the structural support and nutrition of the vascular wall (Majesky et al., 2011). It is also involved in vessel wall pathology and plays a more central role than previously thought (Milutinović et al., 2019). In sooth, adventitial thickening occurs in atherosclerosis prior to any medial or intimal changes (Milutinović et al., 2019). During this process, adventitial fibroblasts produce numerous chemotactic cytokines that recruit various inflammatory cells such as macrophages, neutrophils and lymphocytes, that are delivered to the tunica adventitia via vasa vasora(Libby, 2009). These cells produce inflammatory cytokines as well as reactive oxygen species that are capable of diffusing through the tunica media via elastic lamellae fenestrations to cause damage to the endothelium, resulting in atherosclerosis(Libby, 2009). This has been called the "outside-in" hypothesis (Mulligan-Kehoe, 2010). It is possible therefore, that the adventitial thickening observed in response to letrozole in this study further compounds the risk of cardiovascular disease in patients using the drug as adjuvant treatment of breast cancer.

5.2 COMPOSITION AND DISTRIBUTION OF VASCULAR SMOOTH MUSCLE CELLS

Observations of the current study have revealed an increase in VSMC composition and collagen deposition in the tunica media of the CCA in response to letrozole administration (Table 5, Figures 8 and 9). The increase in VSMC is in keeping with observations in previous studies that reported

VSMC proliferation in response to estrogen deprivation (Kevin Range et al., 2013). *In vitro* studies have shown that estrogen promotes a pro-apoptotic state in VSMC (Dehaini et al., 2018).

Estrogens, especially 17β-estradiol, are known to inhibit VSMC proliferation (Kevin Range et al., 2013). This has been demonstrated *in vitro,* even in culture media that contained the mitogenic factors basic fibroblast growth factor (bFGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Dehaini et al., 2018). Estrogens modulate this effect via the receptor ERα. Activation of ERα leads to dephosphorylation of the proteins Akt and Erk, which results in inhibition of VSMC migration (de Oliveira et al., 2018; Hisamoto et al., 2001; Kevin Range et al., 2013). The molecular underpinning of this phenomenon is linked to the role of estrogen in the phosphorylation of retinoblastoma protein (pRb) (Caligiuri et al., 2013; Takahashi et al., 2003). Phosphorylation of pRb causes its inactivation, permitting E2F transcription factors to stimulate cellular proliferation (Dehaini et al., 2018). 17β-estradiol inhibits the phosphorylation of pRb leading to inhibition of cellular proliferation (Dehaini et al., 2018; Kevin Range et al., 2013). Therefore, in estrogen deficiency, the rate of cellular proliferation increases. It is possible that this is the mechanism underlying VSMCs proliferation. A further mechanism that may explain VSMC proliferation in estrogen deficiency is through Akt. Akt is an antiapoptotic factor that is modulated by estrogen signalling(de Oliveira et al., 2018; Kevin Range et al., 2013). *In vitro* inhibition of VSMCs proliferation has been demonstrated by decreasing Akt phosphorylation, which induces expression of p21, an inducer of G_1/S cell cycle block(Dehaini et al., 2018). Further, estrogens inhibit protein kinase A through the activation of the MAPK/ERK pathway, leading to apoptosis (Cheng et al., 2009). In states of reduced estrogen, it is plausible that these estrogen-activated proapoptotic pathways are inhibited. This could be the mechanism of VSMC hyperplasia observed in the current study.

Vascular smooth muscle cells are key players in vascular wall metabolism and mechanobiology. They regulate vascular tone and in so doing, affect systemic blood pressure (Lacolley et al., 2012, 2017; Wagenseil and Mecham, 2009). They are also involved in vascular wall response to inflammation and play a key role in atherogenesis. An increase in VSMC could result in increased vascular wall thickness that results in overall arterial stiffness(Lacolley et al., 2017; Wagenseil and Mecham, 2012). This is associated with hypertension, vascular calcification and atherosclerosis (Lacolley et al., 2012).

Intimal hyperplasia observed in the current study has been demonstrated in other states of hormonal deprivation(Cheruiyot et al., 2018b). It may be attributed to endothelial dysfunction and

inflammation that results in VSMCs migration into the SEZ followed by the activation of their synthetic phenotype (Milutinović et al., 2019). Synthetic VSMCs deposit collagen in the SEZ and result in intimal thickening. Pertinent to this is the reduction in nitric oxide (NO) coupled with an increase in pro-inflammatory cytokines (Ahanchi et al., 2007) as well as reactive oxygen species (Staiculescu et al., 2014). The resultant oxidative stress may cause damage to the endothelium and lead to further recruitment of VSMCs into the tunica intima.

Intimal hyperplasia precedes atherosclerotic lesions (Nakashima et al., 2008). In atherogenesis, macrophages within the intima scavenge lipoproteins, forming foam cells that constitute a major cell type in atheromatous plaques (Milutinović et al., 2019). It is plausible, therefore to postulate that the increased prevalence and the progression of atherosclerosis in patients taking AIs is due to the intimal hyperplasia observed in the current study. Atheromatous plaque progression causes narrowing of arterial luminae and may expose end organs to ischemic insults should luminal blockade occur (Vinay et al., 2015). The increased VSMC quantity observed in the current study could, therefore, explain the elevated risk of cardiovascular disease in patients taking AIs.

5.3 CONNECTIVE TISSUE COMPOSITION AND ORGANIZATION

5.3.1 ORGANISATION OF COLLAGEN FIBERS

The present study demonstrates that administration of letrozole results in an increase in the collagen fiber content in the CCA tunica media (Table 6, Figures 10 and 11). Similar observations of the change in CCA collagen fiber content have been made in response to induced hypogonadism (Cheruiyot et al., 2018a). The increase in collagen fiber content in experimental rats could be attributed to the reduction in global and local estrogen levels due to aromatase blockade by letrozole. Various pathways involved in increased collagen fibre synthesis could explain this observation. First, estrogen deprivation due to letrozole administration results in the conversion of more VSMCs into a synthetic phenotype(Dehaini et al., 2018; Fardon et al., 2020). *In vitro* studies on VSMCs demonstrate that estrogen blockade results in the downregulation of calponin, a marker of contractile VSMCs (Fardon et al., 2020). The increased synthetic VSMCs lay down collagen within the tunica media and intima (Darby and Hewitson, 2007). Secondly, estrogen deprivation results in selective inhibition of matrix metalloproteinases (MMP), including MMP-1, which is responsible for the breakdown of

collagen (Choi et al., 2008). The consequent reduction in collagen breakdown results in the accumulation of collagen in the vascular wall. A combination of increased synthesis of collagen from VSMCs and reduced degradation leads to higher collagen fiber content in the vessel wall.

The role of collagen in the lamellar unit of elastic arteries is to prevent overstretching during systole (Chow et al., 2014). High collagen fiber content as observed in the current study results in arterial stiffening and reduced vessel compliance (Cecelja and Chowienczyk, 2012). This phenomenon also occurs with aging and is postulated to partly underlie the pathoanatomy of essential hypertension (Cecelja and Chowienczyk, 2012). The increase in collagen content observed with letrozole in the CCA could be expected in other elastic arteries, such as the aorta. This could underlie the greater incidence of cardiovascular disease among patients on treatment with AIs due to the stiffening of these vessels.

5.3.2 ORGANISATION OF ELASTIC FIBERS

Findings of the current study also reveal that letrozole administration results in reduction of vascular elastic fibres coupled with the fragmentation of elastic lamellae in the tunica media (Table 7, Figures 12 and 13). Using animal models of menopause, it has been shown that estrogen administration results in increase in tissue elastic content and reduced synthesis of collagen (Reslan and Khalil, 2012; Rzepecki et al., 2019). On the other hand, it is known that menopause accelerates the rate of arterial stiffening due to a reduction in elastin content within vessel walls (Moreau and Hildreth, 2014). Similar to vascular tissue, estrogen deprivation leads to reduced elastic content in the skin (Rzepecki et al., 2019). The biochemical basis of elastic fiber reduction in estrogen deprivation remains a topic of study. It has been attributed to the activation of MMP-2 and MMP-9 which have high affinity for elastin (Lam et al., 2009; Lee et al., 2003). These MMPs are important in vascular remodelling (Wagenseil and Mecham, 2009). Activation of these MMPs could also be a result of the upregulation of various cytokines such as interleukin 1 and tumor necrosis factor alpha (TNFα) in response to estrogen deficiency (Pfeilschifter et al., 2002). As a result, increased fragmentation of elastic fibers is observed.

The connective tissue content of the CCA and other large elastic arteries is important in maintaining the compliance of the vascular wall(Wagenseil and Mecham, 2009). Indeed, the tension and recoil conferred by these connective tissue fibers are integral in reducing fluxes in blood flow that result from differences in blood pressure during systole and diastole. This

"windkessel" effect is responsible for maintaining constant blood flow in downstream blood vessels and capillary beds (Barrett et al., 2012). Loss of elastic fibers and consequent arterial stiffening occur with age in menopause (Moreau and Hildreth, 2014). The results of this study therefore suggest that the use of letrozole could compound this age-related arterial stiffening and result in an increase in systolic blood pressure.

6 CONCLUSION

Letrozole administration is associated with structural changes to the wall of the CCA. These changes could affect the function of the vessel wall and may explain the greater incidence of cardiovascular disease in patients taking aromatase inhibitors. These observations negate the null hypothesis of the study. Since AIs are associated with an increased survival rate and result in significant prolongation of life-years, their long-term use should be augmented with appropriate measures to maintain the cardiovascular well-being of these patients.

6.1 RECOMMENDATIONS

The study suggests that further research is needed to explore the effects of letrozole and other aromatase inhibitors on different blood vessels and organs, as well as the possible ways to mitigate or reverse these effects. The study also calls for more advanced and sophisticated methods and techniques that could provide more insight and detail into the mechanisms and pathways that mediate these effects, as well as the potential biomarkers and indicators that could predict these effects. The employment of polarized light microscopy, confocal laser microscopy and electron microscopy to compare connective fiber crimp morphology, endothelial cell structure as well as immunohistochemical staining to illustrate specific markers of smooth muscle proliferation is desirable.

The study recommends that patients taking letrozole should be regularly screened for carotid artery disease using non-invasive methods such as ultrasound. This could help detect and prevent potential complications and improve the quality of life. The study also advises that patients taking letrozole should be given appropriate interventions to maintain their cardiovascular health, such as lifestyle modifications, statins, or other drugs that can reduce the risk of atherosclerosis. The study also highlights the need for more personalized and precision medicine for breast cancer patients.

The study raises awareness of the potential cardiovascular risks associated with letrozole and other aromatase inhibitors, which are widely prescribed for postmenopausal women with hormone-responsive breast cancer. The study therefore calls for more education and counseling for these patients and their caregivers, as well as more collaboration and communication among oncologists, cardiologists, and other health care providers.

Some suggestions for further studies are as follows:

The study could be replicated and validated using a larger and more diverse sample of rats, as well as different strains and genders of rats, to increase the generalizability and reliability of the results.

The study could be extended and expanded to include other blood vessels and organs that may be affected by letrozole and other aromatase inhibitors, such as the coronary artery, the aorta, the heart, etc. This could provide a more comprehensive and holistic picture of the effects of these drugs on the body.

The study could be complemented and supplemented by other methods and techniques that could provide more insight and detail into the effects of letrozole and other aromatase inhibitors on the carotid artery, such as molecular, biochemical, or genetic analysis, as well as functional or physiological assessment. This could reveal the underlying mechanisms and pathways that mediate these effects, as well as the potential biomarkers and indicators that could predict these effects.

6.2 STUDY LIMITATIONS AND DELIMITATIONS

Morphometric measurements could be affected by tissue shrinkage during processing. However, these errors caused by tissue processing did not interfere with comparative analysis as they carried through all measurements since the reagents used and the protocols followed in processing tissues were the same.

The use of fewer controls than experimental animals may reduce the accuracy and precision of the estimates of the structural parameters of interest and, may not adequately represent the variability and heterogeneity of the population in each group, and may introduce sampling bias and error. A smaller sample size may also decrease the effect size and power of the study. However, similar studies have drawn similar conclusions.

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APPENDICES

APPENDIX I: DATA COLLECTION SHEET

Study number_____

Study Group_____ Sacrifice Day_____

Weight of the rat_____ Volume_____

PARAMETER	VALUE			
	Measurement 1	**Measurement 2**	**Measurement 3**	**AVERAGE**
IMT*				
AT**				
Volume density of elastic fibers				
Volume density of collagen fibers				
Volume density of VSMCs****				
Density of VSMCs				

*Intima-medial thickness

**Adventitial thickness

****Vascular Smooth Muscle Cells

APPENDIX II: ETHICAL APPROVAL LETTER

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

P.O. Box 30197,
00100 Nairobi,
Kenya.

Tel: 4449004/4442014/ 6
Ext. 2300
Direct Line. 4448648

REF: FVM BAUEC/2020/249

Mr. Chaudhry Talha Hannan,
University of Nairobi
Dept. Human Anatomy
08/01/2020

Dear Mr. Hannan

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

Light microscopic features of the cerebellar cortex of the albino rat following letrozole administration.

Mr. Chaudhry Talha Hannan H31/82900/ 2017

We refer to your proposal submitted to our committee for review and your application letter dated 13th December 2019. We have reviewed your application for ethical clearance for the study **entitled light microscopic features of the cerebellar cortex of the albino rat following letrozole administration.**

The male rats numbers, letrozole administration regime and tissue sampling protocol meets minimum standards of the Faculty of Veterinary medicine ethical regulation guidelines.

We have also noted that a registered veterinary surgeon will supervise the work.

We hereby give approval for you to proceed with the project 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 III: APPROVAL TO NEST STUDY

UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
DEPARTMENT OF HUMAN ANATOMY

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Cell phone: 0510541/0710622310
Fax: 020-4450011

3rd August 2020

Dr Musa Misiani
H56/11117/2018

RE: APPROVAL OF USE OF BIOBANK TISSUES

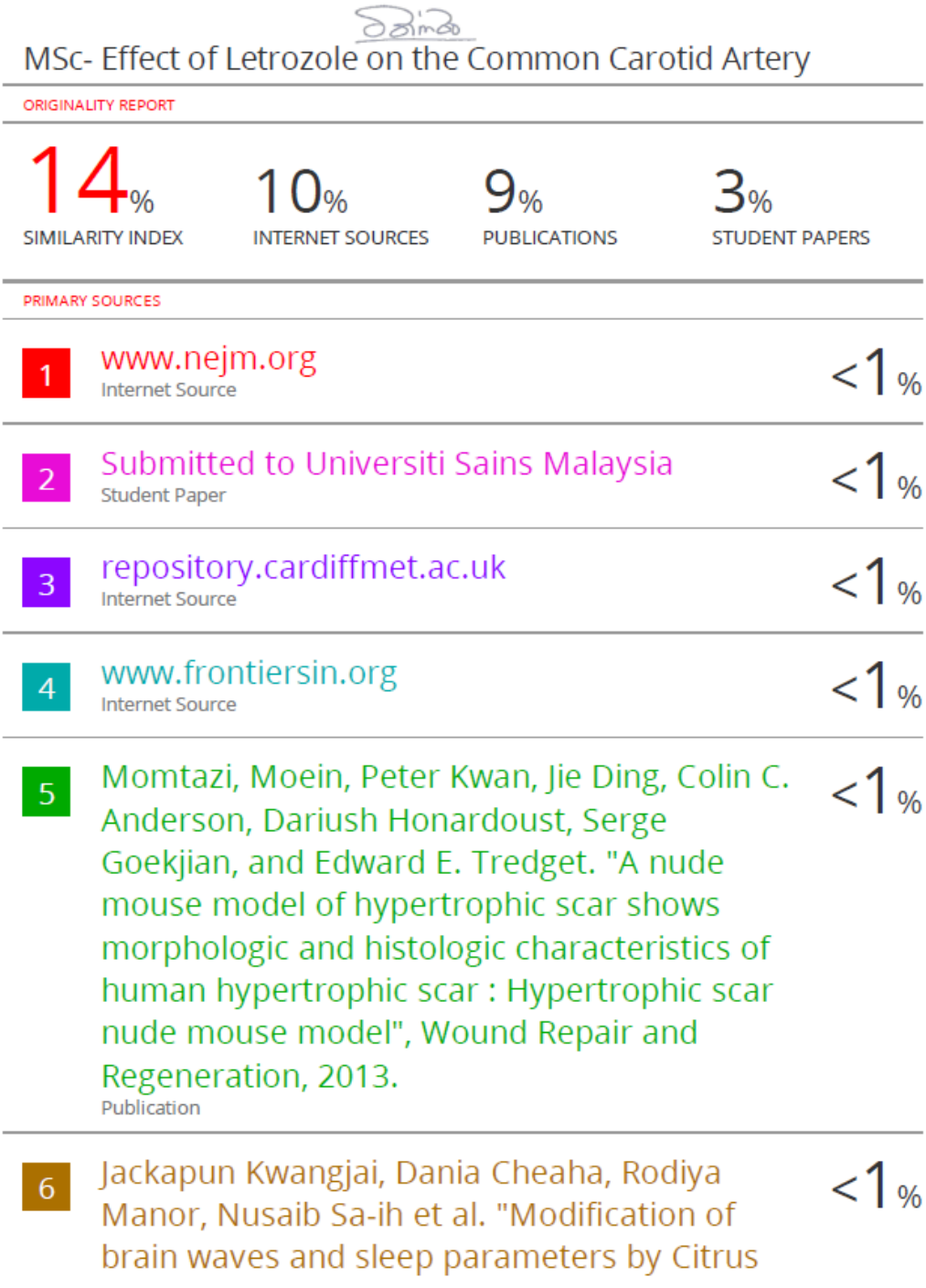
Reference is made to your proposal for use of biobank tissues in your proposal "*Histomorphometry of the common carotid artery of the albino rat (Rattus norvegicus domesticus) following chronic administration of letrozole*".

This is to confirm approval for the use of such tissues as collected in the study "*Histological features of the cerebellar cortex of the albino rat (rattus norvegicus) following letrozole administration*" approved under ethical review FVM BAUEC/2020/249 and stored in the Department of Human Anatomy biobank.

Kind regards,

Prof. Obimbo MM, MD, Ph.D.
Chairman, Department of Human Anatomy

APPENDIX IV: ORIGINALITY REPORT



MSc- Effect of Letrozole on the Common Carotid Artery

ORIGINALITY REPORT

14%	10%	9%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

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5	Momtazi, Moein, Peter Kwan, Jie Ding, Colin C. Anderson, Dariush Honardoust, Serge Goekjian, and Edward E. Tredget. "A nude mouse model of hypertrophic scar shows morphologic and histologic characteristics of human hypertrophic scar : Hypertrophic scar nude mouse model", Wound Repair and Regeneration, 2013. Publication	<1%
6	Jackapun Kwangjai, Dania Cheaha, Rodiya Manor, Nusaib Sa-ih et al. "Modification of brain waves and sleep parameters by Citrus	<1%