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Ultrastructural changes in the common carotid artery in terms of age and gender-related in Rat model

Cambios ultraestructurales en la arteria carótida común en función de la edad y el sexo en un modelo de rata

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ABSTRACT

Sex-related differences were investigated in ultrastructural modifications that occur with ageing in the rat carotid artery in the study. As ageing impacts every system and organ within the human body, it also impacts the circulatory system. The circulatory system serves various functions in the body such as nourishing organs and tissues, oxygenating and eliminating toxic substances. A condition like atherosclerosis or thrombosis in the circulatory system results in permanent harm to structures like the central nervous system, heart and even fatality. Although the pathologies observed in the vessels are commonly attributed to the generation of atheromas, it has been recognised in recent years that changes in the tunica intima and tunica media lead to impaired vascular function without the formation of atheromas. Despite the long-standing belief that women have a advantage in age-related cardiovascular diseases, there has been no ultrastructural examination to support this theory. We explored the sex-related discrepancies in the ultrastructural modifications produced by aging in the carotid artery. A total of 28 Sprague-Dawley rats, 14 males and 14 females, were planned to be used in the study. Of these 28 rats, 4 females and 4 males was constitute the control group. The rats in the control group was approximately 10 weeks old, and the rats in the experimental group, which was represent the aged group, was 19 weeks old. All animals in the study were anaesthetised and then sacrificed by removal of the heart. The right and left common carotid arteries were removed from the sacrificed animals. Collected vessels prepared for Transmission Electron Microscopy (TEM) examination. For each animal, at least four TEM images were taken from four different sections from the same block. As a result, the impact of vascular ageing manifested as apoptosis and age-related dysfunction in endothelial cells, thickening of the subendothelial layer, elastin deterioration, collagen deposition in the matrix, and degradation of the internal elastic lamina. Notably, vascular degeneration is more severe in men than in women. It is clear that the endothelium is subject to accumulated damage with age. We believe that the dissimilarity between males and females is attributed to the estrogen's proliferative and anti-inflammatory impact.

Key words: Ageing; endothelium; electron microscopy; gender-dependent difference; ultrastructural

RESUMEN

Investigamos las diferencias relacionadas con el sexo en las modificaciones ultraestructurales que se producen con el envejecimiento en la arteria carótida de la rata. El envejecimiento afecta a todos los sistemas y órganos del cuerpo humano, y también al sistema circulatorio. El sistema circulatorio desempeña diversas funciones en el organismo, como nutrir órganos y tejidos, oxigenar y eliminar sustancias tóxicas. Una afección como la aterosclerosis o la trombosis en el sistema circulatorio provoca daños permanentes en estructuras como el sistema nervioso central, el corazón e incluso la muerte. Aunque las patologías observadas en los vasos se atribuyen comúnmente a la generación de ateromas, en los últimos años se ha reconocido que los cambios en la túnica íntima y la túnica media conducen a un deterioro de la función vascular sin la formación de ateromas. A pesar de la creencia arraigada de que las mujeres tienen ventaja en las enfermedades cardiovasculares relacionadas con la edad, no se ha realizado ningún examen ultraestructural que respalde esta teoría. Exploramos las discrepancias relacionadas con el sexo en las modificaciones ultraestructurales producidas por el envejecimiento en la arteria carótida. Se planificó utilizar en el estudio un total de 28 ratas Sprague-Dawley, 14 machos y 14 hembras. De estas 28 ratas, 4 hembras y 4 machos constituirán el grupo de control. Las ratas del grupo de control tendrán aproximadamente 10 semanas de edad, y las del grupo experimental, que representará el grupo envejecido, 19 semanas. Todos los animales del estudio serán anestesiados y sacrificados mediante extracción del corazón. Se extrajeron las arterias carótidas comunes derecha e izquierda de los animales sacrificados. Los vasos extraídos se prepararon para el examen TEM. Para cada animal, se tomaron al menos cuatro imágenes TEM de cuatro secciones diferentes del mismo bloque. Como resultado, el impacto del envejecimiento vascular se manifestó como apoptosis y disfunción relacionada con la edad en las células endoteliales, engrosamiento de la capa subendotelial, deterioro de la elastina, deposición de colágeno en la matriz y degradación de la lámina elástica interna. En particular, la degeneración vascular es más grave en los hombres que en las mujeres. Está claro que el endotelio sufre daños acumulados con la edad. Creemos que la diferencia entre hombres y mujeres se atribuye al efecto proliferativo y antiinflamatorio de los estrógenos.

Palabras clave: Envejecimiento; endotelio; microscopía electrónica; diferencia dependiente del sexo; ultraestructural

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INTRODUCTION

Common carotid arteries are the main arteries supplying the head region. The right common carotid artery originates from the arcus aorta and the left side from the brachiocephalic artery [1, 2]. The aorta and its branches are called elastic arteries. Elastic arteries are responsible for the pulsatile progression of blood through the vessel. In other words, they enable the blood pumped by the heart throughout systole to flow during diastole. Elastic arteries consist of tunica intima, tunica media and tunica adventitia. The tunica intima comprises endothelium, subendothelial layer, and internal elastic membrane.

The subendothelial layer contains collagen and elastic fibres synthesised by smooth muscle cells. The tunica media is the thickest layer in the elastic arteries and contains abundant layers of elastic fibres. While the elastic fibre layers in this layer are few or absent at birth, they gradually increase in number with advancing age. The tunica adventitia consists of a connective layer which is considerably thinner than the tunica media. Although collagen fibres are present in all layers, their ratio in the adventitia is higher, thus helping to keep the expansion of the vessel wall within physiological limits during systole [3, 4].

Endothelial cells are the most crucial cellular group within arteries. Endothelial cells maintain the structure of the vessel wall and keep it functional. In addition to acting as a barrier between blood and subendothelial tissues, endothelial cells have many different functions. These include creating a smooth surface that prevents blood cells from adhering to the vascular surface, secreting substances that promote and inhibit clotting when needed, regulating the exchange of substances and fluids between blood and tissues, sending paracrine signals that cause contraction and relaxation of neighbouring smooth muscle cells, secreting cytokines involved in the immune response, secreting growth factors in response to damage, regulating angiogenesis and vascular remodelling, removing hormones and some other mediators [5, 6].

Even though the endothelium has so many important tasks to perform, a disorder that can occur in its structure and function can lead to life-threatening problems. Examples of these are atherosclerosis and thrombosis. These types of diseases tend to occur with age. This is because the accumulation of harmful substances in the body increases with age, and the reduced ability of cells to regenerate makes this situation easier [7, 8].

The common carotid artery, which was used in this study, is one of the arteries with atherosclerosis and thrombosis. Atherosclerosis and thrombosis in the common carotid artery can lead to life-threatening conditions. As mentioned above, the common carotid artery supplies blood to the head, particularly the brain. As a result of narrowing and occlusion in these vessels, conditions such as stroke, death, and cerebral palsy may occur [9]. For this reason, the common carotid artery was chosen in this study. Although previous studies have examined the changes in the vessels with age, differences related to gender have not been elucidated. the changes were examined that occur in the vessels with age differ depending on gender.

MATERIALS AND METHODS

Animals

The experimental studies were performed in accordance with the Declaration of Helsinki, Başkent University Research Center Rules and Başkent University Experimental/Clinical Research Principles. Sprague-Dawley rats (*Ratus norvegicus*) were obtained from Başkent University Experimental Animal Production and Research Center. The rats (*Ratus norvegicus*) were brought to Başkent University Experimental Animal Production and Research Center. Research Unit 10 days (d) before the diet administration to acclimatize them to the environment, where they were divided into old and young groups and housed with 2-3 animals in each cage in an environment with constant temperature (25 ± 2°C) and relative humidity (32 ± 7%), ventilated by a fan and with a 12-h light/dark cycle. During this period, rats were fed standard chow (22% raw protein, 9% raw ash, 7% raw cellulose, 3% raw fat BİL-YEM Gıda Sanayi Ankara) and water was not restricted (ad libitum).

Study Groups

A total of 28 Sprague-Dawley rats, 14 males and 14 females, were planned to be used in the study. Of these 28 rats, 4 females and 4 males were constituted the control group. The rats in the control group were 10 weeks old, and the rats in the experimental group, which was represent the aged group, was 19 weeks old.

Surgical Procedure

All animals in the study were anaesthetised with 60 $mg \cdot kg^{-1}$ ketamine hydrochloride and 10 $mg \cdot kg^{-1}$ xylazine hydrochloride and then sacrificed by removal of the heart. The right and left common carotid arteries were removed from the sacrificed animals.

Histological Analysis

The removed vessels were immediately placed in a 0.1 M phosphate buffered container containing 2.5% glutaraldehyde for 24 h. The following morning, the vessels were postfixed in 1% osmium tetraoxide (OsO4) in 0.1 M phosphate buffer for 1 hour and dehydrated in a graded series of alcohols (25-100%). After passing through propylene oxide, the samples were embedded in Araldyte CY 212, DDSA (2-dodecenylsuccinic anhydride), BDMA (benzyldimethylmethylamine) and dibutylphthalate. Semi-thin sections (1 µm) taken transversely from the vessels were stained with toluidine blue and examined by light microscopy (Carl Zeiss, Göttingen Germany). Ultrathin sections of the arteries were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (TEM) (LEO 906E, Oberkochen, Germany). TEM was preferred because it has advantages over other microscopes in imaging ultrastructural cell architecture. For each animal, at least four TEM images were taken from four different sections from the same block.

RESULTS AND DISCUSSION

As expected in young rats in control group, the endothelial cells continue with subendothelial layer and internal elastic lamina. The nucleus of the endothelial cell appears homogeneously distributed with euchromatin-rich cytoplasm. The basal lamina was continuous with the endothelial cell and retains its normal structure. In the control male, natural looking elastic fibres were also seen in the tunica media (FIG. 1).

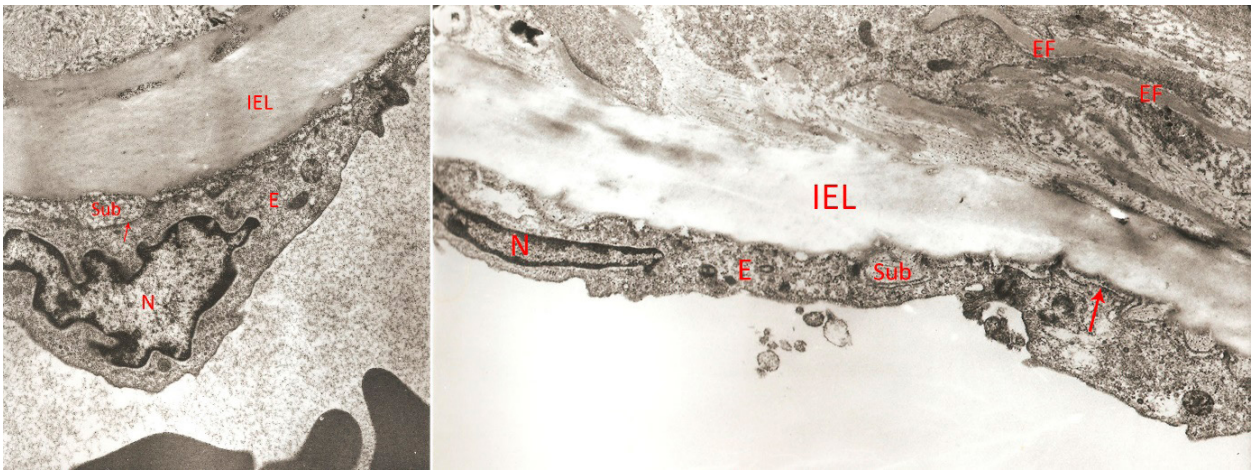

FIGURE 1. Control group electromicrographs (control female on the left X7750, control male on the right X6000) (Sub: Subendothelial layer, IEL: Internal Elastic Lamina, E: Endothelial Cell, N: Nucleus of the Endothelial cell, EF: Elastic Fibres, →: Basal Lamina)

The endothelial cells in the provided observations demonstrate a series of notable changes in old female (OF) rats (FIG. 2) Initially, these cells exhibit separation from the basal lamina in certain areas (FIG. 2 OF–1), while in other instances, they revealed polyploid nuclei and enlargement (FIG. 2 OF–2). This change is accompanied by an increase in heterochromatin and a karyorrhectic appearance in the endothelial cell nuclei (FIG. 2 OF–3), indicating a subsequent shrinkage and degeneration within the nucleus, along with apoptotic characteristics visible in the cell's appearance. Furthermore, a darkening of the cell nuclei is evident (FIG. 2 OF–4). Furthermore, alterations manifest in the subendothelial layer, irregular smooth muscle (SC) cell growth, and excessive disruption of the endothelium (FIG. 2 OF–5). As a result, collagen accumulates due to the compromised integrity of the internal elastic lamina (IEL) extending from the basal lamina (FIG. 2 OF–6). Other notable changes include the cell nucleus degenerating in favour of apoptosis, leading to the formation of bubbles in the plasma membrane and vacuole–like spaces in the cytoplasm. Accumulations of collagen–like material are witnessed under the IEL (FIG. 2 OF–7). The cells exhibit cytoplasmic protrusions towards the lumen, alongside disrupted cell boundaries and the accumulation of vesicles in the cytoplasm (FIG. 2 OF–8). Additionally, the endothelial cells display vacuolation in multiple areas, and a separation is noted between the IEL and the underlying tissue (FIG. 2 OF–9), ultimately resulting in a corrugated appearance of the basal lamina. These observations collectively depict a series of significant and varied alterations occurring within the endothelial cells.

The histological examination revealed multiple various features across the endothelial layers. The endothelial cells in the provided observations demonstrate a series of notable changes in old male (OM) rats, as illustrated in FIG. 3. Large gaps were evident in the endothelium with separation from the basal lamina, accompanied by a dense accumulation of collagen under the IEL (FIG. 3 OM–1). Nuclear changes, such as increased heterochromatin distribution and cytoplasmic vacuolization, were observed alongside intracellular lipid–like structures (FIG. 3 OM–2). Moreover, abnormalities included the formation of large vacuoles in the endothelial cells, irregular folds in the basal lamina surrounding the nucleus, and disruptions in the IEL (FIG. 3 OM–3, OM–4). These changes were accompanied by polyploid nuclei, matrix disturbances in the tunica media, and signs of inflammation, suggested by leukocyte diapedesis and platelet presence, potentially indicating a background of endothelial cell necroptosis (FIG. 3 OM–5, OM–6). Additionally, endothelial degeneration, infiltration of lymphocytes, and the presence of vacuoles with possible lipid precursors were noted (FIG. 3 OM–7). The discontinuity of the endothelial line and basal lamina, along with detached endothelial cells, illustrated structural disarray (FIG. 3 OM–8, OM–9). Furthermore, pyknotic changes in the nucleus, smooth muscle cell irregularities in the tunica media, and subendothelial collagen accumulations due to detachment of the endothelial cells were observed throughout the tissue (FIG. 3 OM–10).

In a study of bovine aortic and microvascular endothelial cells, overgrowth and enlargement of cells were observed in the bovine aortic endothelium with increasing age. It was suggested that this was due to reduced cell renewal or filling of the gap in the endothelium by migrating cells rather than dying cells [10]. In a study on the effect of aging on vasoactivity in monkeys, although thickening of the tunica intima was observed with aging, atherosclerosis did not develop. It has been suggested that apoptosis in endothelial cells may cause loss of endothelial function due to decreased cell density [11]. Another study in mice showed that intense endothelial apoptosis occurs in the

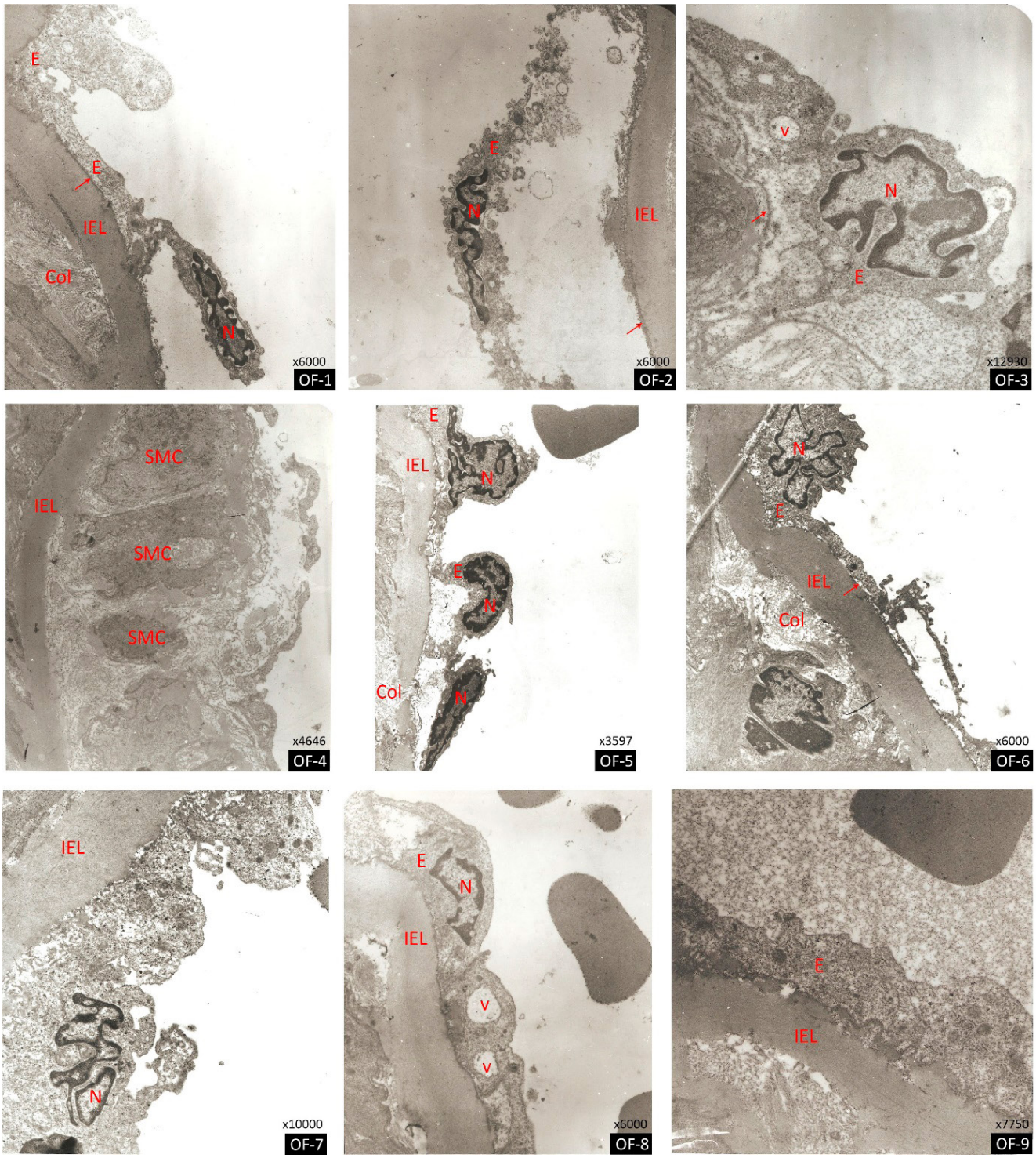

FIGURE 2. Electromicrographs of the old female group.F–1,2,6,8 6000×; OF–3 12930×; OF–4 4446×, OF–5 3597×; OF–7 10000×; OF–9 7750×. In OF–1,2,5,6, endothelial (E) cells are separated from the basal lamina (indicated by arrowhead) and desquamated in places. In OF–5,6,7, endothelial cells have polyploid nuclei and enlarged. OF–1. Heterochromatin increase and karyorrhectic appearance were observed in endothelial cell nuclei. OF–2. Shrinkage and degeneration started in the cell nucleus. The cell exhibits apoptotic appearance with bubbles in the plasma membrane. OF–3. Darkening was observed in the cell nucleus and increased vacuolisation was observed around the plasma membrane close to the basal lamina. OF–4. Thickening of the subendothelial layer and irregular growth of smooth muscle (SC) cells and excessive disruption of the endothelium were observed. OF–5. Collagen accumulated as a result of disruption of the integrity of the IEL protruded from the basal lamina. OF–6. Cell nucleus degenerated by strangulation in favour of apoptosis, bubbles are formed in the plasma membrane, vacuole–like spaces are seen in the cytoplasm. Accumulations of collagen–like material are seen under the IEL. OF–7. Cytoplasmic protrusion is seen towards the lumen with disrupted cell boundaries. Accumulations of vesicles are seen in the cytoplasm. OF–8. Endothelial cell is vacuolated in more than one place. Separation was seen between the IEL and the underlying tissue. OF–9. The basal lamina has a corrugated appearance. (E: endothelial cell, N: nucleus, IEL: internal elastic lamina, SC: smooth muscle cell, v: vacuole, Coll: collagen, →: basal lamina)

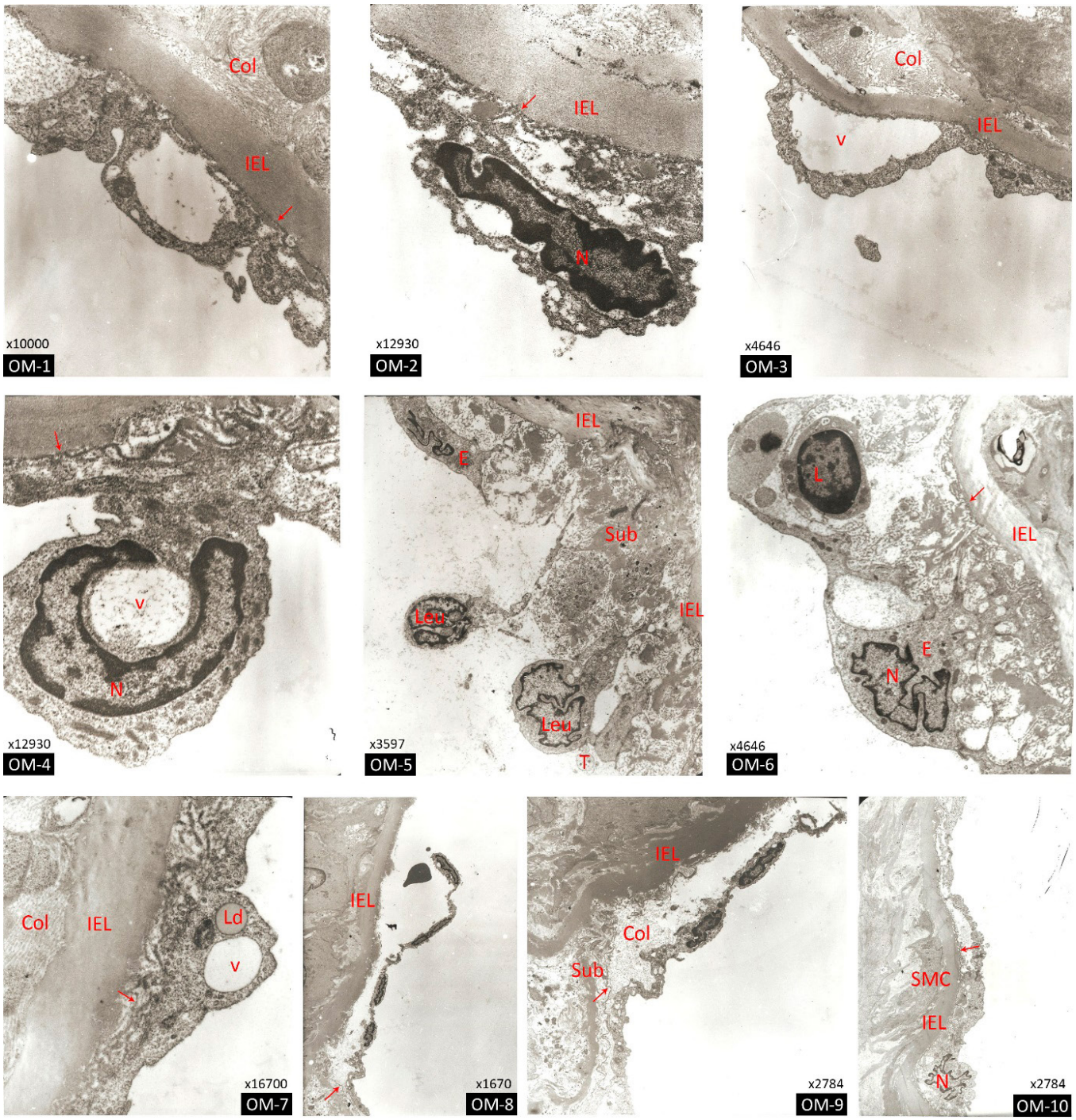

FIGURE 3. Electromicrographs of the old male group OM–1 10000×; OM–2,4 12930×; OM–3,6 4646×; OM–5 3597×; OM–7 16700×; OM–8 1670×; OM–9,10 2784×. Detailed description of the ultrastructural structures of the cells depicted in the figure above. OM-1. Large gaps formation in the endothelium and separation from the basal lamina were observed. There is dense collagen accumulation under the IEL. OM-2. Heterochromatin distribution was observed to increase with increasing nuclear indentation and large cytoplasmic vacuoles were formed. Intracellular accumulations thought to be of lipid structure are seen. OM-3. There is large vacuole formation in the endothelial cell. Collagen accumulation is seen under the IEL. Heterogenic structure is seen in the upper right cytoplasm. OM-4: The nucleus is abnormally surrounded by vacuoles and irregular folds are formed in the basal lamina. OM-5. Endothelial polyploid nucleus and desquamation, disruption in the IEL, disruptions and accumulations in the subendothelial layer, structural disturbances in the matrix in the tunica media are present. The presence of platelets and leukocyte diapedesis were evaluated in favour of inflammation. Apoptotic changes in the endothelium against a background of inflammation suggested necroptosis. OM-6. The endothelial cell has a degenerated appearance with polyploid nuclei and is separated from the basal lamina. The subendothelial layer has a large number of folds and cavities. There was also accumulations in the subendothelial layer. Infiltrated lymphocytes are seen adjacent to the endothelial cells. OM-7. Single vacuole is seen in the cytoplasm with a grey oil droplet. The grey lipid droplet may be a precursor of lipofuscin. Collagen deposition and fluctuations in the basal lamina are seen under the IEL. OM-8. The endothelial line is separated from the basal lamina and does not show continuity. OM-9. Discontinuity in the basal lamina and subendothelial collagen accumulation due to detachment of the endothelial cell. OM-10. The nucleus exhibits a pyknotic appearance. There are places where the endothelial cell is detached from the basal lamina. In the tunica media there is an irregular smooth muscle cell which has lost its spindle shape. (E: endothelial cell, N: nucleus, IEL: internal elastic lamina, DC: smooth muscle cell, v: vacuole, Col: collagen, Leu: leukocyte, T: platelet, L: lymphocyte, →: basal lamina)

capillaries that supply muscle with age [12]. In the study showing that increased fluid shear stress mediates recovery in aged endothelium, it was stated that ageing leads to an increase in polyploid nuclei, irregular cell shape and size growth in endothelial cells. Furthermore, subendothelial matrix thickening, lipofuscin-like structures and apoptotic endothelial cells were observed [13].

In a study investigating the effect of ageing on the common iliac artery in rats, it was found that in old rats, gaps were observed between endothelial cells, collagen increased, elastic fibres decreased, endothelial cells and smooth muscle cells died, and a picture characterised by chromatin degradation and cell lysis was observed [14]. Similarly, endothelial cells with polyploid nuclei, irregular shape and apopitotic endothelial cells were observed in our study. Endothelial cell desquamation explains the decrease in cell density.

A study of the carotid body in the common carotid artery reported that atherosclerosis did not occur despite thickening of the vessel wall and rupture of elastic bands with age [15]. In this study, a deterioration in the integrity of the IEL was also observed. Studies on angiotensin II (Ang II) in rats have reported that Ang II increases the activity of matrix metalloproteinases (MMPs) and increases the collagen ratio in the tunica intima and tunica media. In the same study, morphological changes in the vessels similar to those in old rats were observed as a result of Ang II infusion in young rats [16]. A similar study in rats reported that age-related physiopathological changes such as proinflammation, vasoconstriction, elastin degeneration, collagen accumulation are related to MMPs and lead to atherosclerosis and increased blood pressure [17]. In light of these studies, we thought that the deterioration of IEL integrity, collagen accumulation in the tunica media and tunica intima, and decrease in the amount of elastin in the tunica media observed in our study may be age-related increased MMP activation. In addition, MMP activation stimulates thrombosis of matrix fragments, which may induce inflammation.

In a study investigating the age-related changes in the aortic intima caused by diet-induced hypercholesterolemia in rats, increased lipid droplets and Golgi complexes were observed in 24-month-old fat-fed rats, whereas huge electron dense clusters concentrated around the IEL were observed in 24-month-old control rats. In 24-month-old control rats, endothelial cell junctions were indistinct and irregular in appearance. Cytoplasmic areas were found on the endothelial cell membrane that protrude into the lumen in the study. Slightly increased monocyte adhesion is observed in the endothelium of 24-month-old control rats compared with 24-month-old fat-fed rats [18]. Intercellular disruption, accumulation in the subendothelial layer and monocyte adhesion were also present in this study. This study suggests that age-related deterioration of the vascular wall may occur independently of diet.

Increased tumour necrosis factor-alpha (TNF-α), caspase-9 regulation and decreased NO bioavailability with age stimulate apoptosis in endothelial cells. This may lead to impaired endothelial function and ischaemia in the elderly [19]. Decreased mitochondrial function with age leads to an increase in reactive oxygen species (ROS) and triggers vascular inflammation [20]. One study reports that the number of monocytes infiltrating the intima increases with age. The reason for the increase in monocyte infiltration has been explained by the increased expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells with age [21]. Ageing endothelial cells become more widespread, enlarged and have increasingly polyploid nuclei. These are all effects of cellular senescence. The ageing of endothelial cells leads to a loss of function, a proinflammatory and proapoptotic state, which increases monocyte migration [22]. In comparison with the above information, it can be conclude that similar changes occurred in our study. Decreased number of mitochondria and structural disruption of mitochondria trigger both apoptosis and inflammation.

In an article comparing age-related endothelial dysfunction in women and men, it was explained that women are affected 10 years later than men. The reason for this is not the effect of estrogen on the plasma lipid ratio, but its proliferative effect on the cell. The decreasing amount of estrogen after menopause brings the level of postmenopausal endothelial dysfunction to the same level as in men [23]. A human study investigated the effect of ageing on the aortic wall. In the study, a thickening of the vessel wall with age was observed. In the vascular matrix, elastin decreases with age, while the amount of collagen increases. Depending on gender, collagen predominates in the matrix in women, whereas elastin and smooth muscle cells predominate in men [3].

In this study, it can be said that male rats have more collagen accumulation in the subendothelial layer and leukocyte infiltration in the tunica intima. These may be due to the anti-inflammatory and proliferative effects of estrogen in the cardiovascular system [24]. On the other hand, apoptotic endothelial cells, desquamation, vacuolisation, defects in the basal lamina and disruption of IEL integrity were observed at similar rates in both male and female rats.

In this study, intracellular vacuolisation was observed in both male and female groups as single large vacuole or multiple small vacuoles. A similar situation was not observed in previous studies. It is speculated that this is due to autophagic vacuoles that increase with age.

CONCLUSION

When it was examined the effects of ageing on the common carotid artery ultrastructurally, similar to previous studies, it was observed deviations from the conditions that should be present in both the cellular dimension and the intercellular matrix, even in the absence of atheroma and atherosclerosis formation. Loss of elastin in the matrix, collagen accumulation and loss of elasticity as a result of IEL damage explain arterial stiffness. We know that inflammation in the tunica intima with aging triggers matrix degradation and cellular damage. As seen in our study, we can say that females are less affected by ageing at the vascular level compared to males. Although we think this is due to the anti-inflammatory and proliferative effects of estrogen, we have no data on this. We think that the effects of estrogen should be investigated ultrastructurally in future studies to better understand the difference in vascular ageing between men and women.

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Ethical approval

The study was approved by the Ethical Committee of the Faculty of Medicine of Baskent University. The ethics approval number is 2009/16.

Consent for publication

The author has participated in the design, execution, and analysis of the paper and has approved the final version.

Conflict of interest

No author has a financial or proprietary interest in any part of the study.

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