Original article

Aerobic physical exercise modifies morpho-quantitative features of extracellular matrix of aorta tunica media in ovariectomized female Wistar rats

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Abstract

Introduction: Estrogen deprivation has been related to arterial stiffness in women, and physical exercise is indicated for treatment of cardiovascular disorders and central arterial stiffness after menopause. This morpho-quantitative study evaluated the extracellular matrix of ascending aorta tunica media in ovariectomized female Wistar rats submitted to aerobic physical exercise, compared to intact and sedentary controls. **Material and methods:** Female Wistar rats were studied from six to nine months of age. They were divided into four groups: sedentary intact, sedentary ovariectomized, exercised intact and exercised ovariectomized. Exercised rats were subjected to a motorized treadmill at 60% of their maximum ability for one hour/day, five days/week for 12 weeks. Histological sections of aorta were stained to detect elastic lamellae, elauninic and oxytalan fibers, and collagen fibers by stereological methods. **Results:** In intact rats, exercise was associated with significantly greater volume density of elastic lamellae, oxytalan fibers, and collagen fibers, relative to being sedentary. In contrast, in ovariectomized rats, exercise was associated with decreased collagen fibers, relative to being sedentary. **Conclusion:** Aerobic physical exercise can decrease ascending aorta stiffness in female Wistar rats with low estrogen levels.

Keywords: aerobic physical exercise, aorta, estrogen, extracellular matrix, stereology.

1 Introduction

Estrogen deficiency causes serious morpho-physiological alterations in females, both in humans and animal models. These changes increase disease risk, reducing quality of life and straining the public health system. Cardiovascular system diseases are the most common diseases of aging and therefore create the largest burden (EAKER et al., 1993; CASTILLO, ARIZNAVARRETA, LAHERA et al., 2005).

Similar to arteriosclerosis, estrogen deprivation (e.g., menopause) is associated with a loss of elasticity in the arteries as they become thickening, enlarged and stiffen (IDICULLA and GOLDBERG, 1987; MISZKO and CRESS, 2000). Degeneration of elastin, increased collagen levels and calcium deposition contribute to arterial stiffness, reducing distensibility of the arterial wall and thereby raising systolic blood pressure. Increases in aortic impedance and thickening of the left ventricular wall have also been described (WENGER, O'ROURKE and MARCUS, 1988).

In developed countries, women live more than one third of their lives in the post-menopausal state. In fact, post-menopause is related to enhanced life expectancy, which increased from 50 years at the beginning of the 20th century to more than 80 years in the early 21st century (WENGER, O'ROURKE and MARCUS, 1988; PAOLETTI and WENGER, 2003; NORTH…, 2007). Several therapies have been indicated to treat or prevent cardiovascular diseases and menopausal metabolic disorders, which are the most common health conditions in post-menopausal women.

Pharmacological therapies such as hormone-replacement therapy are widespread, but their effects on cardiovascular disease are still a topic of debate (TATCHUM-TALOM, MARTEL and MARETTE, 2002; BUPHA-INTR and WATTANAPERMPOOL, 2003; MAAS, SHOUW, GROBBEE et al., 2004; GROSS, RITZ, KORSCH et al., 2005). Non-pharmacological treatments such as physical exercise have also been recommended (TCHERNOF, CALLES-ESCANDON, SITES et al., 1998; SIMON, 2006) and are included in public health services (PATE, PRATT and BLAIR, 1995). If associated with changes in lifestyle and diet (alcohol and nicotine), regular, moderate physical exercise can improve cardiovascular health of old-aged, fatty or sedentary individuals, as well as post-menopausal women (BEITZ and DÖREN, 2004; CARELS, DARBY, CACCIAPAGLIA et al., 2004; RACKLEY, 2004). Exercise may also influence hormonal role (PATE, PRATT and BLAIR, 1995; COPELAND, CHU and TREMBLAY, 2004) and reverse diminished arterial compliance (SEALS, 2003).

In the present study, were analyzed the morpho-quantitative features of the ascending aorta (AA) in female rats submitted to estrogen deficiency via ovariectomy (OVX), followed by aerobic physical exercise on a treadmill.

2 Material and methods

This study was conducted according to animal experiment guidelines (number 1168/2007) of the Faculty of Veterinary Medicine and Zootechny at the University of São Paulo, Brazil. A total of 20 adult female Wistar rats (200-240 g) were group-housed (3 animals/cage) and maintained on a 12-h light/dark cycle at 21 °C with free access to a standard rat chow (Nuvital, Parana, Brazil) and fresh water.

Ten animals underwent bilateral OVX. Under aseptic conditions, they were anesthetized (ketamine, 75 $mg.kg^{-1}$, i.p. and xylazine, 5 $mg.kg^{-1}$, i.p.) (WIXSON, WHITE, HUGHES et al., 1987) and the ovaries exposed via a small midline abdominal incision. The ovaries were clamped and removed and the uterine horns were ligated, leaving the uterus intact, then the abdominal wall was sutured. The anti-inflammatory and analgesic flunixin-meglumine (2.5 $mg.kg^{-1}$, s.c.) was administered once daily for three consecutive days (FLECKNELL, 1999).

OVX and intact (INT) control animals were divided into sedentary (sed-OVX and sed-INT) and exercise-trained (ex-OVX and ex-INT) groups. Sample sizes were 5/group.

Each rat was submitted to a maximum effort test (MET) (RODRIGUES, IRIGOYEN and DE ANGELIS, 2006; DE ANGELIS, OLIVEIRA, WERNER et al., 1997) on an adapted motorized treadmill (Inbrasport, Rio Grande do Sul, Brazil) to determine optimal training intensity (RODRIGUES, IRIGOYEN and DE ANGELIS, 2006). The initial test speed was 0.3 km/h, which was increased every 4 minutes in increments of 0.3 km/h until the animal reached exhaustion. The maximal training intensity for each animal was defined as the treadmill speed after which they were unable to run voluntarily (SILVA, BRUM, NEGRÃO et al., 1997). Each rat was submitted to MET monthly to adjust its maximal training speed (FONTINELE, 2007).

Exercise protocol was performed for 12 weeks. OVX and intact controls were subjected to 60% of MET five days/week (Monday to Friday) (DE ANGELIS, OLIVEIRA, WERNER et al., 1997). During the first week, animals ran for 30 min/day (IRIGOYEN, PAULINI, FLORES et al., 2005). Each week, their run time was increased by 10 minutes until they reached 1 h/day in the fourth week (DE ANGELIS, OLIVEIRA, WERNER et al., 1997); this was maintained for the remainder of the exercise period. Sedentary rats were submitted to 30% of MET for 10 minutes once/week to maintain running ability for the periodic METs.

Euthanasia and perfusion were done under heavy sedation (ketamine, 150 $mg.kg^{-1}$, i.p. and xylazine, 10 $mg.kg^{-1}$, i.p.) (WIXSON, WHITE, HUGHES et al., 1987), the thorax was opened. Blood samples were collected from the left ventricle of OVX animals for later analyzis of estrogen levels (by radioimmunoassay test) routinely performed by specialized biochemical laboratories (Rhesus and Provet, Brazil). The estrogen levels after OVX were confirmed to be decreased as expected. Afterwards, the right atrium was cut, a bulbed cannula was inserted into the left ventricle, and a cleaning solution of phosphate-buffered saline (0.1 M, pH 7.4, Sigma) containing 2% heparin (Roche) and 0.1% sodium nitrite (Sigma) was perfused through the AA. Following the perfusion, fixation was performed with a modified Karnovsky solution (5% glutaraldehyde and 1% formaldehyde) in a sodium cacodylate buffer (0.125 M, pH 7.4, EMS). Finally, the AA was excised.

Each arterial ring in a 4 mm section beginning at the base of the AA was immediately isolated and immersed in the fixative solution for 72 hours at 4 °C. Samples were then washed in distilled water, dehydrated in graded ethanol concentrations, diaphanized in xylene, and embedded in Paraplast plus (Sigma).

Every arterial ring of AA was divided into six regions from the ventricle to the aortic arc, and a total of four 6 mm (micrometre) sections from each region, cut via microtome (Leica DMR, Wetzlar, Germany), were taken using systematic and uniform random sampling (SURS) (HOWARD and REED, 2005). Sections were placed on glass slides, deparaffinized at 58 °C in xylene, and hydrated in graded ethanol concentrations (70° to absolute). They were then stained according to Verhoeff's ferric hematoxylin (PROPHET, MILLS, ARRINGTON et al., 1992), Weigert's resorcin, or Weigert's resorcin after oxidation (MONTES, 1996) to detect elastic lamellae, elauninic fibers and oxytalan fibers, respectively. Collagen fibers were visualized using the Picrosirius red method (JUNQUEIRA, BIGNOLAS and BRENTANI, 1979). Finally, sections were dehydrated in graded ethanol concentrations, diaphanized in xylene and mounted under a coverslip with a synthetic resin (Entellan, Merck).

Stereological data were obtained using four fields from each section were acquired via SURS using a semiautomatic device for morphometry (KS 400, Zeiss). Volume density (Vv) was estimated according to Thompson (1930) and Gundersen, Bendtsen and Korbo (1988). This parameter refers to the volume fraction occupied by the interest profile (Sp[interest profile]) divided by the total volume (Sp[ref]), in other words, the number of points hitting in the interest profile divided by the number of points hitting in the reference space (i.e., aorta tunica media). Therefore, volume density was estimated as in Wulfsohn, Nyengaard and Tang (2004): Vv = Sp[interest profile] / Sp[ref].

An unbiased counting grid of crosses (a/p = 2.25 cm^2) and a frame with a solid forbidden line and dashed acceptance line (area of 100 cm^2), as illustrated in Figure 1, were SURS superimposed on section fields of view (BRÜEL, OXLUND and NYENGARD, 2005). The points included in the analysis were those that contacted a point in the top right-hand corner of the counting grid (VAN VRÉ, VAN BEUSEKOM, VRINTS et al., 2007). Interest profiles included elastic lamellae, elauninic and oxytalan fibers, and collagen, as shown in Figure 1.

The volume fraction ranges from 0 to 1 but is often expressed as a percentage (HOWARD and REED, 2005). The volume density of oxytalan fibers was obtained by deducting points counted for elauninic fibers from the total points hitting the material counted, because of impossibility differing oxytalan fibers from elauninics fibers.

Statistical analysis data are presented as mean ± standard deviation. Data were analyzed using two-way ANOVA and Tukey posthoc tests with statistical significance set at $p < 0.05$.

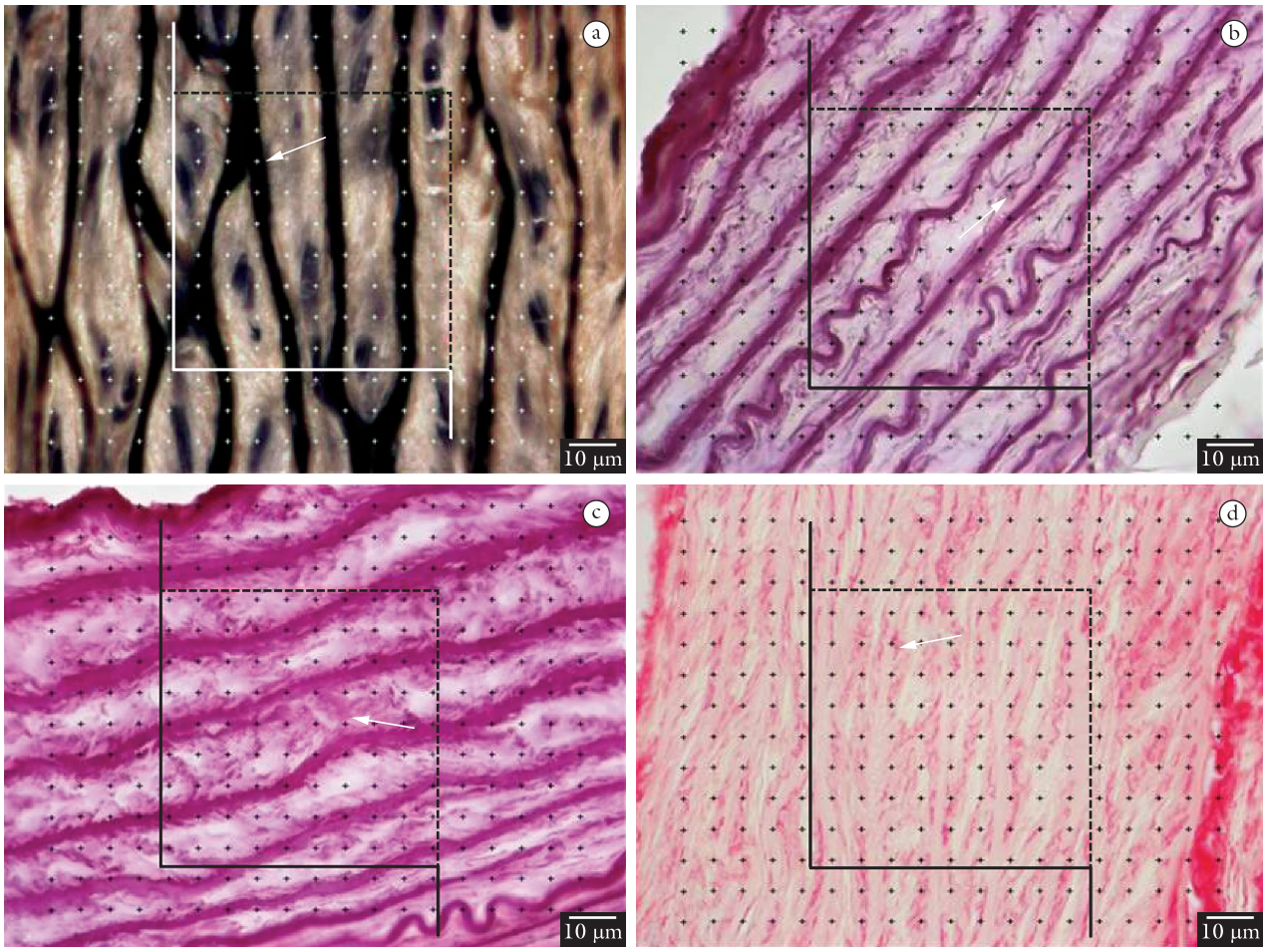

Figure 1. Photomicrograph showing unbiased counting using a grid of crosses and a frame with a solid forbidden line and dashed acceptance line, superimposed upon sections of AA. a) Arrows indicate examples of "mature" elastic fibers visualized by Verhoeff, b) elaunin fibers by Weigert's resorcin, c) oxytalan fibers by Weigert's resorcin after oxidation, and d) collagen fibers by Picrosirius red. Scale bar: 10 mm.

3 Results

The volume densities of elastic fibers and collagen for all groups are shown in Figure 2.

Significant group differences were detected. Specifically, in intact rats, exercise was associated with increased volume densities of oxytalan and collagen fibers and decreased densities of elauninic fibers, relative to sedentary rats. OVX, regardless of exercise, had no effect on elauninic or oxytalan fibers. In OVX rats, exercise was associated with decreased collagen density, relative to sedentary rats.

The current study aimed to investigate structural changes in the AA after estrogen deprivation and exercise. We chose to analyze all components of the extracellular matrix because the elastic and collagen systems together are responsible for the physiological functions of arterial vessels. Elastin provides elasticity (i.e., permits compliance) while collagen promotes resistance (i.e., prevents excessive extensibility). Together, these components produce the appropriate mechanical characteristics of the arterial wall (MATSUDA, NOSAKA, SATO et al., 1989; MATSUDA, NOSAKA, SATO et al., 1993).

Thus, description of structural changes in these components may enhance understanding of the functional consequences of cardiac disease (CABRAL, 2002), which can then be applied in a clinical setting.

In general, OVX had a greater effect than exercise in the current study. OVX rats, regardless of their activity level, had significantly decreased lamellar volume density. This suggests that normal estrogen levels, combined with exercise-induced increases in blood pressure, may be protective by preventing exaggerated tension on AA wall. Our data support previous observations that estrogen deprivation is associated with arterial stiffness (JONASON, HENRIKSEN, KANGRO et al., 1998; KALLIKAZAROS, TSIOUFIS, ZAMBARAS et al., 2002), perhaps due to increased levels of the vasoconstrictor endotelina-1 (YANES, ROMERO, CUCCHIARELLI et al., 2005) and reduced levels of the vasodilator nitric oxide (JIANG, SARREL, LINDSAY et al., 1991).

On the other hand, Park, Omi, Nosaka et al. (2008) found no effect of estrogen deprivation on arterial wall stiffness, either in elastin degeneration or calcium deposition. However, this study was performed in 6-week-old female rats, 12 weeks after OVX, and the researchers do not reject the possibility that estrogen deprivation may promote arterial stiffness during the aging process.

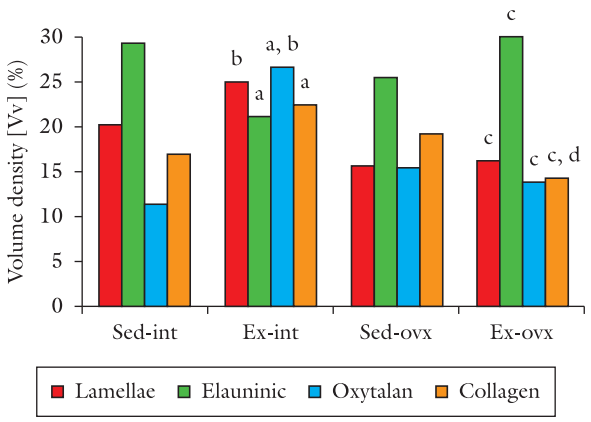

Figure 2. Volume densities (mean ± standard deviation) of elastic fibers and collagen are shown by group. [a] $p < 0.05$ relative to sed-INT, [b] $p < 0.05$ relative to sed-OVX, [c] $p < 0.05$ relative to ex-INT.

The elastic property of lamellae makes blood flow through the vascular bed because it returns to its natural shape after stretching. Tissue with reduced elasticity has decreased ability to reshape (RODRIGUES JUNIOR, 1987). Lower lamellar volume density in OVX rats was observed in AA as well as in branches of the aorta. This suggests interference in blood flow, which is affected not only by pressure differences between points of the vascular bed, but also by vascular peripheral resistance. Thus, stiffer vessels would not enlarge with exercise-induced increases in blood pressure as much as healthy vessels. As a result, total peripheral resistance would not decrease, which would prevent an increase in blood flow through the vascular bed. The present data support this idea, as physical exercise increased the volume of elastic lamellae in ex-INT, but not ex-OVX rats.

Matsuda, Nosaka, Sato et al. (1989) showed significantly more elastin and fewer calcium deposits on the descending aorta wall of nine-months-old male rats that exercised, compared to sedentary rats. This supports the idea that physical exercise, begun at a younger age, promotes increased arterial compliance Matsuda, Nosaka, Sato et al. (1993).

Keeley and Partiridge (1974) observed that the lamellar structure of aortic elastin fibers degenerates with age in six-months-old rats, and these effects were suppressed with exercise. This may explain why we failed to see effects of exercise in intact rats that were six months old at the beginning of the current study.

According to Keeley and Partridge (1974), at this age, animals already have protein alterations in the elastic lamellae. Data from the current study confirm previous findings of Matsuda, Nosaka, Sato et al. (1989), which suggested that starting physical exercise early in life might improve aorta compliance in rats. Similar effects have been observed in human patients (FERREIRA, BOREHAM and STEHOUWERB, 2006). Interestingly, in menopausal women, the extent of arterial stiffness reduction was related to the age at which physical exercise began (MOREA, DONATO, SEALS et al., 2003; SUGAWARA, OTSUKI, TANABE et al., 2006).

Some observations about the components of the extracellular matrix deserve mention. First, normal, exercising rats showed a lower volume density of elauninic fibers, which probably reflects compensation for their increased elastic lamellae. Second, these same animals showed a higher volume density of oxytalan fibers, possibly because exercise-induced tension may have induced a protective mechanism to prevent the increased elastic lamellae from excessively enlarging vessels. As oxytalan fibers promote resistance in areas submitted to mechanical stress (GOLDFISCHER, COLTOFF-SCHILLER and SCHWARTZ, 1983; RODRIGUES JUNIOR, 1987; HORTA, DE CARVALHO and MANDARIM-DE-LACERDA et al., 2005), it seems likely that increasing oxytalan volume density contributes to increased resistance of the vessels. Finally, normal, exercising rats also showed higher collagen levels, which could be explained by exercise-induced increases of blood pressure and heartbeat frequency. This causes higher pressure on arterial vessels, especially on AA, which has the highest pressure when the left ventricle contracts. Xu, Zarins, Pannara et al. (2000) previously showed that increasing blood pressure in the lumen stimulates collagen production.

Matsuda, Nosaka, Sato et al. (1989) also observed increased elastin and collagen in the aorta of exercised rats relative to sedentary rats. The proper proportion of components with high and low elastic capacity may produce an overall more effective elastic capacity. An unknown protective mechanism may block excessive vessel enlargement, promote the correct balance of compliance and resistance to allow high blood volume in the vascular bed, and prevent rupturing of the vessel under high pressure.

An important finding in the current study was the significantly lower collagen volume density in exercised OVX rats, compared to sedentary OVX rats, suggesting that physical exercise reduced arterial stiffness after estrogen deprivation. Decreasing arterial stiffness is important to maintain appropriate vascular peripheral resistance, to diminish pressure on the aorta wall, and to allow appropriate blood flow through the vascular bed. However, the question remains whether reduction of collagen volume density will effectively maintain the elastic properties of the vessel without reducing its resistance.

On the other hand, OVX increased collagen in sedentary animals, which corroborates the arterial stiffness observed in post-menopausal women. Physical exercise is known to be beneficial for arterial vessels. Specifically, long-term treadmill exercise is known to increase nitric oxide (LEWIS, DART, CHIN-DUSTING et al., 1999; HAMBRECHT, ADAMS, ERBS et al., 2003) and endothelium-derived relaxing factor and to decrease plasma levels of the vasoconstrictor endothelin-1 (MAEDA, TANABE, MIYAUCHI et al., 2003), causing vasodilatation. This way, we can assume that arterial compliance factors act on the reduction of vasoconstrictor tone.

There are few human studies investigating the mechanisms underlying exercise-induced alterations in arteries, but exercise is known to reduce hypertension and body fat, which are closely related to arterial stiffness (STEHOUWER and FERREIRA, 2006). Physical exercise may also improve sympathetic activity and endothelial dysfunction, which can induce arterial stiffness (FERREIRA, BOREHAM and STEHOUWERB, 2006). Thus, these factors could improve vessel function individually or in combination.

Similar to exercise, estradiol increases nitric oxide levels. Acute 17-beta-estradiol supplementation causes relaxation of the aorta (JIANG, SARREL, LINDSAY et al., 1991), as well as mesenteric (SHAW, TAGGART and AUSTIN, 2000) and coronary arteries (MÜGGE, RIEDEL, BARTON et al., 1993). After OVX, endothelin-1 increases, thereby increasing blood pressure. These events combine to produce hypertension in OVX rats (YANES, ROMERO, CUCCHIARELLI et al., 2005). Conversely, estrogen deprivation is related to loss of vascular distensibility, which may contribute to cardiovascular disease (BLACHER, GUERIN, PANNIER et al., 1999). Increased arterial stiffness, which is associated with diseases of aging, contributes to the development of hypertension (due to increased total peripheral vascular resistance), left ventricular overload and hypertrophy, coronary perfusion alterations (LONDON, MARCHAIS, GUERIN et al., 2004), endothelial dysfunction and atherosclerosis (NICHOLS, 2005). An increase in systolic pressure by 2 mmHg increases risk of death by coronary artery disease and stroke by 5% and 7%, respectively (LEWINGTON, CLARKE, QIZILBASH et al., 2002).

Diseases that directly affect the aorta may cause quantitative or qualitative alterations of any matrix component, including water, ions, proteoglycans, collagen or elastic fibers. Vascular stiffness is increased by higher collagen-elastin ratios and by calcium and lipid deposition (AGUILA and MANDARIM-DE-LACERDA, 2003). Estrogen deprivation and aerobic exercise-induced pressures would affect on matrix components, changing the collagen-elastin ratio.

Thus, changes in arterial wall structure, such as changes in elastin and collagen levels, characterize adaptive changes induced by physical exercise and aging. Sokolis (2007) states that aorta components adapt based on the pressure to which they are submitted: higher pressure leads to stiffer vessels with more collagen, while lower pressure leads to more distensible vessels with more elastin.

Taken as a whole, we conclude that aerobic physical exercises below 60% of maximal effort can attenuate loss of arterial elasticity caused by estrogen deprivation. Zieman, Melenovsky and Kass et al. (2005) emphasize that increases in arterial compliance are related to cardiovascular risk reduction. In fact, cardiovascular diseases are also that more affect women in post-menopausal period in Brazil (BRASIL, 2008) and around the world. This leads to a high rate of morbidity and mortality in that period.

It is important to continue studies that link morphological changes to physiology and clinical outcome.

4 Conclusion

The current morpho-quantitative study showed that estrogen deprivation reduces the volume density of elastic lamellae of aorta tunica media in female Wistar rats, regardless of whether animals underwent aerobic physical exercise. Nevertheless, under conditions of normal estrogen levels, aerobic physical exercise reduces elauninic fiber volume and increases elastic lamellae, as well as oxytalan and collagen fiber volume in aorta tunica media. Furthermore, after estrogen deprivation, exercise was associated with significantly lower collagen volume density than being sedentary. In conclusion, we suggest that aerobic physical exercise can mediate arterial stiffness after estrogen deprivation, even when begun during adulthood.

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