Aerobic Exercise Modulates Visceral Adipose Tissue of Estrogen Deprived Rats in an Experimental Model of Dyslipidemia

Walkyria Villegas Magalhães[1] Kemily Loren Barros Chucata[1] Nuha Ahmad Dsouki[2] Ricardo Aparecido Baptista Nucci[1,3] Aparecida Gabriela Bexiga Veloso[4] Fernando Luiz Affonso Fonseca[5,6] Laura Beatriz Mesiano Maifrino[5,7]

[1] Laboratory of Morphological and Immunohistochemical Studies, Department of Physical Education, São Judas Tadeu University located at Rua Taquari, Mooca, São Paulo, Brazil
[2] Department of Morphology and Physiology of the ABC District Medical School located at Av. Lauro Gomes, Vila Sacadura Cabral, Santo André, Brazil
[3] Department of Pathology, University of São Paulo Medical School located at Av. Dr. Arnaldo, Cerqueira César, São Paulo, Brazil
[4] Department of Morphology of Oswaldo Cruz School located at Rua Brigadeiro Galvão, Barra Funda, São Paulo, Brazil
[5] Laboratory of Clinical Analysis of the ABC District Medical School located at Av. Lauro Gomes, Vila Sacadura Cabral, Santo André, Brazil
[6] Department of Pharmaceutical Sciences of the Federal University of São Paulo (UNIFESP) located at Rua Sena Madureira, Vila Clementino, São Paulo, Brazil
[7] Dante Pazzanese Institute of Cardiology located at Av. Dr. Dante Pazzanese, Vila Mariana, São Paulo, Brazil

Address for correspondence Ricardo Aparecido Baptista Nucci, PhD, Department of Pathology, Av. Dr. Arnaldo, 455-Cerqueira César, São Paulo 01246-903, Brazil (e-mail: nucci.ricardo.ab@gmail.com).

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Abstract

Introduction Menopausal women have an increase deposition of body fat and changes in the lipid profile, being especially susceptible to cardiovascular diseases, and type 2 diabetes. However, physical activity can mitigate this situation. Thus, the aim of the present study is to evaluate the effects of moderate aerobic exercise on visceral adipose tissue (VAT) of female LDL-receptor knockout ovariectomized mice.

Methods We used 48 animals, divided into six groups ($n = 8$/per group): sedentary control (SC), sedentary ovariectomized control (SCO), trained ovariectomized control (TCO), sedentary non-ovariectomized LDL-receptor knockout (KS), sedentary ovariectomized LDL-receptor knockout (KOS), and trained LDL-receptor knockout ovariectomized (KOT). We analyzed the VAT through morphometric and stereological parameters in hematoxylin and eosin stained sections. Additionally, we evaluated biochemical parameters as glucose, triglycerides, and total cholesterol. Finally, immunohistochemical techniques for matrix remodeling, inflammation, apoptosis, and oxidative stress were evaluated.

Results We observed that menopause is related to increased visceral adiposity, inflammation, oxidative stress, macrophages activity, serum levels of glucose, triglycerides, and total cholesterol. However, exercise was effective in reducing these parameters, as well as being associated with increased vascularization of VAT and interstitial volume density.

Conclusions Moderate exercise is a key factor in mitigating the effects of dyslipidemia in estrogen deprivation. However, further studies are needed to corroborate with our findings.

Keywords
- adiposity
- dyslipidemia
- experimental
- menopause
- treadmill

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Introduction

Menopause is characterized by a decrease in estrogen production, a hormone that stimulates lipolysis and influences the production of lipoproteins leading to an increase in visceral adipose tissue (VAT).[1–3] Thus, weight gain associated with climacteric leads to obesity and related diseases such as dyslipidemia, diabetes, and cardiovascular diseases.[1,3]

Lipid storage is possible due to the increase in number (hyperplasia) and size (hypertrophy) of VAT adipocytes.[4] In obese individuals, the VAT storage capacity is exceeded, and the lipids are deposited in other organs (e.g., liver, heart, pancreas, and kidneys), which can be damaged in a process named lipotoxicity.[5,6] The expansion of VAT compresses the blood vessels leading to a deficit in the supply of oxygen, nutrients, and hormones. The hypoxia caused by VAT stimulates inflammatory markers and oxidative stress, as well as, the accumulation of immune system cells, mainly macrophages.[7–9]

Regular physical exercise promotes positive physiological adaptations and minimizes the effects of aging and menopause by reducing the levels of triglycerides and low-density lipoprotein cholesterol (LDL-cholesterol), aiding in the loss of visceral fat. Aerobic exercise is recommended in a non-pharmacological fashion for the prevention and treatment of diseases associated with weight gain (e.g., dyslipidemia).[1,10–12] However, a few studies evaluated the direct effects of aerobic exercise in adipose tissue when associated with estrogen deprivation and dyslipidemia.

Therefore, this study aims to analyze the effects of aerobic exercise on the visceral adipose tissue, through morphometric, stereological, and immunohistochemical techniques, of female LDL-receptor knockout ovariectomized mice.

Materials and Methods

Division of Animals

This study was approved by the Ethics Committee in Research of the São Judas Tadeu University under protocol number 058/2007. We used 24 genetically modified female mice, knockout of the LDL receptor (LDL-knockout group) and 24 wild female mice (C57BL/6J) (control group) purchased from the Laboratory Animal Center of São Judas Tadeu University, São Paulo, Brazil. Animals were kept in cages in a room with controlled temperature (22–24°C) and a light/dark cycle of 12/12 hours. All mice were fed with standard chow and water '*ad libitum*.' The animals were randomly divided into six groups ($n = 8$/per group): sedentary control (SC), sedentary control ovariectomized (SCO), trained control ovariectomized (TCO), LDL-knockout sedentary (KS), LDL-knockout sedentary ovariectomized (KOS), and LDL-knockout trained ovariectomized (KOT).

Experimental Procedures

Ovariectomy was performed at 9 months as previously reported.[13] Aerobic physical training began 7 days after ovariectomy for 4 weeks on a treadmill with progressive load (1 hour per day, 5× per week, and 50–70% of maximal running speed) as previous reported.[12,13] At the end of the training protocol, animals were euthanized. Blood was collected in tubes without anticoagulant and centrifuged at 3000 rpm at room temperature for 10 minutes to obtain the serum of each animal to determine the following biochemical parameters using enzymatic colorimetric assay with spectrophotometric analysis: (A) glucose, (B) triglycerides, and (C) total cholesterol. Each biochemical parameter was performed in duplicate as previous described.[12]

A laparotomy was performed in which the VAT was removed, weighted, and sectioned. The samples were fixed in 10% buffered formalin for 24 hours. Afterward, the tissue was transferred to a 70% ethyl alcohol solution, dehydrated in increasing ethanol series, diaphanized in xylol, and embedded in paraffin. Four non-serial 6 µm thick sections were obtained per animal and stained with hematoxylin and eosin for light microscopy analysis (Zeiss, ×400 magnifications). Additionally, immunohistochemical stain techniques were used for metalloproteinases 2 (MMP-2, Santa Cruz Biotechnology-sc-13595) and 9 (MMP-9, Santa Cruz Biotechnology-sc-21733), macrophages (F4/80, Abcam-ab6640), cyclo-oxygenase-2 (COX-2, Abcam-ab15191), caspase-3 (Caspase-3, GeneTex-GTX22302), 8-hydroxy-2'-deoxyguanosine (8-OHdG, Santa Cruz Biotechnology-sc-66036), and vascular endothelial growth factor (VEGF, MyBioSource-MBS859873). Thus, sections were washed with a PBS-Tween solution and incubated with biotinylated secondary antibody, avidin-chain enzyme, stained with DAB, and lastly with hematoxylin. Ten images per section were evaluated in each animal (i.e., 40 images per animal). The slides were analyzed using a light microscopy to identify the presence of a dark brown precipitate and photographed for quantitative analysis with the aid of the ImageJ software. We analyzed the numerical density of adipocytes and adipocyte area (μm^2) through morphometric techniques and with the aid of the Axio Vision 4.8 software (Zeiss). Using the area, we classified the adipocytes size as: small ($<1485.47\,\mu m^2$), medium (between $1485.48\,\mu m^2$ and $3921.81\,\mu m^2$), and large ($>3921.82\,\mu m^2$). Stereology was used for the volume densities of adipocytes, interstitium, blood vessels with the aid of the ImageJ software (version 1.47-National Institutes of Health) in a test system of 252 points.[14]

Statistical Analysis

Data are expressed as mean ± standard error (SEM). The variables had their normality and homogeneity verified by the Kolmogorov–Smirnov and Levene tests, respectively. We used one-way analysis of variance (ANOVA) with Tukey's posttest. The generalized linear model (GLzM) was used to establish the influence of independent variables (dyslipidemia, ovariectomy, and physical training) on the differences found between the groups. All statistical analyses were performed with the aid of the SPSS software (version 21). $P < 0.05$ was considered significant.

Results

►**Table 1** shows that there was no significant difference between initial body mass (IM), final body mass (FM), and

Table 1 Initial body mass (IM), final body mass (FM), difference between the masses (FM-IM) and percentage of visceral adipose tissue in relation to final mass (%VAT)

	SC	SCO	TCO	KS	KOS	KOT
IM (g)	22.50 ± 0.3	22.90 ± 0.2	22.40 ± 0.1	22.90 ± 0.4	22.70 ± 0.4	23.40 ± 0.2
FM (g)	24.14 ± 0.2	23.80 ± 0.2	23.40 ± 0.4	23.20 ± 0.4	24.90 ± 0.9	25.50 ± 0.4
FM-IM (g)	1.40 ± 0.1	1.40 ± 0.3	0.90 ± 0.4	0.30 ± 0.2	1.90 ± 0.5	1.20 ± 0.1
VAT %	2.30 ± 0.04	3.20 ± 0.02[a]	1.90 ± 0.04[ab]	2.60 ± 0.1[ac]	3.11 ± 0.02[abcd]	1.70 ± 0.04[abde]

Values represent mean ± SEM. [a]$p < 0.05$ vs. SC; [b]$p < 0.05$ vs. SCO; [c]$p < 0.05$ vs. TCO; [d]$p < 0.05$ vs. KS; [e]$p < 0.05$ vs. KOS.

their difference (FM-IM). In contrast, we observed that ovariectomy promotes a significant increase in visceral adipose tissue (VAT %) in sedentary groups (SCO and KOS) when compared to non-ovariectomized groups (SC and KS) and physical training led to a significant reduction in ovariectomized animals adiposity (TCO and KOT groups).

We observed that ovariectomy led to an increase in glucose, triglyceride, and total cholesterol levels; however, physical exercise contributed to the reduction of these parameters in both control and dyslipidemic animals (►**Table 2**).

In the morphometric analysis, both dyslipidemia, ovariectomy, and exercise significantly influenced the numerical density and area of the adipocytes (►**Table 3**). Regarding size distribution, dyslipidemic groups had a higher percentage of small adipocytes and a lower percentage of large adipocytes when compared with control groups. Additionally, ovariectomy increased the percentage of small adipocytes and decreased medium and large adipocytes, while training reduced small adipocytes and increased medium and large adipocytes (►**Fig. 1**). Stereology showed that training was the main factor that significantly influenced the differences between the groups regarding the density of adipocyte volume (►**Table 3**). However, both dyslipidemia and moderate training were key factors for the volume density of interstitium. In contrast, dyslipidemia significantly decreased the blood vessels density when compared to the non-ovariectomized animals. Representative images are shown in ►**Fig. 2**.

We observed that ovariectomy and sedentarism increased immunohistochemical parameters, as well as, 8-OHdG, caspase-3, COX-2, MMP-9, and VEGF when compared to non-ovariectomized sedentary animals (►**Table 4** and ►**Fig. 3**). Nevertheless, ovariectomized animals

Table 2 Biochemical parameters: glucose (mg/dL), triglycerides (mg/dL), and total cholesterol (mg/dL) in the studied groups

Groups	Glucose (mg/dL)	Triglycerides (mg/dL)	Total cholesterol (mg/dL)
SC	82.4 ± 18.0	30.0 ± 7.8	69.0 ± 14.8
SCO	98 ± 12	73.9 ± 17.0[a]	180.0 ± 69.0[a]
TCO	88.8 ± 13.0	27.0 ± 16.0[b]	83.0 ± 17.9[b]
KS	86 ± 20.0	110.0 ± 20.8[abc]	221.0 ± 52.8[ac]
KOS	128 ± 6.0	177.0 ± 7.5[abcd]	242.9 ± 5.0[ac]
KOT	103.0 ± 44.8	88 ± 20.0[ace]	158.8 ± 2.0[ace]

Values represent mean ± SEM. [a]$p < 0.05$ vs. SC, [b]$p < 0.05$ vs. SCO; [c]$p < 0.05$ vs. TCO; [d]$p < 0.05$ vs KS, [e]$p < 0.05$ vs. KOS.

Table 3 Morphometric and stereological parameters showing the adipocytes numerical density (No.), area (μm^2), and volume density (%), as well as insterstitium (%) and blood vessels (%)

	SC	SCO	TCO	KS	KOS	KOT
Morphometry						
Numerical density (No.)	23.20 ± 1.19	69.76 ± 7.86[a]	37.57 ± 2.55[a]	38.76 ± 2.12[a]	54.97 ± 4.78[ac]	35.36 ± 1.30[a]
Area (μm^2)	2879.12 ± 67.46	1969.36 ± 60.16[a]	2306.58 ± 41.77[ab]	2214.72 ± 45.60[ab]	1686.60 ± 41.62[acd]	2082.92 ± 44.30[abce]
Stereology						
Adipocytes (%)	86.99 ± 0.44	86.31 ± 1.18[a]	92.78 ± 0.27[ab]	91.86 ± 0.43[ab]	89.15 ± 0.98[ac]	89.60 ± 0.57[acd]
Interstitium (%)	11.08 ± 0.44	9.06 ± 0.48[a]	6.01 ± 0.25[ab]	6.32 ± 0.27[ab]	7.48 ± 0.35[a]	8.86 ± 0.28[cde]
Blood vessels (%)	1.87 ± 0.18	1.00 ± 0.15[a]	1.09 ± 0.14	0.62 ± 0.18[abc]	0.39 ± 0.12[abc]	0.61 ± 0.11[ac]

No.: number. Values represent mean ± SEM. [a]$p < 0.05$ vs. SC, [b]$p < 0.05$ vs. SCO; [c]$p < 0.05$ vs. TCO; [d]$p < 0.05$ vs KS, [e]$p < 0.05$ vs. KOS.

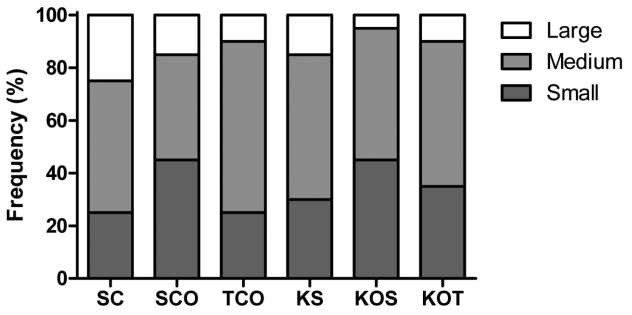

Fig. 1 Frequency distribution of small (<1485.47 μm^2), medium (between 1485.48 μm^2 and 3921.81 μm^2) and large (>3921.82 μm^2) adipocytes.

submitted to a routine of exercise, significantly modulating these parameters.

Discussion

In our study, ovariectomized animals had higher VAT, glucose, triglycerides, and total cholesterol when compared to non-ovariectomized animals. These results corroborate with the literature, which demonstrates the role of estrogen in the regulation of energy homeostasis.[15] Estrogen reduction is considered a risk factor for obesity and other comorbidities such as insulin resistance, type 2 diabetes, dyslipidemia, and cardiovascular diseases.[16,17] Our data also demonstrated that aerobic exercise is an important intervention for significantly decreasing the adiposity of ovariectomized animals and for the reduction of serum levels of glucose, triglycerides, and total cholesterol because exercise promotes increased the lipolysis of adipose tissue and improvements in metabolic homeostasis, regardless of the loss of changes in body weight.[18,19]

The sedentary ovariectomized groups (SCO and KOS) showed an increase in the number of adipocytes and a great variation in the area of these cells when compared to the SC and KS groups, respectively. However, exercise training decreased adipocytes numerical density, and increased adipocytes area. Low levels of estrogen increase the area of adipocytes as previously demonstrated in studies with mice and humans.[4,9] However, physical exercise allows a decrease in the levels of fat stored in the VAT, thus the adipocytes decrease in size.[1,20,21] Rodrigues et al[22] working with Sprague–Dawley females showed that ovariectomy increased adipocyte hypertrophy and that training decreased the area of fat cells.

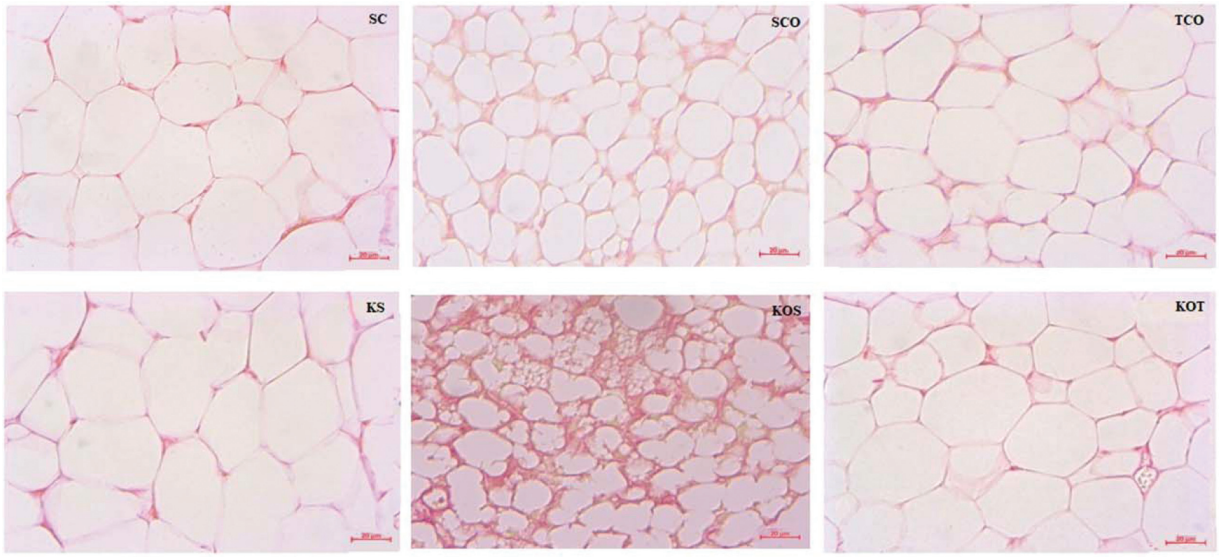

Fig. 2 Representative images stained with hematoxylin and eosin of the visceral adipose tissue (VAT) of the groups. Scale bar = 20 μm.

Table 4 Immunohistochemical (%) analysis between the groups

	SC	SCO	TCO	KS	KOS	KOT
8-OHdG (%)	0.70 ± 0.80	2.30 ± 0.15^a	0.74 ± 0.88^b	1.28 ± 0.19^b	3.20 ± 0.23^{acd}	1.94 ± 0.26^{ace}
Caspase-3 (%)	0.72 ± 0.23	1.38 ± 0.02^a	0.43 ± 0.01^{ab}	0.83 ± 0.14^{bc}	2.68 ± 0.14^{acd}	0.88 ± 0.06^{ace}
COX-2 (%)	0.77 ± 0.86	3.25 ± 0.28^a	1.28 ± 0.15^b	1.67 ± 0.12^{abc}	3.72 ± 0.22^{acd}	2.69 ± 0.19^{ac}
F4/80 (%)	0.57 ± 0.05	2.14 ± 0.16^a	0.53 ± 0.46^b	1.00 ± 0.11^{bc}	1.86 ± 0.13^{acd}	0.84 ± 0.66^{be}
MMP-2 (%)	0.80 ± 0.72	2.49 ± 0.22^a	1.09 ± 0.72^b	1.04 ± 0.79^b	1.23 ± 0.12^b	1.11 ± 0.13^b
MMP-9 (%)	0.80 ± 0.54	1.88 ± 0.15^a	1.84 ± 0.85^a	2.39 ± 0.20^a	3.07 ± 0.20^{abc}	2.05 ± 0.11^a
VEGF (%)	2.18 ± 0.15	1.15 ± 0.83^a	2.32 ± 0.16^b	2.32 ± 0.20^b	0.96 ± 0.72^{acd}	4.35 ± 0.21^{abcde}

Note: Values represent mean ± SEM. $^a p < 0.05$ vs. SC, $^b p < 0.05$ vs. SCO; $^c p < 0.05$ vs. TCO; $^d p < 0.05$ vs KS, $^e p < 0.05$ vs. KOS.

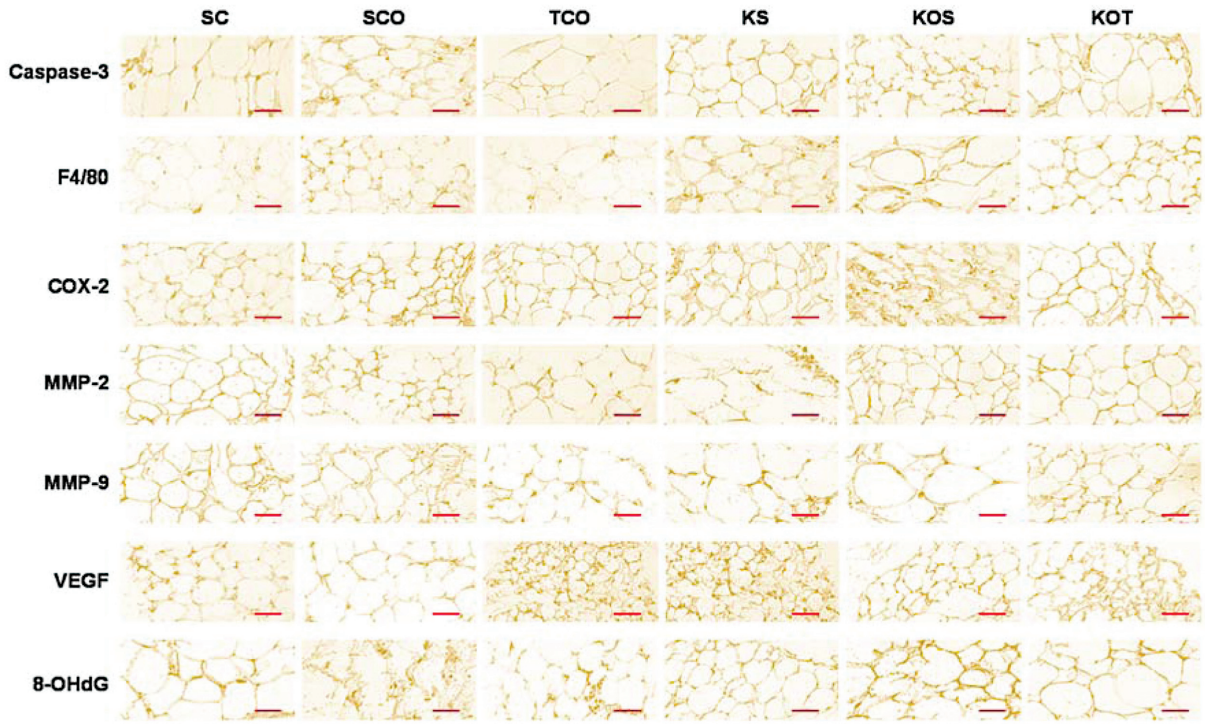

Fig. 3 Representative images of immunohistochemical staining for caspase-3, F4/80, COX-2, MMP-2, MMP-9, VEGF and 8-OHdG between the groups. Scale bar = 20 µm.

It is known that adipose tissue has high plasticity and maintains its ability to expand throughout the life of animals through hypertrophy and hyperplasia.[23,24] The balance between these two mechanisms is an important factor for lipid storage homeostasis and for adipocyte metabolism because inadequate VAT expansion is related to dyslipidemia and other disorders such as insulin resistance and changes in adipocytokine secretion.[25,26]

Hyperplasia was seen as a process that would occur only during early stages of development and that hypertrophy would have a greater influence on the growth of adipose tissue.[23] However, recent research in rodents has proven that during prolonged caloric excess, new adipocytes may emerge contributing to the expansion of the VAT.[24] Smaller adipocytes caused by hyperplasia, in contrast, are associated with increased angiogenesis, which reduces hypoxia and, consequently, inflammation in adipose tissue.[24] In addition, an increase in adipocyte size has been linked to diseases such as insulin resistance, dyslipidemia and impaired adipocytokine secretion that represents a marker for adipose tissue dysfunction.[25–27]

The simultaneous presence of large and small cells is associated with biochemical differences where large and hypertrophic adipocytes are associated with a greater secretion of inflammatory markers (e.g., COX-2) and less secretion of anti-inflammatory factors when compared to smaller adipocytes.[24] However, future studies analyzing the inflammatory markers are needed. According to Welte,[28] small adipocytes are associated with an increased rate of proliferation and large adipocytes, in contrast, are related to the accumulation of fat in mature adipocytes. In addition, small adipocytes are considered especially important to avoid the metabolic decline associated with obesity because adipogenesis not only distributes excess calories among newly formed adipocytes but also reduces the number of hypertrophic adipocytes and, therefore, the secretion of pro-inflammatory factors.[24] Thus, the results of our study showed that ovariectomy, by promoting an increase in the number of adipocytes and inflammatory markers in the SCO and KOS groups, is directly related to the hyperplasia process, whereas physical exercise, by decreasing the number of fat cells, is associated with lipolysis, anti-inflammatory factors, and the maturation of the remaining adipocytes.

Stereological analysis showed that physical exercise decreased the volume density of adipocytes and increased the volume density of interstices in a statistically significant way in the dyslipidemic groups. Ovariectomy did not significantly influence these parameters, but it did contribute to a decrease in vascularization and an increase in the infiltration of macrophages in VAT. The increase in the volume density of vessels, VEGF and the decrease in the volume density of macrophages demonstrate that physical exercise is an effective factor in improving VAT vascularization. However, future studies should analyze the effect of different exercise intensities on VAT.

As the adipocytes expand, they impair vascularization and, consequently, the diffusion of oxygen and nutrients to the adipose tissue via matrix remodeling (e.g., metalloproteinases),[7,9] in addition to triggering immune responses and contributing to chronic low-grade inflammation and oxidative stress.[24,29] Under normal conditions, adipose

tissue is minimally infiltrated by immune cells responsible for maintaining its integrity.[8,30] However, during obesity, overexpression of monocyte chemotactic protein 1 (MCP-1) causes infiltration of macrophages in the VAT, increase oxidative stress, metalloproteinases activity, and greater differentiation of adipocytes.[8,30] In addition, the compression of blood vessels due to the expansion of VAT and the consequent oxygen deficit increases the amount of macrophages (chemotaxis), increasing inflammatory mediators.[8,31] Physical exercise, in addition to reducing VAT by increasing the oxidation of fatty acids through lipolysis, increases vascularity, blood flow, and the expression of anti-inflammatory cytokines and maintain oxidative balance. Such anti-inflammatory effect contributes to the decrease of macrophage infiltration.[1,12,32] Thus, further studies should focus in analyze different exercise intensities to show these parameters.

Conclusion

We can conclude that in animal models exercise is considered a treatment and prevention of dyslipidemia, a condition related to hypoestrogenism due to menopause because the decrease in the area of adipocytes provides an increase in vascularization, which reduces hypoxia and, consequently, chronic inflammation, oxidative stress, and matrix remodeling. However, more biochemical and molecular studies analyzing the inflammatory pathways are needed to corroborate our findings mainly in humans using different intensities of exercise.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

W.V.M. Investigation, writing-review and editing.
K.L.B.C. Investigation, writing-review and editing.
N.A.D. Investigation, writing-review and editing.
R.A.B.N. Data curation, formal analysis, visualization, writing-review and editing.
A.G.B.V. Investigation, writing-review and editing.
F.L.A.F. Resources, supervision, validation, writing-original draft.
L.B.M.M. Conceptualization, methodology, project administration, writing-original draft.

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None.

Conflict of Interest

None declared.

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