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ORIGINAL

Aerobic exercise attenuates the effects of ovariectomy and sedentarism on body composition and food intake in female rats

Exercício aeróbico atenua os efeitos da ovariectomia e do sedentarismo na composição corporal e no consumo alimentar em ratas

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ABSTRACT

Objective

To evaluate the impact of low to moderate aerobic exercise and ovariectomy on body composition and food consumption in female rats.

Methods

Forty adult Wistar female rats (age: 23 weeks; body weight: 275.2±3.6g; mean±SEM) were divided into 4 groups (n=10): laparotomy-sedentary; laparotomy-exercised; ovariectomy-sedentary; and ovariectomy-exercised. The exercised

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groups were submitted to a treadmill running program (16m/min; 30min/day, 5 days/week), for 8 weeks. Body weight and food consumption were monitored during the experiment. Visceral fat and carcass water, protein, ash, fat and carbohydrate fractions were analyzed. Two-way ANOVA plus the Tukey's post hoc test was used for comparisons and $p<0.05$ was considered significant.

Results

The ovariectomized (ovariectomy-sedentary+ovariectomy-exercised) and sedentary (laparotomy-sedentary+ovariectomy-sedentary) animals showed higher ($p<0.05$) weight gain, food consumption, food efficiency ratio and weight gain/body weight ratio than laparotomy animals (laparotomy-sedentary+laparotomy-exercised) and exercised (exercised laparotomy+exercised ovariectomy), respectively. The ovariectomized and sedentary animals showed higher ($p<0.05$) carcass weight, fat percentage and visceral fat than laparotomy and exercised rats, respectively.

Conclusion

Ovariectomy and physical inactivity increase obesogenic indicators, whereas regular aerobic exercise of low to moderate intensity attenuates these unfavorable effects in female rats.

Keywords: Food consumption. Intra-Abdominal fat. Physical exercise. Weight gain.

RESUMO

Objetivo

Avaliar o impacto do exercício aeróbico de intensidade baixa a moderada e da ovariectomia na composição corporal e no consumo alimentar em ratas.

Métodos

Quarenta ratas Wistar adultas (idade: 23 semanas; peso corporal: 275, 2±3, 6g; média±EPM) foram divididas em 4 grupos (n=10): laparotomia-sedentária, laparotomia-exercitada, ovariectomia-sedentária e ovariectomia-exercitada. Os grupos laparotomia-exercitada e ovariectomia-exercitada foram submetidos a um programa de corrida em esteira (16m/mim; 30min/dia, 5 dias/semana) durante 8 semanas. Foram monitorados o peso corporal e o consumo alimentar das ratas durante o experimento. Analisaram-se as frações de água, proteínas, cinzas, gordura e carboidrato da carcaça, bem como a gordura visceral. Empregou-se ANOVA Two-Way, seguida do teste post hoc de Tukey para as análises estatísticas. Adotou-se o nível de significância de p<0,05.

Resultados

As ratas ovariectomizadas (ovariectomia-sedentária+ovariectomia-exercitada) e sedentárias (laparotomia-sedentária+ovariectomia-sedentária) exibiram maior (p<0,05) ganho de peso, consumo alimentar, coeficiente de eficácia alimentar e taxa de ganho de peso/peso corporal do que as ratas laparotomizadas (laparotomia-sedentária+laparotomia-exercitada) e exercitadas (laparotomia-exercitada+ovariectomia-exercitada), respectivamente. A carcaça das ratas ovariectomizadas e sedentárias apresentaram maior (p<0,05) peso, percentual de gordura e gordura visceral do que as ratas laparotomizadas e exercitadas, respectivamente.

Conclusão

A ovariectomia e o sedentarismo elevam indicadores obesogênicos, enquanto que o exercício aeróbico regular de intensidade baixa a moderada atenua esses efeitos desfavoráveis em ratas.

Palavras-chave: *Consumo alimentar. Gordura intra-abdominal, Exercício físico, Ganho de peso.*

INTRODUCTION

Obesity is growing worldwide, being one of the main public health problems, especially in post-menopausal women [1-4]. Menopause is a natural aging process in women that promotes metabolic and body changes by increasing weight and body fat, which may increase the risk of cardiovascular diseases, obesity, diabetes, hypertension and cancer [2-8].

Studies with humans and animals have shown that postmenopausal obesity may occur due to hormonal dysfunction, greater physical inactivity and increased food intake, although some authors have reported an inconsistent impact of the ovarian function loss on food consumption [6,7,9-12].

Although estrogen is known to suppress body fat and to protect against certain chronic diseases, some authors have not observed an effect of ovariectomy surgery on body weight [7,12,13]. Such inconsistencies require further studies on the absence of estrogen caused by ovariectomy on body composition, especially on the accumulation of muscle fat.

Aerobic exercises promote adaptations which are beneficial to the cardiovascular system by increasing energy expenditure and inhibiting weight gain, being a strategy for obesity control [2,4,7,9,14-16]. However, postmenopausal and elderly women usually participate in aerobic exercises programs of very low intensity such as walking, which promote minor effects on body composition [14,17].

Studies report that rats running on the treadmill for 60 minutes at 10 to 12m/min did not experience benefits on body composition, but at 17.5 and 18m/min running showed an increase in aerobic energy markers [7,14,17,18]. In contrast, the 16m/min run at an intensity of 65% promoted more aerobic benefits [14]. However, in the study of Miyatake *et al.* [13] no weight change in mice was observed. This information inconsistency shows the need for further studies on the impact of running intensity on body composition.

Exercises have shown to be effective against weight gain and the deleterious effects of postmenopausal aging [6,8,12,19]. However, the low to moderate 16m/min aerobic running intensity on body composition is not yet known, as well as its effect with the deprivation of the ovarian hormone. Thus, the objective of this study was to assess the impact of low to moderate aerobic exercise and ovariectomy on body composition and food consumption in Wistar rats.

METHODS

The study was carried out with 40 adult Wistar female rats (weight: 275.2±3.6g; mean±MSE). In the 23rd week of life, the rats were submitted to Ovariectomy (OVX) or laparotomy (Sham) surgery. The animals were previously anesthetized with intramuscular ketamine (70mg/kg) and intramuscular xylazine (8mg/kg), receiving subcutaneous administration of the anti-inflammatory substance Ketofen: 2mg/kg/3d and antibiotic (sodium ampicillin: 30mg/kg) kg/5d) [20,21]. After 3 weeks of recovery, the animals were allocated into 4 groups (n=10), in a 2x2 factorial design: SHAM Sedentary (SS), SHAM Exercised (SE), OVX Sedentary (OS) e OVX Exercised (OE).

The animals were housed in individual cages for 8 weeks, kept in an environment at a temperature of 22±2°C, relative humidity of about 60%, and a photoperiod of 12 hours, in addition to receiving daily 18 to 20g of AIN-93M diet and deionized water *ad libitum* [22]. The animals were obtained from the vivarium of the *Centro de Ciências Biológicas e da Saúde da Universidade Federal de Viçosa*, MG (Brazil), and the experimental procedures were approved by the Ethics Committee of the Veterinary Department of UFV MG (Opinion No. 80/2007).

After 2 weeks of recovery from surgery, all rats (20 SHAM and 20 OVX) underwent a process of adaptation to a running exercise on a treadmill (5-7m/min, 8min/day) for one week (Table 1). The 10 rats from each surgery (SHAM and OVX), which were better adapted to the exercise, started to form the ES and EO groups, that were submitted to a progressive program of aerobic workout on a treadmill with no incline (adapted from Ferreira *et al*. [17]); the rats underwent 5 sessions/week workout, for 8 weeks. These animals exercised at progressive speed and duration, and from the 3rd to the 8th week the speed was set at 16m/min and the duration was 30min/day. The other SHAM and OVX rats became part of the SS and SO groups and remained in their cages during the experimental period.

In the last experimental week, the blood lactate of the exercised animals (groups SE and OE) during a training session was determined. Five animals were evaluated per group, chosen at random.

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Table 1 – Exercise program. *Viçosa* (MG), Brazil, 2009.

Variables	Day of the week	Velocity (m/mim)	Time (min)	Running distance (m)
Adaptation (1 week)	Monday to Friday	5 to 7	8	50
1st week	Monday	10	10	100
	Tuesday	10	15	150
	Wednesday	10	20	200
	Thursday	10	25	250
	Friday	10	30	300
2nd week	Monday	10	30	300
	Tuesday	12	30	360
	Wednesday	14	30	420
	Thursday and Friday	16	30	480
3rd to 8th week	Monday to Friday	16	30	480
Performance test		16	30	480

The measurements were carried out at 3 points in time: at rest and after 15 minutes and 30 minutes running [23]. Blood was collected from the animals' tail end to dose the lactate concentration the Accusport BM-Lactate equipment (Roche®, Germany) was used [14].

At the end of the 8th week, all the animals in each group were euthanized; visceral fat was removed and weighed. The empty carcass (muscles and bones) was separated and stored in a freezer at -20ºC to further determine the fractions: water, fat, proteins and ashes. The water content rate was evaluated using the gravimetric method by water evaporation in an oven (Fanem, Brazil) at 105ºC for 24 hours. The fat percentage was determined by the gravimetric method in a Soxhlet apparatus using ethyl ether as solvent in an 8 hours/extraction. The percentage of protein was calculated in triplicate by the indirect method of nitrogen determination (Protein(g)=nitrogen(g)x6.25) by the Kjeldahl method. The percentage of ash was determined by incineration at 600ºC for 6 hours. The percentage of carbohydrates was calculated by the equation, 100% – (% of water, protein, ash and fat). The analyses were conducted according to the official A.O.A.C. methods [24].

The body weight of all animals in each group and the dietary intake of 5 animals in each group, chosen at random, were monitored weekly using a digital electronic scale (Mars, Brazil). Weight gain was determined calculating the weight difference between sacrifice time point and the first week of life. The Food Efficiency Ratio (FER%) was determined by the equation [FER%=(body weight gain/diet consumption) x100]. The Weight Gain Rate by Body Weight was determined by WGR=(body weight gain/final weight) x100. All rates were calculated in five animals/group.

After submitting the data to the Kolmogorov-Smirnov normality test, analysis of variance (two-way ANOVA of repeated measures) was applied to assess performance and body weight between the weeks of study. The Two-Way ANOVA was applied to evaluate the Exercise and Ovary factors and their interactions in the parameters of weight gain, diet consumption, dietary rates and body composition. In the analysis of multiple post hoc comparisons, Tukey's test was used. Pearson's correlation test was used for dietary rates. For statistical analysis, Sigma Stat 3.0 software (Systat Software Inc., USA) was used with a significance level of $p<0.05$.

RESULTS

The Figure 1 shows the blood lactate concentrations. There was no difference in lactate between the rats of the SE and OE groups at the 3 evaluation time points. It was found that blood lactate increased slightly ($p<0.05$) from the resting condition to 15 and 30 minutes in both workout groups. However, despite

the lactate elevation between 15 and 30 minutes, this difference was not statistically significant, suggesting stabilization.

There was no difference in the initial body weight of the animals between the groups and between the factors Ovary and Exercise (p>0.05; Table 2 and Figure 2). Regarding the factors Ovary and Exercise, an increase in body weight and weight gain at the end of the experiment was observed. As for the Ovary factor, from the 2nd week onwards, it was found that the animals in the OE group exhibited greater body weight (p<0.001), compared to the SE. The OS group showed greater body weight (p = 0.003) than the SE group after the 2nd week, revealing interaction between the exercise and ovary factors.

At the end of the 8th week, an interaction was identified between the factors Ovary and Exercise for final body weight and weight gain, as both OVX and physical inactivity increased body weight and weight gain (Table 2 and Figure 2).

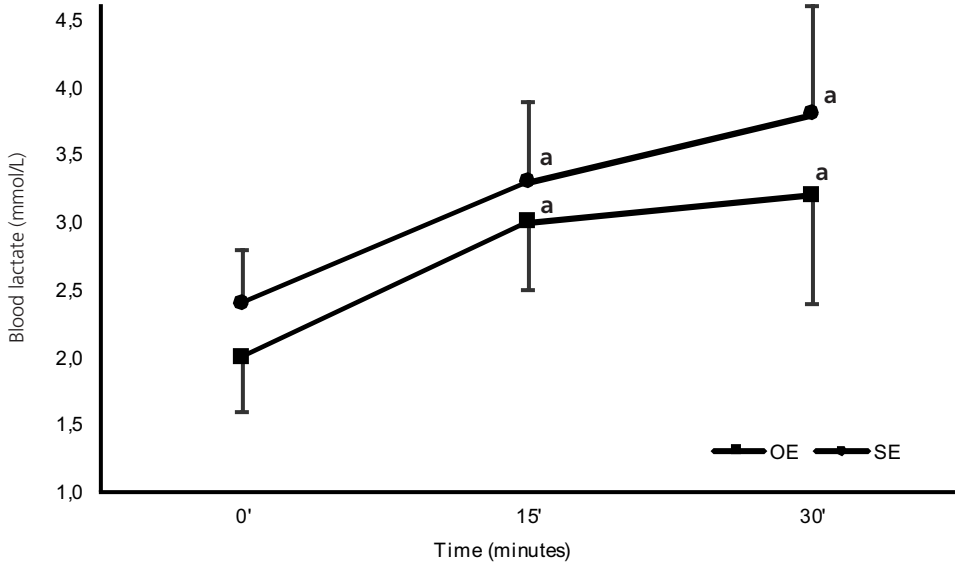

Figure 1 – Blood lactate concentration of the exercised animals (SE and OE groups) in the performance evaluation. *Viçosa* (MG), Brazil, 2009.
Note: Values in mean±SD. Significance (p<0.05): [a]*vs*. Time 0 minutes for the ANOVA Test Repeated Measures. OE: OVX Exercised; SE: SHAM Exercised.

Table 2 – Initial and final body weight (g), weight gain (g) and dietary intake of the animals. *Viçosa* (MG), Brazil, 2009.

	Initial Weight (g)	Final Weight (g)	Weight Gain (g)	Diet Consumption (g)	FER (%)	WGR (%)
Groups						
SS	284.2±6.7	317.8±9.0	33.6±5.7	944.8±14,5	3.6±0,6	10.5±1.6
SE	286.1±7.1	289.9±7.1	2.9±2.8[a]	908.3±9,7	0.4±0,7[a]	1.2±2.1[a]
OS	290.2±10.1	350.0±8.4[b]	59.8±6.2[ab]	999.6±19,0[a]	6.0±0,6[a]	17.1±1.8[b]
OE	296.0±12.0	324.9±14.2[b]	28.9±3.9[bc]	929.8±21.2[c]	3.1±0,4[bc]	8.8±1.0[bc]
Exercise Factor						
Sedentary	287.2±6.5	333.9±7.1	46.7±3.5	972.2±11.8	4.8±0,4	13.8±1.2
Exercised	291.0±6.5	307.4±7.1[d]	13.5±3.7[d]	919.0±11.8[d]	1.8±0,4[d]	5.0±1.2[d]
Ovary Factor						
SHAM	285.1±6.5	303.9±7.1	15.9±3.7	926.5±11.8	2.0±0,4	5.8±1.2
OVX	293.1±6.5	337.5±7.1[e]	44.4±3.5[e]	964.7±11.8[e]	4.5±0,4[e]	13.0±1.2[e]

Note: Values on average ± SEM. Significance (p<0.05): [a]*vs*. SS; [b]*vs*. SE, [c]*vs*. OS, [d]*vs*. Sedentary, [e]*vs*. SHAM for F test. FER: Food Efficiency Ratio; OE: OVX Exercised; OS: OVX Sedentary; SE: SHAM Exercised; SS: SHAM Sedentary; Weight Gain Rate: WGR.

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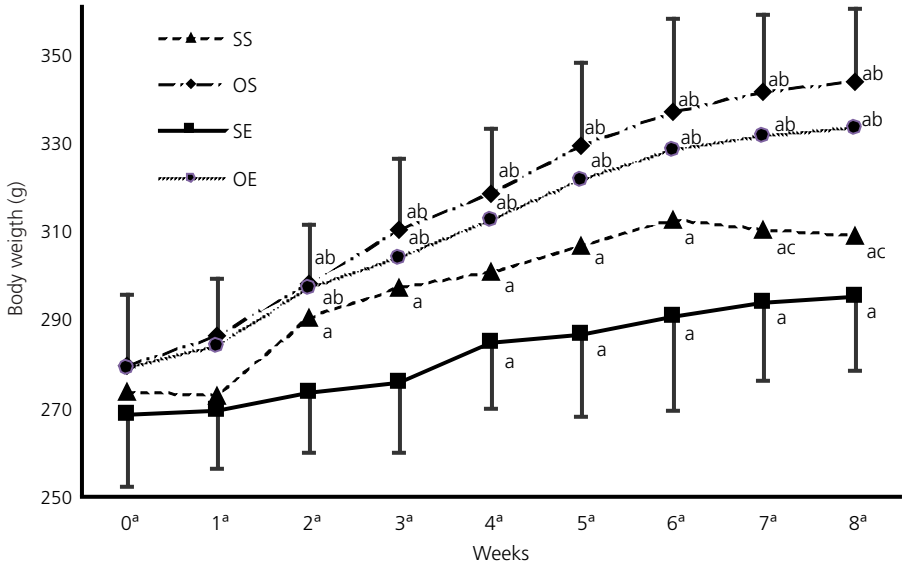

Figure 2 – Body weight of the animals during the experiment. *Viçosa* (MG), Brazil, 2009.

Note: Significance ($p<0.05$): [a]*vs.* 1st week for ANOVA Repeated Measures Test, [b]*vs.* SE, [c]*vs.* OS for Two Way ANOVA Test. OE: OVX Exercised; OS: OVX Sedentary; SE: SHAM Exercised; SS: SHAM Sedentary.

A positive correlation (r=0.620 and $p<0.001$) was observed between body weight gain and dietary intake throughout the experiment. Thus, the data on weight gain were normalized by dietary intake, and the food efficiency ratio (FER%) was calculated. OVX and physical inactivity increased body weight gain, dietary intake and FER% by the end of the experiment (Table 2). An interaction was also identified between factors for dietary intake and FER%.

In order to exclude the interference of body weight in the weight gain and diet consumption, these parameters were standardized by calculating the weight gain rate (WGR). It was found that the WGR was higher in OVX and physical inactivity, an interaction between factors was observed.

A significant difference was observed in the carcass weight (muscle and bones) between groups and the Ovarian factor (Table 3). In this connection, the data of the fractions of water, protein, ash, fat and carbohydrates in the carcass were standardized by the carcass weight and presented as a percentage. No statistical differences were found for the percentages of ash and carbohydrate ($p>0.05$). There was an interaction between the factors Ovary and Exercise for the percentage of fat and for visceral fat, in which these parameters were reduced with exercises and increased with OVX.

As for the Ovary factor, it was observed that the carcass weight, the fat and visceral fat rates were higher in the OVX rats than in the Sham rats. However, the OVX rats exhibited a lower percentage of water in the carcass than the Sham rats. Regarding the Exercise factor, it was observed that the rats undergoing workout exhibited higher % of water and protein content and lower % of fat and visceral fat content than the sedentary rats (Table 3).

DISCUSSION

The aim of this study was to assess the impact of low to moderate intensity aerobic exercises and ovariectomy on body composition and food consumption in Wistar rats. The main results were that OVX

Table 3 – Body Composition in Content and Percentage. *Viçosa* (MG), Brazil, 2009.

	Carcass Weight (g)	Water (%)	Protein (%)	Ash (%)	Fat (%)	CHO (%)	Visceral Fat (g)
Groups							
SS	133.3±3.3	65.8±0.5	20.3±0.4	5.6±0.2	7.8±0.7	0.5±0.1	28.7±2.6
SE	132.5±3.3	67.4±0.5	20.5±0.4	5.3±0.2	6.1±0.7[a]	0.2±0.1	18.1±2.6[a]
OS	140.9±3.3	64.7±0.5	19.2±0.4[a]	5.4±0.1	10.0±0.7[a]	7.0±0.2	34.9±2.6[a]
OE	146.9±3.3[b]	66.3±0.5[c]	20.4±0.4[c]	5.2±0.1	7.9±0.7[bc]	0.2±0.1	27.2±2.6[bc]
Exercise Factor							
Sedentary	137.1±2.3	65.3±0.4	19.7±0.3	5.5±0.1	8.9±0.5	0.5±0.2	31.8±1.9
Exercised	139.7±2.3	66.8±0.4[d]	20.5±0.3[d]	5.3±0.1	7.0±0.5[d]	0.4±0.1	22.7±1.9[d]
Ovary Factor							
SHAM	132.9±2.3	66.6±0.4	20.4±0.3	5.5±0.1	6.9±0.5	0.6±0.2	23.4±1.9
OVX	143.9±2.3[e]	65.5±0.4[e]	19.8±0.3	5.3±0.1	8.9±0.5[e]	0.5±0.2	31.1±1.9[e]

Note: Values on average±SEM. Significance ($p<0.05$): [a]*vs*. SS; [b]*vs*. SE, [c]*vs*. OS, [d]*vs*. Sedentary, e vs. SHAM for ANOVA Two Way. CHO: Carbohydrates; OE: OVX Exercised; OS: OVX Sedentary; SE: SHAM Exercised; SS: SHAM Sedentary.

increased body weight gain, accumulation of visceral fat and the carcass fat rate, in addition to increased food consumption. More importantly, regular low to moderate intensity treadmill workout attenuated these unfavorable ovariectomy effects.

The treadmill aerobic running program used in this study reduced body fat, resulting in lower body weight in the exercised rats. These results are similar to those of Sherk *et al*. [6], in which 17-week-old Wistar rats running 60 minutes/day at 15m/min for 8 weeks experienced a reduction in fat and body weight. Regular aerobic exercise increases energy expenditure and reduces body fat storage in postmenopausal women and in OVX rats, by increasing the rate of fat oxidation due to the activation of the AMPK enzyme, which regulates lipid metabolism [10,11,19]. Another suggested mechanism is that aerobic exercise regulates the use of substrates in the Krebs cycle and electron transport in the respiratory chain], where fatty acids are released from adipocytes through the activation of Hormone-Sensitive Lipase (HSL). This enhances lipolysis and provides energetic substrate to the exercising muscles [7,9,16,18].

Aerobic exercise is considered a strategy to also reduce abdominal and visceral fat [7,16]. The results of this study showed that treadmill aerobic running reduced muscle fat in the same proportion as visceral fat. However, there are still questions about the best intensity and duration of exercise that will promote the highest rate of fat oxidation [16]. Ferreira *et al.* [17] reported that Wistar rats body fat did not change after running exercise on a treadmill at 12m/min for 60 minutes for 10 weeks. Rezaei *et al.* [14] found that running at 10m/min did not increase free fatty acids and lipolysis-inducing glucocorticoids in Wistar rats; however, this occurred with rates running at 17.5m/min. Da Silva Dias *et al.* [7] observed that OVX rats that ran 60 minutes at 18m/min presented with reduced visceral fat and triglycerides, in addition to an improvement in insulin sensitivity. These findings corroborate the American College of Sports Medicine (ACSM) position, that moderate intensity aerobic exercises are more indicated to prevent weight gain. In our study, moderate intensity aerobic running was used for a shorter time than that of previous studies and lower levels of body fat were observed in OVX rats [7,10,11,14,19]. This suggests that exercise of intensity closer to the anaerobic threshold, even for a shorter duration, would promote a higher rate of fat oxidation than very low intensity exercises, showing to be more beneficial in controlling overweight and obesity in individuals with loss of the ovarian function.

One of the benefits of aerobic exercises on body composition seen here was a reduction in body weight. It is believed that this occurred due to the reduction in fat mass as reported in other studies [14,16,17]. Despite the De Brito Vieira *et al.* study [18] suggesting that the ideal speed to obtain benefits

from running on a treadmill is 17.5 m/min, the study by Kang *et al.* [19] demonstrated a reduction in body weight in OVX rats training on 15º incline treadmill at 16m/min for 60 minutes, for 8 weeks. In the present study we observed a reduction in body weight from the third treadmill training week, without incline, lasting only 30 minutes. This confirms that the aerobic exercise program proposed in this study with a shorter duration than those mentioned above can be useful for obese and postmenopausal individuals, who wish to reduce their body weight.

The results presented here demonstrated that OVX increased body and carcass weight, as well as fat in the carcass and viscera. The increase in total body fat in OVX rats was reported by Sherk *et al.* [6] in adult rats 8 weeks after surgery and by Tuazon *et al.* [10] in adult mice 6 weeks after surgery. In addition, Pighon *et al*. [12] found an increase in subcutaneous, visceral and hepatic fats in female rats 6 weeks after OVX surgery. However, in the present study, in addition to the 32.9% increase in visceral fat in OVX rats, there was also a 29.0% increase in the percentage of carcass fat (muscle) thus demonstrating a proportionality in the increase between visceral and muscle fat. This increase in body fat has been reported as being a consequence of aging in postmenopausal women, which could promote insulin resistance and an increased risk of chronic non-communicable diseases [1,2,4,5,8,11,12,16]. More importantly, the present study demonstrated that regular aerobic exercise reduced muscle fat in OVX rats to the levels of sedentary Sham rats. This finding confirms that the muscles are sites of fat deposition after loss of the ovarian function, and this fat deposition provides an energy substrate for low to moderate intensity exercises.

The increase in body fat observed in this study may be the main cause for the increase in final body weight and weight gain after OVX. After 3 weeks of post-surgery recovery and 1 more week of experiment, a significant increase in body weight was already observed in OVX rats, compared to Sham rats. These results are similar to those of previous studies [6,19,25]. The increase in body weight may be due to hormonal dysfunction and to the greater accumulation of fat as well as to the greater food intake of OVX rats, as observed in the present study [6,10,12,19].

The greater weight gain of OVX and sedentary rats compared to the Sham exercised rats may have been caused by food consumption, which was positively correlated with weight gain, as observed by other authors [6]. However, the results of FER% and WGR confirm that, regardless of food consumption and body weight, OVX and sedentary rats showed greater weight gain. It is also conceivable that OVX increased physical inactivity by reducing rats spontaneous activity, which can increase food consumption and contribute to weight gain [7,10]. Such a situation could be related to aging, sedentary condition and loss of ovarian function [11]. However, our study did not evaluate the spontaneous physical activity of the rats to confirm this mechanism.

As for the proportions of water, fat, protein and ash in the rats carcasses in this study, the values were similar to those of other studies [17,26]. However, an unexpected result was that there was no difference in the percentage of ash between the OVX and the Sham rats. The absence of estrogen after OVX should stimulate less vitamin D release, reduce the efficiency of intestinal calcium absorption and promote a negative calcium balance. Consequently, a reduced retention and lower bone calcium levels would be expected [1,21]. In addition to the negative calcium balance, OVX can also interfere with the homeostasis of other minerals, such as magnesium, zinc and phosphorus. The results presented here indicate that the magnitude of this mineral reduction in OVX rats was not sufficient to biologically affect individuals with estrogen deficiency.

Finally, the treadmill aerobic workout program used in the present study is characterized as being of low to moderate intensity. This can be confirmed with the blood lactate concentration observed during a training session, as observed by other investigators [14,23]. Blood lactate levels measured at rest increased

to 3.5mmol/L after 15 and 30 minutes running. However, lactate levels did not extrapolate the values of the anaerobic lactate threshold of 4.0 mmol/L [14,23]. According to Rezaei *et al*. [14], the 16m/min running speed kept lactate below the anaerobic threshold at an intensity of 65% for Wistar rats, which is associated with the aerobic metabolism and the predominance of fat oxidation as an energy substrate for workout. These findings are of clinical relevance, as they indicate regular aerobic workout as an important strategy to control overweight and obesity in individuals deprived of the ovarian function, which is a common physical activity among postmenopausal women.

The present study has limitations. First, the rats spontaneous activity during the period they were housed in the cages was not measured; thus it is not possible to correlate it with weight gain and food consumption. Second, the mineral content in bones to determine the impact of OVX and aerobic workout on their storage levels has not been evaluated, which indicates that new studies with a longer intervention time should be performed to investigate this topic.

CONCLUSION

It was concluded that ovariectomy and physical inactivity increase obesogenic indicators and regular low to moderate intensity aerobic workout attenuates these unfavorable effects in rats.

CONTRIBUTORS

FSC FRANCO participated in the conception, design, execution, writing and revision of the study. NMB COSTA contributed to the conception, design, execution, writing and revision of the study. AJ NATALI contributed to the conception, design, writing and revision of the study.

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