Effect of 12-wk Training in Ovariectomised Rats on PGC-1α, NRF-1 and Energy Substrates

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ABSTRACT

Metabolic diseases are associated with hypoestrogenism owing to their lower energy expenditure and consequent imbalance. Physical training promotes energy expenditure through PGC-1α and NRF-1, which are muscle proteins of the oxidative metabolism. However, the influence of physical training on protein expression in individuals with hypoestrogenism remains uncertain. Thus, the aim of this study is to determine the effect of 12 weeks of moderate-intensity swimming training on the muscle expression of PGC-1α, NRF-1, glycogen and triglyceride in ovariectomised rats. OVX and OVX + TR rats were subjected to ovariectomy. The trained animals swam for 30 minutes, 5 days/week, at 80 % of the critical load intensity. Soleus was collected to quantify PGC-1α and NRF-1 expressions, while gastrocnemius and gluteus maximus were collected to measure glycogen and triglyceride. Blood glucose was also evaluated. Whereas ovariectomy decreased PGC-1α expression ($p < 0.05$) without altering NRF-1 ($p = 0.48$), physical training increased PGC-1α ($p < 0.01$) and NRF-1 ($p < 0.05$). Ovariectomy reduced glycogen ($p < 0.05$) and triglyceride ($p < 0.05$), whereas physical training increased glycogen ($p < 0.05$) but did not change triglyceride ($p = 0.06$). Ovariectomy increased blood glucose ($p < 0.01$), while physical training reduced it ($p < 0.01$). In summary, 12 weeks of individualized and moderate-intensity training were capable of preventing muscle metabolic consequences caused by ovariectomy.

Introduction

Besides the classic role in the reproductive system, the ovaries also secrete steroid hormones with supplementary functions in other tissues [1]. Some of the most relevant hormones secreted by ovaries are oestrogens, whose deficiency can be related to decreased energy expenditure [2], increased insulin resistance [2, 3], and imbalance in the lipid metabolism [4], favouring the emergence of metabolic diseases [5]. Ovariectomy is considered to be a valid animal model for studying the metabolic impacts caused by hypoestrogenism [2, 6]. At physiological levels, oestrogens participate in the energy metabolism by binding to receptors present in the mitochondria [3, 7], triggering several downstream signalling path-

ways [8]. Specifically regarding mitochondrial proteins, the relationship between oestrogens and the activation of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and nuclear respiratory factor 1 (NRF-1) [7] is well established and relevant in the literature.

PGC-1α is widely studied due to its action on lipid metabolism control [9, 10], muscle mass protection, reduction of the oxidative damage and inflammation [11], and fatty acid oxidation [9, 12]. PGC-1α coordinates gene expression, activating factors such as peroxisome proliferator-activated receptors (PPARs) [7, 9, 12], forkhead transcription factors (FOXOs) [7], and nuclear respiratory factors (NRFs). Among the NRFs (1 and 2), PGC-1α interacts with the NRF-1, co-activating its transcriptional activity [7, 9]. NRF-1 induces the expression of mitochondrial transcription factor A, which in turn promotes transcription of mitochondrial DNA (mtDNA) [7, 8, 13]. Both proteins, PGC-1α and NRF-1, are determinant for maintaining mitochondrial biogenesis [7]. Although reported that the knockout of oestrogen receptor α results in a reduction of fatty acid oxidation [13], changes in mitochondrial dynamics [7] and its functionality [14], the influence of oestrogen deficiency (at the systemic level) on the expression of PGC-1α and NRF-1 in the skeletal muscle, are not fully consolidated [2, 3, 8]. However, it is important to emphasize that molecular changes may justify systemic imbalances. Considering that the skeletal muscle plays a determining role in the regulation of the glycolytic and lipid metabolism and is known to contain a high expression of mitochondrial proteins and oestrogen receptors [15], investigations focusing on such muscle (especially muscle contraction) can bring clarifying answers about energy disorders associated with oestrogen deficiency. With this deep understanding, it is possible to look for alternatives to overcome the lack of actions previously triggered by such a hormone when its physiological levels are adequate.

In this sense, it is known that physical exercise is a powerful metabolic modulator of the skeletal muscle [16]. Muscle contraction generated during exercise promotes activation of PGC-1α [11, 16–18], with consequent activation of NRF-1 [7, 9]. Among the energetic metabolic responses produced by exercise, the stimulus on the PGC-1 activity leads to an increase in the fatty acid uptake and oxidation [11] and in the glucose uptake [19] through genes that regulate energy homeostasis [9]. Mainly in the oestrogen deficiency condition, exercise is commonly prescribed due to its beneficial health effects, with the improvement in muscle lipid profile being considered a relevant factor [20]. However, the effect of moderate-intensity physical training on PGC-1α and NRF-1 levels in this adverse situation is not fully consolidated, with the decrease of ovarian hormones considered an unfavourable factor to muscle and systemic energy homeostasis. For this purpose, the aim of this study was to determine the effect of 12 weeks of individualized and moderate-intensity physical training on the expression of PGC-1α and NRF-1 in the skeletal muscle tissue of rats and its repercussions on energy substrates of ovariectomised rats. We hypothesised that ovariectomy would lead to the reduction of PGC-1α and NRF-1 and that 12 weeks of individualized and moderate-intensity physical training would minimize such loss, allowing continuous stimulation of energy consumption by the metabolism, potentially preventing metabolic diseases.

Materials and Methods

Animals

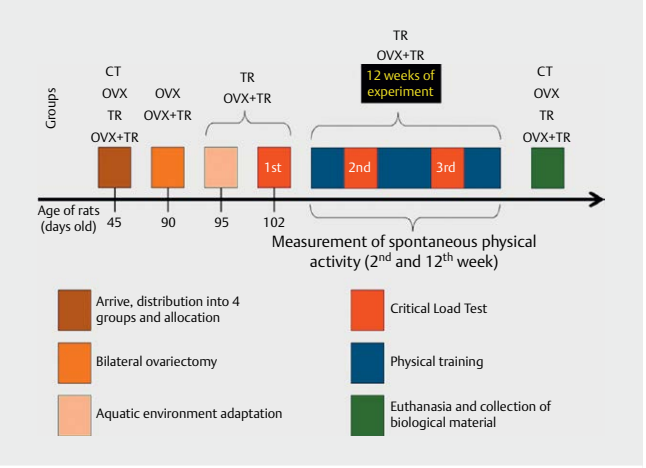
Thirty-eight female Wistar rats (body mass: 285 ± 25 g at the beginning of the experiment) were evaluated and housed in the animal facility, which follows a 10/14 hour light/dark cycle under a controlled temperature (22 ± 2 °C) with free access to water and feed for the rodents (Presence brand - laboratory line, Paulinia, São Paulo, Brazil) throughout the experiment. The experimental procedure was approved by the Ethics Committee on Animal Use (CEUA) under protocol no. 155606417. The study meets the ethical standards of the journal [21].

Experimental design

At 45 days old, the animals were randomly distributed into four groups: control (CT: n = 10), ovariectomised (OVX: n = 10), trained (TR: n = 9), and ovariectomised/trained (OVX + TR: n = 9). After familiarisation with the environment, at 90 days old the OVX and OVX + TR animals were subjected to the surgical procedure of bilateral ovariectomy. At 95 days old, the TR and OVX + TR groups started the aquatic environment adaptation, and at 102 days old these groups were subjected to the first critical load test to determine the exercise intensity for physical training over 12 weeks (▶ **Fig. 1**).

Surgical procedure of bilateral ovariectomy

First, the OVX and OVX + TR animals received intraperitoneal anaesthesia composed of ketamine (10 mg/kg) and xylazine (0.1 mg/kg). The technique was performed according to Zarrow [22], in which a 1.5-cm incision was made on both sides in the skin, lateral to the spine and between the last rib and the knee, allowing the ovaries to be exposed, tied, and removed from the pelvic cavity. The remainder of the tissue was returned to the peritoneal cavity and all the layers were sutured with cotton thread (line 10). The OVX and OVX + TR animals received analgesic (14.2 mg/kg of sodium dipyrone, 1x/day for 3 days) and were kept under observation for 5 days in the post-surgical period.



▶ **Fig. 1** Experimental design of the study.

Aquatic environment adaptation and critical load intensity determination

At 95 days old, the TR and OVX + TR animals were subjected to the protocol of familiarisation with the aquatic environment, adjusted according to the proposed models by Gobatto et al. [23] and De Lima et al. [24]. Our protocol lasted 6 days and involved progressively manipulated parameters, such as exposure time to water of 5–20 minutes, a depth of 10-80 cm, and load weight varying from 0 to 3 % of body mass. Swimming was performed in individual cylindrical PVC tanks with an 80 cm column of water x 30 cm in diameter and with the water temperature maintained at 31 ± 1 °C, following the guidelines of the American Physiological Society [25]. The workload was imposed to the animals by attaching small lead weights to their upper back using elastics according to the body mass of the animals (% body mass). Exercise sessions were conducted at the same time of the day (dark period; 05:00 p.m.). Deeper tanks of water with individual swimming compartments were used to keep the animals from resting at the tank bottom or jumping in an attempt to escape [26]. All training-related variables (prescribed and executed volume as well as workload intensity exercise) were recorded daily.

At 102 days old, the animals were subjected to critical load test, a protocol based on mathematical analysis of the relationship between exercise intensity and time to exhaustion. The test consisted of 4 maximum intensities with different loads (predictive loads) to analyse time to exhaustion between approximately two and ten minutes, performed with 24 hours among them. The criteria to exhaustion were established according to Beck and Gobatto [27]. The time and load data were subjected to linear regression analysis based on the third model proposed by Gobatto et al. [23].

The critical load (% body mass) was assumed as the angular coefficient from the linear fit (linear load vs. time to exhaustion). The coefficient of determination (R^2) accepted was higher than 0.95. Regarding the physical training protocol, the animals were allowed to swim for 30 consecutive minutes daily (5 days per week). The exercise intensity (individualized for each animal) was equivalent to 80 % of the critical load intensity, with weekly load adjustment according to body mass variation. Over the 12 weeks of physical training, the critical load test was reapplied in the fifth and ninth weeks for fine adjustment of critical load.

Spontaneous physical activity

Spontaneous physical activity was measured at the beginning (2nd week) and the end (12th week) using the gravimetric principle employed and described in detail by many researchers [28–31]. Spontaneous physical activity recordings were performed for 24 continuous hours (on non-training days), starting at 6:00 p.m. Considering that single housing (in order to obtain individual values) could be deleterious by disturbing the animals' daily routines, we measured spontaneous physical activity of rats on a per-cage basis where they were normally maintained (5 rats per cage). The daily spontaneous physical activity was determined by the sum of weight variations in absolute values, according to a mathematical strategy proposed by Biesiadecki et al. [32]. Spontaneous physical activity data were expressed as mean and standard deviation using two measures of daily summation for each group.

Obtention and storage of biological material

Euthanasia was performed after the 12-week interventions through decapitation, a method considered acceptable by the American Veterinary Medical Association (AVMA) [33]. In order to avoid acute effect of physical exercise, the euthanasia of the TR and OVX + TR animals (in rest condition) occurred 48 hours after the last physical training session. After euthanasia, the blood was collected (the animals were not fasted) and the samples were centrifuged for 15 minutes at 3000 rpm for serum separation for blood glucose analysis. The soleus muscle was excised, dusted in talcum, frozen in liquid nitrogen (–196 °C) and stored at –80 °C until the histological procedures. Gluteus maximus and a mixed portion of the gastrocnemius skeletal muscles were collected as additional tissues for triglyceride analysis plus liver for glycogen analysis. The uterus was weighed for some animals to confirm the ovariectomy procedure.

Histology and immunofluorescence procedures

The protein expressions were analysed by immunofluorescence [34, 35]. First, the transversal histological frozen sections (6 micrometres) of the soleus muscle were obtained from a cryostat (Leica CM 1850 UV) maintained at –25 °C and collected in glass slides (26 × 76 mm). The slides were stained with haematoxylin-eosin to identify morphological alterations in the tissue through a light microscope.

For NRF-1 quantification, the sections underwent double staining with laminin (for the purpose of demarcating the cell) and anti-NRF-1. The slides were incubated with a mix of NRF-1 primary antibodies (mouse monoclonal; dilution 1:500; Santa Cruz Biotechnology, Inc.; Dallas, TX, USA; Cod. sc-101102) and anti-laminin (rabbit polyclonal; dilution 1:200; Abcam; Cambridge, UK; Cod. Ab11575) diluted in BSA 1 % for 45 minutes at 37 °C. Then, the sections were washed in PBS solution (three cycles of 5 minutes) and incubated for 35 minutes at 37 °C by a mix of secondary antibodies: Alexa Fluor 488 IgG_1 to mark NRF-1 in green colour (polyclonal; dilution 1:1000; Jackson ImmunoResearch, Laboratories, Inc.; West Grove, PA, USA; Cod. 115-545-205) and Alexa Fluor 647 IgG to mark laminin in red colour (polyclonal; dilution 1:200; Invitrogen; Life Technologies; Carlsbad, CA, USA; Cod. A-21244). Subsequently, the sections were washed again with PBS solution (three cycles of 5 minutes).

Likewise, to identify PGC-1α the sections also underwent double staining with laminin (for the purpose of demarcating the cell) and anti-PGC-1α. A mix of PGC-1α primary antibodies (mouse monoclonal; dilution 1:50; Santa Cruz Biotechnology, Inc.; Dallas, TX, USA; Cod. sc-518025) and anti-laminin (rabbit polyclonal; dilution 1:200; Abcam; Cambridge, UK; Cod. Ab11575) was applied on the sections for 45 minutes at 37 °C, with subsequent PBS solution washing. The mix of secondary antibodies used was Alexa Fluor 647 IgG_{2a} to mark PGC-1α in red colour (dilution 1:1000; Santa Cruz Biotechnology, Inc.; Dallas, TX, USA; Cod. sc-24637) and Alexa Fluor 488 IgG to mark laminin in green colour (diluted 1:200; Invitrogen, Life Technologies), maintaining the incubation for 35 minutes at 37 °C. The slides were photographed in an automated fluorescence microscope system (ImageXpress Micro, Molecular Devices; San José, CA, USA) using the 20x objective lens and specific filters for NRF-1/laminin (FITC/Cy5) and PGC-1α/laminin (Cy5/FITC). The

images were saved with identical size and resolution. After image acquisition, the expressions of PGC-1α and NRF-1 were quantified by selecting five distinct and random fields (with standardized height and width) in three sections per animal using ImageJ 1.52a software (National Institutes of Health, Bethesda, MD, USA). The images were selected randomly and without overlay. The mean value of integrated intensity for each sample was calculated and plotted in the graph.

Muscle triglyceride

Firstly, for every 200 mg of muscle tissue (gluteus maximus and gastrocnemius) we added 1 mL of Triton X-100 at a concentration of 0.1 %, with subsequent mechanical homogenization. Thus, the muscle tissue was centrifuged for 10 minutes at 4000 rpm, and 10 µL of supernatant was collected from each sample and pipetted into a microplate with 200 µL of reagent from the commercial triacylglycerol kit (LaborLab; Cod. 1770290; Guarulhos, São Paulo, Brazil). The reading was made using a SpectraMax i3 spectrophotometer (Molecular Devices, San José, CA, USA) operating at a wavelength of 505 nm, following the kit guidelines.

Liver and muscle glycogen

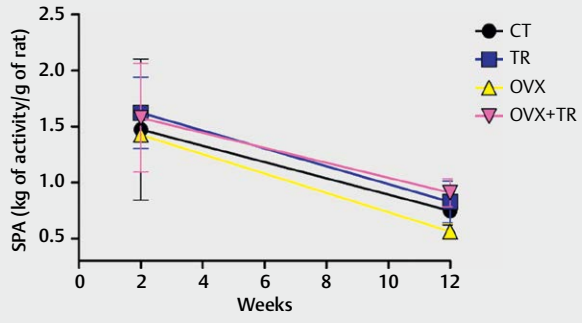
The technique followed the protocol reported by Dubois et al. [36], which consisted of primary tissue digestion (gluteus maximus, gastrocnemius skeletal muscles, and liver) in KOH (30 %). Subsequently, the glycogen was precipitated using sodium sulphate (Na_2SO_4) and ethanol (70 %). From a chemical reaction using phenol (Êxodo Científica, Sumaré, São Paulo, Brazil) and sulphuric acid (Dinâmica Química Contemporânea Ltda, Indaiatuba, São Paulo, Brazil), a glycogen concentration was colourimetrically measured by the sample absorbance (wavelength of 490 nm) in a spectrophotometer (Hach Company, Loveland, CO, USA).

Serum analysis

With regard to the blood glucose analysis, in each sample containing 3 µL of serum 300 µL of reagent from a commercial glucose kit (LaborLab; Cod. 1770130; Guarulhos, SP, Brazil)was added. The reading was made using a spectrophotometer (SpectraMax i3, Molecular Devices; San José, CA, USA) operating at a wavelength of 505 nm, following the kit guidelines.

Statistical analysis

The results were presented as mean ± standard deviation. Data were submitted to Shapiro-Wilk's normality test, allowing the use of parametric statistics. PGC-1α and NRF-1 expressions, muscle triglyceride and glycogen, body mass, blood glucose, and hepatic glycogen were subjected to a two-way factorial analysis of variance for the main effects of physical training (physical training, two levels: CT and OVX vs. TR and OVX + TR) and ovariectomy (ovariectomy, two levels: CT and TR vs. OVX and OVX + TR). Spontaneous physical activity underwent a three-way factorial analysis of variance to evaluate the effects of physical training, ovariectomy and time. To obtain the final value of muscle triglyceride and glycogen, we performed a pooled analysis (gluteus maximus with gastrocnemius). When appropriate, we used the Newman-Keuls post hoc test. The thresholds for small, moderate, and large effects were 0.20, 0.50, and 0.80, respectively. The effect size (ES) was determined according to the formula: (mean1 -mean2)/pooled SD [37]. A significance level of 5 % was established for all analyses, and Statistica 7.0 (StatSoft, Inc.; Tulsa, OK, USA) was used.



▶ **Fig. 2** Data from spontaneous physical activity at the beginning (2nd week) and the end (12th week) of experimental procedures for the control (CT; n = 10), trained (TR; n = 9), ovariectomised (OVX; n = 10), and ovariectomised/trained (OVX + TR; n = 9) groups. The values are expressed as mean and standard deviation of two measures of daily summation for each group. SPA: spontaneous physical activity

Results

Spontaneous physical activity

When evaluating the second and last weeks of the experiment (▶ **Fig. 2**), it was possible to note that ovariectomy did not promote alteration in the spontaneous physical activity ($F = 0.38$; $p = 0.53$), whereas physical training increased it ($F = 5.15$; $p < 0.05$) and time reduced it ($F = 89.95$; $p < 0.01$).

Swimming physical training

The data regarding the time to exhaustion mean verified during the performance of 4 maximum intensity tests with different loads (% body mass) used in the 3 critical load tests throughout the experiment are presented in ▶ **Tab. 1**. For the TR group, Load 1, Load 2, Load 3 and Load 4 corresponded to a mean and standard deviation of 7.5 ± 0.9, 9.0 ± 0.9, 10 ± 1.0 and 12 ± 2.0 % of body mass, respectively, whereas for the OVX group these values were 7.7 ± 1.4, 9.0 ± 0.8, 10 ± 0.9, and 11 ± 1.0 % of body mass, respectively. In relation to critical load, for the 1st, 2nd, and 3rd tests the TR group presented a mean and standard deviation of 7.54 ± 1.01, 8.16 ± 0.89, and 8.01 ± 0.64 % of body mass, respectively, whereas the OVX + TR group obtained a value of 6.01 ± 0.83, 8.09 ± 0.99, 7.85 ± 0.88 % of body mass, respectively. The mean value of R^2 for both groups was 0.98. At the end of the experiment, the total time of swimming physical training was calculated considering all sessions. It was verified that the animals (for both groups) reached an average value of 96.5 % of the total established for the protocol.

PGC-1α and NRF-1 quantification by immunofluorescence in soleus skeletal muscle

Ovariectomy decreased PGC-1α ($F = 4.87$; $p < 0.05$), whereas physical training enhanced the expression of this protein ($F = 8.60$; $p < 0.01$) (▶ **Figs 3a and c**). Regarding the expression of NRF-1, ovariectomy did not alter it ($F = 0.50$; $p = 0.48$), whereas physical training increased it ($F = 5.28$; $p < 0.05$) (▶ **Figs 3b and d**).

Muscle triglyceride and glycogen

Ovariectomy reduced muscle triglyceride ($F = 6.49$; $p < 0.05$), whereas physical training did not promote any alteration in this variable ($F = 3.57$; $p = 0.06$). As to muscle glycogen, ovariectomy promoted a decrease in glycogen storage ($F = 4.53$; $p < 0.05$), whereas physical training increased it ($F = 5.45$; $p < 0.05$) (▶ **Fig. 4**).

Body mass, blood glucose, and hepatic glycogen

Over 12 weeks, ovariectomy increased body mass ($F = 23.39$; $p < 0.01$), whereas physical training was not able to alter this parameter ($F = 0.41$; $p = 0.52$). Regarding blood glucose, ovariectomy increased ($F = 9.57$; $p < 0.01$), whereas physical training decreased it ($F = 27.66$; $p < 0.01$). Ovariectomy caused a decrease in the amount of hepatic glycogen ($F = 5.80$; $p < 0.05$), whilst physical training did not reverse this effect ($F = 1.24$; $p = 0.27$) (▶ **Tab. 2**).

Discussion

Overall, ovariectomy was found to promote significant changes in the muscle metabolism by reducing PGC-1α levels and the energy reserve of triacylglycerol and glycogen – even though it did not change the NRF-1 expression. These factors may have contributed to the reduction in the hepatic glycogen, the increase in blood glucose and body mass gain. On the other hand, to the best of our knowledge this is the first study showing the efficacy of moderate-intensity physical training with individual adjustment of loads in the suppression of these metabolic impairments, allowing OVX + TR animals to present conditions similar to CT animals.

It was also found that ovariectomy reduced the PGC-1α in the skeletal muscle ($F = 4.87$; $p < 0.05$), corroborating some studies in the literature [2, 4, 8]. The decrease in PGC-1α levels has negative consequences for some functions, such as the regulation of transcriptional genes that act on the muscular structure and function [11] and genes responsible for energy transport and oxidation [9]. Considering that PGC-1α transcription is induced after binding of the complex (oestrogen and receptor) to nuclear DNA [7], an adverse situation of oestrogen deficiency can negatively affect the PGC-1α expression, especially after long periods. In addition, PGC-1α is regulated via AMP-activated protein kinase (AMPK), an enzyme that in the presence of oestrogens has its expression increased [8]. Barbosa et al. [8] identified that animals under ovariectomy effect presented a decrease in AMPK and PGC-1α, evidencing this relationship and the importance of oestrogens in the maintenance of these expressions.

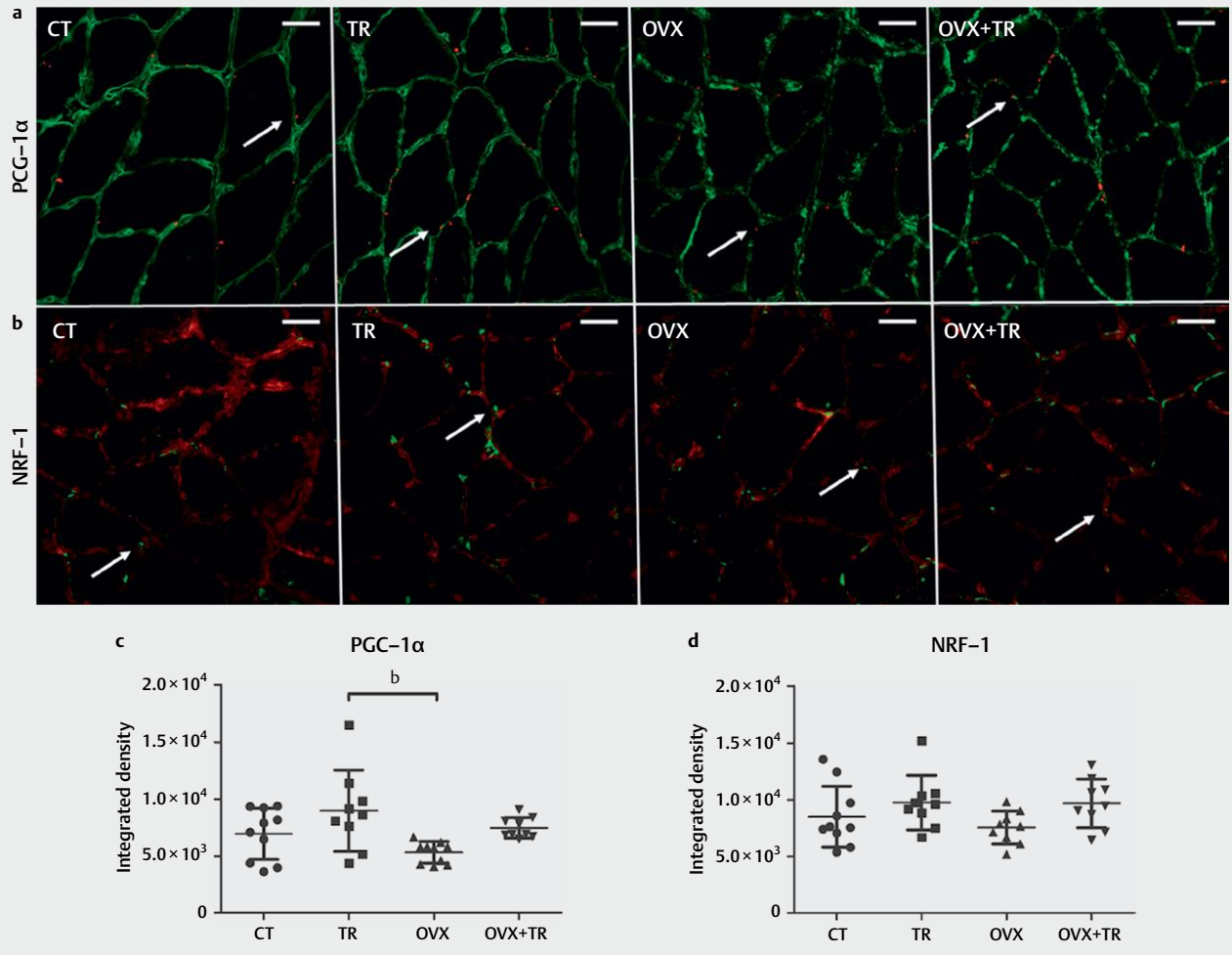
Owing to the participation of the PGC-1α in bioenergetic reactions such as beta oxidation [9, 12], the entry of fatty acid in the skeletal muscle becomes essential [9], being the main carrier the fatty acid translocase CD36 (FAT CD36) [38]. Considering that FAT CD36 is a protein regulated by PGC-1α [9], this may explain why the present study found triacylglycerol reduction in ovariectomised animals ($F = 6.49$; $p < 0.05$). As to carbohydrate reserve, it is known that ovariectomy causes reduction in the glucose transporter 4 (GLUT4) levels [39], justifying the decrease in muscle glycogen storage observed ($F = 4.53$; $p < 0.05$). In addition, following our result about the reduction of PGC-1α in ovariectomised animals, it is reported in the literature that PGC-1α influences the GLUT4 transcription [40], reflecting in the glycogen replacement owing to the role of such a transporter in glucose uptake [18]. This point contributes to the understanding of the reduced blood glucose levels found in this study as a result of physical training ($F = 27.66$; $p < 0.01$). OVX + TR animals showed an 11 % reduction in blood glucose when compared to OVX, considering that this parameter is extremely favourable to the prevention of metabolic diseases in view of the relationship between oestrogen deficiency and insulin resistance [41, 42]. Similarly to Baxi et al. [43], our study pointed to a reduction in the hepatic glycogen level caused by ovariectomy, which can be explained by the action of oestrogens over glucose transporter 2 [44]. These energy impairments in the hepatic tissue may affect systemic balance, favouring the appearance of metabolic diseases.

Given that PGC-1α stimulates NRF-1 [7, 11], our hypothesis that NRF-1 expression would also be affected in the ovariectomy condition was refuted. Conversely, ovariectomy did not alter NRF-1 levels ($F = 0.50$; $p = 0.48$), corroborating other studies in the literature [2, 3] and in contrast to the study by Barbosa et al. [8]. However, when OVX and CT groups are compared, the non-significant 11 % reduction in NRF-1 expression suggests that an extended intervention time could lead to substantial results. Our hypothesis started from the concept that oestrogens would activate NRF-1 [7, 13, 45], while in the opposite situation (in the ovariectomy condition) the pathway would be less activated, culminating in less expression. In this way, it can be deduced that NRF-1 is less sensitive to oestrogen, since in a situation where oestrogen levels are normal there is a smaller number of pathways influencing its activation, whereas PGC-1α is usually more stimulated and has its vulnerable expression in an adverse circumstance of hypoestrogenism.

On the other hand, the physical training chosen is characterized as endurance exercise according to the intensity applied [46], being associated with PGC-1α expression for acting on its transcriptional regulation [47, 48], as well as with NRF-1 [49]. This study found that physical training increased the PGC-1α ($F = 8.60$; $p < 0.01$) and when comparing the mean of the OVX + TR group to that of the OVX and CT groups, it was found that physical training caused an increase of 40 % and 7 %, respectively – a non-significant value. A similar result was reported by Barbosa et al. [8] when subjecting ovariectomised animals to ladder training for 12 weeks. Despite the difference in the training methodology, both can be effective as long as executed in the long term. These same animals presented increased AMPK and cAMP response element-binding protein, signalling pathways responsible for stimulating PGC-1α that are activated during physical exercise [8], which aids in understanding possible activated pathways. In contrast, Zheng et al. [4] did not report any change in the PGC-1α expression after performing ovariectomy in animals under training on a treadmill, which consisted of a gradual increase in speed (12–20 m/min) for 10 minutes, twice a day for 3 days, offering 1 day of rest over 61 days. This comparison allowed

▶ **Table 1** Time to exhaustion data verified in the performance of 4 maximum intensity tests with different loads (% body mass) used in the three critical load tests throughout the experiment in the trained group (TR; n = 9) and ovariectomized/trained group (OVX + TR; n = 9).

	Time to exhaustion (s) – load 1	Time to exhaustion (s) – load 2	Time to exhaustion (s) – load 3	Time to exhaustion (s) – load 4
TR	149 ± 52	245 ± 51	359 ± 107	565 ± 112
OVX + TR	158 ± 43	272 ± 52	432 ± 89	600 ± 112
Data from time to exhaustion. Values expressed as mean and standard deviation. s: seconds.				



▶ **Fig. 3** Representative immunofluorescence images from tissue sections. **a**) Samples of laminin (green) with PGC-1α (red) in soleus skeletal muscle and **b**) samples of laminin (red) with NRF-1 (green) in soleus skeletal muscle in the control (CT; n = 10), trained (TR; n = 9), ovariectomised (OVX; n = 10), and ovariectomised/trained (OVX + TR; n = 9) groups. The graphs represent the mean and standard deviation of the (**c**) PGC-1α and (**d**) NRF-1 expressions by integrated density. Statistical analysis: two-way ANOVA was used to identify the effects of ovariectomy and physical training, whereas Newman Keuls post hoc test was used to trace differences among groups. Statistical significance symbols: [b] $p < 0.05$ in relation to TR. In comparison with significant difference the ES was 1.62 (TR vs. OVX). For illustration, we used objective lens = 20x; bars = 20 µm

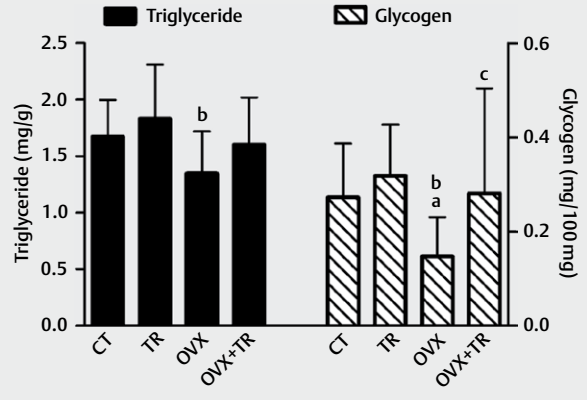
us to infer that the intensity does not necessarily need to be severe to achieve positive results. Instead, weekly frequency and duration of the session can be considered important points in physical training. Another point that must be considered relevant for the stimulation of PGC-1α is the high accumulated amount of muscle contraction (volume rather than exercise intensity).

Based on the data, it can be concluded that PGC-1α is responsible for the increase in the NRF-1 expression as a result of physical training ($F = 5.28$; $p < 0.05$) due to its co-activator role in NRF-1 [7, 8, 11, 50]. It has been reported that rodents have up-regulation of NRF-1 after 12–18 hours of swimming training sessions (two 3-h bouts separated by a 45-min rest), with a concomitant increase in the PGC-1α level [51].

Additionally, the increase in muscle glycogen promoted by physical training ($F = 5.45$; $p < 0.05$) can be associated with the increase in PGC-1α. This is supported by Wende et al. [52], who studied the role of PGC-1α in glycogen replenishment in the post physical training period. The authors found a lower substrate replacement in an-

imals with PGC-1α deficiency. A connection between PGC-1α activity and GLUT4 transcription [40] may explain the mechanisms involved in the management of energy storages, such as glycogen and glucose uptake [18]. Although the present study applied moderate-intensity physical training characterized by fat oxidation rates, it must be mentioned that carbohydrates stored as muscle glycogen are essential for maintaining the Krebs cycle [53], evidencing the importance of energy availability of this substrate for the skeletal muscle.

In addition to the main variables mentioned, it is known that exercise stimulates energy expenditure [54]. In this study, it was found that physical training increased this parameter despite the reduction in spontaneous physical activity of all groups observed in the last week ($F = 5.15$; $p < 0.05$). According to Garland et al. [55] and Kotz et al. [56], such a parameter can correspond to 30 % of the daily energy expenditure of an animal. As physical activity favours energy expenditure primarily by stimulating the skeletal muscle with consequent energy consumption [57], this factor may have influenced the positive results of physical training even though some parameters were compromised by ovarian hormone deficiency.



▶ **Fig. 4** Representative graphs of muscle triglyceride and glycogen contents. Data from muscle triglyceride (left Y axis) and glycogen (right Y axis) for the control (CT; n = 10), trained (TR; n = 9), ovariectomised (OVX; n = 10), and ovariectomised/trained (OVX + TR; n = 9) groups. The values are expressed as mean and standard deviation. Statistical analysis: two-way ANOVA was used to identify the effects of ovariectomy and physical training, whereas Newman Keuls post hoc test was used to trace differences among groups. Statistical significance symbols: [a] $p < 0.05$ in relation to CT; [b] $p < 0.05$ in relation to TR; [c] $p < 0.05$ in relation to OVX in the same parameter. In comparison with significant difference the lower ES was 1.13 for triglyceride (OVX vs. TR) and 0.88 for glycogen (OVX vs. OVX + TR). mg: milligrams; g: grams

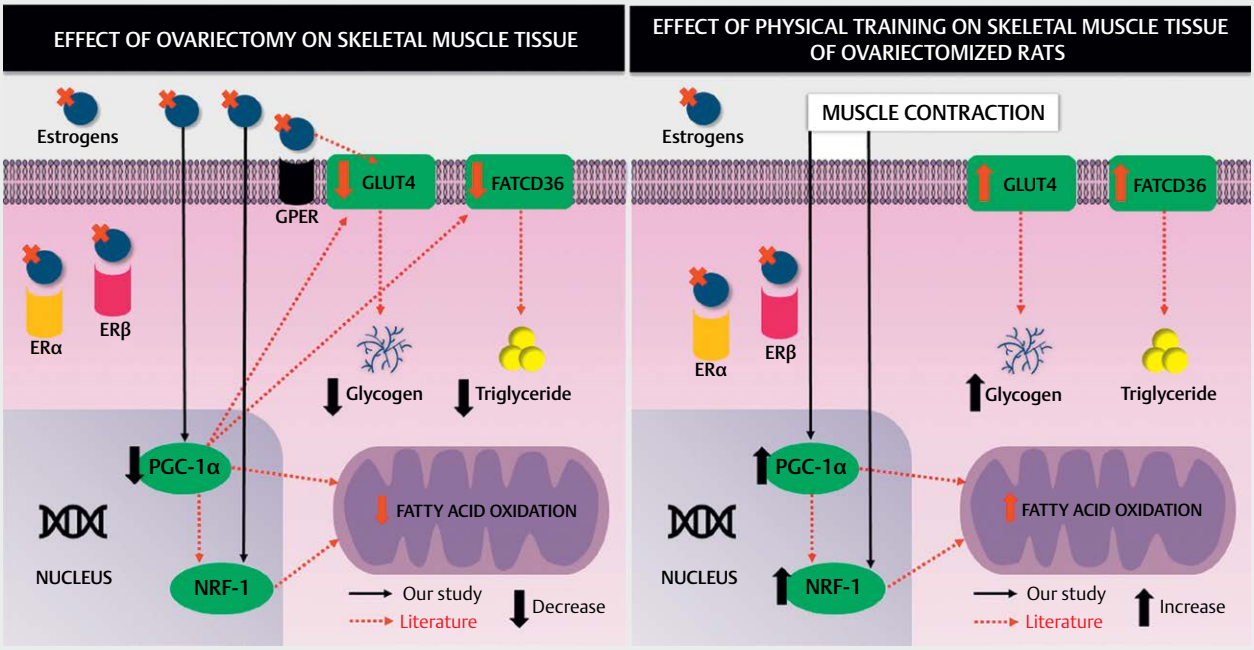
In summary, the skeletal muscle is a potent stimulator of body energy expenditure [2], highly modulated by the action of oestrogens from the activation of proteins, resulting in increased mitochondrial biogenesis, beta-oxidation, and lipid catabolism [6, 15]. Among the proteins responsive to the presence of oestrogens, we can highlight PGC-1α and NRF-1, whose relationship with these metabolic phenomena was already mentioned. On the other hand, it was observed that the abrupt decline in the oestrogen levels mainly compromises the PGC-1α expression. Considering that ovariectomy is known to be associated with metabolic syndrome [6], our findings at the molecular level help in the systemic understanding of the energy imbalance that increases blood glucose and body mass, favouring the appearance of other metabolic complications. In an attempt to maintain the PGC-1α and NRF-1 levels for an adequate functioning of the skeletal muscle from the consumption and oxidation of substrates in ovariectomised animals, this study opted for physical training, a non-pharmacological treatment that may effectively promote a healthy metabolism by means of muscle contractions. Finally, the didactic illustration (▶ **Fig. 5**) was designed to compile the data obtained in this study and the possible mechanisms involved according to the literature.

Despite the satisfactory results supported by the theoretical concepts in the literature, the study is not beyond criticism. We did not measure the food intake, which could aid in the interpretation of energetic data. Indeed, the serum levels of steroid hormones were not measured, with ovariectomy success confirmed through the uterus weight of some animals. Moreover, a group with oestradiol supplementation was not included in the study. Yet, the sham surgery was not performed in the non-ovariectomised groups. Regarding it, we considered that recovery occurs in one week [58] and the surgery effect would not be present after 12 weeks of the experiment. Nevertheless, it is an important issue that must be considered to accordingly interpret the results of this experiment.

▶ **Table 2** Physiological characterization data in the control group (CT; n = 10), trained group (TR; n = 9), ovariectomised group (OVX; n = 10) and ovariectomised/trained group (OVX + TR; n = 9).

	CT	TR	OVX	OVX + TR
Body mass (g)	313.43 ± 17.89	302.51 ± 24.07	349.78 ± 24.01[a,b]	349.62 ± 37.55[a,b]
Blood glucose (mmol/L)	7.13 ± 0.70	6.14 ± 0.40[a]	7.64 ± 0.39[b]	6.74 ± 0.34[b,c]
Hepatic glycogen (mg/100 mg)	3.02 ± 1.23	3.69 ± 1.35	2.16 ± 0.83	2.46 ± 0.37

Data from body mass, blood glucose, and hepatic glycogen in the control group (CT; n = 10), trained group (TR; n = 9), ovariectomised group (OVX; n = 10), and ovariectomised/trained group (OVX + TR; n = 9). Values expressed as mean and standard deviation. Two-way ANOVA was used for identifying the effects of ovariectomy and physical training and Newman Keuls post hoc test was used to trace differences among groups. Statistical significance symbols: [a] $p < 0.05$ in relation to CT; [b] $p < 0.05$ in relation to TR and [c] $p < 0.05$ in relation to OVX in the same parameter and in the comparisons with significant difference, the lower ES were 1.31 for body mass (CT vs. OVX + TR) and 1.61 for blood glucose (TR vs. OVX + TR). g: grams; mmol: millimole; L: litre; mg: milligrams; dL: decilitre.



▶ **Fig. 5** Illustration of ovariectomy actions and muscle contraction in the skeletal muscle. The black arrows correspond to our results, while the dashed red arrows represent the literature data. Ovariectomy resulted in lower PGC-1α activation and transcription, promoting a decline in the fatty acid oxidation. The muscle contraction caused by physical training resulted in greater PGC-1α activation and transcription, with consequent NRF-1 activation and transcription, reflecting in the increase of fatty acid oxidation. PGC-1α regulated FAT CD36 and GLUT4, favouring fatty acid and glucose uptake, respectively. GPER: G-protein-coupled oestrogen receptors; ERα: oestrogen-receptor alpha; ERβ: oestrogen-receptor beta

Conclusion

Ovariectomy was found to reduce PGC-1α in the skeletal muscle, as well as muscle triglyceride and glycogen and hepatic glycogen, while increasing blood glucose and body mass. However, moderate-intensity physical training at 80 % of individual critical load (30 minutes daily, 5 times a week) for 12 weeks was efficient in preventing these metabolic impairments, maintaining the PGC-1α and NRF-1 levels and allowing the maintenance of physiological conditions, even under oestrogen deficiency. Together, such results showed the positive influence of physical exercise as a non-pharmacological treatment for the prevention of metabolic impairments caused by hypoestrogenism.

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Conflict of interest

The authors declare that they have no conflict of interest

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