Physical Inactivity is a Major Contributor to Ovariectomy-Induced Sarcopenia

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Abstract

Since the mechanism(s) underlying menopause-related sarcopenia remain unknown we aimed to investigate the role of physical inactivity in its etiology. Ovariectomized and sham-operated rats were allocated into 2 experimental groups: 1) sedentary-standard housing; and 2) exercise-housed with running wheel. After a 9-month experimental period, soleus muscle structure and biochemical properties were analyzed. No differences existed in muscle fibre size or ultrastructure between sedentary sham and ovariectomized animals housed in standard conditions. In the exercise groups, average daily running distance was 10-fold less in ovariectomized compared to sham-animals. Further, in exercised animals, soleus fibre size was smaller in ovariectomized compared to sham-animals. Nonetheless, compared to both sedentary groups, muscle fibre size was larger in the exercised ovariectomized animals. Our results indicate that ovariectomy-induced sarcopenia is not due to the loss of ovarian hormones per se, but is largely due to physical inactivity.

Introduction

Menopause is a milestone in women's health, as the loss of regular estrogen production is typically followed by an increased occurrence of several detrimental musculoskeletal and cardiovascular changes. Among those, decreases in skeletal muscle mass and function are major outcomes of menopause and they are thought to be directly linked to hypoestrogenemia [9]. Globally, these skeletal muscle changes are termed sarcopenia, and they reflect a decrease in both the size of individual myocytes as well as a relative increase in intercellular connecting tissue, which eventually drives down the force generated per unit area thereby reducing skeletal muscle quality [27].

Skeletal muscle in several animal species, including humans [17] express estrogen receptors (ER) making muscle fibres a target tissue of estrogen action. A decrease in skeletal muscle mass and strength is a well-documented outcome of menopause. [9] This menopause-related loss of skeletal muscle mass and strength is a well-documented outcome of menopause. [9] This menopause-related loss of skeletal muscle mass and strength can be prevented, at least in part, with estrogen replacement therapy [31]. Ovariectomy (OVX) in mice also leads to significant decreases in locomotor muscle strength [21] which can be successfully restored following estrogen treatment [20]. Several mechanisms have been proposed to explain the loss of skeletal muscle mass and strength induced by estrogen deficiency [2–6,16,25,26,33,36,38]. For example, changes in mice soleus muscle contractile properties following OVX are shown to be associated with a significant shift in muscle fibre composition from fast to slow myosin heavy chain (MyHC) isoforms [16]. Ovariectomy is also known to induce alterations on the extracellular matrix of the rat heart by promoting an increase in type I collagen expression and a decrease in matrix metalloproteinase 2 expression, both of which are prevented by estrogen supplementation [39]. Furthermore, low estrogen levels could also have a negative impact on skeletal muscle energy metabolism as mitochondria are an estrogen sensitive organelle [5] as estrogen administration to OVX animals enhances respiratory chain activity [25]. Interestingly, some of the impairment within skeletal muscle of OVX animals has been shown to be prevented by regular bouts of muscular exercise [16]. This observation raises the possibility that the impact of estrogen deficiency on skeletal muscle is not due to estrogen defi-
ciency alone. In this regard, evidence suggests that estrogen plays an important role in voluntary physical activity because low estrogen levels is associated with physical inactivity [11]. Indeed, OVX rats have been reported to run significantly less compared to age matched intact controls [35]. In addition, estrogen replacement can effectively reverse OVX induced physical inactivity in mice in a dose-dependent fashion [8,11]. Collectively, these data suggest that low estrogen levels are associated with a decrease in voluntary physical activity. It follows that the decreases in skeletal muscle mass and strength following meno-
pause could be linked to reduced physical activity (i.e., muscle disuse) and therefore, may not be directly linked to the effect of low estrogen levels on skeletal muscle fibres per se. To investi-
gate this issue we hypothesized that physical inactivity is a
major contributor to menopause related sarcopenia. Therefore, the aim of this study was to test if the loss of ovarian function has a direct effect on skeletal muscle structural and biochemical properties or if the changes in skeletal muscle following loss of ovarian function largely reflect muscle disuse due to decreases in voluntary motor activity.

Materials and Methods

Overview of experimental approach
To determine the role that estrogen plays in skeletal muscle structure and size as well as the role of estrogen in promoting physical activity, we performed the following experiment. Our experimental approach was to study ovariectomized and sham operated female rats that were housed with access to running wheels every day. Distance travelled by each rat in the running wheel was recorded daily and body weight recorded weekly at the end of the light phase. Food intake was also recorded weekly and was determined as the difference in chow weight contained on the rack between the beginning and the end of the week. All animal sacrifices were approved through graded ethanol solutions, cleared in xylene and embedded in Epon (TAAB) at 60 °C until polymerization. Ultra-thin (100 nm) sections were cut in a ultramicrotome (Reichert-Jung Ultracut) with a diamond knife (ultra 45 ° Dia-
mome), contrasted with uranyl acetate and lead citrate (Sigma) and embedded in Epon (TAAB) at 60 °C until polymerization. Ultra-thin (100 nm) sections were cut in a ultramicrotome (Reichert-Jung Ultracut) with a diamond knife (ultra 45° Dia-

tome), contrasted with uranyl acetate and lead citrate (Sigma) and analyzed with a transmission electron microscope (Zeiss EM10A) at an accelerating voltage of 60 kV. Micrographs were recorded with a coupled photographic camera.

Soleus muscle fibre cross-sectional area (CSA)
Soleus muscle sections were stained with H&E and the images analyzed with ImageJ software (NIH, Bethesda, MD). CSA was determined for each animal as the average area of 350 fibres. The

Animal sacrifice and organs collection
Following the 9-month experimental period, all rats were anesthetized with 4% sevoflurane and sacrificed by exsanguination. Note that all animals were 14 months old at time of sacrifice. Blood was collected from vena cava and was later used for bio-
chemical analysis. Parametrial, retroperitoneal, inguinal, and mesenteric fat depots were surgically dissected and weighed for quantification of intra-abdominal fat content. Both hind limbs
soleus muscle were dissected, washed in cold PBS (pH 7.2) and weighed together. Organs weight was determined with a precision balance (resolution 0.01 mg; Kern 870). Right tibia was also collected and its length measured with a digital calliper (resolution 0.01 mm, Powerfix) for correcting weight comparisons to differences in animal size. The right soleus muscle was removed, weighed and transversely sectioned in 2 samples: one processed immediately for light (LM) microscopy and the second for transmission electron microscopy (TEM). The left soleus muscle was minced and homogenized in a ratio of 1:10 in ice-cold extraction medium containing 50 mM Tris/base and 1 mM EDTA (Sigma) pH 7.4 with a Potter-Elvehjem homogenizer and pestle. The homoge-
nate was then collected, sonicated for 1 min in ice-cold water (Sonorex, Bandelin Electronic) and centrifuged at 700g for 10 min at 4 °C. The supernatant was collected for spectrophotometric assessment of citrate synthase (CS) and glyceraldehyde 3-phos-
phate dehydrogenase (GAPDH) activity as well as type I myosin expression by Slot-Blot. Total protein content was determined by the Lowry method using BSA (Sigma) as standard.

Histology

Tissue processing for light and transmission electron microscopy
One of the right soleus muscle samples was fixed overnight in a solution containing 4% paraformaldehyde, 2.5% sucrose (Sigma) and 0.1% glutaraldehyde (TAAB) in PBS (pH 7.2) at 4 °C, dehy-
drated through graded ethanol solutions, cleared in xylene and mounted in paraffin. Transverse 6μm thick sections were cut, and used for assessing fibre cross-sectional area (CSA), fibrous tissue accumulation, apoptotic myonuclei presence and type I myosin heavy chain (MyHC) expression by immunohistochem-
istry. The sections were analyzed with a light microscope and images recorded with a coupled digital camera (Axio Imager A1, Carl Zeiss; Germany). The remaining sample of the right soleus muscle was sectioned into smaller portions (≈3 mm3) and fixed overnight in 2.5% gluteraldehyde in 0.2M sodium cacodylate buffer (Sigma; pH 7.2) at 4 °C. After rinsing with 0.2M sodium cacodylate buffer they were post fixed with 2% osmium tetrox-
ide in 0.2M sodium cacodylate, dehydrated in graded ethanol and embedded in Epon (TAAB) at 60 °C until polymerization. Ultra-thin (100 nm) sections were cut in a ultramicrotome (Reichert-Jung Ultracut) with a diamond knife (ultra 45° Dia-
tome), contrasted with uranyl acetate and lead citrate (Sigma) and analyzed with a transmission electron microscope (Zeiss EM10A) at an accelerating voltage of 60 kV. Micrographs were recorded with a coupled photographic camera.
intra-observer coefficient of variation (CV) was calculated for half of the images and was found to be 4.65%.

Assessment of fibrous tissue accumulation
*Soleus muscle* sections were stained with Picrosirius red (PSR) according to the method of Sweat et al. [34] by incubation on 0.1% sirius red in saturated picric acid for 1 h. Sections were then rinsed in 0.5% acetic acid, dehydrated in ethanol and cleared in xylene. PSR technique stains collagen bright red and muscle tissue yellow. Images were analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Inc.) by quantification of the percentage area covered by collagen (red) and muscle tissue (yellow). The intra-observer coefficient of variation (CV) was determined for a subset of images and found to be 5.27%.

Assessment of apoptosis in soleus muscle
The presence of apoptotic nuclei in soleus muscle was assayed by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) using a commercially available kit (In situ cell death detection kit AP) according to the manufacturer’s instructions (Roche). After deparaffinization, sections were immersed in 0.1M citrate buffer (pH 6.0) and microwave irradiated for 1 min (750W). After rinsing in cold PBS, sections were first blocked with 3% BSA in 0.1M Tris-HCl (pH 7.5) for 30 min at 20°C and then incubated in freshly prepared TUNEL reaction solution (nucleotide mixture+terminal deoxynucleotidyl transferase) in a humidified chamber at 37°C for 60 min in the dark. Negative and positive controls were simultaneously prepared by incubation with label solution only (nucleotide mixture) or by incubation with DNase I (Sigma) prior to the labelling procedure, respectively. Sections were analyzed with a fluorescent microscope coupled to a digital camera (Axio Imager A1, Carl Zeiss) and apoptotic cells were identified as brightly fluorescent in opposition to the pale green background staining.

*Soleus muscle ultrastructure analysis*
Morphometric analysis of *soleus* muscle ultrastructure was performed on the digitized TEM micrographs. Intermyofibrillar (IMF) and subsarcolemmal (SSM) mitochondria size (area) as well as total mitochondria density (n/μm²) were determined using ImageJ software (NIH, Bethesda, MD) considering each supercellular structures was also performed.

Assessment of soleus muscle type I myosin heavy chain (MyHC) expression by immunohistochemistry
After deparaffinization and rehydration, soleus muscle sections were rinsed in 0.1% TBS-T and non specific binding was blocked by incubation with 3% BSA in 0.1% TBS-T for 30 min at 37°C. Sections were then incubated with mouse monoclonal anti type I myosin (M8421, Sigma) primary antibody diluted 1:100 in 0.1% TBS-T overnight in a humidified chamber. After rinsing 3×5 min in 0.1% TBS-T, sections were incubated with goat anti-mouse alkaline phosphatase conjugated secondary antibody (SC-3698, Abcam) diluted 1:100 in 0.1% TBS-T for 1 h at 37°C in a humidified chamber. Detection was performed by incubation with Fast Red TR/Naphthol AS-MX Tablets (SigmaFast, Sigma). Negative controls were performed for each section by omission of the primary antibody. Counterstaining was performed with hematoxylin.

**Biochemistry**

**Determination of serum 17β-estradiol concentration**
Serum was separated from the blood samples by centrifugation at 4°C and estradiol (17β-estradiol) concentration was assayed by solid phase competitive binding ELISA using a commercially available kit (Estradiol ELISA DE2693) and a spectrophotometer (iEMS, Labsystems) according to the manufacturer recommendations (Demeditec Diagnostics) for validating the effectiveness of the OVX procedure. Briefly, 25μl of serum and 200μl of enzyme conjugate were dispensed in each plate well and incubated for 120 min at 25°C. After complete washing, 100μl of substrate solution was added to each plate well and incubated for a further 15 min. Absorbance was read at 450 nm after addition of 50μl stop solution. Duplicates were analyzed for each sample and triplicates for each standard. Inter- and intra-assay CV were lower than 9.4 and 6.8%, respectively. Assay range is between 9.7 to 2000 pg/mL.

**MyHC I immunoblot**
Semi-quantification of MyHC I content was performed by Slot Blot as described previously [14]. Briefly, samples of *soleus* muscle homogenate containing 10μg of protein were loaded into a nitrocellulose membrane (Hybond; Amersham Biosciences) with a slot blot filtration manifold device (Hybri-Slot; Gibco BRL) coupled to a vacuum pump (KNF Neuberger). The membrane was then blocked in 5% non-fat dried milk in 0.1% TBS-T for 1 h, incubated with primary antibody (Mouse monoclonal anti type I myosin; M8421, Sigma) diluted 1:1000 in 5% non-fat dried milk in 0.1% TBS-T for 2 h, washed 3×5 min in TBS-T and then incubated with secondary antibody (Rabbit polyclonal to Mouse IgG HRP; ab6728, Abcam) conjugated with horseradish peroxidase diluted 1:10000 in 5% non-fat dried milk in 0.1% TBS-T for 2 h. The blots were developed by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Pharmacia Biotech), followed by exposure to a photographic film (Kodac Biomax Ligth Film, Sigma). The films were then analyzed with ImageJ software (NIH, Bethesda, MD) using the “Integrated Density” option to measure the intensity of the auto radiographic signal inside a boundary drawn around the bands detected in the films after background subtraction.

**Determination of citrate synthase (CS) activity**
CS activity was determined as described previously [7] by the spectrophotometric measurement at 412 nm of the amount of 5,5-dithio-bis(2-nitrobenzoate) that reacted with acetyl-coenzyme A (CoA) after its release from the reaction of acetyl-CoA with oxaloacetate. Brilfly 150μl of ultrapure water, 20μl of freshly prepared assay buffer containing 1.0 mM DTNB and 1% Triton X-100 (pH 8.1), and 20μl of the soleus homogenate were dispensed to each plate well. The absorbance was read for 5 min with 15s interval in a spectrophotometer (iEMS, Labsystems) at 37°C following the addition of 10μl of 10 mM oxaloacetate. The activity was calculated using CS from porcine heart (C3260, Sigma) as standard and expressed per mg of protein content.

**Determination of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity**
GAPDH activity was measured as described previously [37] as an increase in absorption at 340 nm following the reduction of NAD. Briefly freshly prepared 173μl of assay buffer containing 15mM sodium pyrophosphate and 30mM sodium arsenate pH
was found to be between 0.95 and 0.98 for all variables assayed. The number were performed with the Mann-Whitney non-parametric test due to distribution lack of normality. Differences were considered significant at p<0.05 and results are presented as mean±standard deviation.

Statistical analysis

The Kolmogorov-Smirnov test was used to investigate within-group normality for a given variable. Levene’s test was used to assess homogeneity of variance. Comparisons between groups were performed by 2-way analysis of variance (ANOVA) with Bonferroni post hoc test if normality and equality of variance were verified. Root square transformation was employed (intra-abdominal fat weight) to re-establish the necessary assumptions for ANOVA. Comparisons between groups regarding mitochondria size and fat weight) to re-establish the necessary assumptions for ANOVA.

Results

SERUM 17β-estradiol

Mean serum 17β-estradiol concentration in OVX+C (20.4±5.69 pg/mL) and OVX+VR (16.7±4.78 pg/mL) groups was significantly lower compared to SHAM+VR (86.7±16.57 pg/mL) and SHAM+C (82.4±19.51 pg/mL) groups. No significant differences existed between OVX+C and OVX+VR and between SHAM+VR and SHAM+C.

Physical activity

During the first week of the experiments the average daily distance travelled by OVX+VR and SHAM+VR animals were similar (1.02±0.383 Km.day\(^{-1}\) vs. 1.38±0.631 Km.day\(^{-1}\) respectively, p=0.294; Fig. 1a). However, beginning in week 2, the difference in physical activity began to differ markedly between the experimental groups. Indeed, by the end of the 9 months of differential housing SHAM+VR animals accumulated a 10-fold higher running distance compared to OVX+VR animals (1449.7±308.98 Km vs. 146.0±31.46 Km respectively; Fig. 1b).

Body weight variation and food intake

Detailed information is presented in Table 1. At the beginning of these experiments, body weight did not differ between the experimental groups. However, over time all animals increased body weight significantly (p≤0.003) with larger body weight increases in OVX animals compared to their SHAM counterparts. Further, body weight increases in the OVX+C were greater than the OVX+VR animals (p<0.05). Regardless of their higher body weights, food intake was similar between SHAM and OVX animals but significantly higher (p<0.01) in exercised than in sedentary animals. At the time of sacrifice, while OVX+VR body weight was significantly higher than that of SHAM+VR (400.9±36.61g vs. 303.2±41.27g, p<0.01) no differences were identified between OVX+C and SHAM+C groups (363.5±31.79g vs. 324.7±39.67g, p=0.087). Intra-abdominal fat mass was also higher in OVX+VR animals compared to SHAM+VR (33.6±10.15g vs. 10.5±9.27g, p<0.01) but not between OVX+C and SHAM+C animals (30.9±7.77g vs. 25.3±7.59g, p=0.285).

Soleus muscle weight was significantly higher in exercised animals compared to sedentary controls (p<0.05) but no differ-

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**Fig. 1** Voluntary motor activity recorded on the running wheel throughout the 9 months of the experimental procedure. a As soon as after the first week following surgery, distance travelled by OVX+VR animals was found to be always significantly lower than SHAM counterparts. b Total running distance was 10-fold higher in SAM+VR than in OVX+VR animals. C-control; VR-voluntary running; OVX-ovariectomized; SHAM-sham operated. *Significant differences between groups (p<0.05). Data are mean±standard deviation.
ences existed between OVX + VR and SHAM + VR \( (p = 0.5) \) as well as between OVX + C and SHAM + C \( (p = 1.0) \).

**Soleus muscle fibre CSA and protein content**

Average soleus muscle fibre CSA was 1913.2 ± 269.10 µm², 1048.5 ± 315.81 µm², 2920.4 ± 805.39 µm² and 1175.6 ± 395.59 µm² for OVX + VR, OVX + C, SHAM + VR and SHAM + C groups, respectively. A significantly higher soleus muscle fibre CSA was identified in SHAM + VR animals compared to OVX + VR animals but there were no significant differences between OVX + C and SHAM + C \( (\text{Fig. 2}) \). Moreover, soleus muscle fibre CSA was also significantly higher in OVX + VR and SHAM + VR animals when compared to their respective sedentary counterparts.

Fibre CSA was significantly correlated with the amount of running each animal performed on the wheel \( (r = 0.765; p < 0.05) \). To investigate if fibre size differences reflected true anabolic gains or just increases in non-protein content (i.e. edema), total protein content of the left soleus muscle was determined and expressed as percentage of muscle wet weight. Total protein content was 15.3 ± 3.08 %, 17.4 ± 2.85 %, 15.0 ± 2.24 % and 17.1 ± 2.40 % for OVX + VR, OVX + C, SHAM + VR and SHAM + C groups, respectively. No significant differences in total protein existed between these experimental groups \( (p \geq 0.757) \).

**Myofibre apoptosis and fibrous tissue expression**

No signs of apoptotic nuclei were identified in soleus muscle fibres of any experimental group despite the presence of other positively stained interstitial cells \( (\text{Fig. 3a}) \). Muscle collagen content was greater in both the OVX and SHAM control groups compared to their exercised counterparts \( (\text{Fig. 3b, c}) \). No differences in muscle collagen content existed between animals housed in the same conditions.

**Soleus muscle ultrastructure**

No significant differences existed in soleus muscle fibre mitochondrial density \( (\text{Fig. 4}) \) between the experimental groups despite the slightly higher mean values in exercised animals (Mean ± SD): OVX + VR = 0.30 ± 0.133, µm², SHAM + VR = 0.28 ± 0.094, µm², OVX + C = 0.21 ± 0.071, and SHAM + C = 0.20 ± 0.029, µm². Further, no differences existed in IMF mitochondrial size between the groups \( (\text{Fig. 4f}) \). In contrast, SSM mitochondria were significantly larger in the soleus muscle of SHAM + VR compared to OVX + VR \( (\text{Fig. 4b, f}) \). No differences in SSM mitochondria size existed between the 2 sedentary groups \( (\text{Fig. 4d, h}) \) although SSM mitochondria were significantly smaller in the sedentary groups compared to both exercise groups \( (\text{Fig. 4k}) \). Independent of their estrogen status, exercised animals also showed...
abundant glycogen granules, signs of satellite cell activation (● Fig. 5a), increased myonuclei transcriptional activity (● Fig. 5c) and myofibril splitting (● Fig. 4e). Several OVX + VR and SHAM + VR animals also displayed frequent secondary lysosomes (● Fig. 5b, d), often near large aggregates of SSM mitochondria. In contrast, these features were in general not observed in both control groups. In turn, muscles from sedentary control animals showed increased levels of both adipose and fibrous tissue (● Fig. 4g) as well as thinner myofibrils with evidence of sarcomeric disorganization (● Fig. 4c).

Enzymatic profile
Soleus muscle CS activity was 3.99 ± 0.784, 4.99 ± 0.706, 4.32 ± 1.514 and 5.18 ± 0.828 μmol/min/mg in OVX + C, OVX + VR, SHAM + C and SHAM + VR groups, respectively. Although soleus muscle CS activity tended to be higher in the exercise animals compared to sedentary, these differences did not reach significance (p ≥ 0.052). GAPDH activity in the soleus muscle was 535 ± 124.1, 521 ± 107.1, 531 ± 110.5 and 569 ± 187.6 nmol/min/mg in OVX + C, OVX + VR, SHAM + C and SHAM + VR groups, respectively. No significant differences existed between the experimental groups.

Soleus muscle MyHC I expression
The immunoblot analysis (● Fig. 6a) showed that soleus muscle MyHC I was over expressed in OVX compared to SHAM animals independent of their housing conditions. Access to the running wheel significantly influenced MyHC I expression in SHAM but not in OVX animals. Further, no differences in MyHC I expression existed between OVX + VR and OVX + C animals. SHAM animals MyHC I was over expressed in those with access to the running wheel. In situ findings of MyHC I expression assayed by immunohistochemistry (● Fig. 6b) also revealed a higher number of muscle fibres positively stained in OVX animals compared to SHAM. Note, however, that a quantitative analysis of muscle fibres expressing MyHC I isoforms was not performed since the majority of the fibres were found to be hybrid, revealing several degrees of affinity with the antibody against type I myosin.

Fig. 3 Apoptotic nucleus and fibrous tissue expression on soleus muscle. a TUNEL staining revealed the absence of myonuclei currently undergoing apoptosis. A higher magnification detail from the TUNEL image of OVX + VR group is provided (†) in which it is possible to identify several brightly fluorescent non muscular nuclei (arrows). b Soleus muscle cross-sections stained with Picrosirius red with collagen stained red and skeletal muscle yellow. c-Control; VR-voluntary running; OVX-ovariectomized; SHAM-sham operated. *Significant differences between groups (p < 0.05). All bars correspond to 100 μm.
Overview of major findings

These experiments provide 3 important findings. First, ovariectomy significantly reduces voluntary physical activity in rats. Second, our data also reveal that ovariectomy-induced sarcopenia in female rats is not directly linked to the loss of ovarian hormones (OH) but appears to be due, at least in part, to a decrease in voluntary physical activity. Finally, our results indicate that the surgical removal of the ovaries significantly changes the myosin isoform profile within the soleus muscle as the expression of MyHC I was significantly increased in both exercised and control OVX animals compared to SHAM counterparts. A brief discussion of each of these major findings follows.

Ovariectomy reduces voluntary physical activity in rats

Our findings clearly demonstrated that ovariectomy greatly decreases voluntary physical activity in rats. Note that previous studies have also shown that motor activity is influenced by circulating estrogen levels [1, 8, 11, 16, 32]. Nonetheless, most studies have described variations in physical activity during relatively short experimental periods (i.e., days to 12-weeks) following estrogen loss [22, 23]. Therefore, the current study is unique because we investigated animal activity patterns following ovariectomy for long time periods (i.e., 36 weeks). Moreover, by investigating the long-term effects of ovariectomy, our results reveal that the level of inactivity associated with the loss of ovarian hormones is greater than previous reports that investigated activity patterns during relatively short time periods following...
ovariectomy [11, 16]. Finally, our results reveal that the reduced voluntary physical activity associated with ovariectomy lasted throughout the entire duration of our study, suggesting that the effects induced by OH loss are permanent.

**Ovariectomy does not alter muscle fibre size**

We reasoned that muscle differences between sham operated and ovariectomized animals exposed to standard housing would reflect alterations in skeletal muscle structure resulting from OH loss while differences between animals housed with running wheels would reveal additional muscle alterations that occur due to differences in voluntary physical activity levels. We specifically selected the soleus muscle for study because this muscle has been widely used by others for the study of OH loss effects on skeletal muscle [16, 20, 21]; hence, this enables a comparison of the current data with findings from previous studies.

Our results reveal that the soleus muscle weight and fibre CSA did not differ between the OVX and SHAM control animals (Table 1; Fig. 2). These results indicate that the absence of OH did not impact soleus muscle weight and suggests that estrogen is not a requirement to maintain muscle mass. Further, our data indicate that when given access to the running wheel, soleus muscle mass of OVX animals increased significantly and was not different from that of SHAM + VR animals, indicating that exercise was effective in inducing an anabolic response. This finding further supports the concept that loss of OH per se does not lead to skeletal muscle atrophy, since without the anabolic stimulus provided by exercise, CSA was identical between OVX + C and SHAM + C animals. Nonetheless, soleus muscle fibres CSA from OVX + VR animals were significantly smaller compared to the CSA of soleus muscle fibres from the SHAM + VR animals. Interestingly, the soleus muscle fibre CSA of these 2 groups of animals were highly correlated (r = 0.765; p < 0.05) with the daily distance travelled in the running wheel. Again, these results are consistent with the concept that OH per se, does not directly regulate muscle fibre size but rather muscular activity plays a significant role in determining muscle mass during aging. Hence, monitoring the voluntary physical activity levels should be an important issue following the menopause, and if possible, increased participation in physical activities should be advised for the management of skeletal muscle losses, as progressive sedentary behaviour appears to be a major driving force for post-menopausal sarcopenia.

It is noteworthy that a previous study has reported that ovariectomy results in an increase of skeletal muscle water content [18]. Therefore, we analyzed the total protein content of the soleus muscle and determined that the ratio of protein content/muscle wet weight was similar between all groups. These findings indicate that our group differences in muscle weights and fibre CSAs were not due to differences in muscle water content.

Further, since variations in estrogen status could also influence food intake [10] which could impact skeletal muscle mass independently of receptor mediated estrogen effects, we recorded the animals food intake throughout the experimental period. Our results show that OVX and SHAM animals ingested the same amount of food when housed in equivalent conditions. Therefore, differences in food intake did not contribute to the variance in muscle mass between the experimental groups. In addition,
when access to the running wheel was provided, both OVX + VR and SHAM + VR animals increased their food intake by an identical ≈ 28% compared to control animals.

Ovariectomy does not alter muscle structure

Previous studies provide evidence that estrogen exerts anti-apoptotic effects in skeletal muscle, which raises the possibility that OVX could impact soleus muscle structure and size by increasing the rate of apoptotic cell death [4, 36]. We therefore analyzed soleus muscle fibres for the presence of myonuclei undergoing apoptosis using the TUNEL staining method. Our results revealed no signs of myonuclei undergoing cell death in any of the experimental groups indicating that OH absence was not associated with increases in apoptotic nuclei at the time of the animals sacrifice (Fig. 3). Further, a previous report has demonstrated that estrogen significantly affects the compositions of the extracellular matrix in cardiac myocytes by inhibiting fibrous tissue accumulation [39]. Others have also reported that estrogen deficiency leads to an increase in the collagen/muscle ratio in the rat urinary tract [28] and that estrogen replacement up regulates the synthesis of MMP-2 in mice mesangial cells [12]. Hence, to determine if loss of ovarian hormone affected the extracellular matrix composition we quantified the fibrous tissue accumulation in the soleus muscle by staining with picrosirius red. Our results indicate that OH absence was not associated with an increase in fibrous tissue accumulation as no differences existed between OVX and SHAM animals housed in the same conditions (Fig. 3). Sedentary behaviour, but not ovariectomy, influenced fibrous tissue accumulation with both OVX and SHAM control animals displaying increases in collagen amount when compared to VR counterparts (Fig. 3). This relative increase in intercellular connecting tissue will certainly contribute to the reduction in skeletal muscle quality, with lower levels of force generation per muscle area. Similar results have been reported by others in experiments investigating the impact of inactivity on muscle tissue connective tissue [15].

Our examination of the ultrastructure of muscle also indicates that loss of OH did not impact the structure of the soleus muscle (Figs. 4, 5). However, both the OVX and SHAM animals with access to the running wheel showed signs of metabolic and structural adaptations induced by regular physical activity, which included increased SSM mitochondria size, increased presence of glycogen granules and signs of satellite cell activation as well as signs of highly active protein synthesis, as evidenced by the presence of myonuclei with multiple nucleoli and prominent endoplasmic reticulum. Consistent with these ultrastructural results, our findings in soleus muscle sections stained with H&E (Fig. 2) revealed myofibres with central nuclei suggesting an increase in muscle regeneration. Note, however, that central nuclei were not observed in muscle fibres of either OVX or SHAM control animals. This indicates that exer-

**Fig. 6** Soleus muscle myosin heavy chain I (MyHC I) isoform expression assayed by slot blot a and immunohistochemistry b. In the upper micrograph it is possible to identify muscle fibres with high (arrowhead) together with fibres with no (black arrows) affinity for MyHC I antibody. Most fibres displayed some intermediate degree of affinity with the antibody (white arrows) and were considered to be hybrid regarding MyHC isoform expression. The lower photomicrograph shows a negative control soleus muscle section with some artefactual staining due to alkaline phosphatase activity on blood vessels. C-control; VR-voluntary running; OVX-ovariectomized; SHAM-sham operated; AU–arbitrary units. *Significant differences between groups (p<0.05). Bars correspond to 50 μm.
Exercise was the main stimulus for the increased anabolic activity observed in muscle cells, and that in the absence of exercise, anabolic activity was similar between experimental groups independent of OH status.

Finally, previous studies have reported that mitochondria are an estrogen sensitive organelle and that estrogen administration to OVX animals enhances respiratory chain activity and antioxidant enzyme expression [25]. Therefore, we quantitatively evaluated mitochondria size and density to determine if a lack of OH alters mitochondrial size in skeletal muscle. Our analysis revealed that the SSM mitochondria size was reduced in both control groups, independent of the OH status. Therefore, mitochondria size and density was unaffected by OVX. Moreover, lack of OH did not influence SSM size in OVX animals with access to the running wheel.

Ovariectomy increases the abundance of myosin heavy chain type I in muscle

Our results reveal an increase in MyHC I expression occurring in the soleus muscle of both control and running OVX animals in comparison to their SHAM counterparts (Fig. 6). These results indicate that a loss of OH stimulated a shift in soleus muscle contractile protein toward an increase in MyHC I abundance in the muscle fibres. Previous studies on cardiac muscle also show that a shift in MyHC isoform expression from fast to slow occurs following ovariectomy [29] and that this shift is reversed with estrogen replacement therapy [30]. Nonetheless, studies investigating the impact OH on skeletal muscle have been more inconsistent with some reports describing a fast-to-slow shift in muscle phenotype whereas others report no changes in myosin heavy chain isoform [16,19]. The reasons for this variation in the literature remain unclear.

Interestingly, despite the increased expression of MyHC I isoform identified in OVX animals, this was not accompanied by notorious metabolic profile changes as differences in soleus muscle CS and GAPDH activity did not exist between OVX and SHAM in both the sedentary and running animals. We interpret these findings as an indication that the lack of OH does not have a major impact on the expression of bioenergetic enzymes in skeletal muscle.

Summary and Conclusions

Our main conclusion is that ovariectomy results in a large and permanent decrease in voluntary physical activity in rodents. Further, our results indicate that ovariectomy-induced sarcopenia in female rats is not due to the loss of ovarian hormones (OH) per se, but appears to be due, at least in part, to a decrease in voluntary physical activity. Furthermore, our experiments also reveal that the surgical removal of the ovaries significantly changes the myosin isoform profile in skeletal muscle as the abundance of MyHC I was significantly increased in muscle of both exercised and control OVX animals compared to SHAM counterparts. Our results also point out the importance of monitoring the physical activity levels following the menopause, as progressive sedentary behaviour seems to be on the origin of skeletal muscle loss and perhaps also on other post-menopause related health conditions.

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