

# Estrogen treatment effects on rats soleus muscles' glycogen content, extracellular matrix and cross-sectional area

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## Abstract

**Objective:** To evaluate the effects of estrogen treatment of rats' soleus muscles after denervation on glucose metabolism, muscle mass, glycogen content, cross-sectional area and connective tissue density. **Methods:** Eighteen rats were divided into the following three groups of six animals: control, denervated for 7 days (denervated), and denervated with estradiol treatment for 7 days (denervated and treated). We measured glucose and insulin tolerance, muscle glycogen, mass, cross-sectional area and connective tissue content. **Results:** The denervated only and the denervated and treated groups displayed a significant reduction in glucose uptake (32% and 53% respectively compared with the control group;  $p < 0.05$ ). Soleus muscle denervation reduced muscle glycogen ( $0.25 \pm 0.03$  vs  $0.43 \pm 0.02$  mg/100mg;  $p < 0.05$ ), muscle mass ( $0.33 \pm 0.09$  vs  $0.48 \pm 0.06$  mg/g;  $p < 0.05$ ) and cross-sectional area ( $1626 \pm 352$  vs  $2234 \pm 349$   $\mu\text{m}^2$ ;  $p < 0.05$ ), and increased connective tissue content ( $35 \pm 7$  vs  $10 \pm 5$ %;  $p < 0.05$ ) compared to controls. Estrogen treatment decreased connective tissue density in the denervated and treated group ( $24 \pm 4$ %;  $p < 0.05$ ) compared to the denervated group. It also prevented alterations on muscle glycogen in denervated and treated group. However, estrogen treatment did not prevent muscle atrophy ( $1626 \pm 352$  vs  $1712 \pm 319$   $\mu\text{m}^2$ ). **Conclusion:** Estrogen treatment of rats' soleus muscles after denervation increased muscle glycogen content and minimized connective tissue density increase, but it did not prevent muscle atrophy.

**Keywords:** denervation, estrogen, muscle atrophy, muscle glycogen, connective tissue.

## 1 Introduction

Denervation reduces muscle mass and cross-sectional area, and increases connective tissue density (OZAWA, KUROSE, KAWAMATA et al., 2013). Connective tissue accumulation delays the exchange of substances between the vascular system and muscle fibers affecting recovery (SCHIAFFINO, KUROSE, KAWAMATA et al., 2013). Extracellular matrix remodeling can affect the extent of axonal growth during reinnervation because collagen fibers can be a barrier and delay the diffusion of nerve growth hormone (SCHIAFFINO, KUROSE, KAWAMATA et al., 2013). These physiological events reduce glucose uptake and metabolism, predisposing muscle fibers to atrophy and to peripheral insulin resistance (LO, RUSSELL, TAYLOR et al., 1970; CODERRE, MONFAR, CHEN et al., 1992; HENRIKSEN, RODNICK, MONDON et al., 1997; XU, SONG, ZHANG et al., 2015).

Muscle fibers' estrogen receptors are modulators and regulators of muscle mass and strength (MANGELSDORF, THUMMEL, BEATO et al., 1995; SKELTON, PHILLIPS, BRUCE et al., 1999; MAUVAIS-JARVIS, CLEGG and HEVENER, 2013;

TIIDUS, LOWE and BROWN, 2013). Estrogen is involved in signaling cascades that modify cellular responsiveness to insulin-like growth factor (IGF-1) and growth hormone altering the homeostasis and size of muscle fibers, as well as myosin and protein synthesis (HUSS, TORRA, STAELS et al., 2004; GUO, LIU, KONERMANN et al., 2014). There is a functional relationship between muscle fiber estrogen receptors and carbohydrate metabolism (insulinotropic effect) inducing insulin secretion and minimizing peripheral insulin resistance (TSAI, MCCORMICK, BRAZEAU et al., 2007; GREISING, BALTGALVIS, KOSIR et al., 2011; KAMANGA-SOLLO, WHITE, WEBER et al., 2013).

Estrogen minimizes loss of muscle and bone mass and type I collagen in ovariectomized rats submitted to hind limb suspension (KAWANO, KANDA, OHMORI et al., 1997; MCCLUNG, DAVIS, WILSON et al., 2006). Rats' soleus muscle growth, cell regeneration and extra-cellular matrix remodeling are estrogen-sensitive (MCCLUNG, DAVIS, WILSON et al., 2006).

Estrogen treatment can reduce muscle atrophy and bone mass loss in ovariectomized rats following suspension (KAWANO, KANDA, OHMORI et al., 1997; McCCLUNG, DAVIS, WILSON et al., 2006). However, the effects of estrogen treatment on muscle mass, metabolism and extra-cellular matrix remodeling following denervation have not been determined. Thus, the aim of this study was to evaluate the effects of estrogen treatment of rats' soleus muscles after denervation on glucose metabolism, muscle mass, glycogen content, cross-sectional area and connective tissue density. Our hypothesis was that estrogen treatment would minimize the deleterious effects of denervation.

## 2 Methods

The study protocol was approved by the Institutional Ethics Committee for Animal Experimentation and the study was conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation. Eighteen three-month-old female Wistar rats (average weight =  $215.1 \pm 32$  g) were studied. The rats were housed in plastic cages in environmentally controlled conditions with free access to water and standard chow.

Most changes in skeletal muscles occur during the first seven days of disuse (SCHIAFFINO, KUROSE, KAWAMATA et al., 2013). Therefore, the rats were randomly assigned to three groups of six animals each:

- 1) Control (control group);
- 2) Denervated for 7 days (denervated group), and
- 3) Denervated and treated with estrogen for 7 days (denervated and treated group).

First, the muscles were denervated by removing 5 mm of sciatic nerve from the left hind limb after anesthesia using sodium pentobarbital (50 mg/kg). Then, the animals received general anesthesia and, after 40 minutes, blood samples were collected from the tail to conduct insulin tolerance test (ITT) and glucose tolerance test (GTT). After that, regular insulin Biobrás (1U/Kg) and glucose (1g/kg) were injected, and new samples were collected after 2.5, 5, 10, 15, 20, 30, 60 and 90 minutes. Blood glucose levels were measured using a glucometer (ACCU-CHEK, Roche Diagnostics, Indianapolis, IN, USA). Finally, the animals were euthanized by cervical dislocation and the soleus muscle was removed, isolated, weighted and analyzed to determine glycogen content, cross-sectional area and connective tissue density.

The muscle samples were submitted to digestion with hot 30% Potassium hydroxide (KOH, P5958 Sigma-Aldrich, Saint Louis, MO, USA) and the glycogen was precipitated using ethanol. Samples were centrifuged for 15 min between the precipitation steps. The precipitated glycogen was submitted to acid hydrolysis in the presence of phenol and reported as mg/100 mg of wet weight (LO, RUSSELL, TAYLOR et al., 1970).

For morphometric analysis, the ventral segment of the soleus was fixed in buffered 10% formol solution. Non-serial cross-sections (7µm) of paraffin embedded muscle were cut and stained with hematoxylin-eosin. Image analysis was done using the Image Pro-plus 4.0 software (Media Cybernetics, Silver Spring, Mary Land, USA) and a digital camera

(JVC® manufacturer, Lawrenceville, Georgia, USA) coupled to a microscope connected to a microcomputer (Zeiss, Narberth, Pennsylvania, USA). All images were captured at ten times magnification. Five cross-sectional areas containing 375 soleus muscle fibers were analyzed per animal. A square reticulum was used for randomly choosing 15 fiber-straight intersections per cut. A planimetry system was used to analyze intramuscular connective tissue density using a reticulum with 2500 µm<sup>2</sup> squares containing 56 straight-line intersections (MATHIEU, CRUZ-ORIVE, HOPPELER et al., 1981). Coinciding points in the endomysium and perimysium in five areas/section in five sections/animal were scored as 1400 points. The relative area of connective tissue (area density) was calculated by dividing the sum of coinciding points in the straight-line intersections by the total number of points.

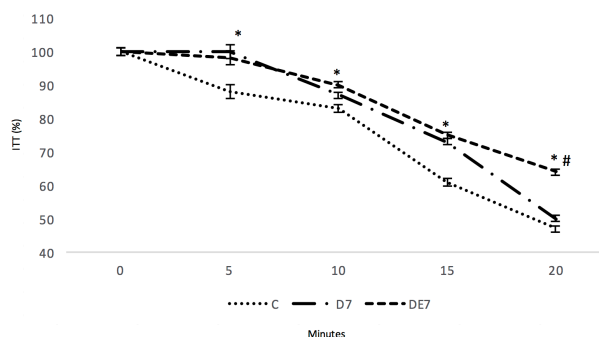
### 2.1 Statistical analysis

The distribution of the data was assessed using the Kolmogorov-Smirnov test. Muscle weight, muscle glycogen, GTT and ITT data were normally distributed and were analyzed using two way-ANOVAs followed by Tukey post-hoc tests. Muscle fibers and connective tissue density were not normally distributed and were analyzed using Kruskal-Wallis tests followed by Tukey HSD post-hoc tests. All analysis were conducted using the Origin 6.0 software (OriginLab, Northampton, MA, USA) with a critical level of 5% ( $p < 0.05$ ).

## 3 Results

The both denervated groups exhibited slower glucose decay at 5, 10 and 15 minutes than the control group (C). Only, the denervated and treated (DE7) group was significantly slower than the control group at 20 minutes. The denervated only (D7) and the denervated and treated (DE7) groups displayed significant average glucose decay reductions of 32% ( $3.25 \pm 0.27$ ) and 53% ( $2.27 \pm 0.25$ ), respectively compared to the control group. There was significant difference between the denervated groups at 20 minutes (Figure 1). There were no significant changes in the glucose tolerance.

Denervation without treatment (denervated only group) resulted in a significant decrease (31%) in muscle mass compared to the control group (Table 1). Estrogen treatment



**Figure 1.** Average glucose decay during ITT (1U/kg) for the control group (C), the denervated for 7 days group (D7), and the denervated and treated with estradiol for 7 days group (DE7). \*Significantly different from the control group ( $p < 0.05$ ); #Significant differences between D7 and DE7 ( $p < 0.05$ ).

**Table 1.** Normalized soleus muscle weight (average  $\pm$  SD), glycogen content, cross-sectional area ( $\mu\text{m}^2$ ), and connective tissue density (%) for the control group, the denervated for 7 days group (denervated), and the denervated and treated with estradiol for 7 days group (denervated and treated).

	Control	Denervated	Denervated and Treated
Muscle weight (mg/g)	$0.48 \pm 0.06$	$0.33 \pm 0.09^*$	$0.39 \pm 0.28^*$
Glycogen (mg/100mg)	$0.43 \pm 0.02$	$0.25 \pm 0.03^*$	$0.50 \pm 0.04$
Cross-sectional area ( $\mu\text{m}^2$ )	$2234 \pm 349$	$1626 \pm 352^*$	$1712 \pm 319^*$
Connective tissue (%)	$10 \pm 5$	$35 \pm 7^*$	$24 \pm 4^{**}$

\*Significantly different from the control group ( $p < 0.05$ ). \*\*Significantly different from the respective denervated group ( $p < 0.05$ ).

did not minimize muscle mass reduction because there was no significant difference between the denervated only and the denervated and treated groups (Table 1).

Glycogen content increased by 16% in the treated group compared to the control group (Table 1). The denervated only group presented a significant reduction (42%) in glycogen compared to the control group. Estrogen treatment significantly increased (100%) the glycogen content in the denervated and treated group compared to the denervated only group.

There was a significant reduction (27%) in muscle cross-sectional area in the denervated group compared to control. Estrogen treatment did not significantly minimize cross-sectional area reduction (Table 1).

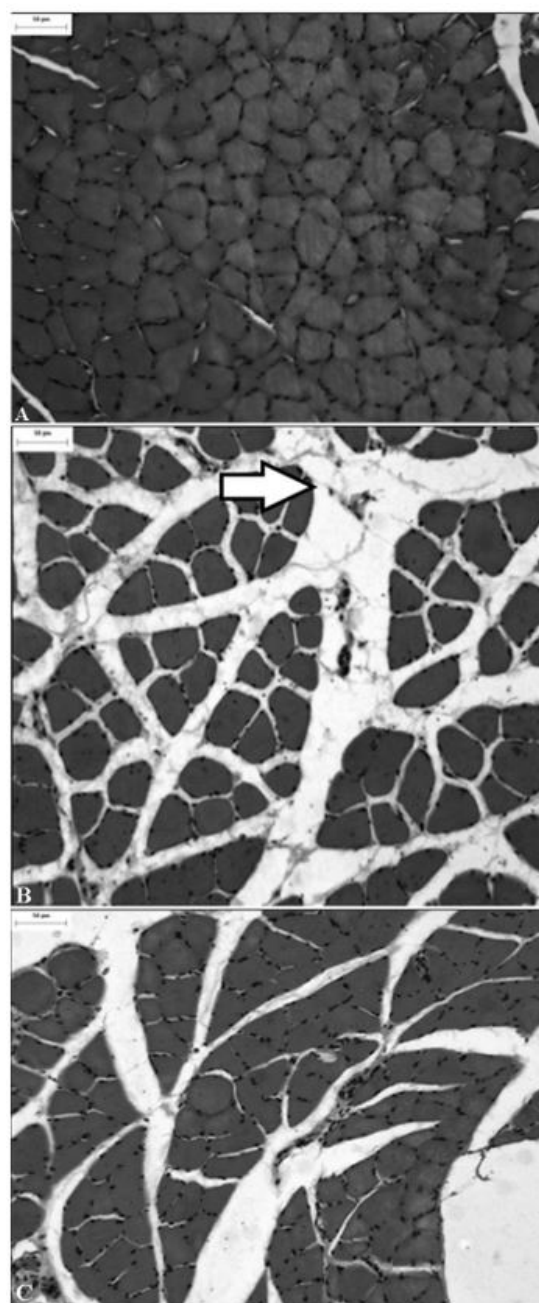
Connective tissue density increased significantly in the denervated only group (250%) and in the denervated and treated group (140%). The denervated and treated group presented 31% lower connective tissue density compared to the denervated only group (Table 1). Figure 2 illustrates the amounts of connective tissue in samples of the three groups.

#### 4 Discussion

We investigated the effects of estrogen treatment following seven days of denervation on muscle cross-sectional area, glycogen content and connective tissue density in rats. The initial hypothesis has been partially confirmed. Estrogen treatment indeed minimized the deleterious effects of denervation; it minimized the accumulation of connective tissue and restored glycogen content, but no effects were found on muscle cross-sectional area.

Soleus muscle denervation reduces glucose uptake within seven days (TURINSKY, 1987; HIROSE, KANEKI, SUGITA et al., 2001; TURINSKY and DAMRAU-ABNEY, 1998). The insulin signaling pathway is compromised after denervation, reducing tissue sensitivity and gene expression of glucose transporters, creating insulin resistance associated with muscle atrophy (WALLIS, APPLEBY, YOUNG et al., 1999; LIN, BRADY, WOLANSKE et al., 2002; XU, SONG, ZHANG et al., 2015). Several days of muscle disuse cause biochemical and morphological changes related to atrophy, as well as changes in proteins isoform, enzymes and metabolic disorders take place (McCLUNG, DAVIS, WILSON et al., 2006; XU, SONG, ZHANG et al., 2015). The present study revealed that denervation reduced the soleus muscle glycogen content, glucose uptake, muscle mass and cross-sectional area, and increased connective tissue density.

Previous studies have demonstrated that estradiol treatment for 6 to 11 days increases insulin sensitivity in the liver and muscles (GONZÁLEZ, ALONSO, GRUESO et al., 2002). Our results showed an decrease in glucose uptake and increase in



**Figure 2.** Soleus muscle fibers (hematoxylin-eosin, 100x); control A: fibers with normal appearance and hexagonal form; denervated only B: increased connective tissue (arrow); denervated and treated with estradiol C: increased in connective tissue, but with lower values than the denervated only group; scale bar 50  $\mu\text{m}$ .



muscle glycogen content in rats treated with estrogen for 7 days following denervation. This may have happened because after denervation the insulin signaling pathways are inactivated by reducing the efficiency of the post-receptor pathway, contributing to insulin resistance (LIN, BRADY, WOLANSKE et al., 2002). Estrogen increases insulin secretion and reduces insulin resistance through the metabolic cascade involving cyclic adenosine monophosphate (AMPc), mitogen-activated protein kinases (MAPK) and PI3K/AKT/MTOR pathways (GONZÁLEZ, ALONSO, GRUESO et al., 2002). Estrogen also induces upregulation of antiapoptotic muscle signaling (RONDA, VASCONSUELO, BOLAND et al., 2010). Our results demonstrated that estrogen treatment for seven days prevented changes in glycogen content in denervated muscles maintaining the muscle fibers' energetic pattern.

Estrogen is also an important regulator of muscle fiber growth. McClung, Davis, Wilson et al. (2006) reported an increase in soleus muscle cross-sectional area and mass after 2 weeks of hormone administration in ovariectomized rats. We observed a reduction in connective tissue density and an increase in glycogen content, but we did not observe changes in cross-sectional area after 7 days of estrogen treatment. The differences between the present study and the McClung, Davis, Wilson et al. (2006) may be related to different time points, treatment duration (2 vs. 1 week), and models of muscle atrophy used (hormonal vs. denervation). Maybe, a week was not long enough to induce the beneficial effects of estrogen in preventing skeletal muscle atrophy. Future investigations will be necessary to evaluate this possibility.

Interestingly, estrogen treatment for seven days reduced connective tissue density, which can sustain the exchange of substances between the vascular bed, muscle fibers and axonal nerve growth (OZAWA, KUROSE, KAWAMATA et al., 2013; SCHIAFFINO, KUROSE, KAWAMATA et al., 2013). Post-menopausal women who take estrogen-based hormone replacement therapy presented lower rates of collagen synthesis in the quadriceps muscle (HANSEN, SKOVGAARD, REITELSEDER et al., 2012). The inhibitory effect of estrogen on collagen/muscle fibrosis has also been found in ovariectomized rats (McCLUNG, DAVIS, WILSON et al., 2006) and in Duchenne muscular dystrophy models (DORCHIES, REUTENAUER-PATTE, DAHMANE et al., 2013). In the present study, estrogen treatment for seven days increased connective tissue density in the soleus muscle. It seems that estrogen is a potent stimulus to regulate connective tissue content in atrophied muscles, but not in control muscles. Movement restriction due to denervation could influence the results potentiating the effects of disuse. However, molecular assessments are necessary to determine the mechanisms by which estrogen affects connective tissue density in denervated muscles.

Although this study was performed in animals, it has clinical relevance because it indicates the potential positive effects of estrogen treatment in the acute phase of muscle atrophy after denervation. Human subject studies are required to evaluate this potential. Future studies should also consider following up animals to access pain and movement restriction during the first days after denervation. Furthermore, we investigated atrophy in the whole soleus muscle, but selective atrophy of different of muscle fiber types may occur and should be investigated.

## 5 Conclusion

Estrogen treatment of rats' soleus muscles after denervation increased muscle glycogen content and minimized connective tissue density increase, but it did not prevent muscle atrophy.

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