

# The effect of a low dose of clenbuterol on rat soleus muscle submitted to joint immobilization

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The aim of the present study was to evaluate the effect of joint immobilization on morphometric parameters and glycogen content of soleus muscle treated with clenbuterol. Male Wistar (3-4 months old) rats were divided into 4 groups (N = 6 for each group): control, clenbuterol, immobilized, and immobilized treated with clenbuterol. Immobilization was performed with acrylic resin orthoses and 10 µg/kg body weight clenbuterol was administered subcutaneously for 7 days. The following parameters were measured the next day on soleus muscle: weight, glycogen content, cross-sectional area, and connective tissue content. The clenbuterol group showed an increase in glycogen (81.6%,  $0.38 \pm 0.09$  vs  $0.69 \pm 0.06$  mg/100 g;  $P < 0.05$ ) without alteration in weight, cross-sectional area or connective tissue compared with the control group. The immobilized group showed a reduction in muscle weight (34.2%,  $123.5 \pm 5.3$  vs  $81.3 \pm 4.6$  mg;  $P < 0.05$ ), glycogen content (31.6%,  $0.38 \pm 0.09$  vs  $0.26 \pm 0.05$  mg/100 mg;  $P < 0.05$ ) and cross-sectional area (44.1%,  $2574.9 \pm 560.2$  vs  $1438.1 \pm 352.2$  µm<sup>2</sup>;  $P < 0.05$ ) and an increase in connective tissue (216.5%,  $8.82 \pm 3.55$  vs  $27.92 \pm 5.36$ %;  $P < 0.05$ ). However, the immobilized + clenbuterol group showed an increase in weight (15.9%;  $81.3 \pm 4.6$  vs  $94.2 \pm 4.3$  mg;  $P < 0.05$ ), glycogen content (92.3%,  $0.26 \pm 0.05$  vs  $0.50 \pm 0.17$  mg/100 mg;  $P < 0.05$ ), and cross-sectional area (19.9%,  $1438.1 \pm 352.2$  vs  $1724.8 \pm 365.5$  µm<sup>2</sup>;  $P < 0.05$ ) and a reduction in connective tissue (52.2%,  $27.92 \pm 5.36$  vs  $13.34 \pm 6.86$ %;  $P < 0.05$ ). Statistical analysis was performed using Kolmogorov-Smirnov and homoscedasticity tests. For the muscle weight and muscle glycogen content, two-way ANOVA and the Tukey test were used. For the cross-sectional area and connective tissue content, Kruskal-Wallis and Tukey tests were used. This study emphasizes the importance of anabolic pharmacological protection during immobilization to minimize skeletal muscle alterations resulting from disuse.

Key words: Immobilization; Clenbuterol; Connective tissue; Glycogen content; Morphometry; Rat soleus muscle

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## Introduction

Skeletal muscle function depends of many factors, such as intact proprioceptive activity and innervation, mechanical load, and joint mobility (1-3). In many adverse situations, such as injury of skeletal muscle, bone and ligaments, immobilization is the most common method of treatment (1-3). However, one of its most preeminent

effects is muscular atrophy (1-3). Several experimental models have been proposed to identify the events initiated by muscle disuse and the onset of precursors of atrophy, which include denervation, tenotomy, prolonged bed rest, hindlimb suspension, or unilateral immobilization (4). Muscular disuse induces insulin resistance and a catabolic state in human skeletal muscles (5) associated with a decrease in GLUT4 transporters (6). Although it is fre-

quently necessary, immobilization is accompanied by side effects, such as muscular cell atrophy, intramuscular fibrosis, loss of muscle extensibility, and limitations in joint motion (7). Several studies have demonstrated that concomitant with muscular hypotrophy, great modifications occur in skeletal muscle homeostasis, affecting myofibrillar protein synthesis, contractile dynamics as well as the effectiveness of metabolic signals, that can lead to a reduction in glycogen reserves, force and fatigue resistance (4,8,9).

Clenbuterol is a  $\beta_2$ -adrenoceptor agonist used as a bronchodilator for treating lung diseases (10). It also has an anabolic muscular effect and lipolytic activity (11). Low doses of clenbuterol (10  $\mu\text{g}/\text{kg}$ ) minimize muscular atrophy in rats, which is partially associated with denervation (12). Other studies on rats also reported that low doses of clenbuterol can cause anabolic effects without myocyte death (13,14). Clenbuterol also presents anabolic effects on the skeletal muscle of non-immobilized mice (15).

Therefore, the objective of the present study was to evaluate the effect of low doses of clenbuterol on morphometric and metabolic parameters of rat soleus muscles submitted to joint immobilization for 7 days.

## Material and Methods

All experimental procedures were approved by the Ethics Committee for Experimental Research of the University Federal de São Carlos (#03/06).

Male Wistar rats (3-4 months old; 250-300 g) were maintained under controlled conditions of temperature and humidity, with free access to food and water. The animals were divided into 4 groups (N = 6 for each group): control, non-immobilized clenbuterol, immobilized, and immobilized treated with clenbuterol for 7 days.

The rats were anesthetized (intraperitoneal injection of sodium pentobarbital, 50 mg/kg body weight) and the left hindlimb was immobilized in neutral ankle position with acrylic resin orthoses (8). Clenbuterol (10  $\mu\text{g}/\text{kg}$  body

weight) was administered subcutaneously daily at 9:00 am (6). After 7 days, the animals were killed and the soleus muscle was isolated and weighed by analytic scale and divided for analysis of glycogen content, cross-sectional area and connective tissue content.

The muscle extremities were used for analysis of glycogen content. Samples were digested in KOH, the glycogen was precipitated with ethanol, centrifuged and submitted to acid hydrolysis in the presence of phenol and measured according to Lo et al. (16).

The soleus belly was processed in paraffin and 5 non-serial 7- $\mu\text{m}$  thick cross-sections were obtained and stained with hematoxylin-eosin for morphometric analysis. Image analysis system was carried out with the Image Pro-plus 4.0 software (Media Cybernetics, USA), a digital camera (JVC, USA) coupled to a microscope (Zeiss, Germany), and connected to a computer. All images were captured at 100X magnification and analyzed as described (8). Cross-sectional area measurements were made on 375 myofibers from 5 fields on each of the 5 muscle sections. A planimetry system was used for intramuscular connective tissue analysis, by the method of Weibel (17) and the density (%) was calculated.

The statistical analysis was performed by Kolmogorov-Smirnov and homoscedasticity tests. For the muscle weight and muscle glycogen content, two-way ANOVA (factors: immobilization x treatment) and the Tukey test were used. For the cross-sectional area and connective tissue content, Kruskal-Wallis and Tukey tests were used ( $P < 0.05$ ).

## Results

The clenbuterol-treated group presented higher soleus glycogen (81.6%,  $P < 0.05$ ) without alterations in weight, cross-sectional area and connective tissue compared with the control group (Table 1, Figure 1A and B).

Rats immobilized for 7 days had 34.2% less soleus muscle weight and 31.6% less glycogen content (Table 1) compared with the control group ( $P < 0.05$ ). The cross-

**Table 1.** Effect of clenbuterol on atrophy caused by immobilization of soleus muscle in the rat.

	C	Cle	I	I + Cle
Weight (mg)	123.5 $\pm$ 5.3	128.1 $\pm$ 5.05	81.3 $\pm$ 4.6*	94.2 $\pm$ 4.3**
Glycogen (mg/100 mg)	0.38 $\pm$ 0.09	0.69 $\pm$ 0.06*	0.26 $\pm$ 0.05*	0.50 $\pm$ 0.17*
Fiber area ( $\mu\text{m}^2$ )	2574.9 $\pm$ 560.2	2531.7 $\pm$ 636.8	1438.1 $\pm$ 352.2*	1724.8 $\pm$ 365.5**
Connective tissue (%)	8.82 $\pm$ 3.55	8.99 $\pm$ 4.19	27.92 $\pm$ 5.36*	13.34 $\pm$ 6.86**

Data are reported as mean  $\pm$  SD (N = 6). C = control; Cle = clenbuterol treatment for 7 days; I = immobilized for 7 days; I + Cle = immobilized for 7 days with clenbuterol treatment. \* $P < 0.05$ , compared with C group; \*\* $P < 0.05$ , compared with I group; # $P < 0.05$ , compared with Cle group.

sectional area of soleus muscle was 44.1% less and there was 216.5% more connective tissue (from  $8.82 \pm 3.55$  to  $27.92 \pm 5.36\%$ ) compared with the control group ( $P < 0.05$ , Table 1, Figure 1C).

When the immobilized group was compared with the immobilized + clenbuterol group, it is clear that the clenbuterol-treated rats had higher muscle weight (15.9%, from  $81.3 \pm 4.6$  to  $94.2 \pm 4.3$  mg), and glycogen content (92.3%, from  $0.26 \pm 0.05$  to  $0.50 \pm 0.17$  mg/100 mg;  $P < 0.05$ ; Table 1).

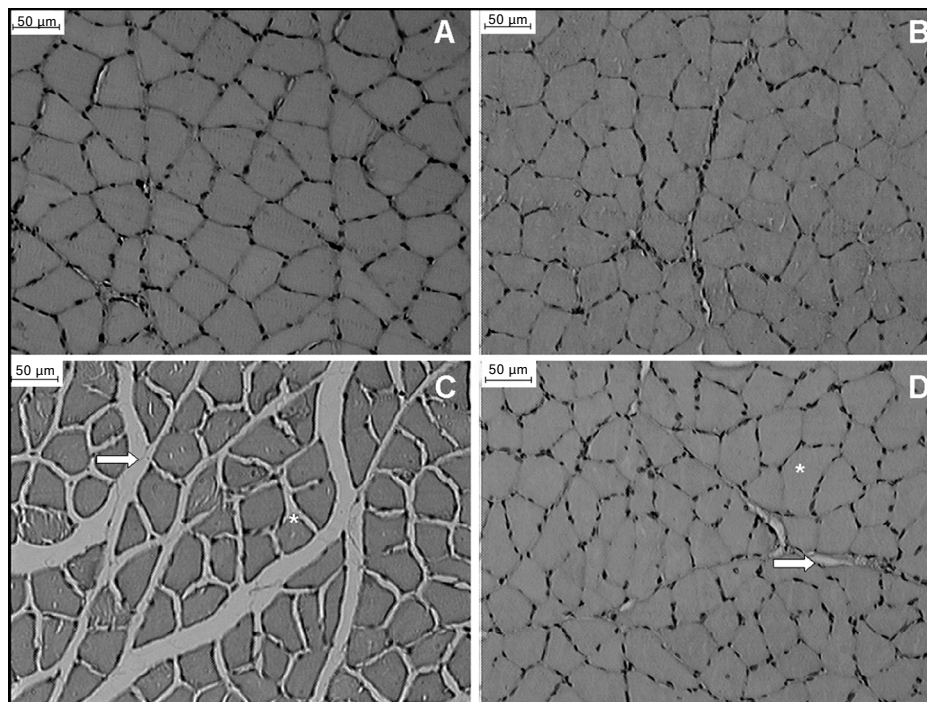
When the clenbuterol group was compared with the immobilized + clenbuterol group, greater cross-sectional area (19.9%,  $P < 0.05$ ) and less connective tissue (52.2%,  $P < 0.05$ ) were observed with clenbuterol (Table 1; Figure 1B and D). Thus, although the clenbuterol treatment increased the cross-sectional area in immobilized animals, it did not revert completely the decrease caused by immobilization, with a significant difference between these groups ( $P < 0.05$ ). Clenbuterol treatment in the immobilized group inhibited connective tissue accumulation compared with non-clenbuterol-treated animals ( $P < 0.05$ , Figure 1D); however, it did not inhibit completely the connective tissue accumulation at control levels.

When the control group was compared with immobilized + clenbuterol (10  $\mu\text{g}/\text{kg}$ ), no difference in glycogen content was found; however, there was a decrease of 23.7% ( $P < 0.05$ ) in weight, this value being lower than that of the immobilized group. The cross-sectional area and connective tissue also presented significant differences ( $P < 0.05$ , Figure 1A and D).

## Discussion

As far as we know, this is the first study that analyzed the effects of a low dose of clenbuterol in a disuse muscle model. Data analysis revealed that clenbuterol prevents muscle loss in soleus muscle, including muscle weight, muscle glycogen content and cross-sectional area of fibers, which could be beneficial in cachexia and disuse conditions.

Selective agonists of  $\beta$ -adrenoceptors, such as clenbuterol, may counter hypotrophy by disuse because of their anabolic effects. However, the mechanisms by which  $\beta$ -adrenergic agents induce anabolism are still not fully understood (18,19). Fitton et al. (6) reported that low doses of clenbuterol have a protective effect on denervated muscle



**Figure 1.** Soleus muscle fibers stained with hematoxylin and eosin. *A*, Control group. *B*, Clenbuterol treatment for 7 days. *C*, Immobilized in ankle neutral position for 7 days. A reduction of muscle fiber area (asterisk) and an increase of intramuscular connective tissue (arrow) can be seen compared with the control group. *D*, Immobilized and clenbuterol treatment for 7 days. An increase of muscle fiber area (asterisk) and a decrease of intramuscular connective tissue (arrow) can be seen compared with the immobilized group. Original magnification: 100X.

before its reinnervation, reducing protein loss and muscular fiber atrophy and partially preserving contractile performance.

The results of the present study demonstrated the anabolic effects of low doses of clenbuterol under conditions of disuse, i.e., prevented loss of soleus weight and cross-sectional area. This correlates with the Canu et al. study (20), in which the anabolic action of clenbuterol was observed in both normal and atrophied muscles. Pellegrino et al. (21) also observed increased cross-sectional area of mouse soleus muscle in the presence of clenbuterol.

Akatsu et al. (18) reported that regulatory mechanisms for skeletal muscle hypertrophy by clenbuterol are unclear; however, they suggest that transforming growth factor  $\beta$  could be involved. Matsumoto et al. (22) suggested that one hypothesis to explain the skeletal muscle hypertrophy induced by clenbuterol is the stimulated production of peptide growth factors such as insulin-like growth factors. However, these studies differ in regard to doses, including low and large doses. Chen and Alway (13) observed that the administration of clenbuterol at 10  $\mu\text{g}/\text{kg}$  body weight reduced atrophy in the slow muscle of senescent rats. Burniston et al. (14) observed that 1 mg/kg clenbuterol increased the protein content associated with myofiber hypertrophy.

Despite glycogen utilization, the immobilized group presented glycogen reduction. Bevan (23) showed that insulin acts in different ways, and that there is participation by subunit PI3-K (phosphatidylinositol 3-kinase), which is responsible for several mechanisms including glycogen synthesis. Muscular disuse induces insulin resistance and a catabolic state in human skeletal muscles (5). In rat hindlimbs immobilized for 7 days, Hirose et al. (24) observed a reduction in intracellular signaling stimulated by insulin and in glucose uptake, suggesting deficit in PI3-K activation, which could explain the results of decrease glycogen in this study; this being one of the mechanisms activated by PI3-K subunit. In the present study, glycogen was increased in clenbuterol and immobilized + clenbuterol groups, which could be explained by clenbuterol causing an increase in tissue glucose uptake, possibly because of the permissive effect of insulin action (25,26). Pan et al. (26) observed that the increased glucose uptake stimulated by clenbuterol was parallel to the increase in

glycogen reserves. According to Hunt et al. (27), the possibility of increased insulin sensitivity in the skeletal muscle after clenbuterol treatment could be due to the reduced influence of epinephrine on insulin action as a result of down-regulation of  $\beta$ -adrenergic receptors.

The connective tissue area density values were lower in the immobilized + clenbuterol group compared with the immobilized group. Because clenbuterol has an anabolic action, an increase in protein synthesis of the extracellular matrix was also expected (28). As this was not observed, it may be that differences in rate of renewal among intramuscular proteins and extracellular matrix might have influenced the results. Because collagen proteins have a slower rate of renewal than muscular tissue (29), clenbuterol did not increase the cross-sectional area (19.9%) as intensely as it decreased the connective tissue (52.2%) in the immobilized + clenbuterol group. This finding is related to a study by Jiang et al. (30), in which the authors observed that clenbuterol (60  $\mu\text{g}$  for more than 3 months) minimized collagen proliferation in denervated skeletal muscle of humans. An important fact in the present study is that the clenbuterol treatment for 7 days led to a decrease in connective tissue using a dose six times smaller than the dose used by Jiang et al. (30).

In contrast, in the study of Patiyal and Katoch (31), high doses of clenbuterol (2 mg/kg) caused collagen content proliferation in the mouse gastrocnemius due to the anabolic effect on protein of clenbuterol in the skeletal muscle. In the Burniston study (14), there was myocyte death and collagen increase in myocardium (100  $\mu\text{g}/\text{kg}$  or 1 mg/kg). Along these lines, collagen proliferation seems to be related to the dose administered since the present study demonstrated that low doses of clenbuterol for a short period induced connective tissue reduction in soleus muscle during 7 days of joint immobilization.

In conclusion, we found that 10  $\mu\text{g}/\text{kg}$  clenbuterol, *sc*, for a short period of time was efficient in minimizing reduction of muscle weight, fiber area and glycogen content during joint immobilization. The extrapolation of our results to clinical practice would suggest that low doses of clenbuterol could be useful during muscle disuse periods, i.e., in joint immobilization before rehabilitation.

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