Six-week anaerobic training improves proteolytic profile of diabetic rats

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ABSTRACT

Objective: To evaluate the effect of six-week anaerobic training on the mRNA expression of genes related to proteolysis *Ubb* (Ubiquitin), *E2-14kDa*, *Trim63* (MuRF1 protein) and *Nfkb1* in the skeletal muscle of diabetic rats. **Materials and methods:** Four groups were established: DE (Diabetes Exercised), DS (Diabetes Sedentary), CE (Control Exercised) and CS (Control Sedentary). The training consisted of 3 sets of 12 jumps in the liquid mean with load equivalent to 50% of BW for 6 weeks. Euthanasia occurred under ip anesthesia, and blood, adipose tissue and skeletal muscles were collected. Gene expression was quantified by RT–PCR in the gastrocnemius muscle. ANOVA one-way was used for comparison among groups, with post-hoc (Tukey) when necessary, considering $p < 0.05$. **Results:** We observed reduction in the body weight and adipose tissue in the diabetic groups. The muscle mass was reduced in DS, which could be reversed by training (DE). Although DS and DE have presented similar body weight, the training protocol in DE promoted reduction in the adipose tissue, and increase of muscle mass. Anaerobic training was efficient to reduce glycaemia only in the diabetic animals until 6 hours after the end of training. The *Trim63* gene expression was increased in DS; decreased *Ubb* gene level was observed in trained rats (CE and DE) compared to sedentary (CS and DS), and DE presented the lowest level of *E2-14kDa* gene expression. **Conclusion:** Six-week anaerobic training promoted muscle mass gain, improved glycemic control, and exerted inhibitory effect on the proteolysis of gastrocnemius muscle of diabetic rats. Arch Endocrinol Metab. 2015;59(5):400-6

Keywords
Ubiquitin-proteasome; anaerobic training; *diabetes mellitus*; skeletal muscle; proteolysis

INTRODUCTION

Characterized as a multifactorial illness, *diabetes mellitus* (DM) represents one of the most studied deleterious metabolic diseases in the world. Among various complications associated to diabetes development, diabetic must handle an elevated rate of protein breakdown, known as sarcopeny. This condition is defined as a non-induced degenerative loss of muscle fiber and, consequently, loss of strength, associated to oxidative stress and reactive oxygen species (ROS) release (1). As a consequence of this dysfunction in diabetics, the ubiquitin-proteasome signaling pathway (UPS – Ubiquitin-Proteasome System) can play an essential role in catalyzing unfolded or unwelcome proteins, which are subsequently catalyzed by proteasome (2,3) considering that in pathological conditions such as DM, gene expression of proteins involved in the regulation of proteolytic processes is elevated, resulting in degradation of myofibrillar structures (4).

Muscle mass can be maintained by the balance between protein synthesis and degradation, being regulated by different regulators, in special insulin. Protein degradation in the skeletal muscle is mainly carried out by the proteasome-ubiquitin system, involving ligases such as Muscle atrophy F-box protein (MAFBx or Atrogin-1) and Muscle Ring Finger-1 (Murf1), whose expression is maintained by PI3Kinase/Akt pathway signaling. Therefore, any impairment in the insulin signaling can impair the muscle mass homeostasis (5). In older women with type 2 diabetes (6) and in animal model of obesity (7), a relationship between the disease and loss of skeletal muscle mass associated to elevated Murf1 and Atrogin expression has been detected. Another important view is the major glucose scavenger tissue is the skeletal muscle. So, the loss of this mass can contribute even more with the alterations in the glycemic homeostasis observed in diabetes and insulin resistance state (6). Among various enzymes compounding the structures responsible for the recognition and protein degradation process, we will highlight two major proteins in the present study: MuRF1, a ligase enzyme E3, related to an accelerated process of proteolysis and a molecular mediator of muscle atrophy, expressed mainly in the skeletal muscle and cardiac tis-

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sue (4); and E2-14kDa protein, a conjugating enzyme, E2, site of regulation on the signaling pathway of the ubiquitin-proteasome in the skeletal muscle (8).

The UPS pathway is essential for activation of nuclear factor kappa B (NFkB), a transcription factor involved in the inflammatory genes expression presented in diabetic state, after degradation of inhibitory IkB proteins. Besides, there is some evidence showing the link between NFkB and atrogene expression induction (9,10).

The exercise is configured as a non-invasive, cheap and easy-accessibility therapeutic method to reduce the complications of diabetes (11). The protocol of jump in the liquid medium have been widely used with experimental models, however, the literature still needs further studies regarding its influence on the protein degradation. The exercise can modulate the pattern of expression of the proteins involved in the proteolysis process (12). It is known that the conjugating enzyme E2-14kDa, when expressed above basal levels, is considered a biomarker of the activation of UPS system (4). This analysis may also be enhanced by binding protein E3 – MuRF-1 (Muscle Ring Finger-1), which is also considered a biomarker of this pathway (13). This study aims to analyze the effects of six-week anaerobic training of jumps in the liquid medium on the gene expression of proteins involved with ubiquitin – proteasome pathway in the gastrocnemius muscle of diabetic trained rats.

MATERIALS AND METHODS

Wistar male rats aged 3-months and weighting 250 g were randomly divided into 4 groups: control sedentary (CS, n = 8), control exercised (CE, n = 8), diabetic sedentary (DS, n = 7) and diabetic exercised (DE, n = 6). The diabetes was induced by alloxan administration (120 mg/kg of body weight), ip. Control groups received physiological solution (0.9% NaCl), ip, as placebo. All animals were kept in the cages located at controlled temperature (25°C) and light cycle (12/12 h, light/dark luminosity) room. All animals received standard chow (Supra Lab, Alisul Ind. Alimentos LTDA, RS, Brazil) and water *ad libitum*. After seven days of alloxan diabetes induction, rats that showed glycaemia above 200 mg/dL were considered diabetic. Blood drops were obtained from distal portion of tale and blood glucose was measured by glucometer Biocheck TD – 4225/Bioeasy Diagnostica Ltda./MG – Brazil). Diabetic groups received insulin therapy (NPH Insulin, 2 U/day, subcutaneous injection) to avoid loss of animals during the intervention, except in the last day of training.

Training protocol

To avoid trauma and/or stress during training, the animals were submitted to a period of adaptation to liquid medium following Ribeiro and cols. (14). The animals were initially kept on shallow water during 5, 10 and 15 minutes, after they were kept in deep water during 5, 10 and 15 minutes, and 5 minutes with a vest attached to the thorax. The training protocol was based on the literature (15). The anaerobic training consisted of jumps in water tank with a load corresponding to 50% of the body weight coupled to the thorax. The session consisted of 3 sets of 12 jumps with 1 minute interval among sets, 3 times a week, for 6 weeks. The counting of repetitions occurred each time the animal was projected toward the surface of the water to breathe. The water temperature was maintained between 30°C and 32°C, as way to be considered thermally neutral on the body temperature of the rat (16).

Samples

Samples of blood were collected from distal region of the tail for glycaemia detection after training and after 6 hours of the last session of training. Then, after 24h, the euthanasia was performed under ip anesthesia with sodium thiopental (60 mg/kg PC). Different samples of muscles were removed and weighted: gastrocnemius, EDL (*Extensor Digitorum Longus*) and Soleus, and periepididymal fat pad. Slices of gastrocnemius muscle were separated for further analysis.

All procedures were approved by Ethical Committee for Animal Use (CEUA, Protocol 02/2012) of Faculty of Sciences and Technology – Sao Paulo State University.

RT-PCR

Total RNA was isolated using Brazol reagent (LGCBio, Sao Paulo, Brazil) according to the manufacturer's instructions. The first-strand cDNA synthesis was carried out using MMLV-RT Reverse Transcriptase (200U; Invitrogen Life Technologies, Carlsbad, CA, USA) with 5 µg total RNA. Specific oligonucleotide primers for *E2-14kDa*, *Nfkb1*, *Trim 63*, *UBb* and *Gapdh* (used as an internal control) were synthesized by Sigma (Sigmaal-

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drich, USA) and are shown in the table 1. Each amplification reaction (25 µl) was performed in the presence of GoTaq DNA Polymerase (Promega, USA), oligonucleotide primers (sense and antisense, 10 mM, Table 1) and reverse transcription (RT) product samples according to the manufacturer's instruction. The annealing temperature was 59°C for *E2-14kDa* (43 cycles), 55°C for *Nfkb11* (36 cycles), 58°C for *Trim 63* (48 cycles), 53°C for *Ubb* (32 cycles), and 58°C for *Gapdh* (24 cycles). After PCR, 8 µl of each sample was electrophoresed in 1% agarose gels and visualized by ethidium bromide staining. The images were photographed by a KODAK System Molecular Imaging Software Version 4.0, 2-User system and Eletronic UV Transilluminator.

Table 1. Sequence of sense and anti-sense oligos

Gene	Oligos sequence	Annealing temperature (ºC)
E2-14kDa	Sense 5' – GTGCACCATCTGAAAACAA – 3' Antisense 5' – ATCGGTTCTGCAGGATGTCT – 3'	59ºC
Gapdh	Sense: 5' – ACATCATCCCTGCATCCACT – 3' Antisense: 5' – GGGAGTTGCTGTTGAAGTCA – 3'	58ºC
Nfkb1	Sense 5' – AAGACTATTGAGCGAACCTT – 3' Antisense 5' – TTGGAATTGACTGACTGACA – 3'	55ºC
Trim63	Sense 5' – GGAGAAGCTGGACTTCATCGA – 3' Antisense 5' – CTTGGCACTCAAGAGGAAGG – 3'	58ºC
Ubb	Sense 5' – TCTTCGTGAAGACCCTGACC – 3' Antisense 5' – CAGGTGCAGGGTTGACTCTT – 3'	53ºC

Statistical analysis

Data are presented as mean ± SEM. All data were normalized by Kolmogorov-Smirnov test, and the data were treated as parametric variables. ANOVA One-way were used for comparison among results of body weight, tissue weight and gene expression. ANOVA repeated measures test were used for comparison of results of glycemia pre-, post- and 6 hours after training. Newmann-Keulls was used as post-test, when necessary, considering $p < 0.05$ as significance level. Software GraphPad Prism 5.0 was used for statistical analysis.

RESULTS

The body weight of diabetic animals was reduced ($p < 0.0001$) compared to control groups (Table 2). DE and DS presented lower ($p < 0.0008$) fat pad compared to CS, but similar to CE. DE presented heavier gastrocnemius muscle ($p < 0.001$) compared to DS, and similar to CE and CS groups. DS presented lighter ($p < 0.0004$) EDL muscle compared to all other groups. CE presented heavier ($p < 0.0004$) EDL muscle compared to CS and DE groups. DS presented lighter ($p < 0.0003$) soleus muscle compared to all other groups, and DE presented lighter ($p < 0.0003$) soleus muscle compared to CE.

Anaerobic training was efficient to reduce glycaemia of the diabetic animals until 6 hours after the end of training (Figure 1) ($p < 0.001$ *vs.* DE Pre-training and DE Post-training), despite of elevated level (≈ 400 mg/dL), when compared to the trained group. On the other hand, the same was not observed in CE group Pre-, Post- and after 6 hours of the end of the training.

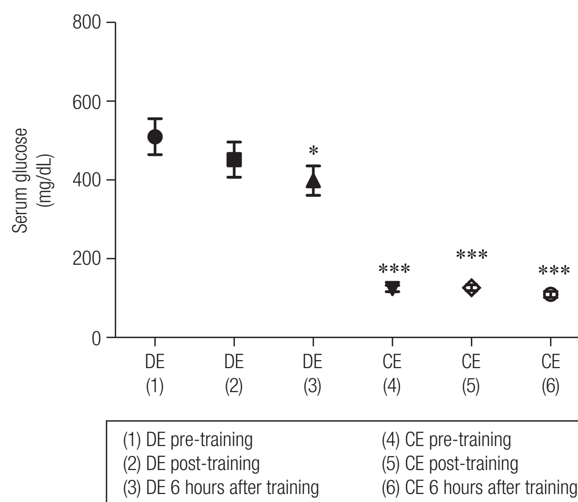

Figure 1. Serum glucose measurements pre-, post-, and after 6 hours of last session of the training in the diabetic animals. Values are expressed as Mean ± SEM. * $p < 0.05$ *vs.* DE pre-training; *** $p < 0.0001$ *vs.* DE pre-training, DE post-training and DE 6 hours after training. DE n = 8; CE n = 9. ANOVA One-way with Newman Keulls as post-test. CS = control sedentary (n = 8), CE = control exercised (n = 8), DS = diabetic sedentary (n = 7) and DE = diabetic exercised (n = 6).

Gene expression levels of *Ubb*, *Trim63*, *E2-14kDa* and *Nfκb1* can be observed in the figure 2. Anaerobic training was effective to reduce ($p < 0.05$) the mRNA of these proteolysis biomarkers in the DE group compared to DS (Figures 2A, 2B and 2C). CE presented reduced mRNA *Ubb* compared to CS ($p < 0.05$). *Trim63* gene level was very elevated in the DS group ($p < 0.019$) compared to all other groups, which was reduced by anaerobic training (DE).

There were no significant difference in the *Nfkb1* gene level among groups (Figure 2D), although a discrete reduction can be observed in the trained groups.

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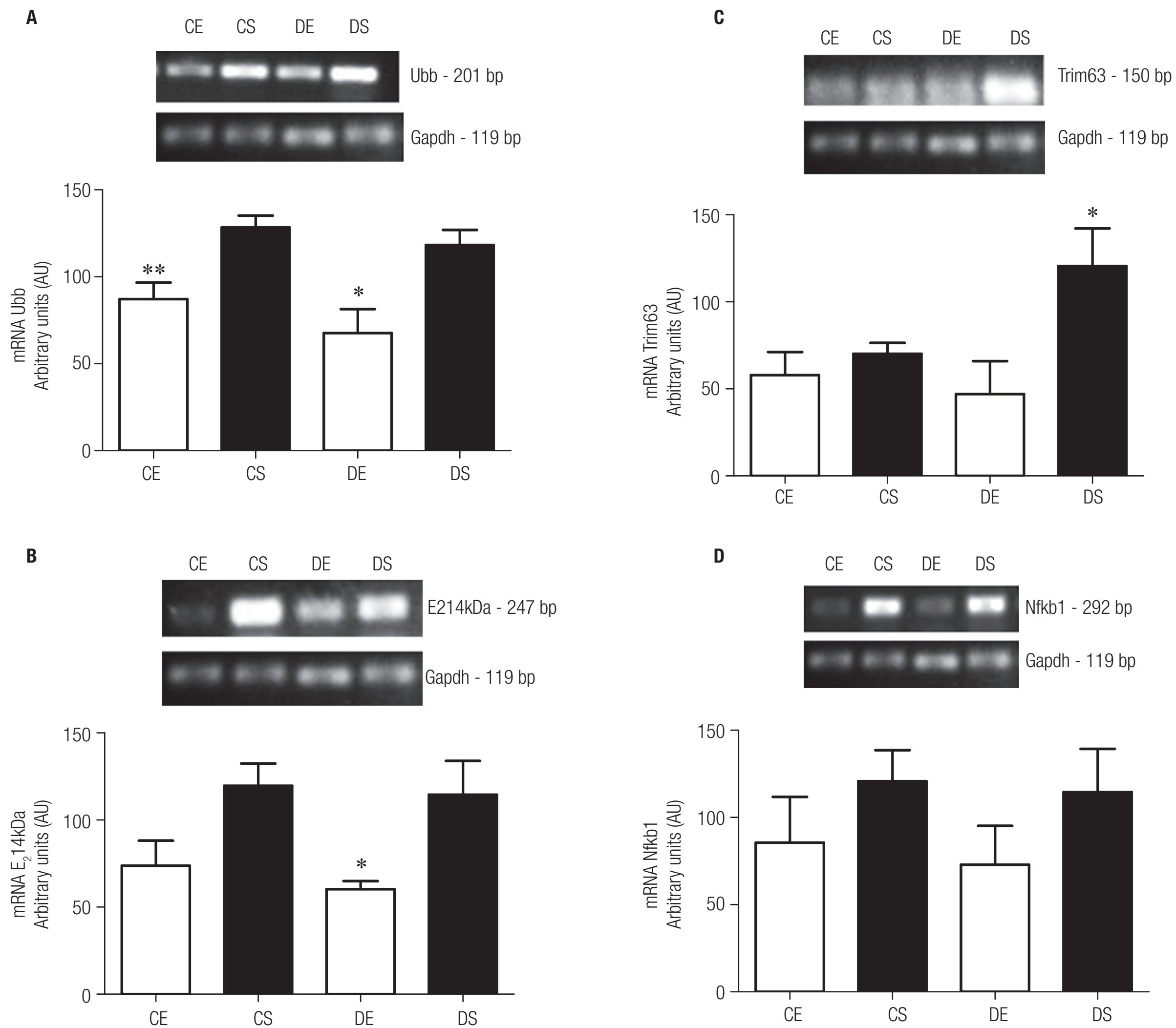

Figure 2. Gene levels (expressed as arbitrary unit, AU) in the gastrocnemius skeletal muscle of diabetic Wistar rats submitted to six-week anaerobic training. (**A**) *Ubb* gene expression. * $p < 0.05$ DE *vs.* DS and CS; ** $p < 0.05$ CE *vs.* DS and CS. (**B**) *E2-14kDa* gene expression. * $p < 0.05$ DE *vs.* DS and CS. (**C**) *Trim63* gene expression. * $p < 0.0191$ DS *vs.* DE, CE and CS. (**D**) *Nfκb1* gene expression. All values are expressed as Mean ± SEM. CS = control sedentary (n = 6), CE = control exercised (n = 7), DS = diabetic sedentary (n = 6) and DE = diabetic exercised (n = 5).

DISCUSSION

Exercise is an efficient, affordable and accessible tool for the diabetic population that prevents the deleterious symptoms of DM. This involves a complex system of structural adaptations including improved insulin sensitivity, glycemic control, reduction of blood pressure, and overall cardiovascular risk (17-19).

In the present study, we observed that diabetic animals (DS) presented considerable loss of weight, which involved loss of lean (muscle) and fat mass (Table 2). This loss can be associated with hypoinsulinemia that occurs due to this disease, generating a catabolic state of proteins and fats. Another contributing factor is associated to elevated glucose excretion observed in the animals (data not shown) and polyuria (20), as expected in this condition. On the other hand, when diabetic animals (DE) were submitted to anaerobic training, we detected a lower weight associated to higher reduction of fat mass associated to increased lean mass compared to the control groups (Table 2), suggesting the positive effect of training to avoid muscle proteolysis.

There was a discrete reduction in the glycaemia of diabetic exercised rats, which was more reduced after 6 hours of the end of the training. On the other hand, this change was not observed in the control group. The reduction in the glycaemia in this condition can be justified by elevated glucose uptake by peripheral tissues, stimulated by contractile activity during the training (15). The training has provoked a hypoglycemic effect, which can be found in different studies involving ex-

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perimental and clinical model of diabetes (20,21), however, few studies have focused on protocols using long-term anaerobic training. Various studies have focused on the training program involving aerobic exercise and/or combined exercise (aerobic plus anaerobic exercise) (17,21-23). In this way, a detailed investigation about anaerobic training's effect on the glycemic profile and physiological mechanisms is very important to understand if and/or how exercise can improve the deleterious condition of diabetic state. In the present study, anaerobic training was practiced, which demands less time for performance favoring the attraction for public in general.

In this study, we observed an improvement in the glucose uptake until 6 hours after the last session of training, which probably occurred due to stable state of adaptation to the training with consequent mobilization of intracellular proteins related to cellular metabolism when exercise is regularly practiced (24). Interestingly, other notable point is that DE group presented a higher reduction of glucose levels after 6 hours of the last session of training when compared to the glycaemia behavior of CE group, suggesting that exercise was more efficient in diabetic animals. Although there are many studies in the literature showing the synergistic effect of insulin plus contractile activity on the increase of glucose transport in the muscle (25-27), in the present study, diabetic rats did not receive insulin in the day of the last session of training, avoiding the insulin effect on this phenomenon and ensuring the training effect on the glucose reduction only. Although it is known that chronic exercise can increase glucose uptake stimulated by insulin, in diabetics without adequate insulin secretion or reduced, this effect, in general, occurs during the exercise. However, it is possible to observe an increase in the post-exercise insulin sensitivity, as an adaptation of hormonal response to the training, involving insulin signaling pathway components (28).

Once the gastrocnemius muscle is composed by mixed muscle fibers (glycolytic and oxidative fibers), which is very similar to the total muscle mass in the body, it was chosen to be evaluated in this study. So we aimed to evaluate the impact of the anaerobic training on the biomarkers of proteolysis in the gastrocnemius muscle of diabetic rats. For this the expression of different genes *Ubb*, *E2-14kDa*, *Trim63* (12,29) related to ubiquitin-proteasome proteolytic pathway was analyzed. We detected that diabetes caused an elevation in the *Trim63* mRNA level (Figure 2C), but the anaerobic training was efficient to reduce this level, suggesting that this gene could be modulated by regular exercise. The levels of the other genes were not so elevated in the diabetic sedentary group; however the anaerobic training reduced the expression independently on the presence of diabetes, suggesting the positive effect of exercise on the maintenance of the muscle mass, and corroborating for the important and therapeutic role of regular exercise to inhibit the protein breakdown of muscle. In the protocols of chronic anaerobic training for hypertrophy in the experimental animal models, significant reductions in MURF-1 e ATROGIN1 in the plantar muscle of healthy, fasted female rats were detected (30). It is known that heterodimers p50/p65 (NF-κB) bind to promoter regions of MuRF-1 DNA inducing its expression, and, consequently protein degradation (31,32). The highest levels presented in the DS group, mainly in *Trim63* expression, proved the efficacy of this disease to accelerate and activate the ubiquitin-proteasome in models of well-stablished DM, without insulin treatment and/or exercise practice.

In the present study no alteration was detected in the mRNA level of *Nfkb*. However, this effect can be reflecting the peak of the *Nfkb* gene expression post-training. In the literature there some information about this, confirming that after 3-12 hours of high intensity of contractile activity there is the peak of gene transcrip-

Table 2. Characteristics of animals: Absolute Body and tissue weight expressed in gramas (g)

Weight (g)	DE	DS	CE	CS
Body weight	239.8 ± 9.86***	240.6 ± 12.1***	304.1 ± 5.88*	340.1 ± 12.82
Fat pad mass	0.1651 ± 0.11***	0.7766 ± 0.30**	1.540 ± 0.36*	2.270 ± 0.24
Gastrocnemius muscle	1.124 ± 0.10	0.859 ± 0.11**	1.430 ± 0.06	1.318 ± 0.05
EDL	0.113 ± 0.009*	0.089 ± 0.01***	0.146 ± 0.005	0.122 ± 0.005*
Soleus	0.133 ± 0.008*	0.110 ± 0.006***	0.159 ± 0.003	0.144 ± 0.007

Values are expressed as Mean ± SEM. ***Body weight***: * $p < 0.05$ *vs.* CS; *** $p < 0.0005$ *vs.* CS and CE; ***Fat pad mass***: * $p < 0.05$ *vs.* CS; ** $p < 0.005$ *vs.* CS; *** $p < 0.0005$ *vs.* CS and CE; ***Gastrocnemius muscle:*** ** $p < 0.005$ *vs.* CS, CE and DE; ***EDL:*** * $p < 0.05$ *vs.* CE and DS; *** p < 0,0005 CE and CS; ***Soleus:*** * $p < 0.05$ *vs.* CE and DS; *** $p < 0.0005$ *vs.* CE and CS. ANOVA One-way with Newman Keulls as post-test. CS = control sedentary (n = 8), CE = control exercised (n = 8), DS = diabetic sedentary (n = 7) and DE = diabetic exercised (n = 6).

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tion in the cells, returning to basal levels in 24 hours (30). In the present study the sacrifice and removal of tissues were performed after 24 hours of the end of the six-week anaerobic training. This result suggests that, in this model, *Nfkb* expression seems not to be involved in the regulation of atrogene expression, contrary to observed in other catabolic states (9,10).

In the literature (12), reduction on the MuRF-1 expression in the muscle of Wistar rats after 8 weeks of aerobic training was found, because of oxidative stress provided by regular exercise. The oxidative stress can promote an unbalance of proteins and enzymes that interferes in the regulation of key-factors for cellular homeostasis. The release of ROS (reactive oxygen species) drives the muscle cells to a catabolic cycle that leads to muscle breakdown, and activates the biochemical pathway of NFkB, directly interfering in the transcription of essential genes for cellular metabolism (1,33). Furthermore, oxidative stress plays a key role in the potentiation of abnormalities generated by DM, since hyperglycemia in the presence of free radicals can lead to auto-oxidation of glucose and protein glycation (34). The metabolism of alloxan promotes intracellular generation of ROS that associated to diabetes potentiates the oxidative stress (35,36). In the literature (37), the exercise was effective in reducing oxidative stress and in improving oxygen uptake, since oxidative stress has direct relation with insulin resistance induced by hyperglycemia, inhibiting the increase of GLUT4, glycogen synthesis and phosphorylation of key-proteins such as IR, Akt and GSK3β.

So, we concluded that six-week anaerobic training of jumps in the liquid medium can promote gain of muscle mass, improvement of glycaemia control, and can promote inhibitory effect on the ubiquitin-proteasome proteolytic pathway in the gastrocnemius muscle of diabetic rats, suggesting that this type of training can be useful and efficient as anti-atrophy treatment for diabetes.

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