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Basic nutritional investigation

## Effects of leucine supplementation and resistance exercise on dexamethasone-induced muscle atrophy and insulin resistance in rats

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### ABSTRACT

**Objective:** We aimed to evaluate the effects of resistance exercise (RE) and leucine (LEU) supplementation on dexamethasone (DEXA)-induced muscle atrophy and insulin resistance.

**Methods:** Male Wistar rats were randomly divided into DEXA (DEX), DEXA + RE (DEX-RE), DEXA + LEU (DEX-LEU), and DEXA + RE + LEU (DEX-RE-LEU) groups. Each group received DEXA 5 mg · kg<sup>-1</sup> · d<sup>-1</sup> for 7 d from drinking water and were pair-fed to the DEX group; LEU-supplemented groups received 0.135 g · kg<sup>-1</sup> · d<sup>-1</sup> through gavage for 7 d; the RE protocol was based on three sessions of squat-type exercise composed by three sets of 10 repetitions at 70% of maximal voluntary strength capacity.

**Results:** The plantaris mass was significantly greater in both trained groups compared with the non-trained groups. Muscle cross-sectional area and fiber areas did not differ between groups. Both trained groups displayed significant increases in the number of intermediated fibers (IIa/IIx), a decreased number of fast-twitch fibers (IIb), an increased ratio of the proteins phospho<sup>Ser2448</sup>/total mammalian target of rapamycin and phospho<sup>Thr389</sup>/total 70-kDa ribosomal protein S6 kinase, and a decreased ratio of phospho<sup>Ser253</sup>/total Forkhead box protein-3a. Plasma glucose was significantly increased in the DEX-LEU group compared with the DEX group and RE significantly decreased hyperglycemia. The DEX-LEU group displayed decreased glucose transporter-4 translocation compared with the DEX group and RE restored this response. LEU supplementation worsened insulin sensitivity and did not attenuate muscle wasting in rats treated with DEXA. Conversely, RE modulated glucose homeostasis and fiber type transition in the plantaris muscle.

**Conclusion:** Resistance exercise but not LEU supplementation promoted fiber type transition and improved glucose homeostasis in DEXA-treated rats.

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### Introduction

Leucine (Leu) supplementation has been a constant focus of investigation of skeletal muscle disorders characterized by muscle wasting owing to its unique non-pharmacologic properties [1,2].

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Several studies have demonstrated its therapeutic role in improving muscle nitrogen balance under experimental conditions such as muscle immobilization [3], aging [4–6], and sepsis [7]. Some reports have also showed that, as an insulin secretagogue, Leu has a potential role in modulating glucose homeostasis [8,9]. Thus, Leu has emerged as an interesting nutritional strategy for treating conditions characterized by skeletal muscle atrophy and disturbance in glucose metabolism.

Similarly, resistance exercise (RE) has been described as one of the strongest and most effective non-pharmacologic treatments against skeletal muscle atrophy [10,11]. In animal models,

despite the limitations of the RE models and protocols, some studies have also observed attenuation of muscle atrophy under catabolic conditions such as muscle disuse [12–16]. Furthermore, we recently demonstrated in a case report that chronic RE with vascular occlusion attenuated the loss of skeletal muscle functionality (strength) in a subject with body inclusion myositis [17]. RE also promotes the therapeutic role of glucose homeostasis, because mechanical stimuli are widely known to improve glucose uptake to skeletal muscle through glucose transporter-4 (GLUT4) translocation to the sarcolemma [18]. In this context, our group observed that type 2 diabetic subjects who submitted to chronic exercise improved glycemic control most likely by enhancing the GLUT4 translocation [19]. In view of these findings, RE may also be considered an effective therapy to counteract simultaneously muscle wasting and insulin resistance.

Conversely, despite their wide therapeutic actions [20], glucocorticoids used in high doses or chronically for longer periods can induce several side effects that may include, among others, diabetes mellitus [21,22] and selective skeletal muscle atrophy [23–26] of fast-twitch fibers [27]. It has been described that steroidal glucocorticoids can stimulate the translocation of specific transcription factors to the nucleus that may enhance the transcription of genes involved in skeletal muscle loss [26]. This effect can be modulated directly, through glucocorticoid receptor translocation, and/or indirectly by the impairment of signaling pathways that are responsive to insulin [28]. The potential role of Leu supplementation and RE in counteracting such effects and the possible mechanisms underlying these responses are not totally elucidated.

Therefore, the aim of the present study was to investigate the effects of Leu supplementation and RE on the skeletal muscle atrophy and glucose homeostasis of rats treated with a high dose of dexamethasone (DEXA). Furthermore, we evaluated the possible molecular mechanisms involved in muscle wasting and glycemic control. Our main hypothesis was that RE exercise could attenuate muscle atrophy and improve glucose homeostasis in this model and that Leu supplementation would exert positive synergic effects through the stimulation of protein synthesis initiation and glucose uptake.

## Materials and methods

### Animals

The experiments were conducted in accordance with the National Research Council's Guidelines for the Care and Use of Laboratory Animals. All methods used were approved by the ethical committee for animal research of the School of Physical Education and Sports, University of Sao Paulo. Adult male Wistar rats (~400 g) were housed under controlled environmental conditions (temperature 22°C, 12-h dark period). They were given free access to commercial laboratory chow and water before the experiments were performed. Twenty-three rats were randomly divided into the following groups: DEXA (DEX;  $n = 06$ ), DEXA + RE (DEX-RE;  $n = 05$ ), DEXA + Leu (DEX-LEU;  $n = 07$ ), and DEXA + RE + Leu (DEX-RE-LEU;  $n = 05$ ). DEXA (a synthetic glucocorticoid analog that does not bind to plasma-binding proteins) was replaced fresh every morning at 09:00 h with the drinking water. Each group received DEXA  $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  for 7 d with the drinking water; DEXA concentrations were adjusted every day based on the intake of drinking water the day before. This protocol was chosen based on our previous study demonstrating that this DEXA dosage can promote significant plantaris muscle atrophy compared with healthy animals [28]. Leu-supplemented groups received  $0.135 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  through gavage [29] for 7 d. Because DEXA has been reported to decrease food intake, all groups were paired to the DEX group. Thus, differences among groups do not originate from different food intakes. Animals were euthanized by decapitation 48 h after the last RE session and a 12-h fast. The plantaris muscles of each limb were isolated, weighed, and frozen at  $-80^\circ\text{C}$  for protein expression analysis. For the GLUT4 translocation experiments, we repeated the experimental protocols and animals had free access to food and water and then were euthanized in the basal state (not fasted;  $n = 4$  per group).

### RE protocol

Resistance exercise was based on the model of Wirth et al. [30]. Briefly, rats performed three sessions of a squat-type exercise in one daily session, with a rest interval of 2 d between sessions. In each session, the rats performed 30 repetitions (3 sets of 10 repetitions) composed of concentric forces [30], which lasted approximately 20 min. The minimum height for collar lifting was fixed at 3.0 cm because we observed that in our training apparatus this was the minimum height required for the animals to perform a full plantar flexion. The rest interval between repetitions was about 10 to 20 s, and the load lifted in each session was 70% of the maximal voluntary strength capacity (MVSC). The eighth repetition test was used to assess the MVSC of each rat and was previously standardized by our group [31–33]. If the rat was successful, the load was increased by 2% until failure. When a lift was not properly performed, the load was decreased by 2% in relation to the MVSC. The load of 70% MVSC was chosen not only because it is within the range of load capable of inducing hypertrophy in human studies [34] but also because it was observed that the rats proved unable to lift the near maximal/maximal loads (>95% MVSC). To obtain the initial MVSC value, the total mass of weights on the lever was gradually increased until the rats proved unable to lift the lever. Therefore, the loads were always lifted in an individualized manner and adjusted according to the MVSC.

### Muscle dry/wet ratio

Muscle tissues were desiccated for 5 d in a drying oven set at  $50^\circ\text{C}$  before determination of the dry weight. The dry/wet weight ratio was determined as previously described [35].

### Histologic analysis

The collected plantaris muscles were also embedded in tissue tek, cooled in isopentane, frozen in liquid nitrogen, and sectioned with a cryostat. The resulting  $10\text{-}\mu\text{m}$  transverse sections were examined with adenosine triphosphatase staining (pH 4.6). To decrease the influence of anatomic area on the fiber type distribution, all plantaris muscles were sectioned in the belly after removal. Fibers without horizontal orientation and/or damaged owing to the section process were not considered for analysis. Cross-sectional areas (CSAs) of 450 muscle fibers of a muscle from each rat were measured using Image Pro-Plus (Media Cybernetics, Bethesda, MD, USA) software.

### Plasma insulin and glucose levels

Blood was collected and serum samples were separated after allowing blood to clot on ice. Serum was stored at  $-80^\circ\text{C}$  for further analysis. Plasma insulin was measured using commercial radioimmunoassay kits (DPC Medlab, São Paulo, SP, Brazil). Plasma glucose levels were measured using an automatized method (Accu-Chek Active System, Roche Diagnostics, Mannheim, Germany). The homeostasis model for assessment of insulin resistance (HOMA-IR) index was calculated as follows:  $\text{HOMA-IR index} (\text{mmol} \cdot \text{mU} \cdot \text{L}^{-2}) = \text{fasting insulin} (\text{mU/L}) \times \text{serum glucose} (\text{mmol/L})/22.5$  [36].

### Cellular fractionation for GLUT4 protein expression

Muscle samples were minced and homogenized in ice-cold lysis buffer (2 mM ethylenediaminetetraacetic acid, 10 mM ethylene glycol bis[2-aminoethyl ether]-N,N,N',N'-tetraacetic acid, 0.25 M sucrose, 1:300 Sigma (Sigma-Aldrich, St. Louis, MO, USA) protease inhibitor cocktail, and 20 mM Tris-HCl at pH 7.5). The homogenate was centrifuged at  $100\,000 \times g$  for 30 min ( $4^\circ\text{C}$ ) to obtain the membrane fraction.

### Western blot

Bradford assays were used to determine the sarcoplasmic protein concentration and then samples were standardized to 1 mg/mL by dilution with 3× Laemmli loading buffer. Briefly, samples were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis in 8% polyacrylamide gels. After electrophoresis, proteins were electrotransferred to a nitrocellulose membrane through a Transblot Semi Dry Transfer Cell Bio-Rad Laboratories (Hercules, California, USA). Equal loading of samples and transfer efficiency were monitored with the use of 0.5% Ponceau S staining of the blot membrane. The blotted membrane was then blocked with 5% low-fat milk (total) or 5% bovine serum albumin (for phospho-antibodies) in Tris-buffered saline with Tween (TBS-T) for 1 h, and then membranes were incubated overnight with the following primary antibodies to evaluate glucose homeostasis and the synthetic and proteolytic signaling pathways: GLUT4 (1:1000; Millipore, Billerica, MA, USA), total mammalian target of rapamycin (mTOR), phospho-mTOR<sup>Ser2448</sup>, total 70-kDa ribosomal protein S6 kinase (p70S6k), phospho-p70S6k<sup>Thr389</sup>, total Forkhead box protein-3a (FoxO3a), phospho-FoxO3a<sup>Ser253</sup>, and muscle-specific RING

**Table 1**

Body weight, skeletal muscle weight, blood glucose, and plasma insulin of each experimental group\*

Variable	Group			
	DEX (n = 6)	DEX-LEU (n = 7)	DEX-RE (n = 5)	DEX-RE-LEU (n = 5)
Basal BW (g)	409 ± 17.9	414 ± 31.3	396 ± 13.4	416 ± 20.1
Final BW (g)	306 ± 0.9	290 ± 33.5	315 ± 20.1	292 ± 32.6
Plantaris weight (mg)	265 ± 40.2	256 ± 38.3	313 ± 29.1 <sup>††</sup>	316 ± 35.8 <sup>††</sup>
Plantaris dry/wet ratio (mg)	0.26 ± 0.04	0.25 ± 0.07	0.25 ± 0.04	0.26 ± 0.04
Plantaris weight/basal BW (mg/g)	0.65 ± 0.09	0.62 ± 0.09	0.79 ± 0.07 <sup>††</sup>	0.77 ± 0.08 <sup>††</sup>
Blood glucose (mmol/L)	7.8 ± 1.3 <sup>§</sup>	16.1 ± 2.0 <sup>§¶</sup>	5.3 ± 0.5	7.6 ± 1.8 <sup>§</sup>
Plasma insulin (mU/L)	47.7 ± 3.3 <sup>§</sup>	65.7 ± 22.4 <sup>§¶</sup>	25.9 ± 2.9 <sup>¶</sup>	49.5 ± 11.2 <sup>§</sup>
HOMA-IR index (mmol · mU <sup>-1</sup> · L <sup>-2</sup> )	12.7 ± 7.4	47.7 ± 11.0 <sup>§¶</sup>	6.2 ± 0.2 <sup>¶</sup>	16.8 ± 3.6 <sup>§</sup>

BW, body weight; DEX, dexamethasone; HOMA-IR, homeostasis model for assessment of insulin resistance; LEU, leucine supplementation; RE, resistance exercise

\* Values are presented as mean ± SD.

† P &lt; 0.05 versus DEX.

†† P &lt; 0.05 versus DEX-LEU.

§ P &lt; 0.05 versus DEX-RE.

¶ P &lt; 0.05 versus DEX-RE-LEU.

finger-1 (MuRF-1; 1:1000; Cell Signalling, Danvers, MA, USA). Membranes were then washed with TBS-T and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:10 000; Cell Signalling), before further washing with TBS-T and incubation for 1 min with enhanced chemiluminescence (ECL). Quantification analysis of blots was performed using Image J (National Institute of Health, Bethesda, MD, USA). Protein expressions were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

#### Data analysis

The dependent variables were tested by one-way or two-way analysis of variance. A post hoc test with a Tukey adjustment was performed for multiple comparison purposes. Student's *t* test was used for comparisons between trained groups. The significance level was set at *P* < 0.05. The results are expressed as mean ± standard deviation.

## Results

### Characterization of RE protocol

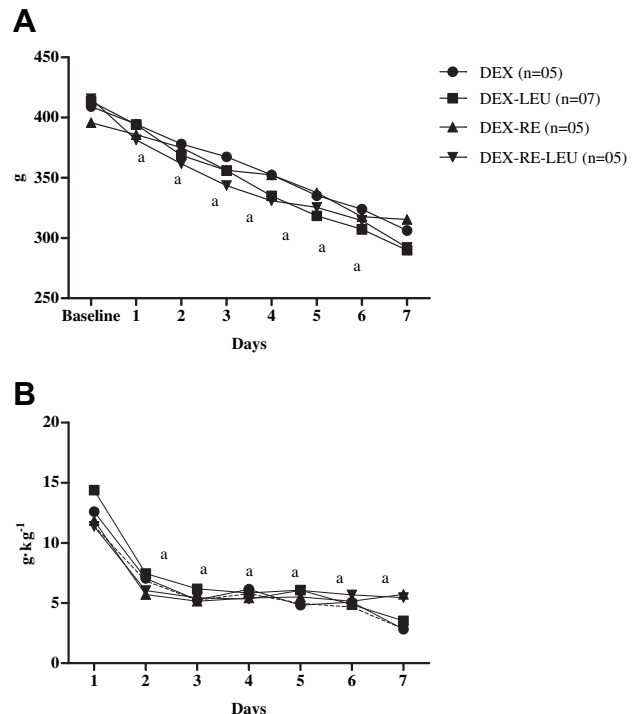
All groups underwent the stages of exercise adaptation as proposed by Wirth et al. [30]. At the end of the apparatus-adaptation phase, the animals from the DEX-RE and DEX-RE-LEU groups were subjected to the MVSC test, which did not differ between these groups (*P* > 0.05; Supplemental Figure S1A). Animals from groups DEX and DEX-LEU were subjected to the same period of environmental stress as the animals from the trained groups. The average length of each exercise session was 1461 ± 225 and 1370 ± 109 s for the DEX-RE and DEX-RE-LEU groups, respectively (*P* > 0.05; Supplemental Figure S1C). The mean time of each set was also measured and did not differ among groups (*P* > 0.05). As observed in Supplemental Figure S1B, the length of the first set (609 ± 192 and 610 ± 80 s for the DEX-RE and DEX-RE-LEU groups, respectively) was quite superior compared with the second set (318 ± 47 and 367 ± 70 s for the DEX-RE and DEX-RE-LEU groups, respectively) and the third set (350 ± 79 and 428 ± 74 s for the DEX-RE and DEX-RE-LEU groups, respectively). This is explainable because the exercise sessions were separated by 48 h, and in the first set of each session, the animals recognized the environment before engaging in the exercise protocol. The height of the collar displacement did not differ between groups and presented average values of 3.02 ± 0.74 for the DEX-RE group and 3.44 ± 0.69 for the DEX-RE-LEU group (*P* > 0.05; Supplemental Figure S1D). Concentric strength also did not differ among groups during the entire experiment (*P* > 0.05; Supplemental Figure S2).

### RE and Leu supplementation did not modulate body weight and food intake in DEXA-treated animals

As presented in Table 1, both groups started and finished the experiment with similar body weights (*P* > 0.05). Despite body weight and food intake being significantly decreased from the second to the last day of treatment (*P* < 0.05; Fig. 1A,B), these variables did not differ among groups (*P* > 0.05), demonstrating that neither RE nor Leu supplementation counteracted the effects induced by the DEXA treatment. Water intake was not different among groups (data not shown).

### RE, but not Leu supplementation, increased plantaris muscle mass in DEXA-treated animals

Of all the interventions applied, only RE was able to increase the plantaris muscle mass in DEXA-treated rats. The DEX-RE and



**Fig. 1.** Body weight (A) and food intake (B) progression data among groups. (A) <sup>a</sup>*P* < 0.05 significant differences among all groups versus baseline. DEX, dexamethasone; LEU, leucine supplementation; RE, resistance exercise.

DEX-RE-LEU groups presented higher values of plantaris muscle mass and plantaris mass/basal body weight compared with the other groups ( $P < 0.05$ ; Table 1) but without significant differences between them ( $P > 0.05$ ). Thus, Leu supplementation did not promote synergistic effects on RE in modulating the plantaris muscle mass. The DEX-LEU group did not show any modification in the plantaris mass compared with the DEX group ( $P > 0.05$ ), demonstrating that Leu supplementation alone also was not able to modulate the plantaris muscle mass. The muscle dry/wet ratio did not differ among groups ( $P > 0.05$ ).

*RE, but not leucine supplementation, did not change plantaris CSA but modulated fiber type profile in DEXA-treated animals*

Figure 2 shows the plantaris muscle CSA and fiber type profile of all groups. Leu supplementation (DEX-LEU) did not change any of these parameters compared with the DEX group ( $P > 0.05$ ; Fig. 2). However, in the DEX-RE group, although the plantaris muscle CSA and fiber areas were unaltered compared with the other groups ( $P > 0.05$ ; Fig. 2F,G), the number of intermediate fibers (IIa/IIx) was significantly increased and the number of fast-twitch fibers (IIb) was decreased compared with the DEX and DEX-LEU groups ( $P < 0.05$ ; Fig. 2E). The DEX-RE-LEU group did not show any difference in the plantaris muscle CSA compared with the other groups ( $P > 0.05$ ; Fig. 2G) and in similar fiber type phenotype compared with the DEX-RE group ( $P > 0.05$ ; Fig. 2E).

*RE improved glucose homeostasis in DEXA-treated animals through GLUT4 translocation and Leu supplementation minimized such responses*

Resistance exercise (DEX-RE group) decreased fasting blood glucose ( $P < 0.05$  versus others) and Leu supplementation (DEX-LEU group) aggravated it ( $P < 0.05$  versus others) and partly blunted the therapeutic effect of RE (DEX-RE-LEU group;  $P < 0.05$

versus others). Plasma insulin and HOMA-IR followed the same result pattern of blood glucose (DEX-LEU = DEX-RE-LEU > DEX > DEX-RE group;  $P < 0.05$ ; Table 1).

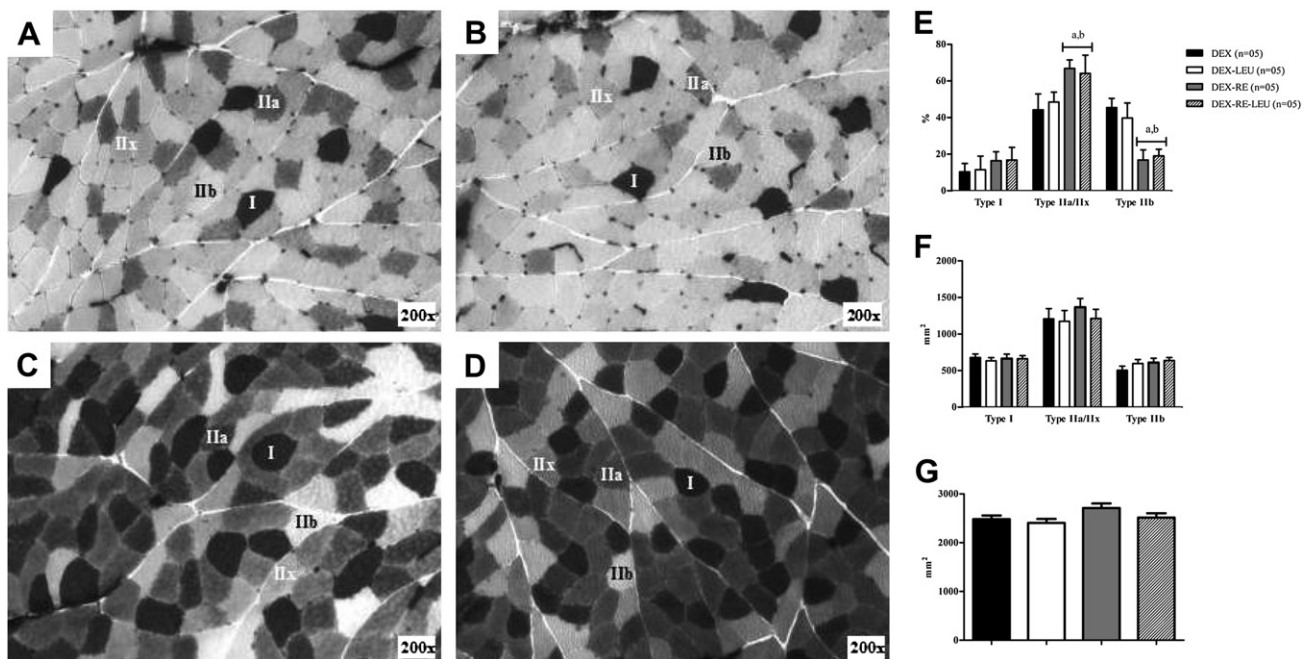
Total GLUT4 protein expression did not differ among groups ( $P > 0.05$ ; Fig. 3A). However, RE (DEX-RE group) significantly increased the basal membrane/total GLUT4 ratio ( $P < 0.05$  versus others; Fig. 3B). Leu supplementation (DEX-LEU group) impaired the GLUT4 translocation to the cell surface ( $P < 0.05$  versus others) and partly decreased (DEX-RE-LEU) the effect induced by RE ( $P < 0.05$  versus others).

*DEXA treatment modulated the expression of proteins involved in muscle remodeling and RE, but not Leu supplementation, improved such responses*

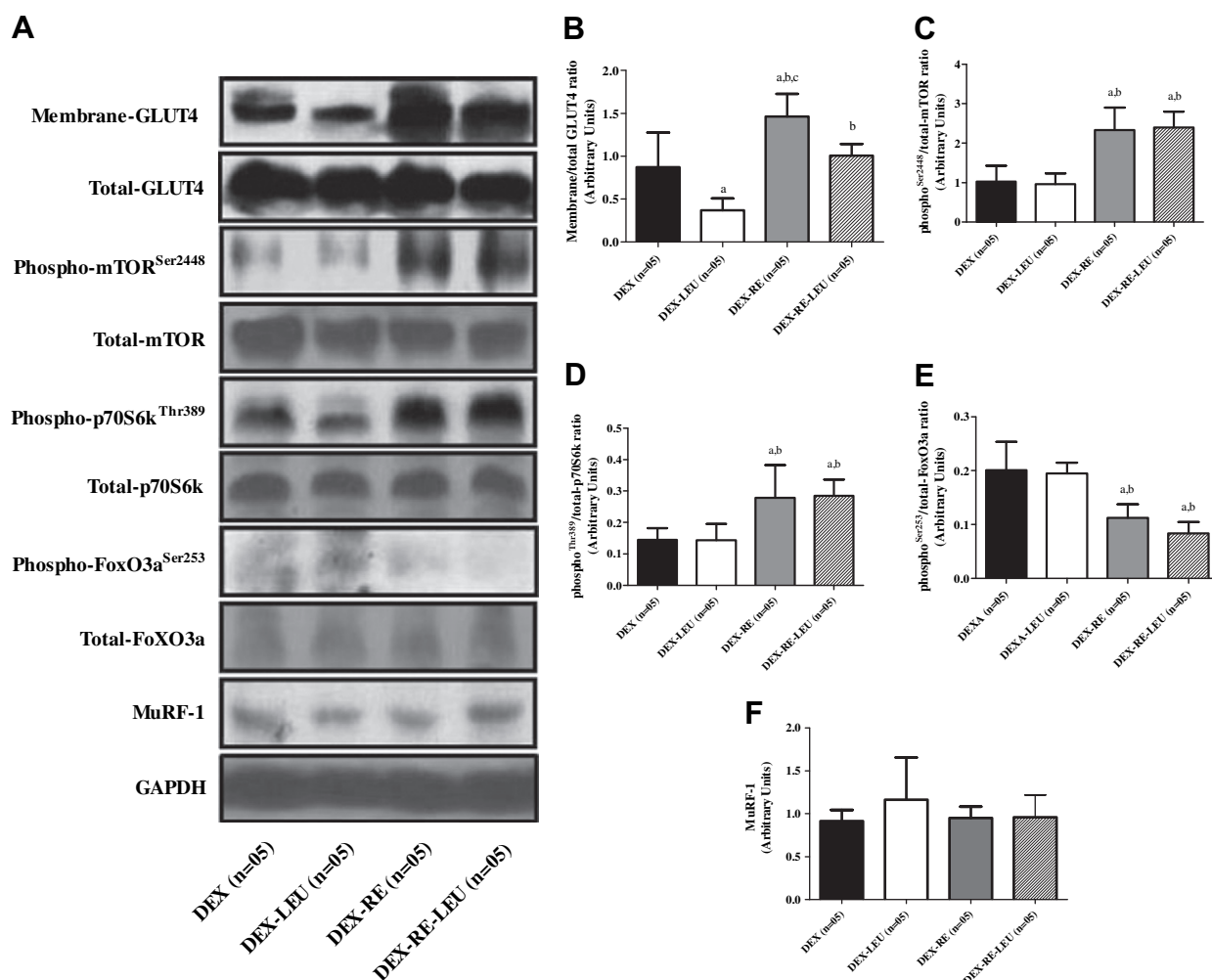
Regarding synthetic machinery, the total protein expression of mTOR, p70S6k, and FoxO3a did not differ among groups ( $P > 0.05$ ; Fig. 3A). However, the DEX-RE and DEX-RE-LEU groups presented significantly increased the phospho<sup>Ser2448</sup>/total mTOR ratio (Fig. 3C) and phospho<sup>Thr389</sup>/total p70S6k ratio (Fig. 3D) compared with the untrained groups ( $P < 0.05$ ) but without differences between them ( $P > 0.05$ ), suggesting no synergistic effects of Leu supplementation on RE responses. Leu supplementation (DEX-LEU group) did not modulate the expression of such protein ratios compared with the DEX group ( $P > 0.05$ ). The phospho<sup>Ser253</sup>/total FoxO3a ratio (Fig. 3E) was similarly decreased in both trained groups compared with the untrained groups ( $P < 0.05$ ). MuRF-1 did not differ between the trained and untrained groups ( $P > 0.05$ ; Fig. 3F).

*Leu supplementation attenuates performance in RE-trained and DEXA-treated rats*

Because the animals were euthanized 48 h after the last exercise session, we were unable to perform an MVSC test at the



**Fig. 2.** Examples of transverse muscle sections with histochemical staining for myosin adenosine triphosphatase, preincubated at pH 4.6 in plantaris muscles from the (A) DEX, (B) DEX-LEU, (C) DEX-RE, and (D) DEX-RE-LEU groups. Fiber types I, IIa, IIx, and IIb were identified. (E) Fiber type profile, (F) fiber area, and (G) muscle cross-sectional area data are presented as mean  $\pm$  SD. <sup>a</sup> $P < 0.05$  versus DEX group; <sup>b</sup> $P < 0.05$  versus DEX-LEU group. DEX, dexamethasone; LEU, leucine supplementation; RE, resistance exercise.



**Fig. 3.** Resistance exercise, but not LEU, improves the expression level of proteins involved in muscle remodeling in the plantaris muscle of DEX-treated rats. (A) Representative blots show the effect of RE and LEU on the phospho/total ratio of proteins related to muscle remodeling. (B) Plantaris membrane/total ratio of GLUT4, (C) phospho/total ratio of mTOR, (D) phospho/total ratio of p70S6k, (E) phospho/total ratio of FoxO3a, and (F) MuRF-1 expression in untrained and RE-trained and DEX-treated rats. Data are presented as mean  $\pm$  SD and normalized against GAPDH. <sup>a</sup> $P < 0.05$  versus DEX group; <sup>b</sup> $P < 0.05$  versus DEX-LEU group; <sup>c</sup> $P < 0.05$  versus DEX-RE group; <sup>d</sup> $P < 0.05$  versus DEX-RE-LEU group. DEX, dexamethasone; FoxO3a, Forkhead box protein-3a; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT4, glucose transporter-4; LEU, leucine supplementation; mTOR, mammalian target of rapamycin; MuRF-1, muscle-specific RING finger-1; p70S6k, 70-kDa ribosomal protein S6 kinase; RE, resistance exercise.

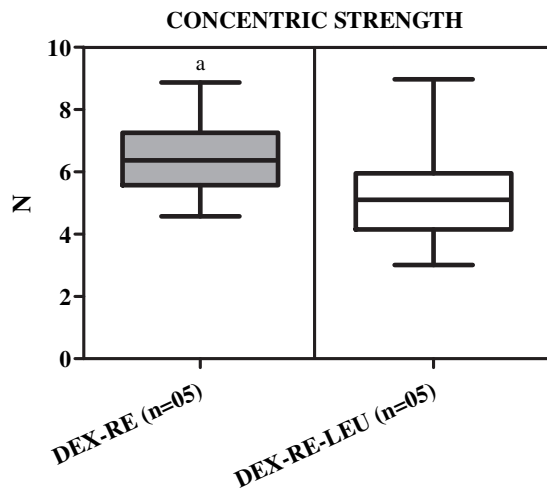
end of the experiment because it can induce severe alterations in the phosphorylation status of several proteins. Therefore, we considered the concentric strength of the last session as a functional parameter. Although the concentric strength of the entire experiment did not differ between trained groups ( $P > 0.05$ ; Supplemental Figure S1A), this variable was significantly altered in the last exercise session. As shown in Figure 4, the DEX-RE group presented higher concentric strength compared with the DEX-RE-LEU group ( $P < 0.05$ ).

## Discussion

To our knowledge, this is the first study that investigated the effects of RE combined with Leu supplementation on skeletal muscle remodeling and glucose homeostasis in a DEXA treatment experimental model. The major findings of the present study were that RE positively modulated the skeletal muscle phenotype and improved glucose homeostasis in DEXA-treated animals. Such an effect can be partly explained by the phosphorylation of proteins involved in the synthetic (mTOR and

p70S6k phospho/total ratio) and proteolytic (FoxO3a phospho/total ratio) machinery and glucose uptake (GLUT4 translocation to the sarcolemma) in skeletal muscle. In contrast, Leu supplementation did not promote any therapeutic effect and worsened glucose homeostasis, as evidenced by the metabolic (insulin sensitivity), molecular (GLUT4 translocation), and functional (concentric strength) data.

Exercise training has been considered a key complementary tool in many different health conditions, mainly aimed at performance. However, more recently, RE also has been used as a therapeutic tool to treat several different diseases to prevent the loss of muscle mass and to improve the metabolic profile of the whole body or specific tissues [37], as in the case of prolonged and high-dose DEXA treatment. In the present study, we demonstrated that even three sessions of high-intensity RE (70% MVSC with intervals of  $\sim 10$  s between repetitions and  $\sim 90$  s between series) were capable of drastically decreasing the consequences of high-dose DEXA treatment in the RE-trained group, improving the metabolic profile linked with impaired glucose homeostasis (as evidenced by decreased fasting plasma



**Fig. 4.** Leucine supplementation attenuates RE performance in RE-trained and DEX-treated rats. Boxplot visualizes the concentric strength of the last exercise session. <sup>a</sup>*P* < 0.05 versus DEX-RE-LEU group. DEX, dexamethasone; LEU, leucine supplementation; RE, resistance exercise.

insulin and blood glucose levels) and the molecular signaling pathway of glucose, such as basal GLUT4 translocation to the cell sarcolemma [18,38,39].

In a previous study by our group, the present protocol of DEXA demonstrated the promotion of significant plantaris muscle wasting and insulin resistance compared with healthy animals [28]. Although the weight of the plantaris muscle was increased in the RE-trained groups compared with the untrained groups, this datum was not reproduced in the histologic analysis, which was probably due to the variability in the dissection of the tendon and connective tissues spared by RE. Thus, three RE sessions were not capable of preventing the DEXA-induced muscle atrophy but increased the phosphorylation of mTOR and p70S6k in key residue amount that contribute to protein synthesis [40–43]. Such a biological response reflects the residual effect of RE because the groups were euthanized 48 h after the last training session. Thus, it is possible to speculate that RE executed during a more prolonged period, even in the presence of high doses of DEXA, could significantly attenuate plantaris muscle loss. Conversely, changes in the phenotype of muscle fibers occurred, and this is a condition that could improve the molecular muscle engine and potentially lead to increases in the strength and power of the muscle fiber [41]. In fact, as expected, there was a transition from type IIb to type IIa/IIx muscle fibers in the RE-trained animals compared with the untrained animals. Such a change might represent a beneficial physiologic effect that was not blocked by DEXA treatment alone, as previously demonstrated [44].

Leucine supplementation has been considered a nutritional non-pharmacologic approach in aging [4–6,45–47]. However, the effect of very high doses of DEXA combined with RE and Leu supplementation (in a dose that is known to be capable of increasing skeletal muscle protein synthesis in healthy conditions) [29] has not yet been demonstrated. Based on these previous experiments, we expected that Leu supplementation combined RE would be capable of promoting the synergistic effects on RE in preventing skeletal muscle atrophy and improving glucose homeostasis. However, we observed that Leu supplementation did not promote any benefits for muscle remodeling and worsened glucose homeostasis compared with

the other groups, as demonstrated by insulin sensitivity, fasting blood glucose, and the HOMA-IR index and the molecular data of basal GLUT4 translocation to the cell surface. These data corroborate with the literature [5]. Leu supplementation per se interacted with DEXA treatment in worsening glucose homeostasis, an effect that was not dependent on muscle mass. Recently, Leu supplementation has been demonstrated to induce a delay in the insulin signaling pathway (IR/PI3K protein expression) in skeletal muscle [48]. Thus, the conclusion that is considered for Leu supplementation is that it does not promote synergistic effects on RE in muscle remodeling, induces a significant degree of insulin resistance in skeletal muscle, and attenuates the therapeutic effect of RE on glucose homeostasis under DEXA treatment (e.g., similar muscle mass, mTOR and p70S6K phospho/total ratio, FoxO3a transcription factor phospho/total ratio, and decreased basal GLUT4 translocation to the sarcolemma). For protein synthesis, we assumed that the Leu effects could be more visible in the postprandial state instead of the postabsorptive state. However, this was not the case for the basal GLUT4 translocation because the animals were not in a fasted state.

In summary, our results are conclusive that RE, even in the presence of high doses of DEXA, improves glucose homeostasis and increases the phosphorylation of key protein kinases (mTOR and p70S6k phospho/total ratio) and the transcription factors (FoxO3a phospho/total ratio) related to RE-induced muscle remodeling. However, unexpectedly, Leu supplementation in a dose known to increase muscle protein synthesis in healthy rats worsened and decreased such beneficial effects of RE. Future studies should address the potential role of other RE protocols, the dose–response effect of Leu supplementation, and the effect of exercise in the death toll in such catabolic conditions.

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#### Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.nut.2011.08.008.

#### References

- [1] Nicastro H, Artioli GG, Costa Ados S, Solis MY, da Luz CR, Blachier F, Lancha AH Jr. An overview of the therapeutic effects of leucine supplementation on skeletal muscle under atrophic conditions. *Amino Acids* 2011;40:287–300.
- [2] Zanchi NE, Nicastro H, Lancha AH Jr. Potential antiproteolytic effects of L-leucine: observations of in vitro and in vivo studies. *Nutr Metab (Lond)* 2008;5:20.
- [3] Baptista IL, Leal ML, Artioli GG, Aoki MS, Fiamoncini J, Turri AO, et al. Leucine attenuates skeletal muscle wasting via inhibition of ubiquitin ligases. *Muscle Nerve* 2010;41:800–8.
- [4] Marzani B, Balage M, Venien A, Astruc T, Papet I, Dardevet D, Mosoni L. Antioxidant supplementation restores defective leucine stimulation of protein synthesis in skeletal muscle from old rats. *J Nutr* 2008;138:2205–11.
- [5] Rieu I, Balage M, Sornet C, Debras E, Ripes S, Rochon-Bonhomme C, Pouyet C, et al. Increased availability of leucine with leucine-rich whey proteins improves postprandial muscle protein synthesis in aging rats. *Nutrition* 2007;23:323–31.
- [6] Combaret L, Dardevet D, Rieu I, Pouch MN, Bechet D, Taillandier D, et al. A leucine-supplemented diet restores the defective postprandial inhibition of proteasome-dependent proteolysis in aged rat skeletal muscle. *J Physiol* 2005;569:489–99.

- [7] De Bandt JP, Cynober L. Therapeutic use of branched-chain amino acids in burn, trauma, and sepsis. *J Nutr* 2006;136:308S–13S.
- [8] da Silva PM, Zoppi CC, Filiputti E, Silveira LR, Quesada I, Boschero AC, Carneiro EM. Preliminary report: leucine supplementation enhances glutamate dehydrogenase expression and restores glucose-induced insulin secretion in protein-malnourished rats. *Metabolism* 2010;59:911–3.
- [9] Filiputti E, Rafacho A, Araujo EP, Silveira LR, Trevisan A, Batista TM, et al. Augmentation of insulin secretion by leucine supplementation in malnourished rats: possible involvement of the phosphatidylinositol 3-phosphate kinase/mammalian target protein of rapamycin pathway. *Metabolism* 2010;59:635–44.
- [10] Little JP, Phillips SM. Resistance exercise and nutrition to counteract muscle wasting. *Appl Physiol Nutr Metab* 2009;34:817–28.
- [11] Phillips SM. Physiologic and molecular bases of muscle hypertrophy and atrophy: impact of resistance exercise on human skeletal muscle (protein and exercise dose effects). *Appl Physiol Nutr Metab* 2009;34:403–10.
- [12] Linderman JK, Gosselink KL, Booth FW, Mukku VR, Grindeland RE. Resistance exercise and growth hormone as countermeasures for skeletal muscle atrophy in hindlimb-suspended rats. *Am J Physiol Regul Integr Comp Physiol* 1994;267:R365–71.
- [13] Adams GR, Haddad F, Bodell PW, Tran PD, Baldwin KM. Combined isometric, concentric, and eccentric resistance exercise prevents unloading-induced muscle atrophy in rats. *J Appl Physiol* 2007;103:1644–54.
- [14] Haddad F, Adams GR, Bodell PW, Baldwin KM. Isometric resistance exercise fails to counteract skeletal muscle atrophy processes during the initial stages of unloading. *J Appl Physiol* 2006;100:433–41.
- [15] Dupont-Versteegden EE, Fluckey JD, Knox M, Gaddy D, Peterson CA. Effect of flywheel-based resistance exercise on processes contributing to muscle atrophy during unloading in adult rats. *J Appl Physiol* 2006;101:202–12.
- [16] Fluckey JD, Dupont-Versteegden EE, Montague DC, Knox M, Tesch P, Peterson CA, Gaddy-Kurten D. A rat resistance exercise regimen attenuates losses of musculoskeletal mass during hindlimb suspension. *Acta Physiol Scand* 2002;176:293–300.
- [17] Gualano B, Ugrinowitsch C, Neves M Jr, Lima FR, Pinto AL, Laurentino G, et al. Vascular occlusion training for inclusion body myositis: a novel therapeutic approach. *J Vis Exp* 2010;5:1894.
- [18] Pereira LO, Lancha AH Jr. Effect of insulin and contraction up on glucose transport in skeletal muscle. *Prog Biophys Mol Biol* 2004;84:1–27.
- [19] Gualano B, de Salles Painelli V, Roschel H, Artioli GG, Junior MN, Lucia de Sa Pinto A, et al. Creatine in type 2 diabetes: a randomized, double-blind, placebo-controlled trial. *Med Sci Sports Exerc* 2011;43:770–8.
- [20] Zanchi NE, Filho MA, Felitti V, Nicastro H, Lorenzetti FM, Lancha AH Jr. Glucocorticoids: extensive physiological actions modulated through multiple mechanisms of gene regulation. *J Cell Physiol* 2010;224:311–5.
- [21] Bernal-Mizrachi C, Weng S, Feng C, Finck BN, Knutsen RH, Leone TC, et al. Dexamethasone induction of hypertension and diabetes is PPAR-alpha dependent in LDL receptor-null mice. *Nat Med* 2003;9:1069–75.
- [22] Giorgino F, Almahfouz A, Goodyear LJ, Smith RJ. Glucocorticoid regulation of insulin receptor and substrate IRS-1 tyrosine phosphorylation in rat skeletal muscle in vivo. *J Clin Invest* 1993;91:2020–30.
- [23] Clarke BA, Drujan D, Willis MS, Murphy LO, Corpina RA, Burova E, et al. The E3 Ligase MuRF1 degrades myosin heavy chain protein in dexamethasone-treated skeletal muscle. *Cell Metab* 2007;6:376–85.
- [24] Gilson H, Schakman O, Combaret L, Lause P, Grobet L, Attaix D, et al. Myostatin gene deletion prevents glucocorticoid-induced muscle atrophy. *Endocrinology* 2007;148:452–60.
- [25] Hasselgren PO. Glucocorticoids and muscle catabolism. *Curr Opin Clin Nutr Metab Care* 1999;2:201–5.
- [26] Waddell DS, Baehr LM, van den Brandt J, Johnsen SA, Reichardt HM, Furlow JD, Bodine SC. The glucocorticoid receptor and FOXO1 synergistically activate the skeletal muscle atrophy-associated MuRF1 gene. *Am J Physiol Endocrinol Metab* 2008;295:E785–97.
- [27] Falduto MT, Czerwinski SM, Hickson RC. Glucocorticoid-induced muscle atrophy prevention by exercise in fast-twitch fibers. *J Appl Physiol* 1990;69:1058–62.
- [28] Nicastro H, Gualano B, de Moraes WMAM, de Salles Painelli V, da Luz CR, dos Santos Costa A, et al. Effects of creatine supplementation on muscle wasting and glucose homeostasis in rats treated with high dose of dexamethasone. *Amino Acids*; 2011 (in press).
- [29] Crozier SJ, Kimball SR, Emmert SW, Anthony JC, Jefferson LS. Oral leucine administration stimulates protein synthesis in rat skeletal muscle. *J Nutr* 2005;135:376–82.
- [30] Wirth O, Gregory EW, Cutlip RG, Miller GR. Control and quantitation of voluntary weight-lifting performance of rats. *J Appl Physiol* 2003;95:402–12.
- [31] Zanchi NE, Lira FS, Seelaender M, Lancha AH Jr. Experimental chronic low-frequency resistance training produces skeletal muscle hypertrophy in the absence of muscle damage and metabolic stress markers. *Cell Biochem Funct* 2010;28:232–8.
- [32] Zanchi NE, Lira FS, de Siqueira Filho MA, Rosa JC, de Oliveira Carvalho CR, Seelaender M, et al. Chronic low frequency/low volume resistance training reduces pro-inflammatory cytokine protein levels and TLR4 mRNA in rat skeletal muscle. *Eur J Appl Physiol* 2010;109:1095–102.
- [33] Lira FS, Koyama CH, Yamashita AS, Rosa JC, Zanchi NE, Batista ML Jr, Seelaender MC. Chronic exercise decreases cytokine production in healthy rat skeletal muscle. *Cell Biochem Funct* 2009;27:458–61.
- [34] Fry AC. The role of resistance exercise intensity on muscle fibre adaptations. *Sports Med* 2004;34:663–79.
- [35] Kauvar DS, Baer DG, Walters TJ. Influence of systemic hypotension on skeletal muscle ischemia-reperfusion injury after 4-hour tourniquet application. *J Surg Educ* 2007;64:273–7.
- [36] Adami GF, Cordera R, Andraghetti G, Camerini GB, Marinari GM, Scopinaro N. Changes in serum ghrelin concentration following biliopancreatic diversion for obesity. *Obes Res* 2004;12:684–7.
- [37] de Salles Painelli V, Gualano B, Artioli GG, de Sa Pinto AL, Bonfa E, Lancha Junior AH, Lima FR. The possible role of physical exercise on the treatment of idiopathic inflammatory myopathies. *Autoimmun Rev* 2009;8:355–9.
- [38] Kennedy JW, Hirshman MF, Gervino EV, Ocel JV, Forse RA, Hoeng SJ, et al. Acute exercise induces GLUT4 translocation in skeletal muscle of normal human subjects and subjects with type 2 diabetes. *Diabetes* 1999;48:1192–7.
- [39] Thorell A, Hirshman MF, Nygren J, Jorfeldt L, Wojtaszewski JF, Dufresne SD, et al. Exercise and insulin cause GLUT-4 translocation in human skeletal muscle. *Am J Physiol Endocrinol Metab* 1999;277:E733–41.
- [40] Terzis G, Spengos K, Mascher H, Georgiadis G, Manta P, Blomstrand E. The degree of p70 S6k and S6 phosphorylation in human skeletal muscle in response to resistance exercise depends on the training volume. *Eur J Appl Physiol* 2010;110:835–43.
- [41] Zanchi NE, Lancha AH Jr. Mechanical stimuli of skeletal muscle: implications on mTOR/p70s6k and protein synthesis. *Eur J Appl Physiol* 2008;102:253–63.
- [42] Terzis G, Georgiadis G, Stratakos G, Vogiatzis I, Kavouras S, Manta P, et al. Resistance exercise-induced increase in muscle mass correlates with p70S6 kinase phosphorylation in human subjects. *Eur J Appl Physiol* 2008;102:145–52.
- [43] Apro W, Blomstrand E. Influence of supplementation with branched-chain amino acids in combination with resistance exercise on p70S6 kinase phosphorylation in resting and exercising human skeletal muscle. *Acta Physiol (Oxf)* 2010;200:237–48.
- [44] Pellegrino MA, D'Antona G, Bortolotto S, Boschi F, Pastoris O, Bottinelli R, et al. Clenbuterol antagonizes glucocorticoid-induced atrophy and fibre type transformation in mice. *Exp Physiol* 2004;89:89–100.
- [45] Nicastro H, Artioli GG, Dos Santos Costa A, Solis MY, da Luz CR, Blachier F, Lancha AH Jr. An overview of the therapeutic effects of leucine supplementation on skeletal muscle under atrophic conditions. *Amino Acids* 2011;40:287–300.
- [46] Rieu I, Balage M, Sornet C, Giraudet C, Pujos E, Grizard J, et al. Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia. *J Physiol* 2006;575:305–15.
- [47] Dardevet D, Rieu I, Fafournoux P, Sornet C, Combaret L, Bruhat A, et al. Leucine: a key amino acid in ageing-associated sarcopenia? *Nutr Res Rev* 2003;16:61–70.
- [48] Balage M, Dupont J, Mothe-Satney I, Tesseraud S, Mosoni L, Dardevet D. Leucine supplementation in rats induced a delay in muscle IR/PI3K signaling pathway associated with overall impaired glucose tolerance. *J Nutr Biochem* 2010;22:219–26.