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ORIGINAL RESEARCH

Effects of leucine supplementation and resistance training on myopathy of diabetic rats

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Diabetes mellitus, leucine supplementation, resistance training, skeletal muscle.

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Abstract

Leucine supplementation and resistance training positively influence the protein translation process and the cell signaling mTOR (mammalian target of rapamycin) pathway that regulates muscle protein balance and muscle remodeling, and thus may be therapeutic to diabetic myopathy. However, the effect of a combined intervention has not been well studied. Forty male Wistar rats were divided into five groups, control (C), diabetic control (D), diabetic + trained (DT), diabetic + L-leucine (DL), diabetic + L-leucine + trained (DLT). The supplementation of 5% leucine in chow, and resistance training were conducted for 8 weeks postweaning of rats. The extensor digitorum longus was used to assess signaling proteins involved in muscle protein synthesis, and the gastrocnemius and soleus were used for determination of muscle weight. Blood samples were collected for biochemical assays. Strength and ambulation tests were employed to evaluate motor performance. Results showed that both leucine supplementation and resistance training elevated the activity of mTOR-p70S6K in diabetic rats ($P < 0.05$). Moreover, though leucine supplementation in combination with resistance training demonstrated synergistic effects on p70S6K ($P < 0.05$), both treatments were capable of recovering motor performance ($P < 0.05$). In conclusion, 5% leucine supplementation combined with resistance training has the potential to attenuate muscle loss and motor performance decrements in diabetic rats, at least in part through increased protein synthesis.

Introduction

Diabetes mellitus is a chronic inflammatory disease that affects nearly every biological process including an altered protein metabolism, decreased muscle mass, and in some cases, impaired ability to carry out daily activities, decreased productivity and diminished quality of life (Workeneh and Bajaj 2013). While the development of each form of diabetes (type 1 or type 2) drastically differs, resultant pathologies often overlap. In each diabetic condition, a failure to maintain healthy muscle is often

observed, and this is termed diabetic myopathy (D'Souza et al. 2013; Krause et al. 2013).

Attenuating the effects of diabetes can be challenging, but recent studies provide important insights into the cellular and molecular etiology of diabetes-associated decreases in muscle mass, and these findings may be useful in the development of new therapies for the disease (Gumucio and Mendias 2013). In addition, studies have shown that leucine supplementation and resistance training confer an anabolic effect by stimulating the beginning of the protein translation process and the cell signaling

pathway known as mTOR (mammalian target of rapamycin), thereby promoting positive effects on strength gains, muscle mass, protein balance, and preservation of functional capacity (Hurley and Roth 2000; Castaneda *et al.* 2002; Dreyer *et al.* 2006).

Resistance training induces an increase in muscle protein synthesis (MPS) that can last for 48 h postexercise (Phillips *et al.* 1997) and the repetitive strain of regimented weekly sessions promotes a positive protein balance that can lead to substantial accretion of muscle proteins over time. A positive protein balance requires that the rate of MPS exceeds the rate of muscle protein breakdown (MPB) (Burd *et al.* 2009). Hypertrophy of skeletal muscle and the resulting concomitant gain in strength are of great interest for people with disease-induced atrophy. There is evidence that resistance training can stimulate and reinforce cellular and molecular processes that lead to a compensatory hypertrophy response in this population (Haddad and Adams 2002). Moreover, resistance training has been shown to augment insulin signaling and glucose transport in skeletal muscle tissue (Takala *et al.* 1999; Holloszy 2005).

The intake of dietary protein after resistance training increases plasma amino acids, which results in the activation of signaling molecules leading to increased MPS and muscle hypertrophy (Phillips 2014). Although essential amino acids are necessary for hypertrophy, L-leucine has been specifically shown to activate mTOR and increase the initiation of the translation of protein synthesis (Guimarães-ferreira *et al.* 2014). On the other hand, the hypothesized beneficial effects of leucine supplementation for the treatment of diabetes mellitus are related to the improvement of energy balance, net protein balance, and glucose homeostasis (Pedroso *et al.* 2015).

In order to better elucidate the actions of leucine supplementation and resistance training (alone or combined) on muscle remodeling processes, the purpose of this study was to investigate the chronic effects of these interventions on muscle atrophy in an experimental model of diabetes mellitus induced by streptozotocin (STZ).

Materials and methods

Animals

Five-day-old male Wistar Hannover rats were separated from their mothers for 8 h. They were divided into two groups: the diabetic group (D group) received an ip injection of STZ [120 mg/kg body weight (b.w.)] freshly diluted in citrate buffer [10 mmol/L Na citrate (pH 4.5)]; and the nondiabetic group (non-D group) received only the vehicle (ip) in an equivalent volume and served as control [control group (C group)] ($n = 8$). After weaning, rats from the D

group were divided into four groups: D group (D, $n = 8$) received an isonitrogenous control diet; DT group (DT, $n = 8$) received an isonitrogenous control diet and performed resistance training; DL group (DL, $n = 8$) was treated with 5% L-leucine; and DLT group (DLT, $n = 8$) was treated with 5% of L-leucine and resistance training. After weaning, the animals presenting glycemia above 150 mg/dl were included in this study.

The animals were obtained from the Animal House of the Faculty of Pharmaceutical Sciences, University of Sao Paulo and the experiments were performed according to the ethical guidelines adopted by the Brazilian College of Animal Experimentation (protocol 451). The animals were maintained in individual cages under standard conditions of light (12-h light/dark cycle), temperature ($22 \pm 2^\circ\text{C}$) and relative humidity ($55 \pm 10\%$), and with full access to food (American Institute of Nutrition) and water. The body weight, food intake and water intake were registered three times per week from weaning to killing (12-week-old). All animals were adapted to the laboratory environment for 1 week, before engaging in the training program. Animals were euthanized under tiletamine-zolazepam and fentanyl anesthesia at 48 h after the last resistance training session and with a 6-h fast. The extensor digitorum longus muscle (EDL), gastrocnemius (GAS), soleus (SOL) muscles of each limb were isolated, weighed, and frozen at -80°C . The protein concentration in EDL muscle homogenate was determined with a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

Diets

The experimental diets were prepared according to the recommendations of the American Institute of Nutrition (Reeves *et al.* 1993). After weight distribution, rats were fed either the control diet (C group), an isonitrogenous control diet (D and DT group), or a leucine-rich diet (DL and DLT group) for 8 weeks. 5% of L-leucine was added to the intervention diet by replacing equal amounts of corn flour, while the isonitrogenous control diet contained an additional 4% of nonessential amino acids (Table 1). The nonessential amino acids meal was used as the control diet to standardize the nitrogen (Vianna *et al.* 2012). The supplemental dose of 5% L-leucine was used because it has been shown in previous work to positively regulate MPS (Rieu *et al.* 2003). Other research used this leucine dose with no toxic effects reported (Tsubuku *et al.* 2004).

Resistance training protocol

The resistance training protocol was adapted from Hornberger and Farrar (2004), which consisted of climbing a

Table 1. Diet composition¹ (%).

Ingredients	Basal diet	Leucine-rich diet	Isonitrogenous control diet
Cornstarch	62%	57%	58%
Casein	14%	14%	14%
Sucrose	10%	10%	10%
Soybean oil	4%	4%	4%
Cellulose	5%	5%	5%
Mineral mixture	3%	3%	3%
Vitamin mixture	1%	1%	1%
L-cystine	1%	1%	1%
Choline bitartrate	0.2%	0.2%	0.2%
Tert-butyl hydroquinone	0.0008%	0.0008%	0.0008%
Leucine	0%	5%	0%
NEAAs	0%	0%	4%

NEAAs- isonitrogenous mixture of nonessential amino acids (alanine, aspartate, glutamate, glycine, proline, and serine).

¹Based on AIN-93M (Reeves *et al.* 1993).

vertical ladder (1.1 × 0.18 m, 2 cm grid, 80° inclined) with progressive loads secured to the base of the rats tail, as described below. The load apparatus was secured to the tail by wrapping the proximal portion of the tail with a self-adhesive foam strip. A Velcro strap was wrapped around the foam strip and fastened. With the load apparatus secured to the tail, the rats were placed at the bottom of the ladder and familiarized with climbing. The initial climb consisted of carrying a load that was 75% of the animal's body mass. After this, an additional 30 g weight was added until a load was reached with which the rat could not climb the entire length of the ladder. Failure was determined when the animal could not progress up the ladder after three successive stimuli to the tail. The highest load successfully carried the entire length of the ladder was considered the rat's maximal carrying capacity for that training session. Training sessions were 9 ladder climbs with 75%, 90%, and 100% of the rat's previous maximal carrying capacity, determined earlier. During subsequent ladder climbs, an additional 30 g load was added until a new maximal carrying capacity was determined.

Motor performance tests functional assessment

In order to evaluate skeletal muscle function, the animals were allowed to grab onto the Grip Strength System (model: DFE-002, San Diego Instruments, San Diego, Cal, USA) with their forepaws as the experimenter gently pulled on their tails. The result is the maximal force before the animal releases the forepaws of the bar (mean

of three measurements of maximum pull) (Anderson *et al.* 2004; Nalbandian *et al.* 2013).

The second motor performance test was the ambulation test, which was performed to determine the mean length and width (cm) of a step measured in hind foot ink prints while mice freely ran along a corridor (length: 100 cm; width: 10.5 cm; height of lateral walls: 20 cm) three different times. Before the test, the animals were permitted to explore the apparatus (Kennel *et al.* 1996; Zanchi *et al.* 2012). Mean values were individually calculated for each test through the mean of three consecutive tests performed.

Serum glucose and insulin levels

Blood was collected and centrifuged in order to obtain the serum fractions. Serum was stored at -80°C for further analysis. Serum glucose was measured using commercial available kits (Labtest®). Serum insulin levels were measured using commercial RECYTMAG-65k MILLIPLEX MAP rat cytoquine/chemoquine magnetic bead.

Western blot analysis

Initially, frozen EDL muscle in ice-cold lysis buffer: 50 mmol/L phosphate buffer (pH 7.0), 0.3 mol/L sucrose, 0.5 mmol/L dithiothreitol, 1 mmol/L EDTA, 0.3 mmol/L PMSF, 10 mmol/L NaF, 1:100 Phosphatase Inhibitor Cocktail 1–2 (Sigma-Aldrich, St. Louis, MO) and 1:100 Protease Inhibitor Cocktail (Sigma-Aldrich) using a polytron homogenizer. After homogenization and transfer to new prechilled tubes, samples were centrifuged at 13,000g for 25 min at 4°C. Supernatants were collected and total protein concentrations were measured using a BCA protein assay kit (Pierce, Rockford, IL). Lysates were combined with a sample buffer: 240 mmol/L Tris (pH 6.8), 40% glycerol, 0.8% sodium dodecyl sulfate (SDS), 200 mmol/L beta-mercaptoethanol, and 0.02% bromophenol blue. Proteins (25 µg) were subjected to SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Membranes were blocked with 5% bovine serum albumin (BSA) and phosphate buffered saline with tween (PBST) and were incubated overnight at 4°C with rabbit polyclonal antibodies (Cell Signaling Technology, Beverly, MA) diluted in PBST buffer: anti-mTOR diluted 1:1000, anti-pmTOR (Ser2448) diluted 1:1000, anti-S6K1 diluted 1:1000, anti-pS6K1 (Thr389) diluted 1:1000. Membranes were incubated for 1 h with horseradish-peroxidase-labeled antirabbit IgG antibodies (Sigma-Aldrich) diluted 1:1000 in PBST buffer and 1% BSA. Blots were visualized for 5 min in an ImageQuant 400 (GE Healthcare, Amersham, UK) image system with

a solution prepared with ECL-Advance Western blotting Reagent (GE Healthcare). After densitometry, phosphorylated proteins were normalized by total protein content. A horseradish-peroxidase-labeled anti- β -actin (Sigma-Aldrich) diluted 1:40,000 was used in order to verify correct protein loading.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software 6.0 (GraphPad Software, Inc., San Diego, CA). 2-way ANOVA test with repeated measures followed by Tukey's test was used for comparison of weekly evolution of body weight and (time \times group) of functional performance tests, and 1-way ANOVA followed by Tukey's test for food and water intake, muscle weight, glucose and insulin and western blot analysis. The a priori significance level was set at $P < 0.05$. The results are expressed as means \pm SEM.

Results

Body weight

The body weight of nontreated diabetic rats (D group) was reduced in final time course of 8 weeks when compared with nondiabetic rats (C group) ($P < 0.05$). Interestingly, the body weight of groups (DT, DL and DLT) that received treatment was not significantly different when compared with group C ($P > 0.05$) (Fig. 1).

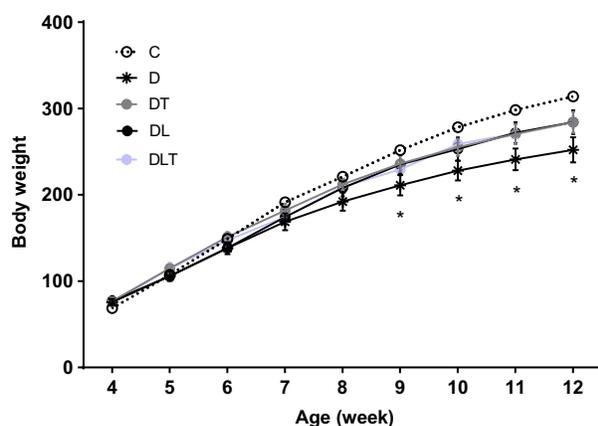


Figure 1. Changes in body weight between eight time-points within each group during the 8 weeks of the experiment ($n = 8$ per group). C: nondiabetic control group; D: diabetic group; DT: resistance training group; DL: leucine supplementation group; DLT: leucine supplementation plus resistance training group. * $P < 0.05$ C group vs. D group.

Food and water intake

In nontreated diabetic rats (D group) food intake was significantly ($P < 0.05$) enhanced as compared with control rats (C group). Both groups (DT, DL and DLT) had lower food intake when compared to diabetic rats (D group). However, only food intake was normalized in diabetic rats with leucine supplementation (DL group) (Table 2). In terms of water intake, the water intake of the D group was significantly ($P < 0.05$) enhanced as compared with C group, DT group, DL group, and DLT group. No statistical differences ($P > 0.05$) were noted in relation to the other groups (Table 2).

Muscle weight

After the 8 week experimental period, the absolute weight of the EDL and GAS muscles were lower in the D group compared to the C group ($P < 0.05$), indicating muscular atrophy in this experimental model. Supplementation with leucine and resistance training did not change the absolute and relative weight of the EDL muscle of diabetic animals. However, there was no statistical difference in the absolute weight of the GAS muscle in the DL, DT, DLT groups compared with C group ($P > 0.05$), suggesting that leucine and/or resistance training may attenuate the muscle mass loss detected in untreated diabetic animals. Moreover, the relative weight of the GAS (absolute muscle weight/100 g of body weight) of the DT and DLT groups was higher compared to the D group, and was higher than the C group, demonstrating the anabolic effect of resistance training ($P < 0.05$). There was no statistical difference in the absolute and relative weights of SOL between the experimental groups ($P > 0.05$), in the absolute and relative weights of SOL between the experimental groups ($P > 0.05$), and between the DL and D groups in the relative weight of the assessed muscles; in other words, chronic supplementation of leucine did not affect this parameter in diabetic rats (Table 2).

Glucose and insulin on serum

The D group had higher fasting glucose compared to other groups, with DT, DL, and DLT showing significant improvements in this outcome ($P < 0.05$). With respect to serum insulin levels, the D group presented impaired fasting insulin compared with nondiabetic rats ($P < 0.05$). Supplementation of leucine and resistance training improved fasting insulin ($P < 0.05$). Although no statistical difference was noted between the diabetic animals, the DT, DL and DLT groups modestly increased insulin compared to the D group. These data are shown in Table 2.

Table 2. Characteristics of the male rats.

Groups	C	D	DT	DL	DLT
EDL (g)	0.137 ± 0.002	0.106 ± 0.007 [§]	0.115 ± 0.002 [§]	0.114 ± 0.003 [§]	0.114 ± 0.002 [§]
EDL (g/100 g)	0.040 ± 0.000	0.039 ± 0.002	0.040 ± 0.001	0.041 ± 0.001	0.043 ± 0.000
GAS (g)	1.591 ± 0.027	1.365 ± 0.072 [§]	1.565 ± 0.54	1.502 ± 0.047	1.502 ± 0.047
GAS (g/100 g)	0.470 ± 0.007	0.467 ± 0.009	0.545 ± 0.007 ^{§#}	0.500 ± 0.007*	0.570 ± 0.024 ^{§#}
SOL (g)	0.116 ± 0.004	0.101 ± 0.005	0.116 ± 0.002	0.112 ± 0.004	0.112 ± 0.007
SOL (g/100 g)	0.035 ± 0.000	0.085 ± 0.046	0.0417 ± 0.002	0.037 ± 0.000	0.040 ± 0.000
Glucose (mg/dl)	85.89 ± 7.29	310.8 ± 57.38 [§]	175.5 ± 10.18 [#]	181.8 ± 21.11 [#]	176.5 ± 27.78 [#]
Insulin (pg/ml)	3022 ± 406	1362 ± 282 [§]	2183 ± 576	1622 ± 399	1911 ± 320
Food Intake (g/24 h)	15.1 ± 5.27	42.8 ± 5.91 [§]	30.1 ± 2.73 ^{§#}	17.9 ± 6.04 [#]	22.6 ± 2.27 ^{§#}
Water Intake (ml/24 h)	33.2 ± 3.28	85.14 ± 27.54 [§]	41.65 ± 5.11 [#]	26.21 ± 1.96 [#]	27.62 ± 1.98 [#]
EDL Protein (mg/g body weight)	0.0910 ± 0.002	0.0639 ± 0.006 [§]	0.0967 ± 0.003 [#]	0.0773 ± 0.000	0.1041 ± 0.011 [#]

Muscle weights in g and g/100 g. Glucose and insulin represents at the last week post euthanasia measured in 6-h-fasted. Food and water intake for the last week of experiment. C: nondiabetic control group; D: diabetic group; DT: resistance training group; DL: leucine supplementation group; DLT: leucine supplementation plus resistance training group ($n = 8$ per group).

[§]Vs. C Group ($P < 0.05$).

[#]Vs. D Group ($P < 0.05$).

*Vs. DLT Group ($P < 0.05$).

Total protein in EDL muscle concentration

Muscle protein content is reported as mg/g of body weight (Adams and Haddad 1996). The muscle protein content in C, DT, and DLT groups were significantly greater as compared with D ($P < 0.05$), but no difference was observed between DT and DLT.

Functional performance tests

To evaluate the effects of training and leucine supplementation on muscle performance, grip strength and ambulation tests were administered. Results for these data are presented in Figure 2. Resistance training (alone or combined) showed statistically greater functional improvements when compared to the D group (grip strength – Fig. 2A, $P < 0.05$ and footprint length and width – Fig. 2B and C, respectively, $P < 0.05$). These results suggest that leucine supplementation alone was able to significantly mitigate the loss of strength in diabetic rats, but resistance training was superior for promoting gains in muscle strength in comparison with leucine-supplemented animals, as shown in Figure 2A ($P < 0.05$). With respect to the 1-repetition maximum (1RM) on the amparate (ladder), the DL group increased strength by 12% increase while the DLT and DT groups showed increases of 102% and 101%, respectively, compared to the D group (data not shown). In the ambulation test, DLT was the only group in which there was attenuation of the motor function worsening in terms of length between its steps when compared to group D. The DT and DLT groups had greater width between their

steps when compared to the group D, and this parameter was superior in DLT compared with DL ($P < 0.05$). Once again, these results indicate that resistance training was fundamental for improvement of physical and motor function in diabetic animals.

Protein expression with relation to muscle protein synthesis

To elucidate potential mechanisms responsible for the anabolic effect of leucine and resistance training on STZ-induced muscle atrophy, we measured mTOR and p70S6K protein levels in EDL muscles. In nontreated diabetic rats phospho^{Ser2448}/total mTOR ratio and phospho^{Thr389}/total p70S6K ratio protein was reduced when compared to the control group (C group) and the DT and DLT groups ($P < 0.05$). Therefore, resistance training enhanced recovery of mTOR and p70S6K proteins ($P < 0.05$), but leucine alone did not affect these parameters (Fig. 3A and B).

Discussion

This study investigated the effects of chronic supplementation with leucine and resistance training on diabetic myopathy in experimental diabetes mellitus induced by streptozotocin. Our results showed that resistance training alone or combined with leucine minimized the deleterious effects of diabetes mellitus on skeletal muscle as determined by an enhanced physical functional capacity muscle as well as improvements in certain metabolic parameters such as fasting glucose and insulin. These

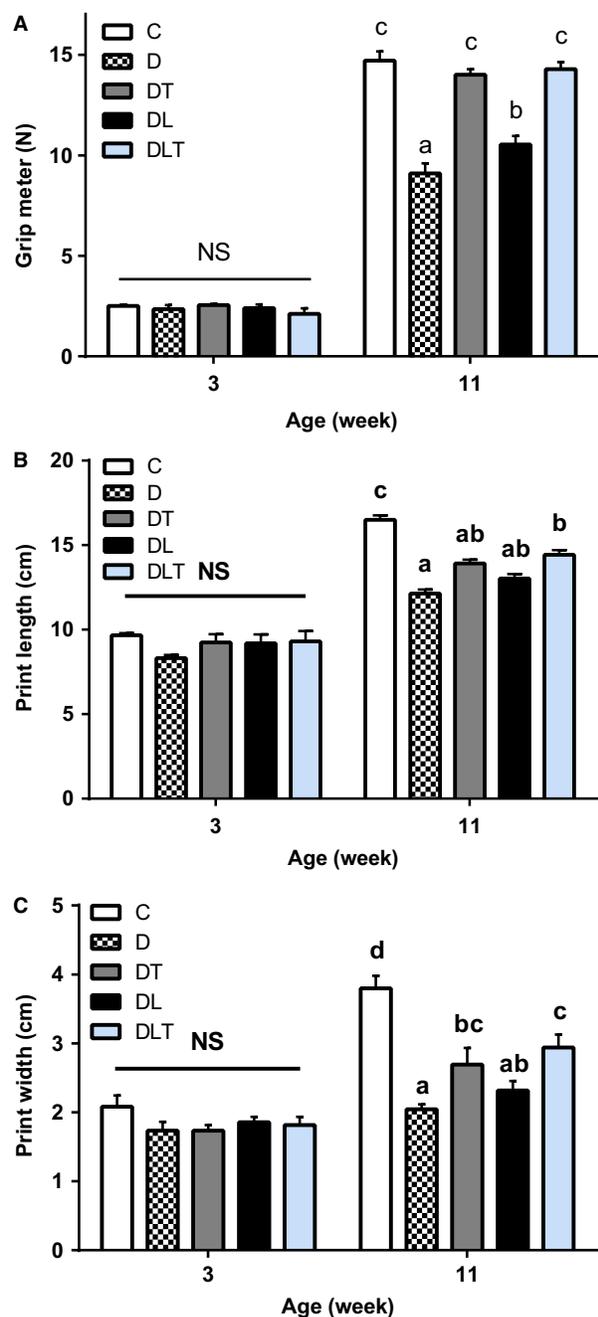


Figure 2. Functional performance tests. (A) grip meter (N); (B) ambulation (length in cm); (C) ambulation (width in cm). C: nondiabetic control group; D: diabetic group; DT: resistance training group; DL: leucine supplementation group; DLT: leucine supplementation plus resistance training group. $N = 8$ per group. Means with different letters are significantly different ($P < 0.05$).

results appear to be mediated by an increased activation of the molecular signaling pathways involved in MPS.

In diabetes mellitus, muscle atrophy associated with diabetic myopathy may be the result of reduced MPS

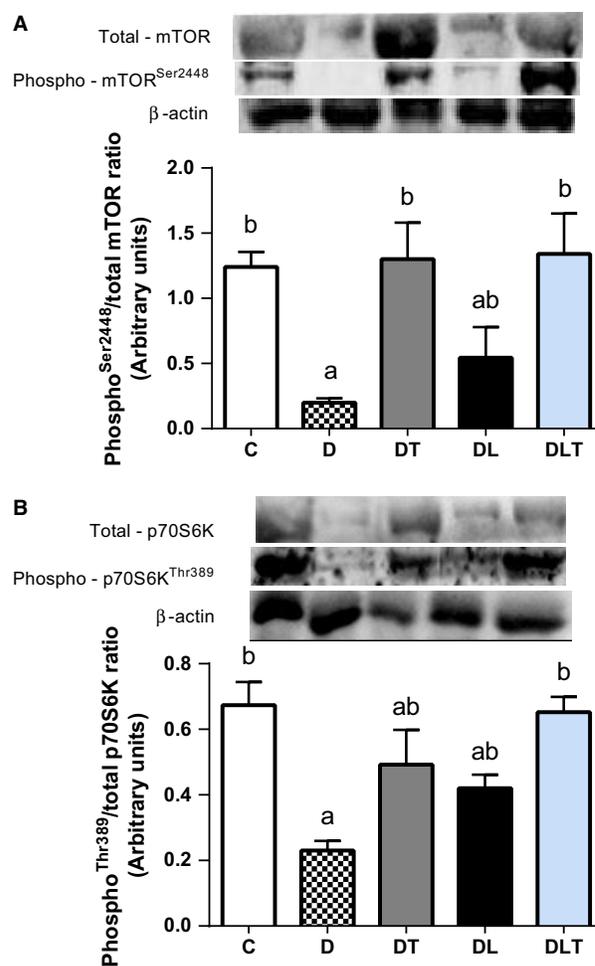


Figure 3. Effects of leucine supplementation and resistance training on protein expression with relation to muscle protein synthesis. (A) Representative blots show the effect of leucine and resistance training on the EDL muscle phospho/total ratio of mTOR, (B) phospho/total ratio of p70S6K. C: nondiabetic control group; D: diabetic group; DT: resistance training group; DL: leucine supplementation group; DLT: leucine supplementation plus resistance training group. $N = 8$ per group. Means with different letters are significantly different ($P < 0.05$).

and/ or exacerbated increase in muscle protein breakdown. It is known that skeletal muscle is approximately 40% of the total body mass (Frontera and Ochala 2015), thus, any degenerative disorder leading to muscle atrophy is associated with a decrease in quality of life along with an increased risk of morbidity and mortality (Goodman et al. 2011; Srikanthan and Karlamangla 2014).

The results of this study show that combined resistance training with leucine supplementation recovered the activity of the mTOR-p70S6K pathway in diabetic animals, which may reflect greater MPS. This suggests that the anabolic stimulation of resistance training is the predominant

factor in the activation of this pathway in diabetic animals, and leucine may enhance this effect as a substrate for muscle protein translation. Evidence shows that leucine directly activates mTOR after a resistance training bout. Pasiakos *et al.* (2011) demonstrated that leucine supplementation after exercise increased MPS by 33% in conjunction with heightened intracellular signaling. Similarly, Dreyer *et al.* (2008) found that a leucine-enriched supplement provided after resistance training increased both mTOR signaling and mixed muscle protein synthesis.

Resistance training induces muscle hypertrophy via a sequential cascade consisting of: (1) activation of muscle fibers; (2) signaling pathway activation resulting from the mechanical deformation of the muscle fibers; (3) release of hormones and myokines involved in the immune/inflammatory response; (4) an increased MPS from the activation of protein transcription and translation, (5) an increase in the cross-sectional area of muscle fibers (Baar and Esser 1999; Kubica *et al.* 2005; Spiering *et al.* 2008; Ogasawara *et al.* 2013). The load-induced activation of mTOR has been shown to be a critical component in this hypertrophic cascade (Burd *et al.* 2010; Goodman *et al.* 2011). Thus, the influence of resistance training on the activation of the mTOR-p70S6K pathway would appear to explain functional improvements in diabetic animals that performed resistance training.

In our study, supplementation of leucine (without mechanical stimulation of resistance training) did not significantly affect the mTOR-p70S6K pathway. It is possible that the duration of treatment or the amount of leucine provided in the diet was lower than necessary to overcome muscle anabolic resistance in diabetes. Leucine stands out among the essential amino acids for its ability to stimulate protein translation and subsequently increase the MPS rate and muscle growth (Norton and Layman 2006; Kelleher *et al.* 2013). However, the dosages of leucine must be sufficiently high to promote optimal anabolic effects and thus mediate the synthesis of myofibrillar proteins, which are impaired in DM (Manders *et al.* 2012). In diabetes, ketoacidosis increases the branched chain amino acid oxidation rate by the branched-chain α -ketoacid dehydrogenase enzyme, particularly leucine, and the inflammatory state increases protein degradation rates (Workeneh and Bajaj 2013). This phenomenon may have hindered the effect of leucine on the activation of the mTOR-p70S6K in diabetic animals.

The activation of mTOR-p70S6K anabolic pathway through resistance training combined with a diet supplemented with leucine was crucial to attenuate the skeletal muscle atrophy in diabetic rats. Our findings showed no statistical difference in the absolute weight of the gastrocnemius muscle of the DL, DT and DLT groups compared with the C group, suggesting that leucine and resistance

training can alleviate the muscle mass loss detected in untreated diabetic animals. The relative weight of the gastrocnemius in the groups that underwent strength training (DT and DLT) increased approximately 16% and 22%, respectively, compared to the diabetic rats without treatment (D group), which highlights the anabolic effect of resistance training on the increase in muscle mass. Although results were not statistically significant, the relative weight of the gastrocnemius muscle in the group that only received leucine supplementation increased by 7% compared with the D group. These data suggest that in diabetic animals, the anabolic effects of leucine depend on the mechanical stimulus of resistance training, unlike what occurs in healthy subjects (Zanchi *et al.* 2012).

Supplementation with leucine and resistance training (alone or combined) did not alter the absolute and relative weights of the EDL muscle and body mass. It is conceivable that the null findings may be attributed to the short study period (8 weeks); a small muscle such as the EDL may require a longer intervention time for significant muscle hypertrophy to be observed. On the other hand, protein concentrations of EDL in C, DT and DLT rats were significantly increased compared with D group. These changes may further suggest that resistance training promotes protein accretion in EDL muscle, without affecting variations in skeletal muscle.

With respect to functional and motor elements, resistance training alone or combined with leucine supplementation recovered muscle strength and significantly improved ambulation capacity (length and width between steps) in the diabetic animals. Our study showed that grip strength was greater in DT and DLT groups compared with the D group, with no statistical difference between the trained groups and the C group, that is, the resistance training increased muscle strength in diabetic rats, even in absence of leucine. Similar findings were reported in studies of Call *et al.* (2010) and Chen *et al.* (2014). On the other hand, Kim *et al.* (2015) found that resistance training did not improve grip strength in diabetic Zucker rats. The reasons for the discrepancies between studies are not clear.

Interestingly, we observed that supplementation with leucine alone was able to attenuate the loss of muscle strength of diabetic animals. However, the change in grip strength of the DT group was greater than that of the DL group, demonstrating that resistance training is the primary stimulus for promoting improvements in this parameter. Contrary to our findings, Zanchi *et al.* (2012) showed that a diet supplemented with leucine impaired motor performance in mice with muscular atrophy induced by dexamethasone.

Supplementation of leucine and resistance training improved glycemia in diabetic animals. In our study, the

treated diabetic animals (DT, DL, and DLT) showed lower fasting and postprandial values compared to the D group, with no statistical difference with the C group. The findings demonstrate that these interventions were able to normalize blood glucose levels in our experimental diabetes model. Interestingly, supplementation of leucine alone induced this effect on blood glucose levels in diabetic animals, similar to results found by Wilkinson *et al.* (2013) in humans with type 2 diabetes. Moreover, when administered in high doses, leucine supplementation may impair insulin signaling and the uptake of glucose in obese rats fed with a high-fat diet, as shown by Ham *et al.* (2014). Due to the controversies over the role of leucine in glucose homeostasis, more studies involving this approach are needed to improve our understanding of the topic.

In this study, untreated diabetic animals displayed moderate hypoinsulinemia that is characteristic of diabetes in the experimental model used. Chronic supplementation of leucine and resistance training did not reverse hypoinsulinemia in diabetic animals, but it was able to improve the concentration of serum insulin in these animals. Some studies have shown that the resistance training is able to increase insulin production and secretion in isolated pancreatic islets of rats with uncompensated type 1 diabetes (Oliveira *et al.* 2010; Huang *et al.* 2011). Moreover, resistance training increases insulin sensitivity in peripheral tissues such as skeletal muscle and glucose uptake, with these effects persisting for up to 24–48 h after an exercise session (Holten *et al.* 2004; Cartee 2015).

Interestingly, we observed that supplementation of leucine alone was able to improve the serum insulin in diabetic animals. This may be explained by the effects of leucine as an insulin secretagogue, which occurs through two potential mechanisms: leucine undergoes deamination to then produce alpha-ketoisocaproate (Gao *et al.* 2003) or it allosterically activates the enzyme glutamate dehydrogenase (Smith and Stanley 2008). Both mechanisms increase the intracellular ATP/ADP ratio and cause the release of insulin (rapid phase secretion of this hormone). This improvement in the concentration of serum insulin in diabetic animals after chronic supplementation of leucine may also have contributed to these animals presenting lower blood sugar levels.

In addition, leucine supplementation as well as resistance training was able to reduce clinical symptoms such as polyphagia and polydipsia present in diabetes. Studies in animals and humans suggest that leucine acts as a nutrient indicator to reduce calorie intake, either by activating mTOR, by increasing concentrations of cholecystokinin, or moderately decreasing antral pressures (Cota

et al. 2006; Blouet and Schwartz 2012; Steinert *et al.* 2015). Strength training in humans can reduce the intake of fat and calories after 8 months of training (Bales *et al.* 2012).

This study provides important information for the development of nonpharmacological strategies for the treatment of diabetes mellitus, particularly in diabetic individuals that depend on exogenous insulin. Note that exogenous insulin alone is not sufficient to stimulate MPS in the postprandial state, which requires a greater intake of protein through a diet supplemented with essential amino acids rich in leucine. In combination with regimented resistance training, this strategy helps to maintain and build strong muscles and bones.

Conclusions

It can be concluded that chronic leucine supplementation and resistance training are effective in improving muscle strength and normalizing fasting plasma glucose and the postprandial phase in diabetic animals. However, resistance training was responsible for major functional adaptations in skeletal muscle of diabetic rats, promoting greater increases in strength and muscle mass compared to leucine supplementation alone. However, the combination of both resistance training and leucine appear to have synergistic effects on MPS, conceivably by regulating the mTOR-p70S6K pathway.

Conflicts of Interest

All authors declare no conflict of interest.

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