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ORIGINAL ARTICLE

A leucine-rich diet and exercise affect the biomechanical characteristics of the digital flexor tendon in rats after nutritional recovery

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Abstract An increase in the capacity of athletic performance depends on adequate nutrition, which ensures optimal function of the musculoskeletal system, including tendon stability. However, little is known about the status of tendons and extracellular matrix modifications during malnutrition and nutritional recovery when leucine is used in response to exercise conditioning. The purpose of this study was to evaluate the collagen content and biomechanical aspects of the deep digital flexor tendon (DDFT) in malnourished rats submitted to nutritional recovery (control diet or leucine-rich diet) and aerobic physical activity. After 60 days of undernourishment (6% protein diet), the malnourished rats were subsequently nutritionally recovered with a control diet or leucine-rich diet and trained or not (swimming, without overload) for 5 weeks. The biomechanical analysis and quantification of hydroxyproline were assessed in the DDFT in all experimental groups. The leucine-rich diet increased hydroxyproline content in the tension region, independently of the training. In the compression region, hydroxyproline content was higher in the malnourished and leucine-trained groups. Biomechanical analysis showed a lower load in the malnourished and all-trained groups. The lowest stress was observed with control-trained animals. The nutritional-recovered groups showed higher strain values corresponding to control group, while the lowest values were observed in malnourished and trained groups. The results suggest that a leucine-rich diet stimulates collagen synthesis of the DDFT, especially when in combination with physical exercise, and seems to determine the increase of resistance and the biomechanical characteristics of tendons.

Keywords Leucine · Aerobic exercise · Extracellular matrix · Tendon · Nutrition

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Introduction

The musculoskeletal system directly depends on the adequate function of tendons and, consequently, on the microstructural integrity of their components (O'Brien 1997; Zhang et al. 2005; Brabaj et al. 2005), particularly during locomotion and for sustaining body weight (Benevides et al. 2004). The mechanical strength of tendons depends on the collagen fibrils, their conformation, genesis and intermolecular interactions (Canty and Kadler 2002). In tendons, fibroblast metabolism synthesizes and maintains various extracellular matrix (ECM) elements, including proteoglycans and collagen itself, which can be influenced by mechanical deformation (MacKenna et al. 2000; Kjær et al. 2009; Fessel and Snedeker 2010) and nutritional state (Oxlund and Andreassen 1992; Lehnert et al. 2006; Smith and Rennie 2007). Focusing the tensile characteristics and efficacy of the muscle–tendon complex, the mechanical behaviour of the tendon determines the efficiency of this complex, and this property depends on the ECM composition and structure (Magnusson et al. 2003). The main tendon components include collagen, which contains 9% proline and is hydroxylated during collagen synthesis, thereby forming hydroxyproline. Therefore, hydroxyproline content can infer collagen synthesis and is directly related to the load that the tendon can support after strain. The

predominant proteoglycan in tensional regions of the tendon is the small proteoglycan decorin, which is related to water content and flexibility and is inversely proportional to hydroxyproline content. Other proteoglycans, aggrecan and biglycan, develop at points where tendons bind under bone and are subjected to further compressive loading. These proteoglycans, along with decorin, are closely related members of the leucine-rich repeat proteoglycan family (Zhang et al. 2005; Kilts et al. 2009). Tendons become more resistant when exercised regularly, with fibre diameter gradually increasing (Enwemeka et al. 1992; Yoon et al. 2003; Fessel and Snedeker 2010; Izu et al. 2010). On the other hand, an inactive state leads to reduced tendon metabolic activity contributing to a slight reorganization of tendon ECM (Benjamin and McGonagle 2009; Banos et al. 2008). Additionally, the swimming exercise protocol can affect collagen metabolism, thereby increasing the tensile strength of rat calcaneal tendons (Magnusson et al. 2003; Buchanan and Marsh 2002; Simonsen et al. 1995). The functional stability of tendons is achieved through a balance between tissue repair and remodelling, even following damage caused by microtraumas (Buchanan and Marsh 2002; Kjaer 2004). In the twenty-first century, many studies are focused on the capacity of athletic performance, especially on how the skeletal-muscle system can achieve optimal performance during certain exercises, thereby producing the best response between muscle stretch and force. With this in mind, well-conducted exercise and proper nutritional intake could protect the musculoskeletal system from injuries. In addition, reduced protein synthesis activity, in situations such as protein malnutrition, could lead to homeostasis impairment (Ventrucci et al. 2004), thus damaging the muscular system, especially tendon tissue. Because malnutrition still remains prevalent in many countries, identifying the molecular improvements following nutritional recovery is extremely valuable, especially when they are associated with exercise. Knowing that a leucine-rich diet stimulates cell activity and increases protein synthesis and lean body mass (Ventrucci et al. 2004), the main objective of the present work was to investigate how a leucine-rich diet in combination with aerobic exercise could improve tendon physical strength during nutritional recovery following a malnourished state.

Materials and methods

Animals

Fifty-six male Wistar rats (*Rattus norvegicus*) that were 21-day-old were maintained in collective cages at 22°C ± 2 under 12-h light/dark cycles with free access to water and semi-purified diets for 11 weeks of the experiment. The Institutional Ethics Committee of the State University of Campinas approved the experimental procedures as protocol number 465-1.

Semi-purified diets

Diets, consisting of a control diet (18% of protein; C), a low-protein diet (6% of protein; M) and a leucine-rich diet (15% of protein plus 3% of L-leucine; L), were prepared according to the recommendations of the American Institute of Nutrition (Table 1) (Ventrucci et al. 2004; Reeves et al. 1993).

Swimming exercise

Rats were submitted to swim training during the 5-week experiment, beginning after the sixth week of the experiment. Animals with no body weight overload were gradually adapted to swim in a 1 m^3 container at 24°C ± 2. Training was performed 5 days per week in the morning, starting with 15 min and progressively increasing 5 min per day until reaching 1 h of exercise per day.

Experimental protocol

Animals were distributed at random into seven experimental groups (8 animals per group) according to nutritional status and swimming exercise (Fig. 1) using the following annotation: C, control diet alone for 11 weeks (sedentary); CT, control diet (11 weeks) with 5 weeks of swim exercise; M, malnourishment alone for 6 weeks; MRC, malnourishment (6 weeks) + 5 weeks control diet (sedentary); MRT, malnourishment (6 weeks) + 5 weeks control diet and swim

Table 1 Composition of the diets (%)

Components	Diet C	Diet L	Diet M
Carbohydrate	39.75 cornstarch	39.75 cornstarch	44.25 ornstarch
	14.2 dextrin	14.2 dextrin	17.8 dextrin
	11 sugar	11 sugar	14.9 sugar
Fat (soy oil)	7	7	7
Fibers[a]	5	5	5
Vitamin mix	1	1	1
Salt mix	3.5	3.5	3.5
Protein (Casein)	18	15	6
Leucine	–	3	–
Cistine	0.3	0.3	0.3
Choline	0.25	0.25	0.25

Diet C control, *diet L* leucine-rich, *diet M* low-protein diet

[a] Cellulose microfiber

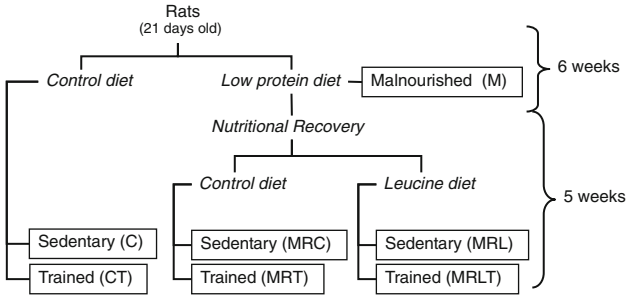

Fig. 1 Experimental groups that were submitted to different diets and training protocols. Young rats (21 days old) were distributed into control and malnourished groups. These rats were treated with a low protein diet for 6 weeks (group *M*) and then redistributed and treated with nutritional recovery with either a control or leucine diet for an additional 5 weeks. Next, the trained animals were submitted to a swimming protocol during 5 weeks, as described in the "Materials and methods". Number of animals per group = 8. Groups: *C* control, *CT* trained, *M* malnourished, *MRC* malnourished and re-fed on control diet, *MRT* malnourished, re-fed on control diet and trained, *MRL* malnourished and re-fed on leucine-rich diet, *MRLT* malnourished, re-fed on leucine-rich diet and trained

exercise; MRL, malnourishment (6 weeks) + 5 weeks leucine-rich diet alone (sedentary); and MRLT, malnourishment (6 weeks) + 5 weeks leucine-enriched diet and swim exercise. Malnourished animals (M group) were sacrificed after 6 weeks of the experiment, and the other rats were killed after 11 weeks of the experimental program. The animals were anaesthetised with sodium pentobarbital (40 mg/kg, iv) and killed by intravenous injection of 1 mL 4 M KCl. The deep digital flexor tendon (DDFT) and the phalanges were removed for the mechanical tensile strain analysis. The DDFT was divided into two regions: the proximal region (p), which is primarily subjected to tensile forces, and the distal region (d), which is subjected to compressive and tensile forces. These two regions were subjected to hydroxyproline quantification after hydrolysis in 6 N HCl (1 mL/10 mg tissue) for 4 h at 130°C. Then, the hydrolysate was treated with 1.41% chloramine T solution and 15% *p*-dimethylaminobenzaldehyde, following incubation for 15 min at 60°C. The hydroxyproline solution was cooled, and the absorbance was measured at 550 nm in a spectrophotometer (Fusion, Packard/Perking Elmer) (Stegemann and Stalder 1967).

Mechanical tensile strain test

Six tendons from each group of animals were used for the mechanical tensile strain test. Prior to the test, the initial length (L_i) and the cross-sectional area (mm^2) of each tendon were measured with a micrometer. The specimens were fixed to the mechanical plate with clutches that fixed the myotendinous junction at one end and the phalanges at the other end. The biomechanical test was assessed by the MTS Teststar II testing machine, available at the Laboratory of Mechanical Properties, Department of Material Engineering, Faculty of Mechanical Engineering, UNICAMP. Each tendon was submitted to a gradual load increase with a constant displacement velocity of 20 mm/min, using a load cell of 1 kN. The stress–strain curve ($\Delta L = L_f - L_i/L_i$, where L_f is the length in tendon failure and L_i corresponds to the initial tendon length) was obtained for each tendon, and the maximum load to tendon failure and failure strength (where T = maximum load/cross-sectional area) was calculated for each tendon sample. The tests were carried out at room temperature using physiological saline to prevent dehydration of the tendons (Benevides et al. 2004).

Statistical analysis

Results were expressed as the mean ± SEM. Data were analysed statistically by two-way ANOVA, followed by Dun's test in order to test the effects of diet and exercise on body weight and biomechanical measures of the tendons. Comparisons between control and exercised groups were performed using one-way ANOVA followed by post-hoc Bonferroni's multiple comparison test (Graph Pad Prism software, v3.00 for Windows 98, USA). Results were considered to be statistically significant when the P value was less than 5% (Gad and Weil 1994).

Results

Body weight evolution of all animals during the experiment was measured twice weekly and is shown in Fig. 2. Note that the control groups (C and CT; Fig. 2a, b, respectively) presented the same body weight gain, even when submitted to swim training. During the 6 weeks of malnutrition, the animals had severe reduction of body weight gain when compared with control groups. Nutritional recovery induced a higher improvement of body weight gain in sedentary groups (MRC and MRL, Fig. 2a) than in trained animals (MRT and MRLT, Fig. 2b). The malnutrition state produced a significant reduction in tendon diameter (cross-sectional area: $M = 1.00 \pm 0.06$ vs. $C = 1.09 \pm 0.01$ mm^2, after 6 weeks of experiment, or $C = 1.26 \pm 0.03$ mm^2, after 11 weeks of experiment), while nutritional recovery increased this parameter (MRC = 1.90 ± 0.05, MRL = 1.80 ± 0.03 mm^2, $P < 0.05$). Exercise, especially when associated with nutritional recovery, increased the diameter of the tendon fibres as compared with the control group (CT = 1.49 ± 0.02, MRT = 1.82 ± 0.09, MRLT = 1.61 ± 0.06 mm^2, $P < 0.05$). The analysis of hydroxyproline content in the tension region of the DDFT showed a higher concentration in leucine-treated

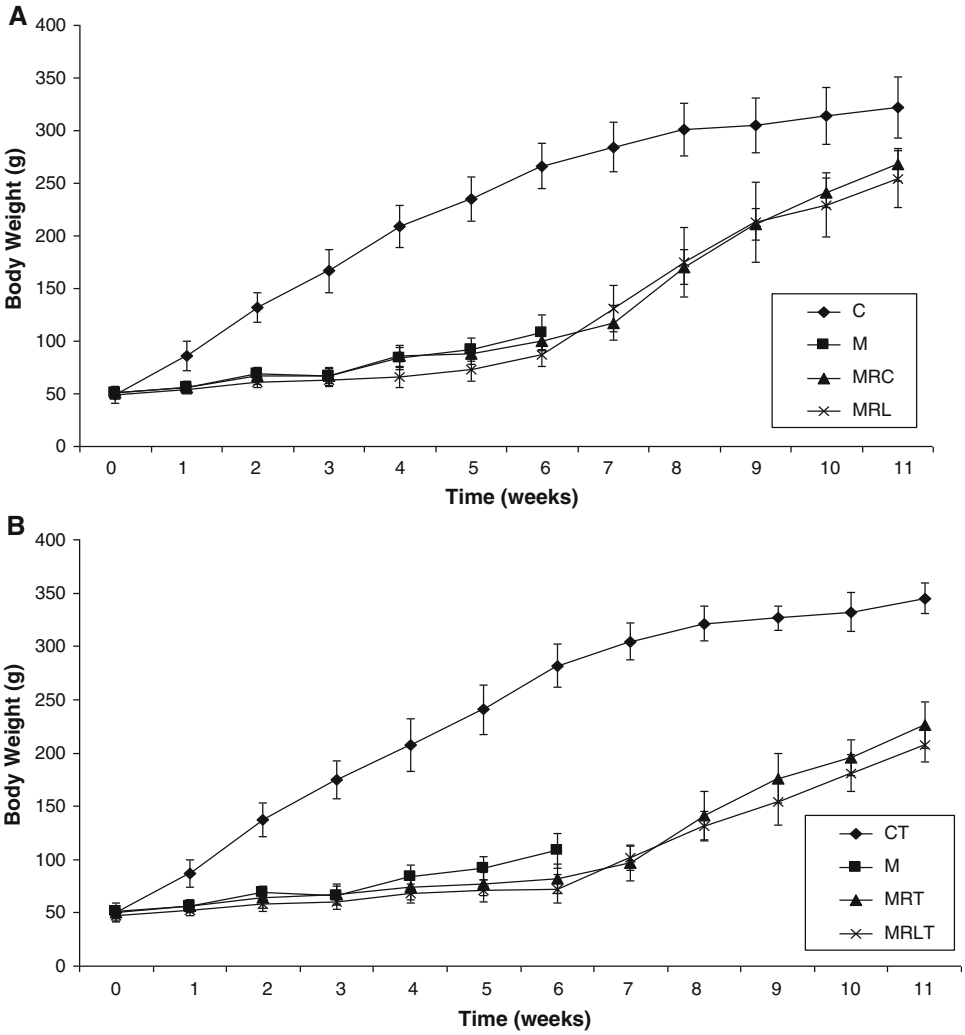

Fig. 2 Body weight gain of animals not submitted to physical training (**a**) and animals submitted to swim-exercise (**b**). Body weight was measured twice weekly. *C* control group, *M* malnourished group, *MRC* malnourished group refed on the control diet, *MRL* malnourished group refed on the leucine-rich diet, *CT* trained control group, *MRT* malnourished group refed on the control diet and trained, *MRLT* malnourished group refed on the leucine-rich diet and trained. Results are expressed as mean ± standard error (SE) for the eight animals per group

groups (MRL and MRLT; Fig. 3a) when compared to sedentary and trained groups. On the other hand, the compression region of the DDFT (Fig. 3b) showed significantly higher hydroxyproline content in groups M and all nutritional recovery animals as compared to group C, except in the MRC group. The tendon biomechanical properties (Fig. 4) indicate how the tendon tissue can support a change in load before being damaged. The CT group presented lower values for all biomechanical parameters (maximal load, displacement, stress and strain) when compared to group C, suggesting a low resistance of this tendon in spite of being trained (the stress–strain curve area, representing the fragility of the tendon tissue, was 364.8 ± 27.8 MJ in CT group vs. 982.4 ± 38.5 in C group). Malnutrition seems to impair the tendon tissue similarly to the CT group (reduced maximal load, displacement and strain, Fig. 4a, b, d, respectively), although the DDFT stress and hydroxyproline content of the compression region were higher in this group (M), which could be associated with a slight improvement in the fragility of the tendon ($M = 422.7 \pm 25.7$ vs. CT = 364.8 ± 27.8 MJ). In nutritionally recovered groups (MRC and MRL), the maximal load, displacement and strain in the DDFT were similar to group C (Fig. 4a, b, d, respectively). Although groups MRC and MRL were initially fed a low-protein diet, following nutritional recovery, the biomechanical parameters of their tendons were significantly different when compared to group M. The tendon resistance was decreased in the MRC group when compared to the control group (MRC = 552.4 ± 20.5; MRL = 756.2 ± 25.9 MJ vs. $C = 982.4 \pm 38.5$ MJ). The trained nutritionally recovered groups (MRT and MRLT; Fig. 4) presented biomechanical properties of their tendons that were similar to group CT. The MRT group had a slight increase in the DDFT displacement, stress and strain values (Fig. 4a, b, d, respectively). Despite having tendon fragility (represented by the stress–strain curve area), the MRT and MRLT groups had more tendon resistance than the CT group (MRT = 501.2 ± 28.3; MRLT = 528.5 ± 31.7 and CT = 354.8 ± 27.8 MJ).

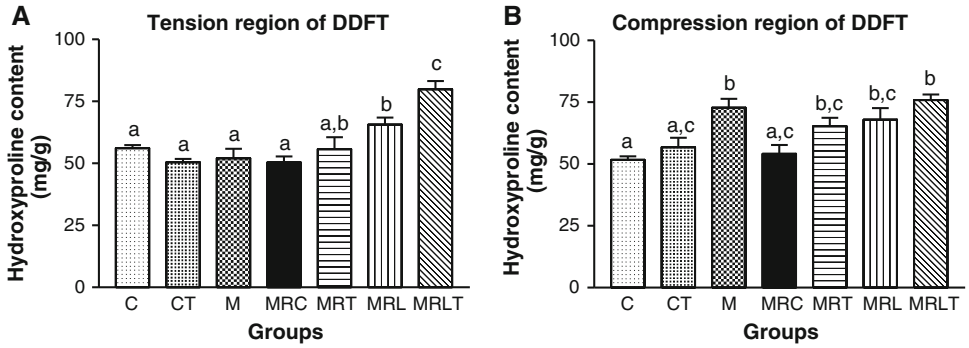

Fig. 3 Hydroxyproline quantification in tension (**a**) and compression region (**b**) of the digital distal flexor tendon in trained rats submitted to malnutrition following nutritional recovery. $N = 6$ animals per group. For a detailed definition of abbreviations, see Fig. 1. Data are expressed as mean ± SE. Different *superscript letters* mean a statistical difference of $P < 0.05$ among the groups, using two-way ANOVA followed by Dun's test

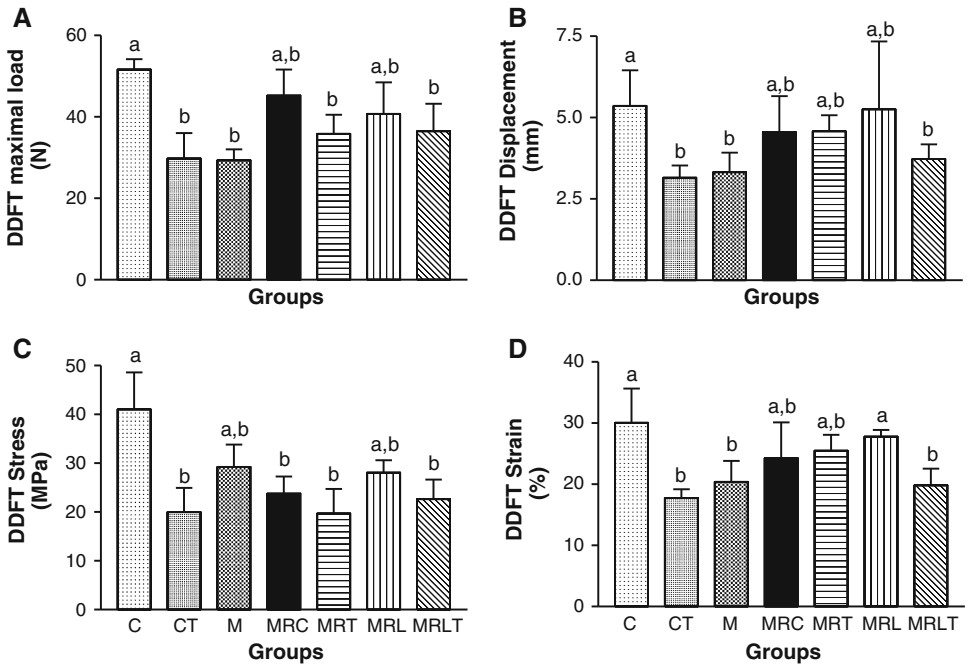

Fig. 4 Mechanical properties of the deep digital flexor tendon (*DDFT*) for the different experimental groups submitted to malnourished and nutritional recovery after the training protocol. Biomechanical test for maximal load (**a**), displacement (**b**), stress (**c**) and strain (**d**) of tendon tissue. $N = 6$ animals per group. Data are expressed as mean ± SE. Different *superscript letters* mean statistical differences among the groups ($P < 0.05$) using two-way ANOVA followed by Dun's test

Discussion

The present work shows that the nutritional recovery process, especially through a leucine-rich diet associated with exercise, improved the tendon biomechanical characteristics imposed by malnutrition. As the musculoskeletal system depends on the adequate function of tendons (Zhang et al. 2005; Brabaj et al. 2005; Benevides et al. 2004), the tendon components and integrity depend on the synthesis of collagen fibrils and their conformation (Zhang et al. 2005; Kjær et al. 2009), which is directly related to protein synthesis and is associated with the metabolism of fibroblasts. The fibroblasts maintain the ECM, including collagen, which can be influenced by mechanical deformation (MacKenna et al. 2000; Kjær et al. 2009; Rennie 2007) and nutritional state (Oxlund and Andreassen 1992; Lehnert et al. 2006; Smith and Rennie 2007; Nicastro et al. 2010). Many countries still suffer from malnutrition. Understanding nutritional recovery, which depends on the quality of the supplemental food and represents a valuable tool in order to treat host tissues after a protein deficiency state, is critical (Balage and Dardevet 2010. The present study showed that malnutrition induced less tendon support, indicating a higher fragility in spite of having higher hydroxyproline content. In parallel, nutritional recovery improved tendon damages induced by malnutrition, especially when combined with a leucine-rich diet and exercise. Malnutrition imposed the least expressive result in the

biomechanical test of the deep digital flexor tendon, withstanding a low load and strain and high stress, suggesting low tendon quality. However, empirically, we observed no impairment of the motor activity of these animals during the experiment, a finding that is possibly related to reduced weight and a lower load on the hind limbs. Thus, the tendon region subjected to compression—typically a fibrocartilage, which contains less collagen than the tensile regions (69 vs. 82% dry weight) (Magnusson et al. 2003; Banos et al. 2008; Rees and Dent 2009)—presented an estimated increase in collagen synthesis in malnourished rats. This increase was probably due to a better withstanding of the tensile strength, as opposed to the compressive force (O'Brien 1997; Kjær et al. 2009). Additionally, the groups submitted to malnutrition and an exercise protocol (M and CT, respectively) had similar results with regard to tendon tension hydroxyproline content and biomechanical properties, except increased tendon stress in group M. During exercise, the organism attempts to repair structures damaged by mechanical requirements, which were lesser in group M due to the reduced weight of the animals from protein malnutrition and sedentary conditions. These results indicate that both protein malnutrition and exercise result in tissue metabolic adaptations in order to maintain active movement necessary for searching for food and homeostasis restoration. Physical exercise influences tendon microstructure and function in the organism, causing collagen fibril hypertrophy and improving tissue quality (Buchanan and Marsh 2002; Bailey 2001). Functional adaptations in tendon structure primarily depend on the imposed mechanical requirements and are not solely restricted to the sites where mechanical stress occurs (Magnusson et al. 2003; Enwemeka et al. 1992; Yoon et al. 2003). Important stabilizing components of collagen bundles that also contribute to tensile strength are crosslinks, formed by the enzyme lysyl-oxidase (Canty and Kadler 2002; Reiser 1994), which keeps the collagen molecules together and guarantees fibril integrity. Evidence indicates that food restriction may significantly affect enzymatic cross-links, nonenzymatic glycosylation and the cross-sectional area of tendon fibrils (Enwemeka et al. 1992; Bailey 2001). These findings suggest that withstanding load and stress generated by the tissue during a biomechanical test not only depends on the total amount of fibres, but also on the interactions between collagen fibrils. This fact may have occurred in the trained groups as indicated by their low performance in the tensile strain test and the high fragility of the tendon, probably due to the occurrence of tendon microtraumas during cyclic exercise (Buchanan and Marsh 2002; Simonsen et al. 1995). Diets rich in leucine play a determining role in stimulating protein synthesis, especially in animals subjected to nutritional recovery (Ventrucci et al. 2004; Layman 2003; Bianchi et al. 2005; Millward 2003; Hutson and Harris 2001; Zanchi 2010). In the catabolic state, protein metabolism, which is mainly modulated by leucine associated with high plasma insulin levels, seems to inhibit proteolysis and stimulate protein synthesis levels (Ventrucci et al. 2004). In the present study, nutritional recovery with leucine seems to have been a determinant in the increase of collagen synthesis as demonstrated by the quantification of hydroxyproline and biomechanical characteristics of tendons. In the two regions analysed (DDFT tension and compression region), the MRL and MRLT groups presented a higher hydroxyproline content indicative of collagen synthesis as well as the resistance of the tendon, when compared with the other groups, especially the groups that received the control diet. The control nutritional recovery, in the absence of leucine, favoured collagen synthesis, especially in the compression region in MRT, probably as a result of exercise because the same was not observed for the MRC group. In addition, the leucine nutritional recovery improved the tendon's collagen synthesis and resistance.

Dietary leucine may have been used as raw material for the production of small proteoglycans that modulate fibrillogenesis, such as fibromodulin and decorin, which consist of approximately 70–80% of repeated groups of 25 residues, preferentially leucine, that act as primary interaction zones with other amino acids (Zhang et al. 2005; Rees and Dent 2009; Fessel and Snedeker 2010). Furthermore, the binding sites for type I collagen present in decorin are preferentially found in leucine-rich zones (Reed and Iozzo 2002; Svensson et al. 1999; Izu et al. 2010). Some studies have demonstrated that supplementation of a protein-deficient diet with certain amino acids, such as methionine, lysine, arginine and proline, may contribute to cross-link repair mechanisms (Simonsen et al. 1995; Banos et al. 2008). The tendon must adjust its metabolic characteristics in response to stress and microtraumas imposed by daily activities under physiological conditions and especially during a physical activity (Magnusson et al. 2003). Thus, different types of physical exercise induce different adaptations of the tendon, and low-strength resistance training in the form of swimming resulted in tendons with a greater tensile strength (Simonsen et al. 1995).

The increased tendon strength observed after resistance exercise may not be associated with the necessary increased strength, but may instead represent an adaptation to withstand tissue injuries caused by mechanical fatigue (Simonsen et al. 1995). However, the same pattern was not observed in the trained groups. Although the hydroxyproline concentration was the same as in C animals, the CT group had small tissue quality in the biomechanical test. Because the tendon adapts gradually to mechanical requirements, the findings presented here are probably due

to the short period of the experiment (5 weeks), which may not have been sufficient for the healing process in order to compensate for the damage suffered (Lin et al. 2003; Natale et al. 2003; Nosaka and Clarkson 1996). Thus, the resting period of the exercised groups (CT, MRT and MRLT) did not allow for the adaptive cellular response from the tendon tissue needed to maintain metabolic homeostasis (Lin et al. 2003), with the cyclic loads imposed by the swimming exercise probably causing cumulative microtraumas. These microinjuries predisposed the tendon to premature rupture during the mechanical tensile strain test (Schechtman and Bader 1997; Banos et al. 2008; Benjamin and McGonagle 2009). Moreover, the leucine-rich diet showed the highest load at high strain despite elevated stress for the MRL group, a finding that was also reported for normal tendons during endurance exercise (Pike et al. 2000; Tsuzaki et al. 1993; Vidal and Carvalho 1990; Nicastro et al. 2010; Zanchi 2010). These results indicate that a leucine-rich diet is an important factor in the production of collagen and improvement of tissue quality (Wayburn and Volk 2009). The hypothesis is that collagen synthesis occurred because the concentration of hydroxyproline was higher in animals refed with leucine, but the formation of collagen fibres may not have been completed. The interaction between collagen fibrils provides the best tendon tensile strength (Canty and Kadler 2002; Benjamin and McGonagle 2009; Vidal and Carvalho 1990), and this interaction requires time and orientation, which could be induced by continuous exercise over long periods of time.

Comparison of the MRL and MRLT groups showed increased tissue quality in the sedentary experimental group submitted to nutritional recovery with leucine (MRL) compared with the exercise group (MRLT). In addition, a higher hydroxyproline concentration was observed in the MRLT group, but this estimation of increased collagen content produced a discrete increment of tissue quality as observed in the mechanical tensile strain test, with these animals withstanding low loads and strains. The higher hydroxyproline content and the consequent higher collagen concentration observed for group MRLT, which supposedly indicates a biomechanical advantage, resulted in a slight improvement in tissue quality, requiring tissue maturation, molecular interaction and the establishment of cross-links to restore normal functional capacity (Lin et al. 2003; Aro et al. 2008). The present results suggest that the leucine-rich diet was effective in stimulating collagen synthesis in tendons and promotes the recovery of body weight. Moreover, some exercise protocol adjustments can allow for the effective functional recovery of tendons, probably improving the capacity and response to stress. Moreover, we believe that high athletic performance depends on the whole homeostatic responses from all tissues, and there are requirements to improve the quality of tendon tissue together with the muscle work. Additional experiments are now underway to better understand the molecular mechanism of tendon injuries and healing after malnutrition and exercise conditions, especially under leucine supplementation.

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