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HEALTH SCIENCES

Swimming exercise changed the collagen synthesis and calcification in calcaneal tendons of mice

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Abstract: Obesity is characterized by the excess of body fat and, therefore, may cause musculoskeletal alterations that can negatively influence the tendons. Such overweight-influenced alterations are exercise sensitive though. Morphological and biochemical alterations were reported in the calcaneal tendon of mice submitted to a lipid-rich diets along with practicing exercises, with the following groups: normal diet without exercise (ND), normal diet with exercise (NDex), lipid-rich diet without exercise (LD), lipid-rich diet without exercise (LDex). The calcaneal tendons were removed and subjected to histological and biochemical analysis. Layers of the tissue were stained with Hematoxylin and Eosin, Picrosirius Red and Von Kossa while a protein dosage was conduce by the Bradford method. The morphologicals analysis there was no statistical difference concerning the number of fibroblasts among the groups. Groups submitted to exercises showed higher amount of collagen and non-collagenous protein deposition. The lipid-rich diet without exercse group had a more disorganized collagen matrix with intense basophilia. The same group had areas of calcification confirmed by Von Kossa technique. Practicing physical activity, such as swimming, can improve the changes caused in the calcaneal tendon in mice submitted to a lipid-rich diets, having a better collagen organization and the synthesis.

Key words: diet, hematoxylin-eosin, obesity, tendon, Von Kossa.

INTRODUCTION

Calcaneal tendon is the largest and the most rigid tendon in the human body and is responsible for fixing soleus and gastrocnemius muscle to the calcaneus bone (Threvendran et al. 2013).This fibrous cord has attracted particular attention, for its importance to Sports Medicine (Benjamin et al. 2004, Shaw & Benjamin, 2007). Either degeneration of the tendon is definited as tendinopathy this can be caused by obesity and lead various symptoms such as pain, edema, and compromised performance (Gaida et al. 2008). The obesity is most often influenced by consumption of high-fat (Feoli et al. 2003). Nogueira et al. (2017), studying effects of animals submitted to lipid-rich diet within animals, found that the animals having a diet with high amount of lipids present glycemic levels, body weight and visceral adiposity increased (Nogueira et al. 2017).

There are two general hypotheses about association of obesity and tendinopathy. The obese individual may develop tendon damage due joint and tendon overload or otherwise the

pathology may develop in response to systemic attributed biochemical changes (Conde et al. 2011). Currently, adipose tissue that leads to weight gain in obese people is related to increased production of pro-inflammatory mediators. Being, this tissue was recognized as one of the main endocrine and signaling organs. Bioactive peptides and hormones are released by the action of adipose tissue such as leptin, lipocalin 2, amyloid serum A3 and adiponectin (Conde et al. 2011). These mediators may influence several cellular activities, among them, the fibroblasts, that exert a direct influence on the structure of the tendon. In particular, adipokines are capable of modulating the production of metalloproteinases, essential enzymes in the degradation of the collagen, a predominant protein in the tendons (Lago et al. 2008, Berry et al. 2011).

Once the tendon is damaged, calcium deposition acts as attempt to compensate for the reduction of tendon force. In this case, tendon rupture release hydroxyapatite crystals in the surrounding soft tissue and leads to an acute inflammatory response (Oliva et al. 2012, Zibis et al. 2013). Clinical manifestations of tendinopathy influenced by calcium deposits can increase in the rupture rate, a shorter recovery time and a greater demand for postoperative problems (Chan et al. 2004). Clinical manifestations of the calcified tendon process include chronic pain related to activity, sensitivity, localized edema, and varying degrees of decreased range of motion. Most of the time, spontaneous reabsorption of calcium deposits occurs which consequently reduces the symptoms, although some authors describe persistent pain in long-term follow-up and persistent reduction in range of motion (Flemming et al. 2003, Maffuli et al. 2003).

Infrequent sport activity may also contribute to changes in tendons, for being directly associated with increased adiposity and obesity in human populations (Franceschi et al. 2014, Wood & Brooks 2015). Daily physical exercise helps weight loss (Cox 2017). Swimming is a moderate-intensity exercise and its practice has been increasing, and it is being stimulated in a variety of countries, even as a non-pharmacological treatment, such as for arterial hypertension, obesity and coronary heart disease (Meredith-Jones et al. 2011, Tanaka 2009). The exercises acute are related to the synthesis of type I collagen in the calcanear tendon in humans. Therefore, the main objective of the present study was to analyze the effects of swimming activities and a lipid-rich diet on the calcaneal tendon.

MATERIALS AND METHODS

Animals

The present study was approved by CEUA/UFU (063/11 protocol) in accordance with the guidelines proposed by the Brazilian College for Animal Experimentation. Male Swiss mice (n=24) were accommodated in Center for Bioterrorism and Animal Experimentation (CBEA) at room temperature (22±1ºC), 12-hour light-dark cycle (inverted), being provided with water and feed ad libitum. When the animals reached five weeks old they were separated into four experimental groups, containing 6 animals each: normal diet and kept sedentary (ND), normal diet and kept practicing exercises (swimming) (NDex), lipid-rich diet and kept sedentary (LD), lipid-rich diet and kept practicing activities (swimming) (LDex).

Diet preparation

In order to induce obesity, it was used the following lipid-rich diet protocol (rich in saturated fatty acids) (Table I).

Swimming activity

Animals kept with either normal or lipid-rich diets along with practicing exercises (swimming), were stored in 280-mm-high, 900-mm-long and 300-mm-wide aquariums for experimental purposes. Each aquarium was divided into 12 150 mm by 150 mm compartments, in order to accommodate the animals separately (Evangelista et al. 2003).

One week before starting the swimming exercise. The animals underwent an adaptation process, for each animal a period of 10 minutes of adaptation to a free diving activity was given in an aquarium with water at 32 ± 3ºC of temperature. After the adaptation, the actual exercise lasted for an hour per day for 5 days a week over 7 weeks. The intensity of the exercise training was set at 50% of the maximum load obtained based on a progressive load test (Fig. 1).

Tissue extractions

Animals were anesthetized with a mix containing diazepam, ketamine and xylazine (2: 4: 4), in order to extract the tissues for analytical purpose. Each animal was transcardially kept in contact with 25 ml of a saline solution and then fixed with 20ml of formaldehyde, consisted of 0.1M of 4% PBS, with a 7,4pH. Tendons were removed and frozen in liquid nitrogen, stored and subsequently used in biochemical analysis.

Body weight and adiposity

During the period in which they were submitted to the swimming exercise. The animals were weighed weekly. Visceral fat was obtained from the evaluation of the kidneys and mesenteric, as well as the evaluation of periepididimal fat (Shimadzu, Kyoto, Japan).

Table I. High-fat diet used in the experiment.

Ingredients	Control Diet (g)	Lipi Rich Diet (rich in saturated fatty acids) (g)
Cornflour	467.5	115.5
Casein	200.0	200.0
Dextrinized Cornflour	132.0	132.0
Sucrose	100.0	100.0
Soybean oil	40.0	40.0
Lard	----	312.0
Cellulose microfiber (fiber)	50.0	50.0
Mineral mixtrure	35.0	35.0
Vitamin mix	10.0	10.0
L-Cystine	3	3
Choline bitartrate	2.5	2.5
Total	1000.0	1000.0

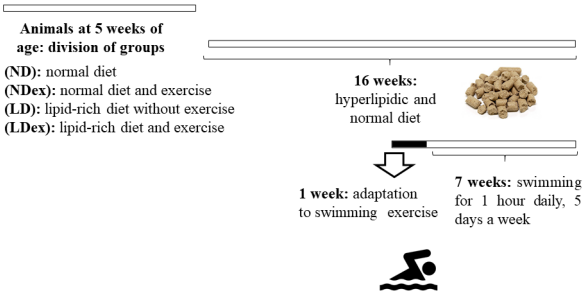

Fig 1. Experimental protocol: division of the groups, period of nutrition and accomplishment of the swimming activity.

Morphological analysis

After fixating the tissue, samples were processed for histological analysis in paraffin. Longitudinal sections were made up with a 5-µm thick layer in the rotary microtome (MICROM / HM-315). Histological slide of tendon from each animal was stained with Hematoxylin and Eosin, Picrosirius Red or Von Kossa to evaluate, respectively, the number of fibroblasts, collagen adhesion and the presence of calcification. Observation and documentation of the images were made in Leica DM 500 microscope. Images were captured under 10x and 40x plan increase, in 5 different sections per slide. Collagen was quantified in pixel / area. First, the Image J program was calibrated with a gray scale. After calibration, all the images obtained with the Picrosirius Red staining were converted to 8 bit (gray scale) and quantified using the Threshold tool. Fibroblast counting was performed with nucleus dermacation in images obtained on slides stained with Hematoxylin and Eosin with the use of Multi-Point tool.

Biochemical analysis

Calcaneal tendons were immediately removed and frozen after animals were euthanized. Fiber clusters were disassembled and kept at about 4ºC. Matrix components were kept in microtubes containing 25 ml of 4 M guanidine chloride (GuHCl), 0.05 M ethylenediamine tetra acetic acid (EDTA), and 1mM of phenyl methane sulfonyl fluoride (PMSF) in 0.05 M acetate buffer along with a 5.8pH. The resultant material was kept for 24 hours a day 7 days per week under constant ice bath. The material was then centrifuged at 10.000 g for 30 minutes. The extract-containing supernatant in GuHCl was used to have the biochemical analysis. The protein extraction dosages in GuHCI were performed by Bradford method, using bovine serum albumin (BSA) as a standard procedure. Readings were carried out

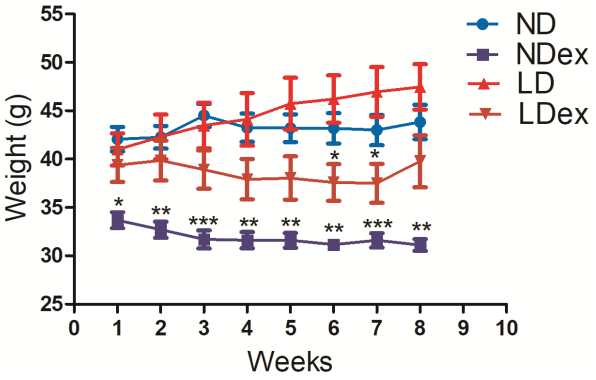

Fig 2. Accompaniment of the body of animals weight during the 7 weeks of practice or not of swimming exercises. The asterisks * represent statistical difference, ANOVA, p ≤0.05.

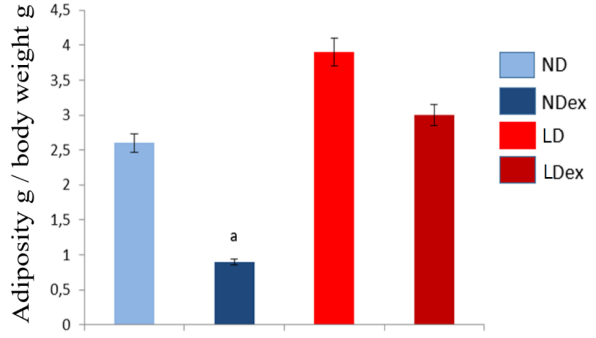

Fig 3. Visceral adiposity values in the groups evaluated. The letter (a) represents statistical difference considering, ANOVA, p ≤0.05.

in 595-nm microplate , VersamaxR with the aid of the Soft Max Pro software.

Statistical analysis

The results were shown along their mean and standard deviation. Groups were compared using a two-way ANOVA ($p<0.05$), along with a posteriori Tukey test.

RESULTS

Body weight and adiposity

Animals had their weights monitored during the period they were submitted to the swimming exercise. The graph below (Fig. 2) represents

the 7 weeks of exercise and demonstrates that the animals in the NDex group presented lower weight in all the measurements, while the animals that received a lipid-rich diet (LDex) presented a reduction only in the final weeks of the period of swimming practice. Regarding the measurement of visceral adiposity, only the group that received normal diet and practiced swimming exercise had the values reduced when compared to the other groups (Fig. 3).

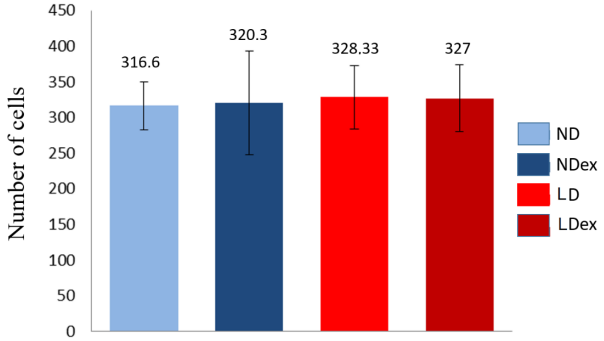

Figure 4. Count of the number of fibroblasts. Groups ND = normal diet without exercise, NDex = normal diet with exercise, LD = lipid-rich diet without exercise, LDex = lipid-rich diet diet with exercise. There were no statistical differences between the groups, ANOVA, p> 0,05.

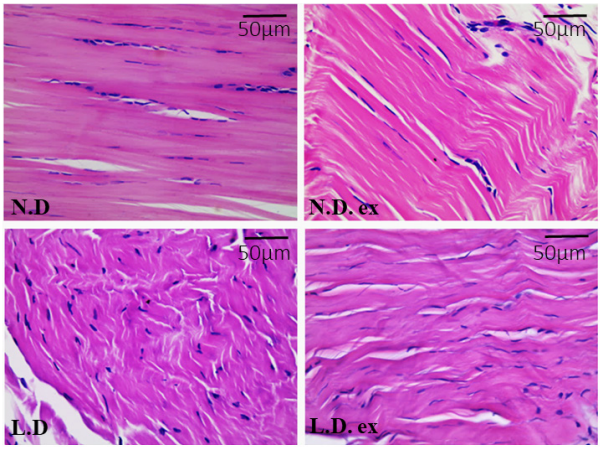

Figure 5. Light microscopy image representation of the calcaneal tendon stained with hematoxylin and eosin. The sections shows as follows: groups ND = normal diet without exercise, NDex = normal diet with exercise, LD = lipid-rich diet without exercise, LDex = lipid-rich diet diet with exercise. It was observed a greater amount of spaces between the collagen fibers and lack of alignment

Fibroblast quantification and calcification

Fibroblasts average count did not have statistically significant differences. The number of cells observed per group were as follows: normal diet group (ND) was 316.66 cells, normal diet with exercise (NDex) group was 320.33 cells, lipid-rich diet (LD) without exercise group was 328.33 cells and for the lipid-rich with exercise group (LDex) was 327.0 (Figure 4).

Hematoxylin and eosin stained layers had a better collagenous aggregation as well as longitudinal orientation in the both groups having a normal diet. As for the groups with lipid-rich diets, there was an increased amount of spaces between the fibers, mainly, in the

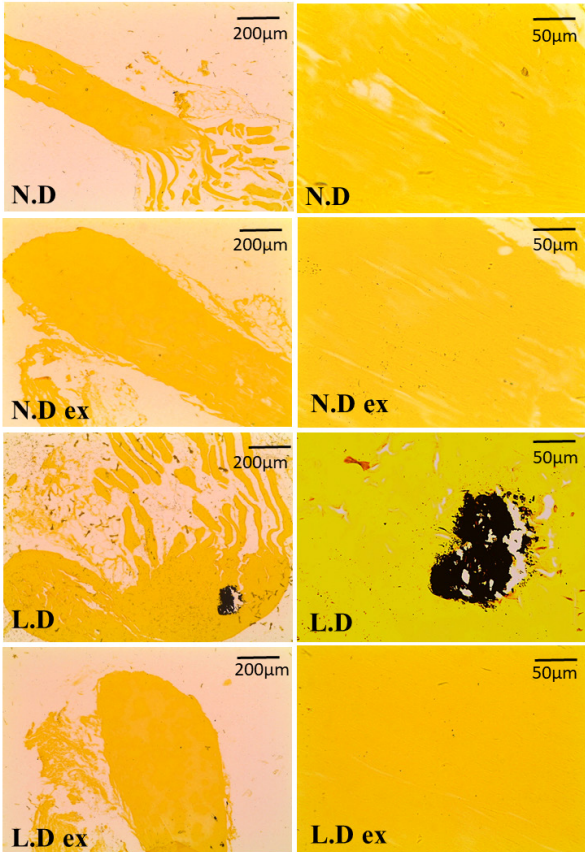

Figure 6. Light microscopy image representation of the calcaneal tendon stained with Von Kossa in all groups evaluated: ~~N.D; N.D ex; H.D and H.D ex.~~ ND = normal diet without exercise, NDex = normal diet with exercise, LD = lipid-rich diet without exercise, LDex = lipid-rich diet diet with exercise.

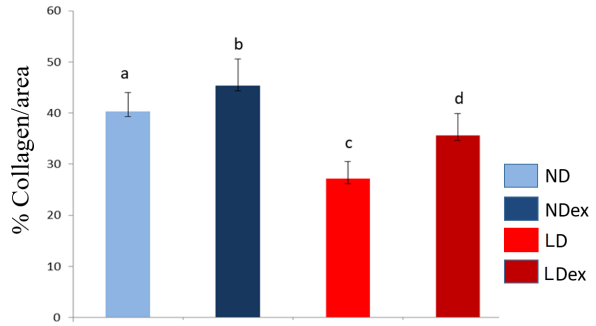

Fig 7. Percentage of collagen under Picrosirius Red staining. Comparison between the tendon of sedentary group with normal diet (ND); the normal diet with exercise group (NDex); lipid-rich without exercise group (LD); and the lipid-rich with exercise group (LDex). NDex had the highest percentage of collagen, and LD the lowest. a,b,c,d: show statistically difference among groups. ANOVA, p<0,05.

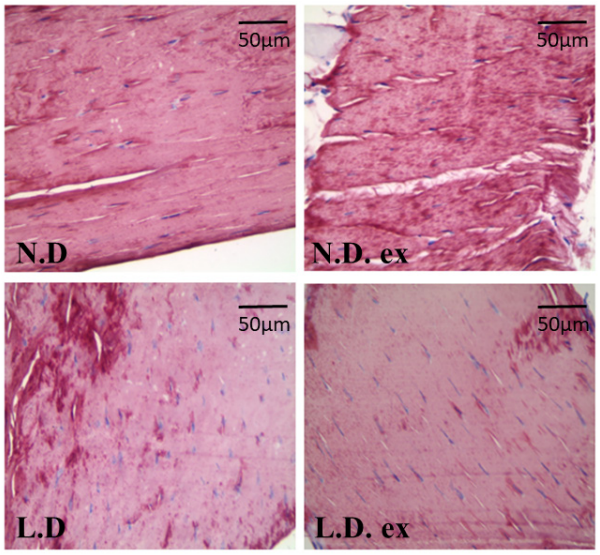

Fig 8. Light microscopy image representation of the calcaneal tendon stained with Picrosirius Red. Comparison between the tendon of the normal diet without exercise group - ND (A); normal diet with exercise group - NDex (B); lipid-rich diet without exercise group - LD (C); lipid-rich diet with exercise group - LDex (D). It was observed that the groups submitted to high-fat diet showed less staining intensity.

lipid-rich group without exercises, besides showing no signs of longitudinal alignment from the collagen fibers (Figure 5).

It was also noticeable the presence of calcifications within the lipid-rich diet groups, in intensely basophilic regions. The confirmation of these calcifications was made by the Von Kossa technique in which the area of calcification presents dark coloration (Figure 6).

Collagen quantification

The quantification of the collagen was accomplished by staining the layers with Pricosirius Red, the dye that detects total collagen and subsequently analyzing the images within Image J software. Collagen count was different among all groups,, having the lipid-rich diet without exercise the lowest percentage of collagens. In contrast, the normal diet with exercises had the highest rate of collagen. In summary, groups with exercise practice included within their routine decreased the amount of collagen deposition, when compared to the other groups. However, when taking the diet into account, the ones with lipid-rich diets had the lowest amount of collages deposited into their tissues (Figure 7). Slides observations corroborated the result of the percentage of the collagen, having both the lipid-rich with and without exercises groups the lowest color insensities, when compared to the other ones (Figure 8).

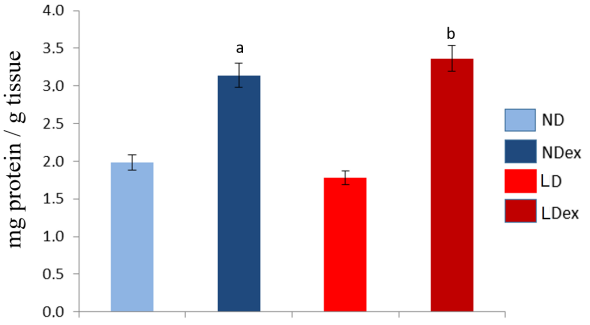

Figure 9. Protein dosages through the Bradford method (detection of total proteins). a and b: show statistically difference among groups, ANOVA, p<0.05.

Total Proteins

Through the usage of the Bradford method to check for protein dosages, it was seen that there were significant differences in the groups with exercise, when compared to groups without exercises. Therefore, both groups with exercises had higher values of non-collagenous proteins, when compared to their sedentary controls. No statistically significant difference was observe within the normal diet groups and lipid-rich diet groups, when analyzed separately (Figure 9).

DISCUSSION

Obesity is considered a worldwide epidemic problem (James et al. 2001, Ogden et al. 2006, WHO 2007), and it costs from 1% to 7% of the total investments in health, which represents a significant expenditure of one national budgets (Visscher & Seidell 2001). Obesity may increase the risk of developing secondary diseases, such as atherosclerosis, diabetes, cancer, liver diseases, immune disorders such as asthma, osteoarthritis, and also tendinopathies (Calle & Kaaks 2004, Wellen & Hotamisligil 2005, Federico et al. 2010). The practice of swimming though is essential for weight loss can be enhanced when coupled with a balanced diet. Here, we observed a marked weight loss in animals that consumed normal ration, while animals that practiced swimming and maintained a high-fat diet had a reduced weight loss. We can infer that in individuals with a high lipid diet, the swimming exercise may have a better influence over a longer period, since the animals in this group only began to present weight reduction in the last weeks of activity.

Tendinopathies is a major concern when dealing with obesity, since it is directly related to the locomotion of one individuals, affecting tendons throughout the body. The main cell found in the tendons is the fibroblasts, which are found not only in adipose tissue but also in lots of other tissues and organs. Fibroblasts are considered versatile cells, being characterized by intense synthetic activity. Such cells can be seen in the ultrastructure by the presence of abundant rough endoplasmic reticulum, Golgi complex, free polysomes and numerous peripheral vesicles (Esquisatto et al. 2003). In the present study, there were no differences detected in the number of the fibroblasts found in the tendons of the tested groups. Nevertheless, significant differences in the percentage of total collagen were observed for both lipid-rich diet without exercise (LD group), normal diet with exercise (NDex) and the lipid-rich diet with exercises (LDex). Regarding the LD group it was possible to observe a decrease in the total collagen content, when compared to other groups. Previous studies have reported that polyunsaturated fatty acids can alter in vitro formation of collagen (Hankenson et al. 2000, Jia & Turek 2005). Therefore, within the present study, when the study object had lipid-rich diet (saturated fatty acid), it was possible to hypothesize that the lipid intake is modifying the fibroblast behavior, decreasing the amount of collagen synthesis, once the number of cells was the different from other related groups. When considering the groups having normal and lipid-rich diet, both with exercises, there was a higher percentage of collagen within the tissue. It demonstrated the importance of exercises to control the amount of fatty acids being deposited in tissues. Studies have reported 100% increase in collagen synthesis in human tendons after 60 minutes of intense exercise and this synthesis has remained high for three days after the exercise (Miller et al. 2005). West et al. (2015) have also reported that exercise increased the concentration of collagen of the tendon and its ligaments strength resistance.

In our findings, LDex group did not show the same concentration of collagen which was seen in NDex group. However, it had a significantly higher rate if compared to the LD group, which indicates that the exercise was effective in preventing the decrease in collagen synthesis observed in the LD group. The presence of non-collagenics proteins was also evident in either the NDex or the LDex groups, following the increase of collagen. It is well known that non-collagenics proteins such as proteoglycans, fibronectin and thrombospondin, are part of fibrillogenesis and of the organization of collagenous fibers (Thorpe et al. 2013, Frolova et al. 2014). Although the identification of such proteins has not been carried out in the present study, it is believed that they are related to the increase of collagen, thereby regulating such phenomena described above. An interesting data found was the presence of calcifications in the tendons of the mice of the LD group. The calcification may suggest that there might be happening the development of an acute tendinitis in response to a chronic tendinopathy as tendinosis, being more commonly found in the tendons of rotator cuff (shoulder tendon) and calcaneal (Castillo-Gonzáles et al. 2014, Bakkegaard et al. 2015). The overuse and aging are common causes of the development of calcification within the tendons. However, obesity has also been reported as a major factor increasing the predisposition for the occurrence of this phenomenon, due to the increase of the body weight and, as a consequence, getting more tension upon the tendons (Rutten et al. 2006, Franceschi et al. 2014, Wood & Brooks 2015). In general, the tendinous tissue close to the calcification becomes inflamed and painful, and this inflammation may lead to the rupture of the tendon (Franceschi et al. 2014).

After observing the calcification, which was seen only in the LD group, probably due to the reasons mentioned above, one can say that the effect of the exercise was positive by reversing this situation in the tendons of mice from LDex group. This happened because the exercise was effective in preventing excessive increase of weight in this group, eliminating one of the causes (overweight) that may lead to the development of calcifications.

CONCLUSIONS

Swimming was effective in improving the negative effects caused by lipid-rich diets in the calcaneal tendon, it being able improve the deposition of extracellular matrix components, especially the collagen and non-collagen type I proteins. In addition, the high fat diet may increase calcification in tendon, favoring the development of tendinitis.

Acknowledgments

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