Original article

Changes in the soleus muscular tissue of rats with experimental periodontitis under physical exercise influences

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Summary

Until the moment, no study explored conjunctively the physical activity relation, using the inflammatory biomarkers, with the periodontitis. This way, the objective of this experiment was to evaluate the muscular tissue behavior of rats submitted to physical exercise in aquatic environment with experimental disease. Twenty-four male Wistar rats were divided in 4 groups: 1) control and sedentary (CS); 2) control and active (CA); 3) with the periodontal disease and sedentary (PDS); with the periodontal disease and active (PDA). On the group that the periodontitis was induced, it was for ligature and the groups with active swimming activity have practiced it for 4 weeks. At the end of 30 days the animals were euthanized and a portion of the gingival tissue and the soleus muscle were removed and underwent analysis by ELISA and morphological and morphometrical analysis of the muscle. Data drawn from the analysis was analyzed through ANOVA and Tukey. Results have shown that there is a higher expression of TNF-α in the gingival tissue and on the muscular tissue of the rats that underwent the induced periodontitis independently of the physical activity (PDS and PDA), as a meaningful decrease on the conjunctive tissue on the groups with induced periodontitis, that have or have not undergone active swimming activity which could suggest a predisposition to muscular injury or difficulty of muscular recovering on these groups. Therefore, it was possible to highlight a correlation between the periodontal disease and the muscle morphological changes, and, moreover, the physical swimming activity promoting an acceleration of the regeneration of the muscle tissue.

Key words:
Physical activity.
Periodontitis.
Soleus muscle.

Cambios en el tejido muscular del sóleo de las ratas con periodontitis experimental bajo influencia del ejercicio físico

Resumen

Hasta este momento, ningún estudio ha explorado conjuntamente la relación de la actividad física con la periodontitis utilizando biomarcadores de inflamación. Siendo así, el objetivo de este estudio fue evaluar el comportamiento del tejido muscular de ratas con periodontitis experimental al realizar ejercicio físico en medio acuático. Veinticuatro ratas Wistar machos fueron divididas en cuatro grupos: 1) control y sedentario (CS); 2) control y ejercicio (CA); 3) con enfermedad periodontal y sedentario (PDS); 4) con enfermedad periodontal y ejercicio (PDA). En los grupos con periodontitis, la enfermedad periodontal fue inducida por ligadura y los grupos con ejercicio realizaron natación durante cuatro semanas. A los treinta días, los animales fueron sacrificados y una parte del tejido de las encías y del músculo soleo se resecaron y utilizaron para análisis con ELISA y para análisis morfológicos y morfométricos. Los datos obtenidos fueron analizados y evaluados a través de los tests ANOVA y TUKEY. Los datos mostraron una mayor expresión de TNF-α tanto en el tejido de las encías como en el tejido muscular de los ratones sometidos a periodontitis inducida independiente del ejercicio físico (PDS y PDA). Se percibió también una disminución significativa en el tejido conjuntivo en los grupos con periodontitis inducida sometidos o no al ejercicio de natación, lo que podría sugerir una predisposición a lesión muscular o una dificultad en la reparación de las lesiones musculares de esos grupos. Por lo tanto, fue posible destacar una correlación entre la enfermedad periodontal y los cambios morfológicos musculares y, además, que la actividad física de natación favoreció una aceleración de la regeneración del tejido muscular.

Palabras clave:
Esfuerzo físico.
Periodontitis.
Músculo soleo.

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Introduction

Physical activity regularly practiced leads to the physiological and morphological adaptation, relevant to the maintenance of the organism homeostasis and these changes influenced on many diseases control, especially those of cardiovascular nature and endocrine-metabolic. This way, several studies have shown that the physical activity is capable to promote changes in various functions of the human organism and of rats[1,2]. Besides, they are capable to promote the acceleration of the process of the inflammation recovery[2,3]. Under this sense, some studies pinpointed that the exercises infer on several stages of the inflammatory process, promoting migration of the leukocytes to the direction of the inflammation focus (chemiotaxis) and the increase of the capacity of phagocytosis of this cells in human beings and animals, besides increasing the antitumor macrophage activity[2,4,5].

The inflammation is an adaptive response, which is broken by stimulus and harmful conditions, such as the infection and the tissue injury. A considered process has been reached on the comprehension of the cells and molecular events, which are involved on the acute inflammatory response and, in a lower scale, the tissue aggression. Still, events that have broken the located chronical inflammation, especially the chronical infections and autoimmune diseases, are partially understood. Little is known, however, about its causes and mechanisms of systemic chronical disease, which occurs in a several variety of diseases, including the diabetes type 2 and cardiovascular diseases. These inflammatory chronical states seem not to be caused by the classical inflammation causes: Infection and aggression. Instead, they seem to be associated with the bad-function of the tissue: a lack of balance of the homeostatic of one of the physiological system, which does not have direct relation to the function of the host defense or tissue repair[6].

The inflammatory response to an infection, while aiming at recovering the homeostasis, can turn out to be harmful if excessive or not balanced. Under these conditions, inflammation plays an essential role in the pathogeneses of many chronical diseases, including periodontitis[6].

Periodontitis is a chronic inflammatory disease triggered by a pathogenic microbe organized by a biofilm that results on the selective destruction of the periodontal tissue that can cause the loss of the tooth. The destruction of the periodontal tissue, however, may not be linear. Periodontitis is actually characterized on alternate phases of the exacerbation and remission of the disease, as well as on the inactivity period. Under this context, it is possible that the cytokines that directs the cellular response during the overlapped inflammatory and healing of the tissues phase that can interact in different levels. Little is known, however, about the interaction between the cytokines and the growth factors that frame the inflammation and the tissue recovering[7].

Tumoral Necrosis Factor-α (TNF-α) is an important inflammatory cytokine expressed during the inflammatory phase of tissue healing and in chronic inflammatory conditions, such as the periodontal disease. The TNF-α inhibits the synthesis of the Extracellular Protein Matrix (EPM) and the active production of the matrix metalloproteinases. Studies have shown that the TNF-α can affect the antagonist upon several induced answers or stimulated by the transforming growth factor of β1 (TGF-β1), such as, the synthesis of the collagen type I and III, and the expression of α- de smooth muscle actin (α-SMA) in fibroblasts of skin and the production of tropo-elastin in fibroblasts on the rats lungs[7].

Epidemiological data suggests that the periodontal diseases can be associated with other systemic pathological conditions[8-10]. Besides that, it is possible to find some evidences of infections caused by odontogenesis origin that has a relation with the damage development caused by the exacerbation and immunological response, which is willing to generate destructions and changes on the expression of myosin[11-13].

Considering that the same proinflammatory biomarkers are involved in both periodontal disease and muscle metabolism, it is plausible that the systemic challenge generated by periodontal disease could also influence physical fitness[2,5].

Although it is possible that the physical activity protects the periodontal area due to the fact that it attenuates the excessive inflammatory response on the host, there are some evidences on the longitudinal studies and a prospective study showing that the adults physically active have lowered the risk of periodontitis. Until the moment, no study has explored conjunctively the relation between the physical activities, using the inflammatory biomarkers, with the periodontitis[14].

Therefore, the objective was to evaluate the muscular tissue behavior of rats submitted to physical exercise in aquatic environment with experimental periodontitis.

Material and method

Obtaining the animals

There were used 24 Wistar Rats weighting an average of 100 g, from the Unioeste central animal research area. These animals were maintained on controlled thermic conditions (23 ± 2º C) and light (cycle of 12 hours light and 12 hours dark – 07:00 A.M – 7:00 P.M) and received water and commercial food all the time. The experimental protocols were approved by the Ethical Committee in Animal Experiment and Practical Classes of UNIOESTE.

Experimental Groups

Animals were divided randomly in 4 groups of 6 animals each, according to Felipetti, *et al*., 2014[15].

- Group (CS): control and sedentary
- Group (CA): control and active
- Group (PDS): with periodontal disease and sedentary
- Group (PDA): with periodontal disease and active

Inducing of the Periodontal Disease

Animals received anesthesia (xylazine 0,04 mL/100 g and ketamine 0,08 mL/100 g), and placed on a proper operatory table, which allowed a maintenance of the buccal opening of the rats making it easier to have access upon their teeth on the posterior jaw region. With the support of a modified pinch and an explorer probe, cotton ligatures number 40 were placed around the lower right first molar. This ligature acted irritating the gingival margin for 30 days, provoking accumulation of bacterial plaque and consequently development of the periodontal disease[16].

Protocol of Aerobic Activity

Previously to inducing the protocol of the periodontal disease on animals of CA and PDA went through a practice of familiarizing on the swimming activity, initiated one week before, with the duration of 15 minutes, 3 times a week.

Two days after inducing the DP, the groups have done an aerobic activity similar to swimming, during 4 weeks, with progressive intensifying over time, from 15 minutes on the first week, 30 minutes on the second, and successively up until reaching 60 minutes on the fourth week, daily with the break of 2 days between the beginnings of the week. The place used was an oval tank, with capacity of 200 L, depth of 60 cm and controlled temperature of 32º ± 1º C[17].

Analysis of Tumoral Necrosis -α Expression (TNF-α)

By the end of the activity period, all the animals were weighted and anaesthetized with ketamine (50 mg/Kg) and xylazine (10 mg/Kg) and beheaded on a guillotine. A portion of the gingival tissue around the teeth submitted or not to the use of the ligature and the soleus muscle, all the experimental groups were removed and used for the by Enzyme-Linked Immunosorbent Assay (ELISA) analysis on the presence of TNF-α cytokine. The total of proteins was extracted from samples of the gingival tissue and the soleus muscle using an extraction buffer to the detergent basis (T-PER, Tissue Protein Extraction Reagent - Pierce), with a cocktail of proteasis inhibitor (Protein Stabilizing Cocktail - Santa Cruz Biotecnology), according to the manufacturer instructions.

The samples were macerated on the buffer (50 µL/mg of tissue) and centrifuged for 5 min to 13.000 rpm to 4 0C. The overflown contend was quantified using a system of Bradford protein (Bio-Rad). As to detect and quantify the TNF-α were used 100 µL of the sample and 100 µL of the buffer in a length of wave of 450 nm according to the Kits ELISA (TNF-α ELISA Kit Biosource), accordingly to the manufacturer instruction.

Collecting the soleus muscle and the Histological Preparation

The right soleus muscle was dissected, fixed on methacarn solution (70% of methanol, 20% of chloroform, 10% of glacial acetic acid) for 24 hours and stored in 70% of alcohol. After that, it went through a process of dehydration in an increasing alcoholic series, diaphanized in xylol and embedded in paraffin. The transversal cut of 5 µm in thickness were obtained by a microatom (CUT 4055 Olympus®, Mainz, Germany) and colored with Hematoxylin and Eosin (HE) for the general analysis of muscular tissue and with Mallory Trichrome (MT) for the conjunctive tissue analysis.

Morphological and Morphometrical Analysis

The following measurement of the soleus muscle was carried: transversal section area, minor fiber muscular diameter, density of the conjunctive tissue and the evaluation of the muscular tissue.

Obtained slides were analyzed under the light of (BX60 Olympus®, Tokyo, Japan). For the measurement of the transversal section area and minor diametrical muscular fiber 10 images were randomly obtained on the objective of 40x, for each image 10 fibers were measured on the program of Image-Pro-Plus 6.0 (Media Cybernetics®, Silver Spring, USA), totalizing 100 measurements for each animal.

To determine the density of the conjunctive tissue, 10 images were randomly obtained through the objective of 40x and 10 measurements carried for each animal, endomysium and perimysium analysis, the GIMP was used (GNU Image Manipulation Program 2.0 - GNU General Public License®, Berkeley, California). The relative area of the conjunctive tissue (density area) was calculated by dividing the total of pixels of the photomicrography by the total of pixels of the conjunctive tissue highlighted.

Data analysis

The data were analyzed and evaluated using the one-way ANOVA test and, when statistically significant differences were found, the Tukey test was used to determine differences between the groups at a 5% significance level. The quantified results were presented on tables and/or in graphics, while the results about the tissue morphology were displayed in images (photomicrography) followed by their description and discussion.

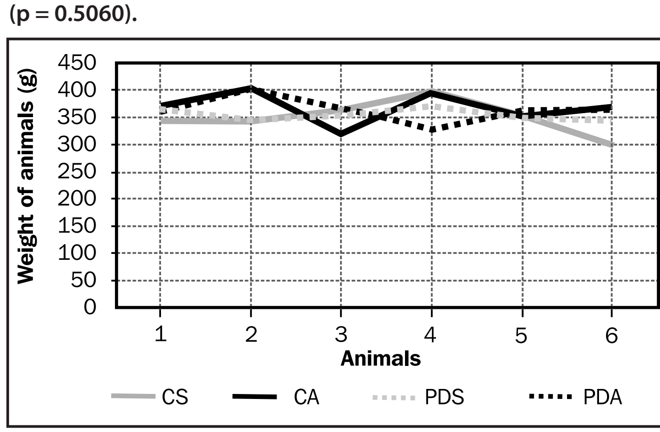
Results

Figure 1 shows the distribution of the weights of the 6 animals of each of the 4 groups. There was no statistically significant difference between the groups at the end of the experimental period (CS = 348.33 ± 30.8, CA = 368.33 ± 28.8, PDS = 353.33 ± 10.5, PDA = 363.5 ± 24.5).

It was observed in Table 1 that, in the groups with induced periodontitis (PDS and PDA), the cement-enamel junction distance was significantly increased, and in the group that performed physical activity on the aquatic environment (PDA) the bone loss was significantly lower than in the group with periodontal disease alone (PDS).

It was possible to observe through Table 2 that the TNF-α expression significantly increased on the gingival tissue happens both on the muscular tissue of rats subjected or not to the physical activity on the aquatic environment, only on the groups with induced periodontitis (PDS and PDA).

Figure 1. Distribution of animal weights of all experimental groups (p = 0.5060).



Results about the area and smaller diameter in soleus muscular fiber, does not show significant statics differences. There was, however, a tendency on the increase of the area and smaller diameter on the CA group and the area of PDA group according to Table 3.

Table 4 shows that the conjunctive is decreasing significantly on the groups of induced periodontitis, subjected or not to swimming activities.

Morphological analysis of the soleus muscle

On the control group and sedentary (CS), the soleus muscle presented an abnormal morphology; the muscular fibers have the polygonal format with nucleus on the periphery position and fascicular pattern (Figure 2A).

The animals subject to the swimming activity (CA) some fiber presented themselves slightly hypertrophic, with a bigger area when compared to the other groups (Figure 2B) and with regular morphological characteristics.

On the periodontal disease group (PDS), the soleus muscle presented morphological changes directly in some fibers. It was observed some fibers with irregular format, disorganization on the cytoskeleton myofibrils (Figure 2C and Figure 2E), with an increase on the number of peripheral nucleus, many of which presented a queued aspect (Figure 2D) and centralized nucleus (Figure 2F). On the conjunctive, it was verified an increase on the endomysium (Figure 2D) and the presence of an inflammatory infiltrate (Figure 2G).

On the periodontal disease associated with activity on the aquatic environment (PDA) was observed few signs of muscle changes, although some fibers present an irregular format with nucleus on the central position and it was not verified any changes on the endomysium, neither the presence of inflammatory infiltrate (Figure 2H).

Table 1. Values of the cement-enamel junction distance to the alveolar bone crest of the established groups.

Groups	Means
CS	47,8 ± 1,2 A
CA	48,7 ± 1,2 A
PDS	84,5 ± 1,2 C
PDA	61,7 ± 2,2 B

Note: Values represent mean ± standard deviation.
Differents letters - statistically different data among the groups (p <0.05).

Table 2. Values of TNF-α expression of all analyzed groups. Values are expressed in pg/ml.

Groups	Gingival tissue	Muscle tissue
CS	4,97 ± 0,17A	3,38 ± 0,88A
CA	4,60 ± 0,43A	3,52 ± 0,59A
PDS	5,35 ± 0,26B	4,39 ± 0,40B
PDA	5,67 ± 0,74B	4,53 ± 0,94B

Note: Values represent mean ± standard deviation.
Differents letters - statistically different data among the groups (p<0.05).

Table 3. Values of area and smaller diameter of muscle tissue of all established groups.

Groups	Area (μm²)	Smaller diameter (μm)
CS	2860,2 ± 210,6	43,1 ± 1,1
CA	3337,7 ± 157,7	45,8 ± 1,5
PDS	2945,2 ± 65,9	40,2 ± 1,5
PDA	3040,4 ± 136,8	43,9 ± 1,9

Note: Values represent mean ± standard deviation.
Differents letters - statistically different data among the groups (p<0.05).

Table 4. Values of conjunctive tissue of all established groups.

Groups	Conjunctive tissue (%)
CS	10,0 ± 3,6 A
CA	6,3 ± 3,1 AB
PDS	3,4 ± 0,5 B
PDA	4,6 ± 1,6 B

Note: Values represent mean ± standard deviation
Differents letters - statistically different data among the groups (p<0.05).

Discussion

The regular physical activity can offer a behavior strategy to limit the inflammation[14]. There is an increasing evidence that, additionally to other benefits, physical activity has an anti-inflammatory effect. The National Health and Nutrition Examination Survey, (NHANES III) verified that the higher the frequency of activity the lesser possibility to increase the C-reactive (PCR) protein and the counting of white cells on the blood[18]. On the other hand, the inverse association between physical activity and several inflammatory biomarkers, including the PCR, were found even in low intensity of activities[19]. Until now, there is no study has explored conjunctively the relation of physical activity with the inflammatory biomarkers during the periodontitis. This was the aim of this study was to evaluate the muscular tissue comportment of rats subjected to experimental periodontitis associated to physical activity.

It was possible to observe that the bone loss evaluated from the distance from the cementum-enamel junction to the alveolar bone crest was significantly increased only in the groups with induced periodontitis (DPS and DPE), demonstrating the efficacy of the experimental periodontal disease induction model (Table 1).

Moreover, it was possible to observe through table 1 that the expression of TNF-α is significantly increased, on the gingival tissue as well as on the muscular tissue of the rats subjected or not to the physical activity, only on groups with induced periodontitis, in accordance to the Arancibia, *et al*. (2013)[7], who reports that the production of TNF-α e TGF-β is increased under inflammatory conditions and could suggest a predisposition to muscular injury or to difficulty to muscular recovering, once the TNF-α inhibits several cellular responses induced by the TGF-β1, including the differentiation of the microfiber blasts, with the activation via Smad signalization (protein involved on inducing the α-SMA under stimulation of TGF-β1) and the production of key-molecules involved on the recovering tissue, such as collagen type 1, fibronectin (FN) and periostin.

Similarly, as to quantified conjunctive tissue (Table 4) it was possible to observe that it decreases significantly on the groups subjected to the experimental periodontitis, independently of physical activity when compared to the controlled group. During the process of wound healing, the initial inflammatory stage is critical to the efficient healing, due to the fact that it promotes the recruitment of phagocytic cells to extinguish the pathogens which could have entered the tissues after reviling. Although the inflammation could delay the process of healing or the wounds or take it to develop an aberrant, granulate formation

Figure 2. Photomicrography of the soleus muscle on the Wistar rats, transversal cut, colored HE (A, B, C, D, F, G, H) and Mallory Trichrome (E). (A), group controlled (CS); (B), group swimming activity (CA); (C, D, E, F e G), Periodontal Disease group (PDS) and (H), Periodontal Disease associated with physical activity (PDA). In A, highlighting the muscle fibers with a polygonal format (F), peripheral nucleus (Np) and blood capillaries (Cs). In B, hypertrophied muscular fibers (F) preserving the structure and muscular organization. In C and E, disorganized presence of myofibrils (Dm). D, increase on the number of peripheral nucleus that presented themselves queued (circle). In F central nucleus (Nu) and G focus on the inflammatory infiltrated (If). In H, pointing out the morphological improvement, but still with the presence of central nucleus on some muscle fibers, with no characteristic format (Nu).

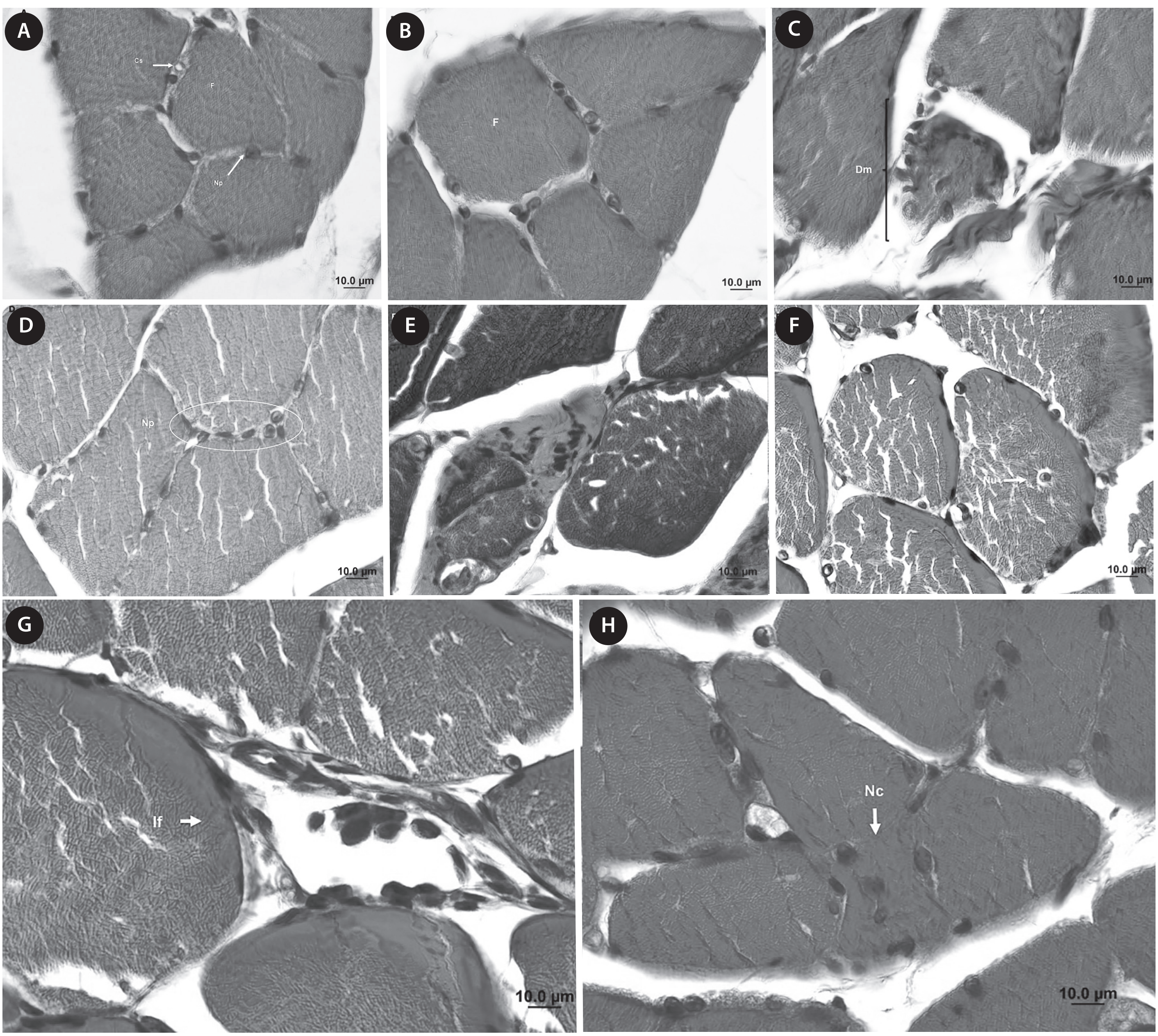
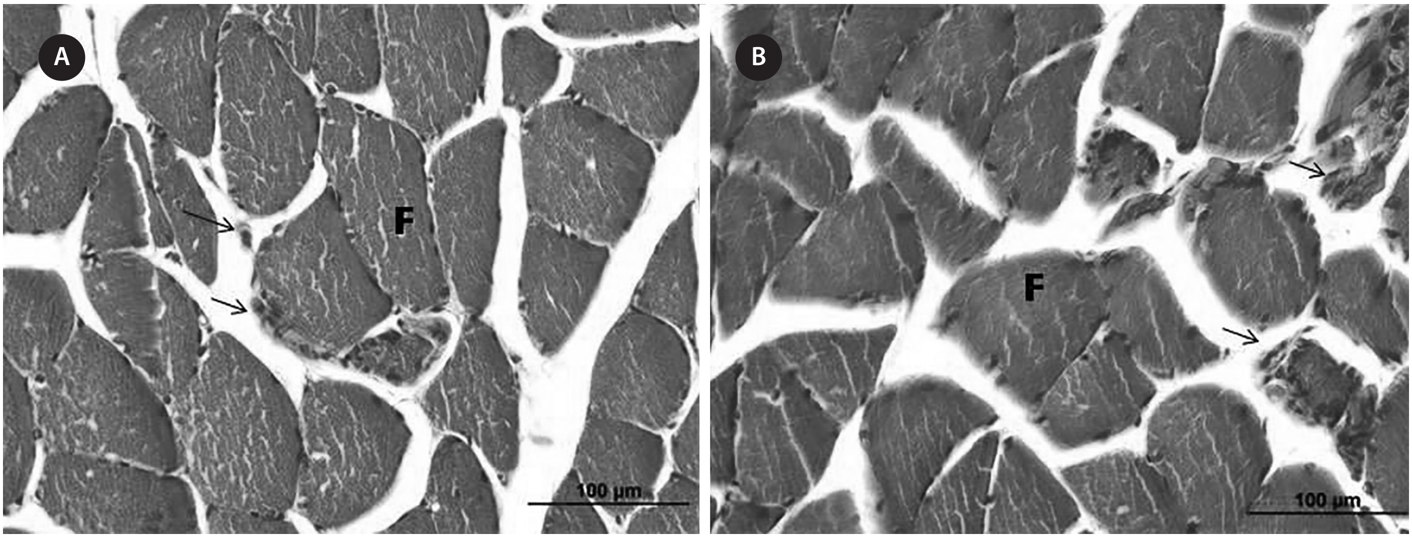


Figure 3. Photomicrography of the soleus muscle on the Wistar rats, colored HE. A – Periodontal Disease group (PDS) and B – Periodontal Disease associated with physical activity (PDA). muscle fibers (F) and inflammatory infiltrated (arrow).



of the tissue. Periodontal disease is considered a chronic inflammatory condition that destroys the tissues and could be prolonged on periods of quiescent or repair. Periodontal inflammation and the recovering of the tissue could actively interact, however, during the natural development of the periodontitis[7].

Morphological data showed that the animals on the group CS presented muscular fibers with regular aspects, as well as the activity group (Figure 2A and Figure 2B)[20,21]. Although the literature brings the swimming activity as to promote morphometric changes, as the increase on the area and the transversal section in muscular fibers[22-24], on this study the used protocol was not able to change significantly this parameters (Table 3). As Oliveira, *et al* (2014)[25] observed on his study, the animals were subjected to swimming activity of high intensity and they did not show morphometric changes on the muscular fibers. The same occurred with the weight of the animals, which corroborated with the same study by Oliveira, *et al*[25], there was no significant alteration between the groups (Figure 1).

Opting for the swimming category is explained because this activity represents an aerobic activity that uses most of the articulation of the body as well as the use of muscles like the soleus, once the animals have to perform an plantar flexion to maintain themselves on the water surface[25], besides the increase on the cardiac and respiratory frequency, due to the viscosity property which offers resistance to movements in any direction, contributing to the muscular resistance training[17]. On the DP group, several morphological detrimental characteristics were found (Figure 2C and Figure 2F). The presence of many big nucleus, as on the periphery as well as centralized on the muscular fibers, presented signs of muscular degeneration, after having suffered changes from the systemic inflammatory process caused by the DP. According to Karalaki, *et al*. (2009)[26], plasticity of the muscular tissue in response to an injury depends on, among other, the functional satellite cells' role. These are on the healthy muscle and are found on the quiescence state. After injury, the support of factor of growing, proliferation and differentiation, create myoblasts that go through the basal sliding the muscular fiber and release enzymes that assist the arrival on the injury place, promoting its recovering[27].

The increase on the inflammatory infiltrated number on the soleus muscle on the group DP (Figure 2G and Figure 3A) confirmed the results found on the analysis of the TNF-α expression (Table 2). These findings point at Souza, *et al*. (2013)[20] who verified on the gastrocnemius and anterior tibia muscles a higher quantity of inflammatory cells on the periodontal disease. The increase of cytokines as the IL-6 and TNF-α indicates that these substances can contribute to the recovering process[28].

The improvement on the morphological parameters on the experimental group DPE (Figure 2H and Figure 3B) can be related to the effects of physical activities upon the muscular physiology, on these animals it was observed that the activity promoted a regeneration on most of the muscular fibers, as well as the decrease on the inflammatory cells number. These data are similar to those found by Faria, *et al* (2008)[29] which evaluated the effect of swimming after a muscular injury, on a protocol of 5 to 8 days with 15 to 45 min sections respectively, and observed an improvement on the muscular morphology. Considering the determined experimental conditions we could observe that muscular tissue's behavior showed an acceleration on the regeneration process after physical activity in aquatic environment in rats with periodontitis experimental and there was a relation between the periodontal disease and the muscular morphological changes.

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