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(RESEARCH ARTICLE)

Musculoskeletal stretch injury shows a faster recovery compared to contusion in rats

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Abstract

Background: The musculoskeletal repair, in most cases, follows the typical inflammatory process. Although the majority of treatments result in good prognosis, the knowledge concerning basic biochemical principles, regeneration and repair mechanisms of skeletal muscles may help in recovery and accelerate the return to sport, helping to carefully choose the more appropriate therapeutic techniques for the injury management.

Objective: This study investigated the effects of contusion and stretch injury on the oxidative damage in the gastrocnemius muscle of rats.

Methods: The biochemical analysis was performed on the 1, 3, 5, or 7 days after the injuries and behavior analysis was performed until 48 hours after injuries to evaluate the healing process changing characteristics.

Design: Experimental animal study.

Results: The contusion and stretch injury increased the oxidative stress markers levels; however, the injuries showed significant differences from each other in the markers of oxidized dichlorofluorescein, superoxide dismutase enzyme activity and N-acetyl-beta-glucosaminidase enzyme activity in skeletal muscle tissue. Furthermore, the injuries showed differences in behavioral analyses in the beam walking test.

Conclusion: In conclusion, we could infer that the stretch injury presents an early increase in the activity of the N-acetyl-β-D-glucosaminidase enzyme, indicating a possible macrophage increase in the first hours after injury.

Keywords: Muscle injurie; Oxidative stress; Inflammatory; Contusion; Stretch injury

1. Introduction

Muscle injuries are the most frequent cause of physical incapacity in sports practice, corresponding to 30-50% of sports injuries. The most frequent injuries are contusions and stretch injury, or lacerations, corresponding to approximately

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90% of muscle injuries in sports practice [1, 2]. The skeletal muscle regeneration is a complex event, which includes changes in generation of reactive oxygen and nitrogen species, interactions between the skeletal muscle and the immune system, as well as satellite cells activation. In sports medicine, the regenerative processes science is decisive for athletes' health and physical performance [3]. Stretch injuries are indirectly resulted from a combination of an intense contraction with a strong stretching, leading to an excessive stress on the skeletal muscle tissue structure. On the other hand, contusions are characterized by muscle cells compression due to impact caused by some weight on the skeletal muscle surface [1.4].

In both injuries, the mechanism of lesion leads to the breakage of some muscle fibers and blood capillaries resulting in an overflow of blood components throughout the injured region [5]. The mechanical impairment of the injured tissue involves the disruption of connective tissues, which can lead to myofiber necrosis, hematomas and inflammation [2, 6, 7]. Our previous studies showed a remarkable oxidative damage in muscular and blood tissues after a contusion [2, 8] or stretch injury [9]. However, it is important to highlight that the oxidative damage and compensatory tissue response to these lesions, as well as comparisons between them are still poorly understood.

In general, the musculoskeletal injury repair involves a typical inflammatory process. Despite their differences (intensity of inflammation, nature, extent and injury strength), in the literature is possible to observe similar treatment protocols [7, 10]. Although the majority of treatments results in a good prognosis for most athletes with muscle injuries, the knowledge about oxidative response basic principles, regeneration and repair mechanisms of skeletal muscles may help in recovery and accelerate the return to sport [1]. Consequently, this knowledge could help clinicians choose techniques that may be more appropriate for the injury management.

Therefore, the present study aimed to compare contusion or stretch injury effects on the animal's behavior and compare the oxidative damage differences in the gastrocnemius muscle of rats. Firstly, we *in vivo* analyzed the possible changes in the animal's behavior according to thigmotaxis behavior, beam walk, hot place, tibial and sciatic functional index. In *ex vivo* analysis the oxidative markers levels, such as thiobarbituric acid reactive substance (TBARS) and oxidized dichlorofluorescein (DCFRS) and the cellular viability index (MTT reduction into formazan) were analyzed as well. Besides, the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) activities and the non-ezymatic non-protein thiol groups (-SH) levels were also analyzed. Finally, as an inflammatory response intensity indicator, we measured myeloperoxidase (MPO) and N-acetyl-β-D-glucosaminidase (NAG) enzyme activities.

2. Material and methods

2.1. Animals

Seventy-eight adults male Wistar rats, weighing 250–340 g, were placed (5 animals each cage) with food and water (ad libitum) in a temperature-controlled room (22 ± 3°C) and at a ratio of 12 hours light/dark (lights were turned on by 7:00 am). The animals were divided into three main groups: (1) Control group: control animals without injury (n = 20); (2) Stretch injury group: the animals submitted to experimental stretch injury model (n = 20), (3) Contusion group: animals submitted to experimental contusion model (n = 20);

The animals of each of these three groups were subdivided into further four subgroups each (n = 5-6), according to the times where the analysis was performed after the muscle injury. In all of these subgroups, the right hind limb of the rats was submitted to one type of skeletal muscle lesion (contusion or stretch injury) and the left hind limb was used as uninjured.

2.2. Skeletal muscle Injury

2.2.1. Contusion injury

Skeletal muscle injury was mostly accomplished according to Martins et al. [11] except for few modifications. After complete anesthetization (ketamine 50 mg/kg, ip and xylazine 10 mg/kg, ip), the animals were placed in prone position to proceed with the right gastrocnemius muscle injury. A 200 g mass was dropped through a polyvinyl chloride tube (used as a guide) with 30 cm of height and 20 mm of diameter placed right onto the right gastrocnemius muscle venter generating an impact force of 0.484 N and two impacts were performed. After injury the rats were left in the coop for anesthesia recovery.

2.2.2. Stretch injury

The skeletal muscle stretch injury was performed according to Ozaki et al. [6] with few modifications. After complete anesthetization (ketamine 50 mg/kg, ip and xylazine 10 mg/kg, ip), the animals were placed in dorsal position to proceed with the right gastrocnemius muscle injury. Gastrocnemius muscle was simultaneously stretching and submitted to tetanic muscle contractions through an electro stimulatory (Globus Elite SII, Globus Corporation, Italy) device, with frequency of 50 Hz, twenty contractions of 10 seconds with intervals of 10 seconds are also performed through self-adhesive electrodes. After the injury, the animals were left in the coop until fully anesthesia recovery.

2.3. Behavioral tests

The following behavioral analyses were developed 6, 24 and 48 hours after lesion:

2.3.1. Thigmotaxis behavior

The animals were introduced into an apparatus consisting of a box (40 × 40 × 19 cm) with a central quadrant (11 × 11). The number of entries and time spent in the central quadrant was then evaluated for 15 min and considered as a thigmotaxis behavior. Spontaneous activity of the animals was recorded for 15 min with a camera (CTO66836, Chin) and the results were obtained by automated analysis (ANY-maze™ software) and further exported as raw data for the statistical analysis. The track plot for each traveled trace was directly exported from ANY-maze™ software after recording [12].

2.3.2. Hot place

Animals were placed individually on a hot plate with the temperature adjusted to 51ºC. Exposure to heat continued until nocifensive reaction of hind paw occurred. The latency of the withdrawal response of each hind paw was determined at 6, 24, and 48 hours. The animals were tested in only one series of measurements and the typical responses were hind paw shaking and/or lifting. The latency to the response was recorded manually with a chronometer, and the time of maximum permanence permitted on the hot surface was 15 s. The experiments were performed in a sound-attenuated and air-conditioned (20-22ºC) laboratory. Hyperalgesia to heat was defined as a decrease in withdrawal latency and calculated as follows: D paw withdrawal latency (s) = right paw withdrawal latency - left paw withdrawal latency [13].

2.3.3. Beam walk

Rats were trained to walk on a wooden beam (2.5×2.5×80 cm), elevated 60 cm above the floor, to get back to their home cage. Before the test, pre-training was carried out in which rats were placed on the beam in front of their home cage and trained to return to the cage. Training was carried out 2 days before the muscle injuries, and performance was video recorded. Ability was measured using a modified scale: score 0, the rat traverses the beam with no foot slip; score 1, the rat traverses with grasping of the lateral side of the beam; score 2, the rat shows disability of walking on the beam but can traverse; score 3, the rat takes a considerable amount of time to traverse the beam because of difficulty walking; score 4, the rat is unable to traverse the beam; score 5, the rat is unable to move the body or any limb on the beam; score 6, the rat is unable to stay on the beam for 10 s. Three trials were recorded for each analysis [14].

2.3.4. Tibial and sciatic functional index

The animals were placed on a wooden trail so that they can only move forward, with a dark shelter at the end. The paws were placed in the stamp ink and the animals walked on a white paper so that the appropriate measures are removed for analysis of the step length, print area (mm^2), box length (mm) and box width (mm) [15].

2.4. Biochemical analysis

After the behavioral tests, the rats were euthanized by anesthetic overdose and then decapitated after analyzes. The biochemical analyses were developed 1, 3, 5, and 7 days after lesion, as following described.

2.4.1. Tissue preparation

The gastrocnemius muscles were dissected, immediately homogenized in saline solution (NaCl 0.9%) and kept in ice. After homogenization, the skeletal muscle samples were centrifuged (at 2000 g and 4°C for 10 min) in order to obtain a slow-speed supernatant fraction (S1). The acquired S1 was used to measure TBARS, DCFRS non-protein thiol(-SH) groups, and also to determine the CAT and SOD enzymes activities. No bone fracture was observed in the dissected rat's legs.

2.4.2. Oxidative stress analysis

TBA-RS levels

Thiobarbituric acid Reactive Substances (TBARS) levels, malondialdehyde (MDA) mainly, were determined as an index of tissue lipid peroxidation according to the method described by Ohkawa et al. [16] Aliquots of 200 µL of skeletal muscle S1 were added to color reaction. TBA-RS levels were measured at 532 nm using a standard curve of MDA and corrected by the protein content.

DCFRS levels

Oxidized dichlorofluorescein (DCFRS) levels were determined as an index of the peroxide production by the cellular components. Skeletal muscle S1 samples (50 µL) were added to a medium containing a Tris-HCl buffer (0.01 mM; pH 7.4) and DCFH-DA (7 µM). After DCFHDA addition, the medium was incubated in the dark for 1 h until fluorescence measurement procedure (excitation at 488 nm and emission at 525 nm and both slit widths used were at 5 nm). DCFRS levels were determined using a standard curve of DCF and the results were corrected by the protein content [17].

MTT reduction levels

Methyl-tetrazolium (MTT) reduction levels were determined as an index of the dehydrogenase enzymes functions, which are involved in the cellular viability. Aliquots of skeletal muscle S1 (500 µL) were added to a medium containing 0.5 mg/mL of MTT and were incubated in the dark for 1 h at 37°C. The MTT reduction reaction was stopped by the addition of 1 mL of dimethyl sulfoxide (DMSO). The formed formazan levels were determined spectrophotometrically at 570 nm and the results were corrected by the protein content [18].

MPO enzyme activity

The MPO enzyme activity was determined in skeletal muscle S1 according to the method proposed by Grisham et al. [19] with some modifications. Briefly, a sample of the skeletal muscle preparation (20 µL) was added to a medium containing potassium phosphate buffer (50 mM; pH = 6.0), hexadecyltrimethylammonium bromide (0.5%), and N,N,N′, N′- tetramethylbenzidine (1.5 mM). The kinetic analysis of MPO was started after H2O2 (0.01%) addition and the color reaction was measured at 655 nm at 37°C.

NAG enzyme activity

The NAG enzyme activity was determined in skeletal muscle S1 according to the method proposed by Luqmani et al. [20] with some modifications. Briefly, a sample of the skeletal muscle preparation (50 µL) was added to a medium containing citrate buffer (0.2M/L; pH = 4.4) and N-acetyl-β-D-glucosaminidase (10mM/L) incubated for 15 min at 37°C. After incubated, interrupt the reaction with sodium carbonate buffer (0.5M/L). The kinetic analysis of NAG was measured at 580 nm at 37°C.

2.4.3. Antioxidant analysis

Non-protein thiol (-SH) groups levels

Non-protein (-SH) groups levels were determined in S1 skeletal muscle and erythrocytes samples according to the method described by Ellman [21], with few modifications. Firstly, the skeletal muscle S1 samples (1 mL) were precipitated with TCA (5%, 0.5 mL) and centrifuged (at 2000g and 4°C, for 10 min), in order to obtain the supernatant fraction S2. Thereafter, samples of S2 fraction (500 µL) were added to a reaction medium containing potassium phosphate buffer (TFK 0.25 mM, pH = 7.4) and DTNB (1 mM). SH non-protein levels were measured by spectrophotometry at 412 nm. The observed values were calculated according to a standard curve built with known GSH concentrations and corrected by the protein content.

CAT and SOD enzyme activities levels

Catalase (CAT) enzyme activity was measured in the skeletal muscle S1 according to the method described by Aebi [22]. A sample of skeletal muscle S1 (50 mL) was added in a mean containing potassium phosphate buffer (TFK 50 mM, pH = 7.4) and H2O2 (1 mM). The CAT kinetic analysis was initiated after the H2O2 addition; the color reaction was measured at 240 nm.

Cytosolic superoxide dismutase (Cu/Zn SOD) enzyme activity was measured in the skeletal muscle S1 according to the method described by Misra and Fridovich [23]. Different samples of skeletal muscle S1 (10 to 50 µL) were added in a

mean containing glycine buffer (50 mM, pH = 10.5) and adrenaline (1 mM). The SOD kinetic analysis was initiated after adrenaline addition; the color reaction was measured at 480 nm.

2.4.4. Protein measurement

Protein content was measured according to the method described by Lowry et al. [24] using bovine serum albumin as the standard measure.

2.5. Statistical analysis

All statistical analyses were done using the software Graph Pad Prism 6.0 for Windows. Kolmogorov-Smirnov and Bartlett's tests were performed for analyzed normality of data and homogeneity of variances, respectively. The effects on behaviors were analyzed by Friedman test followed by Dunn's post-Hoc test, for biochemistry analysis were used to two-way ANOVA followed by Tukey Post-Hoc test. Data are expressed as mean and standard error, and differences were considered significant when $p \leq 0.05$.

3. Results

3.1. Oxidative damage in injured muscle

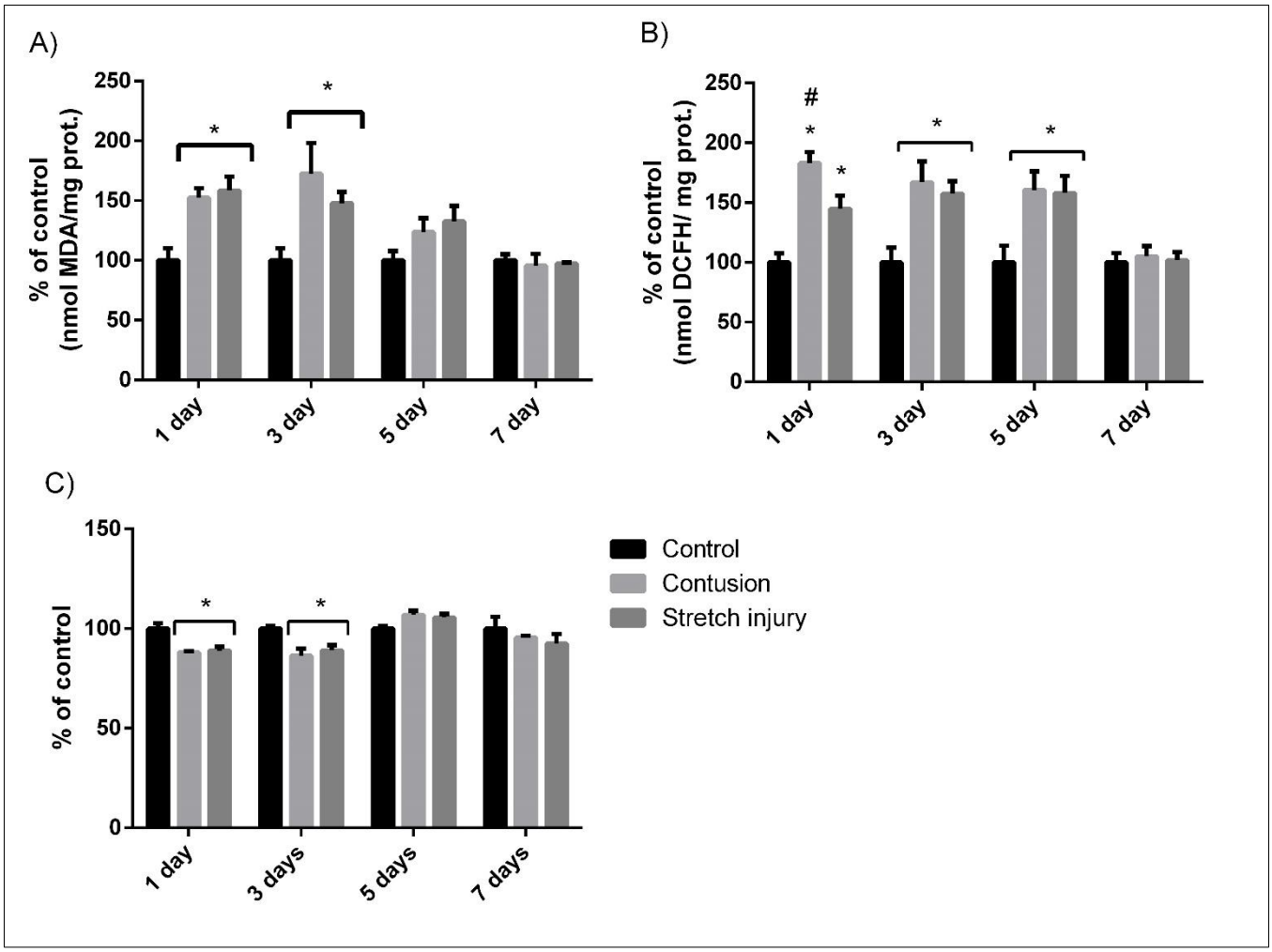

Figure 1 Injured muscle oxidative damage throughout time (1, 3, 5 and 7 days after injury) assessed by means of (A) the MDA levels in muscle, (B) the DCFRS levels and (C) the MTT reduction levels, expressed in percentage of control value. Values are presented as mean ± SEM and were analyzed by ANOVA (two-way), followed by Tukey test. Differences were considered significant when: * P <0.05 * = differences found between the control of their day without injury. # = significant difference found in comparison to the stretch injury group. $ = significant difference found in comparison to contusion group. N= 5/group.

The contusion and stretch injury caused increase levels of TBARS (figure 1A) in 1st day ($p < 0.01$, mean diff. = -52.39, 95% CI of diff. = -93.30 to -11.47 and mean diff. = -58.33, 95% CI of diff. = -99.25 to -17.42, respectively), and maintain high levels until 3rd day compared to control ($p < 0.05$, mean diff. = -72.51, 95% CI of diff. = -113.4 to -31.59 and $p < 0.05$, mean diff = -73.76, 95% CI of diff. = -88.67 to -6.846).

Similarly, in the first day after contusion and stretch injury was observed an increase in the ROS levels when compared to control ($p < 0.01$; mean diff. = -81.12, 95% CI of diff. = -119.6 to -46.65 and $p < 0.05$, mean diff. = -44.98, 95% CI of diff. = -81.44 to -8.51, respectively), and a significant difference between contusion and stretch group was observed ($p < 0.05$, mean diff = 38.14, 95% CI of diff. = 1.676 to 74.60) (figure 1B). Both the lesions maintained the increase of DCFRS levels (figure 1B) in 3rd day ($p < 0.05$, mean diff. = -66.97, 95% CI of diff. = -121.3 to -12.63 and $p < 0.05$, mean diff.= -57.38, 95% CI of diff. = -111.7 to -3.03) and in the 5th day of analysis ($p < 0.05$, mean diff. = -60.43, 95% CI of diff. = -118.3 to 2.58 and mean diff. = -58.07, 95% CI of diff. = -115.9 to -0.22, respectively) when compared to control conditions uninjured.

In the same way, MTT reduction levels (figure 1C) decrease on the 1st day after the injury in both muscle injuries (contusion group: $p < 0.05$, mean diff. = 11.99, 95% CI of diff. = 4.24 to 19.73 and stretch injury group: $p < 0.05$, mean diff. = 11.01, 95% CI of diff. = 3.26 to 18.76) and were lower than control values until the 3rd day after the injury onset (contusion group: $p < 0.05$, mean diff. = 13.60, 95% CI of diff. = 3.03 to 24.15 and stretch injury group: $p < 0.05$, mean diff. = 10.95, 95% CI of diff. = 0.39 to 21.50).

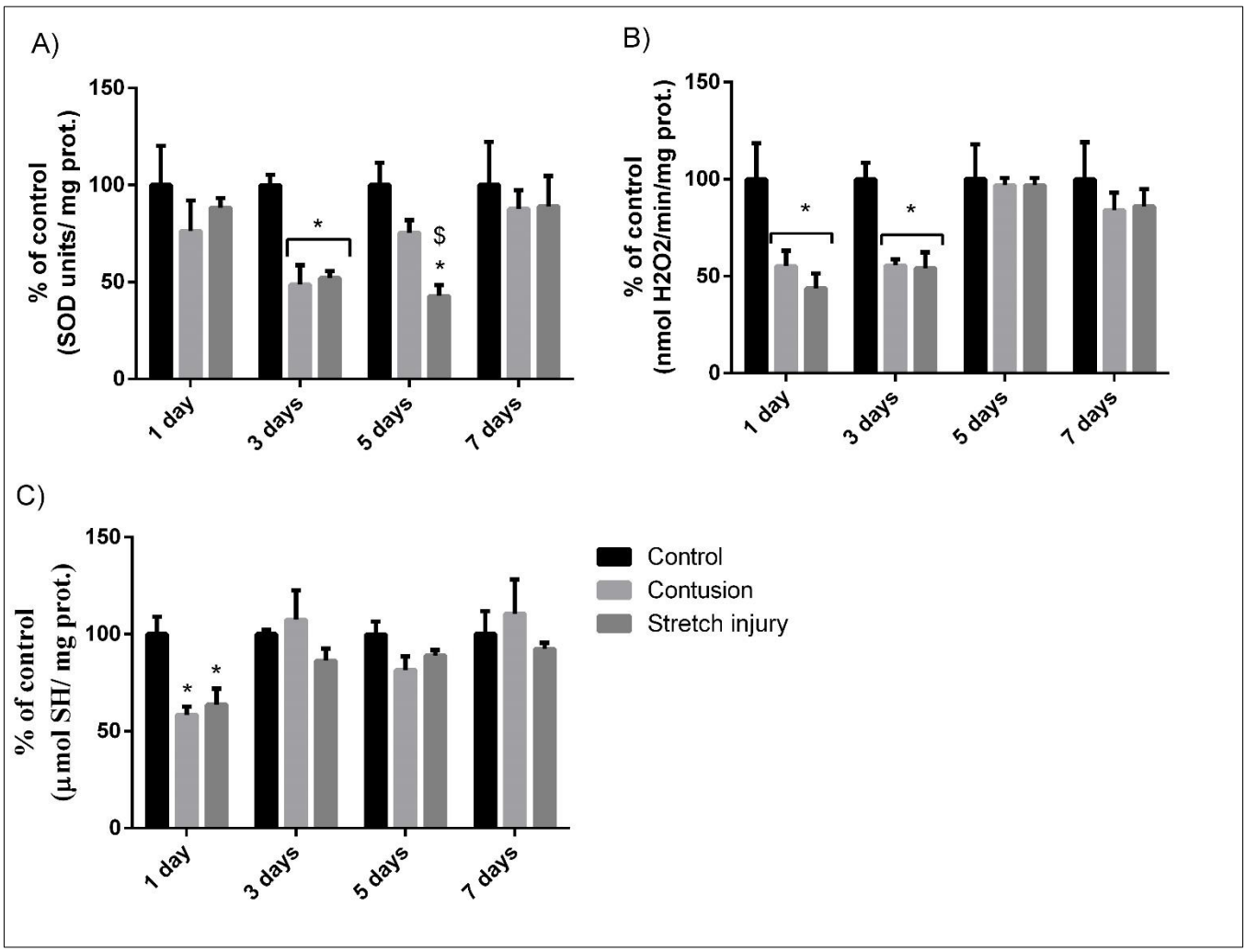

Figure 2 Injured muscle antioxidant profile throughout time (1, 3, 5 and 7 days after injury) assessed by means of SOD enzyme activity and (B) CAT enzyme activity and (C) the SH levels, expressed in percentage of control value. Values are presented as mean ± SEM and were analyzed by ANOVA (two-way), followed by Tukey test. Differences were considered significant when: * $P < 0.05$. * = differences found between the control of their day without injury. # = significant difference found in comparison to the stretch injury group. $ = significant difference found in comparison to contusion group. N= 5/group.

The figure 2A shows the SOD enzyme activity in the skeletal muscle after the muscle injury. The muscle injuries decreased the SOD activity in comparison to control group in the 3rd day after the injury onset (contusion group: p <0.005, mean diff. = 51.35, 95% CI of diff. = 24.37 to 78.33 and stretch injury group: p <0.05, mean diff. = 47.91; 95% CI of diff. = 20.93 to 74.90). Although SOD activity after contusion was normalized in 5th day, the stretch injury maintained lower SOD activity compared to control (p <0.005, mean diff. = 57.25, 95% CI of diff. = 24.62 to 89.87) and contusion (p <0.05, mean dif. =32.65, 95% CI of diff. = 0.01 to 65.27).

Similarly, both muscle injuries determined a decrease in the skeletal muscle CAT activity in relation to control group in 1st day (contusion group: p <0.05, mean diff. = 48.76, 95% CI of diff. = 1.27 to 96.25 and stretch injury group: p <0.05, mean diff. = 56.27, 95% CI of diff. = 8.77 to 103.8); and in 3rd day compared to control uninjured (contusion p <0.05, mean diff. = 44.48, 95% CI of diff. = 16.92 to 72.03 and stretch p <0.005, mean diff. =45.86, 95% CI of diff. = 18.30 to 73.41) (figure 2B).

Moreover, both muscle injuries decreased significantly the non-protein thiol group (-SH) levels (figure 2C) when compared to control in 1st day after injury muscle (Contusion group: p <0.005, mean diff. = 41.41, 95% CI of diff. = 12.39 to 70.64 and stretch injury group: p <0.05, mean diff. = 36.37, 95% CI of diff. = 7.23 to 65.49).

3.2. Inflammation in injured muscle

The figure 3A shows that contusion and stretch injuries increased the MPO enzyme activity in the 1st day (p <0.05, mean diff. = -221.3, 95% CI of diff. = -391.7 to -50.98 and p <0.001, mean diff. = -305.4, 95% CI of diff. = -475,7 to -135,1). Furthermore, the NAG activity was also increased from the first day after the stretch injury (p <0.001, mean diff. = -37.78, 95% CI of diff. = -57.58 to -17.97 and p <0.005, mean diff. =-28.46, 95% CI of diff. = -48.26 to -8.651) to the 3th day (p <0.005, mean diff. = -32.44, 95% CI of diff. = -52.52 to -12.37 and p <0.05, mean diff. = -21.38, 95% CI of diff. = -41.46 to -1.30) when compared to the control and contusion group. Although in the 7th the NAG activity of stretch injury muscle was higher than control (p <0.05, mean diff. = -22.31, 95% CI of diff. = -39.79 to -4.82), the contusion muscle NAG activity was higher than the stretch injury muscle levels (p <0.001, mean diff. = -47.58, 95% CI of diff. = -65.07 to -30.10 and p <0.05, mean diff. =25.27, 95% CI of diff. = 7.78 to 42.76).

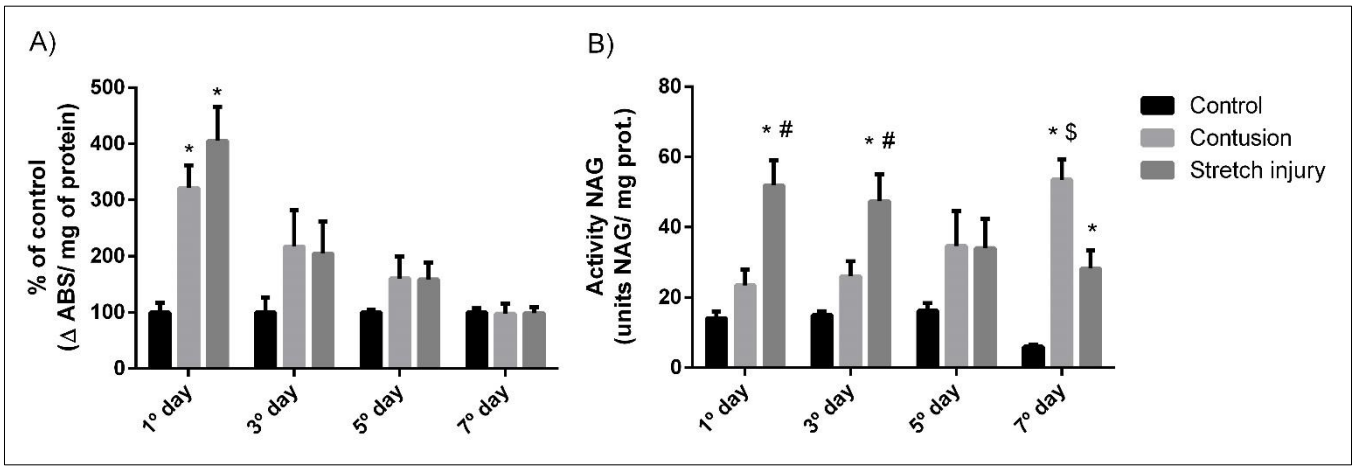

Figure 3 Injured muscle inflammatory enzyme activities throughout time (1, 3, 5 and 7 days after injury) assessed by means of (A) MPO enzyme activity and (B) NAG enzyme activity, expressed in percentage of control value and units NAG, respectively. Values are presented as mean ± SEM and were analyzed by ANOVA (two-way), followed by Tukey test. Differences were considered significant when: * P <0.05. * = differences found between the control of their day without injury. # = significant difference found in comparison to the stretch injury group. $ = significant difference found in comparison to contusion group. N= 5/group.

3.3. Motor behavior impairment after lesion

Contusion and stretch injury increased thigmotaxis behavior in rats since it reduced the number of entries and time spent in the central quadrant of the apparatus (p <0.05) (Fig.4A). Similar results were observed in the mean speed (Fig.4B) and travelled distance (Fig.4C). The stretch injury animals demonstrated a decreased thigmotaxis behavior in the first hours in the 1st day.

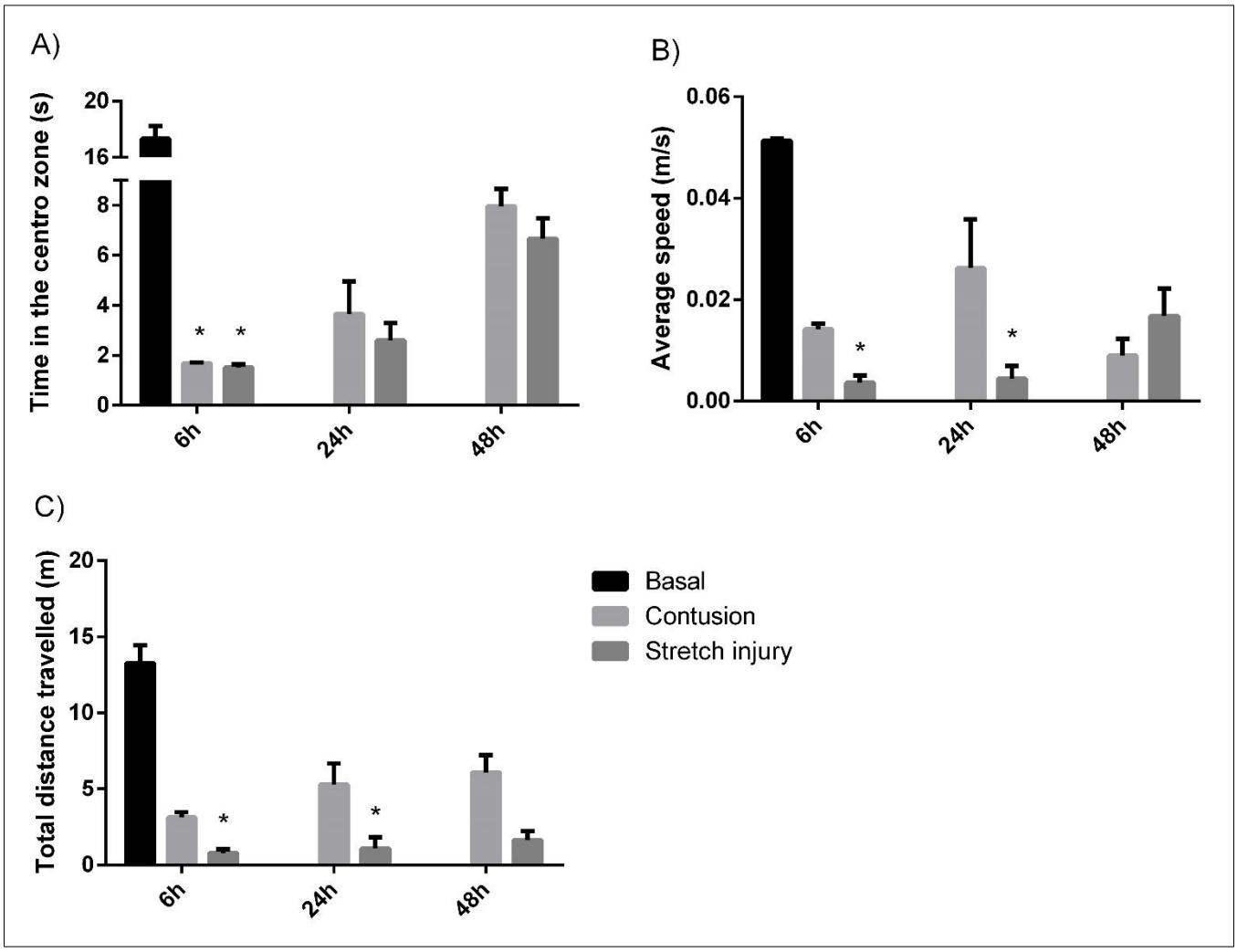

Figure 4 Chances in animal's thigmotaxis behavior along time (6, 24 and 48h after injury) assessed by means of (A) Time in the center zone, (B) average speed and (C) total distance travelled. Values are presented as mean ± SEM and were analyzed by Friedman test followed by Dunn's test. Differences were considered significant when: * $P < 0.05$. * = differences found between the control of their day without injury. # = significant difference found in comparison to the stretch injury group. $ = significant difference found in comparison to contusion group. N= 6/group.

In the figure 5A the results show that contused and stretched animals presented changes in hot sensitivity at 6 h after muscle injuries ($p < 0.05$), decreasing latency of paw licking to 58% and 40%, respectively. However, the beam walk score was increased when compared to control group in 6 and in 24 hours after injury ($p < 0.05$) (Fig. 5B). Moreover, in 6 hours the beam walk score of stretched animals was significantly higher than the contused animals ($p < 0.05$).

The peripheral tibial nerve function index (TFI) (Fig. 6A) was significantly reduced at 6 hours both in contused and stretched when compared to the control animals ($p < 0.05$). At 24 hours only contused animals maintained a reduced TFI levels ($p < 0.05$). Moreover, the peripheral sciatic nerve function index (SFI) was reduced in stretched animals at 6 hours ($p < 0.05$), and in contused animals only at 24 hours ($p < 0.05$) after the lesion onset (Fig 6B).

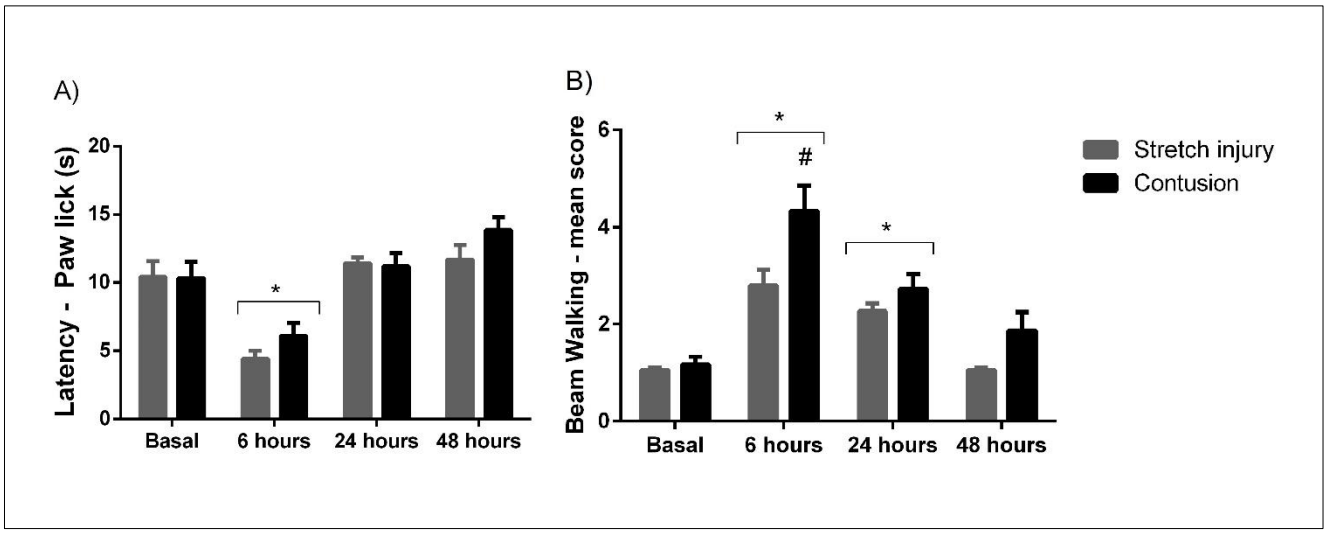

Figure 5 Chances in animal's hot sensitivity along time (6, 24 and 48h after injury) assessed by means of (A) latency – paw lick (s) and (B) Beam Walking – mean score. Values are presented as mean ± SEM and were analyzed by Friedman test followed by Dunn's test. Differences were considered significant when: * $P < 0.05$. * = differences found between the control of their day without injury. # = significant difference found in comparison to the stretch injury group. $ = significant difference found in comparison to contusion group. N= 6/group.

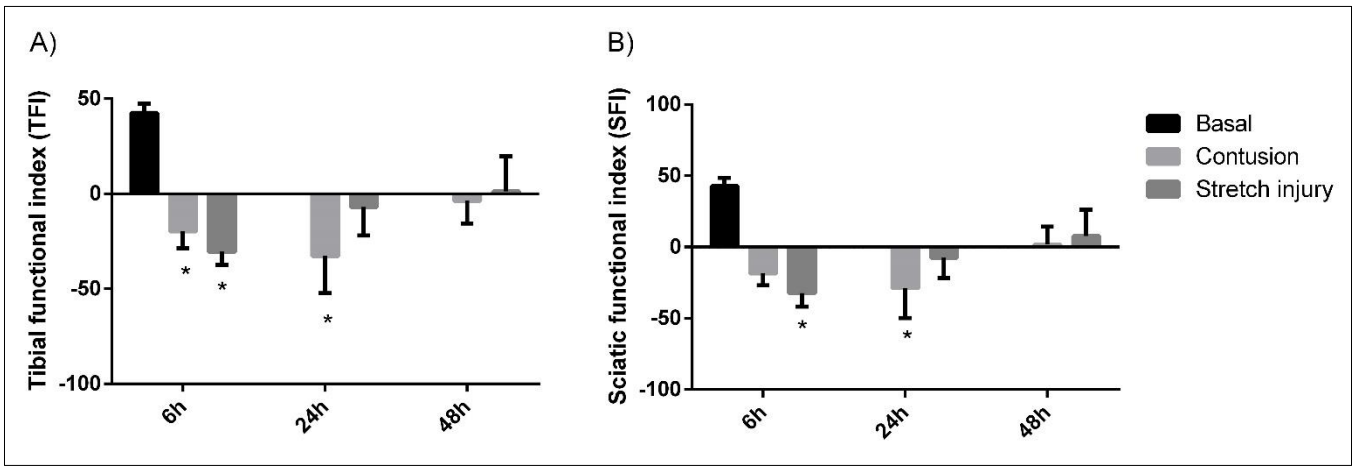

Figure 6 Chances in animal's peripheral nerve function along time (6, 24 and 48h after injury) assessed by means of (A) tibial functional index (s) and (B) sciatic functional index. Values are presented as mean ± SEM and were analyzed by Friedman test followed by Dunn's test. Differences were considered significant when: * $P < 0.05$. * = differences found between the control of their day without injury. # = significant difference found in comparison to the stretch injury group. $ = significant difference found in comparison to contusion group. N= 6/group.

4. Discussion

The major motivation of the experimental protocols proposed in this study was to compare some oxidative, inflammatory and behavioral changes determined by two prevalent sport lesions (contusion and stretch injury). Aiming to mathematically prove each injury specificity, which is fundamental to properly elaborate rehabilitation protocols, we performed the two-way ANOVA test to compare the evolution time of each lesion. Thus, based on our analysis, we observed differences in parameters such as DCFH levels, SOD and NAG activity. In addition, regarding the lesion type, beam walking test results were also analyzed. The stretch injury seems to have less intense biochemical and behavioral changes and its rehab is faster than the contusion.

We observed that both contusion and stretch injuries determined a remarkable oxidative damage in muscle tissue, which was accompanied by changes in animal's motor behavior in the early stage after injury (within the first 24 hours). These types of injury result in functional impairment due to alterations such as capillary rupture or rupture of the sarcolemma and myofilaments [5] generating an inflammatory process and necrosis, being the neutrophils the first inflammatory cells to reach the injury site followed by macrophages. With the neutrophils presence, enzymes such the myeloperoxidase, contribute to ROS generation, which is important to promote the damage tissue destruction. However, the excessive ROS production, over the cellular antioxidant systems capacity to scavenge them, could lead to an inflammatory process exacerbation, determining the damage to myofibrils [2].

Regeneration after skeletal muscle injury generally includes three stages: the first stage, which appears in 1–3 days after the skeletal muscle injury, showing local swelling, hematoma, muscle tissue necrosis and inflammatory reaction associated with ROS increase. The second stage is the repair period, which generally occurs 5–10 days after skeletal muscle injury, when necrotic muscle tissue is phagocyted and starts the myocytes regeneration. The third stage is the shaping stage of muscle tissue, which usually begins 2–3 weeks after skeletal muscle injury, when occurs the myocytes maturation and scar tissue formation [25]. Both contusion and stretch injury determined an increase in the oxidative damage, resulting from an excessive ROS generation determined by muscle injuries that could extrapolate the lesion site and increase the results in the oxidative damage of blood tissue components as well. In this study, we did not measure oxidative damage in blood tissue, but we observed a significant increase in TBARS levels after musculoskeletal lesions, as observed in previous studies [2, 8].

ROS are defined as partially reduced oxygen metabolites that have strong oxidation capacities, that is, they are harmful to cells when presented in high concentrations, damaging DNA and oxidizing proteins and lipids of cellular constituents. However, in low concentrations, these free radicals perform complex signaling functions, responsible for regulating cell growth and adhesion, differentiation, senescence and apoptosis [26]. In the present study, we observed that injured muscles have high levels of DCFH in the initial hours after injury, suggesting an increase in H_2O_2 formation, mainly in muscle contusion. Two-way ANOVA revealed that both musculoskeletal injuries determined a significant oxidative damage up to the 5th day after the onset.

Molecular electron transfer is a tightly controlled process, which in physiological situations, only 1% –2% of the electrons that leak in this process react with oxygen, resulting in a superoxide anion (O2-). The main production sites for O2- in the electron transport chain are complexes I and III with this formation being more prevalent in complex I of skeletal muscle tissues. This O2- generated by mitochondria can react with the SOD enzyme in the mitochondrial matrix to generate H_2O_2, which can cross the outer mitochondrial membrane to access cytosolic targets. This can lead to several functional results, such as activation of transcription factors sensitive to the redox system, such as Hypoxia-inducible factors (HIF-1α) and kappa nuclear factor B (NF-κB), activation of pro-inflammatory cytokines and activation of inflammasomes. Once H_2O_2 is formed, it can also be removed to prevent hydroxyl radical generation. The main route involves the decomposition of H_2O_2 in water by the CAT enzyme and glutathione peroxidase. CAT is mainly found in peroxisomes and, in a small extent, in cytosol. This enzyme is also very important in H_2O_2 metabolism, especially in high peroxide concentrations [26]. In the MTT tetrazolium reduction assay, similar to the results found in previous studies [2; 8; 9], injured muscles had a reduced cell viability in the early lesion stages.

Hartmann et al. [27] show that muscle contusion is able to increase the oxygen flow depending on the synthesis of ATP and OXPHOS in the oxidative and mixed fibers when stimulated by complex III substrates. Furthermore, muscle damage increases the production of H_2O_2 and reduces the activity of the enzyme citrate synthase. In our results, we observed that both injuries decreased activity of CAT enzyme until 3 days after the lesion. The reduction in catalase activity may indicate that, under stress conditions, the H_2O_2 produced can be more consumed in oxidative processes, such as in lipid peroxidation (as observed in TBARS), than eliminated from the metabolism by the action of the catalase enzyme [28]. Another explanation for reduced CAT activity is the decreased H_2O_2 levels, because it is a SOD activity product, which was reduced until the 3th day after contusion, and until the 5th day after the stretch injury.

Oxidative stress is responsible for increasing the expression of inflammatory mediators such as cytokines, chemokines and adhesion molecules. In response, first neutrophils and then macrophages infiltrate the tissue determining a higher ROS production and an inflammatory cascade exacerbation [29]. In the acute phase of skeletal muscle healing an extensive infiltration of inflammatory cells, predominantly neutrophils, is observed in injured tissues. In inflamed tissues, where neutrophils are abundant, H_2O_2 and chloride generate HOCl by the enzyme myeloperoxidase, generally considered as being a neutrophils specific enzyme [30]. In the present study, we observed that both lesions increased MPO activity in the 24 hours after injury, indicating a possible increase in neutrophils presence in the musculoskeletal tissue. Similarly, in other studies, the MPO enzyme activity in injured tissues was used as an intensity indicator in acute inflammatory response [2, 8, 9].

Ghaly and Marsh [29] showed that an increased neutrophils infiltration is evident in the early stages after muscle contusion. Their study indicates that neutrophils start the inflammatory process (which presence is negligible after the third day of lesion) followed by macrophage recruitment and activation, resulting in increased oxidative damage in injured tissues.

NAG, is a lysosomal enzyme activated in the presence of macrophage, constituting a parameter for the assessment of macrophage activity [20]. In the present study, we observed that the NAG activity increased in the first hours after stretch injury while in contusion this increase was observed only after the 7th day of injury onset. These results indicate that the macrophage recruitment was different according to the injury type, being more premature in stretched muscles. Robbertson et al. [31] observed that the chemostatic response of macrophages to injured muscle was pronounced in 24h after injury, which corroborates with the increased NAG enzyme activity in stretch injury.

Although the mechanisms responsible for macrophages chemotaxis are poorly understood, the injured muscle probably releases a variety of signaling molecules responsible for recruiting these cells, but the resulting contributions and interactions of neutrophils and macrophages in tissue repair remains unclear [32]. However, Novak et al. [33] describe that expression of the macrophage-associated cytokines as elevated IL-1β and TNFα in the muscle 1- and 3-days post laceration and subsequently declined towards uninjured levels. 3 days after injury, muscle macrophages can exhibit high levels of IL-10 mRNA expression and protein, a powerful anti-inflammatory cytokine, which may contribute to the macrophage deactivation throughout the inflammatory period [33]. Ghaly and Marsh [29] describe the importance of sufficient macrophage infiltration and TGF-b1- induced activation of COX-2 pathways for muscle regeneration. However, if excessive, the phagocytic activities of neutrophils and macrophages after injury are not benign. The infiltration and activation of neutrophils are especially cytotoxic and have been linked to secondary tissue damage. Thus, the balance between beneficial inflammation that facilitates muscle regeneration and deleterious inflammation that inhibits muscle regeneration and promotes fibrotic scar formation is extremely important. The skeletal muscle regeneration happens because of the satellite cells presence with fusion and differentiation in multinucleated myotubes that contribute for the myofibers formation after muscle injuries [34].

Regarding behavioral analysis, we observed that the stretch injury significantly decreased the animal's locomotor activity in the early stages after injury what was not observed after contusion. However, both injuries decreased the time spent by the animals on the central zone of open field after 6 hours, indicating a possible state of anxiety after muscle injury [35]. The balance beam performance is a useful measure of fine coordination and balance, where it is possible to detect motor deficits after injuries. We observed that the animals submitted to both injuries showed and impairment of balance and coordination in the first 24 hours, but not after 48 hours. To the best of our knowledge, this is the first study addressing motor skills balance and coordination impairment after an injury in rats *through the* beam walking test.

It is well established that pain cannot be monitored in a direct form in animals, but it can only be estimated through the analysis of their responses. Most of what is known about pain mechanisms is derived from rodent models of somatic nociception and hyperalgesia. In tests based on the use of thermal stimuli such as the hot plate, the latency of nocifensive reactions are obtained by heat stimuli of constant supra-threshold intensity. Our findings indicated a decrease in the nociceptive threshold in tissues injured by contusion or stretch, indicating a very rapid hyperalgesic response (within 6h after lesion onset). The sciatic functional index (SFI) and the tibial functional index (TFI) are reliable indexes of functional recovery following peripheral nerve injury and repair in mouse models. Although our study does not generate any type of direct lesion on the peripheral spinal nerves, we observed that both the stretched and contused animals presented a decrease of STF and TFI in the first 24 hours.

5. Conclusion

Macrophages were already observed in the site of skeletal muscle damage in the initial 3h after injury, but their presence significantly increase after 48h. However, the stretch injury apparently seems to present an early macrophage recruitment (in the first hours after injury).

Therefore, we evidenced significant differences in oxidative, inflammatory and behavioral responses in rats according to the specific skeletal muscle injury. More studies are needed to corroborate the molecular specificity of these and other skeletal muscle injuries, giving basis for treatment strategies that may effectively rehab these sports injuries, minimizing unnecessary costs and accelerating the return of athletes to their practice.

Limitations of study

No studies were found comparing types of musculoskeletal injuries to help substantiate the mechanisms generated by the injuries. It is suggested that more studies are needed to elucidate the biochemical and behavioral effects, especially studies investigating the different musculoskeletal lesions in mitochondrial functions, aiming to understand their effects on inflammatory mediators.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors have no competing interests to declare that are relevant to the content of this article.

Statement of ethical approval

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. Animals were kept and used according to the Care Commission and the Experimental Animals Use Guidelines of the Federal University of Santa Maria, Brazil, registered and approved by the committee of ethics of animal use with the number 4694151216.

Author's contributions

Conceptualization, R.P.M; Methodology and Formal Analysis, R.P.M, D.D.H, A.B.V.F, D.F.G and T.C.D; Writing-Original Draft Preparation, R.P.M and D.D.H; Writing-Review & Editing, L.U.S, F.A.A.S and G.O.P; Project Administration, G.O.P. All authors have read and approved the final submitted manuscript.

References

[1] A Fernandes, T. L., Pedrinelli, A., and Hernandez, A. J. Muscle injury – physiopathology, diagnosis, treatment and clinical presentation. Rev. Bra. de Ortopedia. 2015; 46(3):247-55. doi: 10.1016/s2255-4971(15)30190-7.

[2] Martins R. P., Hartmann D. D., Moraes J. P., Soares F. A. A. and Puntel G. O. Platelet-rich plasma reduces the oxidative damage determined by a skeletal muscle contusion in rats. Platelets. 2016; 27(8):784-790. doi: 10.1080/09537104.2016.1184752.

[3] Zembrom-Lancy A., Morawin B., Wawrzyniak-Gramacka E., Gramacki J., Kotlega D., Zieman E. Multiple cryotherapy attenuates oxi-inflammatory response following skeletal muscle injury. Int. Jour. Of environmental research and public health. 2020; 17(21):7855. doi: 10.3390/ijerph17217855.

[4] Smith, C., Kruger, M. J., Smith, R. M., Myburgh, K. H. The Inflammatory Response to Skeletal Muscle Injury. Sports Medicine. 2008; 38(11):947-69. doi: 10.2165/00007256-200838110-00005.

[5] Järvinen T. A., H., Jarvinen T. L. N., Kaariainen M., Aarimaa V., Vaittinen S., Kalimo H., Jarvinen M. Muscle injuries: optimising recovery. Best Practice Res. Clin. Rheumatol. 2007; 21(2):317-31. doi: 10.1016/j.berh.2006.12.004.

[6] Ozaki G., Camargo R. C. T., Koike T. E., Garcia T. A., Castoldi R. C., Camargo Filho J. C. S. Fractal Analysis of Skeletal Muscle Tissue of Rats Subjected to Stretch Injury. Int. J. of Morphology. 2015; 33(3):908-913 doi: 10.4067/s0717-95022015000300017.

[7] Quintero A. J., Wright V. J., Fu F. H., Huard J. Stem cells for the treatment of skeletal muscle injury. Clin Sports Med. 2009; 28(1):1-11. doi: S0278-5919(08)00066-5 [pii]\r10.1016/j.csm.2008.08.009.

[8] Puntel G. O., Carvalho N. R., Amaral G. P., Lobato L. D., Silveira S. O., Daubermann M. F., et al. Therapeutic cold: an effective kind to modulate the oxidative damage resulting of a skeletal muscle contusion. Free Radical Res. 2011, 45: 125–138. doi: 10.3109/10715762.2010.517252.

[9] Martins R. P., Hartmann D. D., Furtado A. B. V., Duarte B. S. L., Signori L. U., Soares F. A. A., et al. Combined platelet-rich plasma and cold water immersion treatment minimize the damage following a skeletal muscle stretch injury in rats. Platelets. 2020; 31(8):1039-1051 doi: 10.1080/09537104.2019.1704718.

[10] Tavares Costa, C. F., Souza, A. C., & Vargas, M. M Injuries physical, psychological stress and coping in football players. RBFF – Rev. Bra. de Futsal e Futebol. 2019; 11(43).

[11] Martins C. N., Moraes M. B., Hauck M., Guerreiro L. F., Rossato, D. D., Varela A. S., et al. Effects of cryotherapy combined with therapeutic ultrasound on oxidative stress and tissue damage after musculoskeletal contusion in rats. Physiotherapy (United Kingdom). 2016; 102(4), 377–383. doi: 10.1016/j.physio.2015.10.013.

[12] Gould, T. D., Dao, D. T., & Kovacsics, C. E. The Open Field Test. Neuromethods, 2009; 1–20. doi:10.1007/978-1-60761-303-9_1.

[13] Espejo E. F. and Mir, D. Strucutre of the rat's behaviour in the hot plate test. Behav. Brain Res. 1993; 56:2 171–176. doi: 10.1016/0166-4328(93)90035-O.

[14] Finger, S. & Stein, D. G. Brain damage and recovery: research and clinical perspectives. Academic Press. New York, 1982; 175-202. doi:10.1001/jama.1983.03340030068038.

[15] Bain, J. R., Mackinnon, S.E, Hunter D.A. Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesion in the rat. Plast. Reconstr Surg. 1989, 83(1):129-136. doi: 10.1097/00006534-198901000-00024.

[16] Ohkawa H., Ohishi N., Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry. 1979; 95(2):351-8 doi: 10.1016/0003-2697(79)90738-3.

[17] Pérez-Severiano F., Rodríguez-Pérez M., Pedraza-Chaverri J., Maldonado P. D., Medina-Campos O. N., Ortíz-Plata A., et al. S-Allylcysteine, a garlic-derived antioxidant, ameliorates quinolinic acid-induced neurotoxicity and oxidative damage in rats. Neurochem. 2004; 45:1175–1183. doi: 10.1016/j.neuint.2004.06.008.

[18] Bernas, T., & Dobrucki, J. Mitochondrial and nonmitochondrial reduction of MTT: Interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry. 2002; 47(4):236-42. doi: 10.1002/cyto.10080.

[19] Grisham, M. B., Jefferson, M. M., Melton, D. F., & Thomas, E. L. Chlorination of endogenous amines by isolated neutrophils. Ammonia-dependent bactericidal, cytotoxic, and cytolytic activities of the chloramines. J. Biol. Chem. 1984; 259(16):10404-13.

[20] Luqmani Y Y., Temmin L., Memon A., Abdulaziz, L. Pakar A., Ali M., et al. Mensurement of serum N-acetyl-b-glucosamidase activity in patients with breast cancer. Acta oncológica. 1999; 38:5, 649-653.

[21] Ellman G. L. Tissue sulfhydryl groups. Arch. Biochem. Biophys; 1952; 82(1): 70-77. doi: 10.1016/0003-9861(59)90090-6.

[22] Aebi H. Catalase in vitro. Method Senzymol. 1984; 105:121–126. doi: 10.1016/s0076-6879(84)05016-3.

[23] Misra H. P., Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. 1972; 247(10):3170-5.

[24] Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J. Protein measurement with the folin phenol reagent. J. Biol. Chem. 1951; 193:265–75.

[25] Xiao W., Liu Y., Chen P. Macrophage depletion impairs skeletal muscle regeneration: the roles of pro-fibrotic factors. Inflam. Oxid. Stress Inflam. 2016; 39, 2016–2028. doi: 10.1007/s10753-016-0438-8.

[26] Schieber M., Chandel N. S. ROS Function in Redox Signaling and Oxidative Stress. Curr. Biol. 2014; 24(10): 453–462. doi: 10.1016/j.cub.2014.03.034.

[27] Hartmann D. D., Gonçalves D. F., Da Rosa P. C., Martins R. P., Courtes A. C., Franco J. L., et al. A single muscle contusion promotes an immediate alteration in mitochondrial bioenergetics response in skeletal muscle fibres with different metabolism. Free Radic. Res. 2020; 54(2-3):137-149. doi: 10.1080/10715762.2020.1723795.

[28] Carmak, I., Horst, W. J. Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase and peroxidase activities in root tips of soybean. Physiologia Plantarum. 1991; 834:463-468. doi: 10.1111/j.1399-3054.1991.tb00121.x.

[29] Ghaly A., Marsh D. R. Ischaemia-reperfusion modulates inflammation and fibrosis of skeletal muscle after contusion injury. Int J Exp Pathol. 2010; 91(3):244-55. doi: 10.1111/j.1365-2613.2010.00708.x.

[30] Mittal M., Siddiqui M. R., Tran K., Reddy S. P., Malik A. B., Reactive oxygen species in inflammation and tissue injury. Antioxid. Redox Signal., 2014; 1126-1167. doi: 10.1089/ars.2012.5149.

[31] Robertson T. A., Maley M. A. L., Grounds M. D., Papadimitriou J. M. The role of macrophages in skeletal muscle regeneration with particular reference to chemotaxis. Exp. Cell Research, 1993; 207, 321-331. doi: 10.1006/excr.1993.1199.

[32] Butterfield T. A., Best T. M., Merrick M. A. The dual roles of neutrophils and macrophages in inflammation: A critical balance between tissue damage and repair. J. Athl. Train. 2006; 41(4): 457-465.

[33] Novak M. L., Weinheimer-Haus E. M., Koh T. J. Macrophage activation and skeletal muscle healing following traumatic injury. J. Pathology. 2014; (232) 3: 344-355. doi: 10.1002/path.4301.

[34] Yin H., Price F., Rudnicki M. A. Satellite cells and the muscle stem cell niche. Physiol Rev. 2013; 93(1):23-67. doi: 10.1152/physrev.00043.2011.

[35] Svennigsen A. F., Dahlin L. B. Repair of the peripheral nerve – Remyelination that Works. Brain Sci. 2013; 3(3):1182-97. doi: 10.3390/brainsci3031182.