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Sonophoresis with Nanostructured Lipid Carrier Gel Containing Quercetin for Muscle Injury Treatment in Rats

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SUMMARY

Background. Quercetin has therapeutic potential in the recovery of musculoskeletal injuries, but orally, this polyphenol presents poor absorption because of its poor water solubility and structural instability. However, its penetration through the skin can be enhanced by quercetin-loaded nanostructured lipid carriers (NLC-Q), even more, when associated with pulsed therapeutic ultrasound (PUT).

Objective. To evaluate the effect of sonophoresis PTU with a gel NLC-Q on biochemical parameters after traumatic muscle injury of the gastrocnemius muscle.

Methods. Forty male Wistar rats were homogeneously divided into five groups: Control; Lesion (no treatment); PTU treated lesion; NLC-Q gel treated lesion and Sonophoresis (PTU with NLC-Q gel) treated lesion. The gastrocnemius muscle was lesioned by mechanical crushing and treatments began 24h after the injury. Interventions were performed at intervals of 12h, making a total of 5 sessions lasting 5 min each. The 1 MHz PUT (0.1 W/cm^{2SATA}, pulsed wave: 20%); NLC-Q gel was spread over the lesion area and sonophoresis used PTU with NLC-Q gel. Serum and muscle creatine kinase (CK) concentrations and muscle oxidative stress (concentration of reactive oxygen species [ROS], lipid peroxidation, protein carbonyls, and the activities of SOD and Catalase enzymes) were evaluated 96h after the lesion.

Results. Blood CK increased only in the untreated Lesion group. Sonophoresis reduced SOD activity, concentration of ROS, lipid peroxidation, and protein oxidation in relation to the Lesion group ($p < 0.05$), but it was similar to the other interventions.

Conclusions. Sonophoresis, PUT, and NLC-Q Gel contributed to the repair of muscle damage and reduced oxidative stress parameter

KEY WORDS

Inflammation; solid lipid nanoparticles (SLN); sonophoresis; skeletal muscle; ultrasonic therapy.

INTRODUCTION

Contusions are the most frequent mechanisms in sports injuries (1) and degeneration/inflammation occurs within the first few minutes after the injury and may last until the second week. At this stage, tissue disruption occurs, leading to the activation of macrophages, and the entry of inflammatory cells (neutrophils and monocytes) into the injured site (2). Chemotactic signals lead to tissue reconstitution via proliferation and differentiation of satellite cells, from the activation of myogenic precursors followed by the formation of scar tissue by fibrin and fibronectin and the activation of more leukocytes to the site (3). This signaling is

due to oxidative stress, in which inflammatory responses to pro-oxidant agents prevail over antioxidants (4). Pro-oxidants are formed by reactive oxygen and nitrogen species, which alter the molecular functions of cells directly (lipoperoxidation, damage to proteins and DNA) and/or indirectly (leukocytes), generating damage to the constitution of myofibrils (5).

At this stage, therapeutic interventions that attenuate the excess production of reactive oxygen and nitrogen species are necessary to minimize the secondary damage derived from the excessive stimulation of pro-inflammatory pathways (6). Thus, exogenous non-enzymatic antioxidants as a therapeutic method, such as quercetin, indicate a favorable action for redox balance restoration (7), for its antioxidant and anti-inflammatory abilities (8). Quercetin ($C_{15}H_{10}O_7$) is a dietary polyphenol that belongs to the flavonoid class. However, due to its low bioavailability, and poor absorption because of its poor water solubility, structural instability, and extensive first-pass metabolism, there is a challenge for it to incorporate high levels of quercetin in its free form in oral therapeutics application (9).

There are several methods for dermal and transdermal drug delivery based on carrier nanotechnology and on the equipment employed to overcome the skin permeability barrier, improving the bioavailability of the active ingredient (10). The use of lipid-based drug carrier vehicles is a solution to overcome the low bioavailability and systemic stability of quercetin (8). Nanostructured lipid carriers are composed of solid and liquid lipids, forming an imperfect crystalline structure. This imperfect structure will support higher drug loading, with higher retention efficiency, and physical stability during storage, which increases apparent solubility, controls the release rate, and improves the bioavailability of encapsulated lipophilic compounds (11).

To effectively achieve transdermal drug distribution and increase drug penetration, nanocarriers can be applied synergistically, with physical methods (12, 13). Sonophoresis is the application of pulsed therapeutic ultrasound (PTU) to facilitate the transport, absorption, and distribution of drugs through the skin and soft tissue. This occurs during or after the influence of a disturbance of ultrasonic waves on the tissues (14). However, this interaction of sonophoresis with quercetin-containing nanostructured lipid carriers gel (NLC-Q) has not yet been tested in musculoskeletal injury treatment, and its mechanisms are still unclear. This research investigates the effects of sonophoresis (PTU with NLC-Q) on creatine kinase concentrations (muscle and blood) and oxidative stress parameters of rats submitted to muscle lesion by contusion.

MATERIALS AND METHODS

Experimental design

Animal handling was performed according to the animal testing guide (according to Brazilian Legislation No. 11,794/2008). All procedures described in this study were approved by the Ethics Committee on Animal Use of the Franciscan University (No. 02/2019 – approval date: February 14, 2024). Forty male *Rattus norvegicus Albinus* were used, approximately 90 days old, weighing between 250 and 300 grams. The animals were kept in collective cages (4 animals per cage), with drinking water and standard commercial feed *ad libitum*. During the study, the animals were kept in a room with a 12h light/dark cycle, conditioned at an average temperature of 22 ± 2 °C. The animals were randomly divided, using a computer program (www.random.org), into five homogeneous groups [Control Group (G1), the animals were manipulated, but there was no injury and no treatment; Lesion Group (G2), the animals were manipulated, but the injury was not treated; PTU Group (G3), injury treated with PUT; NLC-Q Gel Group (G4), injury treated with NLC-Q Gel; Sonophoresis Group (G5), injury treated with PUT and NLC-Q Gel], with 8 animals in each group.

Gel containing a nanostructured lipid carrier loaded with quercetin (NLC-Q)

NLC-Q was developed according to the methodology (15). The high shear rate method used an Ultraturrax® (T-18, IKA® Brazil) to produce the NLC-Q. 100 mL of NLC-Q were produced according to the composition described in the organic phase (Inwitor 900K: 0.8 g [IOI Oleo GmbH Germany]; Crodamol®: 4.2 g [IPP Pharma, Brazil]; Span 60: 1.0 g [ZTCC, China]; Quercetin: 0.1 g [YTBIO, China]) and aqueous phase (Tween 80: 2.0 g [INLAB, Brazil]; MilliQ Water: 92 mL). The formulations were analyzed in triplicate and characterized for size (Values measured in room temperature 0 days: 103.3 ± 1.3 ηm; 30 days after production: 105.7 ± 1.4 ηm), polydispersity index (PdI 0 days: 0.167 ± 0.01; 30 days: 0.165 ± 0.02), zeta potential (ZP 0 days: -6.78 ± 1.25 mV; 30 days: -9.94 ± 0.98 mV), content (0 days: 97.5 ± 0.70 %; 30 days: 90.9 ± 0.18 %) and pH (0 days: 5.46 ± 0.03 30 days: 5.55 ± 0.1) (15). Quercetin was then added to the organic phase and allowed to stir for another 5 minutes, and the aqueous phase was poured over the organic phase and stirred for 10 minutes. The NLC-Q

was taken to the Ultraturrax® (T-18, IKA® Brazil) at 18000 rpm for 30 min, and the formulation was cooled to room temperature and packaged in 100 mL amber flasks (15).
After, a volume of 100 mL Gel-NLC-Q was prepared by the dispersion method. Initially, 0.4 g Carbopol ETD 2020® (Lubrizol, Brazil) (0.4%), 0.3 g Germall (115 Ashland, Brazil) (0.3%), and 0.25 g Triethanolamine (Adcos professional, Brazil) (0.25%) were weighed. These substances were mixed manually one by one using a pistil in a porcelain crucible in the following order: Carbopol ETD 2020® (Lubrizol, Brazil) 0.4 g, Germall™ 115 (Ashland, Brazil), 0.3 g and the Triethanolamine emollient solution (Adcos professional, Brazil) 0.25 g. After that, 99.05 mL of the NLC-Q was added to the mixture being manually stirred with the pistil until its complete homogenization. After preparation, three samples of the NLC-Q gel (8 g) were submitted to the centrifugation test in test tubes (centrifuge model TDL 80-2B, China) for 30 min at 3000 rpm. This test causes an increase in the force of gravity, enhancing the mobility of the particles, which can cause phase separation, sediment or supernatant formation, and coalescence. Any sign of instability indicates the need for product reformulation (15).

Muscular injury

The muscle injury protocol occurred noninvasively in the gastrocnemius muscle (right), according to the specific methodology for muscle injury in rats (16). Before the injury, the animals were anesthetized with the association of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg), administered intraperitoneally. Afterward, the animals were positioned in ventral decubitus on the base of the injury generator equipment with the knee fully extended and the ankle in a neutral position (90°). The gastrocnemius muscle injury was produced by a 200 g weight, which fell through a metal guide from a height of 50 cm. The rats in the Control Group were also anesthetized to ensure standardization, however without muscle trauma. At the end of the study, the gastrocnemius muscles were dissected and no signs of bone fracture were found at the site.

Interventions protocol

Twenty-four hours after the injury, manipulations, and interventions on the animals began, which occurred every 12h (5 interventions) until 72h, and then a 24h interval was respected for euthanasia (96 h). The study design is shown in **figure 1**.
Regarding Control (G1) and Lesion (G2) Groups, the animals were manipulated at the same period as the treated groups. The manipulation consisted of applying the PTU (off) with commercial gel. The PTU Group (G3) underwent

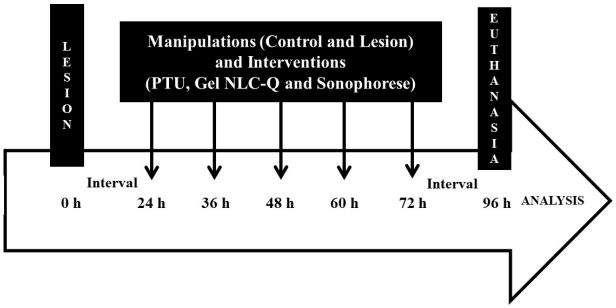

Figure 1. Study design.

h: hour; PTU: pulsed therapeutic ultrasound; Gel NLC-Q: gel with nanostructured lipid carriers with quercetin; Sonophoresis: PTU with Gel NLC-Q.

5 applications of therapeutic ultrasound with commercial gel. In the NLC-Q Gel Group (G4), the gel was spread on the injured area and remained for 5 min on the site. The Sonophoresis Group (G5) had 5 applications of therapeutic ultrasound with NLC-Q gel.
The therapeutic ultrasound equipment used (QUARK model Proseven 977, Amparo, SP, Brazil) was applied at a frequency of 1 MHz, intensity of 0.5 W/cm² ^{SPTA} (spatial peak-temporal average), for a time of 5 min in a pulsed waveform, 20% duty cycle (2 ms time on and 8 ms time off), which corresponds to 0.1 W/cm² ^{SATA} (spatial-averaged temporal intensity) (16-18). The effective radiation area (ERA) of the transducer is 3.8 cm². The equipment was applied in circular movements over the injured gastrocnemius muscle.

Euthanasia protocol and sample preparation for biochemical analysis

After the end of the experimental period (96 h), the rats were euthanized by intraperitoneal anesthesia with an overdose of ketamine (500 mg/kg). Whole blood was collected by intracardiac puncture in heparinized tubes. Blood samples were cooled (5 °C) for subsequent analysis of creatine kinase. The right gastrocnemius muscle was removed and divided into two parts. One part was quickly homogenized in saline solution (150 mM) and kept on ice. After homogenization, the skeletal muscle samples were frozen (-80 °C) to be used for the determination of biochemical markers.

Biochemical analysis

The concentration of creatine kinase was measured in blood plasma samples as an index of muscle contusion injury using diagnostic kits (CK-NAC Liquiform, Labtest, MG, Brazil) on a biochemical analyzer (Mindray BS 200, China). Creatine kinase activity in the gastrocnemius muscle was evaluated by the colorimetric method. After incubation for 20

minutes at 37 °C in a water bath (DeLeo, Brazil) for color development, absorbance reading of the samples was performed in a Synergy HTX spectrophotometer (Biotek, USA) at a wavelength of 540 ηm. The results were expressed as µmol of creatine formed per min per mg of protein.

The activity of the cytosolic superoxide dismutase (Cu/Zn SOD) enzyme was measured in the skeletal muscle S1. Different samples of skeletal muscle (10 to 50 µL) were added to a medium containing glycine buffer (50 mM, pH = 10.5) and adrenaline (1 mM). The superoxide dismutase (SOD) kinetic analysis was initiated after adrenaline addition; the color reaction was measured at 480 ηm (19).

Catalase (CAT) enzyme activity was measured in the skeletal muscle. A sample of skeletal muscle (50 mL) was added to a medium containing potassium phosphate buffer (TFK 50 mM, pH = 7.4) and H_2O_2 (1 mM). The CAT kinetic analysis was initiated after the H_2O_2 addition; the color reaction was measured at 240 ηm. Protein content was measured using bovine serum albumin as the standard measure (19).

The production of reactive oxygen species (ROS) in muscle homogenates was determined by the oxidation of reduced dichlorofluorescein (H_2DCF-DA) (20). Briefly, skin homogenates were added to a standard medium containing Tris-HCl as a buffer (10 mM, pH 7.4) and H_2DCF-DA (1 mM) for 60 min in a lightless condition. Fluorescence quantification was determined at 488 Zm for excitation and 525 Zm for emission, with slit widths of 3 Zm, in a spectrofluorometer (RF-5301 PC; Shimadzu, Japan) using oxidized dichlorofluorescein (DCF) as standard (19).

Thiobarbituric Acid Reactive Substance (TBARS) and malondialdehyde (MDA) levels were mainly determined as an index of tissue lipid peroxidation. Aliquots of 200 µL of skeletal muscle S1 were added to the color reaction. TBARS levels were measured at 532 ηm using a standard curve of MDA and corrected by the protein content (19).

The determination of carbonyl is based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) forming dinitrophenylhydrazone, a yellow compound, measured by spectrophotometer (Biotek Synergy HTX, USA) at 370 ηm (21). Results are expressed as ηmol of carbonyls/mg of protein.

Statistical analysis

Data are presented as mean and standard deviation (± SD). The distribution of variables was tested by the Shapiro-Wilk normality test. Data with symmetrical distribution were compared by one-way ANOVA and followed by Tukey's *post-hoc*. The differences between the groups are presented by the mean difference, the 95% confidence interval (95%CI), and their respective percentages. The significance level was 5% ($p < 0.05$).

RESULTS

The activity of the CK enzyme

The results of CK in gastrocnemius muscle and blood plasma are in **figure 2**. In skeletal muscle, CK activity was higher ($p < 0.005$) in the Lesion, PTU gel NLC-Q, and Sonophoresis groups compared with the Control Group (**figure 2A**). These increases were 152% for the Lesion Group (mean difference 65.5; 95%CI 2.8-128.2 U/L), 156% for PTU (mean difference 70.2; 95%CI 7.5-132.9), 159% for NLC-Q Gel (MD 75; 95%CI 12.3-137.7), and 186% for Sonophoresis (mean difference 107.8; 95%CI 45-170), respectively, but there was no difference between these groups. In blood plasma, CK concentrations increased by 369% (mean difference 1691; 95%CI 619.5-2,762) in the Lesion Group ($p < 0.001$) compared to the Control Group. The PTU, NLC-Q Gel and Sonophoresis groups showed similar results to the Control Group. However, compared to the Lesion Group, plasma CK concentrations decreased -51% (mean difference -497; 95%CI -122 to -2,265) in the PTU Group, -54% (mean difference -1267; 95%CI -196 to -2,338) in the NLC-Q Gel Group and -50% (mean difference -1,149; 95%CI -78 to -2,220) in the Sonophoresis Group. The three treatment groups showed similar results (data shown in **figure 2B**).

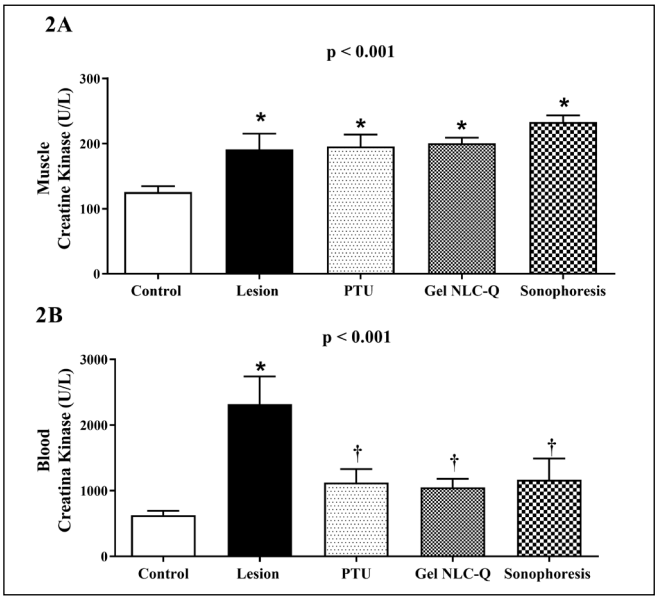

Figure 2. Results of creatine kinase (CK) enzymatic activity in gastrocnemius muscle and blood plasma.

All values are presented as mean ± SD. **(A)** Creatine Kinase – Muscle; **(B)** Creatine Kinase – Blood; PTU: pulsed therapeutic ultrasound; NLC-Q: nanostructured lipid carriers Quercetin; Sonophoresis: PTU with Gel NLC-Q. *$p < 0.05$ *vs* Control; †$p < 0.05$ *vs* Lesion.

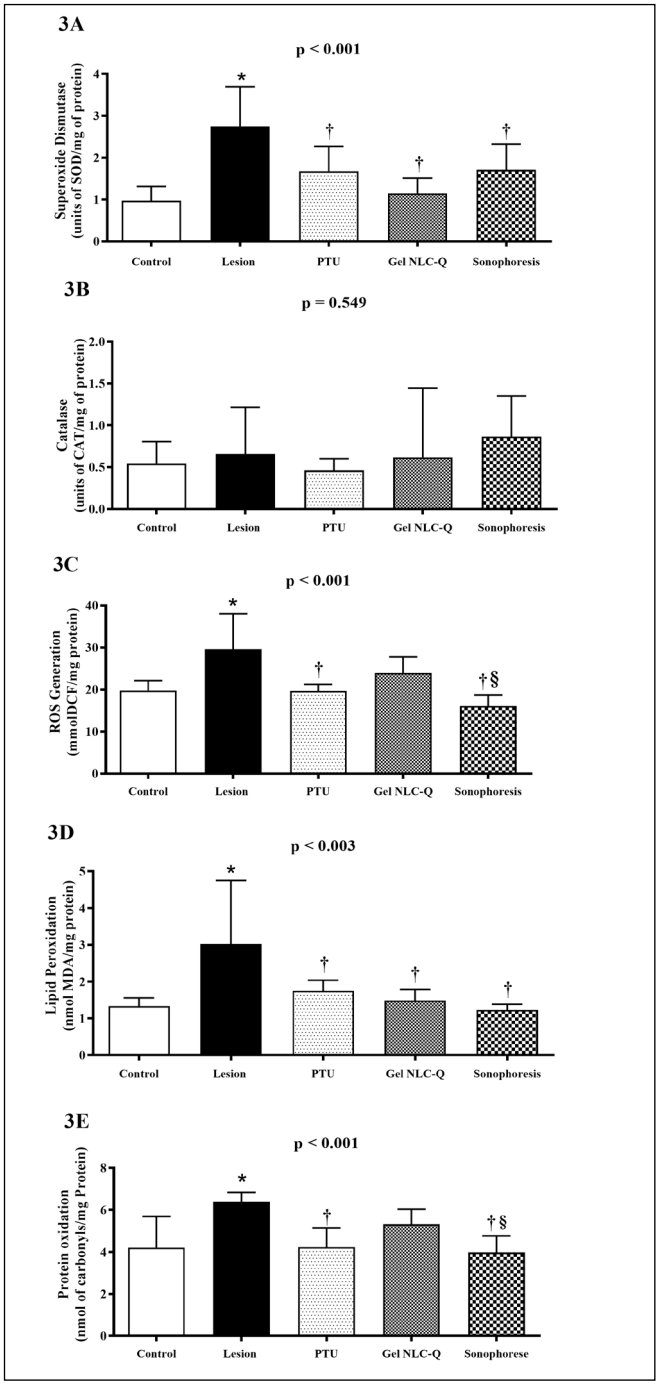

Figure 3. Effect of treatments on oxidative stress parameters in gastrocnemius muscle submitted to contusion injury.

All values are presented as mean ± SD. Superoxide dismutase (SOD) **(A)**; Catalase CAT **(B)**; DCF: Diclorofluoresceína **(C)**; TBARS: Thiobarbituric Acid Reactive Substances **(D)**; Protein Oxidation (E); PTU: pulsed therapeutic ultrasound; Gel NLC-Q: gel with nanostructured lipid carriers with quercetin; Sonophoresis: PTU with Gel NLC-Q.

*p < 0.05 *vs* Control; †p < 0.05 *vs* Lesion. ‡ p < 0.05 *vs* PTU; §p < 0.05 *vs* Gel NLC-Q.

Oxidative stress

The evaluated results of oxidative stress are presented in **figure 3**. SOD activity increased by 282% (mean difference 1.8; 95%CI 0.9-2.6 units of SOD/mg of protein) in the Lesion Group (p < 0.001) compared with the Control Group (**figure 3A**). The three interventions (PTU, NLC-Q Gel, and Sonophoresis) showed similar results to the Control Group (p > 0.05). About the Lesion Group, the PTU, NLC-Q Gel, and Sonophoresis groups reduced SOD activity by -39% (mean difference -1.1; 95%CI -0.2 to -1.9), -58% (mean difference -1.6; 95%CI -0.7 to -2.4), and -37% (mean difference 1.0; 95%CI -0.2 to -1.8), respectively. CAT enzyme activity showed no difference (p = 0.549) among Groups (**figure 3B**). The Lesion Group increased ROS production by 149% (mean difference 9.8; 95%CI 2.9-16.7 nmol DCF/mg of Protein) about the Control Group (p < 0.001) (**figure 3C**). The intervention groups showed no differences compared to the Control Group. Regarding the Lesion Group, PTU and Sonophoresis Groups showed a reduction in ROS production (p < 0.001) of 44% (mean difference -9.9; 95%CI -2.9 to -16.8) and 56% (mean difference -13.5; 95%CI -6.6 to -20.4), respectively. The Sonophoresis Group decreased ROS generation by 33% (mean difference 7.8; 95%CI -0.9 to -14.7) compared with the NLC-Q Gel Group.

Lipid peroxidation (TBARS) increased by 226% (mean difference 1.68; 95%CI 0.54-2.82 nmol MDA/mg of Protein; p < 0.003) in the Lesion Group, compared with the Control Group (**figure 3D**). The intervention groups reduced lipid damage, where PTU was -42% (mean difference -1.27; 95%CI -2.41 to -13), NLC-Q Gel -51% (mean difference -1.53; 95%CI -2.67 to -0.39) and Sonophoresis -59% (mean difference 1.78; 95%CI -2.89 to -0.68) about the Lesion Group. Lipid peroxidation was similar in the Control and the intervention groups (PTU, NLC-Q Gel, and Sonophoresis).

Protein oxidation increased (mean difference 2.2; 95%CI 0.8-3.5 nmol carbonyls/mg of Protein; p < 0.001) by 152% in the Lesion Group compared with the Control Group. The intervention groups were similar to the Control Group. Regarding the Lesion Group, the PTU had a decrease of -34% (mean difference -2.1 95%CI -0.8 to -3.4) and the Sonophoresis Group of -38% (mean difference -2.4; 95%CI -1.0 to -1.6). Sonophoresis reduced protein oxidation by -25% (mean difference -1.3; 95%CI -0.1 to -2.7) compared to the NLC-Q Gel group (**figure 3E**).

DISCUSSION

The present study demonstrates that sonophoresis (PTU with NLC-Q Gel) attenuates oxidative stress and reduces

CK plasma levels in the blood. Muscle injury from contusion induces oxidative stress and the consequent inflammatory response (16, 19, 22, 23).

The activity of antioxidant enzymes (SOD, CAT, and oxidized glutathione) increased after muscle injury, which occurs due to the increased generation of ROS (4). In the present study, the interventions (PTU, NLC-Q Gel, and Sonophoresis) reduced the SOD activity of the injured muscle. This reduction in SOD activity has already been demonstrated after the application of PTU (22-25). In sonophoresis, this reduction in SOD activity is corroborated by previous studies that used PTU with quercetin gel complexed to β-cyclodextrin (22), PTU with gold nanoparticle gel and ibuprofen (25), PTU with dimethyl sulfoxide gel and gold nanoparticles (23) and PTU with dimethyl sulfoxide gel and gold nanoparticles (24). CAT activity did not change at the end of the study, which is corroborated by previous studies with sonophoresis (25, 26).

In the current study, the sonophoresis decreased total ROS generation and reduced lipid and protein oxidation. These results have been demonstrated in previous studies, in which sonophoresis reduced superoxide anion (24) formation, total ROS generation (22, 24, 25), lipoperoxidation (22-24), and protein damage (25), decreasing the oxidative damage that occurs immediately after muscle injury (27). These results possibly occurred due to the phenomenon of acoustic cavitation caused by the sound wave of PTU, which causes disarrangement in the stratum corneum, favoring the penetration of NLC-Q through the skin (28). Thus, this compound can develop its antioxidant and anti-inflammatory activities (9,28), which has already been demonstrated with quercetin gel complexed to β-cyclodextrin (22). However, in the present study, NLCs were used, which favors topical drug delivery (29) since they can support higher drug loading and retention efficiency, physical stability during storage, increase apparent solubility, control the release rate, and improve the bioavailability of encapsulated lipophilic compounds in relation to other carriers (10).

The increased levels of blood and muscle creatine kinase confirm, in the present study, the injury and inflammatory response arising from direct trauma to the gastrocnemius muscle. The treatments did not reduce muscle creatine kinase activity. These results confirm the findings of a previous study that measured creatine kinase levels 50 h after gastrocnemius muscle injury treated with diclofenac and did not observe a decrease in creatine kinase enzyme activity (30). On the other hand, the treatments reduced blood plasma creatine kinase levels. These results agree with a previous study that applied PTU for 72h on muscle contusion injury (16). However, to our knowledge, NLC-Q gel and sonophoresis (PUT with NLC-Q gel) had not yet been shown to have this effect. The inflammatory phase of muscle damage can be measured by the increase in circulating biomarkers a few hours after injury (23, 25, 27), which is demonstrated in the present study by changes in CK. The structures of muscle fibers undergo alteration by oxidative stress, which leads to the disruption of the sarcolemma and the extravasation of specific metabolic enzymes from the sarcoplasm and mitochondria (31). This mechanism of lower extravasation of CK into the blood suggests a reduction of injury by the interventions.

The 1 MHz PTU improves transdermal permeation (13) due to its thermal, mechanical, and acoustic cavitation effects (14, 32, 33). These results corroborate transdermal drug delivery through synergistic combinations with the use of nanotechnology associated with therapeutic ultrasound for the treatment of soft tissue injuries (25, 34). In this study, PTU favored the transdermal passage of NLC-Q, leading to a reduction in total ROS generation, reduction in oxidative damage (lipid and protein damage), and decrease in plasma creatine kinase, which favored recovery from muscle injury. In the tissue, quercetin develops its anti-inflammatory and antioxidant actions (22, 35, 36). However, the effects of sonophoresis depend on the parameters of the PTU (37), the type of active ingredient contained in the nanoparticle, and the interaction of these parameters with the nanoparticles. Among the limitations of the study, there is the absence of histological evaluations of injured muscle tissue, and the assessment of quercetin concentrations in blood plasma. These measures would strengthen the findings of the present study.

CONCLUSIONS

Sonophoresis (PTU with NLC-Q Gel), initiated 24 hours after muscle injury by contusion and applied daily for 5 min for 3 days, reduces the total generation of ROS, attenuates the oxidation of lipids and proteins, and decreases the levels of creatine kinase in the blood. These antioxidant and anti-inflammatory effects favor the myoregeneration process, demonstrating that sonophoresis contributed to the reduction of the oxidative damage caused by contusion, besides tissue repair. Therefore, it is possible to suggest sonophoresis as a therapeutic method for the treatment of these muscle injuries.

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DATA AVAILABILITY

Data are available under reasonable request to the corresponding author.

CONTRIBUTIONS

JPM, LUS, VCR: conceptualization, project administration, writing - review & editing. JPM, RPM, BVCR, GOP, MBM, DLR, REM, JSR, IWS, TDM, CF: investigation, data collection, results analysis, interpretation. JPM, CF, LUS: writing - original draft.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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