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RESEARCH ARTICLE

Effects of phonophoresis with gold nanoparticles on oxidative stress parameters in a traumatic muscle injury model

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Abstract

The aim of this study was to evaluate the effects of therapeutic pulsed ultrasound with gold nanoparticles on oxidative stress parameters after traumatic muscle injury in Wistar rats. The animals were randomly divided into nine groups ($n = 6$ each): sham (uninjured muscle); muscle injury without treatment; muscle injury and treatment with dimethyl sulfoxide (15 mg/kg); muscle injury and treatment with gold nanoparticles (27 μg); muscle injury and treatment with dimethyl sulfoxide + gold nanoparticles (Plus); muscle injury and therapeutic pulsed ultrasound; muscle injury and therapeutic pulsed ultrasound + dimethyl sulfoxide; muscle injury and therapeutic pulsed ultrasound + gold nanoparticles; and muscle injury and therapeutic pulsed ultrasound + Plus. Gastrocnemius injury was induced by a single-impact blunt trauma. Therapeutic pulsed ultrasound (6-min application, frequency 1.0 MHz, intensity 0.8 W/cm^2) was used 2, 12, 24, and 48 h after trauma. Mitochondrial superoxide generation, lipid peroxidation, and protein carbonylation, and the activities of superoxide dismutase, glutathione peroxidase, and catalase were evaluated. The increase in the superoxide production and TBARS and carbonyl levels observed in the control group after muscle damage were reduced in animals exposed to therapeutic pulsed ultrasound plus nanoparticles. Similarly, antioxidants enzymes showed a decreased activity with the same treatment. Our work suggest that therapeutic pulsed ultrasound + dimethyl sulfoxide + gold nanoparticles has beneficial effects on the muscle healing process by inducing a decrease in oxidative stress parameters and most likely decreasing the deleterious effects of the inflammatory response.

Keywords

Gold nanoparticles, muscles, oxidative stress, phonophoresis, ultrasonic therapy

History

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Introduction

The healing of the skeletal muscle in response to trauma depends on the type of injury, such as contusion, strain, or laceration, and on its severity. However, in general, the healing process consists of three phases: the destruction phase, the repair phase, and the remodeling phase. The destruction phase is characterized by necrosis, hematoma formation, and an influx of inflammatory cells (Ten Broek et al., 2010).

Injury induces the increased generation of reactive oxygen species (ROS) in the skeletal muscle, which alters the intracellular oxidant–antioxidant balance in favor of injury and may result in oxidative damage to the traumatized muscle when the production of ROS overwhelms the antioxidant defense systems (Saborido et al., 2011). Multiple potential sites for ROS generation in skeletal muscle have been identified, including mitochondria, NADPH oxidase enzymes, phospholipase A2-dependent processes, and xanthine oxidase (Jackson, 2009).

In this context, therapeutic pulsed ultrasound (TPU) is commonly used in the rehabilitation setting to elicit thermal or nonthermal physiologic effects (Johns, 2002; Markert et al., 2005). TPU is a noninvasive form of mechanical energy transmitted transcutaneously as high-frequency acoustic pressure waves into biological tissues (Lu et al., 2009). The beneficial effects of TPU on a variety of connective tissues and related cellular and molecular mechanisms have been assessed biomechanically (Piedade et al., 2008; Alfredo et al., 2009; Freitas et al., 2010).

At the cell level, it has been hypothesized that changes in diffusion rates and membrane permeability to ions due to acoustic streaming and stable cavitation can stimulate cells

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through the upregulation of signaling molecules (Yang et al., 2008; Maruani et al., 2010).

According to the hypothesis that TPU affects membrane permeability, it could be used together with anti-inflammatory or antioxidant drugs to promote greater absorption and enhance their effects. Using phonophoresis, it is possible to introduce convenient therapeutic concentrations of the drug transdermally into tissues (Goraj-Szczypiorowska et al., 2006).

Gold nanoparticles (GNPs), also called nanogold, have been actively investigated in a wide variety of biomedical applications due to their biocompatibility and facile conjugation with biomolecules (Lévy et al., 2004; Sokolov et al., 2004). Gold compounds have received great attention as anti-inflammatory agents due to their ability to inhibit the expression of NF-κB and the subsequent inflammatory reactions (Jeon et al., 2000; Norton, 2008). According to Tsai et al. (2007), nanogold decreases pro-inflammatory cytokines and macrophage infiltration in a model of arthritis. Barathmanikanth et al. (2010) have also shown that GNPs are anti-oxidative agents that inhibit the formation of ROS and scavenge free radicals, thus assisting anti-oxidant defense enzymes.

In previous studies, our group has used extensively the phonophoresis to facilitate the transport of nanoparticles into the injured tissue. Ours results have shown that 5 and 7 days after the muscle injury the anti-inflammatory and anti-oxidants effects are potentiated from application of nanoparticles plus ultrasound. (Freitas et al., 2007, 2010; Engelmann et al., 2012; Silveira et al., 2010, 2012; Victor et al., 2012). Therefore, considering such results, we decided to evaluate the effects of this therapy on oxidative stress parameters at an earlier stage of the inflammatory response (48 h), when the production of ROS is greater.

Materials and methods

Synthesis and characterization of GNs

GNs were prepared as described elsewhere (Turkevich et al., 1951; Victor et al., 2012). Sodium citrate (Sigma-Aldrich Chemical Co., St. Louis, MO) and hydrogen tetrachloroaurate ($HAuCl_4$) solution (Sigma-Aldrich Chemical Co., St. Louis, MO) were used without further purification. The electronic spectrum was observed with a Shimadzu model UV-1800 spectrophotometer (Shimadzu Corp., Kyoto, Japan). The X-ray diffraction analysis was performed with a Shimadzu LAB-X model XDR-6000 diffractometer with Cu Kα radiation ($\lambda = 1.54056$ Å, 30 kV, 30 mA) (Shimadzu Corp., Kyoto, Japan). The scan rate was 2°/min from 20 to 80°. Transmission electron microscopy (TEM) and field-emission scanning electron microscopy (SEM-FEG) measurements were performed with a Hitachi H-9000-NA (Chiyoda-ku, Japan) and a JEOL JSM-7401F (Akishima, Japan) device, respectively. The Au concentration in the GNP solution was determined by atomic absorption analysis (Sonavane et al., 2008; Singh et al., 2009; Rocha et al., 2011) with a VARIAN model AA 240Z atomic absorption spectrometer (Varian Medical Systems, Inc., Palo Alto, CA). The Zeta potential was measured at 25 °C in a Zeta Potential Analyzer (ZetaPALS; Brookhaven Instruments, Holtsville, NY).

Animals

Male Wistar rats (250–300 g) were obtained from the Central Animal House of the Universidade do Extremo Sul Catarinense, Santa Catarina, Brazil, caged in groups of six, offered commercial rat chow and water *ad libitum*, and maintained under 12-h light/dark cycles. The animals were randomly divided into nine groups ($n = 6$): sham (uninjured muscle); muscle injury without treatment; muscle injury and treatment with dimethyl sulfoxide (DMSO) [15 mg/kg; adapted from Koksal et al. (2003)] mixed in ultrasound gel; muscle injury and treatment with GNPs [27 μg; adapted from Tsai et al. (2007)] mixed in ultrasound gel; muscle injury and treatment with DMSO + GNPs (Plus) mixed in ultrasound gel; muscle injury and TPU (0.8 W/cm^2) + ultrasound gel; muscle injury and TPU (0.8 W/cm^2) + DMSO gel (15 mg/kg); muscle injury and TPU (0.8 W/cm^2) + GNP gel (27 μg); and muscle injury and TPU (0.8 W/cm^2) + Plus gel. All studies were performed in accordance with the guidelines of the National Institutes of Health and with the approval of the Ethics Committee on Animal Use of the Universidade do Extremo Sul Catarinense, Santa Catarina, Brazil.

Muscle injury model

Animals were anesthetized by the intraperitoneal injection of ketamine (70 mg/kg) and xylazine (15 mg/kg). Gastrocnemius injury was induced by a single-impact blunt trauma in a press developed by the Centro Industrial de Equipamentos de Ensino e Pesquisa (CIDEP, Porto Alegre, Rio Grande do Sul, Brazil). Briefly, injury was produced by a metal mass (0.459 kg) falling through a metal guide from a height of 18 cm. The impact kinetic energy delivered was 0.811 J. Sham rats were also anesthetized to ensure standardization but did not undergo muscle trauma (Rizzi et al., 2006).

Treatment

Treatment with therapeutic pulsed ultrasound [IBRAMED, Amparo, São Paulo, Brazil; 6-min duration, frequency of 1.0 MHz, intensity of 0.8 W/cm^2, effective radiating area (ERA) 1 cm^2, 50% duty cycle of 1:2 (5 ms on, 5 ms off) and focused geometry of the ultrasound beam] was used 2, 12, 24 and 48 h after muscle trauma [adapted from Silveira et al. (2010)]. The ultrasound-treated area was ∼2 cm^2 (Freitas et al., 2007). The movement of the beam was circular. The parameters used for the TPU were established in previous studies by our group (Freitas et al., 2007, 2010; Silveira et al., 2010). The groups with muscle injury and treatment with DMSO, GNPs and Plus were also exposed to circular beam movement for 6 min with the ultrasound in off mode (Saliba et al., 2007).

Sacrifice protocol

Two hours after the last application, the animals were euthanized by decapitation and the injured region of the gastrocnemius muscle was surgically removed and immediately processed, aliquoted and stored at −70 °C for subsequent analysis.

Sample preparation

Samples were prepared as previously reported (Silveira et al., 2010). Briefly, the injured region of the gastrocnemius was homogenized in the buffer solution, the homogenates were centrifuged at 1000 *g* for 10 min at 4 °C, and the supernatants were kept at −70 °C until use in the experiments. All biochemical analyses were performed within 5 days.

Biochemical assays

Measurement of mitochondrial superoxide generation

Submitochondrial particles were isolated by differential centrifugation as previously described (Poderoso et al., 1996). Superoxide anion production was estimated by measuring adrenaline oxidation in a buffer containing submitochondrial particles, succinate (electron transfer chain initiator), and catalase (CAT). The results were expressed in nanomoles per minute per milligram of protein.

Lipid peroxidation

The formation of thiobarbituric acid reactive substances (TBARS) during a thiobarbituric acid heating reaction was used as an index of lipid peroxidation (Draper & Hadley, 1989). Briefly, the samples were mixed with 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid. Subsequently, they were heated in a boiling water bath for 30 min. The level of TBARS was determined by absorbance at 532 nm using 1,1,3,3-tetramethoxypropane as an external standard. The results are expressed as nanomoles per milligram of protein.

Protein carbonylation

Oxidative damage to proteins was assessed by measuring carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH) (Levine et al., 1990). Proteins were precipitated by adding 20% trichloroacetic acid and incubating with DNPH. The samples were then redissolved in 6 M guanidine hydrochloride and carbonylation was determined by the absorbance at 370 nm using a molar absorption coefficient of 22.000 M^{-1}. Total protein was determined from the absorbance at 270 nm in the same sample. The results are expressed as nanomoles per milligram of protein.

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was assayed by measuring the inhibition of adrenaline auto-oxidation using the absorbance at 480 nm (Bannister & Calabrese, 1987). The results are expressed in units of SOD activity per milligram of protein.

Glutathione peroxidase assay

Glutathione peroxidase (GPx) activity was measured by using tert-butyl hydroperoxide as the substrate (Wendel, 1981). Enzyme activity was measured by monitoring NADPH disappearance at $\lambda_{max} = 340$ nm in 50 mM potassium phosphate buffer, pH 7.0, containing 1.0 mM EDTA, 2.0 mM GSH, 0.2 U/mL GSH reductase, 1.0 mM azide, 0.2 mM tert-butyl hydroperoxide, 0.2 mM NADPH, and supernatant containing 0.2–0.3 mg protein mL^{-1}. GPx activity is expressed as nanomoles of NADPH oxidized per minute per milligram of protein, using an extinction coefficient of 6.22×10^6 for NADPH.

CAT activity

CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at $\lambda_{max} = 240$ nm. The results are expressed in units of CAT activity per milligram of protein (Aebi, 1984).

Protein determination

The amounts of protein in the samples that were tested for TBARS, protein carbonylation and enzyme activities were determined using the Lowry technique (Lowry et al., 1951).

Statistical analysis

Data were analyzed by one-way analysis of variance followed by Tukey's test when the p values were significant ($p < 0.050$). All analyses were performed using the Statistical Package for the Social Sciences (SPSS, version 17.0; IBM Corp., Armonk, NY) software.

Results

Characterization of GNs

In aqueous solution, the electronic spectrum showed a surface plasmon resonance (SPR) band with λ_{max} at 520 nm, typical of spherical GNs. The mean particle diameter of 25 nm was calculated with Scherer's equation (Suryanarayana & Norton, 1998) from the X-ray spectrum of the GNs. The micrography images from TEM corroborated with the value calculated from X-ray diffraction. A concentration of 36 mg L^{-1} solution was obtained by atomic absorption with theoretical value of 40 mg L^{-1} (Sonavane et al., 2008). The zeta potential revealed a value of −30 mV, indicating the stability of the GNs.

Superoxide anion production

In Figure 1, the group of rats with muscle injury and no treatment had a significant increase in superoxide compared to the sham group; only the TPU + Plus gel significantly

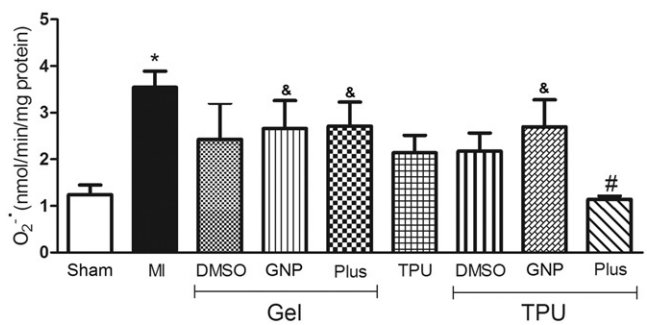

Figure 1. Effect of TPU + Plus gel on superoxide anion production in skeletal muscle after injury (48 h). Data are expressed as the means ± standard error of mean for six animals. *$p < 0.05$ compared to sham, #$p < 0.05$ compared to muscle injury without treatment, &$p < 0.05$ compared to TPU + Plus (Tukey's test).

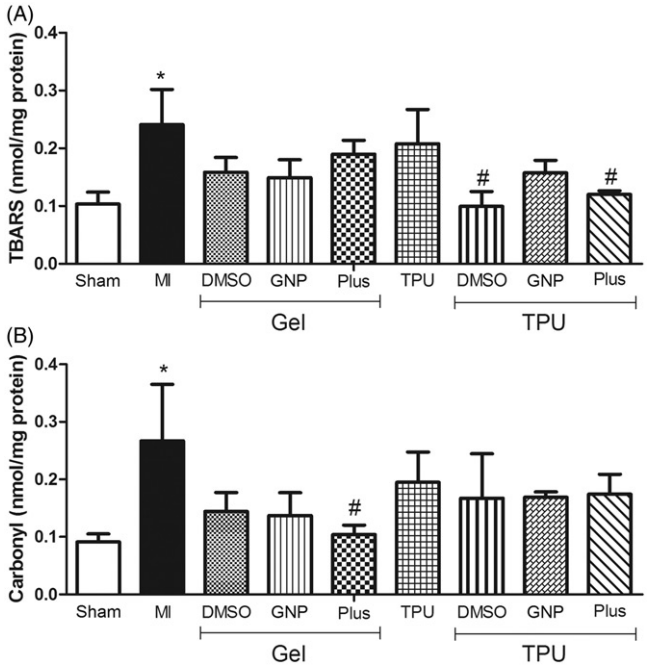

Figure 2. Effect of TPU + Plus gel on TBARS (A) and protein carbonylation (B) levels in skeletal muscle after injury (48 h). Data are expressed as the means ± standard error of mean for six animals. *$p < 0.05$ compared to sham, #$p < 0.05$ compared to muscle injury without treatment, &$p < 0.05$ compared to TPU + Plus (Tukey's test).

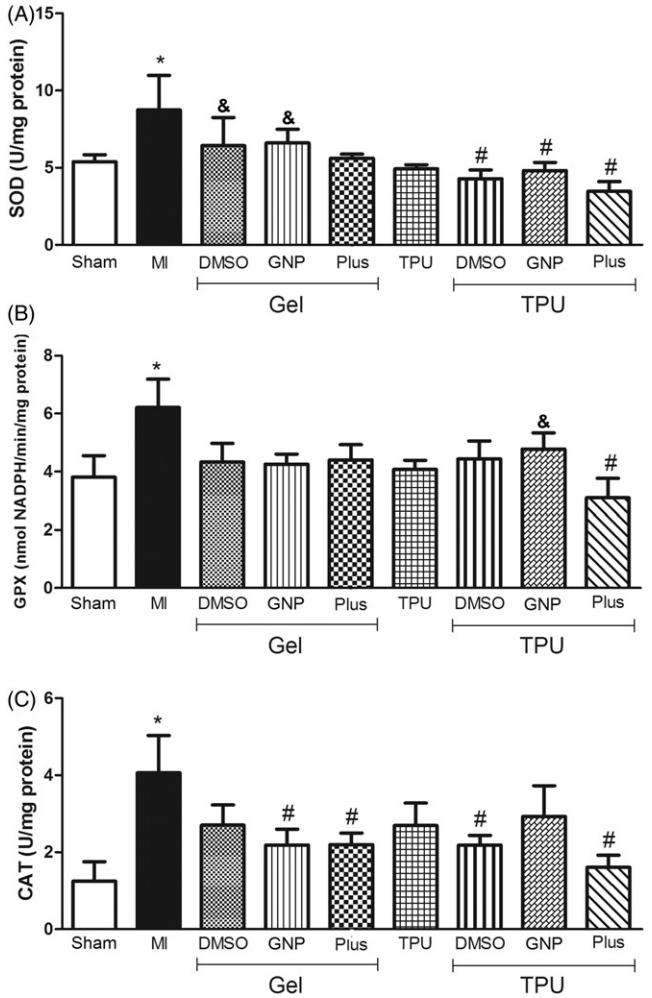

Figure 3. Effect of TPU + Plus gel on SOD (A), GPx (B), and CAT (C) levels in skeletal muscle after injury (48 h). Data are expressed as the means ± standard error of mean for six animals. *$p < 0.05$ compared to sham, #$p < 0.05$ compared to muscle injury without treatment, &$p < 0.05$ compared to TPU + Plus (Tukey's test).

inhibited superoxide production compared to the untreated group [$F_{(8,34)} = 2.32$, $p < 0.05$].

Lipid peroxidation and protein carbonylation

The levels of lipid peroxidation (Figure 2A) in the rats with muscle injury and no treatment were significantly increased compared to the sham group. The TPU + DMSO gel and TPU + Plus gel groups similarly and significantly inhibited peroxidation compared to the muscle injury without treatment group [$F_{(8,33)} = 3.82$, $p < 0.05$].

Protein carbonylation (Figure 2B) was also significantly increased in the muscle injury without treatment group when compared to the sham group. Only the Plus gel group showed significantly decreased carbonylation compared to the untreated group [$F_{(8,32)} = 2.11$, $p < 0.05$].

SOD, GPx, and CAT activities

In Figure 3, a significant increase in the activity of the three antioxidant enzymes was observed in the muscle injury without treatment group. Only the TPU + Plus group significantly inhibited the increased activities of all three enzymes SOD [$F_{(8,30)} = 2.30$, $p < 0.05$], GPx [$F_{(8,34)} = 2.05$, $p < 0.05$], and CAT [$F_{(8,34)} = 2.00$, $p < 0.05$].

Discussion

The aim of this study was to evaluate the effects of TPU with DMSO and GNPs on oxidative stress parameters 48 h after traumatic muscle injury.

Studies conducted over the past 15 years show that ROS (superoxide, hydroxyl radicals, nitric oxide, peroxynitrite, and the free radical-derived product hydrogen peroxide) play an important role in inflammation and or infection-induced alterations in muscle function (Supinski & Callahan, 1985; Tidball, 2005; Brunelli & Rovere-Querini, 2008; Ciciliot & Schiaffino, 2010). ROS are crucial to protect cells against the invasion of infectious agents; however, when ROS production is excessive, biomolecules may be damaged, and the recovery process of the injured tissue could be hindered (Tang et al., 2002; Kerkweg et al., 2007).

The production of superoxide anion at the site of the lesion and its inhibition by the TPU + Plus gel group were evaluated and compared to the untreated injured group. The results show that only the combination of TPU + DMSO and GNP was effective in decreasing this parameter (Figure 1). Low-frequency ultrasound is efficient in enhancing the cutaneous penetration of topical drugs into human skin. The explanation for increased transdermal permeability due to low-frequency ultrasound is linked to the thermal effects and is mainly attributed to the cavitation generated by the high vibrational frequency. Because of this cavitation, there is a micromassage effect that increases membrane permeability and protein synthesis (Tezel et al., 2002; Maruani et al., 2010).

DMSO is commonly used in studies of skeletal muscle as a selective antioxidant or as a solvent for numerous drugs. DMSO has a remarkable and very specific effect on myosin by inhibiting ATPase activity and positively influencing actin, Ca^{2+} and regulatory proteins (Reid & Moody, 1985; Mariano et al., 2001). According to Camici et al. (2006), DMSO is also used as a solvent for chemotherapeutic drugs, and due to its anti-inflammatory properties, it has been successfully used in humans for treating rheumatic, pulmonary, gastrointestinal, muscular, neurologic, urinary and dermatologic disorders. Thus, the effects of DMSO can be enhanced when used with TPU.

Recent advances in gold technology have led to probes with improved properties and performance for cell biologists, including increased labeling density, better sensitivity, and greater penetration into tissues. Factors such as shape, size, surface coating and charge affect the probes' physicochemical properties and are therefore expected to influence interactions with biologic systems. GNPs have an anti-inflammatory effect by decreasing pro-inflammatory cytokine levels and macrophage infiltration in a model of arthritis (Tsai et al., 2007). It is likely that the increase in membrane permeability induced by TPU increased the absorption of GNPs and led to the reduction of superoxide anion levels as noted in our results.

To demonstrate that phonophoresis with GNP + DMSO was effective in reducing oxidative stress after muscle injury, oxidative damage and antioxidant enzyme activities were evaluated. The levels of lipid peroxidation were significantly decreased in the TPU + DMSO and TPU + Plus groups; however, decreased protein carbonylation was only observed in the group that received DMSO + GNP (Figure 2). The TPU + Plus group showed a significant decrease in the activity of SOD, GPx, and CAT (Figure 3). However, we can also see in Figure 3(A) that all groups that received treatment with phonophoresis had a decrease in the activity of SOD, showing that this type of therapy has a greater effect in decreasing ROS production and the consequent effects.

Although TPU alone accelerates the recovery of muscle injuries, these effects can be improved with the combined use of pharmacologic agents, as reported previously by our group (Freitas et al., 2007). Thus, the combined use of ultrasound and anti-inflammatory agents such as DMSO and GNPs represents an important alternative in the treatment of muscle injuries (Rocha et al., 2011). DMSO stabilizes cell membranes from excess free radical formation and abnormal Ca^{2+} entry into cells, while improving muscle healing. DMSO administration during reoxygenation resulted in a significant increase in ATP associated with markedly lower lactate levels and good muscle recovery (Gilboe et al., 1991). Thus, an increase in ATP combined with reduced energy consumption may reflect favorable changes in the balance of energy supply and demand during ischemic/hypoxic events such as in traumatic injury (Jacob & de la Torre, 2009).

GNs have received great attention as anti-inflammatory agents due to their ability to inhibit the expression of NF-κB and subsequent inflammatory reactions (Jeon et al., 2003; Norton, 2008) GNPs block NF-κB activation by interacting with cys-179 of IKK-β and inhibiting the production of proinflammatory cytokines, such as TNF-α and IL-1β (Jeon et al., 2000, 2003). Another study showed that GNPs inhibited lipopolysaccharide (LPS)-induced NO production and inducible nitric oxide synthase (iNOS) expression in RAW264.7 cells. Furthermore, GNPs suppressed the LPS-induced activation of NF-κB through the inhibition of Akt activity. GNPs also inhibited the LPS-induced phosphorylation of signal transducer and activator of transcription-1 via the down-regulation of interferon-β expression (Ma et al., 2010).

Moreover, GNPs are effective in quenching ROS in a dose-dependent manner (Kajita et al., 2007). GNPs catalyzed the oxidation of NADH to NAD, enhanced the antioxidant activity of vitamin E and decreased ROS induced in a hepatoma cell line (Nie et al., 2007; Martín et al., 2010) and inhibited osteoclast formation induced by the receptor activator of NF-κB ligand in bone marrow-derived macrophages (Sul et al., 2010). In another wound healing model, GNP decreased CD68 expression and increased SOD1 expression around the wound area, suggesting anti-inflammatory and antioxidative effects.

The TPU has proved effective and safe in treatment of muscle and tendon injuries in humans. Colloidal gold and some of the compounds have recognized therapeutic properties, especially for the treatment of inflammatory and arthritic processes (Kim et al., 2007). Several studies performed in our group using phonophoresis and/or iontophoresis with topic application of GNP for treating muscle and tendon injuries in rats show promise and indicate the possibility of use this new therapy in the treatment of these diseases in humans (Freitas et al., 2007, 2010; Silveira et al., 2010, 2012; Dohnert et al., 2012; Engelmann et al., 2012; Victor et al., 2012).

Conclusion

Considering results of this study and the previous results from our group, we suggest that TPU + DMSO + GNP has beneficial effects on the muscle healing process by inducing a decrease in oxidative stress parameters and most likely decreasing the deleterious effects of the inflammatory response. Further studies *in vivo* and *in vitro* are being conducted in our laboratory to better understand the phonophoresis properties of GNP transdermal permeation in muscle tissue and the mechanisms involved in the antioxidant and anti-inflammatory effects.

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Declaration of interest

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