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● *Original Contribution*

EFFECT OF PULSED THERAPEUTIC ULTRASOUND AND DIOSMIN ON SKELETAL MUSCLE OXIDATIVE PARAMETERS

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Abstract—Cyclodextrins (CDs) have been widely used as a promising alternative in the formation of inclusion complexes with poorly soluble molecules. From this perspective, the present study aimed to study the inclusion complexes of diosmin in β-cyclodextrin, chemically quantify the diosmin-in-gel preparation and analyze the stability of the gels. Furthermore, we evaluated the effect of therapeutic pulsed ultrasound (TPU) in association with the gel–diosmin complex on the parameters of muscle damage and oxidative stress in rats. Serum creatine kinase (CK) levels were used as an indicator of skeletal muscle injury. Lipid peroxidation (thiobarbituric acid-reactive substances [TBARS]) and superoxide dismutase and catalase activities were used as indicators of oxidative stress. The results obtained indicated that the inclusion complex obtained by co-evaporation had the highest complexation efficiency and stability; there was no change in the features of diosmin on incorporation into the Carbopol gel. Additionally, a significant ($p < 0.05$) decrease was observed in CK levels (TPU plus gel-diosmin: 178.4 ± 85.3 U/L) relative to the untreated group (527.8 ± 46.1 U/L). Levels of TBARS were lower in the TPU plus gel-diosmin group (0.008 ± 0.0004 nmol malondialdehyde/mg protein, $p < 0.05$) compared with the untreated group (0.081 ± 0.011 nmol malondialdehyde/mg protein, $p < 0.05$, n = 6). Catalase activity did not statistically significantly differ between the treatment groups, and superoxide dismutase activity was lower in the diosmin-treated group (0.320 ± 0.11 U/mg protein) compared with the untreated group (0.983 ± 0.40 U/mg protein). These results suggest that TPU in association with the diosmin–gel complex is effective in reducing muscle damage and oxidative stress after mechanical trauma. (E-mail: evaleide@uol.com.br) **© 2017 World Federation for Ultrasound in Medicine & Biology. All rights reserved.**

Key Words: **Inclusion complex, Diosmin, β-Cyclodextrin, Ultrasound, Muscle injury, Oxidative stress.**

INTRODUCTION

Muscle injuries can occur in sports, recreation and even daily life activities. Most muscle injuries occur in sports as a result of direct or indirect impact and usually cause a local inflammatory response. This event involves many tissue processes; for example, muscle fiber intrinsic degeneration and basal lamina damage, which alters myofibrils, disrupt mitochondria and sarcoplasmic reticulum, discontinue sarcolemma, alter calcium levels and can lead to cell death (Oliveira 2004).

Several studies have reported that the muscle injury (Frankiewicz-Jozko et al. 1996) or even muscle fatigue (Barclay and Hansel 1991; Brotto and Nosek 1996) process promotes significant increases in the production of reactive oxygen species (ROS). Endogenous generation of ROS is a consequence of anaerobic metabolism (Michiels et al. 1994). In physiologic concentrations, ROS have a marked biological function, acting as a second messenger (Forman et al. 2004; Linley et al. 2012; Sauer et al. 2001). However, supraphysiological concentrations of ROS may be avoided because of its reactivity, which causes deleterious cellular effects such as impairment of protein, lipid and DNA oxidation and cellular homeostasis (Starkov 2008; Sies 1991).

Various natural substances from plants, such as flavonoids and phenolics, have been found to have

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anti-inflammatory, anti-carcinogenic and antioxidant properties. Diosmin ($C_{28}H_{32}O_{15}$), a member of the flavonoid group, is a natural glycoside (molecular weight = 608.5 g/mol) separated from Rutaceae. It improves lymphatic drainage, protects capillary microcirculation and reduces capillary permeability, thereby assisting in the treatment of chronic venous insufficiency (Silambarasan and Raja 2012). The anti-inflammatory, anti-hypertensive, diuretic, analgesic and hypolipemiant properties of diosmin have been described previously (Gil-Izquierdo et al. 2001).

Diosmin, however, has low solubility in water and other organic solvents, which limits its therapeutic applications. In this context, the complexation of diosmin with β-cyclodextrin becomes an interesting strategy to improve the pharmacologic potential of diosmin (Freag et al. 2013). Cyclodextrins are characterized as cyclic oligosaccharides composed of α-D-glucopyranose subunits connected by α-1,4 glycosidic linkages, forming a ring having hydrophilic and hydrophobic cavity surfaces. This molecular organization is responsible for the ability of cyclodextrins to form inclusion complexes with a wide variety of guest molecules. This phenomenon has received significant attention in the pharmaceutical industry to improve the aqueous solubility, chemical stability, dissolution profile and release of several drugs (Anwe et al. 2014; Menezes et al. 2014).

Therapeutic pulsed ultrasound (TPU) has been widely used in physical therapy to facilitate the cutaneous permeation of drugs, a process known as *phonophoresis* (Campos et al. 2004). Phonophoresis is the enhanced delivery of topically applied drugs though the external layer of the skin (stratum corneum) to underlying tissues (Cagnie et al. 2003). Ultrasound therapy mechanically influences the activity of cells, platelets, mast cells, macrophages and neutrophils involved in the inflammatory phase of tissue regeneration, accelerating the healing process.

Ultrasonic waves increase membrane and platelet permeability, promoting serotonin liberation. In response to the increased intra-cellular calcium levels, mast cells break up and liberate histamine. The capacity for calcium transport though cell membranes can affect cell activity, increasing synthesis and secretion of injury markers (Freitas et al. 2007).

Described in the literature as having anti-inflammatory properties and as a major ultrasound adjunct physiotherapists use to treat muscle injuries, diosmin, when applied in combination with TPU, could enhance the effect of TPU. Very few studies have reported on the effects of oxidative stress in skeletal muscle and its importance in the mechanisms involved in the regulation of muscle disorders. The aim of this study was to analyze the effects of TPU when used with diosmin, compared with the effects of TPU alone with ultrasound gel and diosmin alone applied topically, on treatment of mechanical muscle injury as assessed with the muscle injury markers of lipid peroxidation and antioxidant enzymes.

METHODS

Formulation of gel containing diosmin complexed with β-cyclodextrin

Physical mixture. The physical mixture (PM) was prepared by the addition of diosmin (608 mg, Lot 070 M1250 V, Sigma-Aldrich, St. Louis, MO, USA) to an agate mortar containing powdered β-cyclodextrin (β-CD) under manual agitation, in a 2:1 molar ratio. The PM was stored in sealed glass containers.

Slurry complexation. Slurry complexation (SC) was carried out by the addition of water (20 mL) to a beaker containing 2270 mg of β-CD and 608.5 mg of diosmin, which is an performing 2:1 molar ratio. The mixture was added to the slurry and stirred for 36 h. Thereafter, the suspension was dried in a desiccator. After drying, the sample underwent manual trituration and was stored in amber in sealed glass containers.

Physicochemical characterization of samples

Differential scanning calorimetry (DSC) and thermogravimetry/derivative thermogravimetry (TG/DTG) curves were obtained in the temperature range 25°C to 600°C. and 25°C to 900°C, respectively, using a DSC-60 e TGA-60 (Shimadzu), under dynamic nitrogen atmosphere (50 mL/min) and heating rate of 10°C/min using aluminum (DSC) and platinum (TG/DTG) crucibles containing about 2 mg of sample each. The DSC cell had been calibrated with indium (melting point: 156.6°C, $\Delta H_{melting}$ = 28.54 J/g). The equipment was checked before testing using standard calcium oxalate with a purity of 99.99% (TG) and indium (DSC). Scanning electron microscopy images at magnifications of 250 × (PM) and 500 × (SC) were obtained under 8-kV voltage acceleration using an electron microscope (JEOL Model JSM-6390-LV).

Analysis by high-performance liquid chromatography and encapsulation efficiency

Diosmin ($C_{28}H_{32}O_{15}$, analytical standard) was diluted with methanol to a concentration of 100 μg/mL and sonicated for 10 min. Before high-performance liquid chromatography (HPLC) analysis, the diosmin solution was filtered through a 0.45-μm membrane filter (polytetrafluoroethylene). HPLC analysis was performed with an HPLC system equipped with two LC-20 AD pumps, a SIL-20 A HT auto injector, a CTO-20 A column oven, a SPDM20 Avp photodiode array detector (DAD) and a CBM-20 A controller (Shimadzu, Kyoto, Japan). The system used a Shim-pack C18 analytical column

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