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Anti-Inflammatory Activity of *Eugenia punicifolia* Extract on Muscular Lesion of *mdx* Dystrophic Mice

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ABSTRACT

Eugenia punicifolia known as "pedra-ume caá" is a shrub largely distributed in the Amazon region popularly used in decoctions or infusions as a natural therapeutic agent, which can interfere on cholinergic nicotinic neurotransmission. This work aimed to investigate a putative anti-inflammatory effect of dichloromethane fraction of *E. punicifolia* extract (*Ep*-CM) in the muscular lesion of *mdx* dystrophic mice, considering that activation of cholinergic mechanisms mitigates inflammation. A polymer containing the *Ep*-CM was implanted in *mdx* gastrocnemius muscle before onset of myonecrosis for local slow and gradual release of bioactive compounds and mice sacrificed 7 days or 9 weeks after surgery. Comparing to control muscle, treatment did not alter choline acetyltransferase and acetylcholinesterase enzymatic activities, but decreased metaloproteases-9 and -2 activities and levels of tumor necrosis factor α and NFκB transcription factor. In addition, treatment also reduced levels of bioactive IL-1β form and cleaved caspase-3, related to early events of cellular death and inflammatory activation and further increased myogenin expression without affecting collagen production which is associated with fibrosis. In vivo treatment of *mdx* dystrophic mice with *Ep*-CM caused significant reduction of muscular inflammation and improved skeletal muscle regeneration without inducing fibrosis. J. Cell. Biochem. 111: 1652–1660, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: MUSCULAR DYSTROPHY; *MDX* MICE; *EUGENIA PUNICIFOLIA*; ACETYLCHOLINE RECEPTOR; SKELETAL MUSCLE

Natural products from plant extracts with potential pharmacological activity are extensively pursued for development of compounds or new lead structures with possible therapeutic applications in various pathologies [Rahimi et al., 2010]. One example is the Myrtaceae family constituted by more than 3,000 species and largely distributed in the Brazilian tropical Amazon region with the genus *Syzigium* and *Eugenia* commonly used for diarrhea and stomach disturbance, and as hypoglycemic medicament [Brito et al., 2007; Bopp et al., 2009]. The mechanism underlying pharmacological properties of the genus *Eugenia* may be partly related to flavonoids (myricitrin, quercetin, and quercetrin), steroids, terpenoids, tanines, and anthraquinones [Consolini and Sarubbio, 2002], but *Eugenia punicifolia* aqueous extract was able to enhance cholinergic nicotinic neurotransmission in the rat diaphragm muscle endplate model [Grangeiro et al., 2006].

Duchenne muscular dystrophy (DMD) is a human devastating X-linked recessive inflammatory myopathy in which progressive muscle degeneration is caused by a defect in the gene coding for dystrophin, a large cytoskeletal protein present in skeletal muscles and certain neurons [Voisin and de la Porte, 2004]. Lack of

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dystrophin leads to disruption of dystrophin-associated protein complex and sarcolemmal instability thus rendering activation of inflammatory signaling cascades. *Mdx* mouse, the animal model of human DMD presents similar pathological alterations with prominent muscle inflammation occurring soon after weaning (3–5 weeks) followed by cycles of degeneration and regeneration (8–12 weeks), and persistent fibrosis with accumulation of connective tissue at older age [Lefaucher and Sebille, 1996; McGeachie and Grounds, 1999; Lagrota-Candido et al., 2002]. Recent data from our group showed that activation of nicotinic acetylcholine receptor reduced inflammatory-mediated muscular lesion and improved muscle regeneration [Leite et al., 2010]. Based on relevant studies about activation of the anti-inflammatory cholinergic pathway [Tracey, 2009] and considering that increased expression of the non-neuronal nicotinic α7 acetylcholine receptor subunit (nAChRα7) plays an important role in the physiopathology of *mdx* muscular lesion [Leite et al., 2010], we reasoned that *E. punicifolia* extract could decrease inflammatory activation and improve tissue remodeling. To explore this hypothesis we employed in vivo experiments with a strategy to locally implant in the dystrophic gastrocnemius muscle a polymer containing *E. punicifolia* extract for slow and gradual release of compounds with putative biological activity.

MATERIALS AND METHODS

PLANT MATERIAL AND PREPARATION OF THE EXTRACT

E. punicifolia was supplied by the Centro de Instrução de Guerra na Selva (CIGS, Manaus, AM, Brazil) and identified at the National Museum (Rio de Janeiro Federal University, Brazil). Official authorization for scientific investigation with plant components was given by the government environmental institution (IBAMA, Brazil), registered under the number 16602-1 with a voucher specimen deposited at the National Museum. The plant was successively extracted at room temperature with solvents of increasing polarity beginning with hexane, dichloromethane (CM), and methanol. The obtained extracts were evaporated to dryness and the residues stored at 4°C. Stock solutions (1 mg/ml) were prepared in 10^{-2} M dimethyl sulfoxide (DMSO, Sigma Chem Co, USA).

PREPARATION OF ELVAX CONTAINING *EP*-CM EXTRACT

Elvax, a polymer that allows slow and gradual release of substances was manufactured as described previously with minor modifications [Smith et al., 1995]. Briefly, ethylene-vinyl acetate copolymer (Elvax 40W, DuPont, Pinheiros, SP, Brazil) was washed in 95% alcohol with multiple changes during 7 days. Elvax was dissolved in dichloromethane to give a 10% solution. Fifty microliters of *Ep*-CM or methanolic fractions (2 mg/ml) were dissolved in DMSO or 50 μl of vehicle DMSO with 1% Fast Green (to help visualizing Elvax slices) was added to the solution. Preparation was mixed up for 1 min and immediately placed in dry ice for 30 min, stored at −20°C for 5 days and further submitted to low vacuum pressure for 16 h at 0°C followed by preparation of 200 μm cryostat sections and stored at −20°C until implantation.

ANIMAL CARE

Male *mdx* dystrophic and age-matched C57BL/10J control non-dystrophic mice were maintained at the Cellular Pathology animal house facilities at Fluminense Federal University. Mice were kept at constant temperature (20°C) with a light/dark cycle of 12 h. Each cage housed up to four mice from the same age and offspring to minimize stress. Mice were sacrificed in the period of inflammatory prevalence at 4 weeks age and period of regeneration prevalence at 12 weeks age. All procedures were done within the guidelines established by the Brazilian College for Animal Experimentation (COBEA) and approved by the Institutional Animal Ethics Board.

At least five mice from each strain were separated on the following groups: Control, *mdx* mice without treatment; Vehicle, gastrocnemius muscle implanted with Elvax containing DMSO; CL, contralateral gastrocnemius muscle of *mdx* implanted with Elvax containing *Ep*-CM fraction; and Ep, gastrocnemius muscle of *mdx* mice implanted with Elvax containing *Ep*-CM fraction.

ELVAX IMPLANTATION

Male *mdx* mice with 3-week-old were anesthetized with 0.03 ml ketamine and 0.02 ml xylazine by intraperitoneal injection. A small skin incision was made with sterile steel blade, and Elvax containing 2 mg/ml of *Ep*-CM or vehicle DMSO was carefully placed on the left gastrocnemius muscle and incision closed with cyanoacrylate ester. Mice were killed 7 days or 9 weeks after surgery and both gastrocnemius muscles collected.

CHOLINE ACETYLTRANSFERASE ENZYMATIC ACTIVITY ANALYSIS

Skeletal muscles were homogenized at 4°C with extraction solution (10 mM EDTA, 200 μM Eserin, 0.5% Triton X-100, 7 mM NaCl, 1 mM $NaPO_4$). Choline acetyltransferase (ChAT) activity was determined [Fonnum, 1975] with 0.1 μCi/tube of [^{3}H] acetyl CoA (Amersham Biosciences, Fairfield, CT) as substrate. Briefly, the enzymatic activity at 37°C was measured in pH 7.4 with 10 μl of substrate solution (40 mM EDTA, 200 μM Eserin, 2 mM acetyl CoA, 40 mM choline, 1 M NaCl, 160 mM $NaPO_4$), 0.1 μCi [^{3}H] acetyl CoA and 10 μl of the homogenized sample in extraction solution. As control, samples were incubated only with blank solution (10 mM EDTA, 0.5% Triton X-100, and 200 μM eserin). The enzymatic assay was performed for 15 min at 37°C, the reaction was stopped with 5 ml of 10 mM EDTA and 10 ml of Kalignost solution (5 mg/ml sodium tetraphenilborate in acetonitrile) under constant shaking for 30 s. Tube content was transferred to other containing 10 ml of cintilation solution, and formation of two phases was observed. The Kalignost solution adsorbes all [^{3}H] acetyl CoA and [^{3}H] ACh is released in the cintilation solution. Only [^{3}H] ACh radioactivity counting for 1 min was analyzed (Packard, model 1600 TR) and blank values were discounted. The values were divided by the specific activity of the radioactive substratum used, the time that the reaction lasted (15 min) and the amount of protein present in each tube. As result, we obtained the specific activity of the enzyme in μmoles per minute per milligram of protein.

ACETYLCHOLINESTERASE ENZYMATIC ACTIVITY ANALYSIS

Skeletal muscles were homogenized at 4°C in extraction buffer (0.05 M Tris–HCl, pH 7.6) and acetylcholinesterase (AChE) activity

determined [Ellman et al., 1961] using acetylthiocholine (ATCh) as substrate. Reaction medium consisted of 50 mM Tris–HCl, pH 8.0, 100 mM $MgCl_2$, 10 mM ATCh, 10 mM DTNB (5:5-ditiobis-2-nitrobenzoic acid), and 10 μl of homogenized sample in extraction buffer to a final volume of 1 ml. The method is based on the measurement of thiocholine production as ATCh is hydrolyzed at 25°C in the presence of DTNB producing a yellow anion detected at 412 nm (Hitachi, model U-3300). As result, we obtained the specific activity of the enzyme in μmoles per minute per milligram of protein.

GELATIN ZYMOGRAPHY

Gastrocnemius muscles from *mdx* and control mice were carefully removed, immediately frozen, and preserved in liquid nitrogen (−196°C). Muscles were homogenized (1/10, w/v) in Tris-buffered saline (TBS, 100 mM Tris–HCl, pH 7.6, 200 mM NaCl, 100 mM $CaCl_2$, and 1% Triton X-100). After centrifugation (12,000*g*, 10°C, 10 min), protein concentration in supernatant aliquots was determined [Lowry et al., 1951] and equal amounts of total protein loaded for zymography (60 μg/lane). SDS–PAGE zymography was performed to determine gelatinase activity [Heussen and Dowdle, 1980]. Briefly, zymogram gels consisted of 7.5% polyacrylamide–SDS impregnated with 2 mg/ml type A gelatin from porcine skin (Sigma, St. Louis, MI) and 4% polyacrylamide–SDS for stacking gels. Gels were further washed twice for 30 min in 2.5% Triton X-100 solution, then incubated at 37°C for 24 h in substrate buffer (10 mM Tris–HCl buffer, pH 7.5, with 5 mM $CaCl_2$, 1 μM $ZnCl_2$). Gels were stained with 30% methanol/10% acetic acid solution containing 0.5% brilliant blue R-250 (Sigma) and discolored with the same solution without brilliant blue R-250. Gelatinase activity was visualized as unstained bands on a blue background representing areas of proteolysis.

Metalloproteases are secreted in a latent form and require cleavage of a NH_2 terminus peptide for activation. The exposure of proenzymes to SDS during gel separation leads to activation without proteolytic cleavage [Talhouk et al., 1992] with appearance of bands corresponding to 100-kDa (MMP-9), 66-kDa (pre-pro-MMP-2), 60-kDa (pro-MMP-2), and 55-kDa (active-MMP-2) [Kherif et al., 1999].

WESTERN BLOTTING

Skeletal muscles were homogenized with protease inhibitor buffer (Sigma). Protein extracts were clarified by centrifugation (12,000*g* for 15 min at 4°C), followed by quantification [Lowry et al., 1951], and concentration adjustment with sample buffer pH 6.8 (173 mM Tris, 30% glycerol, 3% sodium dodecyl sulfate, 3% β-mercaptoethanol, and 0.1% bromophenol blue). Samples were denatured by boiling for 5 min and loaded on 12.5% SDS–PAGE for TNFα, interleukin 1β (IL-1β), cleaved-caspase-3 and high mobility group box 1 (HMGB1), and 10% for NFκB detection. Proteins were transferred to PVDF membranes (Hybond-P; Amersham Biosciences) and blots carried out with Snap i.d (Millipore, USA) according to the manufacturer's recommendations. Membranes were blocked with 0.2% non-fat dry milk in 0.05% Tween-20 Tris-buffered saline (TBST), pH 7.4. Thereafter it was individually incubated with the primary rabbit polyclonal anti-NFκB p65 (A at 1:200) and anti-myogenin (at 1:350, Santa Cruz Biotechnology, Santa Cruz, CA), anti-IL-1β (at 1:1,500; Peprotech, Rocky Hill, NJ), and anti-cleaved Caspase-3 (Asp175 at 1:350; Cell Signaling, Beverly, MA); goat anti-TNFα (L-19 at 1:200; Santa Cruz Biotechnology) and monoclonal mouse anti-human HMGB1 (MAb1690 at 1:150 dilution; R&D Systems, Minneapolis, MN).

Peroxidase-conjugated rabbit anti-goat (Molecular Probes, Eugene, OR) was used for TNFα detection, goat anti-rabbit (Zymed, San Francisco, CA) for NFκB p65 and cleaved Caspase-3 detection, and goat anti-mouse for HMGB1 detection (Zymed) at 1:3,000, 1:1,500, and 1:2,000, respectively. Bands were identified using ECL Plus (Amersham Biosciences) for chemiluminescent detection and subsequent film exposure for 5 min. Presence of proteins were verified by comparing protein bands to the Molecular Rainbow Weight Marker (Amersham Biosciences). As negative controls, samples were incubated without primary antibodies. Equal loading of protein was assessed on stripped blots by immunodetection of β-actin using peroxidase-conjugated goat anti-human polyclonal antibody diluted at 1:350 (Santa Cruz Biotechnology).

APOPTOSIS DETECTION IN SITU

Gastrocnemius muscles from *mdx* mice were carefully removed, placed for 4 h in 4% paraformaldehyde solution and submitted to cryoprotection by sequential incubation with a gradient of sucrose solution (10%, 20%, and 30%, w/v, in PBS) for 6 h at 4°C in each solution. Cryostat cross-sections (10 μm, spaced 200 μm) were mounted on poly-L-lysine precoated slides and rinsed for 20 min in PBS. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method was utilized to identify the apoptotic cells (Upstate, Temecula). In brief, after incubation of protein kinase for 30 min at 37°C and further washing steps, sections were labeled with biotinylated nucleotides using terminal deoxynucleotidyl transferase (TdT) enzyme for 1 h in a humidity chamber at 37°C. The incorporated nucleotides were detected using avidin–FITC-labeled solution in the dark for 30 min at 37°C and sections were counterstained with Dapi. Images were randomly obtained with a Nikon Eclipse TE2000-U microscope with identical time exposure and image settings and merged on Adobe Photoshop CS3.

HISTOLOGICAL STAINING AND MORPHOMETRIC ANALYSIS

Gastrocnemius muscles from *mdx* mice were carefully removed and fixed in formalin-buffered Millonig fixative (pH 7.2) for 24 h. Five-micrometer thick sections of wax-embedded material were stained with hematoxilin–eosin and sirius red for collagen. High definition whole area images of all cross-sections from each *mdx* mouse at a time point were obtained from individual photomicrographs with a microdigital camera mounted on a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) using a 20× objective. Images were blended using Adobe Photoshop CS3 Extended software. Total surface area and areas occupied by inflammatory infiltrates and collagen deposition were determined with Image-Pro 4.5 (Media Cybernetics, Inc.). Results were expressed as percentage of total area in the cross-section.

QUANTITATIVE AND STATISTICAL ANALYSIS

All experiments were conducted in at least triplicates. Quantitative analysis was performed using the image-analysis software Scion

Image for Windows (Scion Corporation, National Institutes of Health; Bethesda, MD) for ChAT and AChE enzymatic activity analysis, zymography, western blotting, TUNEL, and morphometry. GraphPad Prism 5 (GraphPad Software, Inc.) was used to calculate mean and standard deviations. One-way ANOVA and unpaired *t*-test were applied to obtain statistical significance of means. Differences were considered to be statistically significant at the 0.05 level of confidence.

RESULTS

EFFECT OF *E. PUNICIFOLIA* TREATMENT ON CHAT AND ACHE ACTIVITIES IN *MDX* SKELETAL MUSCLE

E. punicifolia aqueous extract exerts pro-cholinergic effects [Grangeiro et al., 2006] but in vivo treatment with methanolic or the dichloromethanic fraction of *E. punicifolia* did not modify ChAT and AChE enzymatic activities in the gastrocnemius muscle of *mdx* mice (Fig. 1A,B).

E. PUNICIFOLIA TREATMENT DECREASES MMPS-9 AND -2 ACTIVITIES IN *MDX* SKELETAL MUSCLE

Activities of MMP-9 and -2 in *mdx* gastrocnemius muscle were analyzed as indicators of local inflammation and tissue remodeling, respectively. Muscle of *mdx* mice treated with *Ep*-CM from 3 to 4 weeks of age during the period of prominent muscular inflammation showed significant reduction of MMP-9 ($62 \pm 12\%$, $P < 0.005$) and MMP-2 ($58 \pm 10\%$, $P < 0.005$) activities in comparison with *mdx* muscle implanted with vehicle (Fig. 2). It was not observed any effect with the methanolic fraction.

E. PUNICIFOLIA TREATMENT DECREASES PRODUCTION OF INFLAMMATORY PROTEINS IN *MDX* SKELETAL MUSCLE

Ep-CM implant during 7 days reduced TNFα production ($42 \pm 9\%$, $P < 0.01$; Fig. 3A) and NFκB expression ($48 \pm 7\%$, $P < 0.005$; Fig. 3B) in *mdx* gastrocnemius muscle in comparison with control groups.

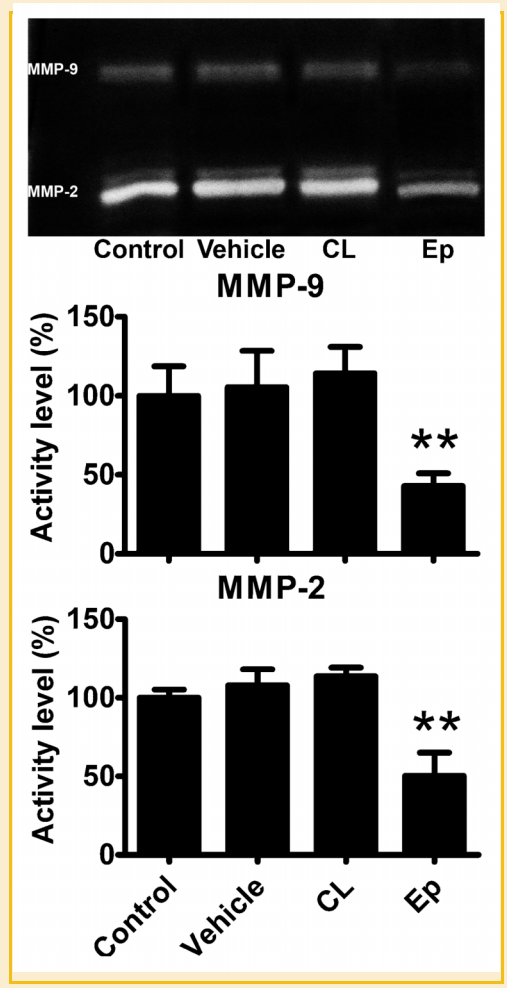

Fig. 2. Local *Ep*-CM treatment reduces MMP-9 and -2 activity level. Zymograms of metalloprotease activity from gastrocnemic skeletal muscles and graphs showing the activity level of MMP-9 and pro-MMP-2 after 7 days of *Ep*-CM treatment at 4 weeks. One-way ANOVA test for both MMPs showed $P < 0.001$. Unpaired *t*-test analyses ($^{**}P < 0.01$). Results are expressed as mean ± SD (n = 6 for each group).

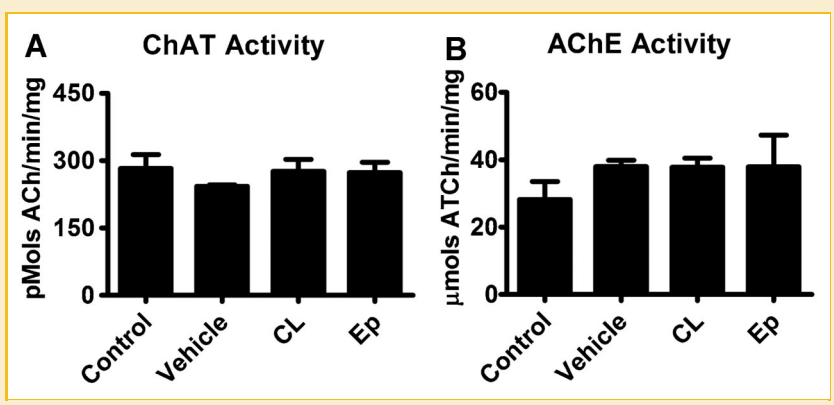

Fig. 1. ChAT and AChE enzymatic activities after local *Ep*-CM treatment. Ten microliters aliquots of the homogenized gastrocnemius muscles indicated were used to determine AChE- and ChAT-specific activities, as described in the Materials and Methods Section. No statistical difference was observed between any of the results in comparison to control groups (One-way ANOVA and unpaired *t*-test analyses). Results are expressed as mean ± SD (n = 4 for each group).

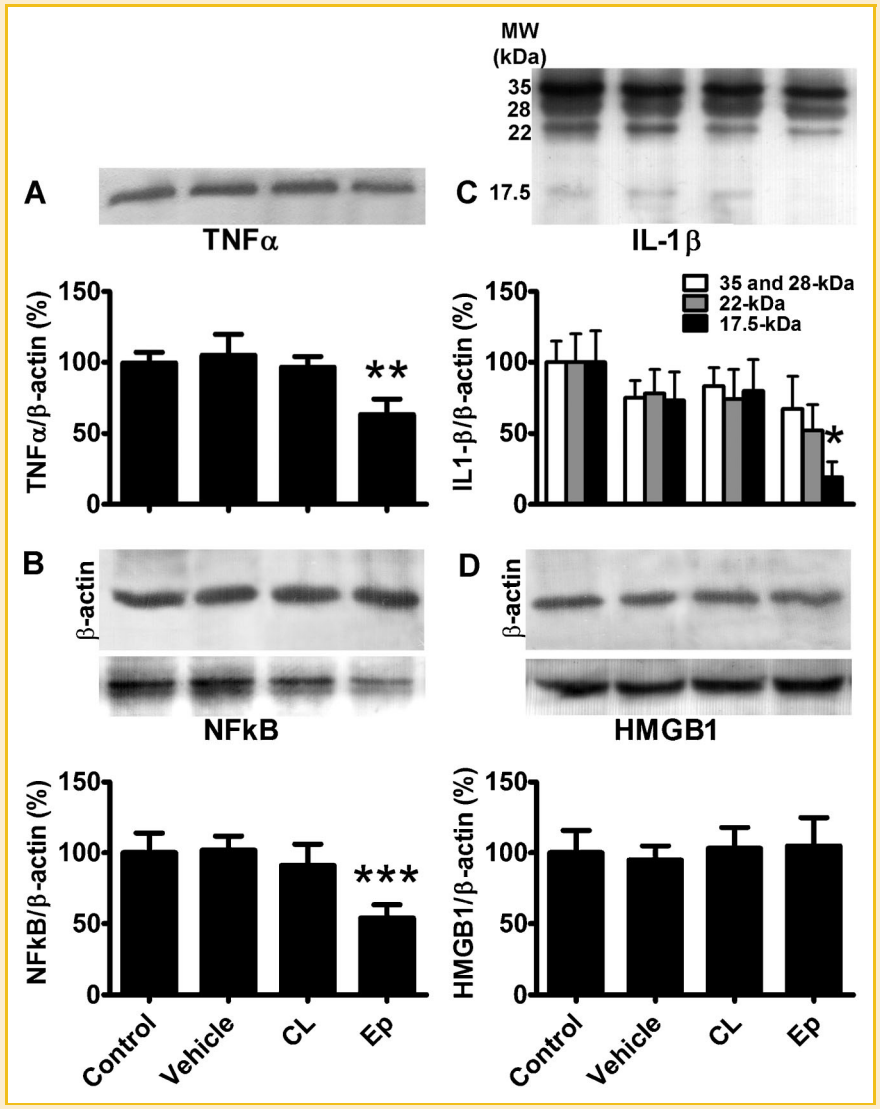

Fig. 3. Local *Ep*-CM treatment reduces inflammatory mediators. Immunoblots and graphs showing (A) TNFα, (B) NFκB, (C) IL-1β forms, and (D) HMGB1 protein expression levels in gastrocnemius muscle after 7 days of *Ep*-CM treatment at the period of inflammatory prevalence at 4 weeks age. The 43-kDa β-actin immunodetection was used as loading control for TNFα and NFκB on the same stripped blots, and also for IL-1β and HMGB1. One-way ANOVA test for TNFα, NFκB, and the bioactive IL-1β form showed $P < 0.01$, $P < 0.001$, and $P < 0.005$, respectively. Unpaired *t*-test analyses ($^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$). Results are represented as mean ± SD (n = 5 for each group).

IL-1β was detected in four forms: 35- and 28-kDa pro-IL-1β, a cleaved 22-kDa inactive, and a cleaved 17.5-kDa bioactive form [Yao et al., 2006]. It was observed reduced expression levels only of the 17.5-kDa IL-1β bioactive form after *Ep*-CM treatment in comparison with *mdx* muscle implanted with vehicle implant (54 ± 13%, $P < 0.05$, Fig. 3C). It was not observed significant alteration of the pro-inflammatory protein HMGB1 (Fig. 3D).

E. PUNICIFOLIA TREATMENT REDUCES APOPTOSIS IN *MDX* SKELETAL MUSCLE

TUNEL was used to assess myofibers undergoing apoptosis. Treatment of *mdx* gastrocnemius muscle at 4 weeks during active myonecrosis with *Ep*-CM caused a reduction of 38 ± 13% ($P < 0.05$) in the number of myofibers with fragmented DNA compared with vehicle implant (Fig. 4A,B). Likewise, reduced expression of cleaved caspase-3 (40 ± 9%, $P < 0.05$) which corresponds to apoptosis signaling cascade activation was consistently observed in muscle homogenates from *Ep*-CM implant in comparison with vehicle implant (Fig. 4C).

E. PUNICIFOLIA TREATMENT DURING 9 WEEKS REDUCES INFLAMMATORY INFILTRATE AND IMPROVES REGENERATION OF *MDX* SKELETAL MUSCLE

In order to assess if prolonged treatment with the *Ep*-CM extract would influence *mdx* inflammatory lesion, it was analyzed whole cross-sections of *mdx* gastrocnemius muscles from five different

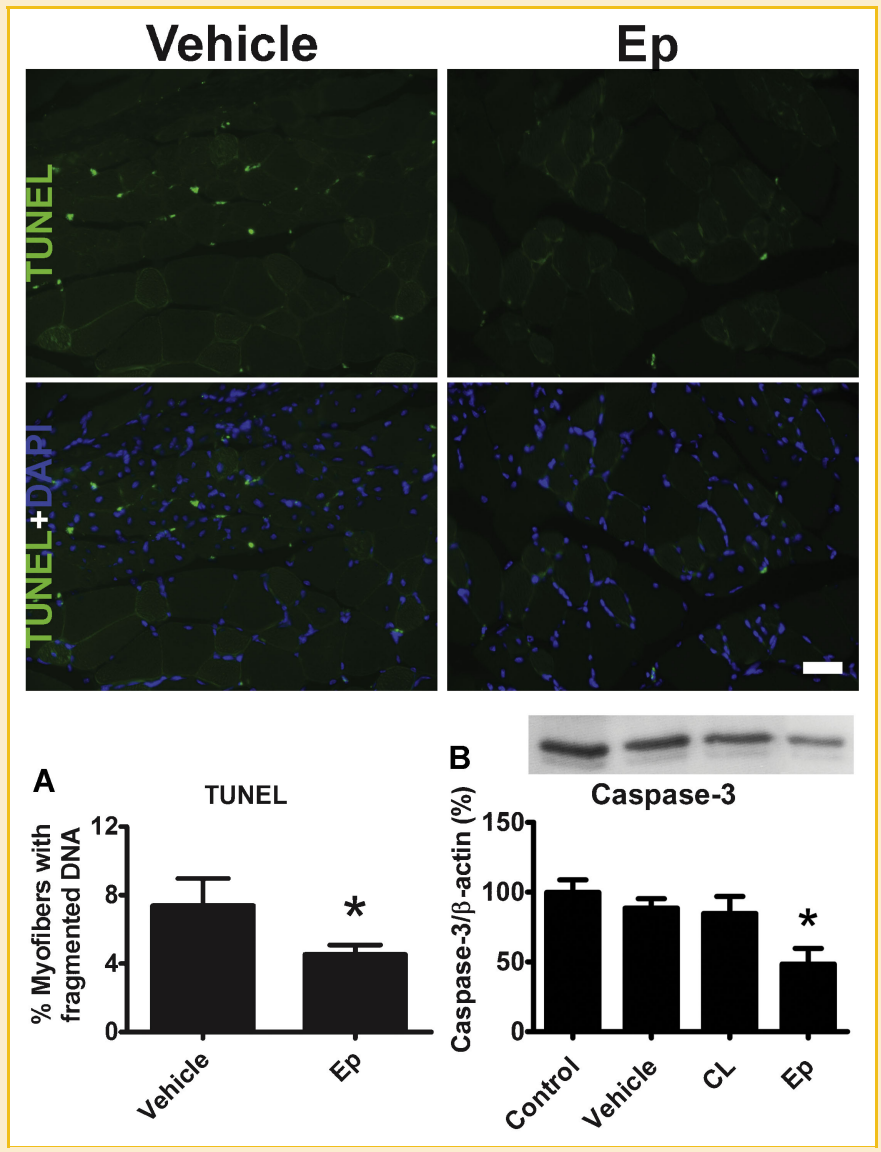

Fig. 4. Local *Ep*-CM treatment reduces apoptosis. On top, incorporated nucleotides FITC-labeled on myofiber nuclei undergoing apoptosis and the same images merged with Dapi from vehicle and local *Ep*-CM-treated *mdx* mice after 7 days of *Ep*-CM treatment at 4 weeks. Scale bar 50 μm. Below, graphs showing quantification of (A) myofibers with fragmented DNA and (B) analysis of cleaved caspase-3 expression. Forty-three-kDa β-actin was used as loading control (image not shown). One-way ANOVA test showed $P < 0.05$. Unpaired *t*-test analyses ($^*P < 0.05$). Results are represented as mean ± SD (n = 3 for TUNEL assay and n = 5 for cleaved caspase-3 immunoblot).

mice. The total area of inflammatory infiltrate in gastrocnemius muscle from vehicle implanted and the contralateral muscle corresponded to 8 ± 1% of the total muscle. In contrast, it was observed a marked reduction of the inflammatory lesion area (86 ± 22%; $P < 0.05$; Fig. 5A) in *Ep*-CM polymer implant in *mdx* gastrocnemius muscle.

Myogenin content was used as a parameter of protein marker of late muscular regeneration. *Mdx* control mice at 12 weeks showed muscle regeneration associated with marked deposition of connective tissue. Skeletal muscles from *mdx* mice implanted with *Ep*-CM showed a significant increase of myogenin content in relation to the contralateral muscle and muscle with vehicle implant (44 ± 12%, $P < 0.05$; Fig. 5B), but no significant alteration in the collagen content (Fig. 5C).

DISCUSSION

Several studies have shown that activation of cholinergic mechanisms mitigate inflammation in several mouse models of diseases [van Westerloo et al., 2005; Osborne-Hereford et al., 2008; Rosas-Ballina et al., 2008; van Maanen et al., 2009]. Previous data showed that *E. punicifolia* aqueous extract used as a natural pharmacological agent with putative action upon cholinergic neurotransmis-

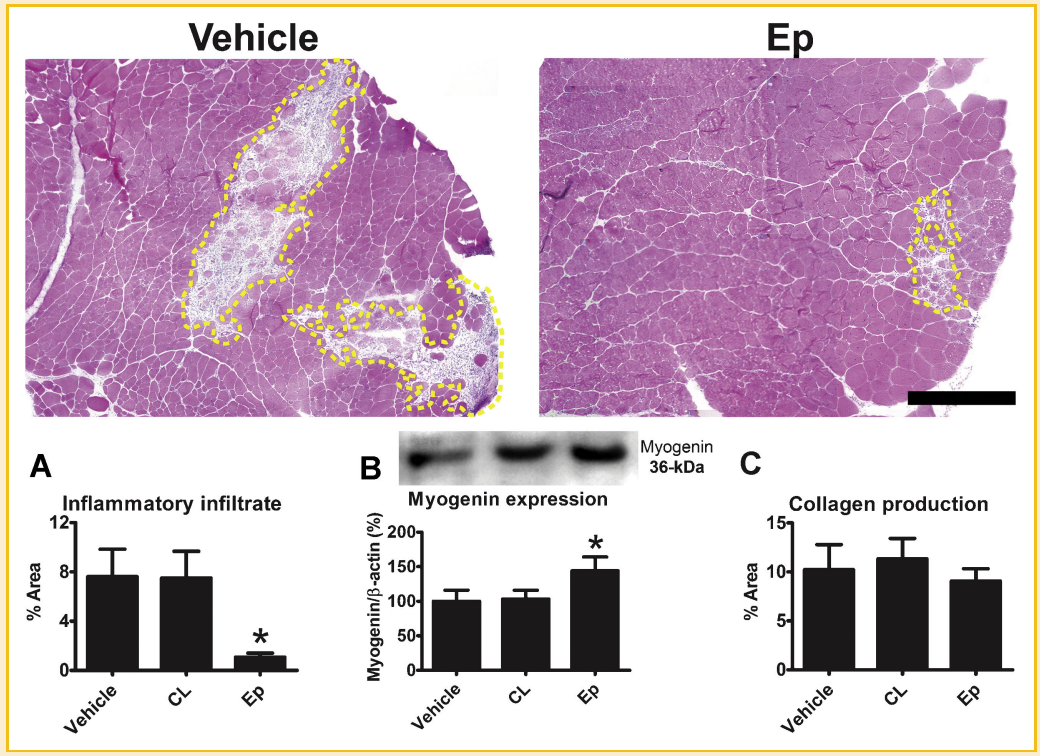

Fig. 5. Histological and regeneration analysis of mdx gastrocnemius muscle after 9 weeks of local *Ep*-CM treatment. On top, high powered images of gastrocnemius muscle from *mdx* mice at 12 weeks of age after local *Ep*-CM or vehicle treatment during 9 weeks. Sections were stained with hematoxilin–eosin for characterization of inflammatory infiltrate depicted by yellow dashed lines. Scale bar 200 μm. Below, graphs showing quantification of (A) inflammatory infiltrate, (B) analysis of myogenin protein expression, and (C) collagen production. Forty-three-kDa β-actin was used as loading control (image not shown). One-way ANOVA test for inflammatory infiltrate and myogenin analysis showed $P < 0.05$. Unpaired *t*-test analyses ($^*P < 0.05$). Results are represented as mean ± SD (n = 5 for each group of all analysis).

sion [Grangeiro et al., 2006] was able to totally recover the effects of the cholinergic nicotinic competitive antagonists pancuronium and gallamine in the neuromuscular junction of rat diaphragm. It was also proposed that the aqueous extract had acetylcholinesterase inhibitory activity. Since then, investigation in other experimental models and the utilization of chemical fractions of the plant extract were carried out in attempt to recognize class of compounds capable to enhance the cholinergic neurotransmission. Recently we showed that nAChR activation influences local inflammatory responses in the muscular lesion of *mdx* mice [Leite et al., 2010]. We fractionated the *E. punicifolia* extract with solvents of different polarities: hexane, dichloromethane, and methanol, but results with statistical relevance were obtained only with the dichloromethane fraction. We present evidence that local in vivo treatment with the dichloromethane fraction exerts anti-inflammatory properties in the gastrocnemius muscle of *mdx* dystrophic mice by mechanisms not related to the pro-cholinergic activation.

In the present work we demonstrate that the *Ep*-CM fraction was not able to induce the synthesis of acetylcholine in vivo, since in situ ChAT and AChE activities were not altered after treatment with the *Ep*-CM. Such results ruled out the possibility that *Ep*-CM would inhibit the action of AChE and amplify acetylcholine receptor interaction. It remains to be elucidated the mechanism by which aqueous *E. punicifolia* extract presents pro-cholinergic properties via activating receptors and/or by the competition for the receptor site with specific antagonists.

TNFα, considered a major indicator of local inflammation amplifies and prolongs the inflammatory response by activating other cells to increase TNFα production, NFκB activation, IL-1β, HMGB1 [Tracey, 2002], and MMP-9 [Kherif et al., 1999]. The protein exists as a 27-kDa membrane-bound precursor that can be processed by TNFα converting enzyme (TACE) and MMP-9 to generate a 17-kDa mature TNFα [Kherif et al., 1999; Mullberg et al., 2000]. TNFα upregulation activates the signaling cascade downstream of the NFκB activation, including activation of I kappa B kinase (IKK) followed by IκB proteins phosphorylation, NFκB translocation, and subsequent gene transcription resulting in increased TNFα protein production [Yoshikawa et al., 2006]. Increased levels of these inflammatory proteins can result in cellular apoptosis [Tracey, 2007] and induce the activation of NFκB, which is a class of protein that responds to stimuli by activating the expression of numerous genes, including those involved with cellular proliferation, migration, angiogenesis, and inflammation. NFκB activation can thus amplify production of several inflammatory proteins.

The present results demonstrate that local treatment with *Ep*-CM reduced MMPs activity, production of the 17-kDa TNFα and 17.5-kDa IL-1β bioactive forms, and NFκB. In addition, treatment reduced apoptosis, determined by cleaved caspase-3 immunodetec-

tion and myofibers with fragmenting of DNA during active myonecrosis stage of *mdx* gastrocnemius muscles. Yet, HMGB1 levels were not altered by treatment. This protein is highly related to induction of inflammation and severe sepsis only when secreted, but has an important physiologic role in the structural dynamics by facilitating gene transcription and DNA repair [Lange and Vasquez, 2009]. This suggests that HMGB1 levels correspond to its normal nuclear content, since wild-type non-dystrophic mice showed similar levels (data not shown). It is conceivable that *Ep*-CM treatment downregulated activation of genes associated with inflammatory proteins.

Ep-CM treatment during 7 days did not increase myogenin expression which is associated with myofiber regeneration (data not shown) but decreased production of inflammatory mediators and further contributed to efficient skeletal muscle repair in *mdx* dystrophic mice evidenced by increased myogenin levels after 9 weeks of treatment. Indeed controlled production of inflammatory mediators are associated with reduction of muscle wasting [Rendon-Mitchell et al., 2003] and NFκB activation [Messina et al., 2006] which can upregulate myogenic transcription factor MyoD, essential for new muscle fiber formation [Guttridge et al., 2000]. In this sense, the local treatment with *E. punicifolia* extract decreased the mentioned inflammatory mediators, and seems to have contributed to efficient skeletal muscle repair in *mdx* dystrophic mice evidenced by increased myogenin levels, without affecting collagen deposition and induce the non-functional fibrosis by a yet unknown mechanism.

The present work shows that local treatment with *Ep*-CM decreased production of inflammatory mediators, reduced apoptosis and contributed to efficient muscular regeneration of *mdx* dystrophic mice, without inducing fibrosis. Future studies are necessary to isolate all bioactive substance(s) from *E. punicifolia* and determine their effects in mitigating myopathy and/or promoting activation of mechanisms related with efficient muscular regeneration, and evaluating its potential use in the treatment of DMD and other inflammatory diseases.

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