



## ORIGINAL ARTICLE

# Laser therapy reduces gelatinolytic activity in the rat trigeminal ganglion during temporomandibular joint inflammation

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**OBJECTIVES:** To investigate whether low-level laser therapy (LLLT) alters the expression and activity of MMP-2 and MMP-9 in the trigeminal ganglion (TG) during different stages of temporomandibular joint (TMJ) inflammation in rats. It also evaluated whether LLLT modifies mechanical allodynia and orofacial hyperalgesia.

**MATERIALS AND METHODS:** Wistar rats ( $\pm 250$  g) were divided into groups that received saline (SAL) or complete Freund's adjuvant (CFA, 50  $\mu$ l) in the TMJ, and that later underwent LLLT (20 J cm<sup>-2</sup>) at their TMJ or not (groups SAL, SAL + LLLT, CFA, and CFA + LLLT). LLLT was applied on days 3, 5, 7, and 9 after SAL or CFA. Mechanical allodynia was evaluated on days 1, 3, 5, 7, and 10; orofacial hyperalgesia was assessed on day 10. Gelatin zymography and *in situ* zymography aided quantification of MMPs in the TG.

**RESULTS:** Low-level laser therapy abolished the reduction in the mechanical orofacial threshold and the increase in orofacial rubbing during the orofacial formalin test induced by CFA. LLLT also decreased the CFA-induced rise in the levels of MMP-9 and MMP-2 as well as the gelatinolytic activity in the TG.

**CONCLUSION:** Low-level laser therapy could constitute an adjuvant therapy to treat temporomandibular disorders and prevent inflammation-induced alterations in the levels of MMP-2 and MMP-9 and in the gelatinolytic activity in TGs.

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**Keywords:** matrix metalloproteinase 2; matrix metalloproteinase 9; temporomandibular joint disorders; low-level laser therapy; orofacial pain

## Introduction

Orofacial pain and temporomandibular disorders (TMDs) are highly prevalent and affect approximately 40–75% of the population (Atsu and Ayhan-Ardic, 2006; De Leeuw, 2008). TMD etiologies have multifactorial origin, including physiological, behavioral, and environmental factors (Cairns, 2010). TMD is involved in stress-related disorders that are characterized by somatic and psychological alterations, such as anxiety and depression (Bush *et al*, 1989; Korszun *et al*, 1996; Nascimento and Leite-Panissi, 2014). The complex characteristics of TMDs and the intense pain reported by patients have led to the emergence of many non-surgical strategies to treat these disorders, including the use of medications such as analgesics and anti-inflammatories, physical therapy, occlusal adjustment, acupuncture, and low-level laser therapy (LLLT) (Petrucci *et al*, 2011).

Application of LLLT effectively relieves pain in the temporomandibular joint (TMJ) region and in the masticatory muscles (Maia *et al*, 2012). LLLT has come into more frequent use because it exerts analgesic and anti-inflammatory activities (Honmura *et al*, 1993; Sakurai *et al*, 2000) as well as muscle relaxation action (Nunez *et al*, 2006). The anti-inflammatory and analgesic properties of LLLT may originate from the fact that laser photobiostimulation reduces the levels of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and interleukin-1 $\beta$  (IL-1 $\beta$ ), two important proinflammatory mediators (Honmura *et al*, 1993; Shimizu *et al*, 1995).

Inflammatory cytokines stimulate the synthesis of matrix metalloproteinases (MMPs), which contribute to the inflammatory process (Shinmei *et al*, 1989). MMPs constitute a family of metalloenzymes that cleave various extracellular matrix components (Cauwe and Opdenakker, 2010). In particular, gelatinases A (MMP-2) and B (MMP-9) are the most often studied MMPs and the most frequently expressed MMPs in the nervous system (Dzwonek *et al*, 2004). Additionally, both MMP-2 and MMP-9 participate in many processes that promote successful neuronal regeneration, such as postinjury de- and remyelination, inflammation, and glial reactivity (Verslegers *et al*, 2013). In the

particular case of MMP-9, it presents rapid and transient upregulation in the injured dorsal root ganglion (DRG) primary sensory neurons, whereas MMP-2 exhibits delayed response in the DRG satellite cells and spinal astrocytes, which is consistent with late-phase neuropathic pain (Kawasaki *et al*, 2008). Again, a previous study has shown that the development of complete Freund's adjuvant (CFA)-induced TMJ inflammation in rats requires the expression of different MMPs in the trigeminal ganglion (TG) (Nascimento *et al*, 2013). MMP-9 expression is greater in the early phase of inflammation (days 1 and 3 following CFA administration into the TMJ), while MMP-2 expression is more pronounced in the late phases of this process (days 7 and 10 following the start of TMJ inflammation) (Nascimento *et al*, 2013). It is noteworthy that satellite glial cells (SGCs) are peripheral glial cells that form a continuous layer around primary sensory neurons within DRGs and trigeminal ganglia (TGs). Furthermore, SGCs have been implicated in the regulation of neuronal homeostasis and neurotransmission in DRGs and TGs (Hanani, 2005).

This investigation aimed to evaluate whether LLLT alters the levels of MMP-2 and MMP-9 (as assayed by gelatin zymography) as well as the gelatinolytic activity in TGs during different stages of temporomandibular inflammation in rats. This study also assessed whether LLLT modifies mechanical allodynia and orofacial hyperalgesia induced by injection of CFA into the TMJ capsule.

## Materials and methods

### Animals

Experiments were performed with Wistar male rats weighing 200–250 g, obtained from the animal facility of the University of São Paulo, Campus of Ribeirão Preto, Brazil. Animals were housed in a room with a controlled temperature ( $24 \pm 1^\circ\text{C}$ ) and a 12-h light/dark cycle with food and water *ad libitum*. The experiments were carried out in compliance with the recommendations of SBNcC (Brazilian Society of Neuroscience and Behavior) and with the approval of the Animal Care and Use Committee of Universidade de São Paulo, campus Ribeirão Preto, SP, Brazil (Protocol # 11.1.888.53.5). All efforts were made to minimize animal suffering.

### Administration of CFA

Initially, rats were anesthetized with an intramuscular injection of ketamine  $10\%$  ( $75 \text{ mg kg}^{-1}$ ) and xylazine  $4\%$  ( $10 \text{ mg kg}^{-1}$ ) followed by bilateral intra-articular administration with  $50 \mu\text{g}$  of CFA (*Mycobacterium tuberculosis*) suspended in a  $50 \mu\text{l}$  paraffin oil (Sigma, St. Louis, MO, USA) or  $0.9\%$  saline solution (SAL). This dose was based on previous reports (Harper *et al*, 2001; Nascimento *et al*, 2013; Nascimento and Leite-Panissi, 2014). A  $26 \text{ G } \frac{1}{2}$  needle attached to a 1-ml plastic syringe was used for the injection. To locate the TMJ for the injection, we palpated the zygomatic arch and the condyle. The needle was inserted immediately below the posteroinferior border of the zygomatic arch and advanced anteriorly to contact the edge of the posterolateral condyle (Zhou *et al*, 1999).

### Low-level laser therapy

A low-level intensity infrared laser (Laser Twin Set MM Optics, São Carlos, São Paulo, Brazil) with a gallium–aluminum–arsenide semiconductor (GaAlAs) was used. Before the administration of intra-articular CFA or SAL, the TMJ regions of the animals were scraped. Immediately after the administration of CFA or SAL, the LLLT session took place. Irradiation was repeated on days 3, 5, 7, and 9 after the induction of persistent inflammation. The rats were physically restrained to avoid light beam diffraction. According to a previous study (Iyomasa *et al*, 2013), an energy dose of  $20 \text{ J cm}^{-2}$  of GaAlAs was used (continuous wave  $40 \text{ mW}$ , time  $20 \text{ s}$ ,  $\lambda = 780 \text{ nm}$ ,  $0.04 \text{ cm}^2$  of spot area), at only one point on the TMJ. The

laser irradiation was performed in direct contact with the skin, on one point on the TMJ (Energy:  $0.8 \text{ J point}^{-1}$ , power density:  $1 \text{ W cm}^{-2}$ ).

### Mechanical orofacial sensitivity

To assess mechanical orofacial sensitivity before (control period) and 3, 5, 7, and 10 days after the bilateral administration of CFA (CFA group) or saline solution  $0.9\%$  (SAL group) into the TMJ, we evaluated the head withdrawal reflex during the application of the mechanical stimuli. To measure the head withdrawal reflex, rats were placed in the testing chamber for a minimum 30-min adaptation period. Progressive, increasing forces from the filament of an electronic von Frey anesthesiometer (Insight Instruments, Ribeirão Preto, São Paulo, Brazil) were applied to the TMJ region until the head was withdrawn. The withdrawal threshold head of each rat was calculated as the mean  $\pm$  standard error of means (s.e.m.) of three values obtained in each session. All experiments were carried out in a quiet room between 9:00 and 11:00 am to minimize diurnal variations. To assess the role of the LLLT in the mechanical orofacial sensitivity, independent groups of rats were administered SAL or CFA injections and submitted or not to LLLT sessions ( $n = 8$  per group). These groups were subjected to mechanical sensitivity evaluations as previously described.

### Nociceptive orofacial sensitivity

To evaluate the orofacial sensitivity, nociceptive front of a noxious stimulus acute located in the orofacial region, we performed the orofacial formalin test in rats after administration of CFA or SAL  $0.9\%$ , intra-articular, bilaterally, in TMJs and submitted or not to the sessions of LLLT ( $n = 8$  per group). Ten days after the beginning of the experiments, the rats were habituated to testing chamber for 20 min, for the administration of  $2\%$  formalin (Nascimento *et al*, 2013). The experimental room had little human activity and a controlled temperature of  $25 \pm 1^\circ\text{C}$ . The animals were removed from the box, and a volume of  $50 \mu\text{l}$  of formalin solution was injected subcutaneously into the orofacial region between the nose and the upper lip. A  $26 \text{ G } \frac{1}{2}$  needle attached to a 1-ml plastic syringe graduated was used for the injections. The administration was done under the briefly anesthesia, with halothane, as quickly as possible, so that the animal was not manipulated for a long time. Immediately after the injections, the rat was returned to the testing chamber, and the time spent by the rat with the behavior of rubbing the area injected, in seconds, was registered for 45 min. The analysis was performed on 15 blocks of 3 min each. In time, the formalin test orofacial is characterized by distinct phases, one initial acute phase, the silent period, and a second phase (Grabow and Dougherty, 2001). For the assessment of nociceptive sensitivity, it is important to note that previous manipulations may interfere in the nociception. In this way, the nociceptive orofacial sensitivity was performed on the tenth day experimental.

### Removal of TG

To quantify MMPs, a separate group of rats that received administration of CFA or SAL submitted or not to the sessions of LLLT ( $n = 6$  per group and per experimental period) as previously described were euthanized with an anesthesia overdose ( $300 \text{ mg kg}^{-1}$   $10\%$  ketamine, plus  $30 \text{ mg kg}^{-1}$   $4\%$  xilazine) 1, 3, 5, 7, or 10 days after the beginning of the experiment. These time points were determined based on a previous study (Nascimento *et al*, 2013). After euthanasia, the TG of each rat was removed bilaterally. The TGs were dissected and one side was frozen in Tissue Tek OCT (*in situ* zymography to assess gelatinolytic activity), while the TG of the other side was frozen at  $-80^\circ\text{C}$  for zymography (gelatin zymography of the TG for the quantification of MMP-2 and MMP-9). Additionally, because some reports have demonstrated that low levels of MMPs can be expressed in the unstimulated brain (Dzwonek *et al*, 2004), distinct groups without any TMJ manipulation (Control groups) were euthanized and the TG were dissected and analyzed in all protocols used in this study.

To make sure that the appropriate anatomical region had been dissected, sections of the TG were stained with hematoxylin and eosin and observed using a light microscope.

### Gelatin zymography of the TG

Gelatin zymography is one of the most commonly employed methods for the quantification of MMP-2 and MMP-9. Under denaturing conditions, enzymes are separated by molecular weight using gel electrophoresis. Then enzymes were refolded and the different molecular weight forms

were visualized in zymograms. In this study, gelatin zymography of MMP-2 and MMP-9 from TG was performed as previously described (Castro *et al*, 2008). Briefly, frozen TG samples (0.08 g) were homogenized in 300  $\mu$ l of extraction buffer containing 10 mM CaCl<sub>2</sub>, 50 mM Tris-HCl pH 7.4, 1 mM Phe (1, 10 ortho-phenanthroline), 1 mM PMSF (phenylmethanesulfonylfluoride), and 1 mM NEM (*N*-ethylmaleimide). These samples were placed in the refrigerator for 20 h on ice to extract the proteins. The samples were then centrifuged at 3000 g for 15 min. The protein content was measured with the Bradford method (Bradford, 1976). The samples were then diluted in sample buffer (2% SDS, 125 mM Tris-HCl pH 6.8, 10% glycerol, and 0.001% bromophenol blue) and subjected to electrophoresis on 12% SDS-PAGE copolymerized with gelatin (1%) as the substrate. After electrophoresis was completed, the gel was incubated for 1 h at room temperature in a 2% Triton X-100 solution, followed by incubation at 37°C for 16 h in Tris-HCl buffer (pH 7.4), containing 10 mmol l<sup>-1</sup> CaCl<sub>2</sub>. The gels were stained with 0.05% Coomassie Brilliant Blue G-250 and then destained with 30% methanol and 10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin. Enzyme activity was determined with densitometry using a Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 (Kodak, Rochester, NY, USA). Gelatinolytic activities were normalized against an internal standard (fetal bovine serum) to allow intergel analyses and comparisons. Based on previous study (Sang *et al*, 1990), the bands at 75, 72, and 64 kDa identified MMP-2, and MMP-9 was identified as the 92-kDa band.

### *In situ* zymography to assess gelatinolytic activity

*In situ* MMP activity was measured in frozen TGs using DQ Gelatin (E12055; Molecular Probes, Eugene, OR, USA) as a fluorogenic substrate. Briefly, TG samples were embedded in Tissue Tek and cut into 5- $\mu$ m sections with a cryostat. Sample sections were incubated with 1.0 mg ml<sup>-1</sup> DQ gelatin in Tris-CaCl<sub>2</sub> buffer (50 mM Tris, 10 mM 177 CaCl<sub>2</sub>, 1 mM ZnCl<sub>2</sub>) in dark humidified chambers for 1 h. The sections were examined with fluorescent microscopy (Leica Imaging Systems Ltd., Cambridge, UK), and the image was captured at magnification of 400 $\times$ . Negative control sections were incubated in the same way as described above, but without DQ-gelatin. Some sections were incubated either with a metalloproteinase inhibitor, 1,10-phenanthroline (Phe), with a serine protease inhibitor, PMSF (Sigma Chemicals, St. Louis, MO, USA), at 2 mM, or with both inhibitors. Proteolytic activity was detected as bright green fluorescence, which indicates substrate breakdown and was evaluated using ImageJ software (National Institute of Health, Bethesda, MD, USA). DAPI (4'-6-diamidino-2-phenylindole) was applied for 3 min, and specimens were washed with phosphate-buffered saline and assayed by fluorescence microscopy to identify cell nuclei. DAPI was detected as blue fluorescence; all images were evaluated using the ImageJ software.

### Statistical analysis

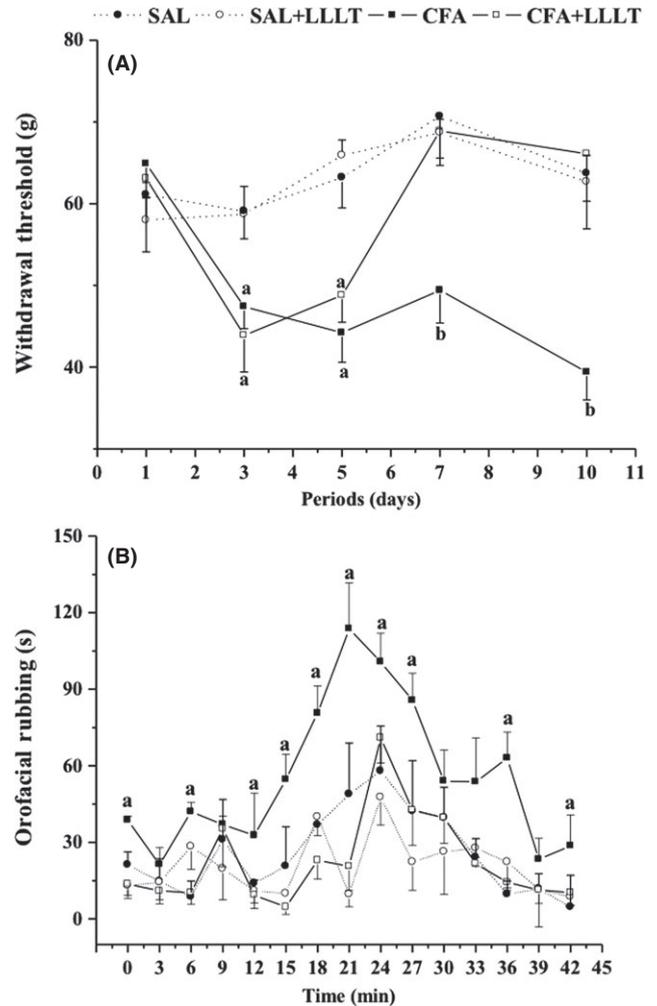
Mechanical sensitivity was determined by the mean  $\pm$  s.e.m. for the threshold (g) of the head withdrawal reflex testing. To analyze the results of the nociceptive sensitivity test, the duration in seconds that animals spent rubbing the vibrissal pad was recorded for 15 intervals of 3 min each. These data are expressed as the mean  $\pm$  s.e.m. for each interval. For both protocols, comparisons between the groups were assessed by a two-way analysis of variance to repeated measures (ANOVA RM) or a two-way ANOVA followed by Newman-Keuls test. A probability value  $P < 0.05$  was considered to be significant.

To evaluate the expression of MMPs, the programs used were GeneTools 8.0 (Synoptics, Cambridge, UK) and ImageJ and it was performed the quantification by means of arbitrary units  $\pm$  s.e.m. of MMP-2 or MMP-9 in each period of survival evaluated. Statistical analysis was performed by two-way analysis of variance (ANOVA) (variables are time and treatment), followed by the Newman-Keuls *post hoc* test, with the level of significance set at  $P < 0.05$ .

## Results

### *Evaluation of mechanical sensitivity in rats with persistent TMJ inflammation submitted to LLLT*

Bilateral CFA administration to the TMJ region reduced the mechanical threshold of the withdrawal reflex (Figure 1A). In groups SAL and SAL + LLLT, injection of



**Figure 1** Mechanical and nociceptive sensitivity. (A) Mean  $\pm$  s.e.m. of the mechanical withdrawal threshold (g) of rats in the control period (day 1) and 3, 5, 7, and 10 days after injection of 0.9% saline solution (SAL) or complete Freund's adjuvant (CFA) into the temporomandibular joint (TMJ) regions; the rats were submitted to low-level laser therapy (LLLT, SAL + LLLT, and CFA + LLLT) or were left untreated (SAL and CFA). (B) Mean  $\pm$  s.e.m. of the orofacial rubbing (s) of rats that were injected with SAL or CFA into TMJ regions; the rats were treated with LLLT (SAL + LLLT and CFA + LLLT) or were left untreated (SAL and CFA). Ten days after injection, 2% formalin was administered to the vibrissal pad of the rats. a:  $P < 0.05$ , Newman-Keuls when compared with SAL, SAL + LLLT, and CFA + LLLT. b:  $P < 0.05$ , Newman-Keuls when compared with SAL and SAL + LLLT. s.e.m.: standard error of the mean

SAL into the TMJ did not significantly decrease the mechanical threshold along the experiment (Figure 1A). Statistical analysis (two-way ANOVA) considering the factors treatment, time, and interaction between treatment and time revealed significant differences in the mechanical orofacial threshold ( $P < 0.001$  for all the factors). The *post hoc* Newman-Keuls test demonstrated that group CFA differed from groups SAL and SAL + LLLT along all the experiment and from group CFA + LLLT on days 7 and 10 after injection ( $P < 0.05$ ). LLLT abolished the reduction in the mechanical orofacial threshold following CFA injection (Figure 1A). Statistical analysis evidenced that group CFA + LLLT differed significantly from

groups SAL and SAL + LLLT only on days 3 and 5 after the beginning of the experiment ( $P < 0.05$ , Newman–Keuls test).

*Evaluation of nociceptive sensitivity in rats with persistent TMJ inflammation submitted to LLLT*

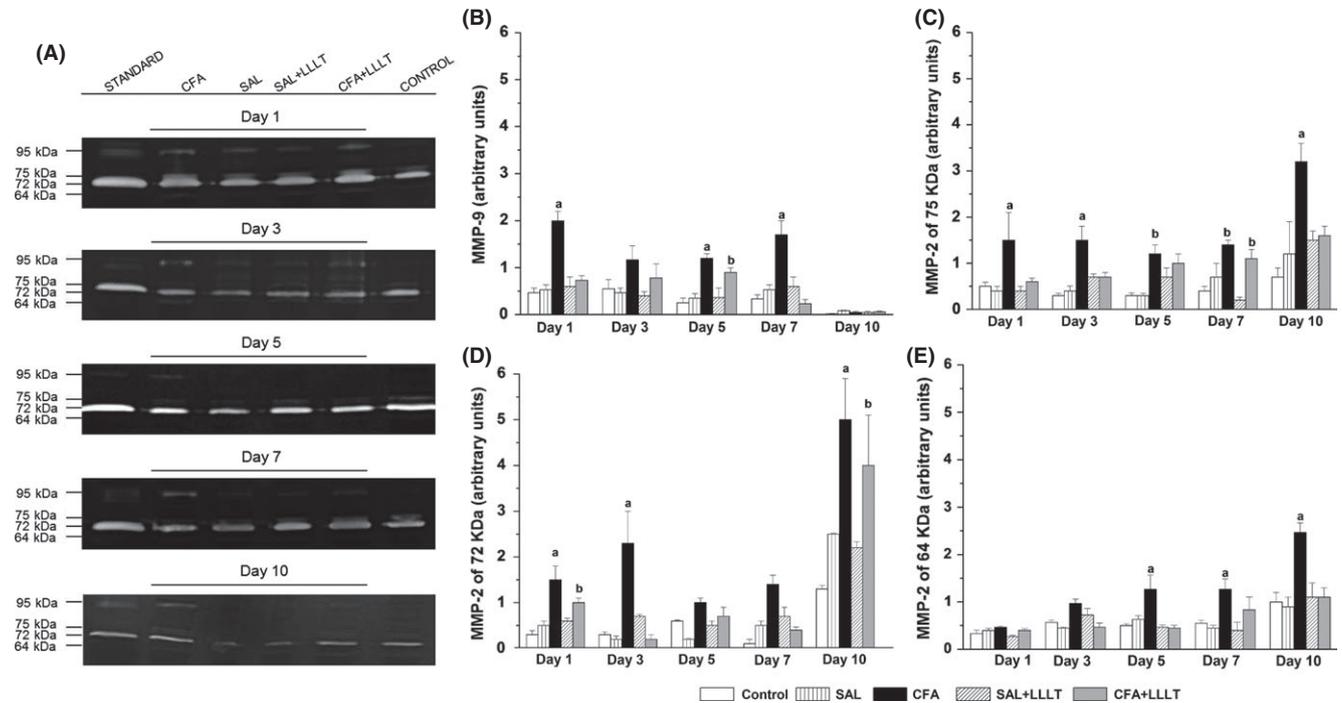
Rats belonging to group CFA exhibited higher frequency of orofacial area rubbing after administration of formalin at both the beginning (phase 1) and the end (phase 2) of the test (Figure 1B). LLLT prevented CFA-induced orofacial rubbing during the orofacial formalin test. Two-way ANOVA revealed that the factors time, treatment, and interaction between time and treatment significantly affected all the experimental groups ( $P < 0.001$  for all the factors). The Newman–Keuls *post hoc* test showed that group CFA presented significantly different mean duration of nociceptive behavior in both phases 1 (0–3 min) and 2 (6–27 and 36–45 min) as compared with groups SAL, SAL + LLLT, and CFA + LLLT ( $P < 0.05$ ) (Figure 1B).

*Quantification of MMP-2 and MMP-9 in the TG using zymography*

The activity levels of MMP-2 and MMP-9 were higher in the rats that received CFA as compared with groups Control, SAL, SAL + LLLT, and CFA + LLLT at distinct periods following the onset of the experiments (Figure 2A–E). Figure 2A illustrates representative SDS-PAGE gelatin zymograms of the different periods analyzed in this work.

Regarding MMP-9, two-way ANOVA analysis showed significant differences among the groups in terms of time ( $P < 0.001$ ), treatment ( $P < 0.001$ ), and interaction between time and treatment ( $P < 0.05$ ). The Newman–Keuls *post hoc* test revealed that MMP-9 expression significantly increased in group CFA on days 1, 5, and 7 after the onset of inflammation as compared with groups Control, SAL, SAL + LLLT, and CFA + LLLT ( $P < 0.05$ ) (Figure 2B). Statistical analyses did not show significant differences in the levels of MMP-9 in the study groups on days 3 and 10 (Figure 2B). Additionally, the levels of MMP-9 in the TG of group CFA + LLLT only differed from the levels of MMP-9 in groups Control, SAL, and SAL + LLLT on day 5 after the initial injection ( $P < 0.001$ , Newman–Keuls test) (Figure 2B).

Statistical analysis revealed differences in the levels of 75-kDa MMP-2; these differences referred to time ( $P < 0.001$ ) and treatment ( $P < 0.001$ ), but no interaction between time and treatment was evident. The Newman–Keuls *post hoc* test pointed to significantly higher levels of 75-kDa MMP-2 in group CFA on days 3 and 10 following the initial injection as compared with groups Control, SAL, SAL + LLLT, and CFA + LLLT ( $P < 0.05$ ) (Figure 2C). Again, group CFA + LLLT only differed from groups Control and SAL on day 10 of the experiment (Figure 2C). Differences were significant for time and treatment (two-way ANOVA,  $P < 0.001$  in both cases), but there was no interaction between time and treatment for the 72-kDa MMP-2 band. Group CFA



**Figure 2** Representative SDS-PAGE gelatin zymograms of trigeminal ganglion extracts (TGs) 1, 3, 5, 7, and 10 days following CFA-induced inflammation (CFA), administration of 0.9% saline (SAL) or without any treatment (Control); the rats were treated with low-level laser therapy (LLLT) of left untreated (Panel A). Std: internal standard. Molecular weights of MMP-9 band (92 kDa MMP-9) and MMP-2 bands (75, 72 and 64 kDa MMP-2) were identified after electrophoresis on 12% SDS-PAGE. Gels were loaded with 40  $\mu$ g of protein in each lane. Panels (B), (C), (D), and (E) show the values for the 92, 75, 72, and 64 kDa molecular weight forms, respectively, in the TGs. a:  $P < 0.05$ , Newman–Keuls when compared with the respective Control, SAL, SAL + LLLT, and CFA + LLLT groups. b:  $P < 0.05$ , Newman–Keuls test when compared with the respective Control, SAL, SAL + LLLT groups. Data are reported as means  $\pm$  s.e.m.: standard error mean ( $n = 8$  per group). CFA, complete Freund’s adjuvant; MMP, matrix metalloproteinase

exhibited increased 72-kDa MMP-2 band on days 1, 3, and 10 after the initial injection as compared with groups Control, SAL, SAL + LLLT, and CFA + LLLT ( $P < 0.05$ , Newman–Keuls test) (Figure 2D). Additionally, the level of the 72-kDa MMP-2 band in group CFA differed from the levels of this same band in groups Control and SAL ( $P < 0.05$ , Newman–Keuls test) at day 5, and in groups Control, SAL, and SAL + LLLT at day 7 (Figure 2D). Group CFA + LLLT did not differ from groups Control, SAL, or SAL + LLLT (Figure 2D). Statistical analysis of the data for 64-kDa MMP-2 revealed differences among the study groups for time, treatment, and interaction between time and treatment ( $P < 0.001$  in all cases, Figure 2E). The Newman–Keuls *post hoc* test evidenced increased level of 64-kDa MMP-2 in the TG of group CFA on days 5, 7, and 10 following the initial injection as compared with groups Control, SAL, SAL + LLLT, and CFA + LLLT ( $P < 0.05$ ) (Figure 2E). However, group CFA + LLLT did not differ from groups Control, SAL, or SAL + LLLT (Figure 2E).

*MMP activity during the development of inflammation in the TMJ in rats submitted to LLLT*

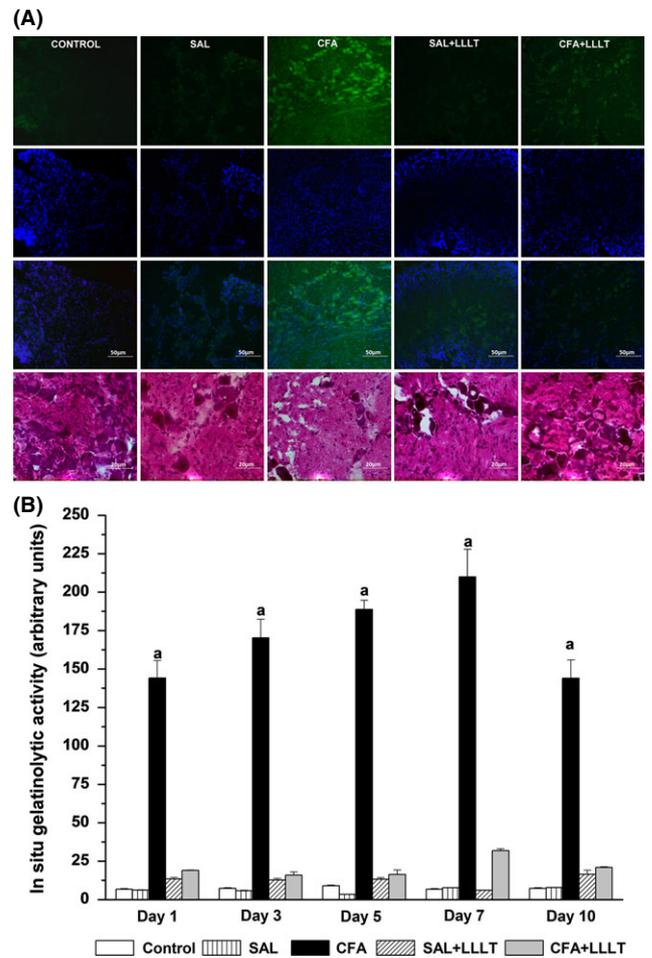
The hematoxylin- and eosin-stained sections in Figure 3A confirmed removal of the TG. It was possible to identify the perikaria of the neurons and surrounding glia cells. Figure 3A depicts representative immunofluorescence photomicrographs showing the total gelatinolytic activity in the TG tissue.

Two-way ANOVA analysis for the total gelatinolytic activity in the TG pointed to differences in terms of treatment and interaction between time and treatment ( $P < 0.001$  in both cases). The Newman–Keuls *post hoc* test revealed that the total gelatinolytic activity was higher in group CFA as compared with groups Control, SAL, SAL + LLLT, and CFA + LLLT ( $P < 0.05$ ) (Figure 3B).

**Discussion**

The results of this study indicate that LLLT reduces orofacial mechanical allodynia and hyperalgesia induced by TMJ inflammation. In particular, the data show that LLLT significantly improves the withdrawal threshold of an innocuous mechanical stimulus (Figure 1A) and reduces the increase in orofacial rubbing during the formalin test (Figure 1B). Moreover, LLLT abolishes the rise in MMP-9 and MMP-2 expression and the increased gelatinolytic activity in the TG following induction with CFA (Figures 2 and 3).

Low-level laser therapy promotes distinct biological effects: it stimulates cell proliferation, increases collagen synthesis and the secretion of growth factors, improves blood circulation, and accelerates epithelialization (Reddy, 2004). Hence, laser therapy probably acts by reducing the local inflammatory response and enhancing tissue repair, thereby suppressing painful symptoms and improving the joint and muscular conditions of patients with TMD. Although LLLT has found wide application in the treatment of inflammatory diseases (Goldman, 1981), the mechanisms of laser irradiation remain unclear. Some authors have found that laser therapy alters the levels of



**Figure 3** *In situ* gelatinolytic activity, MMP-2, and MMP-9 expression in the trigeminal ganglion (TG) from rats. Panel (A) shows representative photomicrographs of gelatinolytic activity on day 7 (*in situ* zymography), DAPI staining and gelatinolytic activity colocalization in the TGs (Merge), and section of TG hematoxylin- and eosin-stained. Panel (B) shows the mean gelatinolytic activity in TGs in each study group assessed by the measurement of bright green fluorescence ( $n = 6$  per group). Data are shown as mean  $\pm$  s.e.m. a:  $P < 0.05$  Newman–Keuls test compared with the respective Control, SAL, SAL + LLLT, and CFA + LLLT groups. The scale bar represents 100  $\mu$ m. CFA, complete Freund's adjuvant; LLLT, low-level laser therapy; MMP, matrix metalloproteinase

PGE<sub>2</sub> and IL-1 $\beta$  *in vitro* (Shimizu *et al.*, 1995; Sakurai *et al.*, 2000) and *in vivo* (Albertini *et al.*, 2004). More specifically, GaAlAs laser irradiation on cultured human periodontal ligament cells significantly decreases the production of PGE<sub>2</sub> and IL-1 $\beta$ , as observed on days 3–5 after application of controlled force to promote orthodontic movement of the lower incisors (Shimizu *et al.*, 1995). Furthermore, a previous study (Albertini *et al.*, 2004) has demonstrated that acute treatment with a GaAlAs (2.5 J cm<sup>-2</sup>, 80 s every hour for 4 h) laser reduces carrageenan-induced footpad edema in rats in the same way that the administration of an anti-inflammatory would work. However, the use of laser therapy does not abate footpad edema in adrenalectomized rats, which suggests that the anti-inflammatory action of the GaAlAs laser may be due to the release of corticosteroids from the adrenal

medulla (Albertini *et al*, 2004). These studies are in line with the present findings, which point to reduced mechanical orofacial allodynia and hyperalgesia in rats, possibly due to decreased levels of intra-articular inflammatory mediators. According to the data of the present work, LLLT significantly lowers CFA-induced MMP-9 and MMP-2 expression in the TG. Evidences exist that inflammation occurs even in the TG, because MMPs play critical roles in inflammation through cleavage of cytokines and chemokines (Parks *et al*, 2004). Additionally, a previous report has shown that MMP expression in the TG involves distinct phases of the inflammatory process. More specifically, MMP-9 mediates the early phase, and MMP-2 participates in the late phase of this process (Nascimento *et al*, 2013).

Regarding the sensory neural pathways, the sensory ganglion neurons constitute the first relay of the central nervous system. Additionally, strong evidence exists that the ganglion cells, including satellite glial cells (SGCs), contribute to the establishment of chronic and persistent pain (Watkins and Maier, 2002; Tsuda *et al*, 2005; Dublin and Hanani, 2007; Berta *et al*, 2012). These SGCs act as a mechanical barrier to the ganglion neurons of the dorsal root and trigeminal ganglia (Hanani, 2005), and they participate in mechanisms of neurotransmitter uptake, thereby promoting the fine control of the neuronal microenvironment (Braun *et al*, 2004). Hence, SGCs take part in the morphological and biochemical modifications that occur after peripheral nerve injury and inflammation (Dublin and Hanani, 2007; Dubovy *et al*, 2010). It is noteworthy that MMPs are expressed in neural tissue, including neurons and glial cells, such as astrocytes and microglia (Gottschall and Deb, 1996). Whereas MMP-2 is a constitutive gelatinase, MMP-9 is highly inducible, and the presence of inflammatory cytokines can trigger its transient expression (Ji *et al*, 2009). Therefore, the main physiological function of MMP-9 in neural tissue probably refers to synaptic plasticity as well as to tissue development and regeneration, which is similar to the function of MMP-2 (Young, 2005). During the development of persistent pain, MMPs can specifically underlie the activation of IL-1 $\beta$  (Parks *et al*, 2004; Kawasaki *et al*, 2008) and other bioactive molecules, including tumor necrosis factor (TNF- $\alpha$ ) and pro-neurotrophins such as pro-NGF and pro-BDNF (Schonbeck *et al*, 1998; Lee *et al*, 2001). Authors have suggested that MMPs cleave pro-IL-1 $\beta$  into IL-1 $\beta$ , which culminates in hyperexcitability of primary sensory neurons (Berta *et al*, 2012) and promotes the allodynia and hyperalgesia observed during inflammatory conditions (Liu *et al*, 2002; Dublin and Hanani, 2007), as shown in the present study. Additionally, by blocking the activation of MMP-9 in SCs, it is possible to suppress IL-1 $\beta$  expression (Berta *et al*, 2012). Thus, LLLT most likely reduces MMP expression in the TG due to its anti-inflammatory effects (Shimizu *et al*, 1995; Sakurai *et al*, 2000; Albertini *et al*, 2004), which in turn decreases orofacial mechanical allodynia and hyperalgesia.

In conclusion, to our knowledge, this is the first study that has reported and characterized the effects of LLLT on MMP expression in the TG following TMJ inflammation. This study has shown that LLLT reduces the orofacial

mechanical allodynia and hyperalgesia induced by CFA administration into the TMJ. Moreover, LLLT abolishes the CFA-induced increase in MMP-9 and MMP-2 expression and the rise in the gelatinolytic activity in the TG. Therefore, LLLT could constitute an adjuvant treatment for TMDs and could help to reduce the alterations that inflammation induces in MMPs activity in the TG.

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### Author contributions

A.C. Desiderá and G.C. Nascimento contributed substantially to the acquisition, analysis and interpretation of data, as well the drafting the article. R.F. Gerlach contributed to conception and design of experiments and revising the article critically. C.R.A. Leite-Panissi contributed to conception and design of experiments, to analysis and interpretation of data, and revising the article critically. All authors read and revised the manuscript.

### Ethical standards

The manuscript does not contain clinical studies or patient data.

### Conflict of interest

The authors declare that they have no conflict of interest.

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