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ORIGINAL ARTICLE

Do electrical current and laser therapies improve bone remodeling during an orthodontic treatment with corticotomy?

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Abstract

Objectives Evaluate the bone remodeling during orthodontic movement with corticotomy when submitted to low-intensity electrical stimulation application (microcurrent—MC) and low-level laser therapy (LLLT).

Material and methods One hundred and fifty Wistar rats were divided into the following 5 groups: (C) submitted to tooth movement; (Cort) tooth movement/corticotomy; (Cort-L) tooth movement/corticotomy/laser AsGaAl 808 nm (4.96J/50s); (Cort-Mc) tooth movement/corticotomy/microcurrent (10 μA/5 min); (Cort-L-Mc) tooth movement/corticotomy and laser/microcurrent alternated. Inflammation, angiogenesis, and osteogenesis were evaluated in the periodontal ligament (PDL) and alveolar bone on the 7th, 14th, and 21st days of orthodontic movement.

Results The quantification of inflammatory infiltrate, angiogenesis and expression of TGF-β1, VEGF, and collagen type I were favorably modulated by the application of therapies such as low-level laser therapy (LLLT), MC, or both combined. However, electrical stimulation increased fibroblasts, osteoclasts and RANK numbers, birefringent collagen fiber organization, and BMP-7 and IL-6 expression.

Conclusions Low-level laser therapy (LLLT) and MC application both improved the process of bone remodeling during orthodontic treatment with corticotomy. Still, electrical current therapy promoted a more effective tooth displacement but presented expected root resorption similar to all experimental treatments.

Clinical relevance It is important to know the effects of minimally invasive therapies on cellular and molecular elements involved in the bone remodeling of orthodontic treatment associated with corticotomy surgery, in order to reduce the adverse effects in the use of this technique and to establish a safer clinical routine.

Keywords Electrical stimulation · Phototherapy · Osteotomy · Corticotomy · Orthodontic movement · Osteogenesis

Introduction

Prolonged orthodontic treatments may promote adverse effects, such as caries, gingival retraction, root resorption, and alveolar bone loss [1]. Therefore, accelerating tooth movement and shortening treatment duration have always been a concern for patients as well for orthodontists [2].

Corticotomy associated with tooth movement may significantly expand treatment options for skeletal discrepancies that promote severe malocclusion usually indicated for orthognathic surgery [3]. The investigation of cellular and molecular changes involved in the corticotomy is important for an adequate development of protocols that minimize the adverse effects of this technique, such as substantial inflammation, which includes pain, edema, and subsequent alveolar bone loss [4, 5].

The use of animals in experimental models allows clinical reproducibility in a standardized way. The in vivo research with animal models provides relevant data on physiological

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and pathological conditions which can be useful in establishing more effective clinical interventions [6].

Orthodontic forces induce responses in tooth-supporting tissues resulting in remodeling the periodontal ligament and alveolar bone due to angiogenesis modulation, synthesis and release of growth factors and cytokines, and cell differentiation [7]. The application of these forces promotes inflammatory response in the periodontal tissue evidenced at the beginning of bone remodeling [8] While in the compression region, there is an increase in osteoclastic activity, in the traction region, the osteoblasts begin to proliferate and mineralize the matrix [9]. Several cell-signaling pathways are activated, which stimulates the *turnover* of the periodontal ligament (PDL), resorption, and localized bone deposition [10]. Yet, orthodontic forces induce numerous molecular events in the periodontal ligament. These events include the synthesis and release of several molecules, such as growth factors, neurotransmitters, and cytokines, involved in the maturation of leukocytes and macrophages with pro-inflammatory property, such as IL-1β, IL-6, IL-8, IL-12, IL-13, and TNF-α. The IL-10 is important for its anti-inflammatory activity, and it also participates in these processes [7, 11–13]. All these molecules promote responses to periodontal tissue, such as cell migration and differentiation, favoring remodeling, resorption, and bone deposition [14–16]. Tooth movement induced by orthodontic forces promotes changes that lead to the remodeling of tooth-supporting tissues and the presence of different biomarkers indicating not only cellular but also metabolic activity [7]. Cytokines, such as IL-1, IL-6, IL-8, and TNF-α, are related to bone remodeling [17] and, also, play an important role in the osteoclastic process [18, 19] through the activation of the kappa B nuclear factor-TNFRS11 (RANK), ligand kappa B nuclear factor-TNFSF11 (RANKL), and osteoprotegerin ligand-TNFRSF11B (OPG) [20].

Interleukin-6 (IL-6) and transforming growth factor β1 (TGF-β1) are important in tooth movement, recruiting inflammatory cells and stimulating angiogenesis and bone remodeling [7, 21]. The soluble IL-6 receptor (IL-6Rα) is suggested to activate the differentiation of osteoclasts by inducing the expression of TNFSF11 on osteoblast surface. Thus, TNFSF11 interacts with TNFRS11 expressed in osteoclast progenitors, immature and mature, inducing the differentiation of these cells [22]. Osteoblasts also control the osteoclastic process, since they synthesize TNFSF11 to promote osteoclast activation [23].

TGF-β1 is a protein that has also chemotactic effects on osteoblasts, promoting the proliferation and differentiation of these cells, and inhibits osteoclastic formation by reducing RANKL and increasing OPG expression [24]. The TGF-β superfamily comprises TGF-βs, activin, bone morphogenetic proteins (BMPs), and other related proteins [25]. BMP and TGF-β signaling pathways play an important role in skeletal development and bone homeostasis. BMPs can trigger a signaling pathway that promotes the differentiation of osteoprogenitor cells and regulates the proliferation, differentiation, maturation, and activity of osteoblasts and chondrocytes in bone and cartilage formation. BMP-7 induces the expression of osteoblastic differentiation markers, such as ALP, and accelerates calcium mineralization [7, 21]. It has been observed that BMP-2 increases the formation and volume of ectopic bone in the presence of TGF-β1 [26]. Tissue molecular analysis collected from a femur fracture of knockout mice has shown that in the absence of BMP-2, the initial repair process involving new chondrogenesis does not occur. The authors consider that this event may possibly be involved with a downregulation of other BMPs which demonstrates the relevance of BMP-2 with osteogenesis [27]. The capabilities of BMP-2 and BMP-7 have been studied in clinical trials of craniofacial deformities and fracture healing [21].

Vascular endothelial growth factor (VEGF) plays a fundamental role in the remodeling of the PDL and bone formation [28]. It is involved not only in bone angiogenesis but also in various aspects of bone development, including chondrocyte differentiation, osteoblast differentiation, and osteoclast recruitment [29]. VEGF is shown to be increased in hypoxia situations by the activation of the hypoxia-stimulating factor (HIF) [30] which also induces RANKL expression in PDL fibroblasts [31].

Accelerating the orthodontic movement has stimulated the research of different auxiliary protocols in treatment including the use of different therapies [32]. According to Kim et al. [33], electrical stimulation may accelerate orthodontic treatment. This effect was also observed in tooth movement submitted to the application of low-intensity electrical current (microcurrent) in rats [34]. The photobiostimulation has also been widely used in the healing process of different tissues and during tooth movement [35–38]. Its action on different biological tissue is vast and important for anti-inflammatory and analgesic effects [39], but it was observed that the periodic low-level laser therapy (LLLT) after corticotomy around moved tooth periodontal tissue decreased the displacement rate of the tooth and the activity of alveolar remodeling [40].

Although orthodontic treatments bring benefits to patients, prolonged periods of this procedure may compromise the final clinical results [1]. The search for an efficient treatment to shorten time in tooth movement has encouraged the investigation of new therapies that aid in orthodontic treatment. Therefore, the purpose of this study was to evaluate the bone remodeling during orthodontic movement with corticotomy submitted to application of low-intensity electrical stimulation and low-level laser therapy, applied alone and alternately. These therapies are important in biostimulation because they are not invasive and may also benefit this clinical procedure.

Methodology

Animals

One hundred and fifty male Wistar rats (*Rattus norvegicus*) were used, weighing 350 g on average and 120 days old. These animals were obtained from the Animal Experimentation Herminio Ometto Foundation (FHO/UNIARARAS). These were kept at constant temperature (23 ± 2 °C) with light/dark cycle (12/12 h) and housed in individual cages with free access to feed and water. They were randomly divided into the following 5 groups ($n = 30$): (C) submitted to tooth movement; (Cort) tooth movement/corticotomy; (Cort-L) tooth movement/corticotomy/laser AsGaAl 808 nm (4.96J/50s); (Cort-Mc) tooth movement/corticotomy/microcurrent (10 μA/5 min); and (Cort-L-Mc) tooth movement/corticotomy/laser/microcurrent. The animals were euthanized on the 7th, 14th, and 21st days with deepening anesthesia and cervical sprain to obtain the samples ($n = 10$ rats/group/experimental period). These samples were used for histomorphometric ($n = 5$ rats/group/experimental period) and molecular analyses ($n = 5$ rats/group/experimental period) (Fig. 1c). The histomorphometric and molecular analyses had as control group only the animals with moved tooth (C) and as positive control group the animals with moved tooth with corticotomy (Cort) [41], since the objective of this study was focused mainly on the therapies used. The procedures were carried out according to experimental standards and biodiversity rights [42] and approved by CEUA/UNIARARAS (020/2015).

Corticotomy and orthodontic movement

After intraperitoneal administration of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (30 mg/kg), corticotomy was performed. After an incision in the mesial of the first left maxillary molar (3.0 mm in length), the gingival tissue divulsion was performed to access the bone tissue. For corticotomy, a truncated conical dental drill (1801 HL) was used in low rotation with saline solution irrigation, performing a cortical osteotomy with a semicircular shape, in the mesial of the moved tooth [43]. After suturing, analgesia with tramadol (1 mg/kg) and sodium dipyrone (10 mg/kg) was added in the water for 72 h. The orthodontic device [34] was installed immediately after the corticotomy procedure (Fig. 1a).

A stainless steel closed coil spring was installed between the left upper first molar (force site) and the upper incisors (anchorage site), which permitted a tip forward movement of the first molar. The coil spring was stretched from 4 to 6 mm, releasing a 0.4 N initial force [34].

Microcurrent application

In the treatment with micro-galvanic current (10 μA/5 min), a transcutaneous electrical stimulator (Physiotonus Microcurrent, BIOSET®, Rio Claro, SP, Brazil) was used [34]. The applications were performed with the help of 2 electrodes (1.0 mm in diameter), placed in the mesial region of the first molar, in two points (vestibular and palatal), under anesthesia and twice a week (Fig. 1b).

Laser application

The low-level laser therapy (LLLT) applications were performed using Photon Lase III® stimulator (DMC Equipamentos LTDA, São Carlos, SP, Brazil) positioned sequentially in the palatal and vestibular regions of the first molar by the standardized time and twice a week.

The laser used was AsGaAl, 808 nm, continuous mode, 100 mW output power, beam area 0.0275cm^2, energy density 90.18J/cm^2, applied for 25 s in two points (palatal and vestibular regions), which received 2.48J/cm^2 each, generating total energy of 4.96J/cm^2. Punctual irradiation was performed at a distance of ± 2 mm and at 90° to the surface of the moved tooth mucosa, placed in the mesial region of the first molar, in two points (vestibular and palatal), under anesthesia and twice a week. The animals from the Cort-L-Mc group received alternating applications, on different days, of laser or microcurrent, twice a week each one (Fig. 1b). Figure 1c shows the development of the experimental protocol with the treatments and analysis carried out.

Measurement of tooth displacement

Clinical analysis ($n = 10$ rats/group/experimental period) measured the distance between the distal face of the 3rd molar and the mesial face of the maxillary 1st molar. A digital caliper was used (Starret, Massachusetts, USA) with a definition of 0.01 mm. In the analysis of tooth displacement, the contralateral side of the same animal was used as a reference only for the amount of tooth displacement on the moved side. The amount of movement was calculated by obtaining the difference between the moved side and the contralateral side. The quantification of root resorption was performed on the 21st day of movement. In the cross-sections, the percentage of root resorption was calculated by dividing the resorption area by the total root area [44].

Histomorphometric analysis

The maxillaes were fixed in 10% buffered formaldehyde solution for 48 h, decalcified in Morse solution, and embedded in Paraplast™ (Histosec®, Merck). Transversal sections

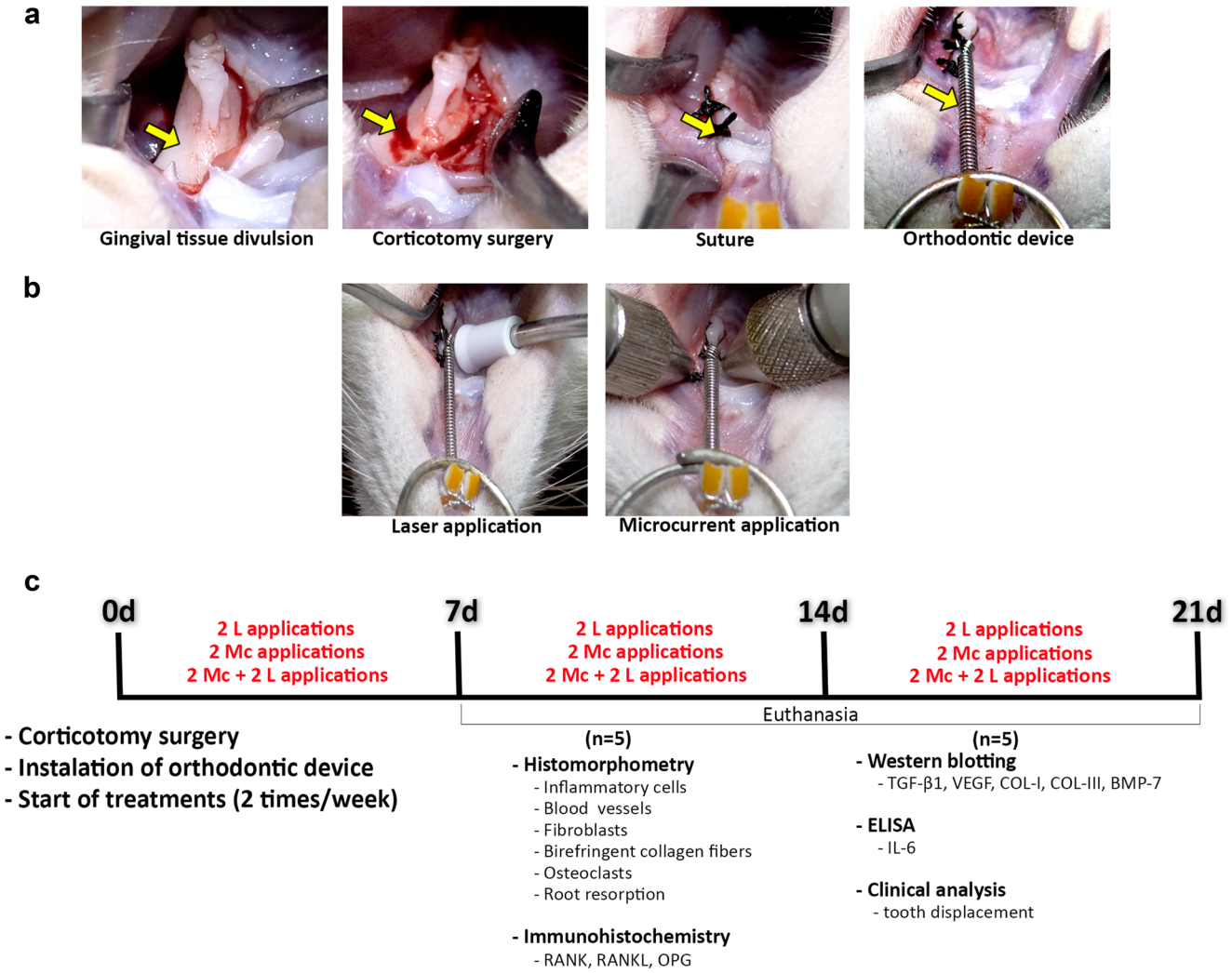

Fig. 1 **a** Methodology of corticotomy surgery and installation of orthodontic device, immediately after corticotomy procedure (first yellow arrow, bone tissue accessed after gingival tissue divulsion; second yellow arrow, cortical osteotomy with semi-circular shape in the mesial of the moved tooth; third yellow arrow, suture in the mesial of the moved tooth; fourth yellow arrow, orthodontic device installed). **b** Treatment with low-level laser therapy (LLLT) and microcurrent. **c** Timeline of follow-up (surgery, treatments, euthanasia, sample harvesting, experimental analysis)

(4.0 μm thick) at the cervical level of the molars were prepared to analyze the mesiobuccal and distobuccal roots. To determine the number of fibroblasts, osteoclasts, and blood vessels, toluidine blue staining was used. The inflammatory infiltrate was determined using the eosin–orange–toluidine blue staining according to Dominici [45]. In both cases, microscopic analysis was done at 400× magnification. The organization and maturation of birefringent collagen fibers (expressed as % of area) were evaluated using the picosirius–hematoxylin technique in bright field and polarized light [34] using a Leica® DM2000 microscope at 100× magnification. In each animal, five sections were obtained, where from three images were captured (n = 15 images/animal). Counts are reported as number of cells per square micrometer (μm^2).

Immunohistochemical analysis

For immunohistochemical staining, 4.0 μm sections were arranged on previously silanized slides. The samples (n = 5/experimental periods) were incubated with the following primary antibodies: anti-OPG (sc-390518, 1:200), anti-RANK (sc-374360, 1:50), and anti-RANKL (sc-377079, 1:200) (Santa Cruz Biotechnology, Dallas, USA). Detection reaction was based in DAB molecule (Novolink™ Max Polymer Detection System (1250 Tests)—RE7280-K, Leica Biosystems Newcastle Ltd, UK) according to the manufacturer's instructions.

The counting of positive cell number for RANK, RANKL, and OPG was performed inside of PDL from the captured images in 400× of magnification using the Sigma Scan

Pro™ 5.0 software for cell counter. From each animal, five sections were obtained, of which three images were captured ($n = 15$ images/animal).

Extraction and quantification of bone and gingival tissue proteins

The alveolar bone in the region of the left upper first molar moved in the different groups was curetted, and the samples were homogenized with Politron (PTA 20S model PT 10/35; Brinkmann Instruments, Westbury, NY, USA). The samples were treated with buffer for Western blotting (100 mM Tris base, #93362, Sigma-Aldrich Inc., pH 7.5, 10 mM EDTA, Triton 10%, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate). For ELISA, the gingival tissue in the region of the left upper first molar moved in the different groups was cut and the samples were homogenized manually. Samples were treated with protein extraction reagent for ELISA (T-PER®-Tissue Protein Extraction Reagent/Thermo Scientific cat. 78510) [46].

The supernatant was collected for the determination of samples' total protein concentration by the Biureto method (RB09695SO—Êxodo Científica, SP, Brazil) and read by spectrophotometry at 540 nm.

Western blotting

The samples were incubated (1:4) at 100 °C for 5 min in Laemmli buffer (0.1% bromophenol blue, 1 M sodium phosphate pH 7.0, 50% glycerol, 10% SDS, 5% β-mercaptoethanol, 200 mM DTT). For polyacrylamide gel electrophoresis (PAGE), a volume corresponds to 50 μg of protein in biphasic gel: stacking gel (acrylamide 4.5%) and resolving gel (acrylamide 12%) [34]. The race was performed at 90 V for approximately 2 h with running buffer (200 mM base Trism, 1.52 M glycine, 7.18 mM EDTA, and 0.4% SDS), diluted 1:4. The samples were transferred to PVDF membranes (Immun-Blot®-BioRad) for 2 h at 120 V at lower room temperature, on wet-blotting with Transfer Buffer (25 mM base Trism, 192 mM glycine). After the transfer, the membranes were blocked with bovine albumin (#A4503, quality level premium; Sigma-Aldrich Inc.) 5% in basal solution (0.01 M Tris base, 0.15 M NaCl, 0.05% Tween 20) for 1 h and 30 min at room temperature. Then, the membranes were washed three times for 10 min with basal solution and incubated overnight under shaking at 4 °C with basal solutions plus 3% serum bovine albumin containing the following primary antibodies: TGF-β1 (sc-52893, 1:500, 25 kDa, mouse monoclonal, Santa Cruz Biotechnology); VEGF (sc-53462, 1:1000, 42 kDa, mouse monoclonal, Santa Cruz Biotechnology); BMP-7 (sc-9305, 1:500, 55 kDa, goat polyclonal, Santa Cruz Biotechnology); collagen type I (sc-8788, 1:500, 132 kDa, goat polyclonal, Santa Cruz Biotechnology); collagen type III (C7805, 1:5000, 70 kDa, mouse monoclonal, Sigma-Aldrich Inc.); and GAPDH (ab8245, 1:500, 36 kDa, mouse monoclonal, Abcam). Subsequently, the membranes were washed three times for 10 min with basal solution and, then, incubated under agitation for 2 h in a solution containing the following secondary antibodies: rabbit anti-goat IgG-HRP (sc-2768, 1:5000, Santa Cruz Biotechnology) and mouse IgGκ BP-HRP (sc-516102, 1:5000, Santa Cruz Biotechnology). Membranes were washed with basal solution and incubated for 2 min with Thermo Scientific® chemiluminescent reagent (#34080) and exposed to the Singene photodocumentator (G: BOX) for documentation. The intensity of the bands was evaluated by densitometry (five times) by the ImageJ program (NIH, USA).

Enzyme-linked immunosorbent assay

The concentration of IL-6 (rat IL-6 ELISA Set, cat. 550319/BD Bioscience) was analyzed by ELISA using monoclonal antibodies to each rat cytokine. A volume corresponding to 30 μg of proteins from each sample was added to the immunoassay plate for IL-6 level determination, according to the manufacturer's instructions (BD Biosciences). Reactions were made in triplicate for each sample. The absorbance was measured at 450 nm on a microplate reader (BioTec, ELx800/USA), and the concentrations of each cytokine were determined on the basis of the linear regression line made for the standard curve obtained as the appropriate recombinant cytokine standard.

Statistical analysis

The data were demonstrated in mean and ±standard error of the mean. All data passed the Kolmogorov–Smirnov normality test and fit the normality curve. The ANOVA two-way was used, and Bonferroni's post-test was performed in GraphPad Prism 5.0, comparing all groups within each segment time, with a pre-established significance level of 5%.

Results

Tooth displacement and root resorption

The results of root resorption are observed and demonstrated only on the 21st day, which corresponds to the last experimental analyzed period (Fig. 2a). No differences were observed in the percentage of root resorption between the experimental groups ($p > 0.05$). Especially in the microcurrent (Cort-Mc, Cort-L-Mc), the tooth displacement increased on the 21st day in relation to C ($p = 0.0007$, $p < 0.0001$, respectively) and Cort-L-Mc in relation to Cort-L ($p = 0.0152$) (Fig. 2b).

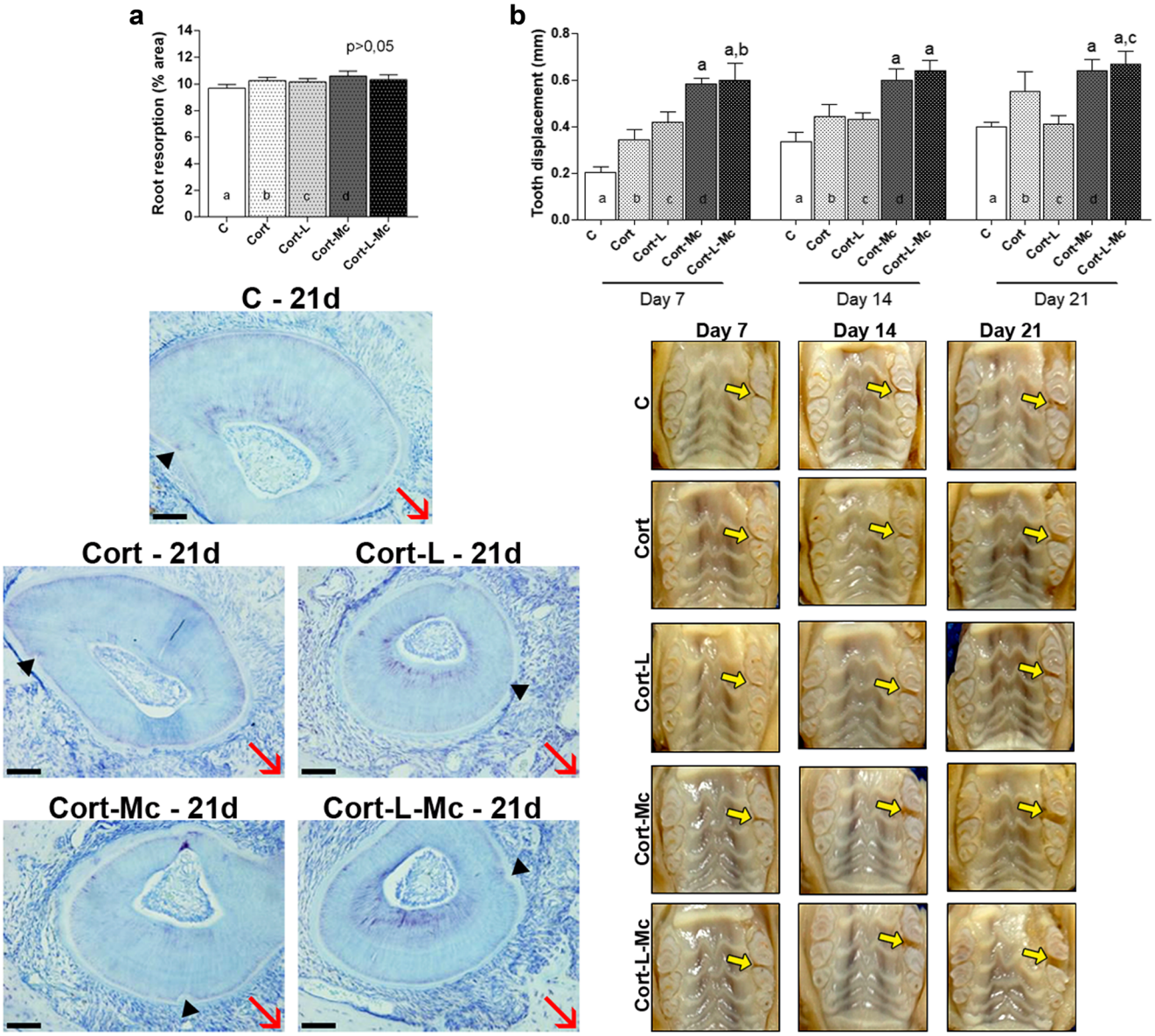

Fig. 2 **a** Root resorption on the 21st with histological analysis. Red arrow, direction of force. **b** Measurement of the tooth displacement (mm) and mesial displacement of the 1st molar in orthodontic movement of rats. Groups: C, tooth movement; Cort, tooth movement and corticotomy; Cort-L, tooth movement/corticotomy/laser; Cort-Mc, tooth movement/corticotomy/microcurrent; Cort-L-Mc, tooth movement/corticotomy/laser/microcurrent. Samples were analyzed on the 7th, 14th, and 21st days after orthodontic movement. The values were compared using ANOVA two-way and Bonferroni's post-test. Results expressed as mean ± standard error of the mean. a, $p < 0.05$ relative to C; b, $p < 0.05$ in relation to the Cort; c, $p < 0.05$ compared to the Cort-L; d, $p < 0.05$ compared to the Cort-Mc

Inflammatory process

The inflammatory infiltrate increased on the 7th day in all experimental groups in relation to group C ($p < 0.0001$), and, in the Cort-L-Mc group, an increase was also observed in relation to the Cort-L group ($p = 0.0004$). On the 14th day, a decrease was observed in the Cort-Mc group in relation to the Cort group ($p = 0.0008$) and in Cort-L-Mc group in relation to C, Cort ($p < 0.0001$), and Cort-L ($p < 0.0057$) groups. It was observed that on the 21st day, groups that received laser or microcurrent treatment had the inflammatory infiltrate decreased in relation to C and Cort groups ($p < 0.0001$), whereas the Cort-L-Mc group was also lower than the Cort-Mc group ($p < 0.0031$) (Fig. 3a).

As for TGF-β1, it was lower on the 21st day in groups treated with laser and microcurrent in relation to C and Cort groups ($p < 0.0195$) (Fig. 3b).

Angiogenesis

Data analysis showed an increase in the number of vessels in Cort-L ($p < 0.0001$), Cort-Mc ($p = 0.0002$), and Cort-L-Mc

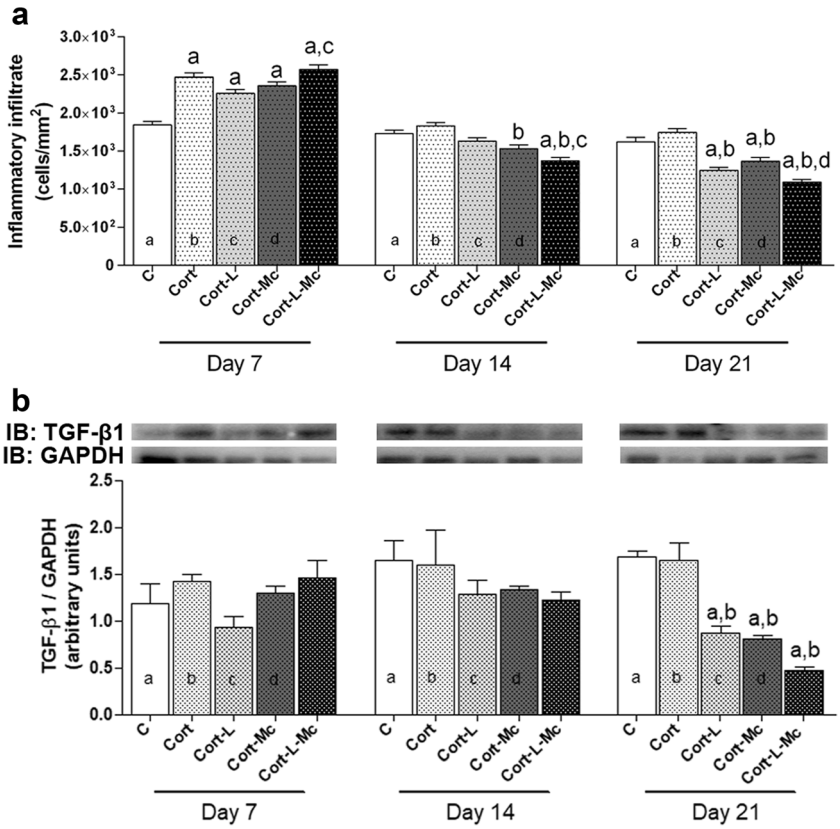

Fig. 3 **a** Inflammatory infiltrate quantification ($n/10^4$ μm^2) by histomorphometry analysis and **b** expression of TGF-β1 in the periodontal ligament in rat orthodontic movement. Groups: C, tooth movement; Cort, tooth movement and corticotomy; Cort-L, tooth movement/corticotomy/laser; Cort-Mc, tooth movement/corticotomy/microcurrent; Cort-L-Mc, tooth movement/corticotomy/laser/microcurrent. Samples were analyzed on the 7th, 14th, and 21st days after orthodontic movement. The values were compared using ANOVA two-way and Bonferroni's post-test. Results expressed as mean ± standard error of the mean. a, $p < 0.05$ relative to C; b, $p < 0.05$ in relation to the Cort; c, $p < 0.05$ compared to the Cort-L; d, $p < 0.05$ compared to the Cort-Mc

($p < 0.0001$) on the 14th day in relation to the C group. On the 21st day, the groups Cort-Mc ($p = 0.0013$) and Cort-L-Mc ($p = 0.0025$) were higher than C (Fig. 4a).

The expression of VEGF was increased on the 7th day in the Cort-Mc and Cort-L-Mc groups and decreased on the 14th day in relation to group C ($p < 0.0001$). Analysis of the 21st day showed a decrease of VEGF in samples from the groups treated with laser and microcurrent applied alone and on alternate days in relation to C and Cort groups ($p < 0.0001$) (Fig. 4b).

Fibroplasia and collagen

The quantification of fibroblasts on the 7th day increased in the group Cort-Mc in relation to groups C, Cort ($p < 0.0001$), and Cort-L ($p = 0.0067$), whereas in the group Cort-L-Mc it increased in relation to the other groups ($p < 0.0001$). On the 14th day, the groups Cort-L, Cort-Mc, and Cort-L-Mc increased in relation to groups C and Cort ($p < 0.0001$). On the 21st day, the groups treated with microcurrent (Cort-Mc, Cort-L-Mc) also increased in relation to the C, Cort, and Cort-L groups ($p < 0.0001$) (Fig. 5a).

The evaluation of birefringent collagen fibers showed an increase in all groups in relation to C in all experimental periods. On the 7th day, the results of groups Cort-L, Cort-Mc, and Cort-L-Mc were higher than those of Cort and the Cort-L-Mc, also in relation to the Cort-L group ($p < 0.0001$). On the 14th day, Cort-L, Cort-Mc, and Cort-L-Mc were increased in relation to the groups C ($p < 0.0001$) and Cort ($p < 0.0004$). The groups Cort-Mc and Cort-L-Mc also increased in relation to the groups Cort and Cort-L ($p < 0.0004$). On the 21st day, the Cort-Mc and Cort-L-Mc groups were higher than Cort and Cort-L groups ($p < 0.0001$) and, also, the Cort-L-Mc group in relation to the Cort-Mc group ($p < 0.0002$) (Fig. 5b).

The expression of collagen III showed a gradual decrease from days 14th to 21st in all groups and had no difference between groups on each day of follow up (Fig. 5c). Whereas collagen I was increased on the 21st day in the Cort-L ($p = 0.0086$), Cort-Mc ($p = 0.0054$), and Cort-L-Mc ($p = 0.0079$) groups in relation to Cort (Fig. 5d).

Osteogenesis

The number of RANK-positive stained cells increased in the groups treated especially with microcurrent in all experimental periods in relation to C, Cort, and Cort-L groups ($p < 0.0010$) (Fig. 6a). Regarding RANKL, only the Cort-Mc group showed a reduction in relation to C on the 7th day ($p = 0.0011$) (Fig. 6b). In positive-stained cells of OPG, no differences were observed between the experimental groups ($p > 0.05$) (Fig. 6c). Figure 6d demonstrates the immunohistochemistry of these markers.

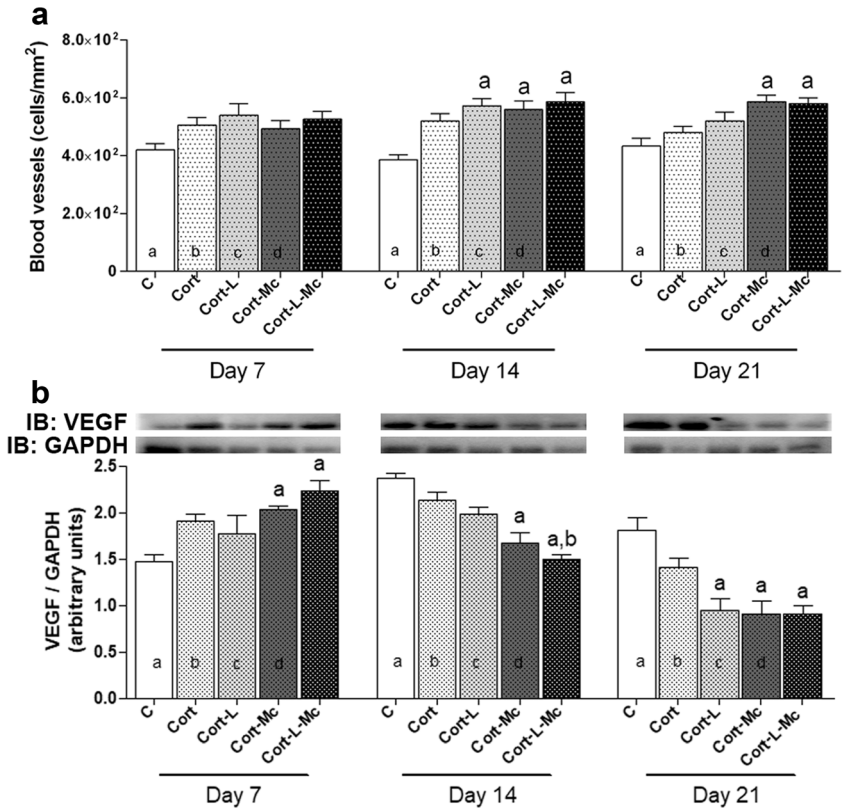

Fig. 4 **a** Blood vessel quantification ($n/10^4$ μm^2) by histomorphometry analysis and **b** of VEGF expression in the periodontal ligament in rat orthodontic movement. Groups: C, tooth movement; Cort, tooth movement and corticotomy; Cort-L, tooth movement/corticotomy/laser; Cort-Mc, tooth movement/corticotomy/microcurrent; Cort-L-Mc, tooth movement/corticotomy/laser/microcurrent. Samples were analyzed on the 7th, 14th, and 21st days after orthodontic movement. The values were compared using ANOVA two-way and Bonferroni's post-test. Results expressed as mean ± standard error of the mean. a, $p < 0.05$ relative to C; b, $p < 0.05$ in relation to the Cort; c, $p < 0.05$ compared to the Cort-L; d, $p < 0.05$ compared to the Cort-Mc

The quantification of osteoclasts showed an increase on the 21st day in groups treated with microcurrent in relation to C ($p < 0.0012$) (Fig. 7a). The increase in BMP-7 protein expression on the 21st day in the Cort-Mc and Cort-L-Mc groups was evidenced in relation to the other groups ($p < 0.0130$) (Fig. 7b). The quantification of IL-6 showed

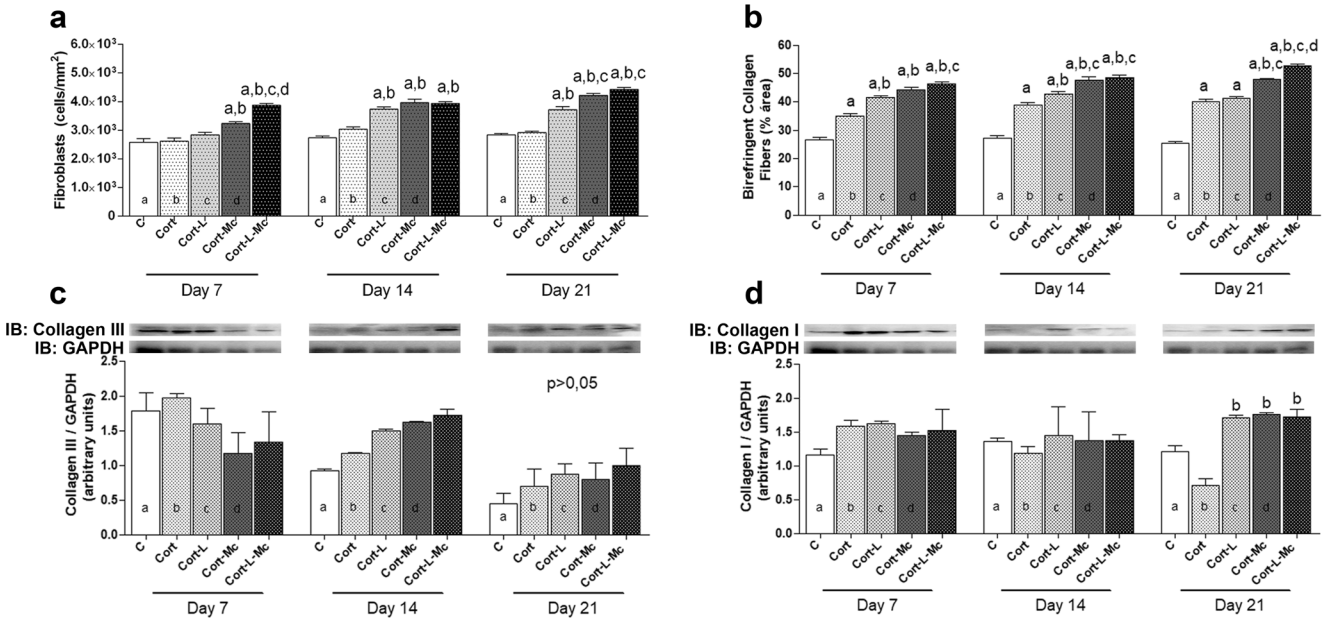

Fig. 5 **a** Fibroblast quantification ($n/10^4$ μm^2) by histomorphometry analysis and **b** birefringent collagen fibers (% area) and expression of **c** collagen III and **d** collagen I in the periodontal ligament in rat orthodontic movement. Groups: C, tooth movement; Cort, tooth movement and corticotomy; Cort-L, tooth movement/corticotomy/laser; Cort-Mc, tooth movement/corticotomy/microcurrent; Cort-L-Mc, tooth movement/corticotomy/laser/microcurrent. Samples were analyzed on the 7th, 14th, and 21st days after orthodontic movement. The values were compared using ANOVA two-way and Bonferroni's post-test. Results expressed as mean ± standard error of the mean. a, $p < 0.05$ relative to C; b, $p < 0.05$ in relation to the Cort; c, $p < 0.05$ compared to the Cort-L; d, $p < 0.05$ compared to the Cort-Mc

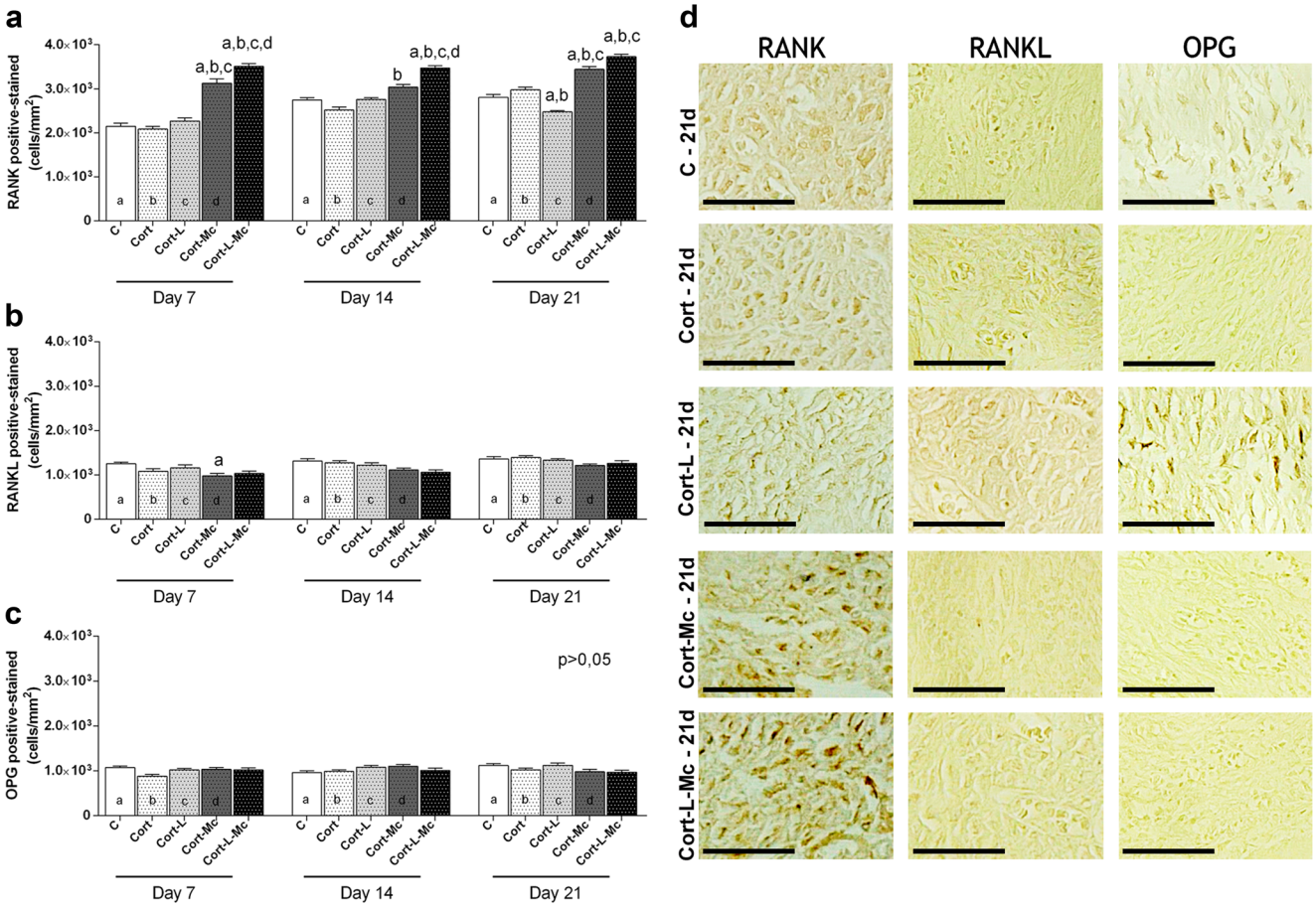

Fig. 6 Quantification of positive stained cells for **a** RANK, **b** RANKL, **c** OPG, and **d** immunohistochemistry in the periodontal ligament and alveolar bone of rats. Groups: C, tooth movement; Cort, tooth movement and corticotomy; Cort-L, tooth movement/corticotomy/laser; Cort-Mc, tooth movement/corticotomy/microcurrent; Cort-L-Mc, tooth movement/corticotomy/laser/microcurrent. Samples were analyzed on the 7th, 14th, and 21st days after orthodontic movement. The values were compared using ANOVA two-way and Bonferroni's post-test. Results expressed as mean ± standard error of the mean. a, $p < 0.05$ relative to C; b, $p < 0.05$ in relation to the Cort; c, $p < 0.05$ compared to the Cort-L; d, $p < 0.05$ compared to the Cort-Mc

an increase of this cytokine in all experimental periods especially in the groups treated with microcurrent (Cort-Mc, Cort-L-Mc) in all periods in relation to the other groups ($p < 0.0001$) (Fig. 7c).

Discussion

Corticotomy surgery alters bone biology by promoting acceleration in tooth movement mediated by the periodontal ligament [47]. The modulation of the inflammatory process is of extreme importance in the phases of tooth movement procedure associated with corticotomy surgery, and it involves the release of a complex cascade of pro-inflammatory signals and growth factors that recruit inflammatory cells and promote angiogenesis [48]. Growth factors and proinflammatory cytokines are released during inflammation by periodontal ligament cells during orthodontic movement [16].

It was observed that the low-level laser therapy (LLLT) did not promote positive tooth displacement results in relation to the electrical current. These data correlate with the results found by Kim et al. [40] who used low-level laser therapy (LLLT) and corticotomy therapies in orthodontic movement in an animal model. These authors observed decreased tooth displacement when they combined the two techniques and concluded that this procedure impaired orthodontic movement. Our results demonstrated that the laser was more effective in modulating the angiogenic and inflammatory processes. On the other hand, we observed that the combination of corticotomy with microcurrent favored tooth displacement, even when alternated to the laser.

The application of electrical current was experimentally tested in animal models and promoted acceleration in tooth movement, as it generates piezoelectric energy and increases the velocity of tooth movement [49]. Although corticotomy surgery is invasive, this technique has numerous benefits in orthodontic treatment, and studies with therapies that improve

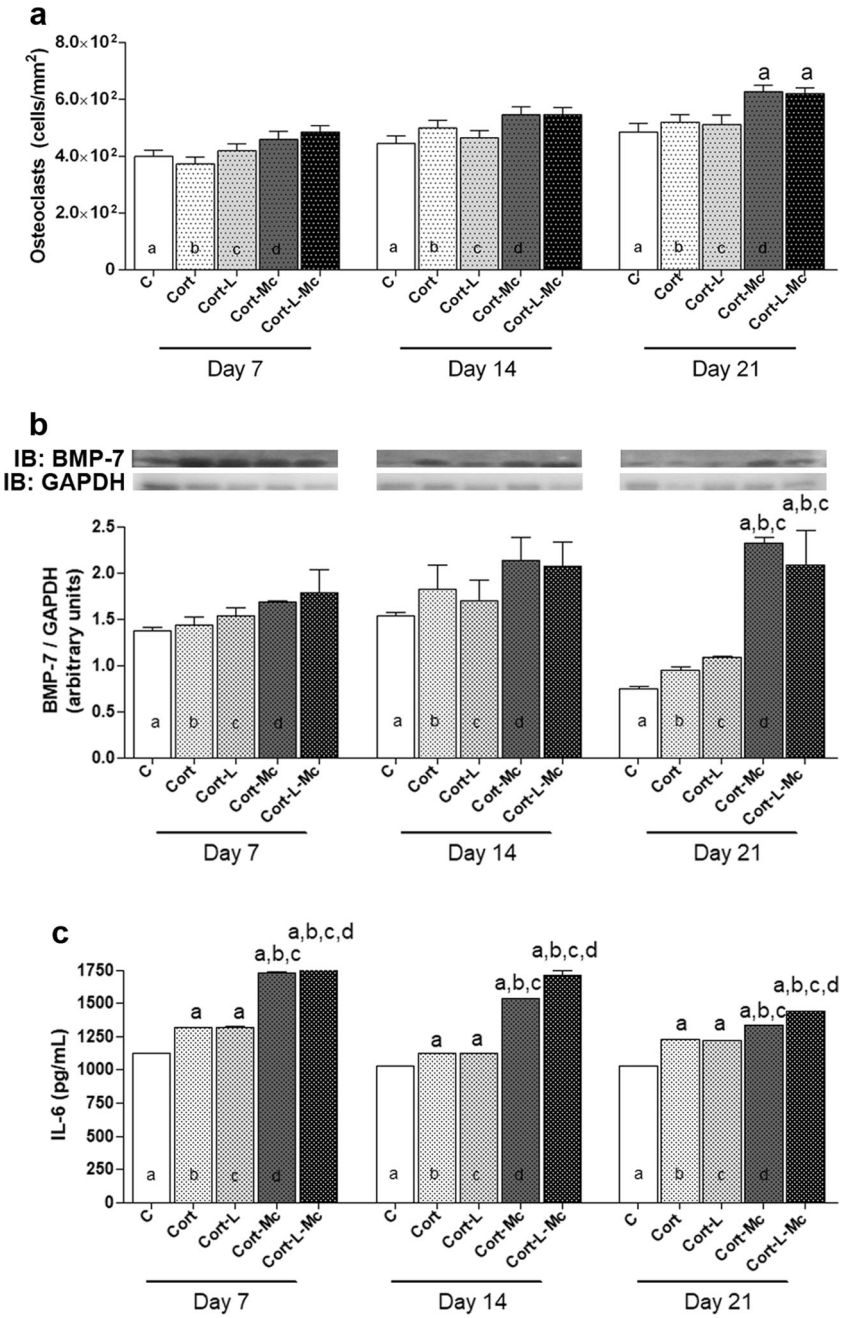

Fig. 7 **a** Quantification of osteoclast number ($n/10^4$ μm^2) by histomorphometry analysis, **b** expression of BMP-7, and **c** quantification of IL-6 by ELISA in the periodontal ligament and alveolar bone in orthodontic movement of rats. Groups: C, tooth movement; Cort, tooth movement and corticotomy; Cort-L, tooth movement/corticotomy/laser; Cort-Mc, tooth movement/corticotomy/microcurrent; Cort-L-Mc, tooth movement/corticotomy/laser/microcurrent. Samples were analyzed on the 7th, 14th, and 21st days after orthodontic movement. The values were compared using ANOVA two-way and Bonferroni's post-test. Results expressed as mean ± standard error of the mean. a, $p < 0.05$ relative to C; b, $p < 0.05$ in relation to the Cort; c, $p < 0.05$ compared to the Cort-L; d, $p < 0.05$ compared to the Cort-Mc

the clinical performance of this procedure or minimize its adverse effects are of great importance in orthodontic practice [5].

On the other hand, accelerating the rate of tooth movement can reduce the duration of orthodontic treatment and be associated with unwanted effects, including root resorption. Non-surgical techniques that assist in this process have been investigated; these include low-intensity laser irradiation, resonance vibration, pulsed electromagnetic fields, electrical currents, and pharmacological approaches [50]. Clinical research on the efficacy of non-surgical interventions to accelerate orthodontic treatment is scarce, with inconclusive results. In light of the critical review by El-Angbawi et al. [50], there is a need for well-established protocols, statistical methods, and randomized clinical trials to determine whether adjunctive therapies can clinically reduce the duration of orthodontic treatment and contribute to accelerate tooth movement without adverse effects. Despite the controversial data presented in the literature [50], in our study, we observed that the use of electrical current as an auxiliary therapy in orthodontic treatment with corticotomy has contributed to tooth movement as demonstrated by the results.

During orthodontic movement, changes occur in levels of RANK, RANKL, and OPG in tooth-supporting tissues, where

RANKL stimulation and OPG inhibition are involved in osteoclastogenesis [51]. It has been shown that increases in RANKL and decreases in OPG can be observed during severe orthodontic root resorption [52]. In our study, even though the use of microcurrent therapy accelerated tooth movement, all groups presented some root resorption. We also did not observe an increase in RANKL levels, which implies a predicted root resorption of orthodontic movement associated with corticotomy [51]. Experimental studies did not observe root resorption in orthodontic treatment with or without corticotomy [53, 54]. Our results are supported by Hassan et al. [55] considering that some root resorption is usually expected during orthodontic movement.

Laser therapy has demonstrated biomodulatory results because of its effects on the inflammatory cell decrease and in the improvement of neovascularization [56]. Some in vitro studies have also demonstrated the biostimulatory effects of laser therapy on the release of cytokines and growth factors in the proliferation process of different cells and reduction of inflammatory cells [57, 58]. These data correlate with the results found in our study where low-level laser therapy (LLLT) and microcurrent applied alone and alternated promoted a decrease of inflammatory infiltrate and the protein expression of TGF-β1 in the last experimental period, suggesting that these treatments were effective in reducing the inflammatory process. The stimulation of inflammatory cytokine expression increases bone remodeling, favoring orthodontic tooth movement [59]. The TGF-β1, a growth factor modulator of the inflammatory process and its isoforms, regulates extracellular matrix synthesis, growth, proliferation, and cell death [60]. In addition, it plays a crucial role in the regeneration of connective tissue and bone remodeling with significant effects on osteogenic differentiation and bone formation, recruiting leukocytes and inducing the inflammatory phase [61]. Osteoblasts can produce TGF-β1, maintaining the balance between dynamic processes of resorption and bone formation [62]. This growth factor also influences the RANKL/OPG system on osteoblasts [24].

Orthodontic forces promote cellular responses in the PDL, which induce bone resorption on the pressure side and bone deposition on the traction side. This process involves the induction of osteoclasts through the RANK/RANKL pathway and several inflammatory cytokines [63]. The RANKL/OPG binding favorably modulates osteoclastogenesis and is considered an important factor in the control of bone resorption [64]. The stimulation of the PDL also increases the speed of bone remodeling and several therapies that are used for this purpose. Among these, the laser and electrical currents have been shown to be promising in orthodontic therapy [34, 65]. In our study, the analysis of the markers indicated that the application of isolated microcurrent when alternated to the laser increased the osteoclastic activity. The increase of IL-6, especially in the groups treated with microcurrent, unto the last period may be related to its role in osteoclastogenesis. IL-6 influences both osteoblast and osteoclast differentiation and its activities through a variety of mechanisms during bone remodeling [66]. Effects of IL-6 on bone formation are determined by the concentrations and combinations of this cytokine with its IL-6Rα receptors present on osteoblasts inducing their differentiation and, thus, osteoclast activity [22]. PGE2, IL-6, and other inflammatory cytokines may also facilitate osteoclastic reabsorption processes [17], regulating immune responses in inflammation sites, and with also an autocrine/paracrine activity that stimulates osteoclast formation and the bone-resorbing activity of preformed osteoclasts [67]. In our study, the IL-6 activation did not indicate expressive bone resorption since RANKL and OPG levels were balanced during all experimental times suggesting a modulation of the osteogenic process, since the increased RANKL promotes bone resorption.

Orthodontic tooth movement and corticotomy can improve bone remodeling, increasing RANK and VEGF expression, with decreased OPG. Bone resorption by osteoclasts and bone formation by osteoblasts are quite obvious around newly formed blood vessels, possibly due to increased VEGF expression [66, 68]. Thus, the increase in RANK and osteoclasts observed in our study may be related to an increase in VEGF in the initial periods and a decrease in the inflammatory process in the final periods as a consequence from therapies used. Although greater expression of RANK was observed in the groups treated with microcurrent in all periods, osteoclast was more evident in the last studied period probably by the presence of mature osteoclasts.

RANKL protein was found to be predominant in inflammatory cells adjacent to areas of pathological bone loss in periodontal disease [69, 70]. In our study, laser therapy was shown to be effective in decreasing the inflammatory process and RANKL, possibly leading to moderate osteoclast with a consequent decrease in OPG expression. It is important to note that the observations of our study correspond to a three-week period where corticotomy was used. A previous study of our research group about bone regeneration showed an increase of OPG from the 30th experimental day [71].

Angiogenesis is fundamental for osteogenesis, and the relationship between these two processes has been called *angiogenic–osteogenic coupling* [72]. The analysis of our results showed that there was an increase in the number of vessels in the groups that were treated with laser and microcurrent applied alone or on alternate days. Different studies have demonstrated the beneficial effects of microcurrent on angiogenic stimulation [73], including orthodontic movement [34]. The development of the vascular network plays a vital role in the osteogenesis process [74]. Spadari et al. [34], using the same intensity and time of electrical stimulation (10 μA/5 min), also

observed an increase in the number of newly formed vessels during orthodontic movement.

An important modulator of angiogenesis is VEGF which is involved in vascular permeability [75], bone formation, including osteoblast differentiation, osteoclast recruitment, and the repair of periodontal ligament injuries during orthodontic movement [61]. This growth factor is increased in the hypoxia situation through the activation of the hypoxia induction factor (HIF) [30]. Our results demonstrated the expression of VEGF decreased in the last experimental periods mainly in the groups that received the therapy with microcurrent and when alternated to the laser. This result is related to the increase in the number of vessels in the same groups, suggesting the importance of these therapies in angiogenesis.

The increase of BMP-7 in the last period evidenced the inductive effect of the microcurrent in the osteogenesis, since the application of the laser separately did not promote an increase in the expression of this protein. BMP-7 also induces the expression of osteoblastic differentiation markers, accelerates calcium deposition, and has osteoinductive potential [76].

For orthodontic treatment success, participation of cells involved in the reorganization and maturation of collagen is, also, important. It was observed that the microcurrent was highlighted by its effects on fibroplasia and the reorganization of collagen. The application of electrical current regulates the secretion of fibroblast growth factor and modulates wound healing [77]. All groups that received different treatments, especially those that received microcurrent application alone or alternated with laser, demonstrated a better reorganization of collagen, since the analysis of the collagen isoforms indicated a growing maturation of these fibers. This was demonstrated by the increase in the expression of type I collagen in the last period in the groups treated with microcurrent and laser. On the other hand, the expression of type III collagen gradually decreased in all groups at the same experimental time. Collagen I and III evaluation has been an important indicator in the repair progress where the synthesis of collagen type III being gradually replaced by collagen type I, an essential process in bone remodeling, can be observed. Collagen type I fibers are presented in a high number in the periodontal ligament, and the *turnover* of these fibers is necessary for bone remodeling in tooth movement [78].

Conclusion

Laser therapy promoted anti-inflammatory and angiogenic effects while the electrical stimulation was more effective in angiogenesis, osteogenesis, and, also, collagen fiber organization in this experimental model. The complements of biological effects on these two therapies when applied alternately, thus improving orthodontic treatment with corticotomy and favoring bone remodeling and tooth displacement, can be observed. However, although electrical current therapy promoted more evident orthodontic movement, it presented a similar expected root resorption to all experimental treatments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and were in accordance with the ethical standards of the Research Ethics Committee of Herminio Ometto University Center (Permit no. 020/2015).

Informed consent For this type of study, formal consent is not required.

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