

Electrical stimulation: Complementary therapy to improve the performance of grafts in bone defects?

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Abstract: The limitations of bone reconstruction techniques have stimulated the tissue engineering for the repair of large bone defects using osteoconductive materials and osteoinductive agents. This study evaluated the effects of low intensity electric current on the inorganic bovine graft in calvaria defects. Bone defects were performed with piezoelectric system in the calvaria of Wistar rats divided into four groups ($n = 24$): (C) without grafting and without electrical stimulation; (E) with grafting; (MC) without grafting and submitted to electrical stimulation; (MC + E) with grafting and submitted to electrical stimulation. Inflammatory, angiogenic and osteogenic events during bone repair at the 10th, 30th, 60th, and 90th days were considered. Several inflammatory markers demonstrated the efficacy of grafting in reducing inflammation, particularly when subjected to electrical

stimulation. Angiogenesis and collagen organization were more evident by electrical stimulation application on the grafts. Moreover, the osteogenic cell differentiation process indicated that the application of microcurrent on grafting modulated the homeostasis of bone remodeling. It is concluded that microcurrent favored the performance of grafts in calvarial rat model. Low-intensity electrical current might improve the osteoconductive property of grafting in bone defects. Therefore, electrical current becomes an option as complementary therapy in clinical trials involving bone surgeries and injuries. © 2018 Wiley Periodicals, Inc. *J. Biomed. Mater. Res. Part B*: 00B: 000–000, 2018.

Key Words: electrical stimulation, bone graft, animal model, bone remodeling, osteogenesis

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INTRODUCTION

Reducing surgical failures and restoring bone structure and function has been the goal of researches involving bone regeneration.¹ Different therapies, used separately or combined, have been proposed for the enhancement or management of these complex clinical situations, which can be often recalcitrant to treatment.²

Bone repair in critical defects in calvaria has been studied in animal models. Despite its limitations, it allows the establishment of a defect such uniform, reproducible, and is adequate for the evaluation and comparison of biomaterials and other agents that aim osteogenesis. In addition, research on bone defects induced in animal models is promising because it presents results with positive perspectives for human applications.^{3–5}

Osteoconduction, osteogenesis, and osteoinduction are important mechanisms to repair bone defects and they involve the interaction of different cell types and molecular signaling pathways.⁶ The use of osteopromotor agents is important in

bone repair therapy since they favorably modulate the synthesis of cytokines involved in inflammation, growth factors, angiogenesis, maturation, and reorganization of collagen, and the receptor activator of nuclear factor-NF- κ B (RANK), receptor activator of NF- κ B ligand (RANKL), and osteoprotegerin (OPG).⁷

Moreover, the development of new osteoinductive and osteoconductive materials and the search for the most effective endogenous stimulation in bone repair have been the goals of different studies.^{8,9} Bone substitutes are directly linked to their biocompatibility and ability to initiate or accelerate the repair process and, when combined with other bioinductive techniques, can provide promising results in the bone repair process and in surgical treatment.¹⁰ Inorganic bovine bone is a well-known bone graft and it has been used in different areas of reconstructive surgery and ambulatory procedures that aim bone repair because it is similar to human bone. Its porosity favors angiogenesis and consequently osteogenesis, which justifies its clinical applicability.¹¹

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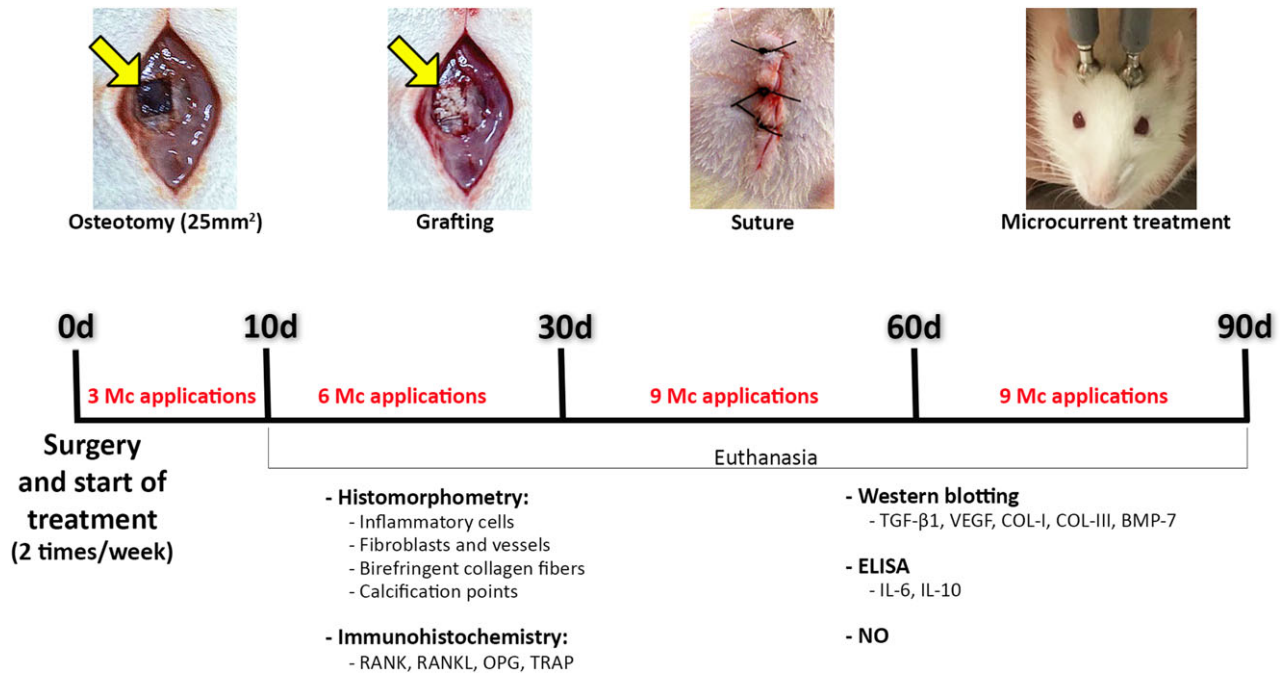


FIGURE 1. Scheme of experimental groups, timeline of follow-up (surgery, treatments, euthanasia and samples harvesting) and experimental analysis.

Osteogenesis induced by electrical stimulation was initially described by Yasuda (1953).¹² Since then, it has been demonstrated that the exogenous application of electric currents at physiological levels play a role in cellular and molecular signaling pathways, stimulating the synthesis, and release of cytokines, growth factors, proteoglycans, proliferation, and differentiation of osteogenic cells.¹³ In addition, the application of low-intensity electric current (microcurrent) activates calcium transport through its voltage-dependent channels.¹⁴ This event triggers an increase of phospholipase A2, prostaglandin synthesis E2, calcium in the cytosol and levels of Calmodulin, promoting cell proliferation through the regulation of nucleotides and enzymatic proteins leading to bone callus formation and maturation.¹⁵

In addition, microcurrent promotes bone formation through the activation of growth factors, such as growth factor and beta-1 transformation (TGF-β1), vascular endothelial growth factor (VEGF), and mRNA expression of BMPs.¹⁶ Electrical stimulation has been shown to be effective in assisting bone repair in a variety of orthopedic conditions, in osteotomies, and in the efficacy of bone grafts.¹⁷ Although electrical stimulation has been used for a long time, its therapeutic acceptance requires further academic and clinical investigations.^{14,18} Despite the lack of clinical evidence, many *in vitro* and *in vivo* studies demonstrate the use of electrical stimulation in bone healing at a cellular level.^{7,16} Masureik and Erikson (1977)¹⁹ demonstrated that electrical current application from 10 to 20 μA stimulated the bone repair in jaw fractures in a preliminary clinical evaluation. Previously, Friedenberget al. (1974),²⁰ observed that electric current above 50 μA promotes bone destruction. Electrical stimulation has been shown to be effective to

enhance delayed fractures, osteotomies and the efficiency of bone grafts. It has been shown electrical stimulation work by alteration of growth factors and transmembrane signaling.¹⁴

It is important to establish the appropriate parameters of intensity, frequency, and time of electrical current application *in vivo* bone defect animal model in order to establish an effective clinical practice. Investigating tissue biostimulation through microcurrent application is advisable, since it is non-invasive, low cost, and may benefit the graft's action during bone regeneration of critical defects in surgical and ambulatory procedures.

Once that electrical stimulation is able to enhance the performance of bone grafts we hypothesized that the association of these two therapies could improve the osteogenesis and the grafting osteointegration during the repair in calvarial defects.

MATERIAL AND METHODS

Animals

Ninety six male Wistar rats (*Rattus norvegicus*—120 days/350 g) were housed in individual polycarbonate boxes (23 °C ± 2 °C, light/dark cycle of 12/12 h), with feed and water *ad libitum*. All surgical and experimental procedures used in this study were performed according to experimental standards and biodiversity rights (NIH Publication 80-23, revised 1996 and Arouca Law-11,794, 2008), approved by CEUA/UNIARARAS (052/2014), and conducted according with the Guide for the Care and Use of Laboratory Animals.²¹ The animals were healthy and procedure did not promote stress and no weight loss or infection was observed.

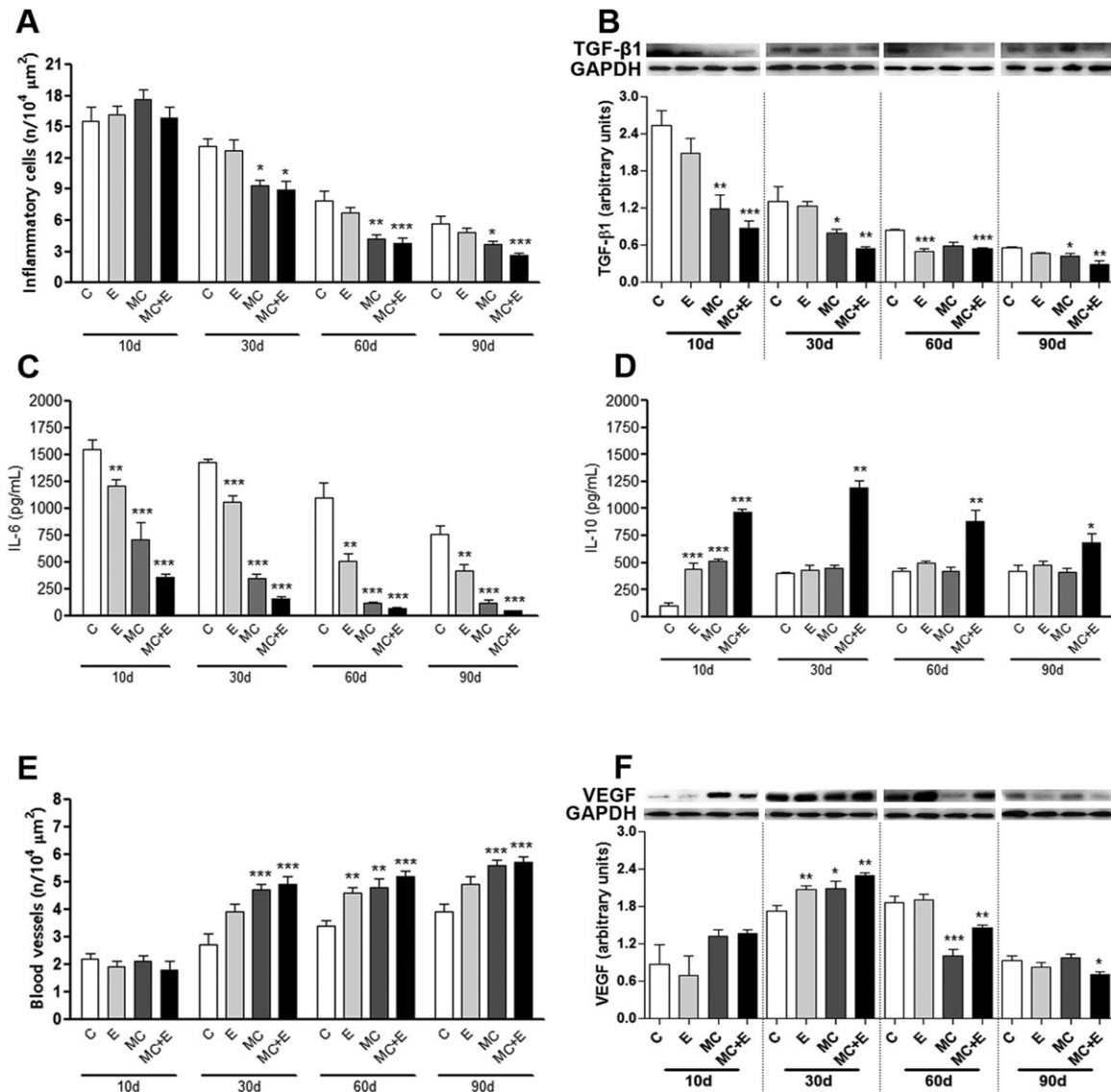


FIGURE 2. (A) Quantification of inflammatory cells, (B) expression of TGF- β 1, levels of cytokines, (C) IL-6 and (D) IL-10, and (E) Quantification of blood vessels, (F) expression of VEGF in defects of the calvaria of Wistar rats. (C)—without treatment; (E)—filled with graft; (MC)—submitted to Microcurrent applications; (MC + E)—filled with graft and submitted to Microcurrent applications. Samples were analyzed at 10, 30, 60, and 90 days after the experimental injury. The values were compared using ANOVA and Tukey post-test. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Results expressed as mean \pm SEM.

Experimental protocol

The animals were anesthetized with Ketamine Hydrochloride (60 mg/kg) and Xylazine Hydrochloride (20 mg/kg). Tricotomy was performed in the occipital region of the animals for induction of the bone defect with piezoelectric system (VK Driller, Piezosonic Esacrom, São Paulo, SP, Brazil). A 5.0 mm saw (SIN 300.0112—50W/100 Hz) under constant irrigation of sterile physiological solution was used to create a critical defect of 25 mm² in calvaria preserving the dura mater. After the surgical procedure, analgesia with sodium dipyrone (500 mg/mL) and tramadol (0.1 mg/kg) was used in the water for 72 hr.¹³

The grafting was performed with Bio-Oss[®] (Geistlich Biomaterials, Wolhusen, Switzerland) particulate in order to fill the entire bone defect. The particulate bone substitute was

chosen due to its capacity of adaptation to the defect, once mixed to the blood. In addition, the periosteum repositioning acts as a biological barrier preventing the dispersion of the material. No need to use another membrane as a barrier was necessary. Subsequently, the skin was repositioned and sutured with 4.0 nylon.

The microcurrent application was performed using a low-intensity transcutaneous electrical stimulator (Physiotonus Microcurrent, BIOSET[®], Indústria de Tecnologia Eletrônica Ltd., Rio Claro, SP, Brazil). Two metal electrodes were placed around the bone defect (10 μ A/05 min).¹³ The applications started after surgery and were performed on alternate days during the experimental period. The intensity of the electric current used (10 μ A) is physiological and imperceptible, causing no discomfort.¹⁴ There was no need to immobilize

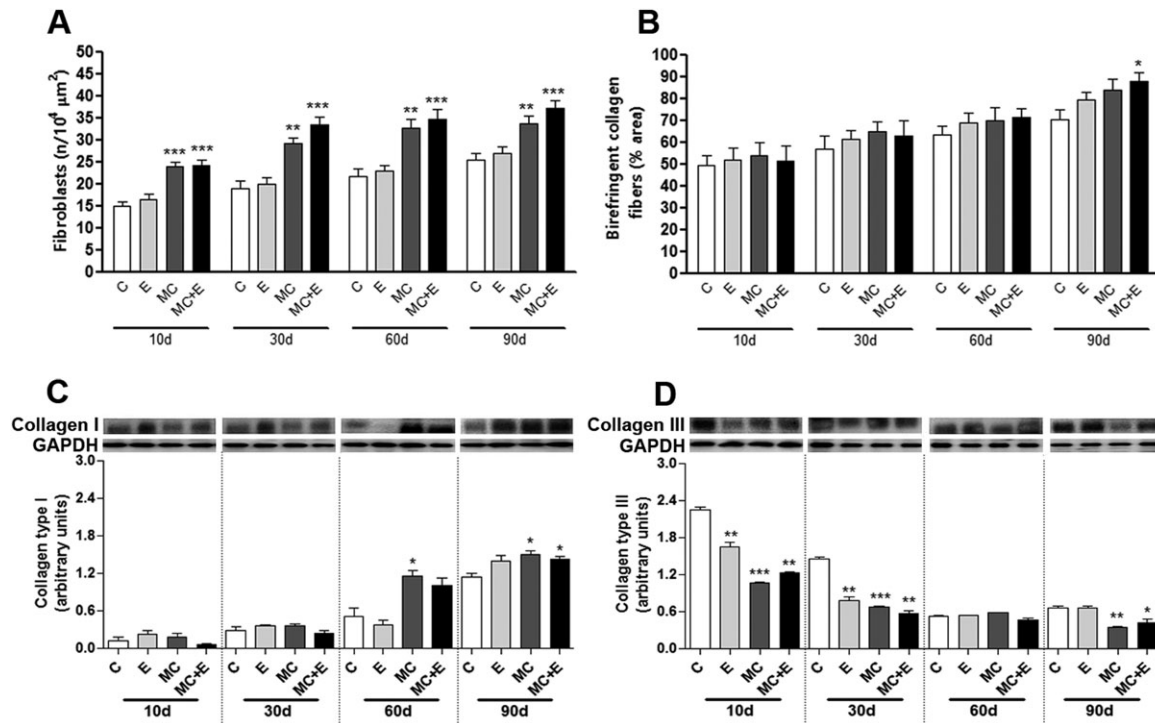


FIGURE 3. (A) Quantification of fibroblasts, (B) birefringent collagen fibers (% area), expression of (C) collagen I and (D) III in defects of the calvaria of Wistar rats. (C)—without treatment; (E)—filled with graft; (MC)—submitted to microcurrent applications; (MC + E)—filled with graft and submitted to microcurrent applications. Samples were analyzed at 10, 30, 60, and 90 days after the experimental injury. The values were compared using ANOVA and Tukey's post-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Results expressed as mean \pm SEM.

the animals or the use of anesthesia. During the experimental period the animals adapted to the treatment which was characterized as not being invasive or stressful.

The rats were divided randomly into four groups ($n = 24$): (C) without grafting and without electrical stimulation; (E) with grafting; (MC) without grafting and submitted to electrical stimulation; (MC + E) with grafting and submitted to electrical stimulation. Six animals each group were euthanized with anesthetic deepening at the 10th, 30th, 60th, and 90th days after the experimental procedure for the collection of the samples (Figure 1). The six samples were cut in half and divided for histomorphometric, immunohistochemistry, histochemistry, and molecular analysis.

Histomorphometric analysis

The tissue fragments were immersed in fixative solution for 48 h and kept in EDTA-based decalcifying solution for 4 weeks. The samples were washed in buffer before paraffin soaking (Histosec®-Merck). The specimens were longitudinally semiserial sectioned in 4.0 μm -thick histological slides and collected from two sites 1.0 mm-distant to each other. Fibroblasts and vessels ($n/10^4 \mu\text{m}^2$) were quantified through Toluidine Blue staining; inflammatory cells ($n/10^4 \mu\text{m}^2$) through Dominici staining; to evaluate the organization and maturation of birefringent collagen fibers (% of area) Picosirius-Hematoxylin staining was used and the Alizarin Red technique to verify the calcification points ($n/10^4 \mu\text{m}^2$). Each sample (five pictures per section) was captured using Leica® DM2000 Microscope.

Immunohistochemical and histochemical analysis

The samples were incubated with primary antibodies (Anti-OPG, Anti-RANK, and Anti-RANKL-Santa Cruz Biotechnology, Dallas, 1:200), secondary antibodies and for detection of a reaction, according to the manufacturer's instructions.²²

For histochemical labeling Tartarate-Resistant Acid Phosphatase (TRAP), the sections were submitted to the specific protocol of the kit (Sigma-Aldrich Inc., Dallas) according to the instructions of the manufacturer. Multinucleated cells showing dark red to purple staining were considered TRAP positive.

The counting of the number of positive cells for RANK, RANKL, OPG, and TRAP was performed from the captured images (five pictures per section) using the Sigma Scan Pro™ 6.0 software.

Extraction of bone proteins

The samples were processed according with adapted protocol.²³ Samples of ± 100 mg of bone tissue were collected and crushed in a crucible with addition of liquid nitrogen, placed in buffer (50 mM Sodium Acetate, pH = 5.8; 4 M Guanidine, 10 V) and maintained at 4 °C/24 h. After that period was centrifuged at 10,000 rpm/30 min/4 °C, the supernatant was collected and treated with 10% absolute ethanol at 4 °C/24 h. Posteriorly were centrifuged at 10,000/15 min/4 °C and the precipitated proteins were aliquoted and treated with protein extraction buffer (Thermo Scientific Inc) for ELISA²⁴ and with protein blotting buffer for Western blotting (Trisma base 100 mM pH7.5; EDTA

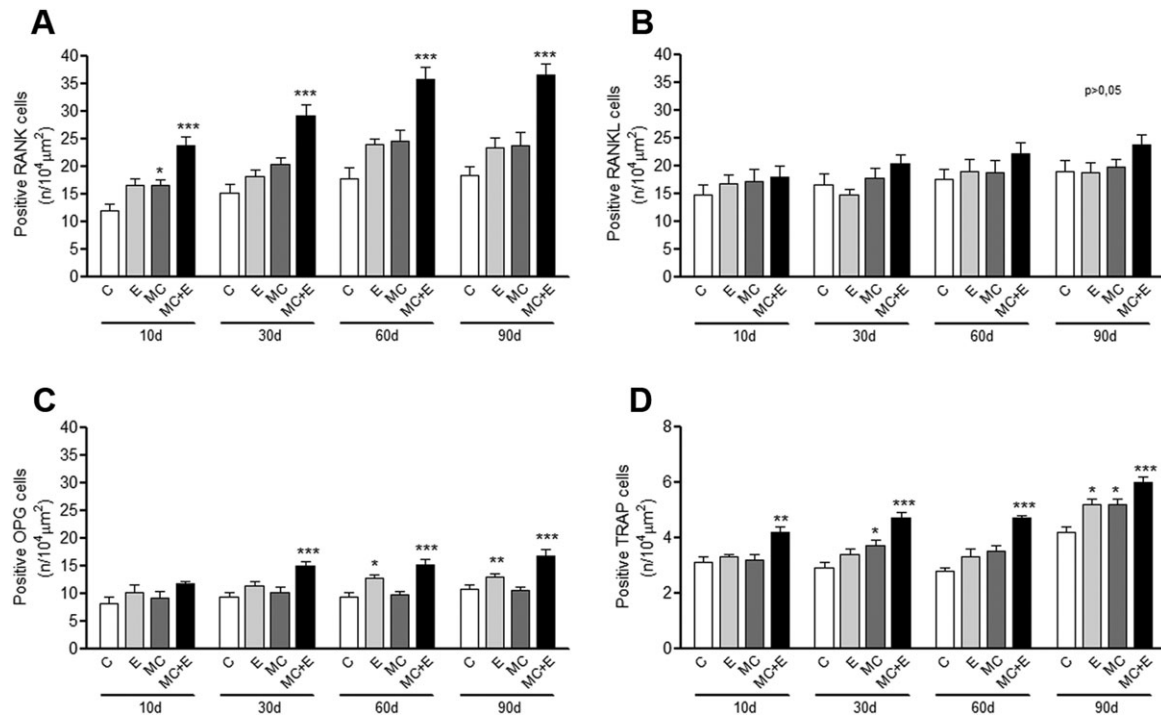


FIGURE 4. Quantification of positive cells to (A) RANK, (B) RANKL, (C) OPG, and (D) TRAP in defects of the calvaria of Wistar rats. (C)—without treatment; (E)—filled with graft; (MC)—submitted to Microcurrent applications; (MC + E)—filled with graft and submitted to Microcurrent applications. Samples were analyzed at 10, 30, 60, and 90 days after the experimental injury. The values were compared using ANOVA and Tukey's post-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Results expressed as mean \pm SEM.

10 mM; SDS 10%; 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate).²⁵

Quantification of bone proteins by Western blotting, ELISA, and determination of nitric oxide (NO)

The TGF- β 1, VEGF, COL-I, COL-III, BMP-7, and β -actin analysis were processed for Western blotting protocol.²³ The intensity of the bands was evaluated using ImageJ software.

Cytokines IL-6 (Interleukin-6) and IL-10 (Interleukin-10) present in the tissue were analyzed by ELISA capture and concentrations of primary and secondary antibodies followed the manufacturer's specifications (BD Bioscience).²³

The production of nitric oxide (NO) was quantified by the accumulation of serum nitrite, using the standard Griess reaction.²⁶

Statistical analysis

The results were typed analyzed in the GraphPad Prism 5 software using ANOVA test and Tukey post-test ($p < 0.05$). The data in the graphs were presented in mean \pm standard error.

RESULTS

Our results evaluated the inflammatory, angiogenic and osteogenic events during bone regeneration. Data from the treated groups were compared with control.

The inflammatory cells and the protein expression of TGF- β 1 decreased gradually from the 30th experimental day especially in the groups that received microcurrent application

(Figure 2A,B). IL-6 presented a gradual reduction in the groups that received treatment with grafting or microcurrent (Figure 2C). On the other hand, was observed that in bone defects with grafting and microcurrent treated (MC + E) high level of IL-10 (Figure 2D).

The results showed an increase in the number of blood vessels in the samples that received microcurrent from 30th to 90th day (Figure 3A). In this study, VEGF expression was increased in the treated groups on the 30th day, but decreased in the MC+E group on the 90th day (Figure 3B).

It was observed an increase in the number of fibroblasts during the follow-up in MC and MC + E groups (Figure 4A). The quantification of birefringent collagen fibers showed an increase on day 90th only in the MC + E group (Figure 4B). The analysis of type I collagen was increased on the 60th and 90th day in groups treated with microcurrent and grafting (Figure 4C). Moreover, the expression of type III collagen decreased gradually in the treated groups throughout the experimental period (Figure 4D).

The analysis of RANK/RANKL/OPG indicated that the application of microcurrent on grafting in bone defects favors and modulates the osteoclastic activation. It can be observed that in the MC + E group there was an increase in the number of positive cells, particularly for RANK throughout the experimental period (Figure 5A,B), and in the E and MC + E groups for OPG from the 60th day (Figure 5C). The histochemical reaction to TRAP demonstrated the positive effects of grafting on repair of bone defects and, particularly, when it received microcurrent applications in all periods studied (Figure 5D).

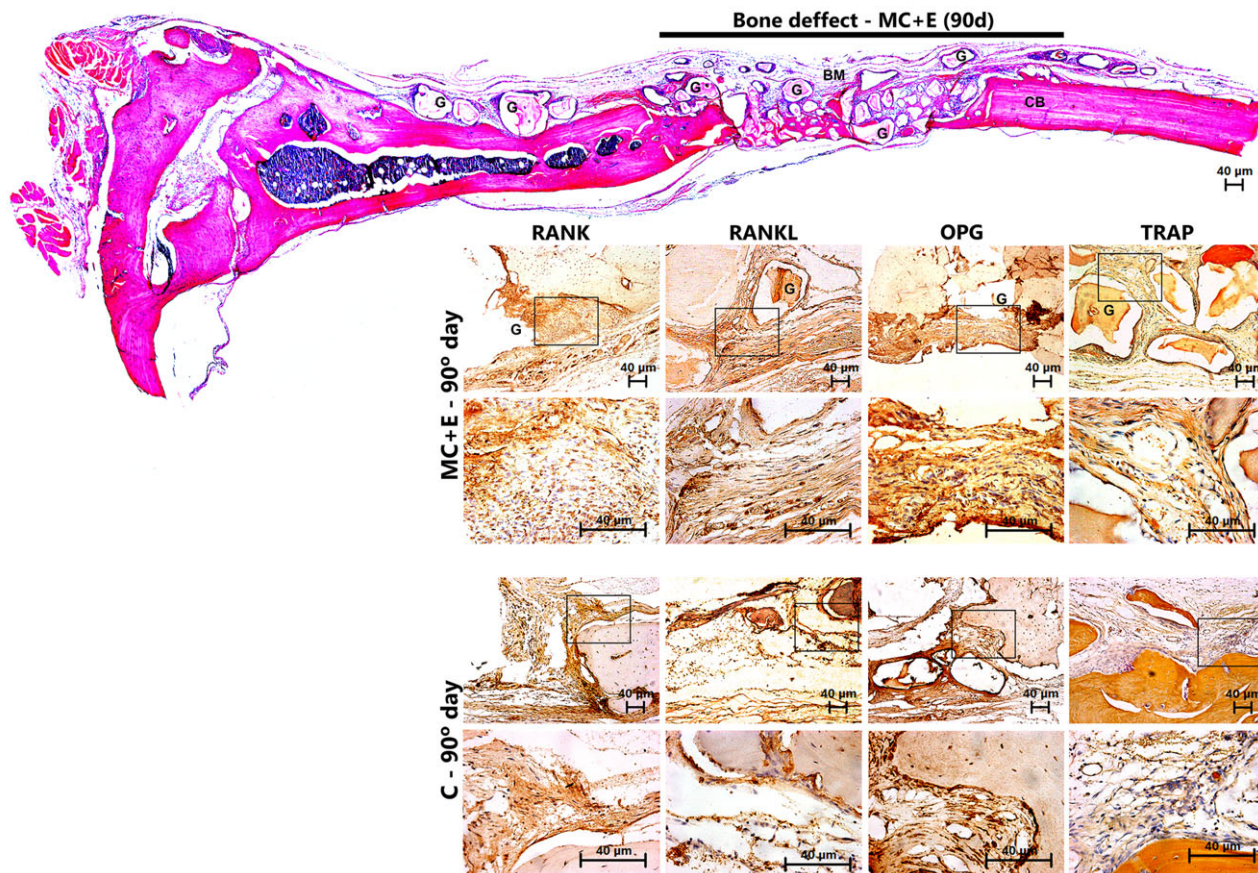


FIGURE 5. Photomicrographs of the films immunolabeled for RANK, RANKL, and OPG, and with TRAP reaction to the 90th experimental day in C and MC + E group. Calvarial defect stained by Hematoxylin–Eosin technique.

Immunostaining for RANK/RANKL/OPG and histochemical marker for TRAP in the MC + E group on the 90th day can also be visualized in photomicrography (Figure 6). NO dosage showed its increase in the MC + E group at all experimental times and, in the other treated groups, only from the 60th day (Figure 6A). There was also an increase in calcification points in the defects that received both grafting and electrical stimulation from the 60th day, indicating the positive participation of these treatments in the osteogenesis process (Figure 6B). The importance of these therapies in bone neof ormation was also evidenced in the increase in BMP-7 expression especially during the last experimental period (Figure 6C).

DISCUSSION

The modulation of inflammatory process is important during bone regeneration and involves pro-inflammatory signals and growth factors that recruit inflammatory cells and promote angiogenesis.²⁷ Our results corroborate with Mendonça et al. (2013)¹³ who observed the reduction of the number of inflammatory cells during the tissue repair in bone defects submitted to the application of microcurrent on the calvarial of rats. This treatment was also effective in reducing the expression of TGF- β 1 during the experimental period, because it modulates the inflammatory process and its isoforms regulate extracellular matrix

synthesis and cell proliferation.²⁸ TGF- β 1 is released by keratinocytes, macrophages, and platelets after injury and is involved in leukocytes recruitment and the decrease of his expression is related with the resolution of the inflammatory process during the repair.²⁹

The interleukin-6 is an important proinflammatory cytokine at the beginning of bone repair, as it recruits inflammatory cells, enhances extracellular matrix synthesis, promotes angiogenesis by stimulating VEGF release, and modulates the differentiation of osteoclasts and osteoblasts.^{30,31} The soluble IL-6 receptor (IL-6R α) is suggested to play a regulatory role in the differentiation of osteoclasts by inducing the expression of the NF- κ B ligand receptor activator (RANKL) on the surface of the osteoblasts. RANKL then interacts with the expressed RANK in osteoclast progenitors, inducing the differentiation of these cells.³²

Gerstenfeld et al. (2003)³³ considered that IL-6 is expressed during the inflammatory phase and its level decrease during the fracture remodeling. In our study, there was also a decrease in IL-6 and TGF- β 1 levels, in the graft-filled defect samples, especially when combined to microcurrent application, demonstrating the beneficial effects of these treatments on the modulation of inflammatory process. The high level of IL-10 during the follow-up point to the efficacy of combined grafting to electrical stimulation in the modulation of inflammation, since IL-10

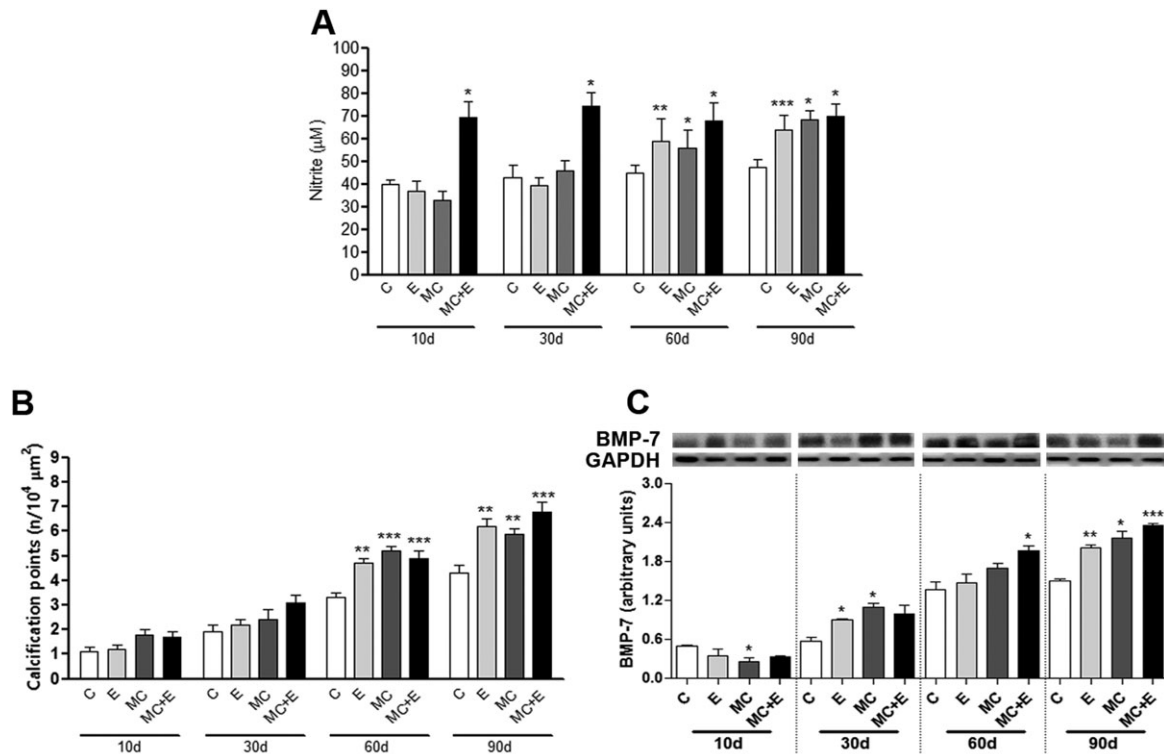


FIGURE 6. (A) Quantification of NO, (B) calcification points, (C) expression of BMP-7 in defects of calvaria of Wistar rats. (C)—without treatment; (E)—filled with graft; (MC)—submitted to Microcurrent applications; (MC + E)—filled with graft and submitted to Microcurrent applications. Samples were analyzed at 10, 30, 60, and 90 days after the experimental injury. The values were compared using ANOVA and Tukey post-test. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Results expressed as mean \pm SEM.

is anti-inflammatory and also modulates osteogenic differentiation, IL-1, IL-6, TNF- α , and NO synthesis being important in bone homeostasis.^{34,35}

Osteogenesis and angiogenesis are involved in bone remodeling and this relationship has been termed angiogenic-osteogenic coupling.³⁶ Vascularization plays an important role in the development, remodeling and homeostasis of bone and it is essential for cell differentiation, presence of cytokines and growth factors that are involved in bone remodeling.³⁷ In this study the observed increase of VEGF on the beginning of bone repair was important, as it is involved not only in angiogenesis but also in various aspects of bone development, including osteoblasts differentiation and osteoclast recruitment.³⁸ This growth factor is shown to be increased in the hypoxia situation by the activation of the hypoxia induction factor (HIF)³⁹ once blood vessels are formed to supply the tissue formation and cell recruitment. It has been shown that electrical stimulation increases vascularization at the injury site,⁷ which was also observed in our results and found by different authors.^{40,41} It demonstrates the importance of this therapy to stimulate angiogenesis and, consequently, osteogenesis.

In addition to angiogenesis, cells proliferation involved in the reorganization and maturation of collagen is important during bone regeneration process. The increases in the number of fibroblasts demonstrate the positive effects of electrical stimulation to factors involved in the collagen content.¹³ Therapies that stimulate the synthesis of type I collagen and lead to increased maturation of collagen are used in the

treatment of injury's in bone repair models.⁴² The evaluation of collagen I and III has been an important indicator of the progress of repair where it can be observed that the synthesis of type III collagen is gradually replaced by that of type I collagen, an essential process during bone remodeling phase, indicating tissue maturation.⁴³

The molecular triad consisting of RANK/RANKL/OPG plays a crucial role in bone remodeling and osteoclastic activation. The increase of RANK expressed on the surface of the osteoclasts and RANKL receptor, indicated an increase in the number of these cells. The RANKL/OPG binding favorably modulates osteoclastogenesis and is considered an important factor in the control of bone resorption.⁴⁴ Both are key regulators of this process and present increased expression after bone injury.^{31,45} In our experimental model, the activation of OPG by grafting and microcurrent indicates its modulatory roles with RANKL, probably down-regulation in osteoclastic activation, favoring homeostasis of bone remodeling once the OPG/RANKL interaction balances the RANK/RANKL binding.⁴⁴

Usually considered only as a histochemical marker of osteoclasts, TRAP is recognized as a molecule that plays an important role in many biological processes including skeletal development, collagen synthesis, bone degradation and mineralization.⁴⁶ The increase of this marker in the groups treated with microcurrent and grafting indicate to a greater proliferation and maturation of osteoclasts. This process may be related to the activation of bone remodeling.

Another factor contributing to bone metabolism is NO. It is involved in fracture healing and stimulates the activity of osteoblasts and osteocytes while maintaining bone resorption mediated by osteoclasts under control. In addition to presenting anti-inflammatory activity, it can act as a modulator of osteoclastic activity and in an auto-crine way in osteoblasts or osteoclasts with cytokine production during the bone remodeling process which was visualized through *in vitro* studies and animal models.⁴⁷ Increased NO serum may influence vascular tone modulation, permeability, and leukocyte migration, favoring osteoblastic differentiation.⁴⁸

The osteogenic capacity of BMP-7 has been studied in human clinical trials of craniofacial deformities and fracture repair.⁴⁹ BMP-7 also induces the expression of markers of osteoblastic differentiation, accelerates calcium mineralization and presents potential osteoinducers.⁵⁰ In our study it was also observed an increase of BMP-7 in all groups treated.

In this context, the results obtained in our study point to the beneficial effects of microcurrent application on the graft that is osteoconductive, since specially the application of the microcurrent provided positive responses in different biomarkers that benefit the osteogenesis. These results corroborate with those found in the literature by different authors. Mollon and Busse (2004)⁵¹ evaluated the positive clinical use of electrical stimulation to treat the delayed union and nonunion of fractures. Kuzyk and Schemistch (2009)⁵² also investigated the effects of electrical stimulation for fracture healing and studied the action mechanism at the cellular level. The authors considered that further studies are need since this technique promotes the differentiation and proliferation of osteoblastic cells. Other clinical studies on bone regeneration were previously performed.^{53,54}

CONCLUSION

Although the animal model is widely used to study cellular and molecular aspects in the repair of different tissues, it may not completely translate to a clinical patient population. However, animal model has strong value for father studies in the events of bone repair. Low-intensity electrical current might improve the osteoconductive property of grafts in bone defects, due to your participation in modulating the inflammation, improving the angiogenesis and osteogenesis process. Therefore, electrical current becomes an option as complementary therapy in clinical trials involving bone surgeries and injuries.

ACKNOWLEDGMENTS

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