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ORIGINAL ARTICLE

Influence of microcurrent on the modulation of remodelling genes in a wound healing assay

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Abstract

The literature has shown the beneficial effects of microcurrent (MC) therapy on tissue repair. We investigated if the application of MC at 10 µA/90 s could modulate the expression of remodeling genes *transforming growth factor beta (Tgfb)*, *connective tissue growth factor* (*Ctgf*), *insulin-like growth factor 1* (*Igf1*), *tenascin C* (*Tnc*), *Fibronectin* (*Fn1*), *Scleraxis* (*Scx*), *Fibromodulin* (*Fmod*) and *tenomodulin* in NIH/3T3 fibroblasts in a wound healing assay. The cell migration was analyzed between days 0 and 4 in both fibroblasts (F) and fibroblasts + MC (F+MC) groups. On the 4th day, cell viability and gene expression were also analyzed after daily MC application. Higher expression of *Ctgf* and lower expression of *Tnc* and *Fmod*, respectively, were observed in the F+MC group in relation to F group ($p < 0.05$), and no difference was observed between the groups for the genes *Tgfb*, *Fn1* and *Scx*. In cell migration, a higher number of cells in the *scratch* region was observed in group F+MC ($p < 0.05$) compared to group F on the 4th day, and the cell viability assay showed no difference between the groups. In conclusion, MC therapy at an intensity/time of 10 µA/90 s with 4 daily applications did not affect cell viability, stimulated fibroblasts migration with the involvement of *Ctgf*, and reduced the *Tnc* and *Fmod* expression.

Keywords Genes · Cell migration · Therapy · Fibroblasts · Microcurrent

Introduction

For decades, low-intensity electrical stimulation therapy, microcurrent (MC), has been reported as an auxiliary therapeutic method to promote wound healing [1], as it triggers multiple events such as increased cell proliferation, collagen synthesis, tissue contraction, neovascularization, cell membrane permeability and normalization of tissue bioelectricity [2]. MC activates and rearranges both ion channels as carrier proteins across the cell, regardless of external chemical gradient and ion flux, contributing to intracellular polarization and cellular response [3]. Each cell type exhibits specific behavior under electrostimulation that may exhibit significant reduction in cell viability or cytotoxic effect [3].

The MC effect has been tested in vitro on different cell types involved in wound healing, such as macrophages, fibroblasts, epidermal cells, endothelial cells and has shown beneficial effects on cell migration and viability, proliferation, orientation, protein augmentation and DNA synthesis [3]. Still in vitro, Uemura et al. [4] observed that MC with 200 µA intensity favored the migration of human dermal fibroblasts within 24 h after application. Data from Sugimoto et al. [5] demonstrated that migration of human dermal fibroblasts treated with 100 µA was greater than in controls. According to a study by Leppik et al. [6] involving osteoblast culture and application of MC therapy (10–150 mV/mm) for 1 h daily for 3 weeks increased cell viability. However, intracellular changes induced by MC are not completely defined.

The literature describes different protocols for applying MC in different tissues. De Campos et al. [7] investigated MC stimulation (20 µA) in the xiphoid cartilage

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repair process and the animals treated with MC showed an increase in the number of chondroblasts and proteoglycans from the 21st day, showing the effect of MC on the acceleration of healing process. In skin, Neves et al. [8] used MC therapy (10 µA/3 min) and observed a reduction in the number of inflammatory cells, an increase in fibroblasts and blood vessels. Data from Belli et al. [9] demonstrated that the treatment of burns with MC (10 µA/3 min) alone or alternating with laser therapy, decreased inflammation, increased angiogenesis and favored tissue reorganization. In bone tissue, Mendonça et al. [10] evaluated MC stimulation (10 µA/5 min) in rat calvary excision and the results demonstrated a reduction in inflammatory cells, an increase in blood vessels and fibroblasts, as well as an increase in the organization of collagen fibers. Zaniboni et al. [11] used MC (10 µA/5 min) during orthodontic movement and observed that MC decreased inflammation, increased angiogenesis and favored bone remodeling.

During healing processes, especially of connective tissues, some genes play fundamental role in the structural and functional tissue restoration, such as *Tgfb* (*transforming growth factor-β1*), *Ctgf* (*connective tissue growth factor*), *Igf1* (*insulin-like growth factor 1*), *Tnc* (*tenascin C*), *Fn1* (*Fibronectin*), *Fmod* (*Fibromodulin*), *Scx* (*Scleraxis*) and *Tnmd* (*tenomodulin*). *Tgfb* acts as a chemotactic agent for neutrophils, monocytes and fibroblasts [12], orchestrates many cellular responses during wound healing as fibroblast proliferation, angiogenesis, and increases extracelular matrix (ECM) production [13], including collagen and fibronectin [14]. *Ctgf* stimulates fibroblast proliferation, ECM production [15], cell adhesion and cell differentiation [16, 17]. *Igf1* enhances healing process through cell proliferation and migration, activity of fibroblasts, collagen production, agiogenesis and re-epithelialization of skin wounds [18–23]. *Tnc* is involved in a range of processes such as mitogenic responses, cell migration, cell attachment, cell spreading, focal adhesion, cell survival, matrix assembly, pro-inflammatory cytokine synthesis [24], and tenascin-C can also interact with diverse growth factors [25]. *Fn1* plays an essential role in development, angiogenesis and in the wound healing promotion [26], and contains a number of binding sites for growth factors [27]. *Fmod* is involved in cell migration, collagen fibrillogenesis [28], angiogenesis [29], osteoclastogenesis [30], myogenic differentiation [31] and modulation of growth-factors signaling [32]. *Scx* facilitates tenocyte mechanosensing [33], it is essential for tendon [34] and musculoskeletal [35] development, regulates proteoglycans expression [36] and fracture callus formation during bone healing [37], and *Scx* also activates *Tnmd* expression to regulate differentiation and maturation of tenocytes [38]. *Tnmd* expression enhances cellular adhesion [39], prevents adipocyte accumulation and fibrovascular scar formation during tendon healing [40], and affects self-renewal and senescence properties of tendon stem/progenitor cells [41].

In addition to the important roles of the previously described genes during development and tissue repair, a study of Zheng et al. [42] have demonstrated the interaction of *Fmod* with *Tgfb* signaling on the fibroblasts function during skin wound healing, with *Ctgf* as dowstream mediator of *Tgfb* signaling. In a previous work performed by our group [43], the MC (10 µA/4 min) therapy applied until the 14th day of tendon repair, increased the expression of *Fmod*, *Ctgf*, *Tnc* and *Fn* genes, suggesting activation of the pathway *Tnmd–Tgfb1–Ctgf*. Therefore, it would be possible to suggest that MC therapy, without systemic influence, could activate the pathway *Tnmd–Tgfb1–Ctgf*? Although the MC therapy at intensity of 10 µA has improved the repair process of various tissues, the molecular mechanisms activated by MC are partially elucidated. We investigated if the application of MC at 10 µA/90 s could modulate the expression of remodeling genes previously mentioned *Tgfb*, *Ctgf*, *Igf1*, *Tnc*, *Fn1*, *Scx*, *Fmod* and *Tnmd* in NIH/3T3 fibroblasts in a wound healing assay.

Materials and methods

NIH/3T3 fibroblast culture and experimental groups

NIH/3T3 mouse fibroblasts used in the present study were donated by the University of São Paulo-USP, Ribeirão Preto. The cultures were plated in 75 cm^2 culture bottles with DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 15% fetal bovine serum (FBS, Nutricell Nutrientes Celulares, Campinas, São Paulo, Brazil), under a humid atmosphere at 37 °C containing 5% CO_2, until reaching 85% confluence. The culture passages were performed approximately every three days. On the 21st passage, the experimental groups were established: (F)-NIH/3T3 fibroblasts without MC application; and (F+MC)-NIH/3T3 fibroblasts + MC application. Thus, NIH/3T3 fibroblasts were plated at a density of 1×10^4 per well for both groups in 24-well culture plates. DMEM supplemented with 15% FBS were used for maintenance of cultures under a humid atmosphere at 37 °C containing 5% CO_2, until reaching 70% confluence. Subsequently, the scratch assay was performed using a 200 µL pipette tip, which was slid over the median extension of the previously marked well, creating a scratch on the cell monolayer. The cultures were washed with DMPBS Flush (Dulbecco's Modified Phosphate-Buffered Saline) to remove cell debris and kept for 4 days until *scratch* closure. Experimental procedures were performed according to approval of the

Animal Use Ethics Committee (CEUA) of the University Center of Hermínio Ometto Foundation/FHO under number 013/2018.

MC application

The intensity of the MC application (10 µA) was based on pre-established studies obtained in the literature [10, 11, 44–47]. To define the application time, the cell viability test was performed by the [3-(4,5-dimethylthiazole-2)-2,5-diphenyltetrazolium bromide] test (MTT) test, for the application times of 30, 90 and 150 s. The application of MC was performed by immersing two electrodes in the culture medium (DMEM supplemented with 15% FBS) present in each well of the culture plate, without maintaining contact with the attached cell monolayer. The device used for the application of MC (biphasic microgalvanic direct current) was the Physiotonus microcurrent© stimulator (Bioset-Rio Claro, São Paulo, Brazil).

The application time of MC for 90s in the F+MC group was selected due to the higher cell viability observed in the 24 h MTT assay. Thus, MC (10 µA/90 s) was applied on days 0, 1, 2 and 3 after scratch assay (Fig. 1). For F group, the disconnected electrodes were placed as described for group F+MC. 24 h after the last application, samples were collected, that is, they were collected on the 4th day. Samples of groups F and F+MC were analyzed for cell viability and gene expression by the Real Time Polymerase Chain Reaction (RT-qPCR) technique.

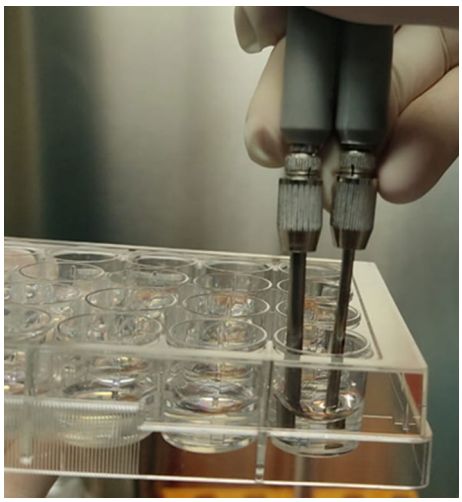

Fig. 1 Microcurrent application (10 µA/90 s): two metal electrodes were placed on the left and right side of the well for half of time (15, 45 and 75 s). Then, the position of electrodes was inverted and the current applied for another half of time (15, 45 and 75 s), considering that total time of application was 30, 90 and 150 s

MTT Test [3-(4,5-dimethylthiazole-2)-2,5-diphenyltetrazolium bromide] for cell viability assay

NIH/3T3 fibroblasts at 21st passage (P) were plated in quadruplicate at 1×10^4 density per well in 24-well culture plates. Cultures were maintained with culture medium (DMEM supplemented with 15% FBS) under a humid atmosphere at 37 °C containing 5% CO_2 for 24 h for cell adhesion. Control wells were established: (1) Only culture medium to be considered as "blank" in absorbance reading; (2) Positive control: NIH/3T3 fibroblasts and culture medium; (3) Negative control: NIH/3T3 fibroblasts, culture medium and 50% DMSO (dimethylsufoxide). On the 4th day, complete culture medium was removed and a solution of 0.5% MTT (Sigma-Aldrich, St. Louis, MO, USA) with DMEM medium without phenol red was used for incubation for 4 h protected from light in a 37 °C oven containing 5% CO_2. After MTT removal, 200 µL of DMSO was added and the absorbance was measured at 570 nm in a microplate reader. For cell viability calculation, the following formula was used: Cell viability (%) = (sample absorbance/positive control absorbance) × 100.

Cell migration assay

Images from the *scratch* on the cell monolayer were documented daily using inverted microscope (Carl Zeiss—Cousin Vert) and Microscope Software ZEN 2012 blue edition. Six wells were used per group, and images were captured in 3 regions (one central and two peripheral) of each well, to analyze cell migration towards the scratch region. Images were captured on day 0 and were used to measure scratch size (cm) to obtain an average value (cm) of scratch width. This average was used to cut the images from days 1, 2, 3 and 4, to isolate only the cells that migrated to the scratch region. After the standardization of the photos, only the scratch area cells were counted by ImageJ 1.46r ("Cell Counter" Plugin) and the average values were calculated for each period.

Real time polymerase chain reaction

Each sample from groups F and F+MC was collected from a 24-well plate, totaling 4 samples per group with 3.1×10^6 cells in each sample. After cells trypsinization and following centrifugation at 1800 rpm for 10 min, the RNA extraction from each sample was performed.

The RNeasy® Plus Mini Kit (Qiagen®, cat. No. 74104) was used for total RNA extraction, according to the manufacturer's instructions. The analysis of quality of the samples was evaluated by reading the absorbance ratios

260/280 nm and 260/230 nm in nanophotometer and the integrity of the RNA was verified by 1% agarose gel electrophoresis under denaturing conditions.

Complementary DNA (cDNA) synthesis was performed from 2.0 µg total RNA using the Applied Biosystems High-Capacity cDNA Reverse Transcription ™ kit (cat. 4368814) following the manufacturer's instructions. The cDNA produced was used as a sample for real time polymerase chain reaction reactions. Reactions, always performed in triplicate, were performed using: cDNAs produced, *TaqMan™Gene Expression Master Mix* (*Life Technologies—PN 4369016*), RNase *free* water and *Taqman* assays (primer + hydrolysis probes) for the genes contained in Table 1 making a final volume of 20 µL. The entire RT-qPCR procedure was performed on the *MX3005P (GE)* instrumentation platform under 95 °C (10 minutes) and 45 cycles of 95 °C (15 s) and 60 °C (1 min). The results were analyzed by the MxPro (GE) program and relative gene expression was obtained by the $2^{-\Delta\Delta CT}$ method. *Ppia* gene was used as endogenous for data normalization.

Statistical analysis

All data presented normal distribution (Gaussian distribution) and parametric tests were employed: for comparisons between groups F and F+MC, data were analyzed using the Student t test ($p < 0.05$); while for the cell viability test after the 30, 90 and 150 s of MC application, the data of the different groups were analyzed using Analysis of Variance (ANOVA One-way), followed by Tukey test ($p < 0.05$). The values were represented by the mean ± standard deviation (SD), using GraphPad Prism® (GraphPad Software, La Jolla, CA, USA), version 3.0 was used.

Table 1 *Taqman* assays used in RT-qPCR: *Ctgf* (*connective tissue growth factor*), *Fmod* (*fibromodulin*), *Fn1* (*fibronectin 1*), *Igf1* (*insulin-like growth factor 1*), *Scx* (*scleraxis*), *Tgfb1* (*transforming growth factor beta 1*), Tnmd (*tenomodulin*), *Tnc* (*tenascin C*), *Gapdh* (*glyceraldehyde-3-phosphate dehydrogenase*), Ppia (*peptidylprolyl isomerase A*)

Gene	Assays	Catalog
Ctgf	Mm01192933_g1	4453320
Fmod	Mm00491215_m1	4448892
Fn1	Mm01256744_m1	4453320
Igf1	Mm00439560_m1	4331182
Scx	Mm01205675_m1	4448892
Tgfb1	Mm01227699_m1	4448892
Tmnd	Mm00491594_m1	4448892
Tnc	Mm00495662_m1	4453320
Gapdh	Mm99999915_g1	4453320
Ppia	Mm02342430_g1	4453320

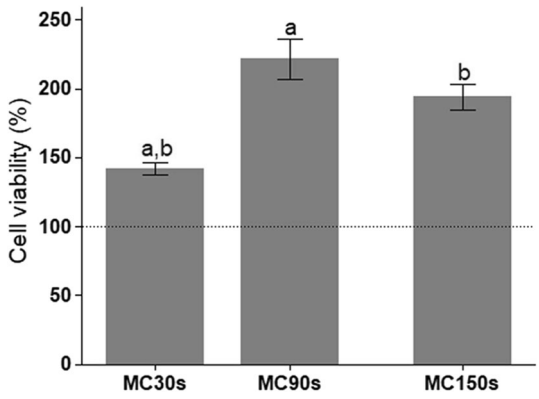

Fig. 2 Cell viability (%) observed 24 h after MC application (10 µA) at times of 30, 90 and 150 s. a, b = equal letters indicate differences between groups. Values represented by mean ± SD

Results

Cell viability

The protocol for applying MC therapy (10 µA) was defined 24 h after the 30, 90 and 150 s times (Fig. 2). All times presented higher cell viability ($p < 0.05$) in comparison to positive control. However, lower cell viability ($p < 0.05$) was observed after the application of MC in the 30 s time in relation to 90 and 120 s times.

On the 4th day after scratch, there was no difference in cell viability between groups F and F+MC (Fig. 3).

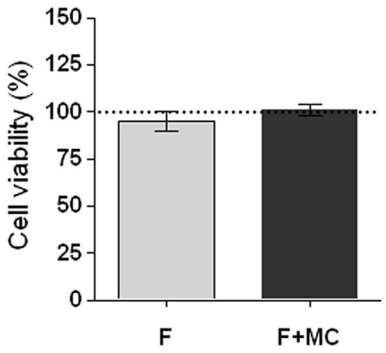

Fig. 3 Cell viability (%) observed on the 4th day: no difference was observed between groups F and F+MC. Values represented by the mean ± SD

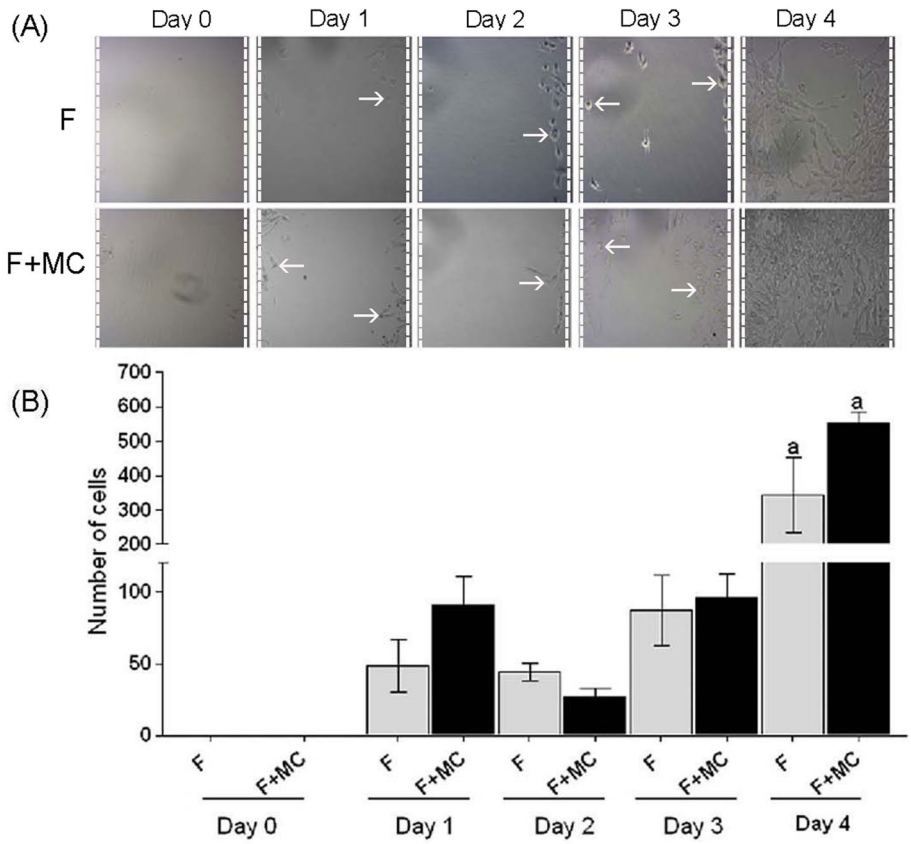

Fig. 4 (**a**) Images showing cell migration (→) to the scratch region on days 0, 1, 2, 3, and 4 in groups F and F+MC. Notice higher number of cells in group F+MC on the 4th day. Bar = 200 µm. (**b**) Cell migration: Until day 3, no marked difference was observed between groups F and F+MC. On the 4th day, there was an increase in cell migration to the scratch region in group F+MC compared to group F. a = difference between groups ($p < 0.05$). Values represented by the mean ± SD

Cell migration

Cell migration analysis was performed on days 0, 1, 2, 3 and 4 by counting the number of cells in the scratch region. On day 0, no cells were observed in the scratch region. On the 1st day, few cells were observed in both groups. On the 2nd day, group F+MC presented lower number of cells in the scratch region compared to group F, however, no statistical differences were observed. On the 3rd day, both groups presented similar number of cells and, on the 4th day, scratch closure was observed in both groups, but with a larger number of cells in the F+MC group ($p < 0.001$) (Fig. 4a, b).

Gene expression

Higher expression of *Ctgf* and lower expression of *Tnc* and *Fmod*, respectively, were observed in the F+MC group in relation to F group ($p < 0.05$). No difference was observed between the groups for the genes *Tgfb*, *Fn1* and *Scx* on the 4th day (Fig. 5). Regarding *Tnmd* and *Igf1*, no gene expression was observed in both groups under the experimental conditions studied.

Discussion

The literature has demonstrated the effectiveness of MC therapy in vivo during the repair of different tissues, such as tendon [43], skin [8], bone [48], heart [49] and cartilage [7]. Thus, the present study aimed to analyze the influence of MC on the modulation of remodeling genes in a wound healing assay, as well to correlate the possible activation of the pathway *Tnmd–Tgfb1–Ctg*, as described by Zheng et al. [42] during wound healing. In a previous work performed by our group [43], the MC (10 µA/4 min) therapy applied until the 14th day of tendon repair, increased the expression of *Fmod*, *Ctgf*, *Tnc* and *Fn* genes. In the present study, higher expression of *Ctgf* and lower expression of *Tnc* and *Fmod* were observed after MC daily application during 4 days, and no difference was observed between the groups for the genes *Tgfb*, *Fn1* and *Scx*. Zheng et al. [42, 50] stated that the wound repair process can be initiated by the activation of *Fmod* expression that can act on the modulation of *Tgfb1* and *Ctgf* genes, inducing cell migration and myofibroblast differentiation, resulting in improved wound healing. Although a reduced expression of *Fmod* was observed, our results point for the involvement of *Ctgf* after MC therapy due to the higher cell migration. *Fmod* and *Ctgf* have other

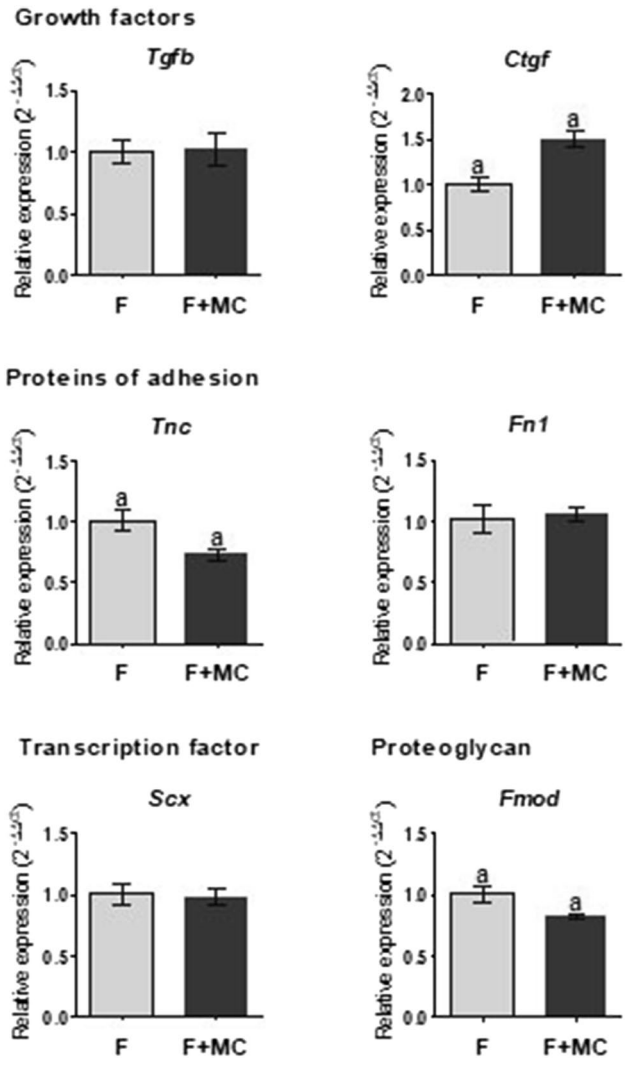

Fig. 5 Gene expression on the 4th day: note higher expression of *Ctgf*, and lower expression of *Tnc* and *Fmod* in the F+MC group in relation to F. No difference was observed between the groups for the genes *Tgfb*, *Fn1* and *Scx*. a = difference between groups ($p < 0.05$). Values represented by the mean ± SD

important roles during tissue repair, in addition to participating in cell migration, such as collagen fibrillogenesis [28], angiogenesis [29], and modulation of other growth-factors signaling [32]. Regarding *Tgfb*, Neves et al. [8] have demonstrated the influence of MC stimulating TGF-β1 expression during wound closure in vivo, while Fonseca et al. [48] reported that MC therapy in the bone reconstruction model reduced TGF-β1 expression and modulated the inflammatory process.

Surprisingly, our results showed a reduced *Tnc* expression after MC therapy on the 4th day, when the closure of scratch occurred. As reviewed by Giblin and Midwood [25], tenascin-C has a diverse range of functions such as the stimulus of the cell migration, attachment and survival, matrix assembly, diverse cytokines synthesis, among others. Tenascin-C transcription may be influenced by a variety of growth factors, cytokines, ECM proteins, biomechanical stimulus [25, 51, 52], and according to our results, also in response to the MC therapy.

An in vivo study of Zuzzi et al. [53] showed that MC 20 µA/5 min accelerated cartilage repair by increasing the deposition of ECM components such as proteoglycans [53], glycosaminoglycans and collagen [54]. It was expected in the present study to show the activation of the *Igf1* and *Fn1* expression, which activate essential signaling pathways that increase deposition and remodeling of ECM components, as well as cell adhesion and migration [55]. However, our results demonstrated the absence of *Igf1* expression and no difference in the expression of *Fn1* after MC therapy on the 4th day.

In the present study, no alteration in the *Scx* expression was observed after MC therapy on the 4th day, and absence of *Tnmd* expression was observed in both groups. According to a study of Shukunami et al. [56], *Scx* activates *Tnmd* expression in vitro to regulate differentiation and maturation of tenocytes, although the *Scx* expression and absence of *Tnmd* expression were observed from the 20th passage of cells. Still in the previously mentioned study [56], no expression of *Scx* and *Tnmd* was found in the NIH/3T3 fibroblasts, partially corroborating our results, in which was observed the expression of *Scx* though no difference after MC application. Kague et al. [35] reported that *Scx* is also related to secretion of ECM components, including some types of collagen, and *Tnmd* is also related to the formation of cytoplasmic extensions that support the organization of ECM.

In vitro studies have shown that electrical currents can influence migration, proliferation, viability and cell function [3, 4]. Our results showed that the application of MC at 10 µA/90 s on the 4th day did not affect the cell viability, corroborating with the in vitro analysis performed by Uemura et al. [4], that demonstrated no difference in the viability of dermal fibroblasts after 24 h of electrostimulation application between 100 and 300 µA (for 8 h). Lin et al. [57] used the intensity of 0.05 to 1.5 mA of MC application in equine tenocytes culture for 8 min and observed no apoptosis after 24 h, and an increase in apoptosis after 48 and 72 h. Thus, the effects of MC on cell viability depends on the intensity and time of application.

Regarding cell migration, a higher number of cells in the *scratch* region on the 4th day observed in our results, demonstrated a stimulatory effect of the MC therapy corroborating with the literature. Our results also showed the involvement of the increased *Ctgf* gene expression with the enhanced cell migration after MC application. Uemura et al. [4] analyzed the migration of dermal fibroblasts 24 h after the application of electrostimulation at 100, 200 and 300 µA intensities, and observed increased

migration only using electrostimulation at the intensity of 200 µA. Tai et al. [2], showed that electrical fields activate multiple cellular signaling mechanisms, such as the PI3K-AKT (phosphoinositide-3 kinase-AKT serine/threonine kinase) mechanism, which alter cell migration, survival and proliferation. It is important to emphasize that, in the present study, on the 2nd day after the application of MC, there was cellular reorientation, since the cells partially retracted to the *scratch* edges and returned to migrate towards the *scratch* center from the 3rd day. Corroborating with our results, Rouabhia et al. [58] reported that dermal fibroblasts when electrically stimulated (50 and 200 mV/mm for up to 6 h) showed a retractable behavior of fibroblasts in a collagen gel matrix. Our study has a translational relevance since research on understanding the molecular mechanisms by which MC regulates cell response, can contribute to the development of protocols for this therapy's clinical use.

Conclusion

The present study demonstrated that the application of MC at an intensity of 10 µA/90 s modulated the expression of some important remodeling genes in a wound healing assay, differently of those results observed in a tendon healing model, and with apparently no involvement of the pathway *Tnmd–Tgfb1–Ctgf* activation on the 4th day. It is important to emphasize that in the in vitro experiments, there is no molecular signaling from other cell types that also respond to the effects of MC therapy, nor cellular interactions and therefore genes expression and protein turnover may be different. Thus, these variables should be considered in development of new protocols using MC, since the effects of the MC depend on the intensity/time of application, of the tissue or cells lineage and of period of the repair process analyzed. In conclusion, MC therapy at an intensity/time of 10 µA/90 s with 4 daily applications did not affect cell viability, stimulated fibroblast migration with the involvement of *Ctgf*, and reduced the *Tnc* and *Fmod* expression.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.The authors declare no conflict of interest.

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