

Electromagnetic Stimulation Combined with *Aloe vera* Increases Collagen Reorganization in Burn Repair

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Abstract: Objectives: Burns are shown as a clinical problem for their severity and multiple complications due to the time required to heal. Therapies that improve their healing are of great importance, especially for being minimally invasive, of low cost and best performance, all related to the speed and quality of healing. This study investigated the effects of the magnetic electro stimulator Haihuá CD9 isolated or in association with *Aloe vera* in rats skin burns. Methods: Experimental groups ($n = 30/\text{group}$) were: (C) Carbopol gel; (F) *A. vera*/Carbopol gel; (H) Haihuá+Carbopol gel; (H+F) Haihuá+A. *vera*/Carbopol gel. Samples were collected on the 7th, 14th, and 21st experimental days for structural and morphometric analysis, hydroxyproline and glycosaminoglycans quantification, zymography for MMP-2 and MMP-9 and Western Blotting for TGF- β 1, VEGF, Collagen I and III. Key findings: The expression of TGF- β 1 in H+F was increased on the 7th day and of MMP-9 on the 7th and 14th days. The expression of VEGF increased in the first experimental periods and decreased in the last for the treated groups. There was an increase in the fibroblasts and birefringent collagen fibers in groups treated with Haihuá isolated or in association with *A. vera* in all periods. The quantification of collagen I increased, while collagen III decreased in H+F. The higher amount of GAGs and MMP-2 active isoform was detected in H and H+F during all periods. Conclusions: Considering the results of the present study, electromagnetic stimulation in association with the *A. vera* extract promoted an increase in the number of fibroblasts, GAGs content, MMP-2 activity, the deposition and organization of collagen fibers, favoring the repair of injuries to second degree burns, and may also present therapeutic potential in this injury type.

Key words: Burns repair, herbal medicine, electromagnetic stimulation, rats.

1. Introduction

Burns are shown as significant clinical problem for their severity and multiple complications due to the time required to heal [1]. Second-degree burns are characterized by an injury to the epidermis and part or deeper layers of the dermis [2]. Therapies that improve their healing are of a great importance, especially for being minimally invasive, of low cost and best performance related to the speed and quality of healing [3, 4].

The early phases in burn repairs are compromised including the formation of marked edema and in the reduction of angiogenesis. Wound repair involves interactions between different growth factors, cytokines, and the ECM (extracellular matrix) [5]. Among the growth factors in the healing process, which were found, the TGF- β 1 (Transforming Growth Factor Beta 1), is important in the inflammatory process, and also the VEGF (Endothelial Growth Factor) is fundamental for angiogenesis [6]. The collagen deposition and other components of the ECM are essential for the repair damaged tissue too [7]. In the early phase of this process an increase occurs in the synthesis of type III collagen which is gradually

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replaced by the collagen I deposition [8]. Among the many essential proteins for tissue repair there are the MMPs (metalloproteinases) that affect the degradation and modulate the ECM [9], the GAGs (glycosaminoglycans) important in the proliferation, migration and differentiation cell [10] and the hydroxyproline involved in the collagen turnover [11].

Facing complications observed in burns and ulcers, several therapeutic approaches are used to achieve an acceptable outcome with a treatment, including the use of medicinal herbs and electromagnetic stimulation [4, 12, 13]. Herbal medicines have shown great potential in the treatment of burns [14] with great antimicrobial and anti-inflammatory potential [4, 15-17]. *Aloe vera* has a healing action that occurs because it maintains the moisture of the wound, stimulates cell migration and proliferation, maturation of collagen and reduces the inflammatory process. Its effects occur by the synergistic action among the various active components that act on the tissue during the new epithelium formation [18-20]. Among its components that have beneficial effects on the repair it can be mentioned the acemannan C-glycosyl chromona, bradikininase, salicylic acid, anthroquinones, tannins, carbohydrates, enzymes, minerals, lipids, amino acids, proteins, vitamins, among others [21-23]. Many studies attribute to *A. vera* the most varied applications in folk medicine because of its great therapeutic potential and bioactivity, especially with regarding its anti-inflammatory, immunomodulating, antioxidant, antiviral, anticancer and healing actions [23-25]. There are also evidences of the antiseptic, antifungal and antibacterial character of the species [26]. Studies suggest that electromagnetic fields produce important effects on a large number of biological processes contributing significantly to tissue repair [27-29]. It is important to note that the effects of electro stimulation magnetic properties depend on the tissue characteristics and its main actions are the deviation of particles with electric charges in motion, with induced currents production, having a normalizing effect on the

membrane potential, diversifying the permeability of different ionic channels constituting a powerful metabolic stimulator of cells, tissues and organs because they conserve the normal electrochemical gradient of the cells, a necessary condition for the production of ATP [30]. It presents anti-inflammatory and analgesic action in the nerve endings, increasing the solubility of the oxygen by the blood [31, 32]. These accelerate wound repair and perform an important role in the mechanisms that determine the migration, adhesion and differentiation cell [33, 34]. It also presents anti-inflammatory, pro-angiogenic and collagenic action [35], constituting non-invasive therapy and of excellent money value [27, 34, 36].

The application of electrical stimulation in association with *A. vera* gel can accelerate healing and reduce inflammation, favoring the penetration of actives into the tissue [37]. Therefore, the purpose of the present study is to investigate the effects of electromagnetic stimulation in burns treated with *A. vera*, considering that this association might be a new therapeutic protocol for skin repair.

2. Material and Methods

2.1 Plant Material

The leaves of *Aloe vera* (L.) Burm. f. (Xanthorrhoeaceae) were collected in the garden located in Araras, São Paulo, Brazil, in geographical coordinates, latitude 22°20'55" S and longitude 47°24'05" W and identified by Dr. Fernanda O. G. Gaspi, according to the exsicata No. UEC183049 deposited in the Herbarium of Campinas University (UEC-Unicamp).

After collection, the leaves were selected and cleaned. To obtain the *A. vera* gel, yellow exudate was removed by gravity, mucilage of its interior was separated and subjected to homogenization in a blender, followed by centrifugation for the removal of the fibers. The obtained gel was incorporated into the carbopol with the ratio of (1:1) under stirring until a complete homogenization [38].

2.2 Animals

All surgical and experimental procedures used in this study were conducted according to the experimental requirements and biodiversity rights of the National Institute of Health for the Care and Use of Laboratory Animals (NIH Publication 80-23, reviewed in 1996). Studies have been done in accordance with the rules established by Arouca Law and approved by the Ethics Committee on Animal Use (CEUA) Hermínio Ometto University Center—UNIARARAS, under number 075/2014.

One hundred and twenty male Wistar rats (*Rattus norvegicus albinus*), aged 120 days and weighing on average 300 g, were obtained from the Center of Animal Experimentation, Hermínio Ometto University Center (UNIARARAS) (Araras, São Paulo, Brazil). The animals were housed in individual polycarbonate cages throughout the experimental procedure at a constant temperature (23 ± 2 °C) and humidity (55%) under a 12/12-h light/dark cycle. The animals had free access to standard chow and drinking water.

2.3 Experimental Groups

The animals were randomly divided into four groups of 30 animals each: (C) treated with Carbopol gel (1 mg); (F) treated with *A. vera* in carbopol gel (1 mg); (H) treated with magnetic electro stimulator Haihuá CD9 and carbopol gel (1 mg); (H+F) treated with magnetic electro stimulator Haihuá CD9 and *A. vera* in carbopol gel (1 mg).

After an overdose of the anesthetic (xylazine hydrochloride and ketamine hydrochloride) wound samples were collected from ten animals of each group on the 7th, 14th, and 21st days of the experimental period for structural and morphometric analysis ($n = 5$) and for analysis of protein expression by Western blotting, quantitative analysis of glycosaminoglycans, hydroxyproline and zymography to metalloproteinases ($n = 5$).

The magnetic electro stimulator Haihuá CD9 has as specifications: pulsed output waveform audio

frequency (approximately sinusoidal); Output frequency: 500-8,000 Hz; maximum output voltage: 80 V, 40 mA; load resistance value: 1,000 ohms; output power: 2.6 w; dimensions: 170 × 120 × 70 mm (length × width × height); weight: 650 g; working temperature: -10 °C to 40 °C; Humidity: < or = 80%; magnetic intensity: > 0.2 T (± 256 G). The electrical power can reach three levels depending on the intensity of the current user and is indicated by the number of illuminated indicators. The electric power intensity can be: small—illuminated indicators 1 or 2; average—lighted indicators 3 to 4; and large—lighted indicators 5 or more; origin: China [39].

For electromagnetic stimulation, two 1 cm diameter electrodes were used, in the output frequency of 500 Hz, applying perpendicular angle to the edge of the injury, for 2 min, with small power intensity (illuminated indicator 1). The electrodes were coated with sterile gauze and immersed in saline. The application of *A. vera* in carbopol gel was carried out with the aid of a *Swab* on the edge of the injury after the application of the electromagnetic stimulation. This protocol was developed by our research group.

2.4 Experimental Procedures

After anesthesia by an intraperitoneal administration of xylazine hydrochloride (1.0 mL/kg) and ketamine hydrochloride (3.0 mL/kg), their backs were shaved. Then, burn injuries were induced on the dorsal skin of all animals with a 2 cm diameter aluminum plate that is an apparatus used to maintain a constant temperature (120 °C). This diameter was chosen in proportion to the dorsal area of the animal. The plate was pressed on the skin for 20 s to induce second-degree burns. To ensure the same pattern of burns in all animals, we used a graduated support rod for sustaining the aluminum plate with the same pressure on the dorsal skin of the animals. This experimental protocol to induce second-degree burn was previously developed by our research group through a pilot study with histological evidence of injury on the epidermis and dermis [2].

After the burn induction, the animals received analgesics: sodium dipyrone, one drop in the postoperative, after 12 and 24 hours. The respective treatments were initiated 24 h after experimental injury. For this purpose, the animals were immobilized without sedation and occurred every other day, three times a week, at the same time for 21 days.

2.4.1 Structural and Morphometric Analysis

The intervals of sampling were selected because they are related to the different phases of tissue repair to be analyzed (on the 7th, 14th, and 21st days that correspond to inflammatory, proliferative and remodeling phases). For this purpose, an area measuring 25 mm in diameter was delimited in the center of the wound to obtain standardized samples. Tissue samples with 10 mm in depth were removed with scalpel use and fixed for structural and morphometric analysis.

The tissue fragments collected were fixed in 10% formaldehyde in Millonig buffer, pH 7.4, for 24 h at room temperature. After this period, the specimens were washed in buffer and submitted to routine procedures for embedding in Paraplast™ (Histosec®, Merck).

Longitudinal sections of the parts with 6 mm thick were treated with the following techniques: Picrossirius-hematoxylin, viewed in common optical microscope to observe the organization of the collagen fibers; TM (Trichrome Masson), to quantify the content of collagen fibers in the repair area (% of total area); AT (toluidine blue) in McIlvaine buffer at pH 4.0 to the epidermis and dermis structural analysis and measurement of the number of fibroblast cells and blood vessels; Dominici, for the quantification of inflammatory infiltrate. The evaluation of the organization and maturation of collagen fibers were carried out by measuring the birefringence of the area to the total area by the method of Picrossirius-hematoxylin under polarized light [2]. Using Masson Trichrome staining, the fields were separated by using the color distribution as a parameter

for discrimination. The color range is the same for each quantized image being defined at the beginning of the analysis. The color band was adjusted until the moment that the representative areas of collagen got separated in the image. The same range was used to identify the collagen fibers in all fields scanning. Subsequently, the percentage was calculated for each field [40]. The images of the sections taken from the central region of the experimental injuries were captured in Light Microscope Leica DM 2000, housed in the Laboratory Micromorphology/Herminio Ometto University—UNIRARAS.

From the captured images of five animals from each group, five samples of $10^4 \mu\text{m}^2$ by cutting the central region of the injuries on the 7th, 14th, and 21st days of treatment were obtained. They were analyzed by using the virtual software Leica Image Measure™ grid and Sigma Scan Pro. 6.0™ for the determination of the following morphometric parameters: total fibroblasts and inflammatory infiltrate number ($n/10^4 \mu\text{m}^2$), the number of blood vessels newly formed ($n/10^4 \mu\text{m}^2$), quantification of birefringent collagen fibers in the repair area (%).

2.4.2 Western Blotting

For the protein expression analysis by Western blotting, wound samples were collected from the injury region of five animals per group on 7th, 14th, and 21st days of treatment after their euthanasia. For protein extraction, the samples obtained were chopped and homogenized with the aid of Polytron (PTA 20S model PT 10/35; Brinkmann Instruments, Westbury, NY, USA) operated at maximum speed for 40 s in buffer (EDTA 10 mM, trizma base 100 mM, sodium pyrophosphate 10 mM, sodium fluoride 100 mM, sodium orthovanadate 100 mM, PMSF 2 mM, aprotinin 0.1 mg/mL, deionized water; Sigma Chemical Co., St. Louis, MO, USA). The extract was centrifuged at 12,000 rpm for 20 min at 4 °C for the insoluble material removal. The supernatant was collected for the protein concentration measurement in the samples by the Biuret method (Portal colorimetric

method, Laborlab, São Paulo, Brazil). Aliquots of the supernatant were treated with Laemmli buffer containing 100 mM DTT (Sigma Chemical Co., St. Louis, MO, USA).

Samples containing 150 µg proteins were boiled for 5 min and submitted to electrophoresis gel 10% (VEGF—40 KDa) and 12% (TGF-β1—25 KDa) SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) in a mini-gel apparatus (Mini-Protean®, Bio-Rad-Richmond, CA, USA). Next, the protein bands were transferred from the gel to a nitrocellulose membrane (Hybond ECL, 0.45 µm) [41]. The membranes were washed in the basal solution (1 M trizma base, 5 M NaCl, Tween 20 a 0.005%, and deionized water) and incubated in blocking solution (basal solution plus 5% Molico® skim milk) for 2 h to reduce nonspecific protein binding. After washing with basal solution, the membranes were incubated overnight at 4 °C with specific antibodies (diluted 1:200): anti-TGF-β1, anti-VEGF (Santa Cruz Biotechnology, USA), anti-collagen type I and anti-collagen type III (Sigma, Saint Louis, Missouri, USA). Next, the membranes were incubated with the secondary goat anti-mouse IgG1: HRP antibody (diluted 1:1,000, Santa Cruz Biotechnology, USA) for 2 h at room temperature. The reaction was developed using a chemiluminescence kit (SuperSignal® West Pico Chemiluminescent Substrate 34080, Thermochemical, Rockford, USA). The membranes were exposed in Syngene G: Box and the intensity of the bands was evaluated by densitometry using the Scion Image 4.0.3.2 software (Scion Co., USA). The densitometry values of TGF-β1, VEGF, collagen type I and type III signals are expressed relative to proteins stained with Ponceau S, which were taken as 100% [42].

2.4.3 Quantitative Analysis of Glycosaminoglycans (GAGs)

The determination of total GAGs content (mg/g dry tissue) samples ($n = 5$ /experimental time) was determined from the release of polysaccharides by

papain digestion (40 mg/g of papain for 1 g of tissue, in sodium phosphate buffer 100 mM pH 6.5 containing 40 mM EDTA and 80 mM β-Mercaptoethanol) at 50 °C for 24 h. The digest was centrifuged at 10,000 rpm and two volumes of ethanol were added to the supernatant. After 24 h at 4 °C, the precipitate was collected by centrifugation at 8,000 rpm, washed with 80% ethanol and acetone and so dried in an oven. The samples were diluted in water. The quantification of GAGs was done through the DMMB (Dimethylmethylene Blue) method [43, 44]. The reading was performed under visible spectrophotometer light at 526 nm.

2.4.4 Quantification of Hydroxyproline

Fragments of tissue ($n = 5$ /time experimental) were immersed in acetone for 48 h and then in chloroform: ethanol (2:1) for 48 h. The samples were hydrolyzed in HCl 6 N (1 mL for each 10 mg of tissue) for 16 h at 110 °C. The hydrolyzate was neutralized with NaOH 6 N and the quantification HO-Pro was performed according to the method described by Stegemann and Stalder [45] and Jorge et al. [46] with some modifications. Hydroxyproline concentrations between 0.2 and 6 µg/mL were used for standard curve and the reading was performed under visible spectrophotometer light at 550 nm.

2.4.5 Zymography to Metalloproteinases

Samples from animals of each experimental group were cut into pieces and the segments were immersed in a solution of 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 10 mM CaCl₂, 0.1% Triton, and 1% protease inhibitor cocktail (Sigma) for protein extraction (100 µL of extraction solution for each 30 µg of tissue) at 4 °C during 2 h. After this first extraction, the samples were incubated adding one-third volume of the same solution described previously, at 60 °C during 5 min. The supernatant from each sample (40 µg protein) was used for the analysis of MMP-2 and MMP-9 activity. The samples were analyzed by electrophoresis in polyacrylamide gel through in 10% containing 0.1% gelatin and held at 4 °C. After the electrophoresis, the gels were washed with 2.5% Triton X-100 and

incubated for 21 h in a solution of 50 mM Tris-HCl (pH 7.4), NaCl 0.1 M, and 0.03% sodium azide at 37 °C. The gels were stained with Coomassie blue R-250 for 1 h to observe the protein bands to negative corresponding to gelatinolytic activity [47]. The gels were washed with a solution containing 30% methanol and 10% acetic acid for observation of the negative bands of protein corresponding to the gelatinolytic activity. Moreover, as a positive control, EDTA 20 mM was used in incubation buffer to inhibit the activity of metalloproteinases. The intensity of the bands from different isoforms, for each group, was determined by densitometry using Alpha 4.0.3.2 software (Scion Corporation, USA).

2.5 Statistical Analysis

The results of the morphometric analysis, western blotting and quantification of hydroxyproline and GAGs were reported by mean and standard deviation ($X \pm SD$) and the values were compared by Two-way ANOVA and Tukey's post-test ($p < 0.05$) using software version 3.0 GraphPad Prism®.

3. Results

3.1. Structural Analysis

Analyses were performed on samples collected from the injury area of the animals in the C group, which presented newly granulation tissue on the 14th day (Fig. 1). In this period, the structural characteristics of the repair tissue indicated a transition between the proliferative and reorganization phases and fibroblasts were presented at the edge of the injury. It was also detected in the period between the 14th and 21st days, areas of intense fibroblasts proliferation, especially in H and H+F groups (Fig. 1). There were no bleeding or edema areas observed in any different experimental times.

Deposition and organization of collagen fibers in the repair area showed increasing levels of deposition, compaction and reorganization during all periods (Fig. 1). Re-epithelialization is observed from the 14th

day and the new layers of cells originating from the basal layer of the injury edge gradually occupied the entire surface of the injury until they covered it entirely on the 21st day, especially in groups H and H+F. Samples from F group showed similar structural characteristics to C group.

The H+F and H groups presented on the 14th day a great deposition of granulation tissue and collagen fibers, which presented to be arranged in an advanced degree of compaction (Fig. 1).

3.2. Morphometric Analysis

The amount of inflammatory infiltrate was reduced gradually in the groups during all periods and no difference among them was observed. There were also no differences observed in the quantification of newly formed vessels among any groups or periods.

Quantitative analysis of fibroblasts was higher in H and H+F ($p = 0.046$) compared to the other groups in all periods (Table 1) and the percentage of birefringent collagen fibers showed increasing values between the 7th and 21st days, and the same groups showed higher values than C and F groups on the 21st day ($p = 0.048$).

3.3. Quantitative Analysis of GAGs and Hydroxyproline

A gradual increase in the GAGs quantification was observed in all groups during the experimental period. On the 14th day, the H and H+F groups showed higher values compared to the other groups ($p = 0.046$) (Table 2).

The hydroxyproline content decreased during the analyzed period among the groups, but no significant differences were observed (Table 2).

3.4. Zymography to Metalloproteinase

The latent MMP-9 isoform was observed in all groups and periods. Higher values for this isoform occurred in the samples of F and H+F groups on the 7th day ($p = 0.045$) and in the F, H, and H+F groups on the 14th day ($p = 0.047$). The MMP-9 active isoform was detected in all groups and periods, with the exception

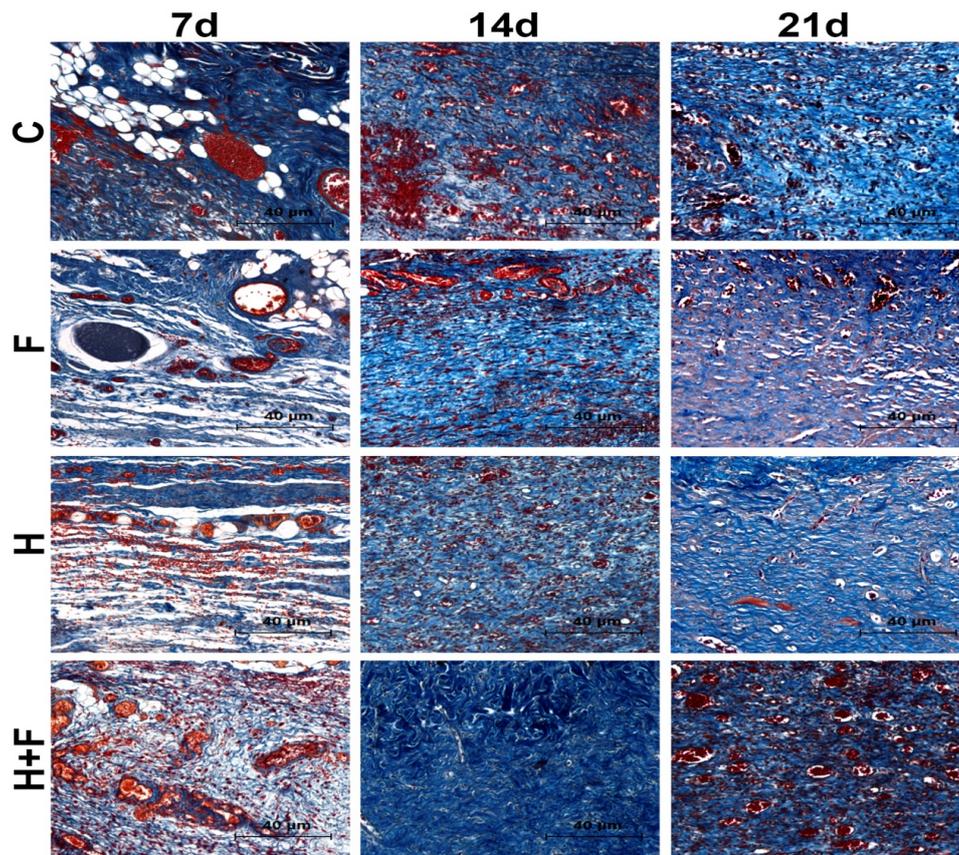


Fig. 1 Cross sections of the wound area of second-degree burns from C, F, H and H+F groups 7 (7d), 14 (14d) and 21 (21d) days after injury.

The sections were stained with Masson's Trichrome. Bar = 150 µm.

Table 1 Morphometric parameters evaluated in the wound healing process in C, F, H and H+F groups, 7 (7d), 14 (14d) and 21 (21d) days after second-degree burns.

Experimental periods		7d	14d	21d
Parameters	Groups			
Number of inflammatory infiltrate (n/10 ⁴ µm ²)	C	22.6 ± 3.2	15.8 ± 2.4	6.9 ± 1.4
	F	19.8 ± 2.7	14.7 ± 1.9	5.8 ± 1.7
	H	20.1 ± 1.9	14.4 ± 2.1	5.7 ± 2.1
	H+F	21.2 ± 2.1	13.5 ± 2.8	6.2 ± 1.5
Number of vessels (n/10 ⁴ µm ²)	C	1.4 ± 0.5	1.9 ± 0.8	2.7 ± 0.7
	F	1.5 ± 0.6	2.2 ± 0.6	2.9 ± 0.6
	H	1.4 ± 0.2	2.5 ± 0.7	3.4 ± 0.5
	H+F	1.2 ± 0.4	2.4 ± 0.4	3.2 ± 0.8
Number of fibroblasts (n/10 ⁴ µm ²)	C	15.2 ± 4.4	24.8 ± 3.4	28.4 ± 4.2
	F	14.8 ± 2.9	22.8 ± 4.1	27.9 ± 1.8
	H	23.7 ± 3.6*	34.1 ± 2.8*	38.4 ± 4.8*
	H+F	24.9 ± 4.1*	39.2 ± 4.7*	40.4 ± 4.1*
Collagen Fibers birefringents (% of area)	C	21.7 ± 4.5	38.9 ± 6.4	58.6 ± 5.5
	F	22.5 ± 3.9	41.1 ± 8.2	61.7 ± 5.7
	H	23.1 ± 4.9	46.7 ± 5.9	75.6 ± 6.5*
	H+F	22.9 ± 3.6	47.8 ± 7.1	74.9 ± 5.1*

Values are the mean and standard deviation of each group and were compared by Two-way ANOVA with Tukey's post-test ($p < 0.05$).

(*) significant differences $p < 0.05$.

Table 2 Biochemical parameters evaluated in the wound healing process in C, F, H and H+F groups, 7 (7d), 14 (14d) and 21 (21d) days after second-degree burns.

Experimental periods		7d	14d	21d
Parameters	Groups			
Glycosaminoglycans ($\mu\text{g}/\text{mg}$ dry tissue)	C	0.43 \pm 0.05	0.63 \pm 0.12	0.82 \pm 0.18
	F	0.49 \pm 0.06	0.61 \pm 0.11	0.96 \pm 0.17
	H	0.35 \pm 0.02	0.95 \pm 0.13*	0.98 \pm 0.15
	H+F	0.38 \pm 0.07	0.79 \pm 0.18*	1.12 \pm 0.21
Hydroxyproline ($\mu\text{g}/\text{mg}$ dry tissue)	C	128.6 \pm 10.2	92.1 \pm 8.9	98.6 \pm 11.3
	F	121.6 \pm 17.6	86.2 \pm 9.7	83.9 \pm 13.4
	H	92.1 \pm 19.3	87.1 \pm 16.5	93.1 \pm 17.4
	H+F	108.9 \pm 18.9	87.9 \pm 10.3	98.4 \pm 9.8

Values are the mean and standard deviation of each group and were compared by Two-way ANOVA with Tukey's post-test ($p < 0.05$).

(*) significant differences $p < 0.05$.

of C group on the 21st day. Higher values were observed for this isoform in injuries of F and H+F groups on the 7th day ($p = 0.040$) and on the 14th day in all treated groups ($p = 0.043$). On the 21st experimental day, there was a reduction of active MMP-9 activity in all experimental groups (Fig. 2).

With relation to the MMP-2, similar results were observed for the intermediate and active isoforms in all groups and periods. Higher values were observed for the intermediate isoform of MMP-2 in the F group only on the 7th day ($p = 0.044$) and in the H and H+F groups in all periods (7d: $p = 0.044$; 14d: $p = 0.046$; 21d: $p = 0.043$). For the active isoform of MMP-2, higher values were also detected in the H and H+F groups (7d: $p = 0.042$; 14d: $p = 0.039$; 21d: $p = 0.041$ Fig. 3).

3.5 Western Blotting

An increase in the TGF- β 1 expression was observed in the H+F group compared to the control group on the 7th day after the injury. On the 21st day, a marked reduction was detected in the samples treated with *A. vera* (F) (Fig. 4).

Regarding the VEGF expression, it was observed an increase of this protein on the 7th and 14th days in all treated groups. On the 21st day, there was a significant decrease especially in H and F groups (Fig. 4).

The type I collagen analysis showed a gradual increase in all experimental groups during the analyzed period, with significant emphasis on the 7th and 14th

experimental days in the H+F group (Fig. 4). In Type III collagen analysis, it was observed a gradual decrease in the deposition of this collagen in the samples of the H+F group throughout the period, with significant emphasis on the 14th and 21st days (Fig. 4).

4. Discussion

Electromagnetic fields are used in therapy in various biological processes [48] and the interest in its clinical application increases for its effects on migration, adhesion and differentiation cell, with great importance in the repair of different injuries and diseases [29, 49]. Medicinal plants are also important therapeutic resources, and *A. vera* is traditionally used for medicinal purposes in many cultures [50]. In this study the *A. vera* pulp was incorporated into the carbopol gel, since it is commonly used as a vehicle of different herbal medicines, not interfering with its properties, nor with the active ingredients of the crude extract used. The gel is suitable because it has a solubility, bioadhesive properties and compatibility with many excipients [51, 52]. The morphometric analysis of inflammatory infiltrate in the present study showed no difference among the groups. However, the treatment with *A. vera* decreased the TGF- β 1 expression in the last experimental period, indicating a positive response to this treatment in the control of inflammation. It is known that the TGF- β 1 initiates the inflammatory phase during the tissue repair [53, 54], and its reduction,

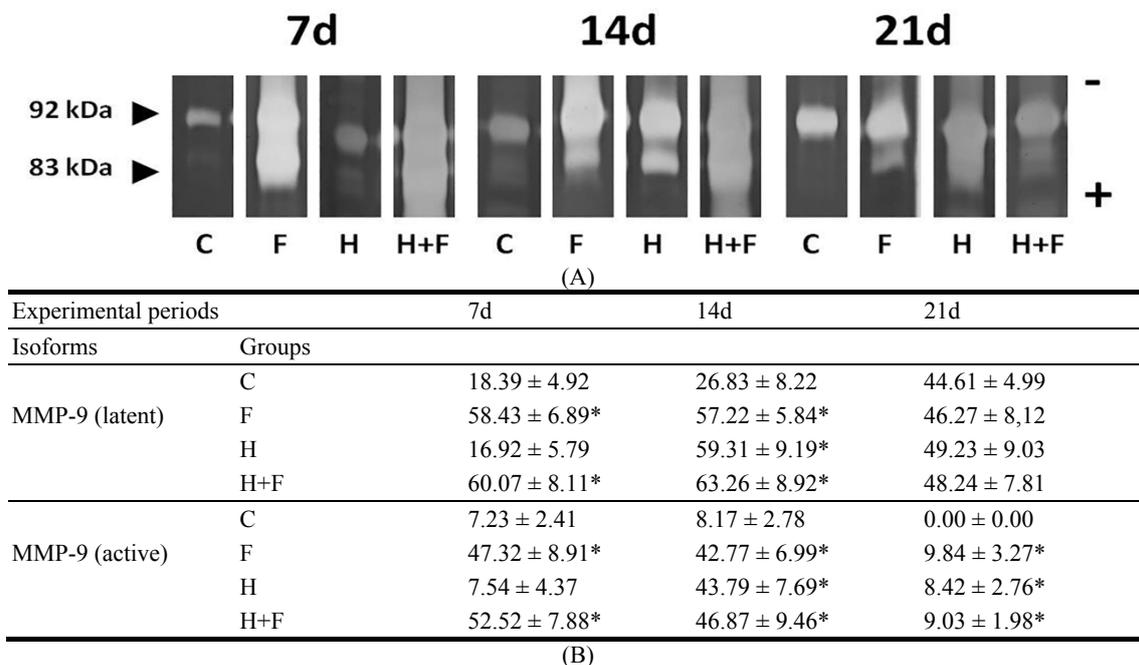


Fig. 2 (A) Zymogram for analyzing the isoforms latent (92 kDa) and active (83 kDa) of MMP-9 in C, F, H and H+F groups 7 (7d), 14 (14d) and 21 (21d) days after injury; (B) Densitometry of the bands (pixels) corresponding the respective isoforms in the injury area.

Values are the mean and standard deviation of each group and were compared by Two-way NOVA with Tukey's post-test ($p < 0.05$). (*) significant differences $p < 0.05$.

indicated a decrease in inflammation with *A. vera* treatment. The literature suggests that the magnesium lactate present in *A. vera* is responsible for the reduction in histamine release in inflammatory response, and this appears to occur through the action of inhibition of arachidonic acid, which interferes with the production of prostaglandins [55]. Davis et al. [20] indicated the mannose-6-phosphate present in this phytotherapy to be responsible for accelerating the process of wound healing in mice. Furthermore, the healing action of this phytotherapy is also attributed to the action of tannin, known for its anti-inflammatory effects, which promotes wound contraction [16, 56].

The electromagnetic fields effect has been widely investigated *in vivo* and *in vitro* on different cell types and animal models involved in tissue repair [57]. The electromagnetic stimulation affects the synthesis and activation of growth factors and cytokines and regulates the gene expression of cytokine by calcium flux modulation [58]. Our results suggest that the treatment with *A. vera* extract and in association with

electromagnetic stimulation favored the inflammation process, with the consequent MMP-9 release. This enzyme is especially enhanced in the early stages of tissue repair, considering that MMP-9 is secreted by macrophages and neutrophils [57-59].

In the first experimental periods, a higher expression of VEGF was observed in all treated groups and decreased in the last in the treated groups, showing the effectiveness of treatments in the stimulation of angiogenesis. VEGF is released during hypoxia [60, 61] and its decreased expression on the 21st day in the H and F groups suggests the effectiveness of both treatments in the formation of new vessels. This growth factor has pro-angiogenic activity and promotes the growth of endothelial cells derived from arteries and veins, contributing to the formation of new blood vessels and increasing vascular permeability [62]. It was established by Moon et al. [63] that the *A. vera* gel induces angiogenesis by the presence of an active compound known as β -sitosterol, and thus contributes to healing. On the other hand, electromagnetic fields are

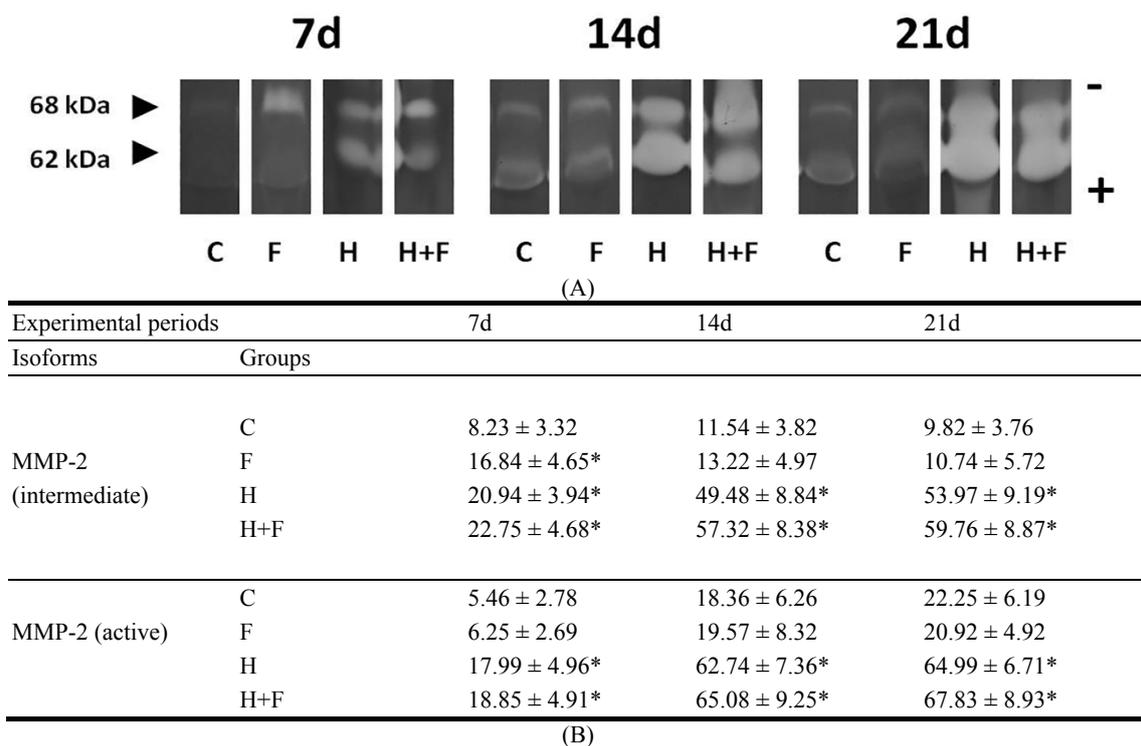


Fig. 3 (A) Zymogram for analyzing the intermediate isoforms (68 kDa) and active (62 kDa) of MMP-2 in C, F, H and H+F groups 7 (7d), 14 (14d) and 21 (21d) days after injury; (B) Densitometry of the bands (pixels) corresponding the respective isoforms in the injury area.

Values are the mean and standard deviations of each group were compared by Two-way ANOVA with Tukey’s post-test ($p < 0.05$). (*) significant differences $p < 0.05$.

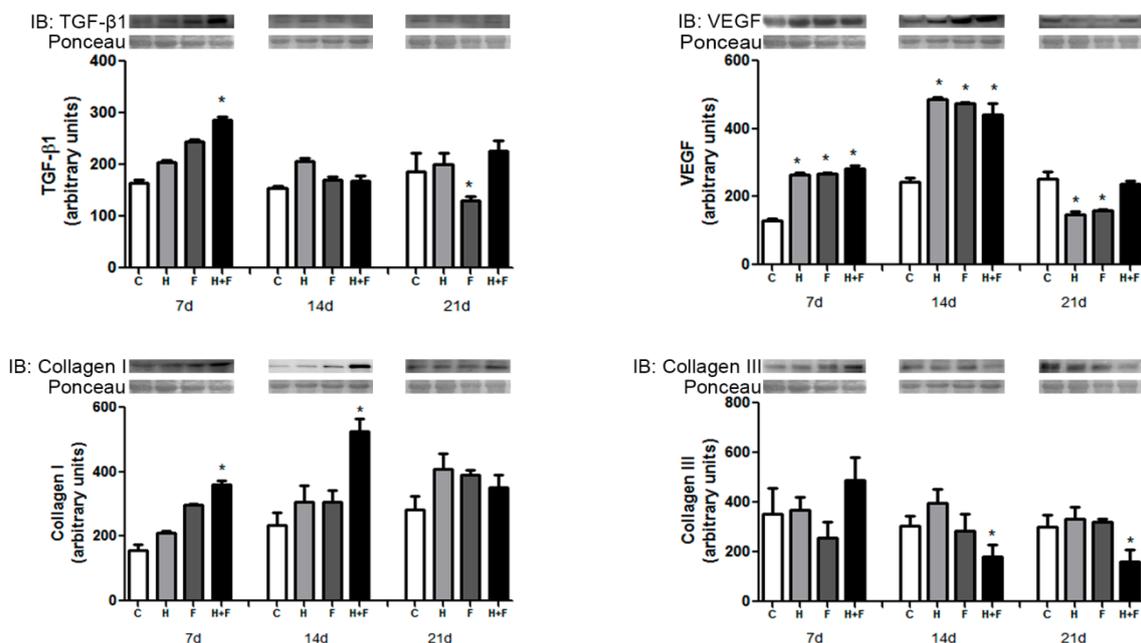


Fig. 4 Immunoblotting analysis of the expression of TGFβ-1, VEGF, collagen I and Collagen III in the wound healing process in C, F, H and H+F groups 7 (7d), 14 (14d) and 21 (21d) days after injury.

Typical blots are shown above average densitometry results.

Values are the mean and standard deviations of each group were compared by Two-way ANOVA with Tukey’s post-test ($p < 0.05$). (*) significant differences $p < 0.05$.

also beneficial for the formation of new blood vessels [27, 29]. Mendonça et al. [37] obtained similar results when associated gel of *A. vera* and microcurrent on the healing of surgical injury in rats.

In the present study, the morphometric analysis showed a gradual increase in the number of fibroblasts and birefringent collagen fibers in the groups treated only with electromagnetic stimulation and in association with *A. vera* extract. The main function of fibroblasts is the maintenance of the structural integrity of connective tissues, starting the synthesis and secretion of ECM components such as glycosaminoglycans and collagen fibers types I and III [10, 11]. Fibroblasts are directly involved in the wound healing process being the principal cell type that establishes the collagen matrix at the wound site [64]. It is important to mention that an increase of GAGs observed in the H and H+F groups on the 14th day, indicates its participation in the collagen fibers alignment [65].

Choi et al. [66] evaluated the effects of electromagnetic stimulation in excisional wounds on the back of rats and found that this treatment significantly increased the number of collagen fibers, as well as the organization of collagen type I in the early stages of repair. Our results showed that during the study period a gradual increase occurred in the expression of collagen type I and a gradual decrease to collagen type III in the groups treated with *A. vera* extract in association to electromagnetic stimulation demonstrating the beneficial effects of this treatment in the fiber collagen reorganization. This increase in the collagen type I reflected both in the greater organization of the collagen fibers, as well as the formation of thicker fibers compared to the type III collagen fibrils [67].

Another factor related to a greater tissue organization in H and H+F groups was a higher amount of active MMP-2 during all periods. It is known that MMP-2 participates in the collagen fibers reorganization [47, 68] and it is possible to relate

the higher MMP-2 activity with greater tissue organization in these groups. It is worth mentioning that the greater occurrence of birefringent areas and the increase of type I collagen in the H and H+F groups, corroborate with the gradual decrease of hydroxyproline during all periods, possibly indicating the lesser amount of degraded collagen and greater fiber formation in the injury region [69]. Thus, electromagnetic stimulation and its association with *A. vera* extract were effective in the collagen deposition and organization.

5. Conclusions

The results of this study showed that electromagnetic stimulation in association with *A. vera* extract increased deposition and organization of collagen fibers, GAGs content and MMP-2 activity, favoring the repair of injuries to second-degree burns. The beneficial effects obtained in this experimental model by the combination of these therapies may suggest a synergistic action among them, with complementation of their biological effects. The physical agent (magnetic electro stimulation) activates specific signaling pathways that provide control of inflammation, angiogenesis and collagenase, but also acts as a supporting facilitating the activation of other pathways, or enhancing them, by the phytochemical agent (*A. vera* extract). Furthermore, more studies should be performed in order to elucidate the molecular mechanisms involved in the use of these techniques.

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

Acknowledgments

We acknowledge Hermínio Ometto Foundation (FHO|UNIARARAS) for supporting this work.

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