Trends in Wound Repair: Cellular and Molecular Basis of Regenerative Therapy Using Electromagnetic Fields

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Abstract: Chronic ulceration of the leg represents a major, underestimated problem of modern health care, involving physical and cosmetic impairment and social stigma along with high community costs for patients' treatment. The increasing prevalence of chronic ulcers, currently reported to be as much as 0.3% in the general population, should stimulate identification of more efficacious therapeutic approaches to achieve complete healing. The strategies of regenerative medicine based on small molecules, biomimetic scaffolds, gene or cell therapy, and electromagnetic field manipulation represent some of the modern therapeutic alternatives for wound healing. Here we review in an integrated, interdisciplinary approach the modern cellular and molecular mechanistic concepts regarding the involvement of extremely low frequency electromagnetic fields (ELF-EMF) in the complex process of tissue repair, with particular focus on chronic wounds. The data analysis supports three main effects of electromagnetic fields on the wound healing pathways: 1) an anti-inflammatory effect, by modulation of cytokine profile that induces the transition of the healing process from a chronic pro-inflammatory to an anti-inflammatory state; 2) a neo-angiogenic effect, by increased endothelial cells proliferation and tubulization and production of fibroblast growth factor (FGF)-2; and 3) a re-epithelialization effect, by stimulation of collagen formation. We believe that utilization of ELF-EMF in larger clinical trials designed to optimize these functional parameters would facilitate a better understanding of ELF-EMF-induced healing mechanisms and lead to improved therapeutic outcomes for this disabling condition which is often totally resistant to treatment.

Keywords: Cellular and molecular mechanisms, chronic ulcers, electromagnetic fields, wound healing.

INTRODUCTION

Disruption of the skin and the subsequent breakdown of the barrier protecting the body are mainly caused by primary tissue injuries that occur following physical, chemical, or thermal insult(s). The anatomic discontinuity of skin is restored by the orderly, integrated and dynamic process of wound healing controlled by multiple molecular and cellular functions that are activated and synchronized to re-establish the homeostasis, integrity, barrier function and tensile strength of the tissue [1].

Clinically, the discontinuity of the skin presented in the format of the open sore (skin ulcer) is most frequently located on the lower extremities and can be caused by a variety of events, such as trauma, exposure to heat or cold, impaired blood circulation, or irritation induced by exposure to corrosive materials. Chronic ulceration of the leg (and/or foot) (leg ulcers) represents a major, yet underestimated problem in modern health care, involving physical and cosmetic impairment and social stigma, along with high community costs for patients' treatment. Chronic ulceration of the lower extremities is facilitated by a range of pathological processes, most commonly identified as venous and/or arterial disease.

The most problematic types of ulcers (initially induced by conditions like neuropathy, ischemia, venous hypertension, pressure, diabetes, etc.) are those that reach the chronic stage and do not follow the natural repair cycle; these ulcers are not responsive to regular care, due to their entrapment in a self-sustaining cycle of chronic inflammation.

Tissue repair is characterized by increased cell proliferation, capillary budding, and the synthesis of extracellular matrix (ECM) to fill in the damaged tissue that has been cleared during inflammation. Regenerative medicine therapy is a new emerging concept in the field of wound healing that has been developing in recent years with the intention of shortening the tedious evolution of the epithelialization process [2]. Regenerative medicine therapy activates latent pathways that induce cellular neogenesis and recovery/restoration of the damaged tissue, rather than leading to eschar formation, a similar process to that described in injured fetal tissues [2]. Its various strategies, based on biomimetic scaffolds, manipulation

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of the electromagnetic environment, administration of small molecules, and use of gene- or cell-based approaches, have recently emerged as therapeutic alternatives [3]. Of these approaches, we will focus on ELF-EMF, which have been previously reported to be effective in healing of chronic wounds. We will discuss the recent concepts of wound repair mechanisms induced by ELF-EMF, supported by experimental evidence provided by clinical, *in vivo* and *in vitro* studies.

CONSIDERATIONS ABOUT ULCERS AND THEIR HEALING PROCESS

Venous ulcers are usually secondary to damaged valves of the veins and their presence leads to sustained venous hypertension (the inability of venous pressure to reach the normal level specific to walking). Arterial ulcers are secondary to atherosclerosis of large vessels. Some leg ulcers are caused by concomitant arterial and venous disease and are called mixed. Diabetic ulcers occur almost exclusively on the foot. They are secondary to either diabetic neuropathy or large vessel atherosclerosis and are characterized by loss of sensitivity. Neuropathic diabetic ulcers occur on pressure areas of the foot and toes and are considered pressure ulcers, while the atherosclerotic type is considered ischemic ulcer. Pressure ulcers, also known as decubitus ulcers or bedsores, are skin ulcers that develop on areas of the body where the blood supply has been reduced due to prolonged pressure. Other wound locations, more specific to post-traumatic or pressure ulcers, are the sacral and gluteal areas.

Underlying medical conditions such as diabetes or vascular diseases often lead to chronic wounds that further become recalcitrant and are therefore of major concern due to their resistance to the regular treatment procedures. The prevalence of leg ulcers varies widely among specific clinical populations, and with the geographic area(s) investigated, from 0.045 in the British population [4] to 0.305% in the Swedish population [5]; higher percentages (3-5%) have been reported in individuals over 65 years [6]. The prevalence of diabetes-related lower extremity chronic wounds varies from ~1%, as reported in studies focused on European and North American populations, to more than 11% in some populations from African countries [7]. In the United States, the reported incidence of pressure ulcers varies widely, from 2% to 40% [8-10].

Treatment of wounds can follow many strategies such as pressure-relieving beds, mattresses, cushions (for pressure sores) and compression (for ulcers of venous etiology). Other potential beneficial alternatives involve adequate debridement, topical antibiotics and inductors of epithelialization like silver preparations, systemic antibiotics (for infected lesions), and modern therapies like low level lasers, therapeutic ultrasound and electromagnetic therapy.

Wound healing is an orderly, integrated, dynamic process that occurs as a cellular response to injury and involves activation of skin cell components (platelets, macrophages, fibroblasts, endothelial cells, and keratinocytes) as outlined in Fig. (**1a**). Many growth factors and cytokines released by these cells coordinate and maintain the healing process [reviewed in 11]. When disruption of the skin appears, the body prevents blood loss and further exposure to pathogens by forming a fibrin-rich clot. Soon after, the growth factors and cytokines released from degranulating platelets act in tandem to attract inflammatory leukocytes that clean the wound of bacterial contamination and to further perform effector functions that orchestrate the healing process. The interplay between the factors involved in cellular migration, adhesion, proliferation, cell matrix interaction and signaling by direct cell-cell contact is the key for guided tissue reorganization and its architecture restoration after an injury. The phases involved in the wound healing cascade - inflammatory, proliferative and remodeling - are partially overlapping as depicted in Fig. (**1b**). In the inflammatory phase, infiltrating monocytes differentiate into macrophages which ingest and remove harmful debris. In the proliferative phase, the growth factors released by macrophages initiate the cellular response, through the involvement of endothelial cells, fibroblasts, and epidermal keratinocytes. In this phase, the rebuilding of viable and functional tissue is supported by an active process of neovascularization, together with an increased activity of epithelial and mesenchymal cells. The remodeling phase of wound healing is coordinated by fibroblasts, which produce and deposit the ECM (collagen and elastin).

Analysis of the chronic wound microenvironment revealed many physiological differences compared to a normally healing wound: prolonged inflammation, an imbalance of regulatory growth factors and cytokines, defective ECM (that loses the capacity to support keratinocyte migration), modified fibroblast function and defective capillary function (inducing inadequate tissue oxygenation), all of which lead to failure of re-epithelialization [12]. In chronic wounds, the normal course of healing is arrested at the inflammatory stage. Subsequently, other abnormal features such as accumulation of devitalized tissue, decreased angio-genesis, increased level of proteases, overproduction of hyperkeratotic tissue, secretion of cellular exudate, and presence of infection at the outer surface prevent adequate cellular response of chronic ulcers to wound-healing stimuli [13].

ELF-EMF - MAIN ASPECTS

Electromagnetic fields (EMF) have an electric and a magnetic field component. The electric field is induced by the presence of charged particles (electrons) and the magnetic field is induced by the movement of charged particles (electron current). Currently, the biological effects of electromagnetic fields of low

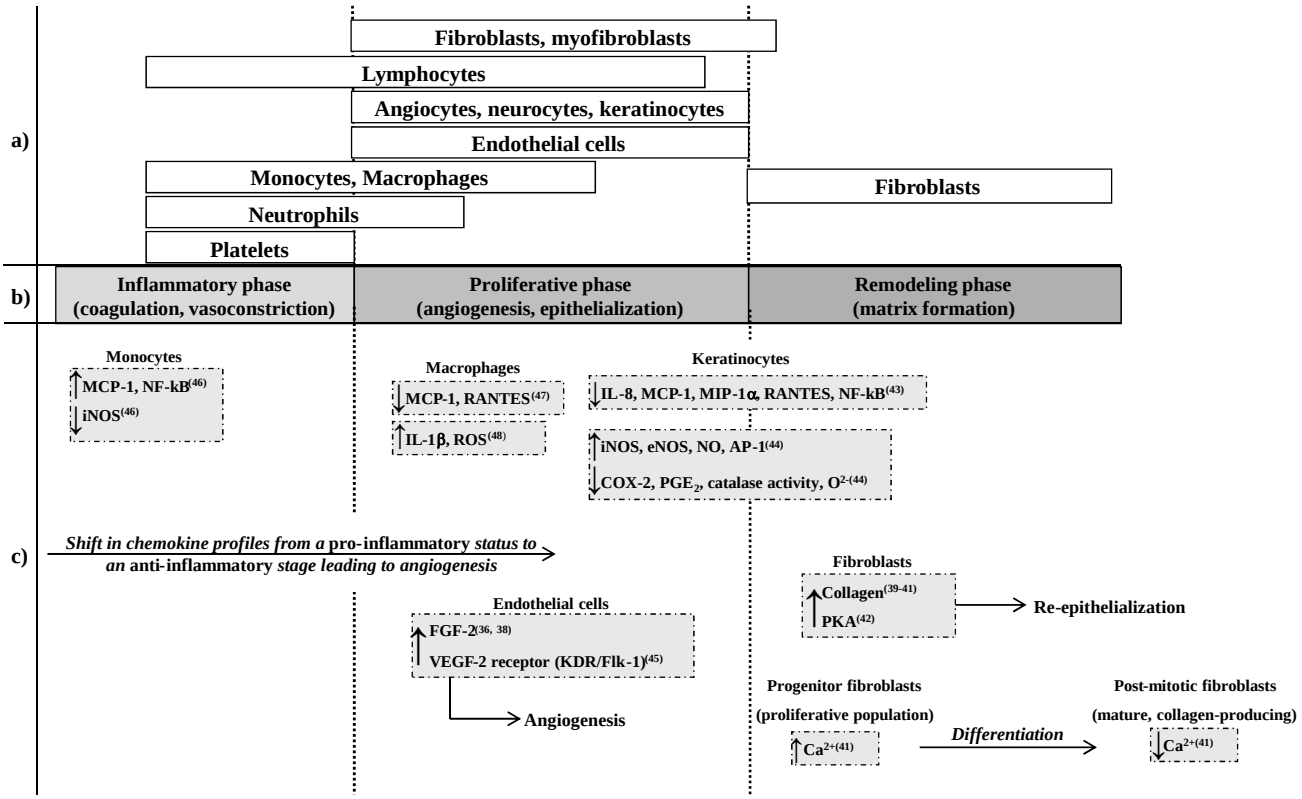

Fig. (1). Wound healing.

a) Cell lineages, **b**) Phases and **c**) Factors involved in the wound repair process. For simplification, only the factors influenced by ELF-EMF (including PEMF) are depicted. **AP-1** = activator protein 1; **COX-2** = cyclooxygenase 2; **eNOS** = endothelial nitric oxide synthase; **FGF** = fibroblast growth factor; **IL** = interleukin; **iNOS** = inducible nitric oxide synthase; **MCP-1** = monocyte chemotactic protein-1; **MIP-1α** = macrophage inflammatory protein-1 alpha; **NF-kB** = nuclear factor-kB; **NO** = nitric oxide; **PGE_2** = prostaglandin E_2; **PKA** = protein kinase A; **RANTES** = regulated on activation normal T cell expressed and secreted; **ROS** = reactive oxygen species; **VEGF** = vascular endothelial growth factor.

Note: NO is responsible for both the up- and down-regulation of the inflammatory phase of wound healing, depending on the timing, its bioavailability, and cell lines considered.

frequency and intensity on living systems are being extensively studied. These weak fields are capable of inducing profound effects *in vivo* and *in vitro*, an ability that represents the basic concept of electromagnetic therapy [14]. It has been reported that EMF influence fundamental cellular processes such as immune and endocrine functions [15, 16], membrane signal transduction [17], cell proliferation [18], differentiation [19], and apoptosis [20]. A number of clinical studies, and experiments *in vivo* and *in vitro* suggested that electromagnetic stimulation can accelerate the wound healing process.

The EMF therapeutic modalities are based on six groups of electromagnetic fields, as reviewed by Markov, 2007 [21]:

1. Static/permanent magnetic fields - created by permanent magnets or by passing direct current through a coil.
2. Low frequency sine waves - frequency of 50 - 60 Hertz (Hz).
3. Pulsed electromagnetic fields (PEMF) - low frequency fields with specific wave shapes and amplitude.
4. Pulsed radiofrequency fields (PRF) - frequencies in the radiofrequency range: 13.56, 27.12, and 40.68 MHz.
5. Transcranial magnetic/electric stimulation - short pulses (≤ 8 Tesla) targeting selected portions of the brain.
6. Millimeter waves - very high frequency range (30 - 100 GHz).

The ELF-EMF, which will be further detailed, represent a form of non-ionizing low-energy electromagnetic field radiation capable of inducing physiological effects at certain parametric “windows” [22]. We will detail herein ELF-EMF of extremely low frequency (1-80 Hz) and low amplitude [0.2-20 miliTesla (mT)] as ELF-EMF have been determined to induce biological effects related to wound healing within these windows. Where specified, we will discuss

the effects of PEMF as a subset of ELF-EMF. On wounds, ELF-EMF act on different loops of healing pathways influencing directly the chronic wound environment, especially the narrow fluid channels that separate the cells and which are essential for cell-cell signaling and for other key intracellular events involving hormones, antibodies, neurotransmitters, etc. [23]. These channels offer a much lower impedance than cell membrane and could potentially mediate the action of electromagnetic fields whose effects are limited (in frequency and intensity) to narrow biologically active windows [24]. This concept explains the influence of ELF-EMF stimulation on the behavior of keratinocytes, fibroblasts and other cell lineages involved in wound healing.

CLINICAL, *IN VIVO* AND *IN VITRO* STUDIES ON ELF-EMF EFFICACY IN TISSUE REPAIR

To examine the potential use of electromagnetic fields in the treatment of leg ulcers, we further summarize the results of clinical (**I**), *in vivo* (**II**) and *in vitro* (**III**) studies included in our analysis and review.

I. Human Clinical Studies

Here we discuss the reported clinical efficiency of ELF-EMF in the process of healing of ulcers based on a total number of five studies identified in the PubMed database [25-29] (Table **1**), four of which used PEMF [25-27, 29]. Three of these studies [25, 27, 28] reported a clinical benefit, while the results of two [26, 29], both having the lowest sample sizes among the five studies, were inconclusive.

Ieran *et al*., 1990, showed that the success rate of healing of venous leg ulcers was significantly higher among patients exposed to PEMF compared to the control group [25]. In addition, PEMF treatment protected patients from ulcer recurrence, compared to the control group. The study by Stiller *et al.*, 1992 reported a significant decrease of wound depth and pain intensity in the group of patients with recalcitrant venous leg ulcers treated with PEMF compared to the placebo group [27]. None of the patients exposed to PEMF exhibited worsening of the lesions, which made a significant difference compared to the placebo group [27]. Canedo-Dorantes *et al*., 2002 reported the first clinical signs of increased vascular network associated with chronic arterial leg ulcers healing after 4-8 weeks from initiation of the electromagnetic therapy. On ulcers of venous etiology, pain, edema and weeping were significantly reduced or eliminated 3-6 weeks after the initiation of the treatment [28]. Some lesions worsened or showed “defective healing” in patients with ulcers associating a concomitant autoimmune disease, or an important arterial occlusion, uncontrolled arterial hypertension, severe lipodermatosclerosis, non-pitting edema, or obesity.

By contrast, no reliable benefits were noticed in two reports that investigated the healing of chronic varicose leg ulcers [26], and spine pressure ulcers [29], although a trend in favor of decrease in ulcer size and of leg circumference was observed in the patients treated with active PEMF [26]. The study by Gupta *et al*., 2009 examined ulcers in advanced stages (III and IV); for a better therapeutic outcome these ulcers would have required surgical reconstructive procedures in addition to the adjuvant electromagnetic therapy [29]. Both reports discussed the need for more focused research designed to determine the best parameters of PEMF that can assure efficient wound healing. These findings indicate that the ELF-EMF treatment is efficient only for a subset of patients with chronic ulcers, particularly of venous origin and is limited by the presence of associated co-morbidities. Efficiency of the treatment may depend on the type of device used and specific combination of parameters. We noticed that the use of electromagnetic fields of low frequency (1-75 Hz) and intensity (up to a maximum of ~4 mT) was associated with the highest efficiency (Table **1**).

The clinical studies discussed herein could not be pooled in a meta-analysis due to the heterogeneous etiological component of the ulcers, variability in therapeutic protocols, work parameters, and treatment duration. The variety of commercially available ELF-EMF/PEMF devices makes it difficult to compare their characteristics and to subsequently analyze the biological and clinical effects they induce in a clinical or laboratory experimental setting.

II. *In Vivo* Animal Studies

We have identified a total number of seven reports investigating the efficiency of PEMF in wound healing in animal models, of which six used a rat model [30-35] and one used a mouse model [36] (Table **2**).

The *in vivo* studies included in the current analysis showed that the area of injury decreased significantly for the group of PEMF-exposed animals compared to the control group [31-32]. Some of the studies reported a significantly faster progression of the overall healing of wounds in animals exposed to PEMF compared to their control counterparts at the end of therapy [32, 36] or particularly during the early stages of tissue repair process (up to 9 days) [34]. Animals exposed to PEMF showed an improved histological organization of the tissues compared to non-exposed controls, early formation of connective tissue and a vascular network, significant decrease in the number of acute inflammatory cells, early collagen synthesis and better maturation, all leading to complete re-epithelialization after 12 days of PEMF exposure [34] (Table **2**). Supportive evidence to these findings were provided in experiments based on a non-wounded rat model exposed to PEMF (25 Hz, 2 mT, 8 days, 2.5 h/day) showing that the electromagnetic stimulation caused increased skin collagen synthesis, as measured by hydroxyproline content, and visualized histologically by intense specific collagen staining [37]. PEMF stimulation increased the superficial vascular network of the skin, supposedly through activation of peripheral

Table 1. Overview of the Clinical Studies Using ELF-EMF (Including PEMF) for Ulcer Treatment

Type of Ulcer/ Body Site	Study		N	Regimen	Duration (weeks)	Results	Refs
	Type	Design					
Venous/ Leg	RCT	DB	37	**Groups**: 19 patients exposed to active stimulators (experimental group); 18 patients exposed to dummy stimulators (control group) **Exposure**: PEMF - 75 Hz, 2.8 mT, 3-4 h/day **Evaluation method**: venous pressure, malleolar edema, granulation tissue, epithelialization	13	The success rate was significantly higher in the experimental group after 90 days ($P < 0.02$) and in the follow-up period ($P < 0.005$) compared to the control group.	[25]
Chronic Varicose/ Leg	NR	DB, C	17	**Groups**: 8 patients received active PEMF therapy; 9 patients received inactive PEMF therapy; all patients received topical therapy throughout the duration of the study **Exposure**: PEMF - 5 Hz, 15 minutes, 2x/week **Evaluation method**: ulcer size, leg girth, degree of pain, presence of infection	5	No statistically significant difference was noted between the two groups in all the clinical parameters tested. A trend in favor of decreased ulcer size and lower leg girth in the group exposed to active PEMF was observed.	[26]
Recalcitrant Venous/ Leg	R	P, DB, PC	31	**Groups**: 18 patients in the active treatment group; 13 patients in the placebo group; all patients received ancillary wound dressing **Exposure**: PEMF - 2.2 mT, 3 h/day **Evaluation method**: wound surface area, ulcer depth and pain intensity, quality and quantity of granulation tissue	8-12	50% of the ulcers in the active treatment group healed or markedly improved versus 0% in the placebo group. A significant decrease in wound depth ($P < 0.04$) and pain intensity ($P < 0.04$) were noted in the active treatment group versus placebo group.	[27]
Chronic Arterial and Venous/ Leg	NR	CS	26	**Groups**: 5 patients exposed to ELF alone; 21 patients exposed to a combination of ELF and SMF **Exposure**: 3.63 mT, 2-3 h/day, 3x/week **Evaluation method**: healing velocity, follow-up of ulcer size and appearance	16	After ELF exposure, 69% of all lesions were cured or healed > 50% in a period < 16 weeks. Healed ulcers remained healed for at least 6 months and up to 2 years after the conclusion of treatment.	[28]
Pressure/ Spine	R	DB, C	12	**Groups**: 6 patients received PEMF therapy; 6 patients received sham treatment; all patients received daily dressing with saline **Exposure**: PEMF - 1 Hz sine wave, 45 minutes, 5x/week **Evaluation method**: wound healing assessed by BJWAT and NPUAP protocol	24	No significant difference in pressure ulcer healing was observed between PEMF- and sham-exposed groups.	[29]

Note: The references are listed in chronological order.
Abbreviations: **BJWAT**, Bates Jensen Wound Assessment Tool; **C**, controlled; **CS**, case series; **DB**, double-blind; **ELF**, electromagnetic field; **ELF-EMF**, extremely low frequency electromagnetic fields; **Hz**, Hertz; **mT**, miliTesla; **N**, number of patients who finished the study; **NPUAP**, National Pressure Ulcer Advisory Panel; **NR**, non-randomized; **P**, prospective; **PC**, placebo-controlled; **PEMF**, pulsed electromagnetic fields; **R**, randomized; **RCT**, randomized controlled trial; **SMF**, static magnetic field.

blood mononuclear cells (PBMC) and their subsequent transportation to the ulcer site *via* humoral route [28].

The studies by Goudarzi *et al.*, 2010 [35] and Callaghan *et al.*, 2008 [36] used a diabetic rat and a mouse model, respectively to study the impact of PEMF on wound healing progression. PEMF significantly increased the rate of wound healing and the tensile strength of scar in exposed diabetic animals compared to their non-exposed counterparts [35]. PEMF accelerated the wound healing process in diabetic and wild-type mice through up-regulation of FGF-2, which is a key angiogenesis factor involved in tissue repair [36]. Furthermore, PEMF prevented tissue necrosis and breakdown in diabetic animals in response to a standardized ischemic insult [36].

Table 2. Overview of the *In Vivo* Studies Focused on the Influence of PEMF on Wound Healing

Animal Model	Field Exposure System		Experimental Setup	Results	Refs
	Hz	mT			
Rat	15	0.2	**Animals**: 96 Sprague-Dawley rats, divided in groups A-D (24 rats/group: 12 PEMF-exposed + 12 controls) **Wound procedure**: excision of a full-thickness 4-cm diameter circle of skin and subcutaneous tissue **Duration**: 28 days **Exposure**: group A was exposed for 12 h every other 12 h; groups B, C, D were exposed for 6 h every other 6 h; groups C and D also received methylprednisolone **Evaluation method**: wound contraction, epithelialization	PEMF did not affect soft tissue healing.	[30]
	50	20	**Animals**: 22 Wistar rats divided in 3 groups: control group received no treatment (n = 8), NF group was treated with topical nitrofurazone solution (n = 7), and PEMF-exposed group (n = 7) **Wound procedure**: circular lesion 3 cm in diameter made on the back **Duration**: 21 days **Exposure**: 35 minutes, 2x/day **Evaluation method**: wound planimetry	The size of the injury decreased in the PEMF-exposed group compared to the control group (beginning with day 7). No difference was found between the PEMF-exposed and NF-treated groups during the first 2 weeks, but a statistically significant difference was found on day 21 ($P < 0.01$).	[31]
	10 20 40 50 60 80	4	**Animals**: 48 Wistar rats equally divided in 8 groups: control, sham and six treatment groups (exposed to six different pulse rates) **Wound procedure**: paired full-thickness incision wound (35 mm in length), parallel to and at a distance of 1.5 cm on each side of the dorsal midline **Duration**: 10 days **Exposure**: 30 minutes/day, 2x/day **Evaluation method**: maximum length, the surface area of the wounds and the healing fractions, full contraction period of the wounds, the tensile strength	The absolute and normalized length of wounds in the PEMF-exposed group (20 Hz) was significantly decreased compared to that of the sham-exposed group ($P < 0.01$). In the 20 Hz-exposed group, the wound healing duration was significantly shorter ($P < 0.02$) and the wound tensile strength was significantly greater ($P < 0.01$) compared to the sham group.	[32]
	5	12.5	**Animals**: 40 Sprague-Dawley rats, equally divided in 2 groups: the experimental group (exposed to PEMF) and control group (exposed to the inactivated PEMF device) **Wound procedure**: full-thickness skin wound of 4 cm^2 **Duration**: 22 days **Exposure**: on day 3, 7, 9 12, 14, 17 and 22; 1,500 pulses/treatment **Evaluation method**: wound contraction, epithelialization, non-healed wound, contraction-epithelialization ratio	PEMF used in this study did not have a significantly beneficial effect on wound healing. Wounds in the PEMF-exposed group were relatively less contracted and showed a compensatory increase in epithelialization in the early stages of wound repair.	[33]
	3	12.5	**Animals**: 48 Wistar rats, equally divided in 2 groups: experimental group (exposed to PEMF) and control group (exposed to the inactivated device) **Wound procedure**: full thickness skin wounds, 2 by 2 cm on the back **Duration**: 22 days **Exposure**: 20 minutes/day **Evaluation method**: wounds' size, healing progress, planimetry, histological examination	Statistically significant acceleration of wound healing in the experimental group versus control group, on days 3, 6, and 9 ($P < 0.02$). At day 22, a complete wound healing was noticed in the experimental group, while in the control group the histological findings were similar to those from the experimental group on day 18.	[34]
	20	8	**Animals**: 28 Wistar rats (14 with induced diabetes and 14 without diabetes) divided in one PEMF-exposed group and one group exposed to the inactivated device **Wound procedure**: full-thickness dermal incision (35 mm) on the right side of the paravertebral region **Duration**: 10 days **Exposure**: 1 h/day **Evaluation method**: measurement of surface area, percentage of healing, duration of healing, wound tensile strength	The rate of healing in PEMF-exposed diabetic rats was significantly higher than in the diabetic control group ($P < 0.001$). PEMF exposure significantly enhanced the tensile strength in diabetic-exposed rats compared to the non-exposed diabetic animals ($P < 0.001$).	[35]

(Table 2). Contd…..

Animal Model	Field Exposure System		Experimental Setup	Results	Refs
	Hz	mT			
Mouse	15	Up to 1.2	**Animals**: groups of 6 mice each (diabetic, wild-type, and FGF-2 knock-out): PEMF-exposed and non-exposed **Wound procedure**: paired 5-mm circular, full-thickness wounds on the dorsum **Duration**: 14 days **Exposure**: 8 h/day **Evaluation method**: gross closure %, time to closure, tissue quality, neovascularization, vascular density, cell proliferation, histology	PEMF significantly accelerated the time to wound closure ($P < 0.05$), granulation and cell proliferation in diabetic and wild-type mice compared to their corresponding control (non-exposed mice) through up-regulation of FGF-2.	[36]

Note: The references are listed in chronological order within the same section of the table (where applicable).
Abbreviations: **FGF-2**, fibroblast growth factor 2; **Hz**, Hertz; **mT**, miliTesla; **NF**, nitrofurazone; **PEMF**, pulsed electromagnetic field.

Supportive data of the role of FGF-2 in wound healing were reported in a study by Tepper *et al.*, 2004 that examined the angiogenic effect of PEMF on non-wounded animals [38]. PEMF significantly stimulated the neoangiogenesis and induced a 2-fold increase in the production of FGF-2 in the exposed animals versus controls.

Two animal studies reviewed herein did not find PEMF exposure particularly useful in the healing of wounds [30, 33]. Glassman *et al.*, 1986 [30] observed no difference in the gross or microscopical appearance of the wounds and in the number and orientation of fibroblasts when the PEMF-exposed and control groups were compared [30]. Similarly, in the study by Milgram *et al.*, (2004) the PEMF-exposed wounds did not heal significantly faster, neither by contraction, nor by epithelialization [33].

The present analysis based on data provided by *in vivo* studies showed that the PEMF have a significant impact on wound closure, acting at three levels in the process of tissue repair: on the inflammatory phase – by reducing the inflammation as reported by Athanasiou *et al.*, 2007 [34]; on the proliferative phase – by increasing angionegenesis [34-36], epithelialization and neovascular network formation [34]; and on the remodeling phase – by increasing collagen formation and inducing better fibers organization [34], which rebuilds the damaged tissue. The review of the data reported in the studies using animal models for wound healing revealed that, like in the case of clinical trials, the benefit of PEMF is limited to a subset of wounds exposed to PEMF of low frequency (3 - 80 Hz) and intensity (up to a maximum of 20 mT) (Table **2**). The rodent *in vivo* studies showed the advantage of providing a whole body response to PEMF exposure, thus better reflecting the setup used in clinical trials for the treatment of ulcers.

III. *In Vitro* Studies - Action of ELF-EMF on Intracellular Pathways Involved in Wound Healing

We have identified a number of twelve studies in the PubMed database reporting on the effect of ELF-EMF on *in vitro* cultures of cells involved in the complex process of wound healing [36, 38-48] (Table **3**), two of which used PEMF [36, 38]. The results reported in these studies support the belief that ELF-EMF contribute to the overall tissue repair process by acting on various secondary messengers as part of several signaling pathways, and by influencing cell growth, proliferation, differentiation, and angiogenesis. We detail here on three groups of effects: **1**) inflammatory effects; **2**) pro-angiogenic; and **3**) effects on growth, differentiation and proliferation of cells involved in matrix formation.

1. ELF-EMF Effects on the Inflammatory Phase of the Wound Healing

Clinical investigations of chronic wound healing showed that advanced stages of wound repair are associated with a shift that transitions the healing process from a chronic, pro-inflammatory state, to an anti-inflammatory state. The pro-inflammatory state is characterized by increased levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-8, regulated upon activation, normal T-cell expressed and secreted (RANTES) and macrophage inflammatory protein (MIP)-1α (known for their effect on the earlier components of the pathway). The anti-inflammatory state is characterized by decreased pro-inflammatory cytokine levels, and induction of anti-inflammatory cytokines that stimulate the resolution of inflammation and favor angiogenesis [49]. The analysis of the *in vitro* studies indicated that ELF-EMF accelerate this shift and have two opposite sequential actions: an early short effect on pro-inflammatory cytokines (**a**), followed by an anti-inflammatory effect (**b**) (Fig. **1c**).

1(a) The stimulatory effects of ELF-EMF on pro-inflammatory cytokines are indicated by the following lines of evidence:

1-(a_1) ELF-EMF up-regulated the expression of monocyte chemotactic protein (MCP)-1 at the mRNA and protein level in exposed, lipopolysaccharide (LPS)-stimulated human monocytes [46]. In general, the tissue injury is followed by the onset of an acute inflammatory reaction that depends on cell proliferation and

migration, and is mediated and modulated by paracrine and/or autocrine production of chemokines. As such, MCP-1 is produced by resident cells (keratinocytes at the wound edge, endothelial cells) and macrophages and represents an important mediator of monocyte/macrophage recruitment and activation at the site of chronic inflammation as part of the wound healing process. In addition, the expression of inducible nitric oxygen synthase (iNOS), responsible for the synthesis of nitric oxide (NO), was down-regulated in stimulated monocytes exposed to ELF-EMF [46]. NO is responsible for both the up- and down-regulation of the inflammatory phase of wound healing; balanced timing and bioavailability of NO production are critical to ensure a beneficial wound closure effect [50]. Finally, the data reported by Reale *et al*., 2006 suggest that the effects of ELF-EMF on MCP-1 and iNOS are mediated by an increase in nuclear factor (NF)-kB expression [46].

1-(a_2) ELF-EMF significantly increased the phagocytic activity of mouse macrophages, and induced free radical release and IL-1β production [48], indicating an activating capacity of these fields to release pro-inflammatory cytokines, and to sustain the inflammatory reaction that occurs at the beginning of the wound healing process. It is well known that resting macrophages have low levels of phagocytic activity and become fully active through binding of pathogens or by local cytokine release. Once activated, macrophages exhibit increased production of reactive oxygen species (ROS) that act as signaling molecules further involved in multiple cellular pathways, like in the activation of IL-1β signal transduction pathway [51].

1(b) The anti-inflammatory effects of ELF-EMF are indicated by the following sets of experiments:

1-(b_1) ELF-EMF strongly inhibited the production of MCP-1 and RANTES in phytohemagglutinin (PHA)-stimulated human macrophages in culture, compared to controls not exposed to ELF-EMF [47]. The electromagnetic fields' inhibitory effect on the production of MCP-1 and RANTES represents a shift in the cytokine profile that accompanies the transition from monocytes to macrophages and further toward the angiogenesis and re-epithelialization stages (Fig. **1c**).

1-(b_2) ELF-EMF significantly down-regulated the production of chemokines specific to the inflammatory phase of wound healing (e.g., IL-8, MCP-1, MIP-1α and RANTES) (Fig. **1c**), induced an early reduction of NF-kB levels and increased cellular proliferative activity of keratinocytes in culture [43]. These results support the hypothesis that ELF-EMF contribute to wound closure by reducing the production of pro-inflammatory mediators and by increasing keratinocyte growth.

1-(b_3) ELF-EMF exposure of keratinocytes in culture induced increased expression levels of iNOS, endothelial nitric oxygen synthase (eNOS), paralleled by increased early NOS activities and NO production [44]. Increased levels of NO may be involved in the down-regulation of the inflammatory phase of wound healing. As such, the suppression of RANTES by NO during wound repair and in cell culture may represent the beginning of the transition from the inflammatory to the regenerative phase of wound healing [52]. Furthermore, the expression of MCP-1 in hyperproliferative keratinocytes at the wound edge also appears to be decreased by NO *in vitro* and possibly *in vivo*, suggesting a secondary mechanism by which adequate levels of NO expressed during inflammation may move the process of wound repair forward [53]. Therefore, the study by Patruno *et al.*, 2010 [44] provides much needed supportive evidence for the results reported by Vianale *et al.*, 2008 and previously discussed [43], showing the influence of NO in the shift of the wound healing process beyond the inflammatory phase. In addition, higher levels of activator protein (AP-1) expression as well as a higher rate of cell proliferation have been observed in the keratinocytes exposed to ELF-EMF [44]. AP-1 is a redox-responsible inducible transcription factor that plays a critical role in the expression of many genes involved in the inflammatory responses and cellular proliferation and differentiation [54].

ELF-EMF decreased cyclooxygenase (COX)-2 expression, prostaglandin (PG)E_2 production, catalase activity and O_2^- production in keratinocytes [44]**.** One of the early responses to inflammatory stimuli in cells involved in the repair processes, including keratinocytes, is the induction of COX-2 whose up-regulation appears to be significantly involved in the persistent inflammation in chronic wounds [55]. These experiments indicated that ELF-EMF exposure accelerates the switching from the inflammatory phase to the final repair phase during the wound healing process. Furthermore, these results are in accordance to the hypothesis that O_2^- production decreases when proliferating keratinocytes become confluent in culture and that antioxidant enzymes may be involved in the regulation of keratinocytes proliferation [56].

2. ELF-EMF Action on Angiogenesis

Local changes in the wound microenvironment such as decreased pH, reduced oxygen tension and increased lactate reflect inadequate tissue perfusion secondary to damaged capillaries, and stimulate the process of angiogenesis [57]. Angiogenesis is a critical

Table 3. Overview of the *In Vitro* Studies Focused on the Influence of ELF-EMF (Including PEMF) on Cells Involved in the Wound Repair Process

Cell Type	Field Exposure System		Experimental Setup	Results	Refs
	Hz	mT			
Skin Fibroblasts	Symmetric, biphasic sinusoidal		**Cells**: HH-8, ELF-EMF-exposed and non-exposed **Exposure**: 2 x 6 h/day, up to 21 days **Cellular and molecular end-points**: total protein and collagen synthesis, cell differentiation	Long-term exposure induced irreversible differentiation of mitotic fibroblasts to post-mitotic fibroblasts. In exposed cells, total protein and collagen synthesis increased significantly compared to non-exposed cells ($P < 0.05$).	[39]
	20	6			
	Symmetric, biphasic sinusoidal		**Cells**: HH-4, ELF-EMF-exposed and non-**exposed** Exposure: 2 x 6 h/day, up to 21 days **Cellular and molecular end-points**: differentiation, collagen synthesis, expression of protein PIVa	Long-term exposure inhibited cell growth and induced collagen synthesis.	[40]
	20	6			
	Sinusoidal		**Cells**: HSF-2, HSF-3, ELF-EMF- and sham-exposed **Exposure**: 1 h **Cellular and molecular end-points**: dynamics of intracellular calcium	Progenitor fibroblasts responded with a stimulation of the dynamics of calcium. Post-mitotic fibroblasts responded with an inhibition of the dynamics of calcium.	[41]
	20	8			
	Sinusoidal		**Cells**: HSF-2, HSF-3, ELF-EMF- and sham-exposed **Exposure**: short-term - 1 h; long-term - a constant 1 h on/1 h off, for 7 days **Cellular and molecular end-points**: PKA activity	Short-term exposure resulted in an increased PKA activity. Long-term exposure induced a transient stimulation of PKA followed by a decrease to the baseline level similar to that observed in sham-exposed controls.	[42]
	20	7-8			
Keratinocytes (HaCaT)	Sinusoidal		**Cells**: ELF-EMF- and sham-exposed **Exposure**: continuous for 1, 4, 12, 24, 48, 72, and 96 h without medium replacement **Cellular and molecular end-points**: cell growth and viability, proliferation and cytokine production (RANTES, MCP-1, MIP-1α, IL-8, NF-kB)	Exposure to ELF-EMF increased the proliferative activity of HaCaT cells and reduced their production of chemokines compared to non-exposed cells. Exposed cells had an almost immediate reducing effect of NF-kB levels compared to its progressive reduction observed in non-exposed cells.	[43]
	50	1			
	Sinusoidal		**Cells**: ELF-EMF- and sham-exposed **Exposure**: continuous for 3, 18, and 48 h without medium replacement **Cellular and molecular end-points**: cell growth and viability, expression of iNOS, eNOS, COX-2 and AP-1, production of NO, O_2^- and PGE_2, catalase activity	Exposure to ELF-EMF increased iNOS and eNOS expression levels, NOS activities and NO production, and induced higher levels of AP-1 expression and cell proliferation rate compared to non-exposed cells. ELF-EMF decreased COX-2 expression, PGE_2 and O_2^- production, and catalase activity compared to non-exposed cells.	[44]
	50	1			
Endothelial Cells	Asymmetric		**Cells**: HUVECs, PEMF- and sham-exposed **Exposure**: 24 h, 48 h, or 7-10 days **Cellular and molecular end-points**: cell proliferation and migration, endothelial cell tubulization, FGF-2, angiopoietin-2, thrombopoietin, EGF, VEGF	PEMF significantly increased endothelial cells proliferation (by 3-fold) and tubulization (by 7-fold) compared to non-exposed cells. Cells exposed to PEMF exhibited a 5-fold increase in FGF-2, and smaller increases in other angiogenic growth factors (angiopoietin-2, thrombopoietin, and EGF).	[38]
	15	1.2			
	Asymmetric		**Cells**: murine, PEMF- and sham-exposed **Exposure**: 18 h **Cellular and molecular end-points**: cell proliferation	PEMF-exposed cells demonstrated a significant increase in proliferation compared to non-exposed cells. PEMF-exposed cells exhibited 3-fold higher levels of FGF-2 compared to non-exposed cells.	[36]
	50	0.2			
	Sinusoidal		**Cells**: HUVECs, ELF-EMF- and sham-exposed **Exposure**: 1, 6, and 12 h **Cellular and molecular end-points**: proliferation and migration, cytoskeleton and VEGF staining	ELF-EMF increased the cell proliferation rate. The ability of wound migration was significantly higher in cells exposed to ELF-EMF compared to non-exposed cells ($P < 0.05$). ELF-EMF increased the phosphorylation state of KDR/Flk-1 (VEGF-2 receptor) in a time-dependent manner compared to non-exposed cells ($P < 0.05$).	[45]
	50	1			

(Table 3). Contd…..

Cell Type	Field Exposure System		Experimental Setup	Results	Refs
	Hz	mT			
Monocytes	Sinusoidal		**Cells**: stimulated or not with LPS, and ELF-EMF-exposed or non-exposed **Exposure**: overnight **Cellular and molecular end-points**: expression and production of iNOS and MCP-1	iNOS was down-regulated at mRNA and protein level following the exposure to ELF-EMF, while MCP-1 was up-regulated. NF-kB expression was increased in basal and LPS-stimulated cells exposed to ELF-EMF compared to their non-exposed counterparts ($P < 0.0005$).	[46]
	50	1			
Macrophages	Sinusoidal		**Cells**: stimulated or not with PHA, and ELF-EMF-exposed or non-exposed **Exposure**: 24 h **Cellular and molecular end-points**: production of MCP-1 and RANTES	ELF-EMF strongly inhibited the production of MCP-1 and RANTES in PHA-activated macrophages compared to the non-stimulated controls ($P < 0.05$).	[47]
	50	1			
	Polarized		**Cells**: mouse, ELF-EMF-exposed or non-exposed **Exposure**: 45 minutes to 48 h **Cellular and molecular end-points**: secretion of IL-1β, production of ROS, phagocytic activity, micronucleus formation	ELF-EMF exposure increased the phagocytic activity, IL-1β release (1.0 mT), and production of ROS.	[48]
	50	0.05 0.1 0.5 1.0			

Notes:
The references are listed in alphabetical order and chronologically within the same section of the table, where applicable.
Sham cells = cells exposed to the same environmental conditions but in the absence of radiation.
Cells are of human origin if not otherwise noted.
Abbreviations: **AP-1**, activator protein 1; **COX-2**, cyclooxygenase-2; **EGF**, epidermal growth factor; **ELF-EMF**, extremely low frequency electromagnetic field; **FGF-2**, fibroblast growth factor-2; **eNOS**, endothelial nitric oxide synthase; **Hz**, Hertz; **HUVECs**, human umbilical vein endothelial cells; **IL**, interleukin; **iNOS**, inducible nitric oxide synthase; **LPS**, lypopolysaccharide; **MCP-1**, monocyte chemoattractant protein-1; **MIP-1α**, macrophage inflammatory protein-1α; **mT**, miliTesla; **NF-kB**, nuclear factor-kB; **NO**, nitric oxide; **PEMF**, pulsed electromagnetic field; **PGE_2**, prostaglandin E_2; **PHA**, phytohaemagglutinin; **PKA**, protein kinase A; **ROS**, reactive oxygen species; **RANTES**, regulated upon activation, normal T-cell expressed and secreted; **VEGF**, vascular endothelial growth factor.

phase of the tissue repair, which involves several growth factors produced during the inflammatory stage of wound healing [e.g., vascular endothelial cell growth factor (VEGF), FGF, angiopoietin, and transforming growth factor (TGF)-β].

The following *in vitro* experiments reflect the stimulatory effect of electromagnetic fields on angiogenesis:

2-1 Exposure of human umbilical vein endothelial cells (HUVECs) to PEMF increased significantly the degree of endothelial cell tubulization and the cell proliferation *in vitro* [38]. Furthermore, the endothelial cells exhibited an increase in FGF-2, as well as smaller increases in other angiogenic growth factors (angiopoietin-2, thrombopoietin, and epidermal growth factor - EGF). These findings demonstrate that PEMF up-regulate angiogenesis and thus contribute to blood vessel formation, as part of the overall wound healing process.

2-2 Murine endothelial cells exposed to PEMF exhibited a 3-fold increase in the levels of FGF-2 [36]; in addition, the media from these exposed cells accelerated the wound healing when applied to diabetic mice that were not exposed to PEMF. These data suggested that FGF-2 up-regulation is the primary mediator of endothelial cell proliferation following PEMF exposure.

2-3 The exposure of HUVECs to ELF-EMF increased endothelial cell proliferation and induced *in vitro* an increase in the rate of capillary like-structures formation [45]; this observation is in good agreement with other studies reporting that ELF-EMF are able to influence biochemical stimuli that induce angiogenesis [34,36,38]. The endothelial cell proliferation was accompanied by an overall increase in expression of VEGF receptor 2 (KDR/Flk-1), suggesting that ELF-EMF can potentially interact with signaling pathways mediated by VEGF and involved in the process of tubule formation [45]. Finally, the data showed that ELF-EMF induced a major reorganization of cytoskeletal fibers and focal adhesion complexes [45] that are in accordance to the observations of accelerated *in vivo* wound healing following exposure to ELF-EMF [32, 36].

While the precise mechanism of action is still under investigation, these studies provide valuable insights on the stimulatory effect of ELF-EMF on intracellular signaling pathways involved in angiogenesis that is a key phase in the normal tissue repair process.

3. ELF-EMF Effect on Growth, Differentiation and Proliferation of Cells Involved in Matrix Formation

The progenitor/post-mitotic fibroblast cell system represents an ideal model for the analysis of wound healing and the influence of ELF-EMF on this complex process, given the particularities of proliferation and differentiation patterns of progenitor population (involved in early stages of wound healing) versus post-mitotic cells (involved in collagen formation occurring later in tissue repair). Several studies have addressed the interaction between ELF-EMF and calcium fluxes [58-61], calcium being a key secondary messenger involved in regulation of cell growth and differentiation. The following sets of data indicate the effect of ELF-EMF on the cells involved in matrix formation during the wound healing process:

3-1 Long-term exposure to ELF-EMF induced the irreversible differentiation of normal human skin fibroblasts to post-mitotic fibroblasts [39]; the post-mitotic fibroblasts are characterized by differentiation-specific proteins (such as PIVa) and differentiation-dependent enhanced metabolic activities that lead to matrix formation through collagen production [40].

3-2 Progenitor fibroblasts responded with a stimulation of the dynamics of calcium, as compared to post-mitotic fibroblasts which responded to ELF-EMF exposure with an inhibition of the dynamics of calcium and a switch to enhanced collagen synthesis responsible for matrix formation [41]. In this cell system, the differentiation state of the cells appears to determine the direction of the ELF-EMF-induced modulation of the mitogen-induced calcium signal.

3-3 Experiments exposing human skin fibroblasts in culture to ELF-EMF identified cAMP-dependent protein kinase A (PKA) as a specific intracellular signal transduction component which may be involved in mediating the growth inhibitory and differentiation-inducing signals of ELF-EMF [42]. The authors showed that ELF-EMF induced an immediate and transient increase in cAMP-dependent protein kinase PKA activity *in vitro*. While still lacking experimental data supporting the modulation of PKA signaling pathway by ELF-EMF, the data reported by Thumm *et al*., 1999 represent a step forward to the identification of an ELF-EMF-sensitive intra-cellular signaling cascade involved in the regulation of growth, proliferation and differen-tiation of cell lineages involved in wound healing [42].

Overall, the current analysis of these *in vitro* studies showed that fibroblasts respond mostly to ELF-EMF of 20 Hz and 6-8 mT [39-42], while keratinocytes [43, 44], monocytes [46], macrophages [47, 48], and endothelial cells [36, 45] are sensitive to electromagnetic fields of 50 Hz and 1 mT. These results support the concept that electromagnetic fields are very efficient for wound healing within extremely low frequency and intensity "windows".

The overview of the *in vitro* studies provided mechanistic evidence for the involvement of ELF-EMF in the complex process of wound healing, with particular emphasis on modulation of those factors responsible for prolonged inflammation, imbalanced production of regulatory growth factors and cytokines, and decreased angiogenesis that contribute to a very slow or defective, incomplete or uncoordinated course of repair of chronic wounds [49, 62].

To clarify the effects of ELF-EMF on wound healing which can improve the therapeutic outcomes, several directions of future research can be considered:

- Additional experiments to confirm the previous results and to identify cellular and molecular effects of ELF-EMF that have not been unveiled yet are needed.
- Future clinical studies should be based on larger numbers of subjects, selected on appropriate criteria and exposed to certain device parameters, previously shown to be effective in the clinical setting. A greater selectivity of patients, with regard to the type of etiological component (arterial, venous, and lymphatic) and degree of severity, is needed.
- An accurate and detailed analysis of the *in vivo* and *in vitro* profiling of cytokines and other critical factors involved in the inflammation/ proliferation/angiogenesis pathways might be performed, in the light of a possible correlation that could better align the characteristics of ELF-EMF used in each experimental setting. This would contribute to a better standardization of the intensity and frequency of electromagnetic fields used for each type of chronic ulcers, animals model or cell line.

CONCLUSIONS

Therapeutic applications of magnetic fields have grown over the last three decades, gaining acceptance in some medical specialties (fracture healing, pain management, etc). In the current analysis we have presented a synthesis of cellular and molecular mechanisms that likely govern ELF-EMF effects in wound healing; the amelioration of clinical features related to wound healing in exposed human subjects and animal models were well reflected by the anti-inflammatory, pro-angiogenesis and collagen formation effects reported by the *in vitro* studies. However, due to variations involving non-linearities in intensity, amplitude, frequency, "windows" of the devices used and wave shapes of the signal, a rigorous rationale regarding extrapolation of *in vivo* and *in vitro* studies to a clinical setup could not be made. Most of the reviewed *in vitro* and *in vivo* studies indicate beneficial results of ELF-EMF stimulation, however due to the heterogeneous etiological component of the ulcers,

variability in therapeutic protocols, work parameters, and treatment duration, a meta-analysis could not be performed. Our review represents a first step to a better understanding of the cellular and molecular mechanisms of ELF-EMF in wound repair and emphasizes the pressing need for clarifications of the chronic ulcers pathways, in order to find better therapies of this invalidant condition.

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