Comparative analyzes of 120 Hz Electromagnetic Field, respect the interferon-β and Transfer Factor effect in the recovery of chronic ulcers measuring the frequency of the lymphocytes CD4+ and CD8+ in an animal model

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Abstract. We analyze the effects of an Electromagnetic Field of 120 Hz, on the frequency total of T Lymphocytes (CD4+, CD8+) in chronic ulcers, provoked on rats, and judging their effect with respect to the known immunomodulators as Transfer Factor and Interferon-β. We employed an animal model based on the use of Wistar type rats. The frequency of T lymphocytes (CD4+, CD8+) was measured and the evolution of the ulcers was observed through analyzes of tissue’s biopsies, calculating the ratio (CD4+)/ (CD8+) as a monitor variable concerning the immunological impact. Comparing with the negative control, the frequency of T lymphocytes CD4+ presented significant increment (P <0.05) starting from the third day of treatment. Lymphocytes CD8+ diminished significantly (P <0.05). The ulcers from the negative control group in 120 h and with Interferon-β in 96 h, were recovered. Otherwise, with Transfer factor and 120 Hz Electromagnetic field, the ulcer’s time recovery was of 72 h. The results in the latest groups suggest that the total frequency of lymphocytes (CD4+, CD8+) present tendencies toward normal levels. Furthermore, the treatment with 120 Hz Electromagnetic fields show less damage in the ill tissue, analyzing the cellularity with electronic sweep microscopy, implicating less chronic immune activation.

1. Introduction
Immune system (IS) represents many selective processes allowing the organisms to detect, confine and remove both pathogens microorganism and abnormal cells, including neoplasias. Failures in its regulation lead to severe infections, allergies, and autoimmune diseases develop [1]. The IS comprises both the innate and the acquired immunity. The IS permit that macrophages capture the antigens, usually employing the major histocompatibility complex (MHC) class I (MHC-I) and class II (MHC-II) to various cytotoxic T lymphocytes called (CD8+). They recognize the antigens associated with MHC-I molecules and helper T cells called (CD4+) exposing the associated antigen with MHC-II molecules. Furthermore, co-stimulatory molecules such as CD3+ and CD29, stimulate the T cells proliferation secreting lymphokines when they are activated. They increase the bactericidal action of the macrophages. This complex set of genes, molecules, and cells alter any of their components [2]. Nevertheless, an immunomodulatory drug is a biological or non-biological substance influencing a
specific function, modifying one or more of the parts of the immunoregulatory network to achieve a
direct or indirect effect like the alpha and beta-Interferon. The expression of antigens from the MHC
decreases, as well as the proliferative response of T lymphocytes. Likewise, the transfer factor (TF) is
a derivative product of leukocytes. It is conferred its biological activity by the phosphate group
contained in its molecule measured by lymphokines production secreted by the (CD4+) lymphocyte.
The TF participate in the cellular immune response conferred by T cells, activated by a specific
antigen presentation by monocytes and macrophages. The TF, provoke the conversion in polarity
of one negative delayed hypersensitivity to a positive one, increasing the lymphocyte proliferation,
among others phenomena [1].

Moreover, TF is a derivative product of leukocytes. It is conferred its biological activity by the
phosphate group contained in its molecule measured by lymphokines production secreted by the
(CD4+) lymphocyte. The TF participate in the cellular immune response conferred by T cells,
activated by a specific antigen presentation by monocytes and macrophages. Thus, an ulcer represents
any lesion in the skin or the mucous membrane provoked by an inflammatory, infectious, or malignant
circumstance. Such conditions include derisory blood supply, tissue anoxia, edema, cell death, and
infection, among other factors. These variations amend the intrinsic interaction between the structural
components of the affected tissues and the immune cells impeding wound healing; prevailing
the hypothesis respect the pathophysiology of chronic ulceration, which focuses on native effects induced
by hemodynamic variations. Between the customary management for this illness, are a direct
application of pharmacological agents, topical usages, and surgical or endovascular repair on the
macrovasculature. New therapies include the use of growth factors and extremely low-frequency
electromagnetic field (ELF-EMF) [3]. The new treatments have their basis in successfully
experimentation concerned the treatment of non-healing skin ulcers with autologous activated
mononuclear cells [4, 5, 6]. It is known otherwise, that the cells have a characteristic physical behavior
as a bioelectrical entity, i.e., like an electric dipole. Grounded in this hypothesis, the researchers in the
world have been performing studies concerning the effects of the ELF-EMF on biological systems,
particularly in the wound healing. Such investigations include changes in cells of the IS through Ca++
signaling [7], from the intracellular deposits originating cellular proliferation [8, 9], including up-
regulated cytokine synthesis [10]. The most commonly found types of ulcers are the so-called ulcers
by decubitus. Such ulcers are located areas with tissue’s necrosis, formed by compressed of soft tissue
against bony prominences [11]. The ulcer eludes the regular sanguine circulation tower the damaged
area provoking ischemia, tissue injury, loss of continuity in the skin with underlying necrosis, favoring
the wound’s infection [12]. The activation of the lymphocytes T is done by a signal on the membrane
of the lymphocyte CD3+, evolving through the phosphorylation process of proteins. The protein
kinase C (PKC) is the crucial enzyme. The signal actives the corresponding antibodies production
(anti-CD3), antagonistic of the lectin, which interacts directly with the complex of lymphocyte CD3+.
Likewise, the ionophores of calcium open ionic channels, allowing the increase in the levels of
intracellular calcium, because PKC needs calcium ions for its activity [13, 14]. Otherwise, the
lymphocyte (CD4+) is a glycoprotein expressed on the surface of T helper cells, regulatory T cells,
and dendritic cells. On T cells, the lymphocytes (CD4+) represent the co-receptor for the T cell
receptor (TCR). They are responsive of the amplification of the signal generated by the TCR, by
recruiting the tyrosine kinase (TCK), which is important because, it acts over many molecules that at
the time, participate in the signaling process of an activated T cell. The T cells are expressing (CD4+)
molecules (and not (CD8+)) on their surface, therefore, are specific for antigen presented by MHC-II
and not by MHC-I (they are MHC-II restricted). For their part, lymphocyte (CD8+) also is a
glycoprotein, which serves as co-receptor expressing it on the surface of the cytotoxic T cells. The
lymphocyte (CD8+) consists of an α and β chain, in which both resemble immunoglobulin-like
molecule which is connected to their membrane by a thin stalk. The α3 portion is attracted to the
MHC-I molecule. This affinity keeps the T cell receptor of the cytotoxic T cell, and during antigen-
specific activation, the target cell bound intimately together [5, 6]. Under this immunological
framework, we use the ratio (CD4+)/CD8+ as an overall immune dysfunction parameter. Both
((CD4+) helper/inducer cells and (CD8+) cytotoxic/suppressor cells) phenotypes of T lymphocytes circulate by the blood torrent. The ratio is sensitive to a vast heterogeneity of factors as sex, age, genetics, exposures types, and infections, and we are going to assess their meaning on the wound healing. A low (CD4+)/(CD8+) rate indicate an immune risk phenotype, associated with an altered immune function, immune senescence, and chronic inflammation. For this reason, the (CD4+)/(CD8+) ratio, can be used as an indication of chronic immune activation reflected into the damage of the ulcer’s tissue. The objective of the work is to determine the effect of 120Hz-EMF on the frequency total of the T lymphocytes (CD4+), (CD8+), evaluating the (CD4+)/(CD8+) ratio. Analyze their relation with the healing of the chronic ulcers in rats, comparing this kind of effect concerning the known effect of immunomodulators like the Interferon-β (INF-β) and the Transfer Factor (TF).

2. Materials and Methods
The animal setup was of 30 Wistar rats of 240 gr (average corporal weight). The animals fed with rodent food of Harlan-Mexico with 18% protein content and water ad libitum, were distributed randomly forming six groups of five rats each one and performed the following schedule immunomodulators’ administration: 1) The negative control (NCG): Intraperitoneally water injection of 0.5 ml. 2) The 120 Hz ELF-EMF group (120Hz-EMF): Treatment with 2.5 mT-120 Hz for 15 min each day for 12 days. 3), 4) TF groups: Were treated with TF, leucocyte extract obtained in the laboratory of National Immunology School of the Biological Sciences, IPN; used in doses of 0.13 mg/kg (TF-I) and 0.26 mg/kg (TF-II) of body weight respectively. 5), 6) The INF-β groups: Were treated with Betaferon (Merck Serono Laboratory) 6x10³ UI, used in doses of 0.13 mg/kg (INF-βI) and 0.26 mg/kg (INF-βII) of body weight respectively. Its calculated the TF and INF-β doses employed in the protocols based on the concentrations administered in patients with sequels of spasticity and a previous study of toxicity [1]. Such doses are not dangerous ones for the animals; their use is to assess the immunological effect on the chronic ulcer formation in the study.

2.1. Preparation of samples
Weighed the animals daily, and peripheral blood samples were taken every third day (from the tail’s rat) for 12 days. They underwent the following technique of staining peripheral blood smear (called “trail” for simplicity) according to Wright [1]: a) Allow drying by air the trail. b) Place the slide with extension up on a bridge staining. c) Cover the slide entirely with the dye solution. d) Let stand for 2 to 5 minutes. e) Add the buffer. f) By slight blows mixing the dye and the buffer solution. g) Rinse the slide in uniform water flux. h) Allow airing dry. i) Read the slides: differential count of total/100-cell lymphocytes, Olympus, objective 100X.

2.2. Characteristics of the 120Hz-MF generation
The magnetic field (MF) and EMF induce several biological phenomena below the thermal threshold [15]. However, with an immense variety in the frequency spectrum, amplitude, and intensity, the own biological mechanism remains unidentified, and the biological effects are assorted and incongruous [16]. It is complicated to compare data from different studies due to precisely this variety of parameters. We use 120Hz because this value is a multiple of one of the Schumann’s resonances, an example of application is the reference [5].

The 120Hz-EMF source employed in the present study, consist of a tri-axial device of three pairs of coils. Each pair was connected in a Helmholtz-like configuration, correlating the three coordinate axes (x, y, and z) according to the electric setup Figure 1. Were used two Helmholtz pair to cancel the geomagnetic field of the earth and those parasites field that comes from a variety of sources in the laboratory. Such pairs of coils were wound with 169 turns of AWG 12-gauge copper enameled magnet wire, having radii of 36 cm and 33 cm. Was used the inner pair of coils as the 120Hz-EMF stimulation, wounded with 600 turns of AWG 20-gauge copper enameled magnet wire having a radius.
of 30 cm. All pairs of Helmholtz coils were able to generate a remarkably homogeneous EMF in a volume of 0.01 m³. The source of current (STAICO model 1005-B) used for the inner pair of coils was able to generate a sufficient EMF in the range 0-3 mT. In the other pair of coils (STAICO model 1010-B), the range was 0-3.5 mT, all at the frequency of 120 Hz, obtained through an electronic bridge of diodes [6].

Figure 1. Electrical circuit of the setup, and the physical device used in the experiment (of reference 6).

2.3. Determination of the total lymphocytes frequency
As a control, the animals were weighed daily, accordingly to display the effect of the different treatments in a body level generalized way, was applied ammonium hydroxy (NH4OH) at 29%, with the previously shaved back part of the rat, every 8 hours during one week to provoke the chronic ulcer. Through the technique of staining peripheral blood smear of Wright, the frequency of total lymphocytes was determined [6].

2.3.1. The T lymphocytes frequency (CD4+, CD8+), and the (CD4+)/((CD8+)) rate determination. A sample of peripheral blood (1 ml) was obtained from the tail of the treated rats to quantify the T lymphocytes frequency (CD4+, CD8+) employing the technique of flow cytometry, and posteriorly was evaluated the ratio (CD4+)/((CD8+)) from the measurements. We took the samples before the treatment and until the twelve days after the treatment, every three days. We carried out the immuno-typification of the lymphocytes T (CD4+, CD8+), using monoclonal antibodies in a flow cytometer Technicon H3. In all the rats, is performed a biopsy to observe the histological and morphological changes with several microscopy techniques, first one, the optical with eosine-hematoxylin (E-H) staining technique plus light and the second one with transmission electron microscopy plus sweep microscopy [6]. In the present study, we only report the ratio (CD4+)/((CD8+)), the rest of the data concern (CD4+) and (CD8+) can found in the reference [6].

2.4. Method of optical microscopy (light+E-H)
Was fixed the skin tissue sample of the rats employing formol at 10%, after that it is placed in paraffin, and stained with the histological technique E-H. It was observed posteriorly, through a Zeiss Axiopt microscope.

2.4.1. Method of sweep and transmission electron microscopy
The skin tissue sample is fixed in glutardialdehyde at 3% by 2 hrs; it is pre-fixed in OSO4 at 1%, dehydration in ethanol is performed and included the sample in Epon 812. Are observed the results in a transmission electron microscope type JEOLJEM-1010. Were treated finally, two fragments of the skin sample of each group in the following way: After fixed in glutardialdehyde at 2.5% by 2 hrs, pre-fixed in OSO4 at 1%, dehydrated in ethanol, the samples were positioned on a slide and covered with gold. The results were analyzed using an electron sweep microscope type JSM-5800LV at high vacuum.
3. Results

**Negative control group:** Was observed in the skin’s sample of the rats, tissue’s granulation with scarce infiltrated inflammatory tissue and low density of both fibrocytes and formation of new tissue (Figure 2). **FT groups:** We observed normal epithelium of the epidermis, papillary dermis with very little collagen tissue, fibrocytes, and scarce infiltrated inflammatory tissue compared with the control group (Figure 3). **INF-β groups:** In healing zone was observed abundant inflammatory cells with fibrocytes predominance; also, infiltrated inflammatory tissue shown multinuclear cell formation of foreign body and great destruction of epithelium manifested by a reddish color (Figure 4). **120Hz-ELF group:** We found in this sample scarce infiltrated inflammatory tissue in a similar way to those from negative control and FT groups. Furthermore, we observed less damage in illness tissue (Figure 5).

**Table 1.** Ratios of the Lymphocytes (CD4+)//(CD8+) that measure the immunological impact of Tx

| Treatment (Tx) | (CD4+)//(CD8+) before Tx X ± SD | (CD4+)//(CD8+) after Tx X ± SD | Change during Tx X ± SD |
|---------------|---------------------------------|-------------------------------|------------------------|
| Water         | 3.02 0.078                      | 3.35 0.088                    | 0.328 ± 0.037          |
| TF-I          | 2.85 0.074                      | 3.61 0.084                    | 0.764 ± 0.035          |
| TF-II         | 3.03 0.063                      | 3.45 0.091                    | 0.418 ± 0.034          |
| INF-βI        | 3.06 0.098                      | 3.99 0.084                    | 0.930 ± 0.034          |
| INF-βII       | 3.00 0.079                      | 4.19 0.083                    | 1.187 ± 0.033          |
| 120Hz-EMF     | 2.89 0.099                      | 4.20 0.094                    | 1.317 ± 0.041          |

4. Discussion and conclusions of the Results

The body weight in all the rats treated increased significantly, data not reported in the literature. In the analysis concerning frequency of total lymphocytes, a significant increment was observed in all treated groups starting from the sixth day comparing with control group. It was obtained similar results by Cadossi [8, 13], Mooney [14], Bersani [19], between other, studying the biological effects of ELF-EMF in the IS, confirming the effect of ELF-EMF at the immunological level. Respect to the determination of frequency in lymphocytes (CD4+), it was observed a notable increment in the treatment with both INF-β and 120Hz-EMF groups, and less increase in both TF groups respect negative control. In references [20, 21] was observed the same behavior. Furthermore, they provide new evidence concerning ELF-EMF effects, in the sense that they can affect the (CD4+) expression in cell surface receptors of human peripheral blood mononuclear cells, describing an additional biological activity. Is observed respect the frequency of lymphocytes (CD8+), a significant decrement during the treatments. Behavior that is important to study the immunological impact of the treatments viewed them from the immune-typification point of view, in which we observe that 120Hz-EMF treatment gives us the best value indicating a clear impact over the IS (Table 1), revealing less chronic immune activation, which means less inflammation, observing less damage in illness tissue (Figure 5).
Nevertheless, in literature, it has not been reported the use of INF-β and TF in chronic ulcers, which we studied in the present work, also the use of the ratio (CD4+)/(CD8+) as an indicator of chronic immune activation. It was evident from results that INF-β has a more immunological impact that TF, despite both together with 120Hz-EMF presented the same time for the recuperation of chronic dermal ulcers. The difference between them was that with 120Hz-EMF the illness tissue showed less damage when it was studied the cellularity with sweep microscopy and that 120Hz-EMF treatment presented the highest immunological impact when the ratio (CD4+/CD8+), was considered during treatments, indicating the impact over the IS diminishing the so-called chronic immune activation.

A future task is to characterize the sub-type (Th-I and Th-II) of the CD4+ lymphocytes. Also, determine their cytokine synthesis content for each one of those substances used in the present work and answer the histological changes concerning all treatments [6].

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