Original Article

Evaluation of an Electrospun Fiber Loaded with Ozonated Oil for the Treatment of Leishmaniasis Lesions in an Experimental Model

Daniela Patrícia Tres[1], Carla Diel Fabrini[1], Janaína Fatima da Roza Cunha[1], Juliana Jurumenha Barreto[1], Mariana Pastri[1], Thaís Kaori Katsumata[1], Gabriel Nardi Fraga[2], Thaís Soprani Ayala[1], Douglas Cardoso Dragunski[2], *Rafael Andrade Menolli[1]

[1]Laboratory of Applied Immunology, Center of Medical and Pharmaceutical Sciences, Western Parana State University, Brazil

[2]Center of Engineering and Exact Sciences, Western Parana State University, Brazil

***Corresponding author:** Dr Rafael Andrade Menolli, E-mail: rafael.menolli@unioeste.br

(Received 28 Mar 2025; accepted 21 Dec 2025)

Abstract

Background: Cutaneous leishmaniasis (CL) is a neglected disease whose lesions may cause severe scarring and psychosocial impact. Current treatments often face poor patient adherence due to side effects and dosing. Electrospinning enables the incorporation of low-cost, bioactive compounds. Ozonated sunflower oil has shown potential to accelerate wound healing. This study aimed to evaluate electrospun fibers containing ozonated sunflower oil combined with standard anti-Leishmania therapy in lesions caused by *Leishmania amazonensis* in an animal model.

Methods: BALB/c mice (n=5/group) were infected with *L. amazonensis* (MHOM/BR/1977/LTB0016). Treatments included: meglumine antimoniate alone (MA), MA plus electrospun fiber with ozonated sunflower oil (MOO), MA plus fiber with non-ozonated sunflower oil (MNO) and an untreated control (CTRL). After 30 days, lesion size, parasite load, histology and systemic/local immune parameters were evaluated.

Results: All the groups exhibited lesion growth (MA 12±0.8%, MOO 24±0.4%, MNO 30±0.8 and CTRL 34±0.9%), but the MA group exhibited less growth. However, in the culture of the paws after the treatment, only in the MOO group, promastigotes did not grow, while the other groups reached titers of 1/6 (MA), 1/192 (MNO) and 1/384 (CTRL). Macroscopically, the MA and MOO groups showed complete healing of the lesion. Histologically, the MA group had a thicker epidermis ($p<0.05$) and the MA and MOO groups presented the lowest amount of cellular infiltration.

Conclusion: Treatment of cutaneous leishmaniasis lesions in a mouse model with an electrospun fiber containing ozonated oil improved drug treatment, ameliorating lesions and could be explored as an alternative treatment for this parasitic disease.

Keywords: Electrospun fiber; Cutaneous leishmaniasis; Ozone therapy; Wound healing; BALB/c

Introduction

Leishmaniasis is an infectious, noncontagious disease caused by different species of the protozoan *Leishmania* sp. It is transmitted to humans by the bite of a female mosquito (genus *Phlebotomus* or *Lutzomyia*) (1).

Leishmaniasis is a neglected disease that affects low-income people with limited access to health services. Cutaneous leishmaniasis (CL) is the most common clinical form of leishmaniasis and has the greatest geographic distribution. Approximately 90% of cases present localized, single or multiple lesions and are associated with 15 species of *Leishmania* (2). Furthermore, cutaneous leishmaniasis is considered a dermatological disorder associated with stigma and psychological suffering. Severe scars reduce quality of life directly and indirectly through a process of social rejection and self-depreciation (3).

Treatment for cutaneous leishmaniasis is complex and depends on the species of *Leishmania*, the site of contamination, the clinical form and the age of the patient (4). Few drugs are available, with the first choice being pen-

http://jad.tums.ac.ir
Published Online: December 30, 2025

tavalent antimonials (meglumine antimoniate and sodium stibogluconate), despite their toxicity, parenteral administration and high cost (5, 6). Miltefosine and amphotericin B can still be used, both of which have been reported to have adverse effects (4).

Ozone is a molecule composed of three oxygen atoms and has several biological activities; it acts as a modulator of the immune system, promoting the recruitment of leukocytes and hydrogen peroxide, the release of cytokines and increased phagocytosis (7). The use of ozone stimulates the healing of skin wounds due to its bactericidal and fungicidal effects, stimulates angiogenesis and increases the supply of oxygen to cells, whether through topical or systemic application (8). Due to the high volatility of ozone in gaseous or aqueous form, it is often used in oily form, ozonizing different types of lipids, mainly vegetables. Furthermore, ozonated oils can act by accelerating the healing via the release of epidermal growth factor (EGF), transforming growth factor β (TGF-β), transforming growth factor, vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) (9).

Nanofibers have been explored in tissue engineering due to their ability to mimic the architecture of the extracellular matrix (10) and electrospinning is a simple experimental technique that allows the production of micro/nanofibers from a polymeric solution (11). Furthermore, the electrospinning technique for producing nanofibers based on a biodegradable polymer and associated with a drug has become a promising alternative for the treatment of diseases and acceleration of wound healing (12).

Considering the wound regeneration potential already demonstrated by ozone and electrospun fibers separately, it is imagined that together they may be able to accelerate the healing of skin lesions caused by cutaneous leishmaniasis. To this end, the objective of this work was to evaluate the use of an electrospun polymer of Ecovio® incorporated with ozonated sunflower oil as an adjuvant to the drug treatment with meglumine antimoniate in lesions of cutaneous leishmaniasis in BALB/c mice.

Materials and Methods

Parasites

Leishmania (*Leishmania*) *amazonensis* (MHOM/BR/1977/LTB0016), in promastigote forms, were cultivated and kept via weekly transplants in 199 medium supplied with 10% fetal bovine serum and hemin (5 µg/mL).

Animals

Twenty female BALB/c mice, 6 to 8 weeks old, received regular feed and water *ad libitum* and were maintained in a common cage under a 12-hour light and dark cycle. All procedures were approved by the Ethics Committee on the Use of Animals of UNIOESTE (n° 11/2022) and followed the Brazilian Law for Scientific Use of Animals (Law 11,794, 10/8/2008). Each animal in the cage received a mark, which was used to randomly select the mouse, setting up the treatment groups at random. The animals were randomly separated into four groups with five animals each for the experiment. To calculate the number of animals, a completely randomized design with a normal distribution was used, employing sample size calculation for mean comparison with a significance level of 5% and a test power of 80%, performed using the Bioestat 5.4 program. An n equal to five per group was defined (4 experimental groups).

Ozonated oil obtainment

Commercial sunflower oil (Liza) received 10% distilled water and Tween 80 (0.5%) as emulsifying agents and was ozonated as described by Moureu et al. (13), with modification. An ozone generator (Ozone and Life, mod. 1.5 RM) was used for 15 hours, bubbling in the oil in an ozone output of 99 ppm. A sufficient volume of the same ozonated oil was used in all the experiments.

http://jad.tums.ac.ir
Published Online: December 30, 2025

Measurement of ozone levels in the oil

The ozone levels were indirectly assessed and the hydrogen peroxide (H_2O_2) levels in the oil were determined via an iodometric method following Martinez Tellez et al. (14). To 0.5 g of oil was added a mixture (18 mL glacial acetic acid and 12 mL chloroform) and 0.5 mL of potassium iodide (K.I.) saturated solution. In the sequence, the mixture was incubated in the dark overnight. Next, 30 mL of distilled water was added and a titration with 0.01 M sodium thiosulfate ($Na_2S_2O_3$) was done until the color disappeared. Finally, 5 ml of starch was added and the mixture was titrated until the blue color disappeared. The peroxide values (Pv) were calculated as follows: Pv= 10×V/W, where V is the volume of $Na_2S_2O_3$ in mL and W is the weight of the oil in grams. At the end of the technique, the Pv obtained, in mEq/kg, was converted into the ozone concentration in mg/g of oil, according to Skalska et al. (15). The measurements were performed at least three times to determine the values.

Fabrication of electrospun fibers

For the electrospinning process, Ecovio® (a polymer composed of Polybutylene adipate terephthalate (PBAT) and polylactid acid (PLA)) solutions were prepared at a concentration of 15% (m/v) of polymer, using as solvent a mixture of chloroform and dimethylformamide in proportions (85/15). Then, 10% (v/v) sunflower oil was added to the solution and the mixture was stirred for 24 h to ensure total solubilization of the polymer. In total, three solutions were prepared: one without sunflower oil (solution 1), one containing 10% sunflower oil (solution 2) and one containing 10% ozonated sunflower oil (solution 3). After the solutions were prepared, they were subjected to electrospinning using the following parameters: flow rate of 1.8 mL/h, voltage of 15 kV, collector needle distance of 15 cm and a rotary type collector at 1800 rpm. A total of 5 mL was electrospun to make nonwovens with final dimensions of approximately 15×20 cm. The ozone concentrations of the electrospun fibers containing ozonated sunflower oil were measured in accordance with the item Measurement of ozone levels in the oil.

In vivo and ex vivo experimental procedures

Lesion development

A suspension of *L. amazonensis* (1×10^6 parasites) in phosphate-buffered saline (PBS) was inoculated intradermically (0,1 mL) into the plantar dorsum of the right hind paw of BALB/c mice under anesthesia (ketamine 50 mg/kg plus xylazine 5 mg/kg). Once the lesions emerged (~8 weeks), the animals were randomly separated into four groups (5 animals per group) and treated as follows: Grupo MA (meglumine antimoniate) – Positive control. Treated intraperitoneally with meglumine antimoniate (Sanofi-Aventis Farmacêutica Ltda) (50 mg/kg) once daily for 30 days. Group MOO (meglumine antimoniate plus fiber containing ozonated sunflower oil). Treated intraperitoneally with meglumine antimoniate (Sanofi-Aventis Farmacêutica Ltda) (50 mg/kg) once daily for 30 days and received the ozonated sunflower oil-loaded fiber, applied once, at the beginning of the treatment. Group MNO (meglumine antimoniate plus the natural sunflower oil (non-ozonated) loaded fiber). Treated intraperitoneally with meglumine antimoniate (Sanofi-Aventis Farmacêutica Ltda) (50 mg/kg) once daily for 30 days and received the natural sunflower oil (non-ozonated) loaded fiber, applied once, at the beginning of the treatment. Group CTRL – Negative control. The untreated group was monitored throughout the 30 days of the study period.

Treatment protocol

After the appearance of lesions (~8 weeks), the animals received the treatments. The fibers containing ozonated or non-ozonated oil were put on the lesions on the first day of the treatment. MA was applied to the animals via IP at a dose of 50 mg/kg daily. All the treatments lasted thirty days.

Lesion measurement and animal weight

Measurements and photographic records of the paws were taken by an operator blinded to the groups. Besides, all the analyses after the euthanasia were conducted by blinded operators to the groups. Paw length (mm) was measured via a digital caliper once a week during the treatment period and on the last day of treatment. Photographic records were obtained weekly to monitor the degree of healing of the lesion.

The animals from different groups were weighed at the beginning and end of the treatments. The weights obtained were used to obtain an index. This index was generated by dividing the average final weight of the animals by the average initial weight in each group. An index higher than one means that animals gained weight and if lower than one, the animals in the group lost weight.

Histologic analysis

At the end of the experiment, the animals were euthanized using xylazine (30 mg/kg) and ketamine (270 mg/kg). During necropsy, the paws were collected, fixed in 10% neutral-buffered formalin, decalcified in 5% trichloroacetic acid, routinely processed for paraffin embedding and stained with hematoxylin and eosin (H and E). Qualitative analysis and cellular counting of dermal and epidermal thickness were performed on H and E-stained paw sections via a photomicroscope (Olympus DP71, Miami, FL, USA) at a final magnification of 400x. Five captures from each lesion using Olympus Viewer 2 were taken. Two segments of each image were evaluated with Image-Pro Plus 6.0 software by measuring the epidermis's cross-sectional area (μm^2) and the number of leucocytes in the dermis after calibrating.

Parasite quantification from the culture of the infected paws

A fragment of the lesions was obtained aseptically after cleaning of the area with povidone-iodine (PVPI) and introduced into a microtube with 550 µL of 199 medium. The fragment was then placed in a well containing 1.5 mL of 199 medium and smashed. After maceration, the contents were homogenized using a 1 mL syringe with a needle. The homogenized cells were transferred to a tube with 2 mL of 199 medium. One hundred microliters were transferred to a well, and 200 µL of medium were added to obtain dilutions ranging from 1/3 to 1/384. The plate was incubated in a BOD (Bio-Oxygen Demand) Incubator at 25 °C. The cells were observed under an inverted microscope for thirty days, every ten days, evaluating the presence of viable promastigote forms.

Leucocyte counting

Before the beginning and at the end of the treatment protocol, blood leukocytes were collected by puncturing the tail using scissors and obtaining aliquots of blood, counting the leukocytes in a hemocytometer on microscope at 400x magnification.

Isolation of mouse peritoneal macrophages

To obtain peritoneal macrophages from the animals, peritoneal lavage was used. Ten milliliters of ice-cold PBS was added to the exposed peritoneum, and the cells were detached through massage. Afterward, with the same needle and syringe used for inoculation, the peritoneal lavage was collected and this material was centrifuged at 420 g for 6 minutes at 4 °C.

The pellet was resuspended in 1 mL of Roswell Park Memorial Institute (RPMI) medium with 10% of Fetal Bovine Serum (FBS). The concentration was altered to 2×10^5 cells/ well (200 µL) and the cells were plated in triplicate in a 96-well plate (37 °C; 5% CO_2) and incubated for two hours. The supernatant was subsequently collected and substituted with fresh RPMI medium. The cells were incubated for 48 h under the same conditions, after which the supernatant was obtained and used to measure Nitric oxide (NO) levels.

Measurement of nitric oxide (NO) levels

The production of NO was determined by measuring the nitrite content in the supernatant of each cell culture as described by Green et al. 1982, with some modifications (16). Briefly, 100 µL from each well was reacted with 100 µL of Griess reagent (1:1 solution of 0.1% (w/v) naphthylenediamine in 5% (v/v) ortho-phosphoric acid and 1% (w/v) p-aminobenzenesulfonamide in 5% (v/v) phosphoric acid). After 10 minutes to stabilize the product formation (azo compound), the plate was read in a microplate reader at 550 nm. The data are presented in micromolar (µM) values obtained from a standard curve of sodium nitrite ($NaNO_2$) in RPMI medium.

Measurement of cytokine levels in the sera of the animals

The cytokines TNF-alpha and interleukin 6 (IL-6) were analyzed in the sera of the animals, using enzyme-linked immunosorbent assay (ELISA) kits (PeproTech, Inc.), following the manufacturer's instructions.

Statistical analysis

Data were subjected to normality assessment via the Shapiro-Wilk test and if considered parametric, comparison between the groups was subjected to analysis for comparison of means via the ANOVA test, with Tukey's post hoc test. Once nonparametric, the Kruskal-Wallis test, with Dunn's post hoc test, was evaluated. The comparison between initial and final measures was evaluated by a Paired t-test if the data were parametric and if they were nonparametric, the Wilcoxon test was applied. Data on ozone levels were evaluated via an unpaired t-test. Data from promastigote culture were analyzed with a Fisher's exact test. Analyses were performed using GraphPad Prism 9.5 and Windows Excel software.

Results

Ozone levels in the sunflower oil and the fiber-containing oil

The ozone concentration applied to the ozonated sunflower oil on the first day was 76.53 ±1.3 (mg ozone/g oil), 72.71±2.1 mg/g on the tenth day and 66.84±1.8 mg/g on the 30th day. The peroxide levels of the fibers used for the treatment were measured after six months of storage, showing higher ozone values than the freshly ozonated oil with 86.96±0.9 mg/g. The same methodology was used for the non-ozonated sunflower oil, for which the concentration of ozone reached 11.74±0.6 mg/g. Values of ozone in the ozonated oil on the 30th day were significantly higher than in the non-ozonated oil ($p < 0.001$).

Measurement and aspects of the lesions after the treatment

Figure 1 shows the measurements of the initial and final thickness in the treated and control groups. Initial and final sizes of the MA and CTRL groups showed differences, while the MOO and MNO groups did not. More importantly, no differences were detected between the final measurements of the different groups, showing similar sizes after thirty days of monitoring/treating. MA group presented an initial size of 4.91±2.55 mm and final of 5.61 ±2.33 mm ($p > 0.05$); MOO group measured initially 5.71±1.82 and completed the treatment with the size of 8.16±0.39 mm ($p < 0.05$); MNO group started the treatment with 5.27±2.23 mm and ended the measurements with 7.99±2.66 mm ($p > 0.05$), finally the CTRL group started treatment with the measures of 5.72±2.55 mm and completed with 7.55±2.94 mm ($p < 0.05$). The group that exhibited the lowest difference between the initial and final measurements was that which received only meglumine antimoniate (MA group), followed by the group treated with the fiber loaded with ozonated oil. The lesions in the MA group were 12±0.8% in growth, while the other three were 24±0.4, 30 ±0.8 and 34±0.9% in size. No differences were observed between the initial or final thickness of the groups ($p > 0.05$).

Despite the absence of a difference in paw size after the treatments, the macroscopic characteristics of the lesions differed after the end of the thirty days. As shown in Figure 2, the

http://jad.tums.ac.ir
Published Online: December 30, 2025

aspects of the lesions were better resolved in the MA and MOO groups. Therefore, wound healing improved in the group treated with MA alone or MA plus the fiber loaded with ozonated oil.

Animal weight

The animals from different groups were weighed at the beginning and end of the treatments and the results are shown in Figure 3. The CTRL group was the only group that gained weight after thirty days, as the index obtained by animals of this group was greater than one. The other groups had an index lower than one (1.0), indicating a decrease in weight between the beginning and end of the treatment. The group with the greatest weight loss was the MNO group (8.23±0.03%), which was significantly different from the control group. The MA and MOO groups lost 2.35±0.008 and 5.56 ±0.03%, respectively, of their initial weight. No significant side effects were observed during the treatment, in addition to the effects caused by cutaneous leishmaniasis.

Blood leucocyte counts

Blood leukocyte counts revealed that all groups exhibited an increase in the number of cells between the beginning of treatment (day 0) and the end of treatment (day 30). All the groups exhibited significant differences between the two observation periods (Fig. 4). Furthermore, no differences were observed between the groups in the initial or final counts, demonstrating a similarity in these numbers.

Culture of promastigotes from infected paws

Paws were removed from the animals after they were euthanized and one piece was subjected to culture. After 10 days of growth, the cultures of paws from the MA group were positive, i.e., parasite growth was observed until a dilution of 1/6. The growth in the MNO group reached 1/192. The culture from the paws of the CTRL groups achieved the highest dilution and parasites were observed until the last dilution (1/384). However, in the MOO culture, no parasites were observed at any dilution, demonstrating that the combination of the reference drug and the membrane loaded with ozonated oil was the most effective at eliminating *L. amazonensis* during treatment. An analysis using Fisher's exact test revealed a significant difference between the groups ($p< 0.0001$).

Histological aspects

The thickness of the epithelium was determined via histological analysis (Fig. 5a) and the group with the greatest thickness was the MA group, which was significantly greater than that of the CTRL group.

The CTRL group presented a thinner epithelium, compatible with active *L. amazonensis* infection accompanied by ulceration and necrosis (Fig. 2). Groups treated with the fiber obtained an intermediate result, with a thickness higher than nontreated, but lower than meglumine-treated, with an increase in those that used a fiber ozone-loaded (MOO group). However, similar results were obtained for cellular infiltration in the dermis between the MA and MOO groups (Fig. 5b), in which cellular infiltration was significantly lower than that in the other two groups: MNO (treated with meglumine antimoniate plus a fiber without ozone) and CTRL (untreated). These histological findings, together with the culture results, showed the positive influence of ozonated oil-loaded fiber on the recuperation of leishmaniasis lesions.

Nitric oxide and cytokine dosages

Systemic interference from the treatments was evaluated in infected animals by measuring the concentration of nitric oxide (NO) in peritoneal macrophages and the concentration of interleukin 6 (IL-6) in the sera of the mice. The NO results are presented in Fig. 6a. A higher concentration of NO was detected in the CTRL group than in the two other fiber-treated groups (MOO and MNO). The quantities in the CTRL group were similar to those in the group treated with only meglumine antimoniate (MA).

IL-6 was produced at higher concentrations in the MA groups than in the control group (Fig. 6b), and despite no significant difference being detected, the MOO group had a lower concentration of this cytokine. Therefore, a similar panel was generated for NO and IL-6 production, in which the two groups treated with ozonated oil-loaded fiber presented lower concentrations of these immune metabolites.

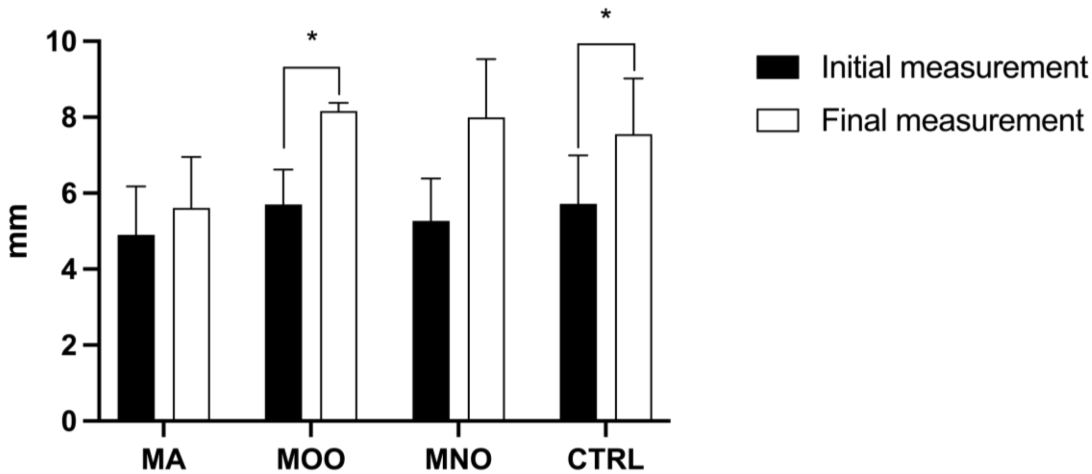

Fig. 1. Initial and final thickness measurements (mm)(mean±SEM) of the infected paws from BALB/c mice (n=5 per group) from the first to the last day (30th) of the experiment. Animals were infected with *Leishmania amazonensis* (1×10^6 parasites) intradermally (0.1 mL). MA: infected animals were treated with meglumine antimoniate; MOO: infected animals were treated with meglumine antimoniate plus fiber combined with ozonated sunflower oil; MNO: infected animals were treated with meglumine antimoniate and fiber combined with nonozonated sunflower oil; CTRL: infected animals were untreated. There was no significant difference between initial and final measures of the different groups (ANOVA one-way). Differences were observed between initial and final measures inside the groups MOO and CTRL (Paired t-test). *p< 0.05. Initial measurement – lesions were measured at the beginning of the treatment (day 0); Final measurement - lesions were measured at the end of the treatment (day 30)

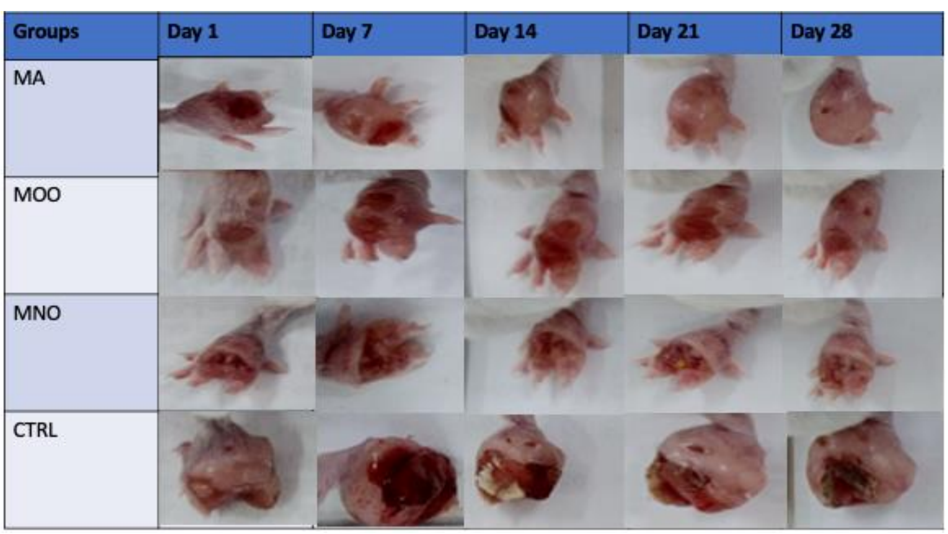

Fig. 2. Macroscopic images of the infected paws from the first to the 28^{th} day of the experiment. These images are representative of each group. Animals were infected with *Leishmania amazonensis* (1×10^6 parasites) intradermally (0.1 mL). MA: infected animals were treated with meglumine antimoniate; MOO: infected animals were treated with meglumine antimoniate plus fiber combined with ozonated sunflower oil; MNO: infected animals were treated with meglumine antimoniate and fiber combined with nonozonated sunflower oil; CTRL: infected animals were untreated

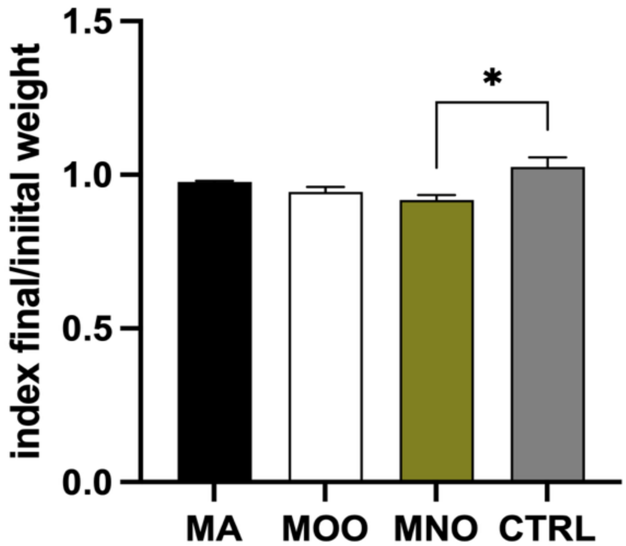

Fig. 3. Index (mean±SEM) generated by a ratio between the average of the animals' weight at the end of the experiment and the average of the animals' weight at the beginning of the experiment in each group (n=5 per group). An index higher than one means that animals gained weight at the end of the experiment, and if lower than one, the animals in the group lost weight. Animals were infected with *Leishmania amazonensis* ($1x10^6$ parasites) intradermically (0.1 mL) and treated as follows - MA: infected animals were treated with meglumine antimoniate; MOO: infected animals were treated with meglumine antimoniate plus fiber combined with ozonated sunflower oil; MNO: infected animals were treated with meglumine antimoniate and fiber combined with nonozonated sunflower oil; CTRL: infected animals were untreated. ANOVA one-way - *p< 0.05

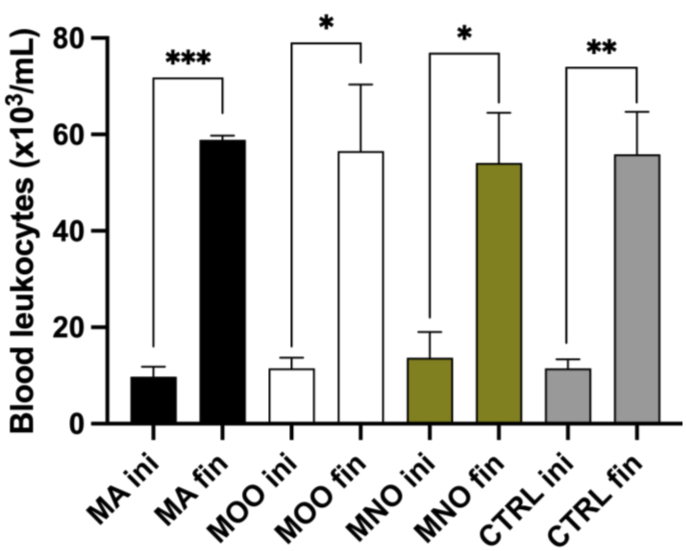

Fig. 4. Initial and final blood leukocyte counting (mean±SEM) of BALB/c mice (n=5 per group) infected with *L. amazonensis* and treated. The initial counting (INI) was done on the first day of the treatment and the final counting (FIN) was done on the last day (30^{th}) of the experiment. Animals were infected with *Leishmania amazonensis* ($1x10^6$ parasites) intradermically (0.1 mL) and treated as follows - MA: infected animals were treated with meglumine antimoniate; MOO: infected animals were treated with meglumine antimoniate plus fiber combined with ozonated sunflower oil; MNO: infected animals were treated with meglumine antimoniate and fiber combined with nonozonated sunflower oil; CTRL: infected animals were untreated. There was no significant difference between initial and final leukocyte counting of the different groups (ANOVA one-way). Differences were observed between initial and final counting inside the groups (Paired t-test). *p< 0.05, **p< 0.01 and ***p< 0.001. INI – initial measurement; FIN – final measurement

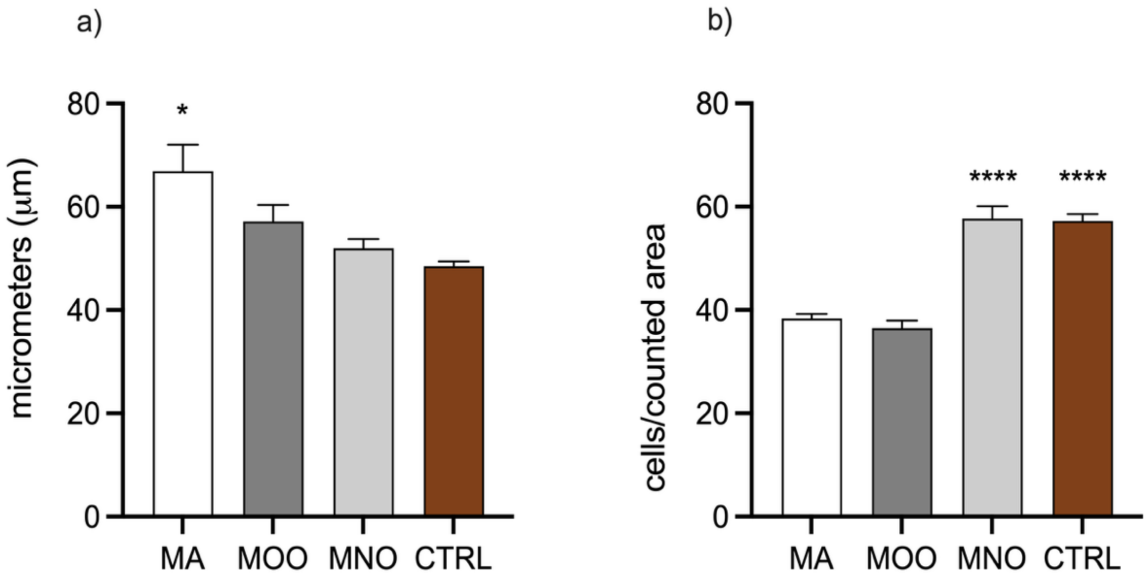

Fig. 5. Histological analysis of the infected paws of the animals. a) Epithelial thickness in micrometres (mean±SEM) (n=5 per group). b) Dermis cellular infiltrate (mean±SEM) (n=5 per group). The histological techniques used to obtain these results are described in the Materials and Methods section. MA: animals infected and treated with meglumine antimoniate; MOO: animals infected and treated with meglumine antimoniate and fiber combined with ozonated sunflower oil; MNO: animals infected and treated with meglumine antimoniate and fiber combined with nonozonated sunflower oil; CTRL: animals infected and untreated. A) * Significant difference compared to the CTRL group. B) ****Significant difference compared to the MA and MOO groups. (*p< 0.05; ****p< 0.0001)

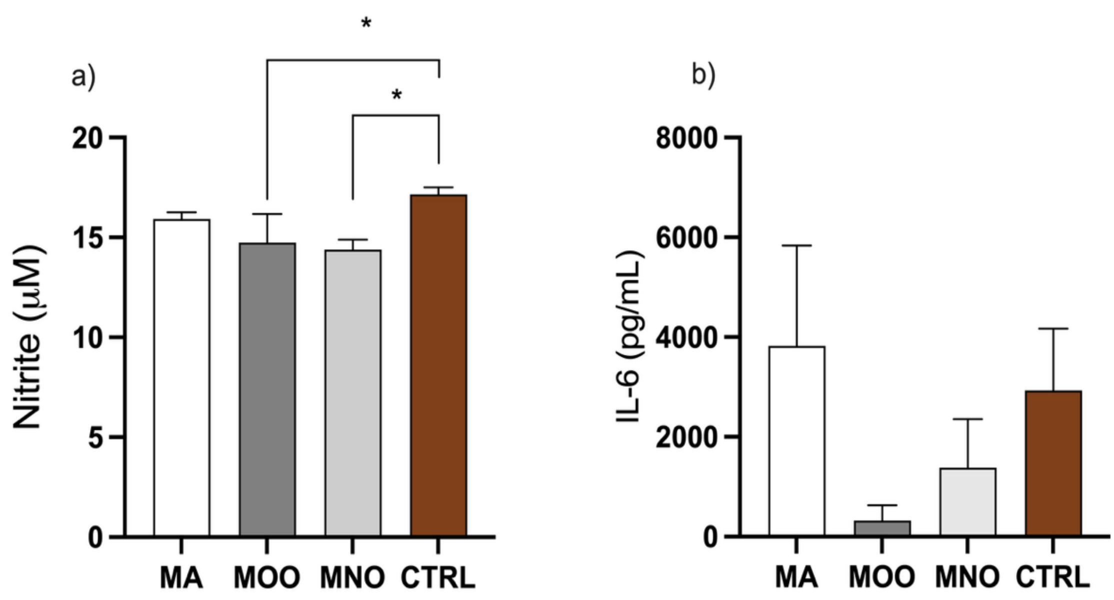

Fig. 6. a) Detection of nitric oxide (mean±SEM) production in the culture of peritoneal macrophages obtained from BALB/c mice infected with *L. amazonensis* and treated (n=5 per group). b) Detection of IL-6 (mean±SEM) production in the sera obtained from BALB/c mice infected with *L. amazonensis* and treated (n=5 per group). Animals were infected with *Leishmania amazonensis* ($1x10^6$ parasites) intradermically (0.1 mL) and treated as follows - MA: infected animals were treated with meglumine antimoniate; MOO: infected animals were treated with meglumine antimoniate plus fiber combined with ozonated sunflower oil; MNO: infected animals were treated with meglumine antimoniate and fiber combined with nonozonated sunflower oil; CTRL: infected animals were untreated. a) *Significant difference compared to the CTRL group (Kruskal-Wallis test). *p< 0.05)

Discussion

Producing polymer nanofibers through electrospinning is one of the most promising methods for promoting wound healing (17), mainly due to its potential to produce materials consisting of biomimetic nanofibers from several natural and synthetic polymers with biological activities; thus, electrospinning has potential for latent application in tissue engineering in addition to being a simple, versatile and economical alternative (18).

The use of fibers against cutaneous leishmaniasis is promising, and the use of several compounds was tested together with standard drugs (19). An example is chitosan, which, combined with meglumine antimoniate (MA), significantly diminished lesions of cutaneous leishmaniasis (20).

It is possible to electrospun nanofibers with compositions, structures and architectures similar to those of the extracellular matrix (ECM) in skin tissue. These fibers can modulate skin cell responses, accelerating the migration, differentiation and deposition. These characteristics allow the production of sutures, dressings, dermal substitutes and skin tissues designed for wound healing (17). Although many studies have focused on electrospun fibers loaded with essential oils, no study has evaluated vegetal oils, which are economical and easy materials for accelerating cicatrization (21).

Ozone is an unstable gas, but it can be stabilized and applied through ozonated oils, creams and lotions. The concentration of ozone detected in the oil remained stable through the thirty days, corroborating the results already found in the literature (22, 23). This non-significant decrease in the ozone in the oil after thirty days is important because the active ingredient is not lost during the treatment period, preventing the patient from using only sunflower oil at the end of thirty days. Furthermore, the ozone concentrations reached by the ozonated and non-ozonated oils were significantly different (data not shown), revealing that the ozonation process was successful. Although no methodology was found to measure oil in the fibers after charging, we detected the same level of ozone in the fiber storage for six months and in the sunflower oil immediately after ozonation. Despite the measurement technique dosing the PV and not the ozone directly, this showed that the incorporation of ozonated sunflower oil in an electrospun fiber remains stable even when stored for six months, showing the potential for popular use of the fiber.

Determining the ozone concentration in a solution is important to evaluating the possible risks or benefits. In this way, using ozone in low concentrations can not initiate oxidative reactions, which will result in cellular pathways or medicinal effects, while a high level could surpass the physiologic antioxidant system, limiting its benefits. An intermediate concentration of peroxide in the oil was determined to be ideal for accelerating wound healing (500–800 mEq/1000 gr), which was the concentration obtained in the present study (24).

Existing treatments for CL require a long period, are painful and are not resolutive in many situations. That way, bring better resolution to the CL lesions necessitating therapies more effective than the current arsenal (25).

The animals treated with ozonated oil-loaded fiber and MA (MOO group) and those treated only with MA (MA group) exhibit improved wound healing, decreased lesion development and decreased parasite growth. The evolution of the lesions shows that after inoculation of the promastigotes by the sandflies, they will infect macrophages and proliferate into amastigotes. The development of the skin lesions is frequently restricted to the site of inoculation, where, at first, a nodule forms and evolves chronically and may ulcerate as the inflammatory response increases (26). The spectrum of CL present in its extremities includes the more severe forms, showing a more vigorous lesion with more parasites in a predominant humoral response; on the other hand, a polarized cellular response may result in an immunopathological injury (27). Therefore, controlling the number of parasites and establishing effective wound healing are essential for CLs, and these can be obtained by modulating therapy. The results showed that only animals treated simultaneously with MA and fiber loaded with ozonized oil eliminated the parasites from the lesions, demonstrating that the incorporation of fiber favors a better resolution of the infection.

We observed that all animals subjected to treatment lost weight, which corroborates one of the adverse events of using meglumine antimoniate, which can lead to anorexia (28) and thus weight loss. The animals that did not receive treatment gained weight at the end of the study. Malnutrition makes individuals vulnerable to infection, consequently worsening their nutritional status, which has been associated with the risk of developing mucosal leishmaniasis and has an influence on the course of American cutaneous leishmaniasis, with a decreased percentage of healing skin lesions and a prolonged healing time for mucosal lesions (29).

Although occasional leukocytosis may occur as one of the main effects of isolated treatment with meglumine antimoniate, which is different from the findings of other studies investigating the hematological profile of patients infected by different species of *Leishmania* before treatment and after treatment with pentavalent antimony, 14 of the 15 patients evaluated had no difference in leukocyte or platelet counts before or after treatment with meglumine antimoniate (30).

Exposure to ozonated oil did not alter leukocytosis in treated animals, which is different from the data in the literature, in which exposure to ozone regulates the inflammatory response, reducing leukocyte infiltration (31). This anti-inflammatory effect occurs due to the increased release of Transforming Growth Factor beta (TGF-β), which, among its functions, reduces the chemotaxis of cells, limiting the inflammatory phase and leading to the proliferative phase and is also responsible for fibroblast proliferation and extracellular matrix production (32). However, as the electrospun fiber containing ozonated sunflower oil was associated with intraperitoneal treatment with meglumine antimoniate, this increase in leukocytes may be related to the second treatment.

Evaluation of histological data demonstrated that the use of ozonated-loaded fiber together with MA improved leishmaniasis lesions, as indicated by decreased cellular infiltration and increased thickness of the epithelium, two clear signals of a more advanced state of wound healing (33). Nevertheless, the presence of many leukocytes does not imply parasite control, since *L. amazonensis* can cause anergy in lymphocytes, impairing their function, a situation observed in the CTRL group (34).

A qualitative analysis of histological sections showed that the MA and MOO groups developed more continuous epithelia, similar to the findings of another study in which leishmaniasis lesions were treated with aqueous ozone (35). Another study revealed that the greatest degree of re-epithelialization occurred in animals treated with ozonized sunflower oil, which is a positive factor for tissue re-epithelialization (36). The low thickness of the epithelium in the MNO group did not corroborate the findings of a study in which the degree of necrosis was evaluated in animals treated with linoleic acid, a component of sunflower oil. This study showed that linoleic acid prevented necrosis in distal and proximal fragments, although the difference was not significant (37). Thus, the presence of oil in the fiber acted differently than it did when applied alone. We can relate these data to the irregularity of the epithelium, as necrosis is a process of cell death that leads to tissue destruction.

In addition to the decrease in cellular infiltration in lesions, treatment with ozonated oil-loaded fiber interfered with systemic parameters, significantly decreasing the level of NO

http://jad.tums.ac.ir
Published Online: December 30, 2025

in peritoneal macrophages in comparison with that in the CTRL group, which was not observed in the MA group (treated only with meglumine antimoniate). Furthermore, the animals treated with the fiber containing ozonated oil had lower IL-6 levels than did the MA and CTRL groups and despite not significantly affecting IL-6 levels, these findings agree with previous data. Therefore, ozone can cause a decrease or increase in NO secretion, acting in its pathway and causing immunomodulation depending on the dose used (38).

One limitation of this study is the absence of amastigote counting on the animals' paws after the treatments ended, which makes it difficult to compare their effectiveness. Although we quantified the promastigotes in the lesion, determining the intracellular form of *Leishmania* would be more conclusive.

Conclusion

Treatment with an electrospun fiber containing ozonated oil, when combined with meglumine antimoniate, led to complete parasite clearance from lesions in a mouse model of cutaneous leishmaniasis, while also improving histological markers of wound healing. This approach holds promise for reducing treatment failure and transmission potential.

Acknowledgements

The authors thank BASF™ for the polymer used for the fabrication of the fiber. C.D.F. received a Master's Scholarship and T.S.A. received a Pos-Doctoral Scholarship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior–CAPES, a Brazilian Governmental Agency.

Ethical considerations

All procedures were approved by the Ethics Committee on the Use of Animals of UNIOESTE (n° 11/2022) and followed the Brazilian Law for Scientific Use of Animals (Law 11,794, 10/8/2008).

Conflict of interest statement

The authors declare there is no conflict of interest.

References

1. Steverding D (2017) The history of leishmaniasis. Parasit Vectors. 10(1): 1–10.
2. PAHO-PanAmerican Health Organization (2018) Leishmaniasis: Epidemiological Report of the Americas. PAHO/WHO Institutional Repository.
3. Bennis I, De Brouwere V, Belrhiti Z, Sahibi H, Boelaert M (2018) Psychosocial burden of localised cutaneous Leishmaniasis: A scoping review. BMC Public Health. 18(1): 1–12.
4. Barba PJ, Morgado-Carrasco D, Quera A (2022) Tratamiento de leishmaniasis cutánea infantil con miltefosina. Actas Dermosifiliogr. 113(8): 827–831.
5. Iranpour S, Hosseinzadeh A, Alipour A (2019) Efficacy of miltefosine compared with glucantime for the treatment of cutaneous leishmaniasis: a systematic review and meta-analysis. Epidemiol Health. 41: e2019011.
6. Castro MDM, Rode J, Machado PRL, Llanos-Cuentas A, Hueb M, Cota G, Rojas IV, Orobio Y, Sarmiento OO, Rojas E, Quintero J, Pimentel MIF, Soto J, Suprien C, Alvarez F, Ramos AP, Arantes RBS, da Silva RE, Arenas CM, Vélez ID, Lyra MR, Saravia NG, Arana B, Alexander N (2023) Cutaneous leishmaniasis treatment and therapeutic outcomes in special populations: A collaborative retrospective study. PLoS Negl Trop Dis. 17(1): e0011029.
7. Pivotto AP, Banhuk FW, Staffen IV, Daga MA, Ayala TS, Menolli RA (2020) Clinical Uses and Molecular Aspects of Ozone

Therapy: A Review. Online J Biol Sci. 20(1): 37–49.
8. Néri J dos SV, Lomba E, Karam AM, Reis SR de A, Marchionni AMT, Medrado ARAP (2017) Ozone therapy influence in the tissue repair process: A literature review. J Oral Diagn. 2: 1–6.
9. Lim Y, Lee H, Woodby B, Valacchi G (2019) Ozonated oils and cutaneous wound healing. Curr Pharm Des. 25(20): 2264–2278.
10. Xue J, Xie J, Liu W, Xia Y (2017) Electrospun nanofibers: new concepts, materials and applications. Acc Chem Res. 50(8): 1976–1987.
11. Miguel SP, Figueira DR, Simões D, Ribeiro MP, Coutinho P, Ferreira P, Correia IJ (2018) Electrospun polymeric nanofibres as wound dressings: A review. Colloids Surf B Biointerfaces. 169: 60–71.
12. Fabrini CD, Ayala TS, Menolli RA (2024) Electrospun polymers in cutaneous wound healing and delivery systems-a review. Int J Polym Mater Polym Biomater. 74 (12): 1157–1167.
13. Moureu S, Violleau F, Ali Haimoud-Lekhal D, Calmon A (2015) Ozonation of sunflower oils: Impact of experimental conditions on the composition and the antibacterial activity of ozonized oils. Chem Phys Lipids. 186: 79–85.
14. Martinez Tellez G, Ledea Lozano O, Díaz Gómez MF (2006) Measurement of Peroxidic Species in Ozonized Sunflower Oil. Ozone Sci Eng. 28(3): 181–185.
15. Skalska K, Ledakowicz S, Perkowski J, Sencio B (2009) Germicidal properties of ozonated sunflower oil. Ozone Sci Eng. 31 (3): 232–237.
16. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. Anal Bioch. 126(1): 131–138.
17. Chen S, Liu B, Carlson MA, Gombart AF, Reilly DA, Xie J (2017) Recent advances in electrospun nanofibers for wound healing. Nanomedicine. 12(11): 1335–1352.
18. Costa RGFF, De Oliveira JE, De Paula GF, De Picciani PHS, De Medeiros ES, Ribeiro C, Mattoso LHC (2012) Electrospinning of Polymers in Solution. Part I: Theoretical Foundation. Polímeros 22 (2): 170–177.
19. Moura LM de, Santos KLM, Passos JAR dos, Silva GF de V, Neres CA da S, Castro WPD de, Santos LGS, Silva DGR (2025) Application of electrospun nanofibers in the treatment of leishmaniasis: a mini-review. Contr L Cienc Socia. 18 (5): e17776.
20. Mohebali F, Aghabarari B, Vaezi MR, Zarei Z, Hassanpour G, Alizadeh Z, Latifi A, Mohebali M (2025) Biomacromolecule chitosan carrying meglumine antimoniate coated on a silver/polyurethane nanocomposite as a wound dressing: Therapeutic efficacy on cutaneous leishmaniasis caused by *Leishmania major* in BALB/c mice. Int J Biol Macromol. 1: 307.
21. Hu X, Liu S, Zhou G, Huang Y, Xie Z, Jing X (2014) Electrospinning of polymeric nanofibers for drug delivery applications. J Control Release. 185(1): 12–21.
22. Orlandin JR, Machado LC, Ambrósio CE, Travagli V (2021) Ozone and its derivatives in veterinary medicine: A careful appraisal. Vet Anim Sci. 13: 100191.
23. Valacchi G, Fortino V, Bocci V (2005) The dual action of ozone on the skin. Br J Dermatol. 153(6): 1096–1100.
24. Valacchi G, Lim Y, Belmonte G, Miracco C, Zanardi I, Bocci V, Travagli V (2011) Ozonated sesame oil enhances cutaneous wound healing in SKH1 mice. Wound Repair Regen. 19(1): 107–115.
25. Rajasekaran R, Chen YPP (2015) Potential therapeutic targets and the role of technology in developing novel antileishmanial drugs. Drug Discov Today. 20(8): 958–968.

26. Noronha LPT, Martins MDA, Castro-Junior AB, Thorstenberg ML, Costa-Soares L, Rangel TP, Carvalho-Gondim F, Rossi-Bergmann B, Savio LEB, Canetti CCA, Coutinho-Silva R (2023) Cysteinyl-leukotrienes promote cutaneous Leishmaniasis control. Front Cell Infect Microbiol. 13: 1192800.
27. Scott P, Novais FO (2016) Cutaneous leishmaniasis: immune responses in protection and pathogenesis. Nat Rev Immunol. 16(9): 581–592.
28. Firdous R, Yasinzai M, Ranja K (2009) Efficacy of Glucantime in the treatment of Old World cutaneous leishmaniasis. Int J Dermatol. 48(7): 758–762.
29. Oliveira AGL, Brito PD, Schubach AO, Oliveira RVC, Saheki MN, Lyra MR, Salgueiro MM, Terceiro BF, Pimentel MIF, Vasconcellos EC, Vlete-Rosalino CM (2013) Influence of the nutritional status in the clinical and therapeutical evolution in adults and elderly with American tegumentary leishmaniasis. Acta Trop. 128(1): 36–40.
30. Espir TT, Guerreiro TS, Naiff MF, Figueira LP, Soares FV, da Silva SS, Franco AMR (2016) Evaluation of different diagnostic methods of American Cutaneous Leishmaniasis in the Brazilian Amazon. Exp Parasitol. 167: 1–6.
31. Pivotto AP, de Souza Lima LB, Michelon A, Ferreira CZP, Gandra RF, Ayala TS, Menolli RA (2024) Topical application of ozonated sunflower oil accelerates the healing of lesions of cutaneous leishmaniasis in mice under meglumine antimoniate treatment. Med Microbiol Immunol. 213(1): 4.
32. Sanguanini RC, Bento MF, de Oliveira E, Arnhold E, Faleiro MBR, Franco LG, Matos MPC, Moura VMBD (2020) Ozonized solutions favor the repair of experimentally induced skin wounds in rats. Pesquisa Veterinaria Brasileira. 40(11): 914–921.
33. Eming SA, Martin P, Tomic-Canic M (2014) Wound repair and regeneration: Mechanisms, signaling and translation. Sci Transl Med. 6(265): 1–36.
34. Sandoval Pacheco CM, Araujo Flores GV, Gonzalez K, Castro Gomes CM, Passero LFD, Tomokane TY, Sosa-Ochoa W, Zúniga C, Calzada J, Saldaña A, Corbett CEP, Silveira FT, Laurenti MD (2021) Macrophage polarization in the skin lesion caused by Neotropical species of *Leishmania* sp. J Immunol Res. 2021: 5596876.
35. Cabral IL, Utzig SL, Banhuk FW, Staffen IV, Loth EA, de Amorim JPA, Negretti F, Gandra RF, Ayala TS, Menolli RA (2020) Aqueous ozone therapy improves the standard treatment of leishmaniasis lesions in animals leading to local and systemic alterations. Parasitol Res. 119(12): 4243–4253.
36. Nakamura AM, Costa MC da, Gómez JLÁ, Paiva FN de, Nardi ABD (2023) Reparação do retalho axial dorsal em ratos tratados com óleo ozonizado. Med Vet (UFRPE) [Internet]. 17(1): 37–45.
37. Silva MLA, Lauz S, Silva VD, Araujo AMW, Silva MDA, Tatekawa LA, Danielski TP (2013) Evaluation of topic linoleic acid application on random-pattern skin flaps in rats. Braz J Plast Surg. 28(4): 643–650.
38. Sagai M, Bocci V (2011) Mechanisms of action involved in ozone therapy: Is healing induced via a mild oxidative stress? Med Gas Res. 1(1): 1–18.