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Effect of the Ozonization Degree of Emu Oil over Healing: An Emerging Oxidation Treatment

Daniel Martin Márquez López, Tomás A. Fregoso-Aguilar, Jorge A. Mendoza-Pérez and Sergio O. Flores-Valle

Abstract

This chapter deals with the ozonization process of the emu oil, the objective of this study was to quantitatively determine the peroxide value (PV) to measure the degree of ozonation, the peroxide value measures the number of peroxide groups in the ozonized oil. The ozone oxidizes the unsaturated chemical functions present in the oil giving a high cure rate for epidermal wounds. The healing process is not completely understood and there are different approaches, therefore, it was determined qualitatively if it has healing and inflammation properties, but the results of our studies have shown that the length and width of the wounds were healing quickly thanks to the peroxidation rate of the oils. In addition, both tests were correlated to obtain a greater appreciation of their functions, the mechanism involves a decrease in the inflammation of the wounds and stimulates the process of scar formation.

Keywords: ozonization, emu oil, peroxide value, healing, inflammation

1. Introduction

The skin is a fibroelastic membrane, considered the “outer covering of the body”; It is an organ that performs a wide range of functions including thermoregulation, protection against external aggressions, the absorption of ultraviolet radiation and the production of vitamin D. Additionally, it has an important function of immune recognition, it is an effective protection barrier against pathogenic microorganisms, being the largest organ of the integumentary system and a powerful receptor of sensory stimuli [1]. The frequent exposure to environmental aggressions makes this organ susceptible to suffer injuries that compromise its integrity altering the normal development of its functions [2]. One of the factors that compromise the continuity of this tissue are chronic wounds such as pressure ulcers (PPU), which have been a public health problem that mainly affects those persons who must remain in bed for long periods of time as consequence of chronic diseases [3] or acute dermatitis [4], which affects all human races and more frequently women. Pressure ulcers (PPU) can be presented at any age; however, it predominates in childhood, being more frequent before 5 years of age and persists in adulthood in up to 60–70% of patients [5]. Emu oil is known for the use that the natives of Australia gave it as a remedy to alleviate different ailments for 200 years [6]. In the First World War, ozonated oils were used for therapeutic purposes, as well as the healing of wounds and fissures [7].

Ozonated oils showed their role as modulators of wound healing [8]. The reaction of ozone with the unsaturations present in the fatty acids and other free acids of natural oils generate products such as ozonides and peroxides with germicidal activity and tissue regeneration [9]. The application of ozonated vegetable oil for wound healing quantification in mice, has shown that this oil must be ozonated until a “mean degree” of peroxidation is reached ($PV = 1631 \pm 64 \text{ mEq/kg}$) [10]. On the other hand, the periodic application of completely ozonated olive oil (iodine value = 0) caused in the skin hypersensitivity to contact and loss of hair in the application zone [11]. Furthermore, it is commonly observed that these ozonated oils present a delayed action [12]. A few crude oils from vegetable origin have the property of healing, once ozonized they acquire this property [13]. The search in the main platforms of scientific information on the ozonization of oils of animal origin throws information that lacks depth to know the effects of the products or by-products in the human body, which does not allow to find a relationship between different ozonized oils, in order to develop a synthetic ozonized oil. Therefore, a need is created to develop research that help to clarify the mechanisms of action of ozonized animal oil, for its greater compression and create the possibility of application. The objective of this work is to quantitatively determine the peroxide value (PV) to measure the degree of ozonation, determine qualitatively if it has healing and inflammation properties, in addition, correlate both tests to obtain a greater appreciation of its functions.

2. Methodology and materials

2.1 Ozonization of emu oil

The experiments were carried out at pressure and room temperature in a semi-continuous type reactor. A constant temperature of $\pm 2^\circ\text{C}$ below the melting temperature of 16°C was maintained.

The ozone is fed continuously and is bubbled to the oil contained in a jacketed reactor, water exert as cooling fluid through a recirculation bath. The reactor is designed in the lower part with a porous ceramic plate (diffuser), an output at the top for monitoring the remain ozone leaving the reactor and has a step valve used to obtain samples at 2, 5, 7 and 10% of ozone.

Ozone is generated from oxygen (acquired from INFRA) with a purity of 99.5%, by means of an ozone generator “AZCO”. The refined grade A Willow Springs emu oil was used as raw material, which was applied the extraction technology as described by Marquez [14]. The ozone/oxygen mixture from the generator is introduced to the reactor from the bottom and is evenly distributed in the aqueous solution by means of the diffuser. In the upper part of the reactor there is an output that is connected to a gas phase ozone analyzer BMT-930 connected to a computer that receives the data to be processed in “MATLAB”, and generates a graph of the concentration of ozone at the exit of the reactor against time (ozonogram) [7, 15]. The working conditions were: oil weight: 9 g, initial ozone concentration: $30 \pm 0.5 \text{ mg/L}$, ozone flow: 0.5 L/min .

2.2 Peroxide value (PV)

This method is based on the determination in the test solution of the amount of peroxides contained by means of a titration based on the ISO 3960: 2017 standard.

The peroxide index indicates the milliequivalents of oxygen in the form of peroxide per kilogram of fat or oil.

A mass of $5.0 \pm 0.05 \text{ g}$ of sample is determined inside the flask, 30 cm^3 of acetic acid-chloroform solution is added and stirred until the sample is completely dissolved.

With a Mohr pipette, 0.5 ml of saturated potassium iodide solution is added; it is stirred and allowed to stand for 1 min, after which 30 ml of water are added. It is slowly and carefully titrated with 0.1 N sodium thiosulfate solution; shake vigorously after each addition, until it has a slightly yellow coloration; then add 0.5 ml of starch indicator solution and titration is continued without stirring until the blue color disappears. If the 0.1 N solution of sodium thiosulfate is less than 0.5 ml, repeat the determination using 0.01 N sodium thiosulfate solution.

Perform a blank test in the same conditions in which the sample test was carried out. In addition, the milliliters of 0.1 N thiosulfate solution used in the titration should be noted in each case and should not exceed 0.1 ml of thiosulfate. The determinations are made in duplicate at least [16].

The peroxide value is calculated by expressing the milliequivalents of peroxide contained in a kilogram of fat or oil by means of the following equation:

$$PV = (A - A_1) \times N \times \left(\frac{1000}{M} \right) \quad (1)$$

where PV = peroxide value; A = milliliters of sodium thiosulfate solution spent in the titration of the sample; A₁ = ml of sodium thiosulfate solution spent in the titration of the blank; N = normality of the sodium thiosulfate solution; M = mass of the sample in grams.

2.3 ³¹P NMR

³¹P NMR analysis was performed in triplicate and was based on the method of Lehnhardt. A detergent solution was prepared containing: sodium cholate (10% w/w), EDTA (1% w/w) and phosphonomethylglycine (PMG) as an internal standard for quantification (0.3 g/l); pH was adjusted to 7.1 using sodium hydroxide. The detergent solution was an aqueous solution containing 20% D₂O for deuterium field-frequency lock capability. Sample was mixed with detergent solution (750 µl) by vortexing, then dispersed by ultrasonication with occasional shaking at 60°C for up to 10 min. The amount of sample used depended on its phospholipid content (lecithin standard 15 mg, cream polar lipid 15 mg, BPC60 powder 50 mg, BPC60 lipid 15 mg, lipid-depleted BPC60 residue powder 70 mg, PC700 20 mg, beta serum powder 60 mg, liquid beta serum 200 µl in 500 µl detergent). When required, pH adjustment was made with aqueous NaOH after the sample was fully dispersed in the detergent. The solution was then transferred to a 5 mm NMR tube for analysis. The mixture was then transferred to a 5 mm NMR tube and the ³¹P-NMR spectra were recorded. The NMR spectral data were acquired in a Bruker 400 MHz system using a delay of 25 s between 90° pulses and a line widening of 4.0 Hz. A minimum of 200 transients were acquired for each sample at room temperature [17]. The ³¹P-NMR had a standard deviation of ±1.20%. A minimum of 200 transients were acquired for each sample at room temperature [17].

The chemical species at approximately 132.2 ppm had two TMDP groups and, therefore, two phosphorus atoms bound to it, doubling the NMR signal. For this reason, to obtain the moles of water, the peak area at approximately 132.2 ppm was halved and then it was added to the peak area at approximately 15.9 ppm.

2.4 Intensive wound healing activity in vivo

2.4.1 Experimental species

Male NIH mice of 60 days of age, weighing between 25 and 30 g, were maintained in separate cages in a room under controlled conditions with temperature

at $23 \pm 2^\circ\text{C}$, in a light cycle for 12 hours: dark cycle for 12 hours with free access to water and full commercial food.

The mice were used after a 3-day acclimation period to the laboratory environment.

Throughout the experiments, the animals were processed in accordance with ethical guidelines for the care of laboratory animals. The study was approved by the Brazilian College of Animal Experimentation (BCAE) protocol n° 050/2012.

2.4.2 Experimental groups

The animals were divided into five groups consisting of 6 animals: Group A: untreated mice (control).

Group B–E: cutaneous wounds treated topically with a single application of approx. 0.074 g of OEO at 2%, OEO at 5%, OEO at 7% and OEO at 10% respectively.

Histomorphometric and histopathological analyzes were performed on the 3rd postoperative day. The animals of each subgroup were euthanized on the pre-established day for the end of the experiment with an intraperitoneal injection of pentobarbital in deep anesthesia.

2.4.3 Model for split skin wounds

This test was carried out according to the methodology described by Pradeep et al. [18] with some modifications. To perform the surgical procedure, the animals were weighed and then anesthetized with a specific dose of pentobarbital (60 mg/kg BW). After being placed in the prone position, the animals were shaved with a comb of the 1.2 mm hair removal machine. The antisepsis was performed with 70% alcohol along the dorsal midline of the cervical region. A cylindrical fragment of the skin was removed from the midline of the dorsal region with a 10 mm diameter biopsy punch. The depth of the wound of the skin included the epidermis, the derma, the hypoderm and the muscular layer, so that the superficial fascia was exposed.

2.4.4 Treatment

After the surgery, the skin wounds were treated topically with approx. 0.074 g of OEO at 2%, OEO at 5%, OEO at 7% and OEO at 10% respectively, the control group does not make any application. The animals were treated every third day, during pre-established periods.

The clinical course of skin wounds was monitored daily for the presence of secretions, scabs, necrosis and secondary infections. At the end of the experiment, the mice were euthanized as previously described.

2.4.5 Statistics analysis

The results obtained from each group were analyzed statistically using a one-way ANOVA test. The GraphPad Prism 4.0 software (GraphPad, San Diego, CA, USA) and SPSS 18.0 (SPSS Inc., Chicago, IL, USA) were used. The differences were considered statistically significant with $P < 0.05$.

2.5 Injury inflammation activity in vivo

NIH male mice between 25 and 30 g were used. For all the 30 mice the feed is removed 12 hours before the test (water ad libitum). On the day of the test

the animals are randomly distributed in batches with six mice each, marked and weighed (BW = body weight).
The saline solution was administered subcutaneously in the bearing of the left leg (LL) while the carrageenan was administered in the right leg (RL), with an indelible nib was marked the site in which the injections were applied, and the measurement was carried out. The injection was applied directing the liquid and the tip of the needle towards the toes of the leg. Great care was taken not to confuse the syringes of saline solution with that of carrageenan or the right and left paw, this could produce deficiencies in the analysis of results [19].
With the help of a Vernier the thickness of each leg was measured in the previously made mark. The first measurement will be made 15 min after carrageenan is administered. This procedure was repeated at times of 30, 50 and 120 min. The difference between the measurements of the right leg minus left leg for each rat will be obtained using:

$$\text{Inflammation} = RL - LL \tag{2}$$

At the end of the test, the animals were returned to their original cage to be delivered to the storekeeper for slaughter in accordance with the norms: NOM-033-ZOO-1995 (humane slaughter of domestic and wild animals), NOM-062- ZOO1-999 (technical specifications for the production care and use of laboratory animals) and NOM-087-Ecol-1995 (final disposal of biological products, excreta and corpses).

3. Results and discussions

3.1 Emu oil composition

Triglycerides are the main components of vegetable oils; their distributions of fatty acid esters differ according to the characteristics of the seeds. The compositions of the avocado and emu oils are shown in **Table 1**. As observed in this table the avocado and emu oils have similar compositions of oleic acid (C18: 1) and linoleic acid (C18: 2), becoming its main components. In the following, “fatty acids” refers to free fatty acids and their dispersed esters in triglycerides.

3.2 Emu oil ozonization

The Emu oil was ozonated until the ozone reaction was completed, that is, when the exit ozone concentration was equal to the inlet concentration (62 min for the

Fatty acids (%)	Avocado oil	Emu oil	Human skin
Palmitoleic C16:1	3–9	3.2	3.8
Stearic C18:0	0.4–1.0	10.1	11.2
Oleic C18:1	56.0–74.0	51.6	30.8
Linoleic C18:2	10.0–17.0	13.2	15.1
Linolenic C18:3	ND-2.0	0.5	0.3
Arachidonic C20:0	ND	0.1	—
Gadoleic C20:1	ND	0.5	—
Phospholipids	1.0	—	—

Table 1.
Lipid composition of oils.

experimental conditions). The latter was controlled by the ozonogram, with the graph representing the ozone that was not consumed during the ozonation process. In **Figure 1**, the ozone of the emu oil can be observed, which indicates that the oil contains oxidizable substrate.

3.3 Peroxide value

Figure 2 shows a gradual increase in the PV with respect to a gradual increase of the ozonation time due to the composition of the fatty chain of triglycerides of the oil and the generation of peroxide bonds.

Ozone reacts with the carbon–carbon double bonds of unsaturated fatty acids and triglyceride present in emu oil to form important products such as trioxolanes and peroxides, which are responsible for decrease of inflammatory activity and help stimulate tissue repair. At low levels, reactive oxygen species (such as H_2O_2) generated by ozonation, which have a short shelf life, are non-radical oxidants capable of acting as ozone messengers responsible for promoting wound healing.

The PVs are obtained after 24 h of reaction. Emu oil only had a PV of 250 mEq O_2 /kg of oil, while the PV of ozonated emu oil was much higher with a range of 2700–2900 mEq O_2 /kg of oil. These results show that during the synthesis of the OEO, almost all the carbon double bonds in the olive oil reacted with the ozone molecules, generating peroxidic species to increase the PV. After 24 h of reaction, the hydroperoxides, the hydrogen peroxides, the polymeric peroxides and other organic peroxides in OEO had reacted with the iodide to form iodine. According to Günaydın, the reaction time is a very important factor in oils with high peroxide content, and must be greater than 2 min [20].

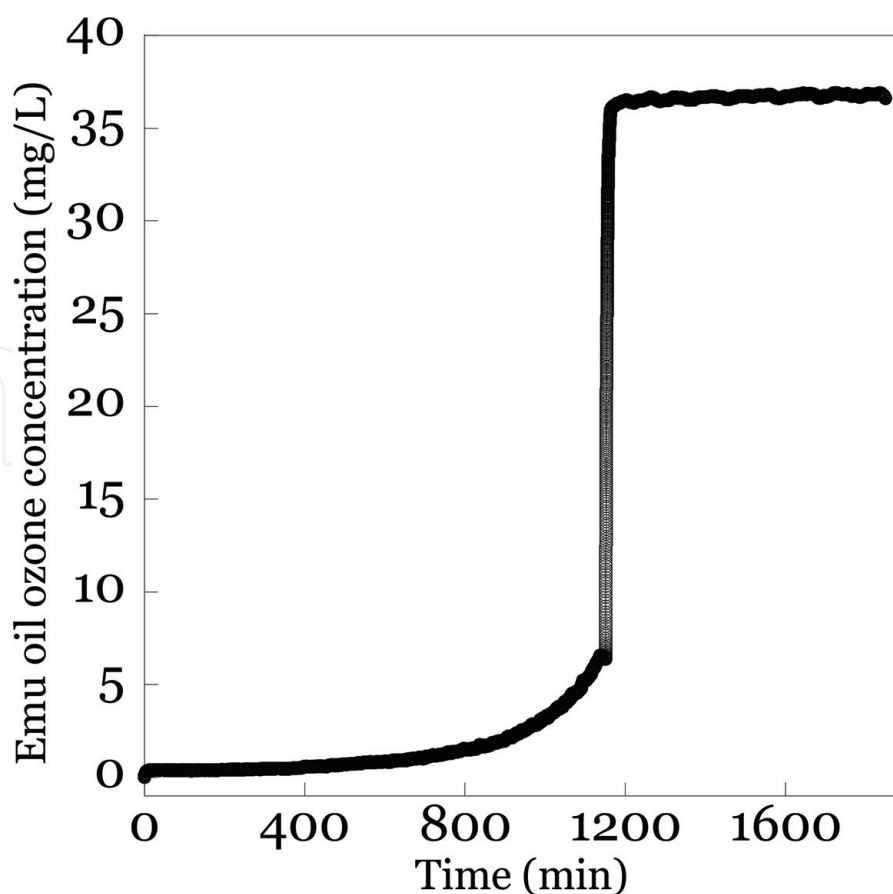


Figure 1.
Ozonogram of emu oil.

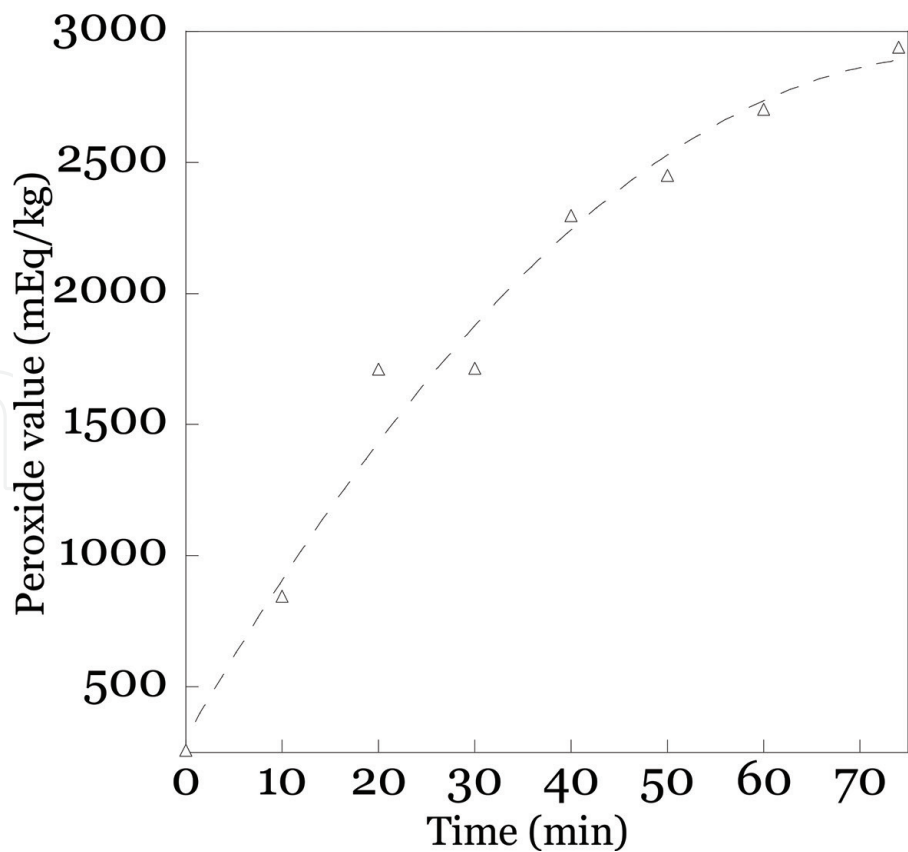


Figure 2.
Peroxide value graph with $R^2 = 0.9798$.

The spectrum of ^{31}P NMR is also shown in **Figure 3**, Where we can observe a peak that is in the range of the chemical change from 0 to -15 which represents the peak of phosphonomethylglycine used as an internal standard for quantification, which indicates that the emu oil does not have any type of phospholipid, the adipose part of the skin has phospholipids, ozonized vegetable-based oils also have phospholipids, once applied these, the phospholipids of the skin come in contact with the phospholipids of the oil of ozonized vegetable origin and send the order to close the pore to avoid invasion.

3.4 Healing

The effect on cleavage and incisional wound of the ozonized emu oil at 2% produced a minimal significant decrease in the period of epithelialization compared to the control. The treatment also showed a significant decrease in wound contraction (50%) compared to the control as shown in **Table 2**. In the incision wound model, it produced an increase in resistance to wound breakage in comparison with the control group as shown in **Figure 4**.

The effect on the excision and the incision wound across the width of the wound produced a decrease in the period of epithelialization compared to the control. The treatment showed a significant decrease in wound contraction (50%) compared to the control. In the incision wound model, both along and across the width of the wound produced an increase in the resistance to rupture of the wound compared to the control group as shown in **Figure 5**.

The weight and body temperature of each mouse were measured, and it was found that in the mice with weight and body temperature over time they ended in mortality.

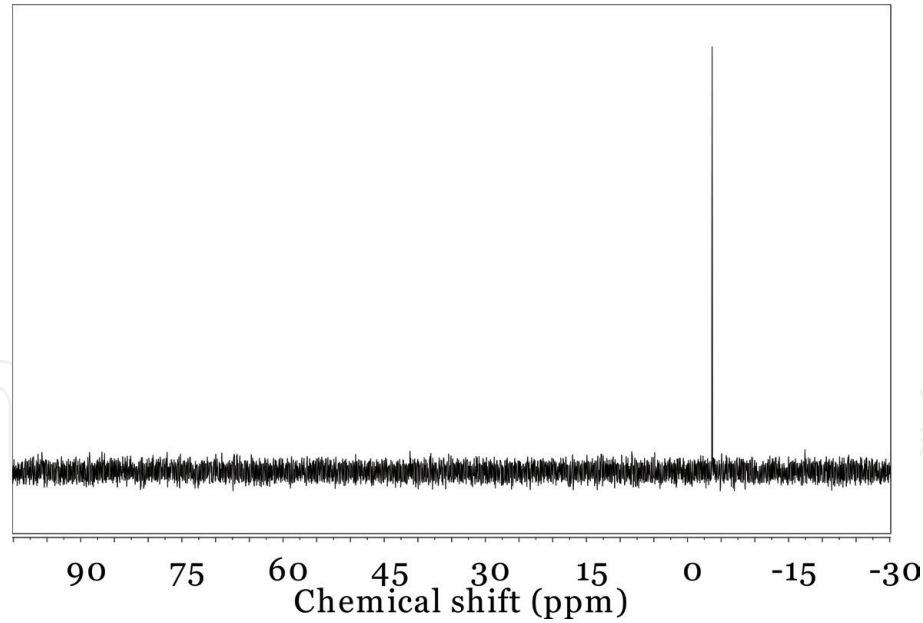


Figure 3.
The ^{31}P -NMR spectra for the refined emu oil.

Emu oil at 2%					
Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Grupo Control					
Day 1	Day 2	Day 3	Day 4	Day 5	Day 6

Table 2.
Healing processes.

3.5 Inflammation

Inflammation studies were carried out in each model, with one leg as control and the other as comparative of inflammation, ozonized emu oils of 2% and 7% concentration were taken, the averages of each mouse were obtained with their respective standard error. A gradual decrease in emu ozonized oil at 2% was observed with respect to 7% emu ozonized oil, as seen in **Figure 6**, in addition to comparing with the control group, which is found in **Figure 7**, a decrease in significant inflammation of the right leg.

In **Figure 7**, the normal decrease in inflammation in the left leg (control) of the animals is noted; aspect that served to evaluate the inflammation of the right leg that were administered with carrageenan and that is observed in Eq. (2) and the **Figure 6**.

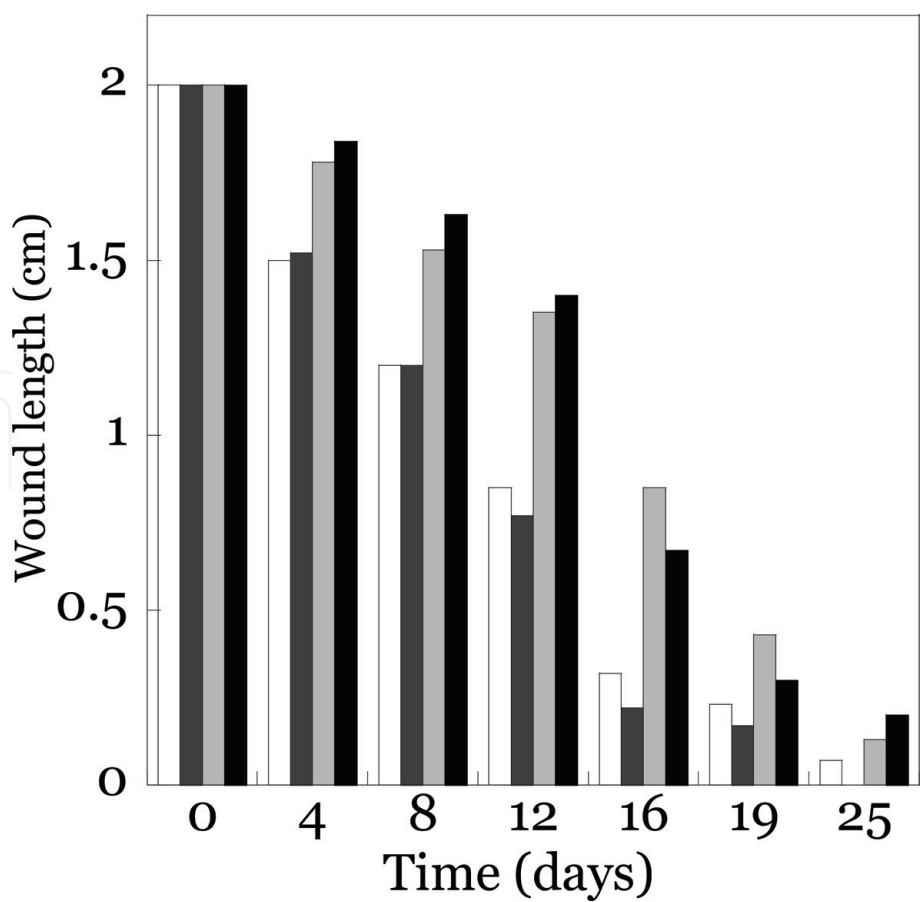


Figure 4.
Wound length for the control group (□) and the ozonized emu oil groups at 2% (■), 7% (▨) and 10% (■).

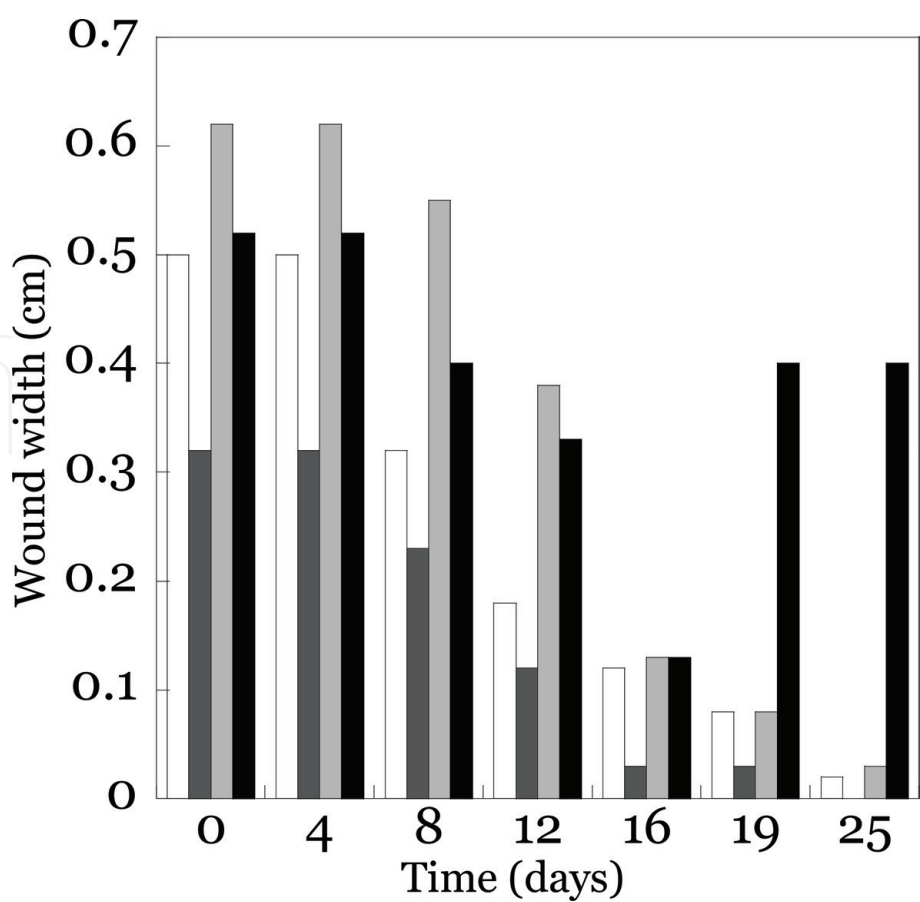


Figure 5.
Wound width healing for the control group (□) and the ozonized emu oil groups at 2% (■), 7% (▨) and 10% (■).

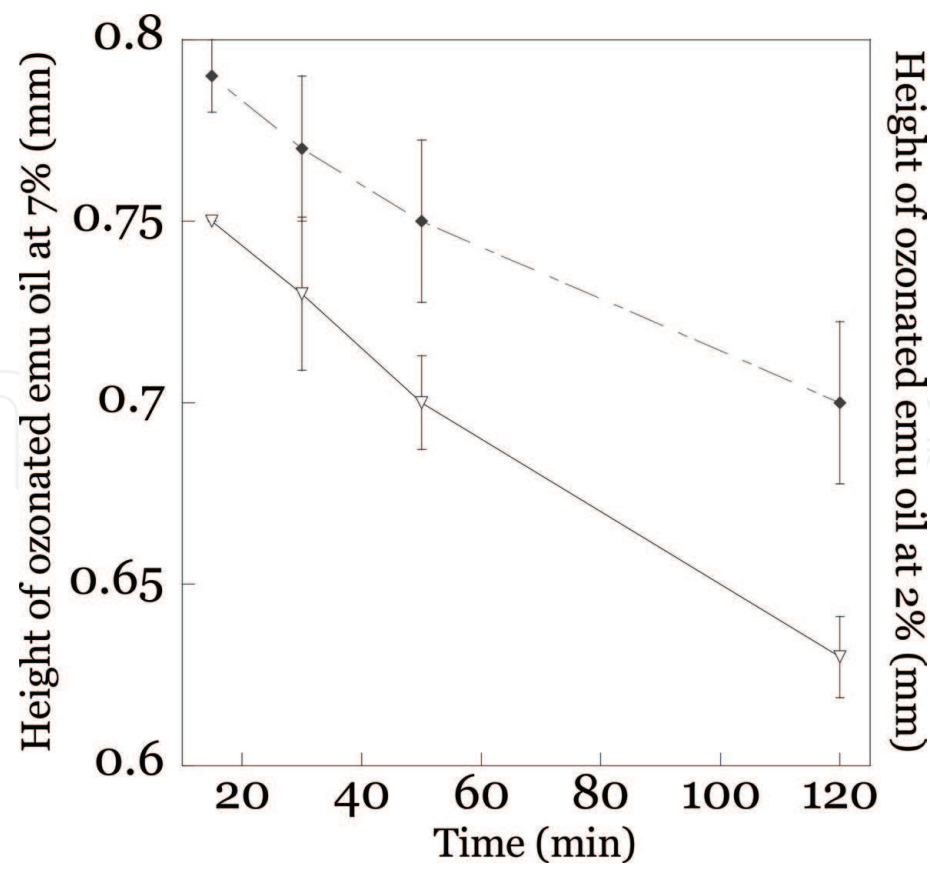


Figure 6.
Comparison of the inflammation height of emu oil at 7% (▽) height of inflammation of emu oil at 2% (◆) on the right leg.

3.6 Discussion of wound healing and inflammation

The reaction of ozone with the unsaturations present in the fatty acids and other free acids of the Emu oil generate products such as ozonides and peroxides. These products may enhance the topical absorption in the mouse skin, because the ozonized emu oil does not have phospholipids.

As it is known, in the processes of tissue damage and inflammation there is a rupture of the phospholipids that constitute the membranes of the cells or their organelles, leading to the activation of the phospholipase A2 which, in turn, is responsible for transforming the membrane phospholipids in arachidonic acid, main precursor through the action of enzymes such as cyclooxygenases, of prostaglandins with proinflammatory activity [21, 22]. This results in the activation of various nociceptor and anti-inflammatory pathways [23]. There are also many drugs aimed for inhibiting the inflammation and pain associated with these damages, but only some are also directed towards the aspect of tissue healing. Consequently, and considering the above, in this work we focus in the study of a biological (natural) product that could present these two characteristics: such as the emu oil subjected to the ozonation method described in the methodology. According to the results shown here, in the healing aspect, only the emu oil concentration at 2% had a significant effect on tissue repair; something that was not observed with the concentrations of 7 and 10%; so, it can be said that the optimal healing concentration was 2%. The fact that an adequate healing does not occur at higher concentrations may depend on the content of other factors not determined in this study, such as factors of plaquetary aggregation, anticoagulants, growth factors, etc. [24], which would be represented in a lesser proportion in the oil, in relation to its content of fatty acids. Obviously, to verify this, more profound studies would have to be done to detect the

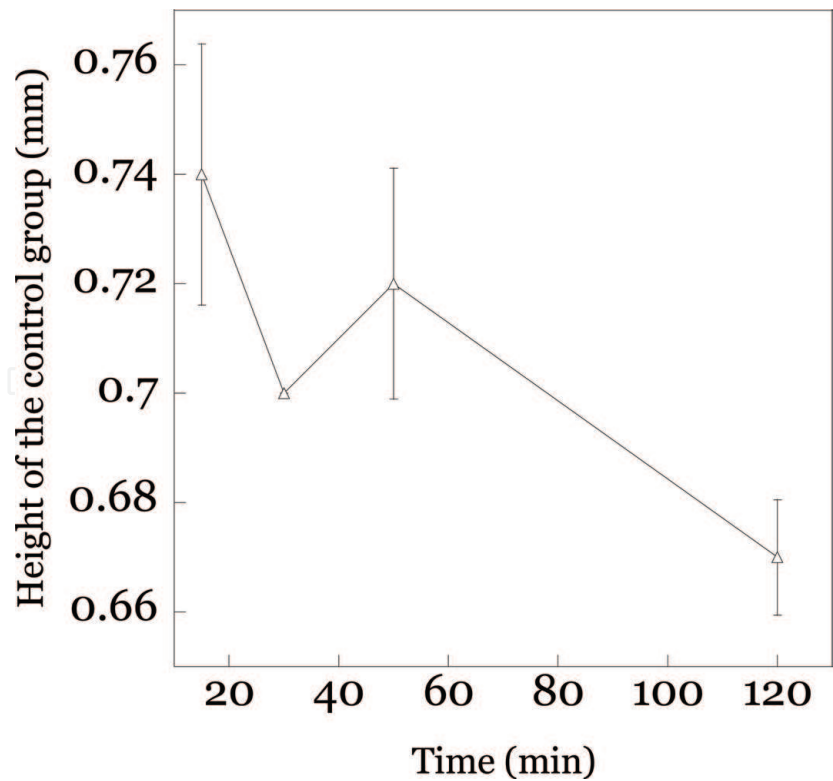


Figure 7.
Inflammation height of the control group (Δ) in the left leg.

presence of such factors that promote an accelerated closing of the wound, increases the neoepithelial thickness and the migration of macrophage-histiocyte cells. However, it is logical to consider that, as with many drugs in allopathic therapy, the dose-response effects do not follow a directly proportional trend, but there is a dose (in our case a concentration) to which the maximum healing response is promoted and none therapeutic effect is observed, although it is increased to dosage, this gives rise to future replications of the experiment with concentrations closer to 2%.

On the other hand, it was remarkable to find that with the two emu oil concentrations tested (2 and 7%), the anti-inflammatory effect was directly related to the concentration used in the mice. This may be related to the content of other fatty acids of emu oil, in addition to the linoleic which is known to participate in the anti-inflammatory actions of some commercial preparations (search for bibliography of this); because having higher content of fatty acids (e.g. stearic, palmitic and even oleic), and be submitted to the ozonation process described in previous paragraphs, it would confer to the tested concentrations a greater capacity to prevent the formation of proinflammatory prostaglandins as some interleukins, prostaglandin E2 and different leukotrienes. There is also the possibility that the ozonizing process conferred inhibitory activity on the inductive cyclooxygenases present in the membrane (COX-2) and that the inhibition of prostaglandin formation was carried out at that level, but for this, they would also have to be make more specific studies; however, the possibility is posed.

4. Conclusions

The present study was conducted to evaluate whether ozonized emu oil could promote wound healing in experimentally induced lesions in mice. The results of the present study also corroborate the use of emu oil for Vedic healing in folk medicine for the treatment of wounds.

Topically applied Vedic healing promoted resistance to wound breakage, wound contraction and period of epithelialization in different models of experimental wounds.

The phase of inflammation, macrophagia, fibroplasias and collagenation are intimately intertwined. Therefore, an intervention in any of these phases by medications could lead to the promotion or depression of the healing phase of the collage.

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Appendices and nomenclature

LL	inflammation in the left leg (mm)
OEO	ozonized emu oil
PMG	phosphonomethylglycine, g/l
PPU	pressure ulcers
PV	peroxide value, mEq peroxide/kg of oil
RL	inflammation in the right leg (mm)

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