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Effect of topical ozonated sunflower oil on second intention wound healing in turtles: a randomised experimental study

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ABSTRACT

Background: Ozone is an antimicrobial agent that in experimental and case-control studies has been found to exert a positive effect on wound healing. Wild and pet chelonians frequently present insidious wounds exhibiting secondary infections and/or delayed healing.
Objectives: Evaluate the effects of topical ozonated sunflower oil on second-intention healing of acute experimental skin wounds in red-eared sliders (*Trachemys scripta elegans*).
Methods: Randomised within-subject controlled study; Group 1 (n = 24) was used to assess clinical healing features; Group 2 (n = 12) was used for histological evaluation in which two sets of wounds were biopsied at 2, 7, 14, 21, 28 and 42 days over the course of the cicatrisation process. A single 6 mm diameter wound was made on each rear limb and topical ozonated (950 peroxide value) and non-ozonated sunflower oil were applied daily for one week on treated and contralateral control wounds, respectively.
Results: Mean wound size was significantly lower in the ozone-treated group at day 28 ($p < 0.0001$) with differences of clinical relevance (74.04% vs. 93.05% reduction of initial wound size). Histologically, the acute inflammatory reaction was enhanced in treated wounds, with significantly higher numbers of heterophils ($p = 0.0016$), lymphocytes ($p < 0.001$) and fibroblasts ($p < 0.001$).
Conclusions: Daily topical application of ozonated sunflower oil over the course of one week improved the healing of acute, full-thickness skin wounds in chelonians. This clinical outcome was histologically correlated with an enhanced acute inflammatory reaction, as well as the production and remodelling of collagen fibres.

Keywords: Wound healing; ozone; sunflower oil; *Trachemys scripta*; chelonians

INTRODUCTION

Ozone (O_3) is widely recognised as an antimicrobial agent and has been investigated for the treatment of chronic and acute skin wounds of various kinds [1,2]. Ozone irreversibly oxidises viral DNA and bacterial cell walls and promotes the formation of reactive oxygen species (ROS), which enhance vasodilation and the production of endogenous growth factors [1,3]. Previous studies have shown that controlled levels of O_3 exert a beneficial effect, promoting the expression of pro-inflammatory cytokines, such as IL-1 and TNF-α, several adaptive inflammatory responses such as cyclooxygenase-2 (COX-2) gene activation in keratinocytes, expression of proliferating cell nuclear antigen (PCNA) and keratin (CK) 10, a cytoskeletal protein expressed in well-differentiated suprabasal skin keratinocytes [1-3]. The recent systematic review of human medical literature conducted by Fitzpatrick et al. [3] concluded that O_3 therapy may improve the proportion of wounds healed in a shorter amount of time, but further research is required because many available reports in human, as well as in veterinary, medical literature involve uncontrolled case studies or the samples were too small to detect clinically meaningful differences.

Currently, there are no widely-established clinical recommendations for the topical O_3 treatment of cutaneous wounds; previous studies have produced heterogeneous findings in terms of dose, vehicle, and duration of treatment, limiting the potential for viable comparisons between studies [3]. The vehicle used to deliver O_3 to the skin is conditioned by the unstable character of the molecule, which quickly breaks down into an oxygen molecule and a single oxygen atom [4]. In this context, ozonated oils have been commonly used for the treatment of cutaneous wounds in clinical and experimental studies, because the O_3 molecule can be stabilised as an ozonide between the double bonds of monounsaturated fatty acids. Fatty acids with two unsaturated bonds (such as sunflower oil) are more reactive to O_3 and remain stable for 2–3 years if kept refrigerated [1,5].

Chelonians are of veterinary clinical interest not only as pets but also as wild animals, especially in terms of conservation efforts. Wild tortoises and turtles are frequently found with wounds complicated by secondary infections and there is a need to find alternative treatments that do not involve antibiotics [6-8]. Compared to mammals, skin healing in reptiles is compromised by reduced wound contraction and reliance on the ambient temperature to maintain body heat [9-11]. In a previous study, the present authors characterised the spontaneous process of second-intention wound healing in the soft skin of the red-eared slider [11]. This is a well-known species and its size and semiaquatic character make it suitable as a useful experimental model for evaluating new therapeutic interventions that could be applied to other reptile species. The goal of this study was to analyse the clinical and histological effects of topical ozonated sunflower oil on second-intention wound healing of acute experimental skin wounds in turtles, maintained in natural temperature conditions and with free access to water. The hypothesis was that the use of ozonated oils could represent a promising alternative wound treatment for chelonians.

MATERIALS AND METHODS

Animals

Thirty-six *Trachemys scripta elegans* adult females were used (straight carapace length ≥ 16 cm; weight range 1.2 to 2.3 kg). All the animals were captive bred and belonged to a zoological garden collection. Prior to being included in the study, a physical examination, packed cell
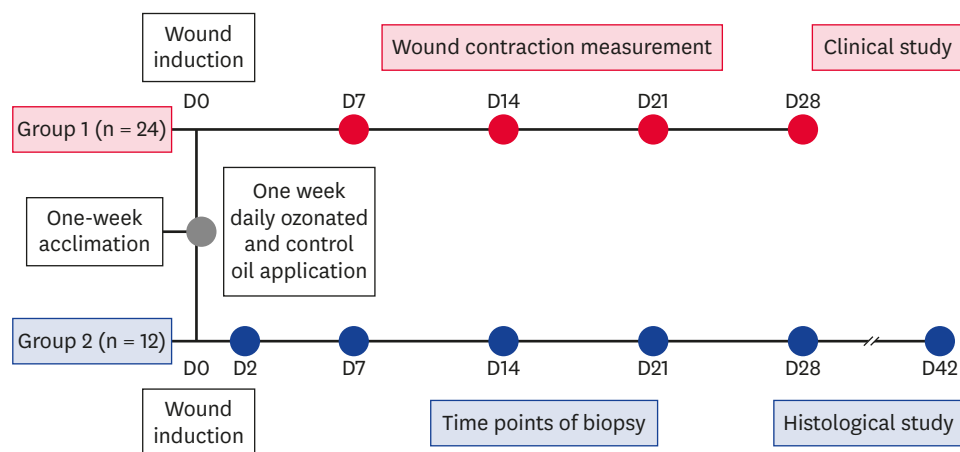
Fig. 1. Study design and timeline diagram showing control points for the clinical and histological variables.

volume measurement and faecal flotation analysis were carried out. Turtles deemed to be healthy were identified with a microchip and housed outdoors in nine vivaria, each with an area of 3 m²; each vivarium included a plastic pool with a capacity of 90 L allowing for complete submersion. The animals had free access to a sunbathing area and food *ad libitum* (Aquatic Turtle Monster Diet, Zeigler Bros, Inc., USA).

After a week of acclimation the turtles were distributed in two groups by tossing a coin: Group 1 (n = 24) was used to assess clinical healing features; Group 2 (n = 12) was used for histological evaluation in which sets of wounds were biopsied at defined time points during the course of the cicatrisation process (**Fig. 1**). Both groups shared the same living quarters and climate conditions (mean ± SD nocturnal and diurnal temperatures 15.6 ± 0.4°C and 26.5 ± 3.3°C respectively; humidity 48%–67%), corresponding to the appropriate temperature range for these freshwater turtles [12].

Skin wound induction

The animals were anaesthetised with ketamine (20 mg/kg IM; Imalgene, Merial, Spain) and medetomidine (0.5 mg/kg IM; Domtor® Lab. Esteve, Spain). Without previous disinfection, a single wound 6 mm in diameter was made on the dorsal aspect of each rear limb using a disposable biopsy punch. The wounds had well-delimited circular to oval edges and reached the subcutaneous tissue, exposing the outermost skeletal muscles and blood vessels (**Fig. 2A and B**). After wound induction, the turtles were housed in individual terrariums at room temperature for approximately 12 h and did not receive anti-inflammatory drugs to avoid interference with wound healing.

Ozonated oil application

Several drops (about 0.5 mL) of ozonated sunflower oil, with a peroxide value (PV) of 950 mEq/Kg, were applied topically 4–6 h after wound induction and daily during the first week post-injury in both groups (**Fig. 1**). Allocation of treatments, to right or left rear limbs of the same subject, was made by tossing a coin; the control wounds received non-ozonated sunflower oil. After each application, the animals were kept out of the water for 6 h.

The sunflower oil was ozonated by bubbling an O_2/O_3 mixture through the oil for 240 h. The O_3 flow rate was kept constant at 1 L/minute and O_3 concentration was 120 µg/mL (Ozonoterapia Veterinaria S.L.; Cádiz, Spain). Determination of PV was made by iodometric

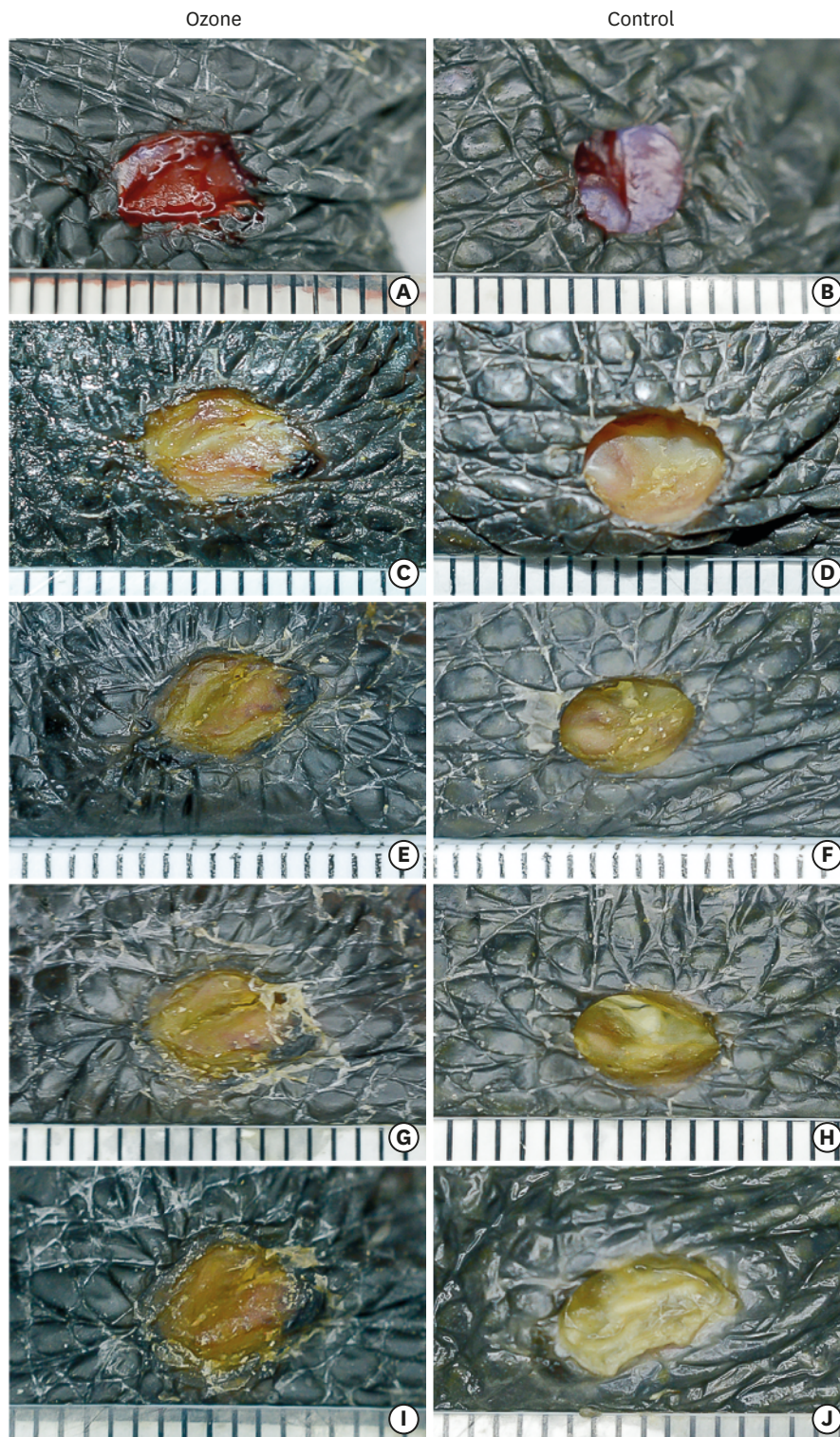**Fig. 2.** Sequence of clinical photographs illustrating wound contraction and macroscopic features in two wounds from the same animal. Wounds were recorded and measured digitally every week from D0 to D28. Figures were rotated to align internal scales in millimetres.

titration [13]. Peroxide value indicates the quantity of peroxide within the oleozone, and is defined as the active oxygen per kilogram of oleozone (mEq/kg).

Clinical evaluation of wound healing

In both groups, wounds were monitored daily and photographed on day 0 (D0) and weekly until 28 days post-wound (time points D0 to D28, respectively) using a macro lens (Nikon AF-S DX 40 mm). Photographs included an internal scale in mm and were code-identified. Wound area was measured, blinded for animal and time point, using image analysis software (ImageJ, U. S. National Institutes of Health, USA). Wound contraction was expressed as the percentage of area reduction from the initial wound. Once the wounds were completely healed, animals were released to their shared pond at the zoological garden premises.

All experimental procedures involving animals were conducted in accordance with the European guidelines for proper use and care of experimental animals and were approved by the Bioethics Committee of the Animal Health Service (Andalucía, Spain) (reference 25/09/2017/134). All necessary permissions were requested and obtained from the Zoological Garden of Córdoba, and all procedures were carried out with the participation and supervision of the Head of Veterinary Services (RG) and within the zoological garden facilities.

Histological study

Turtles from Group 2 were used for the microscopic study (**Fig. 1**); at each of the six time points (2, 7, 14, 21, 28, and 42 days), two animals were selected using a computational random number generator, and their wounds (two treated and their contralateral controls) biopsied under general anaesthesia using a biopsy punch 8 mm in diameter. Wounds were then sutured using Monosyn® 2/0 and the turtles kept in their vivaria. Samples were fixed in buffered 10% formaldehyde for 16 to 20 h, hemisectioned and processed to a paraffin-embedded state. Serial sections, 4 μm thick, were obtained from each block and stained to enable accurate evaluation of the wound healing process [11,14]. Thus, haematoxylin and eosin (HE) stain was used to evaluate microscopic features; methenamine silver (Gomori PAMS) to stain the basement membrane zone (BMZ); Masson's trichrome (TM) to evaluate the collagen fibres and extracellular matrix during the proliferative and remodelling phases; routine Gram stain was applied to evaluate bacterial proliferation. Systematic microscopic evaluation included: acute inflammatory response, re-epithelialisation, BMZ and connective tissue formation, and remodelling during the healing process.

Morphometric analysis

A morphometric analysis was performed using three non-sequential sections from each of the 24 wound biopsies (12 treated and 12 control wounds from Group 2 turtles). In each section, three photographs, with 400× high power fields (HPF), were taken at the lateral edges and wound-bed (nine photographs per wound) to count inflammatory cells (heterophils, macrophages, lymphocytes) and fibroblasts. The mean value for each cell type and each turtle's biopsies were calculated so that 12 paired, control and ozone-treated values, were obtained for comparison.

Statistical analyses

A sample size to provide 95% power to detect a biologically important 25% difference in wound retraction at day 28 ($\alpha < 0.05$; paired t-test) was determined from previous studies using the same experimental model [12] (GraphPad StatMate 2.00 for Windows; GraphPad Software Inc., USA). In order to increase the power of the experiment, the areas of Group 2 wounds, measured before they had been biopsied, were grouped with the same time point measures as Group 1 wounds. The distribution of continuous data was tested for normality using the Kolmogorov-Smirnov test. Mean wound contraction at each time point and mean

inflammatory cells counts in control and ozone-treated wounds (Group 2) were compared by a paired Student's *t*-test. A value of $p < 0.05$ was considered significant (Prism 5.04 for Windows; GraphPad Software Inc.).

RESULTS

Clinical evaluation of wound healing

All procedures were well tolerated by the animals. The topical application of ozonated and control oils did not require further anaesthesia or sedation and wounds healed satisfactorily in all turtles. Macroscopically, the wound area was initially covered with serous or serous-haemorrhagic fluid; this first exudate formed a thin film over the subcutaneous muscles and its thickness increased at different rates between ozone-treated and control wounds. In control wounds, crusts looked thinner at D7 and the subcutaneous muscles could still be perceived in some wounds (**Fig. 2D**). At this time, the ozone-treated wounds showed more consolidated crusts, usually stained with haemorrhagic remains (**Fig. 2C**). At D14 and D21 the macroscopic features of wounds in the same animal were similar except for thicker and darker crusts in the ozone-treated wounds (**Fig. 2E-H**). At D28, many of the control wounds showed crusts with a moistened appearance (**Fig. 2J**), whereas crusts in ozone-treated wounds looked drier and firmly adhered to the wound margins (**Fig. 2I**). Mean wound retraction was slightly lower in ozone-treated wounds during the first two weeks, but subsequently progressed faster than control wounds (**Fig. 3**). At D28, mean wound size in control and ozone-treated wounds was 93.05% and 74.04% respectively (**Table 1**) and the differences were statistically significant ($p < 0.0001$).

Microscopic findings

At D2, the margins of ozone-treated wounds were infiltrated by abundant heterophils and relatively small numbers of macrophages mixed with plasma and fibrin (**Fig. 4A**); the adjacent epidermis was also infiltrated by heterophils (**Fig. 4A**, inset), and the wound surface was covered by a fine serocellular crust. Heterophilic infiltration was lower in the contralateral wounds (**Fig. 4B**). At D7 and D14, the inflammatory exudate, composed of heterophils,
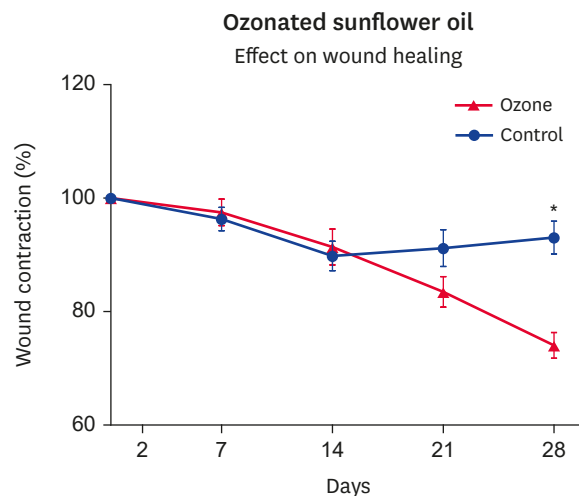

Fig. 3. Evolution of wound contraction during the first 28 days. Each point represents mean wound size expressed as a percentage of the initial wound area.
*Statistically significant differences between groups ($p < 0.001$ paired *t*-test).

Table 1. Descriptive statistics of wound size expressed as a percentage of the initial wound area

Descriptive statistics	D7		D14		D21		D28	
	Control	Ozone	Control	Ozone	Control	Ozone	Control	Ozone
Number of values	34	34	32	32	30	30	28	28
Mean	96.31	97.49	89.80	91.39	91.19	83.47	93.05	74.04
Std. deviation	11.97	13.61	14.72	17.90	18.33	14.73	15.22	12.04
Std. error	2.052	2.334	2.602	3.164	3.346	2.689	2.877	2.276
Lower 95% CI of mean	92.14	92.74	84.49	84.93	84.35	77.97	87.15	69.37
Upper 95% CI of mean	100.5	102.2	95.10	97.84	98.04	88.973	95.40	98.95
Minimum	72.474	75.43	59.96	52.31	58.15	44.65	64.88	43.84
25% percentile	88.69	86.53	78.66	79.74	78.51	75.42	79.93	68.27
Median	97.12	95.67	89.00	91.80	90.42	89.95	96.60	73.84
75% percentile	103.4	106.9	98.01	102.0	102.3	93.59	104.4	83.38
Maximum	126.2	144.4	129.9	146.9	150.1	108.2	117.3	94.76

D, days post-wounding; CI, confidence interval.

macrophages and lymphocytes, was more abundant and the granulation tissue more prominent in ozone-treated wounds than in their control counterparts (**Fig. 4C and D**). Re-epithelialisation progressed similarly from the lateral edges to the beds of ozone-treated and control wounds and was completed in both groups at D14, when the wounds were still covered by thick crusts. From D21 onwards, the proliferative phase of healing predominated over the inflammatory processes; thus, vascular buds, active fibroblasts and collagen fibre production and deposition were the main features at the wound beds, but maturation and remodelling were morphologically more advanced in the ozone-treated wounds (**Fig. 4E and F**). In parallel to granulation tissue maturation, the BMZ was better defined in the ozone-treated wounds (**Fig. 4E and F**, insets). At D42, the ozone-treated wounds showed more consolidated granulation tissue and the remodelling of collagen fibres was more advanced (**Fig. 4G and H**). The new epidermis appeared well differentiated with centripetal re-pigmentation in both the ozone-treated and control wounds. Bacterial colonies were observed, at different time points, within the crusts of many untreated wounds (seven out of 12 wounds) but were uncommon in the treated ones (one out of 12 wounds). Intralesional bacterial infection was not observed.

Morphometric analysis

Topical application of ozonated oil resulted in statistically significant differences in mean heterophil counts ($p = 0.0016$). From D2 to D28 the number of heterophils was markedly higher in the ozone-treated wounds and converged in the wounds biopsied at D28 and at D42 when heterophils were virtually absent (**Table 2**). On the other hand, mean macrophage counts were statistically non-significant. The numbers of lymphocytes and fibroblasts at each time point were higher in ozone-treated wounds and their means were significantly higher than in control wounds ($p < 0.001$) (**Fig. 5**).

DISCUSSION

The present authors' results demonstrate that topical application of ozonated sunflower oil improved the healing of acute full-thickness skin wounds in chelonians with significantly greater wound-size reduction at D28, when differences compared to non-treated wounds were of clinical relevance. This outcome was histologically associated with an enhanced acute inflammatory response characterised by significantly higher counts of heterophils and lymphocytes, as well as fibroblasts and collagen fibre deposition during the proliferative phase of healing.
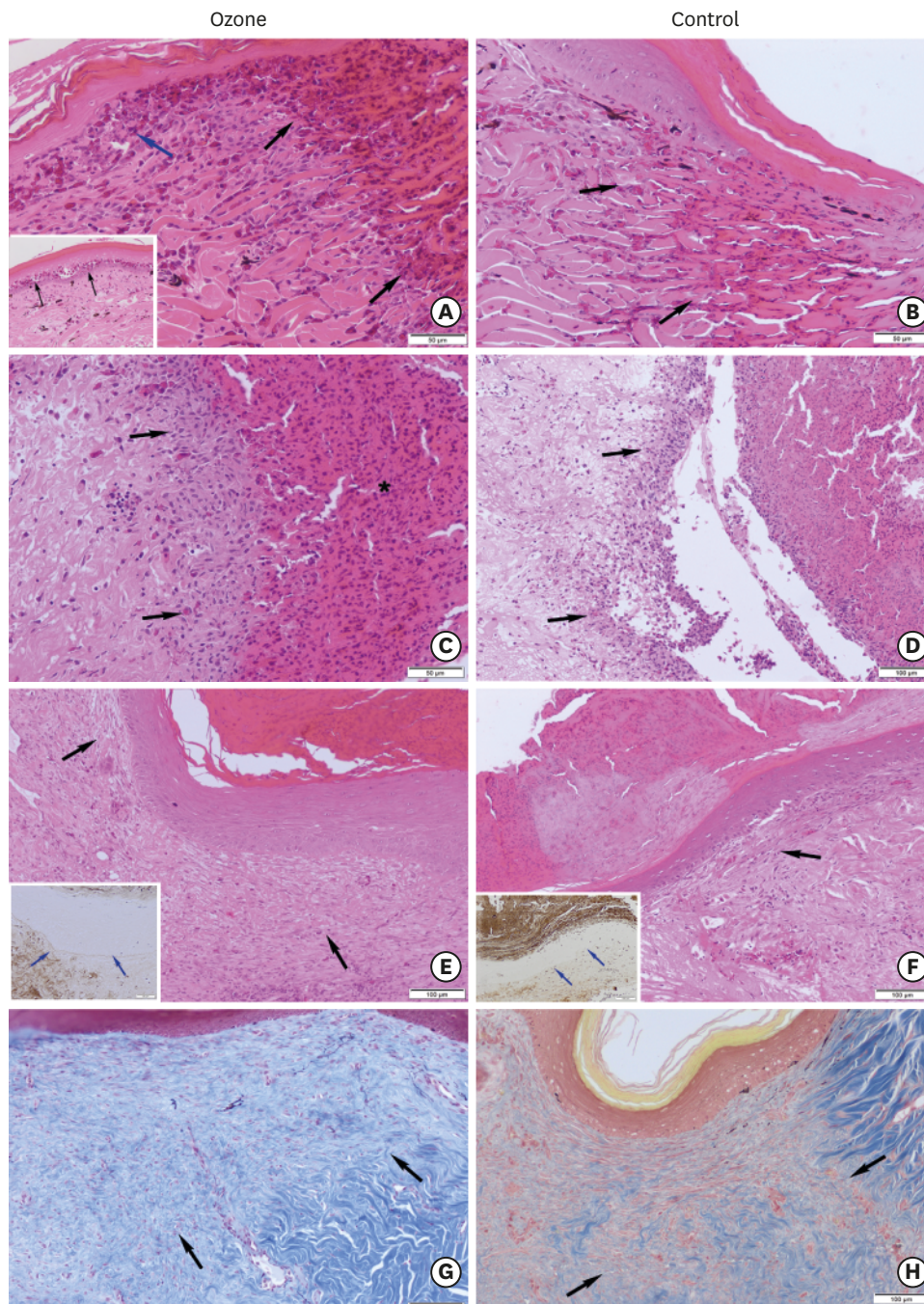
Fig. 4. (A-H) *Trachemys scripta elegans* skin. Sequence of microscopic features of wound healing in ozone-treated wounds and their control counterparts. (A) D2, the treated wound shows a palisade of acidophilic transudate and numerous heterophils demarcating the margins (black arrows), and infiltrating the closest epidermis (blue arrow). Inset: Detail of the intraepidermal heterophilic exudate (arrows). (B) The control wound shows less heterophilic infiltration (arrows) and the adjacent epidermis is not involved. H&E. (C) D7, there is abundant cellular exudate demarcating the wound edges (arrows) from the crust (asterisk). (D) The contralateral wound shows less inflammatory exudate (arrows). (E, F) D28, treated wound shows abundant and mature granulation tissue (comprising numerous fibroblasts and moderate collagen fibres arranged in parallel to the surface; arrows) compared with its contralateral control. (F) H&E. The epidermis is similarly developed and the crusts persist both in treated and control wounds. Insets, (E, F) note that the basement membrane zone (arrows) is better differentiated in the treated wound (Gomori PAMS). (G, H) D42, the treated wound is filled by abundant collagen fibres (blue stained) arranged in parallel to the epidermis (remodelling phase feature), while active fibroblasts and vascular buds persist in the contralateral control (arrows). Trichrome Mason stain.

Table 2. Descriptive statistics of the inflammatory cell counts in the paired wounds biopsied along the 6 successive time points

| Descriptive statistics	Heterophils		Macrophages		Lymphocytes		Fibroblasts	
	Control	Ozone	Control	Ozone	Control	Ozone	Control	Ozone
Number of values	12	12	12	12	12	12	12	12
Mean	14.94	33.24	5.31	5.91	3.45	7.11	11.10	19.42
Std. deviation	14.91	27.70	1.10	3.35	1.48	2.61	8.20	10.28
Std. error	4.31	7.99	0.32	0.97	0.43	0.75	2.37	2.97
Lower 95% CI of mean	5.46	15.64	4.61	3.78	2.49	5.46	5.89	12.88
Upper 95% CI of mean	24.41	50.84	6.01	8.04	4.38	8.76	16.31	25.95
Minimum	0.89	0.78	3.78	1.00	1.33	2.11	1.89	6.67
25% percentile	4.39	9.39	4.25	3.25	2.11	5.47	3.33	10.50
Median	8.45	27.39	5.44	5.89	3.67	6.89	10.11	17.72
75% percentile	22.44	49.78	6.33	8.72	4.56	8.92	14.78	27.31
Maximum	49.56	87.33	7.11	12.42	6.22	12.22	27.78	36.89

CI, confidence interval.
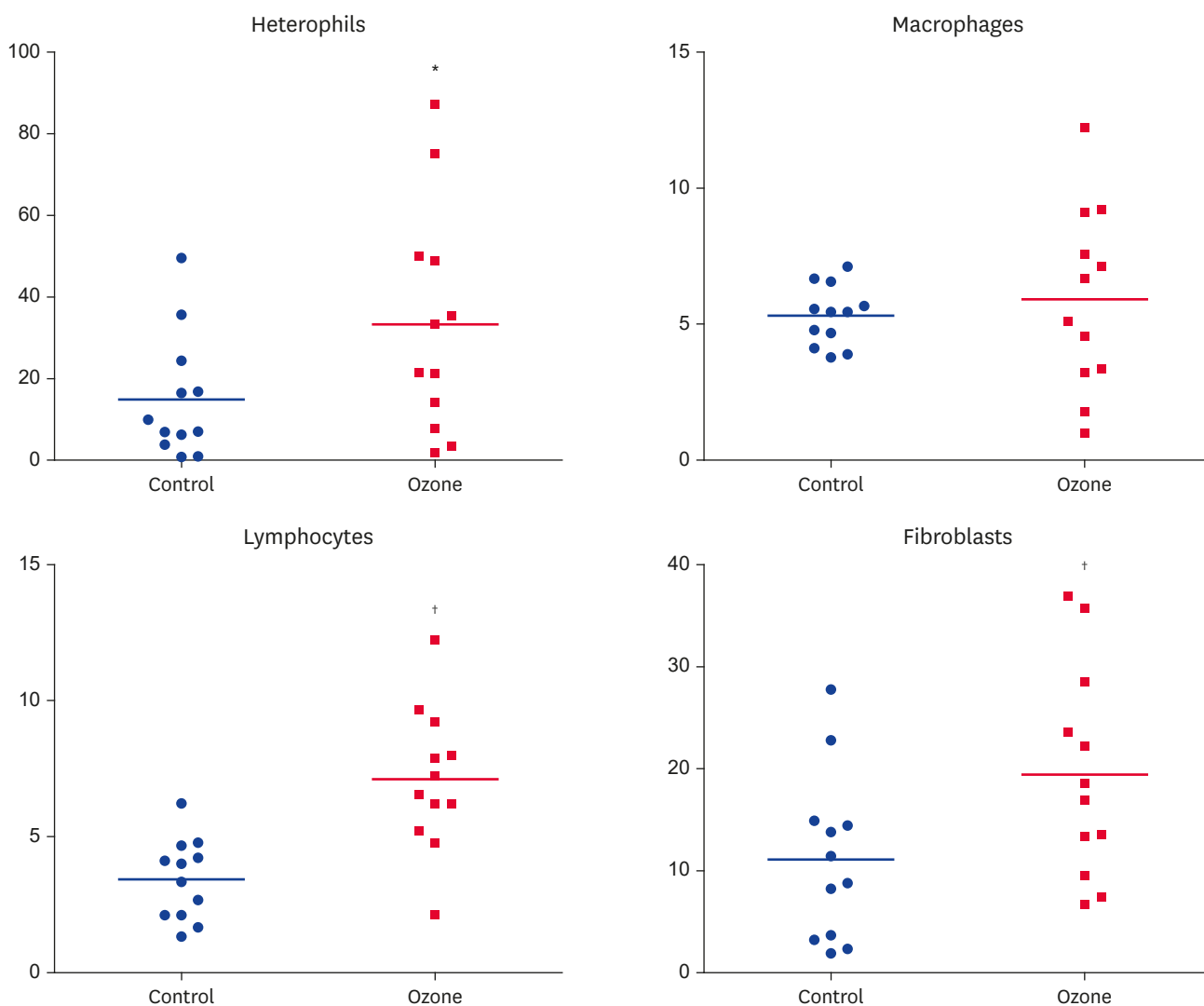
Fig. 5. Scatter plot of inflammatory cell counts from D2 to D42 in the paired control and ozone-treated wounds (n = 12).
*p = 0.0016; †p < 0.001.

This is the first experimental study on O_3 therapy applied to reptile wounds, but numerous experimental and clinical studies in mammals and human beings are available for comparison. A recent metanalysis evaluating O_3 therapy in human patients supported the topical application of O_3 as a treatment for chronic wounds, but there was no conclusive evidence of O_3 as a superior therapy compared to standard treatments [3]. In turtles, one week of topical ozonated oil application to acute wounds was found in the present study to be associated with a significantly smaller mean wound size. Microscopic observations indicated that ozonated oil increased the inflammatory response, which is the first step of wound healing. This biological effect could be even more beneficial in the treatment of the chronic wounds affecting human patients, or when the inflammatory response is inhibited by a systemic condition [3]. An excess of inflammation may be detrimental to normal wound healing and previous reports point out that prolonged exposure to O_3 is certainly deleterious [1]. However, controlled O_3 exposure at appropriate doses can accelerate the cell cycle by inducing synthesis of growth factors, providing a useful therapeutic effect on the skin [1,3].

The effect of O_3 therapy is influenced by the method of delivery, duration of treatment, type of oil used and its peroxide content [3,15,16]. Sunflower oil is one of the richest sources of n-6 polyunsaturated fatty acids (n-6PUFAs), with a 60% content of linoleic acid [5]. In a previous study in SKH1 mice, various types of oils and levels of peroxidation were tested; sesame oil with a medium level of peroxidation (about 1500 PV) was able to significantly accelerate the first phase of acute wound healing, in contrast to olive and linseed oils with the same amount of peroxidation [15]. Sunflower oil was not investigated, but when used at a similar level of peroxidation in the same strain of diabetic mice, provided results comparable to sesame oil [17]. For this first-time study in reptiles, sunflower oil was selected because it is one of the most commonly ozonated oils and several topical presentations are commercially available. The ozonated sunflower oil used had a PV of 950, close to the medium range of peroxidation (1000 to 3000 PV) recommended in the aforementioned study and to the 848 PV of commercially available presentations such as Oleozone, or the 800 PV recommended for the treatment of trophic ulcers in human patients [5]. Treatment duration also influences the effect of O_3 therapies [3]. Some of the biological effects of O_3 on skin cells have been detected *in vitro* after a 6-day period of exposure [18]. In the present study, wounds were treated for one week because skin cicatrisation in turtles is characterised by early crust formation [11], which prevents further application of topical therapies. A more extended period of application would have required removing the crust covering the wounds and thus interfering with the normal cicatrisation process.

Wound closure is a highly regulated physiological process [19]. The histological findings of the present study suggest that the significant increase of heterophils and lymphocytes associated with O_3 therapy could be objectively interpreted as an improved cellular response. Ozone could affect the expression of redox-sensitive transcription factors such as NFκB, which acts as an activator for pro-inflammatory cytokines. Heterophils, macrophages and lymphocytes play a major role by secreting cytokines and growth factors, which regulate, among other processes, the activation and recruitment of fibroblasts, collagen synthesis and angiogenesis during the proliferation phase of wound healing. The histological changes associated with O_3 therapy in turtles are comparable with those obtained by similar full-thickness experimental wound models in mice [18]. The capacity of ozonated oil with a medium level of peroxidation to prompt the inflammatory phase of cicatrisation could be even more useful in the chronic non-healing wounds of chelonians and other reptiles.

The microscopic observations made in the present study also suggest that O_3 treatment promoted the proliferative phase of second-intention wound healing, as supported by the higher number of fibroblasts found in treated vs. untreated contralateral wounds. By releasing oxygen, O_3 therapy has been shown to increase the expression of numerous regulatory cytokines and chemokines, including transforming growth factor (TGF-β1), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), which are critically important in promoting collagen production and deposition, as well as angiogenesis [2,3,18,19]. Reptile wound healing differs from that of mammals in that angiogenesis and granulation tissue production are scarce and slow [9-11]. In mammals, reduction in wound size is mainly accomplished by centripetal motion of the skin surrounding the wound due to well-developed cutaneous muscles [9], which are absent in chelonians; thus, wound-size reduction and overall second-intention wound healing is slower in reptiles [11]. In a previous study on green iguanas (*Iguana iguana*), 5 mm-diameter full-thickness wounds treated by laser had a significantly smaller mean wound diameter at day 14 ($p = 0.002$), but the magnitude of the difference with respect to a non-treated control was 0.4 mm in diameter [20]. At day 28, ozone-treated wounds achieved a 19.01% greater reduction in mean wound size. The magnitude of this difference is lower than the 24.26% obtained in a similarly designed study using topical insulin [14]. Although wound contraction is not a major feature of second-intention wound healing in reptiles, it is an objective outcome used in most studies and the significantly higher wound contraction of the ozone-treated wounds may be an important finding bearing in mind the limited wound contraction reported in reptiles.

Ozone is widely acknowledged as one of the best disinfectants, and one primary indication of O_3 therapy in second-intention wound healing is the elimination of pathogens. In chelonians, Gram-negative bacteria present in the environment are the most common cause of bacterial infections [21]. In the present study, the wounds were exposed to the environment and bacterial colonies were occasionally identified within the crusts covering both treated and control wounds, but never involving the dermis. This lack of bacterial complications was probably due to the acute nature of the wounds, and to an effective immune response, given that the study only included healthy animals. As ambient temperature and stress have been shown to influence the rate of healing in reptiles and chelonians [22,23], the animals were accommodated at an optimum temperature range and had free access to water and sunbathing areas to regulate body temperature and diminish stress. In sick or malnourished chelonians recovered from the wild, bacterial infection of skin wounds is common and the wide antimicrobial spectrum of O_3, in the current scenario of emerging antibiotic resistance is potentially one of its main advantages. Moreover, ozonated oils remain stable for 2–3 years at 4°C, are inexpensive and are well tolerated even in mucosal areas [1,5].

The present study was designed in such a way as to prioritise outcomes that clinicians could judge easily and would help them decide whether or not to introduce topical O_3 therapy in their daily practice. Nonetheless, this first experimental work has several shortcomings that may have affected the results. Sample size was estimated based on available data of wound size reduction and not on data from cells counts, where differences proved to be more marked. Regarding the experimental design, a within-subject controlled experimental design was chosen, with one wound in each rear leg for welfare reasons and to avoid wounds in close proximity. This design minimised any possible bias in the allocation of animals or wounds to each group of interventions and avoided the major high individual variability during wound healing processes observed in previous studies [11]. Comparison with a standard

therapy has been advocated for all clinical studies assessing O_3 as a new therapy in human patients [3]. In reptiles, there are neither previous studies evaluating O_3 therapy nor any well-defined standard topical treatments to manage wound healing by second intention [20,24]. A decision was made not to have a control group given no treatment whatsoever, because vegetable oils, such as sunflower oil, may promote cutaneous wound healing [25]. Thus, in order to isolate the effect of O_3 from the effect of sunflower oil, non-ozonated oil was used as control group. The inclusion of a control group of wounds without any oil treatment would probably have resulted in more clinically relevant differences. Finally, some of the changes associated with ozonated oil administration on the acute experimentally-induced wounds of healthy animals may not occur in sick animals or in chronic wounds.

In conclusion, topical sunflower ozonated oil reduced wound size significantly 28 days after wound induction and differences were of an important clinical magnitude. This improvement was correlated microscopically with a significant increase of heterophils, lymphocytes and fibroblasts. An enhanced early inflammatory response, fibroblast proliferation and collagen fibre remodelling support the hypothesis that, in addition to its wide spectrum of antimicrobial effects, topical O_3 therapy may be useful in the treatment of cutaneous wounds in chelonians.

ACKNOWLEDGEMENTS

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