



Effect of ozone therapy on wound healing in the buccal mucosa of rats

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ABSTRACT

Objective: The objective of this study was to evaluate the effects of ozone therapy on wound healing formed experimentally in the oral cavity of rats.

Design: Two surgical wounds were generated on the cheeks of 24 Wistar rats, bilaterally. Half of the animals were submitted to ozone therapy on both wounds (experimental group) and the other half received no treatment (control group). In the experimental group, wounds were exposed to ozone gas 1, 2 or 3 (60 µg/mL) times. Evaluation of wound healing of the buccal mucosa was followed for 1, 3 and 7 days. The distribution of neutrophils, fibroplasia and angiogenesis were analyzed. Samples were classified in a healing numerical scale according to the inflammatory intensity. Data were submitted to Mann-Whitney and Kruskal-Wallis tests ($\alpha = 0.05$).

Results: On day 1, wounds were similar in both groups, lesions were open and bloody with slightly minor bleeding in the ozone therapy group. On day 3, the group with ozone therapy was almost all refurbished and with higher angiogenesis, while the control group still had more bloody points and lower blood vessels. On day 7, both wounds were remodeled, with higher fibroplasia in the group that received ozone therapy.

Conclusion: It can be concluded that ozone therapy was effective in improving angiogenesis and fibroblasts count in the buccal mucosa of rats.

1. Introduction

Ozone gas is found in the environment as a protective gaseous layer in the terrestrial atmosphere. It prevents the living beings from harmful effects of ultraviolet radiations (Ripamonti et al., 2012). Research and clinical therapies using ozone have increased in the medical and dental fields due to properties such as biocompatibility, antimicrobial activity, disinfectant action, and healing (Naik, Kohli, Zohabhasan, & Bhatia, 2016).

Among several therapies using ozone, its use can be highlighted in: treatment of diabetes and their complications (Martinez-Sanchez et al., 2005), wound healing (Kim et al., 2009), regeneration of articular cartilage (Manoto, Maepa, & Motaung, 2018), and its coadjuvant action in antitumor treatment (Kuroda et al., 2018). In dentistry, ozone therapy has also been promising (Kumar et al., 2016; Naik et al., 2016; Shilpa,

Reddy, Dinapadu, Reddy, & Pasari, 2013). It is effective inhibiting the development of caries lesion (Holmes, 2003), and decreasing inflammation in gingival and periodontal chronic diseases (Naik et al., 2016), and also reducing the microbial biofilm inside root canal (Shilpa et al., 2013).

Ozone therapy has also been used to accelerate the healing process, reducing inflammatory response and edema (Naik et al., 2016). For healing of epithelial mucosa, ozone can be topically administered by three distinct routes: application of gas, ozonized oils, and ozonized water (Naik et al., 2016). Topic application of ozonized oil was effective to increase wound healing in rats' epithelium, promoting proliferation and synthesis of collagen on the injury sites, and increasing growth factors expression such as PDGF, TGF- β and VEGF (Kim et al., 2009). Ozonized water applied topically reduced the number of *S. aureus* colonies in skin injuries and decreased the severity of wounds in people

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with atopic dermatitis (Lu et al., 2018). Ozonized gas has the highest oxidative potential; therefore, it would be more effective if applied directly on the epithelium of mucosa injuries than when associated with oil or water. Just a few studies have evaluated the effect of ozone gas in healing wounds in oral mucosa (Bayer, Kazancioglu, Acar, Demirtas, & Kandas, 2017; Kovach, Kravchenko, Khotimska, Nazaryan, & Gargin, 2017), and these analyses were based on the growth factors concentration without observing cells behavior.

Thus, the aim of this study was to compare the inflammatory response and healing process of wounds produced in the buccal mucosa of rats, when using ozone therapy, with ozone gas topically applied, in three different time-points or leaving them to heal naturally. The study hypothesis is that the use of ozone therapy decreases injury inflammation and healing time.

2. Materials and methods

This Project was submitted and approved by the Ethical Committee for Animals from Institute of Energetics and Nuclear Research (IPEN, n° 224/18) and it is in accordance with the National Institutes of Health guidelines for care and use of Laboratory animals. Twenty-four adult male Wistar rats, with ages from 40 to 60 days and weight between 250 and 300 g were used in this study. They were maintained in cages, under illumination cycles (14 h illuminated and 10 h darkness) in a temperature controlled environment at 20 °C. Diet was solid before surgery and pasty after surgical procedures with no restriction of water. Male rats were chosen to minimize variation as they have no effect of hormonal cycle on the cell response. Animals were divided in six experimental groups (n = 4 animals), according to the therapy used for tissue repair (ozone therapy or natural healing) and with the endpoint (1, 3 and 7 days). Based on the principles of reduction and refinement of animal

studies, in each rat two injuries were induced, one on the right side and another on the left side of their cheeks, totalizing 8 wounds (n = 8 wounds) to be evaluated in each group. The number of lesions was calculated by the software Experimental Design Assistant (NC3Rs, London, England), considering the significance global level of 95 % ($\alpha = 0.05$), sample power 80 % and standard deviation of 0.5. The latter was based on data from a previous published study, with similar methodology to evaluate inflammatory process in mice's back (Alan et al., 2018).

2.1. Surgical procedure

The methodology used for inducing epithelial injury on a rat model was slightly modified from a previous study (Cavalcante et al., 2011). Animals were anesthetized by an intramuscular injection, with ketamine (60 mg/Kg) and xilazine (8 mg/Kg) and received dipyrone (100 mg/Kg) by subcutaneous route before surgical procedure. Anesthetized animals were immobilized in a surgical frame (Fig. 1 A). Two surgical wounds were induced in the buccal mucosa, one on each side, using a hollow polyester matrix with 4 mm diameter to ensure a standardized area (Fig. 1B). Initially, two perpendicular incisions were made using a scalpel blade n° 12 inside the matrix (Fig. 1C) and the area was slightly scratched with a scalpel blade n° 15. Lesions had a circular shape with 4 mm diameter where the epithelium was scratched (Fig. 1D). Half of the animals had their wounds immediately submitted to ozone therapy, bilaterally (experimental group, Fig. 1E), and half of the animals did not receive any therapy (control group).

Ozone therapy was delivered using an ozone generating machine (Medplus, Philozon, Balneário Camburiu, SC, Brazil) attached to a medicinal oxygen cylinder (Air liquid Brasil LTDA, São Paulo, Brazil). A bifurcated hose was coupled in the vacuum pump of the machine to

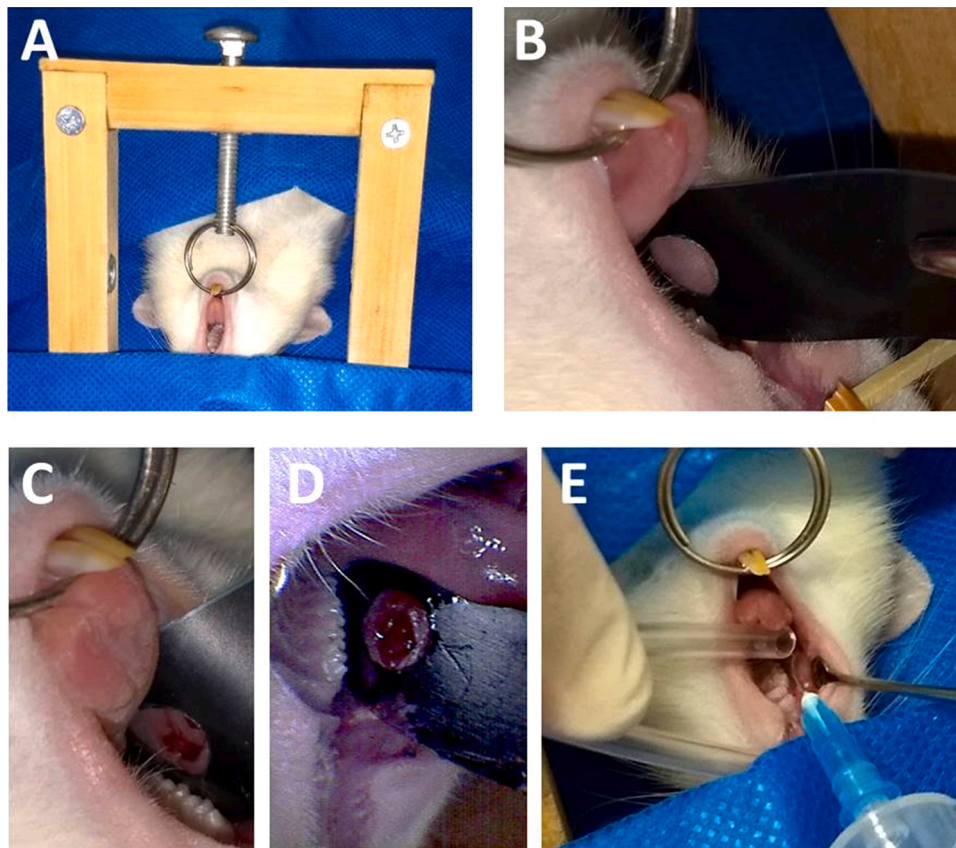


Fig. 1. Experimental animal model. (A) Animal immobilization in the surgical frame; (B) hollow polyester matrix in position to induce wounding; (C) initial induced lesion; (D) ozone application with a plastic syringe and vacuum suction with a bifurcated hose.

absorb any excess ozone, which was destroyed. Each surgical wound received 10 mL ozone gas in the concentration of 60 µg/mL, per session of ozone therapy. A plastic syringe with a 1 mm diameter tip was positioned 2 mm above the wound and used to apply ozone gas. Since ozone is heavier than air, the injured site was positioned downwards during ozone application to avoid accidental aspiration by the animal. Also, a vacuum suction was used to remove any excess gas. Ozone therapy was performed after the surgical procedure and repeated according to the experimental groups, totalizing 1 application (60 µg/mL) in the group with endpoint at 1 day; 2 applications (120 µg/mL) in the group with endpoint at 3 days; and 3 applications (180 µg/mL) in the group with endpoint at 7 days, as described in the Table 1.

In each post-operative session, wounds were photographed using two cameras: an intraoral (M- 989, Super Cam, China) and a regular handheld camera (S9, Samsung Galaxy, China). Animals were euthanized by anesthetic overdose at the endpoints of 1, 3 or 7 days, according to experimental groups.

2.2. Histological analysis

Muscular and mucous tissue were removed and fixed into 10 % buffered formalin solution for 24 h. After rinsing in water, specimens were paraffin-embedded and 5µm-thick-sections were cut and stained with hematoxylin and eosin (HE).

The HE slides were quantitatively analyzed by a blinded pathologist in 400x magnification and scored according to the histopathological parameters (Cavalcante et al., 2011), using the following numerical healing scale: (0) no ulcer and remodeled connective tissue (mature, homogen and parallel collagen fibers); (1) no ulcer and fibrosis with mild chronic inflammation (diffuse and intense deposition of collagen fibers); (2) with ulcer and fibrosis with moderate chronic inflammation; (3) with ulcer and chronic inflammation (granulation tissue); (4) with ulcer and acute inflammation (blood vessels dilated, mixed inflammatory infiltrate containing neutrophils).

Histomorphometry analyses were performed to determine the infiltration of neutrophils, fibroplasia and angiogenesis by selecting aleatory sites in the healing areas of samples. In each site, microscopic fields (250 × 250 µm) were randomly selected to count neutrophils, fibroblasts/myofibroblasts (both cells have the same fusiform morphology, they were counted and presented together in the results section). Quantitative analysis of angiogenesis blood vessels, excluding endothelial sprouts, were counted in images from 4 aleatory fields (magnification 40x), including the area comprising the epithelium to the connective tissue, according to the methodology previously described (Brizeno et al., 2016).

2.3. Statistical analysis

Data of the injury classification according to inflammatory intensity, and also data from histomorphometric analysis (not normal and not

homoscedastic, according Kolmogorov-Smirnov and Levene test respectively) were submitted to non-parametrical tests: Mann-Whitney to compare control and experimental groups, in each endpoint; Kruskal-Wallis and Student-Newman-Keuls to compare the same group in function of the endpoint. A global level of significance of 95 % ($\alpha = 0.05$) was adopted in all the statistical tests.

3. Results

Fig. 2 shows clinical aspects of the wounds in function of post-operative time of control and ozone therapy groups. Immediately after surgical procedure, both ozone therapy (Fig. 2A) and control group (Fig. 2B) showed open bloody wounds, with slightly less bleeding in the ozone therapy group. On day 3, the wound in the ozone group (Fig. 2C) was almost completely remodeled whereas in the control group (Fig. 2D) the wound presented some remodeling but with more bloody points. Finally, 7 days after surgery, wounds were completely healed in both groups (Fig. 2E and 2 F). However, the group with ozone therapy shows a better macroscopic aspect, since no trace of the lesion is observed whereas, in the control group, a reddish mucosa area is seen.

Fig. 3 shows histological images of control and ozone therapy groups, at endpoints of 1, 3 and 7 days. On day 1, ozone group presented ulcer with moderate inflammatory infiltrate of mononuclear cells with predominance of lymphocytes (Fig. 3A). The control group presented moderate mixed inflammatory infiltrate cells with predominance of neutrophils (Fig. 3B). On day 3, ozone group showed mild inflammatory infiltrate of mononuclear cells with predominance of lymphocytes, fibrin net and angiogenesis and inset showing diffuse deposition of collagen fibers and angiogenesis (Fig. 3C). Control group showed mild inflammatory infiltrate of mononuclear cells with predominance of lymphocytes and angiogenesis (Fig. 3D). Finally, on day 7, the group with ozone therapy showed diffuse deposition of collagen fibers within mild inflammatory infiltrate of mononuclear cells (Fig. 3E) and control group presented diffuse deposition of collagen fibers and moderate inflammatory infiltrate of mononuclear cells. Fig. 1S in supplemental material shows one histological image of each animal in 40x magnification for each experimental time, to highlight histological aspects.

Fig. 4 shows the histomorphometric analysis 1, 3 and 7 days after surgery. At the day 1, the ozone experimental group was statically similar to the control group for all the variables measured, to known, healing scale, neutrophils and fibroblasts number, and angiogenesis. On day 3 after surgery, there was an improvement in the healing process and an increase in the angiogenesis process, which were statistically significant for the ozone therapy group in relation to the control group. Angiogenesis can be better observed in the inset of Fig. 3C. No significant difference was observed between the groups regarding fibroblasts count. On day 7 after surgical procedure, both groups were similar regarding healing scale, angiogenesis and neutrophils count. However, the ozone therapy group showed higher fibroblast count than the control group, as also observed in Fig. 3E and 3F. Comparing both therapies in function of time (Fig. 5), ozone therapy showed an improvement in healing at day 3 and was stable at day 7. On another hand, healing improvement was slower in the control group, which was statically different than the other endpoints only at day 7. Neutrophils infiltration was similar in both groups and higher in day 1 and 3 after surgery, and reduced significantly at day 7. Angiogenesis showed a peak on day 3 after surgery for both treatments, but it was 4 times higher in the ozone therapy group. Finally, the fibroblast count was not significantly different along the 7 days for the control group but increased significantly at the day 7 in the ozone therapy group in relation to the other endpoints and in relation to the control group.

4. Discussion

The study hypothesis, that the ozone therapy accelerates healing of wounds in buccal mucosa was accepted. Although no differences were

Table 1

Experimental groups.

Groups	Treatment	Endpoint (days)	Number of ozone applications	Days of ozone application
Group 1_ozone	Ozone therapy	1	1	0
Group 3_ozone	Ozone therapy	3	2	0 and 2
Group 7_ozone	Ozone therapy	7	3	0, 2 and 4
Group 1_control	Natural healing	1	0	–
Group 3_control	Natural healing	3	0	–
Group 7_control	Natural healing	7	0	–

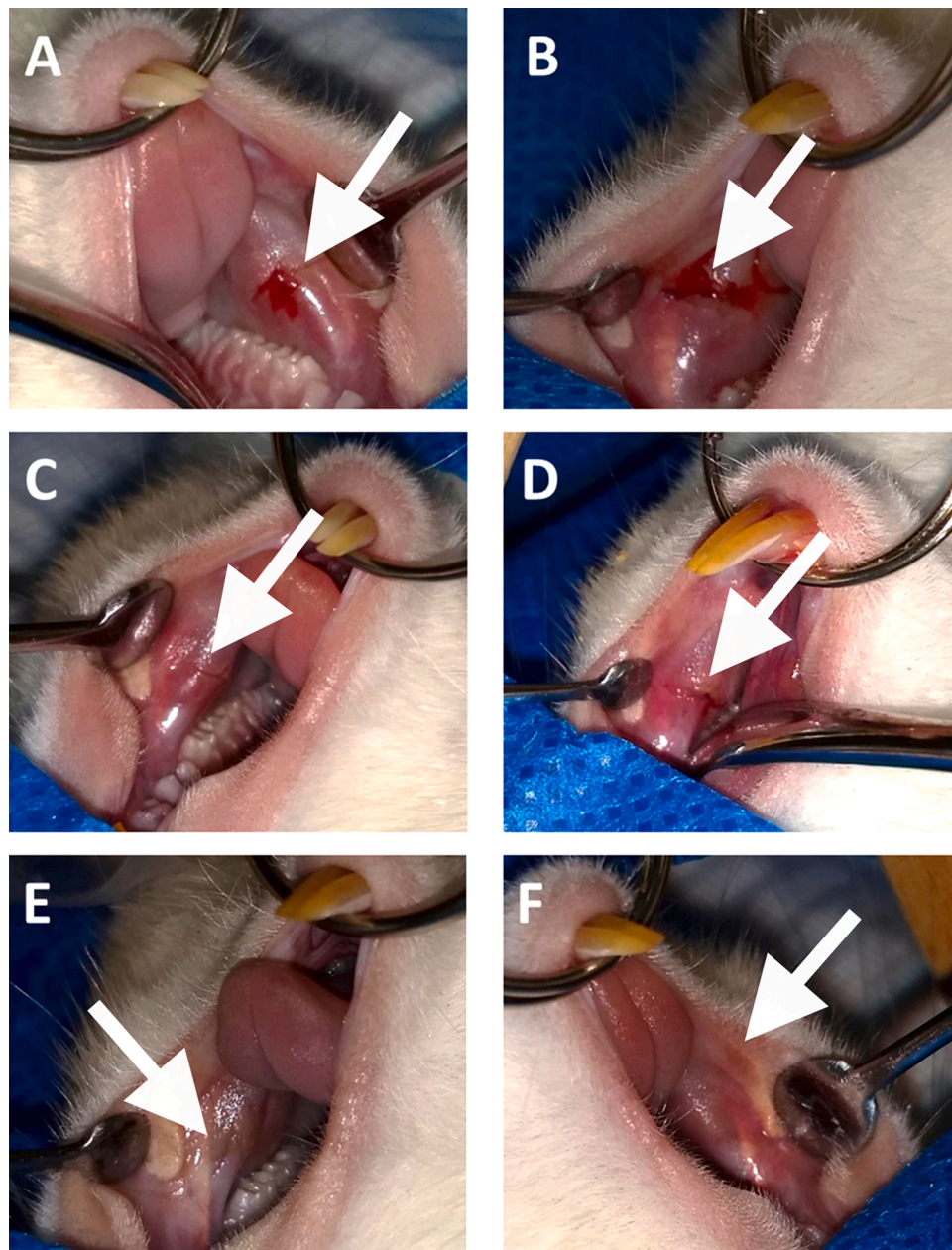


Fig. 2. Macroscopic aspect of lesions (A) ozone therapy group immediately after surgical procedure; (B) control group after surgical procedure; (C) ozone therapy group 3 days after surgical procedure; (D) control group 3 days after surgical procedure; (E) ozone therapy group 7 days after surgical procedure; (F) control group 7 days after surgical procedure. Arrows indicate wound sites.

found in the healing scale between ozone therapy and control group on day 1 after surgery, day 3 showed greater angiogenesis and better healing in the ozone therapy group and day 7 presented a higher fibroblast count, which evidences faster healing.

The action mechanism of ozone in tissues is not completely understood, but studies have pointed that ozone triggers a cascade of reactions in appropriate doses, leading to synthesis of several cytokines and growth factors which results in the increase of local perfusion and oxygen diffusion, optimization of immune response, and also in upregulation of antioxidants enzymes (Di Mauro et al., 2019; Zanardi, Borrelli, Valacchi, Travagli, & Bocci, 2016), which translates as a faster and more efficient wound healing (Smith, Wilson, Gandhi, Vatsia, & Khan, 2017).

No significant histological differences were found in this study on the first day after surgery, between experimental and control groups even though the ozone therapy group presented less bleeding macroscopically compared to the control group. Due to recent surgical trauma, the

wound in both groups is in the inflammatory phase of the repair process, characterized by an intense inflammatory infiltrate. The absence of statistical difference regarding neutrophils count is an indicative that the inflammatory intensity was similar in both groups and possibly the surgical trauma itself was more important in the chemotaxis of these cells than the effect of ozone therapy.

On the third day after surgery, no difference was found between the groups in neutrophils or fibroblast count. The histological analysis showed improvement in the healing process for the ozone therapy group, as certain fibrosis degree were observed in the wounds and it presented less intense chronic inflammation and higher angiogenesis than the control group. The effect of ozone in the angiogenesis occurs due the sum of some factors as, increase of tissue oxygenation, increase of expression of cytokines and of growth factors as VEGF (vessel and endothelial growth factor) (Smith et al., 2017; Zeng & Lu, 2018). In fact, studies with a model of epithelial surgical flaps in rats and lesions on

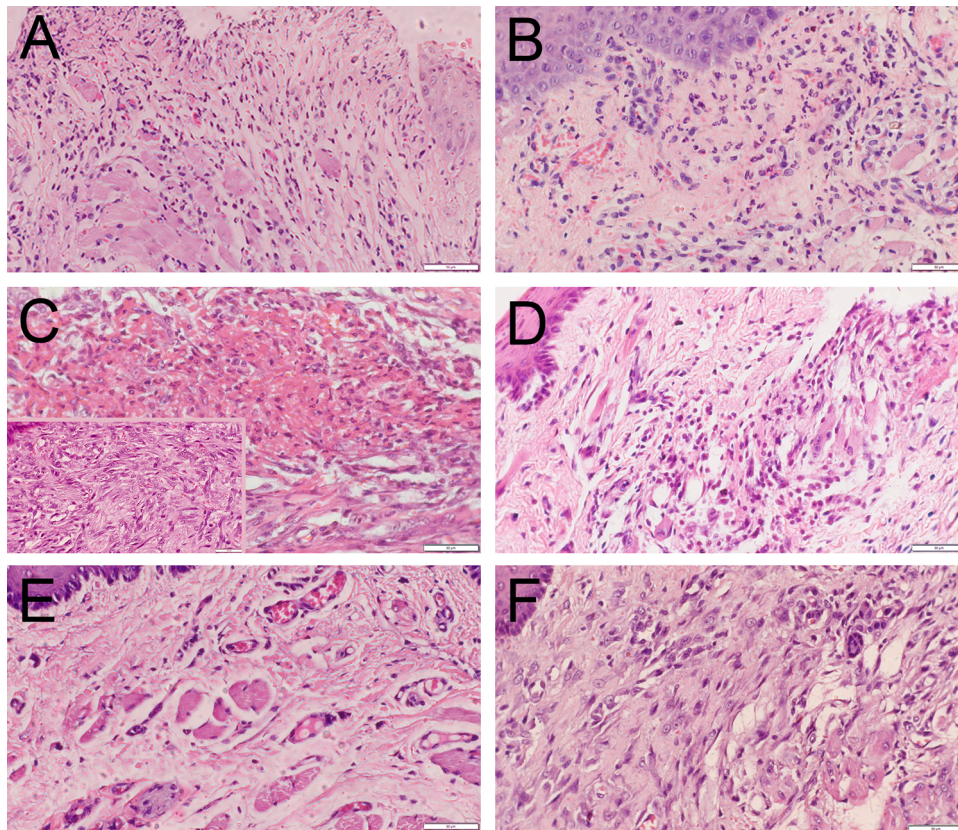


Fig. 3. Histological images with magnification of $40 \times$. (A) ozone therapy group 1 day after surgical procedure; (B) control group 1 day after surgical procedure; (C) ozone therapy group 3 days after surgical procedure; (D) control group 3 days after surgical procedure; (E) ozone therapy group 7 days after surgical procedure; (F) control group 7 days after surgical procedure.

buccal mucosa in pigs have reported an increase of VEGF concentration in animals receiving ozonized oil or ozone gas daily on the wounds for 3 or 7 days (Eroglu et al., 2018; Kim et al., 2009). However, another study examining wounds in the buccal mucosa of rabbits, observed significant reduction of neutrophils count 3 days after surgical procedure using ozone gas. It is believed that such differences in the responses can be attributed to the lesion size (more extensive in the rabbits) and to the fast metabolism and fast healing in the Wistar rats used in the present study.

On day 7 after surgery, no differences were found between the groups regarding healing scale. Cavalcanti. et al (2011) observed that wound healing in rats mucosa can be observed until 10 days for this surgical model. However the lesions in the present study have already been well remodeled at 7 days, possibly due to the depth of the lesions, which were more superficial lesions in this study, where a shorter endpoint is more promising to indicate the differences in healing time. Nevertheless, the number of fibroblast was greater in the ozone therapy group than control. In fact, studies have highlighted the effect of ozone in the migration and activation of fibroblasts, with increase in the expression of collagen I and TGF- β genes (Xiao et al., 2017), and increase in the syntheses of growth factors as PDGF and TGF- β (Eroglu et al., 2018; Kim et al., 2009), which are directly related to fibroblasts proliferation (Barrientos, Stojadinovic, Golinko, Brem, & Tomic-Canic, 2008), and consequently to the syntheses of collagen extracellular matrix.

Satisfactory results have been reported using the application of ozone in a concentration from 27–50 $\mu\text{g/mL}$ in humans (Andreula, 2011; Bocci, Zanardia, Valacchi, Borrelli, & Travagli, 2015). The use of 60 $\mu\text{g/mL}$ improved its effect without producing toxicity (no animal was lost during the experiment) or jeopardizing the healing process. Some studies have developed methodologies with greater lesions and more

sessions of ozone application (Kim et al., 2009; Ripamonti et al., 2012). However, dose and application frequency are parameters that still need to be standardized. In this study, it could be observed that even in lower lesions the effect of ozone therapy in ulcer wounds of rats buccal mucosa was effective from the third day after surgery until the final healing, indicating a better healing process in oral lesions. It is important to highlight that others aspects related to the ozone therapy were not evaluated in this study, as the distance of the syringe tip from wound, ozone concentration, and application speed, among others, that can also contribute to the post-operative results and should be observed. Other outcomes can also be evaluated in future studies such as decrease of symptomatology (Travagli, Zanardi, Valacchi, & Bocci, 2010), antimicrobial effect (Patel & Gujjari, 2013) etc. The protocol presented here mimics real oral conditions, such as bacterial flora, biofilm, pH, and blood supply to the jaw, as well as the influence of masticatory forces, being an efficient pre-clinical model to evaluate the healing process.

According to the exposed, it can be concluded that the ozone therapy was effective to accelerate the healing of wounds in the rat's oral mucosa increasing angiogenesis 3 days after surgery and increasing fibroblast count after 7 days.

Conflict of interest

The authors declare no conflict of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

The authors report no declarations of interest.

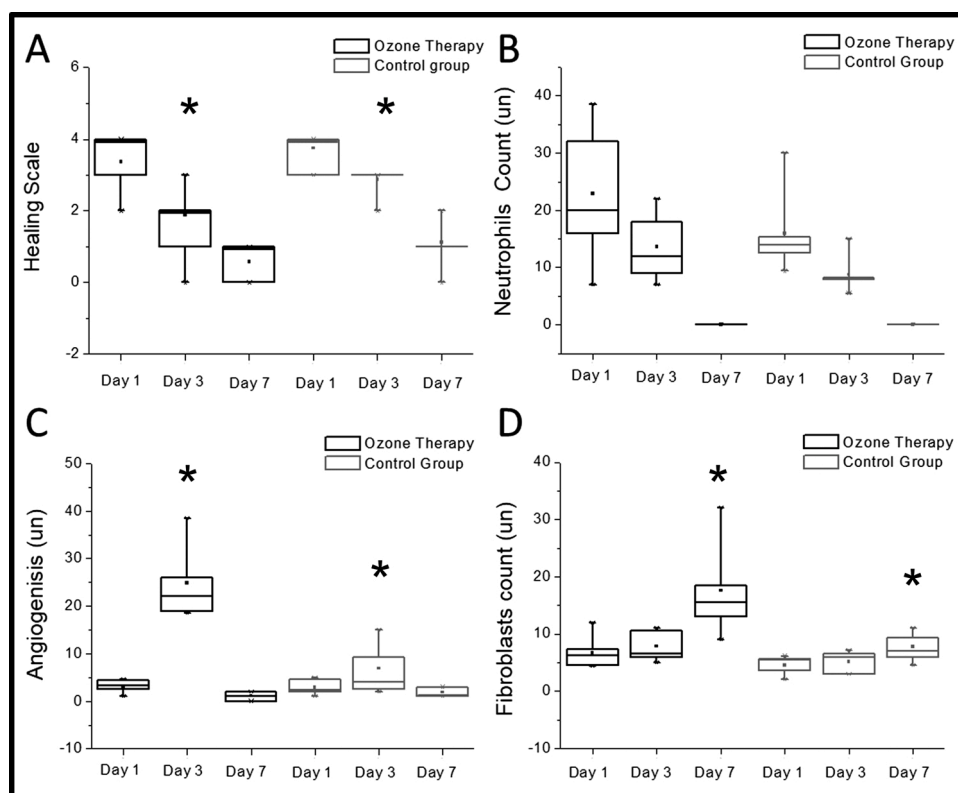


Fig. 4. Median, first and third quartiles, minimum and maximum values and mean (points) of histological and histomorphometric parameters 1, 3 and 7 day after surgical procedure: (A) Healing scale; (B) neutrophils count; (C) angiogenesis; (D) fibroblast count. The (*) indicates significant difference statistics among ozone therapy and control group.

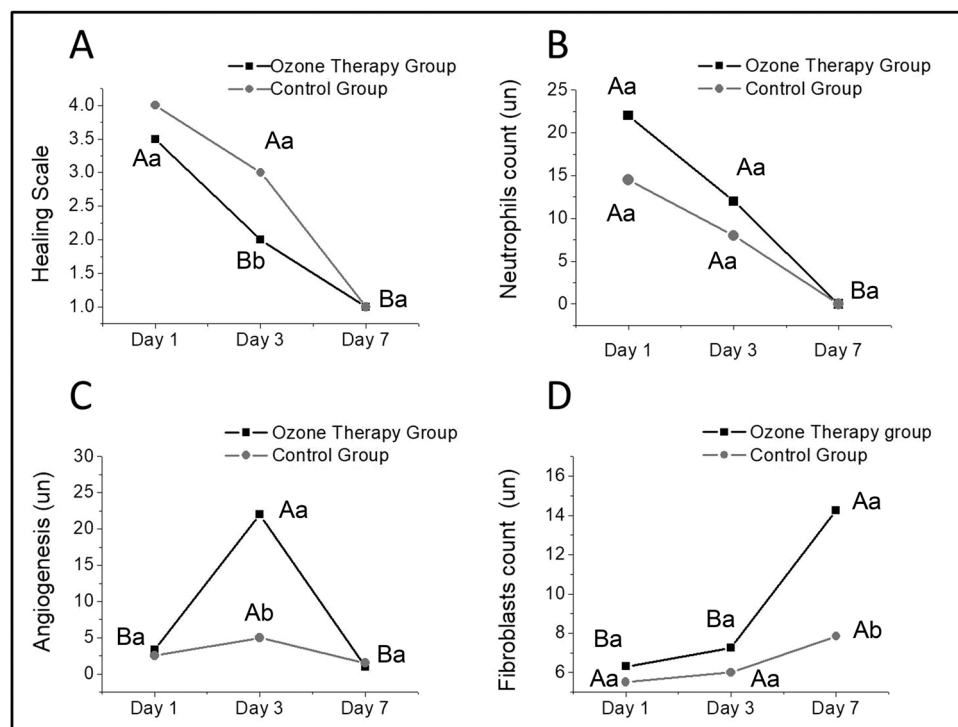


Fig. 5. Comparison of healing scales (A), neutrophils count (B), angiogenesis (C) and fibroblasts count (D) in function of the time after surgery. Capital letters compare one single therapy in function of time and lowercase letters compare control and ozone therapy groups at the same time.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.archoralbio.2020.104889>.

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