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Effectiveness Of Ozonated Black Seed Oil On Full-Thickness Skin Defect Healing

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Abstract

Background: Inadequacies in wound treatment could lead to complications. Black seed oil (BSO) alone has antimicrobial and antioxidant properties. Ozone is known for its potential to improve the wound healing process through the reactive oxygen species (ROS) mechanism. However, the studies of ozonated black seed oil (OBSO) on wound healing are limited.
Objectives: To determine the effects of OBSO in full-thickness skin defect on the dorsal of Sprague Dawley (SD) rats, specifically on wound closure rate, epithelization, number of fibroblasts, and collagen distribution.
Methods: Full-thickness skin defect (10 mm) was created on dorsal of 60 SD rats that were divided into six groups: gentamycin, normal saline, BSO, OBSO at varied doses of 1.4, 1.8, and 2.2 g/dl (n=10). Half of the population in each group were terminated after three days of treatment, while the remain subjects were terminated after seven days. The wound area was measured with ImageJ computerized application and the skin tissue was excised for histopathological examination with HE and Van Giesson staining.
Results: The statistical analysis showed no significant differences in the wound area, reepithelization, and distribution of collagen after 3 and 7 days of treatment ($p > 0.05$), while there was a significant difference ($p < 0.05$) for the number of fibroblasts in the 3 and 7 days of treatment.
Conclusion: The application of OBSO gave no significant difference in enhancing wound healing compared to the control group after 3 and 7 days of treatment.

Keywords: ozonated black seed oil, *full-thickness skin defect*, wound healing

Introduction

The incidence of wounds is increasing every year. Meanwhile, a study in the United Kingdom reported that the wounding patient prevalence is 3-4 per 1000. In Indonesia, the trauma prevalence had increased to 8.2% in 2013 with the highest incidence being abrasion wounds (70.9%).[1,2] Wound is the discontinuation of skin tissue due to various mechanisms, namely, the acute wound caused by physical and mechanical injury, as well as chronic due to failure of the wound healing process, which is caused by physiological conditions such as diabetes mellitus (DM).[3,4] Meanwhile, the wound healing process consists of the inflammatory, proliferative, and maturation phase that usually take several weeks for better tissue integrity restoration.[5]
The inflammation phase begins with the hemostasis process immediately after the injury. In this phase, cytokines stimulate inflammatory factors and activate intrinsic together with extrinsic pathways that invite neutrophils, lymphocytes, and macrophages to fight the infection and clear debris. At the proliferation phase, the angiogenesis process involves the conception of endothelial cells and the rearrangement of basal membranes. This improves the proliferation of the fibroblasts and collagen synthesis, while chemotactic factors and IFN-β production by fibroblast causes granulation tissue formation. In this stage, re-epithelialization occurs where epithelial cells proliferate and migrate to the wounded area to cover the lesion.[6] Maturation or re-modelling phase is the final stage of wound healing, and it involves the balance between the synthesis and degradation of collagen and organized other disposed proteins. In this phase, type III collagens are replaced by type I, the vascularization decreases, and the scar gradually turns grey.[7] Moreover, untreated wound increases the risk of microorganisms infection on wound sites which leads to a prolonged healing process. Therefore, enhancing wound closure is crucial to avoid extended infections and other complications.[8]

Ozone (O_3) has been known for its potent antimicrobial properties since the 19th century, while its use for medical purposes has been widely studied recently.[9] Furthermore, it is an unstable molecule decomposes into a reactive single oxygen to microorganisms, such as viruses, bacteria, and protozoa.[10] When ozone in an effective dose range contacts with damaged tissue, it oxidizes lipid and tissue proteins to produce Reactive Oxygen Species (ROS), which helps activate pro-inflammatory cytokines and enhance wound healing.[11] The activity of ozone produces ROS products, such as hydrogen peroxide, which is vital in wound healing and activates various biochemical pathways in the body.[12] Previous studies have shown that ozone is capable of influencing the level of VEGF *(Vascular Endothelial Growth Factor)*, TGF-β *(Transforming Growth Factor- beta*, and PDGF *(Platelet-Derived Growth Factor)*.[13]

There are three ozone therapies applied for medical purposes: direct exposure of ozone gas in the hyperbaric chamber, ozonated water, and ozonated oil. [14] The ozonated water is widely used in dentistry to inhibit bacterial growth and plaque formation. However, it is unstable and has a short half-life compared to ozonated oil.[14] Meanwhile, topical therapy enables ozone to penetrate the skin and kill microorganisms effectively.[15] Therefore, the study of ozonated oil such as vegetable and black seed oil has been developed recently.[16-19]

Black seed oil is an active compound that improves various diseases due to its antimicrobial and antifungal properties, as shown by the in vitro study.[20] Furthermore, black seed oil contain *Thymoquinone*, flavonoid, and *triterpenoid* that have been proved to enhance wound healing, [20]. At the same time, the topical application of black seed oil on ulcer wounds in diabetic-induced mice showed promising results for the epithelization process.[21]. In the wound healing process, black cumin increases the angiogenesis process, fibroblasts that support the proliferation phase, collagen synthesis, and fibers which are properly arranged from the results of histopathological examination.[22]

There are currently limited studies of ozonated black seed oil treatment at varying doses on wound healing. Therefore, this study aims to determine the effectiveness of ozonated black seed oil on the wound healing in terms of closure, epithelization rates, number of fibroblasts, and collagen distribution.

Methods

Ethical statement

This was an experimental study with only one post-test control group design and was approved by the Ethics Commission of the Public Health Faculty, Diponegoro University, Semarang number 65/EC/H/FK-UNDIP/VII/2020

Animals

The 60 male Sprague Dawley mice (8-12 weeks old) weighing 250±50 gram used as a subject were in a good and healthy condition (active movement) without signs of visible anatomic abnormalities, while the exclusion criteria included mice that died during this study. These mice were randomly divided into six different groups, namely, (n=10) that received gentamycin (K+), normal saline (K-), black seed oil (M), ozonated black seed oil at a dose of 1.4 g/dl (MA), 1.8 g/dl (MB), and 2.2 g/dl (MC). Half of the sample population were terminated after three days of treatment, while the remaining samples were terminated after seven days. Furthermore, the mice were kept in individual laboratory-grade cages at a constant room temperature and 12 hours per day lightning with adequate *ad libitum* food and drinkable water supply. The mice were first acclimated seven days before wound creation.

Production of Ozonated Black Seed Oil (OBSO)

The OBSO production was carried out at the Plasma Research Center (PRC), Diponegoro University, Indonesia, by combining black seed oil and ozone produced from a generator connected to an oxygen cylinder. The ozone was dissolved into 70 cc black seed oil that was facilitated by a magnetic stirrer and a diffuser connected to the ozone generator. In this study, ozone concentrations were 1.4 g/dl, 1.8 g/dl, and 2.2 g/dl.

Full Thickness Wounds

The table surgery and all the apparatus were sterilized and sanitized to minimize contamination risk, and anesthesia was administered through aerosol administration with Ether. After hypoesthesia was assessed by loss of muscle tone and pain stimulation, the hair in the upper dorsal region (2-3 cm around the wound model) was shaved. Afterward, the skin was disinfected by an alcohol swab, and the full-thickness skin excision with a diameter of 1 cm was made using punch biopsy. After the wound was cleaned by normal saline 0.9%, the mice were placed in the individual cage for recovery.

Topical application

Two drops (about 0.1 ml) of black seed oil, ozonated BSO, and normal saline was applied to the wound, while gentamycin was thinly applied to the wound surface. The topical application was carried out once a day for 3 and 7 days with the wound left undressed.

Wound area measurement

The wound area was measured after 3 and 7 days of treatment. Meanwhile, the wound and a ruler placed beside it were photographed with a camera. The wound area was measured by computerized software named ImageJ (National Institute of Health, United States) in cm^2.

Histological analysis

Surgical excision of the wounded area was performed after 3 and 7 days of treatment, while Hematoxylin and eosin staining were conducted for epithelization and the number of fibroblast analyses. Also, the epithelization was assessed

microscopically using 40x magnification, and fibroblast using a 400x magnification binocular microscope. Van Giesson staining was performed for distribution of collagen analysis that was assessed microscopically using 100x magnification. The number of fibroblasts and collagen distribution was observed and counted in 5 fields of view and the results were averaged.

Data analysis

Data analysis was performed using statistic-computerized software (IBM SPSS ver.26). The data normality was tested using the Shapiro-Wilk test. One-Way ANOVA test was chosen to compare means between groups with an alternative of Non-Parametric Kruskal-Wallis. The data were considered significant $p < 0.05$ and this is obtained, it is followed by the *Least Significance Difference (LSD)* Post Hoc test to analyze differences between groups, and the Kruskal Wallis test is followed by the Mann-Whitney test.

Results

This study used 60 male Sprague Dawley mice and 5 mice were excluded during the experimental process. Hence, a total of 55 male Sprague Dawley mice were used as experimental animals.

Wound area analysis

The wound area was measured after 3 and 7 days of treatment in cm^2 and normality and average wound area were shown in Table 1. One-way ANOVA showed no significant difference in wound area for 3 days of treatment ($p = 0.370$). This same result was from non-parametric Kruskal-Wallis, which showed no significant difference in wound area for 7 days of treatment between groups ($p = 0.351$). Wound area is shown in Figure 1, Figure 2 and Figure 3.

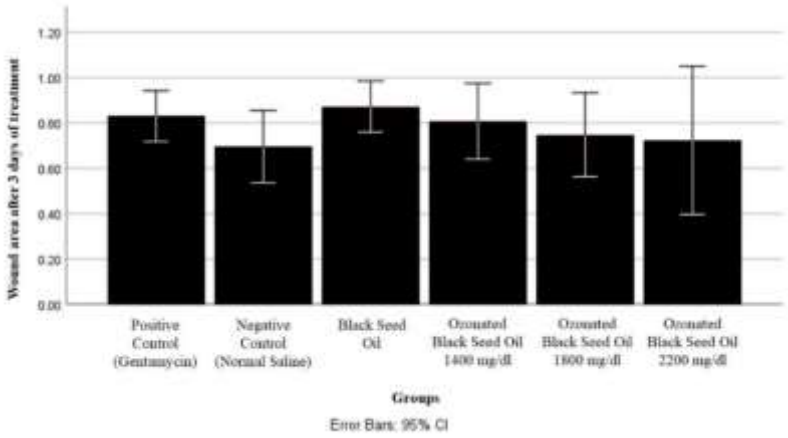


Figure 1. Mean wound area after 3 days treatment

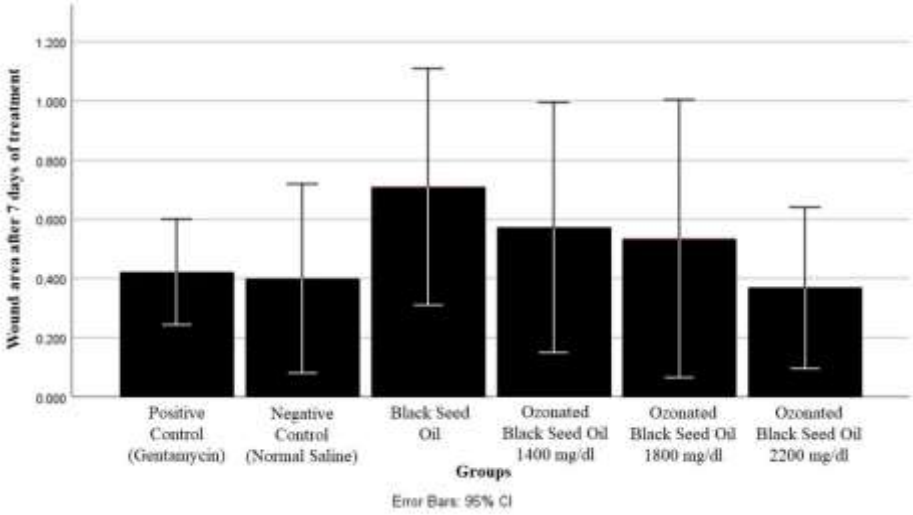


Figure 2. Mean wound area after 7 days treatment

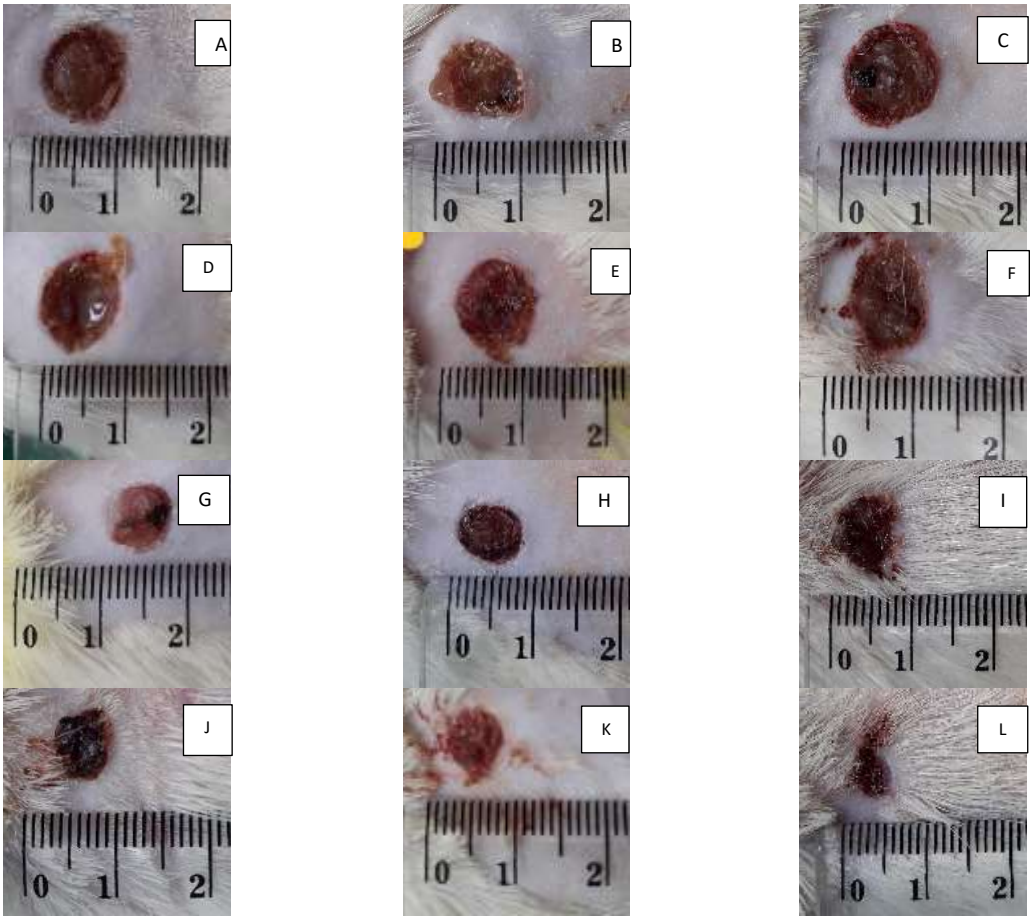


Figure 3. Presentation of wound area after 3 days treatment with gentamycin (A), normal saline (B), black seed oil (C), ozonated black seed oil 1.4 g/ml (D), ozonated black seed oil 1.8 g/ml (E), ozonated black seed oil 2.2 g/ml (F). Presentation of wound area after 7 days treatment with gentamycin (G), normal saline (H), black seed oil (I), ozonated black seed oil 1.4 g/ml (J), ozonated black seed oil 1.8 g/ml (K), ozonated black seed oil 2.2 g/ml (L).

Epithelization analysis

Epithelization was obtained by measuring the distance between novel epithelium formed on both sides of the wound microscopically. The shortest length between epitheliums showed the best reepithelization rate, while the One Way ANOVA test showed $p = 0.809$, indicating the absence of a significant difference of epithelization between study groups after 3 days of treatment. Meanwhile, the non-parametric Kruskal-Wallis test result showed an insignificant difference of epithelization after 7 days of treatment with $p = 0.825$. The results of the data analysis are shown in Table 1, Figure 4, Figure 5 and Figure 6.

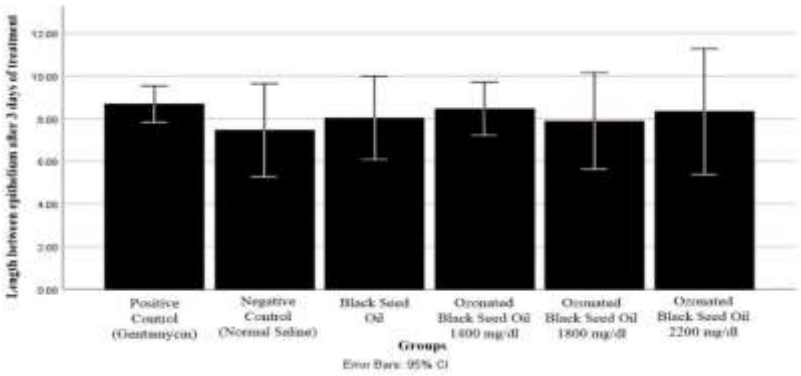


Figure 4. Mean length between epithelium after 3 days treatment

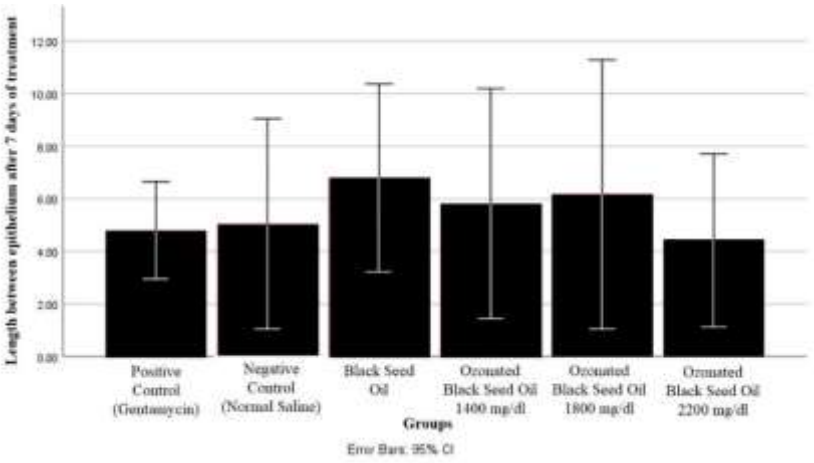


Figure 3. Mean epithelization after 3 and 7 days treatment
Figure 5. Mean length between epithelium after 7 days treatment

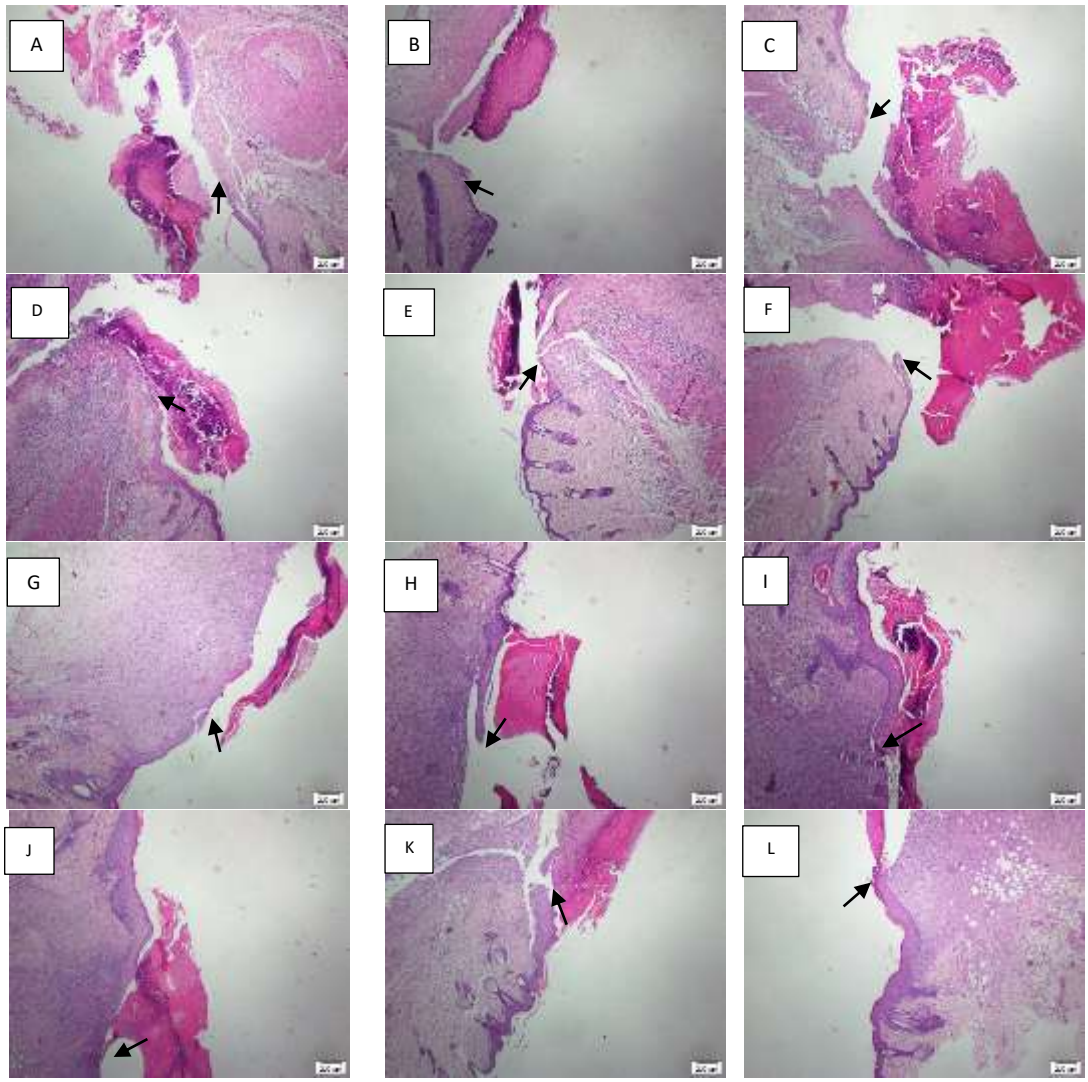


Figure 6. Histopathological findings of epithelial after 3 days treatment with gentamycin (A), normal saline (B), black seed oil (C), ozonated black seed oil 1.4 g/ml (D), ozonated black seed oil 1.8 g/ml (E), ozonated black seed oil 2.2 g/ml (F). Histopathological findings of epithelial after 7 days treatment with gentamycin (G), normal saline (H), black seed oil (I), ozonated black seed oil 1.4 g/ml (J), ozonated black seed oil 1.8 g/ml (K), ozonated black seed oil 2.2 g/ml (L).

The Number of Fibroblast Analysis

The number of fibroblasts was obtained by counting the fibroblast in 5 fields of view and the results were averaged. The normality test for 3 and 7 days of treatment groups showed that the data were normally distributed. One way ANOVA test showed $p = 0.040$ for 3 days treatment and $p = 0.000$ for 7 days group which indicated that there was a significant difference between groups. Post Hoc LSD test showed a value of $p = 0.001$ which indicated that there was a significant difference between the positive control and OBSO group with 1.4 g/ml of ozone after 3 days of treatment while OBSO group 1.4 g/ml of ozone had fewer number of fibroblasts. Meanwhile, for 7 days treatment group, Post Hoc LSD test showed a value of $p = 0.018$ which indicated that there was significant difference between BSO and OBSO with 2.2 g/ml of ozone, while OBSO with 2.2 g/ml of ozone had higher number of fibroblasts. The results of data analysis are shown in Table 1, Figure 7, 8 and 9.

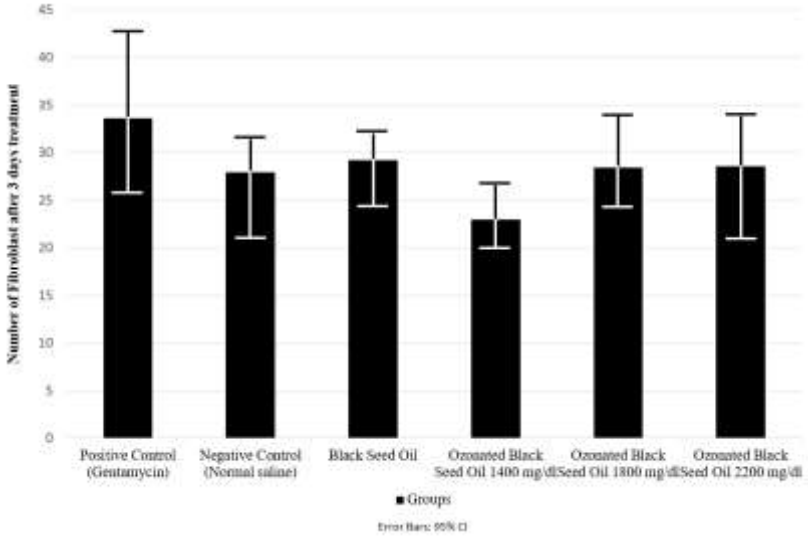


Figure 7. Mean number of fibroblast after 3 days treatment

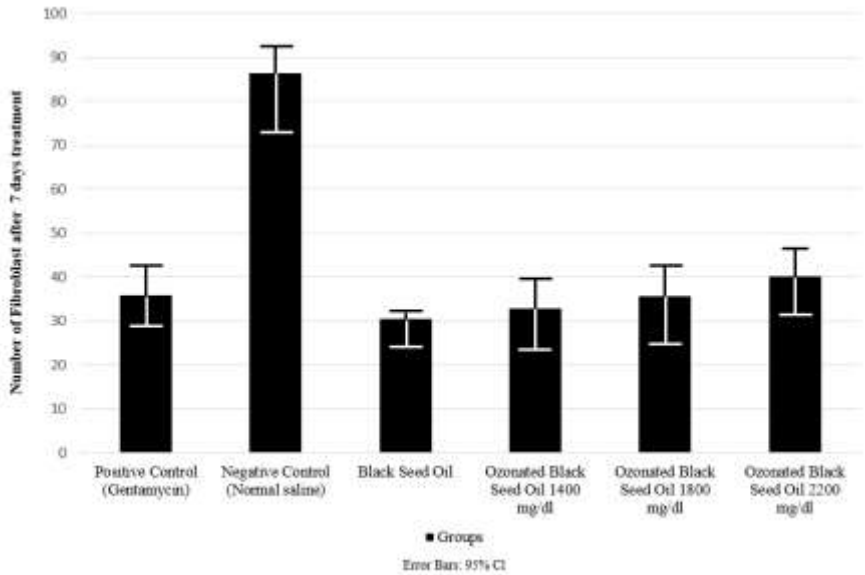


Figure 8. Mean number of fibroblast after 7 days treatment

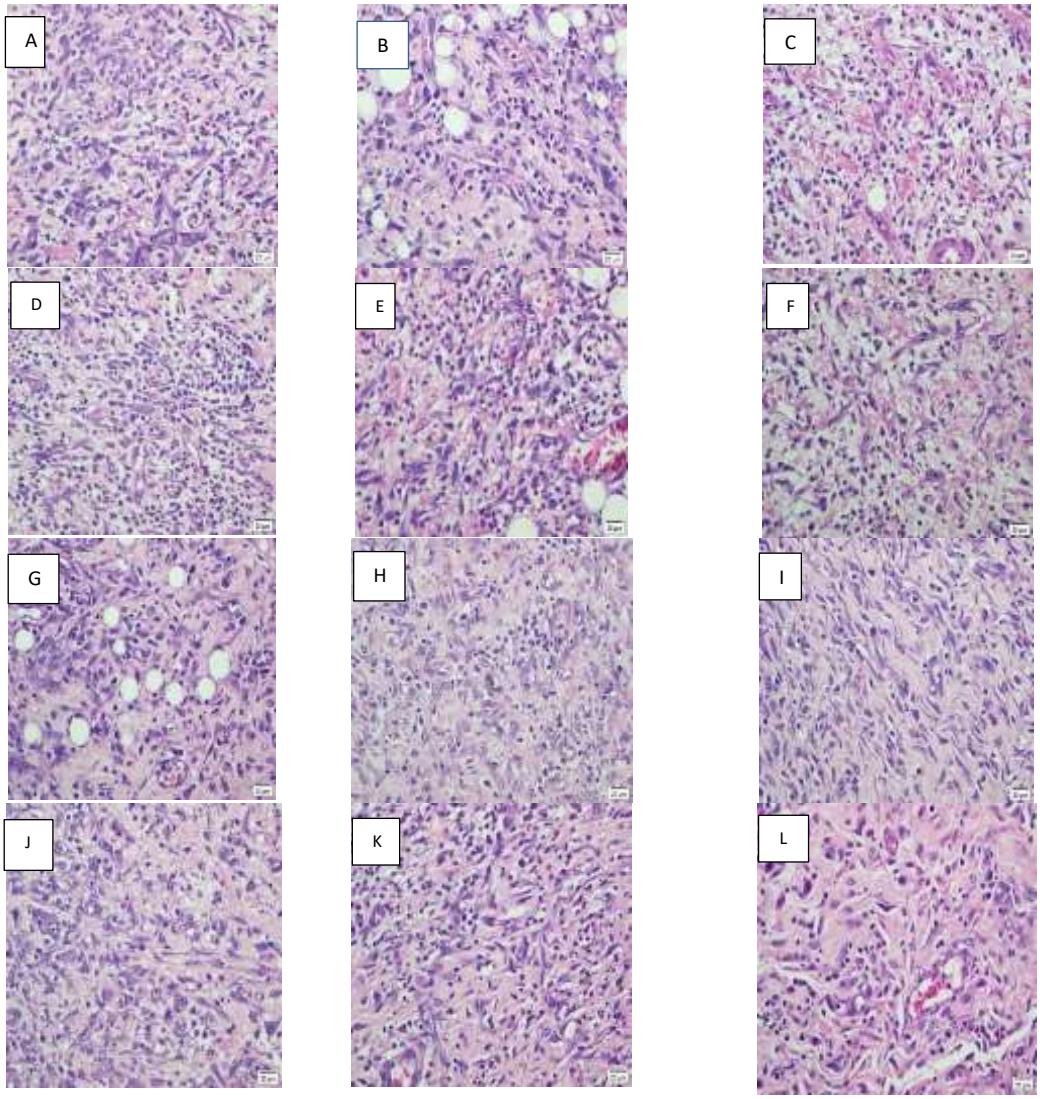
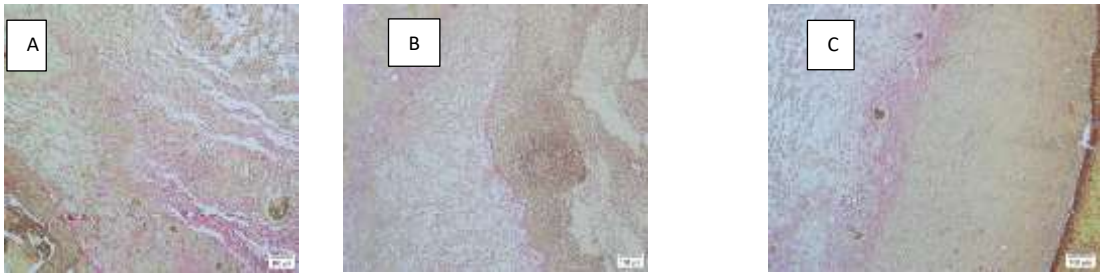


Figure 9. Histopathological findings of fibroblast after 3 days treatment with gentamycin (A), normal saline (B), black seed oil (C), ozonated black seed oil 1.4 g/ml (D), ozonated black seed oil 1.8 g/ml (E), ozonated black seed oil 2.2 g/ml (F). Histopathological findings of fibroblast after 7 days treatment with gentamycin (G), normal saline (H), black seed oil (I), ozonated black seed oil 1.4 g/ml (J), ozonated black seed oil 1.8 g/ml (K), ozonated black seed oil 2.2 g/ml (L).

The Distribution of Collagen Analysis

The normality test showed that the three days of the treatment group was normally distributed while the 7 days group was not normally distributed. One way ANOVA test with of value of $p = 0.242$ showed no significant difference after 3 days of treatment. Meanwhile, the Kruskal Wallis test obtained value $p = 0.517$ which showed that there was no significant difference after 7 days of treatment. The results of the data analysis are shown in Table 1 and Figure 10.



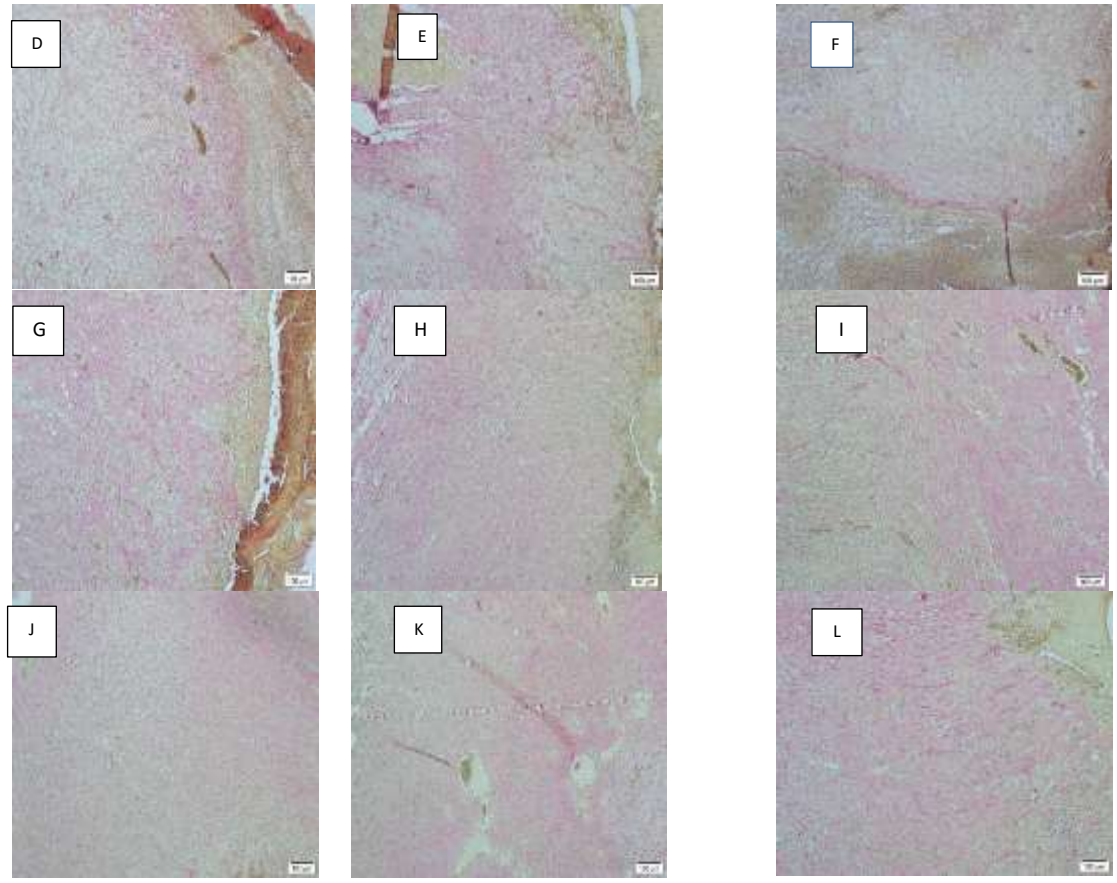


Figure 10. Histopathological findings of collagen after 3 days treatment with gentamycin (A), normal saline (B), black seed oil (C), ozonated black seed oil 1.4 g/ml (D), ozonated black seed oil 1.8 g/ml (E), ozonated black seed oil 2.2 g/ml (F). Histopathological findings of collagen after 7 days treatment with gentamycin (G), normal saline (H), black seed oil (I), ozonated black seed oil 1.4 g/ml (J), ozonated black seed oil 1.8 g/ml (K), ozonated black seed oil 2.2 g/ml (L).

Tabel 1. Mean of Wound Area, Epithelization, Number of Fibroblast, and Distribution of Collagen

Length of Treatment	Groups	N	Mean ± SD			
			Wound Area	Epithelization	Number of Fibroblast	Distribution of Collagen
3 days	K+	5	0.83 ± 0.09	8.67 ± 0.68	33.60 ± 6.17	44.40 ± 10.62
	K–	5	0.69 ± 0.12	7.46 ± 1.75	27.92 ± 4.37	39.20 ± 17.52
	M	4	0.87 ± 0.07	8.02 ± 1.22	29.20 ± 3.50	58.00 ± 20.20
	MA	5	0.80 ± 0.13	8.46 ± 1.00	22.96 ± 2.66	54.40 ± 11.78
	MB	5	0.74 ± 0.14	7.89 ± 1.81	28.40 ± 4.05	55.60 ± 10.14
	MC	4	0.72 ± 0.20	8.32 ± 1.85	28.55 ± 5.35	56.50 ± 10.76
7 days	K+	5	0.42 ± 0.14	0.42 ± 0.14	35.84 ± 4.51	76.40 ± 4.98
	K–	4	0.40 ± 0.20	0.40 ± 0.20	86.50 ± 8.69	77.50 ± 4.12
	M	5	0.71 ± 0.32	0.71 ± 0.32	30.44 ± 2.16	71.60 ± 6.22
	MA	4	0.57 ± 0.26	0.57 ± 0.26	32.90 ± 5.55	71.50 ± 5.26
	MB	4	0.53 ± 0.29	0.53 ± 0.29	35.65 ± 7.99	73.00 ± 8.72
	MC	5	0.36 ± 0.21	0.36 ± 0.21	40.20 ± 6.04	80.40 ± 12.83

Discussion

No significant difference was found in the wound area and epithelization between study groups after 3 and 7 days of treatment. A previous by RIZAOĞLU, et al., stated that there was no significant difference between negative control and experimental groups that received ozonated sesame oil, *Nigella sativa* oil, *and Hipericum perforatum* oil in terms of wound area and epithelization after 7 days of treatment. Meanwhile, the wound healing rates were more significant in the experimental groups after 14 days of treatment, especially the group treated with ozonated *Nigella sativa* oil. However, it is difficult to compare these studies since the study conducted by RIZAOĞLU stated that the ozonation level of ozonated *Nigella sativa* oil was unknown.[19]

This study showed an increase in the number of fibroblasts and distribution of collagen on 7 days treatment group when compared to the 3 days group. Meanwhile, a previous study conducted by Kim *et al.* on ozone therapy for full-thickness wound healing in pigs showed an insignificant increase in fibroblasts and collagen on the 3 days of treatment and a significant increase in collagen and fibroblasts on the wound edges and the middle of the wound compared to the oil group on the 7 days.[23] Furthermore, there was a significant difference in the fibroblasts number after 3 and 7 days of treatment, while a study by Hibono *et al.*, stated that the number of fibroblasts is at maximum limit along with the peak of type III collagen synthesis. When the need for collagen produced by fibroblasts is optimal, it stops synthesizing and is replaced by scar tissue, leading to decreased fibroblasts' number.[21] In this study, the number of fibroblasts increased after 7 days of treatment for all groups, indicating that the number had not yet reached the maximum limit.

A previous study conducted by Nourbar *et al.* on the effect of hydroethanolic extract from black seed on wound healing in mice DM-induced showed the highest epidermal thickness with the maximum number of fibroblast cells and the thickest collagen fibers formed compared to the other groups.[22] This effect might be due to the content of the black seed oil that consists of 34-39% oil, 0.5-1.5% essential oil, 29-37% carbohydrates, 20-23% protein, and thymoquinone 0 - 0.75% which showed that thymoquinone plays an important role in wound healing by acting as an antimicrobial.[19]

The peroxide of ozonated oil gave attribution to the wound healing process. The exposure of ROS on wound increases the proliferative phase through the NFkB *(Nuclear Factor kappa-light-chain-enhancer activated B cells)* redox transcription factor activator that could induce the synthesis of growth factors in the wound epithelization. Meanwhile, the activation of NFkB by O_3 had a biphasic dose-effect relationship between ROS levels and NFkB. Moreover, a previous study by Valachhi *et al.*, stated that ozonated sesame oil with a medium level of peroxidation (1,500 meq/kg) had a higher effect in enhancing wound closure rates with a significant increase in myofibroblasts on the 3rd day and reached its peak on the 7th day. Therefore, there were no significant effects observed with low (949 meq/kg) or high (3,000 meq/kg) levels of peroxidation. The application of ozonated sesame oil with a high level of peroxidation inhibited the activation of NFkB which gave a slower effect on wound healing in SKH1 mice.[24] Also, a previous study stated that ozonated olive oil with a peroxidation level of 2.7-2.9 Eq/kg gave no cytotoxic effect on the L929 fibroblast culture cell line during 48-h in vitro exposure. Meanwhile, further in vivo study has not been conducted to evaluate any complications that occurred.[25] Tamba *et al.*, stated that the topical application of ozonated aloe vera oil with a peroxidation level of 1,772.81 meq/kg had a significant effect on SD mice wound healing in terms of epithelization. Similarly, a study conducted by Vahlepi *et al.*, stated that the rate of wound closure in SD mice was increasing in experimental groups that received ozonated aloe vera with the same peroxidation level. The study also showed that ozonated aloe vera oil was applied twice daily and the wound was covered by an elastomull bandage during the study for 3 and 7 days of treatment.[26,27] However, it was difficult to compare these results due to the different oil media used as ozone carriers, while the various components of PUFA have a different level of peroxidation. Since ozone reacts with carbon double bonds, a higher degree of unsaturation leads to loner ozonation reaction time.[28] Currently, there are limited studies on the cytotoxic or therapeutic effect of ozonated oil treatment with a level of peroxidation ranging from 1.8-3 Eq/kg on wound healing in animal models.

A previous study conducted by Pai *et al.* on Sprague Dawley rats with wounds to the subcutaneous layer with treatment groups of 6 drops ozonated sesame oil per application for 11 days of treatments showed that the quality of wound healing in ozonated oil groups was better than the vehicle-treated group based on histopathological findings.[29] In this study, the wound was treated with two drops of ozonated black seed oil once per day for 3 and 7 days. Moreover, the wound was left open for microorganism contamination and post-operation infection which led to the prolonged inflammatory phase causing inhibition of wound healing.[30] A microbiology identification test was not conducted, therefore, the specific profile of wound-infecting microorganisms was not obtained. However, some literature stated that the most common microorganisms in wound infection were MRSA and MSSA strain of *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa,* and *Listeria monocytogenes*.[31] A previous study on the antibacterial effect of black seed extract against MRSA by Sharikh *et al* showed that the isopropanol extract which indicated the best antimicrobial ability to inhibit the growth of all the bacteria tested significantly. Furthermore, the study also showed that black seed acted better against *Staphylococcus aureu*s than *Pseudomonas aeruginosa*.[32] Although the ozonated oil has antimicrobial properties, there were different effects among bacteria strains. The previous study by Amri, *et al.*, showed that ozonated pomegranate oil acted stronger on gram-positive bacteria than on gram-negative bacteria.[33] Furthermore, Mohammed *et al.*, stated that black seed oil had no antimicrobial effect on gram-negative bacteria in vitro.[34]

The susceptibility of samples to infection was different from one another and influenced by several factors such as the ability of the immune system to fight pathogens, the intensity of microorganisms on the wound site, and the species of wound-infecting microorganism with various virulence factors. Meanwhile, the accumulation of fluid in the wound inhibits the migration of fibroblast cells, promotes infection, and causes ischemia in the wound area which slows the healing process. Some of the classical signs of infected wounds were erythema, increased local warmth, swelling, purulent discharge, and delayed wound healing.[35] Therefore, wound care management with strict aseptic technique, proper tissue

handling, debris, necrosis elimination, tension avoidance, and vascularization maintenance was essential in reducing local negative factors in wound healing.[36] Excessive topical application of black seed oil rich in essential fatty acid on the undressed wound induces crust formation, making it difficult to observe and measure. Therefore, it is important to study the dosage and method of ozonated black seed oil application on wound topically.[37,38]

Moreover, the storage temperature applied on ozonated black seed oil affected its composition. A previous study by Moureu *et al*., (2015), stated that the ozonated sunflower oil kept at room temperature and +37 ºC showed a decrease in peroxide value and an increase in acid value. The samples kept at lower temperature -20 ºC and +4 ºC remained stable even over 1 year and the alteration of composition had no impact on ozonated oil potency against *Staphylococcus uberis.* Therefore, the study stated that the increase in acid value is responsible for antibacterial effects as compensation for the decrease in peroxide value.[39] This study showed that the ozonated black seed oil was stored in a transparent plastic bottle at room temperature, allowing the alteration of peroxide value and affecting the wound healing rate.

The limitation in this study was the short time of the treatment. We only observed the wound healing until 7 days, instead of the wound was successfully closed. We had not find the proper formula and application of ozonated black seed oil on wound topically to prevent excessive crust formation. Moreover, the literature of ozonated black seed oil on wound healing is still limited. Furthermore, peroxide value has to be assessed to determine its correlation with the wound healing process.

Conclusion

The ozonated black seed oil with 2.2 mg/ml dose of ozone was able to increase the number of fibroblast after 7 days of treatment and also increased the distribution of collagen after 3 and 7 days of treatment compared to the control group of gentamicin which had no significant difference. Nevertheless, no significant difference was found in wound closure and epithelization rate in the experimental groups of ozonated black seed oil after 3 and 7 days of treatment. Therefore, further study on the effect of ozonated black seed oil on wound healing with a wound dressing management to minimize bias factors such as wound infections is recommended.

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