RESEARCH ARTICLE

The role of ozonated *Aloe vera* oil in full-thickness skin defects: Macrophage count and epithelization length parameter [version 1; peer review: 2 not approved]

Ukio Salferius Tamba ¹, Muhamad Thohar Arifin^{1,2}, Muhamad Nur ³, Muflihatul Muniroh^{1,4}, Neni Susilaningsih^{1,5}

¹Biomedical Science, Faculty of Medicine, Diponegoro University, Semarang, Indonesia

²Department of Neurosurgery, Diponegoro University, Semarang, Indonesia

³Physic Division, Faculty of Science and Mathematics, Diponegoro University, Semarang, Indonesia

⁴Physiology Department, Faculty of Medicine, Diponegoro University, Semarang, Indonesia

⁵Anatomy and Histology Department, Faculty of Medicine, Diponegoro University, Semarang, Indonesia

V1 First published: 09 Oct 2020, 9:1218
<https://doi.org/10.12688/f1000research.25063.1>
 Latest published: 09 Oct 2020, 9:1218
<https://doi.org/10.12688/f1000research.25063.1>

Abstract

Background: Aloe vera has been reported to enhance keratinocyte proliferation and migration, and thereby accelerate wound healing. Ozone therapy is an alternative medicine with disinfecting properties and strong oxidative stress induction capacity. This study aimed to evaluate the effect of *Aloe vera* oil provided with different dosages of ozone in accelerating the healing rate comparing two-phases; inflammation (day 3) and proliferation (day 7) of full-thickness defect wounds in Sprague Dawley rats as an adjuvant treatment based on macrophage count and new epithelialization length.

Methods: We conducted a study using a post-test only control group design, where a total of 50 Sprague Dawley rats were randomized into ten groups. Two control groups were administered *Aloe vera* oil (P1, P6), while gentamicin ointment was used in the other control group (P2, P7). Ozonated *Aloe vera* oil dose was used: 600 mg/ml (P3,P8), 1200 mg/ml (P4,P9), and 1800 mg/ml (P5,P10). Groups P1, P2, P3, P4, P5 were terminated on the 3rd day, while P6, P7, P8, P9, P10 were evaluated on the 7th day. Macrophage counts were assessed using a 100x magnification microscope, through hematoxylin-eosin (HE) staining, and paraffin block with Masson trichrome staining was used to measure the new epithelialization length.

Results: There were significant differences in the macrophage count on day three between the group-administered gentamicin (P2) and 1800 mg/ml ozonated *Aloe vera* oil (P5) (79.96;131.96, $p<0,05$). The new epithelialization length significantly increased in the group provided with wound treatment up to day 7, using 1800 mg/ml ozonated *Aloe vera* oil (P10), compared to non-ozonated *Aloe vera* (P6) and the gentamicin therapy (P7) (1160.88 μ m; 1367.64; 2451.40 μ m, $p<0,05$).

Open Peer Review

Approval Status

	1	2
version 1 09 Oct 2020	 view	 view
1. Yos Adi Prakoso , University of Wijaya Kusuma Surabaya, East Java, Indonesia		
2. Huzwah Khaza'ai , Universiti Putra Malaysia, Serdang, Malaysia		
Any reports and responses or comments on the article can be found at the end of the article.		

Conclusions: The application of ozonated *Aloe vera* oil to full-thickness skin defects in Sprague Dawley rats resulted in a higher macrophage count and longer new epithelialization length than in controls.

Keywords

Aloe vera oil, Ozone Therapy, Full-thickness skin defect, macrophage count, epithelialization

Corresponding author: Muhamad Thohar Arifin (thohar@fk.undip.ac.id)

Author roles: **Tamba US:** Data Curation, Writing – Original Draft Preparation; **Arifin MT:** Conceptualization, Funding Acquisition, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; **Nur M:** Supervision, Writing – Review & Editing; **Muniroh M:** Supervision, Validation; **Susilaningsih N:** Supervision, Validation

Competing interests: No competing interests were disclosed.

Grant information: This research was funded by Direktorat Riset dan Pengabdian Masyarakat, Deputi Bidang Penguatan Riset dan Pengembangan Kementerian Riset dan Teknologi/Badan Riset dan Inovasi Nasional No 225-30/UN7.6.1/PP/2020.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Copyright: © 2020 Tamba US *et al.* This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Tamba US, Arifin MT, Nur M *et al.* **The role of ozonated *Aloe vera* oil in full-thickness skin defects: Macrophage count and epithelialization length parameter [version 1; peer review: 2 not approved]** F1000Research 2020, 9:1218 <https://doi.org/10.12688/f1000research.25063.1>

First published: 09 Oct 2020, 9:1218 <https://doi.org/10.12688/f1000research.25063.1>

Introduction

Soft tissue wound refers to the damage or loss of tissue continuity, possibly caused by internal factors such as drugs, changes in circulation and metabolic processes, infection, and oxygen transportation failures. External factors include extreme temperatures, injuries, radiation, and the presence of chemical substances¹. The body's physiological response, in terms of wound healing, results from various interrelated processes, including coagulation, inflammation, deposition, extracellular matrix differentiation, fibroplasia, epithelialization, contraction, and remodeling¹. Furthermore, the remediation course is mediated by various cells, cytokines, matrices, and growth factors, through three phases - inflammation, tissue proliferation, and remodeling². The process of acute cutaneous wound healing consists of three stages. The first stage is the inflammatory phase, which occurs following the injury and lasts until day-3. The second stage is the proliferation phase that generally takes place from day -7 until day-14, and the third stage is the remodeling phase that can last for several months or even years. The result of a previous study explained that only at specific doses did ozonated oil show a significant increase in the initial phase of wound healing³.

Macrophages play critical roles in all phases of adult wound healing, which are inflammation, proliferation, and remodeling. As wounds heal, the local macrophage population transitions from predominantly pro-inflammatory (M1-like phenotypes) to anti-inflammatory (M2-like phenotypes). Thus, local macrophages retain pro-inflammatory characteristics⁴.

Aloe vera is a thorny plant that grows in tropical and subtropical climates. It has been used for millennia to improve cutaneous wound healing, with this now being known to be due to its ability to increase the proliferation and migration of fibroblasts and human primary epidermal keratinocytes (HPEKs) flow cytometry analysis revealed that cell surface expressions of $\beta 1$ -, $\alpha 6$ -, $\beta 4$ -integrin, and E-cadherin increased in HPEKs treated with *Aloe vera* gel. These increases may contribute to cell migration and wound healing⁵. Many active ingredients, including vitamins, enzymes, minerals, sugars, lignin, salicylic acids, and amino acids, have been identified from these species. However, therapeutic effects have not correlated well with each component. Recently, a study exploring the biological effects of a single element from *Aloe* species was conducted. Choi *et al.* reported that a 5.5 kDa glycoprotein isolated from *Aloe vera* gel by activity-guided sequential fractionation was found to enhance keratinocyte proliferation and migration, and thereby accelerate wound healing⁵. However, it is currently believed that biological activities associated with *Aloe* arise from the synergistic actions of multiple compounds rather than from a single component⁵.

Ozone therapy is an alternative medicine with disinfecting properties and strong oxidative stress induction capacity. These characteristics have been implicated in the stimulation of protective mechanisms for cells and organs by increasing the efficacy of endogenous oxygen-free radicals scavenging⁶.

Moreover, ozone therapy inactivates bacteria and damages cell walls through the oxidation of phospholipids and lipoproteins, and also inhibits fungal growth, damages viral capsids, and interferes with the bacterial reproduction cycle⁷. This therapy could be applied as an adjuvant or alternative to standard treatments in patients with various types of wounds, particularly when used topical⁶. Previous studies have highlighted the benefits of ozonated sesame oil in wound healing⁷.

Therefore, this study aimed to evaluate the effect of *Aloe vera* oil provided with different dosages of ozone in accelerating the healing rate comparing two-phases; inflammation (day 3) and proliferation (day 7) of full-thickness defect wounds in Sprague Dawley rats as an adjuvant treatment based on macrophage count and new epithelialization length.

Methods

This was an experimental study using a post-test only control group design. The outcome data were recorded after the duration of the treatment. A total of 50 adult male Sprague Dawley rats were distributed randomly into ten groups, with five in each. A total of ten groups were created, where the controls, P1 (received *Aloe vera* oil) and P2 (received gentamicin), respectively, for three days. Furthermore, P3, P4, and P5 was administered ozonated *Aloe vera* Oil at a dose of 600, 1200, and 1800 mg/ml of ozone, respectively for three days. Therefore, P6 (received *Aloe vera* oil) and P7 (received gentamicin), respectively, for seven days, while P8 P9 and P10 received ozonated *Aloe vera* Oil at a dose of 600, 1200, and 1800 mg/ml ozone, respectively for seven days. A full-thickness defect measuring 1 cm in diameter was created on the back of the rat. The rats were sacrificed in each group on the third (P1, P2, P3, P4, P5) (inflammation stage of wound healing) and seventh day (P6, P7, P8, P9, P10) (proliferation stage wound healing), respectively, to obtain the macrophage count and length of new epithelialization (Figure 1).

Animals

The 50 adult male Sprague Dawley rats from a local laboratory-grade rat breeder, with a pure-breeding status, weighing 250 ± 50 grams and lodged in individual stainless-steel laboratory-grade rat cages at room temperature of $28.0 \pm 2.0^{\circ}\text{C}$, which was monitored with a digital thermometer. The rats were acclimated in the laboratory for one week. Twelve hours light was provided with from 06.00–18.00 and darkness from 18.00–06.00 with allowed access food (BioFeed pellets, manufactured by *Karunia Kasih Abadi*, Klaten, Indonesia) and water supply (clean drinkable water). These conditions were to ensure the elimination of stress, and feeding was conducted *ad libitum*. Each cage was inhabited by two to three rats according to the cage size. We did not mix rats from different groups in one cage. We routinely assessed the clinical and behavioral status of the rat every day during routine checks to prevent any clinical or behavioral changes in the laboratory rat. No rats were found dead, having decreased appetite/growth, or exhibited behavioral changes during the study period.

Figure 1. Study flow chart.

Ozonated Aloe vera oil

A previous study⁶ involved the application of ozonated sesame oil (8 microliters) to a wound measuring 3.5 mm in diameter, we employed the same method. The doses used in this investigation were 50.6, 101.3, and 205.5 mg/ml of ozonated *Aloe vera* oil; hence the amount of ozone provided to each 3.5 mm wound was 0.405 mg, 0.81 mg, and 1.62 mg, respectively. Furthermore, the ozonated oil (Cap) capacity was determined using the formula: $\text{Cap} = C \times F_{\text{Rate}} \times T$, where Concentration (C) was set at 45 mg/L, and F_{Rate} (Flow rate) at 1.5 liters/minute,

while Time includes 30, 60 and 120 minutes. The manufacture and testing of ozonated *Aloe vera* were carried out at the Plasma Research Center (PRC), Diponegoro University, Indonesia. The tools used for making ozonated oil were ozone generators (ozone generator manufactured in-house by Plasma Research Center, Diponegoro University) and magnetic stirrers. The ozone outlet is connected to an anti-oxidation hose with a diffuser, which served to increase the effectiveness of ozone absorption in the oil. Chemical characterizations (acidity value (AV), iodine value (IV); and peroxide value (PV))

of ozonated Aloe Vera oil samples have also been performed. As for PV evaluation, it was determined using both iodometric titrations according with the official monograph of the European Pharmacopoeia as well as placing the sample at reflux for 60 minutes⁸. Physical characterization has been performed by viscosity measurements (Viscomate VM-10AL, CBC Europe) at both the temperatures of 22 and 35_0.2 1C. A total of 40 ml *Aloe vera* oil was used in this experiment. Previous studies have used three ozone concentration – low, medium and high – which we replicated⁶. In this experiment the ozone concentration used were 600mg/ml as the low PV concentration; 1200 mg/ml for medium, and 1800 mg/ml as the high PV concentration. **Table 1** shows the peroxide value (PV) concentration in every ozone dose.

Full-thickness wounds

Full-thickness wounds of one-centimeter diameter were made on the back of the rat (**Figure 2**). The procedure involved anesthetizing the Rats with a mixture of Ketamine (100 mg/mL; Ketamin Cas Number: 6740-88-1 Ikapharmindo, Indonesia) -Xylazine (20 mg/mL; Xyla Cat. No.: IX2 Interchemie werken “De Adelaar” BV Local Distributor: PT Tekad Mandiri Citra). A Mixture dose of 80 mg/kg Ketamine;

10 mg/kg Xylazine via the intraperitoneal route, followed by the careful placement of the rat in the left lateral decubitus position. The hair on the dorsum area was shaved, and povidone-iodine was used to clean the skin, followed by ethanol 70%. The wound area was marked, and the lesion was made by cutting the skin using a scalpel to the base of the *panniculus carnosus*. The fatty and connective tissues were cleaned, and the site irrigated with normal saline (if necessary), before wrapping with a band-aid. Furthermore, the non-adhesive cottonoid

Table 1. Calculation of peroxide value based on different ozonated *Aloe vera* doses.

No	Ozone Dose mg/ml	Peroxide Value Milliequivalent per 1000 grams
1	0	66.88 ± 1.71
2	600	654.01 ± 6.44
3	1200	1140.93 ± 6.06
4	1800	1772.81 ± 14.66

Figure 2. Full-thickness defect induction. **A:** Image of the wound creation process on the back of rat; **B:** Example of full-thickness defect wound (round shape in this study); **C:** Placement of non-adhesive segment of Hansaplast plaster on wound model; **D:** Elastomull bandage was used to wrap the thorax and dorsum area of rat, and tightness was adjusted to ensure proper breathing (adequate chest expansion) and movement of extremities before returning to the cage.

was applied to covering the wound. The rats were then placed in a clean and warm cage with adequate lighting to recover from the anesthesia.

Topical applications

Eight microliters of either untreated Aloe vera oil or Ozonated aloe vera were applied twice a day (every 12 hours) on the wounds, and 1 cm gentamycin ointment was used on the control group.

Histological analysis

Surgical excision of the wounded area was performed at days 3 and 7 in each group. Skin tissue was immersion fixed in 10% NBF (neutral-buffered formalin) (Medici, medical labware, Jakarta Indonesia. Cat. NBF-19L) for 24 hours at room temperature. Sections (4 mm) were deparaffinized in xylene (Xylene Cath No 1.08661.2500 MERCK Merck Millipore Germany) and rehydrated in alcohol (Dipanax, Cath No 20501021606, Tangerang, Indonesia) gradients. Hematoxylin and eosin (H&E) staining (Hematoxylin Solution, Harris Modified, MFCD00078111 Merck Germany; Eosin Y Solution, Product No HT110132Sigma-Aldrich USA) were performed for histology⁹. The macrophage count was assessed microscopically, using a 100x magnification binocular microscope. The length of new epithelialization was examined by mason staining to the paraffin block¹⁰, followed by the measurement of epithelial tissue thickness with a microscope, under 40x magnifications. The results were then documented/photographed with optics (Miconas, Indonesia).

Ethical statement

This research was approved and declared ethically feasible by the Ethics Commission of the Public Health Faculty, Diponegoro University Semarang, with ethical clearance number 132/EC/H/KEPK/FK-UNDIP/X/2019. All efforts were made to alleviate harm to animals by administering anesthesia to

all of the study animals before full-thickness wound model creation, keeping the animals in a well-maintained cage, and ensuring graceful termination of animals before we took tissue samples for a histopathology examination.

Data analysis

Data analysis was performed using SPSS 21 version. All data were analyzed using descriptive statistics. Normality tests were performed using the Shapiro wilk test. Homogeneity testing and one-way ANOVA were performed to compare the control with the treatment groups. Post hoc Games-Howell was used to determine the difference between each variable. This process was conducted to investigate the presence of significant differences, and p-value <0.05 was considered statistically significant.

Results

Analysis of macrophage count

Table 2 shows the differences in the mean macrophage count of treatment groups 1-10, and the highest mean value was recorded on day-3, in P5(administered 1800 mg of ozonated Aloe vera oil) as 131.96. Meanwhile, the lowest (79.96) was measured on day-3, in P2, with gentamicin treatment (Figure 3). The result of the data normality test shows the absence of normal data distribution (p=0.028 or p<0.05). Hence, data transformation was performed to generate normally distributed data (with a p-value range of 0.065 to 0.884).

All normally distributed data were tested using the one-way ANOVA to ascertain the presence of significant differences in mean macrophage count between the ten groups. Table 3 shows that the data obtained were not homogenous, at p=0.028, or p<0.05. However, one-way ANOVA test resulted in a p-value of <0,001, indicating the presence of a significant difference between groups. Therefore, a Post hoc Games-Howell test was then conducted to observe the variation in

Table 2. Data normality test result of macrophage count.

Group	Mean ± SD	Median (min – max)	p	Transf.
P1	93.08 ± 20.89	85.6 (78.4 – 129.6)	0.028	0.065*
P2	79.96 ± 11.93	78.2 (63.0 – 95.8)	0.848*	0.759*
P3	88.04 ± 34.18	80.2 (40.6 – 131.8)	0.864*	0.521*
P4	108.92 ± 13.49	106.2 (96 – 131.8)	0.117*	0.186*
P5	131.96 ± 25.95	141.4 (102.2 – 161.8)	0.395*	0.325*
P6	69.08 ± 13.08	68.6 (54.8 – 87.4)	0.834*	0.884*
P7	90.28 ± 36.21	78.6 (56.6 – 132.6)	0.170*	0.216*
P8	90.28 ± 10.67	90.2 (73.4 – 102)	0.553*	0.401*
P9	85.96 ± 17.71	78.2 (72 – 116)	0.104*	0.201*
P10	83.16 ± 25.30	94.8 (51.2 – 108.0)	0.276*	0.222*

* Normal (p > 0.05); † Shapiro-wilk

the mean macrophage count of two groups. Table 4 shows the results of the Post hoc Games-Howell test, and a significant difference ($p < 0.05$) was observed between the group provided with gentamicin (P2) and 1800 mg/ml of ozonated *Aloe vera* oil (P5) on day-3, at 79.96 and 131.96, respectively. Also, there was substantial variation between the groups administered 1200 mg/ml (P4) and 1800 mg/ml (P5) of ozonated *Aloe vera* oil sacrificed on day 3, with those euthanized *Aloe vera* oil on day 7 (P6), at 168.92, 131.96 and 69.08, respectively.

Analysis of epithelialization

Table 5 shows the variation in the mean length of new epithelialization between the ten treatment groups, with the highest value of 2451.40, recorded in group P10 provided with 1800 mg/ml of ozonated *Aloe vera* oil, on day-7. Meanwhile, the least (799.49) was reported on day-3, in group P3 administered 600 mg/ml of ozonated *Aloe vera* oil (Figure 4). The data normality test result shows the normal distribution in the ten treatment groups, with p-value ranging from 0.083 to 0.970. Therefore, all normally distributed data were tested using the one-way ANOVA to investigate any significant differences in mean macrophage count between the ten groups. Based on the normality test, Table 6 demonstrated non-normal distributed data, with $p = 0.013$ or $p < 0.05$. However, one-way ANOVA test showed a p-value of < 0.001 , indicating the absence of any significant differences between treatment groups. Therefore, a Post hoc Games-Howell test was conducted to evaluate the variation in the mean length of new epithelialization between two groups.

The result of the Post hoc Games-Howell test (Table 7) shows a substantial increase in the length of new epithelialization, particularly in groups treated using 1800 mg/ml of ozonated *Aloe vera* oil (P10) for seven days (2451.40 μm), compared to those administered *Aloe vera* oil therapy (P6) and gentamicin therapy (P7), at 1160.88 μm and 1367.64 μm , respectively. Furthermore, ozonated *Aloe vera* oil therapy proved effective in each group of *Sprague Dawley* rats with full-thickness skin defect, based on the increased epithelialization.

This process occurred during the wound healing process with gentamicin (P7), with no significant difference (1367.64 μm), compared to P6 treated with *Aloe vera* oil (1160.88 μm), P8 with 600 mg/ml of ozonated *Aloe vera* oil (2074.48 μm), and P9 with 1200 mg/ml of ozonated *Aloe vera* oil (1984.09). Therefore, the administration of ozonated *Aloe vera* oil at specific doses has a significant positive effect on the healing response of full-thickness defect wounds in *Sprague Dawley* rats, leading to faster new epithelialization length development.

Discussion

The process of acute cutaneous wound healing generally consists of three stages, including the inflammatory phase experienced immediately after the injury, which extends to day-7. The second stage is proliferation, observed to day-14,

and the third is the remodeling phase, known to last for several months or even years⁶. Previous studies have demonstrated a significant increase in the initial phase of wound healing in response to moderate doses of ozonated oil (1500 mg/dl)⁶. The current study showed the effectiveness of 1800 mg/ml ozonated *Aloe vera* oil in promoting the wound healing response of full-thickness defects in *Sprague Dawley*.

According to Valacchi *et al.*⁶, ozone is an efficient bactericidal, anti-fungi, and anti-viral agent, hence the propensity to reduce infection and improve skin unification during wound healing. The effect of ozonated oil on wound closure rate depends on the peroxide content⁶, as ozone possesses powerful oxidative characteristics in both gas and liquid phases, therefore producing microbicidal effects needed to damage the cell wall and cytoplasmic membrane of bacteria and fungi. This cascade of events leads to increased cell permeability, consequently facilitating the entry of ozone molecules¹¹.

The use of ozonated oil also prevents superinfections and stimulates tissue reconstruction through increased cell proliferation and the formation of new vascularization⁶. Also, an increase in oxygen tension at the wound site elevates the tendency of granulation tissue formation, which has implications on the wound closure rate⁴. The augmented expression of transforming growth factor-beta (TGF-beta) and vascular endothelial growth factor (VEGF), known to play critical roles in the wound healing process, has also been achieved in clinical and experimental wound healing research with ozone therapy¹².

The results of data analysis showed differences in the mean macrophage count between groups treated with 600, 1200, and 1800 mg/ml of ozonated *Aloe vera* oil and the control, administered *Aloe vera* oil or gentamicin alone. The evaluation parameter was higher in groups provided with 1200 mg/ml and 1800 mg/ml of ozonated *Aloe vera* oil for three days compared to those given gentamicin for three days and *Aloe vera* oil for seven days. This outcome was probably due to the increased oxidative role of ozone during the early phase of the inflammatory process¹³.

Ozone has been widely recognized as an anti-viral, bactericidal, and anti-fungal agent, and used in clinical therapy for its beneficial wound healing effects. This element is not able to penetrate the cell but is able to bind unsaturated fatty acids, and consequently form reactive oxygen species (ROS)⁶.

One form of ROS compound is hydrogen peroxide, known as a non-radical oxidant and implicated as an ozone mediator in several biological processes, including wound therapy¹⁴. Besides, ROS plays a crucial role in these processes by decreasing blood flow and forming a thrombus.

ROS are known to assist in the transportation of neutrophils through the blood vessels, therefore serving as a protection against bacteria by signaling the immunocytes, including

Figure 3. Histopathological findings of macrophage count by hematoxylin-eosin staining 100x magnification in all groups (P1 - P10).

Table 3. Homogeneity and one-way ANOVA test results of macrophage count.

Group	Mean $\pm$ SD	P	Homogenous
P1	93.08 $\pm$ 20.89	0.029*	0.028
P2	79.96 $\pm$ 11.93		
P3	88.04 $\pm$ 34.18		
P4	108.92 $\pm$ 13.49		
P5	131.96 $\pm$ 25.95		
P6	69.08 $\pm$ 13.08		
P7	90.28 $\pm$ 36.21		
P8	90.28 $\pm$ 10.67		
P9	85.96 $\pm$ 17.71		
P10	83.16 $\pm$ 25.30		

* Significant ($p < 0.05$); ** Homogenous ($p > 0.05$)**Table 4. Post hoc Games-Howell result for the macrophage count.**

Group	P2	P3	P4	P5	P6	P7	P8	P9	P10
P1	0.935	1.000	0.807	0.293	0.429	1.000	1.000	0.999	0.996
P2	–	1.000	0.107	0.046*	0.898	1.000	0.891	0.999	1.000
P3		–	0.899	0.580	0.992	1.000	1.000	1.000	1.000
P4			–	0.755	0.038*	0.911	0.399	0.410	0.658
P5				–	0.015*	0.569	0.140	0.125	0.293
P6					–	0.968	0.306	0.711	0.988
P7						–	1.00	1.000	1.000
P8							–	1.000	0.997
P9								–	1.000

* Significant ($p < 0.05$)**Table 5. Data normality test result of new epithelialization length (in micrometres).**

Group	Mean $\pm$ SD	Median (min – max)	p
P1	1179.20 $\pm$ 235.65	1142.72 (950.12 – 1523.65)	0.606*
P2	1175.14 $\pm$ 343.37	1111.27 (804.10 – 1563.76)	0.370*
P3	799.49 $\pm$ 272.93	789.97 (466.58 – 1141.83)	0.907*
P4	1487.41 $\pm$ 871.70	1230.43 (699.53 – 2962.25)	0.182*
P5	981.11 $\pm$ 236.15	871.14 (769.91 – 1275.64)	0.158*
P6	1160.88 $\pm$ 276.51	1078.29 (867.30 – 1595.27)	0.636*
P7	1367.64 $\pm$ 105.20	1314.88 (1254.88 – 1506.94)	0.427*
P8	2074.48 $\pm$ 531.74	1981.17 (1333.25 – 2604.28)	0.462*
P9	1984.09 $\pm$ 653.72	1675.41 (1397.27 – 2726.21)	0.083*
P10	2451.40 $\pm$ 296.22	2303.98 (2157.46 – 2866.08)	0.403*

* Normal ($p > 0.05$); [†] Shapiro-wilk

Figure 4. Histopathological findings of epithelial tissue length by a microscopic procedure using 40x magnification in all groups (P1 – P10).

Table 6. Homogeneity and one-way ANOVA test results of new epithelialization length.

Group	Mean $\pm$ SD	p	Homogenous
P1	1179.20 $\pm$ 235.65	<0.001*	0.013
P2	1175.14 $\pm$ 343.37		
P3	799.49 $\pm$ 272.93		
P4	1487.41 $\pm$ 871.70		
P5	981.11 $\pm$ 236.15		
P6	1160.88 $\pm$ 276.51		
P7	1367.64 $\pm$ 105.20		
P8	2074.48 $\pm$ 531.74		
P9	1984.09 $\pm$ 653.72		
P10	2451.40 $\pm$ 296.22		

* Significant (p < 0.05)

Table 7. Post hoc Games-Howell result of new epithelialization length.

Group	P2	P3	P4	P5	P6	P7	P8	P9	P10
P1	1.000	0.447	0.996	0.920	1.000	0.798	0.168	0.390	0.002*
P2	–	0.665	0.997	0.978	1.000	0.942	0.191	0.425	0.005*
P3		–	0.773	0.967	0.579	0.079	0.043*	0.130	<0.001*
P4			–	0.928	0.994	1.000	0.928	0.981	0.485
P5				–	0.970	0.185	0.082	0.220	0.001*
P6					–	0.825	0.163	0.382	0.002*
P7						–	0.312	0.601	0.008*
P8							–	1.000	0.897
P9								–	0.869

* Significant (p < 0.05).

monocytes, to fight pathogenic germs¹⁵. Furthermore, ROS stimulates endothelial cells to form new blood vessels, form an extracellular matrix (including collagen synthesis), and keratinocyte proliferation¹⁵, although excessive production leads to oxidative stress. This condition causes adverse effects on the wound healing process, hence the need for antioxidants to achieve a dynamic balance¹⁵.

The mean value of total macrophage produced on day 4 – 7 was lower than day 1 – 3. According to previous research, this variation was affiliated with the presence of vitamins, enzymes, proteins, carbohydrates, minerals (calcium, sodium, magnesium,

zinc, and iron), and amino acids in *Aloe vera*, which play a major role in treating inflammation¹⁶. Also, various anti-inflammatory agents, including salicylic acid, Indomethacin, Manosa-6-phosphate, B sitosterol, as well as components of Lignin, Saponin, and Anthraquinone consisting of Aloin, Barbaloin, Anhranol, Anthracene, Aloetic acid, Aloe-emodin, have been identified as essential ingredients of antibiotics and pain killer drugs. Meanwhile, *Aloe vera* is also rich in vitamins, polysaccharides, and other components with health benefits. The anti-inflammatory constituents can stimulate the immune system, leading to cell regeneration, and the consequent acceleration of healing in the inflammatory phase¹⁶.

Based on the results of new epithelialization length measurement, ozone induces a notable effect on the wound healing process. Furthermore, the administration of 1800 mg/ml ozonated *Aloe vera* oil for seven days had a significant positive mean difference compared to the other groups.

Various studies have been conducted to observe the role of topical *Aloe vera* on the wound healing process. Oryan *et al.*¹⁶ examined a 2x2 cm wound on the back of rats treated with *Aloe vera* gel, and the parameters evaluated include wound surface, size shrinkage, and epithelialization. Teplicki *et al.*⁸ demonstrated increased healing in terms of proliferation and migration of fibroblasts and keratinocytes after treatment. *Aloe vera* shows effectiveness through increased cell migration, keratinocyte function, and epithelialization repair⁸.

The current study results indicate the fastest wound healing rate in the group treated with the highest dose of ozonated *Aloe vera* oil (1800 mg/ml). This was followed by others, in the order of 600 mg/ml and 1200 mg/ml, the positive control administered gentamicin, and then *Aloe vera* oil, respectively. Therefore, further research is required to examine the effect of ozonated *Aloe vera* oil to ascertain the most effective dose for wound healing.

This study has several limitations, including the lack of uniformity in the hygiene level of each rat, which possibly influenced the wound healing process. Also, the wound tissue sampling was not conducted identically, therefore making it impossible to determine the exact phase of wound healing encountered on day seven, as the process in *Sprague Dawley* rats is perhaps different from humans.

Conclusion

Based on the results and discussion, ozonated *Aloe vera* oil applied to full-thickness skin defect in *Sprague Dawley* rats resulted in higher macrophage count and longer new epithelialization length than the control group.

However, uniformity in the hygiene level of each rat is important in order to accelerate wound healing processes and minimize infection. Also, there is a need for further research to investigate the exact component in *Aloe vera* oil responsible for the treatment progression. Furthermore, subsequent studies are also to determine the effective dose to facilitate implementation is not only experimental animals, but also humans, to observe the antioxidant effect of ozonated *Aloe vera* oil.

Data availability

Underlying data

Open Science Framework: The Role of Ozonated *Aloe vera* Oil in Full-Thickness Skin Defect: Macrophage Count And Epithelialization Length Parameter, <https://doi.org/10.17605/OSF.IO/7A82V>¹⁸

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Acknowledgments

The authors would like to express our gratitude to the Center Plasma Research (CPR), Diponegoro University, Semarang.

References

- Robson MC: **Wound healing: biologic features and approaches to maximum healing trajectories.** *Curr Probl Surg.* 2001; **38**(2): 72–141.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Li J, Chen J, Kirsner R: **Pathophysiology of acute wound healing.** *Clin Dermatol.* 2007; **25**(1): 9–18.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Travagli V, Zanardi I, Valacchi G, *et al.*: **Ozone and ozonated oils in skin diseases: a review** *Mediators Inflamm.* 2010; **2010**: 610418.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Krzyszczak P, Schloss R, Palmer A, *et al.*: **The Role of Macrophages in Acute and Chronic Wound Healing and Interventions to Promote Pro-wound Healing Phenotypes.** *Front Physiol.* 2018; **9**: 419.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Moriyama M, Moriyama H, Uda J, *et al.*: **Beneficial Effects of the Genus *Aloe* on Wound Healing, Cell Proliferation, and Differentiation of Epidermal Keratinocytes.** *PLoS One.* 2016; **11**(10): e0164799.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Valacchi G, Lim Y, Belmonte G, *et al.*: **Ozonated sesame oil enhances cutaneous wound healing in SKH1 mice.** *Wound Repair Regen.* 2011; **19**(1): 107–115.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Lim Y, Phung AD, Corbacho AM, *et al.*: **Modulation of cutaneous wound healing by ozone: Differences between young and aged mice.** *Toxicol Lett.* 2006; **160**(2): 127–34.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Teplicki E, Ma Q, Castillo DE, *et al.*: **The Effects of *Aloe vera* on Wound Healing in Cell Proliferation, Migration, and Viability.** *Wounds.* 2018; **30**(9): 263–268.
[PubMed Abstract](#)
- Feldman AT, Wolfe D: **Tissue Processing and Hematoxylin and Eosin Staining.** In: Day C. (eds) *Histopathology. Methods in Molecular Biology (Methods and Protocols).* Humana Press, New York, NY. 2014; **1180**.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Suvik A, Effendy AW: **The use of modified Masson's trichrome staining in collagen evaluation in wound healing study.** *Mal J Vet Res.* 2012; **3**(1): 39–47.
[Reference Source](#)
- Nagayoshi M, Kitamura C, Fukuizumi T: **Antimicrobial effect of ozonated water on bacteria invading dentinal tubules.** *J Endod.* 2004; **30**(11): 778–781.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Saini R: **Ozone therapy in dentistry: A strategic review.** *J Nat Sci Biol Med.* 2011; **2**(2): 151–153.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Danusantoso H: **Peran radikal bebas terhadap beberapa penyakit paru.** *Jurnal Kedokteran Trisakti.* 2003; **22**(1): 31–36.
[Reference Source](#)
- Bocci VA: **Scientific and medical aspects of ozone therapy. State of the art.**

Arch Med Res. 2006; **37**(4): 425–35.

[PubMed Abstract](#) | [Publisher Full Text](#)

15. Dunnill C, Patton T, Brennan J, *et al.*: **Reactive oxygen species (ROS) and wound healing: the functional role of ROS and emerging ROS-modulating technologies for augmentation of the healing process.** *Int Wound J.* 2017; **14**(1): 89–96.
[PubMed Abstract](#) | [Publisher Full Text](#)
16. Carolia N, Sukohar A: **Pengaruh Pemberian Ekstrak Lidah Buaya Konsentrasi 25%, 50%, 75%, dan 100% terhadap Jumlah Makrofag pada Radang Mukosa**

Mulut Tikus Putih Jantan Galur Sprague Dawley. *Jurnal Kedokteran Universitas Lampung.* 2016; **1**: 243–246.

[Reference Source](#)

17. Oryan A, Mohammadipour A, Moshiri A, *et al.*: **Topical Application of Aloe vera Accelerated Wound Healing, Modeling, and Remodeling: An Experimental Study.** *Ann Plast Surg.* 2016; **77**(1): 37–46.
[PubMed Abstract](#) | [Publisher Full Text](#)
18. Muhamad TA: **The Role of Ozonated Aloe vera Oil in Full-Thickness Skin Defect: Macrophage Count And Epithelization Length Parameter.** 2020.
<http://www.doi.org/10.17605/OSF.IO/7A82V>

Open Peer Review

Current Peer Review Status:

Version 1

Reviewer Report 08 July 2021

<https://doi.org/10.5256/f1000research.27651.r86729>

© 2021 Khaza'ai H. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Huzwah Khaza'ai

Biochemistry Laboratory, Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang, Malaysia

The study was to investigate the effects of ozonated *Aloe vera* oil on full-thickness wounds. However, the study is not comprehensive enough to achieve an understanding of the mechanism of healing processes by ozonated *Aloe vera* oil. Theoretically, the healing process is involved in 4 stages which are homeostasis, inflammation, proliferation, and remodelling. The study only focuses on stages 2 and 3 looking at the macrophages and epithelialization. Due to the poor resolution of histological data the counting of macrophages is ambiguous.

1. Are days 3 and 7 really representing stages 2 and 3 of wound healing, which normally can only be observed in a continuous study from day 0 until day 21?
2. The peroxide value/total antioxidant status should also be measured in wound tissues.
3. How is the dosage of *Aloe vera* oil determined?
4. The Conclusion does not focus on the findings. The reviewer has no clue if ozonated oil or *Aloe vera* is involved in wound healing.
5. The author needs to do more comprehensive work before this work can be accepted for indexing. The author also must clearly determine the problem statement.

The study is still in its early stages and therefore I would Not Approve this paper due to the many reasons as stated above.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Wound healing, antioxidant, vitamin E , diabetic and alzheimer's disease

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 03 June 2021

<https://doi.org/10.5256/f1000research.27651.r83934>

© 2021 Prakoso Y. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Yos Adi Prakoso

Faculty of Veterinary Medicine, University of Wijaya Kusuma Surabaya, East Java, Indonesia

Dear Authors

I'm sorry to say that this paper cannot be accepted for indexing due to a lot of errors were observed from the methods and analysis.

1. You were not citing the appropriate references that fell within your scope of the manuscript.
2. You have limited references.
3. The parameters and analysis are not sufficient. For example: how can you count the macrophages within the skin tissue without using immunohistochemistry? I thought that it is impossible to be conducted.
4. The quality of your histological images is low and you make a wrong assessment of your interpretation. This study parameter should be recalibrated by the pathologist or expert of

skin pathologist due to the mismatch of staining with the assessed parameters.

5. The data of normality and homogeneity is not applicable to be embedded within the text.

6. Your discussion is not enough to present the present result.

I hope you can fix the problems.

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Skin immunology, skin wound healing, modified herbal therapy, wound infection expert, histopathology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research