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Ozonated Aloe Vera Oil Accelerates Radiation Dermatitis Healing in Sprague Dawley Rats

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Abstract
Background: Radiation dermatitis is the most common side-effect of radiation therapy. The current daily therapy uses topical corticosteroids to reduce inflammation. However, long-term usage may cause some side effects. Aloe vera could act as an anti-inflammatory and antioxidant agent, and ozone exhibits an antioxidant dan bactericidal effect.
The present study aimed to analyze the effect of topical administration of ozonated Aloe vera oil on inflammatory and growth factors in radiation dermatitis wounds.
Method: This was an experimental study with a "post-test only control group" design. A total number of 36 Sprague Dawley rats were divided into two control and four intervention groups. C1: no intervention, C2: hydrocortisone ointment, P1: Aloe vera oil, P2/P3/P4: ozonated aloe vera oil 300/600/1200 mg/ml. Termination and histopathological analysis of inflammatory and growth factors were performed after seven days of treatment. Statistical analysis was performed using SPSS version 22 (IBM corporation, USA). The normality test of the data was carried out using the Sapphiro Wilk test. Normally distributed data were subjected to the One-Way ANOVA test and continued with the Post Hoc LSD test after a significant difference was obtained. Data that were not normally distributed were tested using the Kruskal-Wallis test, followed by the Mann-Whitney U test. Therefore, all values obtained were considered significant at $P < 0.05$.
Results: Significant differences were found between the control and the treatment groups. Decreased tumor necrosis factor-alpha expression, neutrophil infiltration, endothelial damage, and increased tumor growth factor beta, platelet-derived growth factor, and fibroblast count were seen after seven days of intervention.
Conclusion: Ozonated Aloe vera oil can accelerate wound healing of radiation dermatitis.
***Keywords**:* Aloe vera, Wound healing, Sprague-Dawley

Introduction

Radiation, as one of the causes of dermatitis, is often found in patients who have undergone radiotherapy treatment. Radiotherapy has become the standard treatment option for various types of cancer, including primary curative and palliative care in oncology treatment.[1,2] Technology development has drastically reduced the toxic effect of radiotherapy; yet, most patients suffer from side effects.[3]

Dermatitis is an inflammatory skin reaction. The inflammatory response and oxidative stress reactions in radiation dermatitis are closely related. This reaction is caused by cell damage after radiation exposure, causing the production of various cytokines, cell cycle changes, and DNA damage. Production of pro-inflammatory and profibrotic cytokines, such as tumor necrosis factor-alpha (TNF-α), Interleukins 6 and 1 (IL-6 and IL-1), tumor growth factor beta (TGF-β), platelet-derived growth factor (PDGF), as well as a connective tissue growth factor, begins in the early phase of inflammation. This inflammatory response causes infiltration of eosinophils and neutrophils, increased fibroblast activation, and endothelial damage that triggers domino effects on the inflammatory response.[4,5]

Topical steroids have long been the mainstay therapy to prevent and treat radiation-induced dermatitis. Several studies state that topical steroids can reduce burning, itching, and erythema but impair wound healing. Furthermore, long-term use of steroids can cause local and systemic side effects; many studies have been carried out by choosing herbal plants as replacement therapy for topical steroids.[6] Aloe vera (*Aloe barbadensis*) is widely used as a topical therapy for skin tissue inflammation due to its anti-inflammatory and antioxidant properties.[7] Aloe vera can inhibit cyclooxygenase and cytokine production, thereby reducing inflammation and accelerating skin healing. Aloe extracts could modulate the inflammatory response to promote healing by stimulating re-epithelialization and angiogenesis by increasing TGF-β activity. A study evaluating the effectiveness of wound healing showed that the average time for healing was 8.79 days less in the Aloe vera group compared with the control group.[8] The intricate composition of Aloe vera has been demonstrated to directly influence both the wound and areas of pathology, either independently or in combination with other treatments. Its characteristics as an anti-inflammatory, antimicrobial, antioxidant, antithrombotic, vasodilatory, and tissue-regenerative agent have been proven to notably reduce the time required for skin wound healing and enhance wound contraction and strength.[9]

Ozone therapy increases oxygen supply and regulates antioxidant enzymes to protect against oxidative reactions, thereby supporting wound healing.[10-12] Topical administration of ozone can improve skin conditions without causing significant toxic effects because ozone immediately reacts with fats in the stratum corneum to be absorbed as antioxidants through the skin. Ozonated olive oil, sesame oil, and linseed oil have been shown to accelerate the wound healing process as compared with oils without ozonation, demonstrating that the oil composition is essential in the wound healing process.[11]

The effects of ozonated aloe vera in treating radiation-induced dermatitis have not been studied extensively. Therefore, the present study aimed to elaborate on the ability of ozonated Aloe vera oil to modulate TNF-α, TGF-β, PDGF, and neutrophil infiltration fibroblast count and endothelial

damage, thereby revealing the potential ability to modulate wound healing of radiation dermatitis.

Materials and Methods

Ethical approval

All the subjects used in this study were housed and conducted in a certified facility complying with medical and veterinary research guidelines. The Ethics Committee in charge has approved this experimental animal study, after a thorough review and approval by the Health Research Ethics Committee of RSUP Dr. Kariadi Semarang, in compliance with the principles set out in the Helsinki Declaration. The ethical clearance has been granted with the approval number 66/EC/H/FK-UNDIP/VII/2021.

Study design

This was an experimental study with a post-test-only control group design aimed at assessing the effect of ozonated Aloe vera oil on TNF-α, TGF-β, PDGF, neutrophil infiltration, fibroblasts count, and endothelial damage. The animals used in this experiment were thirty-six male Sprague Dawley rats 2-3 months old, bodyweight 150 ± 50 grams, healthy or active, and with no anatomical abnormality. Rats were acclimatized in the laboratory for one week before intervention. Each rat was kept in separate cages, given 12 hours of light, and fed moderately during the study.

Radiation dermatitis and topical intervention

Rats were anesthetized using ketamine-xylazine injection (ketamine 80 mg/kg, xylazine 10 mg/kg) intraperitoneal; each rat was placed in the prone position to expose the back area measuring 4x4 cm before receiving radiation at a dose of 7 Gy from a linear accelerator (LINAC) (Siemens Primus™). A certified radio-oncologist performs the radiation procedure. The subjects were returned to a clean tray after radiotherapy was given, and then allowed to spontaneously regain consciousness before being delivered topical intervention the next day. The radiation wound was characterized by faint erythema and dry desquamation of grade I dermatitis, according to RTOG/EORTC.[13] Aloe vera oil without ozone and ozonated Aloe vera oil obtained from the Center for Plasma Research, Diponegoro University, were produced using the method described in Taqwim Hidayat et al.[14] The topical intervention was given 24 hours after radiotherapy, according to treatment group. The intervention was given for seven consecutive days, given twice a day.

Treatment groups

C1 (negative control group): rats were radiated without any additional therapy.

C2 (positive control group): rats were radiated, then given hydrocortisone ointment 2,5%

P1: rats were radiated, then given non-ozonated aloe vera oil

P2: rats were radiated, then given 300 mg/mL ozonated Aloe vera oil

P3: rats were radiated, then given 600 mg/mL ozonated Aloe vera oil

P4: rats were radiated, then given 1200 mg/mL ozonated Aloe vera oil

Immunohistology analysis

Immunohistochemical staining was carried out at the Department of Anatomical Pathology, Faculty of Medicine, University of Sebelas Maret Solo, and readings were carried out at the Department of Anatomy, Faculty of Medicine, Diponegoro University, Semarang. Rats were euthanized, and tissue excision was performed after the topical intervention was administered. Tissue excision is performed on the radiation-exposed tissue and a small amount of normal skin along the edges. Tissue fixed with 10% buffered formalin was cut into 3mm slices and prepared into paraffin blocks. Paraffin block tissue was cut into 3-micrometer slices,

followed by staining TNF-α, TGF, and PDGF antibodies.

TNF-α, TGF, and PDGF expression in radiation dermatitis wounds was measured semi-quantitatively using the Allred score: proportion score + intensity. Proportion Score (A), 0 = 0% stained cells; 1≤% colored cells; 2 = 1-10% of colored cells; 3 = 11-33% stained cells; 4 = 34-66% of stained cells, 5 = 67% of stained cells. Intensity Score (B), 0 = colorless, 1 = weak intensity, 2 = moderate intensity, 3 = strong intensity. The proportion score is added to the intensity score and gives a final score of 0-8. The qualitative assessment of TNF-α, TGF-β, and PDGF was taken from each sample's five fields of view to be averaged.

Neutrophil infiltration, fibroblasts count, and endothelial damage in dermatitis wound tissue were counted in 5 visual fields with Hematoxylin Eosin staining. Qualitative assessment of neutrophil infiltration, fibroblasts, and endothelial damage was taken from five fields of view using a binocular microscope magnification (400×) of each sample to be averaged. Two certified pathologists carried out preparation and analysis.

Statistical analysis

Statistical analysis was performed using SPSS version 22 (IBM corporation, USA). The normality of the data was checked using the Sapphiro Wilk test. Normally distributed data were subjected to the One-Way ANOVA test and continued with the Post Hoc LSD test after a significant difference was obtained. Data that were not normally distributed were tested using the Kruskal-Wallis test, followed by the Mann-Whitney U test. All values obtained were considered to be significant at $P < 0.05$.

Results

TNF-α, TGF-β, and PDGF expression

All 36 rats survived the radiation and were terminated on the seventh day of treatment. TNF-α, TGF-β, and PDGF were observed using a binocular microscope with magnification (400x), assessed using Allred scores in five fields of view in each treatment group, and then averaged. Hydrocortisone ointment significantly reduced the TNF-α level on the wound bed but provided no significant increase in TGF-β and PDGF levels compared with the placebo group (Tables 1 and 2). Meanwhile, Aloe vera groups especially ozonated groups, revealed significantly lower TNF-α and higher TGF-β and PDGF levels than the placebo group. Further analysis comparing hydrocortisone and ozonated Aloe vera group revealed that ozonated aloe vera therapy produced higher TGF-β and PDGF levels.

Representative histology of immunohistochemical staining of TNF-α, TGF-β and PDGF antibodies is shown in figures 1-3.

Neutrophil infiltration, fibroblast count, and endothelial damage

Assessment of neutrophil infiltration, fibroblasts count, and endothelial damage was carried out in 5 fields of view using a 400x magnification binocular microscope with hematoxylin-eosin staining, which was then averaged. The hydrocortisone group increased the fibroblast count on the wound bed but did not affect the neutrophil infiltration and endothelial damage. Ozonated Aloe vera at 1200 mg/mL reduced neutrophil infiltration and endothelial damage and increased fibroblast count compared with the placebo group. There were significant differences in mean neutrophil infiltration, number of fibroblasts, and endothelial damage between the C1 group without treatment and the P2-P4 group namely ozonated aloe vera 300/600/1200 mg/mL and the C2 group with P2-P4 group (Table 3 and 4).

Representative histology of haematoxylin-eosin staining appearance of neutrophil infiltration, number of fibroblasts,

and endothelial damage are shown in figures 4-6.

Discussion

In this study, aloe vera and ozone therapy emerged as promising interventions due to their anti-inflammatory, antibacterial, and tissue regeneration properties. Significant differences were found between the control and the treatment groups. Decreased tumor necrosis factor-alpha expression, neutrophil infiltration, endothelial damage, and increased tumor growth factor beta, platelet-derived growth factor, and fibroblast count were seen after seven days of intervention.

Radiation dermatitis induces skin injury, initiating a multifaceted cascade of events involving diverse cell types and growth factors crucial for wound closure and tissue regeneration.[11,15]. This is accordance with a study by Krzyszczyk P. et al. that showed ozone offers several advantages in wound healing. Ozonated oil has demonstrated efficacy in infection prevention and stimulation of tissue reconstruction by promoting cell proliferation and formation of new blood vessels.[16] The elevated oxygen levels at the wound site facilitate increased granulation tissue formation, thereby expediting wound closure.[17]

As compared with placebo, this study showed that ozonated Aloe vera oil decreased TNF-α, neutrophil infiltration, and endothelial damage in radiation dermatitis wounds, which increased the expression of TGF-β, PDGF, and the number of fibroblasts in a dose-dependent manner. These findings elaborate on the mechanism of ozonated aloe vera in accelerating the wound healing process. Previous studies have also shown that topical ozone therapy can reduce inflammation in dermatitis, where the number of inflammatory cells in the epidermis and oedema is significantly reduced.[18] Throughout the wound healing journey, platelets generate a range of growth factors and inflammatory cytokines, including TGF-β, PDGF, EGF, and FGF, all of which are present during the inflammatory phase. Certain among them function as chemokines.[19] In radiation dermatitis, pathological changes such as cellular depletion, matrix changes, and microvascular damage occur, causing hypoxia in the wound tissue, thus disrupting the wound healing process.[15]

Ionizing radiation is used in various clinical practices, especially in cancer treatment with radiotherapy. One of the side effects caused by radiotherapy is radiation dermatitis which causes skin injury.[15] The wound healing process on the skin is complex; this process requires the coordination of several types of cells, such as keratinocytes, fibroblasts, endothelial cells, macrophages, among others. The migration of these cells will lead to an inflammatory response, forming new tissue and ending in wound closure. Growth factors such as TNF-α, TGF-β, PDGF, and others also play an essential role in inflammation, proliferation, epithelialization, granulation tissue formation, maturation, tissue remodeling, and other processes.[11] To achieve optimal wound healing, several inflammatory mediators, such as drugs and medicinal plants, are widely used in wound healing. Aloe vera is one of the medicinal plants often used for its anti-inflammatory, antibacterial, antioxidant, and anti-fungal properties.[17]

Aloe vera aid the wound-healing process because its extract contains acemannan and sitosterol that can activate macrophages in wounds.[20] In addition, ozone plays a role in stimulating TGF-β and other growth factors in the wound area. TGF-β and other growth factors induce fibroblast proliferation, which is affected by oxygen supply.[21] Ozonated aloe vera oil boosts the

presence of reactive oxygen species and reactive nitrogen species in the vicinity of the wound, involving platelets, macrophages, fibroblasts, endothelial cells, and keratinocytes, which serve as agents promoting wound healing.

This study provides well-described insight into the effect of ozonated Aloe vera oil in modulating the inflammatory response on radiation dermatitis wound healing through various inflammatory cytokine and histology changes, particularly neutrophil infiltration, fibroblast count, and endothelial damage.

However, this study only elaborated on the expression of cytokine at a one-time point. Further investigation regarding the dynamic change in the cytokine response and histological changes at a serial time would be beneficial. This study also exposed the subject to the radiation at one time only; further research on multiple exposures to radiation would become essential to a clinical setting as patients often receive multiple exposures to radiation during their treatment.

The limitations of the present study include a singular focus on assessing cytokine expression and histological changes at a single time point, which may limit the understanding of dynamic changes in the wound healing process over time. Additionally, exposure to radiation was conducted only once, whereas in clinical settings, patients typically undergo multiple radiation sessions, suggesting a need for future research reflecting this reality. Finally, while the study provided insights into the mechanisms of ozonated Aloe vera oil in wound healing, further mechanistic investigations are necessary to fully elucidate its therapeutic pathways. Further research on multiple exposures to radiation would become essential to a clinical setting as patients often receive multiple exposures to radiation during their treatment.

Conclusion

Topical ozonated Aloe vera oil promotes wound healing of radiation dermatitis by decreasing TNF-α expression, neutrophil infiltration, and endothelial damage and increasing TGF-β expression, PDGF, and fibroblast count.

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Authors' Contribution

All authors contributed to data analysis, drafting, and revising of the article and agreed to be responsible for all aspects of this work.

Conflict of Interest

None declared.

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Table 1. Results of TNF-A, TGF-B, and PDGF expression (Allred score) in placebo, hydrocortisone ointment, and aloe vera treatment groups

Group	TNF-α	TGF-β	PDGF
	(mean ± SD)		
C1- without therapy	6.76 ± 0.41	3.60 ± 0.40	4.04 ± 0.50
C2-hydrocortisone ointment	3.72 ± 1.13	4.20 ± 1.02	4.52 ± 1.06
P1-non-ozonate aloe vera	6.04 ± 0.09	5.00 ± 0.81	5.60 ± 1.02
P2-aloe vera 300	5.33 ± 0.39	5.33 ± 0.63	5.73 ± 0.39
P3-aloe vera 600	5.37 ± 0.32	5.47 ± 0.78	6.17 ± 0.97
P4-aloe vera 1200	5.83 ± 0.15	5.93 ± 1.17	6.40 ± 0.70

TNF-α: Tumor necrosis factor-alpha; TNF-β: Tumor necrosis factor-beta; PDGF: Platelet-derived growth factor

Table 2. Differences in TNF-α, TGF-β, and PDGF Expression among Intervention Groups: Without Therapy (C1), Hydrocortisone (C2), Aloe vera (P1), OVA 300 (P2), OVA 600 (P3), and OVA 1200 (P4)

Intervention group difference	**TNF-α (*P* value)**	**TGF-β (*P* value)**	**PDGF (*P* value)**
C1-without therapy			
C2- hydrocortisone	0.08*	0.271	0.401
P1-Aloe vera	0.007*	0.014*	0.009*
P2-OVA 300	0.005*	0.002*	0.006*
P3-OVA 600	0.005*	0.001*	0.006*
P4-OVA 1200	0.005*	<0.001*	0.006*
C2-hydrocortisone			
P1-Aloe vera	0.007*	0.146	0,113
P2- OVA 300	0.005*	0.035*	0.026*
P3-OVA 600	0.007*	0.020*	0.028*
P4-OVA 1200	0.005*	0.002*	0.010*
P1-Aloe vera			
P2-OVA 300	0.005*	0.520	0.264
P3-OVA 600	0.005*	0.370	0.081
P4-OVA 1200	0.027*	0.079	0.066
P2-OVA 300			
P3-OVA 600	0.935	0.787	0.681
P4-OVA 1200	0.029*	0.229	0.080
P3-OVA 600			
P4-OVA 1200	0.007*	0.347	0.329

P Value < 0.05 (*) showing significant difference; TNF-α: Tumor necrosis factor-alpha; TNF-β: Tumor necrosis factor-beta; PDGF: Platelet-derived growth factor; OVA: Aloe Vera oil

Table 3. Average results of neutrophil infiltration, fibroblast count, and endothelial damage in placebo, hydrocortisone ointment, and aloe vera treatment groups

Group	Neutrophil infiltration	Fibroblast count	Endothelial damage
	(mean ± SD)		
C1-without therapy	1.68 ± 1.24	8.64 ± 0.26	2.84 ± 0.50
C2-hydrocortisone ointment	0.84 ± 0.30	9.84 ± 0.50	2.64 ± 0.38
P1-non-ozonated aloe vera	0.56 ± 0.30	10.44 ± 0.43	2.68 ± 0.30
P2-aloe vera 300	0.20 ± 0.18	11.50 ± 0.53	2.43 ± 0.20
P3-aloe vera 600	0.17 ± 0.15	12.40 ± 0.58	2.30 ± 0.21
P4-aloe vera 1200	0.12 ± 0.11	12.97 ± 1.01	1.77 ± 0.15

Table 4. Average difference of neutrophil infiltration, fibroblast count, and endothelial damage in placebo, hydrocortisone ointment, and aloe vera treatment groups

Intervention group difference	Neutrophil infiltration	Fibroblast count	Endothelial damage
C1-without therapy			
C2-hydrocortisone	0.399	0.005*	0.240
P1-Aloe vera	0.067	< 0.001*	0.338
P2-OVA 300	0.006*	< 0.001*	0.096
P3-OVA 600	0.005*	< 0.001*	0.080
P4-OVA 1200	0.008*	< 0.001*	0.007*
C2-hydrocortisone			
P1-Aloe vera	0.169	0.134	1.000
P2-OVA 300	0.009*	< 0.001*	0.159
P3-OVA 600	0.007*	< 0.001*	0.096
P4-OVA 1200	0.008*	< 0.001*	0.007*
P1-Aloe vera			
P2-OVA 300	0.040*	0.008*	0.218
P3-OVA 600	0.024*	< 0.001*	0.048
P4-OVA 1200	0.017*	< 0.001*	0.005*
P2-OVA 300			
P3-OVA 600	0.733	0.008*	0.277
P4-OVA 1200	0.431	0.017*	0.003*
P3-OVA 600			
P4-OVA 1200	0.609	0.122	0.004*

P Value < 0.05 (*) showing significantly difference

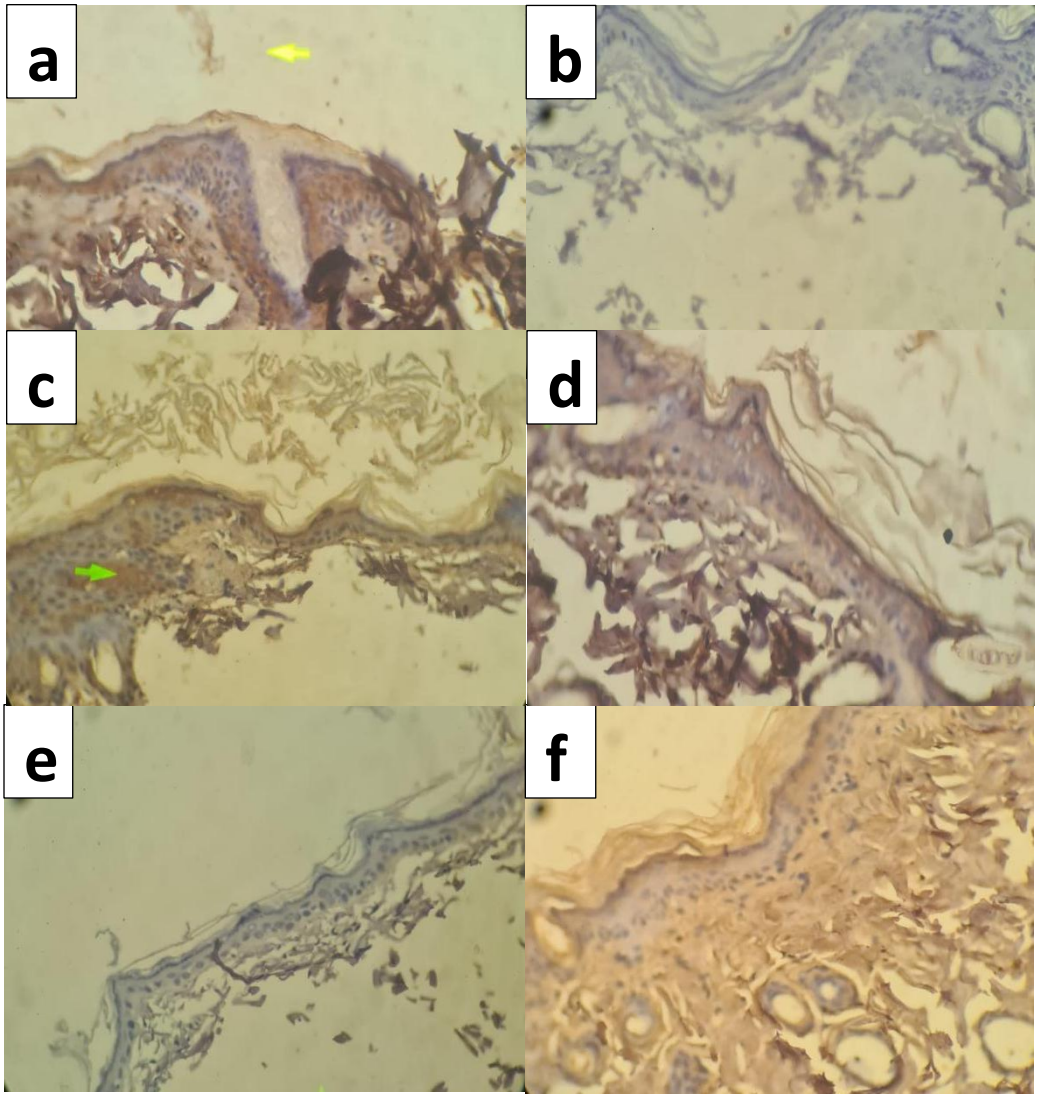

Figure 1. This figure shows the immunohistochemical staining of TNF-α expression in different treatment groups: (a) C1 representing the negative control, (b) C2 treated with hydrocortisone ointment, (c) P1 treated with Aloe vera, (d) P2 treated with Ozonated Aloe vera at 300 mg/mL, (e) P3 treated with Ozonated Aloe vera at 600 mg/mL, and (f) P4 treated with Ozonated Aloe vera at 1200 mg/mL.

TNF-α: Tumor necrosis factor-alpha

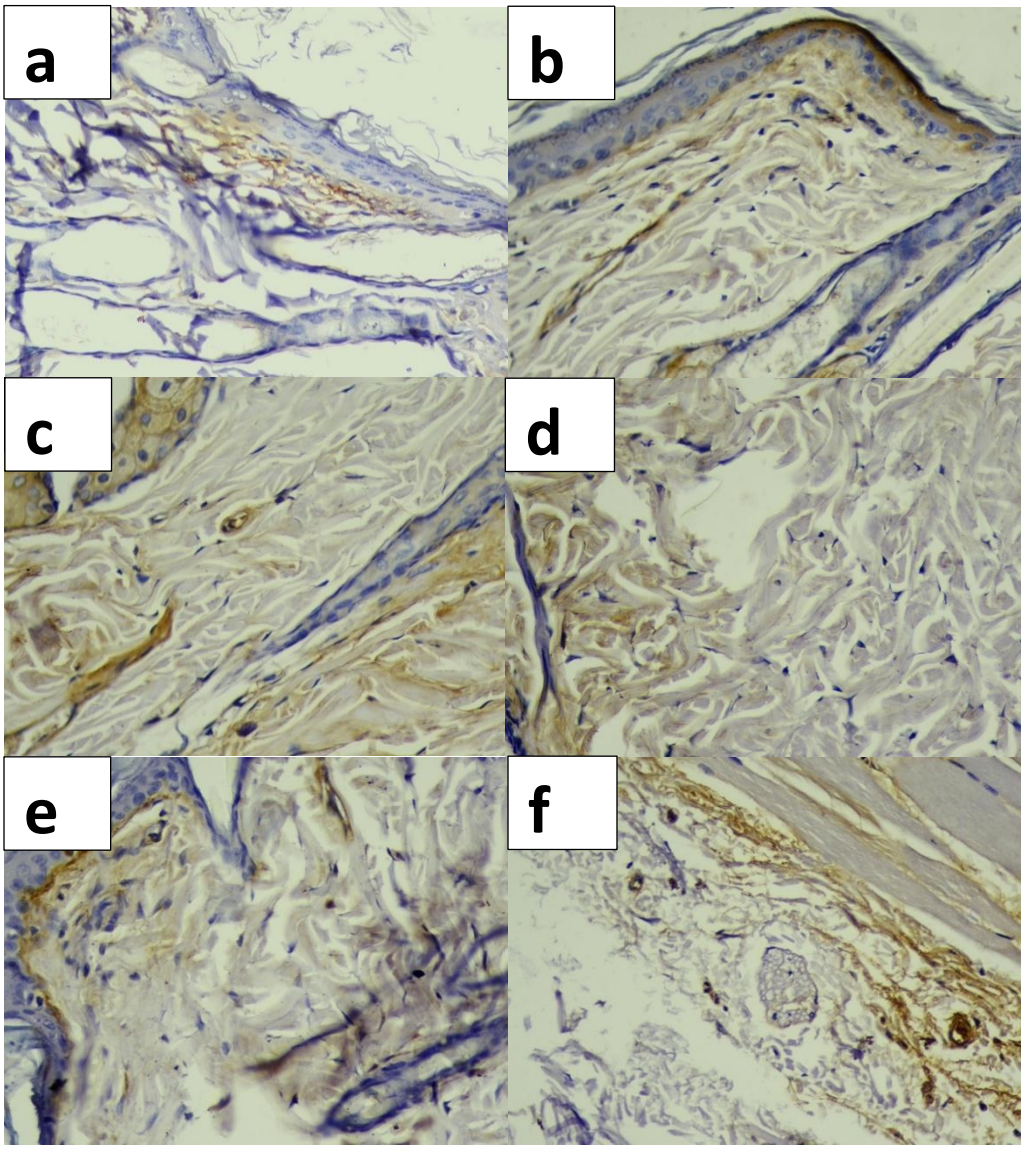

Figure 2. This figure shows the immunohistochemical staining of TGF-β expression across various treatment groups: (a) C1 as the negative control, (b) C2 treated with hydrocortisone ointment, (c) P1 treated with Aloe vera, (d) P2 treated with Ozonated Aloe vera at 300 mg/mL, (e) P3 treated with Ozonated Aloe vera at 600 mg/mL, and (f) P4 treated with Ozonated Aloe vera at 1200 mg/mL

TNF-β: Tumor necrosis factor-beta

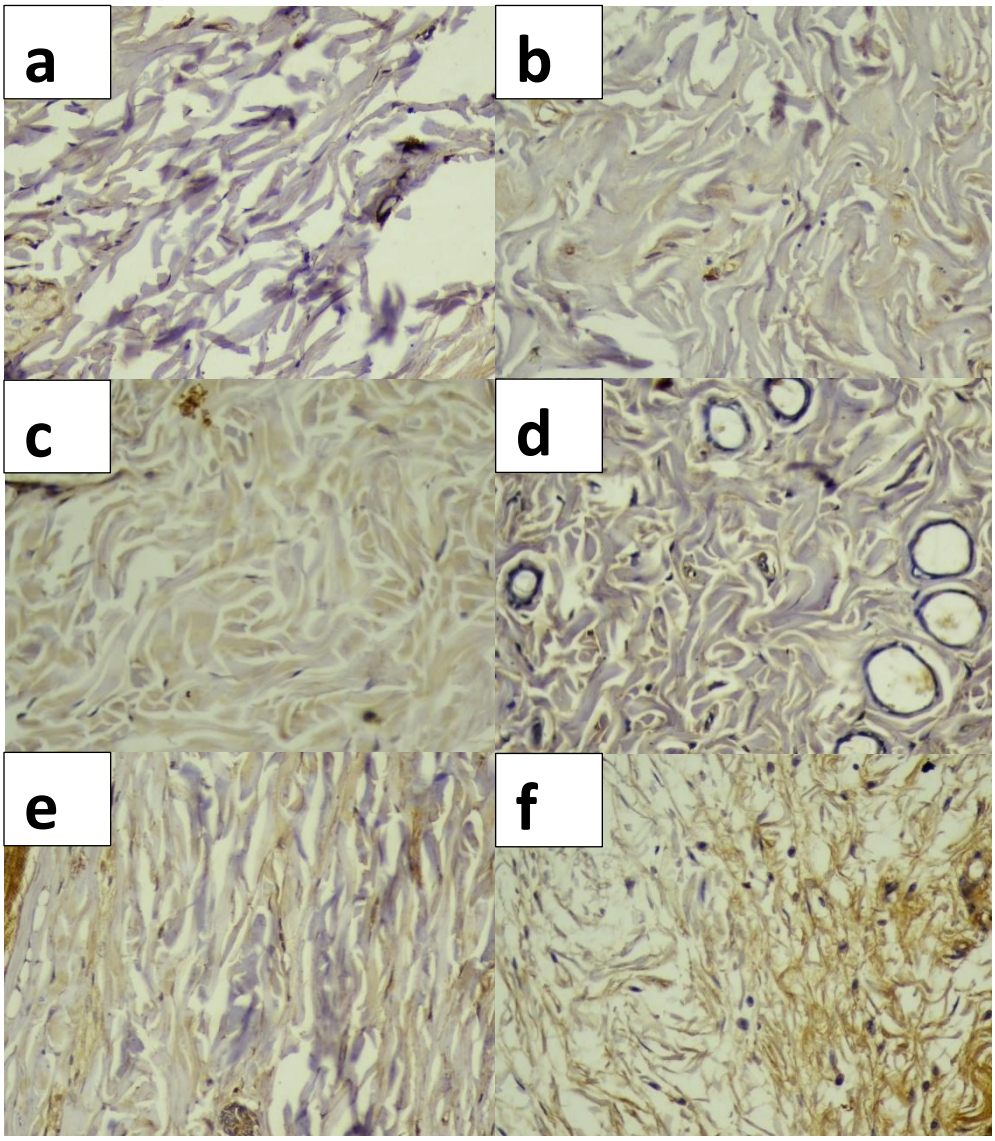

Figure 3. This figure shows the immunohistochemical staining of PDGF expression in different treatment groups: (a) C1 representing the negative control, (b) C2 treated with hydrocortisone ointment, (c) P1 treated with Aloe vera, (d) P2 treated with Ozonated Aloe vera at 300 mg/mL, (e) P3 treated with Ozonated Aloe vera at 600 mg/mL, and (f) P4 treated with Ozonated Aloe vera at 1200 mg/mL. PDGF stands for Platelet-Derived Growth Factor.

PDGF: Platelet-derived growth factor

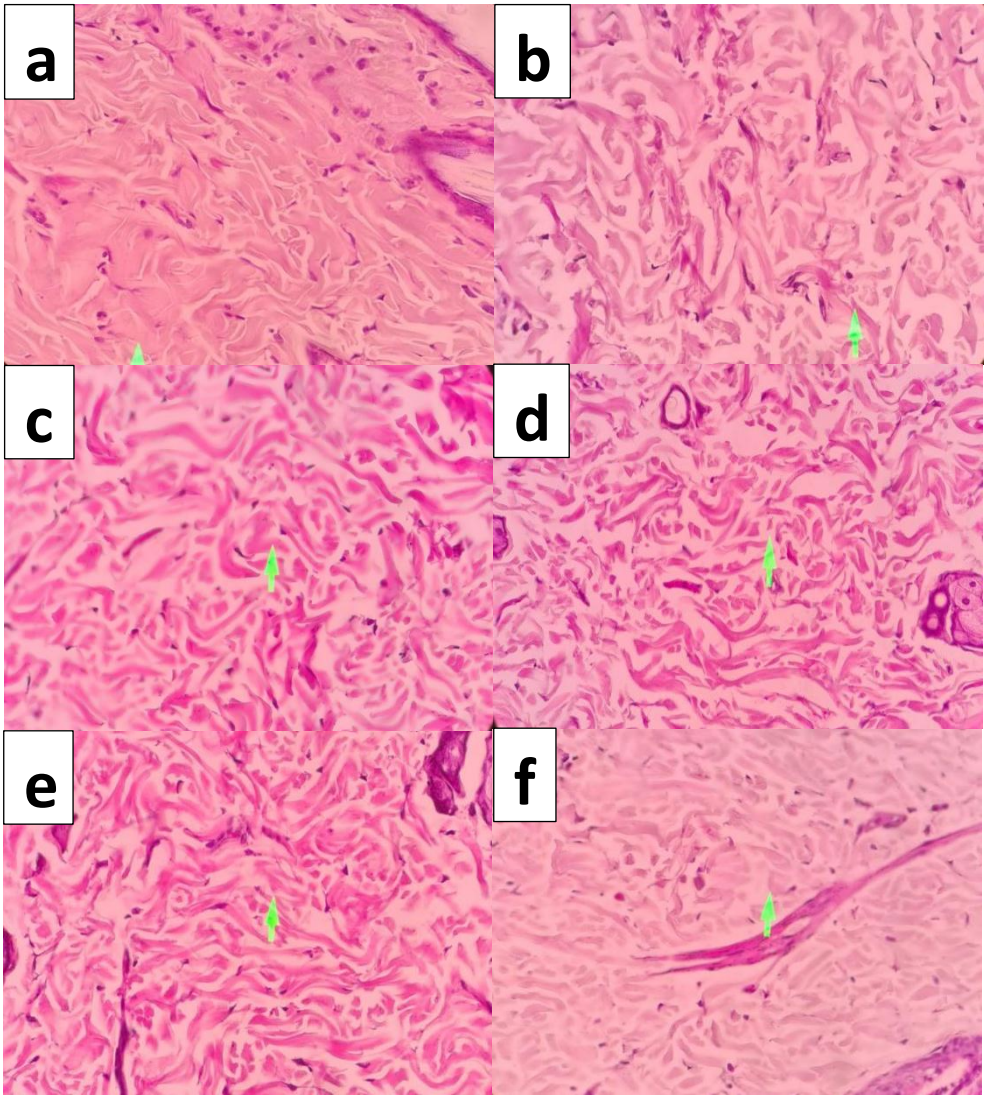

Figure 4. This figure shows the hematoxylin and eosin (HE) staining of neutrophil infiltration in different treatment groups: (a) C1 representing the negative control, (b) C2 treated with hydrocortisone ointment, (c) P1 treated with Aloe vera, (d) P2 treated with Ozonated Aloe vera at 300 mg/mL, (e) P3 treated with Ozonated Aloe vera at 600 mg/mL, and (f) P4 treated with Ozonated Aloe vera at 1200 mg/mL.

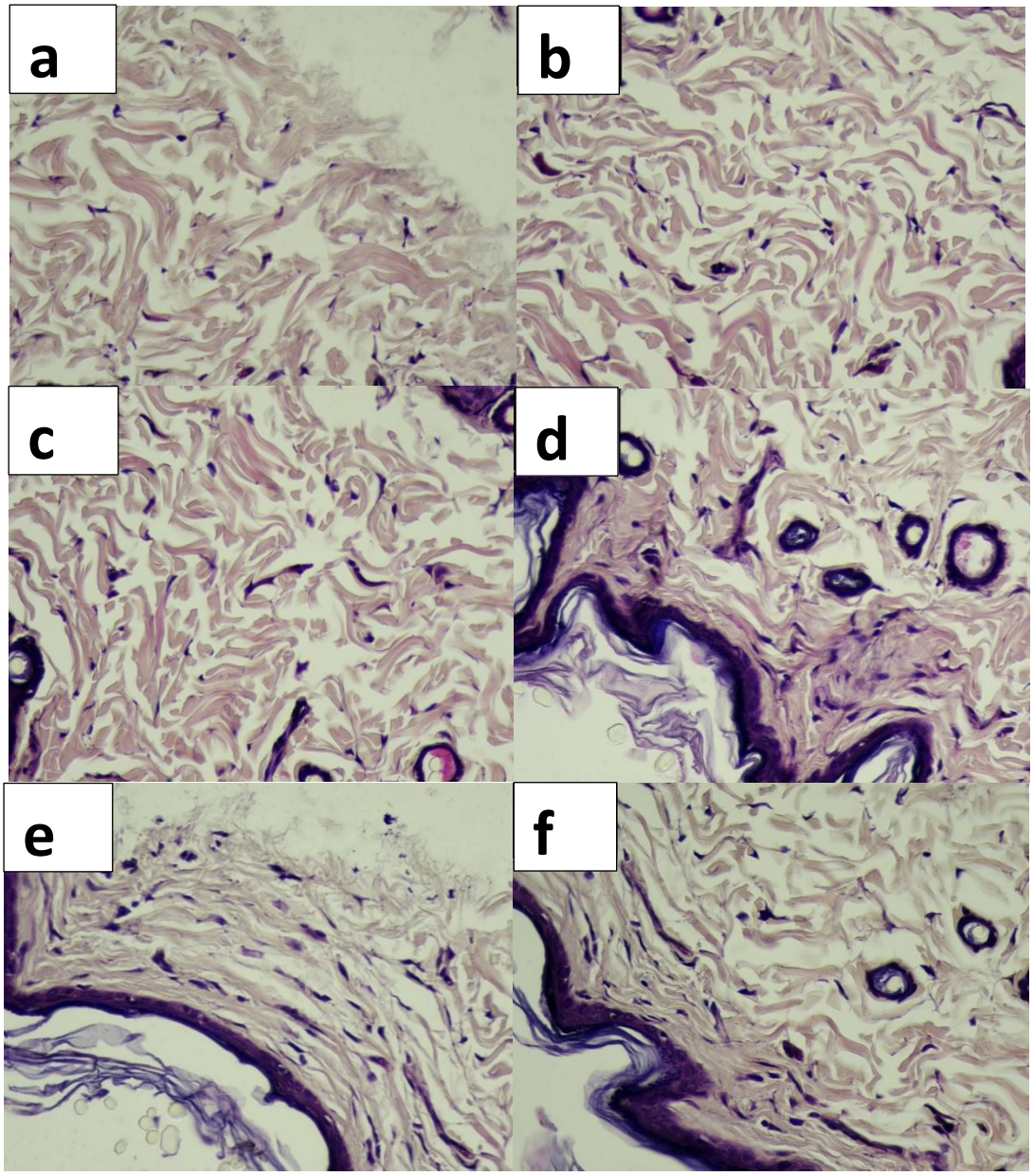

Figure 5. This figure shows the hematoxylin and eosin (HE) staining depicting fibroblast count in various treatment groups: (a) C1 representing the negative control, (b) C2 treated with hydrocortisone ointment, (c) P1 treated with Aloe vera, (d) P2 treated with Ozonated Aloe vera at 300 mg/mL, (e) P3 treated with Ozonated Aloe vera at 600 mg/mL, and (f) P4 treated with Ozonated Aloe vera at 1200 mg/mL.

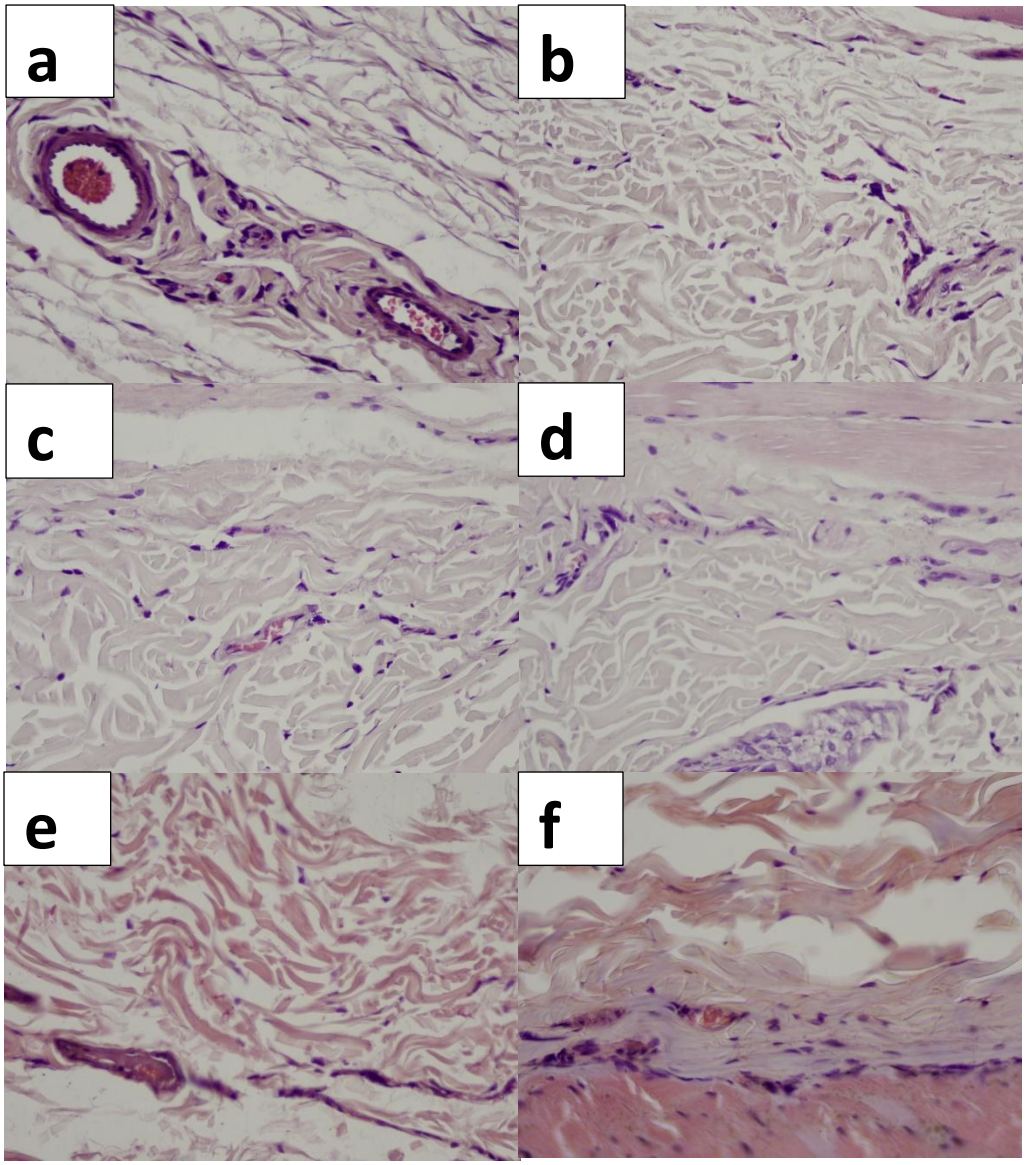

Figure 6. Hematoxylin and eosin (HE) staining illustrating endothelial damage in different treatment groups: (a) C1 representing the negative control, (b) C2 treated with hydrocortisone ointment, (c) P1 treated with Aloe vera, (d) P2 treated with Ozonated Aloe vera at 300 mg/mL, (e) P3 treated with Ozonated Aloe vera at 600 mg/mL, and (f) P4 treated with Ozonated Aloe vera at 1200 mg/mL.