

HASIL CEK_Plasma Jet-Treated Lidah Buaya (Aloe Vera) Influences Proliferative-Phase Wound Healing

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Plasma Jet–Treated *Lidah Buaya* (Aloe Vera) Influences Proliferative-Phase Wound Healing

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ABSTRACT: An *in vivo* experimental study was conducted to evaluate the effect of a plasma-treated aloe vera slice on cutaneous acute wound healing using a small animal model. A nonequilibrium atmospheric plasma pressure jet (N-APPJ) was developed. Aloe vera slices with thicknesses of approximately 2 mm were treated by N-APPJ at distances of 5 mm, 10 mm, and 20 mm before application. Possible reactive species on the surfaces of the plasma-treated aloe vera slices were chemically identified. Forty-five male Balb/c mice, aged 7–8 weeks, were classified into 5 groups: control (C), aloe vera slice alone (Av), plasma-treated aloe vera slice at 5-mm distance (PTAv-5), plasma-treated aloe vera slice at 10-mm distance (PTAv-10), and plasma-treated aloe vera slice at 20-mm distance (PAV-20). Wounds were observed for 14 days. Histological evaluation using general staining for re-epithelialization was also conducted. The “dropped-water method” was able to identify surfatic RONS. Additionally, this investigation revealed that sizes of wound areas in groups containing an aloe vera slice, from days 2 to 14, were significantly smaller compared with the control group. During the proliferative phase, wound size in PTAv-20 was smaller than that in both PTAv-5 and PTAv-10. From days 4 to 7, wound size in PTAv-20 was slightly smaller than that in Av; however, wound size in PTAv-5 and PTAv-10 was greater than that in PTAv-20. On day 7, re-epithelialization percentages in Av and PTAv-20 were significantly higher than in C. It was concluded that plasma-treated aloe vera has the ability to influence the proliferative phase of wound healing.

KEY WORDS: atmospheric plasma jet, plasma medicine, redox, plasma-activated water (PAW), wound, aloe vera, gels

I. INTRODUCTION

In basic, nonchronic wound healing there are three overlapping stages: inflammation, proliferation, and remodeling.¹ Advanced studies showed that signaling biomolecules, namely reactive oxygen and nitrogen species (RONS), have a critical role in support of such processes.^{2,3}

An analysis of Medicare beneficiaries identified that 8.2 million people had wounds with or without infections. Medicare cost estimates for acute and chronic wound treatments range from \$28.1 billion to \$96.8 billion.⁴ Interdisciplinary collaboration to tackle such a problem is important.

In clinical settings, it is well known that there are many treatment options for improvement of wound healing—from natural agents like hormones,^{5,6} honey,^{7,8} and aloe vera⁹ to technological agents like electrical stimulation¹⁰ and ultra violet light.¹¹ The problem is that very few treatments can cope with conditions in the wound bed throughout all stages of healing. Consequently, finding better treatments is crucial. A treatment consisting of a plasma agent in combination with a natural treatment that already exists, such as aloe vera, could lead to insights.

Fundamentally, there are four physical states of observable matter in everyday life: solid, liquid, gas, and plasma. Plasma medicine involves plasma science, pharmacology, life sciences, biomedicine, and other health sciences to functionalize physical plasma for medical treatment.¹² There are nonreactive parts (gases) and reactive parts (ions, energetic and radical particles) in plasma. Conceptually, the medical aspects of plasma are related to its ability to generate biological molecules, such as RONS, which can be controlled physically through their dose and behavior.¹³ RONS in micro-concentrations^{14,15} have efficacy for wound healing, while in high concentrations they can damage living tissue.¹⁶ The efficacy of atmospheric plasma for wound healing has been established.^{17–21} However, harmful effects due to excessive doses have been reported.²² Efforts to combine atmospheric plasma with natural products for wound healing are ongoing.^{23–25}

Although RONS are key players, recent investigations into plasma medicine have shown that the positive biomedical effects of RONS are determined by the presence of liquid around target cells.^{26,27} There are two basic principles in this context: first, the effects of plasma are caused by changes it induces in the liquid zone surrounding cells. Second, liquid phases containing RONS play a pivotal role in plasma-induced biological responses. Woedtke and Weltmann showed that biological plasma effects are primarily mediated through reactive oxygen and nitrogen species influencing cellular redox-regulated processes. Therefore, plasma medicine is considered a field of applied redox biology.²⁸

As a succulent plant, aloe vera (*Lidah Buaya* in Semarang, Indonesia) is a xerophyte, which means that it is adapted to living in areas of low water availability and characterized by its large water storage tissue. The primary characteristic of the aloe vera plant is its huge water content, ranging 99–99.5%.²⁹ The remaining 0.5–1.0% of solid material contains many potentially active compounds, including water- and fat-soluble vitamins, minerals, enzymes, simple/complex polysaccharides, phenolic compounds, and organic

acids. Of the structural components of the aloe vera leaf, the rind comprises 20–30% and the pulp 70–80% of total leaf weight.

This research was conducted to evaluate the effectiveness of plasma jet–treated fresh aloe vera slices on acute wound healing in a small animal model. Prior to wound evaluation, the identification and concentration calculation of surface H_2O_2 and NO_2^- in fresh aloe vera slice after plasma jet treatment was conducted using noninvasive methods.

II. METHODS AND MATERIALS

A. Nonequilibrium Atmospheric Pressure Plasma Jet System

The nonequilibrium atmospheric pressure plasma jet (N-APPJ), developed by Teschke et al.,³⁰ was applied. This reactor has been described elsewhere.¹⁹ Briefly, the inner and outer diameters of the capillary quartz tube were modified to be 0.65 and 1.55 mm, respectively. Two electrodes of conductive rounded materials were applied on the tube. Nonconductive material, purchased from Rumah Tanah Liat Citra, Tangerang Selatan, Indonesia, namely *tanah lempung*, or local clay, was applied to isolate the electrodes. As reported elsewhere,²² characterizations of electrical and optical emission were conducted in the Plasma Laboratory at the Research Center for Sustainable Energy and Technology, Institute of Science and Engineering, Kanazawa University, Japan. The discharge voltage and discharge current were measured with a high-voltage probe (P6015A; Tektronix, Tokyo, Japan) and a current probe (8585C; Pearson Electronics, Palo Alto, CA). When argon gas at flow rates of 0.1, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 standard liters per minute (slm), flowed from one end of the quartz tube, at low frequency (~18.32 kHz) AC high voltage, with a peak-to-peak voltage of 9.58 kV and current of 55.2 mA, was measured at the upper ring electrode. Medical-grade argon gas with 99.999% purity (Samator, Secang, Magelang, Indonesia) was used as a working gas.

Emissions from the plasma jet between 200 and 450 nm were recorded by a photonic multichannel analyzer (PMA-12) (C10029, Hamamatsu Photonics, Hamamatsu, Japan). The emissions were focused on the entrance slit of an optical fiber probe of the PMA-12 using a convex quartz lens with focal length $f = 100$ mm. The exposure time was 19 ms, and the number of accumulations was 30. The light diameter of the optical fiber was 1 mm. The optical emission intensity of the optical system was calibrated using a standard lamp. Optical emission spectroscopy (OES) measurement at approximately 10 mm under the nozzle showed the presence of both hydroxyl radical (OH) and nitrogen-based reactive species in the gas phase during plasma generation (Fig. 1). Relative intensities at wavelengths of 309 and 337 nm reached peaks at a 2-slm flow rate.

B. Preparation of Fresh Aloe Vera Leaves

Fresh aloe vera (*L. Burm. f.*) leaves were collected and authenticated at the Laboratory of Biology, Faculty of Mathematic and Natural Sciences, Universitas Ahmad Dahlan, Yogyakarta, Indonesia. Slices of aloe vera with a thickness of approximately 2 mm and

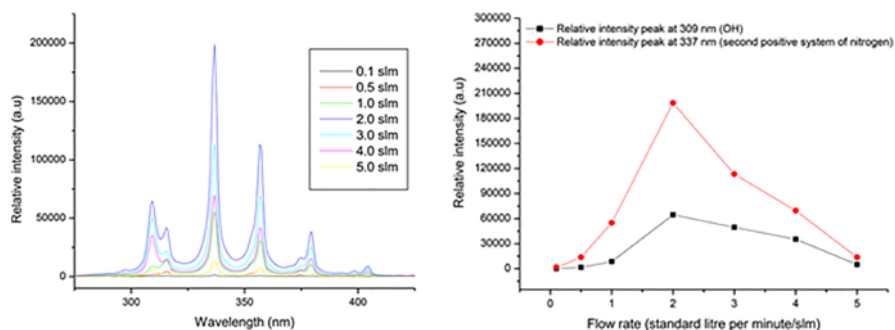


FIG. 1: Left: identification of ROS and RNS by OES in varying flow rates. Right: relationship between flow rates and relative intensity at peaks 309 (OH) and 337 nm (second positive system of nitrogen).

dimensions of 6×6 mm were manually cut with a knife on a daily basis. Afterward, the slices were stored in a 4°C refrigerator overnight for experimental use the next day.

1. Semiquantitative Identification of H_2O_2 on the Surface of Plasma-Treated Aloe Vera Slices

The presence of H_2O_2 on the surface of an aloe vera slice was identified using a peroxide paper test strip (Merck, Darmstadt, Germany) following 2-min plasma jet treatment at distances of 5, 10, or 20 mm. A colorimetric method with a concentration range of 0.5–25 mg/L H_2O_2 was used. To evaluate the possible degradation of the H_2O_2 , the identification was conducted immediately and 15, 30, 45, and 60-min post-treatment.

2. Calculation of Quantitative Concentrations of Hydrogen Peroxide (H_2O_2) and Nitrite (NO_2^-)

Post-treatment concentrations of H_2O_2 and NO_2 on the surface of the plasma-treated aloe vera slices were analyzed with a peroxidase enzyme for H_2O_2 and a naphthylethylenediamine visual colorimetric for NO_2 that used a commercial reagent (Kyoritsu Chemical-Check Lab, Tokyo, Japan, range: 0.05–5.0 mg/L, and Model WAK- H_2O_2 , range: 0.02–1.00 mg/L) after plasma jet treatment. This method has been used by other researchers.³¹ Samples were classified into three groups based on their applied distance between the surface of the aloe vera slices and the plasma jet reactor nozzle tip. Groups PTA_v-5, PTA_v-10, and PTA_v-20 were APPJ-treated at distances of 5, 10, and 20 mm, respectively. The argon gas flow rate was set at 2 slm.

General sample preparation is described in Fig. 2. First, the aloe vera slice was prepared and a plastic ring with a 4-mm diameter was put on its surface. This ring was used for maintaining pure water on the plasma-treated surface area. The visual appearance

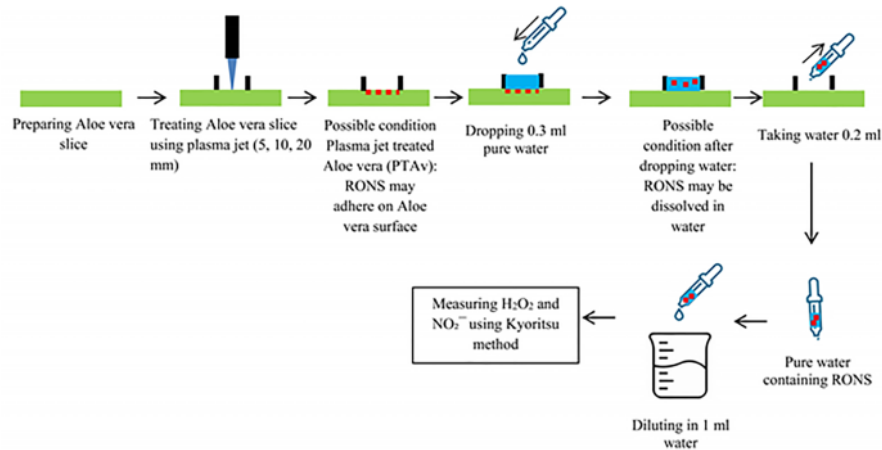


FIG. 2: General preparation for concentration calculation of H_2O_2 and NO_2^- on surface of plasma-treated aloe vera slice

of the aloe vera surfaces after treatment at 5-, 10, and 20-mm distances is shown in Fig. 3. Immediately following treatment, 0.3 mL of pure water was dropped on the surface of the slice. After approximately 15 s, 0.2 mL of the dropped water was recovered and diluted in another 1.0 mL of pure water. Digital packtest devices (Kyoritsu Chemical-Check Lab., Japan; Model DPM- H_2O_2 and Model DPM- NO_2) were used to measure concentrations of H_2O_2 and NO_2 in the 1.2-mL mixed water. All experiments were performed in triplicate for each group.

C. Animals and Investigation Protocol

Forty-five male Balb/c mice, purchased from **Laboratorium Penelitian dan Pengujian Terpadu/Integrated Research and Testing Laboratory (LPPT UGM), Gadjah Mada University, Yogyakarta, Indonesia**, were obtained at 7 to 8 weeks of age and maintained under the following controlled conditions: 12-h light-dark cycle, of $25.0 \pm 2.0^\circ\text{C}$, and regular mouse chow and water ad libitum. All procedures were in accordance with animal welfare guidelines and were approved by the ethics committee for preclinical investigation in LPPT UGM (00028/04/LPPT/VI/2019). This institution uses the standards of ISO/IEC 17025 and the National Accreditation Committee of Indonesia (Komite Akreditasi Nasional/KAN, Indonesia).

D. Experimental Design

The mice were anaesthetized by injection of ketamine-xylazine (KX) at a dose of 50 and 5 mg/kg, respectively, through intraperitoneal administration.³² The working procedure

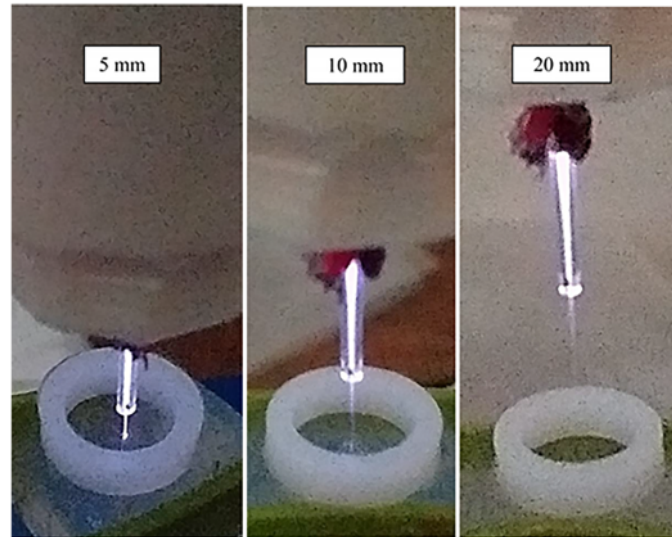


FIG. 3: APPJ treatment on aloe vera surface at 5-, 10-, and 20-mm distances. Ring used for maintaining pure water on surface area following plasma jet treatment.

to create a nonchronic wound on experimental mice was as described elsewhere.²⁵ Rounded wounds of 4-mm diameter full skin thickness were made at the panniculus on both sides of the mouse dorsum. They were created using disposable 4-mm biopsy punches. Wound samples were classified as

- Hydrocolloid dressing alone (C)
- Aloe vera alone (Av)
- Plasma-treated aloe vera at a distance of 5 mm (PTAv-5)
- Plasma-treated aloe vera at a distance of 10 mm (PTAv-10)
- Plasma-treated aloe vera at a distance of 20 mm (PTAv-20)

The experimental protocol is shown in Fig. 4. C wounds were covered daily with a hydrocolloid dressing (HD) alone. Av wounds were covered daily with an aloe vera slice alone. PTAv-5, PTAv-10, and PTAv-20 wounds were covered daily with aloe vera slices plasma jet-treated for 2 min at their respective distances of 5, 10, and 20 mm. The argon gas flow rate was set at 2 slm.

E. Macroscopic Evaluation of Wound

Macroscopic evaluations were conducted daily as described elsewhere²⁵ and documented using a digital camera. This procedure was conducted for 14 days. Day 0 was the day that the acute wounds were made.

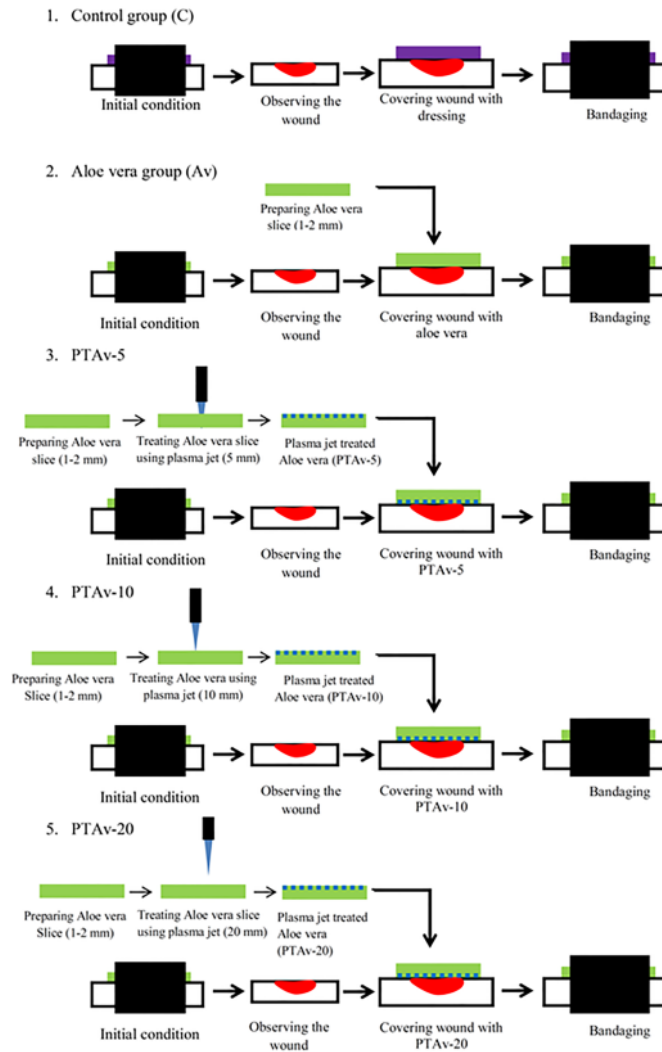


FIG. 4: Experimental procedure days 1–13

F. Tissue Processing and Measuring of Re-Epithelialization

The mice were euthanized by a high-dose IP injection of KZ7, 11, or 14 days after wound creation. The wound and the surrounding normal skin were harvested, and each specimen was bisected at the wound center. Wounds were fixed in neutral buffered 10%

formalin solution, pH 7.4, for approximately 15 h. The samples were then rinsed in 0.01 M PBS for approximately 8 h. Subsequently, they were dehydrated in an alcohol series, cleared in xylene, and embedded in paraffin to prepare serial 5- μ m sections. These sections were stained with haematoxylin-eosin (HE). With the results of the haematoxylin-eosin staining, the percentage of re-epithelialization was calculated using the formula as reported elsewhere:

$$\text{Re-epithelialization (\%)} = \frac{\text{length of new epithelium}}{\text{length of wound between wound edges}} \times 100\% \quad (1)$$

G. Statistical Analysis

Data were subjected to statistical analyses using SPSS 16.0. The ratio between the average and original wound areas, as well as percentages of re-epithelialization, were evaluated by ANOVA, followed by the Tukey-Kramer method. *P* values < 0.05 were considered significant.

III. RESULTS

A. Semiquantitative Identification of H₂O₂

Figure 5 shows a surrogate qualitative condition of H₂O₂ on the surface of an aloe vera slice from 0 to 60 min after plasma jet treatment. The immediate and 15-min timepoints in all plasma-treated aloe vera groups (PTAv-5, PTAv-10, and PTAv-20) had detectable H₂O₂, whereas the 45- and 60-min timepoints did not. In the 30-min timepoint, H₂O₂ was detectable in PTAv-5 but not in PTAv-20. In conclusion, this investigation revealed that the presence of reactive oxygen and nitrogen species produced by an atmospheric pressure plasma jet, as marked by the presence of H₂O₂, could be identified on the surface of the aloe vera slices for several minutes.

B. Quantitative Concentration of H₂O₂ and NO₂

Quantitative concentrations of both H₂O₂ and NO₂ on the surface of plasma-treated aloe vera slices were calculated. As shown in Fig. 6, after treatments at distances of 5, 10, and 20 mm, H₂O₂ concentrations of approximately 1.4, 0.8, and 0.4 mg/L were detected. NO₂ concentrations were approximately 0.7, 0.6, and 0.2 mg/L.

C. Macroscopic Evaluation of Wounds

Figure 7 shows the appearances of wounds on days 0, 3, 7, 11, and 14. On days 3 through 14, wounds in groups containing aloe vera were smaller than those in controls. On days 11 and 14, wounds in groups containing aloe vera appeared more mature compared with controls; the surrounding yellowish appearance was likely due to aloe vera

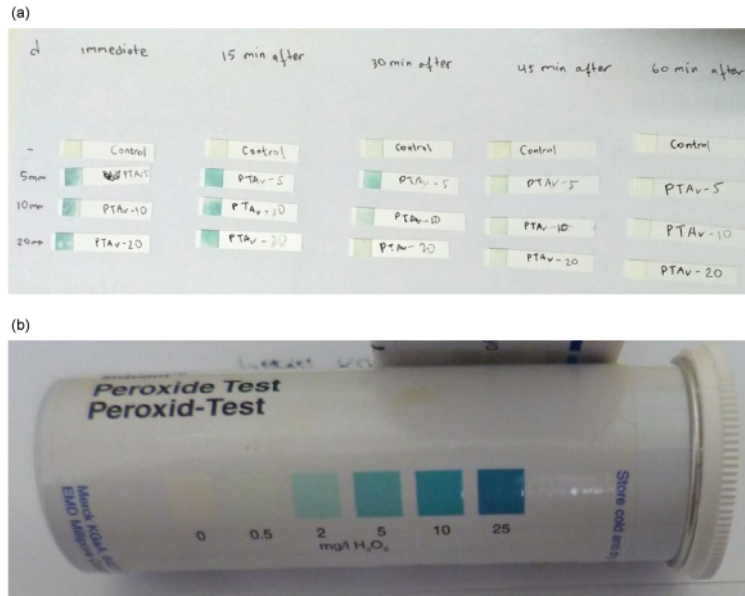


FIG. 5: (a) representative semiquantitative conditions of H₂O₂ on surface of aloe vera slice for 60 min following plasma jet treatment. H₂O₂ detected immediately and 15 min post-treatment in PTAv-5, PTAv-10, and PTAv-20 but not detected 45 min and 60 min post-treatment. At 30-min post-treatment, H₂O₂ detected in PTAv-5 but not in PTAv-20. PTAv-5: plasma jet-treated aloe vera slice at 5-mm distance; PTAv-10: plasma jet-treated aloe vera slice at 5-mm distance; PTAv-20: plasma jet-treated aloe vera slice at 20-mm distance. (b) H₂O₂ indicator.

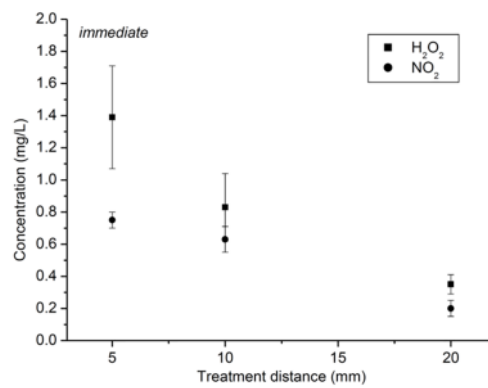


FIG. 6: Relationship between treatment distances and quantitative concentrations of H₂O₂ and NO₂⁻ immediately post-treatment

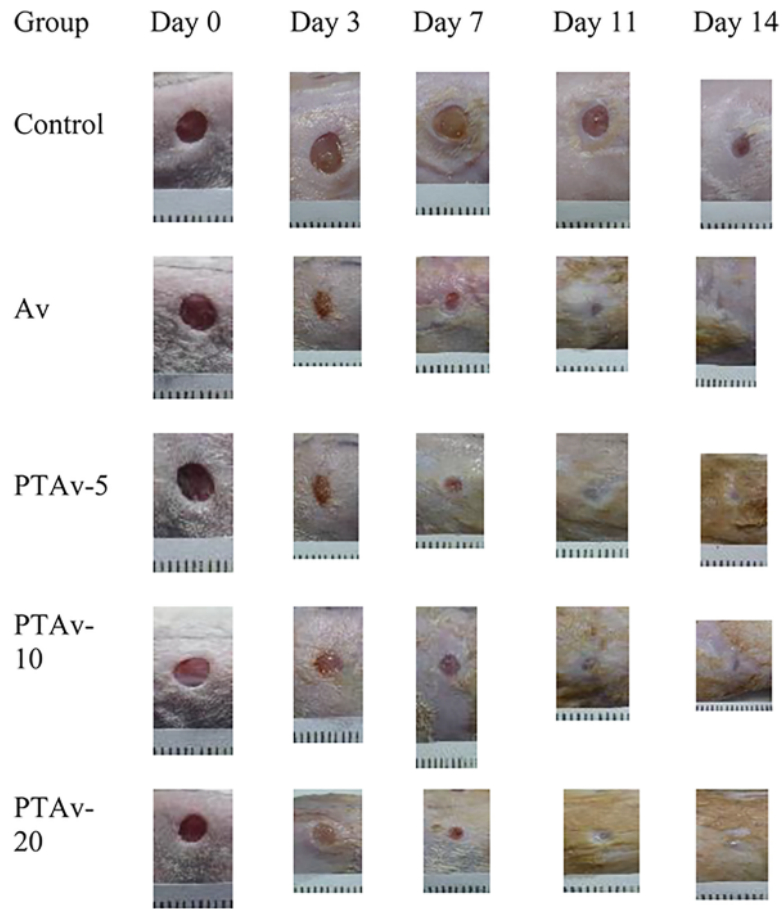


FIG. 7: Wounds on days 0, 3, 7, 11, and 14

gel. Among groups containing aloe vera, there were differences in area size but surface conditions were similar.

D. Evaluation of Wound Area Reduction and Re-Epithelialization

Figure 8(a) shows a graph of wound area reduction from days 0 to 14. It appears that starting from day-2 wound sizes in the groups containing aloe vera, both alone and treated by plasma jet, were significantly smaller than the controls ($p < 0.01$). This indicates that the natural ingredients in aloe vera may have properties to accelerate wound healing.

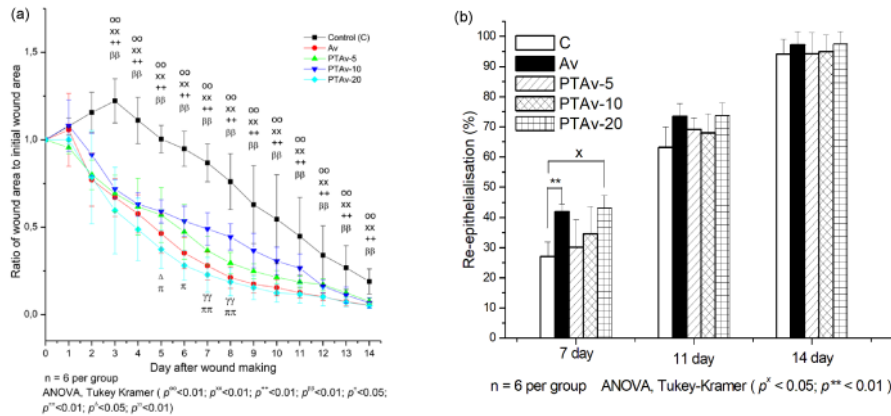


FIG. 8: (a) Ratio of wound area at different timepoints compared to initial wound area. Days 4–14, significantly smaller wound area in aloe vera groups than in control group. Days 4–7, slightly lower ratio in PTAv-20 than in Av. PTAv-20 may accelerate wound healing; PTAv-5 and PTAv-10 appear to impede it. o, x, +, β, γ, Δ, and π = significance level of C to Av, PTAv-5, PTAv-10, and PTAv-20; Av to PTAv-10; PTAv-5 to PTAv-20; and PTAv-10 to PTAv-20, respectively. (b) Percentage of re-epithelialization: day 7 percentages in Av and PTAv-20 significantly higher than in C. x and **, significance level of C to PTAv-20 and Av, respectively.

Furthermore, it appears that, although PTAv-5, PTAv-10, and PTAv-20 were treated with aloe vera activated by plasma jet, their healing rates were not the same. During the proliferation phase, namely 5, 6, 7, and 8, wound size in PTAv-20 was significantly smaller than that in PTAv-10 ($p < 0.05$). On day 5, wound size in PTAv-20 was also significantly smaller than that in PTAv-5 ($p < 0.05$). Figure 8(b) shows that on day 7, the percentages of re-epithelialization in Av, as well as in PTAv-20, were significantly higher than those in controls (Av vs. C: $p < 0.05$; PTAv-20 vs. C: $p < 0.05$). This indicates that the ability of plasma jet-activated aloe vera to improve wound healing during the proliferation phase may be correlated with its ability to accelerate re-epithelialization.

IV. DISCUSSION

This investigation reveals that wound area size in experimental groups treated with aloe vera from days 2 to 14 are significantly smaller compared with controls (treated with hydrocolloid dressing only). This suggests that aloe vera has a critical role in accelerating healing during wound inflammation, proliferation, and remodeling. Interestingly, on the last experimental day, day 14, wound areas in the groups containing aloe vera were significantly smaller compared with controls. So far, aloe vera in raw and fresh form, as used in this study, may be an effective alternative to hydrocolloid dressing, which has a relatively high cost.

In this investigation, differences in wound area sizes between groups treated with aloe vera slices were apparent on days 5, 6, 7, and 8; however, on day 14, sizes were not significantly different. It is suggested that atmospheric plasma jet, as applied in this investigation, influences the effectiveness of aloe vera slices both positively and negatively. It is also indicated that the applied distance of the plasma jet to the aloe vera surface may be a main factor. On one hand, a distance of 5 to 10 mm increased wound area sizes compared to aloe vera only; on the other hand, a distance of 20 mm decreased them.

This investigation also showed that re-epithelialization percentages by day 7 in both Av and PTA_v-20 were significantly higher than in C. However, re-epithelialization percentages between Av and PTA_v-20 were not significantly different. This suggests that the effects of both are similar. Conversely, PTA_v-5 and PTA_v-10 showed less effectiveness compared with PTA_v-20.

Considering that wound healing consists of inflammation, proliferation, and remodeling,¹ it is hypothesized that plasma jet-treated aloe vera slices may modify the physiological mechanism of wound healing during proliferation. Of course, it has been reported that only aloe vera gel with high water content improves wound healing and increases new epithelialization, due to its ability to keep the wound moist.^{33,34} However, plasma jet-treated aloe vera at a distance of 20 mm may be used as a basis in optimizing healing efficacy. Varying plasma jet treatment times may turn out to be the best treatment model. With respect to the recent concept of plasma-liquid interaction,²⁷ modifying the liquid content of aloe vera using plasma may also improve treatment.

This research is the first to evaluate the influence of plasma jet-treated fresh aloe vera slices on acute experimental wound healing using an animal model. Our investigation also identified and calculated H₂O₂ and NO₂ concentrations as markers of RONS. Why plasma jet-treated fresh aloe vera slices at a distance of 20 mm reduce wound area size during the proliferative phase, while distances of 5 and 10 mm act oppositely, may have to do with the RONS concentration rate. Concentration rates for distances of 5 and 10 mm are higher than that for a distance 20 mm, as presented in this investigation. It is well established that RONS in small concentrations^{14,15} have positive wound-healing effects while in excessive concentration are destructive to living tissue.¹⁶

This investigation applied aloe vera leaf in fresh and raw form. Generally, aloe vera leaf consists of three distinct layers:³⁵

- The inner layer containing clear gel, also called the mucilaginous layer
- The middle layer containing latex, a bitter yellow sap containing anthraquinones and glycosides
- The outer layer containing a protective rind of 15–20 cells

Plasma jet-treating aloe vera means that ROS and RNS (RONS) produced by atmospheric plasma are directed to the aloe vera gel on the top of the inner leaf, as shown in Fig. 9(a).

A diagnostic method to monitor the delivery of RONS produced by atmospheric plasma jet in living tissue is a critical development in plasma medicine.³⁶ Szili et al. used

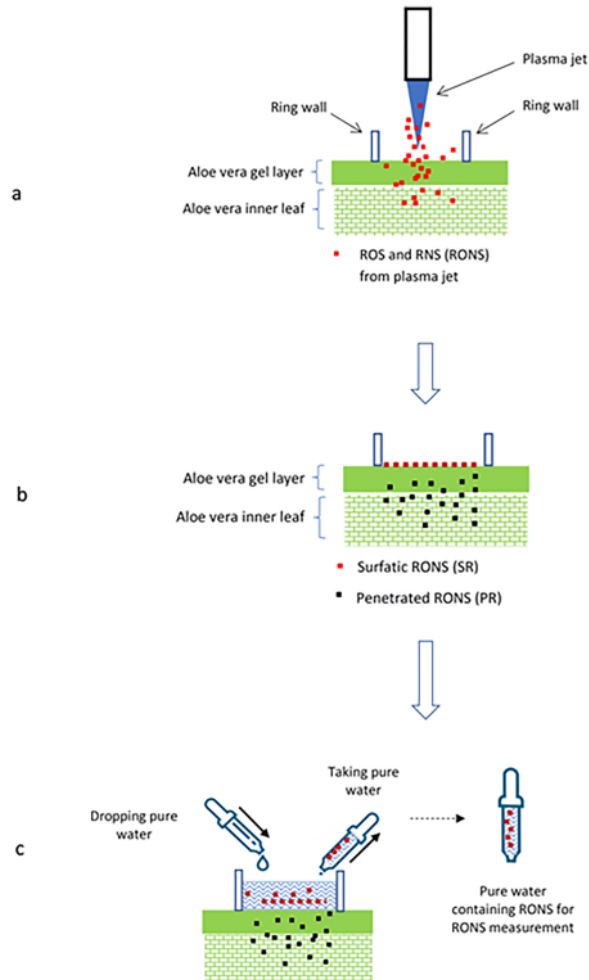


FIG. 9: Possible mechanism of formatting and measuring “surfatic RONS”: (a) aloe vera slice layers receiving RONS generated by atmospheric plasma jet; (b) differentiation of surfatic and penetrated RONS in aloe vera layers; (c) dropped-water method for measuring surfatic RONS

gel (gelatine gel) as a “tissue model” to evaluate the penetration rate of ROS that were produced by atmospheric plasma jet. It was reported that ROS can penetrate the gel to 150–1.5 mm below the surface.³⁷

Assuming that the penetration rate of ROS in this investigation is almost same as that in Szili et al., it is hypothesized that RONS may penetrate the inner leaf [see Fig.

9(b)]. Logically, the shorter the plasma jet distance, the longer the penetration rate. Clearly, the penetration of RONS generated by plasma jet at 20 mm was shorter than that at 10 and 5 mm. However, this study identified and calculated H_2O_2 and NO_2 as RONS markers using a strip tester and the “dropped-water” method, as shown in Fig. 9(c). We hypothesized that water may only react with RONS on the surface or near the surface of the gel. Consequently, H_2O_2 and NO_2 may only represent what can be called “surfatic RONS (SR).” Below such SR may exist “penetrated RONS (PR)” in a larger amount due to a shorter plasma jet distance which we may have missed. The insight into wound treatment gained by this was that, after the plasma-treated aloe vera slice was applied, the layer containing SR was delivered to the wound first and PR after. However, the gap in our investigation was the lack of data on the amount of RONS delivered. This topic requires further investigation.

It was concluded that plasma-treated aloe vera at a distance of 20 mm (PTAv-20) may accelerate acute wound healing, while plasma-treated aloe vera at a distance of 5 mm (PTAv-5) and at a distance of 10-mm (PTAv-10) may impede it. The effectiveness of PTAv-20 is supported by the presence of surfatic as well as penetrated RONS within the aloe vera slice gels.

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