

HASIL CEK_Accelerated Healing of Chronic Wounds under a Combinatorial Therapeutic Regimen Based on Cold Atmospheric Plasma Jet Using Contact and Noncontact Styles

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Accelerated Healing of Chronic Wounds under a Combinatorial Therapeutic Regimen Based on Cold Atmospheric Plasma Jet Using Contact and Noncontact Styles

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ABSTRACT: One critical element for applying atmospheric pressure plasma jet for medical purposes is that it is possible to construct a combinatorial therapeutic regimen based on contact and noncontact styles for the cold atmospheric plasma jet. This study evaluates plasma jet effectiveness for bacteria-infected wounds in a small animal model. In this investigation, we test a novel combinative treatment using contact and noncontact style for plasma jet that was generated at high voltage of ~ 9 kV. We use medical-grade argon gas as a single carrier gas. The object of plasma treatment is BALB/c mouse skin wounds that were infected with *Staphylococcus aureus*. We use four plasma jet treatments, namely, C (control), CP–CP (contact), NCP–NCP (noncontact), and CP–NCP (contact–noncontact). For CP–NCP, from days 0 to 7 we apply a contact style of plasma jet treatment to wounds to kill bacteria; from days 8 to 13, a noncontact style of plasma jet is applied to stimulate wound healing. Our results show that with CP–CP, contact plasma treatment can remove the biofilm layer, but after the biofilm layer disappears contact plasma treatment inhibits the wound-healing process. NCP–NCP is not effective in eliminating bacterial biofilms and impedes the wound-healing process. With CP–NCP, contact plasma exposure during days 0 to 7 is able to remove bacterial biofilms, and irradiation of noncontact plasma during days 8 to 14 accelerates wound healing. Finally, CP–NCP significantly accelerates healing. The combinatorial therapeutic regimen based on contact and noncontact styles of cold atmospheric plasma jet is recommended for chronic wound management, because it effectively removes bacterial biofilms and accelerates wound healing.

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KEY WORDS: animal model, chronic wound, plasma medicine, RONS, combination treatment

I. INTRODUCTION

It is well understood that chronic wounds have several levels of bacterial burden. Stotts¹ stated that bioburden is a microorganism presence on the surface of a wound. Generally, bacterial bioburden persistence on a wound can be divided into five microbial stages: contamination, colonization, critical colonization, biofilm, and infection. Contamination is the appearance of a nonduplicating microorganism on the wound surface without a host defend. Colonization is the appearance of duplicating microorganisms adherent to the wound surface without a host defend system. Critical colonization is the appearance of duplicating microorganisms on the wound and attached to the wound's cells and formations. Biofilm is a complex community of accumulated bacteria embedded in a self-secreted extracellular polysaccharide matrix. Infection is characterized by invading microorganisms into a wound's tissue and leads to local or systematic defense.

Topical management is positive wound care that restores the physiologic wound environment. A healthy environment's main features include adequate moisture level, temperature control, pH regulation, and bacterial burden control.² Recent methods that are known to control bacterial load include (1) debridement, (2) precise wound cleansing, (3) particular infection control precautions, (4) use of antimicrobials, and (5) use of moisture-retentive dressings.

Antimicrobial agent efficacy for eradicating bacterial burden in clinical care was known, but a lack still exists. For example, mupirocin 2% ointment is effective against *Streptococcus pyogenes* and methicillin-resistant *Staphylococcus aureus* (MRSA), but it cannot be used for a large burn. It also contains chemical materials, specifically, polyethylene glycol, which can damage kidneys if absorbed through the skin.¹ Therefore, an effort to find alternative antimicrobial agents is essential. For this point, atmospheric pressure plasma jet may provide a new avenue³ for bioburden eradication.

Plasma is well known as an ionized gas. In this context, plasma is not blood plasma but refers to physical plasma³ as a phase of the fourth state of matter after solid, liquid, and gas. Two parts exist in the plasma phase, namely, stable components (gases) and reactive components (ions and energetic and³ radical particles).³ Conceptually, the main aspect of plasma's medical effectiveness is related to the capability of plasmas to generate biological molecules, namely, reactive oxygen species (ROS) and reactive nitrogen species (RNS). Together, ROS and RNS is called reactive oxygen and nitrogen species (RONS). When accurately controlled and administered in appropriate doses RONS can be efficacious for medical treatment.⁴⁻⁶ RONS that are produced by plasma and have potential for effectiveness include hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), singlet oxygen (1O_2), ozone (O_3), hydroxyl radical ($\cdot OH$), organic radicals ($RO\cdot$, $RO_2\cdot$), nitric oxide ($\cdot NO$), nitrogen dioxide ($\cdot NO_2$), peroxyxynitrite ($ONOO\cdot$), and many others.⁷

Plasma jet is one of the most exciting sources for medical application, and a variety of human-made plasma sources can be developed under the environment of atmospheric pressure. The jet can be lengthened to approach a specific area that is not limited by

electrodes.⁸ Conceptually, plasma jet has two main conditions with particular characteristics. A plasma condition is comprised of radicals with relatively shorter lifetimes, such as N_2^* , O_2^* , OH, and N_2^+ . An afterglow condition contains radicals with relatively longer lifetimes, such as OH, O, O_3 , NO, and some metastable molecules such as O_2 and N_2 .⁹ Differentiation in plasma jet treatment style is contact and noncontact, occurring perhaps as a manifestation of the two conditions.

In our previous study, we reported that the noncontact treatment style of plasma jet (afterglow condition) in animal models was capable of stimulating skin-wound healing³ accelerating re-epithelialization, mimicking modern medical settings.^{10,11} However, the contact style of plasma jet treatment (the plasma condition) has a destructive effect on normal mouse skin.^{11,12} Abnormal epidermal tissue in skin wounds was found to be due to the contact style of plasma jet treatment.¹³ However, applying plasma jet treatment in the contact style may effectively eradicate chronic wound-related bacteria.^{14–16}

Plasma jet treatment is significantly effective in killing bacteria including *S. aureus*, *Pseudomonas aeruginosa*, MRSA, and methicillin-resistant *Staphylococcus epidermidis* (MRSE).^{17–19} Furthermore, Darmawati et al.¹⁷ reported that the contact style of plasma jet treatment was significantly effective in killing bacteria but also has a destructive effect on skin and wounds. On the other hand, noncontact style carries a reduced effect in killing bacteria but effectively stimulates wound healing. Finally, these researchers¹⁷ concluded that the noncontact style of plasma jet is more effective than the contact style. However, a lack of studies exist. In the human body, a chronic wound is clinically impossible to separate out the components of bacteria and wound. To combat this problem, it is important to evaluate plasma jet treatment effectiveness using a chronic wound model, namely, a bacteria-infected wound. Thus, the purpose of this study is to evaluate plasma jet effectiveness for bacteria-infected wounds.

This research was conducted as a development of our previous study.^{17,20} Study differences are shown in Fig. 1. The Darmawati et al.¹⁷ evaluation was carried out separately on two biological materials (bacteria in a media plate and acute wounds), whereas in the present study the evaluation was carried out on mice with chronic wounds using a bacteria-infected wound model. Darmawati et al.²⁰ used different styles based on treatment day. In this study, we introduced a novel treatment style of the plasma jet¹ namely, the combinative treatment of contact and noncontact styles. This means that from days 0 to 7, we applied the contact style of plasma jet treatment to the wound to kill bacteria, and from days 8 to 13 we used the noncontact style of plasma jet on wounds to stimulate healing.

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II. MATERIALS AND METHODS

A. Atmospheric Pressure Plasma Jet

The atmospheric pressure plasma jet system that is used in this experiment was developed on the basis of Teschke et al.²¹ and is explained previously.¹⁷ We made a modification in the dimension of the capillary quartz tube. Tube inner and outer diameters

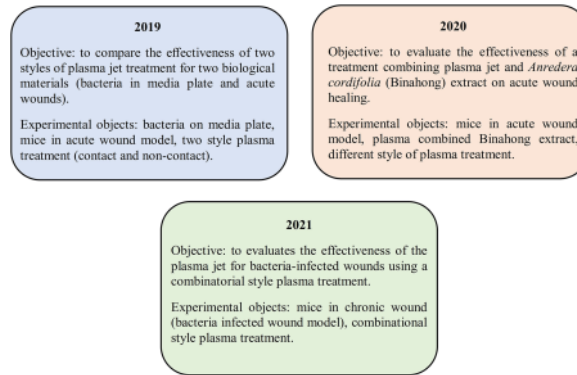


FIG. 1: Development from previous research that occurred in 2019¹⁷ and 2020²⁰

in this experiment were 1.55 and 0.65 mm. Around the quartz tube, we applied two ring-like electrodes. A nonconductor material (clay) was used to isolate the two electrodes.

We identified H_2O_2 and NO_2 using the Kyoritsu chemical method, which was reported previously.¹⁷ Plasma jet effectiveness against bacteria on plate media has already been reported, and that study referred to previous studies by Darmawati et al.¹⁷

B. Microorganisms

The bacteria strains of *S. aureus* (American Type Culture Collection [ATCC] 6538) were obtained from the Laboratory of Microbiology, Department of Medical Laboratory Science, Universitas Muhammadiyah Semarang, Indonesia. Bacteria suspension was done by inoculating a colony of *S. aureus* into brain heart infusion broth and incubating for 24 h at 37°C. The suspension was plated on a blood agar plate (Oxoid Ltd., Hampshire, UK) medium and incubated for 24 h at 37°C. The colony was suspended in saline solutions (0.85%), and turbidity was adjusted to the standard of McFarland 7 solution (21×10^8 colony-forming units [CFU]/mL).

C. Animals and Experimental Protocol

All experimental protocols followed animal welfare guidelines and were approved by the ethics committee for preclinical investigation in Laboratorium Penelitian dan Pengujian Terpadu/Integrated Research and Testing Laboratory (LPPT UGM), Gadjah Mada University, Yogyakarta, Indonesia (certificate approval number 00003/04/LPPT/III/2020). LPPT UGM is accredited by ISO/IEC 17025 and the National Accreditation Committee of Indonesia (Komite Akreditasi Nasional/KAN, Indonesia). We used a total of 72 BALB/c male mice that were aged 8 wk and weighed 35.0–40.0 g. We purchased

the mice from LPPT UGM Indonesia. Mice weights during the observation period are shown in Fig. 2. Mice were individually caged under controlled conditions in an air-conditioned room at $28.0^{\circ}\text{C} \pm 2.0^{\circ}\text{C}$, with light–dark cycle of light from 08:00 am to 8:00 pm, and under ad libitum feeding conditions. All experiments were carried out under anesthesia, using ketamine–xylazine of 50 mg/kg + 5 mg/kg, respectively. Every effort was made to minimize suffering.²²

D. Bacteria-Infected Wound Model

Bacteria-infected wounds developed by infecting *S. aureus* ATCC 6538 in acute wounds of mice that Davis et al.²³ previously explained. Male BALB/c mice were acclimatized for 1 wk, and then a full-thickness acute wound was made on the dorsal part of mice with a punch biopsy that was 4 mm in diameter (Kai Industries Co. Ltd., Gifu, Japan). A 50- μL bacterial suspension, equivalent to the turbidity of a McFarland 7 standard solution (21×10^8 CFU/mL), was inoculated into the acute wounds and rubbed into each wound for 10 s using a sterile loop. The wounds were covered with a dressing (Tegaderm Hydrocolloid Dressing; 3M Health Care, St. Paul, MN) for 72 h to allow wounds to become colonized. Dressings were secured in place with a bandage. This method creates a suitable environment for biofilm formation.

E. Plasma Jet Treatment on Wounds

Evaluations of plasma jet treatment on bacteria-infected wounds were conducted in the Laboratory Plasma Medicine for Experimental Wound Healing, Universitas

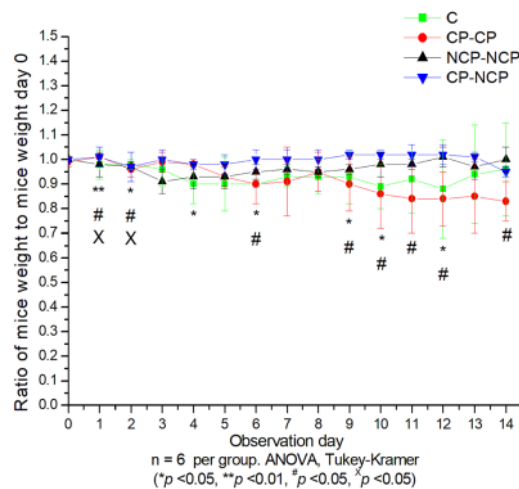


FIG. 2: Weight of mice during observation on days 0–13. ANOVA, analysis of variance.

Muhammadiyah Semarang, Indonesia. Mice were anaesthetized via ketamine–xylazine injection using intraperitoneal administration.²² Three days after wounds were infected with bacteria, plasma jet treatment began (day 0). The experimental procedure after day 0 is explained in Fig. 3. Plasma jet treatment was carried out once daily for 3 min during 14 d. Mice were randomly divided into four groups, with three mice or six wound samples for every group as follows:

- A. Control or untreated group (C): Bacteria-infected wounds were allowed to heal daily under Tegaderm Hydrocolloid Dressing (3M Health Care).
- B. Plasma treatment group with contact style treatment (CP–CP): Bacteria-infected wounds were given plasma jet treatment in contact style for 3 min. In this context,

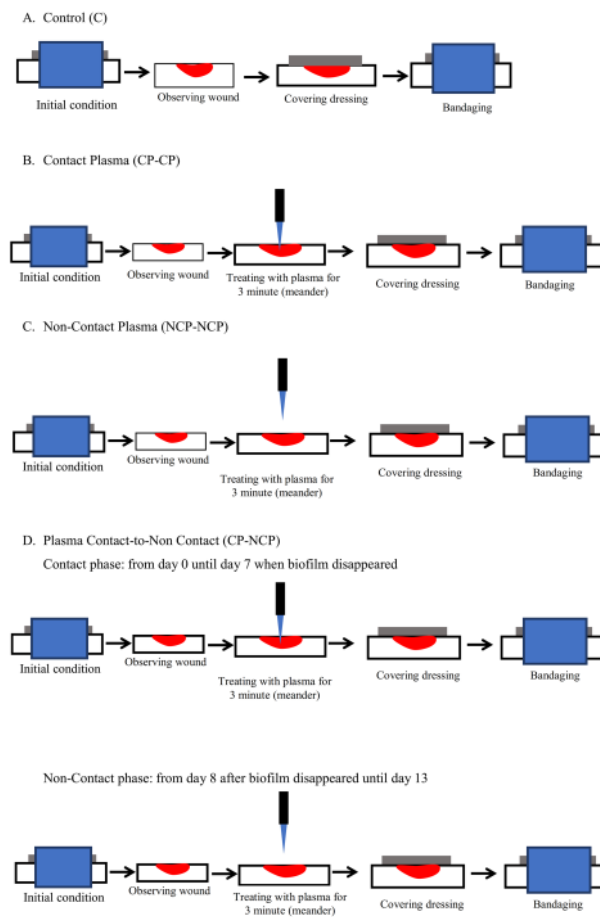


FIG. 3: (A–D) Experiment protocol during days 0–13

the distance from the nozzle of the plasma jet reactor tube to wound surface was 5 mm. In this condition, the plasma jet was visually contacted to the wound surface.

C. Plasma treatment group with noncontact style treatment (NCP–NCP): Bacteria-infected wounds were given plasma jet treatment in noncontact style for 3 min. In this context, the distance from the nozzle of the plasma jet reactor tube to wound surface was fixed at 20 mm. In this condition, plasma jet was visually not contacted to the wound surface.

D. Plasma treatment group with contact and noncontact style treatment (CP–NCP): Bacteria-infected wounds were given plasma jet treatment in contact style for 3 min from days 0 to 7. The following day, from days 8 to 13, noncontact style of plasma jet treatment were applied for 3 min. In this context, the distance from the nozzle of the plasma jet reactor tube to wound surface for contact style was 5 mm and for noncontact style distance, 20 mm.

During the 14-d experimental period, the bandages and wound dressings in all groups were removed daily and renewed for plasma treatment or wound evaluation.

F. Thermal Distribution Evaluation

We measured temperature distribution of the treated wound skin and its surroundings using an infrared (IR) digital camera (FLIR C series; Teledyne FLIR LLC, Thousand Oaks, CA). Approximately five images were taken for each sample. We used FLIR Tools computer software to analyze image data. A schematic of a plasma medicine system with an infrared camera's position is shown in Fig. 4.

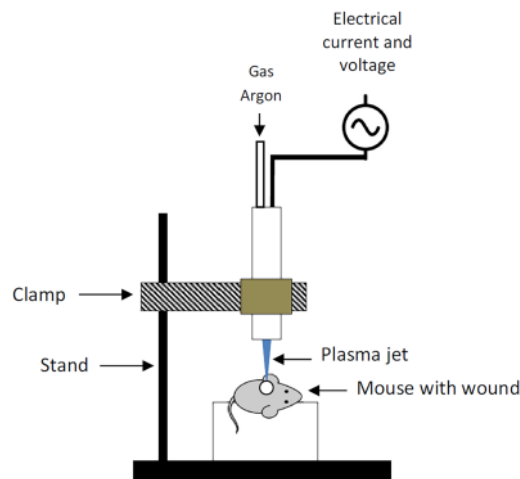


FIG. 4: Experimental setup

G. Macroscopic Evaluation of Wound

First, macroscopic evaluation of the wound was manually conducted, followed by computational processes based on the procedure, as elucidated previously.¹⁷ This evaluation was conducted daily for 14 d. Day 0 is when plasma jet treatment was started. We used a digital camera to document observed wound conditions.

H. New Epithelial Evaluation

On experiment days 7, 11, and 14, the mice were euthanized via excessive ketamine–xylazine injection. The wound and surrounding skin were collected and then bisected at the center of the wound. Tissue processing and hematoxylin and eosin (HE) staining were performed based on the previously elucidated procedure.¹⁷ On the basis of HE staining results, the percentage of re-epithelialization was calculated using the formula $100\% \times (\text{length of new epithelium} / \text{length of wound between wound edges})$.

I. Total Bacteria Evaluation

We evaluated microorganisms by examining total bacteria periodically on days 0, 3, 7, 11, and 14 during the experiments period; this method is referenced in Liang et al.²⁴ Wound exudate was taken using sterile swabs and placed into 5 mL of saline solution. A serial dilution was performed until 10^{-3} , and then 100 μL of suspension were plated on Plate Count agar medium (Oxoid) and incubated overnight at 37°C. After incubation, we counted the number of colonies.

J. Total Neutrophil Evaluation

The hematology evaluation to count the number of neutrophils on days 3, 7, 11, and 14 was conducted using a Hematology Analyzer (Mindray BC-2600, Shenzhen, China). First, mouse blood samples were collected by cardiac puncture. Blood was placed in a microtube with 10% ethylenediaminetetraacetic acid anticoagulant. The neutrophil number was checked automatically with the hematology analyzer.

K. Tumor Necrosis Factor- α level

We conducted tumor necrosis factor (TNF)- α evaluations of the wound skin using an enzyme-linked immunosorbent assay (ELISA) (TNF- α rat ELISA kit; Sigma-Aldrich, St. Louis, MO) method. As mentioned above, on days 7 and 14 of the experimental period, mice were euthanized via injection of excessive ketamine–xylazine. The wound and surrounding skin was collected and weighed to be ± 10 mg. The wounds were then frozen with liquid nitrogen. We pounded the sample on a mortar until it was smooth, added 2 mL of phosphate-buffered saline, and centrifuged at 16,000g for 15 min at 4°C. The separated supernatant was used for the ELISA test. Absorbance

was immediately read at 450 nm, and sample concentration was calculated based on a standard curve.

L. Malondialdehyde Levels

We conducted a wound skin malondialdehyde (MDA) evaluation using a thiobarbituric acid reactive substance method. As explained above, on days 7 and 14 of the experimental period mice were euthanized via injection of excessive ketamine–xylazine and we collected cells of the wound and surrounding skin and blood. Wounds were weighed to be ± 10 mg, and blood was collected using cardiac puncture. Determination of the MDA level was established using the Lipid Peroxidation (MDA) (Sigma-Aldrich), according to Kapusta et al.'s procedure.²⁵

M. Statistical Analysis

Data were subjected to statistical analyses using the Statistical Package for the Social Sciences (IBM, Armonk, NY), ver. 16.0. We evaluated the ratio of wound area to original wound area, percentage of re-epithelialization, amount of total bacteria and total neutrophils, and TNF- α and MDA levels by analysis of variance followed by the Tukey–Kramer method; p values of < 0.05 were considered to be significant.

III. RESULTS

A. Macroscopic Evaluation of Wounds

For every group, we inspected bacteria-infected wounds on mice skin daily from days -3 to 14, as shown in Fig. 5. On day -3 , the wound was created and inoculated with bacteria. Bacterial biofilms as a yellowish layer on the wound's surface began to appear on day 0 for all groups. The wound areas of C and NCP–NCP groups increased from day 0 to 14 and a bacterial biofilm grew on the wound surface.

The wound areas of CP–CP and CP–NCP from days 0 to 3 increased, as did the biofilm layer. During days 7 to 14, the biofilm layer of CP–CP slowly disappeared, but the wound area did not decrease. In contrast, in those of CP–NCP during days 7 to 14 the biofilm layer slowly disappeared, followed by decreased wound area. For the CP–NCP condition at the end of the observational period, the wound surface area looked healthy, almost like normal skin. This result suggests that contact–noncontact plasma treatment is most effective for chronic wound treatment. In contrast to the CP–CP condition for which the wound surface area looked unhealthy, the surface (yellowish red) indicated that contact–contact plasma treatment impairs wound healing.

From days 5 to 14, the area of NCP–NCP was significantly larger than that of CP–CP and CP–NCP. At day 14, CPN–NCP was notably smaller than other groups. It is worth mentioning that (1) α the significance level of NCP–NCP vs. CP–CP, (2) the β significance level of NCP–NCP vs. CP–NCP, (3) the χ significance level of C vs. CP–CP,

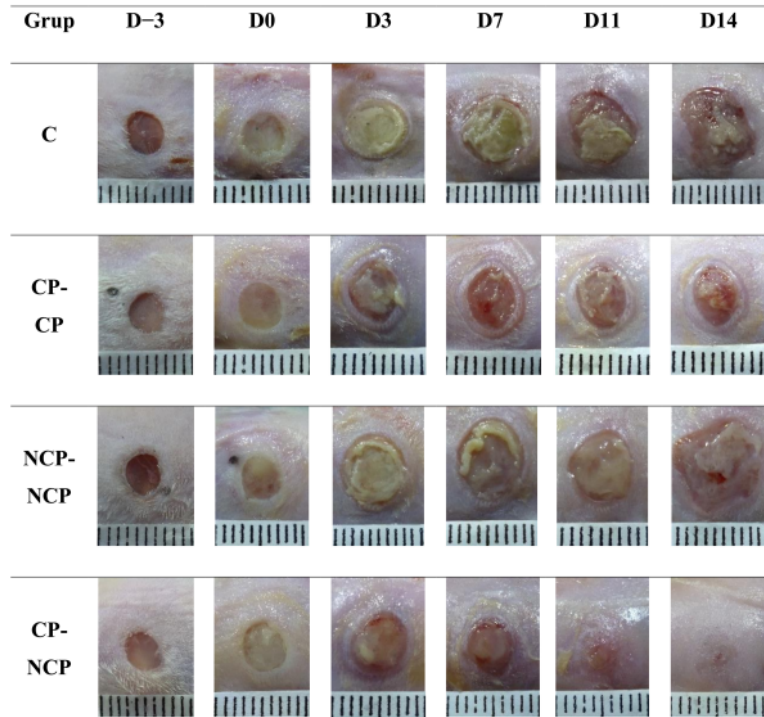


FIG. 5: Wound appearance on days -3, 0, 3, 7, 11, and 14

(4) the δ significance level C vs. CP-NCP, and (5) the ϵ level of significance of CP-CP vs. CP-NCP.

As mentioned above, macroscopic observation was performed by documenting with a digital camera and tracing the wound area to determine the ratio between wound area to initial wound area during the treatment period. Tracing data are shown in Fig. 6. From days 5 to 14, the area for NCP-NCP was significantly larger than that for CP-CP and CP-NCP (NCP-NCP vs. CP-CP: $p < 0.01$; NCP-NCP vs. CP-NCP: $p < 0.01$). CP-NCP at day 14 was significantly smaller than other groups (CP-NCP vs. C: $p < 0.01$; CP-NCP vs. CP-CP: $p < 0.01$; CP-NCP vs. NCP-NCP: $p < 0.01$). From macroscopic evaluation, it can be concluded that CP-NCP is the most effective treatment for chronic wounds.

B. New Epithelial Evaluation

As shown in Fig. 7, the percentage of re-epithelialization with CP-NCP at days 7, 11, and 14 was significantly higher than C, CP-CP, and NCP-NCP (CP-NCP vs. C: $p < 0.01$; CP-NCP vs. CP-CP: $p < 0.01$; CP-NCP vs. NCP-NCP: $p < 0.01$). The percentage

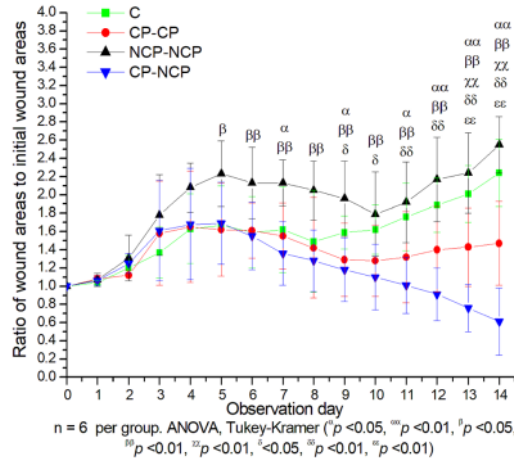


FIG. 6: Ratio of wound area to initial wound area during the 14-d treatment period. ANOVA, analysis of variance.

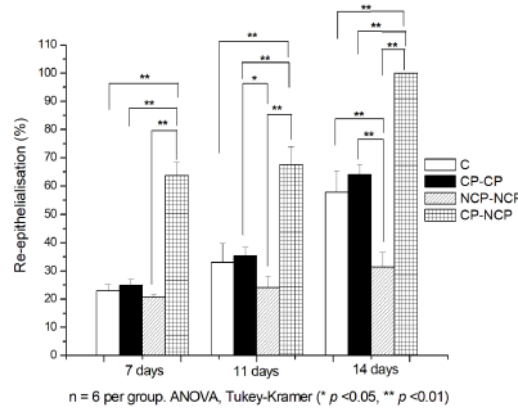


FIG. 7: Percentage of wound tissue re-epithelialization. Percentages of CP–NCP at days 7, 11, and 14 were significantly higher than those of the other groups. Percentages of NCP–NCP at day 14 were significantly lower than those of the other groups. ANOVA, analysis of variance.

of re-epithelialization at day 7 for C, CP–CP, and NCP–NCP did not differ significantly (C vs. CP–CP: $p > 0.05$; C vs. CP–NCP: $p > 0.05$; CP–CP vs. CP–NCP: $p > 0.05$), but NCP–NCP on day 14 was significantly less than for C and CP–CP (NCP–NCP vs. C: $p < 0.01$; NCP–NCP vs. CP–CP: $p < 0.01$). Observation at day 14 revealed that the new epithelium had completely covered the wound in CP–NCP. The lowest re-epithelialization

percentage was found in NCP–NCP, whereas the percentage of re-epithelialization in groups C and CP–CP did not differ significantly (C vs. CP–CP: $p > 0.05$).

C. Total Bacteria Evaluation

Total bacteria on days 0, 3, 7, 11, and 14 is shown in Fig. 8. Starting from day 5, a notable difference in total bacteria among groups could be seen. Total bacteria of NCP–NCP and C from days 0 to 14 continued to increase, but CP–CP and CP–NCP continued to decrease. NCP–NCP at days 7 to 14 were significantly higher than for groups C, CP–CP, and CP–NCP (NCP–NCP vs. C: $p < 0.01$; NCP–NCP vs. CP–CP: $p < 0.01$; NCP–NCP vs. CP–NCP: $p < 0.01$). Observations for C during days 7 to 14 were significantly higher than CP–CP and CP–NCP (C vs. CP–CP: $p < 0.01$; C vs. CP–NCP: $p < 0.01$). Starting from day 7, CP–NCP's total bacteria was significantly lower than for the CP–CP and NCP–NCP groups (NCP–NCP vs. CP–CP: $p < 0.01$; NCP–NCP vs. CP–NCP: $p < 0.01$).

1. Total Neutrophil Evaluation

Blood neutrophil was evaluated for systemic inflammatory response, with results shown in Fig. 9. NCP–NCP's number of neutrophils on day 7 is significantly higher than that for C, CP–CP, and CP–NCP (NCP–NCP vs. CP–CP: $p < 0.01$; NCP–NCP vs. C: $p < 0.01$; NCP–NCP vs. CP–NCP: $p < 0.01$), whereas CP–CP total neutrophils were significantly higher than for C and CP–NCP (CP–CP vs. C: $p < 0.01$; CP–CP vs. CP–NCP: $p < 0.01$). Groups C and CP–NCP did not differ significantly (C vs. CP–NCP: $p > 0.05$).

Observation on days 11 and 14 showed a decreased number of neutrophils in NCP–NCP compared to the count on day 7. Total neutrophils in NCP–NCP, CP–CP, and C

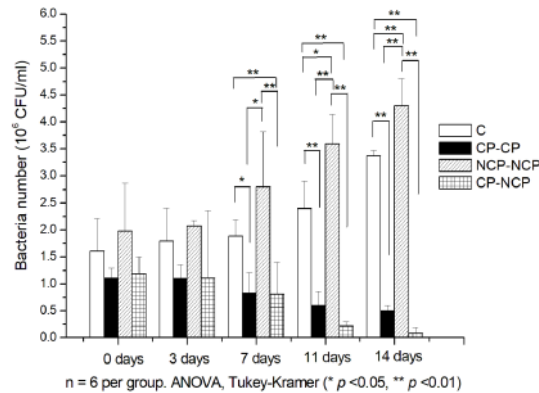


FIG. 8: Total bacteria histogram for days 3, 7, 11, and 14. Starting at day 7, a significant difference in total bacteria between groups occurred. NCP–NCPs during days 7 to 14 were significantly higher than for other groups. From day 7, CP–NCP continued to decline significantly.

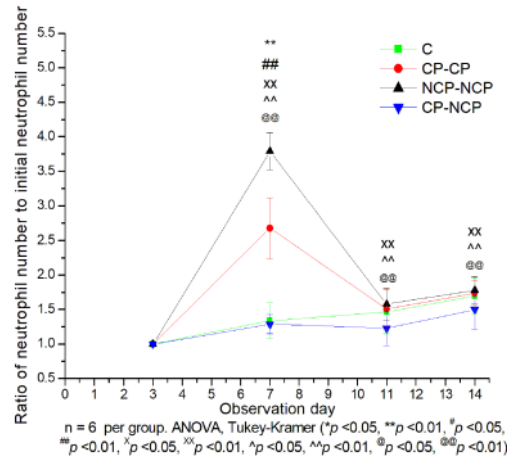


FIG. 9: Tot neutrophil histograms for days 3, 7, 11, and 14. Total neutrophils in NCP–NCP on day 7 were significantly higher than in the other groups. CP–NCP on days 11 to 14 was significantly lower than in the other groups.

did not differ significantly (NCP–NCP vs. CP–CP: $p > 0.05$; NCP–NCP vs. C: $p > 0.05$; NCP–NCP vs. CP–NCP: $p > 0.01$). The number of neutrophils in CP–NCP was significantly less than NCP–NCP, CP–CP, and C (NCP–NCP vs. CP–CP: $p < 0.01$; NCP–NCP vs. C: $p < 0.01$; NCP–NCP vs. CP–NCP: $p < 0.01$).

2. TNF- α Levels

TNF- α levels for each group are shown in Fig. 10. TNF- α levels of NCP–NCP at days 7 and 14 were significantly higher than those of other groups (NCP–NCP vs. CP–CP: $p < 0.01$; NCP–NCP vs. C: $p < 0.01$; NCP–NCP vs. CP–NCP: $p < 0.01$). C was significantly higher than CP–CP and CP–NCP (C vs. CP–CP: $p < 0.01$; C vs. CP–NCP: $p < 0.05$). CP–CP and CP–NCP did not differ significantly (C vs. CP–NCP: $p > 0.05$).

Observation on day 14 showed decreased TNF- α levels in C, NCP–NCP, and CP–NCP groups; except for CP–CP, no significant difference was found. High levels of TNF- α in the NCP–NCP group indicated that bacterial infection was a factor in increased TNF- α levels.

3. MDA Levels

MDA levels for each group are shown in Fig. 11. On day 7, highest MDA levels occurred in the CP–CP and CP–NCP groups. Group C was significantly higher than NCP–NCP (C vs. CP–NCP: $p < 0.01$). Day 14 showed decreased MDA levels for groups C, CP–CP,

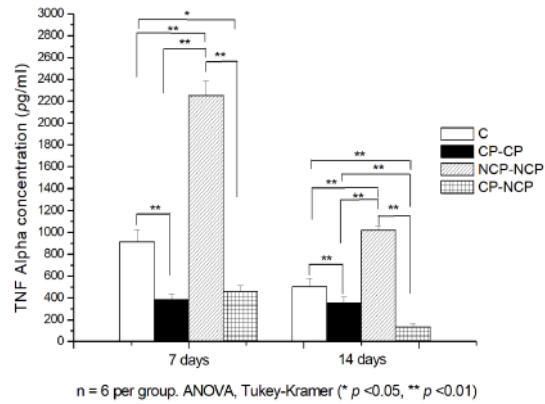


FIG. 10: TNF- α level histogram for days 7 and 14. From day 7, a significant difference is found in TNF- α levels among groups. NCP-NCP on days 7 and 14 were significantly higher than other groups. NCP-NCP, C, and CP-NCP decreased significantly by day 14, whereas CP-CP showed no significant difference between days 7 and 14. ANOVA, analysis of variance; TNF, tumor necrosis factor.

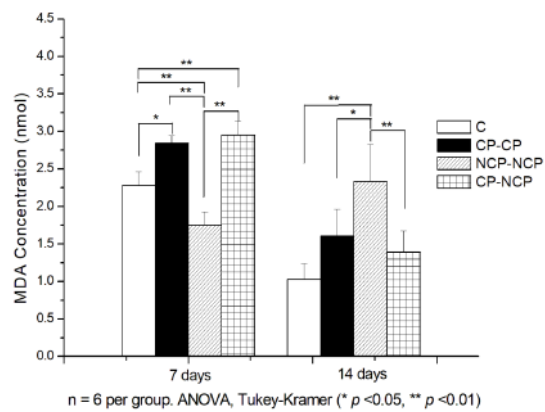


FIG. 11: MDA level histogram for days 7 and 14. On day 7, MDA levels were highest in CP-CP and CP-NCP. A decrease occurred from day 7 to 14 in groups C, CP-CP, and CP-NCP, whereas an increase was present in NCP-NCP. MDA, malondialdehyde.

and CP-NCP. At day 14, an increase occurred in the NCP-NCP group, and MDA levels in NCP-NCP were significantly higher than other groups (NCP-NCP vs. CP-CP: $p < 0.05$; NCP-NCP vs. C: $p < 0.01$; NCP-NCP vs. CP-NCP: $p < 0.01$).

4. Thermal Distribution Evaluation

Thermal images were analyzed using FLIR Software Tools; we used the color palette labeled medical to express the image. Temperature range was set between 20°C and 40°C. T_{\max} is the point of highest skin temperature under plasma treatment.

Visually, distribution of wound temperatures among groups differs under plasma treatment, shown in Fig. 12. T_{\max} in CP-CP and CP-NCP on day 3 was higher than NCP-NCP. T_{\max} of plasma contact treatment was $\pm 33.4^{\circ}\text{C}$ – 34.7°C . Furthermore, T_{\max} of CP-CP on days 7, 8, and 13 was uniform, at $\sim 50.1^{\circ}\text{C}$ – 51.3°C . T_{\max} of CP-NCP on day 7 tended to be the same as that of CP-CP, $\sim 50^{\circ}\text{C}$; at days 8 and 13, T_{\max} decreased to $\sim 30.1^{\circ}\text{C}$ – 34°C . The NCP-NCP group generally had the lowest T_{\max} of all groups on all observation days, $\sim 28.2^{\circ}\text{C}$ – 32.9°C .

IV. DISCUSSION

It is well known that it is simple to switch plasma jet treatment style from contact to non-contact (and vice versa) in the clinical setting. Darmawati et al.¹⁷ reported that contact style for plasma jet treatment could impede acute wound healing, whereas noncontact style can stimulate acute wound healing. Darmawati et al.¹⁷ stated this conclusion on the

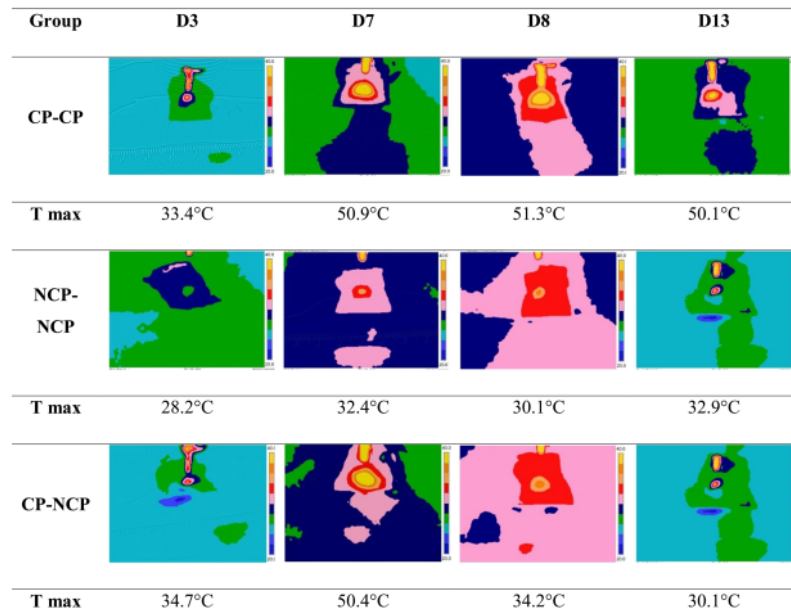


FIG. 12: Distribution of temperature and T_{\max} in skin area under plasma treatment

basis of two data sources, namely, the effect of plasma jet on bacteria that is inoculated on the surface of an agar medium and on acute wound in a small animal model. However, this investigation introduced a new style of plasma jet treatment: a combination of contact and noncontact treatment style (CP–NCP). We used a model of chronic wound or bacteria-infected wound for treatment. Results showed that the combination of contact and noncontact treatment style significantly accelerated healing compared to the control group and other styles of plasma jet treatment. In CP–PNC from days 0 to 7, the contact style was effective in killing bacteria on the wound, and from days 8 to 14 the noncontact style was effective in stimulating wound repair by promoting re-epithelialization. In CP–CP, contact style was also effective in killing bacteria on the wound from days 0 to 14; however, from days 8 to 14 it may damage wound tissue and impede re-epithelialization.

We reported on efficacy of noncontact style plasma jet treatment (NCP–NCP) for acute wounds based on 14 observation days.¹⁷ However, its efficacy for treating chronic wounds in the present investigation was unproven. NCP–NCP tended to fertilize the bacteria burden and impede wound healing. This indicates that noncontact style is not effective in removing bacterial biofilms. Lu reported that noncontact plasma exposure produces radicals with relatively long lifetimes, such as OH, O, O₃, NO, and several diffuse molecules, including O₂ and N₂.⁹ Nitrogen (N₂) in this context may have a role in providing nutrients needed for energy in the growth and formation of bacterial cells.²⁶

Inflammation is a crucial phase in chronic wound healing. A chronic wound is characterized by a longer inflammation phase. We evaluated two biomarkers, neutrophil and TNF- α . It is well known that high levels of neutrophil and TNF- α elevate inflammation. The present study revealed that at the end of observation day 14, the two in the CP–NCP group were significantly lower than in other groups. Contact and noncontact style plasma jet suppressed the inflammation phase of chronic wound healing.

The thermal effect is also an essential parameter of plasma jet. It is commonly accepted that plasma jet should be fixed at a temperature below 40°C for medical therapy. Contact style of plasma jet elevated local skin temperature by > 40°C or caused ΔT by more than 4°C,^{11,17} possibly damaging skin.¹⁷ However, the present investigation indicated that when the bacterial biofilm still visually appears on the wound surface, it is possible to effectively use contact style to kill such bacteria. In addition, contact style must be switched to noncontact style when such bacterial biofilms disappear. Noncontact style produces radicals such as NO with a relatively long lifetime.⁹ Thana et al. stated that the NO that was produced by plasma had a stimulating effect on wound healing and tissue regeneration.²⁷ NO is an important cellular signaling molecule in humans that can affect the immune system, increase growth factors, and stimulate cell proliferation, angiogenesis, and collagen synthesis, resulting in reconstructing damaged skin.²⁷

This study used RONS that was produced by plasma jets to remove biofilms and stimulate wound healing. The dose of RONS produced by the plasma jet can be controlled by regulating plasma treatment time and distance. For comparison, a study by Alkawareek et al.²⁸ used time variations to evaluate use of atmospheric pressure plasma for eradicating bacterial biofilms. The present study used distance

combinations to evaluate use of plasma to remove biofilms and stimulate wound healing. RONS, especially H_2O_2 and NO_2^- , has an important role in the process of removing biofilms and stimulating wound healing. H_2O_2 and NO_2^- that are produced by contact style can kill cells (including bacteria) and normal tissue. H_2O_2 works by producing destructive hydroxyl free radicals that can attack membrane lipids and DNA.²⁹ On the other hand, H_2O_2 and NO_2^- that are produced by noncontact style can stimulate wound healing. The combinatorial therapeutic regimen based on contact and noncontact styles of cold atmospheric plasma jet is recommended for chronic wound management, because it effectively removes bacterial biofilms and can accelerate wound healing.

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