Manuscript ID: PMED-47583

Response to Production Team

Dear, Production Team

Following some information, we confirmed several things:

1. We confirm that all figures, photos, and tables are our original work, and that no permissions are required
2. We have to confirm that this research "TNF-α Expression and New Epithelial Thickness in Mice Skin *(Mus musculus)* Infected MRSA by Medical Plasma Treatment" did not receive funding from the NIH.

Sincerely

Author's team

Manuscript ID: PMED-47583
Response to Reviewers

Dear, Editor Satoshi Hamaguchi

Thank you for giving us the opportunity to submit a revised draft of the manuscript "*TNF-α Expression and New Epithelial Thickness in Mice Skin (Mus musculus) Infected MRSA by Medical Plasma Treatment*". We appreciate the time and effort that you and the reviewers dedicated to providing feedback on our manuscript and are grateful for the insightful comments on and valuable improvements to our paper.

We have incorporated most of the suggestions made by the reviewer. Those changes are marked in red within the manuscript. Please see below, in blue, for a point-by-point response to the reviewers' comments and concerns. All page numbers refer to the revised manuscript file with tracked changes.

Reviewer ' Comments to the Authors

- Comment 1: *I think the authors should describe the details of the plasma treatment methods in "2.5. Plasma Jet Treatment on Wound". For example, how long (time and distance) did they treat the wound area with plasma?*

  Response: Thank you for pointing this out. We have added complete information about how treatment of plasma jet on wound in **2.5. Plasma Jet Treatment on Wound** [Line 15 in page 4]. Moreover, we have added images to provide a clearer illustration in Figure 1 [page 6].

- Comment 2: *I think the authors should describe the details in the Figure legends.*

  Response: Yes, we have added the figure legends for Figure 2 [page 7], Figure 3 [page 8], Figure 4 [page 9], and Figure 5 [page 10].

- Comment 3: *The authors sometimes count the day from Day -3 (Figure 1), while they count the day from Day 0 in Figure 2. This is very confusing, and I think they should count the day from Day 0. I also think they should use not "D0, D1, ,,," but "Day 0, Day 1, ,,"*

  Response: We agree with the reviewer's suggestion. We have count the day from Day 0 until Day 17, and we have replaced the word "D0, D1" to "Day 1 until Day 17" on a whole manuscript.

- Comment 4: *I think the authors should discuss why contact and non-contact exposure is better than contact or non-contact exposure.*

  Response: Thank you for the suggestions. We have added the topic such as reviewer's suggestion in discussions section on [Line 11-19 in page 12].

We look forward to hearing from you in due time regarding our submission and to respond to any further questions and comments you may have.

Sincerely

Author’s team

TNF-α Expression and New Epithelial Thickness in Mice Skin (*Mus musculus*) Infected MRSA by Medical Plasma Treatment

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Abstract

Chronic wounds develop as a result of infection, commonly carried on by bacteria and form biofilms. MRSA is a kind of bacteria that can form biofilms. Recently, medical plasma technology has been applied to accelerate wound healing. The objective of the research was to investigate the response of cutaneous wounds in MRSA-infected animals to contact and non-contact therapy using medical plasma Argon jet-type with histopathological and molecular approaches. Argon gas, with a purity of 99.995%, is utilized as a carrier gas for generating plasma medical at a flow rate of 1 standard liter per minute (slm). This experiment was divided into 4 treatment groups, K (infected wound without plasma treatment), CP (5mm plasma treatment), NCP (20mm plasma treatment), and CP-NCP (infected wound with 5mm and 20mm plasma combination treatment). The result of the observation obtained that contact plasma from Day 3 to Day 10 can remove bacterial biofilm and that non-contact plasma treatment from D11 to D16 is effective to accelerate wound healing. At Day 17, the macroscopic biofilm area in the CP-NCP group began to decrease with an increasing percentage of re-epithelialization, and no necrotic cells were observed. TNF-α levels were observed significantly lower in the CP-NCP group at Day 17 compared to other groups. In conclusion, contact-non-contact (CP-NCP) treatment is suggested for the management of chronic infections since it is beneficial for removing the bacterial biofilm layer and can promote wound healing.

Key words: Animal model, chronic wound, plasma medicine, MRSA, combination treatment.

1. Introduction

The major cause of chronic wounds is bacterial infection, which results in the production of biofilms (1). *Staphylococcus aureus (S. aureus)* is the most common pathogen bacterial detected in patients with wound infection. *Methicillin-resistant Staphylococcus aureus* (MRSA) is one of the clinically identified strains of *S. aureus* that has been reported to be resistant to methicillin. MRSA has been found in the skin tissue of individuals with chronic wounds. MRSA can form biofilms, which are colonies of adhered bacteria to a surface and coated in a matrix of extracellular polymeric substances (EPS) produced by bacteria (2). A wound is considered to be healed if epithelial tissue has developed across a whole wound surface. The formation of biofilm in the wound tissue inhibits the process of skin tissue re-epithelialization (3). The delayed wound healing makes skin tissue difficult to repair (4).

The fourth type of substance after solids, liquids, and gases is plasma. Instead of blood plasma, the plasma being discussed here is the type of plasma known as an ionized gas (5). Conceptually, the function of plasma in medicine is related to its capability to produce biological molecules including ROS and RNS (commonly abbreviated as RONS) (6) (7) (8). RONS includes superoxide (O_2), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), singlet oxygen (O_2), ozone (O_3), organic radicals (RO, RO_2), nitric oxide (NO), nitrogen dioxide (NO_2) dan peroxynitrite ($ONOO^-$). If use correctly at the appropriate dose, RONS can be beneficial for both physical and pathophysiological health therapy (9).

It was determined that medical plasma has a significant role in the inactivation of bacteria by breaking the cell membrane's outer layer in the treatment of bacterial biofilms. The cell membrane ruptures due to the electrostatic forces carried on by the accumulation of charged particles on the membrane. Plasma exposure, whether direct (contact) or indirect (non-contact),

results in varying RONS content. Direct exposure results in plasma containing radicals with a short half-life and high reactivity, such as N_2^-, O_2, OH dan N_2^+. In contrast, indirect exposure refers to sample exposure where sample placement is outside the plasma release area, generating radicals with relatively long lifetimes, such as OH, O, O_3, NO, and some molecules that can disperse, including O_2 and N_2 (10).

Darmawati et al (11) reported that medical plasma has the ability to eliminate wound bacteria. The combination of contact and non-contact plasma treatments was effective in removing *Staphylococcus aureus* bacterial biofilm and accelerating wound healing. The size of the wound can be used to evaluate wound healing. This parameter is critical for monitoring chronic wound healing and determining therapy effectiveness. However, in research, the efficiency of plasma therapy is evaluated using invasive histological techniques, particularly skin biopsies around wounds as specimens to analyze the physiology of wound healing, which is supported by routine staining techniques (Hematoxylin-Eosin).

This study is a continuation of our previous research (11), and the objective is to develop a diagnostic for medical plasma usage in the treatment of chronic wounds on a wound model infected with *Methicillin-Resistant Staphylococcus aureus* (MRSA) bacteria.

2. Materials and Methods

2.1. *Atmospheric Pressure* Plasma Jet

The prototype of atmospheric pressurized jet-type medical plasma technology as created by Teschke (12) with a purity of 99.995% and a flow rate of 1 slm per minute flowing through the end of the quartz tube will be used as the input gas for the plasma generator. This study refers to previous research conducted by Darmawati et al (11).

2.2. MRSA Bacteria Preparation

The bacteria strains were obtained from the Laboratory of Microbiology Department of Medical Laboratory Science, Universitas Muhammadiyah Semarang, Indonesia. Inoculated colony of MRSA was used to prepare bacterial suspension in a test tube containing liquid Brain Heart Infusion (BHI) medium, incubated at 37°C for 24 hours. The suspension was initially cultured on a Blood Agar Plate (Oxoid, UK) for 24 hours at 37°C. Next, the suspension was cultured on Mannitol Salt Agar (MSA) (Oxoid, UK) for 24 hours at 37°C. The collected colonies were homogenized after being suspended in a test tube containing 3 ml of 0.85% NaCl. The turbidity of the suspension was compared to that of Mc Farland 0.5 standard solution (1.5×10^8 CFU/mL) (11).

2.3. Procedure Animals and Experimental Protocol

All research procedures followed animal welfare criteria and were approved by the ethical committee of the Health Research Ethics Commission (KEPK), Faculty of Public Health, University of Muhammadiyah Semarang (Certificate number: 633/KEPK-FKM/UNIMUS/2022). 32 male BALB/c mice weighing 35.0-40.0 g were acquired from the Laboratory of the Faculty of Medicine, Sebelas Maret University, Surakarta, Indonesia. Mice were individually placed in an air-conditioned room at a temperature of 28.0±2.0℃ with a light-dark cycle, light from 08:00 to 20:00, and ad libitum feeding circumstances. All experiments were conducted using ketamine-xylazine (K) 50 mg/kg + (X) 5 mg/kg anesthesia, and every effort was made to reduce pain (11).

2.4. Bacteria Infected Wounds Model

Bacteria infected wounds were developed by infecting MRSA bacteria in acute wounds of mice, as referred to in the study (13). Male Balb/c mice were acclimatized for one week, then a full-thickness acute wound with a diameter of 4mm was made on the dorsal skin of the mice using

a 4mm punch biopsy (Kai Industries Co. Ltd., Gifu, Japan). 50 µL Bacterial suspension equivalent to Mc Farland 7 standard solution (21×10^8 CFU/mL) was inoculated into acute wounds of mice by spreading the suspension for 10 seconds to each wound using a sterile spatula. The wound was covered with a hydrocolloid dressing, then covered with a plaster for three days. This method created an ideal environment for the production of biofilms (11).

2.5. Plasma Jet Treatment on Wound

Mice were anesthetized with a ketamine and xylazine anesthetic solution at a ratio of (2:1) and 0.01 ml/ww mice before being wounded in the dorsal skin using a 4mm disposable punch biopsy (Day 0) (11). Plasma jet treatment began three days after the wounds were infected with bacteria (Day 3), followed by wound care consisting of changing hydrocolloid dressings (Tegaderm Hydrocolloid Dressing; 3M Health Care, St. Paul, MN) and bandages daily (Leukoplast; BNH Medical, Germany) for 14 days.

Figure 1 shows the experimental procedure following Day 3. During 14 days, plasma jet treatment was performed once a day for 3 minutes. Mice were randomized into four groups, with three mice or six wound samples in each group, as follows:

A. Control Group, bacteria-infected wounds not treated with plasma (K): Bacteria-infected wounds were allowed to heal on a daily basis by using (Tegaderm Hydrocolloid Dressing; 3M Health Care, St. Paul, MN)
B. Contact plasma Group, bacteria-infected wounds treated with plasma contact style (CP): Bacteria-infected wounds were treated for 3 minutes with plasma jet treatment in contact style. In this setting, the distance between the nozzle of the plasma jet reactor tube and the wound surface was 5 mm. In this position, the plasma jet made visual contact with the wound surface.

C. Non-contact plasma Group, bacteria-infected wounds treated with plasma non-contact style (NCP): Bacteria-infected wounds were treated for 3 minutes with plasma jet treatment in non-contact style. In this setting, the distance between the nozzle of the plasma jet reactor tube and the wound surface was 20 mm. In this position, the plasma jet not made visual contact with the wound surface.

D. Contact and Non-contact Group, bacteria-infected wounds treated with plasma contact and non-contact style (CP-NCP): Bacteria-infected wounds were treated for 3 minutes with plasma jet treatment in contact style from Day 3 to Day 10. The following day, from Day 11 to Day 16, non-contact plasma jet treatment was used for 3 minutes. In this setting, the distance between the nozzle of the plasma jet reactor tube and the wound surface was 5 mm for contact style and 20 mm for non-contact style.

2.6. Macroscopic Evaluation of Wound

First, the macroscopic evaluation was performed manually, followed by a computational method based on the previously described procedure (11). This evaluation was carried out every day for 14 days. Day 3 is the day when plasma jet treatment is started. A digital camera (Lumix FH6, Panasonic, Japan) was used to record the wound conditions that were observed.

2.7. New Epithelial and Necrosis Cell Evaluation

The level of re-epithelialization and necrosis tissue of wounds was observed microscopically in each group on Day 6 (the third day after bacterial-infected wounding), Day 10 (the seventh day after bacterial-infected wounding), Day 14 (the eleventh day after bacterial-infected wounding), and Day 17 (the fourteenth day after bacterial-infected wounding). The wound on skin mice tissue was collected for tissue processing to produce wound tissue sections, and each preparation was stained with Hematoxylin and Eosin (HE). The percentage of re-epithelialization

was calculated by the formula: 100% × (new epithelial length/wound length between wound edges) and the necrosis percentage was obtained using the formula: 100% × (cell necrosis area/wound length between wound edges) (11)

Figure 1. (A−D) Experiment protocol during Day 3–16

2.8. Tumor Necrosis Factor Alpha (TNF-α) Level

Mice were dissection at Day 6, Day 10, Day 14, and Day 17. Blood was drawn from the cardiac puncture for molecular evaluation using the TNF- ELISA Kit (TNF-α rat ELISA kit; Sigma-Aldrich, St. Louis, MO). Blood samples were collected and processed into serum. Take 2 mL of blood, separate the supernatant, and place it in a new microtube. Curva Expert software version 1.4 was used to process the absorbance standards and samples.

2.9. Statistical Analysis

The results of macroscopic, microscopic (percentage of re-epithelialization), and TNF-α level, were analyzed using the Statistical Package for the Social Sciences (SPSS) program evaluated by ANOVA followed by the Tukey Kramer method; P value < 0.05 was considered significant.

3. Result

3.1. Macroscopic Evaluation of Wound

Bacterial-infected wounds on mice skin were observed daily and macroscopic images of the wounds were documented using a digital camera from Day 3 to Day 17, the results are shown in **Figure 2**.

Figure 2. Wound appearance on days 0, 3, 6, 10, 14, and 17
K: control group, CP: contact group, NCP: non-contact group,
CP-NCP: combination contact and non-contact group

In Day 3, the wound usually passes through an inflammatory phase, which is characterized by an expanded wound size and exudate fluid. The wound progressively enters the proliferative phase, which is characterized by the wound shrinking until Day 17. The wound area ratio decreased rapidly in the plasma treatment group than the control group.

Figure 3. Ratio of wound area to initial wound area during the 14-d treatment period. From Days 5-12, the wound area of group K was significantly larger than CP, NCP, and CP-NCP. At Day 17, there was no significant difference between groups. Notes: (i) α significance level of K to CP, (ii) β significance level of K to NCP, (iii) * significance level of K to the CP-NCP.

The macroscopic area data analyzed by the SPSS program showed normal and homogeneous distribution data ($p < 0.05$), then the One way ANOVA test with Turkey-Kramer continued to see significant differences between groups. Based on the ratio of the wound area to the initial wound area, the results showed in Figure 3, there was an increase in the size of the wound area in the K, CP, NCP, and CP-NCP groups over the 14-day treatment period. From Day 6 to Day 7, there was no significant difference between the groups. From Day 5 to Day 12 the

wound area in K was significantly larger than that of CP, NCP, and CP-NCP (K vs CP: $p<0.01$, K vs NCP: $p<0.01$, C vs CP-NCP: $p< 0.05$). Day 8 to Day 14 the wound area of group K was significantly larger than that of the CP, NCP, and CP-NCP groups. (K vs CP: $p<0.01$, K vs NCP: $p<0.01$, K vs CP-NCP: $p<0.05$).

3.2. New epithelial and Necrosis Cell Evaluation

Figure 4. Percentage of re-epithelialization and cell necrosis of wound tissue.
The percentage of re-epithelialization of Days 10, 14, and 17 CP-NCP was significantly higher than the other groups. Meanwhile, Necrosis cells were not observed in all groups between Day 14 and Day 17, followed by an increase in re-epithelialization.

Figure 4 shows that the percentage of re-epithelialization of Day 10, Day 14, and Day 17 CP-NCP was significantly higher than the other groups. The NCP group D7 was significantly higher than CP and K groups (NCP vs CP: $p<0.01$, C vs NCP: $p<0.01$) whereas the other groups showed no significant difference. Observations on Day 17 indicated that in the CP, NCP, and CP-NCP groups, the new epithelium had fully covered the wound. Group K had the lowest percentage of re-epithelialization, while the percentages of re-epithelialization in the CP, NCP, and CP-NCP groups were not significantly different. The percentage rate of necrosis and re-epithelialization of the wound was evaluated on Days 6, 10, 14 and 17. Necrotic cells started to form at Day 16 and increased at Day 10 in all groups K, CP, NCP, and CP-NCP. However, necrosis cells were not found in all groups from Day 14 to Day 17, at Day 14 to Day 17 necrosis cells were not found in all groups.

3.3. Tumor Necrosis Factor Alpha (TNF-α) Level

Figure 5 illustrates the TNF-α levels for each group. TNF-α levels in the CP group were significantly higher than the other groups on Day 6, while the K group was higher than the other groups on Day 10 (K vs NCP: $p<0.005$, K vs CPNCP: $p<0.005$, CP vs NCP: $p<0.005$). In Day 17, group K was significantly higher than the CP and NCP groups (K vs NCP: $p<0.005$, K vs CPNCP: $p<0.005$, K vs CPNCP: $p<0.005$). On Day 17, K, CP, NCP, and CP-NCP TNF-α levels dropped dramatically.

Figure 5. TNF-levels histogram on D3, D7, D11, D14.

From Day 6 there was a significant difference in TNF-α levels between groups. On Day 10, CP group was significantly higher than the other groups. While, K, CP, NCP, and CP-NCP decreased significantly on Day 17.

4. Discussion

The wound area ratio reduced more rapidly in the plasma-treated group than in the control group. This finding indicates that the application of plasma can accelerate wound healing. The study's findings are in accordance with Darmawati et al (2019), which found that using medical plasma can assist in the healing process (14). The thermal impact of plasma treatment on the skin with plasma jet treatment duration and maximum temperature (T-max) for plasma-affected skin with distances of 5, 10, 15, and 20mm between the skin surface and the plasma jet reactor nozzle. This indicates that a shorter distance leads to a higher T-max. Treatment of plasma jets distanced 5mm apart for 1 to 5 minutes produces T-max value between 56°C and 60°C. Treatment of plasma jets distanced 20mm apart for the same duration of time produced T-max of less than 40°C (14).

The charged particles in plasma play a key role in the inactivation of bacteria in medical plasma treatment for bacterial biofilms by breaking down the outer layer of the cell membrane. The electrostatic forces induced by the accumulating of charged particles on the cell membrane reduce stretching capability, resulting in cell membrane rupture (15). RONS content varied depending on whether plasma was treated direct (CP) or indirect (NCP). Invasive exposure resulted in plasma containing radicals with a short half-life and high reactivity, such as N_2^-, O_2, OH, and N_2^+. Non-invasive exposure, on the other hand, describes sample exposure where the sample is placed outside the plasma release area, producing radicals with relatively long lives such as OH, O, O_3, NO, and some dispersible molecules such as O_2 and N_2 (10). According to Nasruddin et al (2017), and Sibbald et al (2011) re-epithelialization for controls was only supported by moist conditions from dressings usage (16) (18).

Giving contact plasma to the wound area's surface after the biofilm layer has been removed severely impairs the wound healing process. The size of the wound area on Day 14 and D17 showed no significant decrease, and the re-epithelialization rate on Day 3 was only 25%, whereas it was >65% on Day 14. According to Darmawati et al (14), contact plasma exposure has negative effects such as damage to normal skin tissue because an elevation in local temperature slows or impairs new epithelial growth (14). Medical plasma treatment caused a higher level of necrosis in the CP group (bacterial-infected wounds with a plasma exposure distance of 5mm). After the biofilm layer has been removed (Days 10-16), non-contact plasma treatment can accelerate wound re-epithelialization. The re-epithelialization rate reached 98% on Day 17. The remodeling phase is the final stage of wound healing, also known as the maturation phase. During this phase, epithelial cells cover the whole wound's edges, and the collagen fibers' structure changes microscopically (19).

Non-contact plasma exposure leads to the generation of radicals with relatively long lifetimes, and one of those is NO (15). According to Thana et al (20), plasma-produced NO has a stimulatory affect on wound healing and tissue regeneration. In humans, NO is a key biological signaling molecule that can drive cell proliferation, angiogenesis, and collagen formation, leading in skin repair. TNF-α levels above normal indicate local or systemic inflammation (21). TNF-α is a key cytokine involved in the acute inflammatory response to bacteria and other microorganisms. Severe infections can cause high levels of TNF-α to be produced, resulting in a systemic response (22).

The decrease in TNF-α levels in the CP-NCP group at Day 17 indicated that TNF-α levels decreased after exposure to contact and non-contact medical plasma. A variety of components of the wound healing process, according to (23) (24) follow the redox regulator. Active oxygen species (Reactive Oxygen Species / ROS) and active nitrogen species (Reactive Nitrogen Species / RNS) such as O_2^-, H_2O_2, and NO are involved in the redox process. These active molecules are known as oxidants, and they serve as signaling messengers in biological processes. Several investigations have demonstrated that these active species play an important role in critical processes during wound healing, such as inflammation, re-epithelialization, and vascularization.

Plasma treatment with a combination of contact and non-contact styles is suggested for the management of chronic wounds. The basic principles of chronic wound care are (a) eliminating the source of infection, which is a biofilm, and (b) preventing the formation of biofilm without causing tissue damage or the emergence of drug resistance (16) (25). This study using plasma jets to remove biofilms, plasma creates ROS, which in high doses can damage healthy tissue and cells (including bacteria). By customizing the plasma exposure distance, the dose of ROS can be controlled. Contact plasma treatment is first given to remove biofilm and kill bacteria, followed

by non-contact plasma treatment to avoid the negative effects of plasma and accelerate wound healing (11).

5. Summary

Medical plasma treatment with contact and non-contact exposure is an effective treatment for MRSA chronic wounds. Bacterial biofilms can be removed and wound healing can be accelerated by contact and non-contact exposure.

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16/01/2023 07:03:56	System notification	Sri Darmawati	**PMED-47583. Begell House Online Submission System - confirmation** Dear Sri Darmawati. This is a confirmation notice that your article "TNF-α Expression and New Epithelial Thickness in Mice Skin (Mus musculus) Infected MRSA by Medical Plasma Treatment" for the journal "Plasma Medicine" has been successfully submitted and sent to the Editor-in-Chief - Satoshi Hamaguchi. Please use the submission site to track the status of your article. The article ID is "PMED-47583". Begell House Online Submission. Reply
16/01/2023 07:03:55	Sri Darmawati	Satoshi Hamaguchi	**Article PMED-47583. The status has been changed from 'DRAFT' to 'SUBMITTED'** Dear Editor-in-Chief - Satoshi Hamaguchi. The status of article PMED-47583 "TNF-α Expression and New Epithelial Thickness in Mice Skin (Mus musculus) Infected MRSA by Medical Plasma Treatment" in journal "Plasma Medicine" has changed from DRAFT to: SUBMITTED The change in status of the article requires your attention. Please login https://submission.begellhouse.com/usr/login.html?prod_code=journals Notes from Author - Sri Darmawati: Dear Editor, I am sending herewith a manuscript entitled "TNF-α Expression and New Epithelial Thickness in Mice Skin (Mus musculus) Infected MRSA by Medical Plasma Treatment)" by Darmawati et al., which I should like to submit for publication in the journal Plasma Medicine I hereby certify that this paper consists of original, unpublished work which is not under consideration for publication elsewhere. I also confirm that all figures and photos in this paper are our original work, have never been published before and that no permits are

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