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When plasma jet is effective for chronic wound bacteria inactivation, is it also effective for wound healing?

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ABSTRACT

Purpose: This investigation aimed to compare the effectiveness of two styles of plasma jet treatment (i.e., contact and non-contact styles) for two biological materials, namely, wound related bacteria and acute wounds.
Method: An atmospheric plasma jet operated at a frequency of 18.32 kHz and high AC voltage with a peak-to-peak voltage of 9.58 kV and a current of 55.2 mA was applied. Argon gas was used as the carries gas of plasma jet generation and was fixed at a flow rate of 1 standard liters per minute (slm).Two biological materials (i.e., wound related bacteria and acute wound) were applied as experimental objects. The sample groups were based on the two styles of plasma jet treatment: contact and non-contact styles. Microbial inhibition zone calculation and macroscopic and histological observations were also performed.
Results: This investigation emphasized that the contact and non-contact styles of plasma jet treatment had significantly different effects for wounds and wound-related chronic bacteria. On the one hand, the contact style was visually attractive and more effective for inactivate bacteria. On the other hand, it caused negative effects, such as damaging normal tissue, significantly impeding wound healing and impeding the growing of new epithelial tissue. The non-contact style, however, was less effective at inactivating bacteria; however, it could accelerate wound healing.
Conclusion: In the context of wound healing, the non-contact style of plasma jet treatment may be better than the contact style of plasma jet treatment.

1. Introduction

It is well-known that all wounds have several levels of bacterial burden. Stotts [1] defined a bioburden as the presence of microorganisms on or in a wound. Conceptually, the continuum of a bacterial bioburden in a wound can be classified into five microbial forms: contamination, colonization, critical colonization, biofilm, and infection. Contamination is the presence of non-duplicating

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microorganisms on the surface of a wound without a host reaction. Colonization is the presence of a duplicating microorganism adherent to the surface of a wound, without a host reaction. Critical colonization is the presence of duplicating microorganisms on a wound and attached to the cells and structures in a wound. A biofilm is a complex community of aggregated bacteria embedded in a self-secreted extracellular polysaccharide matrix. Infection is the invasion of microorganisms into the wound tissue, resulting in a local or systematic response.

Topical wound management is the positive manipulation of a wound to restore its environment, physiologically. The primary factors of such an environment are an adequate moisture level, temperature control, pH regulation and bacterial burden control [2]. To date, the strategies used control bacterial burden include: (1) debridement, (2), appropriate wound cleansing, (3) appropriate infection control precautions, (4) use of antimicrobials, and (5) use of a moisture-retentive dressing.

A broad range of antimicrobial material is used to reduce the bacterial burden in the clinical setting; however, drawbacks still exist. For example, mupirocin 2% ointment is effective against MRSA; however, it is contraindicated for large burns. This ointment also contains a chemical substance (polyethylene glycol) that may damage the kidneys if absorbed through the skin [1]. Therefore, the importance of finding new antimicrobial agents is clear. For this purpose, bioburden reduction using an atmospheric pressure plasma jet is being developed.

Plasma in this context is not blood plasma but physical plasma and is the phase of the fourth state of matter after solid, liquid and gas. Plasma is commonly called ionized gas. In the plasma phase, there are stable parts (gases) and reactive parts (ions, energetic and radical particles) [3]. It is believed that the main medical aspects of plasma are related to its ability to generate biological molecules, namely, reactive oxygen species (ROS) and reactive oxygen species (RNS), which if carefully controlled and used at the right doses, can be efficacious for medical therapy [4–6]. The ROS and RNS produced by plasma that are suspected to have efficacy are superoxide (O_2^- •), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), singlet oxygen ($^1O_2$), ozone (O_3), organic radicals ($RO^•$, $RO_2^•$), nitric oxide ($^•NO$), nitrogen dioxide ($•NO_2$) and peroxynitrite ($ONOO-$) [7].

It is well known that there are many human made plasma sources that can be generated under in an atmospheric pressure environment, and plasma jet is one of the most attractive sources for clinical applications because such a plasma source can be protracted to reach a specific spot or area that is not limited by electrodes [8]. Generally, a plasma jet consists of a couple of primary conditions with unique characteristics: *a plasma condition* containing radicals with relatively short life times, such as N_2*, O_2*, OH, and N_2^+,*an afterglow condition* containing radicals with relatively long life times, such as OH, O, O_3, NO, and some metastable molecules, including O_2(a) and N_2(A) [9]. The differentiations of the contact and non-contact styles of plasma jet treatment may be a manifestation of these two conditions.

Our previous report found that the non-contact treatment style of the plasma jet (afterglow condition) was able to accelerate skin wound healing in animal models mimicking the modern clinical setting [10,11]. The contact style of plasma jet treatment (a plasma condition) to the normal skin of mice, unfortunately, showed a detrimental effect [11,12]. An abnormality of the epidermal part of mature wounds, due to the contact style of plasma jet treatment, was observed [13]. The contact style of plasma jet treatment, however, may effectively kill chronic wound related bacteria [14–16].

It has been reported that the treatment goal of critical colonization and infection is to reduce the bioburden without causing injury to normal tissue or development of drug resistance [17]. The antimicrobial effectiveness of an atmospheric plasma jet for chronic wound related bacteria has been scientifically reported [14–16]; however, safety reports for wounds using an in vivo model are still lacking. This investigation aimed to compare the effectiveness of the two styles of plasma jet treatment, the contact and non-contact styles, for two biological material objects, wound related bacteria and acute wounds. Considering that the effectiveness of the plasma jet depends on its parameters and treatment style, this investigation tried to answer one simple question: using the same parameters and the same treatments style, when a plasma jet is effective at killing chronic wound associated bacteria, is it also effective at accelerating wound healing? Four types of standard and local microorganisms, *Staphylococcus aureus* ATCC 6538 (SA), *Pseudomonas aeruginosa* ATCC 9027 (PA), Methicillin Resistant *Staphylococcus aureus* (MRSA) isolated from Kariadi Hospital Semarang (Indonesia), and *Carbapenem Resistant Pseudomonas aeruginosa* (CRPA) isolated from Kariadi Hospital Semarang (Indonesia),were used in this investigation.

2. Methods and materials

2.1. Atmospheric pressure plasma jet

This experiment used an atmospheric pressure plasma jet system that was developed based on Teschke et al. [18] and has been previously described [12]. A modification was conducted regarding the dimension of the capillary quartz tube. The inner and outer diameters of the tube in this experiment were 1.55 and 0.65 mm, respectively. A couple of electrode-like rings were applied around the quartz tube. Clay, a non-conductor material, was applied to isolate the two electrodes.

The basic parameters of the plasma jet for medical purposes (i.e., the electrical and optical emission properties) were evaluated at the Research Center for Sustainable Energy and Technology, Institute of Science and Engineering, Kanazawa University, Japan. Medical grade argon gas (99.999% purity) was used as the carrier gas. The voltage was measured using a high-voltage probe (P6015A, Tektronix, Beaverton, OR, USA), and the current passing through the discharge device was measured with a Rogowski coil-type current monitor (2877, Pearson Electronics Inc., Palo Alto, CA, USA) connected to a digital oscilloscope (DSOX3024T InfiniiVision, Keysight Inc., Santa Rosa, CA, USA). The high voltage probe was attached to the upper ring, where a high voltage was applied. A current probe was attached to a cable toward the ground electrode. A high-voltage probe and a current probe (8585C; Pearson Electronics, Palo Alto, CA, USA) were used to monitor the discharge voltage and discharge current during plasma jet generation, respectively. It was found that as argon gas, at a flow rate of 1, 2 and 3 standard litres per minute (slm), was delivered throughout the quartz tube, a low-frequency (~ 18.32 kHz) high AC voltage, with a peak-to-peak voltage of 9.58 kV and current of 55.2 mA, was measured at the upper ring electrode. Fig. 1 shows a current and a voltage waveform at an argon gas flow rate of 1 slm.

A photonic multichannel analyser (PMA-12) (model C10029, Hamamatsu Photonics) was used to record emissions between 200 nm and 800 nm. The emissions from the plasma jet was focused on the entrance slit to an optical fibre probe of PMA-12 using a convex quartz

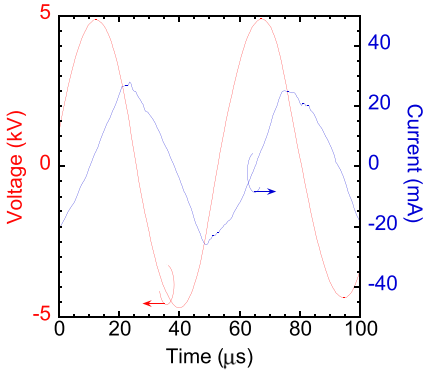


Fig. 1. Current and voltage waveform at an argon gas flow rate of 1 slm.

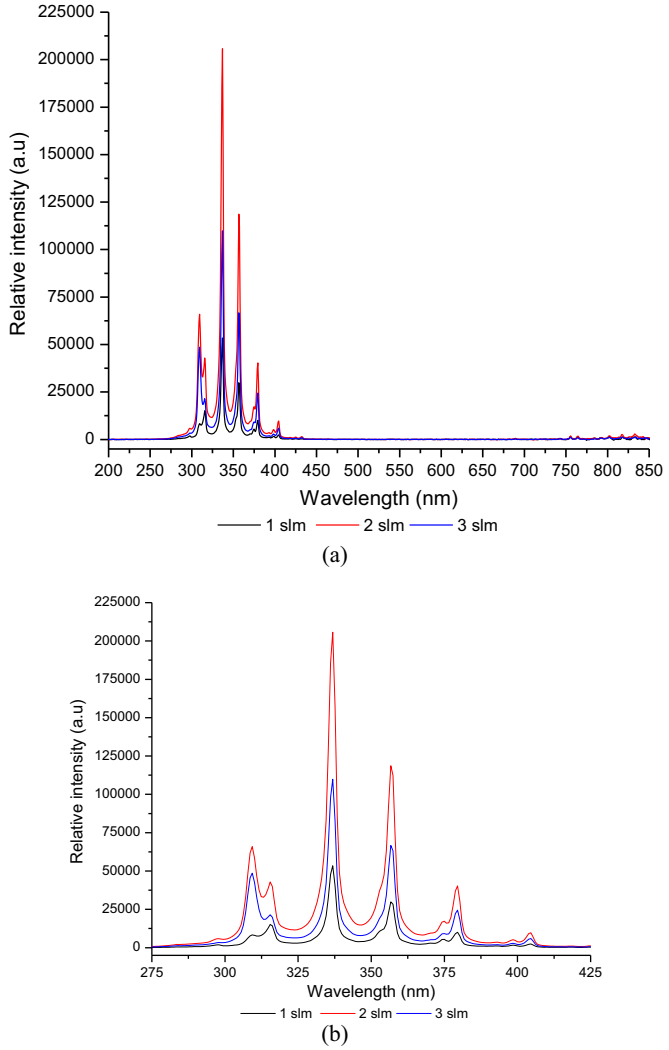


Fig. 2. Optical emission spectroscopy (OES) evaluation of an atmospheric plasma jet near 10 mm under the nozzle of the plasma jet reactor (without mice) with various gas flow rates (1, 2 and 3 slm). OH and nitrogen-based reactive species were detected. (a) OES evaluation between 200 nm and 850 nm; (b) OES evaluation between 275 nm and 425 nm.

lens with a focal length f = 100 mm. The exposure time as 19 ms, and the number of accumulations, was 30. *The light diameter of the optical fibre was 1 mm.* The optical emission intensity of the present optical system was calibrated using a standard lamp.

Optical emission spectroscopy (OES) measurement at approximately 10 mm under the nozzle showed emissions of the OH (A-X transition) transition near 309 nm, N2 (C-B transition) (band head maximum at 337 nm) and Ar I (maximum 763 nm).This measurement showed the presences of both hydroxyl radical (OH) and nitrogen-based reactive species in the gas phase during its generation (Fig. 2). This measurement also showed that the plurality of argon gas flow caused a plurality of the rate of those radical species. Using the graphical results presented in Fig. 2b, it was observed that the radical species rate at an argon gas flow rate of 2 slm was the highest, while that at an argon gas flow rate of 1 slm was the lowest. Finally, argon gas with a flow rate of 3 slm was between these two measurements.

It was difficult to identify the presence of ROS and RNS using OES Spectroscopy in the plasma jet after-glow condition. H_2O2 and NO_2were identified at a distance of 20 mm under the nozzle of the plasma jet reactor (after-glow) using the "Kyoritsu" chemical method as previously reported [12]. The concentrations of H_2O_2 and NO_2 in ultrapure water were analysed using a peroxidase enzyme for H_2O_2 and a

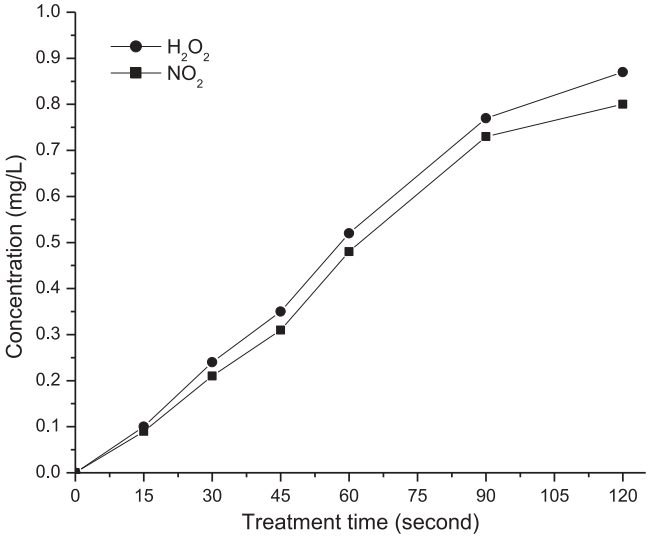


Fig. 3. Relationship between the treatment times and concentrations of H_2O and NO_2 generated in pure water after the plasma jet treatment with a distance of 20 mm.

(Naphthyl) ethylenediamine visual colorimetric reaction for NO_2 with a commercial reagent (Kyoritsu Chemical-Check Lab., Japan; Model WAK-H_2O_2, range: 0.05–5.0 mg/L and Model WAK-NO_2, range: 0.02–1.00 mg/L) after the plasma jet treatment. Ten millilitres of ultrapure water in glass was treated with the plasma jet with treatment times of 15, 30, 45, 60, 90 and 120 s. The distance between the nozzle of the plasma jet reactor to the surface of water was approximately 20 mm. A digital packtest device (Kyoritsu Chemical-Check Lab., Japan; Model DPM-H_2O_2 and Model DPM-NO_2) was used to measure the concentrations of H_2O_2 and NO_2. The results are shown in Fig. 3. A longer treatment time led to higher concentrations of H_2O_2 and NO_2. For every treatment time, the concentration of H_2O_2 was slightly higher than that of NO_2. For the treatment time of 120 s, the concentrations of H_2O and NO_2 were between 0.7 mg/L and 0.9 mg/L.

The thermal effect of the plasma jet treatment on normal skin was evaluated using an infrared thermal camera (FLIR C2, Sweden) with a previously reported procedure [12]. Fig. 4 showed the relationship between the plasma jet treatment time and temperature maximum for plasma influenced skin (T_{max}), with distance d variations between skin

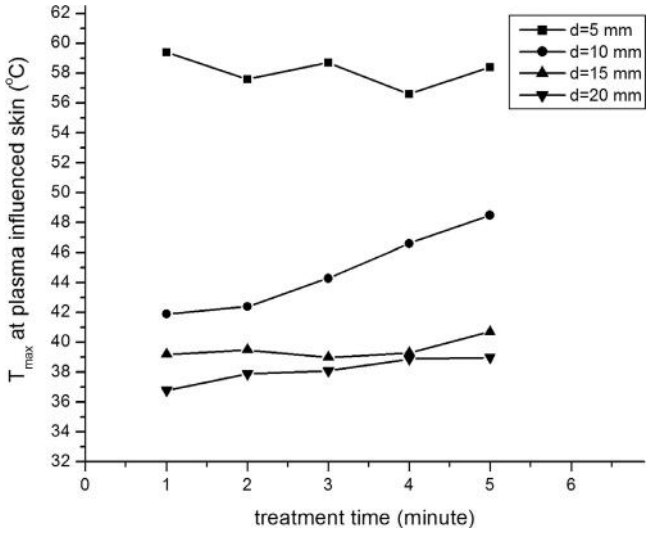


Fig. 4. Relationship between the treatment time and temperature maximum on plasma influenced skin with distance variations between the skin surface and the nozzle of the plasma jet reactor.

surface and the plasma jet reactor nozzle of 5, 10, 15 and 20 mm. It was shown that a lower distance d resulted in a higher $T_{max.}$The plasma jet treatment with a distance of 5 mm for 1 to 5 min caused a T_{max} between 56°C and 60°C. The plasma jet treatment with a distance of 20 mm for the same time duration caused a $T_{max.}$ less than 40°C. Skin injury was observed after plasma jet treatments with distances of 5 mm and 10 mm. The plasma jet treatment with a distance of 20 mm, however, did not cause skin injury.

2.2. Microorganisms

An evaluation of the plasma jet treatment on microorganisms was conducted at the Laboratory of Microbiology, Department of Medical Laboratory Science, Universitas Muhammadiyah Semarang, Indonesia. Four types of standard and local microorganism were used in this investigation: *Staphylococcus aureus* ATCC 6538 (SA), *Pseudomonas aeruginosa* ATCC 9027 (PA), Methicillin Resistant *Staphylococcus aureus* (MRSA) isolated from Kariadi Hospital Semarang (Indonesia), and *Carbapenem ResistantPseudomonas aeruginosa* (CRPA) isolated from Kariadi Hospital Semarang (Indonesia). Fresh cultures were obtained by plating *Pseudomonas aeruginosa* ATCC 9027 and Carbapenem Resistant *Pseudomonas aeruginosa* on a MacConkey agar (OXOID) and *Staphylococcus aureus* ATCC 6538 and Methicillin Resistant *Staphylococcus aureus* on a blood agar plate (OXOID). Following incubation for 24 h at 37 °C, all bacteria were suspended in a saline solution (NaCl 0.9%), and the turbidity was then adjusted to the standard of McFarland 0.5. To evaluate the effect of the atmospheric plasma jet on inactivation of bacteria, 0.1 mL of cells suspended on the surface of a solid culture medium (Mueller Hinton Agar /MHA media, OXOID) was plated on standard Petri dishes a using sterile swab. The plated bacteria were exposed to the plasma jet after the dishes were kept at room temperature for 10 min. An evaluation of the effect of the plasma jet on inactivation of bacteria was conducted by calculating the area of the bacterial inhibition zone on the agar.

2.3. Plasma jet treatment on microorganism

The parameters of the atmospheric plasma jet on bacteria and those on the wound were the same. The atmospheric plasma jet operated at a frequency of 18.32 kHz and a high AC voltage with a peak-to-peak voltage of 9.58 kV and current of 55.2 mA. Argon gas was used as the carrier gas for plasma jet generation, which was fixed at a flow rate of 1 slm. All bacterial samples were classified into two groups: the contact (P5) and non-contact styles (P20) of plasma treatment. The contact style (P5) is a plasma jet treatment with a distance between the agar surface and the nozzle of the plasma jet reactor of 5 mm, perpendicularly. At this distance, the plasma jet was visually observed. The non-contact style (P20) is a plasma jet treatment with the distance between the agar surface and the nozzle of the plasma jet reactor of 20 mm, perpendicularly. At this distance, the plasma jet was not visually observed. To ensure reproducibility, each experiment was performed with four or five replicates. The four bacteria were treated by the plasma jet for different time intervals (1, 2, 3, 4 and 5 min). After the plasma jet treatment, all samples were incubated for 24 h at 37 °C. For a control experiment, the samples were treated with only an argon gas flow at the same flow rate without plasma generation.

2.4. Animals and experimental protocol

The Guidelines for the Care and Use of Laboratory Animals of Laboratorium Penelitian dan Pengujian Terpadu / Integrated Research and Testing Laboratory (LPPT UGM), Gadjah Mada University, Yogyakarta, Indonesia were used as the work protocol in this experiment (Certificate number: 00128/04/LPPT/II/2019). LPPT UGM is accredited under ISO/IEC 17025 and the National Accreditation Committee of Indonesia (Komite Akreditasi Nasional/KAN, Indonesia). Nine BALB/c male mice aged 8 weeks and weighing 21.3–28.0 g purchased from Laboratorium Penelitian dan Pengujian Terpadu/ Integrated Research and Testing Laboratory (LPPT), Gadjah Mada University, Yogyakarta, Indonesia, were individually caged in an air-conditioned room at 28.0 ± 2.0 °C with light from 08:00 to 20:00 h and under ad libitum feeding conditions.

2.5. Plasma jet treatment on wound

The parameters of the atmospheric plasma jet on wounds and those on bacteria were the same. An evaluation of the plasma jet treatment on wound healing was conducted at the Research Center for Experimental Wound Healing, Universitas Muhammadiyah Magelang, Indonesia. After being completely anaesthetised via an injection of ketamine-xylazine, (K) 50 mg/kg + (X) 5 mg/kg through intraperitoneal administration [19], 2 circular (4 mm in diameter) full-thickness cutaneous wounds were created using a sterile disposable biopsy punch of 4 mm (Kai Industries Co. Ltd., Gifu, Japan) following a previously described technique [12]. Considering the results of the antimicrobial evaluation of the plasma jet, contact and non-contact plasma jet treatments were performed once daily for 3 min for 14 days.

The experimental procedure after day 0 is shown in Fig. 5. Generally, laboratory mice were randomly classified into 3 groups, with three mice or six wound samples in every group.

A Control or untreated group (C): wounds were allowed to heal daily under a Tegaderm's hydrocolloid dressing (3 M Health Care, USA).
B Group with plasma treatment with the contact style of treatment (P5): Wounds were given the plasma jet treatment for 3 min with the spot and contact styles of the plasma jet treatment. In this context, the distance from the wound surface to the nozzle of the plasma jet reactor tube was 5 mm. Under this condition, the plasma jet made contact with wound surface.
C Group with plasma treatment with the contact style of treatment (P20): Wounds were given the plasma jet treatment for 3 min with the spot and contact styles of plasma jet treatment. In this context, the distance from the wound surface to the nozzle of the plasma jet reactor tube was fixed at 20 mm. Under this condition, the plasma jet was not contact with the wound surface.

During the 14 days of the experiment, the wound dressings and bandages in all groups were detached daily and reviewed for plasma treatment and/or wound evaluation.

2.6. Macroscopic evaluation of wounds

Macroscopic evaluations of the wounds were first conducted manually, followed by computational processes. This evaluation was conducted daily for 14 days. Day 0 was the day when the wounds were created. Before the evaluation, the normal tissue around the wounds was cleaned with a saline solution. The peripheries of the wounds were traced on polypropylene sheets, and photographs were taken daily. The traced polypropylene sheets were captured with a scanner linked to a computer using Adobe Photoshop Elements 7.0. The wounds areas were then calculated using the image analysis software Scion Image Beta 4.02 (Scion Corporation, Frederick, Maryland, USA).

2.7. Tissue processing

On the 14th day after wound creation, mice were euthanised through a massive injection of ketamine-xylazine. The wound and the surrounding intact skin were harvested, and each sample of wound and surrounding intact skin was bisected at the wound centre. Each wound was stapled onto polypropylene sheets and fixed in a neutral buffered 10% formalin solution for approximately 24 h. Subsequently, they were dehydrated in an alcohol series, cleaned in xylene, and embedded in

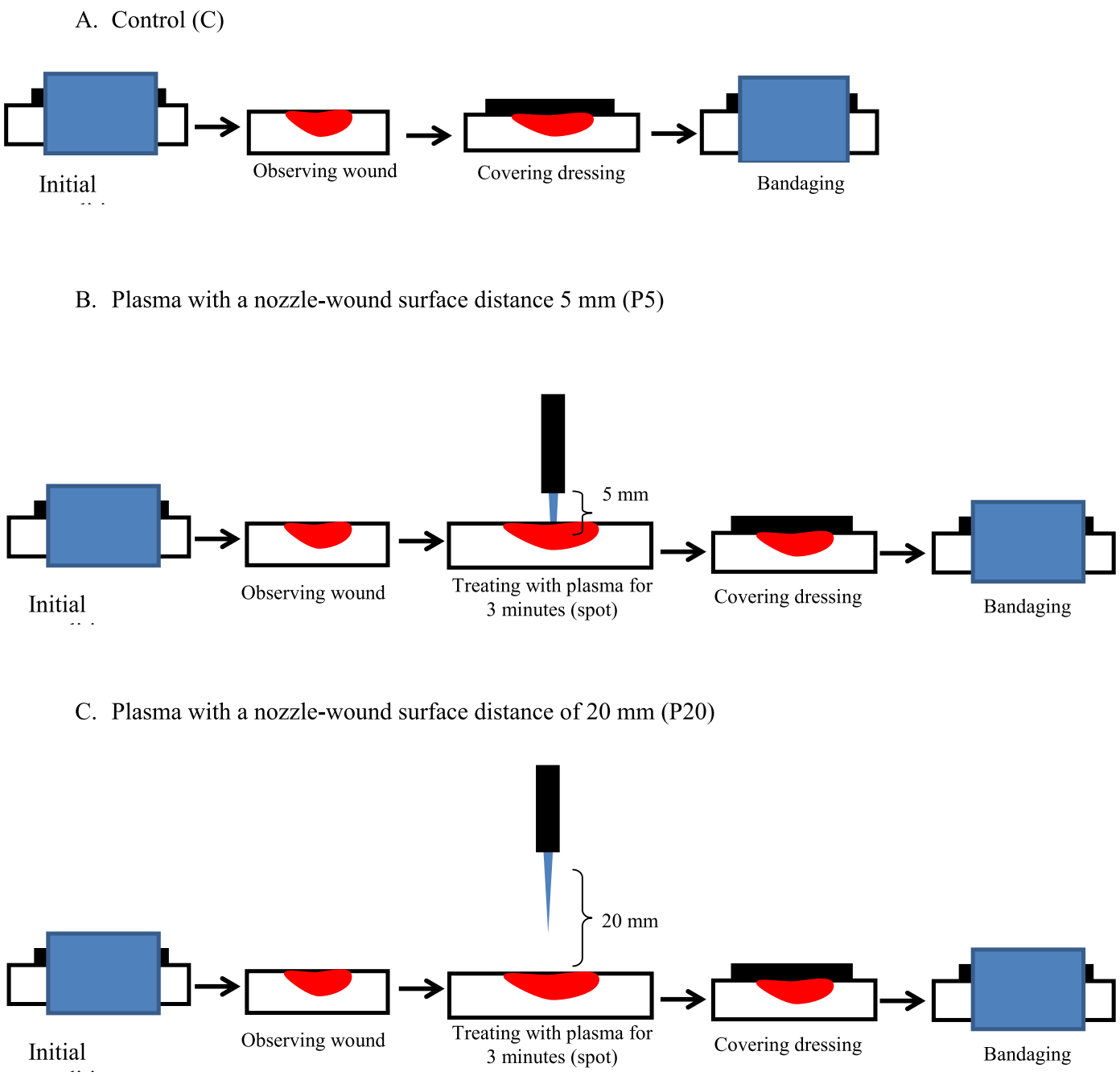


Fig. 5. Experimental procedure.

paraffin to prepare serial 5 μm sections. The tissue sections were then stained with haematoxylin-eosin (HE).

2.8. New epithelial tissue observation

The plurality of the plasma jet treatment may cause numerous types of new epithelial tissue in wound on day 14 in every group. To test this hypothesis, tissue section conditions were classified into two results: (a) the tissue condition with new epithelial 100%; (b) the tissue condition with new epithelial less than 100%. To monitor this condition, the haematoxylin-eosin staining results containing new epithelial was used. Four tissue sections were used from 6 different wounds (mice) for every group.

2.9. Statistical analysis

Data were subjected to statistical analyses using SPSS 16.0. The inhibition zone area, the ratio of the wound area to the original wound area and the absolute wound area were evaluated by ANOVA, followed by the Tukey–Kramer method. P values <0.05 were considered to be significant.

3. Results

3.1. Plasma jet effect on wound chronic related bacteria: inhibition zone evaluation

A relationship between the treatment time and inhibition zone for the contact (P5) and non-contact (P20) plasma jet treatment styles in

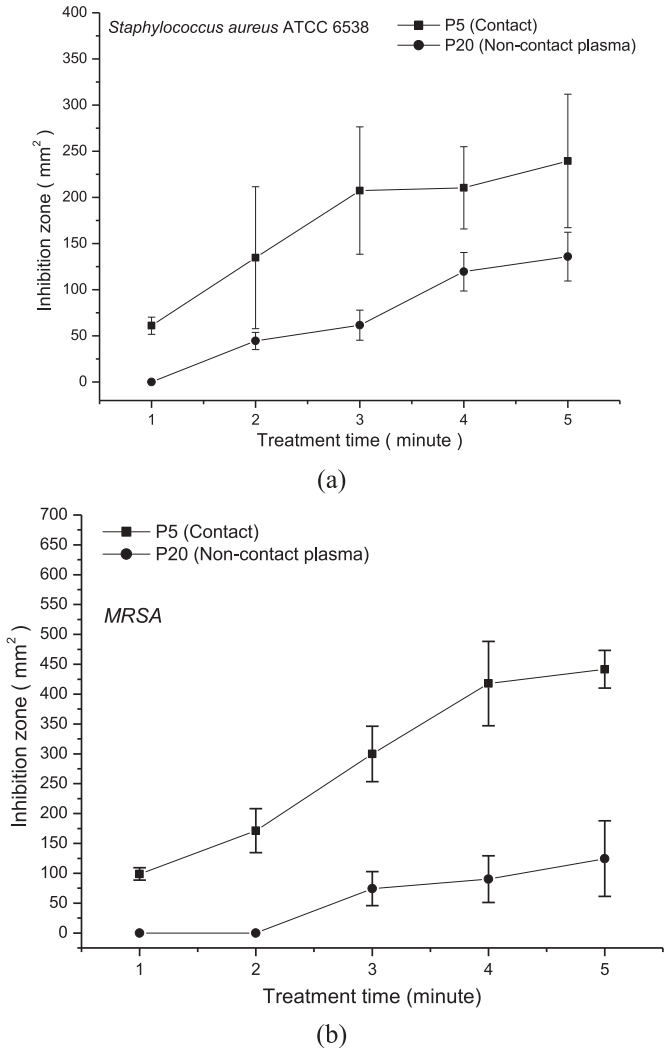


Fig. 6. Relationship between the treatment time and inhibition zone for the contact (P5) and non-contact (P20) plasma jet treatment styles in gram-positive bacteria: a. *Staphylococcus aureus* ATCC 6538 (SA) and b. MRSA.

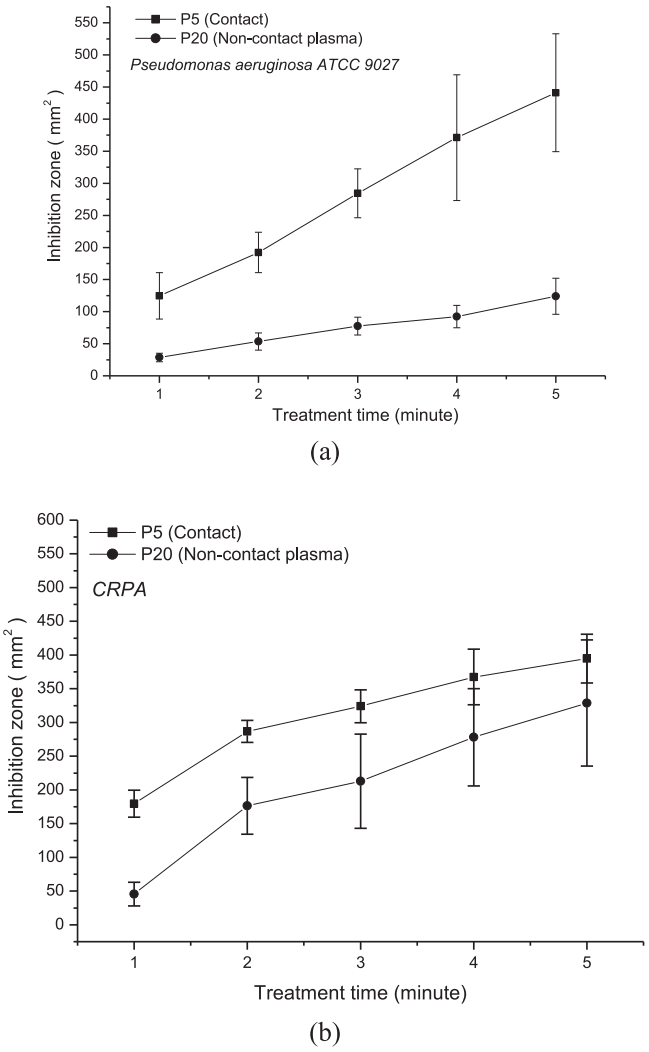


Fig. 7. Relationship between the treatment time and inhibition zone for the contact (P5) and non-contact (P20) plasma jet treatment styles in gram-negative bacteria: a. *Pseudomonas aeruginosa* ATCC 9027 (PA); b. CRPA.

gram-positive bacteria, namely, *Staphylococcus aureus* ATCC 6538 (SA) and MRSA, was observed, as shown in Fig. 6**.** The inhibition zones in the contact groups were significantly larger than those in the non-contact group. The inhibition zone was zero for SA for 1 min of the non-contact treatment. The inhibition zone was also zero for MRSA for1 and 2 min of the non-contact treatments.

A relationship between the treatment time and inhibition zone for the contact (P5) and non-contact (P20) treatment styles in gram-negative bacteria, namely, *Pseudomonas aeruginosa* ATCC 9027 (PA) and CRPA, was observed, as shown in Fig. 7. The inhibition zones in the contact groups were significantly larger than those in the non-contact groups. The inhibition zone was zero in PA for 1 min of the non-contact treatment. Inhibition zones were found in all groups, beginning with 1 min of treatment time.

Fig. 8 shows that inhibition zones were found in all groups for a plasma treatment of 1 min; however, the levels varied. Based on the results for only 1 and 2 min of treatment, the inhibition zones, ranked from highest to lowest, were P5-CRPA, P5-PA ATCC, P5-MRSA and P5-SA. These results indicated that the contact style of the plasma jet treatment was more effective at killing gram-negative bacteria compared to gram-positive bacteria. Within the gram-negative bacteria groups, however, the contact style of the plasma jet treatment was more effective at killing locally isolated CRPA bacteria compared with standard bacteria PA ATCC. Regarding the gram-positive bacteria groups,

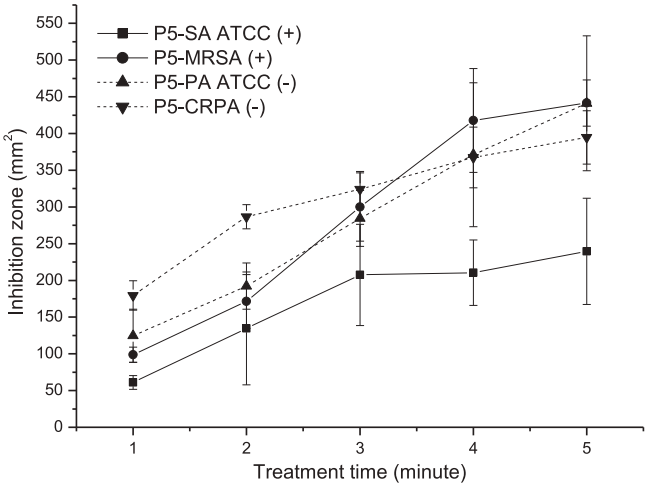


Fig. 8. Relationship between the treatment time and inhibition zone for only the contact style of plasma jet treatment in groups with gram-negative and gram-positive bacteria.

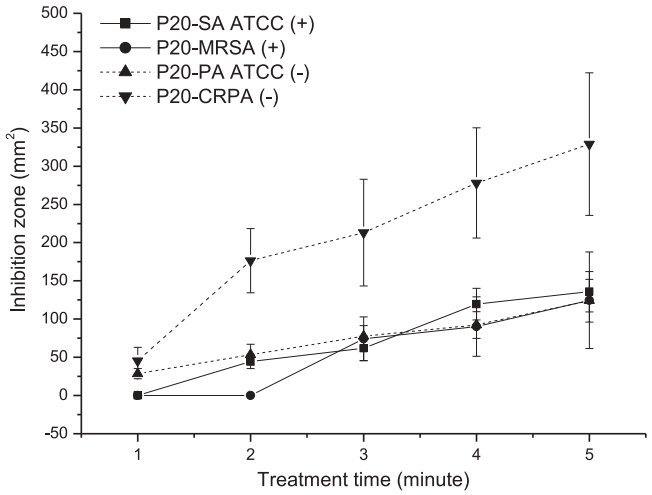


Fig. 9. Relationship between the treatment time and inhibition zone for only the non-contact style of the plasma jet treatment in groups with gram-negative and gram-positive bacteria.

the effectiveness of the contact style of the plasma jet treatment on locally isolated MRSA was higher compared to standard bacteria SA ATCC.

Inhibition zones in groups with gram-negative bacteria, namely, P20-PA ATCC and P20-CRPA, were found after a 1-min treatment time, while in gram-positive bacteria, namely, P20-SA ATCC and P20-MRSA, inhibition zones were not found after the aforementioned treatment time (Fig. 9). The inhibition zone trend for non-contact plasma treatments from1 to 5 min for P20-CRPA (gram negative) was the highest. While the inhibition zones in P20-PA ATCC, P20-SA ATCC and P20-MRSA varied for the 1- and 2-minute treatment times, the trend for those treatments ranging from 3 to 5 min were similar. It is difficult to the rank inhibition zones for all groups; however, if we choose the results for only 1 and 2 min of treatment time, it is shown that the inhibition zones ranked from the highest to the lowest are as follows: P20-CRPA, PA ATCC, P20-SA ATCC and P20-MRSA. This result also highlighted two points. *First*, the non-contact style of the plasma jet treatment was more effective at killing gram-negative bacteria compared to gram-positive bacteria. *Second*, for gram-negative bacteria, the non-contact style of the plasma jet treatment was more effective at killing isolated CRPA bacteria compared to standard PA ATCC bacteria, while for gram-positive bacteria, the non-contact style of the plasma jet treatment was more effective at killing standard SA ATCC bacteria compared to isolated MRSA bacteria.

3.2. Plasma jet effect on wounds

3.2.1. Macroscopic observation of wounds

The wounds on mouse skin were inspected daily for each group from days 0 to 14, as shown in Fig. 10. While the wound areas for the C and P20 groups initially increased and then gradually decreased until the end of the observation days, the wound areas in P5 initially increased but did not decrease until the end of the observation day. On day 14, while the surface textures of the wounds in C and P20 appeared healthy and mature, those in P5 were the opposite. Immature and unhealthy conditions (as demonstrated by a yellowish colour on the wound surface) were still observed in P5 on day 14. The size of the wound area in P5 was approximately the same or larger than that on day 0. The daily plasma jet treatment with a distance 5 mm for 3 min may significantly damage wound tissue.

3.2.2. Reduction graph of wound area

Fig. 11a shows the reduction of the experimental wound during 14 healing days in all groups based on the ratios of the wound areas to the original wound areas. From day 0 until 3, there was no significant different between the groups. From day 4 until 14, the wound areas in C and P20 were significantly smaller than those in P5 (C vs P5: $P < 0.05$; P20 vs P5: $P < 0.05$). On days 10 and 11, the wound area in P20 as significantly smaller than that in C (C vs P20: $P < 0.05$); however, on day 14, that of the former was nearly the same as that of the latter (C vs P20: $P > 0.05$).The comparison of the absolute number of the average of wound area on days 0 and 14 was also calculated, as shown in Fig. 11b. Based on this histogram, it is indicated that in the P5 group, the wound area size on day 14 was nearly the same as that on day 0 (P5

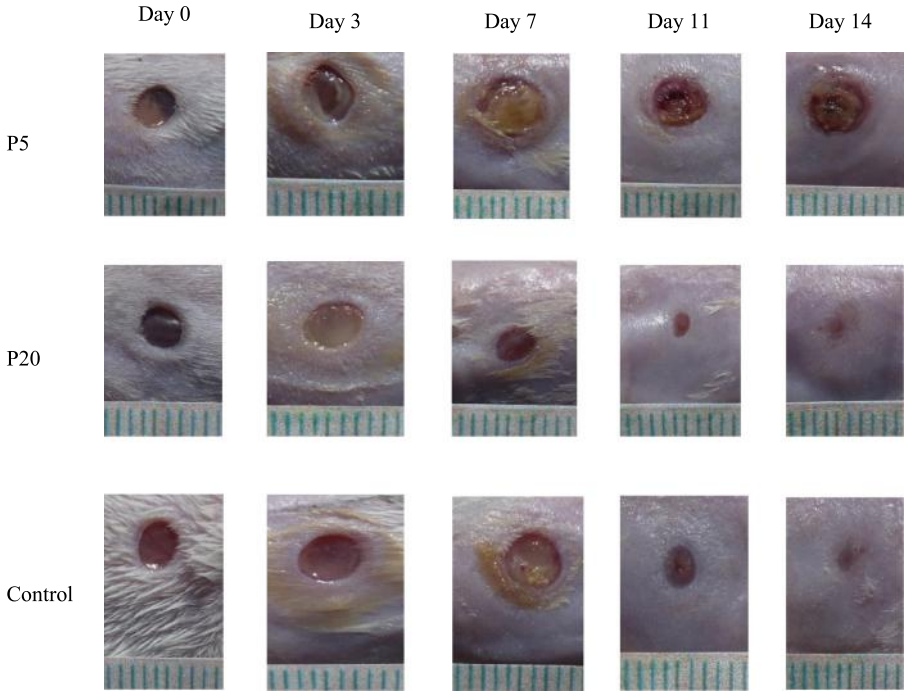
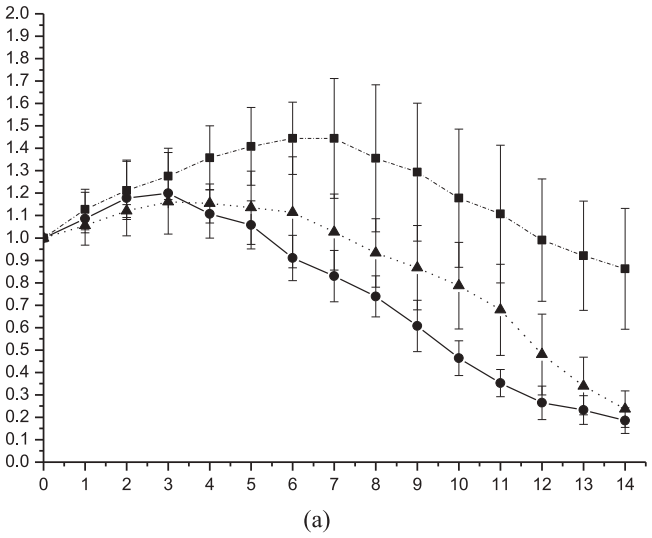


Fig. 10. Appearance of wounds on days 0, 3, 7, 11, and 14.



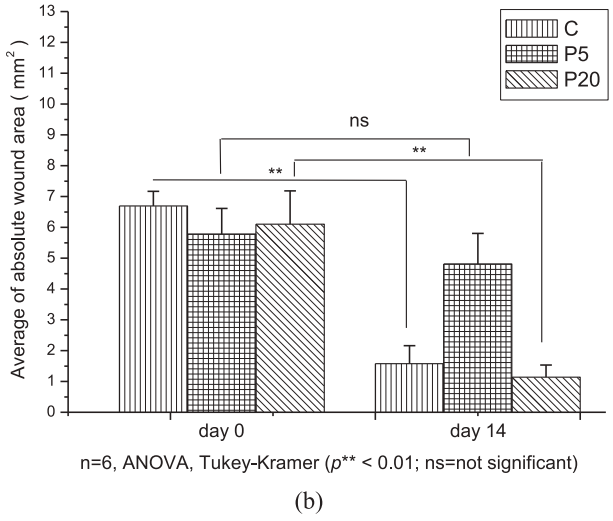


Fig. 11. (a) Ratio of the wound areas to the initial wound areas during healing. From day 4 until day 14, the wound area in P5 was significantly larger than that in C and P20; (b) comparison of the absolute values of the wounds areas in all groups between days 0 and 14. Regarding the wound area between the two days, while they were significantly different in C and P5, they were not significantly different in P5.

day 14 vs P5 day 0:$P > 0.05$). Conversely, in the C and P20 groups, the wound area size on day 14 was significantly smaller than that on day 0 (C day 14 vs C day 0:$P < 0.01$; P20 day 14 vs P20 day 0: $P < 0.01$). It can be concluded that plasma jet treatment with a distance of 20 mm accelerated wound healing, while a distance of 5 mm delayed wound healing.

3.2.3. *New epithelial tissue observation*

As shown in Fig. 12, it was found that tissue samples containing new epithelial tissue 100% were observed in all samples of the C and P20 groups. Conversely, such conditions were not observed in all samples of the P5 group. The P5 group contained wound tissue sample with new epithelial tissue less than 100%.

4. Discussion

Conceptually, the atmospheric plasma jet has two different conditions, namely, the plasma condition and after-glow condition, as stated by Lu [9]. The plasma condition includes radicals, particles and chemical substances with short life spans, while the after-glow condition

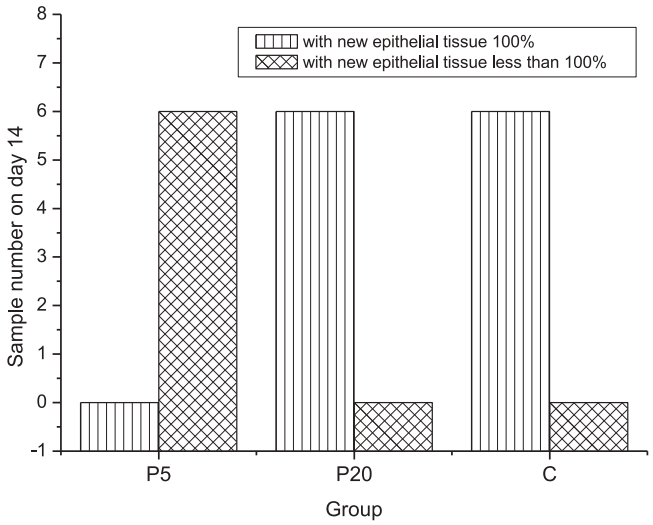


Fig. 12. Number of tissue samples with new epithelial tissue 100% and the number with less than 100% in P5, P20 and C. While new epithelial tissue 100% was observed in all samples for the C and P20 groups, new epithelial tissue100% was not observed in the P20 group.

includes chemical active species with longer lifetimes. Using the optical emission spectroscopy method, the presence of nitrogen- and oxygen-based species within the plasma jet used in this investigation was identified using an applied distance of 10 mm; however, it was difficult to identify those using the same method with an applied distance of 20 mm. Another method (i.e., the chemical-enzymatic method) was applied. The presence of relatively longer lifetime H_2O_2 and NO_2 species was identified. Considering the results, the contact (P5 group) and non-contact styles of the plasma treatment (P20 group), as implemented in this investigation, were hypothesized as being representatives of the plasma condition and after-glow condition, respectively.

Under the parameters developed in this investigation, it was demonstrated that he plasma jet condition and after-glow condition had significantly different effects for wound and wound related chronic bacteria. On the one hand, the plasma jet condition was visually attractive and more effective at inactivating bacteria; on the other hand, it caused negative effects, such as damaging normal skin due to local thermal elevation; significantly impeding wound healing; and impeding or damaging the growth of a new epithelium. The after-glow condition, however, was less effective at inactivating bacteria compared with the first condition; however, it accelerated wound healing.

This study may have one shortcoming because the negative effect of the contact style of the plasma jet treatment, as stated in this study, only includes at plasma jet treatment at a distance of 5 mm with a treatment time of 3 min. Plasma jet treatments at longer distances (6, 7, 8, 9 or 10 mm) with shorter treatment times may also produce the contact style and have positive or negative effects for bacteria and wounds; however, this study did not investigate these treatments. For comparison, the study by Schmidt et al. [20], performed with a CE-certified plasma source (kINPen, Neoplas tools, Germany), showed that wound healing can be positively influenced at a treatment distance of 8 mm ("almost contact style") with a treatment time of 20 s. Thus, it is important to conduct further investigations using the contact style of plasma jet with longer distances and shorter treatment times.

In mice models, several investigators demonstrated accelerated wound healing following a plasma treatment [20–24], and a variety of studies describe the antibacterial effect of plasma treatments on various gram positive and negative bacteria [25–27]. Since there is no standardized method of using plasma to date (Microwave Plasma, Plasma Needle, DBD Plasma, Plasma Gun, SMD Plasma, Plasma jet etc.) and since each research group uses their own plasma parameters (treatment

Table 1
Comparison of the conditions and effects between the contact and non-contact styles of the plasma jet treatment on bacteria and wounds.

	Contact style (P5)	Non-contact style (P20)
Plasma jet condition	1. Plasma jet contact with object 2. Nozzle – object distance of 5 mm 3. Plasma jet condition	1. Plasma jet no contact with object 2. Nozzle – object distance 5 mm 3. After-glow condition
Species reactive/active	Radicals and particles with shorter lifespans	Chemical substance or radicals with longer lifetime
Effect on normal skin	Cause injury	Cause no injury
Effect on local skin temperature	Skin temperature increase more than 50 °C	Skin temperature less than 40 C
Effect on bacteria inactivation	More effective	Less effective but able to kill
Effect on wound	Impede healing	Stimulate healing
Effect on re-epithelialization	Impede or damage re-epithelialization	Stimulate re-epithelialization

time, plasma composition, distance from plasma source to object etc.), the results of this study cannot be directly generalized to other studies using different devices.

It is now the dawning of plasma medicine research, and many plasma researchers are obtaining a large amount of data under different conditions. At same time, this study is important for developing plasma medicine devices. This investigation may produce data that are different from other investigator's data due to the different parameters used. Because plasma devices and conditions are not yet standardized, the data from our research are important for plasma medicine research. This study emphasizes that those who apply and develop plasma jet devices for chronic or infected wound therapy should be aware that in a clinical setting, it is easy to shift from the glow condition to the plasma jet condition by changing the distance between the nozzle of the plasma jet reactor and the object by only few millimetres. This investigation applied a distance of 5 mm for the contact style and 20 mm for the non-contact style; however, the effects of the two were significantly different, both in the context of bacteria inactivation and wound healing, as summarized in Table 1. Finally, as plasma jets involve many parameters, this investigation suggests that the safety and effectiveness aspects of the plasma jet treatment should be considered, first through carefully controlling the distance as well as treatment duration.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cpme.2019.100085.

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