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Evaluation the effectiveness of combinative treatment of cold plasma jet, Indonesian honey, and micro-well dressing to accelerate wound healing



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ABSTRACT

A combinative treatment of cold plasma jet, honey solution, and micro-well dressing to accelerate acute wound healing of mouse skin was evaluated. A honey solution 1% in phosphate-buffered saline (PBS) 20 μ L was dropped into micro-well dressing that is attached on an acute wound before a 2-min cold plasma treatment. An inclined treatment style plasma jet with a 45° gradient was used. An infrared thermal camera was used to monitor the fate of the solution. To evaluate its effect, macroscopic evaluation and general staining were conducted. It was revealed that a combination of that honey and plasma treatment may not be efficacious. However, the related procedure functionalising micro-well dressing may provide a new insight in how to combine plasma jets and other solutions in animal or human models for skin-oriented treatment.

1. Introduction

Generally, wound healing has been divided into 3 overlapping stages: inflammation, granulation tissue formation, and matrix formation and remodeling [1]. In wound care management, it is well known that there are many modalities, beside standard wound care, that have the ability to improve wound healing, from natural products like hormones [2,3] and honey [4,5] to physical tools, like light [6]; however, it is understood that very few can cope with conditions in the wound bed throughout all stages of healing [7]. It is therefore very important to explore a new approach in order to get fine treatment that meets the requirements of the wound. A combination treatment of cold plasma jet and honey may provide new insights.

Cold plasma is a so-called non-equilibrium plasma, in which the gas temperature is much lower than the electron temperature. In the study of biomedical application of plasma (plasma medicine), it is established that the innovative value of plasma treatment may be associated with

its possibility of producing a family of exogenous biological molecules, namely reactive oxygen and nitrogen species (RONS) [8]. It is a remarkable fact that small molecules containing RONS have pivotal roles in biological systems [9]. In a biomedical sense, they not only have physiological effects, like influencing cell proliferation, cell adhesion and spreading, wound healing, and growth hormones, but also pathophysiological effects, like influencing atherosclerosis, diabetes, and cancer [10]. In recent years, cold plasma has demonstrated many possible applications in medical scope such as for disinfection of the human body or teeth [11–14], sterilization of medical equipment [15,16], and cancer therapy [17,18]. The possibility of potentiation of wound healing by cold plasma has also been the focus of attention in many recent investigations [19–23].

Even though there are a broader range of atmospheric plasma sources, cold plasma jets have been chosen one of the most favourite sources for plasma medicine application because the plasma can be extended to regions not limited by electrodes [24]. Referring to Lu, a

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plasma jet consists of 2 main conditions with distinctive characteristics: (a) a plasma condition containing relatively short-lifetime radicals, such as N_2^+ , O_2^+ , OH, and N_2^+ ; and (b) an afterglow condition containing relative long-lifetime radicals, such as OH, O, O_3 , NO, and some metastable molecules, such as $O_2(a)$ and $N_2(A)$ [25]. On the basis of our previous report, it was found that a non-contact treatment style of plasma jet (afterglow condition) was able to accelerate skin wound healing in animal models [20]. Moreover, such wound healing acceleration can be optimised by adding distilled water droplets during the treatment [21]. It was suspected that water is able to enhance the production of reactive species of plasma jet, like hydrogen peroxide (H_2O_2) and nitrogen based species, that, at an appropriate concentration, can improve wound healing [26,27]. Jablonowski et al. [28] stated that the interaction processes phase between plasma and liquid has been identified to be the pivotal key to understanding the detailed mechanisms of the effects atmospheric pressure plasma has on living systems. Considering that water is in a liquid phase, it is hypothesised that the other type of solution, honey, may also be compatible to be combined with a plasma jet to support wound healing.

Honey has been implemented for wound therapy since ancient times but medical-grade honey dressing was developed in the late 1990s [7]. It is now realized that honey, like Indonesian honey [4], is a biologic wound dressing with many biological activities that work in concert to support the healing processes. Honey is a potential source of natural antioxidants that become more active in dilutions [5]. Honey in dilution with a low concentration of H_2O_2 showed the fast cytotoxic effect on mammalian cells [30,31]. Furthermore, its antioxidant and anti-inflammatory activity provide a favourable bioambience for wound healing.

This research was conducted to evaluate the effectiveness of a combinative treatment of cold plasma jet and micro-well dressing containing honey to accelerate acute wound healing in an animal model. A micro-concentration of Indonesian honey solution (1% in PBS) in micro-volume (about 20 μ L) was dropped into a micro-well of hydrocolloid dressing that covered an acute wound before plasma treatment. Micro-well dressing is familiar dressing for clinical wound care that was modified by making a single hole (diameter \sim 1 mm) in it. During treatment, an infrared thermal camera was applied to monitor the fate of the honey solution.

2. Experiment

2.1. Cold plasma jet system

This research used a cold atmospheric pressure plasma jet system with an inclined style of treatment (gradient = 45°) as shown in Fig. 1.

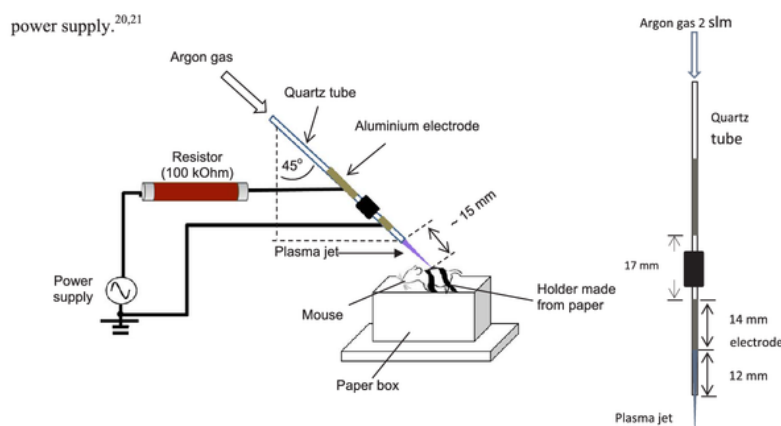


Fig. 1. Experiment setup.

This system was developed based on Teschke et al. [32] Medical-grade argon gas (99.999% purity) produced by the Samator Company (Indonesia) was used as a carrier gas. Two aluminium foil ring electrodes were used around the quartz tube for this system. It had a quartz tube with a 1.5 mm inner diameter and a 2.7 mm outer diameter. The quartz tube was produced by the Fujiwara Company (Japan). The distance between the 2 electrodes was 17 mm. The lower ring electrode was connected to the ground. A low-frequency (\sim 20 kHz) AC high voltage, with a peak-to-peak voltage of 6.67 kV, was applied to the upper ring electrode when argon gas at a flow rate of 2 standard liter per minute (slm) was injected from one end of the quartz tube. A high-voltage probe and a current probe were applied to measure the discharge voltage and discharge current to estimate the consumed power of the power supply [20,21].

2.2. Thermal and safety evaluation of cold plasma jet on normal skin

The hair of an anaesthetized BALB/c mouse was shaved a day before treatment. It was anaesthetized via injection of ketamine-xylazine, (K) 50 mg/kg + (X) 5 mg/kg, into the peritoneal cavity [33]. The mouse was treated with a cold plasma jet under the following conditions: argon gas flow rate = 2 slm; peak-to-peak voltage = 6.67 kV; nozzle tip-skin distances (d) = 5, 10, 15, and 20 mm as shown in Fig. 1; and treatment time = 4 min. The dorsal skin of the mouse was treated with different nozzle tip-skin distances on different spots so that there were 4 spot samples. A digital camera (Panasonic Lumix FH6) was used to document the experimental conditions both during and after treatment. During treatment, the temperature distribution of the treated skin and its surroundings was measured using a low-cost non-contact infrared thermal camera (FLIR C2, Sweden). Using this device, about 5 images from each sample were produced over the 4 min treatment time. The skin condition after treatment was then visually observed.

The relationship between nozzle tip-skin surface distance and ΔT was evaluated. ΔT was calculated as $T_p - T_{ni}$, in which T_p is the peak temperature spot of the skin under plasma treatment and T_{ni} is the temperature spot on the skin with no plasma treatment. T_p and T_{ni} were obtained from thermal images processed using FLIR Software Tools. A colour palette provided by FLIR Software Tools, namely Medical, was used to express images.

2.3. Honey solution

A commercial Indonesian pure honey (Madu Murni Nusantara, Solo, Indonesia) diluted in PBS with a low concentration (1%) was applied in this research.

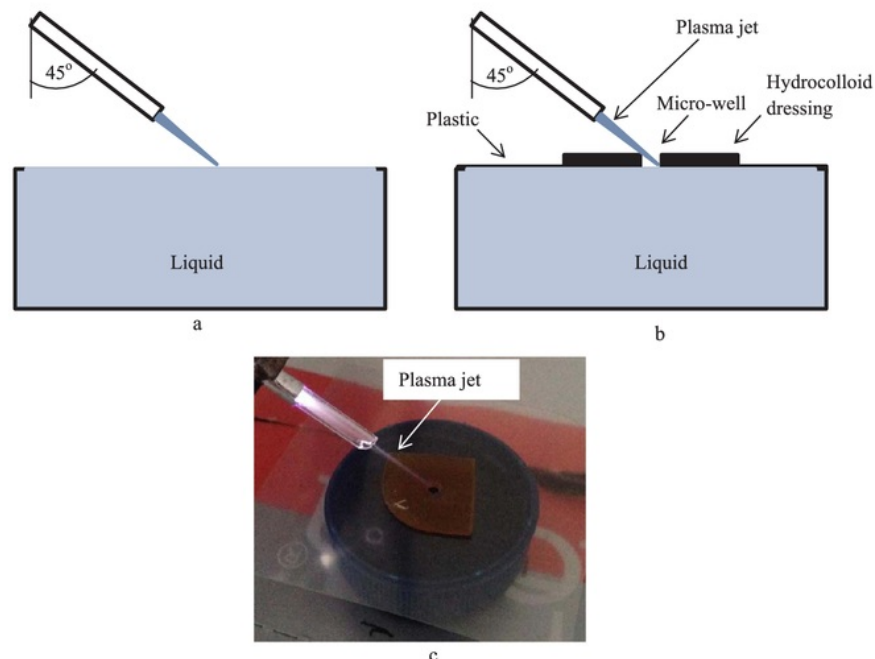


Fig. 2. Two inclined styles of plasma jet treatments for H_2O_2 identification: (a) plasma jet treated on liquid directly (P); (b, c) plasma jet treated on liquid through micro-well dressing containing a 1 mm hole (PMWD).

2.4. Micro-well dressing

Micro-well dressing is hydrocolloid dressing (Tegaderm; 3 M Health Care, USA) that was modified by making a single hole (diameter ~ 1 mm) in it.

2.5. Evaluation of H_2O_2 in the liquid phase

Evaluation of H_2O_2 in the liquid phase was conducted under 2 inclined styles of plasma jet treatment as shown in Fig. 2.: (a) plasma jet treated on liquid directly (P); (b) plasma jet treated on liquid through micro-well dressing containing a 1 mm hole (PMWD). For every style, liquid, namely 5 ml of pure water, in a plastic vessel was treated with a plasma jet under the following parameters: argon gas flow rate = 2 slm; peak-to-peak voltage = 6.67 kV; nozzle tip-solution surface distance = 15 mm as shown in Fig. 1; and treatment time = 1, 2, 3, 4, and 5 min. H_2O_2 in pure water was analysed by a peroxidase enzyme method using a commercial reagent (Kyoritsu Chemical-Check Lab., Model WAK-H2O2, range: 0.05–5.0 mg/L) immediately after cold plasma treatment. This method was also previously used by other researchers [33]. The presence of H_2O_2 was identified based on the change of the liquid's colour and then was confirmed using the absorbance rate change of a UV–vis absorption spectrophotometer using the absorption peak at 540 nm.

2.6. Identification of the possible reduction of reactive oxygen species of a plasma jet by honey

Considering that honey has antioxidant abilities, it is hypothesised that reactive oxygen species produced by a plasma jet may experience a reduction when they immersed in it. To test it, the rate of H_2O_2 in PBS and that of honey 1% solution pursuing plasma jet treatment were compared. PBS 5 ml and honey 1% 5 ml in separate plastic vessels were treated with a plasma jet under the following parameters: argon gas flow rate = 2 slm; peak-to-peak voltage = 6.67 kV; nozzle tip-solution surface distance = 15 mm as shown in Fig. 1; and treatment time = 2, 4,

and 6 min. Only an inclined style of plasma jet treatment without micro-well dressing as shown in Fig. 2a was implemented. The identification procedure of H_2O_2 as described in point 2.5 was then developed.

2.7. Animals and experimental protocol

This research was conducted in Research Unit of Experimental Wound Healing Muhammadiyah University of Magelang, Central Java, Indonesia. The experimental protocol and animal care were in accordance with 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 (certificate number: 00008/04/LPPT/2016). LPPT UGM is accredited under ISO/IEC 17025 and the National Accreditation Committee of Indonesia (KAN Akreditasi Nasional/KAN, Indonesia). Forty-eight BALB/c CrSlc male mice aged 8 weeks and weighing 21.3–28.0 g purchased from LPPT UGM were used. Mice were caged individually in an air-conditioned room at $28.0 \pm 2.0^\circ\text{C}$ with light from 09:00 to 21:00 h and under ad libitum feeding conditions.

2.8. Wound healing model and plasma treatment

After completely anaesthetized via injection of ketamine-xylazine, (K) 50 mg/kg + (X) 5 mg/kg, into the peritoneal cavity [34], 2 circular (4 mm in diameter) full-thickness skin wounds including the panniculus on both sides of the dorsum of the mouse were made with a sterile disposable biopsy punch of 4 mm (Kai Industries Co. Ltd., Gifu, Japan) following a described technique [20]. Considering the evaluation results of H_2O_2 concentrations as shown in Figs. 6 and 7, a plasma jet with an inclined style of treatment was conducted once daily for 2 min over 14 days. The position of the wound surface was about 15 mm from the nozzle tip with a defined slope (45° gradient) as shown in Fig. 1. In the plasma-treated group (P), the tip of the plasma jet touched the wound surface. The daily room temperature and room humidity during the 14 days of the experiment represented a tropical

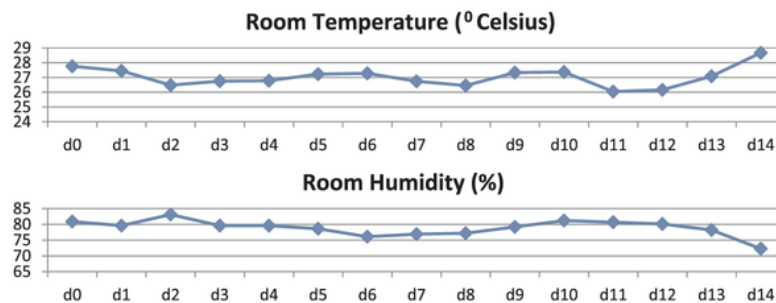


Fig. 3. Room temperature and room humidity during the experiment.

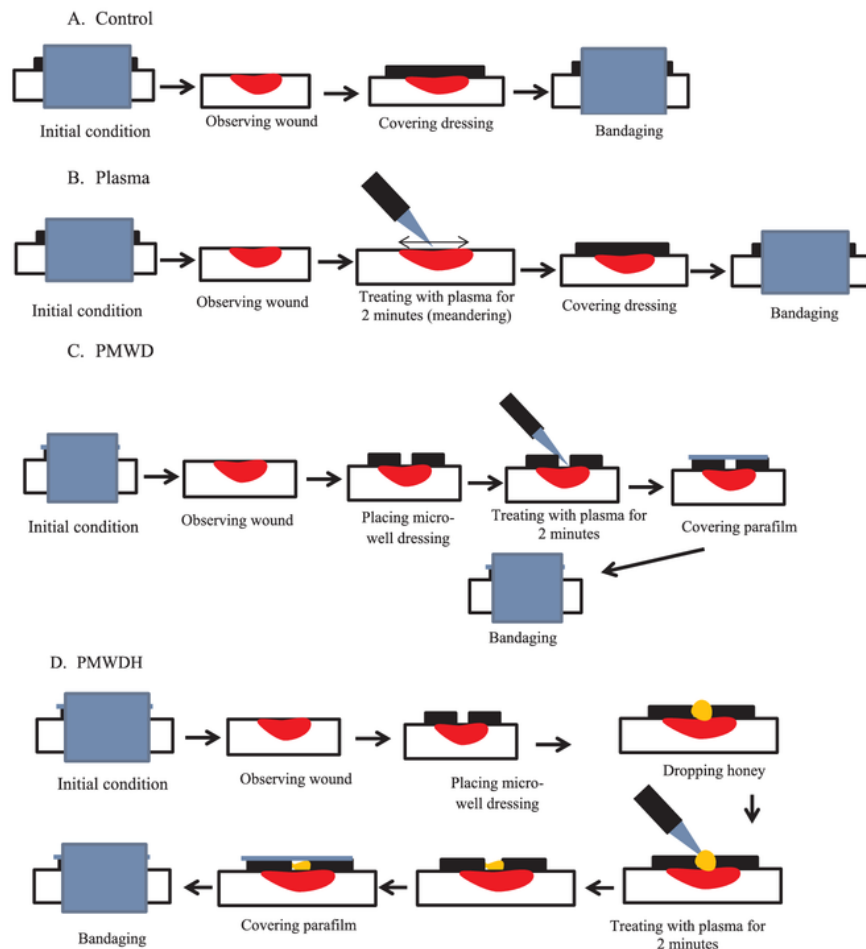


Fig. 4. Experimental procedure.

room condition as detailed in Fig. 3.

The experimental procedure after day 0 is shown in Fig. 4. Generally, the mice were randomly classified into 4 groups:

- Control group (C): Wounds were allowed to heal daily under hydrocolloid dressing (Tegaderm; 3 M Health Care, USA) to maintain their moist environment. To maintain hydrocolloid dressing coverage of the wound, the mouse body was then bandaged.
- Group with plasma treatment only (P): Wounds were given the plasma jet treatment for 2 min with a meandering style to ensure the overall surface received the treatment and then covered with

hydrocolloid dressing. To maintain hydrocolloid dressing coverage of the wound, the mouse body was then bandaged.

- Group with combination treatment of PMWD: Wounds were covered by micro-well dressing. Wounds were then treated by plasma jet for 2 min through a 1 mm hole in the dressing. After treatment, a micro-well was then covered by parafilm (Bemis Company, Neenah, Wisconsin, USA) and the mouse body was then bandaged.
- Group with combination treatment of plasma with micro-well dressing and honey solution (PMWDH): Wounds were covered by micro-well dressing. A 1 mm hole in the dressing was then dropped by a honey solution 20 μ L and treated by plasma jet for 2 min. After

treatment, the micro-well was covered by parafilm and the mouse body was then bandaged.

During the 14 days treatment period, wound dressing and bandage in all groups were removed and renewed every day for plasma treatment and/or wound evaluation.

2.9. Evaluation the presence of the honey solution during cold plasma treatment


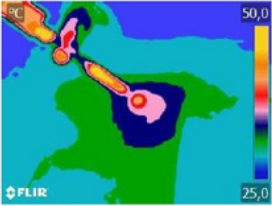
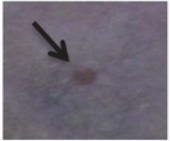
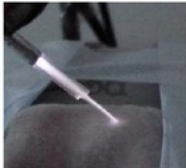
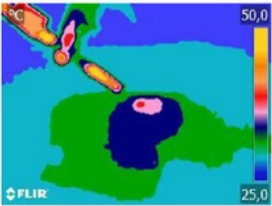
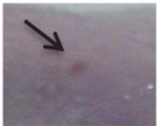
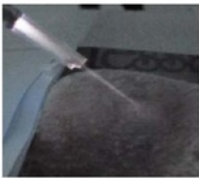
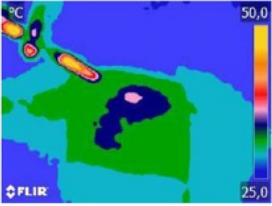

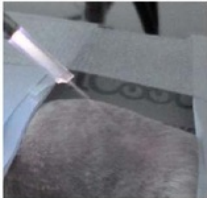
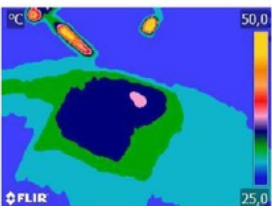

It was reported that plasma jet treatment able to cause the liquid solution to move out from wound surface [21]. In this research, the presence of the honey solution that was dropped on the micro-well dressing was monitored using a low-cost non-contact infrared thermal

camera (FLIR C2, Sweden).

2.10. Macroscopic evaluation of wound

The day when the wounds were created was designated as day 0, and the process of wound healing was observed daily from day 0 to day 14. Before observation, the environment surrounding the wounds was cleaned with saline solution. Wound edges were traced on polypropylene sheets and photographs were taken every day. The traces on the sheets were captured with a scanner onto a personal computer using Adobe Photoshop Elements 7.0 and the areas of the wounds were calculated using the image analysis software Scion Image Beta 4.02 (Scion Corporation, Frederick, Maryland, USA).

Table 1
Representative skin images used to evaluate the safety effects of plasma jet treatment as a function of nozzle tip-skin distance (d). Temperature range adjusted between 25 °C and 50 °C.

d (mm)	During treatment	During treatment (infrared image, visualised using medical mode)	$T_{\max};$ ΔT (°C)	Post-treatment condition
5			$41.64 \pm 0.48;$ 7.50 ± 0.40	
10			$39.06 \pm 0.09;$ 5.44 ± 0.21	
15			$37.02 \pm 0.19;$ 3.56 ± 0.36	
20			$36.48 \pm 0.16;$ 3.30 ± 0.70	

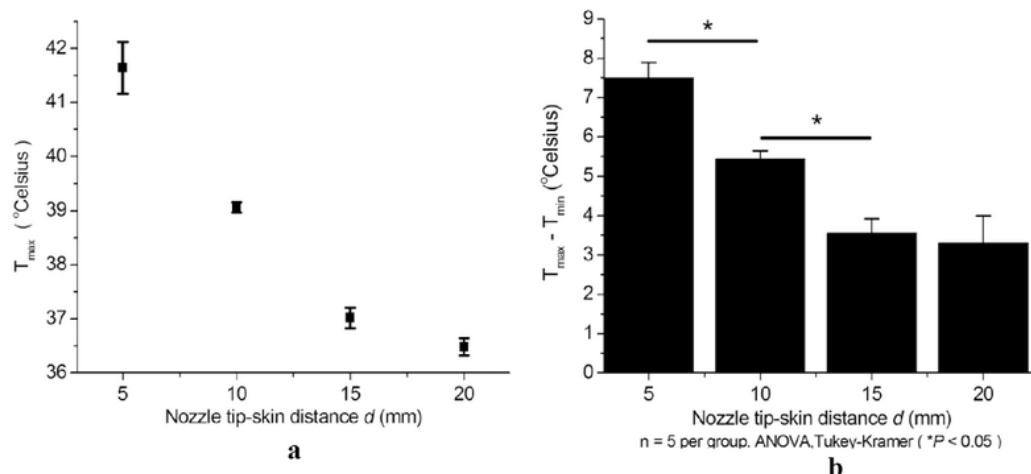


Fig. 5. a. The graph shows the relationship between d and T_{max} . b. The histogram shows the relationship between d and ΔT .

2.11. Prediction of healing day

The day of wound healing was predicted based on visual inspection of the wounds using a graph of the ratio of the wound area to the wound's initial area. Initially, the overall trend of this graph was evaluated. The healing day was plotted on the y-axis when the trend of reduction of the wound size started to become flat, as reported previously [20,21], which was at 0.33 (see Fig. 8a). From that plotted point, line z was made, crossing the lines of reduction for each group. Wound healing days at points P*, PMWD*, PMWDH*, and C* were then made based on the lines from their meeting points to the x-axis.

2.12. Tissue processing

The mice were euthanised by a massive injection of ketamine-xylazine via IP injection on day 3, 7, 11, or 14 post-wounding. 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 to prevent over-contraction of the sample and fixed in neutral buffered 10% formalin solution in 0.01 M phosphate buffer, pH 7.4, for about 15 h. The samples were then rinsed in 0.01 M PBS for about 8 h. Subsequently, they were dehydrated in an alcohol series, cleaned in xylene, and embedded in paraffin to prepare serial 5- μ m sections. The sections involving the wound centre were then stained with haematoxylin-eosin (HE).

2.13. Microscopic observations: measuring re-epithelialisation

On the basis of the results of haematoxylin-eosin staining, the percentage of re-epithelialisation was calculated using formulation [20]:

$$\text{Reepithelialisation(\%)} = \frac{\text{length of new epithelium}}{\text{length of wound between wound edges}} \times 100\%$$

Four to six sections were used from 3 different mice on days 3, 7, 11, and 14 after wounding.

2.14. Statistical analysis

Data were subjected to statistical analyses using SPSS 16.0. The H_2O_2 concentration, the mice skin temperature, the ratio of the average wound area to the original wound area, the number of days of wound healing, and the results of microscopic count were evaluated by ANOVA followed by the Tukey-Kramer method; P values < 0.05 were considered significant.

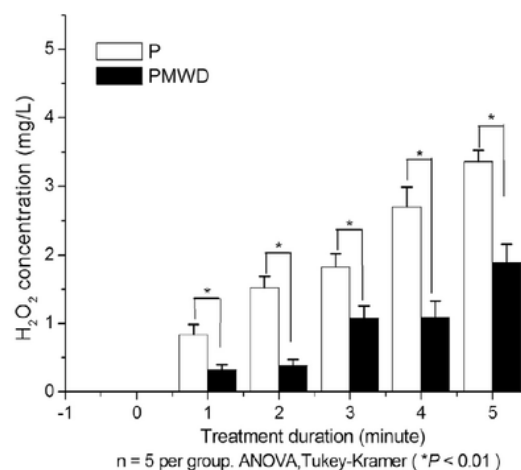


Fig. 6. Relationship between plasma treatment duration both in P and PMWD and H_2O_2 concentration.

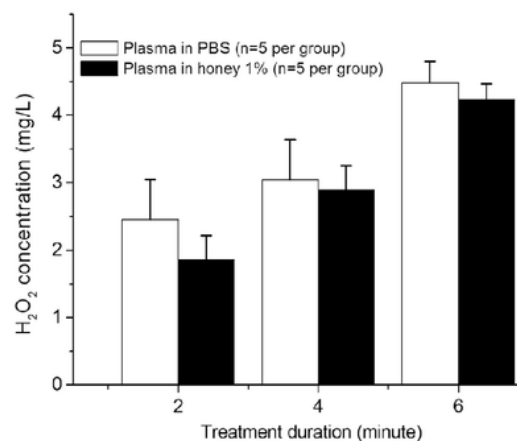


Fig. 7. Relationship between plasma treatment duration both in PBS and honey 1% and H_2O_2 concentration.

3. Results

3.1. Safety aspects of the cold plasma jet on normal mice skin

Visually it was revealed that the shorter d resulted in a brighter plasma jet. Visually it was also shown that at 5, 10, and 15 mm the plasma jet touched the skin, while at 20 mm the plasma jet did not touch the skin. A uniform temperature distribution on the skin under plasma treatment at 15 and 20 mm was observed with peak temperatures of 37.02 ± 0.19 °C and 36.48 ± 0.16 °C (pink band), respectively. On the other hand, a multiform temperature distribution on the skin under plasma treatment at 5 and 10 mm was observed with peak temperatures at their centres reaching 41.64 ± 0.48 °C (yellow band) and 39.06 ± 0.09 °C (red band), respectively. Unfortunately, injury was observed after plasma treatment at 5 and 10 mm, whilst this was not observed at 15 and 20 mm (Fig. 1). Additionally, plasma treatment with nozzle-skin distances of 5 mm, 10 mm, 15 mm, and 20 mm caused ΔT (elevated skin temperature) of 7.50 ± 0.40 °C, 5.44 ± 0.21 °C, 3.56 ± 0.36 °C, and 3.30 ± 0.70 °C, respectively (Fig. 5).

3.2. Identifying the presence of H_2O_2 in the liquid phase

On the basis of the results as shown in Fig. 6, it was indicated that at an applied distance of 15 mm, the plasma jet generated H_2O_2 in pure water. The plasma jet also produced reactive oxygen species in open air that was able to penetrate the micro-well dressing. Additionally, in P and PMWD, the concentration of H_2O_2 tended to increase due to the increase of the plasma treatment duration. But the concentration of H_2O_2 in P was higher than in PMWD.

3.3. Reactive oxygen species of plasma jet in PBS and honey solution

Based on Fig. 7 it was shown that the plasma jet generated H_2O_2 both in PBS and honey 1% solution. Although there were no statistically

significant differences between H_2O_2 concentration in PBS and that in honey 1% ($P > 0.05$), the former was relatively higher than that in the latter. Since honey has an antioxidant ability to reduce reactive oxygen species, it would be reasonable that the concentration of H_2O_2 in honey 1% is lower, suggesting an antioxidant ability of Indonesian honey that reduces the production rate of reactive oxygen species of plasma in liquid.

3.4. Evaluation the presence of dropped honey on micro-well dressing during plasma treatment

During plasma treatment, the honey solution was identified on the single hole of the micro-well dressing in PMWDH (blue dot). Yellowish dot (arrow) shown the hole of microwell dressing without honey. The hole without honey solution was also identified in PMWD (yellowish dot). Additionally, it was shown that the plasma jet was visually brighter in PMWDH than in PMWD (Table 2).

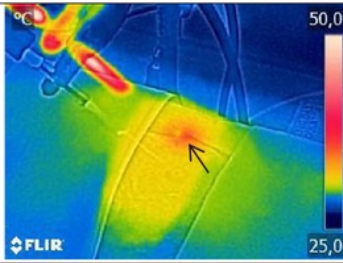
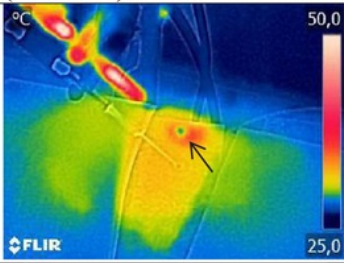

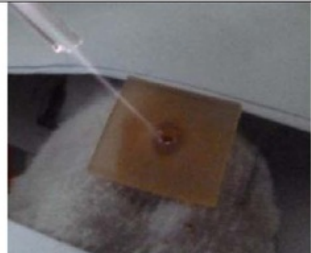
3.5. Macroscopic observation

Wounds in all groups were evaluated from day 0 to day 14, as shown in Fig. 8. They expanded due to oedema during a few initial days and then gradually decreased in size until the end of the observation period. Quantitatively, wound sizes for P, PMWD, and PMWDH on days 7, 11, and 14 were smaller than those for C. From day 12, in PMWD and PMWDH, however, a protrusion was observed in the centre of the wounds. On day 14, the wound surfaces in all groups were mostly fresh.

3.6. Wound area reduction and day of wound healing

Generally, there were resembled patterns with respect to wound area reduction in all groups, as shown in Fig. 9a. They expanded during a few initial days and then gradually decreased in size until the end of the observation period. On observation days 4–13, wound sizes in P are significantly lower compared to those in C ($P < 0.05$). During those

Table 2
Conditions during plasma treatment for the PMWD and PMWDH groups.

Experimental Group	
Plasma treatment with micro-well dressing (PMWD)	Plasma treatment with micro-well dressing and dropped honey (PHMWD)
<p>Conditions during plasma treatment (images produced by an infrared thermal camera using rainbow palette- Thermal MSX)</p> 	
<p>Conditions during plasma treatment (images produced by digital camera)</p> 	

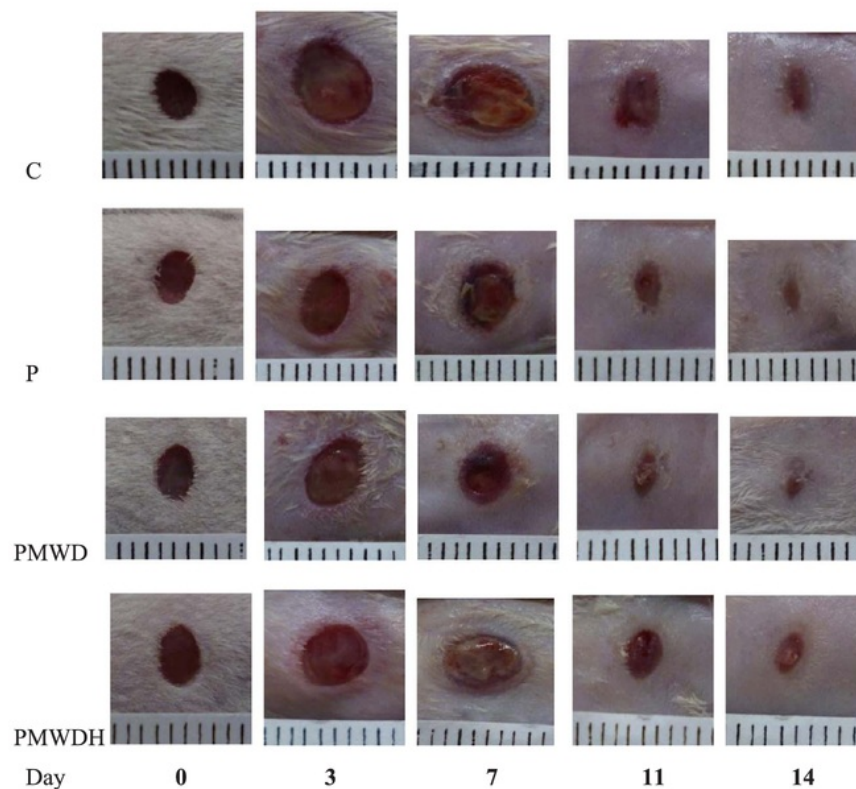


Fig. 8. Macroscopic observation of the wounds on days 0, 3, 7, 11, and 14.

days, wound sizes in P are almost same as those in PMWD and are lower than those in PMWDH in which the 2 means were not significantly different (P vs PMWD: $P > 0.05$; P vs PMWDH: $P > 0.05$). On observation days 3–5 and 12–13, wound sizes in both PMWD and PMWDH are lower than those in C, but the 2 means were not significantly different (PMWD vs C: $P > 0.05$; PMWDH vs C: $P > 0.05$). On the other hand, on observation days 6–9, wound sizes in PMWD and PMWDH are significantly lower compared to those in control (PMWD vs C: $P > 0.05$; PMWDH vs C: $P > 0.05$). At the end of the observation period, wound sizes in P, PMWD, and PMWDH are almost the same. Although there were no statistically significant differences between P and C, or between PMWD and C, or between PMWDH and C, the wound size of P, PMWD, and PMWDH were smaller than C.

Wound healing days for C, P, PMWD, and PMWDH were 14.0 ± 2.8 , 10.0 ± 0.6 , 10.5 ± 1.3 , and 11.8 ± 1.5 days, respectively, as shown in Fig. 9b. The day of wound healing for P was significantly earlier, by 4 days, than that for C ($P < 0.01$). The day of wound healing for PMWD was also significantly earlier, by 3.5 days, than that for C ($P < 0.05$). Although there were no statistically significant differences between PMWDH and C, the day of wound healing for PMWDH was earlier than that for C by about 2 days.

3.7. Re-epithelialisation

Re-epithelialisation during healing was evaluated on days 7, 11, and 14 (Fig. 10). On day 7, the re-epithelialisation percentage for the experimental groups (P, PMWD, and PMWDH) was higher than that of the control group. Two means between C and P and between C and PMWDH were significantly different (C vs P: $P < 0.01$; C vs PMWDH: $P < 0.05$); however, that between C and PMWD was not significantly different ($P = 0.062$). In the experimental groups, the re-epithelialisation percentage for P was higher than that of PMWD and PMWDH, but 2

means were not significantly different (P vs PMWD: $P > 0.05$; P vs PMWDH: $P > 0.05$).

The re-epithelialisation percentage for all groups increased dramatically from day 7 to 11. On day 11, the re-epithelialisation percentage for the experimental groups was higher than that of control group, but 2 means between C and every member of the experimental groups were not significantly different. On day 14, a new epithelium completely covered all of the wounds.

4. Discussion

Studies of plasma medicine for acute wounds using an animal model mimicking a clinical setting, as applied in this experiment, were conducted previously [20,21]. Compared to those, the trend of the healing acceleration following plasma jet treatment for all groups within this experiment may generally be the same. First, the pattern of the reduction graph for all plasma-related groups (P, PMWD, and PMWDH) is similar to that of the hydrocolloid treated group (C). Wound sizes increased during the inflammation phase and then decreased gradually during later phases. Second, it is shown that a plasma jet may have significant effects during inflammation and granulation tissue-formation phases. Plasma also promoted re-epithelialisation as well as wound contraction. Based on recent and previous results, it is indicated that a cold plasma jet can be controlled so that it becomes adaptable with standard treatments of modern wound healing using dressing.

Concerning the prediction of the healing day, however, this experiment produced the best result compared to previous studies. This experiment showed that the day of healing in the plasma-treated group (P) was 4 days earlier than that in the control group. Table 3 reveals the summary of the conditions and results of research previously in comparison with this research. The plurality of the results may be

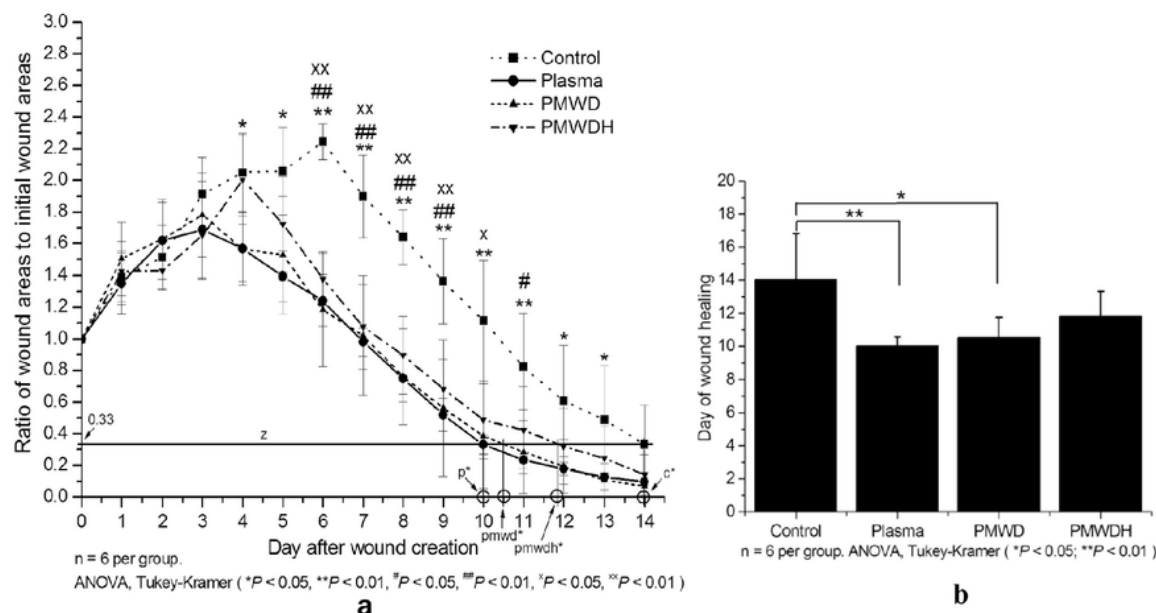


Fig. 9. a) Ratio of wound areas to initial wound areas during healing. On days 6–10, the sizes of the wounds in PMWDH were significantly smaller than in C; however, they were slightly larger than those in PMWD and P. Note that * shows the significance level of P to C, while # and X show that of PMWD to C and that of PMWDH to C, respectively. At point 0.33 on the y-axis, it was assumed that a new epithelium covered the wound surface completely in all samples for all groups. Points P*, PMWD*, PMWDH*, and C* show days of wound healing prediction for P, PMWD, PMWDH, and C, respectively. b) A histogram regarding days of wound healing prediction for every group. C, P, PMWD, and PMWDH were 14.0 ± 2.8 , 10.0 ± 0.6 , 10.5 ± 1.3 , and 11.8 ± 1.5 days, respectively.

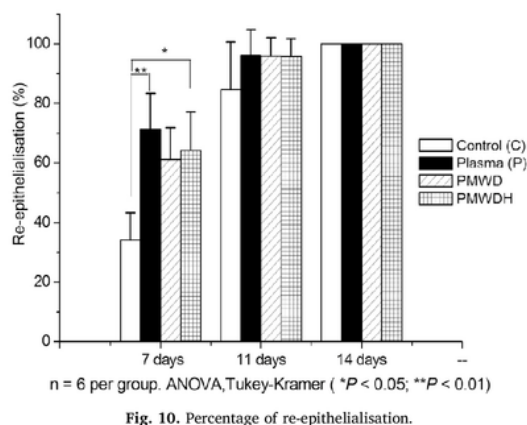


Fig. 10. Percentage of re-epithelialisation.

correlated with the plurality of applied plasma parameters and treatment modes.

In the plasma-treated group (P), especially, the meandering style of treatment was conducted to ensure that RONS produced by the plasma jet could reach all parts of the wound surface. Application at a distance of about 15 mm was preferred after considering the plasma jet's effect on normal skin. At that distance, interestingly, although the tip of the jet touched the skin surface, plasma did not cause detrimental effects. This condition was not reached in our experiment previously [22]. Also, this experiment suggests that plasma jets may not cause detrimental effects on skin if ΔT is less than 4°C .

This research found that on day 7, re-epithelialisation percentages for the experimental groups was higher than for the control group, but varied within their percentages. Only plasma treatment is the most effective group; however, PMWDH was almost same with PMWD. It is thought that those results may be comprehensively influenced by the wound's condition during and after treatments as described in Fig. 11.

The coverage area of plasma jet activation on a wound surface may

become a crucial factor during treatment [20], while moist and dry wound conditions may become dominant factors after treatment [39]. Table 4 details possible logical relationships between conditions, possible effects, and results in connection with re-epithelialisation for every group. Re-epithelialisation for C was only supported by a moist condition under dressing; however, that for P was supported not only by a moist condition during post-treatment but also plasma activation in all parts of the wound surface during its treatment.

During treatment, re-epithelialisation for PMWD and PMWDH was promoted by the plasma jet through spot-like via the 1 mm hole in the dressing, on the centre of the wound surface. After treatment, it was promoted by a moist condition excluding the wound centre. But the dry condition on the wound centre in PMWD impeded it. Re-epithelialisation in PMWDH may be promoted not only by a moist condition, but also by plasma-activated honey on the wound centre. Considering the results in PMWD and PMWDH, Indonesian honey with low concentration 1% in PBS, however, may have no additional supportive effect and a combination of such honey and plasma treatment may not be useful.

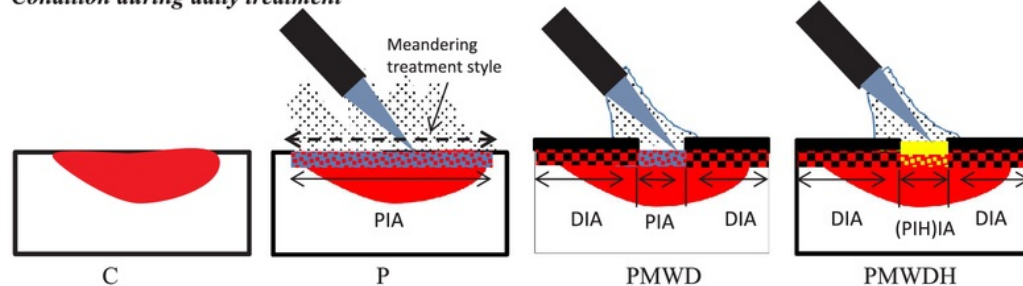
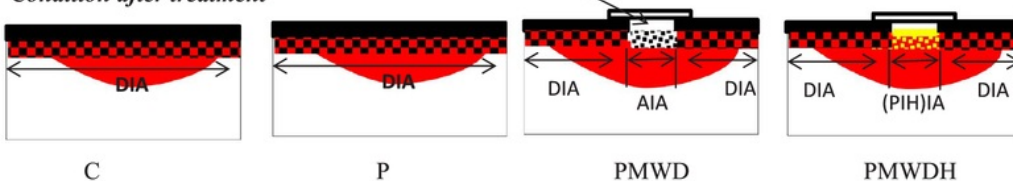
Recently, plasma-activated water (PAW) or plasma-treated water (PTW), that is, water exposed to low-temperature atmospheric pressure plasma, has become one of the most attractive studies in plasma medicine [28,35]. The effectiveness of a combinative treatment of plasma jets and distilled water for wound healing was reported previously [21]. After the inflammation phase, however, it was observed that during plasma treatment, water tended to move out from the wound due to the histological stiffness of the wound during maturation. In this experiment, a micro-volume honey solution was able to be kept using micro-well dressing during 14 days of the experiment. However, while distilled water enhanced the production of hydrogen peroxide, honey reduced it due to its possible antioxidant activity. Although the honey solution did not optimise the plasma jet to significantly accelerate wound healing, the related procedure functionalising micro-well dressing may provide a new insight in how to combine plasma jets and other solutions in animal or human models for skin-oriented treatment.

Table 3

Conditions and results of plasma medicine for wound healing using an animal model mimicking a clinical setting as reported previously in comparison with this research result.

No	Plasma treatment condition	Wound type and treatment mode	Prediction of healing day for each group (days)
Nasruddin et al. [20]	a. Working voltage = 25 kVp-p b. Electrodes distance 1 mm c. SIm = 5 d. Treatment duration = 1 min	a. Acute, 2 mm in diameter b. Perpendicular and on the spot style c. Plasma jet on the spot and not touching the wound	C = 9.0 ± 1.3 P = 8.0 ± 0.6
Nasruddin et al. [21]	a. Working voltage = 25 kVp-p b. Electrodes distance 1 mm c. SIm = 5 d. Treatment duration = 1 min	a. Acute, 4 mm in diameter b. Perpendicular and on the spot style c. Plasma jet on the spot and not touching the wound	C = 12.6 ± 1.6 P = 12.2 ± 1.4 PW = 10.8 ± 1.2
This research	a. Working voltage = 6.67 kVp-p b. Electrodes distance 17 mm c. SIm = 2 d. Treatment duration = 2 min	a. Acute, 4 mm in diameter b. Style with 45° in gradient, meandering style c. For the P group, the plasma jet touches all wound surfaces (meander style) d. For PMWD and PMWDH, the plasma jet focused on the micro-well	C = 14.0 ± 2.8 P = 10.0 ± 0.6 PMWD = 10.5 ± 1.3 PMWDH = 11.8 ± 1.5

C, control or only hydrocolloid dressing treatment; P, plasma or only plasma jet treatment; PW, combinative treatment of plasma and water; PMWD, combinative treatment of plasma and micro-well dressing; PMWDH, combinative treatment of plasma, micro-well dressing, and honey solution 1%

Condition during daily treatment**Condition after treatment**

C, control or only hydrocolloid dressing treatment; P, plasma or only plasma jet treatment; PMWD, combinative treatment of plasma and micro-well dressing; PMWDH, combinative treatment of plasma, micro-well dressing, and honey solution 1%; DIA, dressing-influenced area; PIA, plasma-influenced area; (PIH)IA, area influenced by plasma-influenced honey; AIA, air-influenced area

**Fig. 11.** Wound conditions during and after plasma jet treatments.

It is well known that temperature is one of the most important aspects [7] in respect to clinical plasma treatment [36]. Determination of the safe distance between the nozzle tip of the plasma jet reactor and the target (animal skin) using an infrared thermal camera, namely Nec Avio F30S, was reported previously [21]. Clinically, infrared thermal imaging has been applied to many areas within wound- and skin-related care [37] and plastic surgery [38]. Presently, thermal imaging cameras are becoming smaller, more sensitive, and cheaper. At this time, this

research has shown that a low-cost [7] infrared thermal camera has the capability to determine the safe distance between the nozzle tip of a plasma jet reactor and its target. Additionally, this device can also be used to monitor the fate of a micro-volume of honey solution dropped into micro-well dressing that is attached to a wound surface during its activation using a plasma jet.

Table 4
Possible logical relationship between condition, possible effects, and results in connection with re-epithelialisation for every group.

Group	Conditions	Possible effects		Results from this experiment	
		After treatment	During treatment	After treatment	
C		Wound under dressing (moist condition)		1. Moist condition improved re-epithelialisation [39]	
P	All parts of wound surface (centre and periphery) exposed by plasma	Wound under dressing (moist condition)	All parts of wound surface (centre and periphery) activated by plasma [20]	1. Plasma improved re-epithelialisation [39]	On day 7, re-epithelialisation more improved compared to C, PMWD, and PMWDH
PMWD	Only centre part of wound surface exposed by plasma via micro-well dressing (PIA)	1. AIA under local air (dry condition) 2. DIA under dressing (moist condition)	Only centre part of wound surface activated by plasma	2. Moist condition improved re-epithelialisation [39]	
				1. Moist condition improved re-epithelialisation [39]	On day 7, re-epithelialisation more improved compared to C, but not more improved compared to P and PMWDH
PMWDH	Only centre part of wound surface exposed by honey-influenced plasma in micro-well dressing	1. (PIH)A under plasma-influenced honey 2. DIA under dressing (moist condition)	Possible only centre part of wound surface activated by plasma-influenced honey	3. Dry condition impeded re-epithelialisation [39]	
				1. Moist condition improved re-epithelialisation [39]	On day 7, re-epithelialisation more improved compared to C, but not more improved significantly compared to P and PMWD

5. Conclusion

Combinative treatment of a plasma jet, an Indonesian honey solution, and micro-well dressing on experimental wound was investigated. It was revealed that Indonesian honey in low concentration has no additional supportive effect and a combination of that honey and plasma treatment may not be efficacious. However, the functionalization of micro-well dressing in this experiment may provide a new insight in how to combine plasma jet and other solutions in animal or human models for skin-oriented treatment.

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