Plasma Jet Effectiveness Alteration in Acute Wound Healing by Binahong (Anredera cordifolia) Extract

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ABSTRACT: An atmospheric pressure plasma jet (APPJ) using medical-grade argon gas as the carrier gas was developed and used to investigate wound healing in small animals combined with Binahong (Anredera cordifolia) leaf extract treatment. The experiment was divided into 4 treatment groups, control (C), plasma jet (P), Binahong leaf extract (B), and Binahong leaf extract followed by plasma jet (PB). Plasma jet treatment for wounds was applied in different styles based on the treatment day. Both P and PB treatments were contact-style for days 0–4 (5-mm distance for 1-min duration) and noncontact style for days 5–13 (20 mm for 3 min). The total period of wound observation was 14 days. Histological evaluation using hematoxylin-eosin (HE) staining was performed on days 7, 11, and 14 to evaluate wound re-epithelialization. Meanwhile, the number of neutrophil cells was counted using a hematology analyzer on days 11 and 14, and malondialdehyde (MDA) levels were examined on days 7 and 14. This research revealed that plasma jet successfully improved wound healing; on the other hand, plasma jet treatment preceded by Binahong leaf extract treatment tended to impede wound healing. It is hypothesized that Binahong may reduce plasma jet effectiveness in wound healing.

KEY WORDS: plasma medicine, wound healing, pro-oxidant, antioxidant, wound, natural product, Anredera cordifolia
I. INTRODUCTION

Plasma medicine is a multidisciplinary science that encompasses plasma science, pharmacology, life sciences, biomedicine, and other health sciences to functionalize plasma.\(^1\) The plasma referred to in this case is the fourth state of observable matter after solid, liquid, and gas.\(^1\) In plasma, there are reactive substances, namely energetic particles, radicals, and ions, as well as nonreactive substances, namely gases. Conceptually, the medical efficacy of plasma is related to its ability to generate biological molecules, namely reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can be controlled physically, kinetically, and dose-dependently.\(^2\) Roy et al. reported that ROS and RNS in microconcentrations\(^3,4\) have properties of wound healing, whereas in high concentrations it is clear that they have the potential to damage living tissue.\(^5\) The usefulness of atmospheric plasma for wound healing has been reported\(^6–10\); the hindrance effect due to excessive dosage has also been reported.\(^11\) Currently, methods to pair medical plasma with natural products to heal wounds are also being developed.\(^12–14\)

Generally, three overlapping stages comprise acute wound healing, inflammation, proliferation, and remodeling.\(^15\) ROS and RNS, as signaling biomolecules, have a pivotal role in support of such processes.\(^16,17\)

Although ROS and RNS are key players, a recent investigation has shown that the positive biomedical effects of ROS and RNS are also determined by the presence of liquid around the targeted cells.\(^18,19\) There are two principles in this study. First, the effects of plasma are caused by changes induced by it to the liquid zone surrounding cells. Second, liquid phases containing ROS and RNS play a pivotal role in plasma-induced biological responses. Woedtke and Weltmann\(^18\) showed that biological plasma effects are primarily mediated through ROS and RNS influencing cellular redox-regulated processes. It has been considered that plasma medicine includes in its scope the application of biological redox.\(^20\)

There are many treatments to improve wound healing. Possible application of natural agents, such as hormones,\(^21,22\) honey,\(^23,24\) and aloe vera,\(^25\) as well as nonnatural agents, like electrical stimulation\(^26\) and ultraviolet light,\(^27\) have been investigated. The drawback is that very few agents are suitable for all stages of wound healing. Consequently, an investigation to find a new agent with better efficacy is a critical issue in experimental wound studies. A combination treatment of nonnatural agent of plasma medicine with the extract of a natural agent of Binahong (Anredera cordifolia) may provide an alternative.

All parts of the Anredera cordifolia plant can be used as a medicine, but the most commonly used are the leaves. Anredera cordifolia leaf extract maintains active compounds such as flavonoids and saponins, which have anti-inflammatory, antioxidant, and antibacterial properties.\(^28\) Flavonoids can act directly as antibiotics by destroying bacterial cell walls and have antioxidant and anti-inflammatory properties, while saponins function as antiseptics to prevent the growth of microorganisms that occur in wounds.\(^29\)

This research was conducted to evaluate the effectiveness of a treatment combining plasma jet and Anredera cordifolia extract on experimental acute wound healing in a small animal model.
II. MATERIALS AND METHODS

A. Nonequilibrium Atmospheric Pressure Plasma Jet System

Atmospheric pressure plasma jet (APPJ) was applied as developed by Teschke et al., but with modification of the inner and outer diameters of the capillary quartz tube to 0.65 and 1.55 mm, respectively. Electrodes were two conductive rounded materials applied to the tube. Nonconductor material, namely a local clay, Tanah Lempung, was applied to isolate the electrodes.

Characterization of electrical and optical emission was conducted in the Plasma Laboratory of the Research Center for Sustainable Energy and Technology, Institute of Science and Engineering, Kanazawa University, Japan. The working gas was clinical-grade argon of 99.999% purity (Samator Co., Secang, Magelang, Indonesia). Electrical and optical emission characteristics have been described elsewhere.\textsuperscript{11,14}

B. Extract Preparation

Fresh binahong leaves were collected and authenticated by the Laboratory of Biology, Faculty of Mathematics and Natural Sciences, Universitas Ahmad Dahlan, Yogyakarta, Indonesia. The leaves were dried in an oven at 40°C and then crushed into a powder to which absolute ethanol was added.\textsuperscript{28} The resulting thick extract was dissolved by adding 1 g of extract to 10 ml of 10% dimethyl sulfoxide (DMSO) solution and then adding 100 ml of distilled water to achieve a solution with a concentration of 1%.

C. Animals and Investigation Protocol

Fifty-two male BALB/c mice aged 7 to 8 weeks were acquired from Laboratorium Penelitian dan Pengujian Terpadu/Integrated Research and Testing Laboratory (LPPT UGM), Universitas Gadjah Mada, Yogyakarta, Indonesia. The mice were maintained under controlled conditions: 12-h light-dark cycle, 23.0 ± 2.0°C room temperature, and mouse chow and water ad libitum. All procedures were in accordance with animal welfare guidelines and approved by the ethics committee for preclinical investigation at LPPT UGM (Certificate No. 00005/04/LPPT/III/2020). The guidelines are based on ISO/IEC 17025 standards and the National Accreditation Committee of Indonesia (Komite Akreditasi Nasional/KAN, Indonesia).

D. Experimental Design

The mice were anesthetized using ketamine-xylazine injection at a dose of 50 mg/kg (K) and 5 mg/kg (X) intraperitoneally (IP).\textsuperscript{11} The acute wound development procedure in this experiment has been described elsewhere.\textsuperscript{11} The resulting wound was resulted in a round and of full thickness with a diameter of 4 mm on both sides of the dorsum.
There were four experimental groups as follows:
1. Control, hydrocolloid dressing (C)
2. Binahong leaf extract (spray) (B)
3. Plasma jet (P)
4. Binahong leaf extract (spray) followed by plasma jet (PB)

The experimental protocol is shown in Fig. 1. Wounds in group C were treated by hydrocolloid dressing (HD). Wounds in group B were sprayed with 1.0 mL of Binahong leaf extract and covered by HD. Wounds in group P were treated with plasma jet and covered with HD. Wounds in group PB were sprayed with extract and then administered plasma jet and covered with HD. The Plasma jet irradiation treatments were given once daily with a different style based on the day: contact treatment for days 0 to 4 by irradiating the wound using plasma jet with 5-mm distance for 1 min, and noncontact treatment for days 5 to 13 using 20-mm distance for 3 min.

### E. Macroscopic Wound Evaluation

Macroscopic wound evaluations were conducted daily for 14 days, following a procedure described elsewhere. The observed wound conditions were documented using a digital camera (Lumix FH6, Panasonic, Japan). The day when the acute wounds were created was counted as day 0.

### F. Tissue Processing and Re-Epithelialization Measurement

The mice were euthanized on days 7, 11, or 14 after wound creation. The wound and the surrounding normal skin were harvested and bisected at the wound center. Wounds were fixed in neutral buffered 10% formalin solution, pH 7.4, for ± 15 hours, dehydrated in an alcohol series, cleared in xylene, and embedded in paraffin to prepare thin sections. Sections of 4–5-μm thickness underwent hematoxylin-eosin (HE) staining. The percentage of re-epithelialization was observed using a light microscope and then calculated using the formula:

\[
\text{Re-epithelialization} \times 100\% = \frac{\text{length of new epithelium}}{\text{length of wound between wound edges}} \times 100\% \tag{1}
\]

### G. Neutrophil Count

Blood samples were taken by cardiac puncture. The steps were anesthetizing the mice by ketamine-xylazine injection followed by inserting the tip of a 1-mL syringe into the heart cavity through the thorax and collecting the blood in a microtube with 10% EDTA as an anticoagulant. The neutrophil cells were counted by impedance using a hematology analyzer (BC 2600, Mindray, Shenzhen, China).
Malondialdehyde (MDA) level measurement began with the preparation of skin wound tissue samples by weighing 10 mg of tissue, then adding 300 μL of MDA lysis buffer and 3 μL of butylated hydroxytoluene (BHT). The prepared tissues were
centrifuged for 10 min at 13,000 g; then 200 μL of the supernatant was homogenized. After addition of 600 μL of thiobarbituric acid (TBA) solution, the supernatant was incubated at 95°C for 60 min. MDA was reacted with TBA in the presence of BHT to minimize the formation of artifacts. After that, the solution was cooled on ice and centrifuged at 4,000 g for 5 min; then 200 μL of clear supernatant was transferred to the microplate. This method is based on the reaction of free MDA in the sample with TBA to generate an MDA-TBA adduct. The MDA-TBA adduct was quantified colorimetrically with optical density (OD) = 532 nm. The MDA concentration was expressed as nmol/mL.  

I. Statistical Analysis

Data were subjected to statistical analyses using SPSS 21.0. Ratios of the average wound area to the original wound area and percentages of re-epithelialization were evaluated by ANOVA followed by the Tukey-Kramer method in which P values < 0.05 were considered to be significant.

III. RESULTS

A. Macroscopic Observation

Figure 2 shows the observed wounds on days 0, 3, 7, 11, and 14 for every treatment group. The healing in all groups had an analogous pattern, in which the wounds increased in size during the inflammation phase and then decreased gradually during the proliferative and maturation phases. The wound size on day 11 in the P group was much smaller than that in the other groups. The yellowish color in the B group consisted of residue of the Binahong leaf extract.

B. Wound Area Reduction and Estimated Healing Day

All groups recovered in a similar pattern that shown in Fig. 3, with the injury size increased dramatically in the early stage and then gradually decreased until day 14. The peak days of inflammation for groups P, B, C, and PB were days 2, 4, 5, and 6, respectively. From day 4 to day 14, the observed wound area in group P was significantly smaller than that in group C on day 4 to day 12 and significantly smaller compared to that in group B on day 5 to day 10; it was significantly smaller than that in the PB group on day 4 to day 13. The day needed for wound healing in group P was estimated at day 10, while for groups C, B, and PB it ranged from day 13 to day 14. The results showed that the wound healing day in group P came earlier than in the other groups and the wound area in group B was smaller than in the other groups. Finally, it was indicated that Binahong extract could reduce the effectiveness of atmospheric plasma jets for accelerating healing in acute wounds.
Plasma Jet Effectiveness Alteration by Binahong

C. Re-Epithelialization

On days 7, 11, and 14 after wound creation, the percentage of re-epithelialization for each group was calculated (Fig. 4). On day 7, the higher percentage of re-epithelialization in the P group was clear. It was significantly higher than in the other groups (P vs. C = $p < 0.01$; P vs. B = $p < 0.01$; P vs. PB = $p < 0.01$), whereas in the Binahong extract group it was not significantly different from that in the control group (C vs. B = $p > 0.05$). However, in the PB group it was significantly smaller than in the C group (C vs. PB = $p < 0.05$). From days 7 to 11, the percentage of re-epithelialization for all groups increased dramatically. In group P, it was significantly higher than in groups B and PB (P vs. B = $p < 0.01$; P vs. PB = $p < 0.01$). In group C, it was significantly higher than in group PB (C vs. PB = $p < 0.01$). On day 14, wounds in all groups had reached 100% re-epithelialization.

D. Neutrophils

On days 11 and 14 after wound creation, the neutrophils in each group were counted (Fig. 5). On day 11, the number of neutrophils in group P was significantly lower than
For days 11–14, the number of neutrophils in all groups decreased, but there were no significant differences between groups.

**E. MDA Levels**

On days 7 and 14 after wound making, MDA levels for each group were measured (Fig. 6). On day 7, MDA levels in group P were significantly higher than in groups C, B, and
PB (P vs. C = \( p < 0.01 \); P vs. B = \( p < 0.01 \); P vs. PB = \( p < 0.01 \)). From day 7 to day 14, MDA levels in all groups decreased. MDA levels in the P group were significantly higher than in the C, B, and PB groups (P vs. C = \( p < 0.01 \); P vs. B = \( p < 0.01 \); P vs. PB = \( p < 0.01 \)). This suggests that the level of oxidative stress in the P group was higher due to plasma exposure.\(^{31}\)
IV. DISCUSSION

Research on plasma medicine began in the mid-1990s, with attempts to use nonequilibrium atmospheric pressure plasma for sterilization of living and nonliving material. In 2019 Woedtke reached one of the most important achievements of basic research in plasma medicine, discovering that biological plasma effects are mainly mediated via ROS and RNS influencing cellular redox-regulated processes. Thus, plasma medicine can be considered the implementation of redox biology.

Many aspects of wound healing depend on redox regulators involving ROS and RNS such as O$_2^-$, H$_2$O$_2$, and NO, as reported by Sen et al. Active species, usually referred to as oxidants, act as signaling messengers in biological mechanisms. Some studies have shown that active species have an important part in the major steps of wound healing, such as inflammation, re-epithelialization, and vascularization, among others.

In a previous investigation, acute wounds of mice were directly treated using atmospheric plasma jet alone; other investigations treated acute wounds using atmospheric plasma jet combined with aqueous natural products. Our investigation, however, is the first to evaluate the effectiveness of both contact and noncontact plasma jet with Binahong extract on experimental acute-wound healing. It has been reported that contact administration has a possible detrimental effect on the skin and wound in a mouse model. However, this investigation revealed that combining contact administration during the inflammation phase, from day 0 to day 4, and noncontact administration during the remaining phases accelerates wound healing. These results correct our previous investigation conclusion that noncontact plasma jet has a more positive effect on acute wounds. Here we showed that contact plasma jet during the inflammation phase has a positive effect.

In the current study, plasma jet alone significantly increased MDA levels until approximately 150 nmol at day 11 and 130 nmol at day 14. Plasma jet has the ability to improve wound healing by reducing the duration of the inflammation phase, as indicated by the graph in Fig. 3 of wound healing from day 0 to day 5 (inflammation phase), and by the reduced number of neutrophils at day 11. The level of oxidative stress in the Plasma group was higher because the plasma itself produced ROS; which stimulated healing and new tissue generation. Meanwhile, in the PB group the level of oxidative stress was lower because of the effect of antioxidants from the Binahong extract. Plasma alone also accelerated re-epithelialization, as indicated by the re-epithelialization percentage on day 7. Binahong followed by plasma jet increased MDA at a rate lower than that of plasma jet alone; however, it tended to impede wound healing. Macroscopically, the wound area of the B group was smaller than that of the C and PB groups, but microscopically B group re-epithelialization was less than that in the C and PB groups. This was because the excess dead tissue inhibited the growth of new epithelium.

It is thus hypothesized that, in the context of the plasma jet-Binahong relationship, Binahong, as an antioxidant, may reduce the healing effectiveness of atmospheric plasma jet, as a prooxidant. The oxidants in plasma and the antioxidants in Binahong
when combined have lowered ability to promote wound healing. The reduced effect of a combination treatment of plasma jet with Piper betle has been reported elsewhere.\textsuperscript{14} Although further investigation is needed, the present investigation indicates biomedical incompatibility between plasma jet and a material that contains antioxidant, as in a natural product, to support wound healing.

V. SUMMARY

This study showed that plasma jet improves wound healing but that Binahong extract followed by plasma jet inhibits it. It is thus hypothesized that Binahong reduces plasma jet effectiveness in wound healing.

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