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HISTOLOGICAL EVIDENCE OF WOUND HEALING PROCESS IN CHRONIC WOUND TISSUE WITH MEDICAL PLASMA TREATMENT DURING PROLIFERATIVE PHASE

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Abstract: Chronic wounds are defined as acute wounds that fail to heal within the expected time. The proliferative phase is one of four major phases in healing physiology that occurs on days 8 to 11, characterized by epithelial cell contraction leading to the edges merging and the wound size decreased. New treatments for chronic wounds have been developed, including medical plasma. Plasma is an ionized gas. Plasma generates RONS molecules, which can be effective in healing chronic wounds but histological view has not been reported. The aim of this study was to calculate the number of inflammatory cells, blood vessels, fibroblasts, the percentage of re-epithelialization, and the thickness of necrosis of chronic wound tissue in mice skin treated with plasma in the proliferation phase. The sample used tissue slides of mice skin with chronic wounds on the 11th day which had been treated with plasma daily. A total of 18 samples were divided into 4 groups. Control (K) chronic wound tissue without treatment, Contact Plasma (CP) chronic wound tissue with 5mm distance of plasma treatment, Contact-Noncontact Plasma (CP-NCP) chronic wound tissue with combination plasma treatment, days 0-6 distance of 5 mm and days 7-13 distance of 20 mm, Non-contact Plasma (NCP) chronic wound tissue with 20mm distance of plasma treatment. The results showed that the highest mean number of fibroblast cells, blood vessels, and percentage re-epithelialization were found in CP-NCP. The highest number of inflammatory cells was in K and the highest thickness of necrosis 70.5µm was found in CP. The final conclusion, the highest average number of fibroblast cells, blood vessels, and percentage of re-epithelialization was found in CP-NCP group indicated the CP-NCP group had the fastest healing process compared to the others. Thus, contact-non-contact plasma medical treatment has potential as a new treatment for chronic wounds.

Keywords: chronic wounds, histology, proliferation phase, wound healing

Introduction

Chronic wounds are becoming more common every year (Youssef *et al*., 2023; Haertel *et al*., 2014; Huda *et al*., 2018; Thana *et al*., 2019), and it is estimated that 1% to 2% of the population in developed countries will experience chronic wounds during their lifetime (Zhihao *et al*., 2023). Chronic wounds are defined as acute wounds that do not heal within the time frame expected for the type of wound. An infection that results in the formation of bacterial biofilms can cause disruption.

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Diabetic ulcers, decubitus ulcers, and venous ulcers are the most common types (Youssef *et al*., 2023; Serra *et al*., 2015).

Wound healing physiology through four major stages: hemostasis, inflammatory, proliferative, and maturation/remodeling. The proliferative phase takes from days 8 to 11 and is characterized by epithelial cells contracting, causing the wound's edges to converge and shrink in size. Macrophages and neutrophils are released so that the wound area can synthesize extracellular cell matrix and remodel (Wang *et al*., 2018).

Strategies in chronic wounds treatment are still limited to the use of wound dressings, cell therapy, oxygen therapy and antibiotic therapy (Clinton and Carter, 2015; Ghavaminejad *et al*., 2016; Engkasan, 2018). These strategies is not entirely successful, in the case of mature biofilms antibiotic therapy is the least effective and has only short-term effects on inflammation and healing (Jamal *et al*., 2015). It is necessary to find new therapeutic methods or technologies for chronic wounds treatment, such as medical plasma technology.

In this study, plasma is the fourth phase of matter, following solid, liquid, and gas. Plasma is also known as ionized gas because it contains both stable (gas) and reactive (ions, energetic particles, and radicals) components (Fridman *et al*., 2013). Many potential biomedical applications for medical plasma have been investigated, including wound healing and the ability to kill bacteria. In low doses, ROS molecules can increase endothelial cell migration, proliferation, and growth factors (Lloyd *et al*., 2010). The ability of medical plasma to heal acute skin wounds has been reported by Haertel *et al*., 2014; Nasruddin, 2014, 2015; Bekeschus *et al*., 2016, however, research reports on the effects of plasma jets on chronic wounds have not been widely studied.

Based on this background, researchers are interested in studying the microscopic view of chronic wound tissue in mice skin treated with medical plasma in the proliferation phase with hematoxylin-eosin (HE) staining. The aim of this study was to calculate the number of inflammatory cells, blood vessels, fibroblasts, the percentage of re-epithelialization, and the thickness of necrosis of chronic wound tissue in mice skin treated with medical plasma in the proliferative phase.

Materials and Methods

The research was conducted at the Experimental Medical Plasma Laboratory, Faculty of Nursing and Health Sciences, Universitas Muhammadiyah Semarang. Chronic wounds developed by infecting S. aureus ATCC 6538 in acute wounds of mice. After acclimating the male BALB/c mice for seven days, a full-thickness acute wound was made on the dorsal part of the mice with a punch biopsy 4mm in diameter (Kai Industries Co. Ltd., Gifu, Japan). 50µL of bacteria suspension, equivalent to the turbidity of a McFarland 7 standard solution was inoculated on acute wounds of mice. To allow the wounds to colonize, they were covered with a dressing (Tegaderm Hydrocolloid Dressing; 3M Health Care) for 72 hours. A bandage was used to keep the dressings in place. This method creates an ideal environment for the formation of biofilms. After chronic wound creation in mice, medical plasma treatment was conducted daily for 3 minutes. Mice were anaesthetized via injection before treatment. A schematic of medical plasma system was shown in Figure 1. After 11 days tratment of medical plasma, the wound and surrounding skin of mice were collected and then bisected at the wound's center. Tissue processing and Hematoxylin-eosin (HE) staining were carried out in accordance

previously described procedure to prepare tissue slides of mice skin with chronic wound tissue (Darmawati et al., 2019).

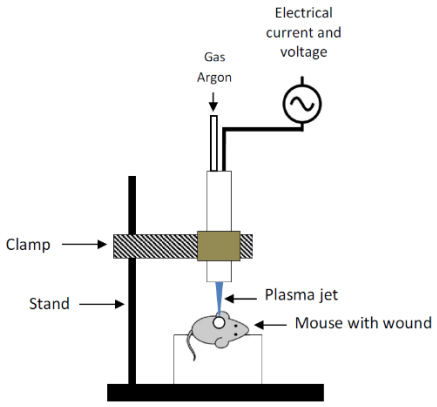

Figure 1: Plasma treatment experimental set-up

The sample used was tissue slides of mice skin with chronic wound tissue on the 11th day which had been treated with medical plasma every day. The total samples are 18 slides, which are divided into 4 groups, including:

Table 1: Samples grouping and medical treatment of each group

Groups	Treatment
Control (K)	Chronic wound tissue without treatment
Contact Plasma (CP)	Chronic wound tissue with medical plasma treatment with a distance of 5mm
Contact-Noncontact Plasma (CP-NCP)	Chronic wound tissue with combination plasma treatment, days 0-6 with a distance of 5 mm and days 7-13 with a distance of 20 mm
Non-contact Plasma (NCP)	Chronic wound tissue with medical plasma treatment 20mm distance.

The equipments used in this study were digital cameras (Panasonic Lumix F2.5), Dinolite Edge microscopes, glass objects and glass decks, timers. The materials used were tissue slide of mice skin wound tissue, distilled water, entelan, xylol, alcohol, hematoxylin, and eosin.

Parameters of the number of inflammatory cells, fibroblasts and blood vessels were counted in 5 fields of view with a magnification of 1000×. Observations of tissue necrosis thickness were calculated using DinoCapture 2.0 software. Observation of re-epithelialization with the help of DinoCapture 2.0 software. The percentage of re-epithelialization was calculated using the formula: 100% × (length of new epithelium/length between the edges of the wound) (Darmawati et al., 2021).

The data obtained included the number of inflammatory cells, fibroblasts, the percentage of re-epithelialization, and the thickness of tissue necrosis. The data obtained was subjected to statistical analysis using the ANOVA test to determine differences in each group of mice skin chronic wound tissue.

Results and Discussion

Result

Microscopic observation of tissue samples from chronic bacterial wound skin of mice on day 11, which was treated with plasma jet with Hematoxylin-Eosin (HE) staining was carried out using a Dinolite Edge microscope. The results are shown in Figure 2.

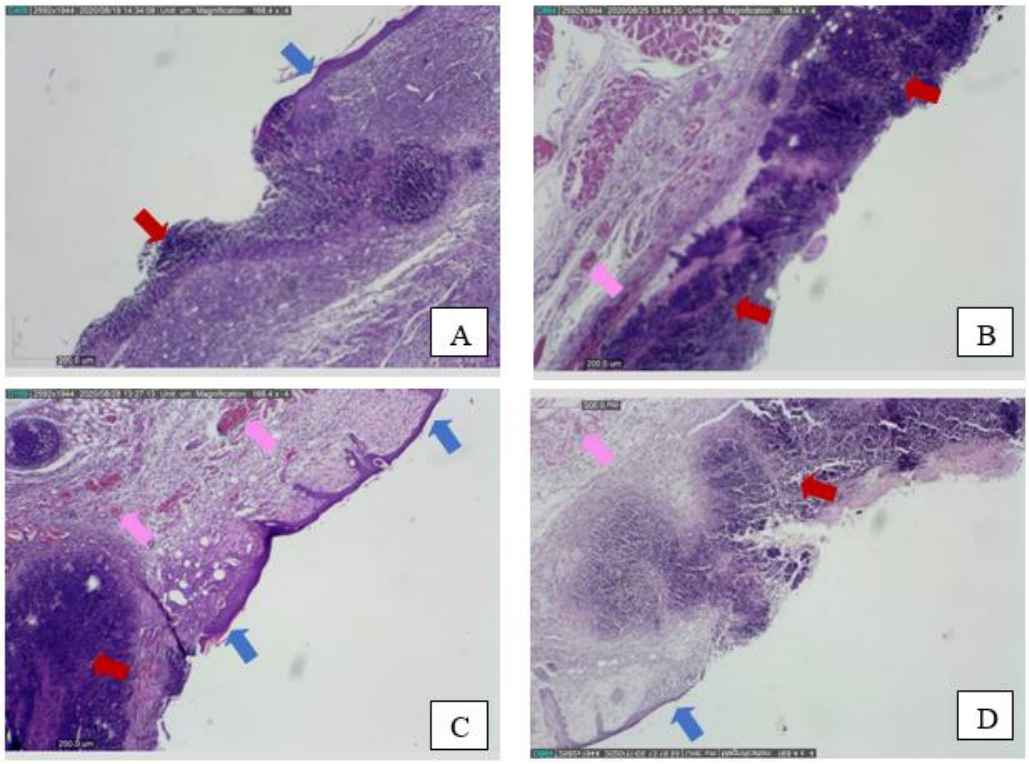

Figure 2: Microscopic observation of the epidermis and dermis of chronic wounds mice skin in the proliferative phase with a 40× objective lens magnification. Description: A. Control (K), B. Contact (CP), C. Contact- Non-Contact (CP-NCP), D. Non-Contact (NCP); Blue arrows: re-epithelialization, Red arrows: necrosis, Pink arrows: new blood vessels

In general, microscopic observations on the chronic wound tissue areas of mice skin in the proliferative phase showed necrotic tissue, new blood vessels, and re-epithelialization. Necrotic tissue was seen in all chronic tissue groups, new blood vessels were found in the medical plasma-treated tissue group, and re-epithelialization was found in all groups except the Contact Plasmas (CP) group.

Data of the average count of inflammatory cells (neutrophils), blood vessels, fibroblasts, the thickness of necrosis, and the percentage of re-epithelialization were counted in 5-field view with 1000× magnification in chronic mice skin wounds. The data is presented in the following Table 2.

Table 2:Results of histological observations of chronic wound tissue slides

Groups	Inflammatory cells (Mean)	Fibroblast cells (Mean)	Blood vessels (Mean)	Necrosis (µm)	e-epithelialization (%)
Control (K)	54.0	11.0	4.0	11.0	22.0
Contact Plasma	53.0	13.0	5.0	71.0*	5.0

(CP)					
Contact-Non contact Plasma (CP-NCP)	30.0	26.0	6.0	20.0	76.0*
Non-contact Plasma (NCP)	44.0	15.0	5.0	34.0	16.0

Notes: * show $p<0.05$ to the other groups (control and treatment group)

In general, the highest average number of fibroblast cells, blood vessels, and percentage of re-epithelialization was found in the contact-non-contact plasma group (CP-NCP). The highest mean number of inflammatory cells was in the control group (K) and the highest thickness of necrosis was found in the contact plasma (CP) group.

Discussion

Based on microscopic observations of the results of HE staining on chronic wound tissue preparations of mouse skin, the cell nucleus, cell membrane, and extracellular matrix were perfectly stained in all preparation groups, except where the cell nucleus and cell membrane were blue due to haematoxylin-eosin staining. Haematoxylin, which is alkaline, will stain the cell nucleus, which is acidic, blue-purple (Khristian and Inderiati, 2017). Parameters number of fibroblast cells, blood vessels, and percentage of re-epithelialization indicated the effectiveness of medical plasma in accelerating healing process. Parameters The number of inflammatory cells and tissue necrosis thickness indicated the effectiveness of medical plasma in removing bacteria.

The contact-non-contact plasma (CP-NCP) group showed the fastest healing phase. Clinical wound healing marker parameters such as fibroblast cells, new blood vessels, and the percentage of re-epithelialization were found to be significantly higher ($p<0.05$) in this group than the others. Fibroblasts are intercellular substance cells that produce collagen, elastin, glycosaminoglycan, reticulum, and glycoprotein fibres. In response to injury, fibroblasts become more active in synthesizing extracellular matrix components by proliferating and increasing fibrinogenesis. Fibroblast cells stained with hematoxylin-eosin stain dark blue, which is based on the hematoxylin-eosin principle. Fibroblast cells are acidic so they will bind to hematoxylin which is alkaline and turning the cell a blue to purple color (Khristian and Inderati, 2017).

It is shown that the combined plasma treatment given to the CP-NCP group is effective for the treatment of chronic wounds. Contact plasma exposure is able to kill bacteria in chronic wound tissue then treatment continued with non-contact plasma exposure that can accelerate re-epithelialization of the wound. Contact plasma exposure produces reactive ROS which can have an antibacterial effect (Alkawareek et al., 2012).

Non-contact plasma exposure generates radicals with a relatively long half-life, one of which is NO. According to Thana et al. (2019), plasma-produced NO has a stimulatory effect on wound healing and tissue regeneration. NO is an important cellular signaling molecule in humans, that can influence the immune system, increase growth factors, and stimulate cell proliferation, angiogenesis (the formation of new blood vessels), and collagen synthesis, resulting in the repair of damaged skin.

Re-epithelialization is the process of epithelial cells covering the wound by resurfacing the wound with new epithelium. This is different from the study by Putri et al (2021) which reported re-epithelialization during the remodeling phase. The remodeling or maturation phase is the final stage in the wound healing process. Epithelial cells in this phase have completely covered the edges of the wound until they reach above 90% (Putri et al., 2021).

The contact plasma (CP) group had the lowest percentage of re-epithelialization. Plasma exposure after the biofilm layer disappears giving it to the surface of the wound area actually hinders the wound healing process. The contact plasma (CP) group had the lowest percentage of re-epithelialization. Giving plasma exposure after the biofilm layer disappears actually inhibits wound healing. According to Darmawati et al. (2019), contact plasma exposure has negative effects such as damaging normal skin tissue due to an increase in local temperature and inhibiting or damaging the growth of new epithelium (Darmawati et al., 2019). This is also proportional to the thickness of the necrosis.

The contact plasma (CP) group had the highest necrosis thickness significantly ($p<0.05$) compared to the other groups. Necrosis is a form of cell death in which cellular cells are destroyed, cellular enzymes leak, and finally the cell is damaged. Necrosis causes a localized host response known as inflammation, induced by substances released from dead cells and performs to remove debris to initiate the the subsequent repair process. Necrosis is the final result of irreversible cell injury. Necrosis mechanisms include failure to generate energy in the form of ATP due to decreased oxygen supply or mitochondrial damage, damage to cell membranes, including plasma and lysosomal membranes, resulting in cellular leakage including enzymes, and permanent damage to cell lipids, proteins, and nucleic acids caused by ROS molecules. High levels of ROS molecules in the skin cause oxidative stress, which causes cell injury and thus damages normal skin tissue. The oxidative processes produced by RONS include O2-, H2O2 and NO2- (Gupta and Kumar, 2015).

Inflammatory cells are cells that play a role in the body's defense response. The control group had a higher number of inflammatory cells than the others due to chronic wound infection factors. Chronic wounds that do not get treatment for bacterial biofilms will grow wider. Staphyllotoxins produced by S. aureus can cause local tissue necrosis, causing the wound area to expand (Bhattacharya et al., 2018). The excess number of bacteria also induces inflammatory cells (McCarty and Percival, 2013), this indicating a prolonged inflammatory phase.

The number of inflammatory cells and tissue necrosis thickness were used as parameters for the presence of inflammation in mice skin tissue. The effectiveness of plasma in eliminating bacteria can be demonstrated by the thickness of tissue necrosis and further proven by the presence of inflammatory cells in the wound area. Staphyllotoxins cause damage or injury to the epidermal structures, causing erosion or ulcers. Erosion or ulcer is a series of processes caused by external trauma or injury. Ulceration will cause the epidermis to be damaged until it disappears causing the dermis to open causing neutrophils to be in the dermis layer (Oztruk et al., 2017).

Conclusion

Histological view of the proliferative phase in chronic wound tissue preparations of mice treated with medical plasma show fibroblast cells, blood vessels, necrotic tissue and re-epithelialization. In the CP-NCP group, the thickness of tissue necrosis was the second lowest after the Control (K) group, this

result is also in line with the low number of inflammatory cells. This shows that CP-NCP treatment is effective in eliminating bacteria that cause infections.

The highest average number of fibroblast cells, blood vessels, and percentage of re-epithelialization was found in the contact-non-contact plasma group (CP-NCP) indicating that the CP-NCP group had the fastest healing process compared to the other groups. Therefore, Contact-non-contact (CP-NCP) exposure of medical plasma has potential as a new treatment for chronic wounds. Further research on chronic wound healing with humans application is needed to explore the potential of medical plasma using contact-non-contact treatment

Declaration of Interest Statement

The authors declare that they have no conflict of interests.

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