An Atmospheric Plasma Jet Induces Expression of Wound Healing Genes in Progressive Burn Wounds in a Comb Burn Rat Model: A Pilot Study

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Burn-related injuries are devastating injuries with a high mortality rate that affect people of all ages worldwide.‡ Fire, flame, scald incidents, or contact with burning objects are the main causes of these injuries.§ Burn injuries occur due to the loss of primary tissue as a result of protein denaturation. Subsequently, the activation of toxic inflammation occurs, and skin and capillary endothelial cells are further damaged by oxidants and proteases, thus increasing the likelihood of ischemic tissue necrosis.¶ Early burn wound excision, skin grafting, and the use of chemical agents have been suggested to improve the outcomes of burn wound healing.∥∥ However, delayed wound healing, bacterial infection, soreness, and scarring remain major challenges for burn management and research.

The rapid development of plasma medicine (medical applications of nonthermal plasma technology) brings a new innovative approach to a broad range of biomedical applications. Plasma medicine has bactericidal properties and can therefore be a beneficial tool in various medical procedures, including tissue disinfection and treatment of chronic wounds with a microbial etiology. Aside from bactericidal effects, plasma medicine has also been reported to have applications in blood coagulation, tissue regeneration, and wound healing.∥∥∥ The induction of cell proliferation, keratinocyte and fibroblast migration, and wound healing-related genes are other properties of plasma medicine.‡‡–‡‡‡ Moreover, plasma medicine has shown its much potential for cancer cell treatment, as it can selectively induce apoptosis in cancer cells while inducing minimal damage to normal cells.‡‡§

Plasma medicine utilizes reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are generated from the collision of gas atoms in a high-strength electrical field, while the gas remains at atmospheric pressure and close to ambient temperature. Electrical stress on the atoms causes electrons to be accelerated and collide with surrounding atoms and molecules, resulting in positively charged atomic and molecular...
ions. The mixture of uncharged atoms, molecules, ions, and electrons is termed “plasma.” Molecules such as ozone (O₃), NO, H₂O₂, and OH radicals are all types of reactive species (RS).⁷⁸²⁸⁰ Regarding the use of ROS, it is important to note that the effect of ROS is dose dependent, meaning that the application dose can either act positively or negatively on the cells. Excessive use of ROS leads to apoptosis, while modest levels of ROS favor growth and improve survivability.⁷⁷³⁷³⁸

In this study, we assessed the effects of plasma jet on the genes responsible for burn wound healing in a brass comb burn wound rat model.

**METHODS**

**Study Setting and Animal Subjects**

The study was performed in the Division of Laboratory Animal Research of Hanyang University. Six male Sprague-Dawley rats (350 g) were used in this study. The rats had access to standard food and water ad libitum, and they were acclimatized to the environment for several days prior to the experiment. The cages were managed in accordance with the National Research Council guidelines⁹⁹ (Approval number: HY-IACUC-18-0011).

**Plasma Device**

A nonthermal atmospheric plasma jet was used in this study. The RADIX plasma generator (MediPL, Seongnam City/South Korea), which is a microwave power signal generator, was developed by MediPL in Korea. This device ejects a single plasma jet from a pen-shaped resonator with an operating frequency of 50/60 Hz. The working gas for the plasma generator was argon, which was delivered at a rate of 5.0 standard liters per minute (slm), and the input power was 2.5 Watts. The plasma produced by this generator formed a stable bright plum –7 mm in length. The temperature produced by this plasma generator is less than 40°C, and it produces less than 0.05 ppm O₃; therefore, the device is ecofriendly.

**Animal Burn Wound Model**

A four-pronged brass comb was used in this study. The prongs were separated by three 5 mm notches, producing three unburned sites (interspaces) between the four burned sites. These interspaces represent zones of stasis that, within hours, progress to ischemia and become necrotic. The dimensions of the brass comb were 20 × 25 × 55 mm rectangular prongs separated by three 5-mm-wide grooves.

**Experimental Protocols**

The experiment was based on a previously established rat contact thermal injury model by Adam Singer.⁸ The rats were anesthetized by inhalation of 0.5% to 2.5% isoflurane. The hair on both sides of the back was clipped using an electric clipper. The brass comb was preheated in a water bath at 100°C for 3 minutes and then perpendicularly applied to the skin on both sides of the back without pressure for a period of 90 s. This resulted in four full-thickness burns separated by three unburned interspaces. One side received plasma treatment (plasma group), while the other side did not (control group). Argon gas flow was used (5 slm), and the input power of the plasma generator was 2.5 Watts. The interspaces of the plasma-treated group were treated using the plasma jet for 2 minutes with a distance of 2 cm between the tip of the plasma jet and the rat skin (Figure 1). An Elizabethan collar was placed on each rat to prevent the rat from licking or scratching its wounds. The treatments were reapplied each day for a total of 7 days. All animals were then euthanized by CO₂ inhalation 7 days after injury.

**Histology and Immunohistochemistry**

The interspaces of the rats were excised at days 0, 4, and 7. The skin tissue was fixed in 4% paraformaldehyde overnight, embedded in paraffin, and cut into serial sections measuring 4 μm. The sections were stained with hematoxylin-eosin (H&E) and Masson’s trichrome (for quantifying collagen deposition) or subjected to immunohistochemistry. The formalin-fixed sections were deparaffinized sequentially through a series of xylene, ethanol, and water treatments. Antigen retrieval was performed by treatment with tenascin-C in a 1% trypsin solution (1:1) at 37°C for 30 minutes. For antigen retrieval of CD31, phospho-S6 235/236, and CD68, the sections were incubated in sodium citrate buffer (10 mM, pH 6.0) for 52, 10, and 10 minutes, respectively, and for retrieval of neutrophil elastase, the sections were incubated in 0.04% pepsin for 10 minutes at room temperature. Antigen retrieval for α-smooth muscle antibody, mouse monoclonal (α-SMA) and fibronectin was not required. To quench endogenous peroxidase activity, the sections were immersed in 3% H₂O₂ in dH₂O for 10 minutes, followed by a blocking step with 5% goat serum in TBST for 1 h. Subsequently, the sections were incubated with anti-αSMA (#M0851, 1:300, mouse monoclonal, AgilentDako, California), anti-fibronectin(Ab23751, 1:1000, rabbit polyclonal, Abcam, London, UK), anti-tenascin-C (4C8MS, 1:50, mouse monoclonal, Novus Biologicals, Colorado), anti-CD31 (ab28364, 1:50, rabbit polyclonal, Abcam, London, UK), anti-phospho-S6 235/236 (#2211, 1:400, rabbit polyclonal, Cell Signaling Technology, Massachusetts), anti-elastase (ab21595, 1:200, rabbit polyclonal, Abcam), and anti-CD68 (ab31630, 1:100, mouse monoclonal, Abcam) primary antibodies in a humidified chamber at 4°C overnight. SignalStain® Boost IHC detection reagents (HRP mouse #8125, HRP rabbit #8114, Cell Signaling Technology) were applied in a humidified chamber for 30 minutes at room temperature. The reaction was developed with diaminobenzidine (DAB; Cell Signaling Technology) for 10 minutes, and the sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted with coverslips.

**Scratch Assay (in vitro assay)**

HaCaT (Keratinocyte) Cells were incubated on six-well plates until being merged into a monolayer. Thereafter, the gap was made by a 100-μl pipette tip, and subsequently the plasma treated on the cells for 30 seconds. The cells were washed with PBS, and thereafter supplied with fresh medium. We observed for 5 days expanding cells around the scratched region. The distances between the edges of the gaps were then measured. The healing of the monolayer’s gaps was photographed under an inverted microscope (Nikon Eclipse TS 100, Japan) at the same observation site.
iNOS and EGF by Enzyme-Linked Immunosorbent Assay (in vitro assay)

After culturing the cold plasma-treated HaCaT cells for 5 days, protein concentration of iNOS and EGF was determined by enzyme-linked immunosorbent assay method according to the instruction (Abcam). Briefly, the serum-free supernatants or extracts of the cells were collected and thoroughly mixed with the samples and antibody cocktail incubated at room temperature for 60 minutes. Thereafter, the plate was washed, and then 100 μl of the working solution was added to each well. The plate was placed in a dark drawer at RT for 10 minutes. One hundred microliter stop solution was added to each well, and the OD value at 450 nm was read on a microplate absorbance reader (Bio-Rad Laboratories, Inc., California).

Analysis of mRNA Expression by qPCR

The mRNA expression of proinflammatory (Tnf-α and Il-1β), anti-inflammatory (Tgf-β, Il-10 and Il-6), and growth factor (Pdgfb, Vegf, Egf, Fgf-2, and Kgf-1) genes and iNOS at days 0, 4, and 7 post-wounding (n = 3 in all groups) was assessed using quantitative real-time polymerase chain reaction (qPCR). RNA from skin tissue biopsies was extracted using TRIzol reagent and then reverse transcribed into cDNA. The qPCR analyses were performed using a cDNA solution, a pair of primers and SYBR Green master mix (Bio-Rad Laboratories, Inc.). qPCR was performed in a final volume of 20 μl under the following cycling conditions: 3 minutes at 95°C, followed by 40 cycles of 10 seconds at 95°C, 10 seconds at 60°C, and 15 seconds at 72°C. All samples were analyzed in triplicate. The gene expression level was normalized to that of Gapdh as a housekeeping gene, and the quantification of the gene expression was calculated relative to the control group. The results were analyzed with CFX Manager (Bio-Rad Laboratories, Inc.) software.

Statistical Analysis

All data were organized in Microsoft Excel 2016 and were statistically analyzed using Minitab 17 software (Minitab Inc., Pennsylvania). We used six rats for this experiment. In a previous study using a brass comb burn wound model, the author indicated that each interspace was counted as a single outcome.5 The brass comb made three interspaces on each side of the rat. Therefore, in this study, the sample size was eighteen samples per group. We analyzed eighteen interspaces for each group on days 0, 4, and 7.

The distribution of the data was tested using the normality test, and the data were normally distributed. Significant differences between two groups were compared using two-way ANOVA with post-hoc analysis. The data are expressed as the means ± SD. P-values of < .05 (*), < .005 (**), and < .0005 (***)) were considered statistically significant.

RESULTS

Altered Inflammatory Cytokine mRNA Levels Induced by Plasma

Inflammation is the first stage of the reaction by which the body achieves wound healing (Table 1). To determine the effectiveness of plasma in preventing burn-induced ischemic tissue necrosis, we extracted and quantified the mRNA levels of inflammatory cytokines, both proinflammatory (Tnf-α and Il-1β) and anti-inflammatory (Tgf-β, Il-10, and Il-6) cytokines, in the ischemic interspaces at days 0, 4, and 7 post-wounding. The levels of both Tnf-α and Il-1β were significantly decreased in the plasma treatment group compared to the control group. Plasma treatment significantly decreased the expression of Tnf-α at day 0 and 4 and also it significantly decreased the expression of Il-1β at day 7 post-wounding compared to the control group. On the other hand, the expression of anti-inflammatory cytokines (Tgf-β, Il-10, and Il-6) was significantly increased in the plasma group compared to the control group. Plasma treatment significantly increased the expression of Tgf-β and Il-10 up to 3-fold compared with that of the control group at day 7. The expression of Il-6 was slightly increased at day 7 of plasma treatment.
Elevated iNOS and Growth Factor Gene Expression

Next, we examined the expression of iNOS and other growth factor genes influenced by plasma treatment (Table 1). To determine whether plasma treatment could alter internal NO production, expression of the iNOS gene was assessed. At days 0 and 4, the expression of iNOS was elevated by 4-fold in the plasma-treated group compared with the control group. However, the expression of iNOS was significantly decreased in the plasma group at 7 days post-wounding.

Each phase of wound healing is controlled and regulated by growth factors. Therefore, to assess the effect of plasma on wound healing, which is regulated by growth factors, the expression of Pdgfb, Vegf, Egf, Fgf-2, and Kgf-1 was measured. The plasma group showed increased expression of each growth factor, except Egf-2, that was tested compared to that of the control group. The increased expression was apparent starting at day 4 and at day 7, and expression was upregulated to 2.319-, 2.018-, 3.675-, and 4.390-fold for Pdgfb, Vegf, Egf, and Kgf-1, respectively.

CD68 Expression

Macrophages are essential cells that can regulate the wound healing process through the production of growth factors. A CD68 antibody was used to detect the presence of macrophages. Immunohistochemical staining results showed that the number of CD68-positive cells observed in the plasma group was significantly higher than that in the control group (Figure 1). This result is supported by the increased expression of growth factors and interleukins, along with the increased number of macrophage (Table 1). These results suggest that plasma treatment could benefit wound healing through macrophages.

Angiogenesis

Angiogenesis is necessary for the development of wound healing. To monitor angiogenesis, an α-SMA antibody was used to detect smooth muscle cells surrounding the blood vessels. There were more blood vessels observed in the plasma group than in the control group (Figure 2). This result was consistent with the increased gene expression of Vegf (Table 1). These data suggest that plasma treatment of burn wounds could facilitate the development of angiogenesis.

Extracellular Matrix Protein Expression

Next, we measured the expression of extracellular matrix ( ECM), which facilitates cellular migration. In an effort to assess ECM deposition, fibronectin and tenasin-C antibodies were used. As shown in Figure 3, the expression of fibronectin in the plasma group was higher than in the control group. The same result was observed regarding the expression of tenasin-C. More intense expression of tenasin-C was observed in the plasma-treated group than in the control group. The increase in both proteins was most evident in the subcutaneous areas. The plasma-induced changes in the ECM protein levels suggest that plasma could affect the development of wound healing by altering ECM protein expression.

| Table 1: Gene expression of inflammatory cytokines, iNOS, and growth factors on both control and plasma group at time points of 0, 4, and 7 days post-wounding |
|---------------------------------------------------------------|
| mRNA Expression (Mean ± SD)                                    |
|                  | Inflammatory cytokines | iNOS | Pdgfb | Vegf | Egf | Fgf-2 | Kgf-1 | EGF | FGF-2 | KGF-1 |
|                  |                          |      |       |      |     |       |       |     |       |       |
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Collagen Deposition

Masson’s trichrome staining was performed to assess collagen deposition in the granulation tissue. Collagen deposition was assessed at day 7 after wounding. At day 7, more intense collagen expression was observed in the subcutaneous and dermis areas in the plasma group than in the control group (Figure 4). The collagen expression in the plasma group reached deeper into the dermis in the plasma group than in the control group. These data suggest that plasma could also facilitate re-epithelialization by increasing collagen production.

Figure 2. Protein expression of α-SMA at day 7. Histological sections were stained with an antibody against α-SMA and counterstained with hematoxylin. There were more α-SMA-positive cells observed in the plasma group (right) than in the control group (left).

Figure 3. Expression of ECM proteins at day 7. Histological sections were stained with antibodies against fibronectin (A–B) or tenascin-C (C–D) and counterstained with hematoxylin. Stronger and more intense staining of fibronectin and tenascin-C was observed after the plasma treatment (B, D) than before plasma treatment (A, C). ECM, extracellular matrix.
Cold Plasma-Mediated Cell Proliferation In Vitro Assay

To investigate in vitro effect of cold plasma on wound healing, we performed the scratch assay in the HaCaT (Keratinocyte) cells. Consequently, the plasma exposure on the scratched region significantly facilitated the recovery of damaged area in a time-dependent manner (Figure 5A). Moreover, we found that the expression of iNOS and EFG protein is increased when exposed plasma for 30 seconds (Figure 5B and C), which is consistent with the transcriptional changes in iNOS and EGF shown in plasma-treated region of rat.

DISCUSSION

In this study, our findings showed a faster wound healing process after plasma treatment than in the control group. The mechanism of action by which this plasma exerts its effects is by using the produced RS to influence the expression of genes that are important for the wound healing process. As a result of plasma treatment, wound healing-related gene expression is altered toward a level that will benefit the wound healing process. Our main goal for this study was to determine whether plasma treatment could lessen the progression of the zone of stasis (interspaces) into full-thickness third-degree burn wounds.

The area that makes direct contact with the source of heat, called the zone of coagulation, is always surrounded by a zone of stasis, which has a chance of progressing to necrosis. This is a characteristic that differentiates burn wounds from other wounds. Necrosis caused by burns could impair homeostasis and ultimately inhibit burn wound healing. Moreover, ischemia could also exacerbate burn wounds. Therefore, a solution to this problem is necessary. The use of plasma in wound treatment is a potential solution, as the mild levels of RS that are produced by plasma have the potential to enhance the wound healing process. To assess the effectiveness of plasma in the development of burn wound healing, we studied the changes in expression of genes that contribute to wound healing in a comb burn wound rat model.

Wound healing consists of several phases. Inflammation marks the start of the wound healing process. Excessive inflammation is the common cause of poor wound healing; therefore, most treatments aim to reduce inflammation. For the body to successfully heal after tissue injury, resolution to inflammation is required. Many mechanisms have been suggested to facilitate inflammation resolution, including the downregulation of proinflammatory cytokines by upregulating anti-inflammatory cytokine expression. Here, we showed decreased expression of the proinflammatory cytokines TNFα and IL-1β and increased expression of the anti-inflammatory cytokines Tgf-β, II-10, and II-6 after plasma treatment. The RS produced by plasma act as second messengers that relay the information arriving at the cell membrane through the binding of ligands and receptors throughout the cells through a series of electron transfer reactions. This signaling is ultimately received by inflammatory cells, which release many kinds of cytokines and induce alterations in the levels of released cytokines. A similar result of ROS-mediated alterations in the expression of inflammatory cytokines was also reported by Lee et al.20

In the wound healing process, nitric oxide is strongly involved due to its functional activities in angiogenesis, inflammation, cell proliferation, matrix deposition, and remodeling. One of the RS that plasma produces is NO. To determine whether delivery of plasma-produced NO could benefit wound healing, the mRNA level of the iNOS gene was measured. Our study showed an increase in the expression of iNOS after plasma treatment. One of the contributions of NO in wound healing is creating a class of biomolecules that are termed electrophilic. One of the most important types electrophilic molecules formed from RNS are nitrated fatty acids (NO2-FAs). In a paper written by Groeger and Freeman, NO2-FAs appeared to have valuable therapeutic benefit by inducing anti-inflammatory gene expression, improving vascular function, and attenuating proinflammatory polymorphological neutrophil and macrophage functions.

Cytokines that are produced by inflammatory cells and growth factors help to recruit macrophages. During the transition between the inflammation phase and granulation tissue formation, macrophages appear to play an important role in mesenchymal recruitment and matrix organization. Therefore, to evaluate the presence of macrophages, a CD68

Figure 4. Masson’s trichrome staining. Stronger collagen protein expression was observed in the plasma group (right) than in the control group (left).
The antibody was used. Our results showed more CD68-positive cells after treatment with the plasma jet than in the control group. Macrophages then secrete several cytokines and growth factors, such as IL-6, VEGF, PDGF, TGFβ, FGFs, and EGF, to not only prepare for entry into the granulation tissue formation phase but also facilitate the pace of angiogenesis.22 Again, increased expression of these cytokines and growth factors was confirmed in this study. During granulation tissue formation, there are high numbers of macrophages and fibroblasts that have their own roles. While continuous secretion of growth factors by macrophages induces fibroplasia, epithelialization and angiogenesis, fibroblasts secrete ECM that is necessary to support cell growth and the development of blood vessels.26 The blood vessels are essential for the success of wound healing, as blood vessels provide oxygen and nutrients that are essential for cell metabolism.21 α-SMA staining was performed, and our results showed that there were more blood vessels formed after plasma treatment than in the control group. These results emphasize that plasma could benefit wound healing by regulating many kinds of genes that are related to wound healing.

The ECM, which is composed of fibronectin and collagen, is produced by fibroblasts at the beginning of their migration toward the wound site.27 Together with tenascin-C, which is produced by keratinocytes, the formation of ECM is supported due to the presence of multiple binding sites for fibronectin.28 In this phase, KGF-1 helps regulate ECM production by mediating keratinocyte proliferation and migration. KGF-1 also has indirect effects, such as mediating angiogenesis.29 In this study, plasma was shown to increase the expression of all of these ECM proteins. Once sufficient matrix has been deposited, fibroblasts alter their phenotype to become myofibroblasts and develop pseudopodia that eventually assist in wound contraction. The collagen produced by...
fibroblasts, collagen type III, is be the primary component that gives strength to tissues. The final phase of wound healing is remodeling, where the initial collagen type III is replaced by collagen type I, which has stronger tensile strength than collagen type III. The remodeling phase can last up to 2 years after wounding.\(^{30,31}\)

This study had several limitations that should be mentioned. First, as this study was conducted in rats, and the results may not be generalizable to humans because of anatomical and physiological differences. However, some effects of plasma jets for the treatment of burn wounds could be confirmed in this study. Thus, this treatment method can be applied as an adjuvant treatment for burn wounds. Second, the validated comb burn model used in this study is primarily a tool to study horizontal injury progression; therefore, it may not resemble the vertical progression of burns in typical human burn injuries.

Third, the sample size in this study seems to be small. However, as mentioned in the statistical analysis section, in a previous study using the brass comb burn wound model, the author indicated that each interspace was counted as a single outcome.\(^5\)

Therefore, in this study, the sample size was 18 samples per group, which might be reasonable. Fourth, the present study did not provide quantified results for proteins involved in inflammation and wound healing in animal level. It did not provide result of protein quantification; however, it was possible to confirm protein quantification results in cell level. Thus, these results could suggest the need for further research.

**CONCLUSIONS**

This study shows that plasma-produced ROS could alter the expression of genes involved in wound healing, including TNFα, IL-1β, TGF-β, IL-10, IL-6, iNOS, VEGF, PDGFβ, FGF-2, KGF-1, and EGF. Moreover, plasma treatment can also increase the expression of CD68, α-SMA, Fibronectin, and tenasin-C and collagen synthesis.

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