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An experimental burn wound-healing study of non-thermal atmospheric pressure microplasma jet arrays

Ok Joo Lee1, Hyung Woo Ju1, Gilson Khang2, Peter P. Sun3, Jose Rivera3, Jin Hoon Cho3, Sung-Jin Park3, J. Gary Eden3 and Chan Hum Park1,4*

1Nano-Bio Regenerative Medical Institute, Hallym University, Chuncheon, Gangwon, Republic of Korea
2Department of BIN Fusion Technology, Department of Polymer Nanoscience and Technology and Polymer BIN Research Centre, Chonbuk National University, Republic of Korea
3Laboratory for Optical Physics and Engineering, Department of Electrical and Computer Engineering, University of Illinois, Urbana, IL, USA
4Department of Otorhinolaryngology–Head and Neck Surgery, Chuncheon Sacred Heart Hospital, School of Medicine, Hallym University, Chuncheon, Gangwon, Republic of Korea

Abstract

In contrast with a thermal plasma surgical instrument based on coagulative and ablative properties, low-temperature (non-thermal) non-equilibrium plasmas are known for novel medicinal effects on exposed tissue while minimizing undesirable tissue damage. In this study we demonstrated that arrays of non-thermal microplasma jet devices fabricated from a transparent polymer can efficiently inactivate fungi (Candida albicans) as well as bacteria (Escherichia coli), both in vitro and in vivo, and that this leads to a significant wound-healing effect. Microplasma jet arrays offer several advantages over conventional single-jet devices, including superior packing density, inherent scalability for larger treatment areas, unprecedented material flexibility in a plasma jet device, and the selective generation of medically relevant reactive species at higher plasma densities. The therapeutic effects of our multi-jet device were verified on second-degree burns in animal rat models. Reduction of the wound area and the histology of the wound after treatment have been investigated, and expression of interleukin (IL)-1α, -6 and -10 was verified to evaluate the healing effects. The consistent effectiveness of non-thermal plasma treatment has been observed especially in decreasing wound size and promoting re-epithelialization through collagen arrangement and the regulation of expression of inflammatory genes. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords non-thermal; microplasma; burn; wound; biomedical; device

1. Introduction

Infections are the primary cause of morbidity and mortality among patients suffering from traumatic burn injuries (Latenser et al., 2007). Even in relatively sterile hospital environments, burn wounds are prone to be colonized by microorganisms and easily infected by fungal pathogens (Horvath et al., 2007) and, furthermore, the growing number of cases of infection by drug-resistant bacteria raises significant concerns for the safety of patients (MacKinnon and Allen, 2000).

For the last decade, the emergence of plasma medicine using atmospheric pressure non-thermal plasmas has paved the way for a powerful disinfection alternative (Feng et al., 2009; Kim et al., 2009; Laroussi, 2002). The effectiveness and versatility of this technology has been reported from extensive laboratory and clinical tests for bacterial inactivation, blood coagulation, tooth whitening, tumour treatment and wound healing (Fridman et al., 2006; Lee et al., 2009; Nosenko et al., 2009; Sun et al., 2010; Vandamme et al., 2010). In particular, controlled
generation of chemically reactive species from the plasma devices serves as a strong disinfectant, as well as simultaneously stimulating the cell–cell signalling responsible for re-epithelialization.

Researchers at the University of Illinois and Hallym University have been collaborating for the development of a large-scale array of microplasma jet devices in the application of non-invasive clinical treatment, including wound healing (Lee et al., 2015; Sun et al., 2012). Microplasma’ or ‘microcavity plasma’ is a term for the spatial confinement of a non-equilibrium plasma to micrometer-scale dimensions operating at atmospheric gas pressure. Extensive research in the past decade has revealed that microplasmas possess unique characteristics as compared to other conventional, macro-scale discharge technology (Eden and Park, 2005). Remarkably, microplasmas exhibit significantly higher plasma densities and power loadings, even at low temperatures, leading to a capability of efficient production of various chemical species (Graves, 2012; Lee et al., 2015). To date, microplasmas have been generated in a wide variety of materials, including silicon, anodized metal and even plastic-based materials (Eden et al., 2003). The prospective use of the latter in the medical field is both promising and exciting, given the large packing densities of jet arrays, material flexibility, and precise control of plasma parameters compared to currently available single pen-type plasma jet devices (Lee et al., 2015).

In this paper, we report the first-time treatment of wounds with second-degree burns by an array of microplasma jet devices fabricated in replica-moulded plastic. We investigated the effectiveness of microplasmas for burn wound treatment, both in vitro and in vivo. Progress of the time-dependent healing was tested through an animal rat model. For further investigation of the wound healing processes, microplasma interaction with either bacteria (Escherichia coli) or fungi (Candida albicans) biofilms has been monitored, and comprehensive histological analysis has also been conducted.

2. Materials and methods

2.1. Non-thermal atmospheric pressure microplasma jet

Microplasma jet devices were fabricated as previously described by Lee et al. (2015). Plasma channels were produced by embedding an array of metal rods in a transparent polymer, which would be removed after assembly was completed and the polymer cured. Figure 1 shows a three-dimensional (3D) view of the fabricated device. To demonstrate the malleability of our fabrication process, microplasma jet devices were prepared in a variety of configurations. Figure 1A shows a 3D view of a $3 \times 3$ array, shown from the side, of microplasma jets emanating in air. Three-layered electrode arrays consisting of four electrode wires, respectively, were built in polymer with a transverse direction of the microchannels. A sinusoidal AC voltage waveform is applied to the electrodes, and each electrode can be operated with individual voltages and polarities, depending on the choice of jet propagation characteristics required. A glass tube 6.35 mm in diameter was attached at the back of the polymer to supply the gas input (not shown here). The polymer was chosen because it is biocompatible and optically transparent throughout the visible spectrum and well into the deep ultraviolet (UV; $\lambda = -250$ nm), so that we can monitor spectroscopically inside the channel. Furthermore, the mechanical flexibility of the polymer can provide versatility and convenience to clinical users, especially for the treatment of the wound area or places difficult to access.

Uniform glow discharges are produced from the cylindrical microchannels by application of an alternative voltage to electrode wire arrays embedded within the silicon polymer. An end-on view of the device structure is shown in Figure 1B; the electrodes are connected at both sides of the device; the relative positions of electrodes and microchannels are illustrated here. In order to provide detailed specifications regarding the relative position between the copper electrode wires and the cylindrical microchannels, Figure 1B shows an end-on view of a $3 \times 3$ microplasma jet array device. Electrode wires with a diameter of 255 $\mu$m are separated by distance of 1 mm with each other. The diameter of the microchannels is 355 ± 5 $\mu$m and they are separated by 1 mm. The closest distance between surfaces of electrode to the edge of microchannel is 195 $\mu$m. With the above parameters, the array packing density is $-120/\text{cm}^2$.

2.2. E. coli and C. albicans culture

Samples of E. coli and C. albicans were cultured in Luria-Bertani medium (1% sodium chloride, 0.5% yeast extract.

Figure 1. Schematic diagram of the structure of a microplasma jet array. (A) General view of a $3 \times 3$ jet device with an electrical configuration. (B) End-on view of microchannels of the device, illustrating the dimensions of the electrodes and their positioning relative to the plasma channels.
array and the surface of the sample was fixed at 3 mm. C. albicans suspension was diluted to 1/100 in sterile saline and 100 μl of the suspension was sampled onto a Sabouraud dextrose agar–chloramphenicol (SDA-C) plate (Hangang, Seoul, Korea). The macroscopic colonies grown after 24 h of incubation were treated by microplasma for 1, 1.5, 2 and 3 min. These plates were incubated for 48 h at room temperature.

2.3. Animal study

Male Sprague-Dawley rats (n = 15), aged 12 weeks, weighing 400 g each, were obtained from the animal centre of Hallym University (Korea). The animals were housed under laboratory conditions at a controlled temperature and maintained under 12 h light–12 h dark cycles (lighting from 07:00 to 19:00 h), with food and water made available ad libitum. The rats were anaesthetized by intraperitoneal injection of zoletil 50 and Rompun, and their dorsal hair was removed. The dorsal skin was exposed for 30 s to a device heated to 60°C to produce a deep second-degree burn of 1 × 1.5 cm size (three wounds/animal) and confirmed by gross pathological change, then treated by microplasma at the wound sites for 1 and 2 min (p1m, and p2m) (Figure 8). The animals were subdivided into five time point groups (1, 3, 7, 9 and 14 days after injury). Treatments twice daily, at an interval of 8 h, were continued for 5 days and the distance between the array and the surface of the tissue was fixed at 3 mm for all treatments. With a helium (He) flow rate of 1.5 standard litres/min (SLM)/microcavity, the length of the visible plume associated with every jet in the array was ~2 mm. Most of the experimental data presented here was obtained with a He flow rate of 0.35 SLM/microcavity, or <14 SLM overall. Eschars were not removed from the wounds because they were thin.

2.4. Wound closure measurements

The extent of the unhealed tissue surface was determined 1, 3, 5, 7, 9 and 14 days after wound formation by imaging each square (control, p1m and p2m) using a charge-coupled device (CCD) camera and analysing the images with INNERVIEW 2.0 software.

2.5. Histological examination

Three rats were sacrificed at each time point. All samples were stored in 10% buffered formalin for at least 24 h, progressively dehydrated in a graded series of ethanols (70%, 80%, 90% and 100% v/v), cleared in Histoclear (AS-ONE, Tokyo, Japan), embedded in paraffin, sectioned at 5 μm thickness, deparaffinized and stained with haematoxylin and eosin (H&E) or Masson’s trichrome (MT). Transforming growth factor-β1 (TGF/β1)- and proliferating cell nuclear antigen (PCNA)-positive cells in the burn wound area were examined by the immunoperoxidase technique, using anti-mouse TGF/β1 and PCNA antibodies, respectively (Bioworld Biotechnology, Louis Park, MN, USA). All sections were observed under an Eclipse 80i microscope (Nikon, Tokyo, Japan).

2.6. Real-time RT–PCR

Total RNA from skin tissues at each time point group was isolated using the RNA-Bee (Tel-test, USA). cDNA was synthesized from total RNA using the cDNA Synthesis Kit for RT–PCR (Maxime RT Premix Oligo(dT), Intron Biotechnology, Korea). Rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal standard. The primer sequences were: IL-1α, forward GAAGATGACCTGGAGGGCATGC, reverse GATGAACTC-CTGCTGACGATCC; IL-6, forward TCTGGTTCTTCTGGAGTTCCGT, reverse GCCACTCTTCTGTGACTCTAAC; IL-10, forward GTTGGCCAAGCTTTGTCAGA, reverse GCGTCGACGTATCCAGA. PCR amplification was performed using a Roter-Gene SYBR Green PCR reagent system (Qiagen, Germany) using a Roter-Gene Q (Qiagen, Germany) at 95°C for 5 s and 60°C for 10 s for 40 cycles.

3. Results and discussion

Microplasma jet devices fabricated from a flexible, transparent polymer were characterized based on number of microchannels, voltage–current analysis and discharge stability and uniformity. Figure 2A shows a series of microplasma jet devices consisting of 1 × 1 to 5 × 5 arrays. The same devices are shown from an end-on view in Figure 2B. The devices were operated with a 20 kHz sinusoidal AC waveform excitation at a pressure of 860 Torr, with He gas feedstock. According to the specification described earlier, the 5 × 5 jet array has a surface area of 16 mm², with jet-to-jet distance of only 1 mm. Uniformity of jet length and luminosity was achieved within 10% and there was no plasma interaction or crosstalk between neighbouring jets in the device dimension described here. All jet devices started operation simultaneously and maintained excellent jet-to-jet operation uniformity over a wide operation range, as also clearly shown in end-on view. Voltage–current characteristics for microplasma jet arrays are shown in Figure 3. Data were given for operation in He at 800 Torr, when the arrays are driven by a sinusoidal voltage waveform at a frequency of 20 kHz. The positive slopes indicate that this microplasma is operating in the abnormal glow discharge mode, and proportionate increase of currents (in similar operational voltages) with regard to the number of microplasma jets indicates that these arrays operate as micro-dielectric barrier discharges.
This is a very important device characteristic, not only for stable operation in glow discharges but also for the scaling-up of arrays for treatment of larger areas by simply increasing the number of microplasma jets. By monitoring the device current while it operates, the power deposition into an individual jet was measured in the range of 3–96 mW, which can be converted to a power density of 1.5–15 W/cm³. Therefore, in this microvolume regime, the power loading to the plasma is a factor of 5–10 times higher than the value reported previously for conventional plasma jets (Kim et al., 2009; Laroussi, 2002). Figure 4A shows device power loading as a function of driving frequency, and it shows increase of current at a linear proportion to the repetition rates of applied voltage waveforms. Figure 4B shows voltage-current curve (V-I) characteristics of the devices with different microchannel diameters, and higher power loading was achieved for the device with smaller diameter as the same trend as other microcavity plasma devices (Eden et al., 2003; Eden and Park, 2005), while the channel diameter increases the current and the length of the emanating jet into the air. The stability of the microplasma jets was optimized by the gas feed pressures (Sun et al., 2012).

Figure 5 shows the emission spectra of a 3 × 3 microplasma jet array in He gas flow. An obvious distinction can be observed between the colour of the plasma fluorescence emanating from the jet (in air) and that from the plasma microchannels within the optically transparent micro-DBDs.
plastic device; Figure 5 highlights the differences between these two regions. The spectrum in blue is that for the plasma with the channels and, aside from OH (A→X) emission at ~308 nm, virtually all of the features arise from atomic He and Rydberg→Rydberg state transitions of He2. However, once the plasmas emerge from the channels and interact with air, the spectrum (shown in red) shows a significant change, that He emission vanishes and strong N2+(B→X) fluorescence in the near-ultraviolet and blue (391.4 and 427.8 nm) appears. The dominance of N2+ emission in the jet is attributed to Penning ionization of N2 by the He (23S) metastables in the plasma flow. As reported earlier, from the research on the relative emission intensities for most of these spectral features with different pressures of He feedstock gas, N2+ is the most intense in the 300–500 nm region, owing to the N2+ [B2Σ_u⁺(v′ = 0)] radiative lifetime, but also to the rate constant for the collisional process: He (23S1) + N2→N2+ (B2Σ_u⁺) + He + e− + ΔE (Sun et al., 2012). Penning ionization of N2 by the He (23S1) metastable is well known (Laroussi, 2002). The linear variation of the N2+ fluorescence intensity with He pressure is consistent with Penning ionization being the dominant process for populating the N2+ (B2Σ_u⁺) state. Similarly, the linear dependence of both the He (43S1→23P) and O (3p5P2→3s5S2) intensities on the He pressure is also expected, because the He (23S1) metastable is the precursor to the production of both the O (3p5P2) and He (43S1) excited atoms.

It is important to note that the intensity of the N2+ line increases along the plasma axis and, more importantly, upon interaction with the surrounding air. Reactive nitrogen species, as well as reactive oxygen species, are commonly cited as being dynamic signalling molecules responsible for therapeutic effects (Graves, 2012). Our multi-jet array device not only enables larger treatment areas but delivers reactive nitrogen species along the axis of a multitude of microplasma jet plumes, thereby increasing the overall number density of reactants interacting with the surface.

To investigate the effectiveness of sterilization by microplasma jets, bacteria on an agar plate were treated with the device. Following the 16 h incubation period, an obvious sterilization effect could be observed. The plates where bacteria had been killed looked like uncontaminated agar, while plates that received no treatment changed colour and appearance significantly as bacteria grew there. The complete sterilization area is easiest to identify, as it corresponds to the agar area that is completely clear from bacteria. As shown in Figure 6, the complete sterilization gradually fades into untreated areas.

Figure 5. Emission spectra of 3×3 array, each obtained from a plasma in the microchannel inside the device (blue) and for the jet interacting with laboratory air (red). In acquiring these data, the He pressure (flow rate) was set to 800 Torr and the voltage was ~2 kV (RMS)

Figure 6. Photographs showing the time-dependent bactericidal (E. coli) effect of microplasma treatment: (A) 15 s; (B) 30 s; (C) 1 min; (D) 2 min
The susceptibility of *Candida* to the microplasma device was determined by applying the *Candida* cells to SDA-C plates. The fungicidal effect of the plasma is shown in Figure 7; the *Candida* cells remarkably decreased at 1.5 min. No fungicidal effect was observed at the samples with untreated controls.

The response to an injury commonly known as wound healing is an innate immune response resulting in inflammation, epithelial proliferation and subsequent remodeling of the affected tissue (Ross et al., 1977). These three activation processes in a wounded tissue give rise to overlapping and complicated cell–cell signalling processes.

![Figure 7](image7.png)  
**Figure 7.** Photographs showing time-dependent fungicidal (*Candida albicans*) effect of microplasma treatment: (A) control; (B) 1 min; (C) 1.5 min; (D) 2 min; (E) 3 min

![Figure 8](image8.png)  
**Figure 8.** Photographs of microplasma treatment on a burn wound (left) and the wound area on the skin of a rat after its creation (right)

![Figure 9](image9.png)  
**Figure 9.** Wound closure in rat burns. (A) Gross findings of control (untreated) and microplasma-treated skin after burning; D, days. (B) Evaluation of wound area vs healing time
Figure 10. Histological analysis of burn wounds stained with H&E at the indicated time points. Re-epithelialization commenced at day 7 for all three groups; by day 9, fibrin clots persisted in the control group, while they were virtually non-existent in the microplasma-treated group. Complete regeneration of tissue occurred at day 14 for the microplasma-treated group, as evidenced by the clear, and therefore healthy, distinction between dermal, epidermal and subcutaneous layers. In contrast, the control group histology showed persistent granulation tissue and infiltration of lymphocytes and neutrophils in the epidermis. Scale bars = 50 μm.

Figure 11. Histological analysis of burn wounds stained with MT at the indicated time points. Collagen deposition (blue) was both dense and continuous on day 14 for the microplasma-treated group as compared to the control; scale bars = 50 μm.
that are primarily regulated by cytokines (Ross et al., 1977). In addition, a plethora of tissue growth factors (TGFs) play an important role in epithelial proliferation. Of equal importance is collagen deposition, in order to keep the wounded area intact during healing. Considering all the possible processes described above, we made a systematic characterization of the wound-healing process through histological staining for growth factors and RT–PCR gene expression of cytokines.

For investigation of the effect of microplasma treatment on burn wounds, the plasma jet array was placed on the top of the samples immediately after the burn wound was made, as shown in Figure 8. After the first treatment on the wound, nine subsequent treatments were made for the first 5 days. Each microplasma treatment to the wounds of two groups were made for 1 and 2 min, respectively. The sizes of the wounds were first measured 6 h after the treatment, and then the diagnosis continued at 1, 3, 5, 7, 9 and 14 days. Figure 9 shows photographs and data of residual wound area change with healing time. It should be noted that the non-treated (control) group shows gradual increase of wound area until day 5, after which the size progressively decreased. In contrast, the groups with plasma treatment shows significant healing progress compared to the non-treated group, so that after day 7 obvious decrease of the wound area (healing) can be observed. The data of Figure 9B also support the observation of wound closure/healing in the

![Figure 12](image.png)

Figure 12. TGFβ1 and PCNA expression in the burn wound site at the indicated time points: (A) TGFβ1; (B) PCNA; scale bars = 50 μm
samples with microplasma treatment, and the microplasma treatment for 1 and 2 min decreased the wound area by 22% and 21%, respectively, while the wound size of the non-treated group remained at 75%.

As shown in Figure 10, the wound-healing process by microplasma treatment was investigated through histological analysis of the burned skin. A second-degree burn was confirmed by the appearance of blistering and oedema without any tissue damage to the underlying fascia and muscle tissue on day 1. Superficial cellularity loss of hair follicles and epithelial blistering were also observed. In all sample groups, the re-epithelialization process began at day 7 after treatment. More importantly, the microplasma-treated group showed faster tissue regeneration in the burn wounds, while fibrin clots still remained on the surface of the wound area in the non-treatment group (C). During the whole healing period, the microplasma-treated group showed a relatively faster re-epithelialization process compared with that of the medical gauze-treated group (C). After 14 days, the wounds treated with microplasma exhibited morphology and histology similar to that of normal skin, and the wound area was completely regenerated without the formation of oedema or granulation tissue. Figure 11 shows the collagen arrangement at the wound site. The collagen arrangement for the microplasma-treated group was observed to be denser and more uniform compared to the non-treated group.

We also report the effects of microplasma on TGFβ1 and PCNA expression in burn wounds. Figure 12 shows TGFβ1 and PCNA-immunoreactive cells at days 7, 9 and 14 after the treatment. At day 7, TGFβ1 in the microplasma-treated groups showed lower expression levels than in the non-treatment group. On the other hand, at day 14, TGFβ1 and PCNA-positive cells in the epidermis markedly increased in the non-treatment group compared with those in the microplasma-treated group.

At the molecular level, burn wound healing depends largely on cytokines and growth hormones (Altavilla et al., 2005; Barret and Herndon, 2003; Bitto et al., 2014; Jeschke et al., 1999). Previous studies have demonstrated that cytokines, including pro-inflammatory and anti-inflammatory cytokines, play critical roles in the burn wound-healing processes (Eming et al., 2007). However, severe burn injury interrupts the homeostasis of the immune system in the body, resulting in excessive production of cytokines, such as IL-1α, IL-6 and IL-10. This overproduction of inflammatory cytokines can attenuate burn wound healing and induce multiple organ failure. We examined the genetic expression of burn-induced cytokines related to wound healing processes using RT–PCR (Figure 13). The microplasma-treated group exhibited expression levels of IL-1α and IL-6 that were relatively lower than those of the non-treatment group (Figure 13A, B). The expression of IL-10, an anti-inflammatory cytokine, in the microplasma-treated group was relatively higher than that of the non-treatment group. This level of expression was further sustained at 9 days for the microplasma-treated group (Figure 13C). These results suggest that non-thermal microplasma treatment facilitates the wound-healing process in burn-damaged tissue via regulation of cytokines, including IL-1α, IL-6 and IL-10.

4. Conclusion

We have successfully demonstrated that an array of low-temperature, microscale plasma jet devices fabricated in flexible polymer are a viable form of medical therapy for burn wounds. The structural flexibility and scalability of our device suggests that plasma devices may be shaped to treat difficult-to-reach areas. In addition, an increased number density of reactive nitrogen and oxygen species are available for reaching the treated sample, due to a multitude of microplasma plumes interacting with the surrounding air. The data reported here consistently show that microplasma treatment results in accelerated wound healing, and both bactericidal and fungicidal effects are observed. Our tissue histology findings indicate collagen arrangement and regulation of TGFβ1 and PCNA expression for the plasma-treated samples. In addition, RT–PCR studies reveal an upregulation of anti-inflammatory genes, along with a decrease in the expression of inflammatory cytokines for the microplasma-treated group. We found that the non-thermal microplasma jet accelerates wound healing in burns via the regulation of anti-inflammatory processing, but further detailed study of the gene expression mechanism is needed.

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Conflict of interest
The authors declare no conflicts of interest.

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