Plasma Medicine, 5(2–4): 237–247 (2015)

Using Helium-Generated Cold Plasma to Control Infection and Healing

Paola Brun,[1,*] Venera Russo,[1] Paola Brun,[2] Elena Tarricone,[2] Simona Corrao,[2] Velika Deligianni,[3] Andrea Leonardi,[4] Roberto Cavazzana,[5] Matteo Zuin,[5] & Emilio Martines[5]

[1]Department of Molecular Medicine, University of Padova, Unit of Microbiology, I-35100 Padua, Italy; [2]Department of Molecular Medicine, University of Padova, Unit of Histology, I-35100 Padua, Italy; [3]Department of Ophthalmology, San Antonio Hospital, I-35100 Padua, Italy; [4]Department of Neuroscience, University of Padova, Unit of Ophthalmology, I-35100 Padua, Italy; [5]Consorzio Ricerca Formazione Innovazione (RFX), Padova 4-35127, Italy

*Address all correspondence to: Paola Brun, PhD, Department of Molecular Medicine, University of Padova, Via A. Gabelli 63, I-35100 Padua, Italy; Tel.: +39 049 8272348 or +39 049 8272343; Fax: +39 049 8272355; paola.brun.1@unipd.it

ABSTRACT: Cold atmospheric pressure plasma has been proposed for sterilization of inert surfaces and disinfection of living tissues. Our recent studies reveal that chemically active species such as reactive oxygen species generated by a low-power atmospheric pressure non-thermal plasma source obtained by ionizing helium gas mixed with ambient air are mandatory for their antimicrobial effects. In addition, they also initiate intracellular signaling pathways required for wound healing in eukaryotic cells. Thus, exposure to plasma sustains healing of tissue injuries both indirectly through microbicidal effects and directly by action on cells such as fibroblasts involved in tissue regeneration.

KEY WORDS: reactive oxygen species, bacteria, fungi, eukaryotic cells, wound healing

I. INTRODUCTION

Wound healing is a dynamic, well-organized process requiring cooperation of different intracellular signaling to repair lesions and damaged tissues.[1] At first, local soluble factors guide proliferation, migration, and transition of tissue resident cells to activated fibroblasts that produce growth factors and a collagen framework that promote tissue regeneration.[2] In this setting, reactive oxygen species (ROS) regulate activation of fibroblasts and have a key role in extracellular matrix deposition.[3] However, the physiological wound-healing process is jeopardized by environmental factors such as microbes infecting the wound, which increases inflammatory cytokine levels and perpetuates fibroblast activation with excessive accumulation of collagen and fibrotic scarring.

Microbiological assays reveal a wide range of infectious agents that contaminate wounds. Thus, gram-positive bacteria (*Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus pyogenes*), filamentous fungi such as *Aspergillus* species, unicellular fungi such as yeasts of the Candida and Trichophyton families, and gram-negative bacteria such as *Pseudomonas aeruginosa* are reported to be the most common pathogens in infected wounds.[4] Nowadays, the success of topical or systemic

1947-5764/15/$35.00

broad-spectrum antibiotic is increasingly weakened by the progressive outbreak of multidrug- or pandrug-resistant microbial strains. Thus, novel therapeutic strategies would be a great breakthrough in the treatment of wound infection. Low-temperature atmospheric pressure plasmas have been successfully used to inactivate various microbes on surfaces and in solution and promote healing of lesions. Moreover, because antimicrobial effects of plasma are mainly ascribed to a space-localized burst of ROS, plasmas are emerging as promising tools for the treatment of localized infection in living tissues.

We recently reported that a low-power atmospheric pressure nonthermal plasma generated by ionizing helium gas intermixed with ambient air slightly increased intracellular ROS levels in primary human keratocytes and conjunctival fibroblasts.[5] The aims of the present study were to assess the microbicidal effects of plasma against the most common pathogens identified in infected wounds and investigate the role of ROS generated by plasma exposure on the proliferation and migration of cultured human primary fibroblasts to accelerate wound healing.

II. MATERIALS AND METHODS

A. Plasma Source

Plasma was produced by applying a radio-frequency (RF) electric field to a flow of helium at atmospheric pressure. This plasma source, also described elsewhere,[5,6] consists of two coaxial tubes, each closed at one end by a double brass grid. The outer tube is copper and electrically grounded and the inner tube is made of insulating material. Plasma is generated between two grids acting as electrodes. The two parallel grids are positioned ~1 mm apart. The electric field is formed in the space between the two grids by applying an RF voltage difference supplied by an RF generator coupled to the source by a matching network. The matching network raises the voltage to the value needed for helium ionization of ~1000 V peak–peak. Despite the high-voltage value, the current flowing in the plasma is so low that the dissipated power is below 1 W. The chosen operational frequency is 4.8 MHz and gas flow rate is 1.5 L/min. The reactive chemical species responsible for the effects described below are formed within the plasma due to the mixing of some ambient air with the helium flow. It is worth mentioning that due to the specific setup, this is an indirect plasma treatment; that is, the sample to be treated is not directly exposed to the plasma but only to the afterglow.

B. Bacterial Strains and Growth Conditions

The effects of plasma were studied on gram-positive bacteria (*S. aureus*, methicillin-resistant *S. aureus*, *S. epidermidis*, and *S. pyogenes*), gram-negative bacteria (*Escherichia coli*, vancomycin-resistant *E. coli*, *P. aeruginosa*, and *Proteus mirabilis*), and fungi (*Candida albicans*, *Aspergillus fumigatus*, and *Trichophyton rubrum*). Microbial strains were isolated from human specimens collected from the Microbiology and Virology Section at the University Hospital of Padova and were identified by gas chromatography

and antibiotic susceptibility tests. All microbial strains were stored at –80 °C and were cultured overnight in Mueller–Hinton broth (MHB) at 37 °C (bacteria) or 35 °C (fungi). Mueller–Hinton agar or Sabouraud agar was used as the solid growth medium where appropriate.

C. Cell Cultures

Primary intestinal fibroblasts were isolated from a nonpathological colonic biopsy of a 56-yr-old male subject. The tissue sample was diced and digested, as previously reported[7] to obtain cell suspension. Cells were grown at 37 °C in culture medium containing Dulbecco's modified Eagle's medium (DMEM; Gibco/Thermo Fisher, Monza, Italy) with 20% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 ng/mL fungizone (Gibco). At confluence, cells were detached using 0.05% trypsin-EDTA (Gibco) and seeded in 24-well tissue-culture plates (Corning, Corning, NY) at 3×10^5 cells/mL. Cells were treated with 5 mM *N*-acetyl-L-cysteine (NAC) for 15 min. The study protocol was approved by the Ethical Committee of the University Hospital of Padova. The tissue donor was provided with detailed information about study aims and protocol and gave his written, informed consent.

D. Plasma Treatment

Microbial suspensions were prepared in sterile phosphate-buffered saline (PBS) from overnight culture and each sample was adjusted to optical density equivalent to 1×10^7 colony-forming units (CFU)/mL. Aliquots (200 μL, 2×10^6 CFU) from each bacterial suspension were exposed for 30, 120, and 300 s to plasma afterglow generated by the plasma tip located 1.5 mm away. After plasma exposure, each sample was properly diluted in sterile PBS and dispersed in solid growth medium for surviving cell viability determination using the colony count method. Samples were tested in triplicate and microbicidal efficacy of plasma was expressed in percentage, calculated according to the following formula:

$$(CFU_{ref} - CFU_{exp})/CFU_{ref} \times 100,$$

where CFU_{ref} and CFU_{exp} are the number of living bacteria colonies in the untreated and plasma-treated samples, respectively.

Human intestinal fibroblasts grown to 70% confluence were incubated with 200 μL of fresh culture medium and exposed to the plasma afterglow generated by the plasma tip located 1.5 mm away for 30, 120, and 300 s. After plasma treatment, cells were incubated for up to 24 h.

E. Intracellular Reactive Oxygen Species

Intestinal fibroblasts were incubated with 10 mM 2',7'-dichlorodihydrofluorescein diac-

etate (H_2DCFDA; Molecular Probes, Invitrogen, Monza, Italy) for 30 min at 37 °C and then exposed to plasma. Intracellular levels of ROS were measured 5 min later using a BD FACS-Calibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ). Ten thousands events were acquired for each sample. The results were analyzed using WinMDI 2.9 (Windows multiple document interface for flow cytometry).

F. Proliferation Assay

Intestinal fibroblasts were incubated at 37 °C for 10 min with 25 mM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen). Staining was quenched by adding 5 volumes of ice-cold culture media. Cells were exposed to plasma and subsequently incubated in fresh culture medium for 72 h at 37 °C. Cell proliferation was then evaluated by partitioning the fluorescent dye between daughter cells using a BD FACS-Calibur flow cytometer.

G. Western Blot Analysis

Plasma-treated intestinal fibroblasts were cultured for 4 h. Cells were then washed with ice-cold PBS and subjected to total protein extraction in nondenaturing radioimmunoprecipitation assay (RIPA) buffer, as described elsewhere.[8] Proteins (40 μg/line) were separated in 10% w/v sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Immun-Blot PVDF membrane (BioRad Laboratories, Inc., Segrate, Italy). Membranes were probed with mouse antiphospho–NF-κβ p65 antibody (cell signaling) for 16 h at 4 °C and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. Anti-β-actin antibody (Sigma-Aldrich, Milan, Italy) was used as the loading control. Immunocomplexes were visualized using enhanced chemiluminescence (ECL; Merck Millipore, Milan, Italy). Images were captured using a Hyperfilm MP (GE Healthcare Life Sciences, Milan, Italy).

H. Wound-Healing Assay

Plasma-treated intestinal fibroblasts were seeded onto glass coverslips and cultured for 24 h at 37 °C. The cell monolayer was then wounded with a plastic tip. Seventy-two hours later, the cells were rinsed twice in PBS, fixed in paraformaldehyde (PFA) 4% w/v for 10 min, and routinely stained with hematoxylin and eosin (H&E). Finally, the slides were examined using a light transmission microscope connected to a camera to capture the images (Leica DMLB, Wetzlar, Germany).

I. Statistical Analyses

The graphics report data as mean ± standard error. An unpaired student's *t*-test was used to compare the data of two groups and a one-way analysis of variance (ANOVA) test followed by the Newman–Keuls post hoc test was used to compare data of three or

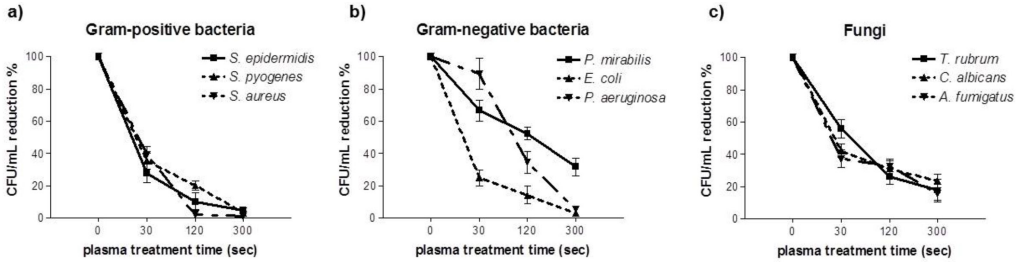

FIG. 1: Inactivation of different microbial strains by helium-generated cold plasma. Microbes (2×10^6 CFU) were treated with plasma for the indicated time. The inactivation (CFU) of (a) gram-positive bacteria (*Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Streptococcus pyogenes*), (b) gram-negative bacteria (*Proteus mirabilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*), and (c) fungi (*Trichophyton rubrum*, *Candida albicans*, and *Aspergillus fumigatus*) was reported as percentages calculated in untreated samples. Data are reported as mean ± standard error; $n = 4$.

more groups. Statistical analysis was performed using GraphPad Prism 3.03 (GraphPad Software, San Diego, CA).

III. RESULTS

A. Antimicrobial Effects of Plasma

As previously reported, the exposure to helium afterglow originating from the plasma source successfully inactivates microbes.[5] We describe the antimicrobial effects of plasma on different gram-negative and -positive bacteria as well as on three different yeast strains, with the aim to demonstrate the broad-spectrum of plasma applications as tool for microbial decontamination. As shown in Fig. 1, plasma treatment induced a rapid and time-dependent inactivation of all tested microbes. However, the decrease in number of colonies differed greatly among the different microbial species. Helium plasma demonstrated higher and reproducible efficacy in destroying different gram-positive bacterial species. Indeed, the plasma-mediated inactivation effect was comparable among the all tested gram-positive bacterial strains, with an antimicrobial efficacy of 97.08% at 300 s (range: *S. epidermidis*, 95.35%; *S. aureus*, 98.95%). The antimicrobial effect of plasma was also comparable among the three fungal strains, with an even lower antimicrobial effect at 300 s (81.11%; range: *C. albicans*, 76.5%, *A. fumigatus*, 84.35%). Dramatic differences in antimicrobial effect, with great interspecies variability, were found among the gram-negative bacteria tested. Indeed, plasma treatment quickly inactivated *E. coli*, substantially decreased the *P. aeruginosa* colony number after only 300 s of exposure, and slightly affected *P. mirabilis*. The diverse effects of plasma treatment can be ascribed to different cell-wall structures and the scavenger response of microbes. Indeed, fungi are endowed with a nuclear membrane to protect genomic DNA against

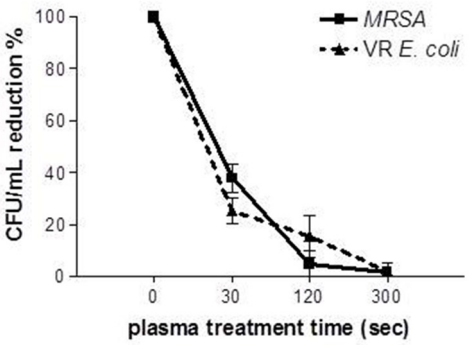

FIG. 2: Inactivation of antibiotic-resistant bacteria by helium-generated cold plasma. Methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant (VR) *E. coli* (2×10^6 CFU) were treated with plasma for the indicated times. Bacterial inactivation (CFU) was reported as percentages calculated in untreated samples. Data are reported as mean ± standard error; $n = 3$.

ROS-mediated damage, whereas *P. mirabilis* and *P. aeruginosa* organize in biofilms and produce a variety of pigments and enzymes that are proven to change the pH of medium and scavenge the chemical reactive species generated by plasma. These considerations strengthen the role of ROS in mediating the antimicrobial effects of plasma. Thus, ROS generation makes plasma an appropriate tool for microbial decontamination, acting via molecular pathways different from those targeted by common antibiotics. This provides a promising technique to overcome the problem of growing microbial antibiotic resistance.

With this in mind, we exposed plasma to two selected antibiotic-resistant bacterial strains: gram-positive methicillin-resistant *S. aureus* (MRSA) and gram-negative vancomycin-resistant (VR) *E. coli*. As reported in Fig. 2, plasma exposure reduced the survival of antibiotic-resistant bacteria at the same degree reported for nonresistant bacteria (see Fig. 1); antibacterial efficacy at 300 s measured 98.32% for MRSA and 97.96% for VR *E. coli*.

To assess the potential occurrence of resistance to plasma antimicrobial effects, aliquots of *S. aureus* and *E. coli* were repeatedly exposed to helium plasma for 30 s once daily for 10 d. As shown in Fig. 3, repeated plasma treatment did not reduce bactericidal effects.

B. Intracellular ROS Generation

Generation of intracellular ROS following 30, 120, and 300 s exposures to the plasma source was evaluated 5 min later in human primary intestinal fibroblasts using the H_2D-CFDA probe. As reported in Fig. 4, plasma treatment increased DCFDA-related fluorescence, with a significant peak following 120 and 300 s of treatment. The burst of intracellular ROS decreased in cells treated with the antioxidant agent NAC.

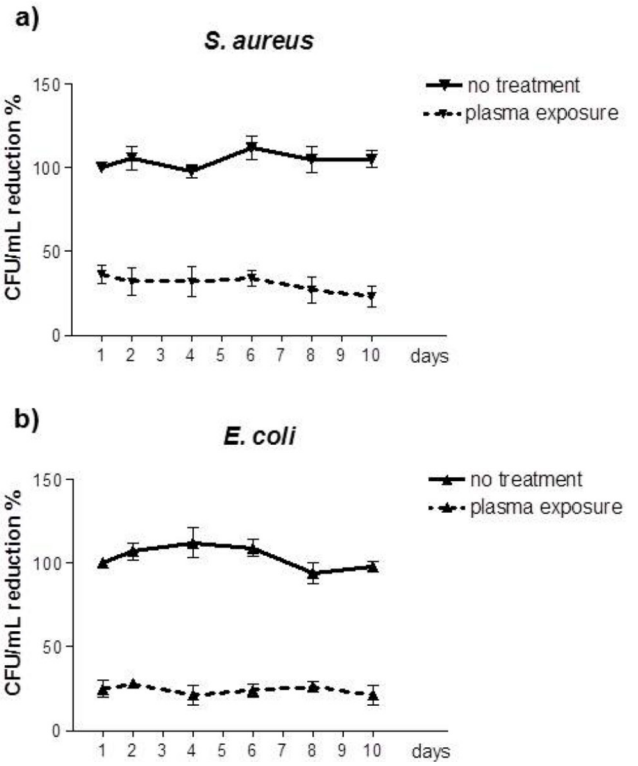

FIG. 3: Repeated treatment with helium-generated cold plasma did not induce resistance in bacteria. *S. aureus* and *E. coli* (2×10^6 CFU) were treated with plasma for 10 d once daily for 30 s. Bacterial inactivation (CFU) was reported as percentages calculated in untreated samples. Data are reported as mean ± standard error; $n = 3$.

C. Plasma Exposure Promotes Cell Proliferation

Cell proliferation was evaluated in human fibroblasts by measuring the partitioning between daughter cells of CFSE probe-related fluorescence. As reported in Fig. 5, fluorescence partitioning occurred in 12.5 ± 1.04% of untreated fibroblasts whereas plasma exposure stimulated proliferation in 44.5 ± 2.1% (120 s plasma treatment) and 63.2 ± 2.7% (300 s plasma treatment) of the cells. Plasma-induced cell proliferation was secondary to intracellular ROS generation because pretreatment with NAC significantly abolished cellular division ($p < 0.05$ vs. plasma-treated cells).

D. Plasma Exposure Increases NF-κβ Expression

Intracellular ROS act as transduction molecules in mammalian cells and engage different signaling pathways.[9] For instance, ROS have been implicated in the activation of the NF-κβ, transcriptional factor that has important roles in cell growth, proliferation, and

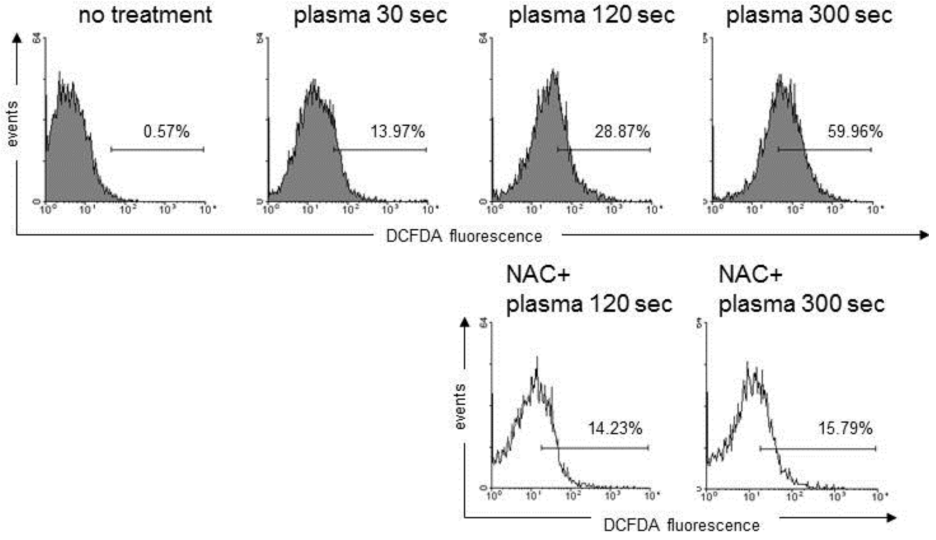

FIG. 4: Helium-generated cold plasma ignited ROS in primary human fibroblasts. Human fibroblasts were labeled with H_2DCFDA and exposed to plasma for 30, 120, or 300 s. Five min later, cells were collected and intracellular ROS levels were detected by FACS cytometric analysis. ROS formation was reported as percentages of fluorescence-positive cells. As indicated, cells were pretreated with 5 mM NAC. Data are representative of three independent experiments with similar results.

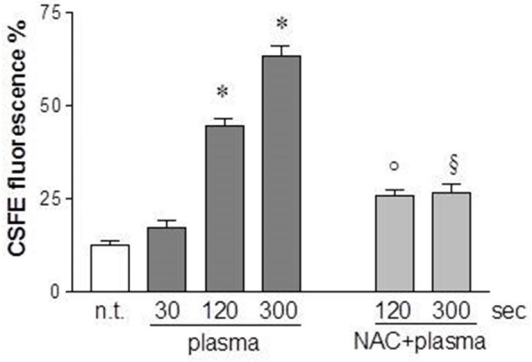

FIG. 5: Helium-generated cold plasma promoted proliferation of human fibroblasts. Human fibroblasts were labeled with CFSE and exposed to plasma for 30, 120, or 300 s. Following 72 h of incubation, cellular proliferation was assessed by FACS cytometric analysis to evaluate the percentage of fluorescent signal partitioned among daughter cells. As indicated, cells were pretreated with 5 mM NAC. Data are reported as mean ± standard error of three independent experiments, each performed in duplicate. *, $p < 0.05$ vs. untreated (n.t.) cells; °, $p < 0.05$ vs. 120 s plasma-treated cells; §, $p < 0.05$ vs. 300 s plasma-treated cells.

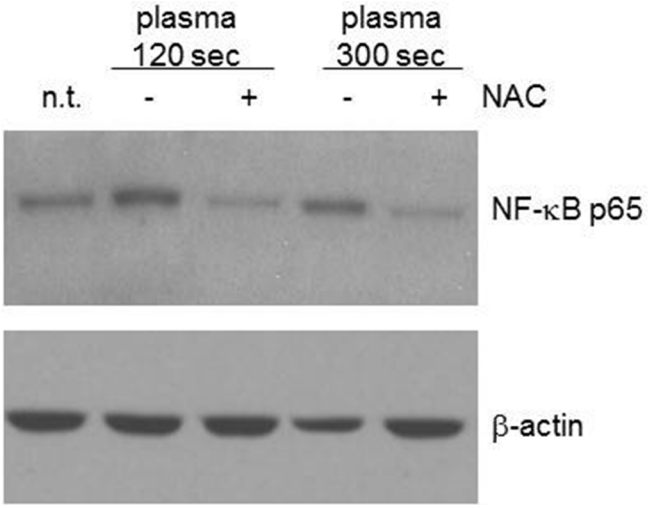

FIG. 6: Helium-generated cold plasma activated the NF-κβ signaling pathway in human fibroblasts. Human fibroblasts were exposed to plasma for 120 or 300 s. Four hours later, phosphorylation of NF-κβ was assessed by western blot analysis on protein extracts. As indicated, cells were pretreated with NAC. β-actin was used as a loading control. Data are representative of two independent experiments with similar results.

survival.[10] As reported in Fig. 6, 120 and 300 s plasma exposure increased NF-κβ phosphorylation levels. This effect was secondary to intracellular ROS generation because pretreatment with NAC significantly counteracted NF-κβ activation.

E. Plasma Exposure Sustains Wound Healing

In addition to increasing cellular proliferation and expression of NF-κβ, plasma treatment sustained the wound-healing process as outlined by the migration of human fibroblasts beyond the edge of the scratch caused in the cell monolayer. Indeed, as reported in Fig. 7, 120 and 300 s plasma treatment clearly fostered closure of the wound. Again, this effect was ROS dependent because pretreatment of cells with NAC abolished wound-healing capacity.

IV. CONCLUSION

Atmospheric pressure nonthermal plasma generated by ionizing a helium flow mixed with ambient air selectively inactivated different microbes without damaging host cells. These data suggest that local treatment with nonthermal plasma represents a great advantage in the treatment of polymicrobial infection. Indeed, systemic antimicrobial agents kill or slow the growth of both commensal and pathogenic microbes, leading to microbial dysbiosis. Moreover, antibiotics and anti-inflammatory agents usually impair

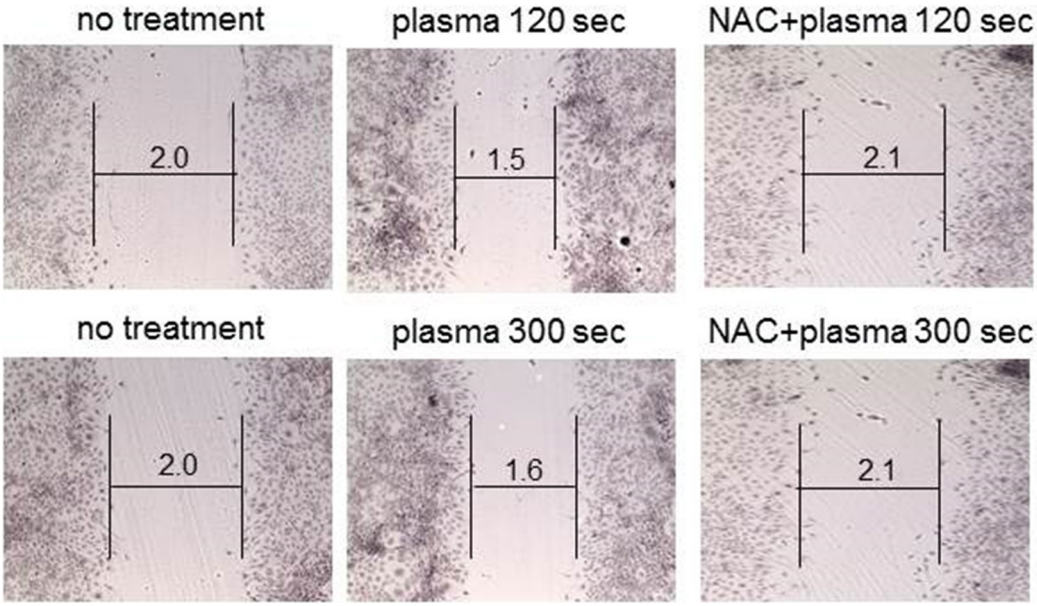

FIG. 7: Helium-generated cold plasma fostered wound healing. Human fibroblasts were exposed to plasma for 120 or 300 s. The cell monolayer was wounded using a plastic tip and cells were then stained with H&E. Images were observed and captured using a light transmission microscope connected to a DMLB Leica camera. Similar results were obtained after two independent experiments.

host-tissue integrity, thus worsening the prognosis of healing. It is now well accepted that the biological effects of nonthermal plasmas are largely mediated by the generation of intracellular ROS.[11] Thus, whereas cellular compartments of organelles protect eukaryotic cells from reactive species burst, bacteria are vulnerable to low levels of ROS. However, this effect is dose dependent. Indeed, increased concentration of ROS leads to different cellular phenotypes and function. These considerations pave the way for a number of different applications of plasma in disinfection and antisepsis of human tissues. Thus, fine modulation of plasma power might result in the increase of antibacterial effects, broadening the spectrum of susceptible microbial species or targeting specific microbial species. At the same time, ROS generated by plasma treatment in host cells may represent an additional source of reactive species, helpful in controlling intracellular pathogens or addressing specific cellular functions.

REFERENCES

1. Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. Nature. 2008;453:314-21.
2. Andoh A, Bamba S, Brittan M, Fujiyama Y, Wright NA. Role of intestinal subepithelial myofibroblasts in inflammation and regenerative response in the gut. Pharmacol Ther.

2007;114:94-106.
3. Rojkind M, Domınguez-Rosales JA, Nieto N, Greenwel P. Role of hydrogen peroxide and oxidative stress in healing responses. Cell Mol Life Sci. 2002;59:1872-91.
4. Giacometti A, Cirioni O, Schimizzi AM, Del Prete MS, Barchiesi F, D'Errico MM, Petrelli E, Scalise G. Epidemiology and microbiology of surgical wound infections. J Clin Microbiol. 2000;38:918-22.
5. Brun P, Brun P, Vono M, Venier P, Tarricone E, Deligianni V, Martines E, Zuin M, Spagnolo S, Cavazzana R, Cardin R, Castagliuolo I, Valerio AL, Leonardi A. Disinfection of ocular cells and tissues by atmospheric-pressure cold plasma. PLoS One. 2012;7:e33245.
6. Martines E, Zuin M, Cavazzana R, Gazza E, Serianni G, Spagnolo S, Spolaore M, Leonardi A, Deligianni V, Brun P, Aragona M, Castagliuolo I, Brun P. A novel plasma source for sterilization of living tissues. New J Phys. 2009;11:115014.
7. Brun P, Pathak S, Castagliuolo I, Palù G, Brun P, Zuin M, Cavazzana R, Martines E. Helium generated cold plasma finely regulates activation of human fibroblast-like primary cells. PLoS One. 2014;9:e104397.
8. Brun P, Castagliuolo I, Pinzani M, Palù G, Martines D. Exposure to bacterial cell wall products triggers an inflammatory phenotype in hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol. 2005;289:G571-8.
9. Chen AC, Arany PR, Huang YY, Tomkinson EM, Sharma SK, Kharkwal GB, Saleem T, Mooney D, Yull FE, Blackwell TS, Hamblin MR. Low-level laser therapy activates NF-kβ via generation of reactive oxygen species in mouse embryonic fibroblasts. PLoS One. 2011;6:e22453.
10. Morgan MJ, Liu ZG. Crosstalk of reactive oxygen species and NF-κβ signaling. Cell Res. 2011;21:103-15.
11. Graves DB. Oxy-nitroso shielding burst model of cold atmospheric plasma therapeutics. Clin Plasma Med. 2014;2:38-49.