33rd Annual International Conference of the IEEE EMBS
Boston, Massachusetts USA, August 30 - September 3, 2011

Non-thermal dielectric barrier discharge plasma induces angiogenesis through reactive oxygen species

Krishna P. Arjunan and Alisa Morss Clyne, *Member, IEEE*

Abstract— **Vascularization plays a key role in processes such as wound healing and tissue engineering. Non-thermal plasma, which primarily produces reactive oxygen species (ROS), recently emerged as an efficient tool in medical applications. Liquids and endothelial cells were treated with a non-thermal dielectric barrier discharge plasma. Plasma treatment of phosphate buffered saline (PBS) and serum-free medium increased ROS concentration in a dose-dependent manner, with a higher concentration in serum-free medium. ROS concentration in cells peaked 1 hour after treatment. 4.2 J/cm^2 increased cell proliferation, 2D and 3D migration, as well as tube formation. A fibroblast growth factor-2 (FGF-2) neutralizing antibody and ROS scavengers for hydrogen peroxide and hydroxyl radicals abrogated these angiogenic effects. Non-thermal plasma may be a potential tool for applying ROS in precise doses to enhance vascularization.**

I. Introduction

Angiogenesis plays a key role in physiological processes [1]. Insufficient vascularization contributes to impaired wound healing in patients with diabetes [2]. *In vitro*, vascularization is essential for engineering complex tissues such as bone, muscle, liver and heart [3]. Over the past few decades, many angiogenic growth factors have been discovered, including fibroblast growth factor-2 (FGF-2) [4]. FGF-2 is associated with cell survival, proliferation, and migration [5]. FGF-2 does not have a recognized signal sequence for secretion and is known to be released by injured cells [6]. Thus, cells that suffer an injury prepare themselves against subsequent injuries through FGF-2. While these growth factors are critical to the angiogenic process, their success depends on timing, dose, and gradients [7]. Efforts to apply growth factors in wounds or in tissue engineered constructs to induce angiogenesis have met with limited success [8].

Plasma medicine is a rapidly expanding interdisciplinary field combining engineering, physics, and life sciences [9]. Plasma is an ionized gas composed of charged particles, excited atoms and molecules, radicals, and UV photons. In non-thermal plasma, electron temperature is much higher than heavy particle temperature. While high temperature electrons interact with gas molecules to create reactive species, overall gas temperature remains close to room temperature and hence non-thermal plasma can be applied directly to cells and tissue without measurable damage [10]. Non-thermal plasma recently emerged as a novel technology for medical applications such as blood coagulation [11], dental cavity treatment [12], malignant cell apoptosis [13] and tissue engineering scaffold treatment [14].

Non-thermal plasma devices, specifically dielectric barrier discharge (DBD) plasma, are used extensively in medicine due to their selectivity, portability, scalability, ease of operation, and low manufacturing and maintenance costs. DBD plasma is generated at atmospheric pressure in air when short duration, high voltage pulses are applied between two electrodes, with one electrode being insulated to prevent a current increase [15]. Non-thermal DBD plasma produces a variety of biologically active reactive species, in particular reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide (O_2-), hydroxyl radicals (OH•), and singlet oxygen ($O_2(1\Delta g)$) [15].

We previously demonstrated that non-thermal DBD plasma promoted endothelial cell proliferation *in vitro* through FGF-2 release [74]. However, the mechanism for this effect was unknown. We hypothesized that FGF-2 release is related to plasma-induced ROS, and that the combination of ROS and FGF-2 further induce endothelial cells to create new blood vessels. In the current work, we measured plasma-produced ROS in liquid and cells over time, and then quantified plasma ROS and cell-released FGF-2 effects on endothelial cell proliferation, migration, and tube formation.

II. Methods

A. Cell culture and materials

Porcine aortic endothelial cells (PAEC) were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine. An intracellular ROS scavenger, N-acetyl cysteine (NAC) and an extracellular ROS scavenger, sodium pyruvate (SP), were used to block plasma-produced ROS.

B. Plasma treatment

Non-thermal DBD plasma was generated by applying alternating polarity pulsed voltage (1.5 kHz, 20 kVpp) between an insulated high voltage electrode and the grounded base holding the cell sample. A variable voltage and current power supply custom was connected to a 25.4 mm diameter copper electrode with a 1 mm thick quartz insulating dielectric covering its bottom surface. The

Manuscript received April 15, 2011.

K. P. Arjunan is with the School of Biomedical Engineering, Science, & Health Systems, Drexel University, Philadelphia, PA 19104 USA.

A. M. Clyne is with the Mechanical Engineering Department, Drexel University, Philadelphia, PA 19104 USA. Phone: 215-895-2366. Fax: 215-895-1478. E-mail: asm67@drexel.edu

discharge gap was fixed at 2 mm. Discharge power density was calculated to be 0.84 W/cm^2 (1.5 kHz) using MATLAB). The plasma treatment dose in J/cm^2 was calculated by multiplying plasma discharge power density with plasma treatment duration. Cells in glass bottom dishes or on coverslips were directly treated with plasma in the presence of 100 µl serum-free medium. There was no significant change in PBS and serum-free medium temperature or pH.

C. Reactive oxygen species detection

Plasma ROS in liquid were quantified with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), which fluoresces following reaction with H_2O_2, OH·, $O_2(1\Delta g)$, and ROO- [17]. The probe was activated by removing acetate groups using an alkaline solution [18]. 150 µl PBS or serum-free medium containing 20 µM DCFH was plasma treated. 100 µl of treated liquid was then transferred to a 96-well plate, and fluorescence was measured at ex/em: 488/525 nm using a microplate reader. H_2O_2 was the positive control.

Intracellular ROS was assessed using the Image-iT LIVE Green ROS Detection Kit (Invitrogen). The assay is based on 5-(and6)-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA), a fluorogenic marker for ROS in live cells [17]. Carboxy-H_2DCFDA is internalized by cells and metabolized by intracellular esterases to carboxy-DCFH, which then reacts with ROS to form fluorescent carboxy-DCF. 100,000 PAEC were plasma treated and were labeled for ROS according to manufacturer's protocol. Tert-butylhydroperoxide (tBHP), which induces intracellular ROS, was the positive control. Samples were imaged using an Olympus IX81 inverted confocal microscope at ex/em: 488/520 nm.

D. Cell proliferation, migration, and tube formation

Endothelial cell proliferation in response to plasma treatment was measured by cell counts. 10,000 cells were treated with plasma or 10 ng/ml FGF-2 on day 0. Medium was changed on days 2 and 4. On days 1, 3 and 5 after plasma treatment, PAEC were trypsinized and counted using a Coulter counter. Cell proliferation was defined as fold change in attached cell number on day 5 compared to day 1.

3D migration was quantified using the QCM cell invasion assay kit (Millipore). 1×10^5 PAEC in 100 µl serum-free medium were plasma treated (4.2 J/cm^2) and then added to the chamber upper compartment. Cells were plasma treated prior to being added to the chamber because the DBD plasma electrode was too large for direct cell treatment in the chamber. 150 µl DMEM with 10% FBS was added to the lower compartment. FGF-2 (10 ng/ml) was used as positive control. Samples were incubated at 37°C for 24 hours. Cells that migrated through the membrane were quantified according to manufacturer's protocol.

An *in vitro* tube formation assay was performed as described [19]. 4 mg/ml rat tail type I collagen was mixed with plasma treated PAEC (4.2 J/cm^2, 3×10^5 cells) in serum-free medium. 200 µl cell-collagen mixture was immediately added to a 24-well plate and incubated for 1 hour at 37°C. Supplemented medium was then added, and medium was replaced every day. Phase contrast microscopy images were taken at 0, 24, 48 and 72 hours. Tube formation was was assessed by measuring tube length from a branch point or between branch points using Image J. Eight tubes were measured in each image, with three images per condition.

E. Statistical analysis

Statistical analysis was performed with Prism software. All experiments were performed in triplicate and repeated three times. Data are expressed as mean ± SD. Comparisons between two groups were analyzed using Student's t-test.

III. Results

A. Plasma ROS in liquid and cells

In both plasma-treated PBS and serum-free medium, ROS concentration increased with plasma dose. ROS levels increased rapidly at low treatment doses and more slowly at higher treatment doses, likely due to ROS reactions with air and liquid components. ROS concentration was consistently 25–35% higher in plasma-treated serum-free medium compared to PBS (Fig 1a).

Plasma treatment also induced a dose-dependent increase in intracellular oxidative stress immediately after treatment (Fig 1b). 4.2 J/cm^2 plasma increased intracellular ROS 14% compared to untreated cells, and 8.4 J/cm^2 plasma produced an 8% further increase. Plasma-induced intracellular ROS peaked by 1 hour after treatment and then returned to near baseline levels by 3 hours after treatment (data not shown).

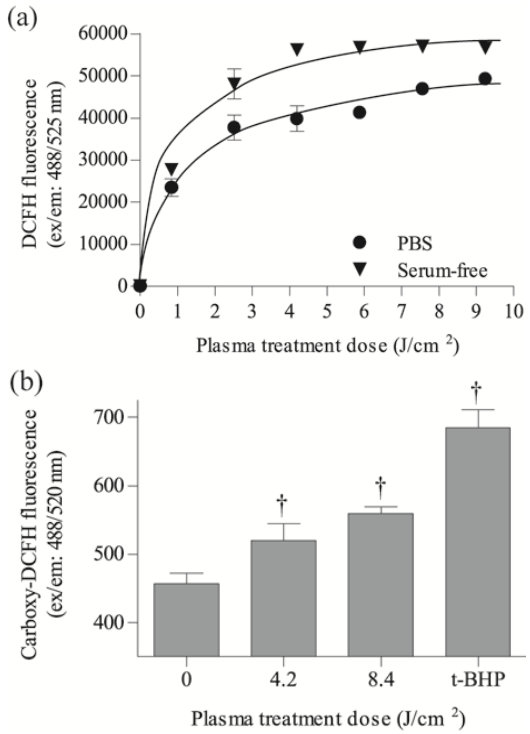

Fig. 1. DBD plasma ROS in liquid and cells. (a) Extracellular ROS in PBS and serum-free medium (b) Intracellular ROS.

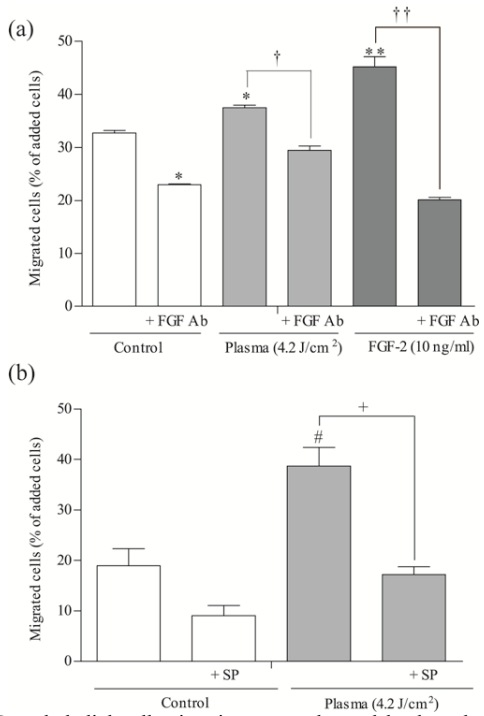

Fig. 2. 3D endothelial cell migration was enhanced by low dose plasma treatment via ROS-induced FGF-2 release. (a) Plasma treated PAEC migration with a neutralizing FGF-2 antibody.*p<0.01 compared to control. †p<0.01. (b) Plasma treated with extracellular ROS scavenger sodium pyruvate. #p<0.05 compared to control, +p< 0.05.

B. Cell proliferation, migration, and tube formation

We previously demonstrated that plasma increased endothelial cell proliferation through FGF-2 release [16]. We now show that plasma-produced ROS are critical to enhanced cell proliferation. Cells treated with 4.2 J/cm^2 plasma on day one had ten times as many cells on day five, whereas control cells had only six times as many cells. An FGF-2 neutralizing antibody and ROS scavengers N-acetyl cysteine and sodium pyruvate abrogated the plasma-induced cell proliferation enhancement.

Since maximum cell proliferation was observed at 4.2 J/cm^2 plasma, 3D cell migration was measured at this dose. 24 hours after plasma treatment (4.2 J/cm^2), the PAEC number that migrated through the chamber increased significantly. An FGF-2 neutralizing antibody reduced the number of plasma-induced migrated cells by 7% (Fig 2a), while sodium pyruvate caused a 22% reduction in migrated cell number (Fig 2b).

Finally, endothelial cell tube formation following plasma treatment was tested. Cells treated with a 4.2 J/cm^2 plasma formed an extensive tube network 24 hours after treatment, while untreated samples formed only tube segments (Fig 5a). Average tube length in plasma treated samples was 121 ± 22 µm, approximately 2.6 times longer than average tube length in control samples (Fig 5b). The FGF-2 neutralizing antibody reduced tube length by 70% (37 ± 8µm). Similarly, a 67% decrease in tube length occurred after plasma treatment in the presence of sodium pyruvate.

IV. Discussion

Vascularization is critical to a variety of processes, including wound healing and tissue engineering. We now show that non-thermal plasma treatment produces ROS in liquid and cells in a dose-dependent manner. These ROS enhance endothelial cell proliferation, migration and tube formation through FGF-2 release and this increase was abrogated by a neutralizing FGF-2 antibody as well as intracellular and extracellular ROS scavengers. These data suggest that plasma may be a useful tool for promoting vascularization.

Plasma produces ROS in gas phase, and these ROS then diffuse into liquid where they react with organic matter. We measured higher ROS levels in plasma-treated serum-free medium as compared to PBS. We believe this occurred because plasma ROS react with medium components such as amino acids, which are not present in PBS. Medium components are readily oxidized by ROS [20] to form organic hydroperoxides, or ROS can also carbonylate medium amino acids such as lysine, arginine, proline and threonine. Since many ROS are short lived, plasma ROS effects on cells may be extended through these ROS interactions with organic medium components.

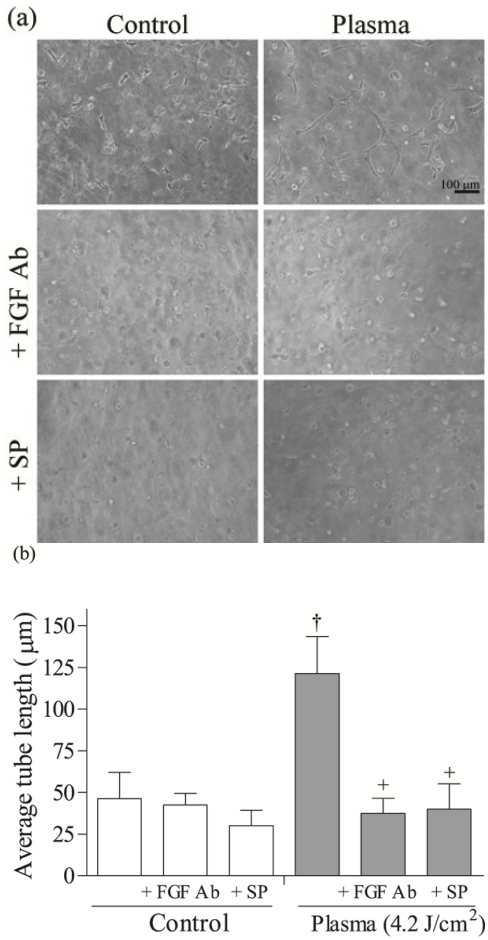

Fig. 3. Endothelial cell tube formation was enhanced by plasma treatment. (a) Phase contrast images of tube formation at24 hours. (b) Tube length †p<0.001 compared to control, +p<0.0001 compared to plasma.

Plasma ROS, whether extracellular or intracellular, can cause sub-lethal cell membrane damage and subsequent FGF-2 release [16]. Extracellularly, ROS such as hydroperoxides or carbonylated amino acids damage the plasma membrane through lipid peroxidation [21]. Intracellularly, ROS may cause cell membrane damage through membrane lipid and protein oxidation. Disruption size may range from 1 nm to greater than 1 μm, thus allowing large molecule movement in and out of the cell [22]. Sub-lethal cell membrane damage by plasma leads to endothelial cell FGF-2 release [16]

Non-thermal plasma has many advantages over other ROS vascularization methods. Plasma is safe, cheap and portable compared to other mechanisms of inducing ROS or releasing FGF-2, such as electrical stimulation, pulsed electromagnetic fields and ionizing radiation. Unlike these technologies, non-thermal plasma does not cause measurable damage to surrounding tissue. Since plasma devices are portable, they can be used in hospitals, disaster areas, and in the military. Plasma devices can also be scaled to meet specific treatment requirements and modified to produce specific reactive species [45]. As shown in our previous work, if direct plasma treatment is not possible, conditioned medium from plasma treated cells containing growth factors can also be used to promote angiogenesis [74].

While plasma can apply ROS to wounds or tissue constructs and thereby promote vascularization, it is unlikely that plasma alone will be produce efficient angiogenesis. Angiogenesis is a complex physiological process involving several angiogenic factors that must be released at specific times and in specific concentration gradients. Hence, it might not be possible to achieve angiogenesis of clinical significance through one treatment technique but rather multiple treatment techniques may be necessary. Growth factor therapy for promoting angiogenesis has been disappointing in clinical studies, even though it showed promise in animal studies [28]. This could be attributed to insufficient growth factor concentrations for the time needed to generate a response due to difficulties in delivery methods and dosing. Non-thermal plasma enables localized ROS application in precise doses, which may be optimized to release sufficient growth factor concentration. Thus plasma may be an adjuvant to conventional vascularization techniques. Adding plasma-treated cells could also accelerate vascularization in wounds and tissue engineering scaffolds.

We now show that non-thermal DBD plasma can stimulate angiogenesis. Plasma dose can be varied by changing treatment time, and plasma composition can be modified by changing electrical parameters. Plasma treatment could be used to promote tissue engineering scaffold vascularization as well as accelerate wound healing via enhanced angiogenesis. For tissue engineering scaffolds, cells could be treated with plasma prior to seeding them in the scaffold. For wound healing, plasma ROS could be used initially at higher doses to sterilize the wound and later at lower doses to promote healing. This novel tool could make ROS a practical, physiological method to create new blood vessels either in vitro or in vivo.

V. References

[1] J. Folkman, "Angiogenesis," *Annu. Rev. Med.* 2006; 57: 1-18.

[2] A. Martin, M.R. Komada, D.C. Sane, "Abnormal angiogenesis in diabetes mellitus," *Medicinal Research Reviews* 2003; 23(2): 117-45.

[3] M. Lovett, K. Lee, A. Edwards, D.L. Kaplan, "Vascularization strategies for tissue engineering," *Tissue Eng Part B Rev.* 2009; 15(3): 353-70.

[4] M. Presta, P. Dell'Era, S. Mitola, E. Moroni, R. Ronca, M. Rusnati, "Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis," *Cytokine & Growth Factor Rev.* 2005; 16(2): 159-78.

[5] M.A. Nugent, R.V. Iozzo, "Fibroblast growth factor-2," *Int J Biochem Cell Biol.* 2000; 32(2): 115-20.

[6] Z. Fuks, R.S. Persaud, A. Alfieri, M. McLoughlin, D. Ehleiter, J.L. Schwartz, et al, "Basic fibroblast growth factor protects endothelial cells against radiation-induced programmed cell death in vitro and in vivo," *Cancer Res.* 1994; 54: 2582-90.

[7] E.R. Edelman, E. Mathiowitz, R. Langer, M. Klagsbrun, "Controlled and modulated release of basic fibroblast growth factor," *Biomaterials*. 1991; 12(7): 619-26.

[8] M.C. Robson, T.A. Mustoe, T.K. Hunt, "The future of recombinant growth factors in wound healing," *The American Journal of Surgery*. 1998; 176(2, Supplement 1): 80S-2S.

[9] M.G. Kong, G. Kroesen, G. Morfill T. Nosenko, T. Shimizu, J. Dijk, J.L. Zimmerman, "Plasma medicine: an introductory review," *New J. Phys*. 2009; 11(11): 115012.

[10] A. Chirokov, A. Gutsol, A. Fridman, "Atmospheric pressure plasma of dielectric barrier discharges," *Pure Appl. Chem.* 2005; 77(2): 487-95.

[11] S. Kalghatgi, G. Fridman, M. Cooper, G. Nagaraj, M. Peddinghaus M. Balasubramanian, et al, "Mechanism of blood coagulation by nonthermal atmospheric pressure dielectric barrier discharge plasma," *IEEE Trans. Plasma Sci.* 2007; 35(5): 1559-66.

[12] R.E.J. Sladek, E. Stoffels, R. Walraven, P.J.A. Tielbeek, R.A. Koolhoven, "Plasma treatment of dental cavities: a feasibility study," *IEEE Trans. Plasma Sci.* 2004; 32(4): 1540-3.

[13] R. Sensenig, S. Kalghatgi, E. Cerchar, G. Fridman, A. Shereshevsky, B. Torabi, et al, "Non-thermal plasma induces apoptosis in melanoma cells via production of intracellular reactive oxygen species," *Ann. Biomed. Eng.* 2010; p 1-14.

[14] E.D. Yildrim, H. Ayan, V.N. Vasilets, A. Fridman, S. Guceri, G. Friedman, W. Sun, "Effect of dielectric barrier discharge plasma on the attachment and proliferation of osteoblasts cultured over poly(ε-caprolactone) scaffolds," *Plasma Process Polym.* 2008; 5(4): 397.

[15] M. Kuchenbecker, N. Bibinov, A. Kaemlimg, D. Wandke, P. Awakowicz, W. Viol, "Characterization of DBD plasma source for biomedical applications," *J. Phys. D: Appl. Phys.* 2009; 42(4).

[16] S. Kalghatgi, G. Friedman, A. Fridman, A. Morss Clyne, "Endothelial cell proliferation is enhanced by low dose non-thermal plasma through fibroblast growth factor-2 release," *Ann. Biomed. Eng.* 2010; 38(10): 748-57.

[17] C.P. LeBel, H. Ischiropoulos, S.C. Bondy, "Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress," *Chem. Res. Toxicol.* 1992; 5(2): 227-31.

[18] R. Cathcart, E. Schwiers, B.N. Ames, "Detection of picomoles of hydroperoxides using a fluorescence dichlorofluorescein assay," *Anal Biochem.* 1983; 134(1): 111-6.

[19] W. Koh, A.N. Stratman, A. Sacharidou, G.E. Davis. "In vitro three dimensional collagen matrix models of endothelial lumen formation during vasculogenesis and angiogenesis," *Methods Enzymol.:* Academic Press; 2008. p. 83-101.

[20] E.R. Stadtman, B.S. Berlett. "Fenton chemistry. amino acid oxidation," *J Biol Chem.* 1991; 266(26): 17201-11.

[21] R.L. Snider, R.B. Moore, "The role of lipid peroxidation in tert-butyl hydroperoxide inhibition of human erythrocyte Ca2++Mg2+-ATPase," *Bios.* 1986; 57(2): 63-74.

[22] P.L. McNeil, R.A. Steinhardt, "Loss, restoration and maintenance of plasma membrane integrity," *J Cell Biol.* 1997; 137(1): 1-4.