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

Krishna Priya Arjunan¹, Gary Friedman², Alexander Fridman³ and Alisa Morss Clyne¹,³,*

¹School of Biomedical Engineering, Science and Health Systems, ²Electrical and Computer Engineering and ³Mechanical Engineering, and Mechanics, Drexel University, Philadelphia, PA, USA

Vascularization plays a key role in processes such as wound healing and tissue engineering. Non-thermal plasma, which primarily produces reactive oxygen species (ROS), has recently emerged as an efficient tool in medical applications including blood coagulation, sterilization and malignant cell apoptosis. Liquids and porcine aortic endothelial cells were treated with a non-thermal dielectric barrier discharge plasma in vitro. Plasma treatment of phosphate-buffered saline (PBS) and serum-free medium increased ROS concentration in a dose-dependent manner, with a higher concentration observed in serum-free medium compared with PBS. Species concentration inside cells peaked 1 h after treatment, followed by a decrease 3 h post treatment. Endothelial cells treated with a plasma dose of 4.2 J cm⁻² had 1.7 times more cells than untreated samples 5 days after plasma treatment. The 4.2 J cm⁻² plasma dose increased two-dimensional migration distance by 40 per cent compared with untreated control, while the number of cells that migrated through a three-dimensional collagen gel increased by 15 per cent. Tube formation was also enhanced by plasma treatment, with tube lengths in plasma-treated samples measuring 2.6 times longer than control samples. A fibroblast growth factor-2 (FGF-2) neutralizing antibody and ROS scavengers abrogated these angiogenic effects. These data indicate that plasma enhanced proliferation, migration and tube formation is due to FGF-2 release induced by plasma-produced ROS. Non-thermal plasma may be used as a potential tool for applying ROS in precise doses to enhance vascularization.

Keywords: vascularization; wound healing; angiogenesis; non-thermal plasma; reactive oxygen species; fibroblast growth factor-2 release

1. INTRODUCTION

Angiogenesis, the growth of new blood vessels from existing vessels, plays a key role in physiological processes including development, growth and wound healing [1]. Insufficient vascularization contributes to impaired wound healing in patients with diabetes [2] and systemic sclerosis [3], the elderly [4], and patients treated with immunosuppressive drugs [5], chemotherapy [6] and radiotherapy [7]. In vitro, vascularization is essential for engineering complex, large tissues and organs such as bone, muscle, liver and heart. Although successful tissue fabrication of a few hundred micrometres has been achieved, tissue engineering is limited by the inability to vascularize constructs to provide nutrients to the tissue core [8–11].

Over the past few decades, many growth factors that stimulate angiogenesis have been discovered, including vascular endothelial growth factor (VEGF) [12,13] and fibroblast growth factor-2 (FGF-2) [14,15]. These growth factors are the standard by which other angiogenic therapies are measured in either in vivo assays (e.g. Matrigel plug, corneal implant) [16–19] or in vitro assays (e.g. cell proliferation, Boyden chamber migration, collagen gel tube formation) [20–24]. While these growth factors are critical to the angiogenic process, their success depends on timing, dose and gradients [25–28]. Efforts to apply growth factors in wounds or in tissue-engineered constructs to induce angiogenesis have met with limited success [28–30].

FGF-2, a pro-angiogenic molecule that binds heparan sulphate proteoglycans as well as cell surface receptors, is associated with cell survival, proliferation and migration [31–33]. FGF-2 is particularly interesting because it does not have a recognized signal sequence for secretion. FGF-2 is known to be released by injured cells, whether the injury is by ionizing radiation, pulsed electromagnetic field, mechanical forces or elevated glucose [34–38]. FGF-2 released during cell injury promotes cell survival and also increases FGF-2 expression in endothelial cells, smooth muscle cells and cardiac myocytes [39–43]. Thus, cells that have suffered an injury prepare themselves against subsequent injuries by synthesizing additional FGF-2. FGF-2 may also induce expression

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of other angiogenic growth factors, such as VEGF, in endothelial cells [44].

Plasma medicine is a rapidly expanding interdisciplinary field combining engineering, physics, biochemistry and life sciences [45]. Plasma, the fourth state of matter, is an ionized gas composed of charged particles (electrons, ions), excited atoms and molecules, radicals, and UV photons. Man-made plasma is created with an electrical discharge and a large electric field. In non-thermal plasma, which is far from thermal equilibrium, 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 plasma can be applied directly to cells and tissue without measurable damage [46]. Non-thermal plasma has recently emerged as a novel technology for various medical applications such as blood coagulation [47–50], wound healing [51], tissue sterilization [48], medical equipment sterilization [52], dental cavity treatment [53], malignant cell apoptosis [54,55] and tissue-engineering scaffold treatment [56].

Non-thermal plasma devices, specifically dielectric barrier discharge (DBD) plasma, are used extensively in medicine because of 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 [57,58]. DBD plasma characteristics in air, including electron density, reduced electric field, gas temperature and active species concentration, vary depending on applied voltage, dielectric material and interelectrode distance [58]. Non-thermal DBD plasma produces a variety of biologically active reactive species, in particular, reactive oxygen species (ROS) [58].

ROS, including hydrogen peroxide (H₂O₂), superoxide (O₂⁻), hydroxyl radicals (OH·) and singlet oxygen (O₂(1Δg)), are highly reactive ions or molecules that contribute to angiogenic signalling [59]. While in an early wound high ROS levels mediate inflammation [60] and may inhibit angiogenesis, later low ROS levels initiate wound healing partially through angiogenesis [61–63]. Low ROS doses may increase endothelial cell migration, proliferation and tube formation in vitro and in vivo through growth factor-related mechanisms. ROS can enhance production of pro-angiogenic factors such as VEGF and FGF-2 [62,64–66], as well as other mitogens such as insulin-like growth factor-1 (IGF-1) [67] and transforming growth factor-α [68]. Low ROS doses may enhance growth factor binding to receptors, induce receptor tyrosine kinase phosphorylation, or act as messengers in downstream signalling along growth factor pathways [59,69–72]. Finally, ROS can damage the cell membrane leading to FGF-2 release, which then has proliferative and survival effects on adjacent cells [37,73].

Our group previously demonstrated that a 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. This study will help identify how plasma can be used to promote vascularization for wound healing and tissue engineering.

2. EXPERIMENTAL PROCEDURE

2.1. Cell culture and materials

Porcine aortic endothelial cells (PAEC) were isolated from swine aorta by the collagenase dispersion method [75]. PAEC were selected because they do not require growth factor supplementation for survival, and there is high cross reactivity between human and porcine growth factors [37,76]. Cells were cultured in low glucose Dulbecco’s modified Eagle’s medium (DMEM, Mediatech) supplemented with 5 per cent foetal bovine serum (FBS, Hyclone), 1 per cent penicillin–streptomycin and 1 per cent l-glutamine (Invitrogen). Cells between passages 4 and 9 were maintained in a humidified incubator at 37°C and 5 per cent CO₂ with medium change every 2 days.

Recombinant human FGF-2 was from Peprotech, and the neutralizing FGF-2 antibody was from Upstate Biotechnology. An intracellular ROS scavenger, 10 mM N-acetyl cysteine (NAC, Sigma) and an extracellular ROS scavenger, 50 mM sodium pyruvate (SP, Sigma), were used to block plasma-produced ROS.

2.2. Plasma treatment

Non-thermal DBD plasma was generated by applying alternating polarity pulsed voltage (1.5 kHz, 20 kV peak-to-peak) between an insulated high-voltage electrode and the grounded base holding the cell sample (figure 1a,b) [74,77]. A variable voltage and current power supply custom made by Quinta Ltd. (Moscow, Russia) was used for treating liquids and cells. The power supply was connected to a 25.4 mm diameter copper electrode with a 1 mm thick quartz insulating dielectric covering its bottom surface. The insulating dielectric prevented current flow between electrodes, creating plasma with high reactive species concentrations but minimal gas heating. The discharge gap between the quartz dielectric and the cell sample was fixed at 2 mm. Voltage and current were measured using a voltage (North Star PVM-4, 100 MHz bandwidth) and current probe (Pearsons Model 2878), respectively (figure 1c). Signals were recorded with an oscilloscope (TDS 5052B, Tektronix Inc.). The pulse waveform had a 1.65 µs width and a 5 V ns⁻¹ rise time. Discharge power density was calculated to be 0.84 W cm⁻² (1.5 kHz) using MATLAB (Mathworks). The plasma treatment dose in Joules per square centimetre was calculated by multiplying plasma discharge power density with plasma treatment duration.

Cells in glass bottom dishes (14 mm, MatTek) or on 18 mm coverslips (VWR) were directly treated with plasma in the presence of 100 µl serum-free medium to prevent sample drying. Plasma was produced...
H\textsubscript{2}O\textsubscript{2} was the positive control.

525 nm using an Infinite 200 Tecan microplate reader.

\[ \text{plate, and fluorescence was measured at ex} \]

plasma dose of 134.4 J cm\textsuperscript{-2}, since a rapid drop in
temperature never rose above 28.5° C even for a high
plasma dose of 134.4 J cm\textsuperscript{-2}, since a rapid drop in
liquid temperature was observed when it was exposed
to room temperature air. Additionally, no significant
change in PBS and serum-free medium pH was observed
up to 9.24 J cm\textsuperscript{-2} plasma.

2.3. Reactive oxygen species detection in liquid
and cells

Since DBD plasma produces a variety of ROS [80],
plasma ROS formed in liquid were quantified with 2',7'-dichlorodihydro-
fluorescein diacetate (DCFH-DA, Cayman chemicals), which fluoresces following reaction
with ROS such as H\textsubscript{2}O\textsubscript{2}, OH\textsuperscript{·}, O\textsubscript{2} (1\Delta\textsubscript{g}) and ROO\textsuperscript{-}
[81,82]. The probe was activated by removing acetate
buffered saline (PBS) or serum-free medium post-
plasma treatment using a thermistor probe and meter
(YSI 427 and 43TA, Yellow Springs Instruments). The
4.2 J cm\textsuperscript{-2} plasma dose increased liquid temperature by
less than 1°C. In PBS and serum-free medium that
were warmed to 37°C before the experiment, liquid
temperature never rose above 28.5°C even for a high
plasma dose of 134.4 J cm\textsuperscript{-2}, since a rapid drop in
liquid temperature was observed when it was exposed
to room temperature air. Additionally, no significant
change in PBS and serum-free medium pH was observed
up to 9.24 J cm\textsuperscript{-2} plasma.

Intracellular ROS was assessed using the Image-iT
LIVE Green ROS Detection Kit (Invitrogen). The assay
is based on 5-(and 6)-carboxy-2', 7'-dichlorodihydro-
fluorescein diacetate (carboxy-H\textsubscript{2}DCFDA), a fluorogenic
marker for ROS in live cells [81,82]. Carboxy-
H\textsubscript{2}DCFDA is internalized by cells and metabolized by
intracellular esterases to carboxy-DCF, which then
reacts with ROS to form fluorescent carboxy-DCF. A
total of PAEC (100,000 cells) were seeded in 50 mm
glass bottom dishes and cultured for 2 days to confluence.
Following plasma treatment (0–8.4 J cm\textsuperscript{-2}), cells were
labelled for ROS according to manufacturer’s protocol.
Briefly, cells were incubated with 25 μM carboxy-
H\textsubscript{2}DCFDA in HEPES buffer (20 mM Hepes, 137 mM
NaCl, 5 mM KCl, 1 mM KH\textsubscript{2}PO\textsubscript{4}, 2 mM CaCl\textsubscript{2}, 1 mM
MgCl\textsubscript{2} and 10 mM glucose, pH 7.4) for 30 min at 37°C.
The \textit{t}-butylhydroperoxide (tBHP, Invitrogen), which
induces intracellular ROS production, was the positive
control. Samples were imaged using an Olympus IX81
inverted confocal microscope at ex/em: 488/520 nm.

2.4. Proliferation assay

Endothelial cell proliferation in response to plasma
treatment was measured by cell counts. Ten thousand
cells were seeded on 18 mm glass coverslips and incu-
bated for 24 h at 37°C. Cells were treated with plasma
(0–8.4 J cm\textsuperscript{-2}, day 0) or 10 ng ml\textsuperscript{-1} FGF-2 (positive
control) and returned to the incubator. 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 with day 1.

2.5. Two-dimensional migration assay

Endothelial cell migration in response to plasma treat-
ment was measured using a two-dimensional migration
assay [84,85]. Thirty thousand cells were seeded inside
4 mm cloning rings on gridded cover slips. A 5 mm glass
bead was used to maintain tight contact between the
ring and the coverslip. After 3 h, the bead and ring were removed, and unattached cells were washed away. This process produced a confluent circle of attached PAEC. Cell samples were then treated with 4.2 J cm\(^{-2}\) plasma, since this dose showed maximal cell response in our previous work [74]. Untreated samples were the negative control, and FGF-2 (10 ng ml\(^{-1}\)) was the positive control. Images were captured at 0, 24, 48 and 72 h after treatment in each direction (0, 90, 180 and 270\(^{\circ}\)) using a Nikon Eclipse TS100 phase contrast microscope with a Nikon DS-Fi1 CCD camera. Migration distance, defined as the difference between cell migration fronts at 0 and 72 h, was analysed using ImageJ. After 72 h, cells were trypsinized and counted using a Coulter counter to determine cell number.

### 2.6 Modified Boyden chamber three-dimensional migration assay

Three-dimensional migration was quantified using the QCM cell invasion assay kit (Millipore). In this assay, cells migrate through a collagen-coated Transwell membrane with 8 µm pores and attach to the membrane bottom surface. Migrated cells are then fluorescently quantified by staining DNA following cell lysis. PAEC (1 × 10\(^{5}\) cells) 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. One hundred and fifty microlitres DMEM with 10 per cent FBS was added to the lower compartment. FGF-2 (10 ng ml\(^{-1}\)) was used as positive control and cells without plasma treatment as negative control. Samples were incubated at 37°C for 24 h. Cells that migrated through the membrane were quantified according to manufacturer’s protocol. Briefly, residual cells from the upper compartment were removed using a cotton swab. Cells on the membrane bottom surface were removed by incubating inserts in cell detachment solution for 30 min at 37°C. Detached cells were incubated in lysis buffer/CyQuant dye solution for 15 min to stain DNA. One hundred and fifty microlitres cell lysis solution was then transferred to a 96-well plate and fluorescence was measured at ex/em: 480/520 nm using a microplate reader. Fluorescence was converted to cell number using a standard curve.

### 2.7. Tube formation assay

An in vitro tube formation assay was performed as described previously [86]. Briefly, 4 mg ml\(^{-1}\) rat tail type I collagen (BD Biosciences) was mixed with plasma-treated PAEC (4.2 J cm\(^{-2}\), 3 × 10\(^{5}\) cells) in serum-free medium. Cell–collagen mixture (200 µl) was immediately added to a 24-well plate and incubated for 1 h at 37°C. Supplemented medium was then added, and medium was replaced every day without disturbing tubes. Phase contrast microscopy images were taken at 0, 24, 48 and 72 h. Tube formation was quantified using ImageJ [87]. Tube formation was assessed by measuring tube length from a branch point or between branch points. Eight tube lengths were measured in each image, with three images per treatment condition.

### 2.8. Statistical analysis

Statistical, linear and nonlinear regression analyses were performed with Prism software (Graphpad). All experiments were performed in triplicate and repeated at least three times. Data are expressed as mean ± s.d. Comparisons between two groups were analysed using Student's t-test, and comparisons between more than two groups were analysed by ANOVA.

### 3. RESULTS

#### 3.1. Reactive oxygen species increased in a dose-dependent manner in liquid and cells following plasma treatment

Previous plasma studies theoretically calculated that a plasma dose of 1 J cm\(^{-2}\) produced 1.88–3.13 × 10\(^{16}\) ROS in gas phase [74]. We now measured plasma-produced ROS in the liquid phase. 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, probably owing to ROS reactions with air and liquid components. ROS concentration was consistently 25–35% higher in plasma-treated serum-free medium compared with PBS (figure 2a). Data were fit to an exponential function by nonlinear regression.

Plasma-produced ROS, as well as products from plasma ROS interaction with serum-free medium, either diffuse through the cell membrane into the cytosol or react with the cell membrane to produce intracellular ROS. We therefore quantified intracellular ROS using carboxy-H\(_2\)DCFDA. Plasma treatment induced a dose-dependent increase in intracellular oxidative stress immediately after treatment (figure 2b). A plasma dose of 4.2 J cm\(^{-2}\) increased intracellular ROS 14 per cent compared with untreated cells, and 8.4 J cm\(^{-2}\) plasma produced an 8 per cent further increase. Cells incubated with positive control tert-butyl hydroperoxide (100 µM) showed 1.5 times more ROS than untreated cells. Intracellular ROS over time following plasma treatment was then quantified for 4.2 J cm\(^{-2}\) plasma. Plasma-induced intracellular ROS peaked by 1 h after treatment and then returned to near baseline levels by 3 h after treatment (figure 2c, d).

#### 3.2. Plasma enhanced endothelial cell proliferation, migration and tube formation through reactive oxygen species-induced fibroblast growth factor-2 release

We previously demonstrated that plasma increased endothelial cell proliferation through FGF-2 release [74]. We now show that plasma-produced ROS are critical to enhanced cell proliferation. Cell proliferation increased in a dose-dependent manner up to 4.2 J cm\(^{-2}\) plasma and then decreased at higher plasma doses (figure 3). Cells treated with 4.2 J cm\(^{-2}\) plasma on day 1 had 10 times as many cells on day 5, whereas control cells had only six times as many cells. Cells incubated with a 10 ng ml\(^{-1}\) FGF-2 bolus on day 1 had 12 times as many cells on day 5. An FGF-2 neutralizing antibody, the intracellular
ROS scavenger N-acetyl cysteine, and the extracellular ROS scavenger sodium pyruvate abrogated the plasma-induced cell proliferation enhancement. Data for FGF-2 Ab, NAC and SP were fit to a linear function by linear regression. Since maximum cell proliferation was observed at 4.2 J cm$^{-2}$ plasma, two-dimensional cell migration was measured at this dose. Endothelial two-dimensional migration increased with plasma as early as 24 h after treatment compared with untreated control (390 versus 190 m$m$), and the difference was maintained up to 72 h after plasma treatment (902 versus 624 m$m$). Plasma-induced cell migration distance at 72 h was less than positive control FGF-2 (1709 m$m$). To elucidate whether enhanced cell migration was attributed to ROS-induced FGF-2 release, cells were plasma-treated in the presence of an FGF-2 neutralizing antibody, N-acetyl cysteine or sodium pyruvate. FGF-2 blockade decreased PAEC migration distance by 23 per cent (figure 4a). Similarly, both N-acetyl cysteine and sodium pyruvate inhibited plasma-induced cell migration distance by 14 and 22 per cent, respectively (figure 4b). No significant difference in cell number was observed between control and plasma-treated samples at 72 h, suggesting that these differences are related to cell migration rather than proliferation.

Three-dimensional cell migration, which provides additional physiological relevance compared with two-dimensional migration, also increased with plasma treatment. Twenty-four hours after plasma treatment (4.2 J cm$^{-2}$), the PAEC number that migrated through the chamber increased significantly. Ten nanograms per millilitre FGF-2, used as positive control, increased migrated PAEC number by 13 per cent. An FGF-2 neutralizing antibody reduced the number of plasma-induced migrated cells by 7 per cent (figure 5a), while sodium pyruvate caused a 22 per cent reduction in migrated cell number (figure 5b).

Finally, endothelial cell tube formation following plasma treatment was tested. Cells treated with 4.2 J cm$^{-2}$ plasma formed an extensive tube network 24 h after

**Figure 2.** ROS were produced by DBD plasma in liquid and cells. (a) Cumulative extracellular ROS in PBS (circles) and serum-free (inverted triangles) medium detected using DCFH (ex/em: 488/525 nm). $p < 0.001$ compared with control for both PBS and serum-free medium, and $p < 0.0001$ between PBS and serum-free medium. (b) Intracellular ROS were measured using carboxy-H$_2$DCFDA (ex/em: 488/520 nm). PAEC were incubated with carboxy-H$_2$DCFDA for 30 min at 37°C and then treated with plasma or positive control tert-butyl hydroperoxide (100 mM, tBHP). (c) Intracellular ROS levels for 3 h following DBD plasma treatment. (d) Sample ROS images taken by confocal microscopy and quantified using IMAGEJ. $^*p < 0.001$ compared with untreated control. (Online version in colour.)

**Figure 3.** DBD plasma treatment enhanced endothelial cell proliferation through ROS-induced FGF-2 release. PAEC were treated with plasma doses of 0–8.4 J cm$^{-2}$ and counted 1, 3 and 5 days after plasma treatment. Cells were treated with an FGF-2 neutralizing antibody to block FGF-2 effects, and 10 mM N-acetyl cysteine (NAC) or 50 mM sodium pyruvate (SP) to block intracellular and extracellular ROS, respectively. Data are represented as fold change in cell number on day 5 compared with day 1. $^p < 0.001$ compared with control. $^*p < 0.0001$ compared with plasma. $^#p < 0.05$ compared with control. Circles, plasma; squares, $^+$FGF Ab; triangles, $^+$NAC; inverted triangles, $^+$SP.

ROS scavenger N-acetyl cysteine, and the extracellular ROS scavenger sodium pyruvate abrogated the plasma-induced cell proliferation enhancement. Data for FGF-2 Ab, NAC and SP were fit to a linear function by linear regression.

Since maximum cell proliferation was observed at 4.2 J cm$^{-2}$ plasma, two-dimensional cell migration was measured at this dose. Endothelial two-dimensional migration increased with plasma as early as 24 h after treatment compared with untreated control (390 versus 190 m$m$), and the difference was maintained up to 72 h after plasma treatment (902 versus 624 m$m$). Plasma-induced cell migration distance at 72 h was less than positive control FGF-2 (1709 m$m$). To elucidate whether enhanced cell migration was attributed to ROS-induced FGF-2 release, cells were plasma-treated in the presence of an FGF-2 neutralizing antibody, N-acetyl cysteine or sodium pyruvate. FGF-2 blockade decreased PAEC migration distance by 23 per cent (figure 4a). Similarly, both N-acetyl cysteine and sodium pyruvate inhibited plasma-induced cell migration distance by 14 and 22 per cent, respectively (figure 4b). No significant difference in cell number was observed between control and plasma-treated samples at 72 h, suggesting that these differences are related to cell migration rather than proliferation.

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Finally, endothelial cell tube formation following plasma treatment was tested. Cells treated with 4.2 J cm$^{-2}$ plasma formed an extensive tube network 24 h after

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treatment, while untreated samples formed only tube segments (figure 6a). Average tube length in plasma-treated samples was 121 ± 22 μm, approximately 2.6 times longer than average tube length in control samples (figure 6b). Samples treated with positive control FGF-2 had an average tube length of 134 ± 16 μm. The FGF-2 neutralizing antibody reduced tube length by 70 per cent (37 ± 8 μm). Similarly, a 67 per cent decrease in tube length occurred after plasma treatment in the presence of ROS scavenger sodium pyruvate.

4. DISCUSSION
Vascularization is critical to a variety of processes, including wound healing and tissue engineering. Low ROS doses promote vascularization through angiogenic growth factor mechanisms [59,88]. Non-thermal plasma produces a variety of ROS in gas phase, and our group previously demonstrated that non-thermal plasma enhanced endothelial cell proliferation through FGF-2 release [74]. 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 when compared with 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 [89–92] to form organic hydroperoxides [91], or ROS can also carbonylate medium amino acids such as lysine, arginine, proline and threonine [92–94]. Since many ROS are short lived, plasma ROS effects on cells may be extended through these ROS interactions with organic medium components.
Plasma-produced ROS (or their reaction products) in liquid then diffuse through the plasma membrane or react with the plasma membrane to produce intracellular ROS through lipid peroxidation. ROS diffusion through the cell membrane depends on molecule size, charge and reactivity. For example, whereas organic hydroperoxides are too large to diffuse through the cell membrane, small molecules such as H$_2$O$_2$ can diffuse through specific membrane aquaporins [95,96]. However, other small molecules, such as O$_2^-$ and OH-radicals, cannot diffuse through the cell membrane owing to negative charge and high reactivity, respectively. Once ROS enter cells, they can damage intracellular components [97] or promote or inhibit intracellular signalling pathways [98,99]. Cells may also activate anti-oxidant mechanisms such as glutathione, catalase, ascorbate and superoxide dismutase, which remove the ROS influx and maintain cell redox state [100]. We observed that the intracellular ROS increase immediately after plasma treatment decreased to control values within 3 h. This could be partially due to ROS removal by cellular anti-oxidants.

Plasma ROS, whether extracellular or intracellular, can cause sub-lethal cell membrane damage and subsequent FGF-2 release [74]. Extracellularly, ROS such as hydroperoxides or carbonylated amino acids damage the plasma membrane through lipid peroxidation [101]. Lipid peroxidation reaction products such as malondialdehyde cause membrane protein and lipid cross-linking, thus disrupting the cell membrane [102]. 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 [103]. Whereas at low ROS doses, endothelial cell membrane damage can be repaired, at high ROS doses irreparable membrane damage and apoptosis occur [104,105]. However, ROS may actually promote membrane damage repair. Wong et al. [93] showed that ROS cause carbonylation of Annexin-A1, an apoptotic protein present on the cytosolic side of the plasma membrane, thus inactivating it and leading to cell survival.

Sub-lethal cell membrane damage by plasma leads to endothelial cell FGF-2 release [74]. Since FGF-2 lacks a classic secretion sequence, it is only known to be released when plasma membrane is damaged [106]. Cell membrane disruption and FGF-2 release are induced in vivo by mechanical forces [107,108], and in vitro by trypsinization [107]. In our previous work, FGF-2 release peaked 3 h after plasma treatment and decreased to half the maximum value within another 3 h [74]. Released FGF-2 promotes damaged cell survival, proliferation, and FGF-2 mRNA and protein expression [37,73,76]. Since ROS are known to enhance FGF-2 affinity to its receptor, the presence of plasma-produced ROS could accelerate released FGF-2 binding [71].

In this study, FGF-2 released following plasma treatment not only induced cell proliferation, but it also induced cell migration and tube formation. Compared to cells treated with FGF-2, plasma-treated cells proliferated 17% more. However, plasma-treated cells released much less FGF-2 (approx. 0.4% of the positive control FGF-2 dose of 10 ng ml$^{-1}$). In typical migration assays, a growth factor gradient is used to induce directional migration. In both our twod- and three-dimensional migration assays, cell FGF-2 release occurred in all directions, therefore, our migration results may not be as high as if a growth factor gradient were induced. We additionally observed a decrease in cell migration when either a neutralizing FGF-2 antibody or a ROS scavenger was added to both plasma-treated and control cells. Both FGF-2 and ROS are constitutively present in medium and cells, therefore, blockade of these factors is expected to affect even untreated samples.

Non-thermal plasma has many advantages over other ROS vascularization methods. Plasma is safe, cheap and portable compared with other mechanisms of inducing ROS or releasing FGF-2, such as electrical stimulation [109], pulsed electromagnetic fields [34] and ionizing radiation [110]. 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 sufficient 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 [28,111]. This could be attributed to insufficient growth factor concentrations for the time needed to generate a response due to difficulties in delivering methods and dosing [111]. 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.

Our goal was to understand plasma-induced FGF-2 release in endothelial cell proliferation, migration and tube formation. However, these studies are not without limitations. The ROS dye DCFH reacts with a wide variety of ROS, thus we are not certain which plasma-produced ROS induce the angiogenic response. Complexity is further enhanced since plasma-produced ROS interact with each other as well as air and liquid components to produce additional reactive species. Other angiogenic factors may also be upregulated by plasma treatment, and these could also play a role in plasma-induced vascularization. The two-dimensional cell monolayer model employed in our research for plasma treatment is significantly different from in vivo conditions. We also used serum-free medium in our studies, since serum is difficult to characterize and may have anti-oxidant properties [112]. Since serum would be present in a wound environment, specific serum and fluid thickness effects on plasma treatment should be determined.

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. We thank Dr Gregory Fridman for the plasma device.

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