

On the Interaction of Non-Thermal Atmospheric Pressure Plasma with Tissues

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Abstract

Non-thermal atmospheric pressure plasma is now being widely developed for various clinical applications such as skin sterilization, blood coagulation, cancer treatment, angiogenesis and wound healing among others. However, understanding of mechanism of interaction between non-thermal plasma and mammalian cells is lacking. Here we investigated the possibility that the dose of non-thermal plasma can be tuned to achieve various results depending on the clinical applications ranging from enhanced cell proliferation to inducing apoptosis in malignant tissue. We also present some of the underlying mechanisms of interaction of non-thermal plasma with mammalian cells.

I. INTRODUCTION

Thermal plasma has been employed in medicine for coagulation and ablation for some time [1]. Treatment of tissues and cells by non-thermal plasma, where the gas temperature is nearly at room temperature, is a recent development [2]. It has been noted that non-thermal plasma applied directly to surfaces of living tissues can coagulate blood; however, it does so without charring the tissue [2, 3]. Similarly, non-thermal plasma appears to kill bacteria on the surface of living tissue without histologically visible damage [2]. It has been reported that non-thermal plasma can also mediate attachment of cells to substrates [4-6], increase transfection efficiency [7, 8] and surface sterilization [9-12]. Ability to tune non-thermal plasma effects together with the simplicity of plasma generating devices and localized nature of plasma application makes it a promising tool in medicine. However, mechanisms of interaction between non-thermal plasma and living systems have been poorly understood.

Here we study the mechanisms of interaction between non-thermal plasma and mammalian cells. Several different methods of non-thermal plasma generation at atmospheric pressure are known. The type of non-thermal plasma employed in this study is called the Dielectric Barrier Discharge (DBD). It was invented by Siemens in 1859 [13]. The plasma in this discharge is created when the time-varying high voltage reaches sufficient magnitude to cause air breakdown. The presence of dielectric layer (dielectric barrier) in the path of the

discharge limits its current which, in turn, limits the energy transferred to ions and neutral gas species keeping their temperature low. Although the plasma gas temperature is low, the presence of charged particles, radicals and electronically excited molecules and atoms makes DBD plasma a potentially active medium whose properties can be controlled to some extent through gas composition as well as waveform of the time-varying applied voltage.

II. METHODS AND MATERIALS

Mammalian Breast Epithelial Cells (MCF10A) were maintained in high glucose Dulbecco's Modified Eagle's Medium-Ham's F12 50:50 mixture (DMEM-Ham's F12 50:50) (Cellgro, Mediatech, VA, USA) supplemented with 5% donor horse serum (Sigma Aldrich, St. Louis, MO, USA), Epidermal Growth Factor (EGF, 100 µg/ml, Sigma Aldrich, St. Louis, MO, USA), Hydrocortisone (1 mg/ml, Sigma Aldrich, St. Louis, MO, USA), Cholera Toxin (1 mg/ml), Insulin (10 mg/ml, and Penicillin/Streptomycin (500 µl, 10000 U/ml penicillin and 10 mg/ml streptomycin). Media was changed every two days. For plasma treatment, cells were washed with phosphate buffered saline (PBS), detached with 0.25% trypsin (GIBCO, Invitrogen, CA, USA), and seeded near confluence (4×10^5 cells/well) on 22 x 22 mm square glass cover slips (VWR, PA, USA) in 6-well plates (Greiner Bio One, NC, USA). Cells were cultured for 24 hours prior to plasma treatment in 2.0 ml supplemented media in a 37°C, 5% CO₂ incubator to allow full attachment and spreading.

N-Acetyl-L-cysteine (2 mM, Sigma-Aldrich, St Louis, MO), an intracellular reactive oxygen species (ROS) scavenger and sodium pyruvate (10 mM, Sigma-Aldrich, St Louis, MO), an extracellular ROS scavenger were used to block the reactive oxygen species produced by non-thermal plasma treatment.

A. Plasma Treatment

Non-thermal atmospheric pressure dielectric barrier discharge plasma was produced using an experimental setup similar to one previously described and schematically illustrated in Figure 1 B. The non-thermal plasma was generated by applying alternating polarity

pulsed (500 Hz – 1.5 kHz) voltage of ~20 kV magnitude (peak to peak), 1.65 μ s pulse width and a rise time of 5 V/ns between the insulated high voltage electrode and the sample undergoing treatment using a variable voltage and variable frequency power supply (Quinta, Russia). 1 mm thick, polished clear fused quartz was used as an insulating dielectric barrier covering the 1 inch diameter copper electrode. The discharge gap between the bottom of the quartz and the treated sample surface was fixed at 2 mm. Discharge power density was measured to be 0.13 Watts/cm² (at 500Hz) and 0.31 Watts/cm² (at 1.5 kHz) using both electrical characterization and a specially designed calorimetric system.

MCF10A cells on glass cover slips were exposed to non-thermal plasma at various doses from 0.13 J/cm² to 7.8 J/cm² (Figure 1). Briefly, each cover slip was removed from the 6-well plate and placed on a microscope slide, which was then positioned on the grounded base of the plasma device. 100 μ l of supplemented media was added to the glass cover slip before plasma treatment to prevent sample drying. Following plasma treatment, the cells were held in the treated medium for one minute and then the cover slip was placed in a new 6-well plate, 2 ml of supplemented media was added to the well, and the samples were returned to the incubator for one hour before analyzing the samples using immunofluorescence or western blot.

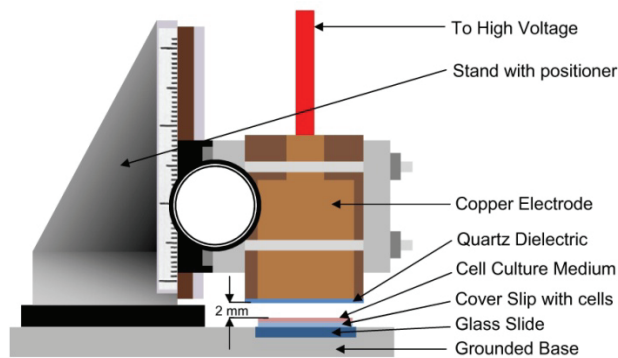


Figure 1. Plasma Treatment Setup

Three different approaches were used for non-thermal plasma-treatment of cells *in vitro*: direct, indirect and separated. In direct treatment, the sample itself was one of the electrodes that created the plasma discharge, as illustrated in. Plasma discharge occurred between the powered high voltage electrode quartz surface and the sample surface, which exposed the sample directly to both neutral reactive species and charged particles. In contrast, for indirect treatment, a grounded mesh was placed between the high voltage electrode and the treated sample to prevent charged particles from reaching the sample surface. In separated plasma treatment, medium alone was plasma treated separately from cells and then immediately applied to cells. In this case, cells were not in direct contact with any plasma component.

B. Immunofluorescence

MCF10A cells were plated onto glass cover slips 24 h before treatment with non-thermal plasma. Immediately after plasma treatment cells were incubated for one hour. Immunofluorescence protocol commenced 1 hour after plasma treatment. Briefly, cells were incubated in pre-extraction buffer for 5 min at 4C, followed by one wash with PBS and incubation in fixation solution for 10 min at room temperature. Cells were then washed in PBS, and incubated in permeabilization buffer for 5 min at 4C. Cells were washed twice with NaN₃ + PBST at room temperature. Primary antibody, mouse monoclonal gH2AX (serine 139, Upstate Biotechnology), diluted 1:1000 in NaN₃ + PBST and cells were incubated in this solution overnight at 4C. Cells were washed thrice in NaN₃ + PBS followed by the addition of secondary antibody, AlexaFluor594 donkey anti-mouse antibody, diluted 1:1000 in NaN₃ + PBST for 1 h in the dark. The secondary antibody solution was removed followed by incubation of slides in 1 μ l DAPI + PBST + NaN₃. Slides were washed thrice in NaN₃ + PBST and mounted using DAPI-free mounting media on glass microscope slides overnight. The slides were then frozen at -20 C for one day prior to imaging them on an upright fluorescence enabled microscope.

C. Western Blot

Protein expression and modification were analyzed by immunoblot. Total cell lysates were prepared by direct lysis of washed cells in 2X SDS sample buffer containing β -mercaptoethanol. Samples were electrophoresed at 150 V in Tris-glycine SDS running buffer [25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS (pH 8.3)]. Following electrophoresis, proteins were transferred on to PVDF (Millipore, MA, USA) membrane for two hours in Tris-glycine transfer buffer [10% SDS, Deionized Water, Tris-Glycine and Methanol (VWR, PA USA)]. Immunoblotting was done by blocking membranes in 1% nonfat dried milk (Carnation) in PBS with 0.1% Tween 20 (PBST) for α -tubulin or 5% bovine serum albumin (BSA, Fraction V, Fisher Scientific) in PBST for g-H2AX followed by incubation with primary antibodies in 1% nonfat dried milk in PBST for α -tubulin and 5% BSA in PBST for γ -H2AX overnight for 10 to 12 h at 4°C with rocking. Primary antibodies used for immunoblot included mouse monoclonal antibodies specific for γ -H2AX [phospho-histone H2AX (serine 139), clone JBW301; Upstate] and α -tubulin (Santa Cruz Biotechnology). The primary antibodies were detected with fluorescently tagged goat anti-mouse Alexa and Fluor 488 (Santa Cruz Biotechnology). Immunoblot was developed using Odyssey Infrared Gel Imaging system (LI-COR Biosciences, NE, USA).

D. Colony Survival Assay

4×10^5 MCF10A cells were seeded on 22 x 22 mm square cover slips in 6-well plates one day before plasma treatment. Cells were plasma treated as described at various doses of plasma and then incubated for one day after plasma treatment. 300 cells were seeded onto 60-mm dishes after exposure to non-thermal plasma or H_2O_2 (positive control). Colonies, which formed 11 days after plating MCF10A cells, were fixed and stained with a crystal violet solution (0.5% in 20% ethanol) and then counted. Assays were done in triplicate.

E. Cell Growth Assay

MCF10A cell proliferation was measured through cell counts on directly treated cells. 10,000 MCF10A cells were seeded on 22 x 22 mm square cover slips in 6-well plates one day before plasma treatment. Cells were plasma treated as described at various doses of plasma and incubated for an additional 3 days with a media change on day 2. Cell number was quantified on days 1 and 3 by counting trypsin-detached cells using a Cell Viability Assay (Guava EasyCyte Plus, Millipore, MA, USA). Fold growth was determined by taking the ratio of cell number on day three to day one.

F. Apoptosis

Apoptosis was measured via annexin V-propidium iodide labeling. Annexin V binds phosphatidylserine translocated from the inner to the outer cell membrane. Cells in early apoptosis are identified as annexin V-positive and negative for the vital dye propidium iodide, which is membrane impermeant and excluded from viable cells. MCF10A cells were prepared for the annexin V-propidium iodide assay by combining floating and trypsin-released attached cells. Samples were centrifuged to pellet cells, washed thoroughly, resuspended in annexin binding buffer, and labeled with annexin V-fluorescein and propidium iodide as per manufacturer instructions (BD Pharmingen, San Jose, CA). Samples were analyzed immediately by flow cytometry (Guava EasyCyte Plus, Millipore, MA, USA).

G. Statistical Analysis

Statistical analyses were performed with Prism software (Graphpad, CA, USA). Data were normally distributed and expressed as the mean \pm S.D. Comparisons between two groups were analyzed by Student's t test, and comparisons between more than 2 groups were analyzed by ANOVA.

III. RESULTS

The initial characterization of non-thermal plasma involved establishing its effect on cell proliferation and survival. Cells were subjected to a range of DBD plasma doses, followed by counting cells. Endothelial cell

proliferation is enhanced by low dose non-thermal plasma treatment (Figure 2). Cells treated with plasma showed greater viable cell number than control up to 30 seconds of plasma treatment.

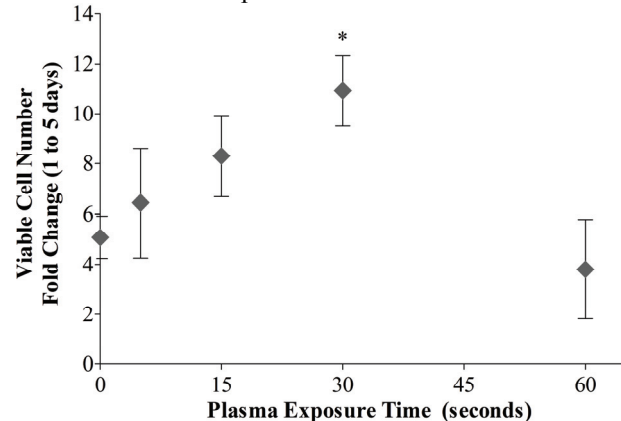


Figure 2. Endothelial cell fold growth is enhanced in non-thermal plasma treated cells 5 days after treatment. Plasma treated cells were counted using a Coulter counter 1 and 5 days after treatment. * $p < 0.01$ as compared to control.

With 30 seconds of treatment, endothelial cells demonstrated twice as many viable cells as untreated controls (Figure 2). However, increased plasma treatment times beyond 30 seconds resulted in decreased cell number.

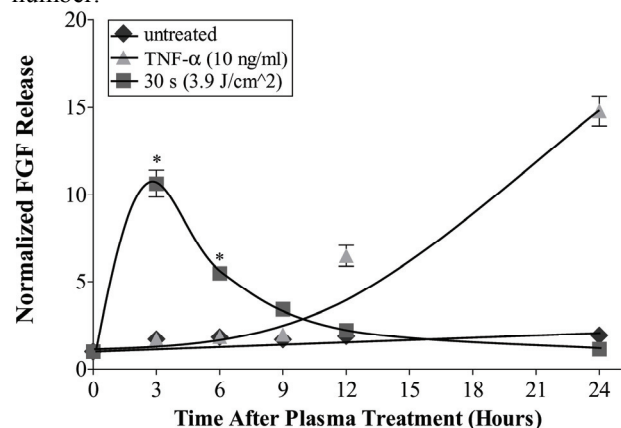


Figure 3. FGF2 release increases up to 3 hours post plasma treatment and then decreases up to 24 hours post plasma treatment. FGF2 was measured in conditioned media samples by ELISA

We next considered whether FGF2 was released from endothelial cells following non-thermal plasma treatment, and whether the released FGF2 contributed to enhanced cell proliferation. FGF2 has no signal sequence for secretion, and therefore is primarily known to be released during sub-lethal cell membrane damage. The FGF2 level in the media increased up to 3 hours after plasma treatment ($3.9 J/cm^2$, 30 s) and then rapidly decreased up to 24 hours after plasma treatment. In contrast, FGF2

media levels for cells treated with 10 ng/ml TNF- α as a positive control rose more slowly but continued to rise up to 24 hours (Figure 3).

Effects on cell survival were determined by measuring colony formation. Cells treated at low doses of plasma (≤ 0.65 J/cm²) showed no significant decrease in survival, whereas survival decreased with increased dose of plasma (Figure 4), suggesting that at higher doses DBD plasma may be inducing cell death.

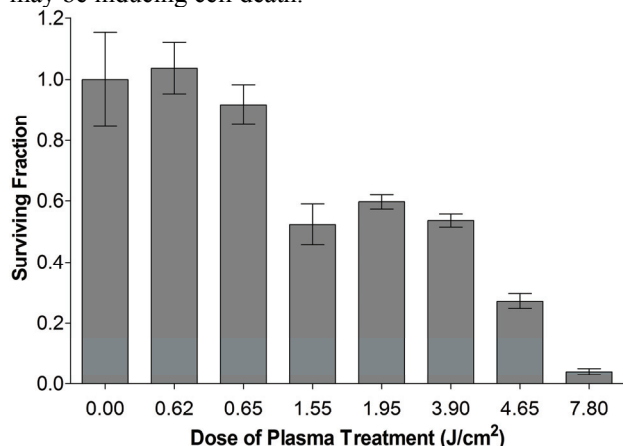


Figure 4. Cells were treated with the indicated dose of DBD plasma; one day after treatment, 300 cells were plated in a 6 cm dish and colonies were counted after 8 days. Data from triplicate samples (\pm S.E.M.) are expressed relative to the # of colonies in the untreated control.

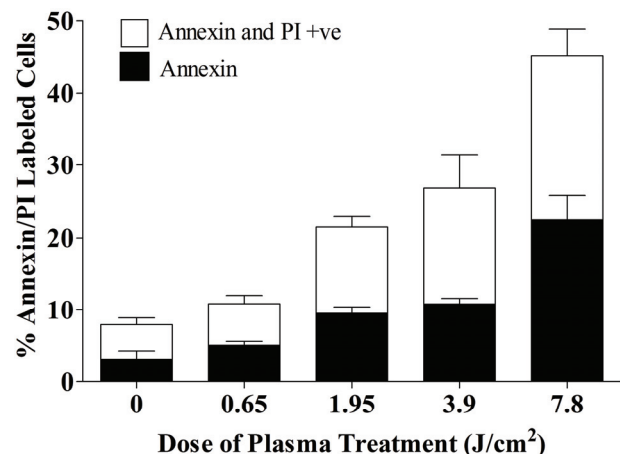


Figure 5. Cells were treated with the indicated dose of DBD plasma as described. 3 days after treatment, cells were harvested and stained with Annexin V/ propidium iodide (PI) and analyzed by Guava. Data from triplicate samples indicate the % of cells that stain positive for Annexin V and PI.

To determine whether cells were dying by apoptosis, cells were treated with 0.65 to 3.9 J/cm², followed by Annexin V/propidium iodide staining; a dose dependent increase in apoptosis was observed (Figure 5), which was corroborated by caspase 3 cleavage. Based on these

results, it appears that cell death resulting from high doses of plasma is largely through induction of apoptosis, rather than necrosis.

One possible mechanism underlying these effects is generation of reactive oxygen species ROS, which at low levels induces cell proliferation and at high levels induces cell death through DNA damage [14]. To determine whether DBD plasma treatment of cells induced DNA damage, we looked at phosphorylation of H2AX, a histone variant that is phosphorylated in response to DNA double strand breaks [15].

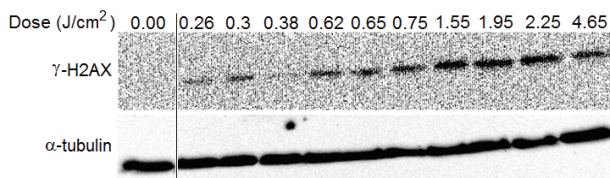


Figure 6. MCF10A cells were treated with the indicated dose of DBD plasma as described. After one hour incubation, lysates were prepared and resolved by SDS-PAGE and representative immunoblots with antibody to γ -H2AX (upper panel) or α -tubulin (lower panel) are shown.

Western blot with an antibody that detects phosphorylated H2AX (γ -H2AX) revealed that plasma treatment of cells induces a dose-dependent increase in the level of γ -H2AX (Figure 6).

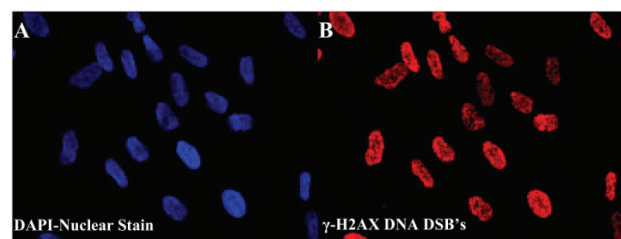


Figure 7 Indirect immunofluorescence of MCF10A cells one hour after treatment with 1.55 J/cm² DBD plasma

Indirect immunofluorescence revealed foci of γ -H2AX (Figure 7), which were increased in number at higher doses (data not shown). These data are consistent with a dose-dependent increase in DNA damage.

We next sought to directly test whether the damage induced by non-thermal plasma is due to ROS (e.g. H₂O₂, OH⁻, singlet oxygen, etc.) generated in the media and/or cells by plasma treatment. Cells were pre-treated with the ROS scavenger, N-acetyl cysteine, which was found to block induction of γ -H2AX even at high doses of DBD (Figure 8), suggesting that the effects are mediated by ROS.

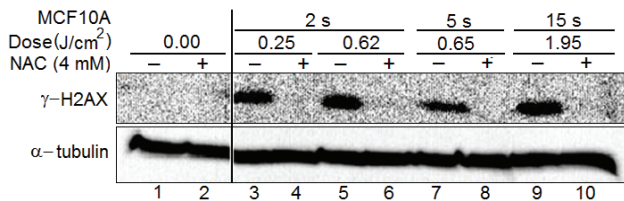


Figure 8. MCF10A cells were incubated for 1 hour with 4 mM N-acetyl cysteine (NAC) (+) or cell culture medium (-), followed by treatment with the indicated dose of DBD plasma. γ-H2AX (upper panel) or α-tubulin (lower panel) was detected by immunoblot of cell lysates prepared one hour after plasma treatment.

Non-thermal plasma produces large amounts of ROS. These ROS may interact with the endothelial cell membrane; leading to damage that allows FGF2 release from plasma treated cells. To test the role of ROS in the plasma-induced cell FGF2 release, endothelial cells were pre-incubated in 4 mM N-acetyl cysteine (NAC) to scavenge intracellular ROS and then plasma-treated in supplemented medium with or without 50 mM sodium pyruvate to scavenge extracellular ROS. Both NAC and sodium pyruvate significantly suppressed FGF2 release from plasma treated cells (Figure 9), suggesting that both intracellular and extracellular ROS may mediate the plasma-induced endothelial cell FGF2 release.

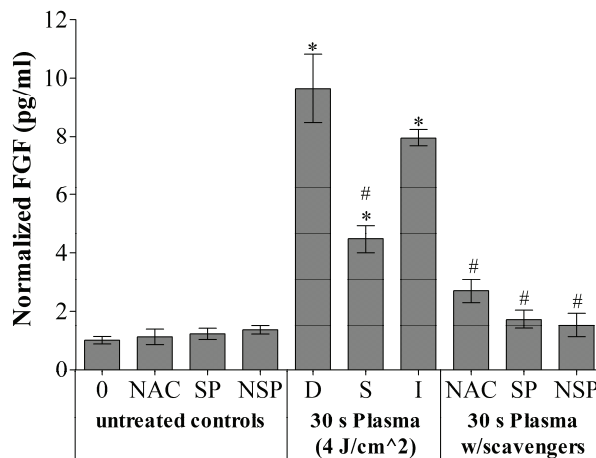


Figure 9. Intracellular (4 mM NAC – N-Acetyl Cysteine) and extracellular (50 mM SP – Sodium Pyruvate) ROS scavengers block FGF2 release from endothelial cells post plasma treatment. * $p < 0.01$ as compared to control (0). # $p < 0.05$ as compared to direct treatment (D). NSP: 4mM N-Acetyl Cysteine and 10 mM Sodium Pyruvate together

As shown in Figure 10. pretreatment with 2.25 mM N-acetylcysteine (NAC), a free radical scavenger, significantly decreased apoptosis in plasma-treated cells as analyzed by Annexin-V/PI staining 24 h after plasma treatment. ($p < 0.001$). We see 12% apoptosis in pre-incubated cells after plasma treatment as compared to 28% in cells which did not receive NAC but were treated

with plasma. Initial results indicate that reactive oxygen species generated by non-thermal plasma somehow penetrate the cellular membrane and may be mediating non-thermal plasma induced apoptosis.

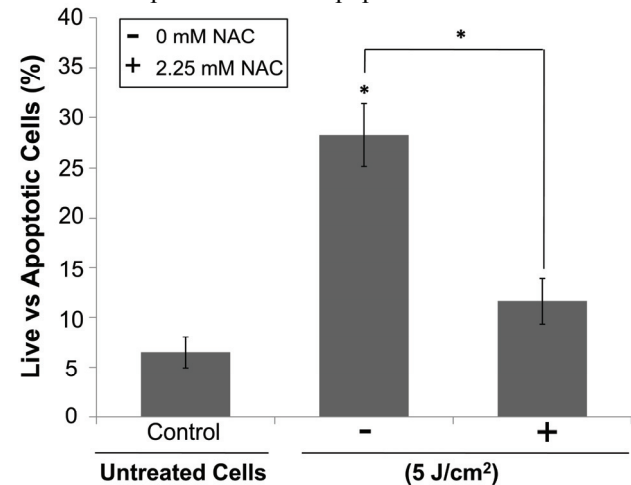


Figure 10. Pretreatment with 2.25 mM N-acetylcysteine (NAC), a free radical scavenger, significantly decreased apoptosis in plasma-treated cells as analyzed by Annexin-V/PI staining 24 h after plasma treatment. ($p < 0.001$). * $p < 0.001$ as compared to untreated control or cells preincubated with N-acetyl cysteine

Non-thermal plasma is known to produce many charged (electrons and ions) and long living and short living neutral species (metastable particles, OH radical, ozone, singlet oxygen, H₂O₂) in gas phase as well as in liquid phase. To determine which of these species are responsible for the induction of DNA damage, non-thermal plasma was applied in different modes. Previous

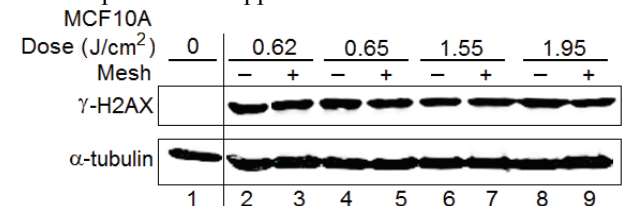


Figure 11. Cells were subjected to DBD as described earlier (direct, -) or a grounded mesh was placed between the electrode and the medium (indirect, +).

experiments have involved direct treatment, in which all the species produced by plasma come in contact with the surface of cell culture medium covering the cells during treatment. Indirect treatment involves placement of a grounded mesh between the high voltage electrode and the medium covering the cells being treated, which blocks charged species and allows only neutral species to come in contact with the surface of the medium. Comparison of DNA damage by direct plasma treatment and indirect plasma treatment shows that phosphorylation of H2AX was not significantly different in indirect vs. direct treatment (Figure 11), indicating that active neutral

species produced by DBD plasma are responsible for the induction of DNA damage.

To determine whether the effects of DBD plasma are due to modification of the cell medium by plasma treatment, the medium was treated in the same way without cells and then added to cells (separated treatment). As shown in Figure 12, damage induced by the treatment of the medium separately from cells was not less than that produced by direct treatment. This suggests that ROS generated in the medium by plasma treatment are responsible for the induction of DNA damage. These ROS species must survive long enough to remain active while being transferred to the cells.

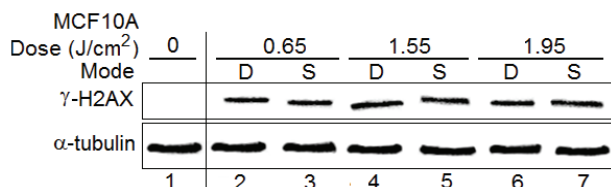


Figure 12. Cells were subjected to DBD plasma as described earlier (direct, D) or media (100 μ l) was subjected to DBD plasma and then transferred to the cells (separate, S).

To determine how long living are these ROS species, cell medium was separately treated as described above and then held for increasing times before being added to cells. Induction of DNA damage by the medium treated with DBD plasma was not significantly reduced by holding media up to one hour prior to adding it to cells (Figure 13), suggesting that neutral species (as shown above charges do not play a major role) may react with organic components in the cell medium to produce long living organic peroxides which are known to have a half life on the order of 12-24 h [16].

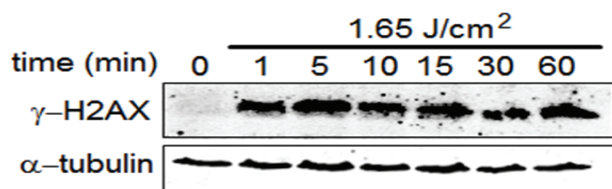


Figure 13. Media (100 μ l separated treatment) was subjected to DBD and was transferred to cells after holding for 1 to 60 min. After 1 minute incubation with cells, cover slips with treated media and cells were transferred to a dish with 2 ml of media

IV. CONCLUSIONS

Use of DBD plasma for clinical applications requires an understanding of its interaction with living tissues. We have shown here that non-thermal plasma interacts with cells indirectly by modifying the surrounding environment of the cells during treatment. We also confirmed that long living reactive oxygen

species (ROS) produced by plasma in cell culture medium mediate interaction between non-thermal plasma and mammalian cells. The amount of ROS produced by plasma can be tightly controlled by varying the applied voltage, allowing fine tuning of therapeutic effect, from stimulating cell proliferation to inducing apoptosis. Future work will focus on establishing whether the effects of plasma are through membrane lipid peroxidation or due to uptake of long living organic hydroperoxides by active transport mechanisms in the cells. The potential clinical applications of DBD plasma include treatment of wounds to enhance healing and sterilize wound surfaces or controlled ablation of tissue, including benign lesions or cancers

V. ACKNOWLEDGEMENTS

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