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Selectivity of Non-Thermal Atmospheric-Pressure Microsecond-Pulsed Dielectric Barrier Discharge Plasma Induced Apoptosis in Tumor Cells over Healthy Cells

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ABSTRACT: Non-thermal plasma is now being widely investigated for various clinical applications ranging from surface sterilization to blood coagulation to wound healing to cancer therapy. We have shown previously that reactive oxygen species (ROS) generated by non-thermal dielectric barrier discharge (DBD) plasma in the medium surrounding the cells induce DNA damage in mammalian cells, and by tuning the dose, plasma has various effects, from enhancing proliferation to inducing apoptosis in cancer cells.[1–3] Although non-thermal plasma primarily produces ROS extracellularly, we hypothesize that it can induce apoptosis in malignant cells similar to ionizing radiation or photodynamic therapy, which primarily produce ROS intracellularly. Unlike ionizing radiation, which damages healthy tissue surrounding the malignant tissue,[4,5] or photodynamic therapy, which causes scarring and burning of nearby healthy tissue, non-thermal plasma, due to its non-penetrating nature, may provide a safer means to induce selective apoptosis in malignant tissue by providing precise control of treatment area and depth.

KEY WORDS: Apoptosis, dielectric barrier discharge, DNA damage, non-thermal plasma, plasma medicine, reactive oxygen species

I. INTRODUCTION

The non-functioning of a tumor-suppressor gene that facilitates apoptosis and the over-expression of an anti-apoptotic protein are both important pathways in cancer development. Many anti-cancer therapies are aimed at modulating these factors with various bioactive agents as well as radiation in an attempt to target components of the apoptotic pathway.[6,7] Electrical and optical therapies are being considered as well.[8,9] However, many of these approaches remain in preclinical development due to either low efficacy or tumor drug resistance.[10,11] Our current research seeks to develop novel techniques to modulate apoptotic activity in cancer cells by evaluating an electro-chemical approach to induce apoptosis.

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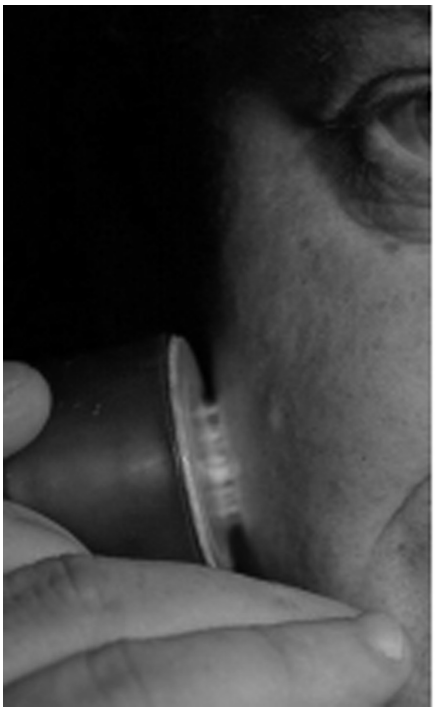

FIGURE 1: Non-thermal plasma can be safely applied to living tissue.

In this study, we sought to explore the effects of exposure of malignant cells to non-thermal atmospheric pressure plasma. It has been shown that non-thermal plasma induces apoptosis like behavior in melanoma cells,[12] but the precise mechanisms of induction of apoptosis by non-thermal plasma were not studied carefully, and the selectivity of non-thermal plasma induced apoptosis in malignant cells over healthy cells has not been studied.

Non-thermal atmospheric pressure DBD plasma has recently emerged as a novel tool in medicine.[13] The operating principle of this plasma discharge is similar to the DBD introduced by Siemens in 1862.[14] Non-thermal plasma occurs at atmospheric pressure in air or other gases when high voltage of sinusoidal waveform or short duration pulses is applied between two electrodes, with at least one electrode being insulated.[15] The insulator prevents build-up of current between the electrodes, creating electrically safe plasma without substantial gas heating. This approach allows direct treatment of living tissue (Figure 1) and biological systems without the thermal damage observed in more conventional thermal plasma therapies.[16,17]

Non-thermal plasma has been widely studied for sterilization of inert surfaces[2–7] and treatment of inert substrates with the purpose of modulating cell attachment.[8–10] Only recently it has been shown that non-thermal plasma can be applied to cells in sub-lethal doses to elicit specific biological effects, including gene transfection,[18–20] cell detachment,[21–24] cell proliferation,[25] and wound healing.[17,26–29] Non-thermal plasma can even

have selective effects. In recent studies on plasma-initiated blood coagulation,[17,28] skin sterilization,[17,30] and tissue toxicity after plasma treatment,[31,32] plasma did not demonstrate measurable toxicity in the surrounding living tissue.

Our floating-electrode DBD system is constructed similarly to conventional DBD and is inherently non-thermal; it is able to operate at room temperature and atmospheric pressure.[14,15,17] Plasma operates under conditions in which one of the electrodes is an insulated high-voltage electrode and the second active (floating since it is not grounded) electrode is human (Figure 1) or animal tissue having a high dielectric constant.[17] Plasma is composed of charged particles (electrons, ions), electronically excited atoms and molecules, radicals, and UV photons. Plasma treatment exposes cells or the tissue surface to active short- and long-lived neutral atoms and molecules, including ozone (O_3), NO, OH radicals, and singlet oxygen (O_2 $^1\Delta_g$), as well as a significant flux of charged particles, including both electrons and positive and negative ions (e.g., super oxide radicals). Non-thermal plasma density, temperature, and composition can be changed to control plasma products.[33–35]

II. MATERIALS AND METHODS

A. Cell Lines and Reagents

Mammalian breast epithelial cells (MCF10A, normal) and transformed mammalian breast epithelial cells, which constitutively over-expressed ERBB2 (MCF10A-NEUT, malignant) were maintained in high glucose Dulbecco's modified Eagle's medium/ Ham's F12 50:50 mixture (Cellgro, Mediatech, VA, USA) supplemented with 5% horse serum (Sigma Aldrich, St. Louis, MO, USA), epidermal growth factor (EGF, 100 μg/ml, Sigma Aldrich), hydrocortisone (1 mg/ml, Sigma-Aldrich), cholera toxin (1 mg/ml, Sigma-Aldrich), insulin (10 mg/ml, Sigma-Aldrich) and penicillin/streptomycin (500 μl, 10000 U/ml penicillin and 10 mg/ml streptomycin, Sigma-Aldrich). For plasma treatment, cells were washed with phosphate buffered saline (PBS), detached with 0.25% trypsin (GIBCO, Invitrogen, Grand Island, NY, USA), and seeded near confluence (4×10^5 cells/well) on 22 × 22 mm square glass cover slips (VWR, Radnor, 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% CO_2 incubator to allow full attachment and spreading.

N-Acetyl-L-cysteine (NAC, 4 mM, Sigma-Aldrich), an intracellular ROS scavenger was used as a scavenger for the ROS produced by non-thermal plasma treatment.

B. Non-Thermal Plasma Treatment of Cells

Non-thermal DBD plasma was produced using an experimental setup similar to one previously described and schematically illustrated in Figure 2.[17] 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-μs pulse width and a rise time of 5 V/ns between the

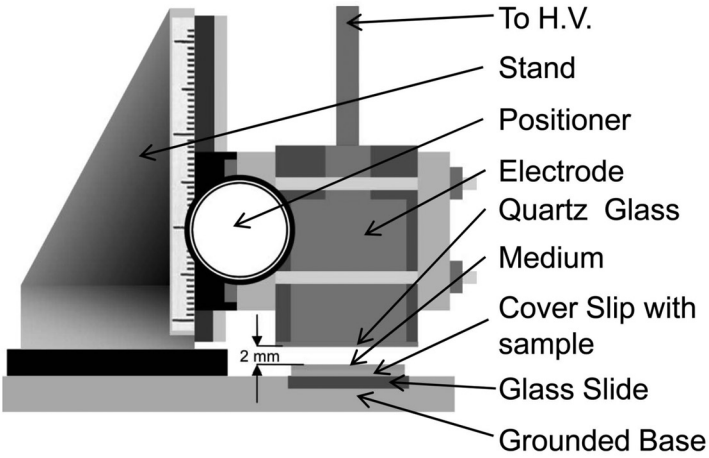

FIGURE 2: Setup used for non-thermal plasma treatment of mammalian cells.

insulated high voltage electrode and the sample undergoing treatment using a variable voltage and variable frequency power supply (Quinta, Russia). A 1-mm-thick, polished, clear fused quartz layer 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/cm^2 (at 500Hz) and 0.31 Watts/cm^2 (at 1.5 kHz) using both electrical characterization and a specially designed calorimetric system.[36] The dose in J/cm^2 of the plasma treatment was calculated by multiplying the power density (W/cm^2) with the duration of plasma treatment (s).

MCF10A or MCF10A-NEUT cells plated on glass cover slips were exposed to non-thermal plasma at various doses (0.13–7.8 J/cm^2). Briefly, each cover slip was removed from the 6-well plate, drained, and placed on a microscope slide, which was then positioned on the grounded base of the plasma device. A volume of 100 µl of complete 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 1 hour before analysis by immunofluorescence or western blot. Induction of apoptosis was measured 48 h after plasma treatment.

C. Cell Proliferation Assay

MCF10A cell proliferation was measured through cell counts on directly treated cells. 10,000 MCF10A cells were seeded on 22 × 22-mm square cover slips in 6-well plates 1 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 the number of attached cells on day 3 to day 1.

D. Colony Survival Assay

Approximately 4×10^5 MCF10A cells were seeded on 22 × 22-mm square cover slips in 6-well plates 1 day before plasma treatment. One day after treatment with DBD plasma or H_2O_2 (positive control), approximately 300 cells were seeded onto 60-mm dishes. Eleven days after plating, cells were fixed and stained with a crystal violet solution (0.5% in 20% ethanol) and colonies were counted. Assays were done in triplicate.

E. Apoptosis

Apoptosis was measured in both MCF10A and MCF10A – NEUT cells 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 in response to the vital dye propidium iodide. Floating and trypsin-released cells were collected and centrifuged, 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).

F. Western Blot (Immunoblotting)

Protein expression and modification were analyzed by immunoblotting. Total cell lysates were prepared by direct lysis of washed cells in 2X SDS sample buffer containing b-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 to PVDF (Millipore, MA, USA) membrane for 2 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 a-tubulin or 5% bovine serum albumin (BSA, Fraction V, Fisher Scientific) in PBST for g-H2AX followed by incubation with primary antibodies overnight for 10 to 12 h at 4ºC with rocking. Primary antibodies used for immunoblot included mouse monoclonal antibodies specific for a-H2AX [phospho-histone H2AX (serine 139), clone JBW301; Upstate] and a-tubulin (Santa Cruz Biotechnology). The primary antibodies were detected with fluorescently tagged goat anti-mouse Alexa and Fluor 488 (Santa Cruz Biotechnology). An immunoblot was created using an Odyssey Infrared Gel Imaging system (LI-COR Biosciences, NE, USA).

G. Immunofluorescence

MCF10A cells were plated onto glass cover slips 24 h before treatment. One hour after plasma treatment, cells were subjected to *in situ* cell fractionation as described,[37] by incubation in pre-extraction buffer (1X PBS + 0.2% Triton-X + 1:50 PMSF) for 5 min at 4°C followed by one wash with PBS and incubation in fixation solution (3% para-formaldehyde + 2% sucrose in PBS) for 10 min at room temperature. Cells were then washed in PBS and incubated in permeabilization buffer (1X PBS + 0.5% Triton-X) for 5 min a 4C. Cells were washed twice with NaN_3 + PBST at room temperature and incubated overnight at 4°C in primary antibody (mouse monoclonal g-H2AX (serine 139, Upstate Biotechnology, 1:1000). After three washes in NaN3 + PBS, cells were incubated for 1 h in the dark in secondary antibody (AlexaFlour594 donkey anti-mouse antibody, diluted 1:1,000). The secondary antibody solution was removed followed by incubation of slides in 1 μl DAPI + PBST + NaN_3 , three washes in NaN_3 + PBST and mounting using DAPI-free mounting media (Vector Labs) on glass microscope slides overnight. The slides were then frozen at -20°C for 1 day prior to imaging them on an upright fluorescence enabled microscope.

III. RESULTS

To test the effects of plasma treatment on mammalian cells, DBD plasma was applied to a culture of human breast epithelial cells (MCF10A). Cells were grown on glass cover slips and exposed to non-thermal plasma as illustrated (Figure 2). Cells were treated in 100 ml of media and incubated for 1 minute prior to placement in 2 ml of media. Typical power density of the DBD plasma is 0.1–1 W/cm^2 and the doses were varied from 0.2 J/cm^2 to 5 J/cm^2. The initial experiments involved establishing the dose dependent effects of plasma treatment on cell proliferation and cell survival by direct cell count and colony formation. This was necessary to determine at which dose non-thermal plasma induced significant cell death. At low doses, cell number increased, whereas at higher doses, cell number decreased (Figure 3); low doses of plasma ($\leq$0.65 J/cm^2) showed no significant effect on cell survival, whereas survival decreased with increased dose of plasma (Figure 4), suggesting that at higher doses DBD plasma induces significant cell death.

One possible mechanism underlying these dose-dependent effects is generation of ROS, which at low levels is known to increase cell proliferation and at high levels induces cell death through DNA damage.[38] DNA damage induced by IR has been shown to result from formation of ROS.[4,5] To determine whether DBD plasma treatment of cells could induce DNA damage, we looked at phosphorylation of H2AX, a histone variant that is phosphorylated in response to DNA damage.[39] Western blot with an antibody that detects H2AX phosphorylated at Ser139 (g-H2AX) revealed that treatment of cells with DBD plasma induces a dose-dependent increase in g-H2AX that indicates a dose-dependent increase in DNA damage (Figure 5). Indirect immunofluorescence also revealed foci of g-H2AX (Figure 6) that increased in num-

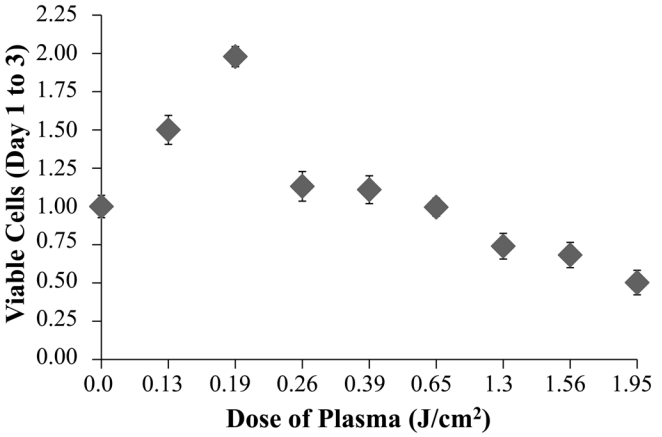

FIGURE 3: Approximately 10^4 MCF10A cells plated on glass cover slips were treated with the indicated dose of DBD plasma as described. Cells were counted 24 and 72 hours after treatment. Data are plotted relative to the number of cells in the untreated plate at 24 hours relative to the number of cells at 72 hours, which was set at 1.0.

ber at higher doses. These data are consistent with a dose-dependent increase in DNA damage.

We next sought to directly test whether the damage induced by DBD plasma is due to ROS (Non-thermal plasma is known to produce H_2O_2, $OH^{\bullet}$, singlet oxygen, atomic

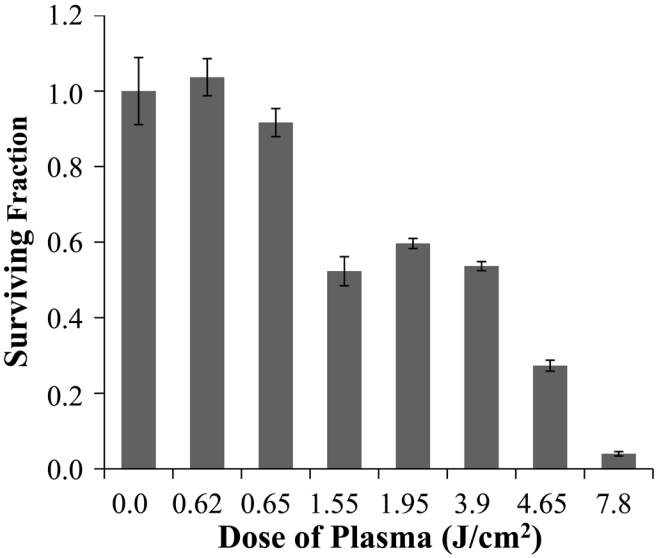

FIGURE 4: Cells were treated with the indicated dose of DBD plasma, and colony survival assays were performed as described. Data are expressed relative to the number of colonies in the untreated control. Data from triplicate samples (± S.D.) are plotted.

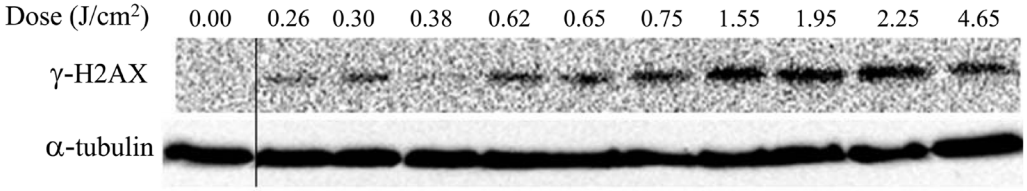

FIGURE 5: Representative blot with antibodies towards g-H2AX (top panel) and a-tubulin (bottom panel, loading control) showing non-thermal DBD plasma induces dose-dependent DNA damage in mammalian cells.

oxygen, superoxide radical, etc.)[40] generated in the media and/or cells by plasma treatment. Pre-treatment of cells with the ROS scavenger, N-acetyl cysteine (NAC), blocked induction of g-H2AX even at high doses of DBD plasma (Figure 7), suggesting that the induction of DNA damage as measured by g-H2AX is mediated by ROS.

Because effects of non-thermal plasma on mammalian cells seem to be mediated by ROS similar to ionizing radiation or photodynamic therapy, we wanted to compare the effects of non-thermal plasma on normal cells compared to malignant cells. Ionizing radiation and photodynamic therapy are effective anti-cancer treatment therapies because they exploit the susceptibility of malignant cells over healthy cells to oxidative stress by inducing formation of ROS primarily intracellularly that ultimately lead to significant DNA damage that cannot be repaired by malignant cells.[4,5,41–43]

Non-thermal plasma on the other hand produces ROS primarily extracellularly. To determine whether malignant cells (MCF10A – NEUT) were more susceptible than healthy cells to ROS produced extracellularly by plasma, normal mammary epithelial cells (MCF10A) and transformed malignant cells (MCF10A-NUET) were exposed to increasing doses of plasma and level of apoptosis was measured Annexin-V/PI assay. At low doses up to 4 J/cm^2 the level of apoptosis in both cells lines (Figure 8a and b) was similar, but at higher doses malignant MCF10A-NUET cells (Figure 8b) were more vulnerable to plasma treatment; almost twice the number of apoptotic cells were counted compared to normal cells (Figure 8a). These data suggest that malignant cells are more vulnerable to plasma than normal cells.

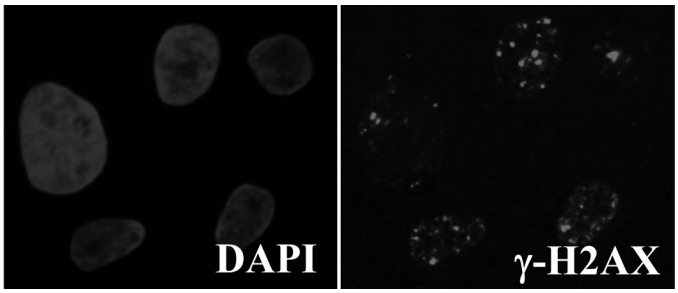

FIGURE 6: Indirect immunofluorescence of MCF10A cells was performed as described 1 hour after treatment with 1.55 J/cm^2 DBD plasma.

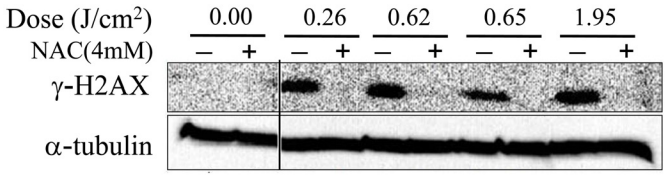

FIGURE 7: MCF10A cells were incubated for 1 hour with 4 mM N-acetyl cysteine (NAC) (+) or cell culture medium (-), followed by treatment with the indicated dose of DBD plasma. g-H2AX (upper panel) or a-tubulin (lower panel) was detected by immunoblot of cell lysates prepared 1 hour after plasma treatment.

To determine whether cells were dying by apoptosis or necrosis, annexin V/ propidium iodide (PI) staining was performed on cells treated with DBD plasma at doses ranging from 0.65 to 7.8 J/cm^2; a dose-dependent increase in annexin V +ve, PI –ve cells was observed (Figure 8a and b). Apoptotic cells exhibit only annexin V positive staining (refer to Figure 8a and b where annexin V positive cells are labeled annexin) while not staining for PI. Based on these results, it appears that cell death resulting from higher doses of plasma is largely through induction of apoptosis, rather than necrosis. Apoptosis is programmed cell death initiated by physiological signals including oxidative stress. Apoptotic cells are broken up into apoptotic bodies, which are engulfed by neighboring cells, leading to clean cell death without significant inflammatory response which is an important consideration in cancer treatment.[44,45] On the contrary, necrosis is cell death accompanied by swelling, blebbing and increased membrane permeability leading to cytosolic content spillage. This typically leads to inflammation in surrounding tissue.[44,45] By controlling plasma dose, we may be able to selectively kill malignant cells without significant necrosis and subsequent inflammation.

IV. DISCUSSION

Non-thermal plasma treatment dose can be tuned to induce selective apoptosis in malignant cells over healthy cells. ROS produced extracellularly by non-thermal plasma likely induces apoptosis in malignant cells by induction of significant DNA damage in malignant cells that have compromised DNA repair as compared to normal healthy cells and cannot repair DNA damage effectively ultimately dying via apoptosis.

Ionizing radiation[46–48] and chemotherapy[49–51] are the most commonly used treatments for various types of malignancies, but both of the treatments have severe side effects since they not only kill the tumor but also damage healthy tissue surrounding the malignant tissue. Many cancers are notoriously resistant to radiation and chemotherapy. Recently, pulsed electric fields have also been shown to be able to initiate apoptosis in malignant cells.[8] Photodynamic therapy (PDT), which combines a photosensitizing drug with a specific wavelength of light generated by lasers, is now being

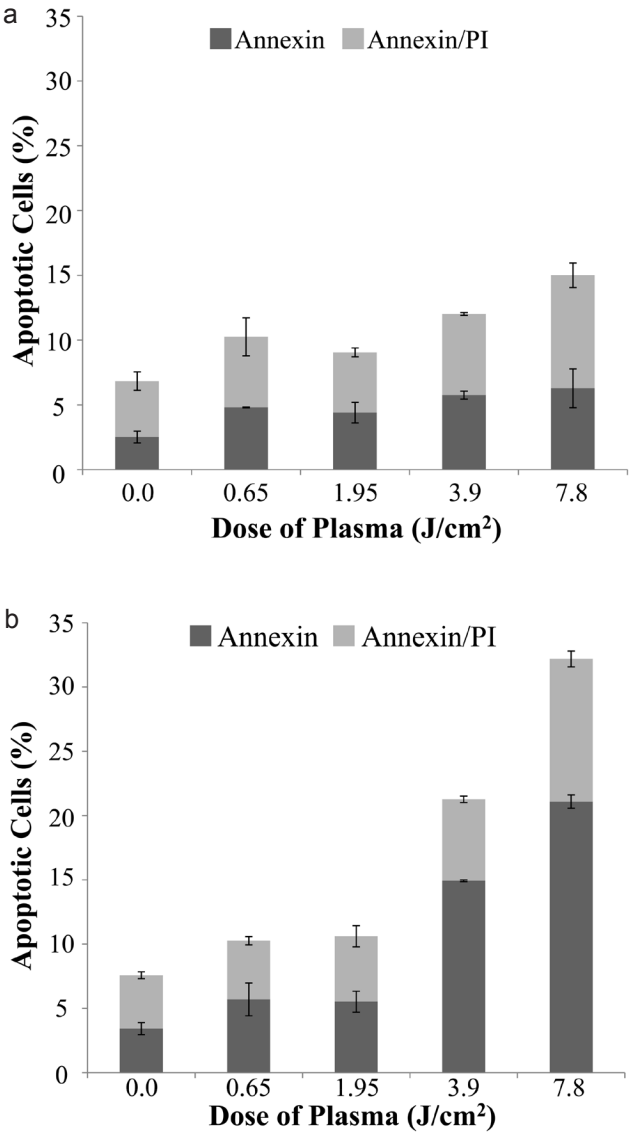

FIGURE 8: (a) 48 h after treatment with the indicated dose of DBD plasma, MCF10A cells were harvested and stained with Annexin V/ propidium iodide (PI) and analyzed by Guava. (b) 48 h after treatment with the indicated dose of DBD plasma, MCF10A - NEUT cells were harvested and stained with Annexin V/ propidium iodide (PI) and analyzed by Guava. Data from triplicate samples (± S.D.) are plotted.

developed for treatment of melanoma and other skin cancers and has been shown to be somewhat successful.[9,52] Non-thermal plasma differs from radiation, chemotherapy and electromagnetic fields. Chemotherapy induces severe systemic side effects while

electromagnetic fields are penetrating and therefore injure surrounding tissue. Ionizing radiation requires extensive and expensive equipment for administration, while PDT requires prior administration of a photosensitizer and efficacy of treatment is limited to the depth of penetration of light. Various studies also suggest that malignant cells frequently acquire radiation resistance[53] and chemoresistance[54] by exploiting their intrinsic resistance to apoptosis and by reprogramming their proliferation and survival pathways during cancer progression. Non-thermal plasma, on the other hand, provides a novel and safer means to induce apoptosis because it is non-thermal, and it provides precise control of treatment area and depth as it is non-penetrating. Non-thermal plasma devices are also relatively inexpensive, small, and simple to manufacture and operate. Non-thermal plasma can be applied directly to specific areas of interest, potentially avoiding systemic side effects, and it is limited only to the depth of penetration of reactive oxygen species. Becuase non-thermal plasma can be attached to the end of a probe, it has the added benefit of potentially being able to target areas deep in the body.

V. CONCLUSION

Initiation of apoptosis is an important issue in cancer treatment because cancer cells frequently have acquired the ability to block apoptosis and thus are more resistant to chemotherapeutic drugs. Targeted and selective destruction of cancer cells are desirable for many reasons, including limiting systemic side effects and preventing damage to nearby healthy tissue. We demonstrated in this study the selectivity of the induction of apoptosis in transformed malignant epithelial cells over normal epithelial cells *in vitro* by exposure to non-thermal atmospheric-pressure plasma. We have demonstrated that the exposure of cells in tissue culture to atmospheric-pressure non-thermal plasma induces apoptosis, and this effect is likely related to production of ROS by non-thermal plasma. We also showed that malignant cells are more susceptible to non-thermal plasma than normal cells. Future investigations are warranted to evaluate the detailed mechanisms by which non-thermal plasma can induce apoptosis. A better understanding of the molecular mechanisms of non-thermal, plasma-mediated apoptosis will facilitate the introduction of this modality as an additional resource in anti-cancer treatment.

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