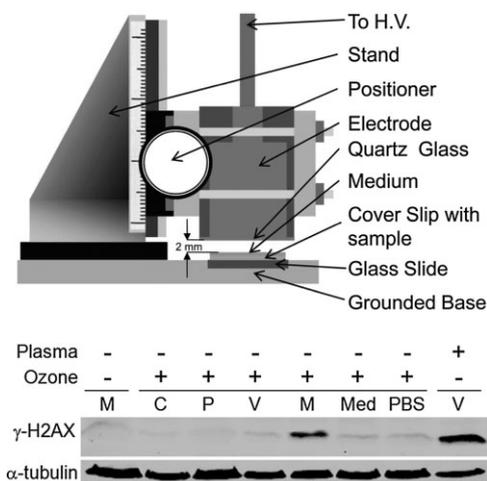


Damage in Mammalian Cells by Non-thermal Atmospheric Pressure Microsecond Pulsed Dielectric Barrier Discharge Plasma is not Mediated by Ozone

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Non-thermal dielectric barrier discharge (DBD) plasma is now being widely developed for various clinical applications^[1,2] but the mechanisms of interaction of such plasmas with mammalian cells are still not well understood. It is known that DBD plasma produces ozone and it is possible that the effects of plasma may be mediated purely by ozone. Although typically requiring much higher treatment time than the typical plasma treatment times reported here, ozonation is, in fact, widely employed to kill microorganisms in water and is also used for wound healing. It has been shown earlier that plasma treatment of mammalian cells submerged in a shallow layer of culture medium can result in dose dependent DNA damage. We wanted to understand the role of ozone in mediating the interaction of non-thermal plasma DBD plasma with mammalian cells. Upon comparing plasma treatment of mammalian breast epithelial cells with ozone treatment we found that ozone treatment is qualitatively different from non-thermal DBD plasma and in fact does not play a role in mediating the observed effects of plasma on mammalian cells.



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1. Introduction

Non-thermal atmospheric pressure plasma is now being widely developed for various applications like surface sterilization,^[3–9] cell proliferation,^[10] modulation of cell attachment,^[11–13] blood coagulation,^[14,15] apoptosis,^[16,17] enhancement of cell transfection,^[18] etc. Previously, we have shown using mammalian cells in culture that non-thermal dielectric barrier discharge (DBD) plasma has dose-dependent effects from increasing cell proliferation to inducing apoptosis. It has been shown that non-thermal plasma treatment of mammalian cells submerged in a shallow layer of culture medium results in dose dependent DNA damage. We have specifically examined the induction of DNA damage by DBD plasma and show that DNA damage is induced by neutral active species.^[19] The goal of this paper was to determine if ozone, a neutral particle, produced in the gas phase of plasma could be responsible for the observed effects of plasma on mammalian cells.

Several different methods of non-thermal plasma generation at atmospheric pressure are known. The type of non-thermal plasma discharge employed in this study is called DBD, which was invented by Siemens in 1859.^[20] DBD occurs at atmospheric pressure in air or other gases when high voltage of sinusoidal waveform or short duration pulses are applied between two electrodes, with at least one electrode being insulated.^[21,22] The insulator prevents current build-up between the electrodes, creating electrically safe plasma without substantial gas heating. This approach allows direct treatment of biological specimens without the thermal damage observed in more conventional thermal plasma.^[9,23] The human body, with its high capacity for charge storage as a result of its high water content and a relatively high dielectric constant can function as the second electrode.^[24,25]

Non-thermal DBD plasma is known to generate significant amounts of ozone^[22,26,27] and it is possible that ozone may play a major role in mediating the interactions of non-thermal plasma with mammalian cells. Ozone is widely used for sterilization of water.^[28] Ozone therapy is also being studied and developed for wound healing, healing of diabetic ulcers and is known to have various medical and physiological effects in mammalian tissue.^[29–32] The goal of this paper was to test the hypothesis that ozone mediates the interaction of non-thermal atmospheric pressure DBD plasma with mammalian cells.

2. Experimental Section

2.1. Cell Lines and Reagents

Mammalian breast epithelial cells (MCF10A) 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 $\mu\text{g} \cdot \text{ml}^{-1}$, Sigma–Aldrich, St. Louis, MO, USA), hydrocortisone (1 $\text{mg} \cdot \text{ml}^{-1}$, Sigma–Aldrich, St. Louis, MO, USA), cholera toxin (1 $\text{mg} \cdot \text{ml}^{-1}$, Sigma–Aldrich, St. Louis, MO, USA), insulin (10 $\text{mg} \cdot \text{ml}^{-1}$, Sigma–Aldrich, St. Louis, MO, USA), and penicillin/streptomycin (500 μl , 10 000 U $\cdot \text{ml}^{-1}$ penicillin and 10 $\text{mg} \cdot \text{ml}^{-1}$ streptomycin, Sigma Aldrich, St. Louis, MO, USA). 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 $\cdot \text{well}^{-1}$) on 22 mm \times 22 mm square glass cover slips (VWR, PA, USA) in 6-well plates (Greiner Bio One, NC, USA). Cells were cultured for 24 h prior to plasma treatment in 2.0 ml supplemented media in a 37 °C, 5% CO₂ incubator to allow full attachment and spreading. Amino acids; serine, methionine, cysteine, arginine, leucine, lysine, isoleucine, valine, proline, glutamic acid, and glutamine (100 μM , Sigma–Aldrich, St. Louis, MO, USA) were used to treat cells directly and separately to determine their role in inducing DNA damage after plasma treatment. *N*-acetyl *L*-cysteine (NAC, 4 mM, Sigma, St. Louis, MO, USA) was used as an intracellular reactive oxygen species (ROS) scavenger. Cells are incubated with 4 mM NAC in growth medium for 2 h at 37 °C. After incubation the cells are washed to remove excess extracellular NAC and then subjected to plasma treatment.

2.2. Non-thermal Plasma Treatment

Non-thermal atmospheric pressure DBD plasma was produced using an experimental setup schematically illustrated in Figure 1 and described by Fridman et al.^[15] 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 $\cdot \text{ns}^{-1}$ between the insulated high voltage electrode and the sample undergoing treatment using a variable voltage and variable frequency power supply (Quinta, Moscow,

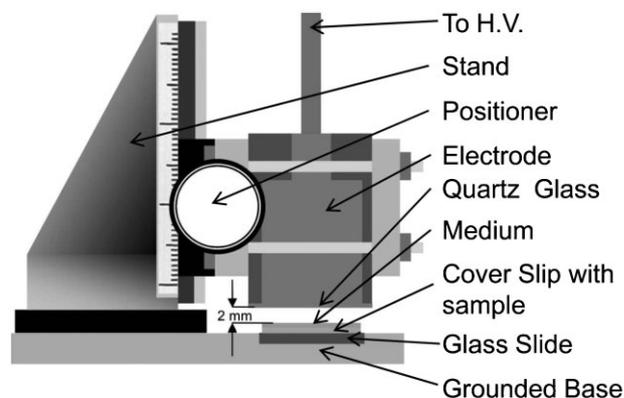


Figure 1. Schematic of setup used to treat mammalian cells in vitro. MCF10A cells plated on a glass cover slip with 100 μl of media overlaying cells were placed on a microscope slide on a grounded base; non-thermal plasma is generated in the gap between the high voltage electrode and the surface of medium covering the cells.

Table 1. Typical concentrations of ROS in gas phase produced by DBD plasma.^[9,34,36]

Plasma generated species	Density [cm^{-3}]
Superoxide ($\text{O}_2^{\bullet-}$)	10^{10} – 10^{12}
Hydroxyl (OH^{\bullet})	10^{15} – 10^{17}
Hydrogen peroxide (H_2O_2)	10^{14} – 10^{16}
Singlet oxygen ($^1\text{O}_2$)	10^{14} – 10^{16}
Ozone (O_3)	10^{15} – 10^{17}
Nitric oxide (NO)	10^{13} – 10^{14}
Electrons (e^-)	10^9 – 10^{11}
Positive ions (M^+)	10^{10} – 10^{12}

Russia). 1 mm thick, polished clear fused quartz was used as an insulating dielectric barrier covering the 1-inch diameter copper electrode encased in ulem. 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 $0.13 \text{ W} \cdot \text{cm}^{-2}$ (at 500 Hz) and $0.31 \text{ W} \cdot \text{cm}^{-2}$ (at 1.5 kHz) using both electrical characterization and a specially designed calorimetric system.^[33] Characterization, including waveforms, details of the electrode and inner circuitry of the non-thermal plasma and power system used in this paper has been described elsewhere.^[15]

MCF10A cells on glass cover slips were exposed to non-thermal plasma at various doses from $0.25 \text{ J} \cdot \text{cm}^{-2}$ (2 s exposure) to $1.95 \text{ J} \cdot \text{cm}^{-2}$ (15 s exposure). The plasma treatment dose in $\text{J} \cdot \text{cm}^{-2}$ was calculated by multiplying the plasma discharge power density by the plasma treatment duration. For example, plasma treatment at a power density of $0.13 \text{ W} \cdot \text{cm}^{-2}$ for 15 s would correspond to a dose of $1.95 \text{ J} \cdot \text{cm}^{-2}$. Non-thermal DBD plasma produces various ROS in gas phase whose typical concentrations are provided in Table 1.^[34–36] 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 μl 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 1 min 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 h before analyzing the samples using immunofluorescence or Western blot.

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. 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.

2.3. Ozone Treatment

Ozone was generated using a proprietary microsecond pulsed DBD plasma ozonator (Quinta, Russia). 100 ml of amino acids methionine, cysteine, proline, and valine dissolved in PBS, PBS alone, and growth medium were exposed to ozone flow on a glass coverslip for 15 s and then immediately applied to cells. We ensured that the entire surface area of the solution being treated was exposed to ozone flow evenly (by flowing the ozone through a tube with the same diameter as the plasma electrode and placing it exactly the same distance away as the plasma electrode) as is the case in the plasma treatment where the entire surface is treated uniformly. Concentration of ozone produced by DBD plasma in the gas phase and by the plasma ozonator was measured using an optical ozone meter MedOzon-245/5 (MedOzone, Russia). The concentration of ozone measured from DBD plasma was 182 ppm at 500 Hz and 30 ppm at 1.5 kHz which is exactly what the plasma ozonator was tuned to produce by controlling the power input and frequency enabling us to compare plasma treatment to ozone treatment.

2.4. Immunoblotting

Protein expression and modification were analyzed by immunoblot. Total cell lysates were prepared by direct lysis of washed cells in $2 \times$ sodium dodecyl sulfate (SDS) sample buffer containing β -mercaptoethanol. Samples were electrophoresed at 150 V in Tris-glycine SDS running buffer [25 mmol $\cdot \text{l}^{-1}$ Tris, 192 mmol $\cdot \text{l}^{-1}$ glycine, 0.1% SDS (pH 8.3)]. Following electrophoresis, proteins were transferred on to PVDF (Millipore, MA, USA) membrane for 2 h 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 γ -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 10–12 h at 4 °C with rocking. Primary antibodies used for immunoblot included mouse monoclonal antibodies specific for α -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). This Western blotting technique was described by Neal^[37] and Towbin et al.^[38]

3. Results and Discussion

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 damage.^[39] 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 2). These data are consistent with a dose-dependent increase in DNA damage after non-thermal plasma treatment of mammalian cells.

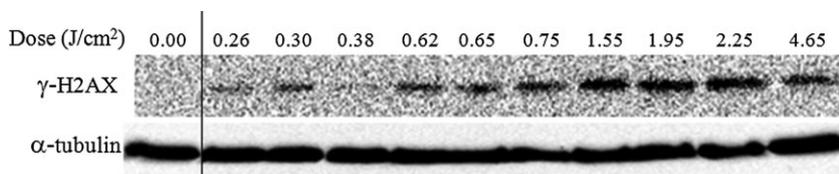


Figure 2. Representative blot with antibodies towards γ -H2AX (top panel) and α -tubulin (bottom panel, loading control) showing non-thermal DBD plasma induces dose-dependent DNA damage in mammalian cells. Alpha-tubulin is used as a loading control (to make sure same amount of protein is added to each lane to have a valid comparison) as it is not modified by plasma treatment.

From here on we use DNA damage as a marker for the effects of plasma treatment.

We next sought to directly test whether the damage induced by DBD plasma is due to ROS (e.g., H_2O_2 , OH^\bullet , singlet oxygen, atomic oxygen, superoxide radical, etc.) generated in the media and/or cells by plasma treatment.^[19,36] Cells were pre-treated with the ROS scavenger, *N*-acetyl cysteine for 2 h and then subjected to plasma treatment, which was found to block induction of γ -H2AX even at high doses of DBD (Figure 3), suggesting that the effects are mediated by ROS.

Ozone being a neutral species, we first sought to establish whether charged species or neutral species produced by plasma in the gas phase are responsible for the observed DNA damage. To determine whether the effects of plasma were due to charged or neutral species, a grounded mesh was used to exclude charged particles (ions and electrons). Insertion of a grounded mesh between the plasma source and the media interface (referred to as indirect treatment) did not significantly affect H2AX phosphorylation (Figure 4), indicating that ions and electrons do not play a significant role and that neutral species including ozone produced in the gas phase maybe responsible for the observed effects on mammalian cells.

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 5, damage induced by the treatment of the medium separately from cells was comparable to that produced by

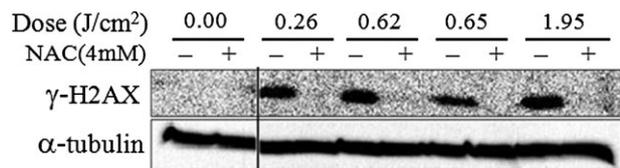


Figure 3. MCF10A cells were incubated for 2 h with 4 mM *N*-acetyl cysteine (NAC) (+) or cell culture medium (-), followed by treatment with the indicated dose of DBD plasma. γ -H2AX (upper panel) or α -tubulin (lower panel) was detected by immunoblot of cell lysates prepared 1 h after plasma treatment.

direct treatment. This suggests that long-lived ROS generated in the medium due to 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. In order 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 1 h prior to adding it to cells,^[19] suggesting that neutral species 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.^[40]

To determine whether the effects of DBD plasma are mediated by long living organic peroxides produced in medium, we compared the effect of separated treatment of medium vs. PBS, which is comprised of inorganic salts. We observed no DNA damage in cells exposed to separately treated PBS (Figure 6), whereas separately treated medium induced DNA damage as shown in previous experiments. Taken together, these data suggest that organic peroxides

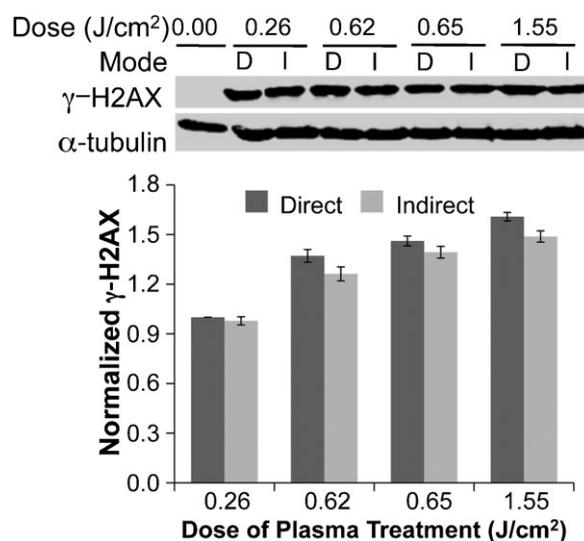


Figure 4. Cells were subjected to plasma as described earlier (direct, D) or a grounded mesh that filters charged particles was placed between the electrode and the medium (indirect, I). Representative immunoblots with γ -H2AX (upper panel) or α -tubulin (lower panel) are shown. The graphs below the immunoblots show quantification from three independent experiments using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). The γ -H2AX signal was normalized to the amount of α -tubulin and data are expressed relative to lowest dose that was set at 1.0.

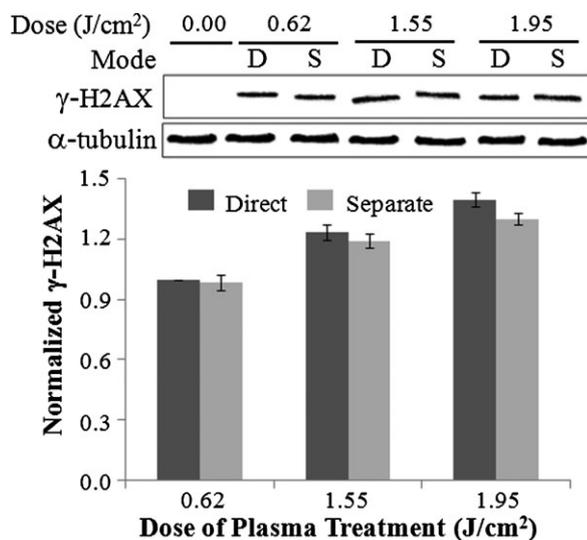


Figure 5. Cells were subjected to DBD plasma as described earlier (direct, D) or media (100 μ l) was exposed to DBD plasma separately and then transferred to the cells (separate, S). Representative immunoblot with γ -H2AX (upper panel) or α -tubulin (lower panel) is shown. The graph below the immunoblot shows quantification using Odyssey of triplicate samples in three separate experiments. The γ -H2AX signal was normalized to the amount of α -tubulin. Data (\pm S.D.) are expressed relative to lowest dose of direct treatment that was set at 1.0.

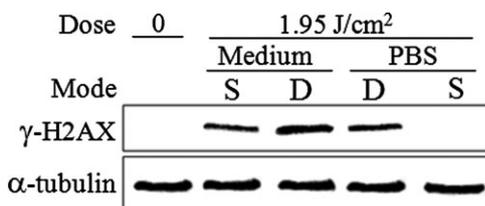


Figure 6. Cells overlaid with 100 μ l of PBS were treated directly with DBD plasma (direct, D) or were treated with PBS that was separately exposed to DBD plasma prior to addition to cells (S). Separately treated PBS does not induce DNA damage in mammalian cells.

formed as a result of ROS produced by neutral active species induce DNA damage. It should be noted that cells in PBS directly treated with plasma exhibit DNA damage, in contrast to cells exposed to separately treated PBS which do not. We presume that the damage in cells after treatment in PBS is the result of short-lived species interacting directly with oxidizable organic substrates (including DNA) in cells. That the separated treatment of PBS does not induce damage suggests that in absence of organic substrates, the ROS that are generated are short-lived probably due to their recombination and consequently are no longer active when added to cells after the separated treatment. The only possible explanation for the observed effects of direct plasma treatment in PBS is that ROS coming directly from

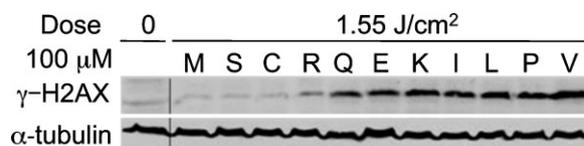


Figure 7. Amount of DNA damage induced by non-thermal plasma is proportional to the peroxidation efficiency of the amino acid. Solutions containing the indicated amino acid (100 μ M) were separately treated with DBD plasma and then added to MCF10A cells. After 1-min incubation, cells on cover slips were diluted in 2 ml media, followed by lysis and Western blot for γ -H2AX or α -tubulin. Refer to Table 1 for the abbreviations and explanations of amino acids.

plasma interact with organic substrates in cells producing stable ROS that include long-lived organic peroxides.

Non-thermal plasma produces a large ROS concentration in the extracellular medium^[19] during treatment as the active species produced in the gas phase of plasma^[36] during treatment enter the medium covering the cells. However, it is unclear how these ROS go inside cells. Pre-treatment for 2 h with *N*-acetyl cysteine, an intracellular ROS scavenger completely blocked phosphorylation of H2AX after non-thermal plasma treatment of MCF10A cells, which indicates that, ROS produced by plasma extracellularly may move across the cell membrane through lipid peroxidation, opening transient cell membrane pores, or signaling pathways which modify the concentration of ROS inside cells. Active species produced by plasma including ozone may also modify the cell medium, which in turn interacts with cells.

Since many of active species including ozone have a short life span, they may immediately interact with medium components including amino acids and proteins, leading to production of long lived reactive organic hydroperoxides.^[40] Cell culture medium used in the experiments described above is composed of amino acids, glucose, vitamins, growth factors and inorganic salts, as well as serum. Gebicki and Gebicki have shown that γ -radiation (IR) induces formation of amino acid and protein hydroperoxides in aqueous solutions containing BSA or amino acids.^[40]

To determine whether active species produced by non-thermal plasma in medium do indeed lead to production of long lived organic hydroperoxides that in turn lead to the observed DNA damage we subjected solutions of single amino acids to separated plasma treatment and then applied them to cells to measure DNA damage. As shown in Figure 7, DNA damage was proportional to the peroxidation efficiency (see Table 2) of the amino acid in the solution with valine producing the most significant level of damage. Moreover, exposure to plasma-treated solutions of PBS containing amino acids that are not as efficiently peroxidized, including glutamine and arginine, induced signifi-

Table 2. Peroxidation efficiency of various amino acid components of cell culture medium when treated with ionizing radiation.^[40]

Amino acid	Peroxidation efficiency [%]
Methionine (M)	0
Serine (S)	0
Cysteine (C)	0.4
Arginine (R)	13
Glutamine (Q)	16
Glutamic acid (E)	28
Lysine (K)	34
Isoleucine (I)	43
Leucine (L)	44
Proline (P)	44
Valine (V)	49

cantly less DNA damage, whereas DBD plasma treatment of solutions containing cysteine, serine, and methionine, which are known to have very low *g*-factors (not peroxidized), produced no detectable DNA damage.

Since interaction of plasma produced neutral active species with amino acids leads to DNA damage it is possible that ozone may play a role in mediating the interaction of non-thermal plasma with mammalian cells by modifying amino acids in cell culture medium, producing long living organic hydroperoxides. Pryor et. al. have shown that ozone is capable of reacting with various amino acids including methionine and cysteine with high rates of reaction at physiological values of pH.^[41] Although typically requiring much higher treatment time than the typical times reported here, ozonation is, in fact, widely employed to kill micro-organisms in water^[28] and ozone therapy is now being widely studied as a novel treatment for various pathologies.^[29,31,42,43] Since plasma produces significant amounts of ozone,^[27,44] it is possible that the DNA damaging effects of plasma may be mediated by ozone to some extent.

To test the possibility that ozone plays a major role in mediating the DNA damage induced by plasma, we exposed cells to 100 μ L of PBS containing cysteine, methionine, proline and valine dissolved in PBS, serum-containing medium or PBS alone that were separately treated with ozone. PBS containing amino acids cysteine, valine or proline, cell medium or PBS, separately treated with ozone resulted in no detectable DNA damage in MCF10A cells as measured by phosphorylation of H2AX (Figure 8), while we observed DNA damage in cells exposed to methionine separately treated by ozone. Plasma treatment induces DNA damage via amino acids that are easily

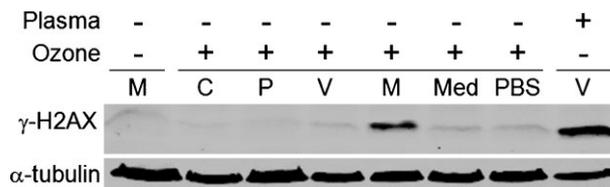


Figure 8. Ozone treatment is qualitatively different from Plasma treatment. Solutions containing the indicated amino acid (100 μ M) or medium or PBS were ozonated for 15 s and then added to MCF10A cells. After 1-min incubation, cells on cover slips were diluted in 2 ml media, followed by lysis and Western blot for γ -H2AX or α -tubulin. Cells exposed to separately plasma treated valine (V) were used as control. Refer to Table 1 for the abbreviations and explanations of amino acids.

peroxidizable, while ozonation induces DNA damage via amino acids that are not peroxidizable. Thus, ozone treatment is qualitatively different from non-thermal DBD plasma treatment and ozone does not play a major role in these studies since it affects mostly methionine and cysteine, while not affecting valine and leucine,^[41] which are most strongly affected by DBD treatment. Long lived neutral organic peroxides produced by non-thermal plasma treatment of highly peroxidizable amino acids in extracellular medium mediate the interaction with mammalian cells which is not observed by ozonation of the same amino acids indicating that ozone produced by DBD plasma does not play a major role in mediating the effects of plasma with mammalian cells.

4. Conclusion

Non-thermal plasma has been shown to induce DNA damage in mammalian cells. Long living neutral organic hydroperoxides mediate the DNA damaging effects of non-thermal plasma in mammalian cells. It was hypothesized that non-thermal plasma induces DNA damage via ozone. The results presented in this paper show that ozone treatment is qualitatively different from plasma treatment and it does not play a major role in these studies. In this study we show that ozone produced by non-thermal atmospheric pressure DBD plasma is not responsible for the observed DNA damage. Further investigation are necessary to determine which of the neutral species play a major role in the interactions of non-thermal plasma with mammalian cells.

5. List of Abbreviations

DBD	Dielectric barrier discharge
ROS	Reactive oxygen species
PBS	Phosphate buffered saline

NAC	N-acetyl L-cysteine
PVDF	Polyvinylidifluoride
M	Methionine
S	Serine
C	Cysteine
R	Arginine
Q	Glutamine
E	Glutamic acid
K	Lysine
I	Isoleucine
L	Leucine
P	Proline
V	Valine

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