DNA Damage in Mammalian Cells by Atmospheric Pressure Microsecond-Pulsed Dielectric Barrier Discharge Plasma Is Not Mediated Via Lipid Peroxidation

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ABSTRACT: Lipid peroxidation in mammalian cells by nonthermal atmospheric pressure microsecond-pulsed dielectric barrier discharge plasma was investigated. It has been shown previously that plasma treatment of mammalian cells submerged in a shallow layer of culture medium can result in dose-dependent DNA damage. We show that nonthermal plasma induces lipid peroxidation, measured by release of malondialdehyde, in cells treated under medium and not under phosphate-buffered saline. N-Diphenyl-phenylenediamine, a lipophilic antioxidant, was used to block nonthermal plasma–induced lipid peroxidation. Although nonthermal plasma induces lipid peroxidation in mammalian cells, this peroxidation does not lead to DNA damage in mammalian cells. Thus, one needs to consider other possible mechanisms for plasma-induced DNA damage in mammalian cells such as reactive oxygen species signaling or active transport of long-lived organic hydroperoxides.

KEY WORDS: reactive oxygen species, nonthermal plasma, plasma medicine, dielectric barrier discharge, malondialdehyde, organic hydroperoxides

I. INTRODUCTION

Nonthermal atmospheric pressure plasma now is being widely developed for various applications such as surface sterilization,[1–6] cell proliferation,[7] modulation of cell attachment,[8–10] blood coagulation,[11,12] apoptosis,[13,14] and enhancement of cell transfection.[15] Using mammalian cells in culture, we previously have shown that nonthermal dielectric barrier discharge (DBD) plasma has dose-dependent effects from increasing cell proliferation to inducing apoptosis and that these effects primarily are due to the formation of reactive oxygen species (ROS).[16] It is known that nonthermal plasma treatment of mammalian cells submerged in a shallow layer of culture medium can result in dose-dependent DNA damage. We specifically have examined the induction of DNA damage by DBD plasma and have shown that DNA damage is induced by organic peroxides formed as a result of ROS produced by neutral active species, which are generated by DBD plasma in cell culture medium.[16] In this sense, plasma can cre-

ate effects similar to ionizing radiation (IR). However, whereas IR penetrates through cell membranes, creating ROS in the immediate vicinity of DNA, plasma treatment acts on cells through the cell medium.[16] The question arises, By what mechanisms do nonthermal plasma effects reach across cell membrane? There are several possibilities. One involves peroxidation of lipid membrane with malondialdehyde (MDA) as a by-product. The by-products of lipid peroxidation (MDA) have been known to create bulky adducts on DNA, which are a form of damage requiring repair. Other mechanisms may involve ROS signaling and transport across the membrane. The goal of this article is to test the hypothesis that nonthermal plasma–induced DNA damage in mammalian cells is created through lipid peroxidation.

II. MATERIALS AND METHODS

A. Nonthermal Plasma Treatment

Nonthermal DBD plasma was generated and applied to cells as described elsewhere.[7,12] Plasma was generated by applying alternating polarity pulsed (500 Hz to 1.5 kHz) voltage of ~20 kV magnitude (peak-to-peak) between the insulated high-voltage electrode and the sample using a variable voltage and frequency power supply (Quinta is located in Russia). One-mm-thick clear quartz was the insulating dielectric barrier covering the 1-inch-diameter copper electrode. The discharge gap between the quartz and the sample was fixed at 2 mm. Discharge power density was 0.13 Watts/cm^2 (at 500 Hz) and 0.31 Watts/cm^2 (at 1.5 kHz) using electrical characterization and a custom calorimetric system.[17] The dose of plasma treatment was calculated as follows: Power density of the plasma at the lowest frequency (500 Hz) of operation during plasma treatment is 0.13 W/cm^2.[17] The dose in J/cm^2 is, then, dose of treatment = power density × duration of treatment. For example, for an exposure of 15 s, the dose of treatment = 0.13 W/cm^2 × 15 s = 1.95 J/cm^2.

   Mammalian breast epithelial (MCF10A) cells on glass cover slips were exposed to nonthermal plasma at various doses, from 0.13 to 7.8 J/cm^2. 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. Then, 100 μL of supplemented media was added to the glass cover slip before plasma treatment to prevent sample drying. After 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 hour before analyzing the samples using immunofluorescence or Western blot.

   Two different approaches were used for nonthermal plasma treatment of cells in vitro: direct 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 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. Cell Culture

MCF10A cells 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), 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). Media was changed every 2 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- × 22-mm square glass cover slips (VWR International, Radnor, PA, USA) in 6-well plates (Greiner Bio One, Monroe, NC, USA). Cells were cultured for 24 hours prior to plasma treatment in 2.0 mL supplemented media in a 37°C, 5% carbon dioxide incubator to allow full attachment and spreading.

Bromotrichloromethane (BrCCl$_3$; Sigma-Aldrich) was used as a known inducer of lipid peroxidation. N-acetyl-cysteine (Sigma-Aldrich) was used as an intracellular scavenger of ROS and diphenyl-phenylenediamine (DPPD; Sigma-Aldrich), a lipophilic synthetic alternative to vitamin E, was used as a lipid peroxidation inhibitor.

C. Lipid Peroxidation Assay

MDA-thiobarbituric acid (TBA) levels were used as a measure of lipid peroxidation after nonthermal plasma treatment of mammalian cells. Cells were treated either directly or separately for 15 s at a dose of 1.95 J/cm^2. Cells were held after plasma treatment for either 1 min or 10 min before adding butylated hydroxtoluene (BHT). The cells were scrapped with a rubber policeman and homogenized at 4°C. Whole lysates were used to measure the level of MDA following the manufacturer's protocol (OxiSelect Thiobarbituric Acid Reactive Substance [TBARS] Assay kit, Cell BioLabs, San Diego, CA, USA). The TBA test was carried out under acidic conditions (pH 3.5) with a colorimetric 96-well microplate assay, and the level of TBARS was expressed relative to the response of the assay to MDA using a plate reader at 532 nm.

D. Western Blot

Protein expression and modification were analyzed by immunoblot. Total cell lysates were prepared by direct lysis of washed cells in 2X sodium dodecyl sulfate (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 polyvinylidene

fluoride (PVDF; Millipore, Billerica, MA, USA) membrane for 2 hours in Tris-glycine transfer buffer (10% SDS, deionized water, Tris-glycine, and methanol [VWR International]). Immunoblotting was done by blocking membranes in 1% nonfat dried milk (Carnation, Nestlé, Wilkes-Barre, PA, USA) in PBS with 0.1% Tween 20 (PBST) for α-tubulin or 5% bovine serum albumin (BSA; Fraction V, Fisher Scientific, PA, USA) 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 to 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; Millipore) and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The primary antibodies were detected with fluorescently tagged goat antimouse Alexa and Fluor 488 (Santa Cruz Biotechnology). Immunoblot was developed using Odyssey infrared gel imaging system (LI-COR Biosciences, Lincoln, NE, USA).

III. 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.[18] 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 (Fig. 1). These data are consistent with a dose-dependent increase in DNA damage after nonthermal plasma treatment of mammalian cells.

We next sought to test directly whether the damage induced by DBD plasma is due to ROS (e.g., H_2O_2, OH•, singlet oxygen, atomic oxygen, superoxide radical) generated in the media and/or cells by plasma treatment. Cells were pretreated with the ROS scavenger N-acetyl cysteine, which was found to block induction of γ-H2AX even at high doses of DBD (Fig. 2), suggesting that the effects are mediated by ROS.

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 Fig. 3, damage induced by the treatment of the medium separately from cells was not significantly less than that produced by direct treatment. This suggests that ROS generated in the medium by plasma treatment
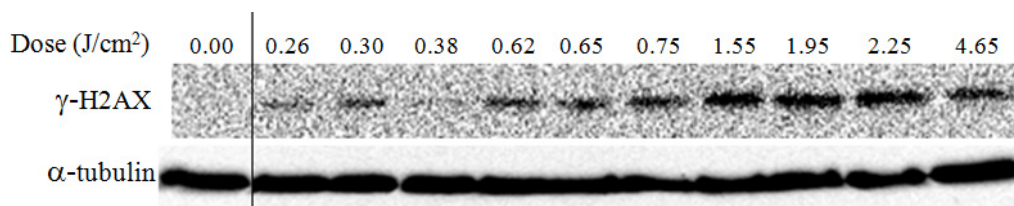

FIGURE 1. Representative blot with antibodies toward γ-H2AX *(top)* and α-tubulin (*bottom*, loading control) showing nonthermal dielectric barrier discharge plasma induces dose-dependent DNA damage in mammalian cells.
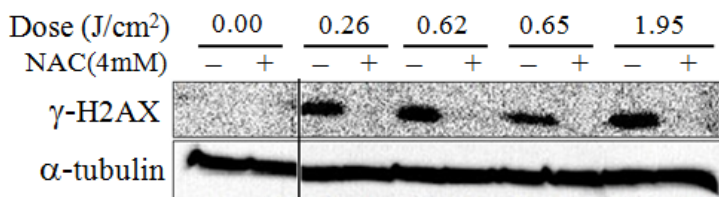
FIGURE 2. MCF10A cells were incubated for 2 hours with 4 mM N-acetyl cysteine (NAC) (+), followed by treatment with the indicated dose of DBD plasma. γ-H2AX (top) or α-tubulin (bottom, loading control) was detected by immunoblot of cell lysates prepared one hour after plasma treatment.
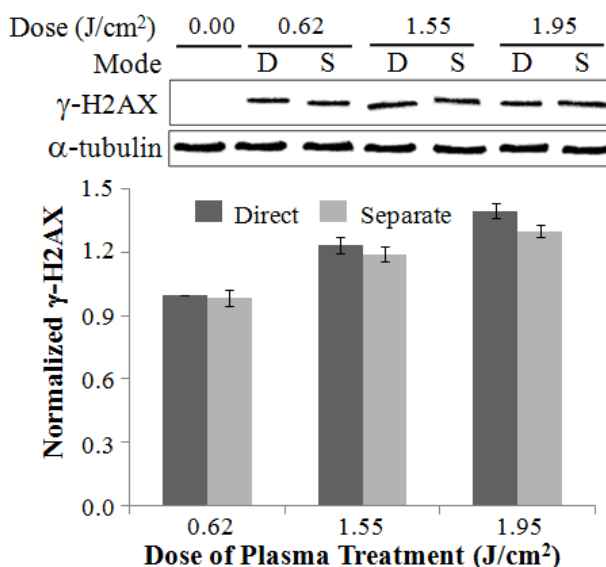

FIGURE 3. Cells were subjected to dielectric barrier discharge (DBD) plasma (direct [D]) or media (100 µL) was exposed to DBD plasma separately and then transferred to the cells (separate [S]). Representative immunoblot with γ-H2AX *(top)* or α-tubulin *(bottom)* is shown. The graph shows quantification, using the Odyssey infrared gel imaging system, of triplicate samples in three separate experiments. The γ-H2AX signal was normalized to the amount of α-tubulin. Data (±standard deviation) are expressed relative to lowest dose of direct treatment that was set at 1.0.

are responsible for the induction of DNA damage. These ROS species must survive long enough to remain active while being transferred to the cells.

To determine how long these ROS species live, the 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[16], suggesting that neutral spe-

cies may react with organic components in the cell medium to produce long-living organic peroxides that are known to have a half life on the order of 12 to 24 h[19].

Further, to test 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 versus PBS, which is comprised of inorganic salts. We observed no DNA damage in cells exposed to separately treated PBS (Fig. 4), whereas separately treated medium did induce DNA damage, as shown in previous experiments. Taken together, these data suggest that DNA damage is induced by organic peroxides formed as a result of ROS produced by neutral active species, which are generated by DBD plasma in cell culture medium.

Nonthermal plasma produces a large ROS concentration in the extracellular medium during treatment. However, it is unclear how these ROS go inside cells. N-acetylcysteine, 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 that modify the concentration of ROS inside cells. Active species produced by plasma also may modify the cell medium, which in turn interacts with cells.

Because many active species 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.[19] These hydroperoxides may then induce lipid peroxidation, and the by-products of lipid peroxidation such as MDA[20] may lead to DNA damage or they may bind to cell membrane receptors and activate intracellular signaling pathways, leading to subsequent DNA damage.

It is well known that ROS such as OH radicals, superoxide radicals, atomic oxygen, singlet oxygen, organic protein, and amino hydroperoxides react with polyunsaturated fatty acid residues in membrane phospholipids to initiate lipid peroxidation, which results in the production of a plethora of by-products, many of them reactive toward protein and DNA.[21] One of the most abundant carbonyl products of lipid peroxidation induced by endogenous and exogenous ROS-inducing chemicals is MDA.[22] It reacts with DNA to form adducts to deoxyguanosine and deoxyadenosine. The major adduct to DNA is a py-
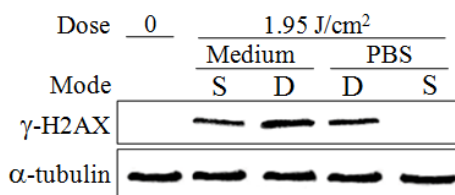

FIGURE 4. Cells overlaid with 100 μL of phosphate-buffered saline (PBS) were treated directly with dielectric barrier discharge (DBD) plasma (D) or were treated with PBS that was separately exposed to DBD plasma prior to the addition to cells (S). Separately treated PBS does not induce DNA damage in mammalian cells.

rimidopurinone called M1G. Site-specific mutagenesis experiments indicate that M1G is mutagenic and is repaired by the nucleotide excision repair pathway.[20,21,23,24]

As described earlier, DNA damage induced by nonthermal plasma was mediated by ROS, which are known to induce lipid peroxidation in mammalian cells. Plasma produces ROS in the medium covering the cells during treatment. These ROS are likely to encounter the cell membrane before DNA, and it is possible that they induce lipid peroxidation either directly or by modifying organic content in the medium. To determine whether DBD plasma treatment induced lipid peroxidation in cells, we looked at the formation of MDA, which is commonly used as an index for measuring lipid peroxidation.[5]

As shown in Fig. 5, we see that plasma indeed induces lipid peroxidation in cells immediately after treatment, when treated under medium either directly or separately. In contrast, nonthermal plasma does not induce lipid peroxidation in mammalian cells treated under PBS, either directly or separately. This suggests that long-living organic hydroperoxides—produced as a result of the interaction of neutral active species produced by nonthermal plasma in medium with organic components of the medium such as proteins and amino—may induce lipid peroxidation. These data are consistent with the fact that DNA damage is induced by organic peroxides formed as a result of ROS produced by neutral active species, which are generated by DBD plasma in cell culture medium.[16]

We next sought to test whether plasma-induced lipid peroxidation led to the observed DNA damage. To block lipid peroxidation in MCF10A cells after plasma treatment, we used DPPD, a synthetic lipophilic antioxidant, and BrCCl$_3$ a known inducer of lipid peroxidation as a pro-oxidant. DPPD is frequently used in cell culture and in in-vivo studies to inhibit lipid peroxidation by various chemical agents.[26–29] MCF10A cells were prein-
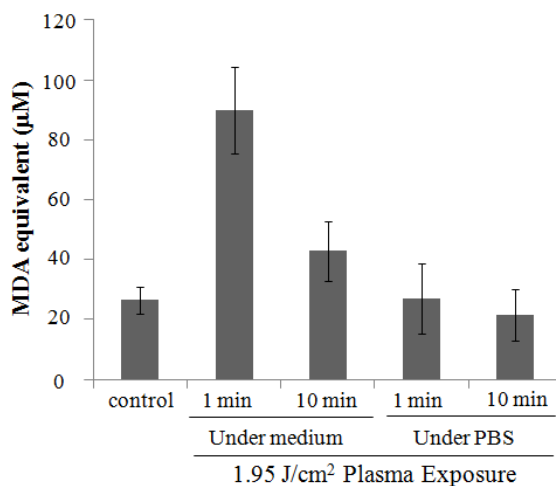

FIGURE 5. Nonthermal plasma treatment of MCF10A cells leads to release of malondialdehyde (MDA), a commonly used marker for measuring lipid peroxidation in mammalian cells. Plot shows the MDA equivalent for untreated cells and cells treated at the indicated dose. Data from triplicate samples (±standard deviation) are plotted.

cubated with DPPD for 15 min at 37°C before plasma treatment or the addition of 1 mM BrCCl$_3$. As shown in Fig. 6, DPPD significantly inhibits lipid peroxidation in MCF10A cells, both after plasma treatment and after incubation of cells with BrCCl$_3$ for 2 hours. Thus, DPPD is a potent inhibitor of nonthermal plasma–induced lipid peroxidation.

With DPPD proving to be a potent blocker of lipid peroxidation, we next sought to investigate the role of nonthermal plasma–induced lipid peroxidation in the observed DNA damage. As stated earlier, MCF10A cells were preincubated with 10 micro molar DPPD for 15 min before exposing the cells to non-thermal plasma at a dose of 1.95 J/cm^2 (15 s at low frequency) or 1 mM BrCCl$_3$ for 2 h, after which plasma treated cells were incubated for 1h at 37°C prior to lysing for analyzing DNA damage. DNA damage was analyzed using the Western blot technique for measuring γ-H2AX. As shown in Fig. 7, DNA damage after plasma treatment of MCF10A cells with or without preincubation of DPPD was the same, whereas DNA damage induced by BrCCl$_3$ was significantly reduced by DPPD. Nonthermal plasma–induced lipid peroxidation does not lead to nonthermal plasma–induced DNA damage. Nonthermal plasma produces significant
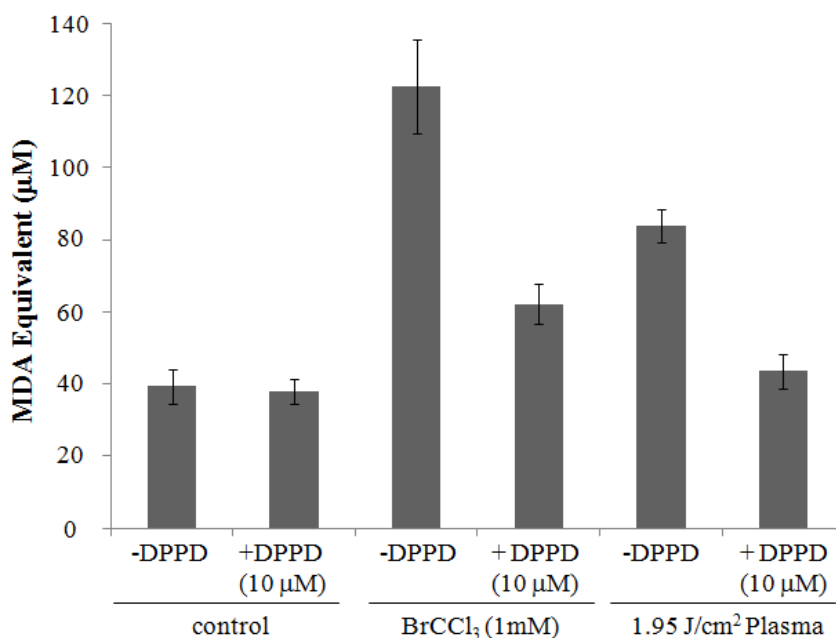

FIGURE 6. MCF10A cells were incubated for 1 h with 10 μM N-diphenyl-phenylenediamine (DPPD) (+DPPD) or cell culture medium (-DPPD), followed by treatment at the indicated dose of dielectric barrier discharge plasma or with 1 mM bromotrichloromethane (BrCCl$_3$). Lipid peroxidation was measured via release of malondialdehyde (MDA). The graph shows the MDA equivalent for untreated cells (control) and cells treated at the indicated plasma dose or with 1 mM BrCCl$_3$. Data from triplicate samples (±standard deviation) are plotted.
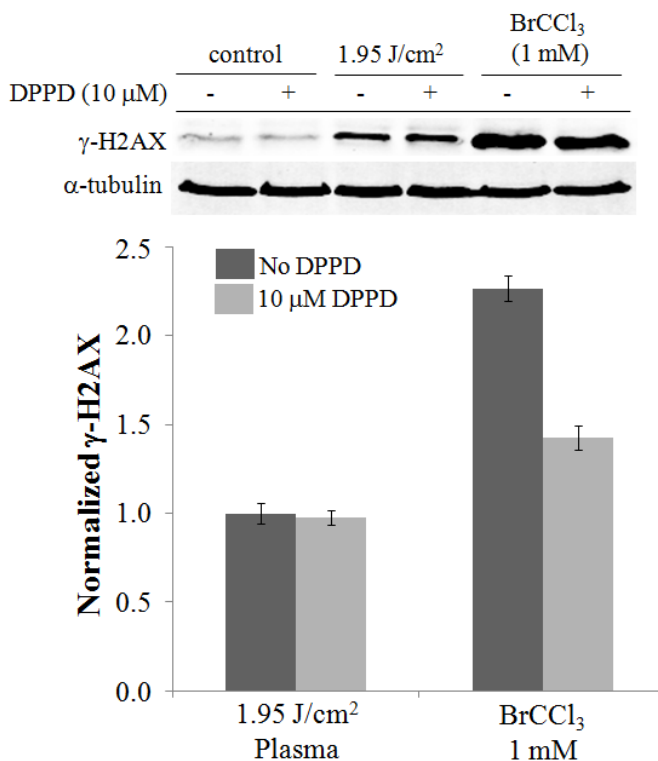
FIGURE 7. MCF10A cells were incubated for 1 h with 10 μM N-diphenyl-phenylenediamine (DPPD) or cell culture medium (No DPPD), followed by treatment at the indicated dose of dielectric barrier discharge plasma or with 1 mM bromotrichloromethane (BrCCl$_3$). Representative immunoblot with γ-H2AX *(top)* or α-tubulin *(bottom)* is shown. The graph shows quantification, using the Odyssey infrared gel imaging system, of triplicate samples in three separate experiments. The γ-H2AX signal was normalized to the amount of α-tubulin. Data (±standard deviation) are expressed relative to plasma treatment in the absence of DPPD, which was set to 1.0.

concentration of ROS in the medium covering the cells during treatment, which leads to lipid peroxidation and DNA damage simultaneously, but DNA damage is not induced as a result of lipid peroxidation.

IV. CONCLUSIONS

It has been shown that ROS mediate nonthermal plasma–induced DNA damage in mammalian cells. They also interact with the cellular membrane, leading to lipid peroxidation. We investigated the role of ROS produced by nonthermal plasma in inducing lipid peroxidation in mammalian cells. It was hypothesized that nonthermal plasma induces DNA damage in mammalian cells via lipid peroxidation. The results presented here

indicate that nonthermal plasma does indeed lead to lipid peroxidation, but nonthermal plasma–induced lipid peroxidation is not what induces DNA damage. Further investigations are necessary to determine other pathways of nonthermal plasma–induced DNA damage in mammalian cells, which may include ROS-mediated cellular signaling or uptake of long-lived ROS via active transport.

ACKNOWLEDGMENTS

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