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Production of intracellular reactive oxygen species and change of cell viability induced by atmospheric pressure plasma in normal and cancer cells

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The effects of atmospheric pressure plasma jet on cancer cells (human lung carcinoma cells) and normal cells (embryonic kidney cells and bronchial epithelial cells) were investigated. Using a detection dye, the production of intracellular reactive oxygen species (ROS) was found to be increased in plasma-treated cells compared to non-treated and gas flow-treated cells. A significant overproduction of ROS and a reduction in cell viability were induced by plasma exposure on cancer cells. Normal cells were observed to be less affected by the plasma-mediated ROS, and cell viability was less changed. The selective effect on cancer and normal cells provides a promising prospect of cold plasma as a cancer therapy. © *2013 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4824986]

In recent years, non-thermal plasmas and their applications in biomedical treatments have developed due to their extensive capability. Many groups have successfully demonstrated plasma's ability by using various targets.[1–5] Generally, ultraviolet (UV) radiation, charged particles, and free radicals such as reactive oxygen species (ROS) that exist in and around plasma can cause various effects. One of the most important factors is the plasma-generated reactive species.[6,7] Overproduction of ROS results in oxidative stress, a harmful process that can be an important mediator of damage to cell structures, including lipids and membranes, proteins, and DNA.[8] Regarding plasma effects on living cells, different behavior under plasma treatment was found depending on cell type and plasma source power and composition.[9,10]

Several types of atmospheric plasma sources have been utilized for plasma-cell interactions. In the dielectric barrier discharges (DBDs), the current can flow to the treated surface and charged particles will generally impact the surface.[11] In the floating electrode DBD (FE-DBD), the living tissue surface act as an electrode at floating electrical potential. It was reported that FE-DBD plasma generated a large amount of ROS, leading to the formation of DNA damages and resulting in a multiphase cell cycle arrest and a subsequent apoptosis induction.[3] In addition, it was shown that non-thermal plasma created by DBD had dose-dependent effects that range from increasing cell proliferation to inducing apoptosis and those effects were primarily due to formation of ROS.[12] Indirect plasmas are produced between two electrodes and are then transported to the area of application entrained in a gas flow. They range from "plasma needles" to larger "plasma torches," including our plasma jet source.[13] In this category of plasma source, ROS are generally created at the boundary between the jet and the adjacent air through various mechanisms. These configurations tend to create electrically safe plasma compared with direct mode.[11] The atmospheric pressure plasma jets are among the most plausible candidates able to destroy cancer cells without damage of surrounding healthy cells.[2,13–15]

There have been reports that the cold plasma jet can selectively ablate some cancer cells while leaving their corresponding normal cells essentially unaffected, suggesting that plasma effect is related to generation of ROS with possible induction of the apoptosis pathway.[13] The ability to treat cancer cells is based on the synergy of several biologically active plasma components.[6] It is important to explore the interactions between the production of plasma-induced reactive species and cellular responses. Since plasma–mediated oxidative stress may bring about harmful or perhaps even beneficial cellular responses, one should examine carefully the plasma-dependent effects within target cells by the parallel comparison of cancer and normal cells from the same tissue having similar characteristics. In this work, various cancer and normal cells maintaining typical epithelial characteristics were treated by atmospheric pressure plasma jets and intracellular ROS generation were measured and quantified. And the cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. To further examine the involvement of plasma-induced ROS in cellular response, the inhibition assay was performed.

Figure 1(a) shows the photograph of the plasma plume and the schematic of the atmospheric pressure plasma jet device. The plasma jet consists of a wire electrode, a Teflon fitting, a glass confinement tube (8 mm inner diameter and 10 mm outer diameter), and a pencil-shaped nozzle (2 mm inner diameter at the exit). A tungsten pin wire (0.3 mm diameter) with a sharpened tip was placed on the tube axis. The glass tube placed between the wire electrode and stainless steel holder makes this device resemble a dielectric barrier discharge. A pencil-shaped nozzle was attached to the end of the glass tube. The distance between the end of the tip and the glass tube exit was approximately 10 mm. The applied voltage, excitation frequency, and gas flow rate were 0.7–1.1 kV_{rms}, 35 kHz, and 0.1 L/min, respectively. The helium gas controlled by a flow meter (RK1600R Kofloc) was fed to a glass tube, and the plasma plume was formed around the tip and expelled out of the nozzle. The sinusoidal voltage source of several tens of kilohertz (HPSI200 FTLab) is applied to the tungsten wire. The voltage and the current

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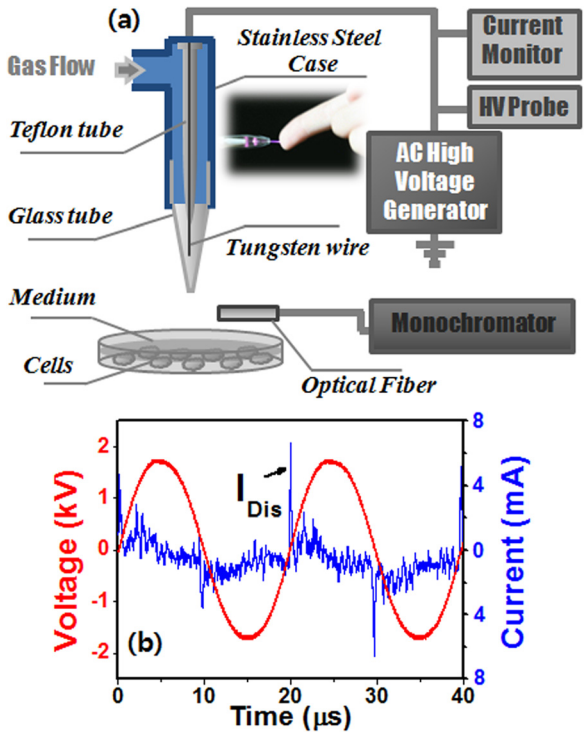

FIG. 1. (a) Schematic of the experimental setup and (b) the waveforms of the voltage and the discharge current.

characteristics were measured using a real time digital oscilloscope (WS44Xs-A LeCroy) via high voltage probe (P5100, Tektronix) and current probe (3972 Pearson). Figure 1(b) shows the waveforms of the voltage and the discharge current of the plasma jet. The discharge current was obtained by subtracting the displacement current from the total current. The discharges occur in the rising and falling periods of the voltage waveform. It is worth noting that this plasma jet operates in low-voltage and low-current mode, well suited for plasma-cell interactions.

To identify reactive species that are generated in the discharge, optical spectra were recorded for emission from the jet in the wavelength range from 200 to 900 nm. The light emitted by the plasma was focused by means of optical fiber into entrance slit of 0.75 m monochromator (1702, SPEX), equipped with a grating of 1200 grooves per millimeter and slit width of 100 μm. Figure 2 shows the emission spectrum observed in the helium plasma jet. The emission spectrum reveals the presence of excited helium, atomic oxygen, and some excited air molecules. In the discharge area, the electron impact dissociation of N_2 and O_2 molecules leads to the formation of atomic oxygen and the breaking of the strong bond in the N_2 molecule by vibrational excitation and dissociation. In the plasma jet, the presence of nitric oxide (NO) at 283 nm is due to the chemical conversion of N and O_2 (or N and O). The N_2^+ emission at the spectral region from 391 to 428 nm is strong since highly energetic helium metastables are able to produce molecular nitrogen ionization when the ionized helium gas interacts with ambient air. The hydroxyl (OH) radical at 309 nm appears as a result of the water molecules' dissociation caused mainly by collision with electrons or long-lived plasma species, in our case helium metastables. The atomic line of hydrogen (H_α) at 656 nm is due to the excitation of hydrogen atoms generated by dissociation of water molecules under the action of the energetic electrons in the plasma.[16]

The interaction of the plasma jet with cells was examined on human lung carcinoma cells (A549), embryonic kidney cells (HEK293T), and bronchial epithelial cells (BEAS-2B). The cells were maintained in RPMI 1640 (Rosewell Park Memorial Institute Medium; A549), BEGM (Bronchial Epithelial Cell Basal Medium and the growth supplements; BEAS-2B), and DMEM (Dulbecco's Modification of Eagle's Medium; HEK293T) supplemented with 10% fetal bovine serum and 100 U/ml penicillin. Cells were incubated at 37 °C with humidified air and 5% CO_2. Then cells were seeded in dishes (for 2′,7′-dichlorofluorescein diacetate (DCF-DA) assay) at a density of 1×10^5 cells and 24-well plates (15.62 mm in diameter, for MTS assay) at a density of 1×10^4 cells per well, incubated overnight, and exposed to the plasma plume (and/or gas flow only) for 10 s on 6–9 points per well and/or dish. Prior to plasma treatment, media from each chamber was almost removed, and a small amount of media (a few hundred microliters) was left to keep cells wet during treatment. The distance from the nozzle to the cell surface was 10 mm. The plasma plume reached the cell directly.

Intracellular ROS generation after plasma treatment was detected by fluorescence microscopy using DCF-DA assay and quantified by measuring pixel intensity with MetaMorph software (Molecular Devices, Sunnyvale, CA). Intracellular ROS assay is a cell-based assay for measuring reactive oxygen species activity within a cell. The assay employs the cell-permeable fluorogenic probe DCF-DA (Molecular Probes®: Cat. No. D399), which diffuses into cells and is deacetylated by cellular esterases to the non-fluorescent dichlorofluorescin (DCFH). In the presence of ROS, DCFH is rapidly oxidized to highly fluorescent DCF.[17] Generation of ROS can be detected by monitoring the increase in fluorescence. Fluorescence was measured with excitation and emission wavelengths set at 488 nm and 520 nm, respectively.

Figure 3(a) represents the fluorescence images of intracellular ROS generation induced by plasma treatment in cancer (the bottom row of figure: A549) and normal (the upper row of figure: HEK293T) cell lines. The cells were pretreated with 10 μM DCF-DA for 5 min at 37 °C in the dark and exposed to plasma. We marked 9 points on a dish

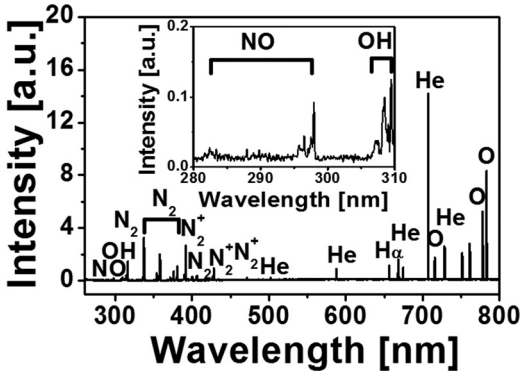

FIG. 2. Emission spectra from the helium plasma jet with the applied voltage of 1.0 kV_{rms} and frequency 35 kHz.

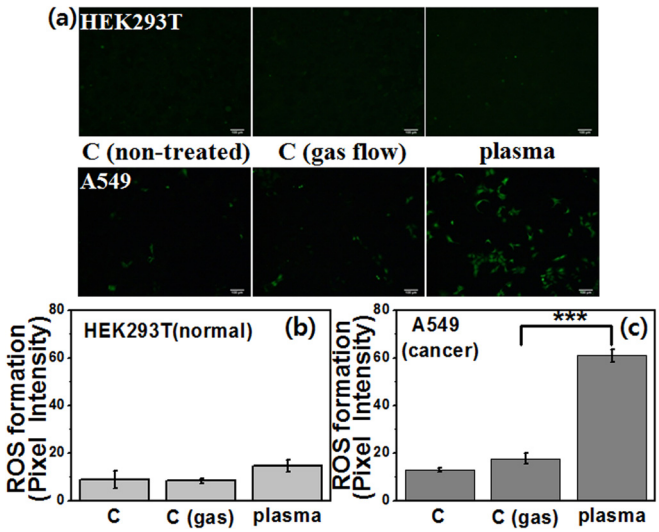

FIG. 3. (a) Fluorescence images of intracellular ROS generation in normal (the upper row of figure; HEK293T) and cancer (the bottom row of figure; A549) cell lines (1.1 kV_{rms}, 35 kHz, and 0.1 L/min). The quantification by measuring fluorescence pixel intensity with MetaMorph software: (b) HEK293T and (c) A549 cell lines. Each point represents the mean ± SD of three replicates. ***$p < 0.001$, compared with cells treated with the gas flow alone.

(51.4 mm in diameter) and treated those points for 10 s with plasma. We observed the production of ROS in those marked points. The cells didn't undergo the necrotic death such as plasma-induced void. Fluorescence-activated cells were detected using fluorescence microscopy (Nikon TS100-F). In the cancer cells after plasma treatment, the intensity level of the fluorescence was higher than that of non-treated and gas-treated cells. Figures 3(b) and 3(c) show the quantified values by measuring pixel intensity with MetaMorph software. The results were expressed as means ± SD. Statistical significance of difference between groups was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using statistical software (Prism, version 4; GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was regarded as significant. As shown in Fig. 3(b), no significant intracellular ROS generation was observed in normal cells. On the other hand, the results show a plasma-dependent elevation of intracellular ROS production in cancer cells [Fig. 3(c)]. It was reported that ROS are the major players in the cell response to plasma treatment in vitro and in vivo.[18,19] Different cells have different sensitivities to plasma depending on cell types and components of plasma plume. Since cancer cells produce high levels of ROS and are under increased oxidative stress, it is reasonable to speculate that compared to normal cells, the malignant cells would be more responsive to ROS-mediated damage.[20] However, one of the difficulties in this hypothesis arises from a necessity of a comparable "normal cell" to use as a control. Addressing this problem requires a control cell line, preferably one that has the similar characteristics aforementioned. So we also examined the plasma effects in a human normal bronchial epithelial (BEAS-2B) cell line.

A549 cells were considered to be alveolar epithelial cells with properties of type II cells, as they were isolated from an alveolar cell carcinoma. BEAS-2B cells were originally established from healthy human bronchial epithelium and transformed by an adenovirus 12-SV40 hybrid virus. They have been shown to maintain typical epithelial morphology and many epithelial functional characteristics.[21] As shown in Figs. 4(a) and 4(b), intracellular ROS generation was quite different between normal and cancer cells. A high intensity level of DCF-DA fluorescence was observed in the plasma-treated cancer cell populations indicating a higher level of ROS concentration than those of normal cells. Moreover, it was observed that plasma could have a different influence on cancer cells in two different applied voltages (plasma doses). As shown in quantification of fluorescence intensity [Fig. 5(a)], the intracellular ROS generation in the plasma-treated cancer cells was dramatically increased compared to the gas flow-treated control. The results obtained

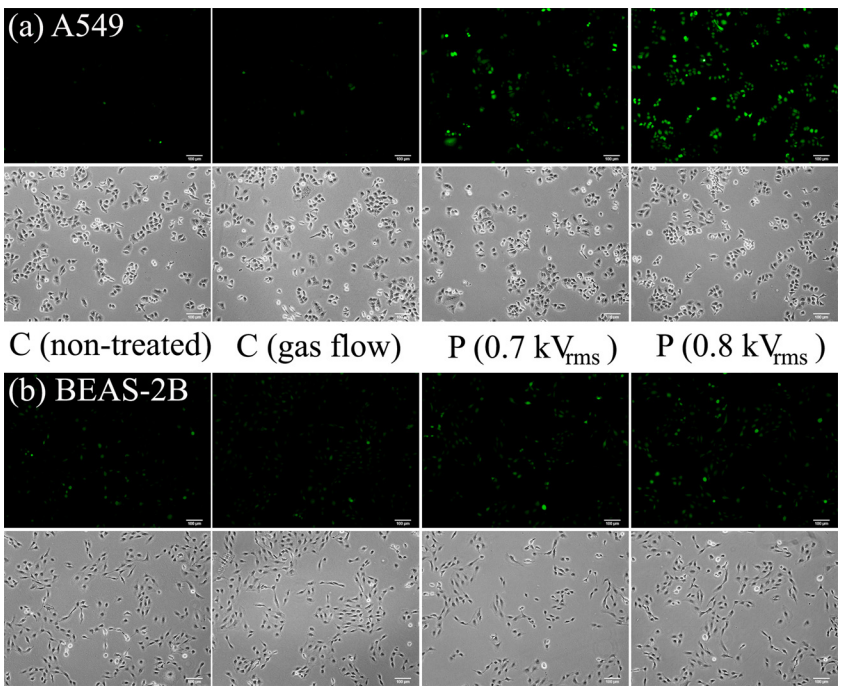

FIG. 4. Fluorescence images of intracellular ROS generation and bright-field images: (a) cancer (the upper row of figure; A549) and (b) normal (the bottom row of figure; BEAS-2B) cell lines (0.7 kV_{rms}, 0.8 kV_{rms}).

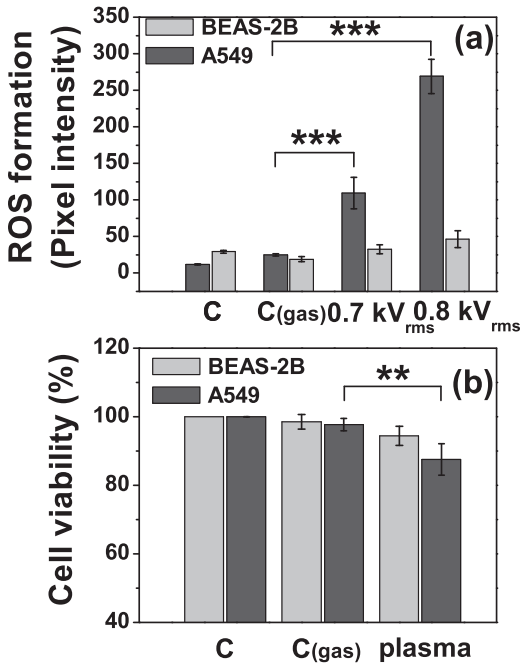

FIG. 5. (a) The quantification by measuring fluorescence pixel intensity with MetaMorph software. (b) Measurement of cell viability by MTS assay: cancer (A549) and normal (BEAS-2B) cell lines (0.75 kV_{rms}). Cell viability was determined 48 h after the plasma treatment. Each point represents the mean ± SD of three replicates. $^{**}p < 0.01$, $^{***}p < 0.001$.

with BEAS-2B cells were similar to those with HEK293T cells. Normal cells have less ROS generation and profound antioxidant systems. Cancer cells normally produce more ROS than do normal cells.[20] This might make them vulnerable to chemotherapeutic agents that further augment ROS generation. Also, some cancer cell types have higher metabolic activities than others (including the normal quiescent cells), and these differences may easily lead to translate into higher rates of ROS formation.

Next, cell viability was assessed by MTS assay with the use of a kit (Promega: Cat. No. G3582) according to the manufacturer's instructions. The MTS tetrazolium compound is bio-reduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH (nicotinamide adenine dinucleotide phosphate; reduced form) or NADH (nicotinamide adenine dinucleotide; reduced form) produced by dehydrogenase enzymes in metabolically active cells.[22] Assays are performed by adding a small amount of the solution reagent directly to culture wells, incubating for 2–4 h and then recording the absorbance at 490 nm with the Victor 3 spectrophotometer (Perkin-Elmer, CT).

Figure 5(b) represents the measurement of cell viability by MTS assay on A549 and BEAS-2B cell lines. The assay was performed 48 h after exposure to plasma. Interestingly, it was observed that the reduction in cell viability rate was different between cancer and normal cells. The viability rate of cancer cells was reduced on the plasma-treated cells. It was lower than that in the gas flow-treated control (87.54 ± 4.5% versus 97.7 ± 1.7%). In the normal cells, the results exhibit a slight decrease in cell viability. It is generally accepted that ROS can function as true second messengers and mediate important cellular functions such as proliferation. The ROS, which contribute to the energy landscapes in and around cells, play numerous roles in maintaining normal cell homeostasis. On the other hand, the high levels of ROS can lead to pronounced DNA damage and a variety of cellular responses, including cell cycle arrests, senescence, and apoptosis. ROS are constantly generated and eliminated inside a cell and regulate ROS-sensitive signaling events.[23,24] The balance of ROS may be dramatically affected by many environmental stimuli, including plasma treatment. The ROS generation and subsequent oxidative damage to the cell membrane are one of the major mechanisms of cell death in cancer therapy.[25] When intracellular ROS production is further increased by plasma treatment, ROS in cancer cells may easily reach the death threshold through aftereffect. These different aspects between cancer and normal cells provide a prospect for ROS-promoting cancer therapy.

To further examine the involvement of plasma-induced ROS in cellular response, we evaluated whether intracellular ROS generation and reduction in viable cell density could be inhibited by various antioxidant agents, such as rotenone (a mitochondrial electron transport chain inhibitor (Sigma:

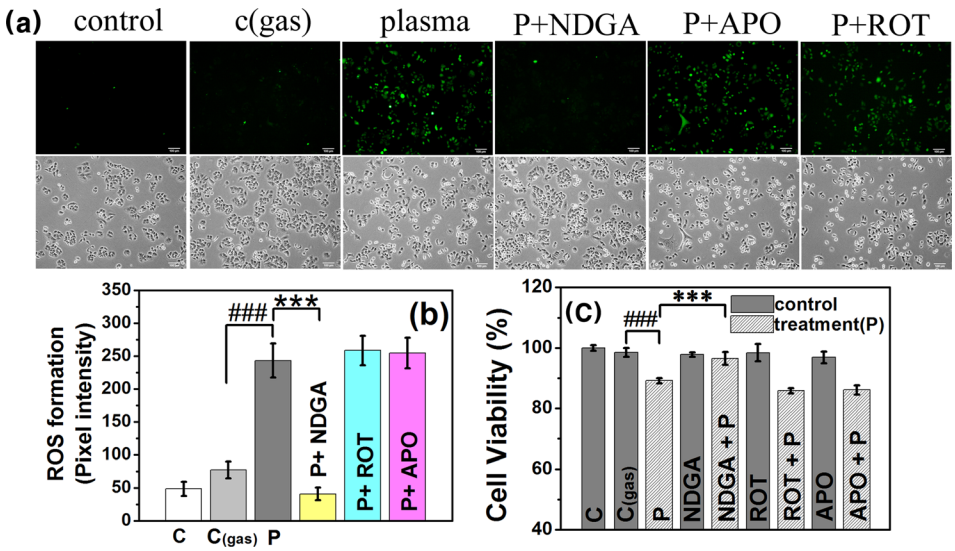

FIG. 6. Effects of antioxidants on intracellular ROS generation induced by plasma (0.7 kV_{rms}): (a) the fluorescence images and bright-field images and (b) the quantification of intracellular ROS generation in cancer (A549) cell lines. (c) Measurement of cell viability by MTS assay. Cells were pretreated for 1 h with antioxidants before plasma treatment (P): 1 μM rotenone (ROT), 10 μM NDGA, and 300 μM apocynin (APO). Each point represents the mean ± SD of three replicates. $^{***}p < 0.001$, $^{###}p < 0.001$.

Cat. No. R8875), 1 μM), NDGA (nordihydroguaiaretic acid: an antioxidant and lipoxygenase (LOX) inhibitor (Sigma: Cat. No. 74540), 10 μM), and apocynin (NADPH oxidase inhibitor (Calbiochem: Cat. No. 178385), 300 μM). The inhibitors were added 1 h before A549 cells were exposed to plasma. As shown in Figs. 6(a) and 6(b), it was observed that plasma could effectively induce ROS generation in A549 cells. However, NDGA blocked the plasma-induced ROS, whereas other antioxidants had no obvious effect on those. NDGA is a recognized inhibitor of LOX. LOX are enzymes that catalyze the addition of oxygen to polyunsaturated fatty acids. Products of LOX are involved in diverse cell functions. It was demonstrated that LOX metabolites influenced ion movement and fluid balance in isolated rabbit tracheal epithelial cells.[26] Our results suggest that plasma-induced ROS generation may be related to a specific pathway associated with LOX in lung cancer cells. NDGA has antioxidant and free radical scavenging properties. It has been reported that NDGA was a potent scavenger of ROS such as peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$), hydroxyl radical ($^{\bullet}OH$), superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) in vitro and could prevent ozone (O_3)-induced lung tyrosine nitration in vivo.[26] Our results indicate that NDGA may be a potent antioxidant that can block plasma-induced oxidative radicals. In addition, it was examined whether the presence of various antioxidants could cause recovery from plasma-mediated reduction in viable cell number. Figure 6(c) represents the measurement of cell viability by MTS assay. The plasma treatment mixed with NDGA tended to mitigate the viability reduction (compared to pure plasma, apocynin, and rotenone). In general, increased ROS stress can induce various biological responses, ranging from a transient growth arrest and adaptation, increase in cellular proliferation, permanent growth arrest or senescence, apoptosis, and necrosis. On the condition that dividing cells are exposed to greater oxidative stress, they can be forced into a permanently growth-arrested state.[27] These results suggest that an abnormal increase of ROS by plasma treatment can affect the cellular viability.

In conclusion, a significant ROS generation was induced by plasma exposure on cancer cells and the overproduction of ROS may partially contribute to the reduced viability. In contrast, it was observed that the plasma-mediated ROS was slightly increased in normal cells and cellular viability tended to be less changed compared to cancer cells. Due to these differences in cell sensitivity to plasma exposure between cancer and normal cells, non-thermal plasma can be one of the promising tools in terms of ROS-promoting cancer therapy. In addition, NDGA could effectively block the plasma-induced ROS production in cancer cells. The presence of NDGA could cause recovery from the reduction in cell viability by plasma treatment in cancer cells. This indicates that plasma may induce oxidative stress through a specific pathway affected by NDGA. These results could become potentially valuable information in medical applications of non-thermal plasma. Since there has been only limited information on ROS generation and mechanism of subsequent oxidative damage after plasma treatment, additional functional studies are now needed to comprehend the plasma action on cells.

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[1]A. Fridman, A. Chirokovo, and A. Gutsol, J. Phys. D: Appl. Phys. **38**, R1 (2005).
[2]N. Georgescu and A. R. Lupu, IEEE Trans. Plasma Sci. **38**, 1949 (2010).
[3]M. Vandamme, E. Robert, S. Lerondel, V. Sarron, D. Ries, S. Dozias, J. Sobilo, D. Gosset, C. Kieda, B. Legrain, J. M. Pouvesle, and A. L. Pape, Int. J. Cancer **130**, 2185 (2012).
[4]M. Moreau, N. Orange, and M. G. J. Feuilloley, Biotechnol. Adv. **26**, 610 (2008).
[5]J. Y. Kim, Y. Wei, J. Li, P. Foy, T. Hawkins, J. Ballato, and S.-O. Kim, Small **7**, 2291 (2011).
[6]M. G. Kong, G. Kroesen, G. Morfill, T. Nosenko, T. Shimizu, J. van Dijk, and J. L. Zimmermann, New J. Phys. **11**, 115012 (2009).
[7]X. Yan, Z. Xiong, F. Zou, S. Zhao, X. Lu, G. Yang, G. He, and K. Ostrikov, Plasma Process. Polym. **9**, 59 (2012).
[8]M. Valko, D. Leibfritz, J. Moncola, M. T. D. Cronin, M. Mazura, and J. Telser, Int. J. Biochem. Cell Biol. **39**, 44 (2007).
[9]E. Stoffels, M. V. Erofeev, I. E. Kieft, and S. E. Kunts, IEEE Trans. Plasma Sci. **32**, 1544 (2004).
[10]S. Kalghatgi, G. Friedman, A. Fridman, and A. M. Clyne, Ann. Biomed. Eng. **38**, 748 (2010).
[11]D. B. Graves, J. Phys. D: Appl. Phys. **45**, 263001 (2012).
[12]S. Kalghatgi, C. M. Kelly, E. Cerchar, B. Torabi, O. Alekseev, A. Fridman, G. Friedman, and J. Azizkhan-Clifford, PLoS One **6**, e16270 (2011).
[13]M. Keidar, A. Shashurin, O. Volotskova, M. A. Stepp, P. Srinivasan, A. Sandler, and B. Trink, Phys. Plasmas **20**, 057101 (2013).
[14]X. Zhang, M. Li, R. Zhou, K. Feng, and S. Yang, Appl. Phys. Lett. **93**, 021502 (2008).
[15]X. Zuo, Y. Wei, L. Chen, Y. Meng, and Plasma Medicine Team, Phys. Plasmas **20**, 083507 (2013).
[16]R. Brandenburg, J. Ehlbeck, M. Stieber, T. Woedtke, J. Zeymer, O. Schlüter, and K. D. Weltmann, Contrib. Plasma Phys. **47**, 72 (2007).
[17]T. M. Johnson, Z. X. Yu, V. J. Ferrans, R. A. Lowenstein, and T. Rinkel, Proc. Natl. Acad. Sci. U.S.A. **93**, 11848 (1996).
[18]H. J. Ahn, K. I. Kim, G. Kim, E. Moon, S. S. Yang, and J. Lee, PLoS One **6**, e28154 (2011).
[19]G. E. Morfill, T. Shimizu, B. Steffes, and H. U. Schmidt, New J. Phys. **11**, 115019 (2009).
[20]T. Ozben, J. Pharm. Sci. **96**, 2181 (2007).
[21]C. Schulz, L. Farkas, K. Wolf, K. Kratzel, G. Eissner, and M. Pfeifer, Scand. J. Immunol. **56**, 294 (2002).
[22]J. A. Barltrop, T. C. Owen, A. H. Cory, and J. G. Cory, Bioorg. Med. Chem. Lett. **1**, 611 (1991).
[23]K. R. Martin and J. C. Barrett, Hum. Exp. Toxicol. **21**, 71 (2002).
[24]B. E. Lehnert and R. Iyer, Hum. Exp. Toxicol. **21**, 65 (2002).
[25]S. M. Bhosle, B. N. Pandey, N. G. Huilgol, K. P. Mishra, S. M. Bhosle, B. N. Pandey, N. G. Huilgol, and K. P. Mishra, Methods Cell Sci. **24**, 65 (2002).
[26]J.-M. Lü, J. Nurko, S. M. Weakley, J. Jiang, P. Kougias, P. H. Lin, Q. Yao, and C. Chen, Med. Sci. Monit. **16**, 93 (2010).
[27]H. Pelicano, D. Carney, and P. Huang, Drug Resist. Updates **7**, 97 (2004).