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Effects of the pulse width on the reactive species production and DNA damage in cancer cells exposed to atmospheric pressure microsecond-pulsed helium plasma jets

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Plasma-liquid and plasma-cell interactions were investigated using an atmospheric pressure dc microsecond-pulsed helium plasma jet. We investigated the effects of the electrical parameters such as applied voltage and pulse width (determined by the pulse frequency and duty ratio) on the production of reactive species in the gas/liquid phases and on the DNA damage responses in the cancer cells. The densities of reactive species including OH radicals were estimated inside the plasma-treated liquids using a chemical probe method, and the nitrite concentration was detected by Griess assay. Importantly, the more concentration of OH resulted in the more DNA base oxidation and breaks in human lung cancer A549 cells. The data are very suggestive that there is strong correlation between the production of OH in the plasmas/liquids and the DNA damage. © 2017 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

I. INTRODUCTION

Since atmospheric pressure plasma jets (APPJs) can produce non-thermal plasma, characterized by reactivity, compactness, and high efficiency,1–3 their applications have rapidly expanded to biology and medicine.4–8 In particular, the use of APPJs in cancer therapies is drawing considerable attention because plasmas contain short lived free radicals, including reactive oxygen and nitrogen species (RONS), charged species, and electric fields, that can induce apoptosis in cancer cells. One of the most important factors is the plasma-generated reactive species including RONS. Over the past few years, a considerable number of studies have tried to control the RONS in atmospheric pressure plasma medium for plasma-cell interactions.4–8

Plasma generated from atmospheric air contains chemically reactive H, N, O, O3, NO, NO2, and OH radicals. When solution is exposed to plasma, some of these RONS dissolve from the gas phase into solution. The final products of these unstable species in the liquid phase might be O2 (1Δg), H2O2, O3, NOx, N2O, and HNOx, which are quite stable and interact with cells resulting in the production of intracellular RONS.4,5,7–10 These chemical species exhibit strong oxidative stress and/or trigger signaling pathways leading to DNA damage and apoptotic cell death. It is widely known that OH radicals are the building blocks of H2O2 which is considered as an important agent in the chemical reactivity of plasma in and in contact with liquids, and thus, OH plays an important role in plasma chemistry among others.5 Moreover, of special interest is nitric oxide owing to its crucial role in cell death.11 There is substantial literature on the role of NO and related compounds as a direct cancer therapy.12,13

We previously reported the effects of electrical parameters of APPJs on the apoptotic rate of cancer cells-in vitro,7 and on the production of the OH and nitrite in the plasma-treated liquid.14

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However, the experiments were performed at a few data points, and the experimental results were not discussed completely, and detailed explanation was not provided in terms of the extracellular and intracellular RONS. The electrical parameters of pulse dc power supply (applied voltage, pulse frequency, and duty ratio) influence the production of RONS in the gas phase. Especially, the duty ratio determines the pulse width, which affects the efficiency of the discharge process. It was shown that the pulsed discharge can be divided into three phases related to the production of active species: (i) primary discharge, (ii) the earlier part of secondary discharge and (iii) the later part of secondary discharge. Aforementioned reactive species are mainly produced in different phases depending on the species type. Especially, many reactive species responsible for biological response such as O, OH, and NO are mainly produced in phases (ii) and (iii). This result indicates the existence of an optimal pulse width for efficient biochemical processes using APPJs. Plasma generated RONS react with media component and this has a profound impact on the RONS concentration in the media. Then the cell culture media mediates the plasma effect on DNA damage and cell death. Reportedly, RONS can induce DNA base modifications including 8-oxoG which is associated with biological processes such as mutagenesis, carcinogenesis and senescence because it exhibits ambiguous coding properties in the biosynthesis of nucleic acids. In addition, RONS-induced DNA damage responses can subsequently facilitate generation of single- or double-strand breaks and DNA cross links. To determine whether the amount of RONS generated in the gas and liquid phases directly evokes DNA lesions, we evaluated the effects of electrical parameters of APPJs on the production of the RONS and explore the correlation between the RONS production and the DNA damage responses within human lung cancer A549 cells.

II. RESULTS AND DISCUSSIONS

Here, we describe the effect of electrical parameters of APPJs on the production of the RONS and explore the correlation between the RONS produced in both the gas and liquid phases and the DNA damage within cancer cells. A schematic diagram is shown in Fig. 1(a). The details of the APPJ can be found in our earlier studies. The helium plasma jet was generated by a pulsed unipolar source of up to 2 kV with a pulse frequency of several tens of kilohertz. The helium gas was delivered at a flow rate of 1 L/min. In this work, the operating range of the electrical parameters is the applied voltage 1.3 - 1.7 kV, the pulse frequency 20 - 50 kHz, and the duty ratio 10 - 50%. The temporal evolutions of the applied voltage, current, and wavelength-integrated optical emission intensity for three different duty ratios are presented in Fig. 1(b). It is observed that the current and the main optical emission occur at the rising period of the voltage waveform (primary discharge (i)), while the weak light emission signal occurred at the falling period of the voltage waveform (secondary discharge (ii)). The RONS produced in the gas and liquid phases are quantified by optical emission spectroscopy (USB-2000+XR-ES OceanOptics) and by RONS-reactive fluorescent probes, respectively.

The optical emission spectrum shows strong nitrogen molecular bands (N$_2$ and N$_2^+$) as well as a few helium and oxygen atomic lines (Fig. 2(a)). The OH (309 nm) band is produced from water vapor in the helium flow and air. The presence of nitric oxide (NO) at 283 nm is due to the chemical conversion of N and O$_2$ (or N and O). These highly reactive species such as O, OH, and NO are considered to be the most effective agents in attacking cells or organic material in general. Figures 2(b) – 2(d) show the variations of the emission intensities of NO, OH, O and H$_\alpha$ with the applied voltage, pulse frequency, and duty ratio, respectively. The effect of increasing the applied voltage is that the faster, more intense ionization wave penetrates further into the plume, and this creates more reactive species. It is also observed that as the pulse frequency is increased the emission intensity of most of the peaks increase. This trend is in an overall agreement with other experiment.

In atmospheric-pressure non-equilibrium plasma the plasma is generated intermittently. The intensity at each wavelength observed using a spectrometer is the time-averaged effective intensity. For a fixed exposure time of the spectrometer, the time-averaged effective intensity at each wavelength is expected to increase linearly with the pulse frequency. However, it should be noted that as can be seen in Fig. 2(c), the increase rates of the emission intensities at each wavelength with the pulse frequency slightly decrease in the range of 35 – 50 kHz.
FIG. 1. (a) Schematics of the experimental setup and the photographs of the plume. Diagnostics include current-voltage measurement, optical emission spectroscopy, and chemical probe method (TA was used as an OH radical trap). (b) Temporal evolutions of the applied voltages, the current, and the wavelength-integrated optical emission intensities for the APPJs driven with three different duty ratios (10%, 30%, and 50%) at 50 kHz (the pulse repetition period = 20 µs).

FIG. 2. (a) Emission spectra from 200 nm to 900 nm observed in the He jet ($V_a = 1.7$ kV$_{pp}$). Variations of the emission intensities from NO, OH, H and O as functions of (b) applied voltage, (c) pulse frequency, and (d) duty ratio ($V_a = 1.5$ kV$_{pp}$).

The effect of the duty ratio is more complicated. To maximize the ratio of active species mainly produced in phase (ii) such as OH and O, the pulse width should be moderately short to cut off the later part of the secondary streamer. To maximize the ratio of active species mainly produced in the later part of the secondary streamer such as OH (A), the pulse width should be long. It is observed that the duty ratio of 25% (the pulse width of 5 µs) results in slightly higher emission intensities from most of species (some remains not much changed). These may be attributed to that although...
a short pulse is desirable for the efficient production of some radicals and excited species such as \( \text{N}_2^+(B) \), the decrease of duty ratio (to 10%) makes the time span between the primary discharge and the secondary discharge shorter (2 µs), thus having the effect equivalent to cutting off the secondary discharge, consequently reducing or saturating the production of H, O, OH, and NO. As mentioned earlier, the increase rate in the intensities is observed to become lower as the pulse frequency is increased to 50 kHz. This may be attributable to the fact that the pulse width effect is added to the

FIG. 3. Measurement of OH concentration using fluorescence spectra of aqueous TA solutions exposed to the APPJ. The dependence on: (a) applied voltage, (b) pulse frequency \((V_a = 1.5 \text{ kV}_{pp})\), and (c) duty ration \((V_a = 1.5 \text{ kV}_{pp})\).
normal frequency effect. The 50 kHz case with the fixed duty ratio of 10% corresponds to a very small pulse width (2 μs) (while the pulse width of the 35 kHz case with the fixed duty ratio of 10% corresponds to ∼3 μs). This small pulse width (2 μs) corresponding to the 50 kHz case may hinder the OH production in the gas phase (on the same physical reason to that discussed in the duty ratio effect) although the emission intensity from OH is higher at 50 kHz.

FIG. 4. Measurement of nitrite concentration with increasing (a) applied voltage, (b) pulse frequency ($V_a = 1.5 \text{ kV}_{pp}$), and (c) duty ratio ($V_a = 1.5 \text{ kV}_{pp}$) (with non-treated case and the gas only treated case).
OH is one of the most active species generated in moist gas mixtures. Since the OH radicals in liquid contribute to cell death, a strong correlation between the production of OH in liquid phase and the DNA damage of cancer cells is expected. As a method of OH radical detection, we utilized the hydroxylation of terephthalic acid (TA). The OH radical reacts with TA to form hydroxyterephthalic acid (HTA) which fluoresces. When the solution containing TA and HTA molecules is irradiated by UV light, the HTA molecules emit light at $\lambda = 425$ nm. From the fluorescence intensity emitted from the post-exposure solution, the amount of OH radicals trapped by TA in plasma-treated liquids could be estimated. Aqueous solution of TA was prepared by dissolving TA (Sigma) in distilled water containing NaOH (Wako). The initial concentrations of TA and NaOH were 3 mM and 10 mM and the initial value of pH was 8. The dish plate (diameter 20 mm) including liquids was irradiated with plasma (for 5 min) and samples of the liquids from dish after plasma treatment were taken using a cuvette to observe the fluorescence after exciting by light source.

Figure 3 shows the OH concentration as functions of applied voltage, pulse frequency and duty ratio. In our previous experiment utilizing different jet device, as the applied voltage and pulse frequency were increased, the fluorescence intensity increased, indicating an increase in the total amount of OH radicals. In this work, the OH concentration was found to depend on the electrical parameters as shown in Fig. 2(b)–(d). The cases of the 10% duty ratio with 50 kHz frequency (pulse width: 2 $\mu$s) and the 35 kHz pulse frequency with duty ratio 10% (pulse width: 3 $\mu$s) were observed to have larger OH concentration. These observations exhibit a little different trend to those of the intensity level of OH in optical spectra (Fig. 1(b)–(d)). A slight reduction of OH at 50 kHz may partly be related to the aforementioned pulse width effect, and be partly caused by the OH kinetics in the liquid (most of the absorbed OH radicals into liquid media react to form $\text{H}_2\text{O}_2$).

Moreover, of special interest is nitric oxide due to its crucial role in both cell death and proliferation. In order to determine the NO levels delivered to growth media, the total nitrite concentrations is often measured. Nitrite concentration is determined using the Griess assay. Figure 4 shows the measurement of nitrite concentration in deionized water after plasma treatment by Griess assay. Since the production mechanism of NO have a lot of routes closely connected to

![Figure 5](image-url)
those of OH, the nitrite concentration was expected to have a similar dependence on the electrical parameters to that of the OH concentration. The detected nitrite concentration became higher with the increase of the applied voltage as expected. However, its dependence on the pulse frequency and the duty ratio was found to be a little different from those of OH concentration. Here, the case of the 10% duty ratio with 50 kHz pulse frequency (pulse width: 2 µs) was observed to have larger nitrite concentration. Nitrite concentration was observed to increase with pulse frequency and decrease slightly with the increase of the duty ratio (with increasing the pulse width).

To explore correlation between the RONS amounts and the extents of DNA damage, A549 cells treated with plasma (or gas alone) were fixed and subjected to immunofluorescence detection for 8-oxoG. For immunofluorescence staining, A549 cells were cultured to 70% confluency onto gelatin coated cover slips (Sigma-Aldrich). After plasma treatment, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min at room temperature and permeabilized with 0.5% Triton X-100 (Bio Basic). Specific antibodies against phospho-histone H2AX (Ser139; Millipore), 8-OxoG (Abcam) were used for visualization of the proteins. Hoechst 33342 was added to counterstain nuclei. The images were captured using a fluorescence microscope (Nikon) equipped with the NIS-Elements 4.0 Nikon imaging software. For quantitative analysis, over 500 cells were analyzed.

FIG. 6. Effects of electrical parameters on genomic DNA breaks. Measurement of γH2AX positive levels with increasing (a) applied voltage, (b) pulse frequency ($V_a = 1.5$ kV pp), and (c) duty ratio in A549 cells after plasma treatment ($V_a = 1.5$ kV pp). The pulse width corresponding to the specific pulse frequency and duty ratio is indicated in parenthesis. (d)–(f): The quantification of γH2AX positive levels for (a), (b), and (c). The bars and error bars are presented as the mean ±SEM from three independent experiments. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. 


from each of three independent experiments. We could observe that as the applied voltages were increased, the number of 8-oxoG-positive cells was increased too (Figure 5(a)). The strongest 8-oxoG responses were found when the pulse frequency of 35 kHz (with the duty ratio of 10%) and the duty ratio of 25% (with the pulse frequency of 50 kHz) were applied (Figures 5(b) and 5(c)). Excessive 8-oxoG if not repaired can cause DNA breaks, an overt indicative of cell apoptosis.\textsuperscript{18} The extents of DNA breaks were investigated using an immunofluorescence analysis of $\gamma$H2AX.\textsuperscript{18} As similar to 8-oxoG results, the more $\gamma$H2AX-positive cells were detected as the applied voltages increased (Figure 6(a)), and 35 kHz frequency (with the duty ratio of 10%) and 25% duty ratio (with the pulse frequency of 50 kHz) resulted in the most severe DNA breaks (Figures 6(b) and 6(c)). Figures 6(d) – 6(f) represent the quantification of $\gamma$H2AX positive levels for Figures 6(a), 6(b), and 6(c). The bars and error bars are presented as the mean ± SEM from three independent experiments. * = p < 0.05; ** = p < 0.01; *** = p < 0.001. From these graphs, we confirm that the DNA damage depends on the applied voltage, pulse width and pulse frequency. The significant DNA damages occur at the higher applied voltage and at the pulse width of 3 $\mu$s (35 kHz) and 5 $\mu$s (50 kHz).

III. CONCLUSION

The consequences of applied voltage, pulse frequency, and duty ratio on the RONS production and on the DNA damage responses were investigated. Our results suggest that there is a robust correlation between the production of OH in the gas/liquid phases and intracellular oxidative stress or the DNA damage responses in cancer cells, implying that optimization of the electrical parameters of APPJ can increase therapeutic efficacy for cancer cell killing. Under the conditions of pulsed plasma experiments in this study, it can be concluded that the application of high voltage with 35 kHz frequency (at the duty ratio of 10%) and duty ratio 25% (at the pulse frequency of 50 kHz) results in the efficient production of OH species and the most severe DNA damages for cancer cell treatment. The correlation of intracellular oxidative stress (or the DNA damage responses) with nitrite concentration needs to be explored further by various methods. Although the induction of apoptosis by plasma treatment has proven to be mediated through the formation of intracellular RONS, other factors such as the charged current into cells and the electric field may contribute to apoptosis. Therefore, the effect of the pulse width on the charged currents into cells and the electric field should also be investigated in future studies.

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