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DNA damage in oral cancer cells induced by nitrogen atmospheric pressure plasma jets

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The nitrogen atmospheric pressure plasma jet (APPJ) was applied to induce DNA damage of SCC-25 oral cancer cells. Optical emission spectra were taken to characterize the reactive species produced in APPJ. In order to explore the spatial distribution of plasma effects, cells were placed onto photo-etched grid slides and the antibody H2A.X was used to locate double strand breaks of DNA inside nuclei using an immunofluorescence assay. The number of cells with double strand breaks in DNA was observed to be varied due to the distance from the irradiation center and duration of plasma treatment. © 2013 AIP Publishing LLC.

As early as the middle of 19th century, dielectric barrier discharges had already been used to generate ozone for water sterilization.1 It is not until recent years that plasma sources operating at the room condition have been developed, opening more possibilities in biomedical applications thus initiating the innovative field called plasma medicine. Due to the significant advantages of avoiding costly complex vacuum systems and high temperatures, an atmospheric pressure plasma jet (APPJ) offers many opportunities for applications at the interface of plasma physics, radiation chemistry, and biomedicine.2 Many reports have shown that APPJ can be a promising method for cancer treatment,3 blood coagulation,4,5 wound disinfection6 and healing,7 tooth bleaching,8 sterilization.1 It is not until recent years that plasma sources had already been used to generate ozone for water treatment.

Since APPJ offers a unique gas environment consisting of radicals, photons, and charged particles, all of which can interact with treated samples directly, the effect of APPJ on cancer cells could be explained at the molecular level. Generally, APPJ contains copious quantities of radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). It is reported that signaling initiated by ROS/RNS activates redox signal pathways through a series of electron transfer processes, leading to cell cycle arrest and cell apoptosis.11,12

Most APPJ sources are operated in the noble gases environment, e.g., helium or argon, which are characterized by a relatively low breakdown voltage and long plasma sustainability. Since they are commonly mixed with a small amount of other gases (e.g., O2) to increase the chemical reactivity of APPJ, and are more costly for long term operation, the use of nitrogen as a feeding gas is a preferable choice. Experiments using N2 gas have only been reported by a few research groups.13–20

In this work, one type of APPJ with N2 gas21 was applied to explore its effect on inducing DNA double strand breaks (DSB) in SCC-25 oral cancer cells. These studies were stimulated by our previous work on APPJ interacting with DNA molecules, resulting in high yields of DSB within a short plasma irradiation time.22,23

A schematic view of the plasma source used for present studies is shown in Fig. 1(a). Two copper ribbon electrodes of 0.6 mm thick separated by a distance of 1.8 mm are spirally and alternatively wrapping around a quartz tube, whose outer diameter is 3 mm. One of the helical electrodes is connected to a high voltage (HV) power supply and the other is grounded. The electrode wrapping zone, with a vertical length of 35 mm along the quartz tube, is the major region of plasma ignition. A wider glass tube was sealed outside the quartz tube and a fluid with a high dielectric constant was filled within the volume between these two tubes. This design not only avoids arcing outside the quartz tube but also cools down the electrodes. When N2 is introduced into the quartz tube and HV is applied, plasma is ignited and forms a plasma jet of a few centimeter long to the open atmosphere (Fig. 1(b)).

Operational parameters of this plasma source were carefully chosen to obtain a stable plasma jet.21 An alternative sine wave high voltage generator (PVMP 500 Plasma Driver)
was used to drive the powered electrode at a frequency of 28 kHz. From a digital oscilloscope (Tektronix TDS2004B, 60 MHz), the electrical characteristics were read as 22.4 kV peak to peak (V rms = 7.75 kV) and 59 mA peak to peak (I rms = 17 mA) using voltage (Tektronix TCP A300) and current (Tektronix P6015A) probes, respectively. The read current showed multiple spikes, indicating microdischarges in the source with basically a capacitive impedance (capacitance of 2.2 pF). Considering the living conditions of cells during plasma treatment, the position of a dish with cells was set at the axial distance of 20 mm down from the orifice, with 1.5 l/min N2 flow rate to avoid heating and drying issues.

Optical emission spectroscopy was also applied to determine the reactive species generated in APPJ. The optical emission spectrum in the range of 200–850 nm obtained for experimental conditions at which cancer cells were irradiated is presented in Fig. 2. Nitrogen oxide bands (NO-γ) were detected between 200 and 300 nm, generated possibly by the collision of reactive nitrogen species in APPJ with surrounding air. The bands of the second positive system (2nd PS) of molecular nitrogen were recorded in the range of 300–420 nm. The existence of these species was due to the excited state of molecular nitrogen caused by discharged electrons. The dominant peak around 557 nm represents an excimer of ON2 (O(1D)N2), which was produced by the interaction between metastable oxygen (O(1S)) and nitrogen molecules (N2). Ozone (O3) was also detected in APPJ with an amount around 6.3 ppm at 15 mm rapidly decreasing to 0.3 ppm at 40 mm along the axial distance from the orifice of the plasma source. The detailed characterization of these ROS and RNS particularly at the plasma-liquid interface is essential for exploring the mechanism of APPJ interacting with biological samples.

To evaluate cell viability, most groups use flow cytometry of detached cells to obtain statistical data of the population of damaged cells or DNA content in a certain phase of the cell cycle induced by plasma treatment. In our studies, cells remained adherent and immunofluorescence was used to identify and quantify cells with DNA damage. Double strand breaks in the DNA can be used as a surrogate marker for a high level DNA damage within the cells. As histone H2A.X becomes phosphorylated in response to DSB, in our experiments, anti-phospho-histone H2A.X primary antibodies were chosen to localize DNA DSB inside the nuclei. Use of a fluorescently labeled secondary antibody enabled visualization and relative quantitation of DNA DSB by means of immunofluorescence microscopy.

Before plasma irradiation, SCC-25 oral cancer cells were grown on a grid slide (Bellco Glass, Photoetch Coverslip, 23 × 23 mm) with a marked dot at the center (Fig. 1(c)) placed inside a P35 cultural dish. To explore the interaction of plasma with cells, a simple chemical liquid environment for cells was used for irradiation purpose. Thus, 2.4 ml phosphate buffered saline (PBS) (1×, Mediatech, Inc., 3 mm thickness in P35 dish) was added instead of a culture medium to prevent cell desiccation. Irradiation trails of 10 s, 30 s, 60 s, and 120 s were conducted, along with control samples. A gas flow control sample was treated only with N2.
gas without plasma ignition and an additional cellular control sample was untreated (i.e., neither plasma nor gas flow). Since the secondary antibody carries the fluorophore used to detect DNA DSB, a secondary antibody control was also conducted, in which cells were incubated with this secondary antibody in the absence of the primary antibody to correct for background binding. To enable visualization of DNA damage, a 1 h incubation was performed right after plasma treatment by replacing PBS with a culture medium and transferring the cells back to the incubator (37 °C, 5% CO2). Afterwards, medium was removed and 4% paraformaldehyde in PBS was added for 20 min to fix the cells, followed by two PBS washes. Then, 0.3% triton-X-100 in PBS was used for 5 min to permeabilize cell membranes, enabling antibody molecules access into the cells. To block nonspecific bindings, 3% bovine serum albumin (BSA) in PBS was then added to the fixed, permeabilized cells, followed by PBS washing for 3 times. After 1 h, primary antibody Anti-phospho-Histone H2A.X (EMD Millipore Corp., 250× diluted) was added to the cells on the slides and incubated overnight in 4 °C room. After washing with PBS 5 times, cells were incubated with the secondary antibody (Alexa Fluor 488 Goat Anti-Mouse IgG (H + L), Life Tech. Corp, 400× diluted) for 20 min in dark, followed by DAPI (or 4',6-diamidino-2-phenylindole) (mounting medium for fluorescence with DAPI, Vector Laboratories Inc.) staining of the cell nuclei. After sealing the slides, green fluorescence (a representative of cells with DNA damage), DAPI-stained nuclei, and phase transmitted images of cells were obtained. All images were acquired at standardized spots on the slides in a defined horizontal and vertical pattern relative to the central marked dot (Fig. 1(c)) using a fluorescence microscope (Advanced Microscopy Group, EVOS FL, 20× projector) (Fig. 3). By comparing the number of cells with positive green fluorescence to the total number of DAPI-stained nuclei, a ratio of cells with DSB in comparison to all cells was obtained using MATLAB by setting the brightness of the untreated control sample as the threshold.

To confirm that the cell damage was a result of plasma species and not due to any temperature effect, non-irradiated samples were warmed up to 43 °C in a water bath for 15 min. Following the same immunofluorescence protocol mentioned above, no damage was observed at elevated temperatures.

Since the diameter of the plasma jet tip is relatively small (~1 mm), it is interesting to investigate how large area can be affected by plasma irradiation. This could provide significant information about a precision of plasma therapy when only a tumor area is expected to be targeted. In this case, visualization of damaged cells distinguishable from intact cells is the key reason for choosing an immunofluorescence microscopy as the technique to analyze treated areas after plasma irradiation.

In order to explore an effective area of plasma irradiation, the ratio of counted cells with DSB versus total cells is plotted in terms of spatial parameters for the cases of four different plasma treatment durations. The horizontal axis in Figs. 4(a)–4(d) indicates both x direction from left to right (black solid curve) and y direction from bottom to top (red dashed curve) relative to the central place on the slide as a zero point (Fig. 1(c)). There has been a difficulty to fix the plasma tip at the marked dot accurately when conducting measurements, thus, the highest ratio of the cells with DSB for each plot appears not necessarily at 0 mm position (e.g., an actual irradiation center is shifted to the top right region for 10 s plasma irradiation in Fig. 4(a)).

The trends of all curves suggest a lower cell number with DSB farther from the irradiation center. As it is seen from a comparison of widths for each peak, a longer irradiation exposure contributes to a wider width, indicating larger plasma effective areas. Since the diameter of the plasma jet tip is very small, the damaging effects acting on distant locations from the center is not likely due to direct interactions of APPJ with cells. Rather, the damaging effects are likely due to secondary interactions as a result of diffusion of reactive radical species and electrons generated in APPJ, followed by complex chemical reactions activating cells to cause DNA DSB. This assumption is also supported by the work of Laroussi group, who found a similar effect of plasma acting on the bacteria: a wider inactivation area of E. coli cells was observed with a longer plasma treatment.

The ratio of cells with DSB as a function of irradiation time at the central marked spot on the slide is presented in Fig. 5. It has been observed that only 30 s irradiation can induce about 60% DSB damage, which indicates relatively fast chemical reactions and biological processes leading to DSB initiated by the reactive species from APPJ. After 30 s, the damage fraction appeared slightly increasing and reaches nearly 80% at 2 min that is a high efficiency of cell damage by APPJ.

The increase of effective areas and a number of DSB with plasma irradiation time can be due to the fact that the reactive species have been continually accumulating on a
surface at the plasma-liquid interface. Due to a low penetration of plasma (~a few μm in liquids), products below “plasma-liquid interface” are mainly originated from those at the interface and lead to a cascade liquid-based chemistry. Since all our samples were incubated for 1 h after various plasma treatments, the concentration of these reactive species at the plasma-liquid interface mainly determines the amount of cells with DSB damage. Among these species, NO is generally believed to be an essential factor in a variety of biological processes. Though NO has the inclination of favoring cell survival and resisting apoptosis, it is only the case for a low concentration of NO. When its concentration is higher, NO radicals play an important role in cell-cycle arrest or apoptosis. Interestingly, it is true not only for NO but also for other ROS/RNS in terms of a similar manner: if an elevated level of the reactive species is kept for a long time, it is more likely that impairment will occur. It has been reported that the initiation of cell proliferation and survival pathways favor a relatively low concentration of ROS, while cell death will occur in the high level condition. Moreover, the sensitivity of cancer cells to cellular ROS is higher than normal cells. When ROS concentration is elevated, antioxidants “in reserve” regulate the balance between generation and elimination of ROS to keep the ROS concentration from reaching the threshold for inducing cell death. However, lacking of this excess antioxidant capacity in cancer cells result in more vulnerable behavior like inducing further oxidative stress, where the threshold can be easily reached and the cells would be forced to perform apoptosis.

Due to high reactivity of ROS generated upon irradiation, the conversion from oxidative stress signals into nitrosative signals has also been observed. It has been proposed that ROS are initiators but NO or other derivatives are the subsequent effectors for the signaling activation.

Investigations on diffusion distances of ROS/RNS and their lifetimes below the “plasma-liquid interface” would be essential for better understanding of processes induced by APPJ interactions with liquids and cells and need to be studied further.

In conclusion, the effectiveness of a nitrogen atmospheric pressure plasma jet for inducing damage of oral cancer cells was explored in terms of both spatial and time factors. By using an immunofluorescence assay, detailed distributions of damaged cells were obtained, indicating possible fast chemical reactions and diffusion process of reactive radicals. A high ratio of cells with DSB obtained in short duration of plasma irradiation supports the potential of plasma

FIG. 5. The ratio of counted cells with DSB as a function of irradiation time at the position of central marked spot with a gas flow control sample (only treated with N₂ gas for 120 s, no plasma ignition) and an untreated control sample (no treatment with either plasma irradiation or gas flow)
cancer therapy using APPJ, which is rich in ROS and RNS formed in the jet and in a liquid. Moreover, the nitrogen APPJ is used to effectively treat cancer cells, which brings additional benefits like lower costs of usage in comparison to APPJ operated with noble gases.

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