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Cold atmospheric-pressure air plasma treatment of C6 glioma cells: effects of reactive oxygen species in the medium produced by the plasma on cell death

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Abstract

An atmospheric-pressure air plasma is employed to treat C6 glioma cells *in vitro*. To elucidate on the mechanism causing cell death and role of reactive species (RS) in the medium produced by the plasma, the concentration of the long-lived RS such as hydrogen peroxide, nitrate, and ozone in the plasma-treated liquid (phosphate-buffered saline solution) is measured. When vitamin C is added to the medium as a ROS quencher, the viability of C6 glioma cells after the plasma treatment is different from that without vitamin C. The results demonstrate that reactive oxygen species (ROS) such as H_2O_2, and O_3 constitute the main factors for inactivation of C6 glioma cells and the reactive nitrogen species (RNS) may only play an auxiliary role in cell death.

Keywords: atmospheric pressure air plasma, reactive species, cell death

(Some figures may appear in colour only in the online journal)

1. Introduction

A cold atmospheric-pressure plasma (CAP) consists of a partially ionized reactive gas at anambient temperature in addition to electrons, positive/negative ions, reactive species, various atoms, excited molecules, and transient electric fields. Suggested as a promising approach to diseases including cancer, various types of CAPs based on different power sources and configurations have been developed because they interact with tissue or cell without a significant temperature increase [1–5]. In particular, the atmospheric-pressure plasma jet is often used to treat cells *in vitro* and *in vivo* [6–10]. It has also been demonstrated that CAPs can induce cell apoptosis or necrosis. Although the exact mechanisms governing the plasma interaction with cells are not fully understood, it is generally agreed that reactive oxygen species (ROS) are potentially detrimental to the cellular metabolism by affecting cell functions such as cell development, growth, survival, as well as tumorigenesis [11–15]. In many studies *in vitro*, the cells are immersed in a liquid like nutrient solution and the penetration depth of the plasma is about ten micrometers [16]. In these cases, neither charged particles nor ROS generated from the plasma can interact directly with the cells. Nevertheless, ROS and RNS in the cell nutrient solution induced by the plasma may play an important role in killing of cancer cells [17]. Kim *et al* have produced evidence that ROS/RNS penetrate the cellular membrane and cause physical changes in the membrane by measuring the changes in the electrical conductivity, lipid oxidation, and morphology [18]. Plasmas generated in air or using air as the working gas can produce

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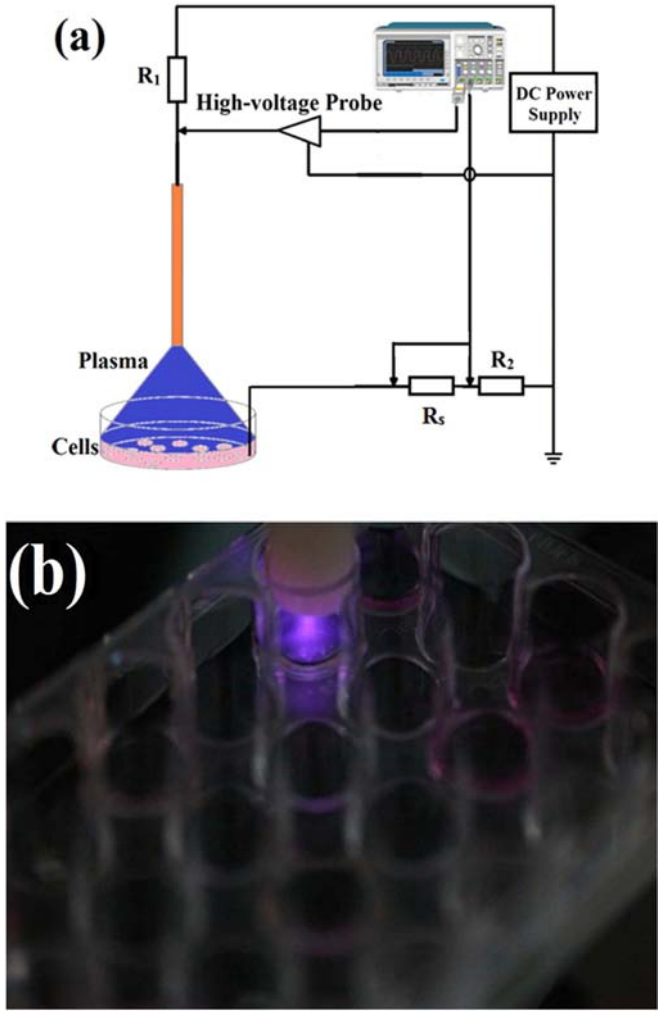

Figure 1. Schematic of the atmospheric-pressure air plasma source and photograph of the air plasma.

large quantities of ROS and RNS in the nutrient solution that seem to be the most efficient biocidal agents in cell apoptosis. Moreover, it is speculated that the plasma changes the ROS/RNS in the nutrient solution which causes cell apoptosis or cell damage. Hence, it is important to investigate the role of RS in the nutrient solution in cell interactions. In this study, an air plasma source is employed to treat C6 glioma cells. The characteristics of the plasma, active species formation in the nutrient solution caused by plasma, and effects of these active species on cells are investigated.

2. Experimental details

2.1. Plasma source

The diagram and photograph of the air plasma device developed in our laboratory are shown in figure 1. The air plasma is driven by a DC high voltage and does not require any external gas feed. Unlike other DC air plasma devices [19–21], the plasma is not generated by one or several fine needles but rather a copper rod. A 2 mm diameter copper rod is connected to the DC supply as a single electrode. The ballast resistors R_1 and R_2 (both 30 MΩ) shown in figure 1 limit the discharge current. Hence, the gas temperature of the plasma source is within 20 °C–30 °C range and it can be touched safely. The voltage and current measurements are performed with a high-voltage probe (P6015A) and sampling resistance (non-inductive R_s in figure 1) via a digital oscilloscope (Tektronix MSO 5104). The discharge peak is less than 10 mA with a repetitive frequency of 10–30 kHz at an input voltage 10 kV. The optical emission from the plasmas is measured using the AvaSpec-2048-8-RM spectrometer with a grating of 2400 grooves/mm.

2.2. Cell culture and plasma treatment

The C6 glioma cells (TCR 1), a rat glioma cell line, was provided by the Chinese Academy of Sciences Committee on a type culture collection cell bank. The C6 glioma cells were cultured in the media supplemented with the Ham's F12K medium (SIGMA Chemical Co) (80%) and the fetal bovine serum (20%) at pH of 7.4 under 5% CO_2 and 95% humidity at 37 °C on 24 well-plates for two or three days. When the cells reached 10^5 per well, the culture medium was removed and washed three times with phosphate-buffered saline (PBS, pH 7.4, Boster, China) solution at 37 °C. PBS was added to the culture plate and the liquid depth was 2.0 mm. The cells immersed in PBS were exposed to the air plasma at a distance of 1.5 cm from the high voltage electrode for different time.

2.3. Measurement of RS in PBS induced by the plasma

RS are produced in liquid inevitably when the air plasma reacts with the PBS. However, the direct measurement of short-lived RS in the liquid is difficult due to the short half-life and high reactivity. In this study, we only measured the concentration of the long-lived RS such as hydrogen peroxide, nitrate, and ozone in the plasma-treated liquid (PBS solution) spectrophotometrically on the PhotoLab 6100 (WTW, Germany). The test kits were 18 789, 09 713, and 00 607, respectively, and the test methods were the same as those described in previous reports [17, 22].

2.4. Antioxidants preventing plasma-induced RS in PBS

As an antioxidant, vitamin C (ascorbic acid, Sigma Chemical Co) is able to inhibit the lipid peroxidation and oxidative DNA damage of cells [23]. In this study, 0.2 mmol l^{-1} of vitamin C was added to the cells immersed in PBS to protect against plasma-induced oxidation before the treatment. After vitamin C was added and mixed 2 min, cells were not washed and then directly treated by plasma. In order to distinguish the effects of different RS on the cells, the concentration of the RS was determined with and without vitamin C.

2.5. Assessment of the viability of C6 glioma cells using Tali viability kit-dead cell red reagent and WST-1 cell proliferation and cytotoxicity assay kit

After the plasma treatment, the C6 glioma cells were stained with 100 μg ml^{-1} of the Tali® dead cell red reagent at room temperature in darkness for 5 min. The Tali® dead cell red reagent only penetrated nonviable cells with damaged/perturbed cell membranes and the cells were examined directly by laser scanning confocal microscopy (Leica, German, TCS SP5).

The viability of the cells treated with the air plasma was assessed by the WST-1 Cell proliferation and cytotoxicity assay kit (Beyotime, Haimen, China) according to the manufacturer's instructions. After plasma treatment for a different time, 10 μl of the reagent were added to each well containing 100 μl of the cell suspension and incubated for an additional 1 h. The absorbance of samples was monitored and the reference wavelength was set at 630 nm on the Rayto RT-2100C microtiter plate reader (Rayto Life and Analytical Sciences Co. Ltd, Shenzhen, PR China). The percent viability of the cells was calculated by comparison with the untreated control cells.

3. Results and discussion

3.1. Reactive species generated and induced in PBS by the atmospheric-pressure air plasma

As a common technique in plasma diagnostics, optical emission spectroscopy (OES) is carried out to study the RS in the air plasma. The typical survey spectra between 200 nm and 900 nm are shown in figure 2. As shown in figures 2(a) and (b), the emission of the second positive system of the nitrogen molecule (N_2(C-B) (300–450 nm) and second order emission from N_2(C-B) (600–800 nm)) are the main contributors in the spectra. The emission bands of the nitric oxide c-system at 200–300 nm (NO (A-X)) as well as reactive atomic oxygen at 777.2 nm and 844.6 nm are also observed from the air plasma. Although these RS generated in the gas-phase plasma cannot directly react with the cells due to the protection rendered by the nutrient solution, they can initiate various chemical reactions at the gas-liquid interface and then form large amounts of primary and secondary RS such as NO_2•, NO•, NO_2^- and NO_3^- in the liquid phase. Nitrogen oxides generated from the air plasma dissolve in the liquid forming nitrites and nitrates in the liquid by the reaction ($_{(aq)}$ means an aqueous species) [24–26]:

$$N_xO_y + H_2O \rightarrow N_xO_{y(aq)} + H_2O. \quad (1)$$

$$N_xO_y = NO_2, NO_3, N_2O_3, N_2O_4, \text{ and } N_2O_5$$

$$HNO_x + H_2O_{(aq)} \rightarrow HNO_{x(aq)} + H_2O_{(aq)} \quad (2)$$

$$HNO_x = HNO_2 \text{ and } HNO_3$$

$$NO_{2(aq)} + OH\bullet \rightarrow HNO_{3(aq)} \quad (3)$$

$$N_2O_{3(aq)} + H_2O \rightarrow HNO_{2(aq)} + HNO_{2(aq)} \quad (4)$$

$$NO_{(aq)} + NO_{2(aq)} + H_2O_{(aq)} \rightarrow HNO_{2(aq)} + HNO_{2(aq)} \quad (5)$$

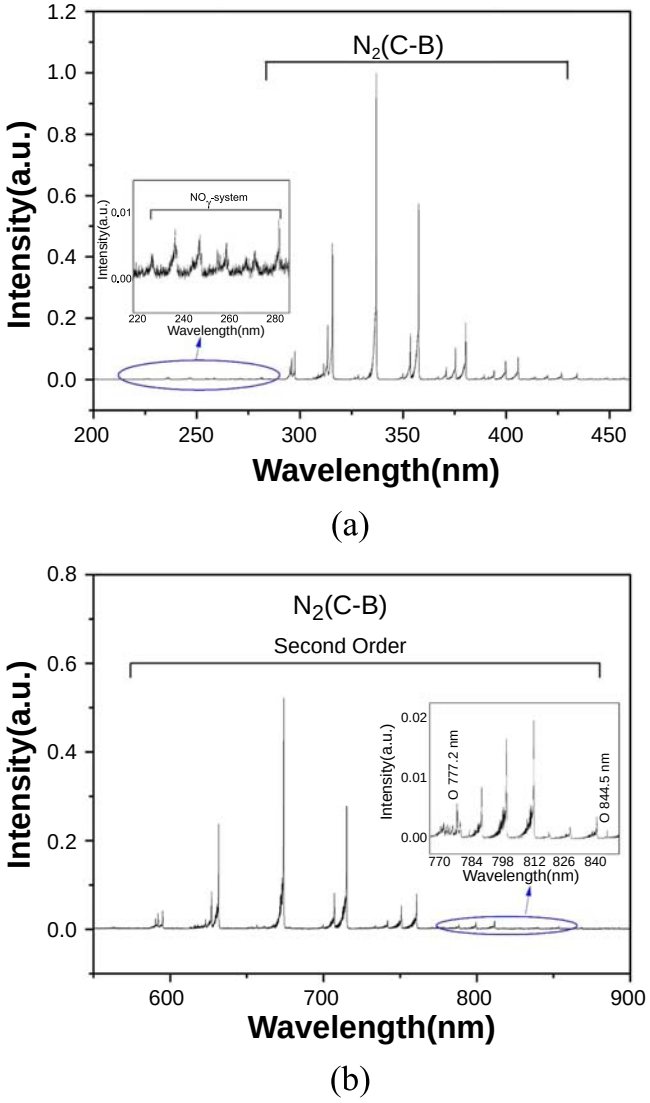

Figure 2. Optical emission spectrum (OES) of the air plasma.

$$NO_{(aq)} + HO_{2\cdot(aq)} \rightarrow HNO_{3(aq)}. \quad (6)$$

Ozone is formed in plasma-treated liquid as follows:

$$O_2 + O\bullet \rightarrow O_3 \quad (7)$$

$$O_{3(gas)} \rightarrow O_{3(aq)}. \quad (8)$$

OH• and H_2O_2 are formed by excitation and/or ionization of water molecules by energetic electrons in the plasma [26–28]:

$$H_2O_{(aq)} + e \rightarrow H\bullet_{(aq)} + OH\bullet_{(aq)} + e \quad (9)$$

$$H_2O_{(aq)} + e \rightarrow H_2O^+_{(aq)} + 2e \quad (10)$$

$$H_2O^+_{(aq)} + H_2O_{(aq)} \rightarrow OH\bullet_{(aq)} + H_3O^+_{(aq)} \quad (11)$$

$$2H_2O_{(aq)} + e \rightarrow H_2 + H_2O_{2(aq)} + e \quad (12)$$

$$OH\bullet_{(aq)} + OH\bullet_{(aq)} \rightarrow H_2O_{2(aq)}. \quad (13)$$

It is difficult to detect the concentration of short-lived reactive species in the plasma-treated liquid (PBS) due to the short half-life and high reactivity. However, the presence of the long-lived reactive species in the aqueous phase can provide indirect proof of the existence of short-lived reactive species

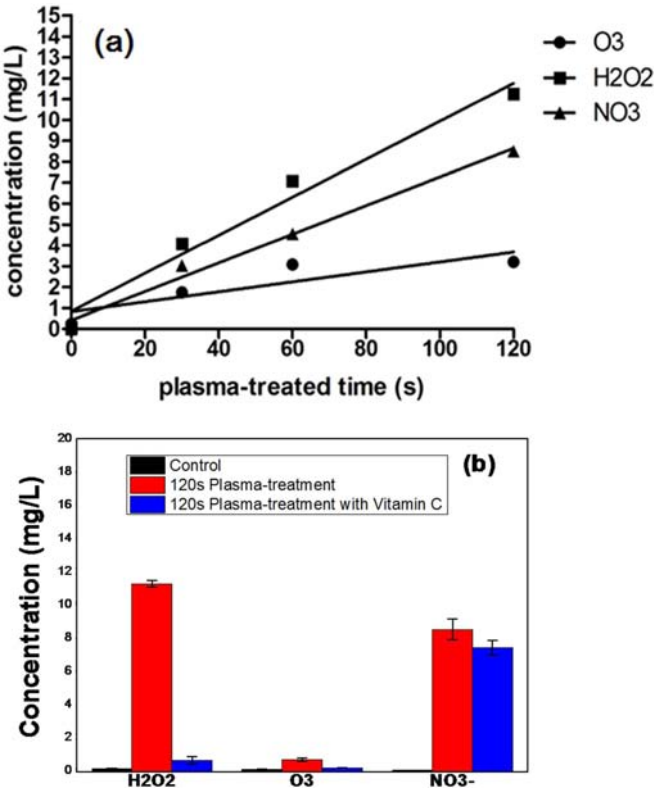

Figure 3. (a) Concentrations of RS in the PBS as a function of treatment time between 0 and 120 s and (b) concentrations of RS in the PBS with and without vitamin C after plasma exposure for 120 s.

according to the aforementioned reactions (1–13). Figure 3(a) shows the concentrations of hydrogen peroxide, nitrate ion, and ozone in the PBS after the air plasma treatment. The RS concentrations increase with plasma exposure time in the initial 60 s and then ozone approaches saturation. The hydrogen peroxide, nitrate ion, and ozone concentrations reach 11.25 mg l^{-1}, 8.5 mg l^{-1}, and 0.6 mg l^{-1}, respectively, after 120 s. When vitamin C is added to the PBS, the hydrogen peroxide and ozone concentrations decrease sharply to 0.6 mg l^{-1} and 0.16 mg l^{-1} respectively, but the nitrate ion concentration decreases by less than 13% to 7.4 mg l^{-1} (shown in figure 3(b)).

3.2. Change in cell inactivation and damage after plasma treatment

The C6 glioma cells are stained with the Tali® dead cell red reagent which is a ready-to-use solution of propidium iodide (PI). PI is impervious to live cells, but easily enters dead cells and stains them with red fluorescence upon binding with nucleic acids consequently increasing the fluorescence by 20 to 30 folds. As shown in figure 4, the control C6 glioma cells are transparent without showing fluorescence, but there are red-fluorescent cells after the plasma treatment, indicating that the cells become nonviable and the cell membranes are damaged by the plasma. The plasma cannot act directly on the cells in the PBS solution and so it is reasonable to assume that the ROS/RNS produced by the plasma in PBS render the cells nonviable and damaged. To confirm it, we analyze the

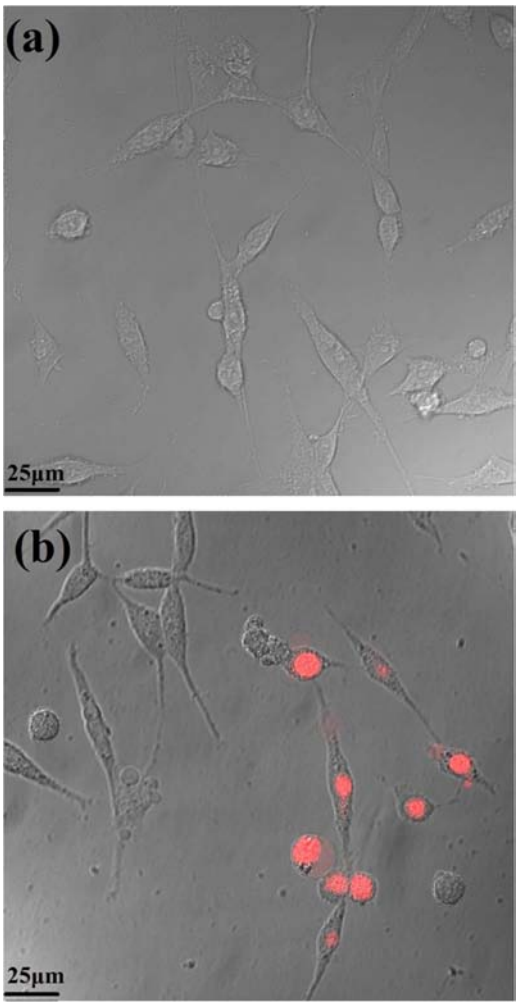

Figure 4. Representative photomicrographs of the C6 Glioma cells stained with PI fluorescent dye before and after exposure to the air plasma.

confocal images of the stained cells after plasma treatment for different time with and without addition of vitamin C. Figures 5(a) to (e) show a gradual increase in the red fluorescence with plasma treatment time without vitamin C, and almost all cells become red after plasma exposure for 120 s. However, when the cells are exposed to the plasma in the presence of vitamin C for 120 s, significant reduced red fluorescence is observed from figure 5(f) compared to figure 5(e). Hence, vitamin C produces protective effects against oxidation caused by RS in the PBS and RS plays a major role in cell inactivation and damage.

The viability of the C6 glioma cells is examined by two methods. The first one is to compare the number of dead cells stained by PI to the total number of cells to derive the cell death rate. We captured up to 20 images (i.e., fields of view) per sample and analyzed the images cell counting and fluorescence detection algorithms. Cell viability (%) = (total number of cells observed − PI stained cells)/the total number of cells observed × 100%. The second one is by using the 'WST-1 Cell Proliferation and Cytotoxicity Assay Kit'. WST-1 is reduced by dehydrogenases in cells to give an orange colored product (formazan), which is soluble in the

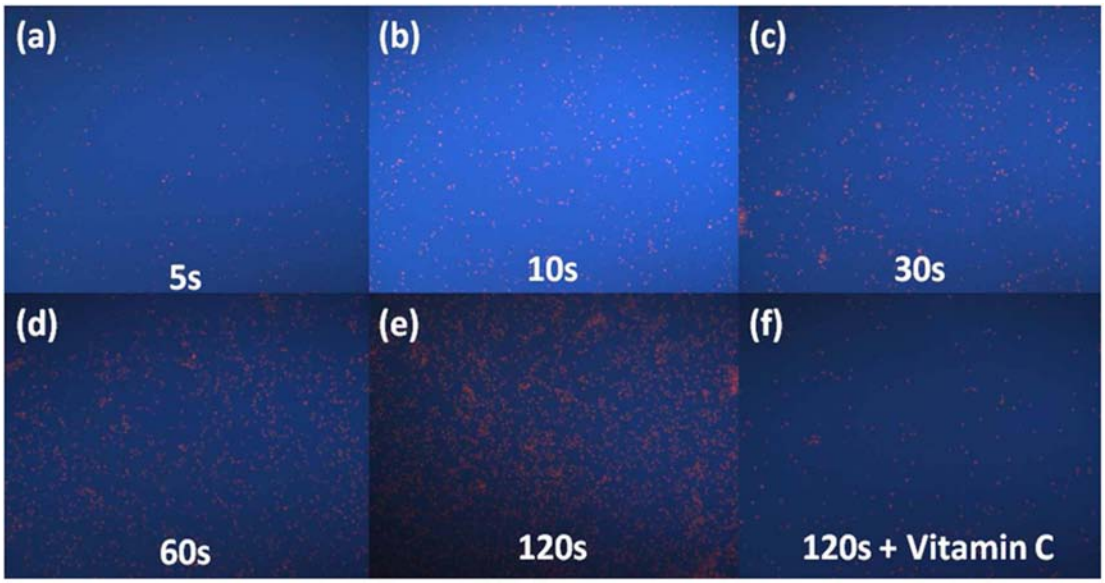

Figure 5. Photomicrographs of the C6 Glioma cells stained with the PI fluorescent dye after air plasma exposure for different times.

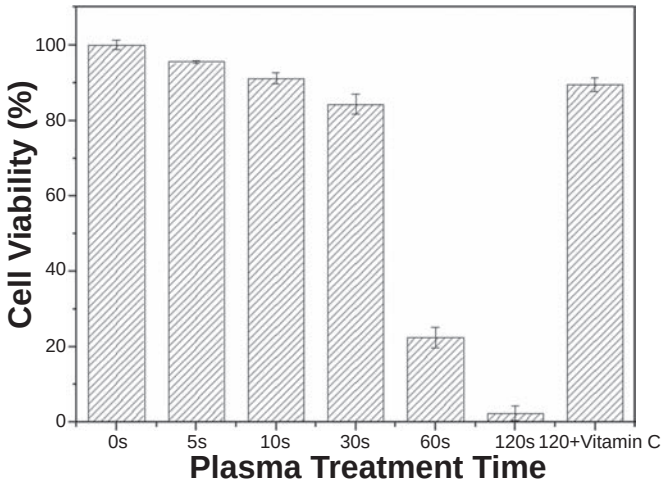

Figure 6. Effects of the plasma on the viability of C6 Glioma cells.

tissue culture medium. The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells. The detection sensitivity using WST-1 is higher than assays using other tetrazolium salts such as MTT. Cell viability (%) = [(sample OD − blank OD)]/(control OD − blank OD)] × 100%. In this study, the OD values were 0.28–0.70. The results from the two methods are generally consistent and so only the results obtained by the second method are shown here. Figure 6 shows that the viability of C6 glioma cells exposed to the air plasma for 5 s, 10 s, 30 s, 60 s, 120 s, and 120 s with vitamin C. The survival ratios are 95.49%, 91.07%, 84.24%, 22.53%, and 2.36%, respectively, at the respective time points without addition of vitamin C. In comparison, after plasma exposure for 120 s, only 10% of the cells are nonviable after vitamin C has been added to the PBS.

In this study, because the C6 glioma cells are immersed in the PBS solution, the plasma cannot affect the cells directly due to the limited penetration depth. However, the plasma can initiate chemical reactions at the gas-liquid phase forming some short-lived or long-lived RS in the PBS. As shown by equations (1) to (13), the plasma interacts with H_2O to form OH• and H_2O_2 as ROS in the PBS and dissolution of nitrogen oxides generated by the air plasma in the PBS leads to formation of nitrates (NO_3^-) and nitrates (NO_2^-). Some previous studies [4, 13, 18, 26, 29–31] have shown that these ROS/RNS in the liquid produced by the plasma are important intermediates in biological reactions, but it is not clear what exact roles these ROS/RNS play in cell apoptosis. As shown in figure 3, the concentrations of ROS (H_2O_2 and O_3) and RNS (NO_3^-) in the PBS increase with plasma treatment time reaching the maximum at 120 s. Accordingly, the viability of the C6 glioma cells decreases gradually without vitamin C and after plasma exposure for 120 s, the viability drops to less than 3% as shown in figure 6. Figure 5 shows that the number of cells producing red fluorescence increases with plasma treatment and they are almost all red after plasma exposure for 120 s without vitamin C. The results suggest that cell

inactivation and damage are closely related to the increase in the RS concentration in the PBS. As a polar compound with a relatively large molecular weight, vitamin C cannot readily cross the cell membrane by simple diffusion. The flux of vitamin C in and out of the cell is controlled by specific mechanisms, including facilitated diffusion and active transport. In two minutes for mix, vitamin C was not taken up by the cells but remained in the supernatant. At physiological concentrations, vitamin C is a potent free radical scavenger, protecting cells against oxidative damage caused by ROS. The antioxidant property of vitamin C is attributed to its ability to reduce the formation of potentially damaging ROS, so in this study vitamin C was selected to act as a ROS quencher. As shown in figures 5 and 6, after vitamin C is added to the PBS, cell inactivation and damage are obviously abated due to the antioxidant effects rendered by vitamin C against RS in the PBS. In this study, the concentrations of H_2O_2 and O_3 in the plasma-treated PBS decrease significantly after addition of vitamin C (figure 3) but the concentrations of nitrates only decrease slightly. It may be because vitamin C reacts with OH• and O• (equations (7) and (9)) to prevent the creation of H_2O_2 and O_3, or vitamin C reacts with H_2O_2 and O_3 directly to consume these two ROS. Nonetheless, vitamin C does not seem to damage the precursor of nitrates (NO_3^-) and hence, the ROS in the PBS produced by the plasma is mainly responsible for cell apoptosis and damage, whereas the RNS only plays an auxiliary role in cell death. Xu *et al* have reported that OH radicals in the plasma-treated medium can induce cell death [32] and suggested that even though the highly reactive OH has a very short lifetime on the order of nanoseconds, it can reach the cells by the Haber-Weiss reaction ultimately causing cell damage and death. In our experiments, OH may be produced by the following reactions:

$$H_2O_{2(aq)} + O_{3(aq)} + OH^-_{(aq)} \rightarrow OH^{\bullet}_{(aq)} + HO_2{}^{\bullet}_{(aq)} + O_{2(aq)} + OH^-_{(aq)} \quad (14)$$

$$H_2O_{2(aq)} \rightarrow OH^{\bullet}_{(aq)} + OH^{\bullet}_{(aq)}. \quad (15)$$

Because H_2O_2 and O_3 have long lifetime, a certain concentration of OH can exist for a long time in the plasma-treated PBS. Therefore, the OH radical can influence the cell viability but the exact contributions require further studies.

4. Conclusion

The effects of RS produced by an atmospheric-pressure air plasma on C6 glioma cells are investigated. The concentrations of H_2O_2, O_3, and NO_3^- in the plasma-treated medium were determined and the concentrations of these RS increase with plasma treatment time. After addition of vitamin C, the H_2O_2 and O_3 concentrations decrease sharply, but the NO_3^- concentration only decreases by less than 13%. Our results show that the viability of C6 glioma cells is related to the concentration of ROS (H_2O_2 and O_3) while RNS (NO_3^-) only plays an auxiliary role in cell death.

Acknowledgments

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References

[1] Yan X *et al* 2012 *Plasma Process. Polym.* **9** 59
[2] Keidar M *et al* 2011 *British Journal of Cancer* **105** 1295
[3] Arjunan K P *et al* 2012 *J. R. Soc. Interface* **9** 147
[4] Sato T, Yokoyama M and Johkura K 2011 *J. Phys. D: Appl. Phys.* **44** 372001
[5] Xu Z *et al* 2015 *Plasma Process. Polym.* **12** 827
[6] Li W *et al* 2016 *Sci. Rep.* **6** 19720
[7] Hou J *et al* 2015 *BMC Genomics* **16** 435
[8] Lin A *et al* 2015 *Plasma Process. Polym.* **12** 1117
[9] Shi X *et al* 2016 *Plasma Sci. Technol.* **18** 353
[10] Joh H M *et al* 2012 *Appl. Phys. Lett.* **101** 053703
[11] Kalghatgi S *et al* 2011 *PLoS One* **6** e16270
[12] Balzer J *et al* 2015 *PLoS One* **10** e0144968
[13] David B G 2012 *J. Phys. D: Appl. Phys.* **45** 263001
[14] Priya A K and Morss C A 2011 *Plasma Process. Polym.* **8** 1154
[15] Kim S J, Joh H M and Chung T H 2013 *Appl. Phys. Lett.* **103** 153705
[16] Xiong Z *et al* 2011 *Appl. Phys. Lett.* **98** 221503
[17] Shen J *et al* 2015 *Plasma Process. Polym.* **12** 252
[18] Kim K *et al* 2014 *Appl. Phys. Lett.* **104** 013701
[19] Wu S *et al* 2011 *IEEE Trans. Plasma Sci.* **39** 2292
[20] Pei X *et al* 2012 *J. Phys. D: Appl. Phys.* **45** 165205
[21] Xu Z *et al* 2015 *Appl. Phys. Lett.* **106** 213701
[22] Zhang H *et al* 2015 *Sci. Rep.* **5** 10031
[23] Joshi S G *et al* 2011 *Antimicrob. Agents Chemother.* **55** 1053
[24] Machala Z *et al* 2013 *Plasma Process. Polym.* **10** 649
[25] Tian W and Kushner M J 2014 *J. Phys. D: Appl. Phys.* **47** 165201
[26] Liu Z C *et al* 2015 *J. Phys. D: Appl. Phys.* **48** 495201
[27] Van G *et al* 2013 *J. Phys. D:Appl. Phys.* **46** 175203
[28] Du C, Sun Y and Zhuang X 2008 *Plasma Chem. Plasma Process.* **28** 523
[29] Tresp H *et al* 2013 *J. Phys. D: Appl. Phys.* **46** 435401
[30] Winter J *et al* 2014 *J. Phys. D: Appl. Phys.* **47** 285401
[31] Kurake N *et al* 2016 *Arch. Biochem. Biophys.* **605** 102e108
[32] Xu D *et al* 2015 *PLoS One* **10** e0128205