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NO$_2^-$ and NO$_3^-$ enhance cold atmospheric plasma induced cancer cell death by generation of ONOO$^-$

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Cold atmospheric plasma (CAP) is a rapidly developed technology that has been widely applied in biomedicine especially in cancer treatment. Due to the generation of various active species in plasma, CAP could induce various tumor cells death and showed a promising potential in cancer therapy. To enhance the biological effects of gas plasma, changing the discharging parameters is the most commonly used method, yet increasing discharging power will lead to a higher possibility of simultaneously damage surrounding tissues. In this study, by adding nontoxic concentration of additional nitrite and nitrate in the medium, we found that anti-tumor effect of CAP treatment was enhanced in the same discharging parameters. By microplate reader and cell flow cytometer we measured several extracellular and intracellular RONS and found that ONOO$^-$ was mostly correlated with the enhanced cancer cell killing effect. We proposed that more nitrogen supplies such as nitrite and nitrate could increase the production of RNS especially ONOO$^-$ and resulted in a better killing effect to cancer cells. Our results provided a new strategy to enhance the antitumor effect by plasma jet treatment without changing the discharging parameters. © 2018 Author(s).

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I. INTRODUCTION

Plasma, which is often regarded as the fourth state of material besides solid, liquid and gas, is mainly consisted of free electron and charged ions. The emergence of cold atmosphere plasma (CAP) makes it possible to study the influence on cells and biological tissues due to its generation under atmospheric pressure at low temperature.$^{1,2}$ Thus, plasma medicine has become a hot topic in interdisciplinary courses recently, including sterilization, dentistry, cosmetology, wound healing, dermatology application and cancer therapy.$^{3-5}$ CAPs could efficiently induce various tumor cells death, and by far it is considered that the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in plasma are the main factors.$^6$ Among these species, hydroxyl radical (OH), hydrogen peroxide (H$_2$O$_2$), ozone (O$_3$), superoxide anion (O$_2^-$), nitric oxide (NO), and peroxynitrite anion (ONOO$^-$) are the main components related to biological effects induced by CAPs.$^5-9$ Hirst AM et al. reported that H$_2$O$_2$ formed in the medium after CAP treatment was a facilitator of DNA damage and cell death.$^{10}$ Naresh Kumar et al. found that deuterium oxide (DO) generated from CAP

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could induce apoptosis on melanocytes G361 tumor cells via DNA damage signaling cascade.\textsuperscript{11} Yan X et al. showed that plasma induced NO accumulation resulted in more effective lipid peroxidation and finally, HepG2 cell death.\textsuperscript{7} Our previous work found that $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ induced by CAP could convert to the highly reactive OH radical with the presence of iron containing proteins, thus resulting in myeloma cell death.\textsuperscript{9} It is reported that RNS was also important in the plasma induced cell death. Panngom K et al. found that the cytotoxicity to human lung cancer cells was strongly mediated by the large amount of $\text{H}_2\text{O}_2$ and NOx in medium generated by dielectric barrier discharge (DBD) plasma.\textsuperscript{8} ONOO$^-$ is a potent oxidizing and nitrating specie formed from a diffusion-controlled reaction between $\text{O}_2^-$ and NO, which could penetrate bilayer lipid membrane and disturb the function of mitochondrion and consequently influence cell metabolism and cause DNA damage leading to cell death.\textsuperscript{12-14} Our previous study also demonstrated the involvement of ONOO$^-$ in the induction of apoptosis by $\text{N}_2/\text{O}_2$ plasma jet.\textsuperscript{15} In normal condition, RNS was produced by plasma mostly through the miscellaneous air in the ambient environment. In order to give more nitrogen supplies for better production of RNS, we added additional nitrite and nitrate in the plasma interaction system and found that it had a better toxic effect in tumor cells. Our results provided a new strategy to enhance the killing effect in tumor cells by plasma jet treatment.

II. MATERIALS AND METHODS

A. CAP generation system

The CAP used in this study was generated by a plasma jet system consisted of a gas flow controller, high-voltage power supply, oscilloscope and plasma jet device. A gas flow of 2 slm He was used at voltages of 10 kHz/8 kV for He plasma generation. Applied voltage, current and average power were monitored real-time to ensure the stability of the gas plasma.

B. Cell culture and plasma treatment

We utilised the LP-1 multiple myeloma cell\textsuperscript{16} and Molm-13 human acute myeloid leukemia cell lines in this study. These cell lines were all grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal calf serum, 100 U/mL penicillin, and 50 µg/mL streptomycin (Corning, Ithaca, NY, USA). Cells were cultured at 37 °C in an incubator (Thermo Scientific Varioskan Flash, Waltham, MA, USA) containing 5 % CO$_2$. Cells were refreshed 24 h before performing experiments. For plasma treatment, $2 \times 10^5$ cells were cultured in 24 well plates in 300 µL RPMI1640 complete medium and were treated with plasma jet 1.5 cm away from the bottom of the plates. After treatment, cells were continually cultured for further experiments.

C. Optical emission spectroscopy

The emission spectra of the plasma was measured using a UV/Visible spectrometer (Maya pro 2000, Ocean Optics, China) within a wavelength range of 200-800 nm. The emission spectra of He plasma was analyzed in the vertical direction 2 cm in front of the plasma jet.

D. Cell viability assay

The effects of plasma on cell viability of LP-1 and Molm-13 cells were investigated using the CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA), which was based on the production of ATP in viable cells. CellTiter-Glo® Buffer was mixed with CellTiter-Glo® Substrate to form the CellTiter-Glo® Reagent before experiment. 100 µL of cell suspension and 100 µL of CellTiter-Glo® Reagent were added to a 96-well opaque plate, then the plate was incubated at room temperature for 10 min after mixing for 2 min on an orbital shaker. The luminescence was determined using the microplate reader (Thermo Scientific) with the protocol of “luminometric” measurement.

E. Measurements of ROS and RNS

Fluorescent probes were used to detect the relevant ROS and RNS in our experiments. DAF-FM DA (Beyotime Institute of Biotechnology) was used for NO detection,\textsuperscript{17} Dihydroethidium (DHE,
Beyotime) for \( \mathrm{O}_2^- \) detection\(^{18} \) and Coumarin Boronic Acid (CBA) for ONOO\(^-\).\(^{19} \) Cells were treated with plasma jet for different durations and the extracellular and intracellular ROS and RNS were measured 6 h after treatment by adding the fluorescent probes according to the instructions. Cells were harvested and the suspension was used to detect extracellular concentration by a microplate reader (Thermo Scientific) with excitation/emission at 495/515 nm for NO, 535/610 nm for \( \mathrm{O}_2^- \) level and 332/410 nm for ONOO\(^-\). Cells were treated with plasma jet for different durations and the extracellular and intracellular ROS and RNS were measured 6 h after treatment by adding the fluorescent probes according to the instructions. Cells were harvested and the suspension was used to detect extracellular concentration by a microplate reader (Thermo Scientific) with excitation/emission at 495/515 nm for NO, 535/610 nm for \( \mathrm{O}_2^- \) level and 332/410 nm for ONOO\(^-\) using the protocol of “Fluorometric” measurement. The cell pellets were resuspended in 400 \( \mu \)L PBS after washing with 1 mL PBS for 3 times and intracellular fluorescence was detected by flow cytometry (BD, C6, Franklin Lakes, NJ, USA) with green fluorescence channel (FL1) for NO, ONOO\(^-\) and red fluorescence channel (FL3) for \( \mathrm{O}_2^- \) level. In addition, peroxynitrite was purchased from Cayman Chemical (Michigan, Ann Arbor, USA), which could produce ONOO\(^-\) and usually supplied as a solution in 3 M NaOH, to investigate the effect of ONOO\(^-\) in cancer cells.

**F. ROS and RNS scavengers**

Several ROS and RNS scavengers were used to distinguish the component efficacy in the plasma. These scavengers were purchased from Sigma-Aldrich (St. Louis, MO, USA), mainly including CPTIO for NO;\(^{20} \) Tiron for \( \mathrm{O}_2^- \);\(^{21} \) Ebselen for ONOO\(^-\);\(^{22,23} \) and N-acetyl cysteine (NAC) as a general ROS scavenger.\(^{24,25} \) Scavengers were added prior to plasma treatment to guarantee their effectiveness at a final concentration of 100 \( \mu \)M for NO; 10 mM for Tiron; 100 nM Ebselen for ONOO\(^-\) and 10 \( \mu \)M for NAC.

**G. Statistical analysis**

All values were presented as mean ± SD of three independent experiments. Differences between controls and treated groups were evaluated using the Mann-Whitney U test. \( P < 0.05 \) was considered statistically significant.

**III. RESULTS**

**A. Characteristics of He plasma generation**

He plasma was generated at the voltage of 10 kHz/8 kV with a He gas flow of 2 SLM. Fig. 1(a) shows the structure of the plasma jet device and photograph of He plasma. Fig. 1(b) shows the corresponding applied voltage, current and average power during He plasma generation. To investigate the different reactive species in the plasma, we used a spectrometer to measure the emission spectra. There were several spectral lines (e.g., OH (A) 309 nm, \( \mathrm{N}_2 \) (C) 337 nm, \( \mathrm{N}_2^+ \) (B) 391 nm) presented in the plasma as shown in Fig. 1(c).

**B. Cytotoxicity of \( \mathrm{NaNO}_2 \), \( \mathrm{NaNO}_3 \) and their synergies with plasma on tumor cells**

Myeloma LP-1 and leukemia Molm-13 tumor cells were treated with different doses of \( \mathrm{NaNO}_2 \) and \( \mathrm{NaNO}_3 \), and cell viability was determined after incubation for 24 h and 48 h. Cell viability assay showed that treatment with different doses of \( \mathrm{NaNO}_2 \) (0~1000 \( \mu \)M) had no effect on LP-1 (Fig. 2(a)) and Molm-13 (Fig. 2(b)) tumor cells. Similar results were obtained by \( \mathrm{NaNO}_3 \) (0~1000 \( \mu \)M) treatment in LP-1 (Fig. 2(a)) and Molm-13 (Fig. 2(b)) tumor cells, indicating that neither \( \mathrm{NO}_2^- \) nor \( \mathrm{NO}_3^- \) had a cell cytotoxicity to LP-1 and Molm-13 tumor cells. Next, we added 10 \( \mu \)M and 50 \( \mu \)M of \( \mathrm{NO}_2^- \) and \( \mathrm{NO}_3^- \) (nontoxic concentration) separately prior to He plasma treatment and investigated whether they could enhance the plasma-induced tumor cell death. The results showed that both \( \mathrm{NO}_2^- \) and \( \mathrm{NO}_3^- \) (at two different concentration) could significantly enhance cell viability reduction that was induced by He plasma in myeloma LP-1 cells (Fig. 2(c)). Similar results were found in leukemia Molm-13 tumor cells (Fig. 2(d)) that \( \mathrm{NO}_2^- \) and \( \mathrm{NO}_3^- \) could enhance the cytotoxicity of He plasma treatment. Furthermore, 50 \( \mu \)M of \( \mathrm{NO}_2^- \) and \( \mathrm{NO}_3^- \) showed a better synergistic effect with He plasma than that of 10 \( \mu \)M, indicating that providing more \( \mathrm{NO}_2^- \) and \( \mathrm{NO}_3^- \) may somehow interact with reactive species in the plasma and result in more cell death.
**FIG. 1.** Characteristics of the He plasma. (a) Schematic representation of the plasma jet device and the photograph of He plasma. (b) Monitoring of the applied voltage, current and average power during He plasma generation. (c) Emission spectra of He plasma detected by the spectrometer. Several unique spectral lines (e.g., OH (A) 309 nm, N$_2$ (C) 337 nm, N$_2^+$ (B) 391 nm) in the plasma are marked.

**FIG. 2.** Cytotoxicity of NaNO$_2$, NaNO$_3$ and their synergies with plasma on tumor cells. (a, b) Cytotoxicity of NaNO$_2$ and NaNO$_3$ with different concentration was measured by cell-titer-glo viability assay in myeloma LP-1 cells (a) and leukemia Molm-13 cells (b) after incubation for 24 h and 48 h. (c, d) The combination effects of He plasma treatment for 30 s with nontoxic concentration of NaNO$_2$ and NaNO$_3$ in LP-1 cells (c) and Molm-13 cells (d) determined by cell viability assay 24 h after treatment. n=3, * indicates $P < 0.05$, ** indicates $P < 0.01$. 

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C. Detection of NO and O$_2^-$ levels after plasma treatment and its' combination with NaNO$_2$ and NaNO$_3$

As demonstrated in our previous study, NO and O$_2^-$ are two of the main substrate to produce ONOO$^-$, so we first monitored intracellular and extracellular NO level by He plasma treatment and the interaction with NaNO$_2$ and NaNO$_3$. As shown in Fig. 3(a), NO level was elevated by plasma treatment in LP-1 myeloma cells and in Molm-13 leukemia cells, while ROS scavenger, NAC, could reverse the increasing of the fluorescent intensity. However, the combination of He plasma with NaNO$_2$ and NaNO$_3$ did not increase the intracellular fluorescent intensity compared to He plasma alone (Fig. 3(b)). The extracellular NO level was significantly increased by He plasma treatment for different durations in LP-1 and Molm-13 cells (Fig. 3(c)). These results showed that NO could be produced by plasma in the liquid and further transferred into the cells.

We next detected the O$_2^-$ level by flow cytometer using dihydroethidium dye after plasma treatment in LP-1 and Molm-13 cells. O$_2^-$ level was significantly increased after plasma treatment for 60 s in LP-1 and Molm-13 cells while NAC could block the elevation of O$_2^-$ level (data not shown). Additional of NaNO$_2$ and NaNO$_3$ did not significantly increase the intracellular O$_2^-$ level when treated with He plasma for 30 s (Fig. 3(d)). Extracellular O$_2^-$ level fluorescent intensity was also measured by microplate reader, showed that He plasma treatment for different durations could significantly increase extracellular O$_2^-$ level in LP-1 and Molm-13 cells (Fig. 3(e)).

![Detection of NO and O$_2^-$ concentration by plasma treatment and nitrite addition](image)

**FIG. 3.** Detection of NO and O$_2^-$ concentration by plasma treatment and nitrite addition. (a) The fluorescence of intracellular NO was assayed by flow cytometer 6 h after He plasma treatment for 30 s, 60 s or pretreated with 10 mM NAC in LP-1 and Molm-13 cells. (b) Measurement of intracellular NO levels 6 h after He plasma treatment and its combination with 50 µM of NaNO$_2$ and NaNO$_3$. (c) Extracellular NO levels of LP-1 and Molm-13 cells were detected by microplate reader 6 h after He plasma treatment for 30 s, 60 s and 90 s. NAC group denoted 10 mM of NAC was added prior to 90 s of He plasma treatment. (d) Measurement of intracellular O$_2^-$ levels 6 h after He plasma treatment and its combination with 50 µM of NaNO$_2$ and NaNO$_3$. (e) Extracellular O$_2^-$ levels of LP-1 and Molm-13 cells were detected by microplate reader 6 h after He plasma treatment for 30 s, 60 s and 90 s. NAC group denoted 10 mM of NAC was added prior to 90 s of He plasma treatment. n=3,* indicates P < 0.05. ** indicates P < 0.01.
FIG. 4. Detection of ONOO⁻ concentration and the effects of ROS scavengers on tumor cell viability. (a) ONOO⁻ level was detected 6 h after He plasma treatment for 30 s, 60 s, and 90 s, with or without cells. (b) Measurement of ONOO⁻ level 6 h after combination treatment of He plasma (30 s/60 s) with 50 µM of NaNO₂ and NaNO₃ in LP-1 cells. (c) Dose dependent cell death induced by peroxynitrite in LP-1 and Molm-13 cells. (d, e) Effects of pretreating with scavenger of NO (CPTIO), O₂⁻ (Tiron) and ONOO⁻ (Ebselen) respectively, on the cytotoxicity of LP-1 cells induced by (d) 30 µM of peroxynitrite or (e) 10 s, 30 s and 60 s of He plasma treatment. n=3, ∗ indicates P < 0.05. ** indicates P < 0.05.

D. Measurement of ONOO⁻ concentration and the effect of ROS scavenger on tumor cell viability

We quantified the effects of CAP on cellular ONOO⁻ levels of LP-1, Molm-13 cells and RPMI 1640 medium using coumarin boronic acid pinacolate easter, a redox-sensitive fluorescent probe.²⁷,²⁸ The ONOO⁻ levels were all significantly raised with the increase of CAP treatment time while 10 mM NAC could fully reverse the induction of ONOO⁻ by CAP treatment (Fig. 4(a)). Additionally, ONOO⁻ level induced by CAP was higher in RPMI 1640 medium than in LP-1 and Molm-13 cells. Fig. 4(b) showed that ONOO⁻ level in LP-1 cells was promoted by the addition of NaNO₂ and NaNO₃ compared to 30 s or 60 s of CAP treatment alone. Peroxynitrit, an exogenous ONOO⁻ reagent was used to test the cytotoxicity of ONOO⁻ to cancer cells. Fig. 4(c) demonstrated that cell viability was decreased at a dose dependent manner in LP-1 and Molm-13 cells after peroxynitrite treatment for 24 h. According to the references 20–23, we chose Carboxy-PTIO (CPTIO), Tiron and Ebselen as the inhibitor of NO, O₂⁻ and ONOO⁻ respectively. The outcomes showed that cell viability was decreased after 30 µM of peroxynitrite treatment, and only Ebselen could reverse the effects (Fig. 4(d)). Then, we detected whether the scavenger of NO, O₂⁻ and ONOO⁻ could attenuate CAP-induced tumor cell death. The results showed that 30 s and 60 s of He plasma treatment had a significant reduction on cell viability, however, the reduction could partially be abrogated when pretreated with ROS scavengers especially for Ebselen (Fig. 4(e)).

IV. DISCUSSION

Different ROS and RNS in gas plasma are mostly depended on several aspects, such as: plasma device and structure, applied voltage, frequency, working and feeding gases, and humidity. In order to get a better inactivation of tumor cells by plasma, we could increase the applied voltage or frequency which may directly increase the input power energy. However, the current will simultaneously increase which might be dangerous in the clinical trials. It is reported that additional O₂ in the working gas might enhance the killing effects of gas plasma on tumor cells, yet O₂ is an electric negative gas that will decrease the intensity of the plasma.²⁹,³⁰ Water vapor in the working gas will increase the generation of OH which is important to plasma induced cell apoptosis,⁹ but excess humanity will extinct the discharging and eliminate the plasma. In our experiments, we observed several unique spectral lines (e.g., OH (A) 309 nm, N₂ (C) 337 nm, N₂+ (B) 391 nm) in He plasma, that is because the He plasma
was produced in the ambient air and some nitrogen and water vapor were mixed in the plasma, such as water molecules in the air, which should be the source of OH radicals. Besides, in the medium, we could detect several RONS after He plasma treatment because some of them could directly permeate from the gas phase to the liquid phase. Some of the species could be generated by second reaction of these species and also the interaction with liquid. For example, $O_2^-$ may be produced in air by electron attachment of oxygen molecules due to the negative affinity of oxygen, and ONOO$^-$ may be generated by the reaction from $O_2^-$ and NO. In this study, we tried to enhance the effect of gas plasma by changing the components in the solution. As demonstrated by several groups that ONOO$^-$ is important in plasma induce tumor cell death, we added additional nitrite and nitrate in the medium to produce more RNS. Indeed, ONOO$^-$ could interact with proteins and biological molecular of the cells, and resulted in a lower concentration of ONOO$^-$ level in LP-1 and Molm-13 cells than in RPMI 1640 medium without cells. Furthermore, our results showed that NO$^2^-$ and NO$^3^-$ could enhance the cytotoxicity of He plasma treatment on myeloma and leukemia tumor cells by the accumulation of ONOO$^-$.

Girard et al. reported that NO$^2^-$, acts in synergy with $H_2O_2$ to enhance cell death in normal and tumor cell lines combined with plasma treatment. They assumed that peroxynitric acid can be formed by the interaction of $NaNO_2$ and $H_2O_2$. Peroxynitrite can induce both cellular apoptosis and necrosis depending on the production rates, endogenous antioxidant levels and exposure time. We have already demonstrated that plasma treatment has a selective inactivation of tumor cells compared to normal cells, partly because of tumor cells have a higher expression of CD95, which is a target of plasma treatment. Combined with the previous reports that peroxynitrite has an important role in plasma induced tumor cell death, this work gives a potential application to enhance plasma biological effects without changing the plasma discharging parameters or increasing the input power.

In conclusion, we found that additional supplement of NO$^2^-$ and NO$^3^-$ could enhance the cytotoxicity of He plasma treatment on myeloma tumor cells without reinforcing the gas plasma discharging. In addition, the synergistic effects were mostly mediated by the production of ONOO$^-$.  

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