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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.
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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, dermology 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_2O_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.[7–9] Hirst AM et al. reported that H_2O_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.[11] Yan X et al. showed that plasma induced NO accumulation resulted in more effective lipid peroxidation and finally, HepG2 cell death.[7] Our previous work found that O_2^- and H_2O_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.[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 H_2O_2 and NOx in medium generated by dielectric barrier discharge (DBD) plasma.[8] $ONOO^-$ is a potent oxidizing and nitrating specie formed from a diffusion-controlled reaction between 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.[12–14] Our previous study also demonstrated the involvement of $ONOO^-$ in the induction of apoptosis by N_2/O_2 plasma jet.[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[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, 2×10^5 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,[17] Dihydroethidium (DHE,

Beyotime) for O_2^- detection[18] and Coumarin Boronic Acid (CBA) for $ONOO^-$.[19] Cells were treated with plasma jet for different durations and the extracelluar 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 extracelluar concentration by a microplate reader (Thermo Scientific) with excitation/emission at 495/515 nm for NO, 535/610 nm for O_2^- level and 332/410 nm for $ONOO^-$ using the protocol of "Fluorometric" measurement. The cell pellets were resuspended in 400 μL 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 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 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 μM for NO; 10 mM for Tiron; 100 nM Ebselen for $ONOO^-$ and 10 μM 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, N_2 (C) 337 nm, N_2^+ (B) 391 nm) presented in the plasma as shown in Fig. 1(c).

B. Cytotoxicity of $NaNO_2$, $NaNO_3$ and their synergies with plasma on tumor cells

Myeloma LP-1 and leukemia Molm-13 tumor cells were treated with different doses of $NaNO_2$ and $NaNO_3$, and cell viablity was determinated after incubation for 24 h and 48 h. Cell viability assay showed that treatment with different doses of $NaNO_2$ (0~1000 μM) had no effect on LP-1 (Fig. 2(a)) and Molm-13 (Fig. 2(b)) tumor cells. Similar results were obtained by $NaNO_3$ (0~1000 μM) treatment in LP-1 (Fig. 2(a)) and Molm-13 (Fig. 2(b)) tumor cells, indicating that neither NO_2^- nor NO_3^- had a cell cytotoxicity to LP-1 and Molm-13 tumor cells. Next, we added 10 μM and 50 μM of NO_2^- and 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 NO_2^- and 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 NO_2^- and NO_3^- could enhance the cytotoxity of He plasma treatment. Furthermore, 50 μM of NO_2^- and NO_3^- showed a better synergistic effect with He plasma than that of 10 μM, indicating that providing more NO_2^- and NO_3^- may somehow interact with reactive species in the plasma and result in more cell death.

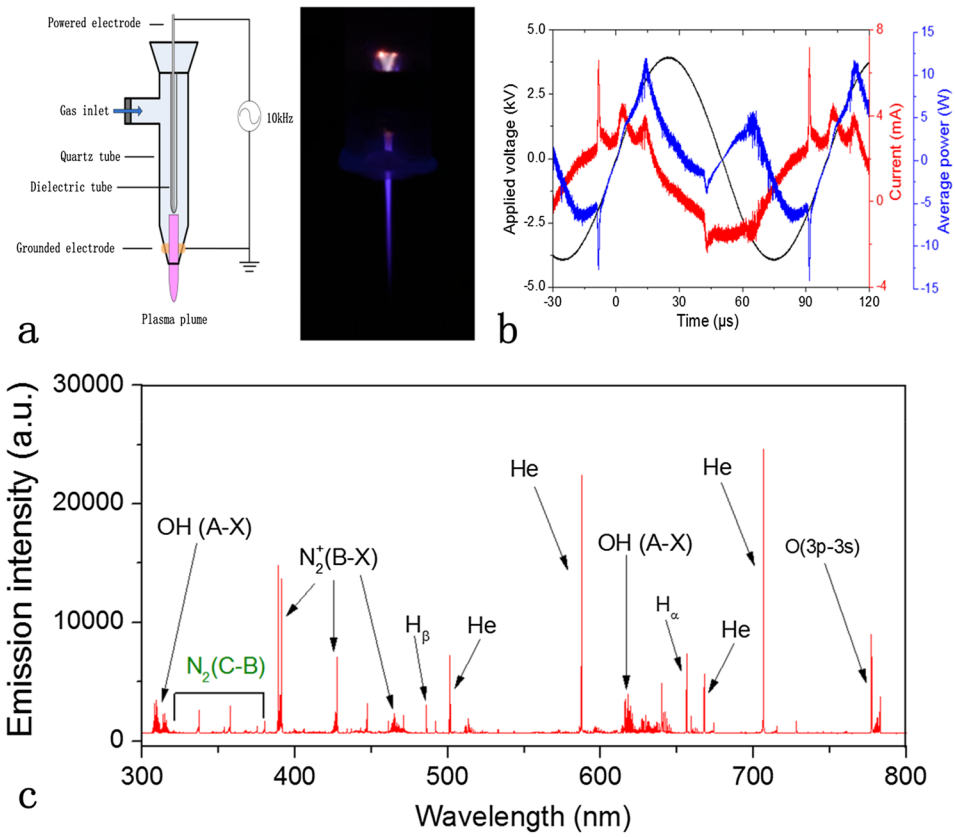

FIG. 1. **Characteristics of the He plasma**. (a) Schematic representation of the plasma jet device and the photograph of He plasma. (b) Monitoring of the applied voltage, current and average power during He plasma generation. (c) Emission spectra of He plasma detected by the spectrometer. Several unique spectral lines (e.g., OH (A) 309 nm, N_2 (C) 337 nm, N_2^+ (B) 391 nm) in the plasma are marked.

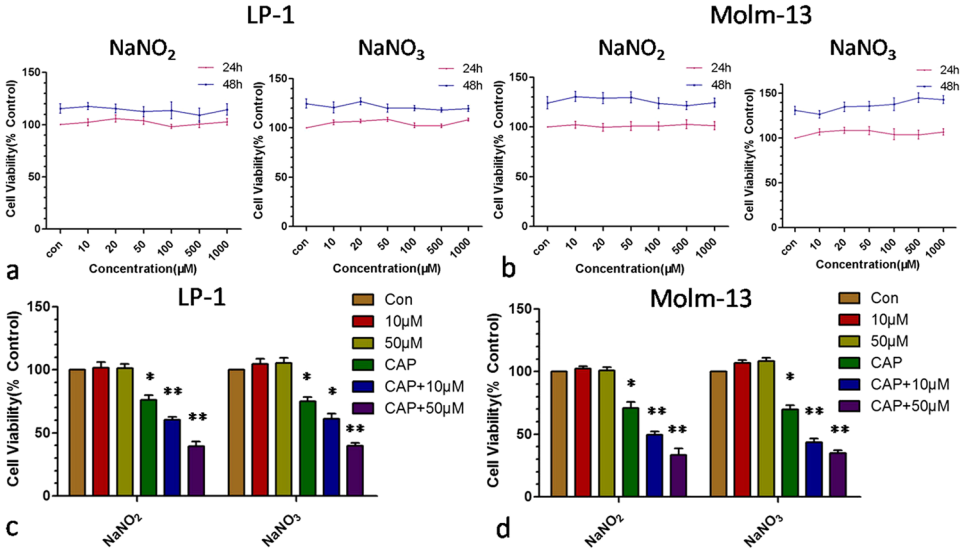

FIG. 2. **Cytotoxicity of $NaNO_2$, $NaNO_3$ and their synergies with plasma on tumor cells**. (a, b) Cytotoxicity of $NaNO_2$ and $NaNO_3$ with different concentration was measured by cell-titer-glo viability assay in myeloma LP-1 cells (a) and leukemia Molm-13 cells (b) after incubation for 24 h and 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 $P < 0.01$.

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 $ONOO^-$,[26] 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 scanvenger, 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)).

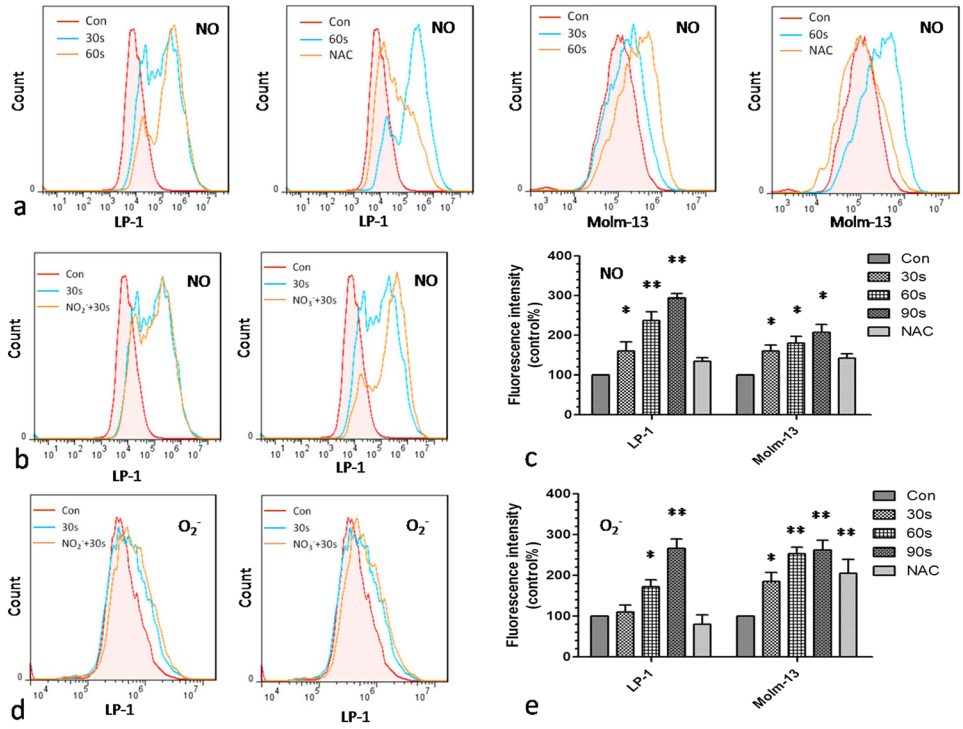

FIG. 3. **Detection of NO and O_2^- concentration by plasma treatment and nitrite addition**. (a)The fluorescence of intracellular NO was assayed by flow cytometer 6 h after He plasma treatment for 30 s, 60 s or pretreated with 10 mM NAC in LP-1 and Molm-13 cells. (b) Measurement of intracellular NO 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 $P < 0.01$.

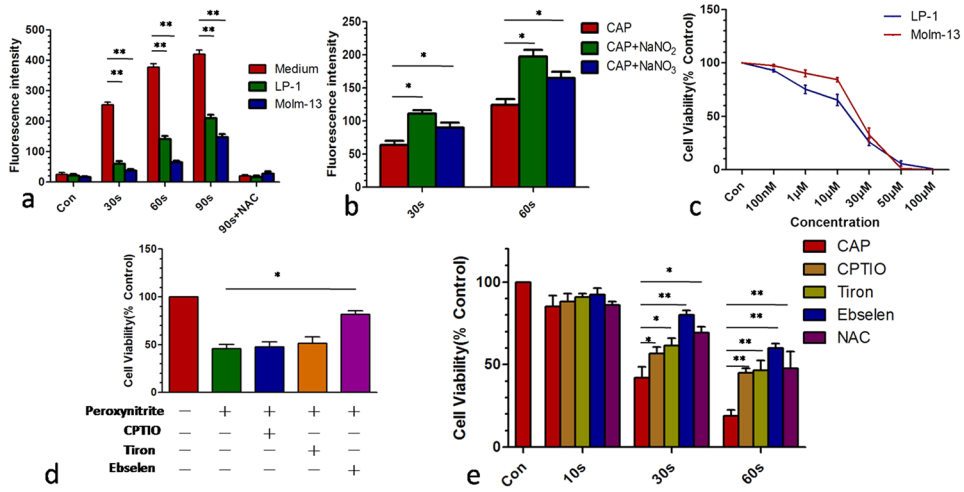

FIG. 4. **Detection of ONOO⁻ concentration and the effects of ROS scavengers on tumor cell viability**. (a) $ONOO^-$ level was detected 6 h after He plasma treatment for 30 s, 60 s, and 90 s, with or without cells. (b) Measurement of $ONOO^-$ level 6 h after combination treatment of He plasma (30 s/60 s) with 50 μM of $NaNO_2$ and $NaNO_3$ 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), O_2^- (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 $NaNO_2$ and $NaNO_3$ 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, O_2^- 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, O_2^- 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 O_2 in the working gas might enhance the killing effects of gas plasma on tumor cells, yet O_2 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, N_2 (C) 337 nm, N_2+ (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 cytotoxity 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.[31] 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.[32] 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.[33] Combined with the previous reports that peroxynitrite has an important role in plasma induced tumor cell death,[15,34] 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 cytotoxity 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 $ONOO^-$.

ACKNOWLEDGMENTS

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