PAPER

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Cold plasma-based control of the activation of pancreatic adenocarcinoma cells

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Abstract

Cold atmospheric plasma (CAP) has shown strong anti-cancer capability *in vitro* and *in vivo*. The interaction between CAP and cancer cells build the foundation to understand the anti-cancer effect of a direct CAP treatment. The CAP-based activation phenomenon is a key factor to understand the CAP cancer treatment. The CAP-treated pancreatic adenocarcinoma cells instantaneously entered a specific activation state, in which these cancer cells become very sensitive to the cytotoxicity of both reactive oxygen species and reactive nitrogen species. Here, we present a roadmap of control the basic operational parameters of the CAP jet (the helium flow rate, the discharge voltage, and the discharge frequency) on the activation. Among three parameters, the discharge voltage shows the largest impact on the activation phenomenon. The maximum activation effect occurred when the discharge voltage reached a medium level. A 0D chemical simulation revealed that such maximum activation effect may be due to the maximum densities of several short-lived reactive species in CAP jet at a certain level of discharge voltage.

Keywords: cold plasma, activation state, cancer treatment

Supplementary material for this article is available online

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

Introduction

CAP is a promising anti-cancer method, which has shown strong anti-cancer capability *in vitro* and *in vivo* [1–4]. Understanding the interaction between CAP and cancer cells is a core step to reveal the anti-cancer mechanism of CAP treatment, which will further guide the design of new CAP devices. The important role of the CAP-originated reactive species including ROS and RNS has been well studied and established [5–7]. These reactive species at least can be divided into two groups, the short-lived reactive species $OH^{\cdot}$ and the long-lived reactive species such as H_2O_2 and NO_2^-. Over the past decade, the long-lived reactive species particularly H_2O_2 have been regarded as the key anti-cancer factor during the CAP treatment. However, just the long-lived reactive species such as H_2O_2 and NO_2^- in the extracellular environment cannot explain the many observed phenomena in plasma medicine, particularly the noticeably stronger anti-cancer effect of the direct CAP treatment compared with the indirect CAP treatment based on the CAP-treated solutions [8–10]. In other words, though the long-lived reactive species

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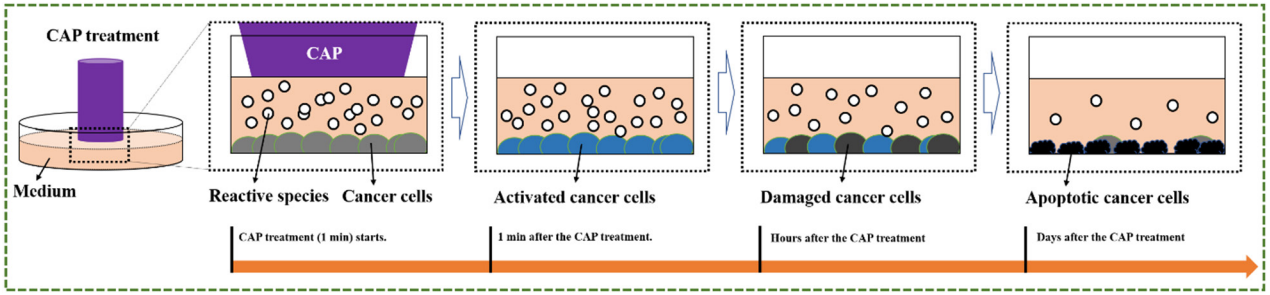

Figure 1. The concept of the activation phenomenon during a direct CAP treatment. The observed anti-cancer effect of CAP treatment is due to two factors. The first factor is the CAP-originated long-lived reactive species, which gradually accumulates in the extracellular environment during CAP treatment (for example, 1 min of treatment at here). The second factor is the activation phenomenon on the CAP-treated cancer cells, which may due to the short-lived reactive species. The activated cancer cells become more sensitive to the CAP-originated ROS and RNS compared with the cancer cells without the activation.

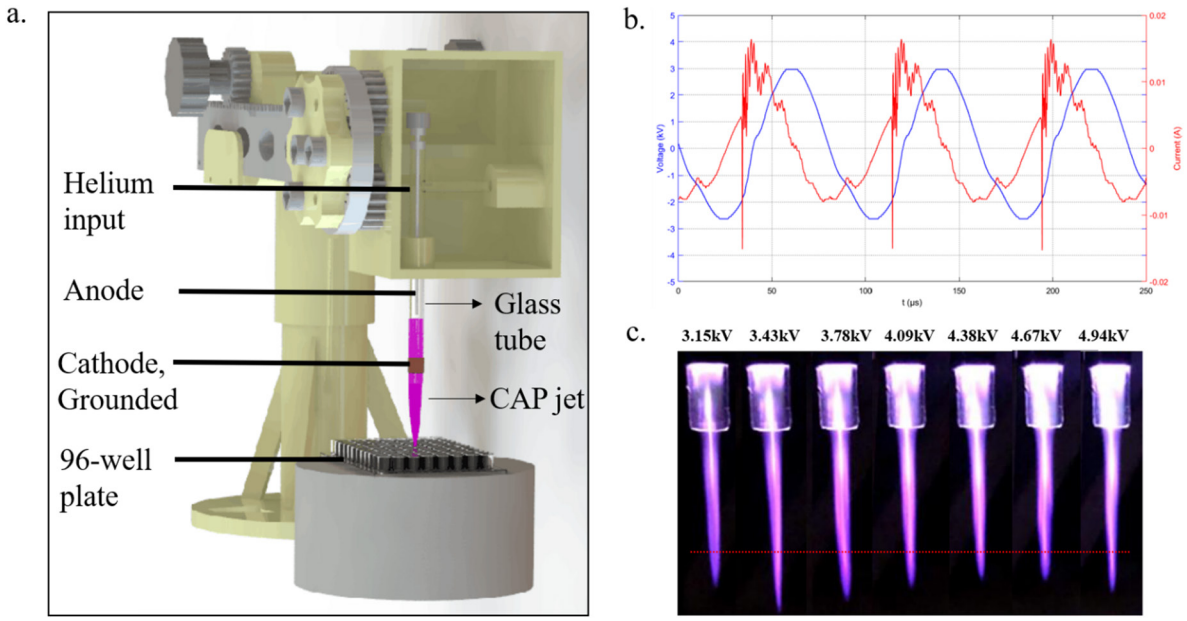

Figure 2. The helium CAP jet device. (a) Schematic illustration of the setup. (b) An example of the discharge voltage and the discharge current under the experiment condition: a peak value of the discharge voltage of 3.15 kV, a flow rate of 1.53 lpm, a discharge frequency of 12.5 kHz. (c) The photos of the morphologies of the CAP jet during the increase of discharge voltage (peak value) from 3.15 kV to 4.94 kV. In this case, the flow rate and the discharge frequency were 1.53 lpm and 12.5 kHz, respectively. The red dotted line represents the bottom of a 96-well plate during the treatment.

concentration in the extracellular environment are the same among these two cases, the CAP-treated pancreatic cancer cells will show much stronger sensitivity to the cytotoxicity of the long-lived reactive species than the pancreatic cancer cells without the direct CAP treatment. The difference between the indirect and the direct treatment may be just due to the short-lived reactive species if we just consider the chemical factors during CAP treatment.

Until recently, we found the clues to understand these puzzles. The CAP-treated pancreatic adenocarcinoma cells (PATU-8988) could quickly enter a specific sensitive state during CAP treatment, in which they became sensitive to the cytotoxicity the long-lived reactive species such H_2O_2 and NO_2^- [11]. We named such a phenomenon as the activation on the CAP-treated cancer cells (figure 1). The short-lived reactive species may cause the activation. The demonstration of the activation phenomenon is specifically explained in [11]. The activation on the cancer cells will not cause noticeable cytotoxicity. The activation will start just 2 s during CAP treatment. The activation will become more obvious when a CAP treatment lasts longer, and the activated cells will be gradually desensitized over the initial 5 h after CAP treatment and finally lose the activation completely. The activation on the CAP-treated cancer cells may be the primary mechanism for the stronger cytotoxicity of the direct CAP treatment compared with the indirect CAP treatment or the treatment based on the CAP-activated solution such as medium. The activation phenomenon also explains the puzzle that a CAP treatment can achieve a strong anti-cancer effect even any single reactive species can just cause a weak effect [12]. The underlying mechanism of the activation phenomenon is still unknown.

In this study, we further demonstrate that the activation on the cancer cells can be modulated by controlling three basic operational parameters of the CAP jet, which include the flow

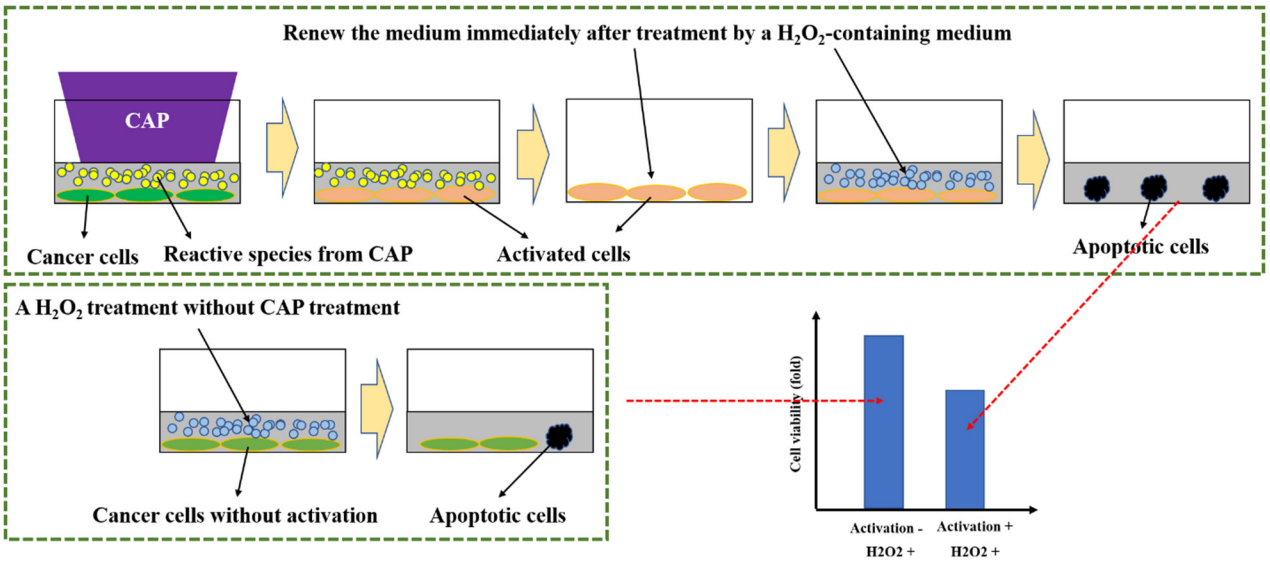

Figure 3. The basic research strategy used in this study. An important ROS in plasma medicine, H_2O_2, was used as the probe to demonstrate the existence of the activation effect on PA-TU-8988T cells throughout the entire study. The activated cancer cells were obtained by the CAP treatment followed by an instant removing of the medium containing the CAP-originated reactive species (yellow dots). All other tests were performed on such activated cells. The comparison between the cytotoxicity of H_2O_2 on the activated cells and the cells without such activation.

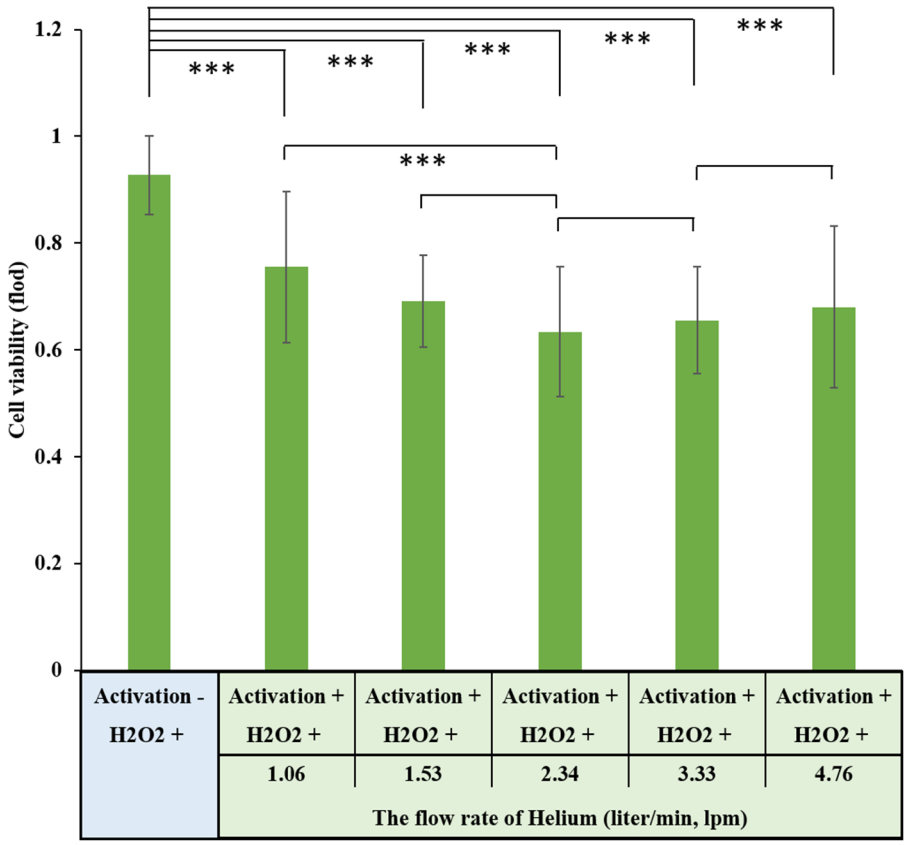

Figure 4. The effect of the flow rate on activation. 1 min of CAP treatment was performed when the discharge voltage was 3.43 kV. With or without activation is represented as activation + and activation -, respectively. With or without H_2O_2 treatment (10 μM) is represented as H_2O_2 + and H_2O_2 −, respectively. All experiments were performed in quadruplicate and were independently repeated for at least three times. The results were shown as the mean ± sd. Student's *t*-tests were performed and the significance was indicated as $*** p < 0.005$, $** p < 0.01$, $* p < 0.05$, respectively.

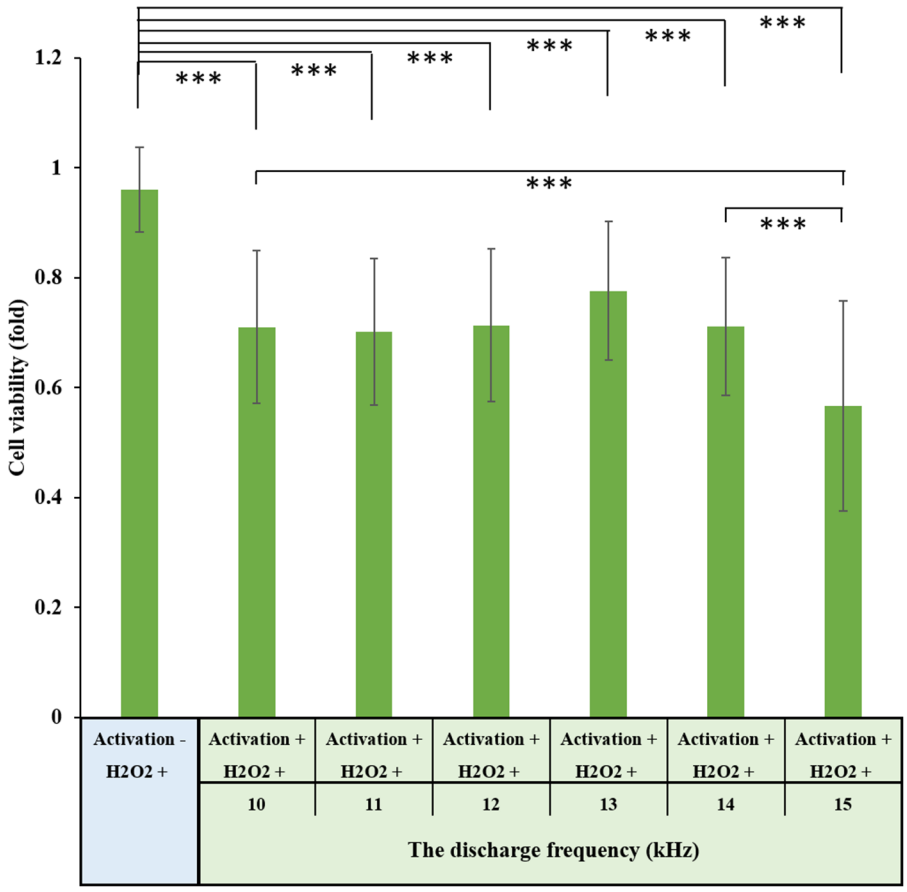

Figure 5. The effect of the discharge frequency on activation. The discharge voltage and the flow rate were 3.43 kV and 1.53 lpm, respectively. With or without activation is represented as activation + and activation −, respectively. With or without H_2O_2 treatment (10 μM) is represented as H_2O_2 + and H_2O_2 −, respectively. All experiments were performed in quadruplicate and were independently repeated for at least three times. The results were shown as the mean ± sd. Student's *t*-tests were performed and the significance was indicated as $*** p < 0.005$, $** p < 0.01$, $* p < 0.05$, respectively.

rate of the carrying gas, the discharge voltage, as well as the discharge frequency. In addition, a 0D chemical simulation was used to analyze the generation of reactive species in the CAP jet as the change of the discharge voltage based on the measured optical emission spectrum (OES), which provides the clues to understand the observed correlation between the discharge and the activation effect on the pancreatic adenocarcinoma cells. This study builds a foundation to optimize the anti-cancer effect of CAP treatment based on the activation phenomenon.

Methods and materials

Human pancreatic adenocarcinoma (PA-TU-8988T) cells provided by Dr Murad's lab at the George Washington University. PA-TU-8988T cells were cultured in the standard Dulbecco's modified Eagle's medium (DMEM, 11965-118, Life Technologies) with 10% (v/v) fetal bovine serum (FBS, ThermoFisher Scientific) and 1% (v/v) antibiotic solution (penicillin and streptomycin, Life Technologies). The cells for CAP treatment were seeded in a 96-well plate (61406-081, Corning). 100 μl of the cells-harvesting solutions (3×10^4 cells ml^{-1}) were seeded in each well. Cancer cells were grown for 1 d under the standard culture condition (a humidified, 37°C, 5% CO_2 environment) before experiments. The media that had been used to culture cells overnight were removed before experiments.

The helium CAP jet device was designed and assembled in Dr Keidar's lab at the George Washington University. It has been used in many studies *in vitro* and *in vivo* [13, 14]. The experimental setup was introduced in figure 2(a). Helium was used as the carrying gas to trigger the in-equilibrium discharge and the formation of CAP jet. The discharge voltage (peak value) between the ring grounded cathode and the central anode was ranged from 3.15 kV to 4.94 kV (figure 2(b)). The discharge process was driven by a pulsed generator with a frequency ranged from 10 kHz to 15 kHz. The flow rate was ranged from 1.062 liter/min (lpm) to 4.763 lpm. The highest temperature of the CAP jet was 40 °C during all experiments. The distance between the down edge of class tube and bottom of 96-well plate was 27.5 mm. The length of the CAP jet was always larger than 27.5 mm during the change process of all parameters in this study. For example, the lengths of CAP jets during the change of the discharge voltage from 3.15 kV to 4.94 kV is shown in figure 2(c).

To investigate the activation phenomenon, at least the effect of the CAP-originated long-lived ROS were precluded. We have confirmed that a 1 min of the direct CAP treatment

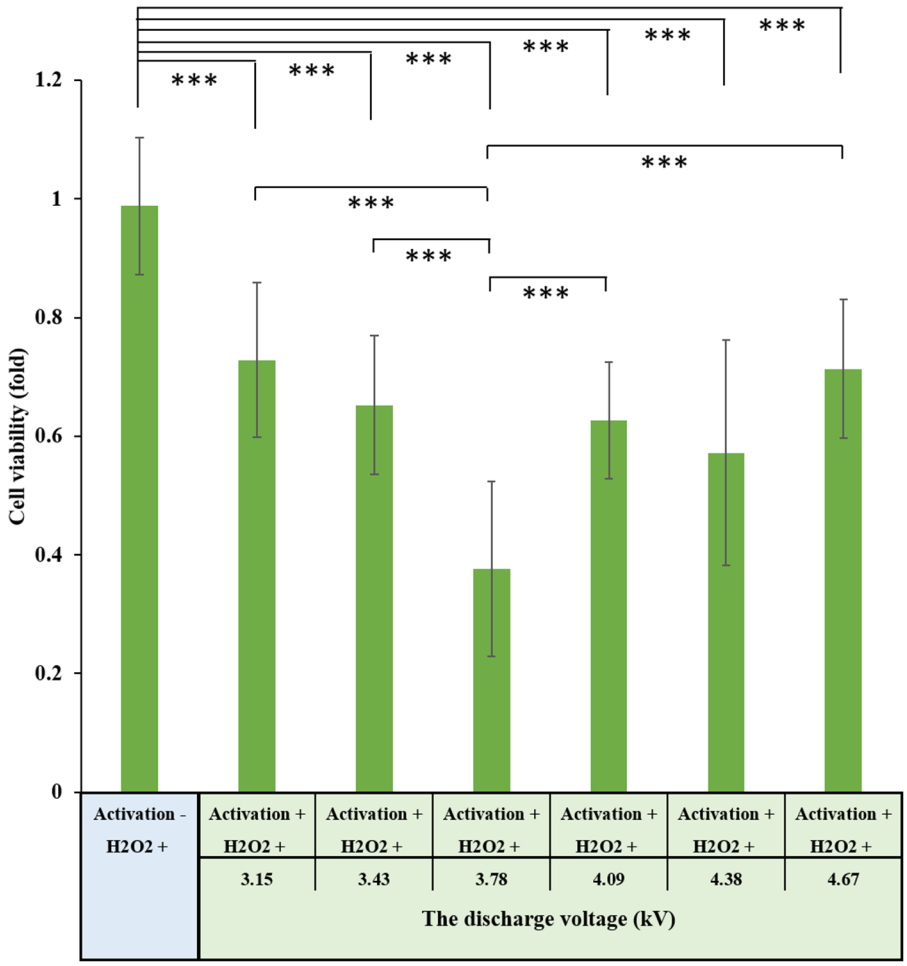

Figure 6. The effect of the discharge voltage on activation. With or without activation is represented as activation + and activation −, respectively. With or without H_2O_2 treatment (10 μM) is represented as H_2O_2 + and H_2O_2 −, respectively. 1 min of experiment was performed when the frequency and the flow rate were set as 12.5 kHz and 1.53 lpm, respectively. All experiments were performed in quadruplicate and were independently repeated for at least three times. The results were shown as the mean ± sd. Student's t-tests were performed and the significance was indicated as $*** p < 0.005$, $** p < 0.01$, $* p < 0.05$, respectively.

will cause a strong killing effect (about 80%) on PA-TU-8988T cells [11]. A quick removing the medium containing ROS and RNS after CAP treatment will counteract the cytotoxicity of CAP treatment [11, 15]. In such case, the activation of cancer cells still exists and will last about 5 h. When reactive species were removed, the cytotoxicity of CAP treatment would also be nearly completed inhibited, because the activation will not cause noticeable cytotoxicity on cancer cells [8, 11]. After the removing of the long-lived reactive species in the medium, the activated cells are obtained. We designed the following protocols (figure 3). The direct CAP treatment (1 min) was first performed on a well of 96-well plate. The cancer cells were immersed in 50 μl of DMEM. The DMEM was quickly (<1 min) removed after CAP treatment. Then, the CAP-activated (treated) cancer cells were obtained. We compared the cytotoxicity of H_2O_2 (10 μM) treatment on the CAP-treated (activated) cancer cells with that on the cancer cells without CAP treatment. The H_2O_2-containing DMEM was made by adding H_2O_2 standard solution (Sigma-Aldrich, 216763) in DMEM with a designed concentration (10 μM). All cells were cultured for 3 d before the final cell viability assay using MTT assay (Sigma-Aldrich, M2128). The cell viability assay was performed following the standard protocols provided by the manufacturer. Finally, the absorbance at 570 nm was processed to be a relative cell viability (fold) by the division of absorbance between the experimental group and the control. In the control, cancer cells were just cultured in the untreated DMEM. Here, we need to point out that because the experimental groups involve twice removing the medium during the whole experiment, the medium in the control has also been removed for twice.

Results and discussion

The effect of the flow rate of helium on the activation was first investigated. The flow rate of helium was ranged from 1.06 lpm to 4.76 lpm (figure 4). 10 μM H_2O_2 treatment will not cause killing effect on PA-TU-8988T without CAP treatment (activation). However, 10 μM H_2O_2 treatment will have significant killing effect on the CAP-activated PA-TU-8988 T cells. This is a typical feature of the activation phenomenon [11]. The activation of PA-TU-8988T cells will be enhanced when the flow rate increases from 1.06 lpm to 1.53 lpm. The activation does not

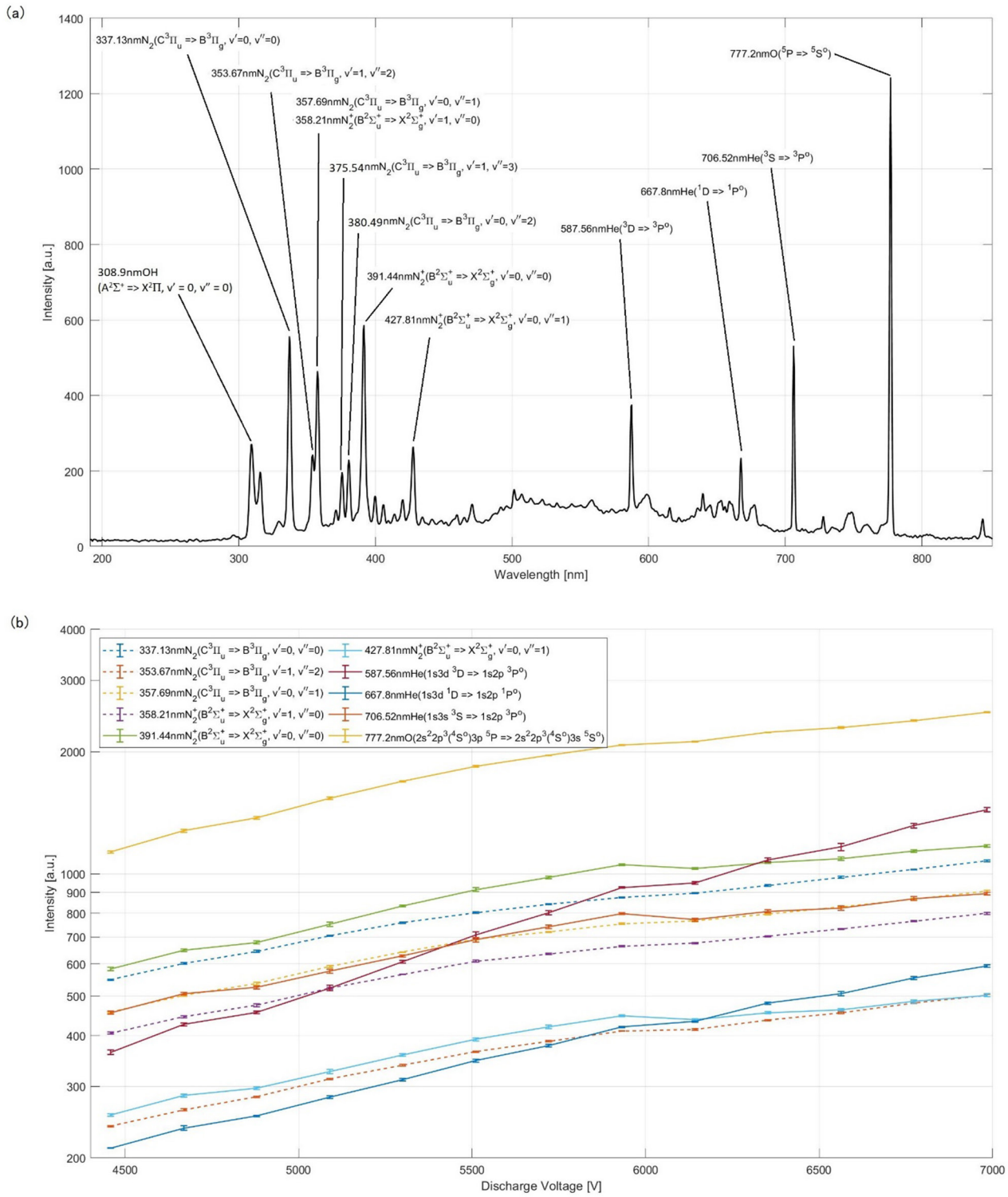

Figure 7. The OES measurement of the CAP jet. The amplitude of discharge voltage increased from about 4500 V to 7000 V (pk–pk). (a) An OES spectrum example of 4500 V (pk–pk). (b) How the top ten identified peaks vary with the increment of discharge voltage.

change when the flow rate increases from 1.53 lpm to 4.76 lpm. Controlling the flow rate is an effective method to enhance the activation effect on the CAP-treated PA-TU-8988T cells.

The effect of the discharge frequency on the activation was further demonstrated. The discharge frequency was changed from 10 kHz to 15 kHz. Similarly, 10 μM H_2O_2 treatment will not cause noticeable killing effect on PA-TU-8988T without CAP treatment (activation), while 10 μM H_2O_2 treatment will have significant killing effect on all the CAP-activated PA-TU-8988T cells (figure 5). Thus, the activation phenomenon can be observed in all cases during the change of the discharge voltage. It is found that the activation on PA-TU-8988T cells will not be enhanced until the discharge frequency reaches a relative high level, 15 kHz. To achieve a strong activation effect, a higher discharge frequency is recommended within the considered range.

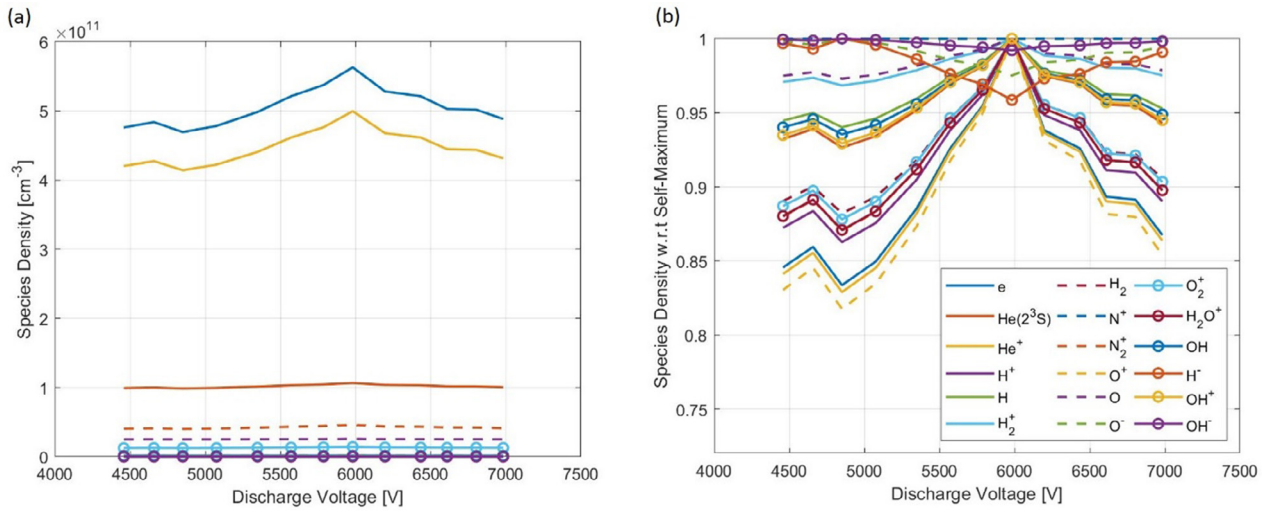

Figure 8. The resulting species densities versus discharge voltage (pk–pk). (a) The actual values of density; (b) the normalized values that each density curve is divided by its own maximum to illustrate the curvature.

The effect of the discharge voltage was finally investigated. The discharge voltage between the two electrodes was ranged from 3.15 kV to 4.67 kV (peak value). The activation effect on the all cases still exists. 10 μM H_2O_2 treatment will not cause killing effect on PA-TU-8988T without the CAP activation but will result in significant killing effect on the CAP-activated PA-TU-8988T cells (figure 6). The maximum activation phenomenon has been observed when the discharge voltage is at the middle of the considered discharge voltage range, i.e. 3.78 kV. The activation effect corresponds to other discharge voltage is noticeable weaker than such maximum case. For example, the cell viability decreases from 0.73 to just 0.38 when the discharge voltage increases from 3.15 kV to 3.78 kV. On the contrary, the cell viability increases again from 0.38 to 0.71 when the discharge voltage increases from 3.78 kV to 4.67 kV. Compared with other two operational parameters, the discharge voltage at the 3.78 kV shows the strongest activation effect on the CAP-treated PA-TU-8988T cells.

As a new concept and observation in plasma medicine, the activation mechanism is still far from clearly understood. The effect of three operational parameters on the anti-cancer effect of CAP treatment has been investigated by different groups [16–18]. In these studies, the discharge voltage has a stronger impact on the anti-cancer effect of the direct CAP treatment than the flow rate does [16]. The anti-cancer effect of the direct CAP treatment is proportional to the discharge voltage [16–18], and such correlation is believed to relate with the formation of reactive species in the extracellular environment such as in medium [16–18]. Reactive species particularly the long-lived reactive species play a core role in the current understanding of the interaction between CAP and cells. We further investigated the chemical composition change due to the increase of discharge voltage by using the OES measurement on the CAP jet. The OES data are shown in figure 7.

The OES data alone cannot explain the observed strongest activation effect under a medium discharge condition. In fact, concentration of all species observed by OES increase with the discharge voltage increase. Based on the accepted conclusions and the simple analysis on OES data, it is hard to understand the strongest activation effect when the discharge voltage is with a medium level, rather than corresponds to the largest discharge voltage. However, the ratio of some of the OES intensities is proportional to the mean electron temperature, which determines the chemical reaction rate coefficients in the CAP jet. Therefore, the OES results provide a relation between the discharge voltage and mean electron temperature. To analyze the chemical composition in the CAP jet, we used a set of zero-dimensional (0D) chemical simulations with electron temperature (T_e) swept from 2 eV to 20 eV to investigate how the discharge voltage affect the generation of reactive species in the CAP jet [19]. Each time, the simulation was run with a constant T_e. As shown in table S1 in the online supplementary material (stacks.iop.org/JPhysD/52/445202/mmedia), 451 chemical reactions with 44 species were considered in this simulation. The details about the simulation were illustrated in the supporting materials. The simulation results provide the relation between the mean electron temperature and species densities. Finally, combining the information from OES and chemical simulation, we revealed how the species densities are related to the discharge voltage as shown in figure 8. Differ from the curves shown in figure S1, these patterns clearly displayed peaks and valleys at 6000 V (pk–pk), which implied that a certain discharge voltage could maximize some species and minimize others. Most reactive species such as O^+, O_2^+, OH, OH^-, as well as OH^+ would have a maximum at a medium discharge voltage, while few reactive species, such as O^-, OH^-, as well as H^-, would have a minimum at the same medium discharge voltage. This trend may explain the strongest activation effect just occurred under a medium discharge voltage level (figure 6), which indicates that the activation effect may be due to the short-live reactive species in CAP jet.

A possible mechanism of the mean electron temperature decrement at higher voltage is the electron-impact collisions on diatomic, triatomic, and other molecules with higher atom numbers. These species are massively generated when the discharge voltage is increased, such as OH, HO_2, and the N_xO_y family, etc. These molecules have complicated rotational and

vibrational degrees of freedom, comparing with He and the homonuclear diatomic species N_2 and O_2. As a result, when those molecules are colliding with electrons, along with the momentum transfer, extra degrees of freedom absorb the electron energy and finally decrease the mean electron temperature. Some evidence is the significant slope decrements of all N_2^+ curves shown in figure 7, while no such slope decrement for N_2 curves. This is because the former ones require ionizations while the latter ones require excitation only. Note that the total energy of the system is still increased by adding more discharge voltage, but such energy increment does not appear as the increment of electron temperature. Therefore, adding the discharge voltage, decreases the mean temperature difference between the electron and other species. In other words, the plasma is thus closer to its thermal equilibrium state.

Nonetheless, other mechanisms may trigger the activation during CAP treatment. Further studies will be performed to investigate whether the activation phenomenon will still appear in the absence of the CAP-originated reactive species. The achievement of strongest anti-cancer effect by modulating the activation effect on the cancer cells rather than changing the extracellular ROS concentration, we provide a new strategy to optimize the anti-cancer effect of CAP treatment. In contrast, nearly all previous studies obtained the stronger anti-cancer effect by directly or indirectly increasing the reactive species in the extracellular environment [13, 15, 20].

Conclusions

The activation phenomenon of the CAP-treated cancer cells is a new concept in plasma medicine. The activation phenomenon explains the stronger anti-cancer effect of a direct CAP treatment compared with an indirect CAP treatment based on the CAP-treated solutions. The flow rate of helium, the discharge voltage and the discharge frequency can affect the activation state of the CAP jet-treated cancer cells. Among three basic operational parameters, a medium discharge voltage (3.78 kV) can cause the strongest activation effect. This study provides a new strategy to optimize the anti-cancer effect of CAP treatment without increasing the extracellular long-lived reactive species concentration but just enhancing the activation effect of the CAP treatment on the cancer cells.

Acknowledgments

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