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The Strong Cell-based Hydrogen Peroxide Generation Triggered by Cold Atmospheric Plasma

Dayun Yan¹, Haitao Cui¹, Wei Zhu¹, Annie Talbot², Lijie Grace Zhang¹, Jonathan H. Sherman³ & Michael Keidar¹

Hydrogen peroxide (H₂O₂) is an important signaling molecule in cancer cells. However, the significant secretion of H₂O₂ by cancer cells have been rarely observed. Cold atmospheric plasma (CAP) is a near room temperature ionized gas composed of neutral particles, charged particles, reactive species, and electrons. Here, we first demonstrated that breast cancer cells and pancreatic adenocarcinoma cells generated micromolar level H₂O₂ during just 1 min of direct CAP treatment on these cells. The cell-based H₂O₂ generation is affected by the medium volume, the cell confluence, as well as the discharge voltage. The application of cold atmospheric plasma (CAP) in cancer treatment has been intensively investigated over the past decade. Several cellular responses to CAP treatment have been observed including the consumption of the CAP-originated reactive species, the rise of intracellular reactive oxygen species, the damage on DNA and mitochondria, as well as the activation of apoptotic events. This is a new previously unknown cellular response to CAP, which provides a new prospective to understand the interaction between CAP and cells *in vitro* and *in vivo*. The short-lived reactive species in CAP may activate cells *in vivo* to generate long-lived reactive species such as H₂O₂, which may trigger immune attack on tumorous tissues via the H₂O₂-mediated lymphocyte activation.

H₂O₂ is an important signaling molecule in cancer cells¹. The production of nanomolar (nM) level of H₂O₂ by several cancer cell lines including melanomas, neuroblastoma, colon carcinoma, and ovarian carcinoma have been observed two decades ago². H₂O₂ may increase the genetic instability of cancer cells by inducing DNA strand breaks, damage on guanine or thymine bases, and the sister chromatid exchanges, which may facilitate the malignant process of cancer cells, such as proliferation, apoptosis resistance, metastasis, angiogenesis and hypoxia-inducible factor 1 activation^{1,2}. On the other hand, H₂O₂ alone with a relative high concentration or as the mediator of a series of anticancer drugs can selectively induce apoptosis in cancer cells^{1,3-5}. H₂O₂ may have promising application in cancer treatment at least as a mediator of series of physical or chemical strategies.

Cold atmospheric plasma (CAP), a near room temperature ionized gas composed of charged particles, neutral particles and electrons, has shown its promising application in cancer treatment over the past decade⁶⁻¹¹. CAP not only effectively decreases the growth of many cancer cell lines *in vitro* through reactive species-triggered cell death but also significantly inhibits or halts the growth of subcutaneous xenograft tumors or melanoma in mice by the direct CAP treatment just above skin^{8,12-15}. The reactive oxygen species (ROS) and the reactive nitrogen species (RNS) have been regarded as the main factors contributing to the complicate interaction between CAP and cancer cells *in vitro* and *in vivo*^{16,17}. Many studies conclude that the death of the CAP-treated cancer cells *in vitro* is due to the apoptosis triggered by the significant rise of intracellular ROS, DNA damage, as well as mitochondrial damage^{7,11,18-21}.

Among dozens of CAP-originated species in aqueous solutions, H₂O₂ has been proven to be a main factor triggering the death of cancer cells *in vitro*²²⁻²⁶. H₂O₂ has not been detected in the emission spectra of CAP in gas phase¹⁹. The H₂O₂ in aqueous solution may be due to the recombination reactions between short-lived species OH^{27,28}. To date, CAP is the only confirmed extracellular H₂O₂ source in plasma medicine. Cells have just been

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regarded as a consumer for the CAP-originated H_2O_2 ^{11,24}. However, using a solution with H_2O_2 alone does not cause the same decrease in cancer cells viability as seen following the CAP treatment^{29,30}. Thus, the CAP treatment on cancer cells cannot be simply regarded as a H_2O_2 -treatment.

In addition to the direct CAP treatment, another strategy using the CAP-stimulated solutions (PSS) to inhibit the growth of cancer cells *in vitro* or to inhibit the growth of tumorous tissues in mice through injection has been also demonstrated recently^{31–34}. PSS is also named as the indirect CAP treatment or the CAP-activated solutions^{24,35}. For the direct CAP treatment *in vitro*, a thin layer of cell culture medium is used to cover cancer cells³⁶. This medium layer facilitates the transition of the reactive species in the gas phase into the dissolved reactive species in medium³⁶. The direct CAP treatment can be regarded essentially the same as the indirect CAP treatment if we assume that the finally formed long-lived reactive species such as H_2O_2 , NO_2^- , ONOO^- in the CAP-treated solution is the sole factor leading to decreased tumor cell viability. However, considering the potential interaction between the short-lived species such as superoxide (O_2^-), hydroxyl radicals ($\text{OH}\cdot$) in CAP and cells, the direct CAP treatment should be different from the indirect CAP treatment. PSS will not contain short-lived species. So far, no direct experimental evidence has been found to describe such essential difference neither qualitatively nor quantitatively.

In this study, we first provide the experimental evidence that the 1 min of CAP treatment can trigger μM level cancer cell-based H_2O_2 generation through comparing the H_2O_2 generation in the CAP-treated cell-free medium and the CAP-treated cancer cells covered by a thin layer of medium. Such cell-based H_2O_2 generation can be regulated by controlling the volume of the medium layer, the cell confluence, as well as the discharge voltage. This study provides the first evidence that cells can not only quickly consume H_2O_2 but also significantly generate H_2O_2 through CAP treatment. This is a novel perspective to understand the interaction between CAP and cells.

Results

Consuming the extracellular H_2O_2 is a basic response of cancer cells cultured in an H_2O_2 -containing environment^{11,24}. We have demonstrated that the CAP-originated H_2O_2 could be completely consumed just several hours after using the CAP-stimulated DMEM to culture glioblastoma (U87MG) cells¹¹. In this study, we investigated the evolution of extracellular H_2O_2 in PSM and H_2O_2 -DMEM, which have been used to culture cancer cell lines. All solutions contained $36.3 \mu\text{M}$ H_2O_2 . The H_2O_2 concentration in PSM and H_2O_2 -DMEM decreases as the culture time increases (Fig. S1). At a higher cell confluence, a faster H_2O_2 consumption rate is noted. To date, all three cancer cell lines we studied have shown a similar feature that cells quickly consume the extracellular H_2O_2 in just 3 hr. This observation is consistent with the observation that the rise of intracellular ROS started immediately after the CAP treatment and ended just about 3 hr post the CAP treatment³⁷.

To date, the direct CAP treatment is the main strategy to investigate the toxicity of CAP on cancer cells *in vitro*^{8,31,32}. In this study, we measured the H_2O_2 concentration in a thin layer of DMEM which has been used to immerse cells during the direct CAP treatment. Without the protection of such medium layer, cancer cells are killed immediately due to the dehydration caused by the helium flow from the CAP jet tube. Based on the preliminary test, $20 \mu\text{L}$ per well on a 96-wells plate was the minimum volume to preclude the impact of helium flow (Fig. S2). In addition, the volume effect of this thin DMEM layer was also investigated through increasing the volume from $20 \mu\text{L}$ to $100 \mu\text{L}$. Three general trends have been observed when we immediately measured the H_2O_2 concentration in DMEM after the treatment. First, the concentration of H_2O_2 in all cases increases as the volume of DMEM decreases (Fig. 1a). This result may be due to the principle that a salute will form in a larger concentration when the volume of solvent (reactive species) is smaller. Second, the direct CAP treatment on pancreatic adenocarcinoma cells (PA-TU-8988T) and breast adenocarcinoma cells (MDA-MB-231) rather than on glioblastoma cells (U87MG) generates significantly more H_2O_2 than the same CAP treatment on DMEM in the same volume (Fig. 1a). This trend is more obvious when the volume of DMEM is smaller. U87MG cells will show similar feature only at some specific conditions such as at a medium volume of $80 \mu\text{L}$ and $100 \mu\text{L}$. The generation of H_2O_2 is also illustrated as the change in H_2O_2 concentration between the CAP-treated DMEM with and without cells (Fig. 1b). The volume of DMEM is also a key factor to affect the cell-based H_2O_2 generation. The maximum relative H_2O_2 generation from MDA-MB-231 cells, PA-TU-8988 cells, and U87MG occurs when the volume of DMEM is $50 \mu\text{L}$, $100 \mu\text{L}$, and $80 \mu\text{L}$, respectively (Fig. 1b). More importantly, only the direct CAP treatment on cancer cells can cause such cell-based H_2O_2 generation. The CAP-stimulated DMEM cannot generate the similar response on all three cell lines (Fig. 1c). The response of cancer cells to PSM is just consuming H_2O_2 , as seen in Fig. S1. In addition, all three cell lines do not generate H_2O_2 without the CAP treatment.

To investigate whether the CAP-treated cancer cells will continue to generate H_2O_2 post the CAP treatment, the evolution of H_2O_2 concentration in DMEM after the direct CAP treatment on cancer cells and the CAP-treated DMEM has been studied. For all cell lines, the H_2O_2 concentration in DMEM gradually decreases post CAP treatment (Fig. 2). Such a decay trend has not been observed during the evolution of the CAP-treated DMEM. Thus, the decreased H_2O_2 concentration is due to the consumption of cancer cells on extracellular H_2O_2 . The cell-based H_2O_2 generation occurs only when the direct CAP treatment is performed. Once the CAP treatment ceases, the H_2O_2 generation stops. When the H_2O_2 concentration measurement was performed at the tenth min after CAP treatment, the cell-based H_2O_2 generation was no longer observed (Fig. 2). This may explain why the cell-based H_2O_2 generation has not been observed in all previous studies.

In addition to the volume of DMEM used to cover cancer cells during CAP treatment, the cell confluence and the discharge voltage can also affect the cell-based H_2O_2 generation. Under the same experimental conditions, the maximum of the cell-based H_2O_2 generation of MDA-MB-231 cells, and PA-TU-8988T cells appears when the cell confluence is 1×10^4 cells/mL and 3×10^4 cells/mL, respectively (Fig. 3a). For U87MG cells, the significant effect of the cell confluence on the cell-based H_2O_2 generation was not observed (Fig. 3a). The discharge voltage is an important physical factor during the CAP treatment. The change of discharge voltage in a short range can significantly change the chemical composition of CAP. According to prior research, the increase of output

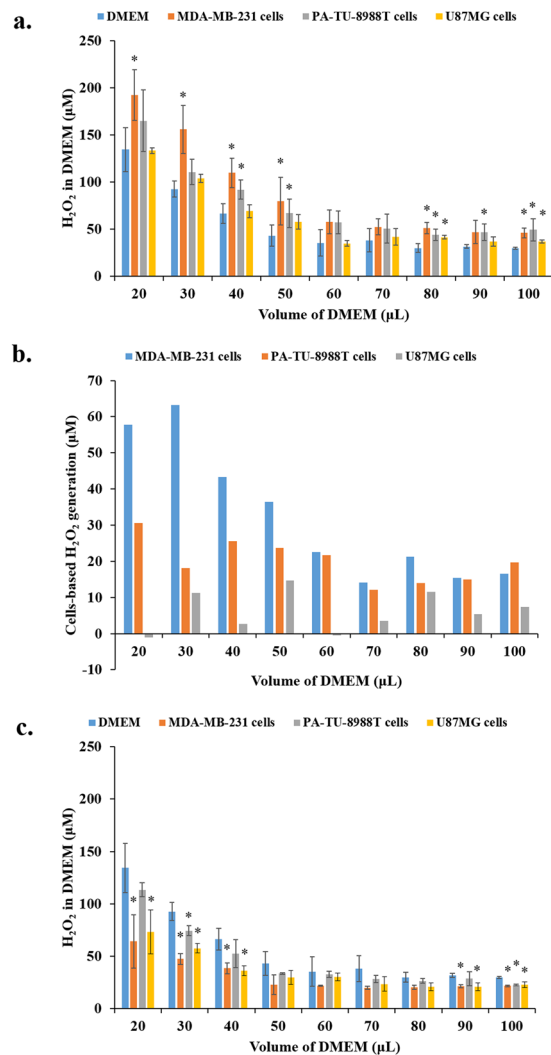


Figure 1. Only the direct CAP treatment can trigger the H₂O₂ production of specific cancer cell lines *in vitro*. **(a)** The H₂O₂ concentration in DMEM after the CAP treatment on just DMEM (control), on pancreatic adenocarcinoma cells (PA-TU-8988T) immersed in DMEM, on breast adenocarcinoma cells (MDA-MB-231) immersed in DMEM, as well as on glioblastoma cells (U87MG) immersed in DMEM. **(b)** The cells-based H₂O₂ concentration. The data is calculated based on the following formula. Cells-based H₂O₂ concentration = H₂O₂ concentration in the DMEM which has been used to immerse cancer cells during the CAP treatment – H₂O₂ concentration in the CAP-treated DMEM (control). The H₂O₂ concentration corresponds to the mean of each bar shown in 1a. **(c)** The indirect CAP treatment will not stimulate cancer cells to generate H₂O₂. All H₂O₂ concentration was measured immediately (about 1 min) after the treatment. Results are presented as the mean ± s.d. of three independently repeated experiments. Student's t-test was performed between the data based on cells immersed in specific volume of DMEM and the data just based on the DMEM with the same volume. The significance is indicated as *p < 0.05.

(discharge) AC voltage from 2.56 kV to 3.80 kV significantly increased the generation of the nitrogen-based species such as N₂⁺ and NO/N₂ in the gas phase of CAP³⁸. The generation of the oxygen-based species such as O and OH[•] just slightly increased during the same process³⁸. Thus, the ratio between the oxygen-based species and the nitrogen-based species decreases as the output voltage increased. We observed the same trend in the CAP-stimulated PBS that the concentration ratio between H₂O₂ and NO₂⁻ decreases from 11.2 to 5.5 as the output voltage increased from 3.02 kV to 3.85 kV. In this study, the increase of output voltage significantly weakens the cell-based H₂O₂ generation (Fig. 3b). A large output voltage will completely inhibit the cell-based H₂O₂ generation for all three cell lines.

H₂O₂ has been regarded as a main anti-cancer reactive species in the CAP treatment *in vitro*^{24–26}. Because the observation that the direct CAP treatment on cancer cells immersed in a thin layer of DMEM causes a significantly stronger H₂O₂ generation than that seen with the indirect CAP treatment on DMEM, it is reasonable to speculate that the direct CAP treatment will cause a stronger anti-cancer effect on cancer cells than the indirect CAP treatment does under the same experimental conditions. Such a comparison was performed on MDA-MB-231 cells and PA-TU-8988T cells. As shown in Fig. 4a and b, both cell lines are much more vulnerable

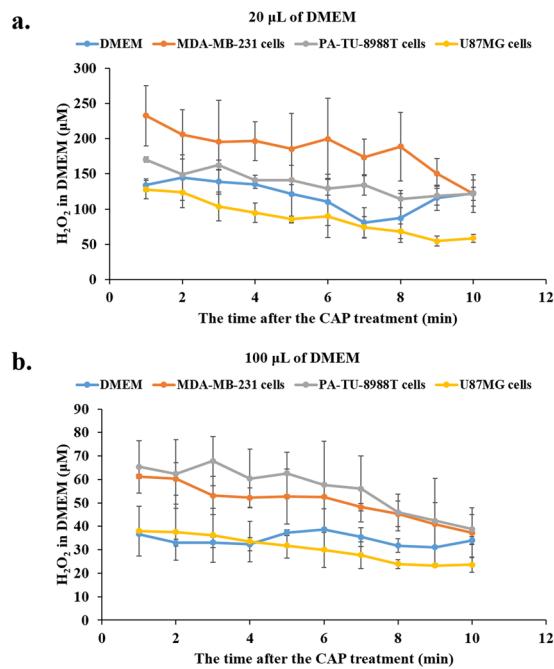


Figure 2. H_2O_2 evolution in the DMEM surrounding cancer cells after the direct CAP treatment. **(a)** Cancer cells immersed in $20\ \mu\text{L}$ of DMEM. **(b)** Cancer cells immersed in $100\ \mu\text{L}$ of DMEM. CAP was used to treat DMEM (control) or cancer cells (MDA-MB-231 cells, PA-TU-8988T cells, and U87MG cells) immersed in DMEM. In each experiment, the 1 min of CAP treatment was performed on the cells immersed in $20\ \mu\text{L}$ or $100\ \mu\text{L}$ of DMEM. The H_2O_2 concentration was measured very minute after the CAP treatment until the tenth minute. Cancer cells in 96-wells plate were cultured overnight before the CAP treatment. The cell confluence was 3×10^4 cells/mL. Results are presented as the mean \pm s.d. of three independently repeated experiments.

to the direct CAP treatment than the indirect CAP treatment. In addition, it was further demonstrated that the strong anti-cancer effect of the direct CAP treatment on the two cell lines could nearly be completely counteracted by pre-treating cancer cells with 6 mM intracellular ROS scavenger NAC or by removing the DMEM immersing cancer cells and replacing it with new untreated DMEM immediately after the direct CAP treatment (Fig. 4c and d). Thus, the anti-cancer effect of the direct CAP treatment on cancer cells is still based on a rise of intracellular ROS due to the extracellular reactive species dissolved in DMEM. Moreover, the volume of DMEM does not show a noticeable impact on the anti-cancer effect of the direct or the indirect CAP treatment, though corresponding H_2O_2 concentration in DMEM varies drastically with volume (Fig. 1). This difference indicates that other factors such as the whole reactive species amount rather than the concentration of reactive species may dominate the anti-cancer effect. In addition to the general trend introduced above, a slight difference on the anti-cancer effect due to the volume of DMEM still can be observed on MDA-MB-231 cells. The strongest anti-cancer effect of the direct CAP treatment on MDA-MB-231 cells occurs when the volume of DMEM is $50\ \mu\text{L}$.

Discussion

Since the first observation that CAP treatment could cause apoptosis in lung cancer cells line in 2004 and the first complete study on the anti-cancer effect of dielectric barrier discharge (DBD) on melanoma cells in 2007, the research on the application of CAP in cancer treatment experienced an exponential development^{7, 8, 11}. Despite the understanding on the anti-cancer mechanism at the molecular and cellular level is far from clear, it is widely acknowledged that the CAP-originated reactive species are the main anti-cancer factors inhibiting the growth of cancer cells *in vitro*^{6-8, 10, 11}. In all previous studies, however, cancer cells have just been regarded as having a passive role in plasma medicine to sustain the attack from the reactive species such as H_2O_2 ^{6-8, 10, 11}. This study first demonstrates that specific cancer cells such as pancreatic adenocarcinoma cells and breast adenocarcinoma cells will generate significant amount of H_2O_2 as a response to the direct CAP treatment. For example, MDA-MB-231 cells generate about 85% more H_2O_2 than the CAP treatment does when the volume of DMEM is $50\ \mu\text{L}$. Despite only two cell lines has shown noticeable cell-based H_2O_2 generation capacity in this study, such cellular response may commonly exist in other cancer cell lines which has not been studied. And, it is completely possible that such feature may also be shared by other cells including normal mammalian cells, bacteria, as well as yeasts. These cells have been investigated in plasma medicine for decades^{37, 39, 40}.

We hypothesize that the short-lived species in CAP may stimulate the H_2O_2 production by cancer cells. First, the H_2O_2 generation by cancer cells occurs only during CAP treatment. The cessation of CAP treatment will immediately cause the consumption of H_2O_2 to be the dominate response of cancer cells (Fig. 2). Consuming H_2O_2 is a basic response of cancer cells in an H_2O_2 -containing environment (Fig. S1). It is possible that H_2O_2 consumption capacity and the H_2O_2 generation capacity coexist during CAP treatment. U87MG cells show a

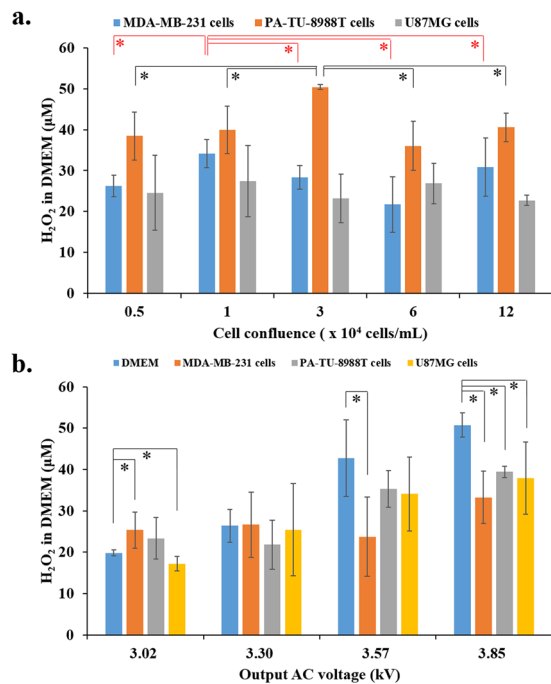


Figure 3. The cell confluence and the output voltage affect the cell-based H₂O₂ generation. **(a)** A relative small confluence can achieve the maximum cell-based H₂O₂ generation. **(b)** High output AV voltage significantly weakens the cell-based H₂O₂ generation. In each experiment, a 1 min of CAP treatment was used to treat DMEM (control) or cancer cells (MDA-MB-231 cells, PA-TU-8988T cells, and U87MG cells) immersed in 100 μL of DMEM. The H₂O₂ concentration was measured immediately (about 1 min) after the treatment. Cancer cells were cultured overnight before the treatment. The cell confluence for **(b)** was 3 × 10⁴ cells/mL. Results are presented as the mean ± s.d. of three independently repeated experiments. Student's t-test was performed and the significance is indicated as *p < 0.05.

much stronger H₂O₂ consumption capacity compared with breast cancer cell lines^{11,24}. Thus, the weak cell-based H₂O₂ generation by U87MG cells may be due to its too strong H₂O₂ consumption capacity which dominates the interaction between U87MG cells and CAP.

The short-lived species in CAP has been regarded as a main source to form H₂O₂ in the CAP-stimulated solutions, though the corresponding mechanism is still disputable^{41,42}. Such H₂O₂ formation is an aqueous solution-based reaction. Hydroxyl radicals may form H₂O₂ by a simple reaction: OH· + OH· → H₂O₂^{27,28}. H₂O₂ may also be formed by the recombination reaction based on hydroperoxyl radicals (HO₂): HO₂ + HO₂ → H₂O₂ + O₂²⁷. HO₂ may be formed by a reaction involving O₂⁻: O₂⁻ + H⁺ → HO₂²⁷. For the cell-based H₂O₂ generation, H₂O₂ may be formed by a dismutation reaction catalyzed by the extracellular superoxide dismutase (Ex-SOD, SOD3) on the cytoplasmic membrane of cancer cells^{22,23}. Superoxide is provided by CAP³⁹. The expression of SOD3 have been found in human pancreatic adenocarcinoma tissues, estrogen-induced breast cancer tissues, and breast carcinoma cells (MCF-7, MDA-MB-231), but not in glioblastoma cells, which may explain why cell-based H₂O₂ production has not been noticeably observed in the CAP-treated U87MG cells⁴³⁻⁴⁵.

The discovery of cell-based H₂O₂ generation provides a new feature which distinguishes CAP treatment and simple chemical treatments such as a H₂O₂ treatment. The direct CAP treatment causes a much stronger anti-cancer effect on pancreatic adenocarcinoma cells and breast adenocarcinoma cells than the indirect CAP treatment does (Fig. 4). Such difference may be at least partially due to the cell-based H₂O₂ generation during CAP treatment (Fig. 1). Nonetheless, it is necessary to emphasize that the weak anti-cancer capacity of the indirect CAP treatment revealed in this study is mainly due to the small diameter of the wells in 96-wells plate. As we demonstrated in previous study, the diameter of multi-wells plate was proportional to the reactive species concentration and the anti-cancer effect of PSS²⁴. Consequently, the PSS made on 6-wells plate will be much more toxic to cancer cells than the PSS made on 96-wells plate²⁴.

More importantly, this study provides a new perspective to understand the potential cellular interaction during CAP treatment, which also provide clues to understand the anti-cancer capacity of CAP *in vivo*. In addition to the proven selective anti-cancer capacity of CAP *in vitro* to cancer cells, another attractive feature of CAP is its promising anti-cancer effect seen *in vivo*. A series of investigations have achieved a similar conclusion that the growth of subcutaneous xenograft tumors or melanoma in mice can be significantly halted by a CAP treatment just above the skins of mice or on the exposed cancerous tissues in mice^{13-15,46}. The mechanism governing these descriptions is nearly completely unknown. ROS and RNS may also be the main factors contributing to the decreased tumor cell viability *in vivo* by CAP treatment through directly attacking tumor or indirectly activating immune response *in vivo* to further kill tumor cells^{18,47,48}. The trans-skin motion (diffusion, transportation or other physical ways) of reactive species may be a key to understand the anti-cancer capacity *in vivo*. Several

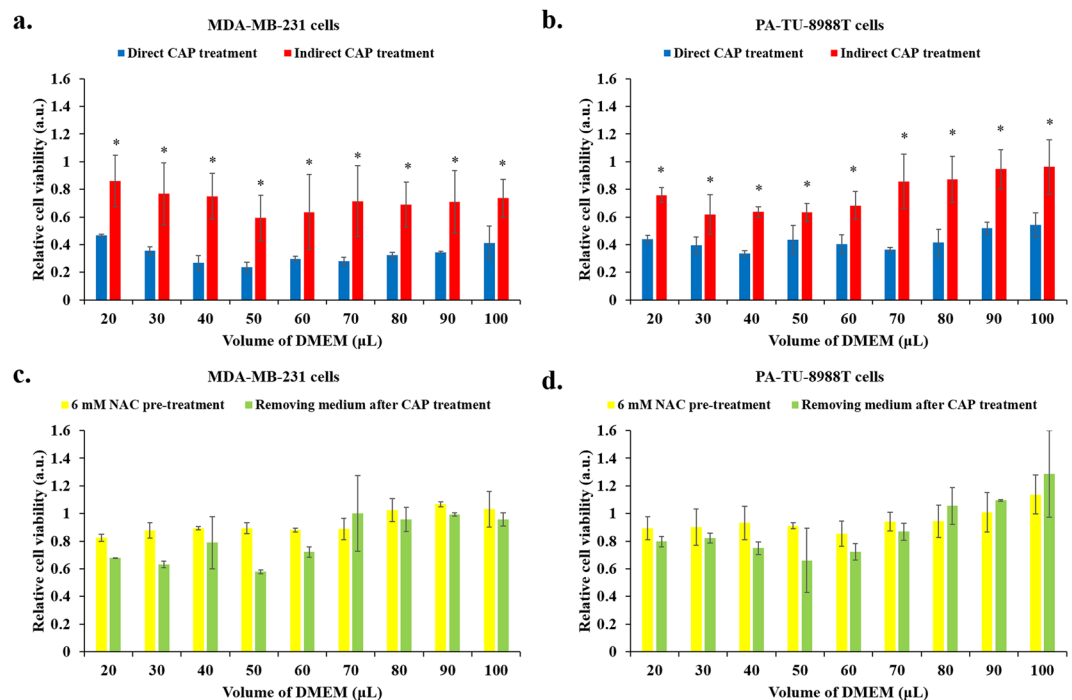


Figure 4. Under the same experimental conditions, the anti-cancer capacity of the direct CAP treatment is significantly stronger than the indirect CAP treatment. For the indirect CAP treatment, the CAP-stimulated DMEM was used to affect the growth of cancer cells. Comparison between the direct CAP treatment and the indirect treatment is performed on (a) MDA-MB-231 cells and (b) PA-TU-8988T cells. Pre-treating cancer cells with 6 mM NAC an intracellular scavenger for 3 hr or immediately (about 1 min) renewing the DMEM after the direct CAP treatment can effectively weakens the anti-cancer capacity of the direct CAP treatment on (c) MDA-MB-231 cells and (d) PA-TU-8988T cells. Results are presented as the mean \pm s.d. of three independently repeated experiments. Student's t-test was performed between the results from the direct CAP treatment and the results from the indirect CAP treatment. The significance is indicated as * $p < 0.05$.

attempts have been performed to understand such processes. The diffusion of reactive species across the skin analogue has been observed^{49,50}. Our study provides a novel explanation for the trans-skin motion of reactive species. The short-lived reactive species, may activate cells to generate long-lived reactive species such as H_2O_2 through the similar way revealed in this study. The tumorous tissue may be immersed in a H_2O_2 -rich environment even the CAP treatment does not directly generate a significant amount of H_2O_2 on the relative dry skin. The cells in skin and the cells in tumor may have similar mutual interaction through generating toxic chemicals such as H_2O_2 to neighbor cells. H_2O_2 is a second messenger in lymphocyte activation^{51,52}. μM level of H_2O_2 rapidly induced the activation of the transcription factor NF- κ B and early gene expression of interleukin-2 (IL-2) and the IL-2 receptor α chain⁵¹. The H_2O_2 -generating tumor tissues may be a potential target for immune system. The anti-cancer capacity of CAP treatment *in vivo* may involve the H_2O_2 -activated immune attack on tumorous tissues.

Conclusions

A new previously unknown basic cellular response to CAP treatment is demonstrated in this study. Only direct CAP treatment on breast adenocarcinoma cells and pancreatic adenocarcinoma cells immersed in a thin layer of medium results in a μM level of cell-based H_2O_2 generation. The measured maximum H_2O_2 generation based on the CAP-stimulated MDA-MB-231 cells immersed in a thin layer of DMEM is about 85% more than that formed in the CAP-stimulated same medium but lacking cells. Controlling the volume of medium, the cell confluence, and the plasma discharge voltage can regulate the cell-based H_2O_2 generation. The abundant short-lived reactive species in CAP may trigger this unique cellular response, which gives a new perspective to understand the interaction between CAP and cells *in vitro* and *in vivo*.

Materials and Methods

CAP device. The CAP device used in this study was a typical CAP jet generator using helium as the carrying gas. The noticeable anti-cancer effect of this device has been demonstrated through a series of previous investigations from our lab^{24,53}. The detailed introduction for this device was illustrated in previous reports^{24,53}. Here, a short introduction is given. A violet plasma jet was generated between a central anode and a ring grounded cathode. The discharge was driven by an alternating current high voltage (3.16 kV) with a frequency of 30 kHz. The generated CAP was ejected out from a quartz tube with a diameter of 4.5 mm. The flow rate of helium was about 4.7 L/min. The input voltage of DC power was 11.5 V. According to the emission spectrum, the CAP in the gas phase was mainly composed of ROS (OH, O), RNS (NO, N_2^+), and helium (He)³⁸.

Cell cultures. Human pancreas adenocarcinoma cells (PA-TU-8988T) and human glioblastoma cells (U87MG) were provided by Dr. Murad's lab at the George Washington University. Human breast adenocarcinoma cells (MDA-MB-231) were provided by Dr. Zhang's lab at the George Washington University, and cultured in the same protocol as previous studies. The Dulbecco's modified Eagle's medium (DMEM) was purchased from Life Technologies (11965-118). DMEM was mixed with 1% (v/v) penicillin and streptomycin solution (Life Technologies, 15140122). The medium used in the cell culture and seeding was composed of DMEM supplemented with 10% (v/v) fetal bovine serum (ThermoFisher Scientific, 26140079) and 1% (v/v) penicillin and streptomycin solution (Life Technologies, 15140122). In each experiment, 100 μ L of cancer cells harvesting solutions were seeded in a well on a 96-wells plate (Corning, 62406-081) a day prior to the CAP treatment. These cancer cells were cultured 24 hr under the standard cell culture conditions (a humidified, 37°C, 5% CO₂ environment). All wells on the margins of 96-wells plate were not used.

Making (N-Acetyl-L-cysteine) NAC-DMEM and pre-treating cancer cells. 6 mM NAC-DMEM was made by dissolving NAC powder (A7250, Sigma-Aldrich) in DMEM. The whole medium which has been used to culture cancer cells overnight were removed first. Then, 100 μ L of NAC-rich DMEM was used to culture cancer cells in the well of a 96-wells plate. After 3 hr, 6 mM NAC-DMEM was removed before the further treatment.

The direct and the indirect CAP treatment on cancer cells. The gap between the bottom of 12-wells plate and the CAP source was set to be 3 cm. All DMEM used in the CAP treatment was made of mixing 1% (v/v) penicillin-streptomycin solution (Life Technologies, 15140122) with standard DMEM (Life Technologies, 11965-118). The schematically illustration of the protocols was presented in Fig. S3. Prior to the direct CAP treatment, the medium which has been used to culture cells overnight was removed. After this step, for the direct CAP treatment, the DMEM with a specific volume such as 20 μ L was transferred to cover the cancer cells in a well on a 96-wells plate. After that, the CAP jet was used to vertically treat the cancer cells for 1 min. For the indirect CAP treatment, the CAP-stimulated DMEM (PSM) was used to affect the growth of cancer cells cultured in a 96-wells plate. To make PSM, the DMEM with a specific volume such as 30 μ L in a well on a 96-wells plate was treated by CAP jet vertically for 1 min. Such PSM was then transferred to affect the cancer cells which have been cultured overnight. For both direct and indirect CAP treatment, the control group corresponded to the cancer cells grown in the new DMEM with a specific volume without the CAP treatment. Then, cancer cells were cultured in the incubator under the standard culture conditions for 3 hr. Subsequently, the thin layer of DMEM around the cancer cells in each well was removed. 100 μ L of new untreated DMEM was added in the well and cultured cancer cells for 3 days before the final cell viability assay. In this study, the treatment just using helium rather than CAP was also performed. The protocols were the same as the description above except that no discharge was used. In another case, the DMEM covering the cells needed to be replaced immediately after the CAP treatment. After removing the CAP-treated DMEM, 100 μ L of new untreated DMEM was added to culture cells for 3 days before the final cell viability assay.

Cell viability assay. MTT (3-(4,5-Dimethyl-2-thiazol)-2,5-Diphenyl-2H-tetrazolium Bromide) assay was performed following the protocol provided by manufacturer (M2128, Sigma-Aldrich). The original experimental data about the cell viability was the absorbance at 570 nm measured by a H1 microplate reader (Hybrid Technology). The original absorbance at 570 nm was processed to be a relative cell viability through the division between the absorbance of the CAP-treated cancer cells and the absorbance of the cancer cells without the CAP treatment.

Extracellular H₂O₂ assay. The H₂O₂ concentration in DMEM or PBS was measured by using Fluorimetric Hydrogen Peroxide Assay Kit (Sigma-Aldrich, MAK165-1KT) following the protocols provided by manufacturer (Sigma-Aldrich). The fluorescence was measured by a H1 microplate reader (Hybrid Technology) at 540/590 nm. The final fluorescence of the experimental group was obtained by deducting the measured fluorescence of the control group from the measured fluorescence of the experimental group. Based on the standard curve, the H₂O₂ concentration in DMEM or PBS was obtained. Because the recommended volume of the sample solution was 50 μ L, when the volume of sample solution was less than 50 μ L but larger than 10 μ L, we just collected 10 μ L of the sample solution and mixed it with 40 μ L of the untreated DMEM to make a 50 μ L of the diluted sample solution. The H₂O₂ concentration of such case was 5 times larger than the measured concentration.

Measuring the H₂O₂ consumption speed by cancer cells. The protocols for different cancer cell lines are the same. Here, we just use PA-TU-8988T cells as an example. First, 100 μ L of the PA-TU-8988T cells harvesting solution was seeded in a well on a 96-wells plate with a confluence of 6×10^4 cells/mL. 3 wells were seeded as 3 samples for one experiment. Cells were cultured in incubator for 6 hr under the standard conditions. Then, 1 mL of DMEM or PBS in a well on a 12-wells plate was treated by CAP for 1 min. After that, the medium which has been used to culture cells for 6 hr was removed. 120 μ L of the CAP-treated DMEM or H₂O₂-containing DMEM (H₂O₂-DMEM) was transferred to culture PA-TU-8988T cells in one well on a 96-wells plate. 36.3 μ M H₂O₂-DMEM was made by adding 9.8 M H₂O₂ standard solution (216763, Sigma-Aldrich) in DMEM. Since then until the third hour, 50 μ L of the DMEM which has been used to culture cells was transferred to a well on a black clear bottom 96-wells plate in triplicate every hour. Ultimately, the residual H₂O₂ concentration in DMEM was measured using fluorimetric hydrogen peroxide assay kit illustrated above.

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Author Contributions

D.Y. and M.K. designed all experiments. D.Y., H.C., W.Z. and A.T., performed all experiments. L.Z. and J.S. contributed to the materials preparation. All authors contributed to the data analysis. D.Y. and M.K. wrote the manuscript.

Additional Information

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