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A comparative study of cold atmospheric plasma treatment, chemical versus physical strategy

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Abstract

To date, reactive oxygen species and reactive nitrogen species have been regarded as the key factors causing the observable cellular death of cold atmospheric plasma (CAP)-treated cancer cells. The chemical basis of the conventional CAP treatment highlights apoptosis as the main CAP-triggered cell death mechanism. However, we recently demonstrated a strong anti-melanoma effect based on physically-based CAP treatment. In this study, we compared the anti-cancer effect of chemically-based versus physically-based CAP treatment on four typical cancer cell lines *in vitro*. Three of these cancer cell lines were more sensitive to the physical factors in CAP than the chemical factors in CAP, while the fourth cell line followed the original trend of being more sensitive to chemical factors. This study demonstrated that the physically-based CAP treatment could be an effective anti-cancer approach, which might have a deep impact on the future application of CAP in medicine.

Keywords: cold atmospheric plasma, cancer treatment, physical treatment, cell death

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

1. Introduction

Cancer has been a large threat to human health for dozens of years [1]. To find a new, non-invasive anti-cancer modality is a challenge. Cold atmospheric plasma (CAP) is a near room temperature ionized gas composed of ionized reactive species, neutral particles, and electrons [2]. For a long time, only the chemical factors, the reactive species in CAP, have been regarded as the key players during the biological response to the CAP treatment [3, 4]. However, the success of this treatment strategy mainly relies on the sensitivity of the particular cancer cells to the reactive species, predominantly the long-lived reactive species such as H_2O_2 [5–8]. In addition, a layer of solution or medium covering the cells is also necessary for the treatment's success. This layer facilitates the transition of short-lived reactive species into long-lived reactive species, which then go on to affect the cells residing beneath it [9, 10]. Based on this rationale, plasma medicine is regarded as reactive species medicine, and in many cases even being referred to as H_2O_2-medicine.

However, this rationale brings up two questions when evaluating the results of *in vivo* experiments. One question is that there is no medium or solution layer on the skin or surrounding

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the tumor tissue in animal studies. This leads to the question of understanding how the anti-tumor effect that is witnessed *in vitro* is able to occur *in vivo*. Another point of concern involves the process of cell death. Apoptosis is the main form of cell death seen as a result of reactive species generated from CAP [5, 11]. Apoptosis does not trigger an immune response. How can we understand the immune response or inflammation seen in the *in vivo* treatment [12]?

Furthermore, the ultimate goal of CAP cancer treatment should be its clinical application. The way to use CAP as a clinical tool should be the guideline for the basic research in laboratories. Though the answers to this core question are not quite clear so far, we can get some clues in the current several clinical tests, which have been performed by various organizations where CAP treatment was incorporated into the patients' cancer treatment plans. One test was done by US Medical Innovation, LLC, and the CAP jet was used to treat the stage 4 colon tumor remnants after the surgery of removing the bulk tumor at Baton Rouge General Medical Center in Louisiana, USA [13]. Another test was performed by treating six patients with locally advanced squamous cell carcinoma of the oropharynx, who were suffering from open infected ulcerations in Germany [14]. In both two tests, the bulk CAP was used to directly touch the tumorous tissues or the remnants may have tumorous tissues. No water layer or medium layer covered these tumorous tissues when they contacted the bulk CAP. These two examples suggest that the direct contact of CAP with the tumorous tissue may be an important working condition in the clinical treatment. The physical effect from the bulk CAP may play an important or even a core role to determine the therapeutic efficacy of CAP treatment in these clinical cases.

Our recent discovery demonstrated that the physical factors, particularly the electromagnetic emission, of the CAP jet could also cause strong growth inhibition of a typical melanoma cell line B16F10 and glioblastoma cell line U87MG through a physical barrier approximately 1 mm thick [15, 16]. The physically-triggered cell death could also be observed even there was a macroscale gap (∼8 mm) between the tip of the jet and the bottom of the cell culture dish [16]. This study is a continuation of our recent work. Here, we compared the anti-cancer capacity of CAP treatment on four other typical cancer cell lines (breast cancer, lung cancer, and bladder cancer) using a physically-based strategy and a chemically-based strategy. The growth inhibition of cancer cells was observed to be a result of a new physically-triggered cell death. This new CAP-triggered cell death is characterized by rapid bubbling on the aggregated cytoplasmic membrane within 2 to 10 min after the treatment. We focused on the long-term features of this novel CAP-triggered cell death to observe the changes which take place days after physically-based CAP treatment.

2. Methods and materials

2.1. Cell culture

One orthotopic murine bladder cancer cell line MBT2, one mouse bladder carcinoma cell line MB49, one human triple-negative invasive ductal breast carcinoma cell line MDA-MB-231 cells, and one human lung carcinoma cell line A549 were used in this study. All of them were cultured in the medium composed of Dulbecco's Modified Eagle Media (DMEM, Life Technologies) supplemented with 10% (v v^{-1}) fetal bovine serum (GE Healthcare, SH30396) and 1% (v v^{-1}) penicillin and streptomycin solution (Life Technologies, 15140122). For each cell line, cancer cells were seeded (1 ml/well) in a 12-well plate with a density of 5×10^4 cells ml^{-1}. The cells were cultured 24 h under the standard culture condition (a humified, 37 °C, 5% CO_2 environment).

2.2. CAP jet device

The CAP jet was designed and manufactured at Dr. Keidar's lab at the George Washington University (figure 1(a)). The discharge was initiated between a copper ring grounded cathode and a central stainless anode in helium gas with a 99.995% purity (Roberts Oxygen, grade 4.5, size 300). The pulse frequency to trigger the discharge was 12.5 kHz. Helium was used to trigger the non-equilibrium discharge at atmospheric conditions. The ionized gas was flowed out (1.53 lpm) by the helium through a glass tube with a diameter of 4.5 mm. The ionized gas touched the surrounding air and finally forms a stable violet ionized gas jet, composed of an abundance of reactive species and other reactive components. The discharge voltage and current were 2.786 kV (rms) and 24.930 mA (rms), respectively (figure 1(b)). The maximum temperature of the jet tip was less than 40 °C. The gap between the nozzle and the target was 27.5 mm.

2.3. Two treatment approaches

In this study, we compared the anti-cancer efficacy of two different treatment approaches. To perform the traditional chemically-based treatment, the medium used in the overnight culture was removed first. Then, 1 ml/well of fresh medium (90% (v v^{-1}) DMEM with 10% (v v^{-1}) fetal bovine serum (FBS)) was added to cover the cells in a 12-well polystyrene plate (Stellar Scientific, TC10-012). The bottom thickness of the 12-well plate was 1 mm. The chemically-based treatment was subsequently performed by treatment the cells in the middle two columns (figure 1(b)). The cancer cells in the two side columns were chosen as the control group, without experiencing the treatment. The medium layer blocked all physical factors in CAP; thus, only the chemical factors affected the cells in this approach [15, 16]. All cells were cultured for 3 d before the final cell viability assay. To perform the physically-based treatment, the medium used in the 24 h of culture was removed first. The physical treatment was performed on the bottom of an inverted 12-well plate (figure 1(c)). The bottom plate can block all chemical factors and also UV in practical. Similarly, the center six wells were chosen to perform the treatment. The six wells in the two outer columns were chosen as the control group. A medium of 1 ml/well was quickly added to culture cells after the treatment. The final cell viability assay was carried out after 3 d of culture. In the case of

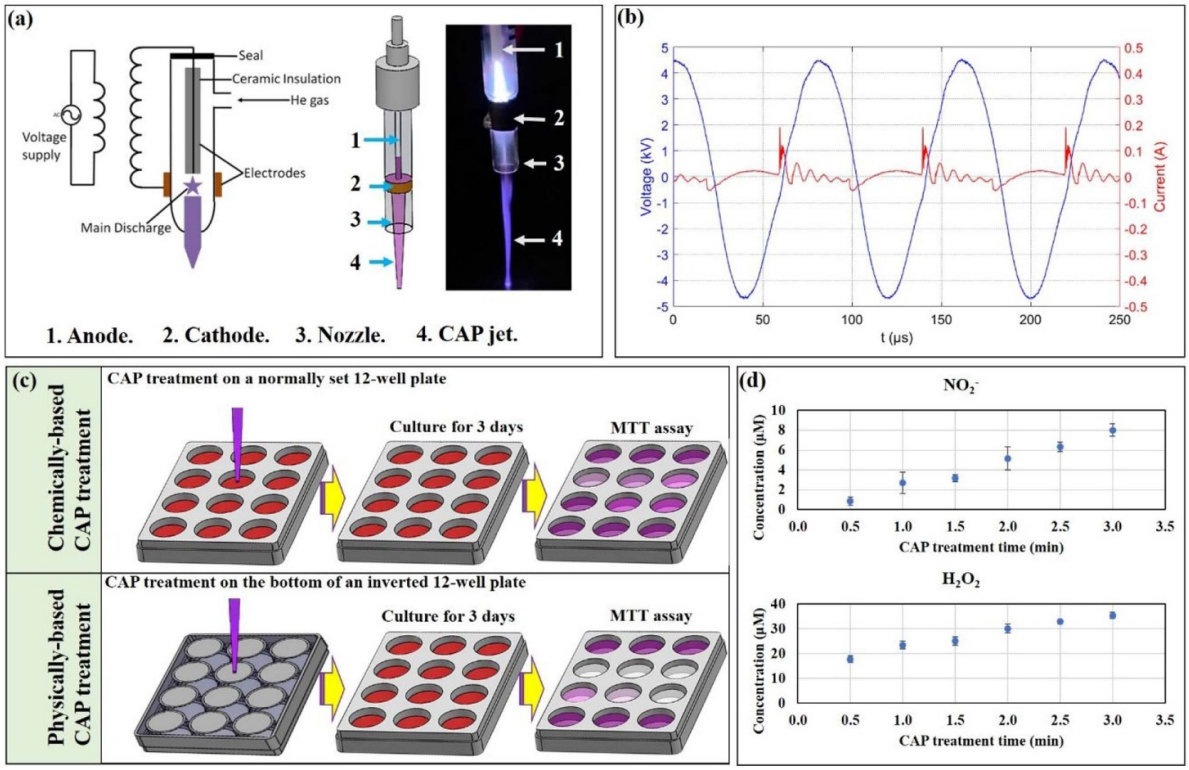

Figure 1. The CAP jet and two different treatment methods. (a) The schematic illustration of the CAP jet device and corresponding photo. (b) The discharge voltage and the discharge current in the operation. (c) The schematic illustrations of the physically-based treatment and chemically-based treatment. (d) The NO_2^- and H_2O_2 generation in the CAP-treated DMEM. Results are presented as the mean ± s.d. of the repeated tests.

the physically-based treatment, there may still be a very thin medium layer covering the cells due to the surface tension of water during the treatment on the inverted plate. As shown in the following figures, the robust growth of cells in the control group demonstrated that the cell viability of cancer cells would not be affected during the inverted setting. Our recent studies also confirmed this trend [15, 16].

The concentration of two important reactive oxygen species/reactive nitrogen species (NO_2^- and H_2O_2) in the CAP-treated DMEM is shown in figure 1(d). One milliliter of DMEM in the 12-well plate was treated by CAP for 0.5–3.0 min. The experimental conditions were as same as the treatment was performed on the cancer cells immersed in the medium. NO_2^- and H_2O_2 were measured using the Griess Reagent System (Promega) and Fluorimetric Hydrogen Peroxide Assay Kit (Sigma-Aldrich), respectively. The operation was carried out following the standard protocols provided by manufacturers. Both the absorbance and the fluorescence were measured by a H1 microplate reader (Hybrid Technology) at 530 nm and 540 nm (ex)/590 nm (em), respectively. The final data were obtained by deducting the signal of the control groups from the CAP-treated groups. Both NO_2^- and H_2O_2 concentrations were calculated based on the standard curves. The tests have been repeated three times.

2.4. The microscopic observation

The microscopic observation was performed by using a Nikon TS100 inverted phase-contrast microscope on the 12-well plates. As revealed in the previous studies, the typical features of the physically-triggered cell death include the obvious and quick bubbling on the CAP-treated cancer cells [15, 16]. Here, as an example, we gave a time-lapse observation of the growth of bubbles on the CAP-treated MB49 cells (figure 2). Such a quick growth of bubbles typically lasts 8–11 min. It is unknown whether growth always lasts with such a length. After this stage, the bubbles will not change until they detach from the cellular membrane over the following 1–2 h [15, 16]. In this study, we focused on the cellular change days after the treatment, which explained the cell viability data obtained using the chemical assay.

2.5. Cell viability assay

The cell viability assay was used to quantify the growth inhibition due to the treatment. The assay was performed using MTT assay according to the standard protocols suggested by the manufacturer (Sigma-Aldrich, M2128). The absorbance at 570 nm was read using an H1 microplate reader (Hybrid Technology). The value of the control group in each case was the mean value of six wells chosen as the control (always the wells in the two side columns). The normalized cell viability was obtained by the division between the experimental group and the control group. The intrinsic growth rate of the cell lines may not affect the comparison of the sensitivity of different cell lines using a proliferation-based assay such as MTT assay [17].

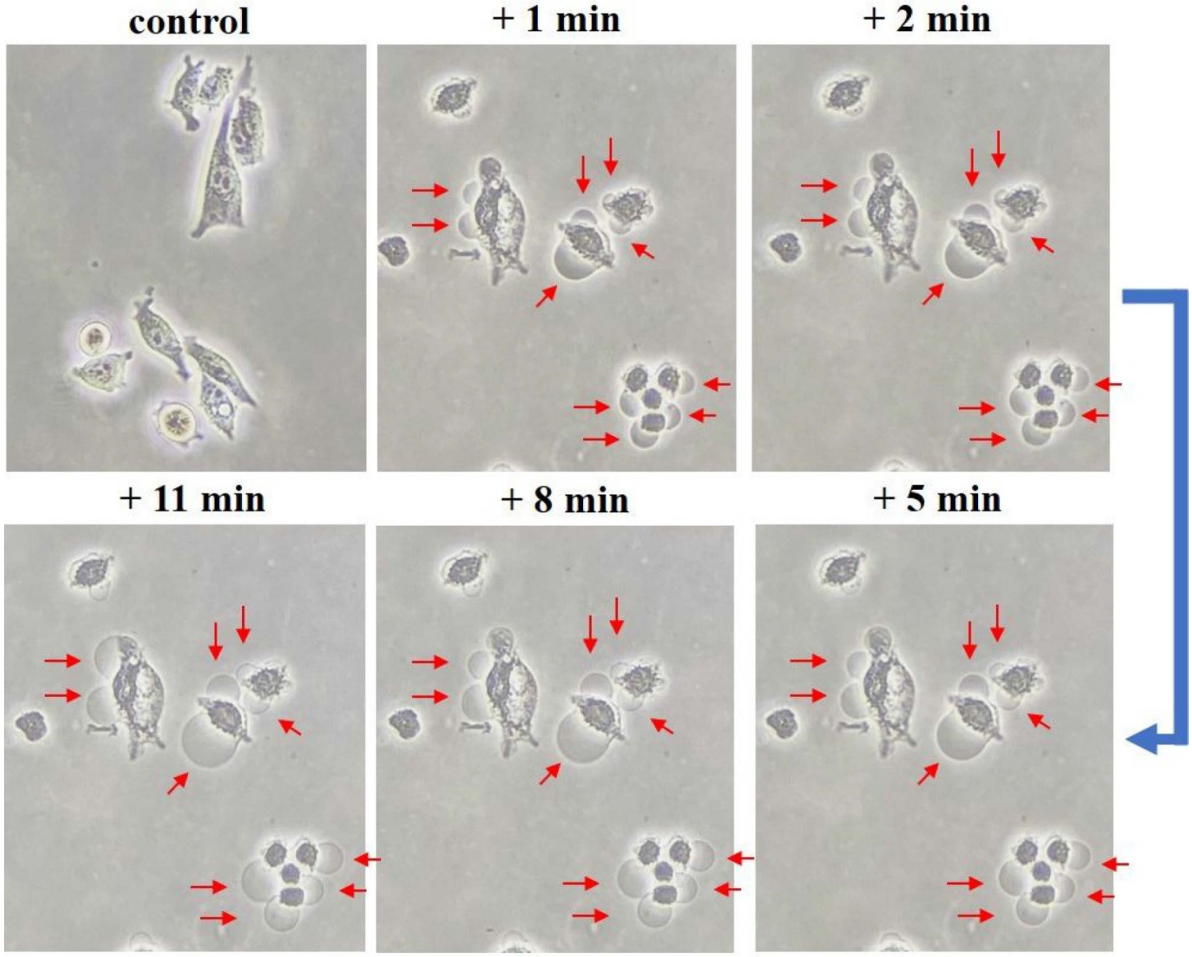

Figure 2. The typical cellular change immediately after the physically-based treatment. The cells in the control group did not experience the CAP treatment. Here, we used MB49 cells as an example. The red arrows mark bubbles on the cellular membrane. MB49 cells (5×10^4 cell ml^{-1}) of 10 ml were seeded in 100 mm dishes and cultured for 24 h in the incubator. A 6 min of physically-based treatment was performed on the inverted 100 mm dish according to the protocols illustrated in section 2. '+x min' meant the photo was taken at x min after the treatment. The scale bar was 50 μm (black). The photos in the experimental group were taken in situ.

3. Results and discussion

The sensitivity to the chemically-based treatment and the physically-based treatment are cell line specific. The physically-based treatment is not definitely better than the chemically-based treatment. It will be determined by not only the treatment time length but also the different cellular response nature to the physical factors and chemical factors in CAP. The cell viability changes and the microscopic observation of cells at 1 d and 2 d after each of the two treatment methods are shown in figures 3–6. Since the results shared some similar features, we have summarized the basic features to draw a comparison between the two treatment approaches.

For breast cancer cell line MDA-MB-231, lung cancer cell line A549, and bladder cancer cell line MBT2, the physically-based treatment caused a stronger anti-cancer effect than the chemically-based treatment at least when the treatment lasted a specific length. For MDA-MB-231 cells, both the physically-based treatment and the chemically-based treatment caused strong growth inhibition (figure 3). Two approaches did not show a noticeable difference except when the treatment time was 1.5 min, 2.0 min, and 2.5 min (figure 3(a)). In that range, the physically-based treatment showed better performance. Thus, for MDA-MB-231 cells, the physically-based treatment just presented stronger but limited anti-cancer capacity compared with the chemically-based treatment. For A549 cells and MBT2 cells, the physically-based treatment showed much better growth inhibition than the chemically-based treatment over nearly the whole treatment range, from 0.5 min to 3.0 min (figures 4 and 5). The cell viability of the MBT2 cell line was not altered by the chemical factors in CAP, suggesting that it is a reactive species-resistant cell line (figures 5(a) and (b)). In contrast, MB49 cells were very sensitive to the chemical factors in CAP and showed strong resistance to the physically-based CAP treatment (figure 6). Overall, the two bladder cancer cell lines that were studied showed greater resistance to the physically-based treatment than the breast cancer and lung cancer cell lines. One minute of physically-based treatment did not cause growth inhibition on the bladder cancer cell lines; however, just 30 s of physically-based treatment could significantly kill MDA-MB-231 cells and A549 cells.

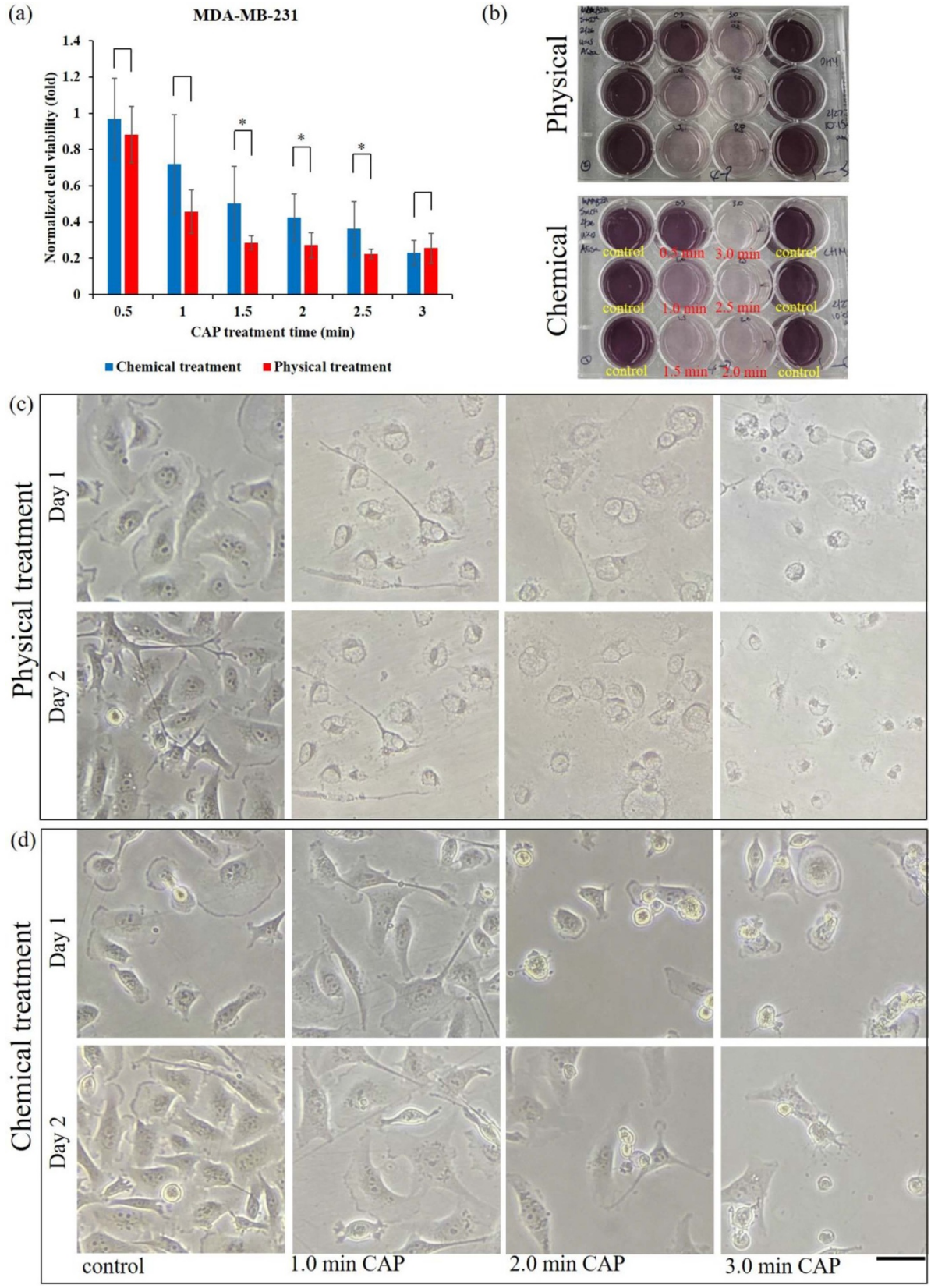

Figure 3. MDA-MB-231 cells. (a) The growth inhibition by two treatment approaches. Results are presented as the mean ± s.d. of the experiments repeated three times. Student's *t*-test was performed, and the significance was indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. (b) A representative MTT assay photo. The experimental design is marked on the bottom panel in yellow and red letters. (c) The microscopic photos of cells after the physically-based treatment. (d) The microscopic photos of cells after the chemically-based treatment. 'Day *x*' signifies the photo was taken at *x* day after the treatment. The scale bar (black) is 50 μm.

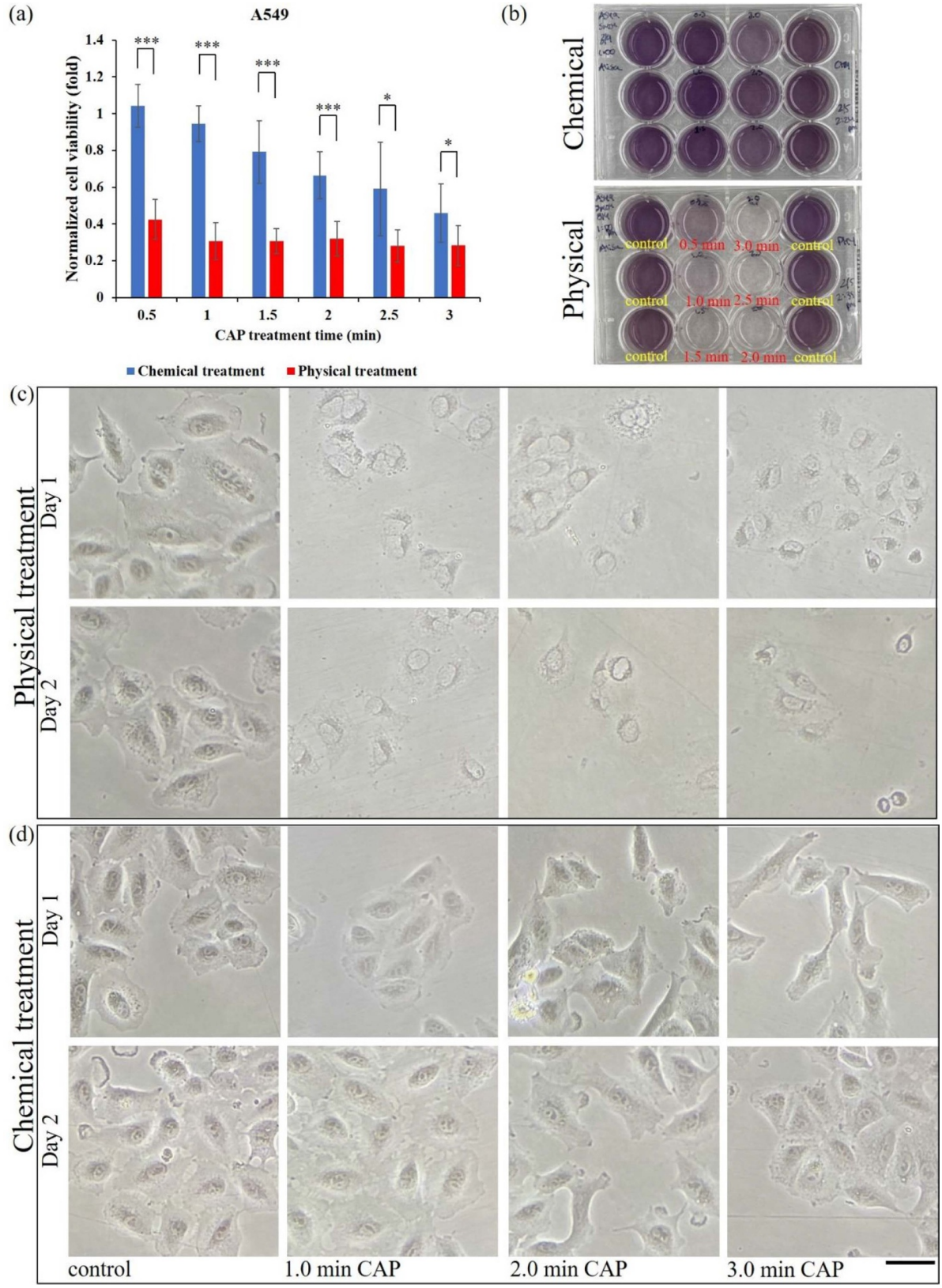

Figure 4. A549 cells. (a) The growth inhibition by two treatment approaches. Results are presented as the mean ± s.d. of the experiments repeated three times. Student's *t*-test was performed, and the significance was indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. (b) A representative MTT assay photo. The experimental design is marked on the bottom panel in yellow and red letters. (c) The microscopic photos of cells after the physically-based treatment. (d) The microscopic photos of cells after the chemically-based treatment. 'Day *x*' signifies the photo was taken at *x* day after the treatment. The scale bar (black) is 50 μm.

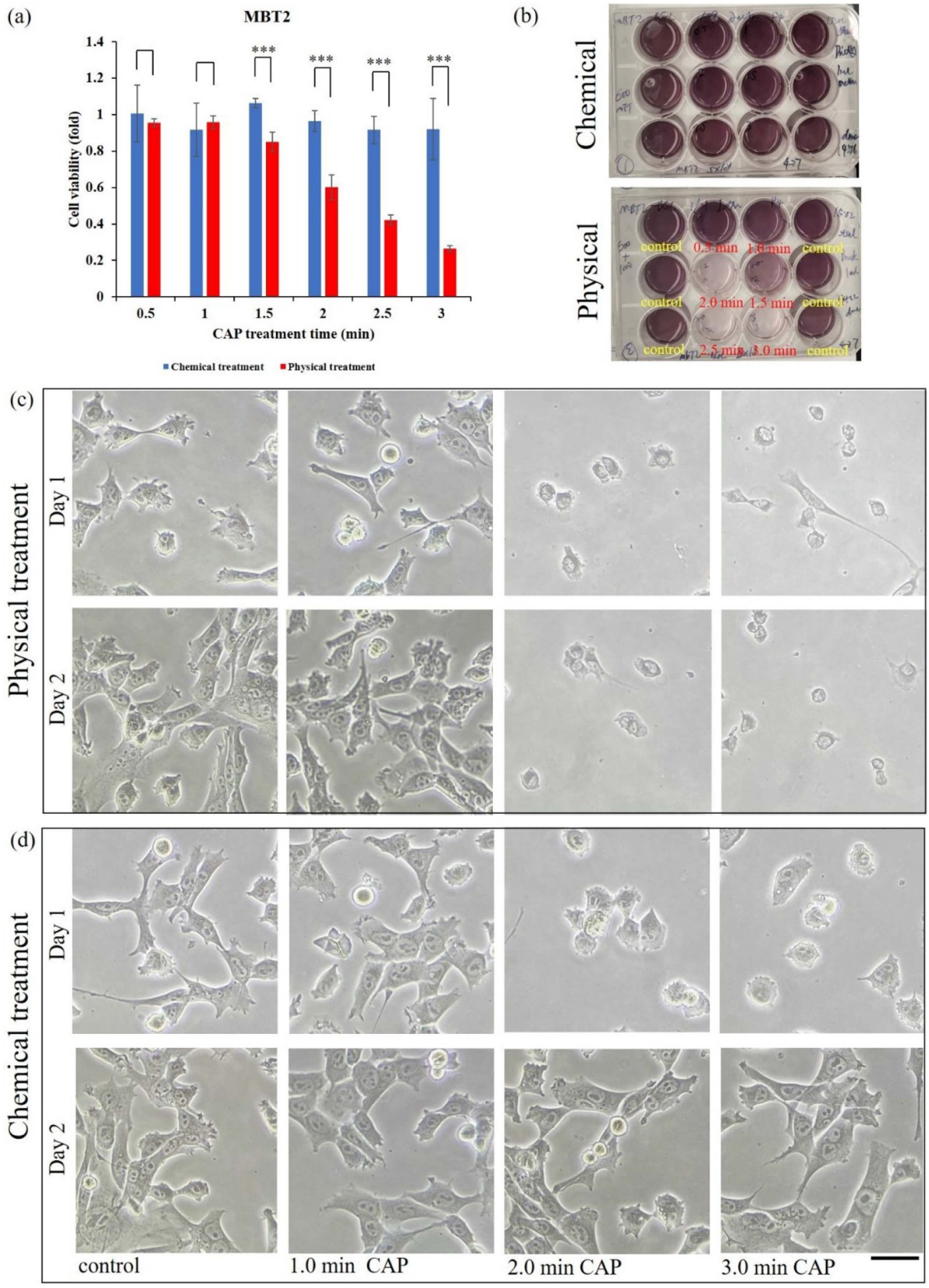

Figure 5. MBT2 cells. (a) The growth inhibition by two treatment approaches. Results are presented as the mean ± s.d. of the experiments repeated three times. Student's *t*-test was performed, and the significance was indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. (b) A representative MTT assay photo. The experimental design is marked on the bottom panel in yellow and red letters. (c) The microscopic photos of cells after the physically-based treatment. (d) The microscopic photos of cells after the chemically-based treatment. 'Day *x*' signifies the photo was taken at *x* day after the treatment. The scale bar (black) is 50 μm.

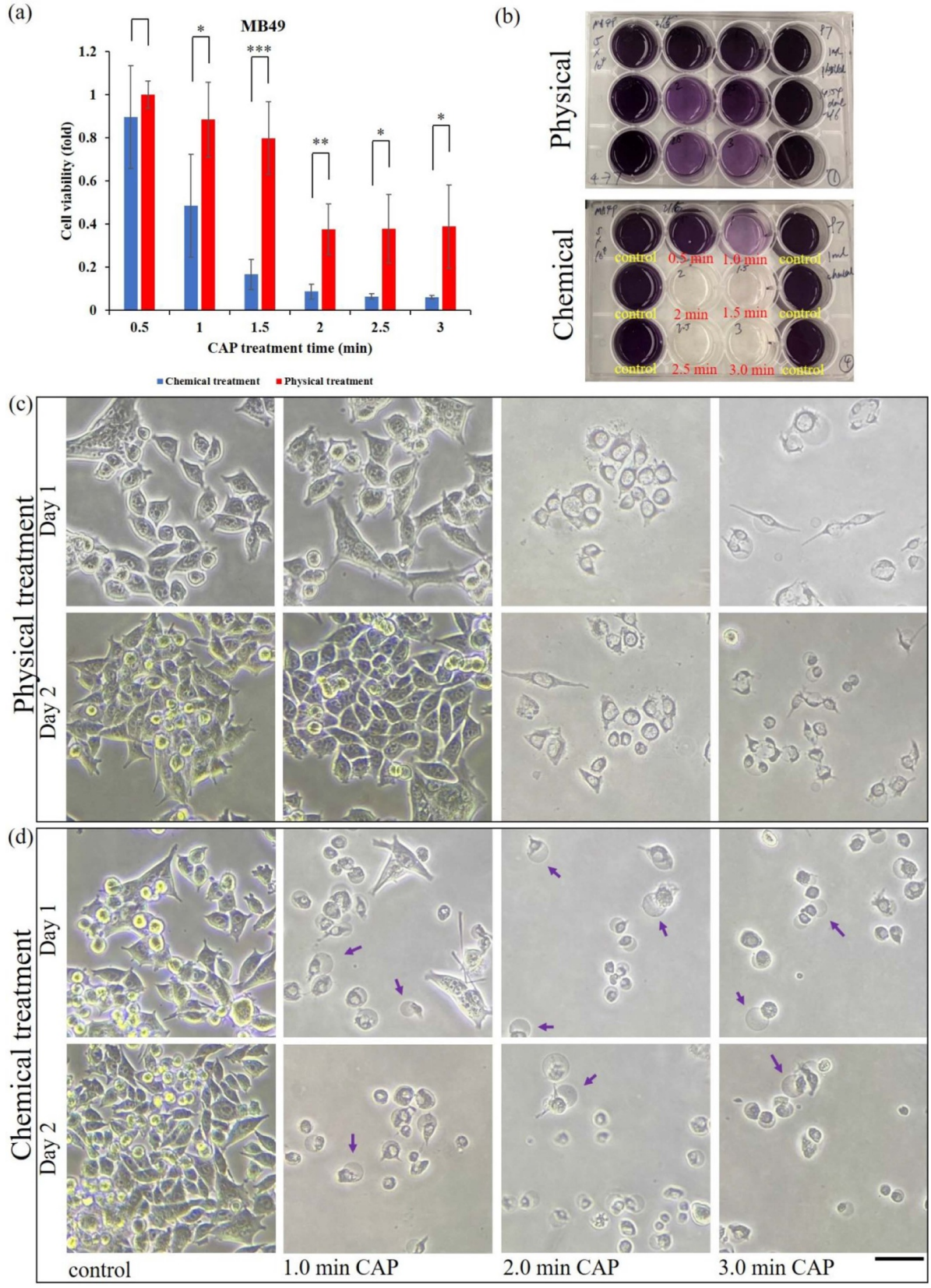

Figure 6. MB49 cells. (a) The growth inhibition by two treatment approaches. Results are presented as the mean ± s.d. of the experiments repeated three times. Student's *t*-test was performed, and the significance was indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. (b) A representative MTT assay photo. The experimental design is marked on the bottom panel in yellow and red letters. (c) The microscopic photos of cells after the physically-based treatment. (d) The microscopic photos of cells after the chemically-based treatment. The bubbles were marked by violet arrows. 'Day *x*' signifies the photo was taken at *x* day after the treatment. The scale bar (black) is 50 μm.

The chemically-based treated cells showed the typical morphological changes previously noted such as the appearance of a 'bright sphere' of highly aggregated cells (figures 3(d) and 5(d)), typically which will be followed by the detachment of cells or apoptosis. In most of these images, the chemically-based treatment only caused a decrease of cell density, which could have been due to the disappearance of or detachment of the dead cells. In contrast, the physically-based treatment could effectively inhibit the growth of all three cell lines with cell death features differing from apoptosis, but consistently present among all three cell lines (figures 3(c), (d), 4(c), (d), 5(c), (d), 6(c) and (d)). Compared with the control group, there were three prominent features of this cell death in the experimental group. First, the cytoplasm drastically shrank towards the nucleus, and in many cases, the cytoplasm appeared as just a thin shell surrounding the nucleus. This feature can be explained by the quick bubbling on the cytoplasmic membrane in the CAP-treated cells at the initial stage of the physically-based treatment (figure 2). Next, the nucleus became white or transparent, suggesting the loss of matter from within the nucleus. This feature has not been seen in the initial stage of bubbling. Therefore, the loss of nuclear components most likely occurs during the culture of the treated cells. The slow loss of DNA in the CAP-treated melanoma cells B16F10 has been confirmed using fluorescent imaging in our recent study [16]. These two features resulted in the cells appearing like empty shells days after CAP treatment.

Lastly, the third feature was that all of the features noted after physically-based CAP treatment appeared to withstand any further change for days after the treatment. No cellular activities, such as division, migration, or apoptosis, were observed among the treated cells. We referred to this feature as the physically triggered 'fixation' of cells. This led us to theorize that since such fixation results in a stagnant cell number, a stronger anti-cancer effect could be achieved after physically-based treatment. The underlying molecular mechanism is totally unknown at the current stage, the further biochemical and cellular analysis should be performed systematically in continuous studies.

It is necessary to point out the bubbling cannot simply be used to discriminate the physically-triggered cell death from the chemically-triggered cell death. As shown in figure 6(d), the bubbling was also observed on the chemically-treated MB49 cells. Similar chemically-triggered bubbling has been noted in previous studies on other cell lines such as HeLa cells [18]. Such a bubbling has been regarded as a feature of pyroptosis, a programmed necrosis [19]. However, bubbling following chemically-based treatment is different from the bubbling seen after physically-based treatment. After chemically-based treatment, it was observed that the bubbling formed slowly, compared to the rapid bubble formation seen after physically-based treatment. The bubbles did remain on the cytoplasmic membrane for days after chemically-based treatment (figure 6(d)) whereas most of the bubbles disappeared from the membrane of cells which underwent physically-based treatment within hours. However, a few bubbles, as well as bubble debris, were observed in specific cell lines such as MB49 days after physically-based treatment (figures 3(c) and 6(c)). In addition, the cytosol and the nucleus of the chemical CAP-treated cells did not show the same features that physically-based CAP treated cells exhibits which were described above.

In addition to the cytotoxicity, the physically-based treatment shows another favorable advantage when compared to chemically-based treatment. Physically-based CAP treatment allows for a treatment modality that is non-invasive, contactless, and transbarrier. The traditional chemically-based treatment relies on reactive oxygen and nitrogen species which require direct contact with the target cells and/or tissues as well as the presence of an aqueous layer to facilitate the formation of the long-lived reactive species such as H_2O_2. An air plasma source was used to directly treat the skin of a mouse with a thickness of 0.75 mm. Some authors did not observe the formation of H_2O_2 or NO_2^-/NO_3^- in the deionized water underneath the skin after the direct CAP treatment on the skin [20]. However, the EM emission of the CAP jet in physically-based CAP treatment can penetrate a dielectric material [15, 16].

Furthermore, we propose that the physically-based cell death seen *in vitro* may be present *in vivo* and be responsible for the anti-tumor effect seen in CAP treatment of subcutaneous tumors in animal studies. The bubbling resulting from physically-based treatment is characterized by the bulk and rapid leakage of cellular solution from the CAP-treated cells. This feature of the novel CAP-triggered cell death provides a reasonable connection between CAP treatment *in vitro* and the immune response or inflammation observed at the CAP-treated site *in vivo*. Nearly all previous studies concluded that apoptosis was the main cell death triggered by the chemically-based CAP treatment. Apoptosis will not trigger an immune or inflammation response *in vivo*; however, several animal studies found a noticeable immune response after CAP treatment [21–23]. The newly found CAP-triggered cell death, clearing necrosis involving the leakage of cellular solutions, will likely trigger an immune response *in vivo* if the same damage occurs *in vivo*.

4. Conclusions

Conventional plasma medicine has been roughly based on the reactive species and the corresponding biological effect. Demonstration of the universal efficacy of the physically-based treatment among four cell lines provides a novel strategy to use CAP as a non-invasive anti-cancer therapy, independent of reactive species and other chemical factors. The sensitivity of cancer cells to the physical factors and the chemical factors in CAP was found to be cell line specific. For most cell lines in this study, the physical factors caused a more significant killing effect on cancer cells than the chemical factors of CAP did. We theorize that the physically-based treatment can be more successful by causing a new type of CAP-triggered cell death as well as by 'fixing' the treated cells to the substrate. For the reactive species resistant cell line, the physically-based treatment could still achieve a promising growth inhibition effect. This study provided an approach to

overcome a natural limitation presented by the reactive species in traditional chemically-based CAP treatment by introducing a new physically-based CAP treatment method into the field of plasma medicine.

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