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Distinct biological actions of electrical and chemical factors of cold atmospheric pressure plasma and their synergistic cytotoxic effects

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Abstract

Cold atmospheric pressure plasma (CAP) is recently regarded as a novel means for cancer therapy, because of its prominent cytotoxicity. The combined effects of electrical and chemical factors of CAP have been considered to achieve the cytotoxicity. Whereas reactive species are well known to be important for the CAP cytotoxicity, the biological significance of electrical factors of CAP remains largely unclear. In this study, we analyzed cytotoxic effects of a combination of electrical and chemical factors of CAP on the human fibrosarcoma HT-1080 cells. When cells were incubated in CAP-exposed solution that contained substantial levels of H_2O_2 and NO_2^-, their viability was markedly decreased. In the absence of effects of reactive species, electrical factors of CAP did not show any adverse influences on cells. Interestingly, CAP-exposed solution exhibited more cytotoxicity on the electrically stimulated cells than unstimulated ones. This observation suggests that the electrical stimulation altered the nature of cells and increased cell susceptibility to CAP-exposed solution. Taken together, our findings reveal a novel aspect of the biological action of electrical factors, which is mechanistically distinct from that of reactive species, and demonstrate that electrical and chemical factors of CAP synergistically act on cells to achieve high cytotoxicity.

Keywords: Cold atmospheric plasma, reactive species, electrical properties, cancer cells.

1. Introduction

Cold atmospheric pressure plasma (CAP) is a partially ionized gas generated at near room temperature. CAP contains a variety of physical and chemical components, including UV ray, photons, charged particles, electrically excited atoms, and chemically reactive species [1–4]. Exposure of human cells to CAP has been reported to elicit various cellular responses in an intensity-dependent manner. Strong CAP efficiently induces cell death, and relatively weak treatment with CAP can stimulate cell proliferation and tissue regeneration [5,6]. CAP is recently considered to be a novel physical approach for various medical applications, such as wound healing, blood coagulation, and cancer therapy, which are collectively called plasma medicine [7–12]. Among various medical applications, CAP holds great promise for cancer therapy, because of its remarkable cytotoxicity.

It has been postulated that both electrical and chemical properties of CAP may contribute to the biological action of CAP [13]. As for the chemical properties of CAP, many preceding studies have clearly demonstrated the cytotoxic action of CAP-derived reactive species [14–19]. CAP could provide a wide array of reactive species such as superoxide anions ($O_2^{\cdot -}$), hydroxyl radicals ($\cdot OH$), hydrogen peroxide (H_2O_2), and nitrite (NO_2^-). These reactive species are considered to primarily account for the cytotoxic action of CAP [20]. When CAP is applied to aqueous solution (e.g., cell culture medium), such CAP-exposed solution is called "plasma-activated medium" or PAM [21, 22]. PAM contains a distinct set of reactive oxygen and nitrogen species (hereafter, referred to as RONS). Typically, PAM contains elevated levels of H_2O_2 and NO_2^- that are maintained over several hours [23]. PAM is well known to exhibit significant cytotoxicity for long periods

after CAP exposure, which is attributed to the persistent presence of elevated H_2O_2 and NO_2^- [24, 25]. Based on its cytotoxicity, PAM is considered to be a new indirect way of CAP usage for cancer therapy.

In contrast to the well-documented cytotoxic effects of CAP-derived RONS, the biological influence of electrical properties of CAP has remained largely elusive. A major reason for the limited information on the electrical aspect of CAP action is attributed to the strong cytotoxicity of RONS, which easily overwhelms the effects of electrical stimulation under CAP-generating conditions. To circumvent this difficulty, we have developed a novel experimental device that allowed to sequester cells from RONS during electrical stimulation under CAP-generating conditions. Using this device, we have demonstrated that relative weak electrical stimulation under CAP-generating conditions caused elevated cell motility along with characteristic morphological changes [26, 27]. Because the stimulated cells moved randomly and did not show oriented migration along the axis of electricity, we speculated that the electrical action of CAP induces the activation of certain intracellular mechanism(s), eventually leading to elevated cell motility in random directions. Importantly, we did not detect any signs of cell death induction during the electrical stimulation under CAP-generating conditions. Collectively, these observations suggested that the electrical action of CAP on cells is mechanistically distinct from that of CAP-derived RONS.

In this study, we attempted to understand the contribution of electrical and chemical factors to the cytotoxicity of CAP. To this end, we treated cells with the electrical properties of CAP in combination with PAM. Intriguingly, the electrical stimulation under CAP-producing conditions did not show apparent cytotoxicity, but cells became more susceptible to PAM. This observation suggests that the electrical properties of CAP can alter the nature of cells, which is not manifested as cytotoxicity. Our findings cast light on the biological significance of electrical factors of CAP and serve as a new clue for further investigation toward a more comprehensive understanding of biological actions of CAP.

2. Materials and methods

2.1 Experimental Setup

Fig. 1 (a) shows the experimental setup that was composed of three units, a power reservoir, a culture chamber, and a ground reservoir. The power reservoir and the ground reservoir contained Hanks' Balanced Salt Solution (HBSS, Gibco, cat. no. 14025092). A glass-bottomed slide with four small compartments (W10.4 × L20.6 × H11.5 mm each, Eppendorf, cat. no. 0030742028) was used as culture chambers. The culture chamber was placed in a CO_2 incubator that maintained 37°C and 5% CO_2 conditions. The culture chamber was connected to the power reservoir and the ground reservoir through agarose bridges that were made of 2% agarose (Sigma-Aldrich, cat. no. A7002) and phosphate-buffered saline (PBS, Gibco, cat. no. 20012050).

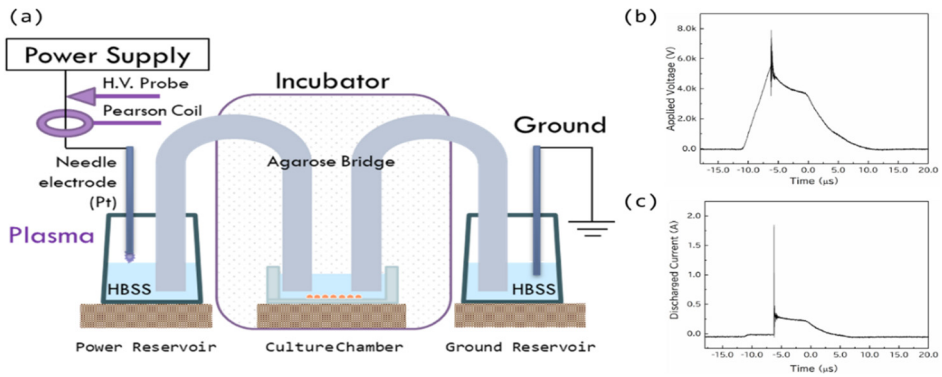

Fig.1. Experimental setup and typical electric waveforms. (a) Schematic representation of experimental setup used in this study. Streamer discharge was generated on the surface of HBSS in the power reservoir. Cells were cultured in the culture chamber that was maintained at 37°C and 5% CO_2. Agarose bridges connected the culture chamber to the power reservoir and ground reservoir. (b, c) Typical electric waveforms of streamer discharge. Applied voltage with a repetitive frequency of 100 Hz was kept at 7.8 kV (b), and the maximum streamer pulsed current was measured to be typically 1.8 A with a rise time of less than 15 ns (c).

The agarose bridge was made with the agarose gel filled in an inverted U-shaped glass tube with 262 mm in center length, 5.4 mm in inner diameter and 8 mm in outer diameter. The end faces of the agarose bridge tubes were located at the both sides of chamber wall with 5 mm in height, and the distance between the centers of tubes in the chamber was 12 mm. The voltage drop in the culture chamber was nearly zero because the resistance in the agarose bridges is much greater than that in the culture chamber.

The high voltage power amplifier (High Voltage Amplifier model P05034, Trek) was connected to a needle-type electrode that was made of platinum and sharpened to a tip radius of 15 μm. Output from the high voltage power amplifier was controlled by a function generator (Multifunction Generator WF1968, NF). Streamer discharge was generated in ambient air on the surface of HBSS in the power reservoir (Fig. 1 (a)). The distance between the needle tip and the HBSS surface was kept at 1 mm, so that the streamer discharge directly hit the HBSS surface. Electric waveforms of the discharge were measured using an oscilloscope (Wave Surfer 104MXs-B) through a high voltage probe (HV–P30A, Iwatsu electric co., ltd.) and a Pearson current monitor (model 6585, Pearson electronics. Inc.). Applied voltage with a repetitive frequency of 100 Hz was kept at 7.8 kV (Fig. 1 (b)), and the maximum streamer pulsed current was measured to be typically 1.8 A with a rise time of less than 15 ns (Fig. 1 (c)).

2.2 Cell culture

The human fibrosarcoma cell line HT-1080 was provided by Japanese Collection of Research Bioresources Cell Bank. Cells were cultured in minimum essential medium eagle (Sigma-Aldrich, cat. no. M4655-500) containing 10% fetal bovine serum (Gibco, cat. no. A3160601) and 1% penicillin/streptomycin (Nacalai Tesque, INC., 26253-84) in a humidified incubator at 5% CO_2 and 37°C. For passage, 0.25 w/v % trypsin 1 mmol L^{-1} EDTA·4Na solution with phenol red (Wako, 201-16945) was used for dispersing cells. For cell stimulation experiments, 2.5×10^5 cells in 1 mL culture medium were placed in each culture compartment and incubated for 24 h to allow cell attachment. After replacement of culture medium with 1500 μL of HBSS, cell stimulation was carried out. All experiments were conducted in a humidified incubator at 5% CO_2 and 37°C.

2.3 Fluorescence microscopy and viability analysis

For fluorescence microscopy, cells were costained with three fluorescent dyes, i.e., ethidium homodimer III, Hoechst 33342, and FITC-Annexin V, using an apoptotic/necrotic/healthy cells detection kit (PromoKine, PK-CA707-30018). Fluorescence images were obtained using an inverted fluorescence microscope (Axio Observer D1, Carl Zeiss). To assess cell viability, cells stained with ethidium homodimer III (red) were regarded as dead cells. Total cell numbers were obtained from Hoechst 33342 staining (blue). A ratio of red cells to blue cells was obtained from at least 1000 cells.

2.4 Measurement of H_2O_2, NO_2^-, and other factors of HBSS

Concentrations of H_2O_2 and NO_2^- in HBSS were analyzed by colorimetric methods using Pack test kits (WAK-H_2O_2 and WAK-NO_2, Kyoritsu Chemical-Check Lab. Corp.) with a digital colorimeter (DPT-MT, Kyoritsu Chemical-Check Lab. Corp.). Electrical conductivity, pH, and temperature of HBSS were measured using a conductivity tester (ECTestr11+, Oakton), a pH meter (B-71X, HORIBA), and a thermometer (E5CN-H, Omron), respectively.

2.5 Fluorescence microscopy and viability analysis

Enzymatic activity of lactate dehydrogenase (LDH) in HBSS was quantified by a colorimetric assay using an LDH cytotoxicity detection kit (Takara, cat. no. MK401) and a Multiskan FC microplate photometer (Thermo Scientific, model: 51119100) according to the manufacturer's instructions.

2.6 Fluorescence microscopy and viability analysis

Each experiment was repeated at least three times, and all data were expressed as mean ± standard deviation (SD). Student's t-test analysis was performed, and statistic significance was presented as follows; *: $p < 0.05$; **: $p < 0.01$.

3. Results

3.1 Measurement of long-lived RONS and other factors

In this study, we used an experimental setup that was composed of three units, a power reservoir, a culture chamber, and a ground reservoir (Fig. 1 (a)). Streamer discharge was generated on the surface of HBSS in the power reservoir. Cells were cultured in the culture chamber that was electrically connected to the power reservoir through an agarose bridge but sequestered from RONS generated by streamer discharge in the power reservoir.

To confirm this, we measured concentrations of H_2O_2 and NO_2^- along with pH fluctuations in three units during 60-min streamer discharge (Fig. 2). In the power reservoir (Fig. 2 (a)), we detected significant increases in H_2O_2 and NO_2^- concentrations that were assessed to be 13 ± 2 ppm and 8 ± 1 ppm, respectively, at 60 min. Slight decreases of pH were also observable, presumably due to the increasing NO_2^- concentrations. In the culture chamber, on the other hand, H_2O_2 and NO_2^- were virtually absent (below the detection limits), and pH was relatively constant during 60-min streamer discharge (Fig. 2 (b)). Similar to the culture chamber, very low H_2O_2 (0.18 ppm) and the absence of NO_2^- were measured in the ground reservoir (Fig. 2 (c)). Temperature and conductivity of all three units remained constant during 60-min streamer discharge (33.4−34.7°C and 13.3−14.8 mS cm^{-1}, respectively. Data not shown), indicating ignorable joule heating under our experimental conditions. Taken together, these observations demonstrated that our experimental setup allowed us to dissect the combined effects of electrical factors and long-lived RONS on cell physiology.

3.2 Microscopic observation of cytotoxicity of PIE, PAM, and their combinations

We next performed fluorescence microscopy to analyze the effects of electrical factors and long-lived RONS on cells. To investigate cell states, cells were costained with three fluorescent dyes. Ethidium homodimer III is a membrane impermeant dye and stains the nucleus red when the cell membrane is severely damaged. FITC-Annexin V is a green fluorescent dye for the cell membrane of severely damaged cells. Hoechst 33342 stains nuclear DNA blue irrespectively of healthy or damaged state, and thus we used this dye for measuring total cell numbers.

Procedures for cell treatment were summarized in Supplementary material. First, streamer discharge was generated in the power reservoir for 60 min. Because the culture chamber was connected with the power reservoir through an agarose bridge, the cells in the culture chamber were electrically stimulated without the influence of RONS (hereafter, called "plasma-induced electrical properties" or PIE). After PIE stimulation for 1 h, the cells were cultured for 24 h and subsequently subjected to staining with three fluorescent dyes. As seen in Fig. 3 (b), a majority of the PIE-stimulated cells were stained blue, but not red or green, which was indistinguishable with staining of untreated negative control cells (Fig. 3 (a)). This observation indicated that PIE stimulation did not cause apparent cytotoxicity, which was in accordance with our previous studies [26,27]. Next, we examined the effect of plasma-activated HBSS. HBSS was exposed to streamer discharge for 60 min and utilized as plasma-activated medium (PAM). PAM was directly added to cells that were cultured in an isolated chamber (without PIE treatment). When cells were cultured in PAM for 1 h, we observed red and green fluorescence in several cells (Fig. 3 (c), up panel). When cell culture in PAM was continued for 24 h, many cells exhibited red and green fluorescence (Fig. 3 (c), down panel), indicating the cytotoxicity of PAM.

Next, cells were stimulated with PIE in HBSS for 1 h, and HBSS in the culture chamber was replaced with that in the power reservoir (PAM). Cells were subsequently incubated in PAM for 24 h (referred to as "PIE→ PAM"). As shown in Fig. 3 (d), we observed that many cells had red and green fluorescence after 24 h incubation. When compared to PAM-only treatment, more cells appeared to be stained with red and green fluorescence. Finally, we combined PIE and PAM for cell treatment. Cells were placed in PAM, stimulated with PIE in the presence of PAM for 1 h, and further incubated for 24 h in PAM (hereafter "PAM+PIE"). As seen in Fig. 4 (e), PIE stimulation in PAM for 1 h resulted in strong staining of red and green fluorescence, indicating that cells were severely damaged at this point. After 24 h incubation in PAM, many cells appeared to be dead, as judged by compromised cell structures, such as round cell shape, and many detached cells. In summary, PIE showed no apparent effects on cell viability, and the combined use of PIE and PAM exhibited more cytotoxicity than PAM-only treatment.

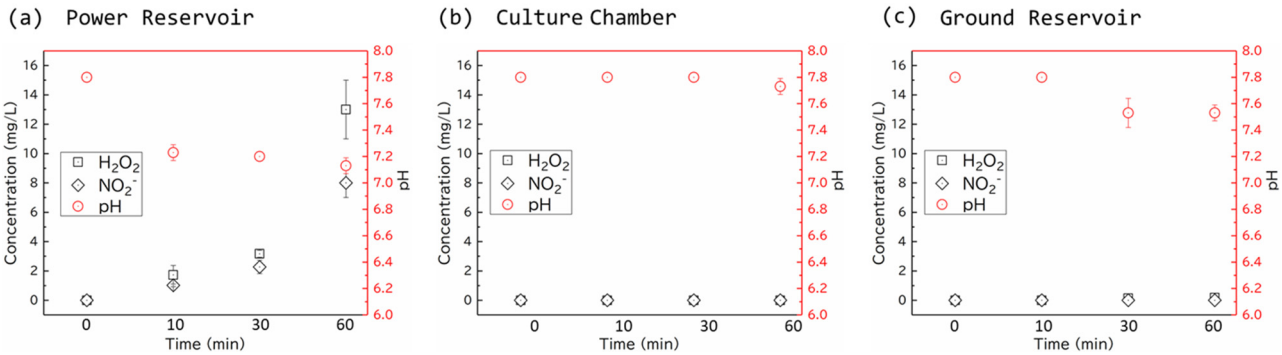

Fig. 2. Measurement of H_2O_2, NO_2^-, and pH. Concentrations of H_2O_2 and NO_2^-, and pH in the power reservoir (a), culture chamber (b), and ground reservoir (c) were measured during 60-min streamer discharge. Mean values and SD were calculated from three independent experiments.

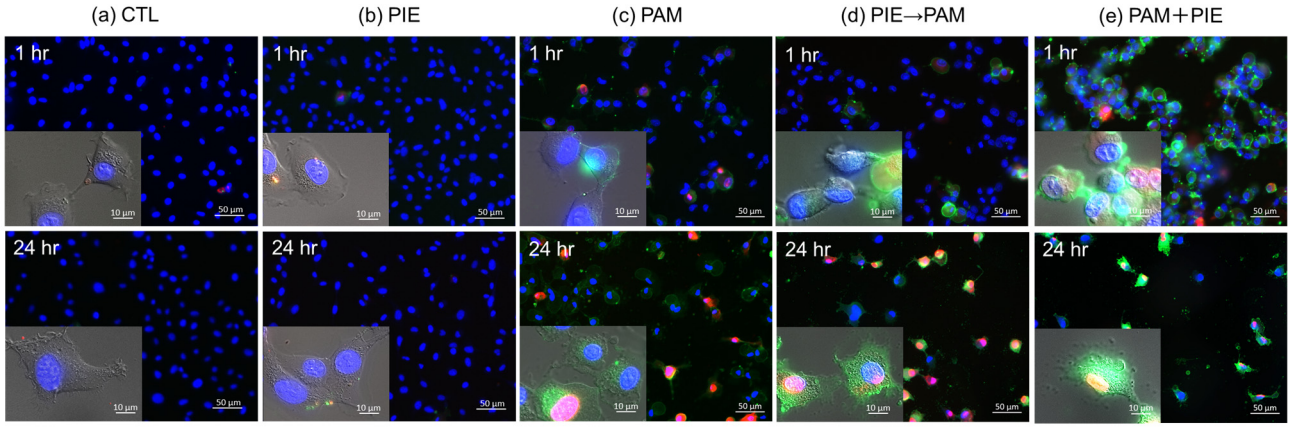

Fig. 3. Fluorescence microscopy of cells treated with PIE, PAM, and their combination. Cells were costained with Hoechst33342 (blue), FITC-Annexin V (green), and ethidium homodimer III (red). (a) CTL: Untreated control cells were incubated for either 1 h (up) or 24 h (down). (b) PIE: Cells were treated with PIE for 1 h and immediately stained (up). Another set of cells were treated with PIE for 1 h and cultured for 24 h (down). (c) PAM: Cells were incubated in PAM for either 1 h or 24 h. (d) PIE→PAM: Cells were treated with PIE for 1 h and immediately stained (up). Another set of cells were treated with PIE for 1 h and subsequently cultured in PAM for 24 h (down). (e) PAM+PIE: Cells were stimulated with PIE in the presence of PAM for 1 h. Cells were immediately stained (up), or cell culture was continued in PAM for 24 h (down).

3.3 Quantitative analysis of cytotoxicity of PIE, PAM, and their combinations

To gain further insight into the combined effects of PIE and PAM, we calculated cell viabilities from fluorescence images of cells. Cells with red fluorescence were counted to be dead, and total cell numbers were obtained from blue staining. As shown in Fig. 4, PIE stimulation did not show an apparent negative effect on cell viability at 24 h, whereas PAM treatment markedly decreased cell viability. Intriguingly, although PIE stimulation itself did not cause apparent cytotoxicity, PAM incubation of the PIE-stimulated cells resulted in lower cell viability than PAM-only treatment (Compare between "PAM" and "PIE→PAM" at 24 h in Fig. 4. $p < 0.05$). This observation suggests that pretreatment of cells with PIE augmented the cytotoxic effect of PAM. As expected, the maximum cytotoxicity was achieved by the simultaneous treatment with PAM+PIE (Fig. 4). By the FITC-Annexin V results shown in the Fig.3, those dead cells in the case of PAM+PIE were probably caused by induction of apoptosis.

To verify the effects of PIE and PAM, we employed another measurement. LDH plays a catalytic role in living cells and normally exists in cells. Because damaged cells leak LDH extracellularly, LDH activity in medium is widely utilized as a biochemical index for cell damage. As shown in Fig. 5, we observed that the LDH value of the PIE-stimulated sample after 24 h incubation ("PIE" in Fig. 5) was quite low and

indistinguishable with that of the untreated control (CTL). PAM treatment caused high LDH activity at 24 h, as expected from the result in Fig. 4. When cells were stimulated with "PIE→PAM" (namely, PIE for 1 h and then incubated in PAM for 24 h), the LDH value was significantly higher than that of the PAM-only sample ($p < 0.01$). The highest value was obtained from the "PAM+PIE" sample. These results on LDH in Fig. 5 were in good agreement with those in Fig. 4, further supporting the notion that PIE stimulation augmented the cytotoxic effect of PAM, even though PIE stimulation itself did not cause apparent cytotoxicity.

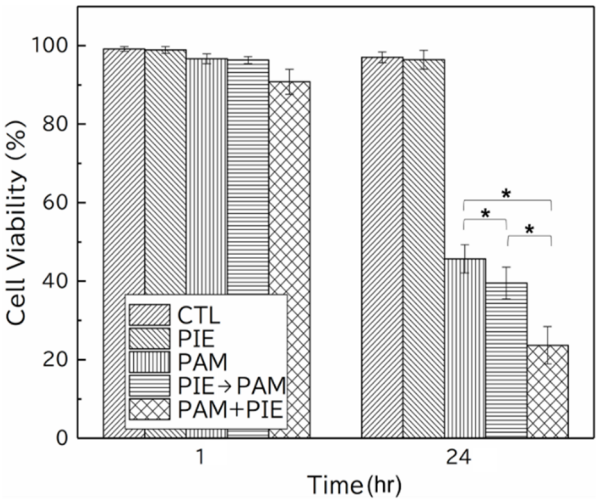

Fig. 4. Cell viability after treatment with PIE, PAM, and their combination.
Cells were treated as described in Fig. 3. (*: $p < 0.05$).

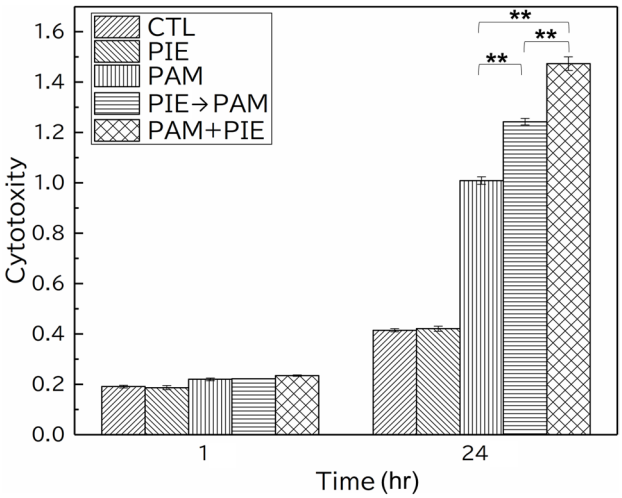

Fig. 5. Cytotoxicity after treatment with PIE, PAM, and their combination.
Cells were treated as described in Fig. 3. Cytotoxicity was measured as an extracellular LDH activity. (**: $p < 0.01$).

4. Discussion

CAP exerts various biological actions through its chemical and electrical factors. Whereas cytotoxic actions of CAP-derived RONS have been well documented so far, a biological contribution of electrical factors of CAP remains largely elusive. In this study, we utilized the experimental setup that enabled to treat cells with a combination of RONS and electrical factors (called PIE in this study) of CAP. Although PIE itself did not generate detectable levels of RONS nor did it have apparent cytotoxic effects, PIE-exposed cells exhibited increased susceptibility to PAM that contained H_2O_2 and NO_2^-. This finding reveals that PIE contributes to biological action of CAP in a way that has not been previously appreciated.

We speculate that there are at least two possible mechanisms by which PIE treatment causes the augmented cytotoxicity of PAM. First, PIE may elicit structural and/or chemical alterations in the cell membrane, leading

to increased PAM cytotoxicity, presumably through increased RONS permeability. The cell membrane is known to be the primary site of action of electrical impacts, such as pulsed electric fields. Milli- to micro-second pulsed electric fields generally act on the cell membrane and produce membrane pores [28]. This phenomenon is called electroporation and widely utilized for DNA transfection [28]. Ultrashort pulsed electric fields in duration of nanoseconds also act on the cell membrane and produce very small membrane pores that elicit Ca^{2+} influx [29] and intricate cellular responses in a Ca^{2+}-dependent manner [30, 31]. Assuming that the cell membrane is the primary sites of electrical impact, PIE may cause subtle structural and/or chemical alterations in the cell membrane, which are not manifested as a reduction in cell viability but enhance RONS permeation.

Another plausible mechanism underlying the synergistic action of PIE and PAM involves intracellular signaling pathways that participate in the modulation of cell death induction. Human cells can undergo multiple modes of cell death, including apoptosis, necrosis, and many atypical cell death modalities [32], all of which are influenced by various intracellular signaling pathways [33]. Electrical impacts, such as nanosecond pulsed electric fields, have been reported to induce several intracellular signaling pathways, including the MAP kinase pathways and the stress response pathway [34–36]. Activation of these signaling pathways frequently exerts facilitatory effects on the execution of cell death. We have previously demonstrated that weak PIE treatment for long periods induces increased cell motility and its associated morphological changes, presumably through the activation of certain intracellular signaling [26, 27]. We assume that PIE treatment may activate certain signaling pathway(s), leading to enhanced cell death induction. Further investigation of molecular details of cell death and its associated intracellular responses will provide better understanding of the observed synergy between PIE and PAM. Of note, the aforementioned two plausible mechanisms for the augmented susceptibility of PIE-exposed cells to PAM are not mutually exclusive.

5. Conclusion

In summary, we demonstrated that PIE-exposed cells exhibited increased susceptibility to PAM, even though PIE itself did not have apparent adverse effects on viability. Although electrical factors of CAP have received much less attention compared to RONS, our findings shed new light on their biological significance. Further investigation will extend our understanding of the importance of electrical factors in CAP action and will ultimately ensure better therapeutic applications of CAP to the human body.

Acknowledgement

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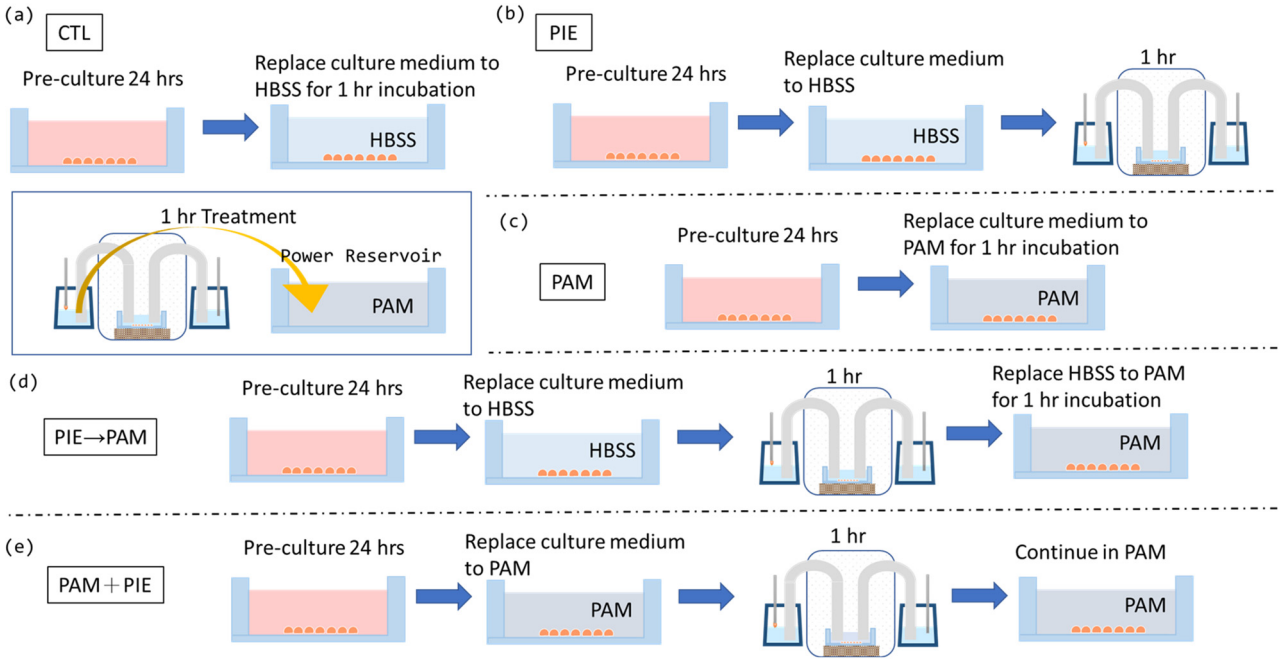
Supporting Information

(a) CTL: Untreated control cells were cultured in HBSS.
(b) PIE: Cells in the culture chamber were electrically stimulated without the influence of RONS. This treatment was referred to as "plasma-induced electrical properties" or PIE in this study. Cells in HBSS were treated with PIE for 1 h. After PIE treatment, cell culture was continued in HBSS for 24 h.
(c) PAM: HBSS in the power reservoir was exposed to streamer discharge for 1 h and utilized as plasma-activated medium (PAM). Cells were cultured in PAM.
(d) PIE→PAM: Cells in HBSS were treated with PIE for 1 h. After PIE treatment, HBSS in the culture chamber was replaced with PAM. PIE-treated cells were cultured in PAM for 24 h.
(d) PAM＋PIE: Cells were treated with PIE in the presence of PAM for 1h. Cell culture was continued in PAM for 24 h.



Supplementary Fig. 1. Schematic representation of cell treatment.