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# Enhancement of cell death by combination of cold atmospheric plasma irradiation and pulsed electric field application

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Cold atmospheric pressure (CAP) can generate highly reactive species at ambient temperature and pressure with minimal thermal loading, making it possible to directly irradiate cells and living tissues. In recent years, there has been growing interest in medical applications such as sterilization, cancer treatment, wound healing, and hemostasis. These effects are thought to occur when reactive oxygen and nitrogen species (RONS) generated in gas and liquid phases during CAP irradiation induce oxidative stress on cells.

Pulsed electric fields (PEF) are widely used as a technique to form pores in cell membranes and artificially introduce membrane-impermeable molecules into cells. In addition, research on calcium ion introduction and cancer cell elimination using nanosecond pulsed electric fields (nsPEF) has also expanded, suggesting potential applications in cancer treatment.

Oshin et al. reported that combining CAP irradiation with nsPEF significantly decreases cell viability compared to either treatment alone [1]. However, the mechanism behind this effect has not been sufficiently explained. This study hypothesized that pore formation from PEF application enhances the membrane permeation of RONS generated around cells by CAP irradiation, thereby amplifying cell death, and experimentally verified this mechanism.

In this study, we used HeLa cells, a human cervical cancer-derived cultured cell line, suspended in Dulbecco's phosphate-buffered saline (D-PBS) to prepare a cell suspension. 300 µL of this cell suspension was placed in a 96-well plate and irradiated with an argon plasma jet (Ar-APPJ). The applied voltage was 18 $kV_{p-p}$, frequency 17 kHz, Ar gas flow rate 0.7 L/min, distance from the APPJ outlet to the liquid surface 10 mm, and irradiation time 5 minutes. Subsequently, 150 µL of the cell suspension was transferred to a cuvette electrode (electrode distance 2 mm), and positive rectangular wave pulses (maximum voltage 200 V, pulse width 5 ms, pulse interval 50 ms, 2 pulses) were applied. After treatment, the cells were collected in medium (DMEM/FBS/PS), cultured for 24 hours, and then cell viability was measured. For intracellular reactive species level measurement, cells loaded with CM-$H_2$DCFDA, a RONS-reactive fluorescent dye, were suspended in D-PBS, subjected to Ar-APPJ irradiation and PEF application, and changes in intracellular RONS levels were measured by flow cytometry. Additionally, cell membrane damage was measured using calcein leakage as an indicator. Cells were stained with calcein-AM, subjected to the same treatment, and calcein leakage from cells was measured by flow cytometry.

When cell viability was measured 24 hours after Ar-APPJ irradiation and PEF application, the untreated (Ar gas exposure) group showed 94.0±0.4% viability, PEF application alone 79.5±4.3%, Ar-APPJ irradiation alone 66.6±14%, while the combined Ar-APPJ irradiation and PEF application resulted in 29±19% viability, demonstrating enhanced cell death. Figure 1(a) shows the results of intracellular RONS level measurements. Compared to the untreated group, PEF application alone showed no change in fluorescence intensity, Ar-APPJ irradiation increased RONS levels, and combined Ar-APPJ irradiation and PEF application showed enhanced RONS level elevation. Figure 1(b) shows the results of calcein fluorescence intensity measurements. Compared to the untreated group, Ar-APPJ irradiation alone did not decrease calcein fluorescence intensity, PEF application caused a decrease in fluorescence intensity, and combined Ar-APPJ irradiation and PEF application resulted in a further decrease. These findings suggest that the enhanced cell death from combined Ar-APPJ irradiation and PEF application involves not only RONS membrane permeation but also enhanced cell membrane damage.

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References

[1] E. Oshin, *et al*., Sci. Rep., **14**, 8852024 (2024)

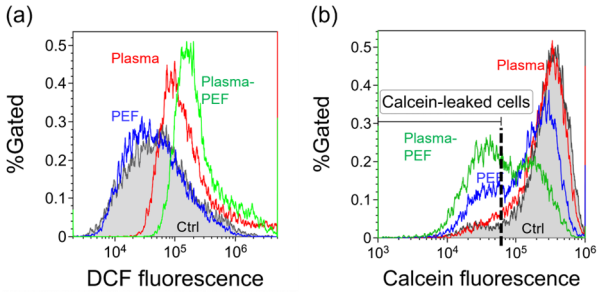


Figure 1 Flow cytometry analysis of (a) intracellular RONS levels and (b) calcein leakage as indicators of cell membrane damage following CAP irradiation and/or PEF application.