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Combinatorial Anticancer Effects of the Non-Invasive High Voltage Micro-Second Pulse Electric Field with Low-Dose Curcumin on A549 Cells

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Novel, alternative, and combinatorial approaches to combat cancer with minimal side effects are imperative due to the significant adverse effects associated with conventional therapies. The natural molecule curcumin has been reported to exhibit substantial anticancer activity against cancers, which are a leading cause of mortality worldwide. However, its clinical application is constrained by poor bioavailability. High Voltage Microsecond Pulse Electric Field (HV-µsPEF) therapy has emerged as a promising alternative in cancer treatment and may serve as an effective adjuvant anti-cancer modality. In the present study, the combinatorial effects of HV-µsPEF and low-dose curcumin on the A549 lung cancer cell line were evaluated with the primary objective of minimizing the curcumin dose required for its anticancer efficacy. HV-µsPEF was generated using a previously reported pulse generator and combined with low doses of curcumin to assess their effects on the A549 cell line. Cellular morphology was analyzed through phase-contrast microscopy, while flow cytometry was employed to evaluate the mode of cell death, curcumin uptake, Reactive Oxygen Species (ROS) levels, and Mitochondrial Membrane Potential (MMP) loss. The results demonstrated a highly synergistic induction of cell death in A549 cells, as observed through phase-contrast microscopy and flow-cytometry analyses, attributed to the enhanced uptake of curcumin by cancer cells in the presence of HV-µsPEF. This combinatorial treatment resulted in increased ROS production and significant MMP loss in A549 cancer cells. The findings indicate that combining HV-µsPEF with low doses of curcumin holds promising anticancer potential, effectively reducing the reliance on high doses of curcumin, which are often impractical to achieve in therapeutic applications.

Keywords: Apoptosis, Electroporation, Flow cytometery, Mitochondrial membrane potential, Reactive oxygen species

Introduction

Pulsed electric field treatment has been widely recognized for its applications in biology and medicine, including gene delivery, electrochemotherapy, and cancer therapy. Electric fields with durations ranging from microseconds to milliseconds and intensities of a few hundred volts per centimeter induce reversible changes in cell membranes, while shorter pulses with intensities in the range of tens of kilovolts per centimeter cause irreversible changes. The reversible breakdown of cell membranes under the influence of electric fields was first documented in 1958.[1] Furthermore, the phenomenon of increased permeability of the plasma membrane of biological cells, termed "electroporation," was first reported in 1972.[(2)] Electroporation is not limited to the physical formation of pores; the cellular responses in electro-permeabilized cells may vary, enabling electrical pulses to modulate additional biological mechanisms.

Electrochemotherapy is a tumor treatment technique that combines electrical impulses with anticancer drugs. The drugs employed in electrochemotherapy are typically non-permeable or have low permeability, yet they exhibit significant intracellular cytotoxicity. Electrical pulses are employed to permeabilize cells, thereby enhancing the uptake of these anticancer drugs. Commonly used drugs in electrochemotherapy include bleomycin (non-permeable) and cisplatin (low permeability).[3] Noteworthy aspects of electrochemotherapy include its capacity to selectively eliminate the most active and dividing cells, primarily cancer cells, while leaving normal tissue unaffected.

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Natural products have been increasingly studied due to their low toxicity and potential therapeutic effects against various cancer types. Curcumin, a natural phenolic compound derived from the rhizome of the Curcuma longa herb, has been utilized in traditional medicine and as a dietary component for thousands of years.[4] Its diverse pharmacological properties, including antimicrobial, antioxidant, anti-inflammatory, and anticancer activities against various cancer cell types, have been extensively documented.[5] Its efficacy has been observed in multiple cancers, such as breast cancer.[6] Curcumin has been shown to inhibit cell proliferation and regulate numerous signal transduction pathways involved in apoptosis, including the modulation of Bcl-2 family proteins, promotion of cytochrome c release, and activation of caspases.[7]

While bleomycin and cisplatin in combination with electrochemotherapy have demonstrated excellent efficacy, they are also associated with severe side effects. Therefore, an alternative approach using electrochemotherapy with a natural compound like curcumin presents a more appealing option compared to synthetic drugs. Furthermore, curcumin exhibits increased cytotoxicity, cost-effectiveness, and minimal side effects. The uptake of curcumin into cells is synergistically enhanced through the application of electrical field pulses, significantly augmenting its anticancer effects.[8]

A seven-fold increase in the cytotoxicity of curcumin has been reported in previous studies when combined with electrochemotherapy in MDA-MB-231 cells.[9] Notably, minimal cytotoxicity was observed in MCF10A, a non-cancerous epithelial cell line.[10] Although the precise mechanisms underlying the synergy between curcumin and electroporation have not been fully elucidated, recent studies have shown that the application of electrical pulses can significantly influence various cellular pathways and processes, including protein synthesis, intracellular dynamics, inflammation, and apoptosis.[11] Additionally, changes in the cell cycle profile and p53 expression have been observed when curcumin was used in combination with electrical pulses in MDA-MB-231 cells.[12]

The initiation of cell signaling pathways has been observed in melanoma cells, where structural changes induced in the cell membrane by the application of electrical field pulses have resulted in increased expression of heat shock proteins.[13] The translocation of calreticulin to the cell membrane in murine colon cancer cells, both in the presence and absence of bleomycin, has also been demonstrated.[14] The application of electrical pulses has been shown to trigger the release of ATP from cancer cells,[14–16] which can subsequently generate Reactive Oxygen Species (ROS) and potentially activate apoptotic pathways.[17] These findings indicate that electrical field pulses can activate multiple cell death pathways in cancer cells.

In this study, the impact of exposing the A549 lung cancer cell line to a High-Voltage Microsecond Pulsed Electric Field (HV-µsPEF) in the presence of a low dose of curcumin is investigated. The combined treatment of HV-µsPEF and low-dose curcumin was shown to enhance the anticancer effect on A549 cells. Various analyses were performed, including flow cytometry to evaluate apoptotic (sub G1) cell death, the assessment of curcumin uptake by the cells, the DCFDA assay to quantify Reactive Oxygen Species (ROS), and the evaluation of Mitochondrial Membrane Potential (MMP) loss. These investigations aimed to elucidate the effects of combining curcumin with electrical pulses on A549 lung cancer cells. The findings revealed that curcumin uptake by cancer cells was significantly increased by HV-µsPEF, accompanied by elevated ROS production. Furthermore, the combination of HV-µsPEF and curcumin induced MMP loss in cancer cells, ultimately leading to apoptotic cell death.

Materials

Dulbecco’s Modified Eagle's Medium (DMEM, HiGlutaXL, AL007G) and Bovine Serum Albumin (BSA) were obtained from Himedia Labs, Mumbai, India. Trypsin and Fetal Bovine Serum (FBS) were acquired from Invitrogen, USA. Propidium iodide, RNase, Dichloro-fluorescein-diacetate (DCFDA), and JC-1 dyes were purchased from Sigma, MO, USA. All other unspecified chemicals were sourced from Sigma, MO, USA.

Method

Cell Culture

Lung adenocarcinoma cells (A549) were obtained from the National Centre for Cell Science, Pune, Maharashtra, India. The cell lines were maintained in Dulbecco’s Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic-antimycotic solution under controlled conditions in an incubator with 95% relative humidity, 5% CO_2, and a temperature of 37°C.

Pulse Generator

To generate a microsecond-duration electrical pulse, a capacitor C is charged using a DC power supply (HV4800E, ECIL India) and discharged through the load via an IGBT switch (SKM800GA176D, SEMIKRON). The load is considered to be capacitive; therefore, the condition C ≫ Load Capacitance must be satisfied to ensure efficient charge and energy transfer from the charging capacitor C to the load capacitor. To meet this requirement, a charging capacitor with a value of 100 µF was selected. The IGBT was triggered using a rectangular low-voltage pulse generator. To vary the voltage, the capacitor C was charged with variable DC voltage source ranging from 100 V to 1200 V in 100 V increments, in accordance with the maximum rating of the IGBT (1200 V). Additionally, to adjust the pulse duration, a variable-duration low-voltage square pulse generator was employed to trigger the IGBT for the desired duration. The experimental setup is illustrated in Fig. 1, while Fig. 2 presents the electrical circuit diagram (Fig. 2a) and the output voltage waveform corresponding to a charging voltage of 300 V (Fig. 2b).[18]

Pulse Electric Field Exposure to Cancer Cells

The electrical pulse parameters were set to 5 and 10 pulses at 300 V with a duration of 50 µs, allowing for the delivery of pulses at a repetition rate of one pulse per second (frequency 1 Hz). These output pulses were used to expose cancer cells within a 0.2 cm gap electroporation cuvette (volume of 400 µL or 0.4 cm^3) (BioRad, Inc., Hercules, CA), thereby generating an electric field within the cuvette. The pulse duration was maintained at 50 µs, with a consistent repetition rate of one pulse per second. Cancer cell suspensions in complete media were placed in the 0.2 cm electroporation cuvette for treatment. An output pulse of 300 V generates electrical fields of 1.5 kV/cm between the electrodes of the 0.2 cm gap electroporation cuvette. The current passing through the load was measured using a current shunt (R=0.005056 Ω) and averaged approximately 15 A at 300 V. The energy delivered to the load per pulse for a 50 µs duration (VIt) was approximately 0.225 J at 300 V. Therefore, 5 and 10 pulses resulting in a total energy exposure of 1.125 J and 2.25 J to the cancer cells respectively.

Light Microscopy

A cell suspension containing 1.5×10^5 cells/mL of A549 cells subjected to various treatments, including HV-µsPEF, curcumin, and their combination, as depicted in Fig. 3a.

Following treatment, the cells were seeded into a 6-well plate. After 48 hours of incubation, the treated cells were analyzed using a light microscope. Images were captured to facilitate a morphology-based analysis of the impacts of the separate pulse and curcumin treatments, as well as the combined effect of the two treatments.

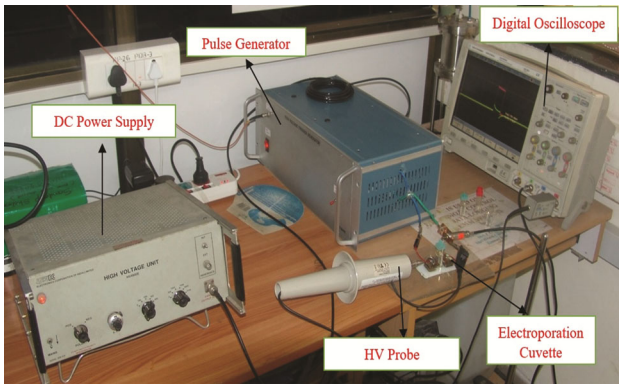

Fig. 1 — Setup showing electrical field exposure to A549 cells in electroporation cuvette

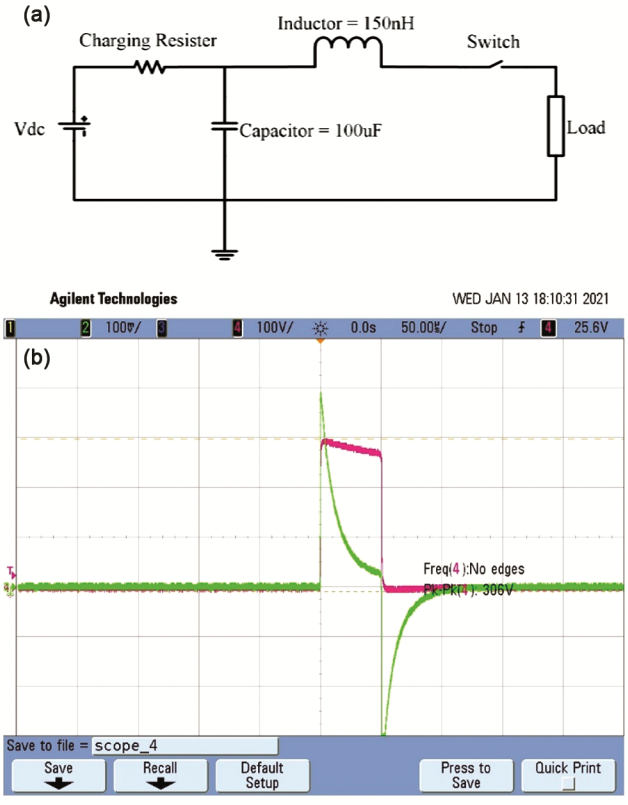

Fig. 2 — Pulse operation: (a) Pulse generator circuit, (b) Output voltage under load (at 300 V)

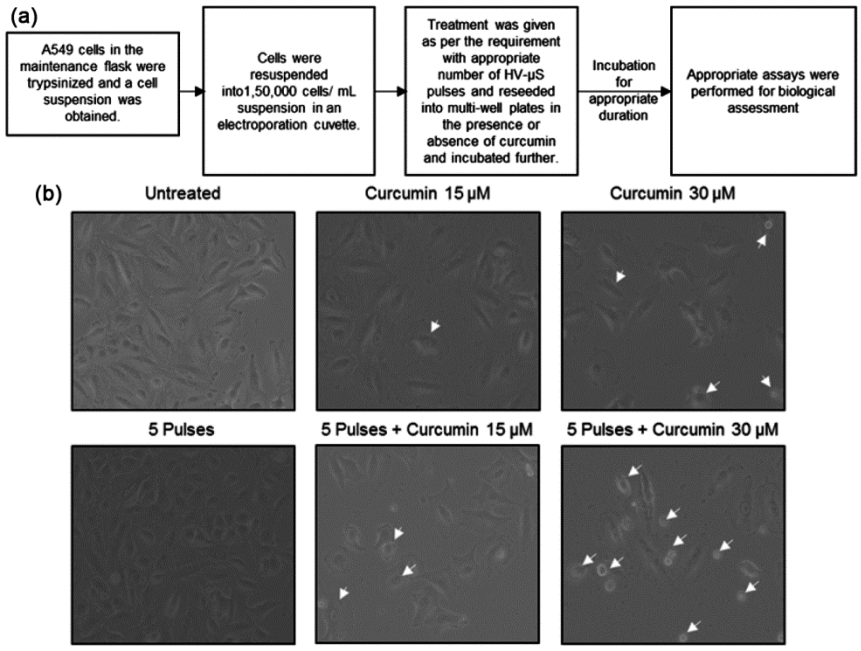

Fig. 3 — Combination treatment of HV-µsPEF pulse and curcumin: (a) Scheme of treatment protocol, (b) Morphological changes resulting from the combination treatment of HV-µsPEF pulse and curcumin

Assessment of Apoptosis by Sub-G1 Population Analysis by Flow Cytometry

The healthy and exponentially growing culture of cells were washed with PBS and trypsinized, followed by resuspension in the cell culture medium. A cell suspension containing 1.5×10^5 cells/mL was subjected to treatment in a cuvette with HV-µsPEF pulses, curcumin, and their combination, as illustrated in Fig. 3a, before being seeded into a 6-well plate. After 72 hours, the spent media in each well was collected. Adherent cells were washed once with 1X PBS, trypsinized to dislodge the cells, and subsequently collected in separate tubes. The cells were pelleted by centrifugation at 2500 RPM for 3 minutes using a tabletop centrifuge (Remi, Mumbai, India). The supernatant was discarded, and the pellet was resuspended in a hypotonic sodium citrate buffer containing 50 µg/mL propidium iodide and 50 µg/mL RNase. Following a 10-minute incubation, the samples were analyzed by flow cytometry. The acquired data were processed using FlowJo software (FlowJo LLC, USA).

Assessment of Curcumin Uptake by Flow Cytometry

A cell suspension containing 1.5×10^5 cells/mL underwent treatment in a cuvette using HV-µsPEF, curcumin, and the combination of both. The treated cells were subsequently seeded in a 6-well plate. Curcumin uptake was evaluated by measuring the increase in intensity in the FL1 channel (green fluorescence) using flow cytometry analysis. This assessment was conducted 2 hours post-treatment, with the cells resuspended in PBS during the analysis process.

Assessment of ROS Using DCFDA Assay

The cell suspension containing 1.5×10^5 cells/mL was treated in a cuvette with HV-µsPEF, curcumin, and their combination, and then seeded in a 6-well plate. After the specified time durations, the cells were incubated with 10 µM DCFDA in DMEM without FBS for 30 minutes in the dark. Subsequently, the cells were washed with 1X PBS, trypsinized, and collected in a tube, and then resuspended in 1X PBS for flow cytometry analysis. The data was analyzed using FlowJo software. The change in FL1 (green) fluorescence was analyzed under different treatment conditions.

Assessment of MMP by JC1 Dye Assay

The cell suspension containing 1.5×10^5 cells was treated in a cuvette with HV-µsPEF, curcumin and their combination, as depicted, before being seeded into a 6-well plate. After the designated incubation periods, the cells were exposed to 10 µM JC1 in DMEM without FBS for 30 minutes under dark conditions. Following incubation, the cells were washed with 1X PBS, trypsinized, collected into a

tube, and resuspended in 1X PBS for flow cytometry analysis. Data acquisition was followed by analysis using FlowJo software, with the fluorescence ratio of FL1 to FL3 being evaluated.

Statistical Analysis

An ANOVA test was performed, and the mean of each group was compared with the means of all other groups. Inter-group comparisons were indicated where relevant. A comprehensive description of the analysis is provided in the legend section of the respective figures. To assess statistical differences between means, Student's t-test was employed, with a significance level set at $p < 0.05$.

Results

Morphological Changes Due to the Treatment of HV-µsPEF Pulse and Curcumin Combination

Insights into the nature of cell death experienced by cancer cells can be obtained through the observation of morphological changes. Distinct phenotypes are associated with apoptosis, which is the preferred form of cell death in cancer therapy.[19] The combined effect of HV-µsPEF and curcumin on the morphology and subsequent growth of lung cancer cells was evaluated by treating A549 cells with five pulses of HV-µsPEF in combination with curcumin (15 µM or 30 µM) for a duration of 48 hours (Fig. 3a). Following treatment, the cells were examined under a light microscope. Untreated A549 cells, serving as controls, exhibited a healthy phenotype after 48 hours. A population of cancer cells showing cell shrinkage, condensed nuclei, and detachment from the substrate was observed with 15 µM curcumin treatment. These observations were intensified with 30 µM curcumin treatment. The combined treatment of curcumin with 5 pulses of HV-µsPEF further increased the population of dead or damaged A549 cells within 48 hours (Fig. 3b). The maximum cytotoxicity was observed with the combined treatment of 5 pulses of HV-µsPEF and 30 µM curcumin. These microscopic observations were further explored.

HV-µsPEF Pulse and Curcumin Combination Induces Synergistic Apoptosis in A549 Cells

The apoptotic population was studied in A549 cells with the combinatorial treatment, as damaged and dead cells were observed microscopically. A549 cells were treated with varying concentrations of curcumin (0 µM, 15 µM, and 30 µM) and different numbers of HV-µsPEF pulses (0 pulses, 5 pulses, and 10 pulses), as specified in Fig. 4a, following the established protocol. The apoptotic sub-G1 population was evaluated 48 hours post-treatment using flow

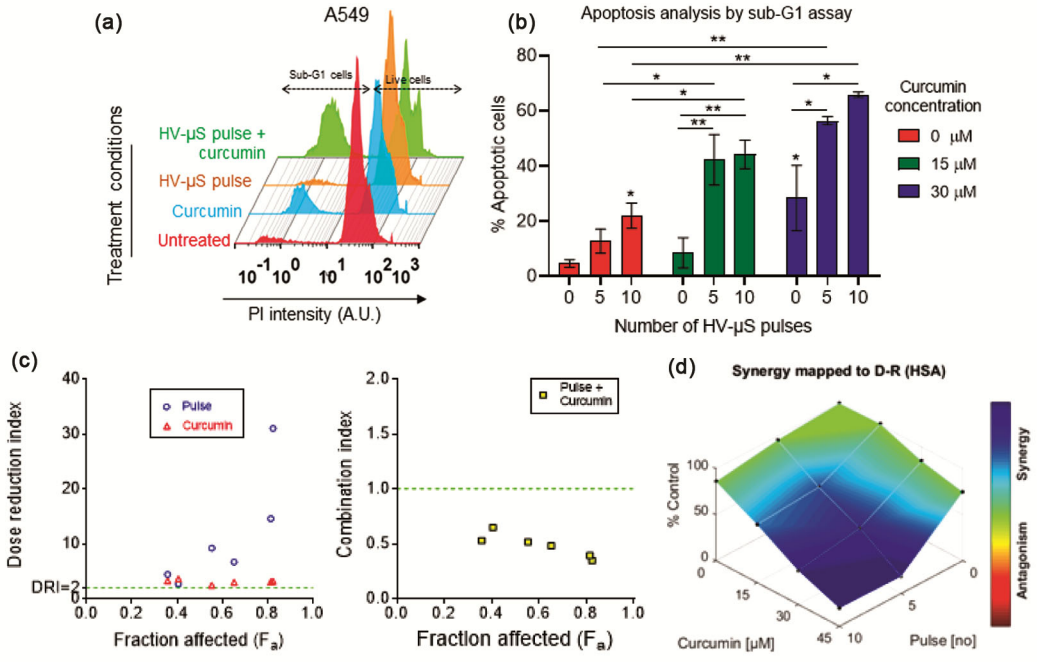

Fig. 4 — Apoptosis analysis by Sub G1 assay: (a) Illustrates the flow cytometry profiles for distinct treatment conditions, (b) Apoptotic population quantification, (c) Dose Reduction Index (DRI) and Combination Index (CI), enabling synergy assessment of both agents' activation of apoptosis and (d) Combenefit plot (using combenefit software) illustrates one of the sub-G1 analysis experiments; values indicated are mean ± S.D,*$p < 0.05$, **$p < 0.01$ compared to the untreated group. Inter-group comparisons are indicated (ANOVA)

cytometry. Cells at different stages of the cell cycle exhibit varying DNA content. The fluorescent molecule propidium iodide (PI), which intercalates with DNA at a specific intensity ratio, is directly proportional to the DNA content in the cells. Apoptotic cells, in comparison to living cells, have reduced DNA content. The flow cytometric analysis demonstrated an increased population of apoptotic cells in the combinatorial treatment of HV-μsPEF with curcumin (Fig. 4a and b). A direct correlation between apoptosis and the number of HV-μsPEF pulses was observed, with an increase in apoptosis corresponding to the number of applied pulses (i.e., 5 pulses and 10 pulses). A significant increase in the percentage of apoptosis was observed following HV-μsPEF pulse treatments in combination with curcumin. The 5-pulse treatment of HV-μPEF resulted in approximately 15% apoptotic cells. However, when applied in combination with 15 μM and 30 μM curcumin, a significant increase in apoptotic cells was noted. Approximately 40% of apoptotic cells were observed in the 5 pulses of HV-μsPEF +15 μM curcumin group, and approximately 55% of apoptotic cells were observed in the 5 pulses of HV-μsPEF +30 μM curcumin group (*$p < 0.05$). A similar trend was observed with the combinatorial treatment using 10 pulses of HV-μsPEF with 15 μM and 30 μM curcumin concentrations (**$p < 0.01$) (Fig. 4b) ($n = 3$ biological replicates).

The effect of the combinatorial treatment of HV-μsPEF and curcumin on the activation of apoptosis was assessed using CompuSyn software, which evaluates the interaction between the two treatment modalities. The flow cytometric data inputs were used to generate dose reduction index (DRI) and combination index (CI) plots to examine the synergy of HV-μsPEF pulses and curcumin treatment (Fig. 4c). The synergy analysis of the combinatorial treatment was further investigated using the Combenefit software with the flow cytometric apoptotic dataset (Fig. 4d).[20] The CI value for the treatment against A549 cells was calculated using the Chou-Talalay equation.[21] CI values equal to one indicate an additive effect, values less than one indicate synergy, and values greater than one indicate antagonism. A CI value of less than 1 indicated a synergistic effect of the combination therapy of HV-μsPEF pulses and curcumin on A549 cells in our experiments (Fig. 4c). The CI values were subsequently used to calculate the dose reduction index (DRI) for the combinatorial treatment. The DRI measures the extent to which the dosage of one or more drugs in a combination can be reduced while achieving effect levels comparable to those obtained with single agents. Drug combinations exhibiting synergy are characterized by significant dose reduction values, indicating that a measurable effect can be achieved at doses substantially lower than those expected based on the activity of single agents. The mean DRI for curcumin in the combination therapy was approximately 2, suggesting a two-fold reduction in dosage when HV-μsPEF pulses were combined with curcumin, as compared to curcumin alone. Given the poor solubility of curcumin in water, these findings highlight the potential to enhance the bioavailability of curcumin at tumor sites. The CI plot of the treatment combination in A549 cells, generated using CompuSyn software (version 1.0; ComboSyn, Paramus, NJ, USA), plotted CI against the apoptotic population, revealing a synergistic pattern (CI < 1) (Fig. 4d).

HV-μsPEF Pulses Increase Curcumin Uptake in A549 Cells

Expanding upon the observed antiproliferative and apoptotic effects of electrochemotherapy, the impact of HV-μsPEF on the cellular uptake of curcumin was investigated. A549 cells were treated following the protocol detailed in Fig. 3a, utilizing specified concentrations of curcumin and varying numbers of HV-μsPEF pulses. The cellular uptake of curcumin was evaluated by assessing the increase in green fluorescence intensity two hours post-treatment using flow cytometry (Fig. 5a). No green fluorescence signals were detected in the control and HV-μsPEF groups; however, cells treated with curcumin exhibited

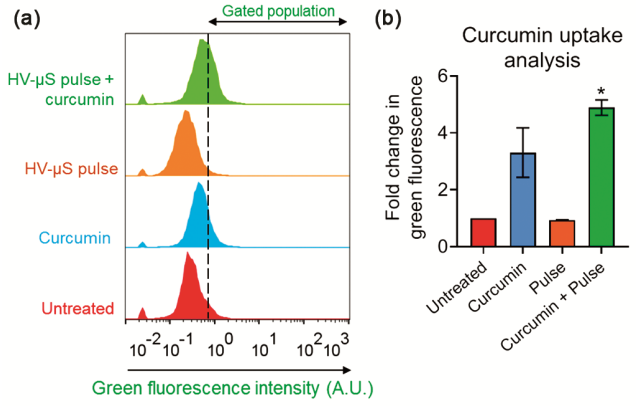

Fig. 5 — Enhanced Curcumin Uptake in A549 Cells by HV-μsPEF Treatment: (a) Illustrates the flow cytometry profiles for distinct treatment conditions, (b) Fold change of green fluorescence compared to the untreated control group; values indicated are mean ± S.D. *$p < 0.05$ compared to the untreated group (ANOVA)

noticeable green fluorescence signals. Flow cytometric analysis demonstrated a marked enhancement in fluorescence intensity with the combinatorial treatment of HV-µsPEF pulses and curcumin (Fig. 5a). The fluorescence intensity in cells subjected to this combination treatment was significantly higher compared to other groups. Quantitative analysis revealed a substantial increase in the fold change of green fluorescence, with statistical significance (*p < 0.05) (n=2 biological replicates) (Fig. 5b). Consistent with prior experimental observations, it was inferred that electrochemotherapy facilitated the cellular uptake of natural anticancer compounds across the cell membrane, thus potentially contributing to the observed enhancement of antiproliferative activity within the combinatorial therapy.

HV-µsPEF Pulse and Curcumin Combination Induces ROS in A549 Cells

The generation of reactive oxygen species (ROS) in lung cancer A549 cells was evaluated following treatments with curcumin, HV-µsPEF pulses, and their combination using the ROS-specific fluorescent dye DCFDA. The cell-permeable fluorogenic probe 2',7'-DCFH-DA enters cells and is deacetylated by intracellular esterases into non-fluorescent 2',7'-DCFH, which is rapidly oxidized to the highly fluorescent DCF in the presence of ROS. The fluorescence of DCF is detectable with maximum excitation and emission wavelengths of 488 nm and 525 nm respectively. The intensity of green fluorescence is directly proportional to the ROS levels within the cytosol.[22] The image displays the flow cytometry profile for various treatment conditions (Fig. 6A). Treatment of A549 cells

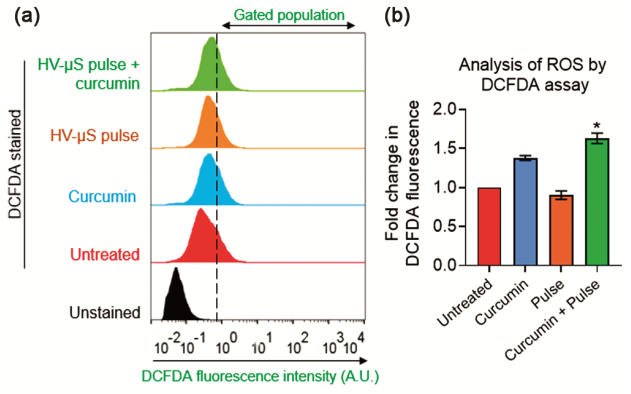

Fig. 6 — HV-µsPEF pulse and curcumin combination induces ROS in A549 cells: (a) Indicates the flow cytometry profile for different treatment conditions, (b) Fold change of green fluorescence compared to the untreated control group; values indicated are mean ± S.D. *p < 0.05 compared to the untreated group (ANOVA)

with curcumin resulted in a 1.4-fold increase in ROS production compared to the untreated group and the HV-µsPEF pulse-treated group (Fig. 6b). The combination treatment further elevated ROS generation to approximately 1.6-fold (*p < 0.05) (n=2 biological replicates) (Fig. 6b).

HV-µsPEF and Curcumin Combination Induces Loss of MMP in A549 Cells

Expanding upon the observed increase in apoptotic cells following the combinatorial treatment in A549 cells the MMP (ΔΨm) was further investigated in these lung cancer cells subjected to curcumin, HV-µsPEF, and their combined dosage. Depolarization of the mitochondrial membrane is a hallmark of the mitochondria-mediated apoptotic pathway, and the MMP serves as a key indicator of cellular health and functionality.

The cyanine dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) was employed to assess mitochondrial status. In energized mitochondria, the green fluorescent JC-1 dye forms red fluorescent JC-1 J-aggregates, signifying a high membrane potential. Conversely, as MMP decreases, JC-1 remains in its monomeric form, indicating membrane depolarization (refer to Fig. 7a).[23] Flow cytometric analysis of the FL3/FL1 fluorescence ratio of JC-1 in A549 cells was performed 24 hours post-treatment to evaluate MMP changes (refer to Fig. 7b). The cell population exhibiting high green fluorescence and no or low red fluorescence intensity was indicative of MMP loss. An enhanced loss of MMP was observed following the combinatorial treatment of HV-µsPEF pulses and curcumin (refer to Fig. 7c). Treatment with curcumin alone led to an approximately twofold reduction in MMP, whereas the combined treatment with curcumin and HV-µsPEF resulted in a more than fourfold reduction in MMP (*p < 0.05) (n=2 biological replicates) (refer to Fig. 7c).

Discussion

Lung cancer remains a significant contributor to global cancer incidence and mortality. According to GLOBOCAN 2020, approximately 1.8 million deaths worldwide are attributed to lung cancer annually. In India, lung cancer ranks as the second most prevalent cancer among males, accounting for 8% of newly diagnosed cases in 2020.[24] The reported statistics indicate a survival rate of only 18.6% for lung cancer patients, markedly lower than those for other prominent cancers such as prostate (98.2%), breast (89.6%) and colorectal (64.5%).[25]

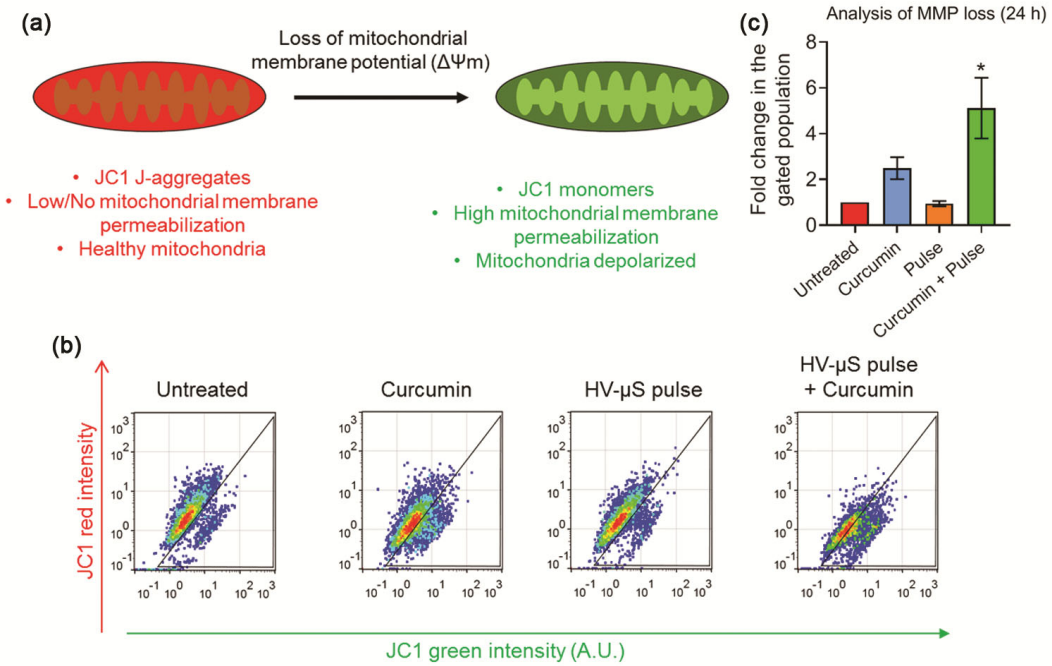

Fig. 7 — HV-μsPEF pulse and curcumin combination induces loss of MMP in A549 cells: (a) Cartoon showing the scheme of JC1 dye-based assessment of mitochondrial membrane potential (MMP) in A549 cells, (b) Indicates the flow cytometry profile for different treatment conditions and (c) Fold change of MMP loss compared to the untreated control group; values indicated are mean ± S.D. *$p<$ 0.05 compared to the untreated group (ANOVA)

The potential of HV-μsPEF to induce tumor cell death through unique mechanisms has garnered significant interest among researchers. In Irreversible Electroporation (IRE) treatments, electric field intensity and pulse number are critical parameters that determine cell viability.[26] However, the response of cancer cells to electric pulses varies significantly depending on the unique properties of each cancer cell type in both *in vitro* and *in vivo* studies.[27] Consequently, further investigations into pulsed electric field effects on cancer cells under diverse physiological conditions are essential. The differential sensitivities between malignant and normal skin cells exposed to Nanosecond Pulsed Electric Fields (nsPEF) were demonstrated in a study by Yang *et al.*[28]

Simulation studies have demonstrated that extremely short nsPEF of 30 ns elicit differential responses in cancerous and normal cells. These variations are attributed to differences in morphological characteristics and the distribution of potential differences across the nucleoplasm and cytoplasm. The simulation findings were subsequently validated through *in vitro* experiments using B16 and L929 cell lines.[29]

To enhance treatment outcomes while minimizing adverse effects, it is essential to consider the selective sensitivity of normal and malignant cells to nsPEF. Previous research has indicated that various chemical compounds, including cytotoxic agents used in electrochemotherapy and chemotherapeutic drugs, have the potential to augment the efficacy of IRE.[30,31]

The mechanisms underlying the use of steep pulse IRE technology to induce cell death in lung cancer cells (L9981) were investigated. IRE with steep pulses was applied to human large cell lung cancer L9981 cells, and the effects on apoptosis, mitochondrial membrane potential, intracellular pH, and calcium ion concentration were examined. The results from apoptosis assays indicated that the cancer cells predominantly underwent necrosis and apoptosis.[32] Additionally, research has demonstrated that pulse electric fields, in combination with low doses of curcumin, can inhibit the proliferation of PANC-1 pancreatic cancer cells in a dose and field strength-dependent manner, leading to apoptotic cell death.(11) Electroporation has also been shown to enhance the effectiveness of curcumin in MCF-7 and HL-10 cells.[33,34] However, reports have highlighted the undesirable side effects associated with intense PEF and invasive electrodes, emphasizing the need for novel approaches that enable low concentrations of curcumin to achieve cytotoxic effects.

Ren *et al.* demonstrated that nsPEFs could prevent cyclin expression by blocking the NF-kB signalling pathway, which in turn prevented cell proliferation in an in vitro pancreatic cancer experiment.[35] Studies showed the role of mitochondria-dependent pathways in nsPEF mediated treatment of human HepG2 cells.[36] Esser *et al.* theoretically predicted the membrane permeabilization of voltage-sensitive organelles such as the mitochondria when exposed to the exogenous electric fields. Nuccitelli *et al.* have shown ROS generation is triggered by nsPEF treatment in human pancreatic cancer cells. This ROS generation can be inhibited by antioxidants or by preventing increase in intracellular Ca^{2+} concentration.[37]

In the present study, the combination treatment of curcumin and non-invasive HV-μsPEF on the human non-small cell lung cancer cells, A549, was investigated. Various concentrations of curcumin were used to treat the cells, which were then exposed to different pulse numbers of HV-μsPEF. A synergistic effect of the combined treatments on cell proliferation, cellular uptake of curcumin, ROS generation, and a decrease in mitochondrial membrane potential was observed. The apoptotic cell population in the treatment groups was assessed using flow cytometry. It is demonstrated in this study that synergistic combinatorial treatment can be effective in the treatment of lung cancer with low-dose curcumin with reduced thermal effect.

Conclusions

The concurrent application of a high-voltage microsecond pulsed electric field (HV-μsPEF) and curcumin was demonstrated to have a synergistic effect on cell viability through electroporation, emphasizing the potential of this combination for inducing apoptosis in cancer cells. While promising, the findings are currently limited to *in vitro* experiments on A549 cancer cells, and further studies are required to validate its applicability across other cancer cell types. Future research should focus on the optimization of PEF parameters and curcumin dosage for different cell types, as well as the development of specialized delivery systems for treating internal carcinomas. The potential applications of this approach include the treatment of external cancers, such as carcinomas and melanoma, along with nonsurgical tumor removal. Despite its potential, the clinical translation of PEF technology for human cancer treatment is recognized as a significant challenge, particularly for cancers of internal organs.

Conflicts of Interest/Competing Interests

None, the authors have no relevant financial or non-financial interests to disclose.

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