

• Basic Research •

Effects of energy controllable steep pulses on intracellular calcium concentration and cell membrane potential

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Grant: National Natural Science
Foundation of China (No.
30371619, No. 30271484)

This paper was translated into English
from its original publication in Chinese.
Translated by: Guangzhou Liheng
and Wei Liu

The original Chinese version of this paper
is published in: *Ai Zheng (Chinese Journal
of Cancer)* 28(9); [http://www.cjcsysu.cn/cn/
article.asp?id=15462](http://www.cjcsysu.cn/cn/article.asp?id=15462)

Submitted: 2008-10-06
Revised: 2009-05-26

[Abstract] **Background and Objective:** Our previous experiments showed that steep pulses could kill tumor cells, but the mechanism is unclear. This study was to probe the effects of different dosages of energy controllable steep pulses (ECSP) on intracellular concentration of dissociative calcium ion ($[Ca^{2+}]_i$) and cell membrane potential. **Methods:** The breast carcinoma MDA-MB-231 cells were divided into control group and five ECSP (different dosages) groups. Ca^{2+} was labeled by Fluo-3/AM and cell membrane potential was labeled by DiBAC₄(3). The mean fluorescence intensity in MDA-MB-231 cells was observed by laser confocal microscopy after ECSP treatment. The changes of calcium concentration and cell membrane potential after ECSP treatment were analyzed. The changes of intracellular $[Ca^{2+}]_i$ after ECSP treatment were also observed either with or without Ca^{2+} outside of the cells. **Results:** Ca^{2+} outflow was observed when the cells were treated with lower dosage of pulse in quiet state; the outflow was enhanced with the dosage increase. In real-time kinetic detection, intracellular Ca^{2+} concentration was increased with the increase of pulse electric field intensity when cells were treated with lower dosages of ECSP. When the voltage was 285 V, frequency was 100 Hz, $[Ca^{2+}]_i$ decreased obviously. The intracellular Ca^{2+} concentration was obviously lower in the cells without outside Ca^{2+} than in cells with outside Ca^{2+} , but it still increased gradually. Low dosage of ECSP induced the increase of cell membrane potential, indicating the depolarization of cell membrane. With increase of the dosage, cell membrane potential was attenuated, indicating the superpolarization of cell membrane. **Conclusion:** Lower dosage of ECSP can induce the depolarization of cell membrane and the inflow of outside Ca^{2+} ; higher dosage of ECSP can directly destroy the cell membrane and induce the superpolarization of cell membrane, then induce the outflow of intracellular Ca^{2+} which causes the necrosis of tumor cells.

Key words: steep pulse, Ca^{2+} concentration, cell membrane potential, laser confocal microscopy (LCM), MDA-MB-231 cell

Endogenous electric fields exist widely in organisms, and have important roles in cellular metabolism. As an exogenous electric field, pulse electric field has different effects on tumor cells depending on intensity, treatment time, and target cells. Cell membrane, as a contact surface between the cell and exterior environment, is one major action target for exogenous electric field.

Meanwhile, the fluidity and the activation/deactivation of ion channels and voltage-gated channels of cell membrane, as well as the transmembrane signal transduction systems of cells, are also essential for the regulation of cellular metabolism. As a second messenger substance of cells, calcium ion is sensitive to exterior stimulation and has important role in cellular metabolism as well. Membrane potential is a most important biophysical feature of cell membrane. Maintaining a certain potential gradient between both sides of cell membrane is indispensable for the transmembrane movement of numerous ions.

The electric fields used in our earlier studies were more intense than those needed to induce depolarization of cell membrane,^{1, 2} and had exerted their effect by directly destroying cell membrane. In our present study, we used a weaker electric field to act on tumor cells, observed the variation, distribution and location of intracellular and extracellular calcium ions after treatment of steep pulses using laser confocal microscopy (LCM), and analyzed the dynamic variation of ions in single cells by real-time quantitative analysis, so as to determine whether downstream signal transduction pathways were activated by the cascade reaction resulted from membrane potential variations effected by steep pulses, and whether intracellular metabolism was interfered and intracellular environment was impaired, and subsequently induced cell apoptosis.

Material and Methods

Main reagents and apparatuses. Designed and developed independently by the Key Laboratory of Voltage Engineering and Electrical New Technology of Ministry of Education, Chongqi University, steep pulse generator produced energy-controllable steep pulses (ECSP) by storing and releasing capacitance with varied combinations of voltages (20-250 V), capacitance (0.01-0.10 F) and frequency (10-1000 Hz).³ The variations in both intracellular dissociated Ca^{2+} and cell membrane potential were detected using LEICA TCS-SP2

laser confocal microscope (Germany). Cell apoptosis was detected using FACS-Calibur flow cytometer by BD Bioscience Corporation (US). Both fluo-3/AM and DiBAC4(3) were produced by Sigma Company (US).

Cell culture and steep pulse treatment. Breast cancer MDA-MB-231 cells were provided by the Department of Pathophysiology in Chongqi Medical University, and were incubated in 35 mm culture dish. When cells were adherent and at logarithmic growth phase, the front-end portion of the electrode plates (with a distance of 1.5 cm between two plates) in the ECSP generator was placed into the bottom of the culture dish to exert steep pulses at different dosages, while in control group, the electrode plates were placed in but no steep pulse treatment was given.

Variations of intracellular dissociated Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) observed by laser confocal microscopy. Intracellular Ca^{2+} was labeled with fluorescent probes by incubating the cells in 10 mol/L fluo-3/AM at 37°C for 60 min. The cells were treated by ECSP at varied dosages (Table 1). Intracellular $[\text{Ca}^{2+}]_i$ was measured by the single channel method described by Wei et al.⁴ at the stimulation wavelength of 488 nm and emission wavelength of 530 nm. The mean fluorescent intensity, which indicated Ca^{2+} concentration, on the X and Y surfaces of cells was calculated. A point scanning was used to reveal the real-time dynamic variation of $[\text{Ca}^{2+}]_i$ during ECSP treatment: intracellular fluorescent intensities before and during electronic stimulation were continuously recorded. The results of point scanning were described by the relation between the ratio of fluorescent intensity

Table 1 The grouping of MDA-MB-231 cells with different dosages of energy controllable steep pulses (ECSP) treatment

Group	Voltage (V)	Frequency (Hz)	Width (μs)	Rising time (ns)	Action time (min)
1	100	100	8.5	50	3
2	100	200	22	60	3
3	150	100	17	60	3
4	200	200	15	70	3
5	285	100	12.5	90	3

after electric stimulation to that before electric stimulation (F/F_0) and time. The increase rate of F/F_0 referred to the variation of F/F_0 in unit time. Subsequently, 3 single cells were selected from different visual fields in each group, and the variations of intracellular Ca^{2+} concentration were recorded by an in-built CCD imaging system in the fluorescent microscope. A fluorescent intensity analyzing software (LEICA TCS image analyzing software by LEICA, Germany) was used to generate fluorescent intensity curves that reflected the dynamic variations of intracellular Ca^{2+} concentration.

Membrane potential variations observed under laser confocal microscope. After stimulation of steep pulses at different dosages, the cells were incubated with 1 μ mol/L membrane potential-sensitive negative-ion fluorescent probe DiBAC4 (3) at 37°C in dark for 20 min. The stimulation wavelength was 488 nm and emission wavelength was 530 nm. Main parameters were as follows: pinhole, 1600 m; PMT, 170%; PMT, 270%; scanning strength, 20%; laser power, 10 mW. Subsequently, three single cells were selected from different visual fields in each group and the intracellular fluorescent intensity, which indicated membrane potential, was calculated by TCS-tools image analyzing system. Increased fluorescent intensity indicated membrane depolarization whereas decreased intensity indicated hyperpolarization.

Cell apoptosis detected by flow cytometry. Adherent MDA-MB-231 cells at logarithmic growth phase were digested by 0.25% trypsin and were then centrifuged at 800 r/min. Serum-free RPMI-1640 medium was added to prepare single cell suspension at a density of 3×10^6 cells/mL. The front-end portion of the platinum electrode needles (with a distance of 1 cm between two needles) in the ECSP generator was placed into the cell suspension to exert steep pulses at different dosages; in control group, the electrodes were placed in but no steep pulse treatment was given. An amount of 100 L of cell suspension was placed into 5 mL flow cytometric tube and was added with 5 L of Annexin V/FITC and 10 L of 20g/mL propidium iodide solution, incubated in dark at room temperature

for 15 min, then analyzed by flow cytometry.

Statistical analysis. The SPSS10.0 software was used; the data were obtained by real-time measurements using the ACAS-570 computer system. Numerical data are shown as mean \pm SD and processed by analysis of variance; significance level of the data was analyzed using students t test. A P value of <0.05 indicated significance.

Results

Variations of intracellular Ca^{2+} concentration as observed by laser confocal microscopy. When the cells were stimulated by ECSP, intracellular $[Ca^{2+}]_i$ varied with the intensity of the electric fields (Fig. 1). With lower energy levels (as in Groups 1, 2 and 3), mean fluorescent intensity of intracellular Ca^{2+} was increased, indicating that influx of extracellular Ca^{2+} was triggered by such dosages; with increased energy, mean fluorescent intensity of intracellular Ca^{2+} was gradually decreased, indicating outflux of large amount of intracellular Ca^{2+} when cell membrane was impaired by increased energy.

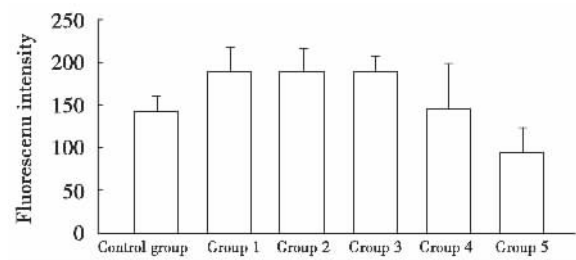


Figure 1 Mean fluorescence intensity of calcium ion (Ca^{2+}) in MDA-MB-231 cells in different energy controllable steep pulse (ECSP) groups

The fluorescence intensity was significantly higher in Groups 1, 2, and 3 than in Groups 4 and 5 ($P < 0.05$); no significant difference was observed between Groups 4, 5 and control group ($P > 0.05$).

With point scanning by laser confocal microscopy, the real-time dynamic observation on intracellular $[Ca^{2+}]_i$ revealed that the magnitude and rate of $[Ca^{2+}]_i$ variation were related to intensity of pulse electric field. When cells were treated by low dosages of steep pulses, fluorescent intensity of intracellular Ca^{2+}

concentration was increased as the pulse electric intensity was elevated and the increase was also accelerated as pulse electric intensity increased. However, when pulse energy was elevated to a certain level, fluorescent intensity gradually decreased. At the voltage of 200 V and frequency of 100 Hz, fluorescent intensity was significantly decreased.

When dissociated Ca^{2+} in electric stimulation solution was complexed by a specific calcium-complexing reagent EDTA, variations of intracellular $[\text{Ca}^{2+}]_i$ in the cells treated by ECSP of different dosages were observed by point scanning. With the presence of extracellular Ca^{2+} , intracellular Ca^{2+} was first increased and was then maintained at a stable level; without extracellular Ca^{2+} , intracellular $[\text{Ca}^{2+}]_i$ was increased slowly. Without extracellular Ca^{2+} , intracellular Ca^{2+} concentration was increased with treatment of ECSP although the increase was significantly slower as compared with that seen with extracellular Ca^{2+} .

Variations of cell membrane potential as observed by laser confocal microscopy. The influx amount of DiBAC4 (3) in ECSP-treated cells (presented as mean fluorescent intensity) varied with ECSP dosages. Low dosage of ECSP induced the increase of DiBAC4 (3) fluorescent intensity which was accelerated with increased dosages (Fig. 2), suggesting that ECSP at low dosage triggered a decrease in negative value of cell membrane potential and induced depolarization in the cells. However, when the dosage was further increased, the effect was obviously different and was seen as weakened DiBAC4 (3) fluorescent intensity in the cells, indicating that the negative value of membrane potential was increased to induce hyperpolarization in the cells.

Cell apoptosis detected by flow cytometry. Flow cytometric detection revealed necrosis in most breast cancer cells treated by ECSP at varied dosages, but apoptosis was hardly seen (Fig. 3).

Discussion

By observing the variation, distribution and

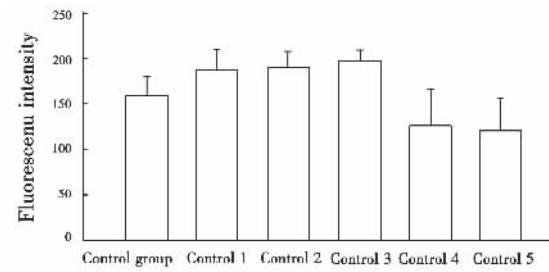


Figure 2 The mean fluorescence intensity of cell membrane potential in different ECSP groups

The fluorescence intensity was significantly higher in Groups 1, 2, and 3 than in Groups 4 and 5 ($P < 0.05$); no significant difference was observed between Groups 4, 5 and control group ($P > 0.05$).

location of intracellular and extracellular Ca^{2+} , as well as the variation of cell membrane potential in ECSP-treated cells using laser confocal microscope, we found that low-energy steep pulse induced depolarization in cell membrane and influx of extracellular Ca^{2+} ; high-energy steep pulse impaired cell membrane directly, resulting in hyperpolarization in cell membrane and outflux of intracellular Ca^{2+} and subsequent necrosis of tumor cells.

Influence of ECSP on concentration of intracellular dissociated Ca^{2+} . In this study, we detected the variations in intracellular dissociated Ca^{2+} concentration after ECSP treatment. At low energy levels, the pulses induced an increase in intracellular Ca^{2+} concentration; when energy level was gradually elevated to a certain degree, Ca^{2+} concentration decreased, rather than increased. This might be because that low-energy pulse leads to the formation of micro pores on cell membrane, through which Ca^{2+} flow into the cells along concentration gradient; the pulse activates potential-operated calcium channel (POC) and results in an increase in intracellular Ca^{2+} concentration. In addition, pulse electric field might also activate the L-type and N-type calcium channels on cell membrane and lead to calcium overload within the cells, which, in turn, induces damage and necrosis in cells by the following pathways: (1) promoting mitochondrial calcium uptake to inhibit ATP synthesis and result in energy metabolism disorder; (2) activating calcium-dependent phospholipase to degenerate membrane phospholipids; (3) activating calcium-dependent

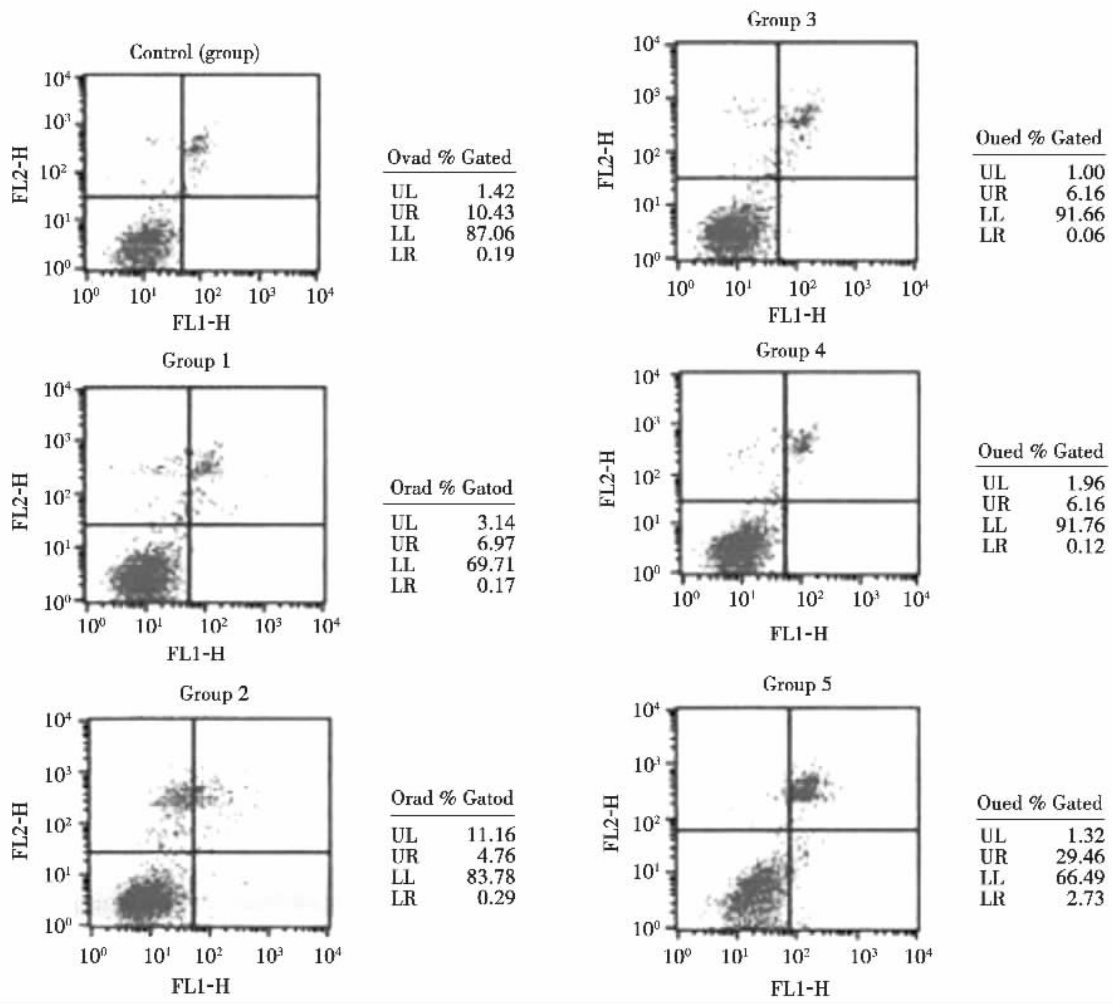


Figure 3 Cell apoptosis after ECSP treatment detected by flow cytometry
No apoptotic peak appears after ECSP treatment.

protease and endonuclease to hydrolyze DNA. When pulse energy is increased gradually to a certain level, irreversible electroporation occurs in cell membrane which is then completely disintegrated, thus large amount of intracellular calcium Ca^{2+} are released, which is seen as decreased intracellular Ca^{2+} concentration.

The elevation of intracellular Ca^{2+} concentration triggered by exogenous electric field may be the result of the influx of extracellular Ca^{2+} and the calcium release from intracellular calcium store. When treated by exogenous electric field, cell membrane potential is altered to activate voltage-sensitive Ca^{2+} channels, through which extracellular Ca^{2+} flows into the cells, and thus intracellular Ca^{2+} concentration is elevated. The real-time dynamic observation on intracellular dissociated Ca^{2+}

concentration after ECSP treatment revealed that pulse electric field triggered an increase in intracellular dissociated Ca^{2+} concentration even without the presence of extracellular Ca^{2+} . Herein, the increase of dissociated Ca^{2+} concentration might be the result of calcium release from intracellular store. As with other excitable cells, tumor cells have two basic intracellular calcium signal pathways: the IP3 pathway and the RYRs pathway. As a calcium-dependent calcium-releasing pathway, RYRs pathway depends on the influx of extracellular Ca^{2+} ; therefore, it is reasonable to believe that the increase of intracellular dissociated Ca^{2+} concentration triggered by pulse electric field, even when extracellular Ca^{2+} is complexed or Ca^{2+} channels on cell membrane are blocked, may be related to the activated IP3

pathway in the cells.⁹ Dibirdik et al.¹⁰ and Kristupaitis et al.¹¹ treated lymphoma DT40 cells with 60 Hz low-energy electric field and found significantly increased IP₃ in the cells, but Miller et al.¹² demonstrated that IP₃ in DT40 cells was not significantly increased by low-energy electric field treatment. These results suggest that the underlying mechanisms of variation in intracellular Ca²⁺ concentration under electric field might be complicated, and the variation could not be simply interpreted as calcium influx or calcium release, but should also be considered in the context of the interactions between the electric fields of ions. The exact mechanisms have yet to be further explored.

In earlier studies, we found apoptosis in steep pulse-treated tumor cells *in vivo*.^{13,14} But in the present study no apoptotic peak was detected in ECSP-treated cells by flow cytometry, while most cells were necrotic. This inconsistency might lie on the following reasons: (1) In earlier studies, apoptosis of *in vivo* tumor cells was found in the margin of treated area. When using electrode needles, pulse energy distributes in concentric circles and weakens gradually with the distance from the center of treated area, therefore, the apoptosis in the margin of treated area might be related to low pulse energy. (2) In this present study, apoptosis was detected in single cell suspension. Since pulse energy was distributed evenly without attenuation, no apoptotic peak was thus detected. (3) Since single cell suspension was added with electric stimulation solution, the inconsistency of electric conductivity might have altered the energy of electric field and cell membrane potential, and thus induced necrosis in the cells; whereas no such issue was seen in *in vivo* tumor cells. (4) During the preparation of single cell suspension, the digestion by trypsin might have also altered the response of cell membrane to ECSP. In conclusion, whether ECSP triggers apoptosis in single cell and the exact mechanisms (if any) need to be further investigated.

Influence of ECSP on cell membrane potential. In this study, the analysis on intracellular fluorescent intensity suggested that ECSP induced the increase of DiBAC₄ (3)

fluorescent intensity in the cells even at low energy levels, and that the effect was strengthened with elevated treatment dosages. This indicates that low energy ECSP could cause a decrease in the absolute value of cell membrane potential and induce depolarization in the cells. Depolarization in cell membrane might be one early marker of cell damage. The underlying reasons for such effect might be that, first of all, ECSP impairs membrane lipid structures of tumor cells and alters membrane permeability to ions, thereby, influx of positive ions, such as K⁺ and Ca²⁺, is increased and maintained at a new level; in addition, we speculate that ECSP impairs tumor cells and membrane proteins, especially zymoproteins, and the decrease of Na⁺-K⁺ ATP enzyme activity may weaken the ability of cell membrane to maintain a depolarized state, result in depolarization of cell membrane and increased influx of extracellular calcium, therefore, take part in the occurrence of cell apoptosis. It is confirmed that ECSP impairs tumor cells and induces depolarization in cell membrane, thus depolarized membrane could also serve as a marker for electric damage on tumor cells. When ECSP dosage was further increased, the effect was significantly changed and was seen as decreased DiBAC₄(3) fluorescent intensity in the cells, indicating that ECSP at this dosage level promoted an increase in the negative value of cell membrane potential and induced hyperpolarization in the cells, influx of extracellular calcium was therefore reduced. This effect might be because the overwhelming pulse energy impairs lipid bilayer membrane of the cells and results in irreversible electroporation, therefore, induces cell death. Since the damage of tumor cell membrane caused by ECSP at this dosage is irreversible and not amendable, the membrane function thus maintains at a low status. Previous studies revealed that membrane depolarization could attenuate cell responsiveness to exterior environment and inhibit cell proliferation,^{15,16} and that accompanying changes in intracellular and extracellular ion content were closely related to intracellular signal transduction as well as subsequent cell metabolism and functions. Hence, at both low and high energy

levels, steep pulses could kill malignant tumors by altering cell membrane potential. However, the exact mechanisms need to be studied.

Possible mechanisms underlying the cytotoxic effect of ECSP on tumor cells.

Theoretically speaking, the mechanisms of ECSP-induced damage rest with the direct damage on cell membrane, which alters the liquid-crystalline phase of cell membrane and thereby changes membrane fluidity and permeability; in the meantime, ECSP induces activity attenuation, deactivation, and exfoliation of membrane proteins, and alters transmembrane proteins. With cell membrane being impaired, cell membrane potential, which is closely related to cytomembrane structures and functions, is therefore changed. Radosevic et al.¹⁷ and Taichman et al.¹⁸ suggested that, when tumor cells were stimulated by chemical agents or other lethal factors, impaired cell membrane could induce membrane depolarization and trigger a decrease in absolute value of membrane potential. The changes in transmembrane potential induced by pulse electric field can influence influx of extracellular Ca^{2+} , whose concentration is an important signal in intracellular signal systems. Among these systems, the initiation of programmed cell death is dependent on Ca^{2+} influx. The executor of programmed cell death, DNA endonuclease, is also directly activated by Ca^{2+} in part, or is dependent on Ca^{2+} in the synthesis process. Low energy pulse electric field induces depolarization of tumor cell membrane, increases influx of extracellular Ca^{2+} by activating potential-operated calcium channel (POC), which elevates intracellular dissociated Ca^{2+} concentration. In turn, increased Ca^{2+} concentration activates DNA endonuclease, which induces apoptosis in the cells that escape from programmed cell death. High energy ECSP causes such irreversible and non-amendable damage on cytomembrane of tumor cells that membrane functions are maintained at a low status and that necrosis is thereby induced in the cells. Moreover, steep pulses may also block Ca^{2+} influx by altering calcium channel, induce hyperpolarization in cytomembrane of tumor cells and inhibit POC, therefore, suppress influx

of extracellular Ca^{2+} and proliferation of tumor cells. Hence, we deduce that steep pulse electric field may induce apoptosis or even necrosis in tumor cells and achieve a treatment efficacy in tumors by altering transmembrane potential in tumor cells, certain proteins on cell membrane and subsequently POC.

The influence of steep pulses on structures and functions of cytomembrane of tumor cells is worthy of investigating. Further experiments and mechanism investigations on inhibiting malignant tumors by steep pulse electric fields are under way, which will provide a new option for research on cancer treatment.

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