

## Original Article

# Mechanisms for steep pulse irreversible electroporation technology to kill human large cell lung cancer cells L9981

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**Abstract:** To explore the mechanisms for steep pulse irreversible electroporation technology to kill the lung cancer cell L9981. The apoptosis, cells mitochondrial membrane potential, internal PH changes and the intra-cellular calcium ions concentration were detected after steep pulses acted on the human large cell lung cancer cell L9981. Apoptosis test results indicated that cancer cells mainly experienced necrosis and apoptosis. Along with the increase of electric parameters, the proportion of the necrotic cells increased rapidly; the detection of cells mitochondrial membrane potential indicated that membrane potential occurred depolarization. Steep pulse can cause cancer cells to produce death and apoptosis. The PH value indicated that intracellular PH level down jumped. Internal PH became more acidic and led to cell death. The detection of intra-cellular calcium ions concentration showed that the number of free calcium significantly increased, and this change had killing effects on cell death and apoptosis. Steep pulse could induce cell apoptosis.

**Keywords:** Steep pulsed, irreversible electrical breakdown, large cell lung cancer, necrosis, apoptosis

### Introduction

Previous our study showed that steep pulse irreversible electroporation technology has a better role in the killing large cell lung cancer cells L9981, and determined the optimal interaction parameters of steep pulse technology ruling in large cell lung cancer cells L9981: voltage amplitude 2000 V/cm, pulse width 100  $\mu$ s, pulse frequency of 1 Hz, pulse number 10 and repeated six times for lung cancer cells L9981 [1]. With this group of parameters, steep pulse could have the best tumor cell-killing effects. To further study the mechanisms for steep pulse killing cancer cell, we also use large cell lung cancer cells L9981 as experimental subjects. With steep pulse, we can investigate the mechanism of apoptosis by flow cytometry to detect apoptosis, mitochondrial membrane potential, Intracellular PH value changes and Intracellular free calcium concentration.

### Materials and methods

#### Materials

Human highly metastatic large cell lung cancer cell lines L9981, which screened to establish through application of a single cell clones from human large cell lung cancer cell lines WCQH-9801 by Prof Zhou Qing-Hua, the large cell lung cancer cell lines L9981 have highly invasive and highly metastatic; RPMI-1640 medium and newborn calf serum bought from GIBCO Co.; Trypsin, HEPES, EDTA bought from Amresco Co. Annexin V-FITC & PI apoptosis kit bought from BD Co. of America; BCECF AM, Fluo-3 AM bought from Beijing Biyuntian reagent Company; Other conventional reagents were supported by domestic companies.

#### Equipment

We use energy controllable steep pulse therapeutic apparatus designed and manufactured

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**Figure 1.** Steep pulse therapy instrument.

by Ministry of Education Key Laboratory of Chongqing University, which combine the different pulse parameters, and produce energy controllable steep pulse by capacitor energy storage and discharge (**Figure 1**). The electrode needle is made from platinum. Electrode cup of 4-mm are presented by The State Key Laboratory of High Voltage and Electrical New Technology of Chongqing University; Vi-Cell Cell viability analyzer bought from Beckman Coulter, USA. FACSaria™ Flow Cytometry bought from Becton Dickinson Co. of America.

### *Cell culture*

Putting human large cell lung cancer cell lines L9981 in RPMI-1640 medium including 10% fetal bovine serum, conventional paste-wall culture on the condition of 37°C, 5% CO<sub>2</sub>. When the cell growth confluence to 80% or so, 0.125% trypsinized cells which include 0.2% EDTA routinely passaged.

### *Cell suspension equipment using for steep pulse technology processing*

When the cell in logarithmic growth phase and in good condition, 0.125% protease will be digest and percuss for a single cell suspension,

and adjust the concentration of living cells to the 1×10<sup>6</sup> cells/ml, putting into the electrode cup (700 ml), stand-by.

### *Parameter settings of steep pulse*

Voltage amplitude 2000 V; Pulse width 100 μs; Repetition rate 1 Hz; The number of pulses 10; Repetitions 0, 1, 2, 3.

### *Detection of apoptosis after steep pulse processing*

Centrifuged cell suspension (Concentration 1×10<sup>6</sup> cells/ml) after steep pulse processing. In accordance with the procedure, followed by adding buffer and Annexin V-FITC Dye, PI Dye. After incubation, Detected by the flow cytometry, excitation is 488 nm, detection channels are FL-1 and FL-3. Data are analyzed by WinMDI 2.9.

### *Potentiometric detection of mitochondrial membrane after steep pulse processing*

Centrifuged cell suspension (Concentration 1×10<sup>6</sup> cells/ml) after steep pulse processing. In accordance with the procedure, followed by adding JC-1 dye stock solution, we can centrifugal and re-suspended after dark incubation. After incubation, detected by the flow cytometry, excitation is 488 nm, detection channels are FL-1 and FL-3. Data are analyzed by WinMDI 2.9.

### *pH change detection in cells after steep pulse processing*

Cell suspension before processing, concentration 1×10<sup>6</sup> cells/ml, 1 ml/tube, dark added 0.1 ul BCECF AM dye stock solution, incubating 20 min at 37°C; get 700 ul from per tube to be processed steep pulse; after that, detected by the flow cytometry, excitation is 488 nm, detection channels are FL-1 and FL-3. Data are analyzed by WinMDI 2.9.

### *Free calcium concentration detection in cells after steep pulse processing*

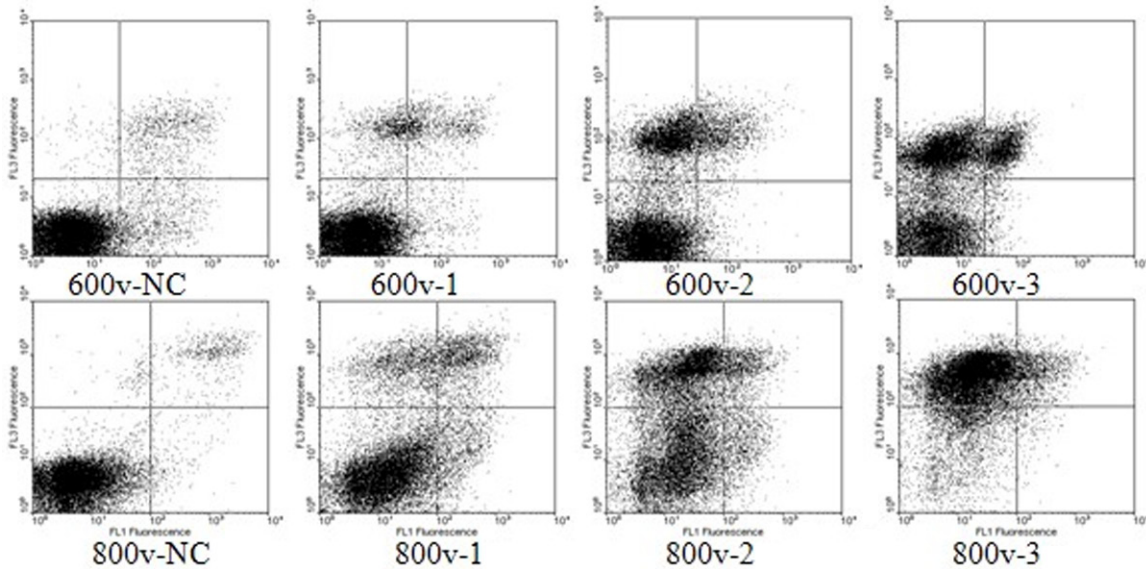
Centrifuged cell suspension (Concentration 1×10<sup>6</sup> cells/ml) after steep pulse processing. In accordance with the procedure, followed by adding 0.02 ul Fluo-3 AM dye stock solution, incubating 15-20 min at 37°C at dark; centrifugal 5 min at room temperature; throw away the

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**Table 1.** Apoptotic changes in the results of flow cytometry in the different parameters of steep pulse electric treatment on human high-metastatic large cell lung cancer cell line L9981

Voltage amplitude	Repetitions	Living cells (%)	Early apoptotic cells (%)	Late apoptotic or necrotic cells (%)	necrotic cells (%)
600 V	0	89.67	5.49	4.34	0.50
	1	87.73	2.43	4.78	5.06
	2	68.16	2.13	7.33	22.38
	3	49.81	1.62	10.90	37.67
800 V	0	95.65	1.10	2.72	0.54
	1	74.52	6.69	8.91	9.88
	2	53.72	5.72	6.86	33.71
	3	19.58	0.99	7.89	71.54

Parameter settings: voltage amplitude 1500 V/cm and 2000 V/cm, pulse width 100  $\mu$ s, pulse frequency 1 Hz, the number of pulses 10; 0: means no deal with; 1 means repeat once, 2 means repeat twice, 3 means repeat three times.



**Figure 2.** Apoptotic changes in the results of flow cytometry in the different parameters of steep pulse electric treatment on human high-metastatic large cell lung cancer cell line L9981. When the voltage amplitude is at 2000 V/cm and repetitions is at 3, most of cells (> 70%) go into the necrotic area.

up apart, and resuspended; detected by the flow cytometry, excitation is 488 nm, detection channels are FL-1 and FL-3. Data are analyzed by WinMDI 2.9.

### Results

#### *Apoptosis detection results after steep pulse processing*

By steep pulse parameter processing for large cell lung cancer cell lines L9981, flow cytometry detected the apoptosis changing results (Table 1; Figure 2). We considered, from the result of apoptosis detection, that L9981 cells

processed by steep pulse experienced apoptosis and necrosis; and with the increase of electrical parameters, the proportion of necrotic cells increased rapidly. When the voltage amplitude is at 2000 V/cm and repetitions is at 3, most of cells (> 70%) go into the necrotic area.

#### *Mitochondrial membrane potential results after steep pulse processing*

From the JC-1 mitochondrial membrane potential changing result (Table 2; Figure 3), 65.91% mitochondrial membrane potential had been depolarization when the voltage amplitude is at 1500 V/cm and repetitions is at 3; and 89.15%

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**Table 2.** Mitochondrial membrane potential changes in the results of flow cytometry in the different parameters of steep pulse electric treatment on human high-metastatic large cell lung cancer cell line L9981

Voltage amplitude	Repetitions	FL1+ FL2- (%)	FL1- FL2+ (%)
NC	0	2.38	68.48
600 V	3	65.91	17.47
	6	90.12	1.12
800 V	3	89.15	0.82
	6	86.22	0.43

Parameter settings: voltage amplitude 1500 V/cm and 2000 V/cm, pulse width 100  $\mu$ s, pulse frequency 1 Hz, the number of pulses 10; 0: means no deal with; 3 means repeat three times, 6 means repeat six times.

depolarization when the voltage amplitude 2000 V/cm and repetitions 3. It is usually considered that the cells are in apoptosis when mitochondrial membrane potential had been depolarization, so that we consider that the steep pulse processing can leads to the apoptosis of tumor cell.

### *Intracellular PH changing results after steep pulse processing*

Cell light scattering detected by the flow cytometry are the parameter values which reflects cell granularity, it are correlated with the changing of cell granules. We discuss here that the colony should be homogeneous community that particle size meets the normal distribution, we can get it from the "side scatter" subgraph. The test results of PH changing in intracellular showed that the light intensity "level hop" decline by detection channels FL-1 with the increasing of the electrical parameters, (**Figure 4**) that to say, the intracellular PH value decline "level hop" correspondingly. If considered the intracellular PH neutral in NC group, the intracellular PH tend to acidity with the increases of electrical parameters. The cells under steep pulse processing come to apoptosis and necrosis.

### *Intracellular free calcium concentration detection results after steep pulse processing*

By steep pulse parameter processing for large cell lung cancer cell lines L9981, flow cytometry detected the number of intracellular free calcium (**Figure 5**). From graph 5, with the increase of steep pulse parameters, number of intracellular free calcium in the cell increased

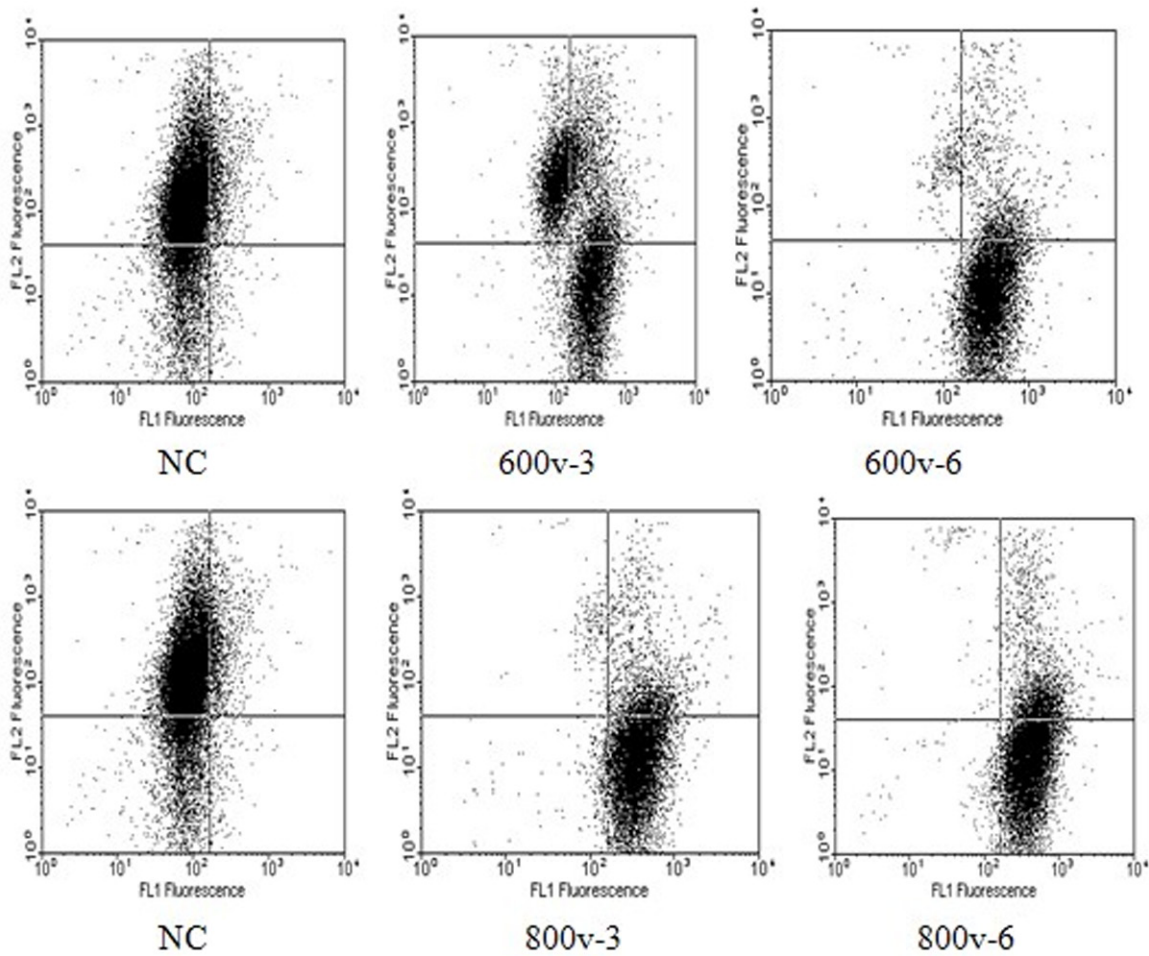
significantly, and can reach a saturation stage basically after three times processing. Above all, it is consistent with Vi-Cell cell activity analysis results and with the test results of apoptosis.

## Discussion

Lung cancer is the highest morbidity and mortality of malignant tumor in the world, about 25.4% of all tumors. In recent years, the trend of its morbidity has been upward [2-4]. In lung cancer, the NSCLC (Non-small cell lung cancer) is the main types, 80~85% of the total lung cancer, it include three histological types -- squamous cell carcinoma, adenocarcinoma, large cell carcinoma. It is the main reason of the threat to the survival of patients with lung cancer. The data published by WHO in 2005 showed that lung cancer mortality is 30.83/100000, male 41.343/100000, female 19.84/100000. It is in the first place of mortality in all malignant tumors. A clinical statistics, which include 1742 cases of Chinese IV NSCLC, show that the survival rate of 1, 2, 3, 4, and 5 years were 44%, 22%, 13%, 9% and 6% [5]. Because of occult incidence of lung cancer, the majority of patients for treatment were so late that lost the opportunity of surgical treatment. In recent years, long-term survival of patients with lung cancer have no significant improvement, survival of patients with lung cancer in 5 years is only 15.8% [6], even though a variety of treatment techniques have significant development and comprehensive cancer treatment has been greatly improved by the method of surgery, radiotherapy and chemotherapy. Therefore, the integrated treatment showed an important role increasingly in lung cancer treatment strategies.

In recent years, the minimally invasive ablation therapy, aimed for inactivated tumor cells and elimination of tumor burden, attracted wide attention. New targeted ablation therapy technology carried out extensively, e.g. freezing [7, 8], focused ultrasound [9, 10], seed implantation [11, 12], microwave [13, 14], radiofrequency [15, 16]. We made the great progress to cancer patients who lose opportunities in surgical treatment. It provides a new method for comprehensive cancer treatment. Comparing with traditional chemotherapy, radiotherapy and

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**Figure 3.** Mitochondrial membrane potential changes in the results of flow cytometry in the different parameters of steep pulse electric treatment on human high-metastatic large cell lung cancer cell line L9981.

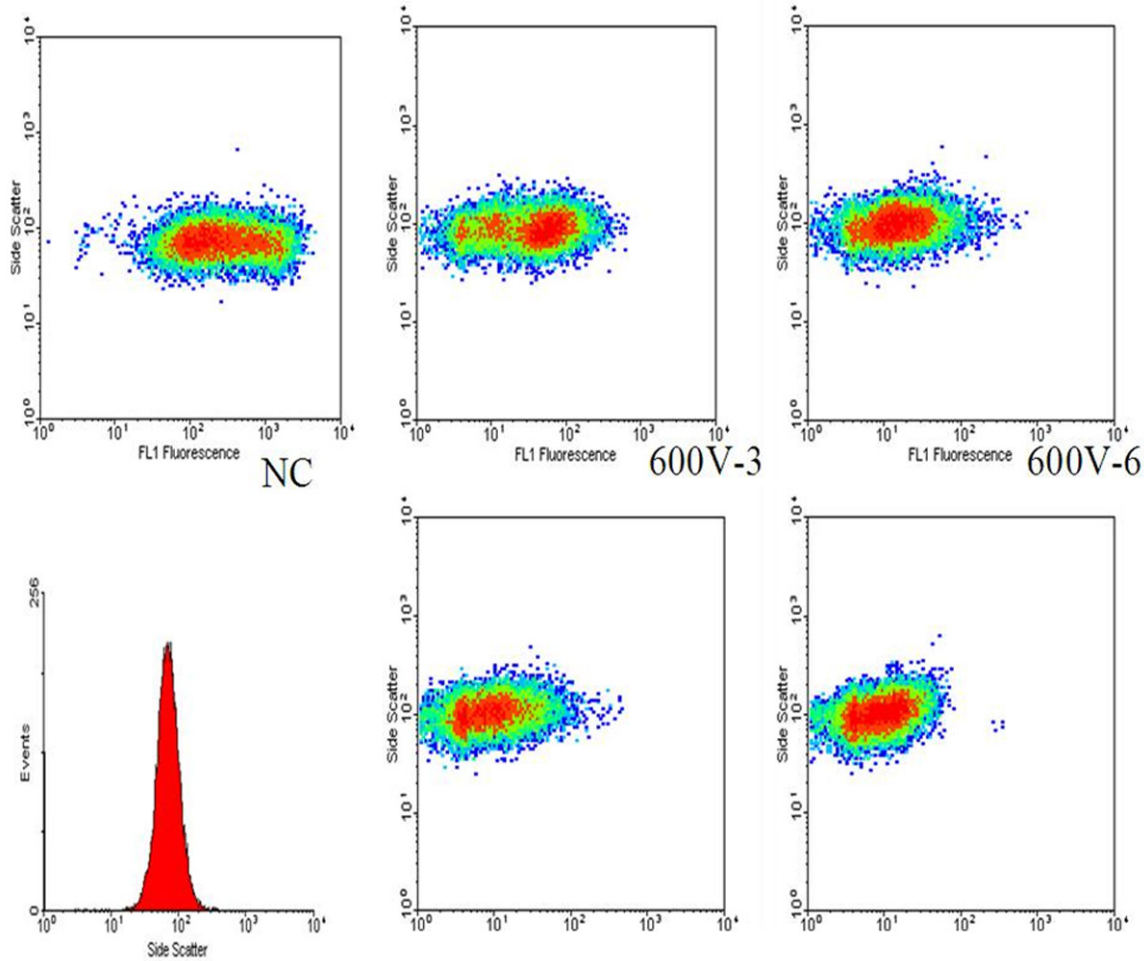
physiotherapy, irreversible electrical breakdown characteristics of SPEF has unique advantages in cancer treatment [17]. It expected to become a new kind of local physical therapy to the tumor, and become one of effective complementary means in comprehensive cancer treatment.

Steep pulse technology is the integration of biomedical, electrical new technology and microelectronics technology and different areas of interdisciplinary. In recent years, as a new possible new method to treat cancer, it attracted the attention of scholars at home and abroad, and in the ascendant [18]. The main mechanism of steep pulse is the irreversible electrical breakdown: membrane formed on a temporary, reversible microporous under the instantaneous high voltage pulse stimulation. After the pulse to be cancelled, the majority of microporous will close at the same time and does not

impact on cell [19]. With the increase of the pulse dose, cell membrane, nuclear membrane are in a continuous dynamic process of charging and discharging, forming the electric field in the membrane, dielectric constant of the cell membrane is different from the surrounding protoplasm dielectric constant. Electric force of the membrane makes the membrane thickness and the thinning. Ultimately, it leads to cell to be irreversible electrical breakdown and death in the end [20-22] (**Figure 5**). Studies in recent years have shown that the steep pulses have a good development prospects in the field of cancer treatment.

Previous our study and determined the optimal interaction parameters of steep pulse technology ruling in large cell lung cancer cells L9981: voltage amplitude 2000 V/cm, pulse width 100  $\mu$ s, pulse frequency of 1 Hz, pulse number 10 and repeated six times for the best treatment

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**Figure 4.** Intracellular PH changes in the results of flow cytometry in the different parameters of steep pulse electric treatment on human high-metastatic large cell lung cancer cell line L9981.

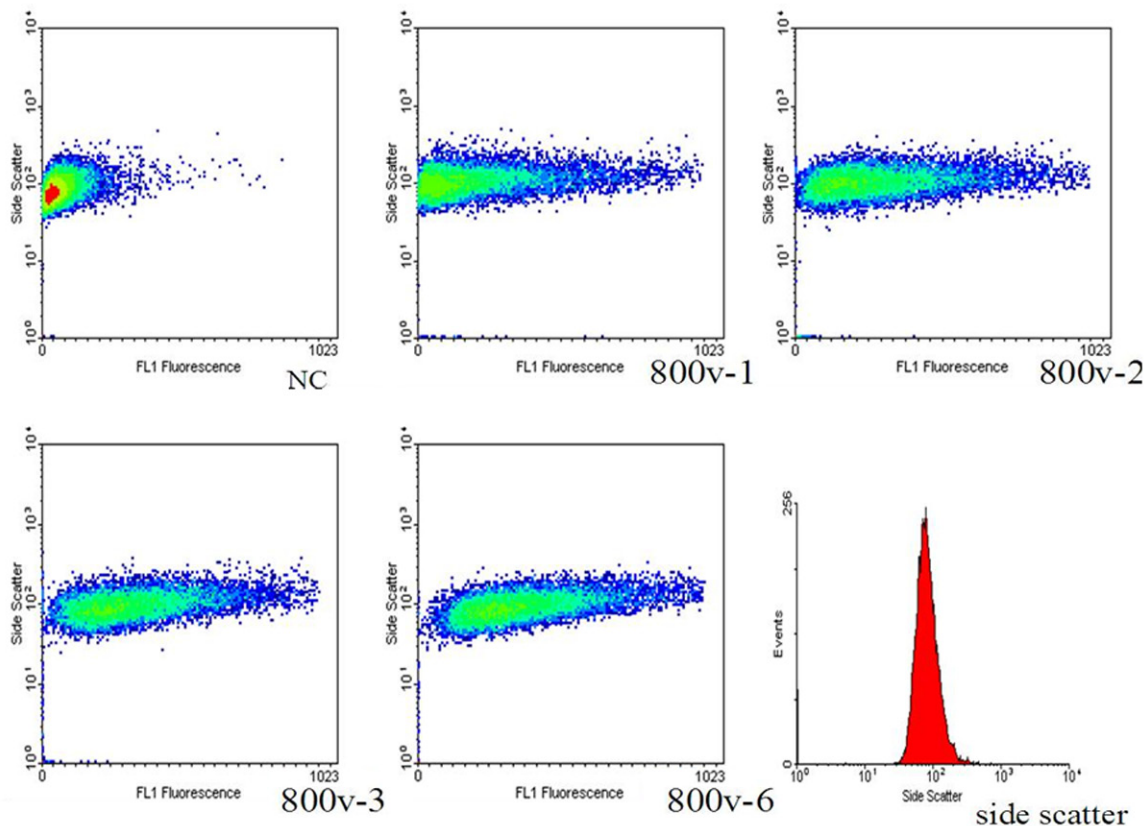
parameters. With this group of parameters, steep pulse could have the best tumor cell-killing cells effects. At present, the SPEF induced tumor cell apoptosis-related experiments become the focus gradually at domestic and foreign steep pulses biological applications.

Cell apoptosis is the responses to the inside and outside stimulating signal, which is one of subjecting to genetic control and programmed cell initiative to death, also called Programmed Cell Death, PCD. At last, the cells exfoliated in vitro or cleavage of apoptotic bodies, and were swallowed by other cells. Study found that the apoptosis is closely related to tumorigenesis and tumor regression [23-25]. If the anti-tumor technology can induce cancer cells apoptosis in large number, and activate programmed cell initiative to death; that will be a new breakthrough to the treatment of tumors. So, it

becomes a research focus that trying to find the new technology inducing cancer cells apoptosis.

We use the flow cytometry to detect the apoptosis, the L9981 cells processed by steep pulse experienced apoptosis and necrosis; and with the increase of electrical parameters, the proportion of necrotic cells increased rapidly. When the voltage amplitude is at 2000 V/cm and repetitions is at 3, most of cells (> 70%) go into the necrotic area. From the mitochondrial membrane potential changing result, there be 89.15% depolarization when the voltage amplitude 2000 V/cm and repetitions 3. It is usually considered that the cells are in apoptosis when mitochondrial membrane potential had been depolarization, so that we consider that the steep pulse processing can leads to the apoptosis of tumor cell [26]. The test results of PH

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**Figure 5.** Intracellular free calcium concentration changes in the results of flow cytometry in the different parameters of steep pulse electric treatment on human high-metastatic large cell lung cancer cell line L9981.

changing in intracellular showed that the light intensity “level hop” decline with the increasing of the electrical parameters. The intracellular PH tend to acidity with the increases of electrical parameters. The cells under steep pulse processing come to apoptosis and necrosis [27].

With the increase of steep pulse parameters, number of intracellular free calcium in the cell increased significantly, and can reach a saturation stage basically after three times processing. Shown by flow cytometry detected the number of intracellular free calcium, with the increase of steep pulse parameters, number of intracellular  $\text{Ca}^{2+}$  in the cell increased significantly, and can reach a saturation stage basically after three times processing. Domestic and foreign scholars believed that, it is one of the important mechanism of apoptosis of the increasing of  $\text{Ca}^{2+}$  in cells [25], and  $\text{Ca}^{2+}$  is an important second “messenger”; with the  $\text{Ca}^{2+}$  concentration to rise continually, endonuclease can be activated directly, leading to apoptosis;

and also mitochondrial transmembrane potential was decreased, and mitochondrial membrane permeability increased, permeability transition pore was opening, cytochrome C and etc. penetrated to the cytoplasm, so that the apoptotic pathway was opened and led to induction of apoptosis.

In summary, steep pulse technology, which leading to tumor cell necrosis by induction of apoptotic effect, that is programmed death, can achieve the purpose of killing tumor cells. This may be a steep pulse mechanism to kill tumor cells.

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