

PRELIMINARY STUDY OF STEEP PULSE IRREVERSIBLE ELECTROPORATION TECHNOLOGY IN HUMAN LARGE CELL LUNG CANCER CELL LINES L9981

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Abstract – Our aim was to validate the effectiveness of steep pulse irreversible electroporation technology in human large cell lung cancer cells and to screen the optimal treatment of parameters for human large cell lung cancer cells. Three different sets of steep pulse therapy parameters were applied on the lung cancer cell line L9981. The cell line L9981 inhibition rate and proliferation capacity were detected by Vi-Cell vitality analysis and MTT. Steep pulsed irreversible electroporation technology for large cell lung cancer L9981 presents killing effects with various therapy parameters. The optimal treatment parameters are at a voltage amplitude of 2000V/cm, pulse width of 100 μ s, pulse frequency of 1 Hz, pulse number 10. With this group of parameters, steep pulse could have the best tumor cell-killing effects.

Key words: Steep pulsed, irreversible electrical breakdown, large cell lung cancer

INTRODUCTION

A steep pulse can cause unique intracellular effects that induce irreversible electrical breakdown, effectively killing tumor cells. Steep pulse can suppress tumor growth and proliferation. It shows a good application prospect in comprehensive treatment of the tumor (Luo et al., 2012). It has opened up a new method for the study and control of biological cells. Previous studies have shown that a steep pulse has a role in killing different types of tumor cells that has not been extensively reported in the literature (Luo et al., 2012). Research will verify the validity of steep pulse in killing the lung cancer cell line L9981. We

screened the best treatment parameters for L9981, and provide a theoretical basis for researching the mechanism of action of steep pulsed and irreversible electrical breakdown.

MATERIALS AND METHODS

Human, highly metastatic, large cell lung cancer cell lines L9981 were screened to establish a single cell clone from human large cell lung cancer cell lines WCQH-9801 by Prof Zhou Qinghua. The large cell lung cancer cell line L9981 is highly invasive and highly metastatic. RPMI-1640 medium and newborn calf serum were obtained from GIBCO Co.; Trypsin,

Table 1. Parameter 4 of steep pulse in L9981 cells (Vi-cell activity)

Single pulse number	Cell death rate % ($\bar{x} \pm s$)	T Value	P Value
10	80.4±5.07	5.065	.000
15	67.9±3.28		

HEPEs and EDTA were from Amresco Co. Other conventional reagents were obtained from domestic companies.

Equipment

We used an energy controllable steep pulse therapeutic apparatus (Fig. 1), designed and manufactured by the Ministry of Education Key Laboratory of Chongqing University, which combines different pulse parameters, producing an energy-controllable steep pulse by capacitor energy storage and discharge. The electrode needle is made from platinum. A Vi-Cell Cell viability analyzer was obtained from Beckman Coulter, USA.

Steep pulse processing

L9981 cells were grown in RPMI-1640 medium which contained 10% fetal bovine serum at 37°, 5% CO₂. When the cells reached 80% growth confluence, they were trypsinized. The cell concentration was adjusted to 1×10⁶ cells/ml and placed in an electrode cup (700 μL). The setting the steep pulse parameters was for L9981. Parameter settings were: voltage, 600V/cm; pulse width, 100 μs; repetition rate, 1 Hz; number of pulses, 10; grouping, NC; does not handle, T1; by parameter processing, 1; T2, repeat the processing according to the parameters, 2; T3, repeat the processing according to the parameters, 3.

Parameter screening of steep pulse technology

Parameter sets for steep pulses that were used for irreversible electroporation of large cell lung cancer cells were as follows: parameter setting 1: voltage: 600V; number of pulses: 10; repetitions: 0-8; set-

ting 2: voltage: 400V/600V/800V/1000V; number of pulses: 10; repetitions: 6; setting 3: voltage: 800V; number of pulses: 10; repetitions: 0-8, setting 4: voltage: 800V; number of pulses: 10/15. At every setting, the frequency was 1 Hz and pulse width was 100 μs.

Cell procedures

Cell counting and analysis of cell activity was performed by using Vi-Cell cell viability analyzer. In order to prepare samples for analysis 100 μL of cell suspensions were mixed with 900 μL of 1×PBS buffer. The proliferation rate of large cell lung cancer cells was evaluated by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. Samples were incubated with MTT (5mg/ml in PBS) for 4 h. The MTT-formazan precipitate was dissolved in DMSO. The microplate reader was used to measure absorbance of cell samples at 490 nm. The relative inhibition rate of cell growth was evaluated according to formula (Long et al., 2012):

$$\text{Cell growth inhibition} = \frac{(\text{OD}_{\text{repeat 0 time}} - \text{OD}_{\text{repeat n times}})}{\text{OD}_{\text{repeat 0 time}}} \times 100\%.$$

Statistical analysis

Statistical software SPSS 11.0 was used to analyze the effect of different sets of steep pulse therapy parameters on lung cancer cell viability, proliferative capacity and cell cycle inhibition. The statistical level of significance was set at 0.05.

RESULTS AND DISCUSSION

Our research shows that cell death was optimal at 2000 V/cm, a pulse width of 100 μs, a frequency of 1 Hz, 10 pulses and 6 repeats (Figs. 5, 6; Table 1).



Fig. 1. Steep pulse therapy instrument.

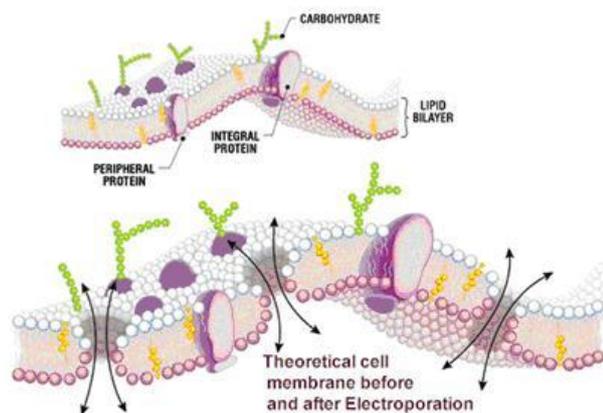


Fig. 2. The mechanism of steep pulses on cell.

Lung cancer has the highest morbidity and mortality of all malignant tumors (about 25.4%). In recent years, the trend of its morbidity has been upward (Siegel et al., 2011; Chen et al., 2011; Wang et al., 2011). NSCLC (Non-small cell lung cancer) is the most frequently diagnosed type of lung cancer,

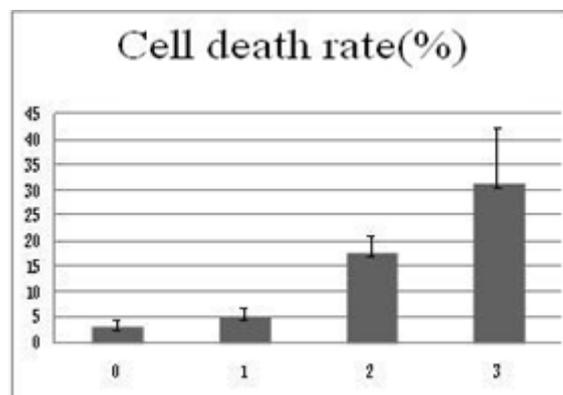


Fig. 3. Four groups of steep pulse parameters after treatment the death rate of lung cancer cell line L9981.

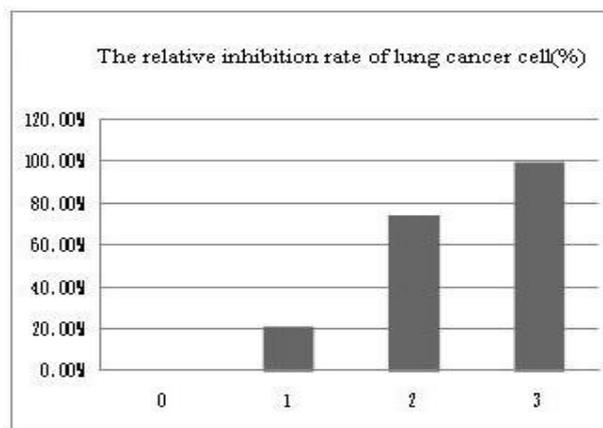


Fig. 4. Four sets of parameters of steep pulse deal with the relative inhibition rate of lung cancer cell line L9981 cell growth. (Parameter settings: voltage amplitude 1500V/cm, pulse width 100μs, pulse frequency 1Hz, the number of pulses 10; 0 means no deal with 1 means repeat once, 2 means repeat twice, 3 means repeat three time)

(80~85% of total lung cancer cases). It is manifested in the following histological types: squamous cell carcinoma, adenocarcinoma and large cell carcinoma. NSCLC is the main cause of cancer related death in patients with lung cancer; the data published by WHO in 2005 show that lung cancer mortality is 30.83/100000 among the general population, 41.343/100000 in males and 19.84/100000 in females. Clinical statistics, which include 1742 cases of Chinese NSCLC, show that 1, 2, 3, 4, and 5-year survival rates were 44%, 22%, 13%, 9% and

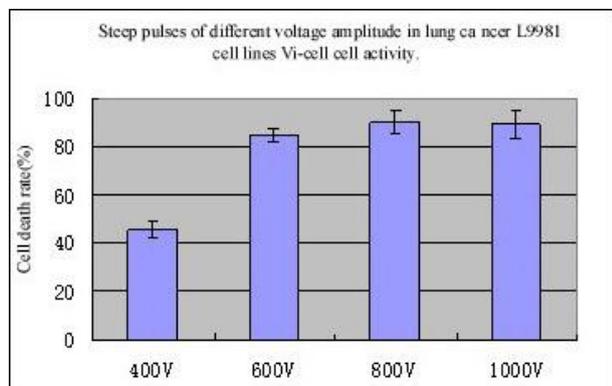


Fig. 5 The effect of steep pulses of different voltage amplitude on Vi-cell activity of lung cancer L9981 cells.

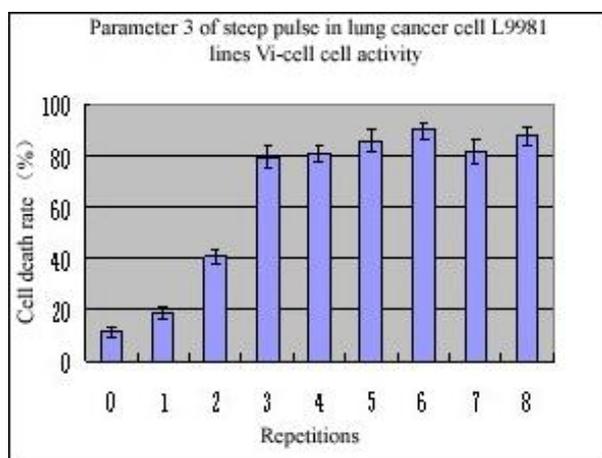


Fig. 6. Parameter 3 of steep pulse in L9981 cells (Vi-cell activity)

6% (Peng et al., 2011). Unfortunately, many patients already develop advanced stage of lung cancer at diagnosis, which is associated with lower efficiency of treatment (Wei et al., 2012). In recent years, long-term survival of patients with lung cancer has not improved significantly. Five-year survival of patients with lung cancer was found to be 15.8% (Siegel et al., 2011), even though a variety of treatment techniques was developed, and comprehensive cancer treatment has been greatly improved by surgery, radiotherapy and chemotherapy. An integrated treatment has showed an increasingly important role in lung cancer treatment strategies (Zhang et al., 2011, 2012). In recent years, the minimally invasive ablation therapy, aimed at inactivating tumor cells and eliminating tumor burden has attracted attention (Nurwidya et al., 2012; Hu et al., 2012). New targeted ablation therapy technology has applied freezing (Sabel, 2009; Nishida et al., 2011; Silva et al., 2010), exposure to focused ultrasound (Hwang and Crum, 2009; Klatte and Marberger, 2009), seed implantation (Morris et al., 2009; Wang et al., 2010), microwaves (Ong et al., 2009; Boutros et al., 2010) and radiofrequencies (Minami and Kudo, 2010; Tacconi et al., 2011). Compared to traditional chemotherapy, irreversible electrical breakdown characteristics of SPEF has unique advantages in cancer treatment (Hofmann and Evans, 1986). It is expected to become an complementary means of comprehensive cancer treatment. It was developed by integrating biomedical, electrical and microelectronic technologies (Yao

et al., 2007). 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 do not impact on the cell (Weaver 2000) (Fig. 2). With the increase in pulse dose, the cell membrane and nuclear membrane are in a continuous dynamic process of charging and discharging, forming an electric field in the membrane; the dielectric constant of the cell membrane is different from the surrounding protoplasm dielectric constant. Ultimately, it leads to irreversible electrical breakdown and death in the end (Zimmermann et al., 2000; Beebe, 2001; Chenguo et al., 2004) Fig. 2.

The destruction rate of liver cancer cells is over 80% after exposure to 1500 V/cm, pulse width 100 μ s, pulse frequency 1 Hz, 10 pulses 10, 6 repeats 6 (Mi et al., 2007). We observed that cell death with the increase in the number of repetitions. The cell mortality rate is lower than 50% after three repetitions and it cannot effectively kill lung cancer L9981 cells. There are many factors that contribute to the perforation of tumor cells, such as the composition of the tumor tissue, bioelectrical impedance, the type and volume of the tumor cells, electrode shape and arrangement. Statistical analysis showed that the best

parameters were: 2000 V/cm, pulse width: 100 μ s, pulse frequency 1 Hz, 10 pulses 10, 6 repeats.

From the results of MTT data, under the condition of Vi-Cell cell viability detection in the electrical parameters of the cell death rate was only less than 40% of the steep pulse, MTT results shows the inhibitory rate more than 99%. We think that, the steep pulse electric treatment could affect the paste-wall capacity and (or) Re-growth capacity. In this experiment, we studied cells in suspension, which differ greatly compared to living tissue. Further studies are needed that parameters the steep pulse electric treatment of cell in cell suspension have what kind of guiding role in the future.

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REFERENCES

- Beebe, S. J., Fox, P. M., Rec, L. J., Somers, K., Stark, R. H. and K. H. Schoenbach (2001) Nanosecond pulsed electric field (nsPEF) effects on cells and tissues: apoptosis induction and tumor growth inhibition. In: *PPPS-2001 Pulsed Power Plasma Science 2001. 28th IEEE International Conference on Plasma Science and 13th IEEE International Pulsed Power Conference. Digest of Technical Papers, 17-22 June 2001*, Vol. vol.1, pp. 211-215. IEEE, Piscataway,NJ, USA.
- Boutros, C., Somasundar, P., Garrean, S., Saied, A. and N. J. Espat (2010) Microwave coagulation therapy for hepatic tumors: review of the literature and critical analysis. *Surgical oncology*, **19**, e22-32.
- Chen, W.-Q., Zheng, R.-S. and H.-M. Zeng (2011) Bayesian age-period-cohort prediction of lung cancer incidence in China. *Thorac. Cancer*, **2**, 149-155.
- Chenguo, Y., Caixin, S., Yan, M., Lan, X. and W. Shibin (2004) Experimental studies on Killing and inhibiting effects of steep pulsed electric field (SPEF) to target cancer cell and solid tumor. *IEEE Transactions on Plasma Science*, **32**, 1626-1633.
- Hofmann, G. A. and G. A. Evans (1986) Electronic genetic inverted question mark physical and biological aspects of cellular electromanipulation. *IEEE engineering in medicine and biology magazine : the quarterly magazine of the Engineering in Medicine & Biology Society*, **5**, 6-25.
- Hu, R., Zhang, X., Liu, X., Xu, B., Yang, H., Xia, Q., Li, L., Chen, C. and J. Tang (2012) Higher temperature improves the efficacy of magnetic fluid hyperthermia for Lewis lung cancer in a mouse model. *Thorac. Cancer*, **3**, 34-39.
- Hwang, J. H. and L. A. Crum (2009) Current status of clinical high-intensity focused ultrasound. *Conference proceedings: Annual International Conference of the IEEE Engineering in Medicine and Biology Society. IEEE Engineering in Medicine and Biology Society. Conference*, **2009**, 130-133.
- Klatte, T. and M. Marberger (2009) High-intensity focused ultrasound for the treatment of renal masses: current status and future potential. *Current opinion in urology*, **19**, 188-191.
- Liu, L. J., Zhao, D. Y., Wang, J., Yao, C. G., Sun, C. X. and J. Y. Tang. (2011) [Ca(2+) is an important mediator of nanosecond steep pulse-induced apoptosis in human ovarian cancer SKOV3 cells]. *Nan fang yi ke da xue xue bao = Journal of Southern Medical University*, **31**, 772-776.
- Long, H., Zhang, S., Liu, C., Shi, J., Tao, L., Situ, D., Ma, G., Zheng, Y. and L. Fu (2012) Characterization of a stem cell population in lung cancer cell line Glc-82. *Thorac. Cancer*, **3**, 8-18.
- Luo, X. D., Sun, J. C., Liu, F., Hu, L. N., Dong, X. J., Sun, D. N. and J. Xiao (2012) Energy controllable steep pulse (ECSP) treatment suppresses tumor growth in rats implanted with walker 256 carcinosarcoma cells through apoptosis and an antitumor immune response. *Oncology research*, **20**, 31-37.
- Mi, Y., Sun, C., Yao, C., Li, C., Mo, D., Tang, L. and H. Liu (2007) Effects of steep pulsed electric fields (SPEF) on mitochondrial transmembrane potential of human liver cancer cell. In: *29th Annual International Conference of IEEE-EMBS, Engineering in Medicine and Biology Society, EMBC'07, August 23, 2007 - August 26, 2007*, pp. 5814-5817. Inst. of Elec. and Elec. Eng. Computer Society, Lyon, France.
- Minami, Y. and M. Kudo (2010) Radiofrequency ablation of hepatocellular carcinoma: Current status. *World journal of radiology*, **2**, 417-424.
- Morris, W. J., Keyes, M., Palma, D. et al. (2009) Population-based study of biochemical and survival outcomes after permanent 125I brachytherapy for low- and intermediate-risk prostate cancer. *Urology*, **73**, 860-865; discussion 865-867.

- Nishida, H., Yamamoto, N., Tanzawa, Y. and H. Tsuchiya (2011) Cryoimmunology for malignant bone and soft-tissue tumors. *International journal of clinical oncology*, **16**, 109-117.
- Nurwidya, F., Takahashi, F. and K. Takahashi (2012) Meeting report: Current cancer perspectives from the 9th Annual Meeting of the Japanese Society of Medical Oncology. *Thorac. Cancer*, **3**, 94-97.
- Ong, S. L., Gravante, G., Metcalfe, M. S., Strickland, A. D., Denison, A. R. and D. M. Lloyd (2009) Efficacy and safety of microwave ablation for primary and secondary liver malignancies: a systematic review. *European journal of gastroenterology & hepatology*, **21**, 599-605.
- Peng, H., Ma, M. and B. Han (2011) [Survival analysis of 1,742 patients with stage IV non-small cell lung cancer]. *Zhong-guo fei ai za zhi = Chinese journal of lung cancer*, **14**, 362-366.
- Sabel, M. S. (2009) Cryo-immunology: a review of the literature and proposed mechanisms for stimulatory versus suppressive immune responses. *Cryobiology*, **58**, 1-11.
- Siegel, R., Ward, E., Brawley, O. and A. Jemal (2011) Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA: a cancer journal for clinicians*, **61**, 212-236.
- Silva, R. G. M., Gross, J. L., Silva, R. A., Haddad, F. J., Younes, R. N., Cruz, V. M. and A. B. M. Avertano-Rocha (2010) Primary monophasic synovial sarcoma of the pleura: Neoadjuvant chemotherapy followed by complete resection. *Thorac. Cancer*, **1**, 95-101.
- Tacconi, F., Ambroggi, V., Pompeo, E., Sellitri, F. and T. C. Mineo (2011) Substernal hand-assisted videothoroscopic lung metastasectomy: Long term results in a selected patient cohort. *Thorac. Cancer*, **2**, 45-53.
- Wang, J.-B., Fan, Y.-G., Jiang, Y. et al. (2011) Attributable causes of lung cancer incidence and mortality in China. *Thorac. Cancer*, **2**, 156-163.
- Wang, J. J., Yuan, H. S., Li, J. N., Jiang, Y. L., Tian, S. Q. and R. J. Yang (2010) CT-guided radioactive seed implantation for recurrent rectal carcinoma after multiple therapy. *Medical Oncology*, **27**, 421-429.
- Weaver, J. C. (2000) Electroporation of cells and tissues. *IEEE Transactions on Plasma Science*, **28**, 24-33.
- Wei, S., Hao, C., Gong, L., Hu, C., Lian, J. and Q. Zhou (2012) Survival and bronchial carcinoid tumors: Development of surgical techniques in a 30-year experience of 82 patients in China. *Thorac. Cancer*, **3**, 48-54.
- Yao, C., Sun, C., Mi, Y. and C. Li (2007) Progress in research of irreversible electrical breakdown of steep pulse for tumor treatment and its key techniques. *High Voltage Engineering*, **33**, 7-13.
- Zhang, Q.-N., Wang, D.-Y., Wang, X.-H., Hui, T.-j., Yang, K.-H., Li, Z., Li, H.-Y. and L.-Y. Guo (2012) Non-conventional radiotherapy versus conventional radiotherapy for inoperable non-small-cell lung cancer: A meta-analysis of randomized clinical trials. *Thorac. Cancer*, **3**, 269-279.
- Zhang, X.-H., Li, C., Dai, C.-F. and B.-S. Zhou (2011) Epidermal growth factor receptor mutations and their correlation with epidermal growth factor receptor-tyrosine kinase inhibitor therapy and association with the characteristics of patients with non-small-cell lung cancer: A meta-analysis. *Thorac. Cancer*, **2**, 101-108.
- Zimmermann, U., Friedrich, U., Mussauer, H., Gessner, P., Hamel, K. and V. Sukhorukov (2000) Electromanipulation of mammalian cells: fundamentals and application. *IEEE Transactions on Plasma Science*, **28**, 72-82.