Effect of Steep Pulsed Electric Field on Proliferation, Viscoelasticity and Adhesion of Human Hepatoma SMMC-7721 Cells

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Abstract. *It has been proven that steep pulsed electric field (SPEF) can directly kill tumor cells and plays an important role in anticancer treatment. The biorheological mechanisms, however, that destroy tumor cells are almost unknown. To resolve this issue, here, an SPEF generator was used to assess the effects of high- and low-dose SPEF on the proliferation of human hepatoma SMMC-7721 cells by MTT assay, and on the viscoelasticity, adhesion of SMMC-7721 cells to endothelial cells by micropipette aspiration technique. Viability and proliferation of SPEF-treated SMMC-7721 cells were significantly inhibited. Cell cycle analysis indicated that SPEF arrested the cell cycle progression of SMMC-7721 cells at the G0/G1 transition to the S-phase. Viscoelastic data fitted by a standard linear solid model showed that viscoelasticity of SMMC-7721 cells changed after treatment with SPEF. Moreover, the adhesive force of low-dose SPEF-treated SMMC-7721 cells to endothelial cells markedly decreased compared to that of control cells. These results suggest that the suppressant effects of SPEF on the proliferation of SMMC-7721 cells appeared to be mediated, at least in part, through arresting cell cycle progression and altering the viscoelastic and adhesive properties of the cells, which provides a novel biorheological mechanism for the antitumor therapy of SPEF.*

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Key Words: Steep pulsed electric field (SPEF), hepatoma, proliferation, biorheology, micropipette aspiration.

Cancer is a malignant disease characterized by the disorganization of the cell cycle and uncontrolled proliferation. How to prevent and treat it is still a challenge for the therapist. In recent years, a new anticancer approach, named electrotherapy, has developed rapidly and become an effective means of tumor treatment. It is a treatment that generates electrical pulses through electrodes placed in the tumor tissue to enhance the ability of anticancer drugs to enter tumor cells. Short, high-voltage electrical pulses create micropores in the membrane of tumor cells (reversible electric breakdown), allowing the entrance of poorly permeating agents such as macromolecules, proteins, drugs and genes (1). Thus, the antitumor effectiveness of a cytotoxic agent markedly increases in individual cells (2). Previous studies have proven successful in applying this protocol, combined with anticancer drugs, to treat head and neck, skin and pancreatic cancer, as well as hepatoma (3). It was also demonstrated that a pulsed electric field could not only increase the effectiveness of an anticancer agent, but also inhibit angiogenesis of the tumor tissue and activate the immunoregulation system of the body (4, 5). Theoretically, by increasing the 'dosage' of the pulsed electric field, the reversible electric breakdown will change to irreversible electroporation, called irreversible electric breakdown. Interestingly, Stephen *et al*. found that high intensity (26-300 kV/cm) nanosecond (10-300 ns) pulsed electric field (nsPEF) treatment without anticancer drugs significantly inhibited growth of tumor tissue and induced apoptosis of tumor cells (6). Moreover, it was reported that nsPEF induced apoptosis in p53-wild-type and p53-null HCT116 colon carcinoma cells (7) and changes in cellular dielectric properties of Jurkat cells, a malignant human T-cell line (8).

In our early experiments, we investigated the effects of a pulsed electric field, of which the rising edge is at the 100 ns level (named steep pulsed electric field, SPEF) in human ovarian carcinoma cells and solid tumor. We demonstrated

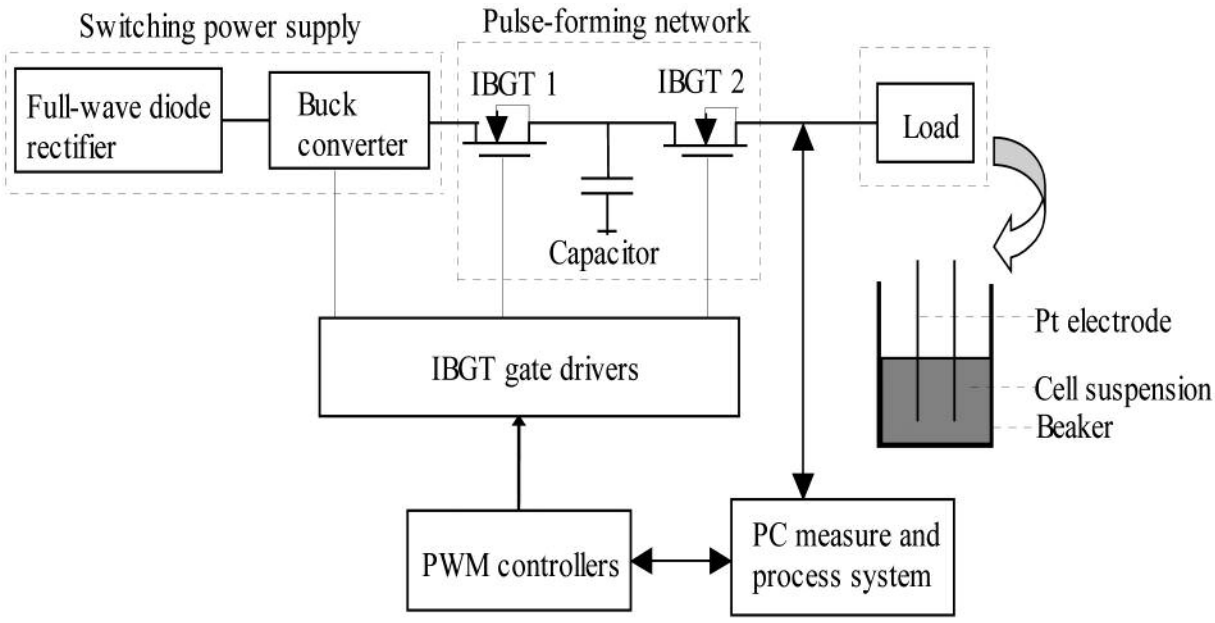

Figure 1. *Schematic diagram of steep pulsed electric field generator. The diameter of the beaker was 3 cm; height was 5 cm. Distance between the two electrodes was 1 cm.*

that SPEF was able to generate irreversible electric breakdown in tumor cells and directly killed tumor cells and destroyed tumor tissue without the use of anticancer drugs, which avoided the harmful side-effect of anticancer drugs in the body (9). Moreover, Tang *et al*. reported that SPEF could trigger apoptosis in liver cancer cell line and demonstrated the relationship between the apoptosis and the change of intracellular calcium concentration (10). Although it was proven that SPEF is a potential approach to treat cancer in the clinic, the mechanisms by which SPEF destroys tumor cells are not fully understood. In the present study, we focused on assessing the effects of SPEF on the proliferation of and the viscoelasticity and adhesive force of human hepatoma SMMC-7721 cells to endothelial cells, and explored the antitumor mechanism of SPEF from a biorheological point of view.

Materials and Methods

Cell culture. Human hepatoma cells (SMMC-7721) and human umbilical vein endothelial cells (HUVECs) were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academic of Sciences, China. SMMC-7721 cells were routinely cultured in suspension of RPMI-1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 units/ml penicillin and 100 units/ml streptomycin (North China Pharmaceutical Group Corporation, China) in a humidified atmosphere of 5% CO_2/95% air at 37˚C. HUVECs were cultured with Dulbecco's modified Eagle's medium (DMEM; HyClone) supplemented with 15% FBS, 100 units/ml penicillin and 100 units/ml streptomycin in the same standard incubator. The cells were detached by a brief exposure to 0.25% trypsin/0.02% EDTA and HUVECs from passages 3-5 were used for experiments.

SPEF generator and tumor cell treatment. The SPEF generator was self-assembled by the Laboratory of High Voltage and Electrotechnics New Technology, Chongqing University, China. The apparatus was integrated with power electronics and a conventional high-voltage pulse generator. As shown in Figure 1, it consists of a switching power supply (Buck converter) generating a high DC voltage, a charge-discharge capacitor forming the pulse, a switch (IGBT), a pulse-width modulation (PWM) controller, and the load (tumor cells). PWM controllers drive the Buck converter and two IGBTs to produce an exponential decay pulse in the load. The pulse frequency (0-2 kHz) and its peak value (0-300 V) can be adjusted by PWM, while the pulse duration (5-100 μs) can be adjusted by switching different capacitors (0.01-0.47 μF). The full waveform and rising edge of the SPEF produced by this generator are shown in Figure 2, where the applied voltage is a function of the duration of time. The rising edge was modified to a nanosecond level (approximately 100 ns) by selecting appropriate capacitors, IGBT and PWM controller.

The SMMC-7721 cell suspension (approximately 5×10^4 cells/ml) was harvested into a sterile beaker (diameter: 3 cm, height: 5 cm), and the platinum (Pt) electrodes of the generator were inserted into the suspension. The distance between two electrodes was 1 cm and the SPEF treatment time was 10 minutes. Experiments were divided into two groups: low-dose SPEF and high-dose SPEF, each receiving the same pulse parameters except for the capacitance. The applied pulse frequency (100 Hz), peak value (200 V) and treatment time (10 min) were chosen in accordance with our previous study (9). The discharge capacity for the low-dose SPEF group was 0.01 μF while that for the high-dose SPEF group was 0.1 μF. Cells without exposure to SPEF were used for control purposes.

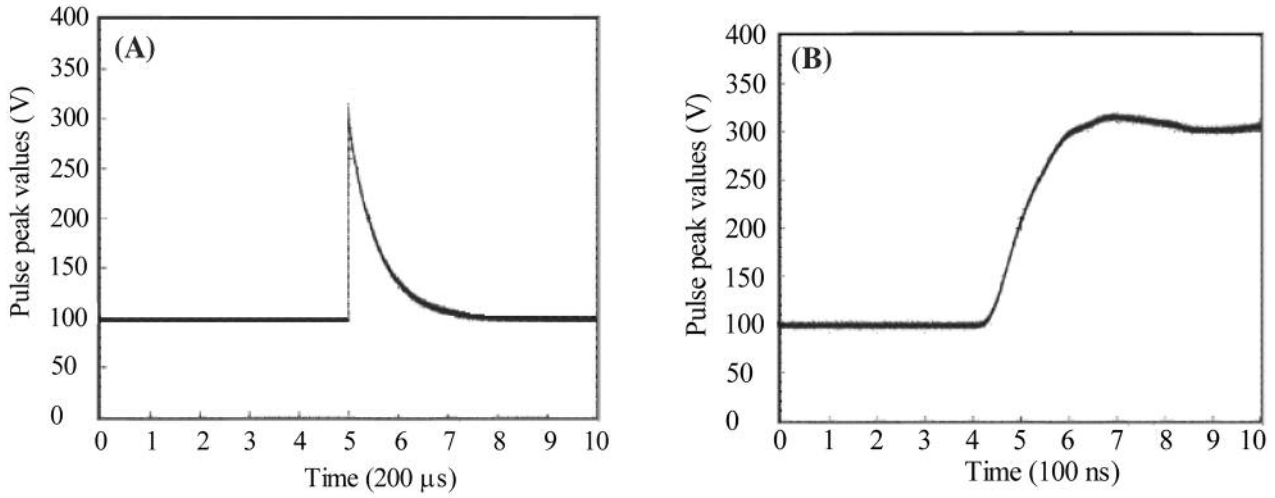

Figure 2. *Full waveform (A) and rising edge (B) of steep pulsed electric field (SPEF) produced by the generator.*

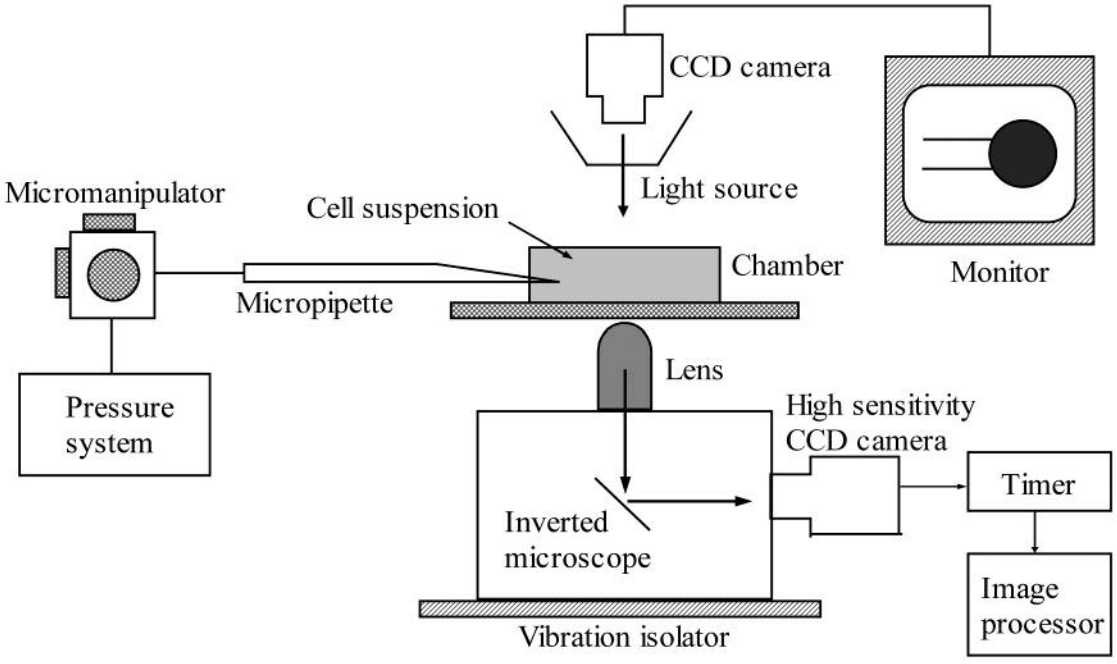

Figure 3. *Schematic diagram of micropipette aspiration system*.

Proliferation and cell cycle assay. Analysis of the cell proliferation was performed with a standard 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) kit (Sigma-Aldrich, USA) for spectrophotometry following the manufacturer's protocol. SPEF-treated SMMC-7721 cells were seeded in 96-well microplates and cultured for different times. The optical density (OD) value after those times was measured at 570 nm using a microplate reader (Model 550; Bio-Rad, USA). The inhibition rate was calculated from the OD values of treated and control samples. The formula is defined as follows:

Inhibition rate=[$OD_{570\ nm}$ (control)–$OD_{570\ nm}$ (SPEF)] /$OD_{570\ nm}$ (control)

Analysis of the cell cycle distribution was carried out after the following procedures: SPEF-treated SMMC-7721 cells were cultured for 24 hours, then approximately 200,000 cells were harvested and rinsed three times with phosphate-buffered saline (PBS; pH 7.4, 0.1 mol/l), fixed in 70% cooled ethanol and dyed with propidium iodide (PI) for 30 minutes. The cell cycle distribution was examined by flow cytometry (BD FACSCalibur, BD Bioscience, USA).

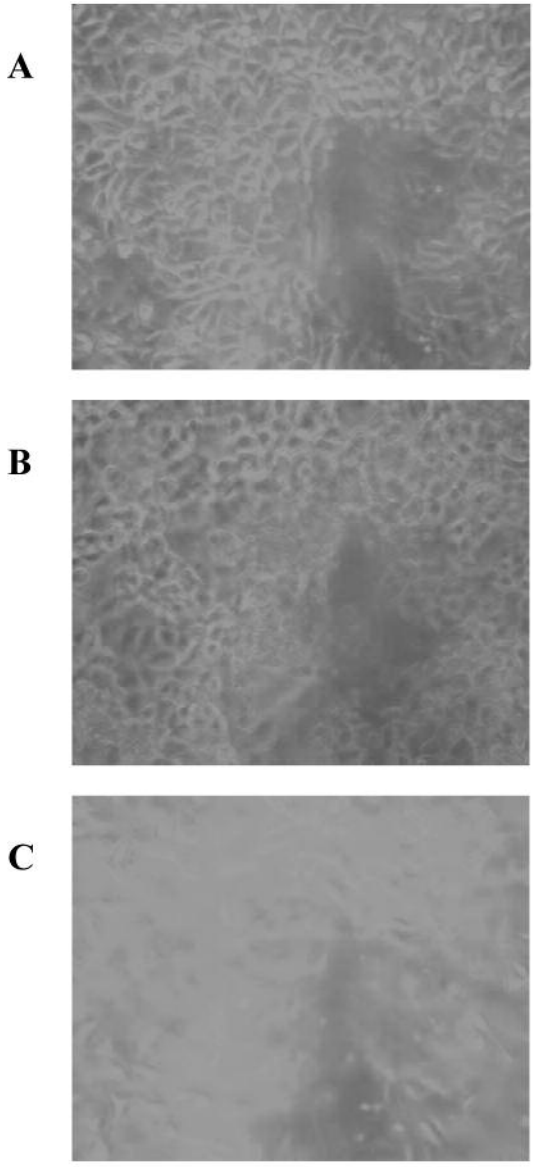

Figure 4. *Phase-contrast microscopic appearance of cultured SMMC-7721 cells after exposure to steep pulsed electric field (SPEF). SMMC-7721 cells were treated with SPEF for 10 minutes, reseeded and cultured for 24 hours. A: control, B: low-dose SPEF group, C: high-dose SPEF group (original magnification ×100).*

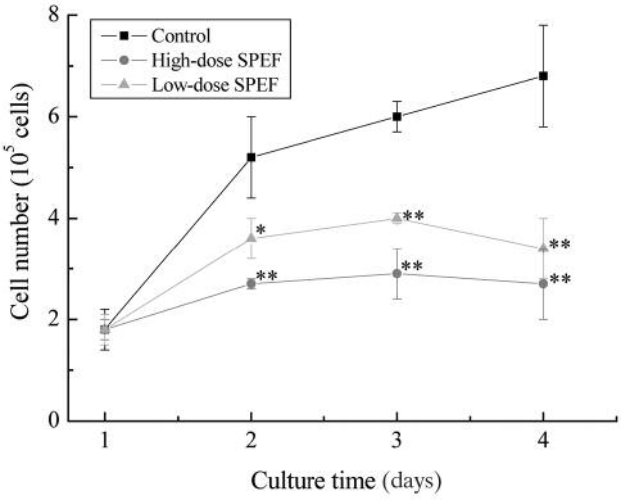

Figure 5. *Effects of steep pulsed electric field (SPEF) on viability of SMMC-7721 cells. SMMC-7721 cells were exposed to SPEF for 10 minutes and then cultured for the designated times. The cell viability was determined by the cell count number. Data represent means±standard deviation of three separate samples per group. *p<0.05 vs. control; **p<0.01 vs. control.*

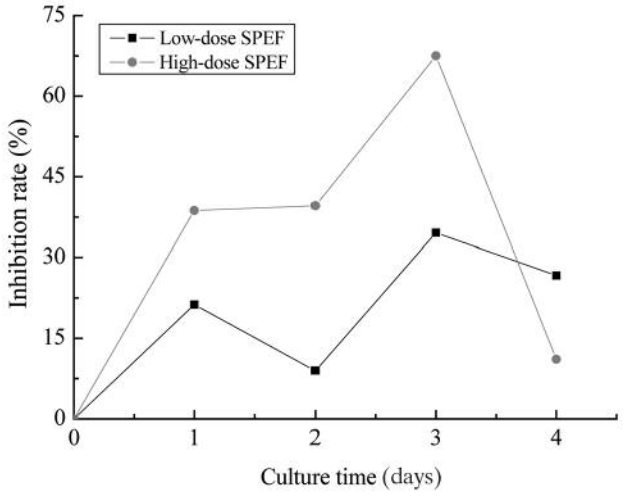

Figure 6. *Inhibitory effects of steep pulsed electric field (SPEF) on proliferation of SMMC-7721 cells. SMMC-7721 cells were exposed to SPEF for 10 minutes and then cultured for the designated times. MTT assay was used to examine the proliferation of SMMC-7721 cells. OD values were obtained at 570 nm with a 96-well micro test spectrophotometer; the inhibition rate was calculated using the OD values.*

Micropipette aspiration system. Viscoelasticity and the adhesive force of SMMC-7721 cells to HUVECs were determined using a micropipette aspiration system (Figure 3). This system is composed of an inverted microscope (Axiovet 35; Zeiss Co., Germany), a chamber (self-assembled), a micromanipulator (MR5170; Eppendorf Co., Germany), an image recording system (NV-HD100MC; Panasonic Co., Japan), an image processing system (Vidas21; Kontron Co., Germany), a video timer (VGT33; For. A Co. Ltd., Japan), a video monitor (12M310; Tokyo Electronic Industry, Japan), a pressure controlling system (self-assembled by Bioengineering Laboratory, Chongqing University, China) and a glass micropipette. The micropipette was made from a glass tube using a micropipette puller (P87; Sutter Instrument Co., USA). The inner radius of micropipettes used here was approximately 2.5-3.0 μm.

Measurement of viscoelasticity and data processing. Approximately 0.5 ml of SMMC-7721 cell suspension (1×10^4 cells/ml) was added to the chamber. The tip of the micropipette was positioned close to surface of a cell by the micromanipulator;

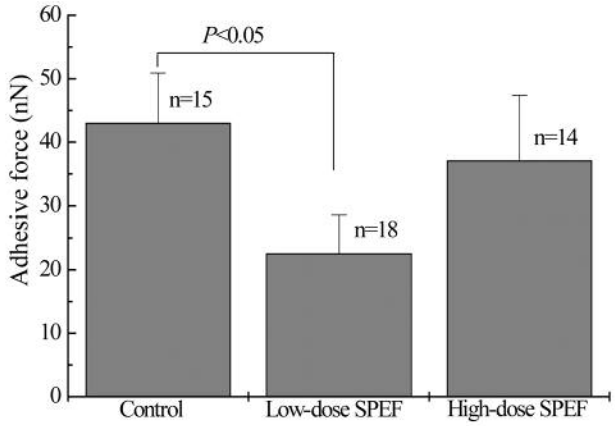

Figure 7. *Effects of steep pulsed electric field (SPEF) on the adhesive force of SMMC-7721 cell to HUVECs. SMMC-7721 cells were exposed to SPEF for 10 minutes and then cultured for 24 hours prior to the adhesive experiment. Micropipette aspiration technique was employed to determine the adhesive force of SMMC-7721 cells to HUVECs. n: Number of cells measured.*

a small portion of the cell was aspirated into the micropipette with a step-rise negative pressure produced by the pressure system. Cell deformability was recorded and stored on the video tape recorder continuously, and the experimental data then measured through the image processing system (11). Data were analyzed using the standard linear solid model, in which an elastic element K_1 is in parallel with a Maxwell element composed of another elastic element K_2, in series with a viscous element μ (12). The combination of elastic coefficients K_1 and K_2 determine the initial cell deformation, which is proportional to $1/(K_1+K_2)$. The viscous coefficient μ in combination with K_1 and K2 determine the rate of slow phase of creeping deformation, with the time constant given by $\mu(K_1+K_2)/K_1K_2$. The maximum deformation as time tends to infinity is proportional to $1/K_1$.

Measurement of adhesive force. A HUVEC monolayer was cultivated in a special chamber. Approximately 0.5 ml of SMMC-7721 cell suspension (1×10^4 cells/ml) was added to the chamber and the chamber was then placed in an incubator at 37˚C for 30 min prior to the experiments. Regulated pressure was set to zero (zero-pressure state) through the pressure controlling system. An SMMC-7721 cell was selected under the microscope, the tip of the micropipette was then positioned close to the surface of the SMMC-7721 cell using the micromanipulator and a small portion of the cell was aspirated into the micropipette by application of negative pressure (*P*). The micropipette was then carefully pulled away from the cell using the micromanipulator. If the cell was not detached from the surface of the adhered HUVEC, the negative pressure was increased by 5 mm H_2O. This process continued until the SMMC-7721 cell was finally detached from the adhered HUVEC, thus giving the critical negative pressure for detachment. The recorder continuously recorded the experimental process. The formula for calculating the adhesive force was defined as:

$$F = \pi \times R_p^2 \times P$$

Table I. *Changes in cell cycle distribution of SMMC-7721 cells after exposure to steep pulsed electric field (SPEF). SMMC-7721 cells were exposed to SPEF for 10 minutes, and then cultured for 24 hours. The harvested cells were fixed with cooled ethanol, stained with propidium iodide and analyzed cycle distribution by flow cytometry.*

Treatment	Cell cycle (%)		
	G0/G1-phase	S-phase	G2/M-phase
Control	61.21±0.44	24.47±1.47	14.33±1.08
Low-dose SPEF	71.22±0.81**	19.73±1.29*	9.04±1.72*
High-dose SPEF	72.02±1.24**	19.35±0.77*	8.96±0.72**

Data represent means±standard deviation of three separate samples per group. Compared with control, *p<0.05, **p<0.01.

where, R_p is the inner radius of the micropipette and *P* the critical negative pressure (13, 14).

Statistical analysis. Data from the experiments are presented as mean±standard deviation (SD) and were analyzed using Student's unpaired *t*-test. A value of p<0.05 was considered significant.

Results

Inhibitory effects of SPEF on viability and proliferation of SMMC-7721 cells. After exposure to SPEF, SMMC-7721 cells were reseeded and cultured for different times and the assessment of effects of SPEF on viability and proliferation of SMMC-7721 cells was performed. Figure 4 shows phase-contrast microscopic images of SPEF-treated SMMC-7721 cells at 24 hours. SPEF exposure gave rise to a significant decrease of the growth in the high-dose group, with few dead cells being observed on the second day (Figure 4C). These changes were also observed in the low-dose SPEF group, although they were less severe than in the high-dose SPEF group (Figure 4B), but were not detected in the control group (Figure 4A). The cell count revealed that the cell density was lower in cultures exposed to low- and high-dose SPEF compared with the control at day 2, 3 and 4. Moreover, high-dose SPEF exposure led to a lower cell density compared with the low-dose SPEF group (Figure 5). MTT assay demonstrated the application of both of high- and low-dose SPEF yielded notable inhibitory effects on the proliferation of SMMC-7721 cells. Higher inhibition rates were observed in the high-dose SPEF group at day 1, 2 and 3. The low-dose SPEF group, however, yielded an increased inhibition rate at day 4 (Figure 6). Furthermore, cell cycle analysis after treatment with SPEF demonstrated an obvious increase in G0/G1-phase (p<0.01) and decreases in S- and G2/M-phase cells (p<0.05) in cell cycle distribution, but the cycle distribution did not differ significantly between the high- and low-dose SPEF groups (Table I).

Table II. *Effects of steep pulsed electric field (SPEF) on viscoelasticity of SMMC-7721 cells. SMMC-7721 cells were exposed to SPEF for 10 minutes, and then cultured for 24 hours before the viscoelastic experiment. Viscoelastic coefficients were measured using micropipette aspiration technique and the data were fitted using the standard linear solid model.*

Treatment/parameter	Control	Low-dose SPEF	High-dose SPEF
K1 (N/m^2)	2395.5±1613.2	2605.3±1049.4	1099.9±692.9*
K2 (N/m^2)	2830.8±1195.7	2472.5±727.5	3865.4±1644.5*
μ ($N.s/m^2$)	219.7±135.5	309.4±150.8*	379.4±242.1*
$[1/(K1+K^2)]$ (m^2/N)	1.91×10^{-4}	1.96×10^{-4}	2.01×10^{-4}
1/K1 (m^2/N)	4.17×10^{-4}	3.84×10^{-4}	9.09×10^{-4}
$[\mu(K_1+K_2)/(K_1K_2)]$ (s)	0.1695	0.2436	0.4426
n	16	22	18

Viscoelastic characteristic values: $1/(K_1+K_2)$ is proportional to the initial cell deformation; $\mu(K_1+K_2)/K_1K_2$ determines the rate of the slow phase of creeping deformation; $1/K_1$ is proportional to the maximum deformation as time tends to infinity. n: Number of cells measured. *Compared with control, $p<0.05$.

Changes in viscoelasticity of treated SMMC-7721 cells. Results of viscoelasticity by micropipette aspiration are shown in Table II. In the low-dose SPEF group, no significant differences of elastic coefficients K_1 and K_2 were observed in comparison with control cells. However, viscous coefficient μ significantly increased after application of SPEF in SMMC-7721 cells. Analysis of the characteristic values of the cell viscoelasticity showed that low-dose SPEF led to an increase trend of the rate of slow phase of creeping deformation $[\mu(K_1+K_2)/K_1K_2]$, but did not yield pronounced differences in the initial cell deformation $[1/(K_1+K_2)]$ or the maximum deformation $(1/K_1)$.

In the high-dose SPEF group, a significant increase of elastic coefficients K_2 and viscous coefficient μ was observed. Elastic coefficients K_1, however, showed a distinct decrease after treatment with high-dose SPEF. A stronger increase of the rate of slow phase of creeping deformation and an increase of the maximum deformation $(1/K_1)$ in this group were detected. The initial cell deformation slightly increased but this difference was not significant.

Changes in the adhesive force of SMMC-7721 cells to HUVECs. After application of low-dose SPEF, the adhesive force of SMMC-7721 cells to HUVECs significantly decreased to 22.5±6.06 nN compared to untreated control cells (42.97±7.95 nN) ($p<0.05$). High-dose SPEF exposure resulted in a mildly lower adhesive force (37.06±10.30 nN), however, no significant differences could be detected in comparison with the control group (Figure 7).

Discussion

The present study is the first to determine the changes in biorheological properties of SPEF-treated human hepatoma SMMC-7721 cells and demonstrates that treatment of SMMC-7721 cells with SPEF results in changes in the cell cycle distribution, in cell viscoelasticity and attenuates adhesive forces to HUVECs. These changes lead to reduced viability and proliferation of SMMC-7721 cells *in vitro*.

Our data showed that there was a rapid decrease in cell viability and proliferation after treatment with high- and low-dose SPEF. These findings are good in agreement with the results of Nuccitelli *et al.* who found nanosecond pulsed electric fields cause effective growth and proliferation inhibition of melanomas (15). Normal cell proliferation is strictly governed by cell cycle progression. In all eukaryotic cells, passage through the cell cycle is a highly regulated process involving ordered transition of G0/G1- to S- and G2/M-phases (16). Cell cycle analysis showed that treatment with SPEF increased the proportion of cells in the G0/G1-phase and decreased the proportion of cells in the S-phase and/or G2/M-phases of the cell cycle, which indicates that SPEF blocks cell cycle progression of SMMC-7721 cells at the G1- to S-phase transition. Kuo *et al.* found VC-CH (ethanolic extract from *Vandellia cordifolia*) arrested the cell cycle progression of several tumor cell lines at the G1 transition to the S-phase (17). Our findings are in keeping with the data of their study. Our results suggest that SPEF suppresses SMMC-7721 cell proliferation by interfering with some regulatory events required for the entry of SMMC-7721 cells into the S-phase.

Viscoelasticity is one of the important biophysical properties of cells and is involved in cell motility, migration and deformation. Cell adhesive and rheological properties play a key role in cell transmigration through the endothelial barrier, in particular in the case of inflammation (leukocytes), or cancer metastasis (cancer cells) (18). We observed that while both low- and high-dose SPEF induced an increase in viscous coefficient μ, no significant changes in elastic coefficient K_1 and K_2 were observed in the low-dose SPEF group. However, high-dose SPEF induced a decrease in K_1 and an increase in K_2, which suggested that application of SPEF altered the rigidity and increased the viscosity of SMMC-7721 cells. Further analysis also found that SPEF treatment increased the rate of the slow phase of creeping deformation and the maximum deformation in SMMC-7721 cells, but significant changes in the initial deformation were not detected. These results are consistent with the data regarding the viscoelastic coefficients, and suggest that SPEF induces an alteration of viscoelasticity which lead to inhibitory effects on viability and proliferation in SMMC-7721 cells.

Cell adhesion is regulated by specific adhesion molecules that interact with molecules on the opposing cell or surface. Adhesion of tumor cells to vascular endothelial cells is one of the key steps in tumor metastasis *via* blood circulation. We found that low-dose SPEF yielded a significant decrease

in the adhesive force of SMMC-7721 cells to HUVECs. These findings indicate that low-dose SPEF may inhibit the expression of some adhesive molecules in SMMC-7721 cells. Interestingly, no significant changes of the adhesive force in high-dose SPEF were observed compared with control cells. This result suggests that high-dose SPEF may not affect expression of adhesive molecules in SMMC-7721 cells, or that it suppresses the expression of some adhesive molecules as may low-dose SPEF, but simultaneously induces an increased expression of other adhesive molecules. However, changes in expressions of adhesive molecules in SMMC-7721 cells after application of SPEF and reasons for their occurrence remain unclear and are a worthwhile topic of future study.

In summary, we have shown that treatment of human hepatoma SMMC-7721 cells with a steep pulsed electric field results in changes in rigidity and viscosity of the cell membrane and attenuates adhesive forces to endothelial cells. These changes lead to decreased viability and proliferation of the treated SMMC-7721 cells *in vitro*. Our results provide an insight into the antitumor mechanism of steep pulsed electric fields from a biorheological point of view.

Acknowledgements

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