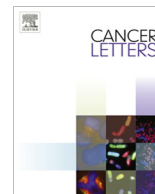




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## Nanosecond pulsed electric field (nsPEF) treatment for hepatocellular carcinoma: A novel locoregional ablation decreasing lung metastasis <sup>☆</sup>

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### ABSTRACT

Hepatocellular carcinoma (HCC) is a highly aggressive malignancy. Nanosecond pulsed electric field (nsPEF) is a new technology destroying tumor cells with a non-thermal high voltage electric field using ultra-short pulses. The study's aim was to evaluate the ablation efficacy of nsPEFs with human HCC cell lines and a highly metastatic potential HCC xenograft model on BALB/c nude mice. The *in vivo* study showed nsPEFs induced HCC cell death in a dose dependent manner. On the high metastatic hepatocellular carcinoma cell line (HCCLM3) xenograft mice model, tumor growth was inhibited significantly in nsPEF-treated groups (single dose and multi-fractionated dose). Besides a local effect, the nsPEF treatment reduced pulmonary metastases. The nsPEFs also enhanced HCC cell phagocytosis by human macrophage cell (THP1) *in vitro*. The nsPEF is efficient in controlling HCC progression and reducing its metastasis. nsPEF treatment may elicit a host immune response against tumor cells. This study suggests nsPEF therapy could be used as a potential locoregional therapy for hepatocellular carcinoma.

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### 1. Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal cancers in the world [1,2]. Radical resection is the first line of treatment but in clinical practice surgery is not often feasible because of an unresectable size or underlying liver cirrhosis. Liver transplantation is curative treatment but limited by organ shortage.

**Abbreviations:** nsPEF, nanosecond pulsed electric field; SD, single dose; MFD, multi-fractionated doses; HCC, hepatocellular carcinoma; TEM, transmission electron microscope; FCM, flowcytometry; LSCM, laser scanning confocal microscope; RFA, radiofrequency ablation; PEI, percutaneous ethanol injection; TACE, transarterial chemoembolization.

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When surgical options cannot be achieved, locoregional therapies including radiofrequency ablation (RFA), percutaneous ethanol injection (PEI) and transarterial chemoembolization, are recognized as appropriate choices and are radical treatments choices for selected patients [3–5].

Nanosecond pulsed electric field (nsPEF) is an emerging bioelectric technology that has showed its potential in cancer therapy [5,6]. nsPEF can generate pulsed high voltage electric field in ultra short nanosecond duration to produce immediate power, which could ablate targeted tumor [7–9]. nsPEF mediates cell destruction by multiple mechanisms [5]. The nanosecond pulses with a high intensity electric field changes the permeability and electric properties of the plasma membrane and intracellular organelle membrane, which eventually result in apoptosis or apoptosis-like cell death. Unlike RFA or PEI, nsPEFs does not rely on heat production or chemical ablation, thus producing a lower risk of local complications, such as thermal or chemical injuries [5,6]. Moreover, nsPEFs can obliterate tumor capillaries with no concurrent impact on larger vessels with thick walls in the targeted area [10]. So nsPEFs can be safely delivered near a large bile duct or vascular vessel [5]. In previous studies, nsPEF has been tested in various malignant

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tumors, including melanoma [10–13], pancreatic cancer [14], basal cell carcinoma [15], hepatocellular carcinoma [16], cutaneous papilloma and squamous cell carcinoma [17].

For nsPEF application in HCC, the following questions need further investigation for the future clinical use. First, the parameters of nsPEF need to be optimized because the treatment effects are highly dependent on duration, intensity and distribution of electric field, dielectric constant and conductivity [4,5,9]. Second, a considerable proportion of HCC cases are in the late stage. These tumors need locoregional treatment to downstage tumor for future surgical resection. Therefore, the ablation strategy, including electrode design, as well as spatial and temporal dose distribution, may be critical for nsPEF treatment of HCC.

This study investigated the use of nsPEF on a human HCC cell lines and a high pulmonary metastatic potential HCC xenograft model using on BALB/c nude mice. We demonstrated nsPEF treatment, in both single and multiple fractionated dosage modes, inhibited HCC tumors and metastases. nsPEF can also enhanced phagocytosis of HCC cells by mononuclear cell *in vitro*, which suggests the involvement of the host's immune system in nsPEF cancer treatment.

## 2. Materials and methods

### 2.1. Cells and animals

Human hepatocellular carcinoma cell line SMMC7721 and macrophage cell line THP1 were purchased from the Chinese Academy of Science. High metastatic HCC cell line HCCLM3 was purchased from the Liver Cancer Institute, Zhongshan Hospital, Fudan University. HCCLM3 and SMMC7721 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, penicillin (100 units/mL), and streptomycin (0.1 mg/mL). THP1 cells alone and the co-culture of THP1 with SMMC7721 cells in 1640medium with 10% FBS. All cells were incubated at 37 °C in a humidified incubator under an atmosphere of 5% CO<sub>2</sub>/95% air.

BALB/c nude mice were purchased from Shanghai Experimental Animal Centre, Chinese Academy of Science. All studies on mice were conducted in accordance with the Guide for the Care and Use of Laboratory Animal of Zhejiang University.

PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling (PKH67GL-1KT) and PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (PKH26GL-1KT) were purchased from Sigma, U.S.A.

### 2.2. Pulse generator and nsPEF parameters

A pulser with Blumlein line configuration generated 100 ns pulses. Pulses were applied at 40 kV/cm with a rate of 0.5 Hz. The application electrodes, pulse generator, voltage and pulsing pattern of the nsPEF were described previously [18]. The energy is written as formula is:

$$W = \tau \frac{V^2}{R} N$$

where  $W$  is the energy (J),  $\tau$  is the pulse duration (ns),  $V$  is the voltage across the electrodes (V),  $R$  is the tissue resistance ( $\Omega$ ), and  $N$  is the pulse number. The dose effect was studied by varying the pulse numbers while keep other parameters constant.

### 2.3. Electrode design for tumor ablation experiments

The nsPEF were delivered to subcutaneous tumors with a pair of electrodes where the anode was placed within the tumor mass and the cathode was placed on the tumor periphery (Fig. 1). The anode was a needle and the cathode was a semi-ring. A semicircular-shaped of electric filed was formed between the two electrodes. One half of the tumor was treated with the initial semi-circular ring placement and then the ring was rotated 180° to complete the tumor treatment. Thus the semi-circular ring touched the tumor securely avoiding electrical breakdown.

### 2.4. Tumor implantation and tumor volume measurement

A pulmonary metastatic human HCC animal model was established by implanting tumor tissue subcutaneously into nude mice as previously described [19,20]. Briefly, suspended HCCLM3 cells were injected into the nude mouse to form a primary tumor. Once formed the primary tumors was excised and cut into cubic blocks about 1 mm<sup>3</sup> to be transplanted subcutaneously into 3-week old nude mice. Four weeks after implantation, tumors were visible at 5 mm in diameter. Mice were anesthetized on a warming bed during each procedure by inhalation anesthesia using 1.4% isoflurane mixed in 100% O<sub>2</sub>. Before and after the treatment, tumors

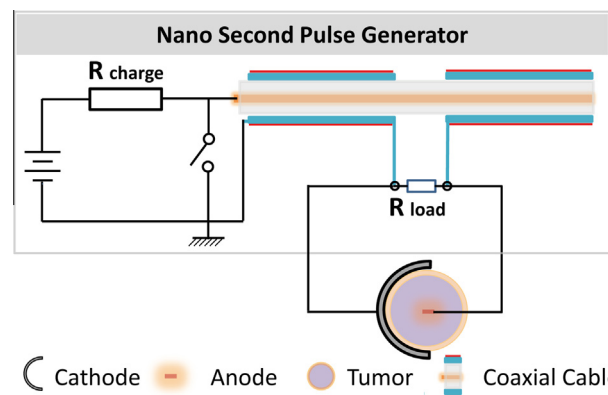


Fig. 1. Schematic diagram of experimental setup for nsPEF on HCC tumors implanted on nude mice.

were measured and imaged by photography. Tumor volumes were calculated using the following formula:  $V = 0.52 \times D_1 \times D_1 \times D_2$  ( $D_1$  and  $D_2$  are short and long tumor diameter respectively). The relative tumor volume was calculated as (tumor volume post-treatment/tumor volume pre-treatment)  $\times$  100%.

### 2.5. Experimental group and nsPEF treatment

On day 21 after birth BALB/c nude mice were implanted with HCCLM3 tumor blocks. Twenty seven mice were randomly divided into four groups to deliver the first treatment on day 49: 7 mice in control group, in which mice were anesthetized and the electrode was placed on the tumor, but no nsPEF was applied; 6 mice in the surgical resection group, in which the whole subcutaneous tumors were resected; 7 mice in the single dose nsPEF group (nsPEF-SD), in which tumors were treated with a nsPEF of 40 kV/cm amplitude, 100 ns ns duration for 300 pulses in a single treatment; 7 mice in the multiple fractionated dose group (nsPEF-MFD), in which tumors were treated with nsPEFs of 40 kV/cm amplitude, 100 ns duration for 100 pulses over three-dose regimen at 48 h intervals. Mice in this group had the second and the third treatment while the other two groups had only anesthesia. The tumor volume was measured in every 3 days. If tumor volume exceeded 2 cm<sup>3</sup>, the mouse was euthanized.

### 2.6. Histology and immunohistochemistry of tissue section

The subcutaneous tumor, lung, bowel, liver, brain, heart, spleen and kidney were excised, fixed in 4% formaldehyde dehydrated, cleared and then embedded in paraffin blocks to cut into 4  $\mu$ m sections for H&E staining and immunohistochemistry. The expression of CEA, PCNA, CK19 and CK18 was detected by an immunohistochemical assay. Briefly, the endogenous peroxidase activity of sections was inactivated with H<sub>2</sub>O<sub>2</sub>. The sections were first incubated with the rabbit anti-human primary antibodies against CEA (Sigma 1:100), PCNA (Abcam 1:1000), CK18 (Abcam 1:100) and CK19 (Abcam 1:250), and then the secondary antibodies of biotinylated goat anti-rabbit IgG (Boster, China) and avidin-biotin-peroxidase complex (Boster, China). After staining with diaminobenzidine (DAB), sections were observed under a light microscope. The results were evaluated by two independent pathologists.

### 2.7. Cell survival analysis after nsPEFs

Cells were harvested and  $1 \times 10^6$  cells were placed in 0.2 cm gap cuvettes (Bio-smith, Biorad) and exposed to nsPEF with 100 ns duration and 40 kV/cm amplitude. After 24 h, these cells were counted with a CCK8 Cell Counting Kit (Merck-Millipore) and the surviving number counted with Biotekmicroplate reader at OD450. The relative cell survival was calculated as (OD450 treated cell/OD450 control cell)  $\times$  100%.

### 2.8. Phagocytosis with the confocal fluorescent microscopy

An *in vitro* co-culture system was developed to investigate the phagocytosis. THP-1 cells ( $0.2 \times 10^5$  cells/well) were differentiated into macrophages in 24-well plate containing 1 mL medium/well with phorbol ester (80 ng, PMA, Sigma USA) over 72 h. After incubation for 72 h, the cells were washed twice with the medium and incubated with a human HCC cell line SMMC7721 in a 1:1 ratio with total number of  $1 \times 10^6$  cells. SMMC7721 were labeled with a red fluorescent dye (PKH26) and THP1 with a green fluorescent dye (PKH67).

### 2.9. Transmission electron microscopy

HCCLM3 cells or co-cultured SMMC7721 cells and THP1 cells were processed according to the standard technical procedure for transmission electron microscopy. Cell microstructure was observed on a JEM-1200EX electron microscope.

### 2.10. Statistical analysis

Statistical analysis was performed with SPSS 15.0 for windows (SPSS, Chicago, IL, USA). Quantitative variables were expressed as means  $\pm$  SD and analyzed by ANOVA. Survival difference was evaluated by the log-rank test and the Kaplan–Meier method. Results were considered statistically significant at  $P < 0.05$ .

## 3. Results

### 3.1. Nanosecond PEF ablation effectively induced HCC cell death in a dose dependent manner in vitro

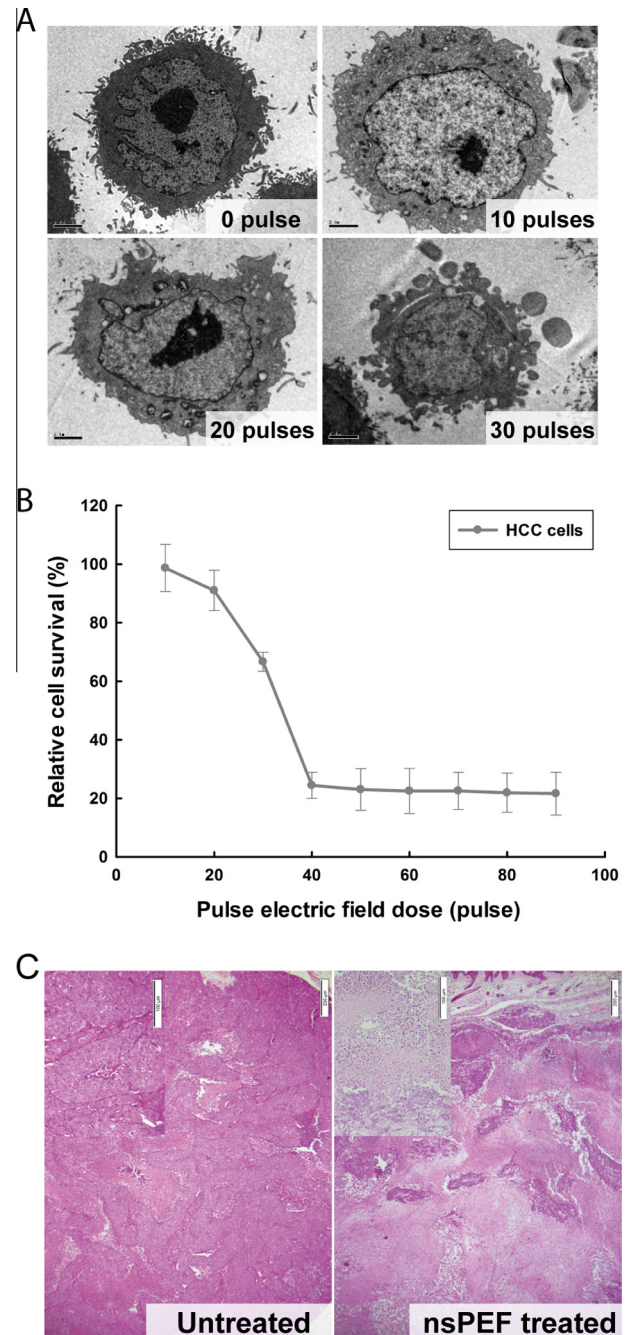
We first examined the effect of nsPEFs at varied dose on HCCLM3 HCC cells. The nsPEFs were supplied by a nanosecond pulse generator and delivered to HCC cells in cuvettes, as previously described by Wang et al. [18]. The morphological changes of treated HCC cells were observed by TEM. When HCCLM3 cells were pulsed with 30 pulses of nsPEFs, they showed apoptosis-like characteristics including nuclear condensation, oversized cytoplasmic particles with integral cellular membranes and organelles (Fig. 2A). Twenty-four hours after treatment, the cell survival was determined by CCK-8 assay and nsPEFs produced dramatically decreasing *in vitro* cell survival when the pulse number exceeded 20 (Fig. 2B). When the pulse number exceeded 100 *in vivo* (Fig. 1), the histological analysis showed cellular destruction throughout the tumor (Fig. 2C).

### 3.2. NsPEFs suppressed tumor growth of subcutaneous implanted human HCC in the mouse model

The effect of nsPEFs on murine hepatoma has been reported [16]. Here we have developed an nsPEF ablation model for human HCC xenograft in nude mice. HCCLM3 tumor blocks were implanted in mice. An nsPEF treatment was started when the tumor had an average volume of 400 mm<sup>3</sup> four weeks post implantation. Two experimental pulsing parameters were used: (1) a single dose ablation (nsPEF-SD) group of HCC tumors were treated with 300 pulses of the nsPEF one time; (2) a multi-fractionated dose (nsPEF-MFD) group of HCC tumors were treated with 100 pulses of the nsPEF for 3 times at of 48 h intervals. Control group mice were anesthetized but without nsPEF treatment. After treatment, the tumor volumes were measured. If the tumor volume exceeded 2 cm<sup>3</sup>, that mice was euthanized. As shown in Fig. 3A, nsPEF-treated tumors regressed. On post treatment days 7 and 14, tumor growth in nsPEF-SD and nsPEF-MFD groups was significantly inhibited compared to the control group ( $P < 0.01$ , Fig. 3B). There were no complications caused by nsPEF treatment and no significant mouse weight loss post-nsPEF treatment.

The survival of the mice was followed the 11th week. The end-point was defined as tumor volume exceeding 2 cm<sup>3</sup>. Kaplan–Meier survival curve is shown in Fig. 3C. The mean post-treatment survival was 23.1 days (range, 15–33 days) in the control group, 38.1 days (range, 21–52 days) in the nsPEF-SD group and 49.7 days (range, 36–52 days) in the nsPEF-MFD group. The survival time of both nsPEF treatment groups was significantly longer than that of the control group ( $P < 0.05$ ).

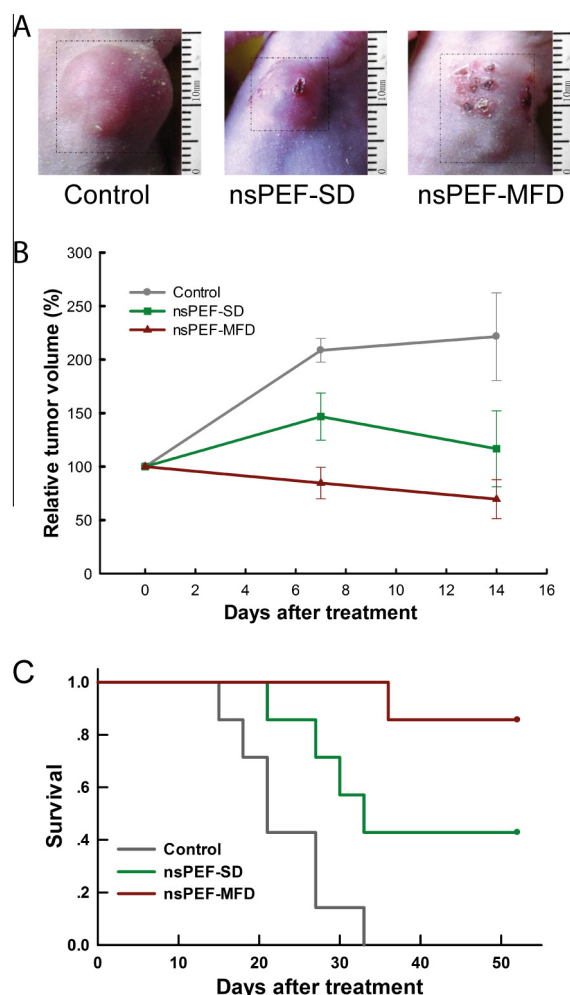
This experiment is capable of inhibiting tumor growth, reducing tumor burden and prolonging animal survival. No severe adverse effect was found in the nsPEF-treated groups.



**Fig. 2.** nsPEF effectively induced HCC cell death *in vivo* and *in vitro*. (A) Morphological changes of HCC-LM3 cells treated with 0 pulse, 10 pulses, 20 pulses and 30 pulses of 40 kV/cm amplitude 100 ns duration PEF. (B) Relative cell survival was evaluated by CCK-8 at 24 h after 10, 20, 30, 40, 50, 60, 70, 80 and 90 pulses of 40 kV/cm 100 ns nsPEF treatment. (C) Histological analysis of tumors formed by HCC-LM3 cells on nude mice with or without 100 pulses of 40 kV/cm 100 ns PEF. A large area of cell death is seen in the treated tumor compared to the untreated tumor. Magnification: 40 $\times$  for original image and 100 $\times$  for views in left upper corners.

### 3.3. nsPEF treatment reduced pulmonary metastasis of HCC

To investigate whether nsPEF cause tumor seeding, we further examined lung metastases from HCCLM3 xenograft in nude mice. HCCLM3 is a human hepatocellular carcinoma cell line with high metastatic potential, which was established from a human HCC metastatic tumor. HCCLM3 produces lung metastases when implanted subcutaneously in BALB/c nude mice [19,20]. In our examination, metastatic nodules were found in the control group, the nsPEF-SD group and resection group as shown in Fig. 4A. The



**Fig. 3.** nsPEFs suppressed tumor growth of subcutaneously implanted human HCC on mice model. Photographs of HCC xenograft tumors in control, nsPEF-SD and nsPEF-MFD group on day 14 after treatment. Tumors treated by single dose of nsPEF and multiple fractionated dose of nsPEF decreased in volume, while tumors in the control group continued growing. (B) Relative volume changes of HCC xenograft tumors in control, nsPEF-SD and nsPEF-MFD group. Both nsPEF treated groups showed decrease in relative tumor volume compared to control ( $p < 0.05$ ). (C) Kaplan–Meier survival curve for mice in control, nsPEF-SD and nsPEF-MFD group. The mean post-treatment survival was 23.1 days (range, 15–33 days) for the control group, 38.1 days (range, 21–52 days) for the nsPEF-SD group and 49.7 days (range, 36–52 days) for the nsPEF-MFD group. nsPEF-SD and nsPEF-MFD group showed prolonged survival as compared with the control group ( $p < 0.05$ ).

immunohistochemical analysis showed that pulmonary nodules were expressing CK18 [21], CK19 [22,23], CEA [24] and PCNA [25], which is consistent with the expression pattern of the HCCLM3 cell line (Fig. 4B).

CK19 (keratin 19) is generally considered the marker of intrahepatic bile duct cells or hepatic progenitor cell. CK19 in HCC is usually associated with recurrence, metastasis and poor prognosis [22,23]. CK 19 is also a potential marker of circulating tumor cells. CK18 is the marker of liver parenchymal cells and hepatoma cells express CK18 [21]. Proliferating cell nuclear antigen (PCNA) is a key factor for DNA replication and cell cycle regulation. PCNA is usually expressed in the cells with fast growth and proliferation [25]. Carcinoembryonic antigen (CEA) is expressed in related malignant cells such as metastatic colorectal cancer related [24]. The immunohistochemical results show that implanted subcutaneous tumor expressed CK18, CK19, CEA and PCNA, the same markers which HCCLM3 cells express before inoculation (Fig. 4). The strong positive expression of CK19 in lung metastatic nodules

verified the origin of the lung metastases to be from the subcutaneously-implanted HCCLM3 cells.

The number of animal with lung metastases was 7 of 7 animals in the control group, 2 of 7 in the nsPEF-SD group, 0 of 7 in the nsPEF-MFD group and 1 of 6 in the resected surgery group, respectively (Fig. 4C). Mice with a single dose of nsPEF ablation showed reduction in the lung metastases rate compared to the control group, and the nsPEF-MFD treatment achieved a metastatic rate as low as resection group. It showed nsPEF decrease lung metastasis from the primary tumor.

### 3.4. nsPEF enhanced phagocytosis of HCC cells by macrophage cell *in vitro*

Locoregional therapy like radiofrequency ablation can elicit specific or non-specific immune response against tumor by various mechanisms. By histological analysis of the HCC xenograft tumor treated by nsPEFs, we found more anti-phagocyte positive cells present in tumor capsule, which suggested a possible role of host defense against tumor cells (Fig. 5A and B).

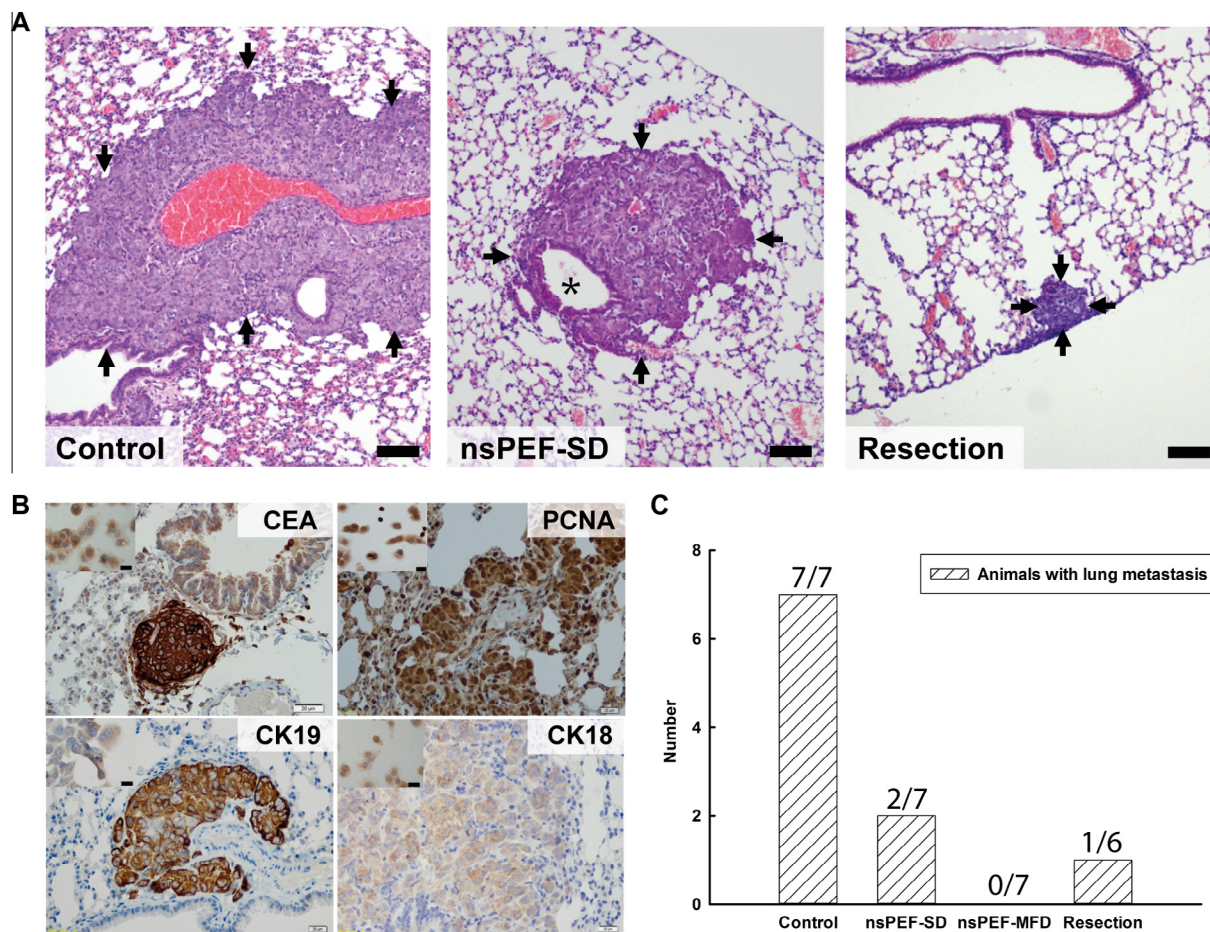
We designed an experiment to observe whether nsPEF treatment increases HCC cells phagocytosis by immune cells *in vitro*. The HCC cell line SMMC7721 was used in this experiment since it is optimal for a phagocytosis study and flow cytometry analysis. As shown in Fig. 2B, 40 kV/cm amplitude of 100 ns duration PEFs with less than 20 pulses was not lethal to HCC cells *in vitro*. When SMMC7721 cells were treated with 18 pulses and then co-cultured with the macrophage cell line THP1 cell line [31]. Phagocytosis was seen with transmission electron microscope within 4 h (Fig. 5C). In the control group the phagocytized HCC cells were found only after 6 h post-treatment. To confirm the result, we performed a cell tracking assay by labeling HCC cells (SMMC7721) with red fluorescent dye PKH26 and THP1 cells with green fluorescent dye PKH67 [32,33].

After 4 h following 18 pulses of 40 kV/cm at 100 ns, SMMC7721 [34] cells with red fluorescence were engulfed by THP1 cell with green fluorescence (Fig. 5D). In flow cytometry, the G region (marked in plots) represented cells with dual fluorescence. A red stained SMMC7721 cell phagocytized by green stained THP1 cell. When co-cultured for 4 h, a significant greater proportion of dual labeled cells in the G region were detected in the nsPEF-treated group ( $23.00 \pm 7.35\%$ ) than the control group ( $6.04 \pm 0.73\%$ ) (Fig. 5E and F). Thus nsPEF treatment increased the phagocytosis of SMMC7721 HCC cells by THP1 macrophage cells.

## 4. Discussion

The management of patients with HCC usually requires a multidisciplinary team to make a treatment plan according to the patient's tumor characteristics and liver function. Surgical resection and liver transplantation have been considered as curative therapy [1,2], but surgical treatments are limited by poor liver function or organ availability. Locoregional therapies, including direct tumor ablation techniques and transcatheter chemoembolization, play an important role in the non-surgical management of HCC. The RFA therapy, one of the local ablation treatment, has been recognized as comparably effective as surgical resection for small sized HCC [3,4].

nsPEF ablates tumor cells by non-thermal and non-chemical effects. Different from conventional ablation techniques, nsPEF does not result in direct necrosis in targeted tissue but induces cell death in an apoptosis or apoptosis-like pathway [5–8,26]. Ultra-short pulsed high voltage electric field affects plasma membranes and membranes of intracellular organelles, which disrupt cellular physiological homeostasis and eventually causes



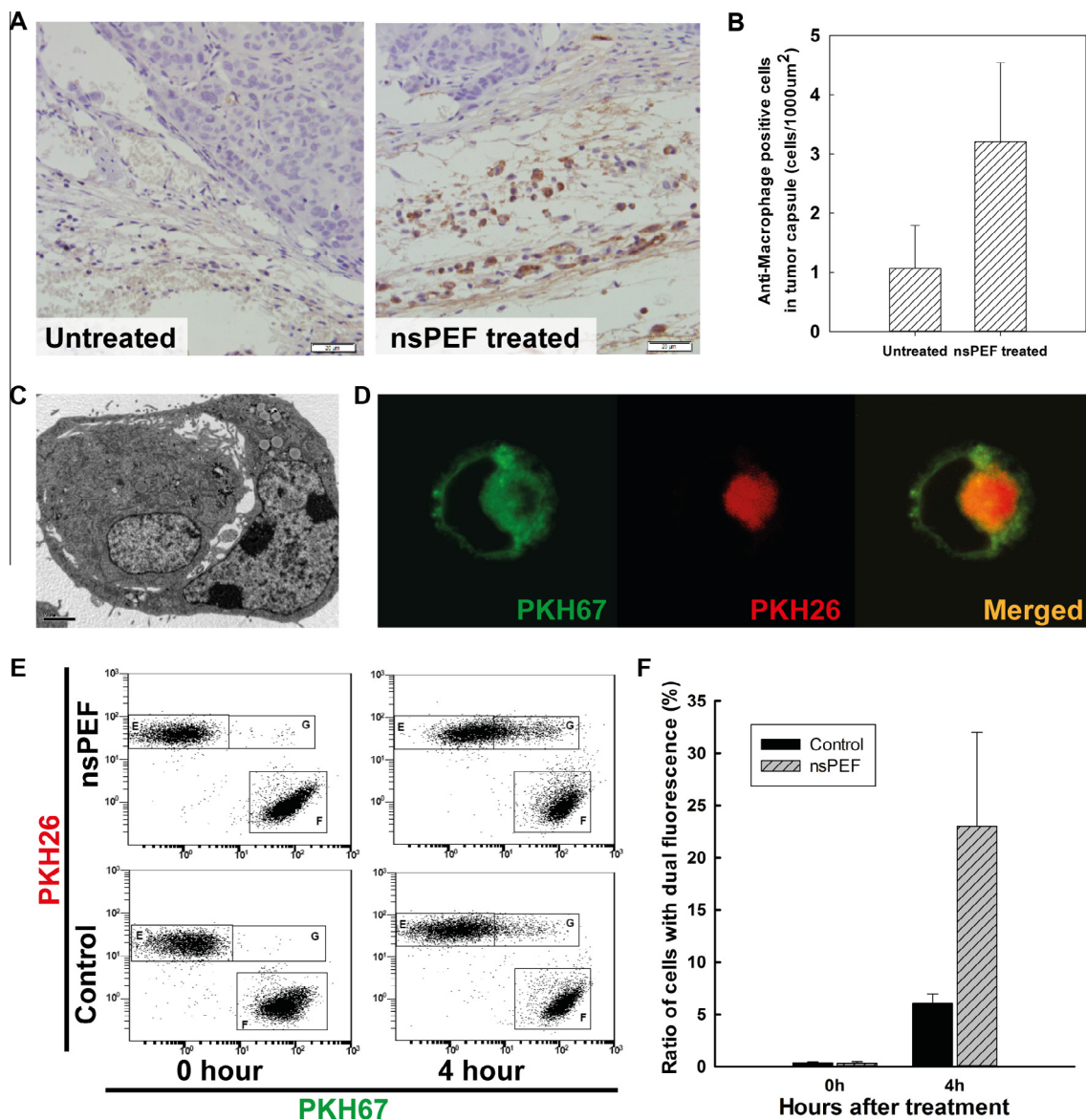
**Fig. 4.** Lung metastases examined in HCCLM3 implanted nude mice. (A) Metastatic nodule found in the lung of a control mouse, nsPEF-SD mouse and a resectioned mouse, while no metastases were found in nsPEF-MFD mice. (B) Immunohistochemical staining of the HCCLM3 cell line cells and representative pulmonary metastases in mouse model (HCCLM3 cells immunostaining was shown in the upper left corner of the images of tumor tissues). The staining pattern of CEA, PCNA, CK18 and CK19 in metastatic nodules was in consistent with cells of the HCCLM3 cell line cells. (C) Number of nude mice with pulmonary metastases in control, nsPEF-SD, nsPEF-MFD and resection group. Compared with control group, nsPEF-SD group and nsPEF-MFD group had significantly reduced lung metastases.

cell death. As demonstrated by TEM in this study, HCC cells treated by a lethal nsPEF dose *in vitro* did not show necrotic morphological changes. In consistence, HCC tumor ablated by nsPEFs underwent gradual shrinkage with minimal bleeding or necrosis. The target area of nsPEFs can be strictly confined by a precisely designed electrode. The vessels with different conductivity such as bile ducts, thick blood vessels or the urethra in the treated area can be preserved [5]. When the nsPEF duration is shorter and the energy carried by single pulse is lower, the energy release is more controllable. These features facilitate nsPEF treatment in HCC: oversized tumors can be safely ablated by multiple doses of nsPEF. The tumors near major vascular vessels which are hard to be ablated by conventional heat producing techniques can be ablated by nsPEFs. In this study, we compared the efficacy of nsPEFs in single dose and multiple fractionated doses on subcutaneous implanted HCC tumors. The result showed multiple fractionated dose nsPEFs inhibited tumors more effectively than that with a single treatment. Therefore, nsPEFs can be used multiple times as a downstage treatment, especially for large tumors that are unresponsive to a single nsPEF dose.

Beside the local ablation effect, nsPEF's usefulness for metastases was also studied. A highly metastatic HCC xenograft model was set up. This model showed that the multi-fractionated dose group

had no pulmonary metastasis (0 for 7) which was even better than the rate of metastasis in the surgical resection group (1 of 6). The risk of metastasis can be minimized by the efficient ablation of the primary tumor.

Local treatment of a tumor can induce an immune response [35–39]; radiotherapy can induce a tumor-specific CD8<sup>+</sup> T cell response [39–41] and then cause a by-stander effect. In our study, we found the non-lethal nsPEFs greatly increases the ratio of HCC cells engulfed by the macrophage cell THP1. Thus nsPEF stimulate macrophages to engulf tumor cells. The multiple dose strategy of nsPEFs extended the treatment period and it prolonged the duration to induce a host defense against the tumor cells. The mechanism of this phenomenon needs further investigation. Electric field induced phosphatidyl serine (PS) externalization on cell membrane [26], which had been reported to be a vital signal to immune cells [27], such as macrophages [28], neutrophils [29] and dendritic cells [30] and this PS flip could signal the phagocytic clearance of target cells [27–30]. PS externalization was recognized as an early event that occurs in cells undergoing apoptosis. The low dose nsPEFs merely changed cell's membrane permeability and rearranged PS distribution without initiating an apoptosis cascade. Therefore, the immune system may identify nsPEF-treated cells by the externalized PS on the cell membrane. The recognition and phagocytosis of tumor cells



**Fig. 5.** nsPEF treatment enhanced phagocytosis of HCC cells by mononuclear cells *in vitro*. (A) An IHC analysis found increased anti-macrophage staining positive cells in the capsule of tumor treated by nsPEFs. (B) Quantization of anti-macrophage positive staining cells in the tumor capsule of control and nsPEF treatment groups at day 1 after nsPEF treatment. (C) Transmission electron microscopy showed phagocytosis of a nsPEF-treated SMMC7721 cell by a THP1 cell after 4 h of co-culture. (D) Laser confocal microscopy showed an nsPEF-treated SMMC7721 cells labeled by red PKH26 was phagocytized by THP1 cells labeled by green PKH 67 after co-culture. (E) Flow cytometry analysis of co-cultured SMMC7721 and THP1 cells with or without nsPEF treatment. G region represents dual fluorescent cells with both PKH 26 and PKH 67 labeling, which indicated phagocytosis of SMMC-7721 HCC cell by THP1 cell. After nsPEF treatment, dual fluorescent cells undergoing phagocytosis increased compared with control. (F) Quantitation of dual fluorescent cells in flow cytometry analysis of control and nsPEF-treated SMMC-7721 cells co-cultured with THP1 cells.

by immune cells play a pivotal role in breaking the balance of immunological reaction and tolerance within tumor [42–44]. The nsPEF-stimulated engulfment of tumor cells may or may not be the trigger for a tumor-antigen specific immune responses [45] or tumor-specific CTL [46]. However our study suggests that nsPEFs induce tumor eradication by an immune response with an unlethal dosage.

In conclusion, our study showed the nsPEF treatment is an efficient means to control HCC growth in an animal model. The multiple fractionated dose of nsPEFs efficiently inhibited tumors without increasing the risk of secondary metastasis. Beyond a local ablation effect, the nsPEF also elicit tumor cell removal by a host defense. Therefore, nsPEF can be used as a locoregional therapy for hepatocellular carcinoma.

## Conflict of Interest

The authors declare that there are no conflict of interest.

## Acknowledgements

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.canlet.2014.01.009>.

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