Nanosecond pulsed electric fields as a novel drug free therapy for breast cancer: An in vivo study

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ABSTRACT

Nanosecond pulsed electric fields (nsPEFs) is a novel non-thermal approach to induce cell apoptosis. NsPEFs has been proven effective in treating several murine tumors, but few studies focus on its efficacy in treating human tumors. To determine if nsPEFs is equally effective in treatment of human breast cancer, 30 human breast cancer tumors across 30 Balb/c (nu/nu) mice were exposed to 720 pulses of 100 ns duration, at 4 pulses per second and 30 kV/cm. Two weeks after treatment, the growth of treated tumors was inhibited by 79%. Morphological changes of tumors were observed via a 3.0T clinical magnetic resonance imaging (MRI) system with a self-made surface coil. Pulsed tumors exhibited apoptosis evaluated by TUNEL staining, inhibition in Bcl-2 expression and decreased blood vessel density. Notably, CD34, vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) expression in treated tumors were strongly suppressed. To evaluate the might-be adverse effects of nsPEFs in healthy tissues, normal skin was treated exactly the same way as tumors, and pulsed skin showed no permanent damages. The results suggest nsPEFs is able to inhibit human breast cancer development and suppress tumor blood vessel growth, indicating nsPEFs may serve as a novel therapy for breast cancer in the future.

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1. Introduction

Breast cancer is one of the most commonly diagnosed cancers among women, accounting for nearly 30% of all newly diagnosed cancer cases in women. Among women between 20 to 59 years old, breast cancer ranks first as a cause of death out of all cancers [1].

For localized breast cancer, mastectomy was the mainstay treatment in several decades [2]. When the concept of breast-conserving therapy was put forward, doctors and scientists started to pursue less invasive methods. Breast cancer patients are now encouraged to undergo a multi-modality treatment consisting of surgery, radiotherapy and chemotherapy, minimizing the side effects and esthetic damages [3,4].

Also from the point of view of breast conservation, several physical approaches have been developed. Ultrasound energy can elevate the temperature of the focal spot to 55–90 °C, inducing cellular death in tumor and normal tissues at the spot [5]. Electric fields of radiofrequency and microwave can heat the tumors as well, killing tumors by hyperthermia [6]. However, such hyperthermia might have some dissatisfaction, as radiofrequency ablation of tumor may induce abscess and damages in healthy vessels even arteries adjacent to tumors [7].

Actually, there are several non-thermal therapies applied in clinics or being tested in laboratory. Many of them are based on electroporation, via electric pulses of approximate 100 μs duration, which introduces impermeable macromolecules into cells. Electrochemotherapy uses such electric pulses to increase drug delivery [8]. Electro-gene therapy also employs electric pulses to transfer toxin genes into tumors, inhibiting tumor growth [9].

Apart from electroporation, nanosecond pulsed electric fields (nsPEFs) as an alternative drug-free and non-thermal technology is emerging as a novel treatment for cancer [10]. Generally, nsPEFs can be regarded as an extension of conventional electroporation, with pulse duration of only 10 ns to several hundred ns, and electric field intensity of 10 kV/cm to several hundred kV/cm [11]. Because of the ultra-short duration of pulses, nsPEFs can induce a series of unique biological effects such as apoptosis [11–14], calcium fluctuation and phosphatidylserine translocation [15–17], but has little hyperthermal effects. NsPEFs induced cell apoptosis has been observed in various types of cells, including pancreatic cancer, melanoma and Hela cells [11–14]. Besides, nsPEFs has been proven effective to eliminate murine melanoma and murine basal cell carcinoma in vivo [18–21].

In this study, we induced tumors of human breast cancer in Balb/c (nu/nu-) mice, and treated both tumors and normal skins
with pulses of 100 ns at 30 kV/cm, attempting to demonstrate the efficacy and to evaluate the might-be side effects of nsPEFs in human breast cancer treatment.

2. Material and methods

2.1. Cell line

Human breast cancer cell line MCF-7 was a gift from Prof. Jianzhong Xi from Dept. of Biomedical Engineering, Peking University. Cells were cultured in Dulbecco’s Modified Eagle’s Medium (ATCC Cat. No. 30-2002), supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin. The temperature of the incubator was maintained at 37 °C with 5% CO2 in air. Cells were passaged with 0.25% trypsin when reaching 70–80% confluency. All cells used for inoculation were between passages 5 and 15.

2.2. Animal model

Four- to five-week-old female Balb/c (nu/nu) mice were purchased and kept in Peking University Laboratory Animal Centre. Mice were housed for 1 week before inoculation. 100 µL cell suspension of 1 × 10⁶/mL MCF-7 cells was injected subcutaneously on the back of the mouse. Each mouse was induced only one tumor. Tumors were then treated when grew to a diameter of 4–5 mm, 12 mice served as control and treated with sham pulse (with electrode clipped on the tumor but no pulse was applied), while 30 mice were treated with nsPEFs. All mice were anaesthetized with isoflurane inhalation during the treatment.

2.3. nsPEFs application

The self-made nsPEFs generator was established on the basis of transmission line circuit. A clamp electrode was designed for applying nsPEF therapy on tumors (Fig. 1A). The main body of the clamp electrode was made of insulating material. Two copper stripes were pasted oppositely on each inside of the clamp with 10 mm × 3 mm, connected by wires with the nsPEFs generator. A 30 kV/cm electric field generated between two pads of the clamp. The opening angle could reach 90°. To avoid soft tissue over squeezed, which may lead to air breakdown, a piece of dressing was placed between the clamps to keep the distance fixed. This ensured clamped tissues being restricted totally in the electric field. The operating voltage was maintained 12–15 kV and operating current kept 100 mA. The pulse duration was 100 ns, and each tumor was pulsed for 1 min at 4 pulses per second per day on 3 consecutive days.

2.4. Tumor volume and weight measure

Each tumor was measured in vivo with caliper every other day in short axis and long axis, as the tumor was considered as an ellipse. Tumor weight was measured on day 1, day 7 and day 14 when 10 mice were randomly chosen and their tumors were removed and fixed with 4% paraformaldehyde.

2.5. Histological assay

After tumors or pulsed skin were removed, they were fixed in 4% paraformaldehyde and put on shaking table overnight at room temperature. The fixed tissues were then embedded with paraffin and cut into 4-µm sections. All samples were stained with hematoxylin and eosin. 20 fields of each sample without overlap were selected randomly at 40× magnification under microscope, in which 100 nuclei were randomly chosen and immunohistochemical positive cells were counted. Quantitative analysis showed the mean number of immunohistochemcial positive cells in 100 cells and indicated with SD.

2.6. Tumor blood vessel analysis

Blood vessel number was counted according to H&E staining. A field of 0.44 mm² was randomly chosen under microscope and blood vessel number was counted in the area. Red stained lumen with endothelial cells or clusters separated from other tumors cells or elements were taken into consideration as a blood vessel. 20 such areas were randomly chosen on one slide and 3 slides are chosen on each tumor. The mean value of the 60 fields was considered the blood vessel number [20].

2.7. TUNEL staining for cell apoptosis

TUNEL staining was performed with TUNEL Apoptosis Assay Kit (Beyotime Institute of Biotechnology, China). Fixed tumors samples were cut into 4-µm sections, then deparaffinized and rehydrated. The sections were treated with 20 µg/mL proteinase K for 20 min at 37 °C. According to the instruction, sections were treated with biotin-dUTP and TdT enzyme for 60 min at 37 °C. Slides were sealed with glycerol and observed under Olympus fluorescent microscope.

2.8. Immunohistochemistry

After deparaffinization, antigen was retrieved by microwaving for 10 min in citrate buffer. The primary antibody, Bcl-2 (anti-human, BioWorld) diluted as 1:500, VEGF (anti-human, BioVision) as 1:1000, CD34 (anti-human, Abcam) as 1:100, and VEGFR (anti-human, Abcam) as 1:1000, were added to the sample and incubated at 4 °C overnight. The secondary antibody was added then and incubated for 1 h at room temperature. 3,3-diaminobenzidine (DAB) was added as chromogen. 20 fields of each sample without overlap were selected randomly at 40× magnification under microscope, in which 100 cells were randomly chosen and immunohistochemcal positive cells were counted. Quantitative analysis showed the mean number of immunohistochemical positive cells in 100 cells and indicated with SD.

2.9. MRI evaluation

All mice with treated or untreated tumors were scanned in a GE Sigma 3.0T MRI scanner under anesthesia with 2% isoflurane on day 1, day 7 and day 14. Mice were immobilized on a specialized surface coil. To gain a better signal-to-noise ratio (SNR), manual tuning and matching of the coil’s resonance circuit was performed for each mouse individually. Each mouse was selected for a whole-body examination employed T2 weighted (T2WI) MRI. Parameters of T2WI are: Fast Spin Echo, TR/TE = 3000/102.6 ms, NEX = 4, Slice Thickness = 4.0 mm, FOV = 18. The scan time was 3 min 18 s.

2.10. Statistical analysis

Statistical analysis was performed with Origin Professional 8.0 software, and significance was considered when p < 0.05.

3. Results

We injected 10⁶/mL human breast cancer MCF-7 cells into one mouse and generated 1 breast cancer tumor on its back. 12 mice
served as control and treated with sham pulse, while 30 mice were treated with 1-minute’s pulse of 30 kV/cm, at the frequency of 4 Hz per day on 3 consecutive days (roughly 720 pulses in total). DayN was set as the Nth day after nsPEFs treatment, and day0 was the exact day when nsPEFs treatment was just finished. All treated tumors showed a retarded growth and suppression in tumor blood vessel development.

3.1. NsPEFs treatment inhibited tumor growth

We tracked the volume and weight of all tumors for 2 weeks after nsPEFs treatment (Fig. 1B and C). All tumors were similar in size before nsPEFs exposure. Pulsed tumors almost stopped growing in the first week, but it began to grow slowly after 10 days. Compared to control tumors, however, growth of pulsed tumors was significantly inhibited even though it did not show a complete remission. On day14, the mean volume of pulsed tumors was 75% less than control tumors (8.37 cm³ ± 0.75 cm³ in control and 2.30 cm³ ± 0.75 cm³ in pulsed tumors). Tumor weight changed consistently. On day1, pulsed tumors were only slightly smaller than control tumors (0.05 g ± 0.01 g in control and 0.049 g ± 0.008 g in pulsed tumors), while on day7, pulsed tumors were remarkably smaller (0.30 g ± 0.08 g in control and 0.11 g ± 0.03 g in pulsed tumors). And on day14, pulsed tumors weighed 79% less than control tumors (0.64 g ± 0.08 g in control and 0.13 g ± 0.07 g in pulsed tumors).

3.2. MRI observation

As a commonly used strategy for cancer screening in clinics, MRI can provide pathological changes of soft tissue with high resolution non-invasively. From the obtained T2 weighted MRI images, shown in Fig. 2A and B, a remarkably delayed growth in pulsed tumors was observed. Moreover, the boundaries of the tumors were clear and no sign of metastasis was observed.

3.3. NsPEFs treatment induced apoptosis in tumor cells

1 h, 3 h and 6 h after nsPEFs treatment, tumors were harvested and dyed with H&E (Fig. 3). Untreated tumor cells showed a well dispersed cytoplasm, and cells were arranged closely. Pulsed tumor cells show a condensed chromatin and reduction of the cytoplasm, which were typical morphological indicators of apoptosis. Apoptosis became more significant till 6 h after nsPEFs exposure, with cell nuclei in more severe shrinkage (65% ± 6% shrinkage in nuclei of treated tumor cells compared to control), and greater intercellular spaces.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is able to determine DNA fragmentation and more likely to mark apoptotic cells rather than dead cells. As shown in Fig. 4, TUNEL positive cells were observed 1 h after nsPEFs treatment and increased at 3 h and 6 h post pulse. The expression of an anti-apoptosis Bcl-2 marker was also inhibited in pulsed tumors, shown in Fig. 5C and D.

3.4. Normal skin showed slight and reversible damage after nsPEFs treatment

To evaluate the might-be damages of nsPEFs to normal skin, we pulsed healthy skin along with subcutaneous tissues and muscle underneath it with exactly the same electric field strength, pulse duration and pulse frequency as the tumors were pulsed. The pulsed tissue was 4–5 mm-thick, and burn-like mark was observed immediately after pulse (Fig. 6A–C). One week after pulse, the burn-like mark was gone and only shallow scars were left in stratum corneum. Subcutaneous tissues and muscles were removed on day7 for H&E staining (Fig. 6D and E). On day14, no apparent damage was observed and the shallow scars in stratum corneum faded out completely.

3.5. NsPEFs decreased tumor blood vessel density

For the breast cancer model we established in nude mice, rich blood vessels surrounding the tumor were observed 7 days after injection of tumor cell suspensions. One week after nsPEFs treatment, it was observed from H&E staining that blood vessel density decreased (Fig. 3C). The existing blood vessel number in treated tumors decreased 50% on day7, and 65% on day14. Interestingly, although apoptosis occurred within hours after nsPEFs, the significant difference in blood vessels number was observed until days after nsPEFs treatment.

3.6. NsPEFs treatment suppressed angiogenesis in tumor

VEGF, VEGFR and CD34 expression in tumor cells were assayed with immunohistochemistry. Treated tumors exhibited suppressed expression of VEGF, VEGFR and CD34, compared to untreated tumors. Quantitative analysis showed that expression of VEGF and CD34 decreased by 75%, and VEGFR by 20%. Demonstrated in Fig. 7, the three markers were dyed with DAB and indicated with red arrows.

4. Discussion

In the study, we applied nsPEFs to breast cancer treatment in vivo with field strength of 30 kV/cm. Tumor was exposed to 1-minute’s treatment of such pulse at 4 pulses per second on 3 consecutive days. Via MRI scanning, it was observed that tumor growth was inhibited. H&E and TUNEL staining of tumor samples

![Fig. 2. MRI evaluation for treated and untreated tumors. Axial view (A) and coronal view (B) of T2 weighted images of control group and experimental group on day0, day7, day14 after nsPEFs treatment.](image-url)
showed apoptosis, and immunohistochemistry assay showed expression tumor blood vessel growth markers, CD34, VEGF and VEGFR, were suppressed. To evaluate the influence of nsPEFs on normal tissues rather than tumors, normal skin, subcutaneous tissues and muscles beneath them were also pulsed exactly the same way as tumors are pulsed. The experimental results suggested that

Fig. 3. (A) Tumor cell structure and morphology changes evaluated by H&E staining in 6 h post 720 pulses at 30 kV/cm with 100 ns duration. Nuclei shrinkage is indicated with yellow arrows respectively. (B) Normalized nuclei area after treatment. Nuclei area is calculated with NIH ImageJ software. Statistical significance is indicated with * when p < 0.05 compared with control tumors. (C) Blood vessel number around pulsed and control tumors on day1, day7 and day14. The number of blood vessels was in a field of 0.44 mm². The blood vessel number is counted according to H&E staining. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Representative images of terminal deoxynucleotidyl tranferase dUTP nick end labeling (TUNEL) of tumor cells after nsPEFs treatment. TUNEL positive cells were indicated in green fluorescence.
Fig. 5. (A) Bcl-2 expression in pulsed and unpulsed cells evaluated by immunohistochemistry. IHc positive cells were indicated with red arrows. (B) Quantitative analysis of Bcl-2 expression in pulsed and unpulsed cells. * Indicates p < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. (A–C) Typical photos of skin pulsed with 720 pulses at 30 kV/cm with 100 ns duration on day0, day7 and day14. (D–E) H&E staining of subcutaneous tissues and muscles underneath the pulsed skin.
such treatment did not create permanent damage to skin or normal tissues, which only left shallow marks on the skin that faded out completely in two weeks.

The 30 kV/cm nsPEFs were chosen mainly based on experiments. We once applied 50 pulses with field strength of 20 kV/cm to breast cancer MCF-7 cell suspension, and it was proved effective to eliminate the cancer cells. As for solid tumors, a stronger nsPEFs treatment is needed to achieve the elimination. NsPEFs of different intensity were tested, including 300 pulses of 25 kV/cm for one-day’s treatment and 100 pulses of 30 kV/cm on 3 days. We realized that treatment of 30 kV/cm at 4 Hz for 1 min on 3 consecutive days (roughly 240 pulses per day on 3 days) can induce an obvious inhibition in tumor growth. Unfortunately, such treatment did not induce a complete remission. Optimized nsPEFs parameters would be further studied.

In our study, 240 pulses of 30 kV/cm and 100 ns on 3 consecutive days’ treatment induce a delayed growth of breast cancer in nude mice. H&E and TUNEL staining showed apoptosis was induced by nsPEFs. Nuclei condensation occurs almost immediately after nsPEFs treatment, which might indicate that chromatin is one of the intracellular elements that respond to nsPEFs sensitively. Previous reports have proven DNA damage occurs after nsPEFs exposure. It was also reported that Jurkat cells exhibited DNA fragmentation immediately after 5 pulses of 60 ns in duration and 60 kV/cm in field strength [22]. DNA fragmentation was induced in mouse B10-2 fibrosarcoma tumor in vivo [23]. DNA double stranded breaks also occurred in nsPEFs-treated B16F10 melanoma [20]. Nuclei condensation is also a morphological marker for apoptosis, which has been proved widely to be one of the targets of nsPEFs [20]. Recently, centrosome, an intracellular element that connects tightly to DNA damage, cell cycle and apoptosis attracts researchers’ attention. It is reported that in nsPEFs treatment would induce over duplication of centrosomes, and down-regulate the expression of PLK1, a major signaling molecule related to centrosome duplication and cell apoptosis [24] in HeLa Cells. This implies that centrosome might play an important role in cell response to nsPEFs.

Although some chemotherapeutic drugs also trigger apoptosis and DNA damages, what is different from chemotherapeutic drugs is that nsPEFs offer a localized treatment that mostly affects the areas that exposed to nsPEFs. As a matter of fact, most approaches involving electric field, such as radiofrequency hyperthermia therapy, are localized. However, healthy tissues close to tumors would be damaged in such hyperthermia treatment [7]. Out of the consideration that normal tissues adjacent to tumors might be affected by nsPEFs, we pulsed normal skin, subcutaneous tissues and muscle beneath them as exactly the same way we pulsed the tumors, to evaluate the possible damages caused by nsPEFs. Only temporary burn-like marks were left in the skin immediately after nsPEFs treatment, and faded out completely in two weeks, although no particular medical attention was employed to these marks. This might be resulted from that normal cells are more able to recover from such slight damage caused by nsPEFs. Actually, it is reported that nsPEFs are more cytotoxic to human basal cell carcinoma than its normal sister cell line [25]. Thus, to some extent, nsPEFs have selectivity in cancer treatment.

Besides the apoptosis triggered by nsPEFs, tumor blood vessel growth suppression might also account for the tumor growth inhibition. Blood vessel growth plays a very important role, as highly developed blood vessel system would facilitate the growth and metastasis of solid tumors [26]. In our study, nsPEFs exposure not only induced a reduction in tumor blood vessel density, but also suppressed the expression of VEGF and CD34 significantly. VEGF is one of the most important proangiogenic factors, and CD34 is a biological marker for micro blood vessel density. This indicates that nsPEFs would have profound effects in intracellular signaling. Particularly for anti-angiogenesis effects, one possible mechanism is due to the nanopore induced by nsPEF formed in fragile endothelial cell membranes of small blood vessels, especially capillaries [20]. Tumor growth inhibition is also likely to be resulted from lacking supporting blood vessels.

Despite a delayed growth instead of a complete remission that the solid tumors of breast cancer showed in the study, it suggests that nsPEFs could be used as an assisting method in modern multimodality treatment. For instance, nsPEFs may be administrated in combination with regular chemotherapy to offer a more effective treatment with lower amount of chemotherapeutic drugs, as it is reported that nsPEFs have synergistic effects with Gemcitabine in oral cancer treatment in vitro [27]. Besides, as nsPEFs can effec-
tively inhibit tumor growth, nsPEFs might also be served as a drug-free alternative for neoadjuvant chemotherapy to achieve the same goal of reducing tumor size before surgery and minimize the adverse effects.

In conclusion, we employed nsPEFs in breast cancer treatment and proved that nsPEFs can inhibit solid tumor growth and tumor blood vessel development. Furthermore, experiments suggested that nsPEFs exposure resulted in suppression of VEGF, VEGFR and CD34 expression. Notably, nsPEFs exhibited certain selectivity for tumor, as normal skin did not show permanent damage. NsPEFs might be served as a promising drug-free and non-thermal strategy in cancer therapy, though the underlying mechanisms still remain further study.

**Conflict of Interest**

None.

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