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Comparative Study of Long- and Short-pulsed Electric Fields for Treating Melanoma in an *In Vivo* Mouse Model

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Abstract. *A mouse melanoma model was set up with green fluorescent protein (GFP) expression in vivo. With the same energy, long- (1 ms) and short- (300 ns) pulsed electric fields were delivered to two melanomas injected into the same mouse. The tumor growth and green fluorescence were followed up to compare the different treatment efficacy of long and short pulses. After two days post treatment, short pulse-treated tumors showed a significantly lower tumor volume compared with long pulse-treated tumors (n=8, p<0.05). On 8 experimental animals, a short nanosecond pulsed electric field (nsPEF) had lesser or delayed effects on GFP quenching and greater effects in reducing tumor size. Short pulses produced by nsPEFs can cause melanoma regression with less effect on the plasma membrane.*

A nsPEF is a chemical-free, non-ionizing physical therapy emerging recently for cancer treatment (1), but few *in vivo* studies have been done to confirm the treatment efficacy. Furthermore, differences between short- and long-pulsed electric fields have been unknown to this present time.

To test nsPEFs' anti-tumor *in vivo* effect and further compare the difference between the long- and short-pulsed fields, our current project designed a fluorescent tumor model in mice to measure regression in melanoma treated by short- (300 ns) and long-pulse (1 ms) electric fields.

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Key Words: Green fluorescent protein, melanoma, pulsed electric field, nanosecond.

Materials and Methods

Animal study. In vivo experiments were set up in conformity with IACUC guidelines under applicable international laws and policies (Animal Care and Use Committee of Eastern Virginia Medical School IACUC #04-011, #04-013). B16F10 melanoma cells were transfected with GFP and kept in the Frank Reidy Research Center for Bioelectrics (FRRCBE, Norfolk, VA, USA). The cells were implanted subcutaneously in the right and left flank of eight SKH-1 female mice (CharlesRiver,Wilmington, MA, USA).

Electric conditions for nanosecond pulse and millisecond pulse. Pulses were generated using 40 kV/cm, with a rise time of approximately 30 ns by a Blumlein pulse generator designed and assembled at the Frank Reidy Research Center for Bioelectrics (Norfolk, VA, USA). The 5-needle electrode was also designed and produced by Frank Reidy Research Center for Bioelectrics as previously described (2). The outer needles were electrically connected to ground (cathode) while the center needle delivered the positive high-voltage pulse (anode). Identical electric energy was delivered to 2 tumors developed on the left and right flanks of the same mouse. One tumor was treated with long- (1 ms, 150 V, 48 pulses) the other with short-pulse (300 ns, 6 kV 100 pulse) electric field.

Fluorescent tumor model. Five days after the subcutaneous injection of 1×10^6 B16F10 GFP cells, tumors had a mean diameter of 5 mm and were treated. With GFP expression, the melanomas were clearly detected under the animal's skin upon fluorescence microscopy.

Sample collections and analysis. 0, 1, 3, 6, 24 and 72 hours after treatment, tumors were viewed and photographed using an inverted fluorescence microscope with a high-quality narrow band GFP filter (Chroma Technology Corp., Brattleboro, VT, USA). Quantification of the fluorescence intensity of GFP was analyzed using analySIS software (Soft Imaging System GmbH, Muenster, Germany). Tumor volume was calculated as described in (3): $V=0.52\times D1^2\times D2$, where D1 and D2 are short and long tumor diameters measured by tranillumination and surface photography.

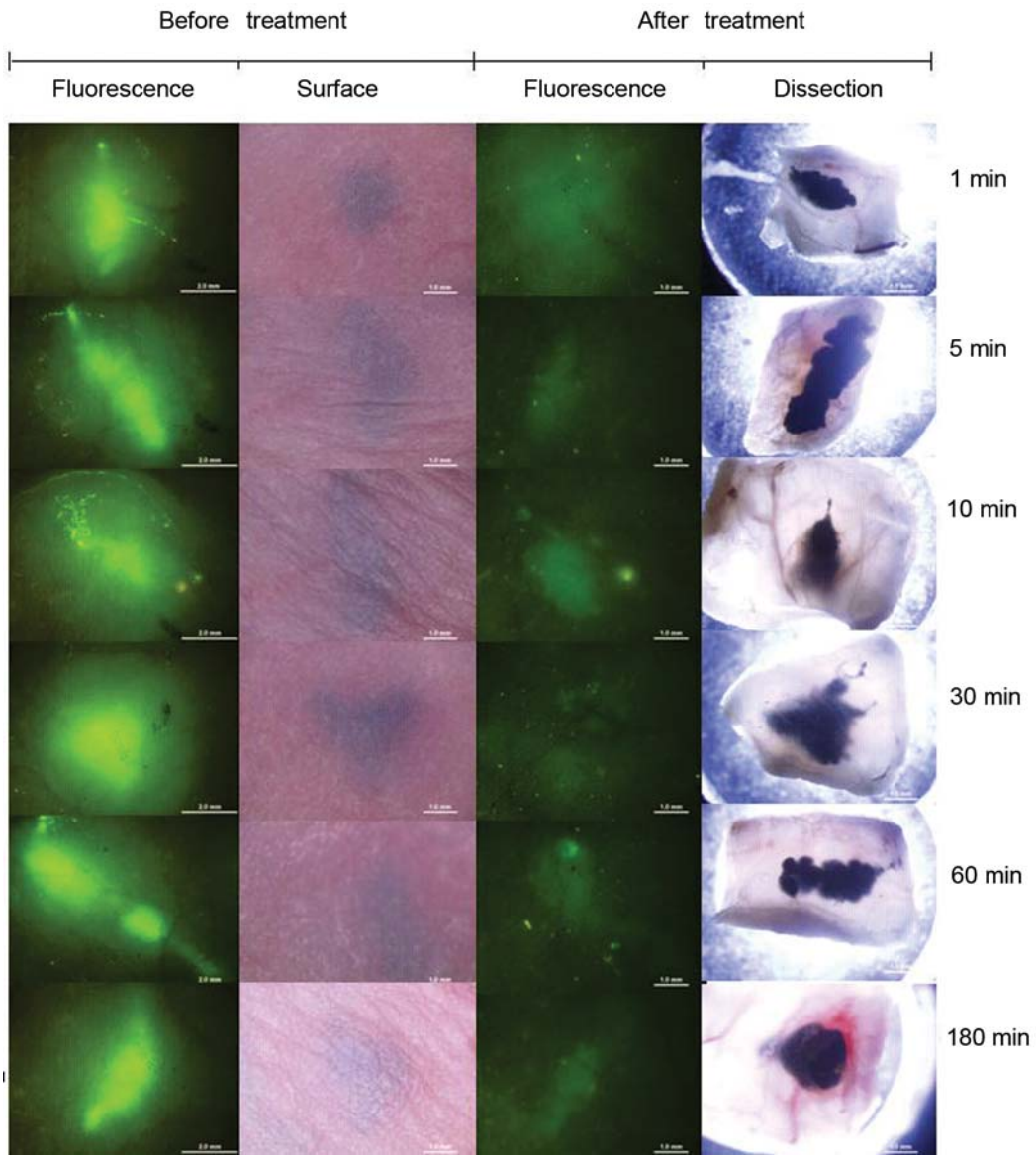

Figure 1. *Long-pulse effects on fluorescent melanoma tumor cells in vivo. Digital imaging was used to quantify the relative fluorescence of B16F10 GFP tumors at different time points. The images show GFP fluorescence at times between 1-180 minutes. The surface view and the dissected view are also shown over the same time course. Scale bar is 2 mm.*

Statistical analysis. Results were presented as the mean±SE and were evaluated using Students *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Comparison of long- and short-pulse electric fields. Before pulses were delivered, tumors had bright green fluorescence. When the 1-ms PEF (150 V, 48 pulses) was applied to the tumor, GFP quenching occurred immediately (Figure 1). In contrast, when the 300-ns PEF (6 kV, 100 pulses) was applied to the tumor, the GFP began to fade gradually (Figure 2). After 3-6 hours, most of the green fluorescence had disappeared. After 24 hours post-nsPEF treatment the fluorescence had comletely vanished. Both long and short pulses had quenching effects on GFP fluorescence, but long-pulse effects were immediate, suggesting a direct effect on GFP fluorescence. In contrast, short-pulse effects on quenching were delayed and coincident with apoptosis, suggesting that GFP degraded with other proteins in response to apoptosis. Furthermore, short pulse conditions inhibited tumors more effectively than longer pulse conditions.

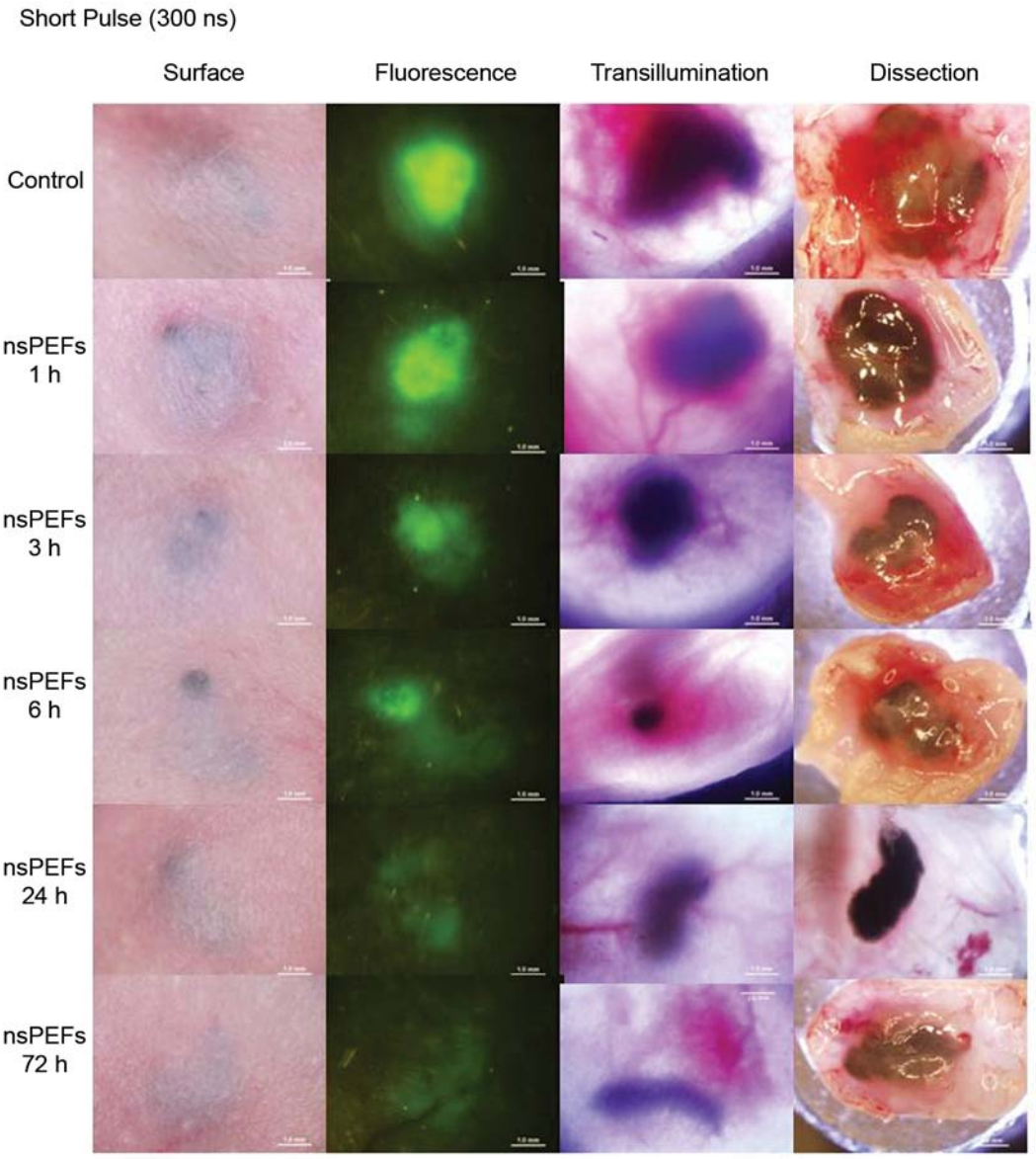

Figure 2. *Short-pulse effects on fluorescent melanoma tumor cells in vivo. Digital imaging was used to quantify the relative fluorescence of B16F10 GFP tumors at different time points. The images show GFP fluorescence at times between 1-72 hours. Surface views, dissected views, and transillumination views are also shown over the same time course. Scale bar is 2 mm.*

After two days post treatment, short-pulse-treated tumors showed a significantly lower tumor volume compared with long-pulse- treated tumors (Figure 3) (n=8, $p<0.05$).

Discussion

Melanoma cancer is now the leading cause of death amoung skin diseases. Solid tumors develop from malignant transformation of melanocytes which are specialized pigmented cells residing in the epidermal basement membrane of the skin (4). Malignant melanoma is a very aggressive disease with a high metastatic rate and very poor overall prognosis. The median survival rate is 6 months and the 5-year survival rate is below 5%. Deaths have increase 15-fold over the past four decades according to the epidemiological data from the American Cancer Society's website (5).

Melanoma has traditionally been approached systemically with chemotherapy, or locally with either surgery or radiotherapy. Researchers have recognized the lack of effective therapies and are looking for improved treatments. Electric fields produce a proven physical effect on the cell membrane. Theese fields serve as a chemical-free, non-ionizing physical

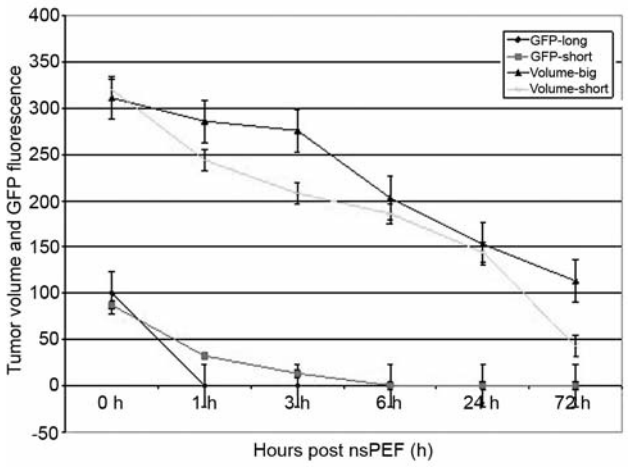

Figure 3. *Tumor volume and GFP fluorescence changes before and after PEF treatment. Effects of long and short pulses on tumor volume and GFP fluorescence as a function of time after pulse treatment are shown as indicated in the color code (n=8, p<0.05). The long and short pulses were adjusted to the same energy density.*

therapy that may trigger apoptosis by a different mechanism or pathway (6). The recent development of pulsed electric fields produce unique characteristics that may solve the problems caused by over heating. Nanosecond-pulsed electric field treatment utilizes high electric fields (kV/cm) applied for ultra-short durations (ns). Quite different from classical plasma membrane electroporation, nsPEFs can produce highly compressed power (billions of watts), of ultra-short pulse durations (ns), rapid rise times (ns), with the high electric fields (kV/cm). The resulting nanosecond pulse is so short that it can penetrate into the cell before the plasma membrane is fully charged, allowing nsPEF to have minimal affect on the plasma membrane, therefore not causing electroporation (7).

Production of nsPEFs has not only had military, but also biological applications creating a new field: Bioelectrics, i.e. using ultra-short pulsed electric fields on living cells, tissues, and even organs. This new burgeoning branch of research analyzes how biological systems react to high electric fields applied with very short-pulse nanosecond duration. Applied in fighting against bacteria, nsPEFs have demonstrated decontamination potential. Only in recent years have nsPEFs been applied to mammalian cells following careful modeling research and studies using non-mammalian cultured cells (6). The main characteristics of nsPEFs are their high power and low energy leading to very little heat production and their ability to enter the cell to penetrate intracellular organelles (8). nsPEF are unique because of their non-electroporation effect on the plasma membrane (9).

Recently, a number of studies have been carried out to determine biological effects of nsPEF in cultured cells. The results showed that nanosecond pulse stimulation of a variety of cells produces a wide range of physiological responses. Cells treated by nsPEF *in vitro* studies include: p53 wild-type and p53-null HCT116 (human colon carcinoma) (10); HL-60 (human promyelocytic leukemia) (11); Jurkat (human peripheral blood, leukemia) (12); B16F10 (murine melanoma) (13) and BS-LCL (human B-cell lymphoblastoid line from a patient with bloom syndrome) (14).

Effects of nsPEFs on cells *in vitro* include: apparent direct electric field effects; induced apoptosis leading to caspase activation and then cell death; nuclear changes and modified cellular functions with delayed plasma membrane effects becoming smaller as the pulse duration is shortened; release of calcium from internal calcium pools and activation of plasma membrane calcium influx channels or capacitative calcium entry (like ligand-mediated responses); induction of DNA and cell cycle anomalies; and diminished cell survival. The biological effect of nsPEFs on cells *in vitro* is directly related to the electric field strength, the pulse number and the pulse duration.

The effect of nsPEF treatment on multicellular tissue or human patients was not tested until very recently. An *ex vivo* study on mouse embryonic fibroblasts was the initial approach for testing nsPEF effects on tissues. Fibrosarcoma tumors (B10.2) were injected in the flanks of C57Bl/6 mice and then excised and exposed to nsPEF. Fibrosarcoma B10.2 cells *ex vivo* became reduced in size after nsPEF treatment (15). Because the tumors were removed from the animals before therapy then sliced and exposed to nsPEF in cuvettes, the experiment was *ex vivo* rather than *in vivo*. Therefore, the data cannot substitute for an *in vivo* experiment.

Based on the previous *in vivo* and *ex vivo* work, nsPEF treatment is hypothesized as a highly localized, drug-free, non-thermal physical technique which would be a new therapy for tumor treatment. To apply nsPEF to tumors *in vivo* is an important bridge to relate the individual *in vitro* cellular response with future clinical application. This emerging field has many unknowns surrounding the nsPEF mechanism of actions on tumors *in vivo*.

In the current study, the same total energy was delivered to two tumors on the same mouse. One treatment was long pulse (150 V, 1 ms, 48 pulses) and the other was short pulse (6 kV, 300 ns, 100 pulses). The energy administered is given by:

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

where W is the energy (joules), τ is the pulse duration, V is the voltage across the electrodes (volts), R is the tissue resistance (Ω), and N is the pulse number. Although there is not an absolute value of energy, for long pulses (1 ms) at a lower voltage (150 V), 48 pulses were calculated as delivering the same energy to the tissue as 100 short pulses. This was based on differences between the electrodes used.

Several interesting effects were observed in the study of conventional plasma membrane electroporation pulses defined

as long pulses and nsPEF pulses as described as short pulses. The first observation was that decreases in tumor volumes occured on both long- and short-pulsed treatment. However, the application of short pulses resulted in greater tumor size decreases than those observed for long pulses. This indicates that nsPEF treatment of tumors is different from conventional electroporation pulse treatment and is thus more effective. Long pulses are known to have predominant effects on the plasma membrane, with few or no effects on the intracellular membrane (16). In contrast, for shorter pulses, as the pulse duration decreases, greater effects occur on intracellular organelles and membranes (16). Nevertheless, short pulses have effects on the plasma membrane creating nanopores in a process termed supra-electroportation. These nanopores are much smaller (~1 nm) and more numerous than larger pores formed by classical plasma membrane electroporation pulses or longer pulses. For 300 ns pulses it is not clear where this condition fits into the paradigms of plasma membrane or intracellular membrane effects. Based on *in vitro* effects of long and short pulses on propidium iodide (PI) uptake in Jurkat cells (16), 60 ns pulses had significantly delayed effect on PI uptake compared to 300 ns pulses, suggesting the absence of direct effects on plasma membranes with shorter pulses. In addition, PI may be too large a molecule to enter nanopores caused by supra-electropoation. For 300 ns pulses, there were immediate effects as well as delayed effects on PI uptake, suggesting a mixture of classical plasma membrane electroporation and supra-electroporation. In contrast, 10- and 100-μs pulses caused immediate PI uptake, suggesting conventional electroporation effects. It should be noted that effects of these pulses *in vitro* and *in vivo* may be different and that pulses in the referenced study were not corrected for energy density. Nevertheless, this provides an initial understanding of differences between conventional plasma membrane electroporation and nsPEFs yielding this supra-electroporation event.

A second interesting observation was made regarding effects of long and short pulses on GFP fluorescence. The long pulses had immediate effects on GFP, causing rapid quenching effect within minutes. This suggests a direct effect of the long pulses on GFP fluorescence (quenching).

In summary, long and short pulses differ in their effects on tumor growth and GFP fluorescence quenching). Short nsPEF pulses have lesser and/or delayed effects on GFP quenching and greater effects in decreasing in tumor size. Nanosecond pulses have less effect on the plasma membrane and may have a role in cancer therapy.

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