Published in final edited form as:

Melanoma Res. 2009 December ; 19(6): 361–371. doi:10.1097/CMR.0b013e32832f1558.

Histopathology of normal skin and melanomas after nanosecond pulsed electric field treatment

Xinhua Chen^{a,b}, R. James Swanson^{b,c}, Juergen F. Kolb^b, Richard Nuccitelli^b, and Karl H. Schoenbach^b

^a Department of Hepatobiliary Surgery, the First Affiliated Hospital Zhejiang University School of Medicine, Hangzhou, Zhejiang, China

^b Frank Reidy Research Center for Bioelectrics, Old Dominion University, Norfolk, Virginia, USA

^c Department of Biological Sciences, Old Dominion University, Norfolk, Virginia, USA

Abstract

Nanosecond pulsed electric fields (nsPEFs) can affect the intracellular structures of cells *in vitro*. This study shows the direct effects of nsPEFs on tumor growth, tumor volume, and histological characteristics of normal skin and B16-F10 melanoma in SKH-1 mice. A melanoma model was set up by injecting B16-F10 into female SKH-1 mice. After a 100-pulse treatment with an nsPEF (40-kV/cm field strength; 300-ns duration; 30-ns rise time; 2-Hz repetition rate), tumor growth and histology were studied using transillumination, light microscopy with hematoxylin and eosin stain and transmission electron microscopy. Melanin and iron within the melanoma tumor were also detected with specific stains. After nsPEF treatment, tumor development was inhibited with decreased volumes post-nsPEF treatment compared with control tumors ($P < 0.05$). The nsPEF-treated tumor volume was reduced significantly compared with the control group ($P < 0.01$). Hematoxylin and eosin stain and transmission electron microscopy showed morphological changes and nuclear shrinkage in the tumor. Fontana–Masson stain indicates that nsPEF can externalize the melanin. Iron stain suggested nsPEF caused slight hemorrhage in the treated tissue. Histology confirmed that repeated applications of nsPEF disrupted the vascular network. nsPEF treatment can significantly disrupt the vasculature, reduce subcutaneous murine melanoma development, and produce tumor cell contraction and nuclear shrinkage while concurrently, but not permanently, damaging peripheral healthy skin tissue in the treated area, which we attribute to the highly localized electric fields surrounding the needle electrodes.

Keywords

melanoma; nanosecond pulsed electric fields; nucleus; skin; vascular disruption

Introduction

The treatment of melanoma requires new therapeutic modalities beyond chemotherapy and ionizing radiation. We have investigated the effect of nanosecond pulsed electric field (nsPEF) exposures, that is, extremely high electric fields that are applied for ultrashort durations. As a result, the electrical power applied is extremely high (billions of watts)

while, because of the short pulse duration, the energy delivered is very low, consequently having no thermal effect on tissue or cells.

Earlier in-vitro research has proven that such high-intensity pulsed electric fields can affect intracellular structures producing a wide range of physiological responses (e.g. induction of apoptosis, stimulation of calcium fluxes, changes in mitochondrial membrane potential) in different cell lines such as human promyelocytic leukemia cells [1], human T-cell leukemia cells [2], and colon carcinoma cells [3], but the in-vivo proof is still in the beginning stages.

Materials and methods

B16-F10 cells

We have used a mouse melanoma model to investigate the direct tumor response of nsPEF on subcutaneous murine melanoma B16-F10. We describe tumor growth and histopathological characteristics in response to nsPEF treatment (T_x). Murine melanoma B16-F10 cells were obtained from ATCC (Manassas, Virginia, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, Georgia, USA), 4 mmol/l l-glutamine (Cellgro, Lawrence, Kansas, USA), and 2% penicillin–streptomycin solution (Cellgro) at 37°C in 5% CO₂.

Animals

In-vivo experiments were set up in conformity with the IACUC guidelines under applicable international laws and policies (Animal Care and Use Committee of Eastern Virginia Medical School). B16-F10 cells were implanted subcutaneously in the right and left flank of SKH-1 female mice using 0.1 ml of cell suspension with 1×10^6 B16-F10 cells prepared from in-vitro cell cultures. Three tumors were induced per mouse; the treated tumor had electrode placement with nsPEF treatment; the sham tumor had electrode placement without nsPEF treatment; the control tumor had no electrode placement or nsPEF treatment.

In-vivo imaging

Before and after the treatment, melanomas were imaged daily by both transillumination and surface photography at $\times 1.2$ magnification. Tumor volume was calculated from these photographs. Tumors were measured daily after the treatment using the formula for a prolate spheroid [4], $V = 0.52 \times D_1^2 \times D_2$, where D_1 and D_2 are short and long tumor diameters, respectively, imaged *in vivo* using transillumination and surface photography.

Pulsed electric field application

Pulsed electric fields of 300-ns duration (rise time 30 ns) and nominal field strength of 40 kV/cm were provided by a Blumlein line pulse generator designed and assembled at the Frank Reidy Research Center for Bioelectrics. Three tumors induced in the same mouse were randomly selected as control, sham, or treated tumors. One hundred pulses were applied to the treated tumor at a frequency of 2Hz with needle electrodes.

The delivery device consisted of four hypodermic needles (30 gauge, i.e. 300 μ m diameter) that were placed at the corners of a square ('box') with a diagonal distance of 4mm from a fifth needle in the center. The needles protruded by 2mm from a flat Teflon base. The outer needles were electrically connected to ground, whereas the center needle was biased with the positive high-voltage pulse (hence constituting the anode, whereas the outer needles assumed the role of the cathode).

The electric field is not homogeneously distributed across the exposed volume in this configuration. The field strength is close to the center needle and drops rapidly with

increasing radial distance to a nominal value, which approximates to the ratio of the applied voltage and the distance to an outer needle. The electric field was 40 kV/cm. Closer to the outer needles, the field strength increases again. As the electrode geometry was the same in all experiments, the biological effects can be directly related to pulse duration and applied voltage (or nominal electric field) despite the inhomogeneous field distribution.

The electrode array was inserted perpendicularly through the skin, and fully into the tumor. As breakdown through air, that is, spark formation, can occur with these extremely high electric fields, the skin was coated with vegetable oil to increase the breakdown strength along the skin surface.

Nanosecond pulsed electric field treatment

B16-F10 cells were injected and monitored for tumor growth to 5mm in diameter, which generally took 5–7 days. The nsPEFs were applied by inserting the 5-needle array electrode into the tumor. The central needle was inserted into the top of the tumor and the outer four needles were outside the tumor edge. The tumors were thus completely surrounded within the cathode portion of the electrode array when exposed to the nsPEF.

After the 100-pulse nsPEF treatment, the melanoma tumors began to shrink within 2 days without the tumor growing back (seven of 12 mice). If the tumor grew back, a second 100-pulse nsPEF treatment (three of 12 mice) and even a third 100-pulse nsPEF treatment (two of 12 mice) was given. In all 12 animals, the treated melanomas were virtually invisible after the various treatment regimens.

Histology study

After euthanasia, tumors were removed and fixed in 10% neutral-buffered formalin before paraffin processing. Sections were stained with hematoxylin and eosin stain (H&E) and assessed microscopically for abnormal cell morphology. One hundred nuclei were randomly selected and outlined in 10 nonoverlapping fields of each section at $\times 600$ magnification. The nuclear area was calculated by MATLAB software (The MathWorks Inc., Natick, Massachusetts, USA) and summed as the mean $\pm$ SD for statistical analysis. Fontana–Masson stain and iron stain (hemosiderin) (International Medical Equipment Inc., Oceanside, California, USA) followed routine protocols [5,6].

Transmission electron microscopy

After euthanasia, treated and control tumors, along with normal skin samples, were excised and immediately processed for TEM. Results were analyzed with a Jeol electron microscope (JEOL USA Inc., Peabody, Massachusetts, USA) at 160 kV.

Safety and damage study

To study safety of and damage because of nsPEF treatment, an area of normal skin on each mouse was also exposed to nsPEFs identical to those described above. The pulsed skin samples were fixed for histopathological analysis and TEM. To monitor mouse general reactions during nsPEF treatment, breath rate, heart rate, and body temperature were recorded with surface plate electrodes connected to a data acquisition system (THM100, Indus Instruments, Texas, USA). To measure intratumoral temperature change during the nsPEF treatment, a fiber optic temperature gauge (FOTM/7M, FISO Technologies, Québec, Canada) was inserted into the tumor, allowing the recording of changes in temperature.

Results

Tumor growth was inhibited by nsPEF treatment

A self-comparison model was set up to avoid the heterogeneity derived from differences between mice. Three melanomas were induced in every SKH-1 mouse, and randomly chosen as control ($n=12$), sham ($n=12$), or treated ($n=12$) tumors. Treated skin around the tumor was also collected to compare with normal skin for further nsPEF safety evaluation. Altogether, 12 mice were included in the 7-day-long tumor growth observation. Histological data showed that 100% of the treated tumors (Fig. 1e–h) responded to the nsPEF treatment compared with the control tumors (Fig. 1a–d).

Tumor growth was inhibited with nsPEF compared with control tumors

One week after nsPEF treatment, the volume of the treated tumor showed a significant difference from controls ($P<0.001$). Control tumors in all mice grew faster and larger than the treated tumors. Skin in the tumor area was also treated. Edema and bleeding appeared after nsPEF treatment and lasted for 3 days. The injured skin completely recovered over a period of 7 days.

Growth after nsPEF treatment recorded by transillumination and surface photography

Pulsed skin undergoes an acute edema with local bleeding, which is evident both 10min and 2 days posttreatment (Fig. 2). On day 2, a shallow eschar was observable on the surface (Fig. 2o), which recovered by day 7 (Fig. 2q). Control tumor size increased continuously on days 2 and 7 maintaining a rich blood supply (Fig. 2j and l). Treated tumor growth was inhibited and a surface eschar developed and tumor blood vessels disappeared as the tumors shrank (Fig. 2p and r).

Tumor volume changes during 7-day post-nsPEFs treatment

Tumor volume changes during the first week after nsPEF treatment are shown in Fig. 3. Local treatment with nsPEF decreased the tumor volume significantly compared with the control tumors. The sham tumors had the electrode inserted into the tumor without delivering any pulses. The sham tumor volumes and growth curves were not significantly different from control tumors.

Tumors collected posteuthanasia

After euthanasia, all tumors ($n=36$) were removed and photographed from the epidermal and subcutaneous surfaces (Fig. 4a). All control group tumors were enlarged and rich in blood supply. The sham tumors were similar to the control group. All nsPEF-treated tumors showed dramatic decrease in size without an extensive blood supply around the tumors. Histology allowed comparison of the vasculature changes (Fig. 4b). The control tumors had a rich blood supply, whereas the nsPEF-treated tumors showed collapsed blood vessels and hemorrhage.

Decreased tumor weight during 14-day post-nsPEF treatment compared with control and sham tumors from the same mouse

nsPEF-treated tumors showed the significantly decreased volume in comparison with control tumors ($P<0.001$). No difference was observed between control and sham tumors (Fig. 5).

Melanoma tumor cellular and nuclear structure post-nsPEF treatment

Tumor histological features were shown by H&E staining. Control tumors showed aggressive growth, regular nest shape, and rich blood supply. The solid tumors were bounded by a thin fibrous capsule and contained internal fibrous bands, demarcating multinodular characteristics. Nests of tumor cells were delineated by well-formed basal lamina composed of cellular lobules separated by oligocellular, fibrous bands. Nested patterns of growth were identified within these lobules. Tumor cells featured clear and regular nuclei with prominent nucleoli. The cytoplasm was characteristically pink and clear. Pigment suggestive of melanin was identified in an organized shape. Treated tumor nuclei shrank dramatically. Tumor cell nests were broken down, losing the cord-like supporting structure on which tumor cells extend. Individual cells were elongated and condensed with decreased nuclear/cytoplasm ratio. Dense cytoplasmic staining makes the field dark, unclear, and disordered. In Fig. 6, the arrows point to tumor nests in low magnification and typical tumor cell nuclei and melanin in high magnification.

Melanoma tumor nuclear area was decreased by post-nsPEF treatment

Nuclear area of control tumors and treated tumors were compared (Fig. 7). Nuclear area decreased significantly 7 days after nsPEF treatment ($P<0.05$).

Melanoma subcellular structures post-nsPEF treatment

Subcellular structures were analyzed by TEM. Full-thickness biopsy was made from normal skin, control, and nsPEF-treated tumor. TEM showed the untreated skin has the typical subcellular organelle. The control tumor had normal nuclei with prominent nucleoli in the center, extensive rough endoplasmic reticulum, Golgi stack, and ribosome. In contrast, the nsPEF-treated melanoma showed: (i) decreased nuclear size, but increased nuclear/cytoplasmic ratio; (ii) dense cytoplasmic bodies, (iii) degenerative tumor cells with fragmentation of nuclei and contracted nuclear outline (Fig. 8).

Fontana–Masson staining of melanin

Fontana–Masson stained the melanin dark brown as shown in the positive control (Fig. 9a, e and i). Control tumors had a thick melanin layer that outlined the malignant melanoma (Fig. 9b, f and j). Treated tumors were almost invisible, but melanin still remains (Fig. 9c, j and k).

Iron stain for red blood cell hemoglobin release caused by nsPEF treatment

The extracellular hemoglobin stained iron blue (Fig. 10a, e and i). Control tumors had no iron–hemoglobin combination, therefore no staining (Fig. 10b, f and j). Treated tumors had positive iron staining (Fig. 10c, g and k). The micrographs indicate capillary damage post-nsPEF treatment with hemoglobin accumulated within the treated area (7-day post-nsPEF treatment).

Safety evaluation and damage of normal skin post-nsPEF treatment

Using isoflurane inhalational anesthesia during the 100 pulses, mice had a higher heart rate and respiratory rate, whereas the body temperature and systolic blood pressure did not change significantly (Table 1). The intratumoral temperature rose 3°C, 10°C lower than the minimum temperature required for a hyperthermia effect, indicating that the nsPEF effect was nonthermal.

Discussion

The application of electric fields to living cells caused a number of significant biological effects [7]. The most common application of pulsed electric fields is classical plasma membrane electroporation that allows foreign drugs to be introduced into cells through temporary formation of pores in the plasma membrane [8]. Electroporation pulses charge the plasma membrane for microsecond to millisecond durations at low electric fields (generally less than 1 kV/cm), without significant effects on intracellular membranes. Quite different from classical plasma membrane electroporation, nsPEF exposures are characterized by much shorter nanosecond pulse durations, rapid nanosecond rise times, and high electric fields (several tens to hundreds of kV/cm). As a result, nsPEF are short enough to penetrate the cell interior before the plasma membrane is fully charged [9–11]. During the charging of the plasma membrane, the cell interior will also be exposed to the strong electric field. If the field strength is high enough, the exposure will then charge internal structures to significant values (e.g. electroporation thresholds) before the same value is reached across the outer membrane [12]. For exposure times shorter than the charging time of the outer membrane, the effect on the plasma membrane is negligible and intracellular effects will predominate.

nsPEF treatment are characterized by high power, but low energy input, leading to very little heating. Pulses of nanosecond duration in our earlier trials showed that for 100 pulses, the temperature in the treated region increases by only 3°C, 10°C lower than the minimum increase for hyperthermia effects [13].

For a single pulse of 300-ns duration and 40-kV/cm nominal electric field strength, the energy that is deposited in the exposed volume will increase by 1.2 K under ideal, that is adiabatic, conditions. The calculation assumes a specific heat of 4.2/J/gK and conductivity of 1 S/m, that is salt water, as a substitute for the unknown values of the tumor tissue. When several pulses are applied, as in our experiments, the overall temperature asymptotically approaches an equilibrium value, which is defined by the ability of the tissue to dissipate the repetitive input of energy, mainly through blood perfusion, heat conduction, and radiation. In our case, repetition rates are slow, with a ‘relaxation time’ of about 500 ms between subsequent exposures; corresponding to 2 Hz. We measured this value to be 3°C higher than the initial value before pulse application. Any potential biological thermal response will have to be related to this equilibrated value rather than possible hotter transients during the application of a pulse, which is too short to interact on biological or biochemical time scales (or for hyperthermic ablation, which usually requires at least several minutes of elevated temperatures). Similarly, transient hotter zones, because of the field inhomogeneity, will not account for any thermal effects on the bulk of the exposed volume, either. Electric fields drop rapidly with distance from the center needle, so will the energy input. At the same time, every local increase in temperature within any layer a few cells thick would be dissipated rapidly through adjacent tissue layers and also the metal of the needles. As a consequence, the temperature across the tumor can be represented (at least within a few milliseconds) by an average value, which is better suited to evaluate temperature effects. Conversely, the concept of ‘temperature’ requires, by its very nature, the interaction of particles, which equilibrate over a given volume in their (kinetic) energies and in this way define the temperature. Therefore, any local deposition of energy, for example, affecting molecular bonds, has rather to be considered an electric field effect than a temperature effect. In conclusion, we hypothesize that the observed effects on tumor cells and tumor mass, on the whole, are nonthermal.

The consequence of applying intense 300 ns pulses was examined on melanoma tumor-bearing mice. This study analyzed melanoma growth and ultrastructure post-nsPEF treatment. The hypothesis that nsPEFs are a highly localized and drug-free physical

treatment technique was confirmed. nsPEF could serve as a promising new therapy for tumor ablation.

After nsPEF treatment, the tumor showed delayed development and sharply decreased volume on the first, second, and third days compared with control tumors ($P<0.05$). The nsPEF-treated tumor weight was reduced by 85.3%, significantly smaller than the control group ($P<0.001$).

Transillumination and surface photography showed a consistent change after nsPEF treatment, whereas the untreated melanomas were grossly recognizable under the skin surface with a round black enlarged appearance on the back. The treated tumors were small and dry with structural shape changes and reduction of blood vessels.

Both H&E and TEM images showed that without nsPEF treatment the melanomas kept a regular outline of tumor cells with a pale nucleus and prominent round nucleolus. Cell cytoplasm was finely dusted with melanin and the cells often formed a tumor nest with an active growing center marked by a good blood supply and a well-organized cancer cell cord marked by invading vessels, dermis or muscle fibers. Lymphatic and vascular invasion was present in some control samples, but not as prominent as in the treated group. In nsPEF-treated melanomas, solid tumor nest construction was detached, tumor cords were broken and the space between tumor cells enlarged, with shrinking spindle-shaped nuclei inside. Regression in size of tumors occurred within 24 h with surrounding tissue swelling and bleeding. Damaged subcutaneous tissue and skin recovered within 7 days. Skin pulsed with nsPEFs showed evidence of typical inflammation in the treated area during the first 3 days, but resolved in 1 week.

Fontana–Masson stain was used to assess depigmentation of melanomas after nsPEF treatment. As melanoma develops from the malignant transformation of melanocytes, specialized melanin-producing cells that reside in the epidermal basement membrane of the skin, melanogenesis is regarded as a functional marker associated with differentiated melanocytes. The data show the effects of nsPEF on organized melanogenesis and retention of intracellular melanins.

Iron staining can show hemosiderin, which is the storage molecule for iron granules, therefore iron staining can mark recent bleeding by staining the iron–hemoglobin complex released by lysed red blood cells. The data showed iron stain in the treated melanomas, a sign of hemorrhage indicating that nsPEFs caused acute blood vessel rupture and bleeding inside the tumor.

To evaluate the safety of nsPEF treatment, vital organ physiological parameters were recorded and evaluated during 100 pulses. Treated mice had higher heart and respiratory rates, whereas body temperature and systolic blood pressure showed no significant changes. The intratumoral temperature rose 3°C, which represents an accumulated 0.2 J of heat. Therefore, the intratumoral temperature during nsPEF treatment was approximately 10°C lower than the minimum temperature required for hyperthermia treatment. The small temperature variation indicated that nsPEF penetrated cells through a nonheating mechanism.

After nsPEF treatment, the melanomas begin to shrink within 3 days. Blood flow to the tumor was disrupted after pulsing and red blood cells leaked out of the capillaries surrounding the tumor (Fig. 4b). nsPEF treatment disrupted the vasculature within the tumor, reduced blood flow, and deprived the tumor of oxygen and nutrients, resulting in tumor cell death.

Besides analyzing tumor cells, we also studied normal skin post-nsPEF treatment. We treated normal skin without melanomas and observed superficial eschar formation and resolution over 1 week (Fig. 1i, j and k). Results suggest that, similar to tumor cells, normal skin epidermal cells surrounding the electrodes were also damaged by the nsPEF treatment. However, this damage resolved in 7-day post-nsPEF treatment (Fig. 1l). We propose that nsPEFs produce their effects in a nonselective manner; the mild damage we have observed in healthy skin is because of the highly localized effect of the needle electrode design.

We also set up a sham control with electrode insertion only. The sham tumors were grown to the same volume as controls, indicating that electrode insertion did not account for the tumor reduction.

Our animal trial proved that nsPEF could inhibit melanoma growth *in vivo*. nsPEF can significantly delay subcutaneous murine melanoma development by directly damaging the tumor structure and nuclei without affecting the peripheral skin because of the high localized needle electrode. nsPEF is different from therapies based on temperature effects. A single human case report has recently been published applying nsPEF treatment to basal cell carcinoma [14]. Depending on electric fields alone, nsPEF therapy could be used for melanoma treatment without chemical drugs so that the serious side effects from chemotherapy would be avoided.

Acknowledgments

The study was supported by the National Natural Science Foundation of China (No. 3070078), Zhejiang Medical Grant (No. 2007QN006) and Juliette Reidy Fellowship in Bioelectric Cancer Research. All authors thank Dr Antoinette F. Hood, the Chair and Professor of the Department of Dermatology Eastern Virginia Medical School for reviewing our histology slides.

References

1. Tekle E, Oubrahim H, Dzekunov SM, Kolb JF, Schoenbach KH, Chock PB, et al. Selective field effects on intracellular vacuoles and vesicle membranes with nanosecond electric pulses. *Biophys J*. 2005; 89:274–284. [PubMed: 15821165]
2. Hall EH, Schoenbach KH, Beebe SJ. Nanosecond pulsed electric fields (nsPEF) induce direct electric field effects and biological effects on human colon carcinoma cells. *DNA Cell Biol*. 2005; 24:283–291. [PubMed: 15869405]
3. Hall EH, Schoenbach KH, Beebe SJ. Nanosecond pulsed electric fields induce apoptosis in p53-wildtype and p53-null HCT116 colon carcinoma cells. *Apoptosis*. 2007; 12:1721–1731. [PubMed: 17520193]
4. Terris MK, Stamey TA. Determination of prostate volume by transrectal ultrasound. *J Urol*. 1991; 145:984–987. [PubMed: 2016815]
5. Ro JY, Lee SS, Ayala AG. Advantage of Fontana-Masson stain in capsule-deficient cryptococcal infection. *Arch Pathol Lab Med*. 1987; 111:53–57. [PubMed: 3541845]
6. Gaitanis G, Chasapi V, Velegraki A. Novel application of the Masson-Fontana stain for demonstrating *Malassezia* species melanin-like pigment production in vitro and in clinical specimens. *J Clin Microbiol*. 2005; 43:4147–4151. [PubMed: 16081962]
7. Kartashev AH. Biological mechanism of long-term effect of alternating electric field on the development of mice. *Fiziol Zh*. 1992; 38:81–85. [PubMed: 1499766]
8. Mori K, Hasegawa T, Sato S, Sugibayashi K. Effect of electric field on the enhanced skin permeation of drugs by electroporation. *J Control Release*. 2003; 90:171–179. [PubMed: 12810300]
9. Vernier PT, Sun Y, Chen MT, Gundersen MA, Craviso GL. Nanosecond electric pulse-induced calcium entry into chromaffin cells. *Bioelectrochemistry*. 2008; 73:1–4. [PubMed: 18407807]

10. Smith KC, Weaver JC. Active mechanisms are needed to describe cell responses to submicrosecond, megavolt-per-meter pulses: cell models for ultrashort pulses. *Biophys J.* 2008; 95:1547–1563. [PubMed: 18408042]
11. Kotnik T, Miklavcic D. Theoretical evaluation of voltage inducement on internal membranes of biological cells exposed to electric fields. *Biophys J.* 2006; 90:480–491. [PubMed: 16239325]
12. Pakhomov An G, Kolb JF, White JA, Joshi RP, Xiao S, Schoenbach KH. Long-lasting plasma membrane permeabilization in mammalian cells by nanosecond pulsed electric field (nsPEF). *Bioelectromagnetics.* 2007; 28:655–663. [PubMed: 17654532]
13. Entin I, Plotnikov A, Korenstein R, Keisari Y. Tumor growth retardation, cure, and induction of antitumor immunity in B16 melanoma-bearing mice by low electric field-enhanced chemotherapy. *Clin Cancer Res.* 2003; 9:3190–3197. [PubMed: 12912972]
14. Garon EB, Sawcer D, Vernier PT, Tang T, Sun Y, Marcu L, et al. In vitro and in vivo evaluation and a case report of intense nanosecond pulsed electric field as a local therapy for human malignancies. *Int J Cancer.* 2007; 121:675–682. [PubMed: 17417774]

Fig. 1.

Histology comparison of the melanomas with or without nanosecond pulsed electric field (nsPEF) treatment from the same mouse. Tumor growth was inhibited 7-day post-nsPEF treatment compared with control tumors. In this figure, the micrographs are from treated and control tumors. No sham micrographs are shown. (a) and (e), (b) and (f), (c) and (g), (d) and (h) are comparison sets of 7-day tumors in four separate mice ($\times 40$). Control tumors are on the top row; treated tumors are on the middle row, and skin samples are in the bottom row. (i) pre-pulse skin ($\times 200$); (j-l) post-pulse skin: (j) first day post-nsPEF treatment ($\times 200$), (k) third day post-nsPEF treatment ($\times 200$), (l) seventh day post-nsPEF treatment ($\times 40$).

Fig. 2.

Growth after nanosecond pulsed electric field (nsPEF) treatment recorded by transillumination and surface photography. The images ($\times 1.2$ magnification) show pulsed skin (top row), untreated control tumors (middle row), and treated tumors with nsPEFs with 100 pulses at 300 ns and 40 kV/cm (bottom row). Images were taken 10 min, 2-day and 7-day post-nsPEF treatment as labeled.

Fig. 3.

Tumor volume during 7-day post-nanosecond pulsed electric fields (nsPEFs) treatment. Tumor volume was determined using calipers on days 1, 3, and 7 after treatment with 100 pulses at 300 ns and 40 kV/cm. Treated ($n=12$) and sham ($n=12$) tumor volumes were compared with controls ($n=12$). * $P<0.001$.

Fig. 4.

(a) Tumors dissected posteuthanasia. Tumors were removed from the mice after euthanasia. All tumors ($n=36$, 12 per group) were removed and photographed from the epidermal and subcutaneous surfaces. Tumors were dissected from the surrounding skin and then weighted for statistical analysis. The first frame shows control tumors; the second frame shows sham tumors; and the third frame shows nsPEF-treated tumors. (b) Antivascular effects of nanosecond pulsed electric fields (nsPEFs) on melanomas. Control tumors rapidly increased in size accompanied by a rich blood supply within. Melanomas expanded aggressively and were surrounded by capillaries and larger blood vessels. The yellow arrows in the control micrographs point to the formation of medium-sized blood vessels with well-walled endothelium-lined structures as well as new angiogenesis. In the nsPEFs-treated tumor, an organized tumor structure was no longer evident and little melanin was left. The yellow arrows in the treatment micrographs point to the vascular collapse and hemorrhage under the damaged epidermal layer.

Fig. 5. Tumor weight comparison. The tumor weight of treated mice decreased significantly compared with control and sham which had no nanosecond pulsed electric field treatment (* $P<0.05$).

Fig. 6.

Melanoma tumor cell structure and nuclei in control and nanosecond pulsed electric fields (nsPEF)-treated tumors. Melanoma cell structure and nuclear changes were analyzed by hematoxylin and eosin (H&E) stain. All tumors are stained with H&E stain and then photographed at $\times 40$ and $\times 600$ magnification. The arrows point to the tumor nests in low magnification and typical tumor cell nuclei and melanin at high magnification. Bar scale: 2 cm on top and 50 μm on bottom. In the control group, we saw a regular cell outline with a pale nucleus and prominent round nucleolus. The cytoplasm was finely dusted with melanin. Cells often formed a tumor nest with an active growing center marked by good blood supply, organized cancer cell cords, invading vessels and dermis or muscle. The treated groups exhibited larger lateral diameter, aggregating nests and solid growth pattern with detached nest construction and broken cords. Intracellular spaces were enlarged and nuclei became spindle-shaped with rapid regression over time. Swelling and bleeding were also evident, but recovered within 1 week. Lymphocyte infiltration was limited to the local area. The arrows point to tumor nests in low magnification and typical tumor cell nuclei and melanin in high magnification.

Fig. 7.

Melanoma nuclear area was decreased post-nanosecond pulsed electric field (nsPEF) treatment. Specimens were collected on the 7-day post-nsPEF treatment. Control tumors ($n=12$) and treated tumors ($n=12$) were routinely stained with hematoxylin and eosin and analyzed by a software MATLAB. The nuclear area decreased significantly after nsPEF treatment (* $P<0.05$).

Fig. 8. Melanoma subcellular structures. The upper row showed the full-thickness biopsy from normal skin, control tumor, and treated tumor from the same mouse 7-day post-nanosecond pulsed electric field (nsPEF) treatment. The lower row shows the corresponding transmission electron microscopy pictures. The yellow arrows show the nucleoli. In the normal skin and control tumor, the arrows point to a nucleolus that is located in the center. It is round, big and complete. In the nsPEFs-treated tumor, the arrow points to a nucleolus that has broken into pieces and is disrupted along the nuclear edge.

Fig. 9.

Fontana–Masson stains for melanin in the tumors with or without nanosecond-pulsed electric field (nsPEF) treatment. (a), (e) and (i) are Fontana– positive controls from human melanomas; (b), (f) and (j) are control tumors; (c), (g) and (k) are treated melanomas; (d), (h) and (j) are normal mouse skin. Yellow arrows indicate melanin. In the positive control human melanoma samples (a, e and i), there are thick and dark layer of melanins. In the melanomas without nsPEF treatment (b, f and j), melanins are as rich as in the positive control, whereas in the nsPEF-treated samples (c, g and k), melanomas were already dead and there was very few melanin. Normal skin samples (d, h and l) serve as the negative control of Fontana stain.

Fig. 10.

Iron stains for residual bleeding from the original nanosecond pulsed electric field (nsPEF) treatment in melanoma tumors with and without nsPEF treatment. (a), (e), and (i) are iron stain-positive controls from human melanoma. (b), (f), and (j) are control tumors, which had no blue stained iron–hemoglobin combination. (c), (g), and (k) are the treated tumors, which had iron–hemoglobin staining. (d), (h), and (l) are normal mouse skin as the iron stain-negative control. The arrows point to cells indicating positive iron stain.

Table 1

Physiological parameters of mice during nsPEF treatment

	Without nsPEF treatment	During nsPEF treatment
Heart beat/min	327 ± 43	475 ± 56
Respirations/min	66 ± 8	96 ± 12
Systolic blood pressure (mmHg)	114 ± 7	116 ± 5
Body temperature (°C)	39.3 ± 0.5	39.1 ± 0.2
Intratumor temperature (°C)	30.3 ± 0.3	33.3 ± 0.3

Data are presented as mean ± SEM ($n=12$). nsPEF treatment can cause edema and bleeding in normal skin as shown in the Fig. 1i, j, k. The damage resolved after 7-day post-nsPEF treatment (Fig. 1l).

nsPEF, nanosecond pulsed electric field.