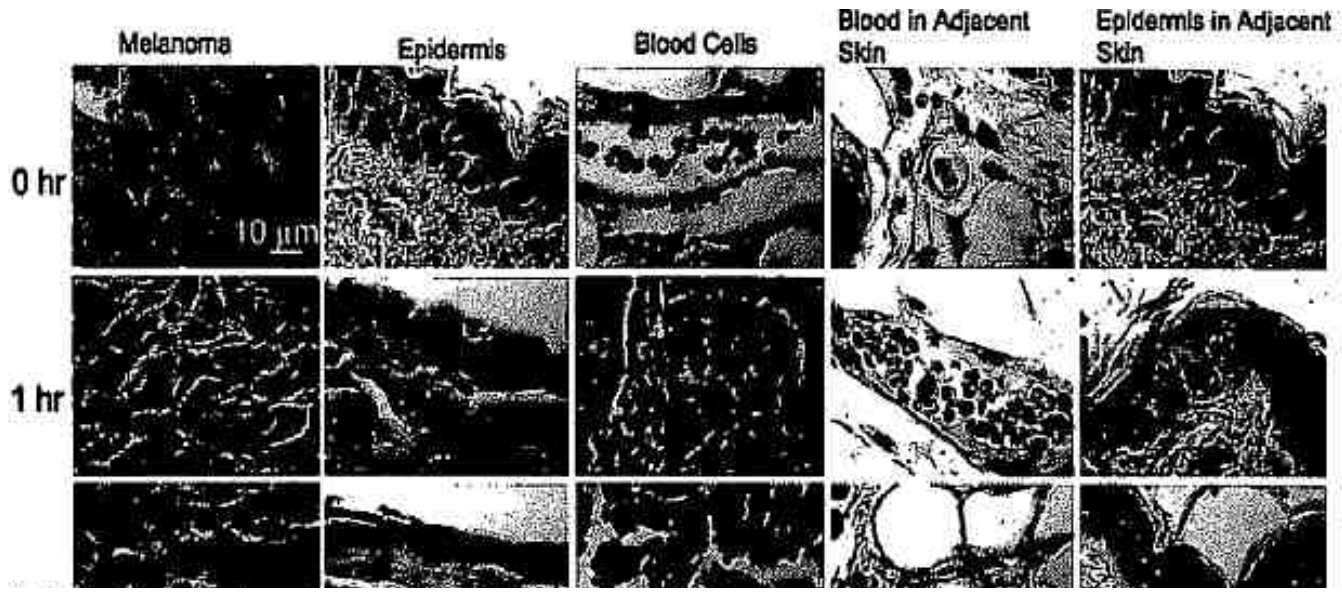
Karl SCHOENBACH, *et al.*
Nanosecond Pulsed Electric Fields [nsPEFs] vs Cancer

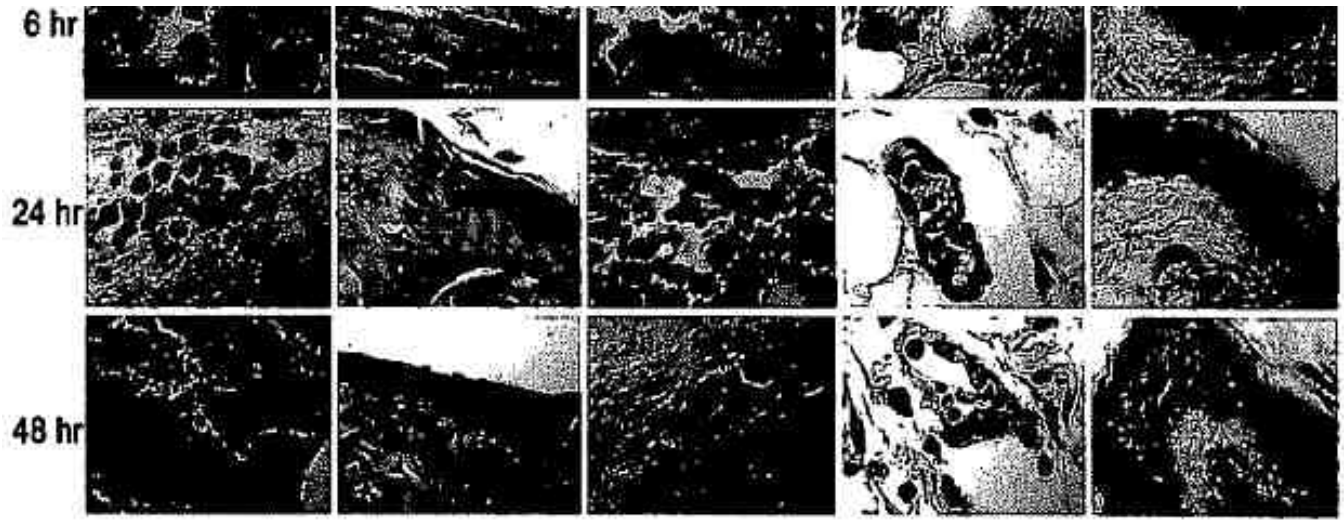
Single-treatment destruction of tumors by nsPEFs -- Articles & patents

Nanosecond pulsed electric fields induce apoptosis in p53-wildtype and p53-null HCT116 colon carcinoma cells

by **Emily H. Hall, Karl H. Schoenbach, Stephen J. Beebe**

Non-ionizing radiation produced by nanosecond pulsed electric fields (nsPEFs) is an alternative to ionizing radiation for cancer treatment. NsPEFs are high power, low energy (non-thermal) pulses that, unlike plasma membrane electroporation, modulate intracellular structures and functions. To determine functions for p53 in nsPEF-induced apoptosis, HCT116p53+/+ and HCT116p53-/- colon carcinoma cells were exposed to multiple pulses of 60 kV/cm with either 60 ns or 300 ns durations and analyzed for apoptotic markers. Several apoptosis markers were observed including cell shrinkage and increased percentages of cells positive for cytochrome c, active caspases, fragmented DNA, and Bax, but not Bcl-2. Unlike nsPEF-induced apoptosis in Jurkat cells (Beebe et al. 2003a) active caspases were observed before increases in cytochrome c, which occurred in the presence and absence of Bax. Cell shrinkage occurred only in cells with increased levels of Bax or cytochrome c. NsPEFs induced apoptosis equally in HCT116p53+/+ and HCT116p53-/- cells. These results demonstrate that non-ionizing radiation produced by nsPEFs can act as a non-ligand agonist with therapeutic potential to induce apoptosis utilizing mitochondrial-independent mechanisms in HCT116 cells that lead to caspase activation and cell death in the presence or absence of p-53 and Bax.





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

Shan Wu, et al.

Highlights

NsPEFs treatment at 30kV/cm can inhibit human breast cancer growth, and suppresses VEGF expression and tumor blood vessel growth.

Such nsPEFs treatment does not cause permanent damage to healthy skins and tissues.

NsPEFs could serve as a novel cancer therapy alone or in combination with other treatment.

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 30Balb/c (nu/nu) mice were exposed to 720 pulses of 100ns duration, at 4pulsespersecond and 30kV/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.

Nanosecond pulsed electric fields activate intracellular signaling pathways

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Exposing cells to nanosecond pulsed electric fields causes a rapid increase in intracellular calcium, enabling a pathway that activates protein kinase C for various physiological functions.
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In cellular electrochemistry, ions respond to stimuli by constantly shuffling across cellular membranes to perform their physiological roles. This flow of ions, the electromotive force, leaves cells vulnerable to exogenous electromagnetic fields that can stimulate and/or modulate cellular activity. An irreparable link exists between changes in ionic concentration and the electric gradient of the cell (or its potential energy). Consequently, we can manipulate the physiology of the cell by altering its permeability to various ions, thereby modulating its electrical gradient. Only a few millivolts in excess of the resting membrane potential can stimulate a dramatic change in ion distribution within the cellular microenvironment. In excitable neural-type cells, electrical-stimulation-induced changes in membrane potential lead to the generation or inactivation of action potentials (AP). These AP trigger activities, such as nerve impulses in neurons or contraction in muscle cells. Within neural networks, targeted alteration of AP can prompt physiological changes that selectively stimulate or inactivate specific signals along nerve fibers. On the whole-organism level, electromagnetic fields applied directly to neural tissue, or transversely through the skull, produce profound effects that range from altered sensory perception to deviations in motor movement. Given this wealth of observable electromagnetic effects on neurological tissues, it is no surprise that other forms of electrical stimuli may elicit novel responses in an exposed biological system.

Our research team is currently exploring the cellular response to high-amplitude, short-duration electrical pulses termed nanosecond pulsed electric fields (nsPEF). Seminal studies showed that nsPEF exposure can elicit changes in membrane potential, plasma membrane phospholipid scrambling, mitochondrial depolarization, calcium uptake, platelet aggregation, and, at intense or repeated exposures, cause cell death.1–7 Notably, these observations show no substantial uptake of propidium iodide, a common indicator of pore formation in the plasma membrane when electric pulses are applied for longer periods (μs to ms).8 Thus, we assume that nsPEF exposure causes the formation of small, ion-permeable pores, or nanopores, in the plasma membrane.2, 9,10 Unlike the larger pores, nanopores retain ion selectivity when exposed to electrical pulses, acting more like a channel, and persist for many minutes after only a single pulse exposure.9, 11 Most notably, the formation of nanopores in the plasma membrane elicits an acute and prolonged increase in intracellular calcium, an ion critical to many neurological and cellular processes.

We believe that nsPEF exposure is an ideal tool for the prolonged and non-invasive modulation of cell electrophysiology. Based on the hypothesis of nanopore formation, we investigated the dynamics of calcium entry into neuroblastoma cells. We used a highly sensitive electron multiplied CCD camera and precisely timed laser excitation to acquire high-resolution, spatiotemporal images of a single cell12 (see Figure 1). We visualized calcium entering from the sides nearest the electrodes in less than 1ms after perturbation by a single 600ns pulse, and filling the cell within 100ms. With extracellular calcium excluded from the bathing buffer, the intensity of the signal was reduced and the signal emanated from within the cell, suggesting calcium release from intracellular stores. By pre-exposing cells to the inhibitor thapsigargin in an effort to deplete intracellular calcium, we saw no change in signal, validating the intracellular origin of the signal. This finding was the first to definitively show, spatially, that nsPEF caused both extracellular uptake and intracellular

release of calcium.

We hypothesize that the release of intracellular calcium is due, in part, to nsPEF-induced activation of intracellular pathways derived from the plasma membrane, namely the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) or PIP2. PIP2 is a well-characterized intracellular pathway that originates on the inner surface of the plasma membrane. It ultimately causes intracellular calcium release from the endoplasmic reticulum via inositol trisphosphate (IP3) receptors (see Figure 2), activating protein kinase C (PKC). To validate our hypothesis, we used a widely accepted optical probe of PIP2 hydrolysis and diacylglycerol (DAG) sensor GFP-C1-PKC? (green fluorescent protein labeled C1 domain of protein kinase C): see Figure 3.13,14 We validated that a single nsPEF exposure can cause hydrolysis of PIP2, ultimately leading to increased DAG on the plasma membrane, and activation of PKC.

PKC triggers many physiological responses, including hormone secretion, AP propagation, and muscle contraction. Thus, by manipulating the electrochemistry of the cell with nsPEF, we can potentially elicit and control a number of biological responses. This single, exogenous, non-chemical stimulus can cause a prolonged activation of intracellular signaling cascades at a similar level to that of pharmaceutical treatment, but without the need for a specific cell surface receptor. The responses can last for minutes and can be delivered locally, precisely and without systemic drug administration. Electrical pulse delivery to cells offers scientists a new, instant, and simplified means of studying cellular physiology through direct, drug-free activation of cellular pathways. Non-invasive activation of PKC could be used to stimulate cognitive function or treat pain without pharmaceuticals or surgery. Future efforts will focus on validation of this effect in primary neuron cultures and evaluation of ion channels regulated by PIP2 hydrolysis.13

References:

1. T. P. Napotnik, Nanosecond electric pulses cause mitochondrial membrane permeabilization in Jurkat cells, Proc. IEEE Int'l. Power Modulators and High Voltage Conf. 2008, p. 60, 2008.
2. A. G. Pakhomov, Long-lasting plasma membrane permeabilization in mammalian cells by nanosecond pulsed electric field (nsPEF), Bioelectromagnetics 28, p. 655-663, 2007.
3. K. H. Schoenbach, Bioelectrics--new applications for pulsed power technology, IEEE Trans. Plasma Sci. 30(1), p. 293-300, 2002.
4. K. S. Schoenbach, Bioelectric effects of nanosecond pulses, IEEE Trans. Dielectrics and Electrical Insulation 14(5), p. 1088-1109, 2007.
5. P. T. Vernier, Nanosecond electric pulse-induced calcium entry into chromaffin cells, Bioelectrochem. 73(1), p. 1-4, 2008.
6. P. T. Vernier, Nanoelectropulse-induced phosphatidylserine translocation, J. Biophys 86(6), p. 4040-8, 2004.
7. P. T. Vernier, Calcium bursts induced by nanosecond electric pulses, Biochem. Biophys. Res. Commun. 310, p. 286-295, 2003.
8. E. S. Buescher, K. H. Schoenbach, Effects of submicrosecond, high intensity pulsed electric fields on living cells--intracellular electromanipulation, IEEE Trans. Dielectrics and Electrical Insulation 10(5), p. 788-794, 2003.
9. A. G. Pakhomov, O. N. Pakhomova, Nanopores: a distinct transmembrane passageway in electroporated cells, Advanced Electroporation Techniques in Biology in Medicine, CRC Press, 2010.
10. P. T. Vernier, Nanopore formation and phosphatidylserine externalization in a phospholipid bilayer at high transmembrane potential, J. Am. Chem. Soc. 128(19), p. 6288-

6289, 2006.
11. A. G. Pakhomov, Lipid nanopores can form a stable, ion channel-like conduction pathway in cell membrane, Biochem. and Biophys. Res. Commun. 385(2), p. 181-186, 2009.
12. H. T. Beier, Resolving the spatial kinetics of electric pulse-induced ion release, Biochem. Biophys. Res. Commun. 423(4), p. 863-866, 2012. doi:10.1016/j.bbrc.2012.06.055
13. N. Gamper, M. S. Shapiro, Regulation of ion transport proteins by membrane phosphoinositides, Nat. Rev. Neurosci. 8(12), p. 921-34, 2007.
14. E. Oancea, Green fluorescent protein (GFP)-tagged cysteine-rich domains from protein kinase C as fluorescent indicators for diacylglycerol signaling in living cells, J. Cell Biol. 140(3), p. 485-98, 1998.

Nanosecond Pulsed Electric Fields: A New Stimulus to Activate Intracellular Signaling

Stephen J. Beebe and Karl H. Schoenbach

When new technologies are introduced into the scientific community, controversy is expected and both excitement and disappointment enrich the lives of those who initiate the new ideas. It becomes the mission of the “inventors” to embrace the burden of proof to establish their ideas and convince the skeptics and disbelievers who will undoubtedly temper their enthusiasm and test their patience. While open mindedness is generally a scientific motto, those who review patents, manuscripts, and grants do not always readily practice it, even when the evidence is convincingly presented; old ideas and concepts often die hard. So it has been and still is in many instances as engineers, physicists, biologists, and physicians pursue innovative ideas and novel technologies.

So what is “Bioelectrics”? It is the application of ultrashort pulsed electric fields to biological cells, tissues, and organs. More specifically, it is the analysis of how these biological systems respond to high electric fields (10–100 s of kV/cm) when applied with nanosecond (1–300) durations. Compressing electrical energy by means of pulsed power techniques allows the generation of ultrashort (billionth of a second) electrical pulses [1]. Because the pulses are so short the energy density is quite low and therefore nonthermal. However, the power is extremely high generating billions of watts. This can be compared to a coal power plant, which generates less than billion watts, but does it continuously. For example, for a 10 ns, 40 kV, 10 O pulse generator, the power provided by the pulse is 160 MW, however, the energy is only 1.6 J. Depositing this energy into one milliliter of water causes an increase in temperature by just one third of one degree Celsius. We have referred to these pulses as ultrashort, high-voltage pulsed electric fields or nanosecond pulsed electric fields (nsPEFs). These conditions are most certainly unique and do not exist in nature. Thus, this provides an opportunity to determine how cells respond to stimuli that they have not evolved to recognize. Undoubtedly, cells respond to nsPEFs in diverse and cell-type-specific ways. This suggests that nsPEFs represent a distinctive, non-ligand stimulus that can disclose basic cell-type-specific differences for responses to the external environment and can also be investigated for potential therapeutic and/or diagnostic applications. A patent has been issued and several provisional patents have been filed for devices and applications of nsPEFs to cells and tissues for a wide range of applications.

The use of electric fields on biological systems is not new, but it has been a common

misconception that nsPEFs are also not new. A method called electroporation has been used for decades to introduce drugs and/or DNA into cells for basic science or for therapeutic purposes. These electric fields charge the plasma membrane causing the temporary formation of “pores” or breaching of plasma membrane integrity that allows the entry of “foreign” molecules into the cell interior. However, compared to nsPEFs classical plasma membrane electroporation pulses are relatively long (microseconds to milliseconds) and with lower electric fields (= 1 kV/cm). Thus, nsPEFs can be orders of magnitude shorter in duration and higher in electric field. For example, during a 1ms electroporation pulse light travels 982,000 feet (186 miles). During a 10 ns pulse, light travels about 10 feet. Nevertheless, because nsPEF applications are an extension of classical plasma membrane electroporation, the effects of nsPEFs are often confused with effects of electroporation on the plasma membrane.

Not so! Especially as the pulse duration is decreased below the charging time of the plasma membrane. The exclamation point serves a special note that cell response phenomena have now significantly changed as the rise-time and the pulse duration are below times required to fully charge the plasma membrane. As opposed to responses to classical plasma membrane electroporation, nsPEF affect intracellular structures (membranes) and functions (cell signaling), which may or may not involve measurable responses from the outer plasma membrane. This primarily depends on the pulse duration, pulse rise time, and electric field. nsPEFs enter new biological and cellular vistas with dimensions, dynamics, and kinetics focused more on intracellular mechanisms [2, 3]. Nevertheless, nsPEFs have effects on the plasma membrane that are direct electric field, nonbiological effects, as well as secondary biological effects. While biological effects on the plasma membrane, such as phosphatidylserine (PS) externalization associated with apoptosis are readily measured, they can be confused with PS externalization resulting from direct electric field effects. Direct electric field effects on plasma membrane integrity are often harder to determine because they occur at levels that are often below the level of detection by fluorophores and/or molecular probes are too large for small pores, referred to as nanopores, which are believed to be present with nsPEFs. Such nanopores and nanochannels for phosphatidylserine externalization are predicted based on modeling and simulation studies [4]. What effects nsPEFs might have on proteins and ion channels are not yet investigated. How diminishing effects on plasma membrane structures and functions interface with increasing effects on intracellular structures and functions as the pulse duration decreases remain the basis for continued research in bioelectrics.

We have referred to the nsPEF-induced occurrence of greater effects on intracellular membranes and lesser effects on plasma membranes as intracellular electro-manipulation (IEM). We used the term “manipulation” instead of “electroporation” because it is yet to be determined whether nsPEF-induced effects on intracellular structures are similar to classical electroporation on the plasma membrane. Since nsPEFs applications are an extension of classical plasma membrane electroporation, it is reasonable and prudent to consider membrane charging as a mechanism for nsPEF effects. While the membranes may not be fully charged for a 10 ns pulse, they are charging during the pulse. However, as the pulse duration decreases to and below 1 ns, charging may not be a major factor. Here pure physics meets biology head on and new dimensions and other mechanisms may be encountered.

This introduction to nsPEFs provides all of the superlatives and fervor that could be expected from physicists and biologists with a brand new toy. So what data support the unique effects of nsPEFs on biological systems? Most of the work has been done on cells in cultures, however an increasing number of studies are being conducted on tissues, including fibrosarcoma and melanoma tumors, and more recently on adipose tissue and skin as an organ (see [2, 3] and references within). Cell culture models include HL-60, Jurkat, and HCT116,

the later including clones that are wildtype and null for p53. However, a number of normal human leukocytes and a wide range of cancer cells have been tested. Cell responses have been measured for nsPEF effects on plasma membranes (integrity, potential, and phosphatidylserine externalization), endoplasmic reticulum (calcium mobilization), mitochondria (respiration, cytochrome c release), and nucleus (fluorescence changes, DNA damage, roles for p53, and gene expression). We have also measured responses of adipose tissue, skin, and tumors. Studies with tumor tissues have determined responses from slowed tumor growth to tumor regression. These cell and tissue responses are distinct from response to classical plasma membrane electroporation.

A major question that remains to be fully investigated is the potential for nsPEF-induced cell-specific effects. There are two generalizations for cell-type-specific nsPEF-induced effects that have been defined. First, nsPEF-induced cell effects are not cell size-dependent as shown for classical plasma membrane electroporation where larger cells are more readily affected. While a well-controlled, extensive study has not been carried out for in vitro cell types, nsPEFs effects on the plasma membrane are more readily demonstrated in smaller cells compared to larger cells. Second, for a number of cell types tested, adherent cells have higher threshold for nsPEF-induced effects than cells that grow in suspension. Studies in progress are beginning to demonstrate selective effects in adipose tissue and skin.

It appears that nsPEFs can affect cells as a double-edged sword; that is at relatively high electric fields nsPEFs recruit apoptosis mechanisms, but at lower electric fields they recruit nonapoptotic signaling mechanisms [2, 3] . It is now becoming clear that proteins that regulate apoptosis are also involved in regulating nonapoptotic processes. For example, we have shown that nsPEFs can modulate caspase activity and caspases have been shown to modulate apoptotic and nonapoptotic cell functions [2, 3] including proliferation, cell cycle, differentiation, as well as programmed cell death. So at higher electric fields nsPEFs can induce apoptosis resulting in cell death and size reduction and/or ablation of tumors. This is observed as direct electric field effects or biological responses to electric fields in the absence of drugs. This is distinct from electrochemotherapy where classical plasma membrane electroporation allows the entry of chemotherapeutic drugs such as bleomysin, which is toxic to the tumor.

At lower electric fields and shorter pulse durations, nsPEF recruit cell signaling mechanisms that induce calcium mobilization and modulate calcium-mediated functions [2, 3] such as platelet activation and aggregation, which is important for blood clotting. Activation of human platelets, Jurkat cells, and HL-60 cells mimic responses to hormones that act through G-protein-coupled, plasma membrane receptors that involve IP3 receptors in the endoplasmic reticulum. Furthermore, abrupt calcium mobilization has been shown to immobilize human neutrophils, presumably due to interruption of signals that direct specialized and specific mobilization in response to chemotactic signals. The mechanism(s) for these calcium mobilization responses remain to be determined. Nevertheless, since calcium is an ubiquitous second messenger signal, nsPEF-induced calcium modulation could have a wide range of applications.

We hypothesized that if nsPEFs affected the nuclear membrane, plasmids and transfected genes may enter the nucleus more readily and gene transcription may be enhanced. We demonstrated that when a green fluorescent protein (GFP) expression plasmid was transfected into cells with a classical plasma membrane electroporation pulse and then followed by a nonapoptotic nsPEF in a low-conductivity media, the level of expression and the number of cells expressing GFP were increased significantly [2, 3] . The mechanism(s) for this result is not yet clear, but nsPEF-induced effects on the nucleus and DNA have been

reported. While some studies suggest that direct electric field effects damage DNA, other studies indicate effect at the nucleus, some of which are reversible and nonlethal. Other studies indicate that nsPEF affect expression of endogenous genes. It remains to be determined if effects on transcription are due to actions on the nuclear membrane, gene transcription mechanisms, or both.

While these observations are exciting, only a few groups have carried out experiments with nsPEFs, but the numbers are growing. This is because generating pulses with such short durations, rapid rise times, and high electric fields is not a common skill. Funding from the Department of Defense through the Air Force Office of Scientific Research by a Multi-University Research Initiative has enhanced the growing number of studies using this new technology. These include investigators at Old Dominion University, Norfolk, where the technology originated and the MURI is administrated; Eastern Virginia Medical School, Norfolk; Harvard/MIT, Cambridge; Washington University, St Louis; University of Texas Health Science Center, San Antonio; Wisconsin University, Madison; Purdue University, Calumet; and Northwestern University, Evanston. A group at University of Southern California with Martin Gundersen and Tom Vernier, also funded by AFOSR, has also been productive with this technology. Furthermore, two Centers of Excellence Programs have been funded in Japan to include investigations of nsPEF effects on biological cells. In addition, groups in England, France, and Germany have begun to establish programs related to bioelectrics research, seeking help from the Center of Bioelectrics in Norfolk. Furthermore, all of the funded groups are training students, some of whom will continue studies in bioelectrics. Moreover, an undergraduate/graduate course in Bioelectrics is now offered at Old Dominion University and a wider range of bioelectrics-related courses may be offered in the future. Thus, it is likely that as work continues in this field, it will expand to other groups with other methodologies and expertise, and enhance our understanding of mechanisms that cells use to respond to unique nsPEF stimuli.

So!! Where do we go from here? Our initial strategies were to do preliminary studies to investigate a number of hypotheses based on understandings from classical plasma membrane electroporation. This approach revealed that a number of projects were worthy of pursuit based on peer-review publications and a wealth of unpublished data. However, a “rich kid in the candy store” strategy will not be successful. First, we are not rich. This technology requires a funding stream that is not easily acquired, especially with a new technology that must build a respected foundation in the peer-review processes. Thus, personnel and resources are limited. Second, acquisition of funding from national foundations such as the NIH, among others, requires focus in areas that are well defined, supported by unshakable preliminary data, and assured of success. Some funding may be available from venture capitol groups and startup companies, but the risk/benefit ratio must be favorable and this is not immediate with a new technology. Therefore, we have developed a strategy that is based on available funds, resources, and personnel to carry forth a series of studies that will provide for the future of bioelectrics. Bioelectrics research will remain stimulating because there is plenty of intracellular territory to explore.

Towards Solid Tumor Treatment by Nanosecond Pulsed Electric Fields (p. 289-306)

Local and drug-free solid tumor ablation by large nanosecond pulsed electric fields leads to supra-electroporation of all cellular membranes and has been observed to trigger nonthermal

cell death by apoptosis. To establish pore-based effects as the underlying mechanism to inducing apoptosis, we use a multicellular system model (spatial scale 100 μm) that has irregularly shaped liver cells and a multiscale liver tissue model (spatial scale 200 mm). Pore histograms for the multicellular model demonstrate the presence of only nanometer-sized pores due to nanosecond electric field pulses. The number of pores in the plasma membrane is such that the average tissue conductance during nanosecond electric field pulses is even higher than for longer irreversible electroporation pulses. It is shown, however, that these nanometer-sized pores, although numerous, only significantly change the permeability of the cellular membranes to small ions, but not to larger molecules. Tumor ablation by nanosecond pulsed electric fields causes small to moderate temperature increases. Thus, the underlying mechanism(s) that trigger cell death by apoptosis must be non-thermal electrical interactions, presumably leading to different ionic and molecular transport than for much longer irreversible electroporation pulses.

Tissue ablation techniques to treat all cells of unwanted tissue for surgical, cosmetic, or other reasons are potentially valuable as minimally invasive clinical tools (1). Tissue ablation may be achieved by the application of RF electric fields that lead to cell death by overheating the targeted tissue. Strong electric field pulses that cause electroporation (EP) of cellular membranes, a universal mechanism by which cellular membranes become permeable to drugs, molecules, and genetic material, may alternatively be used for tissue ablation. Drug-assisted methods such as electro-chemotherapy (ECT) (2) and electro-genetherapy (3) are examples of such EP-based tissue ablation methods. There is now evidence, however, that drugs actually may not be necessary, since the impact of certain strong electric field pulses that cause EP itself is sufficient to trigger cellular mechanisms that lead to cellular death.

In a previous paper (4) we considered some fundamental mechanistic aspects that are relevant to solid tumor treatment by irreversible electroporation (IRE) (5-10). In short, IRE involves the application of pulses with a duration of typically hundreds of microseconds and an electric field strength on the order of a few kilovolts per centimeter (kV/cm). These IRE pulses are sufficient to cause what is known as conventional EP, for which pores in the plasma membrane (PM) of the cells are sufficiently large to facilitate molecular uptake and release. While those pulses reseal in most EP-based applications, IRE pulses are designed such that pores in the PM do not reseal - hence the PM is said to be irreversibly electroporated. Therefore, the barrier function of the PM is lost, leading to cellular death in a treated tissue region. Specifically, solid tumor treatment by IRE is observed to cause necrosis in the targeted cells (9).

Using Blumleln circuit-based or MOSFET-based pulsed power technologies, solid tumor treatment by much shorter nanosecond pulsed electric fields (nsPEFs) with durations of ten to several hundred nanoseconds and field strengths of tens or even hundreds of kV/cm has been demonstrated (11-14). In particular, Nuccitelli et al., (12, 14) used electric field pulses with durations of about 300 ns and with electric field strengths of 20 kV/cm and 40 kV/cm to demonstrate self-destruction of melanomas. Garon et al., (13) used electric field pulses that were even one order of magnitude shorter, namely 20 ns or less and field strengths up to 60 kV/cm. Their results showed decreased cell viability of a variety of human cancer cells in vitro, induction of tumor regression in vivo, and successful treatment of a human subject with a basal cell carcinoma for which they found a ?complete pathologic response?. Cell death does not appear to be due to immediate PM destruction, as expected for IRE pulses. Instead, it appears to be the result of delayed effects, which may be caused by the efflux of Ca2+ from intracellular stores (e.g., endosplasmic reticulum) that eventually cause apoptosis (13).

The concept of using nsPEF pulses as a therapeutic tool to treat solid tumors was first

demonstrated by Beebe et al., (11) who reported the induction of apoptosis in solid tumors (mouse fibrosarcoma) ex vivo and the reduction of fibrosarcoma tumor size in vivo by nsPEF pulses up to 300 kV/cm and durations from 10 ns to 300 ns. Signs of apoptotic cell death comprise cell shrinkage, activation of caspases, persistent externalization of phosphatidylserine (PS) at the PM, and fragmentation of DNA (11). Apoptosis induction by nsPEF has also been observed for mammalian cancer cells in vitro (15).

There have been several other interesting observations about the responses of cells and tissues to nsPEF pulses. First, EP markers such as propidium iodide (PI) and ethidium homodimer, whose uptake has been traditionally used to indicate the membrane integrity and permeability changes in the PM were reported to be taken up by cells only in very small amounts. However, we have noted that no measurement sensitivity was established and that uptake may be below the detection limit (16). Second, effects in the cell interior have been observed that have not generally been reported for conventional EP conditions. Calcium release from the endoplasmic reticulum, cytochrome-c release from mitochondria, phosphatidylserine translocation at the PM, and caspase activation are examples of such observed intracellular effects (11, 17-22), Those effects can be readily understood in terms of the supra-EP hypothesis (23-26) which leads to a different degree of EP as will be described in the present paper.

The often stated motivation for the recent focus on nanosecond pulses is the low energy density that is delivered per pulse despite the much larger field than for IRE pulses. This argument appears misleading, however, since it is certainly not the dissipative energy that causes cell death, as is the case for RF tumor ablation, but a non-linear biophysical mechanism such as EP. It is arguably more important that nsPEF pulses lead to strong electric fields both inside and outside the cells and thereby significantly perturb organelle membranes in addition to the PM (17, 25).

The consideration of both IRE and nsPEF pulses thus provides the tantalizing prospect of designing specific electric field pulse protocols and treatments that lead to different cell death mechanisms, for example necrosis with IRE pulses and apoptosis with nsPEF pulses. The ability to determine the cellular death mechanism by an appropriate choice of electric field parameters may seem like an unnecessary choice for a patient in urgent need of tumor treatment. Yet, the apoptotic cell death pathway may provide certain advantages. Specifically, if secondary necrosis can be avoided then it should be possible to bypass non-specific damage to nearby tissue due to e.g., inflammation and/or scarring. It might also be possible to avoid the tumor lysis syndrome resulting from massive tumor necrosis.

As the cellular and tissue response to nsPEF pulses is so distinctively different from the response to IRE pulses, the object of the present paper is to seek an underlying mechanistic understanding of the tissue response to nsPEF pulses. This is achieved by using exactly the same tissue models as used previously for IRE pulses (4), for which we determine the local electric fields and currents in the treated tissue and then study the biophysical response in terms of pore densities and pore sizes that lead to cell permeability changes, as well as the resulting temperature change in the tissue model. Throughout, we attempt to make objective comparisons between responses to nsPEF pulses and the much longer and weaker IRE pulses.

Methods

The basic methods and tissue models are the same as used previously (4) only the electric field pulse waveforms are different. In this way we can straightforwardly compare the mechanistic response at the cellular and tissue level to IRE pulses that lead to necrotic cell

death with nsPEF pulses that lead to apoptotic cell death. To facilitate understanding we have restated our methods here.

As previously (4) we use the transport lattice (TL) method, which allows for a convenient description of electrical, chemical, and thermal behavior in a complex biological geometry that may contain tissue inhomogeneities and anisotropies. Basic features of the TL method have been presented elsewhere (23-25, 27-29). For the study of nanosecond pulses to treat solid tumors, we use two system models, a multicellular model of irregular cells and a tissue model, and consider their electrical and thermal responses to two representative nsPEF pulses. Each system model represents rabbit liver tissue but on a different spatial scale. Although experiments with nsPEF pulses on tumors have been performed for different cells and tissues, a liver tissue model is adopted here in order to compare the distinct features of tissue responses due to nsPEF pulses with those of IRE pulses.

Discussion and Conclusion

The treatment of solid tumors by nsPEF pulses leads to supra-EP at the PM, a universal mechanism by which only small nanometer-sized pores at large densities perforate cell membranes, and a strong interplay of conduction and displacement currents inside the cells and the interstitial space of the tissue. As a consequence, the cell interior is affected by intracellular electric fields that are almost as strong as the applied field strength. What is often neglected in the discussion of EP is the resulting exposure of the interior of the cell, i.e., both the intracellular electrolyte and the intracellular organelles, to an electric field. The inevitability of creating an intracellular field should address the magnitude of the intracellular field, which may vary from unimportant to tremendously important. It is therefore understandable that nsPEF pulses cause pronounced intracellular effects, which presumably are responsible for triggering a different cell death mechanism (apoptosis) than longer IRE pulses (necrosis). Supra-EP at the PM leads to a conductance change of the membrane that exceeds that of longer IRE pulses...

Synergistic Effects of Nanosecond Pulsed Electric Fields Combined with Low Concentration of Gemcitabine on Human Oral Squamous Cell Carcinoma In Vitro

Jing Wang, et al.

Abstract

Treatment of cancer often involves uses of multiple therapeutic strategies with different mechanisms of action. In this study we investigated combinations of nanosecond pulsed electric fields (nsPEF) with low concentrations of gemcitabine on human oral cancer cells. Cells (Cal-27) were treated with pulse parameters (20 pulses, 100 ns in duration, intensities of 10, 30 and 60 kV/cm) and then cultured in medium with 0.01 μg/ml gemcitabine. Proliferation, apoptosis/necrosis, invasion and morphology of those cells were examined using MTT, flow cytometry, clonogenics, transwell migration and TEM assay. Results show that combination treatments of gemcitabine and nsPEFs exhibited significant synergistic activities versus individual treatments for inhibiting oral cancer cell proliferation and inducing apoptosis and necrosis. However, there was no apparent synergism for cell invasion. By this we demonstrated synergistic inhibition of Cal-27 cells in vitro by nsPEFs and gemcitabine. Synergistic behavior indicates that these two treatments have different sites of action and combination treatment allows reduced doses of gemcitabine and lower nsPEF

conditions, which may provide better treatment for patients than either treatment alone while reducing systemic toxicities.

Diverse Effects of Nanosecond Pulsed Electric Fields on Cells and Tissues

Stephen J. Beebe, Jody White, Peter F. Blackmore, Yuping Deng, Kenneth Somers, and Karl H. Schoenbach.

ABSTRACT

The application of pulsed electric fields to cells is extended to include nonthermal pulses with shorter durations (10-300 ns), higher electric fields (=350 kV/cm), higher power (gigawatts), and distinct effects (nsPEF) compared to classical electroporation. Here we define effects and explore potential application for nsPEF in biology and medicine. As the pulse duration is decreased below the plasma membrane charging time constant, plasma membrane effects decrease and intracellular effects predominate. NsPEFs induced apoptosis and caspase activation that was calcium-dependent (Jurkat cells) and calcium-independent (HL-60 and Jurkat cells). In mouse B10-2 fibrosarcoma tumors, nsPEFs induced caspase activation and DNA fragmentation ex vivo, and reduced tumor size in vivo. With conditions below thresholds for classical electroporation and apoptosis, nsPEF induced calcium release from intracellular stores and subsequent calcium influx through store-operated channels in the plasma membrane that mimicked purinergic receptor-mediated calcium mobilization. When nsPEF were applied after classical electroporation pulses, GFP reporter gene expression was enhanced above that observed for classical electroporation. These findings indicate that nsPEF extend classical electroporation to include events that primarily affect intracellular structures and functions. Potential applications for nsPEF include inducing apoptosis in cells and tumors, probing signal transduction mechanisms that determine cell fate, and enhancing gene expression.

Effect of nanosecond pulsed electric field on Escherichia coli in water: inactivation and impact on protein changes

A. Guionet1, et al.

Abstract
Aims

This article shows the effect of nanosecond pulsed electric field (nsPEF) on Escherichia coli, which could imply a durable change in protein expressions and then impacted the phenotype of surviving bacteria that might lead to increase pathogenicity.

Methods and Results

The effects of nsPEF on E. coli viability and membrane permeabilization were investigated. One log10 reduction in bacterial counts was achieved at field strength of 107 V m-1 with a train of 500 successive pulses of 60 × 10-9 s. Incubation of germs after treatment with propidium iodide showed that membrane permeabilization was reversible. Possible protein changes of surviving bacteria were checked to assess potential phenotypical changes using

two-dimensional electrophoresis. In our study, after 40 generations, only UniProt #P39187 was up-regulated with P = 0·05 compared with the control and corresponded to the uncharacterized protein YtfJ. Antibiograms were used to check whether or not the pattern of cultivable bacteria after nsPEF deliveries changed.

Conclusions

The results tend to show that nsPEFs are able to inactivate bacteria and have probably no serious impact in E. coli protein patterns.

Significance and Impact of the Study

The use of nsPEF is a safe promising new nonthermal method for bacterial inactivation in the food processing and environmental industry.

Nanosecond pulsed electric fields cause melanomas to self-destruct
WO2007100727

[Excerpt]

Abstract

Methods for a new, drug-free therapy for treating solid skin tumors through the application of nanosecond pulsed electric fields ('nsPEFs') are provided. In one embodiment of the invention, the cells are melanoma cells, and the applied nsPEFs penetrate into the interior of tumor cells and cause tumor cell nuclei to rapidly shrink and tumor blood flow to stop. This new technique provides a highly localized targeting of tumor cells with only minor effects on overlying skin.

Inventors Richard Nuccitelli, Stephen J Beebe, Karl H Schoenbach

BACKGROUND OF THE INVENTION

[0001] Electric fields have been employed in several different types of cancer therapy. Some of these involve radio frequency or microwave devices that heat the tumor to greater than 43 °C to kill the cells via hyperthermia (K.K.Tanabe, S.A.Curley, G.D.Dodd, A.E.Siperstein, S.N.Goldberg (2004) Cancer. 100:641-650; D.Haemmerich, P.F.Laeseke (2005) Int. J. Hyperthermia. 21 : 755-760). Others use pulsed electric fields to permeabilize the tumor cells to allow the introduction of toxic drugs or DNA (M.L.Lucas, R.Heller (2003) DNA Cell Biol. 22:755-763; Y.Kubota, Y.Tomita, M.Tsukigi, H.Kurachi, T.Motoyama, L.M.Mir (2005) Melanoma Res. 15:133-134; A.Gothelf, L.M.Mir, J.Gehl (2003) Cancer Treat.Rev. 29:371-387). Previous studies have shown that fibrosarcoma tumors, treated in situ with nanosecond pulsed electric fields, exhibited a reduced growth rate compared to control tumors in the same animal (S.J.Beebe, P.Fox, LJ.Rec, K.Somers, R.H.Stark, K.H.Schoenbach (2002) IEEE Transactions on Plasma Science. 30:286-292). [0002] The main characteristics of nanosecond pulsed electric fields (nsPEF) are their low energy that leads to very little heat production and their ability to penetrate into the cell to permeabilize intracellular organelles (K.H.Schoenbach, S. J.Beebe, E.S.Buescher (2001) Bioelectromagnetics. 22:440-448; E.S.Buescher, K.H.Schoenbach (2003) IEEE Transactions on Dielectrics and Electrical Insulation. 10:788-794) and release calcium (P.T. Vernier, Y.H.Sun, L.Marcu, S.Salemi,

C.M.Craft, M.A.Gundersen (2003) B B R C. 310:286-295; E.S.Buescher, R.R.Smith, K.H.Schoenbach (2004) IEEE Transactions on Plasma Science 32:1563-1572; J.A. White, P.F.Blackmore, K.H.Schoenbach, S.J.Beebe (2004) J.Biol.Chem. 279:22964-22972) from the endoplasmic reticulum (J.A. White et al. (2004) J.Biol.Chem). They provide a new approach for physically targeting intracellular organelles with many applications, including the initiation of apoptosis in cultured cells (S.J.Beebe, P.M.Fox, L.J.Rec, EX. Willis, K.H.Schoenbach (2003) FASEB J. 17:1493-1495; S.J.Beebe, J.White, P.F.Blackmore, Y.Deng, K.Somers, K.H.Schoenbach (2003) DNA Cell Biol. 22:785-796; SJ.Beebe, P.F.Blackmore, J.White, R.P.Joshi, K.H.Schoenbach (2004) Physiol Meas. 25: 1077-1093) and tumors (S.J.Beebe et al. (2002) IEEE Transactions on Plasma Science) enhancement of gene transfection efficiency (S.J.Beebe et al. (2003) DNA Cell Biol; SJ.Beebe et al. (2004) Physiol Meas.) and reducing tumor growth (S.J.Beebe et al. (2002) IEEE Transactions on Plasma Science).

[0003] The use of electric fields on biological cells to rupture the cell membrane can lead to cell death via necrosis, a nonphysiological type of cell destruction, while the use of nsPEFs on biological cells to permeabilize intracellular organelles can initiate cell death via apoptosis. When treating biological cells within tissue in situ, being able to initiate cell death via apoptosis would allow the destruction of specific undesired cells in situ without engendering the non-specific damage to surrounding or nearby tissue in the body due to inflammation and scarring that is normally observed with necrosis. Investigations of the effects of ultrashort, high intensity pulsed electric fields or nanosecond pulsed electric fields (nsPEF) on mammalian cells have demonstrated distinct differences on cell structure and function compared to classical plasma membrane electroporation. It was previously demonstrated that nsPEF invoked signal transduction mechanisms that initiate apoptosis cascades in several human cell lines including HL-60 cells (Beebe, S. J., et al. (2002) IEEE Trans. Plasma ScL 30, 286-292; Beebe, SJ., et al. (2003) FASEB J. 17, 1493-1495).

[0004] The efficacy of this nsPEF treatment is believed to depend on two separate electric field parameters: pulse duration and amplitude. The effect of pulse duration can be understood by considering the process of membrane charging when the cell is placed in an electric field. Ions in the cell interior will respond to the electric field by moving in the field direction and charging the highly resistive membrane until they experience no further force. By definition this will only occur when their redistribution establishes an equal and opposite field so that the net electric field in the cell interior is zero. However this redistribution takes a certain amount of time that is characterized by the charging time constant of the plasma membrane, typically in the 0.1 to 1 microsecond range. If the nsPEF is shorter than this charging time, the interior charges will not have sufficient time to redistribute to counteract the imposed field and it will penetrate into the cell and charge every organelle membrane for a duration which is dependent on both the charging time constant of the cell's plasma membrane as well as that of the organelle membrane (K.H.Schoenbach, R.P.Joshi, J.F.Kolb, N.Chen, M.Stacey, P.F.BIackmore,E.S.Buescher, S.J.Beebe (2004) Proc. IEEE. 92:1122-1137).

[0005] A second critical nsPEF parameter is the amplitude of the pulse. Both the force exerted on charges and the electroporation of lipid membranes depend on the strength of the electric field. When the electric field across a cellular membrane exceeds about 1 volt (2 kV/cm for a cell 10 μm in diameter), water-filled pores form in the membrane's lipid bilayer and the size and lifetime of these pores are dependent on the strength and duration of the electric field pulse. For amplitudes exceeding 2 kV/cm and pulse durations in the millisecond range, large pores form resulting in electroporation of the membrane that has been used to introduce normally impermeant anticancer drugs into targeted tissues (M.L.Lucas et al (2003)

DNA Cell Biol.; Y.Kubota et al (2005) Melanoma Res.; A.Gothelf et al (2003) Cancer Treat.Rev.; J.Teissie, M.Golzio, M.P.Rols (2005) Biochim.Biophys.Acta 1724:270-280). For these long pulses, the pulse amplitude is limited to about 2 kV/cm to avoid thermal effects. Since heating is proportional to pulse duration and the square of the field strength, the much shorter pulses in the nanosecond range can have a higher field strength while delivering the same low level of thermal energy to the tissue. A 20-fold higher field strength of 40 kV/cm can be employed to generate structural changes in the plasma membrane that result in a smaller electrical barrier as well as higher voltage gradients across cellular organelles for die duration of the pulse (Q.Hu, S.Viswanadham, R.PJoshi, K.H.Schoenbach, SJ.Beebe, P.F.Blackmore (2005) Phys.Rev.E Stat.Nonlin.Soß.Matter Phys.7?:031914-l-0319l4-9). A typical tumor cell nucleus measuring 10 μm in diameter will experience a voltage gradient of roughly 40 V across its diameter during each pulse. This electric field is large enough to cause electrodeformation (R.PJoshi, Q.Hu, K.H.Schoenbach, H.P.Hjalmarson (2002) Phys.Rev.E Stat.Nonlin.Soft.Matter Phys. 65:021913).

[0006] Previous studies provided direct evidence for cellular DNA as a direct or indirect target of nsPEF. Using a comet assay, Stacey, et al. (M.Stacey, J.Stickley, P.Fox, V.Statler, K.Schoenbach, SJ.Beebe, S.Buescher (2003) Mutat.Res. 542:65-75) found that ten 60 ns pulses of 60 kV/cm caused a rapid 2.6-fold increase in the mean image length of DNA electrophoresis tracks in Jurkat cell extracts and a 1.6-fold increase in the comet assay from HL60 cell extracts. In both cases this was a very significant change ($p<0.001$). This elongation in DNA electrophoresis tracks is normally interpreted to indicate fragmentation of the DNA into smaller pieces that is associated with apoptotic cell death. An indication of changes in the DNA following nsPEF treatment comes from images of the nucleus labeled with acridine orange, a vital fluorescent dye that intercalates into DNA and RNA, Chen et al. (N.Chen, K.H.Schoenbach, J.F.Kolb, S.RJames, A.L.Garner, J.Yang, R.PJoshi, SJ.Beebe (2004) Biochem.Biophys.Res.Commun. 317:421-427). A single 10 ns pulse of 26 kV/cm caused a dramatic decrease in fluorescence intensity in the nucleus evident as early as 5 min after the pulse. This change could be due to an outflow of DNA or to conformational changes in the DNA. [0007] The ability to selectively modify specific cells in ways that lead to apoptosis could provide a new method for the selective destruction of undesired tissue (e.g., cancer cells, fat cells or cartilage cells) while minimizing side effects on surrounding tissue. An electrical method of treatment that results, not only in tumor growth inhibition, but in complete tumor regression, without hyperthermia, drugs, or significant side effects, would be a great advancement in the field of cancer therapy and other in situ therapies. These and various other needs are addressed, at least in part, by one or more embodiments of the present invention.

BRIEF SUMMARY OF THE INVENTION

[0008] One or more aspects of the invention provide a method for selectively initiating apoptosis in target cells in a tissue. The method comprises applying at least one nsPEF to said tissue. The at least one nsPEF has a pulse duration of at least about 10 nanoseconds and no more than about 1 microsecond and an electric field pulse strength of at least about 10 kV/cm and no more than about 350 kV/cm. In one or more embodiments of the invention, the method is carried out in situ.

[0009] In one aspect, at least one nsPEF has a pulse duration of about 300 nanoseconds and an electric field pulse strength of at least about 20 kV/cm and no more than about 40 kV/cm.

[0010] In one or more embodiments of the invention, at least 100 nsPEFs are applied to said tissue. In one aspect, at least 300 nsPEFs are applied to the tissue. In another aspect, at least

400 nsPEFS are applied to the tissue. In yet another embodiment of the invention, the method of treatment of at least one nsPEF is repeated.

[0011] In one or more aspects of the invention, the target cells are fat cells. In one or more aspects of the invention, the target cells are bone cells. In one or more aspects of the invention, the target cells are vascular cells. In one or more aspects of the invention, the target cells are muscle cells. In one or more aspects of the invention, the target cells are cartilage cells. In one or more aspects of the invention, the target cells are stem cells. In one or more aspects of the invention, the target cells are a combination of the above cells. [0012] Also provided in the invention is a method for inhibiting blood flow in a tissue. The method comprises applying at least one nsPEF to said tissue. The at least one nsPEF has a pulse duration of at least about 10 nanoseconds and no more than about 1 microsecond and an electric field pulse strength of at least about 10 kV/cm and no more than about 350 kV/cm. In one or more embodiments of the invention, the method is carried out in situ. [0013] The invention also provides a method for inducing tumor regression. The method comprises applying at least one nsPEF to said tumor. The at least one nsPEF has a pulse duration of at least about 10 nanoseconds and no more than about 1 microsecond and an electric field pulse strength of at least about 10 kV/cm and no more than about 350 kV/cm. In one or more embodiments of the invention, the method is carried out in situ...

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WO2010107947
Nanosecond pulsed electric field parameters for destroying tumors with a single treatment

[Excerpt]

Nanosecond pulsed electric field (nsPEF) parameters for destroying tumors with a single treatment are described. A nsPEF generator may be used with an electrode assembly to apply the pulses to one or more tumors where the parameters for the nsPEF are optimized for treating such tumors. The system may also be used to treat tumors on or within internal organs by using an expandable bipolar electrode assembly that can be imaged by ultrasound and can penetrate, e.g., the stomach, intestine or bowel wall, etc. and be positioned in or around the tumor on an internal organ while being guided by an operator who visualizes its position with ultrasound imaging. It utilizes an electrode assembly that extends down an internal cavity in the endoscope to allow the operator to spread the electrodes for pulse delivery of a nanosecond pulsed electric field (nsPEF) to the tumor.

FIELD OF THE INVENTION

[0002] This application is directed to systems and methods for treating tumors on internal organs that have been identified using endoscopic ultrasound by precisely positioning a pulsed field delivery device on or in the tumor guided by ultrasound imaging.

BACKGROUND OF THE INVENTION

[0003] Endoscopic ultrasound (EUS) combines endoscopy and ultrasound in order to obtain images and information about the digestive tract and the surrounding tissue and organs.

Endoscopy refers to the procedure of inserting a long flexible tube via the mouth or the rectum to visualize the digestive tract, whereas ultrasound uses high-frequency sound waves to produce images of the organs and structures inside the body such as ovaries, uterus, liver, gallbladder, pancreas, aorta, etc.

[0004] In EUS a small ultrasound transducer is installed on the tip of the endoscope. By inserting the endoscope through the esophagus into the stomach, the ultrasound transducer can be placed against the inner surface of the stomach or gastrointestinal tract so that sound waves can be beamed through the stomach wall to obtain high quality ultrasound images of the organs on the other side of the stomach wall such as the kidney, pancreas and liver. Because of the proximity of the EUS transducer to the organ(s) of interest, the images obtained are frequently more accurate and more detailed than the ones obtained by traditional ultrasound where the transducer in placed on the skin. Tumors on internal organs have ultrasound reflection properties that are different from the organ so that they can be easily detected with EUS. An example of EUS is shown and described in U.S. Pat. 7,318,806, which is incorporated herein by reference in its entirety.

[0005] Some of these ultrasound imaging endoscopes have been designed with an open channel down the center into which a fine needle aspirator or other instruments can be inserted to allow the sampling of tumor tissue by poking through the stomach wall and into the tumor tissue for aspiration. The aspirated tissue sample can then be stained and observed by a pathologist to obtain an immediate diagnosis of malignancy.

[0006] Nanosecond pulsed electric fields (nsPEF) have been found to trigger both necrosis and apoptosis in skin tumors. Treatment with nsPEF independently initiates the process of apoptosis within the tumor cells themselves causing the tumor to slowly self- destruct without requiring toxic drugs or permanent p[epsilon]rmeabi[iota]ization. In addition to initiating apoptosis in the tumor cells, nanosecond pulsed electric fields halt blood flow in the capillaries feeding it which in turn reduces blood flow to the tumor and activation of apoptosis pathways causing the tumor to slowly shrink and disappear within an average of 47 days.

[0007] An example of nsPEF is shown and described in U.S. Pat. 6,326,177, which is incorporated herein by reference in its entirety.

[0008] Various devices utilizing ElTS are known yet they are generally insufficient to treat tumors accessible via endoscopic access utilizing nsPEF. Accordingly, there exists a need for methods and devices which are efficacious and safe in facilitating the treatment of tumors in patients.

SUMMARY OF THE INVENTION

[0009] In delivering nanosecond pulsed electric fields (nsPEF) to a region of tissue. such as a tumor, it is possible to precisely control the number of pulses delivered as well as the frequency of those pulses to deliver electrotherapy via an electrode assembly designed to draw tissue into a recessed cavity in order to immobilize the tissue and position the electrodes firmly against or within the tissue. The recessed cavity may be varied in its size to match a size of any particular rumor to be treated such that the treated tumor may be received within the cavity in close proximity or in direct contact against the electrodes.

[0010] The electrode assembly may be configured into a variety of configurations for delivering electrotherapy and may also utilize suction to fix in place the tissue being treated.

For example, six (6) spaced apart planar electrodes may be positioned circumferentially about the recessed cavity. In other variations, the electrode assembly may comprise a support member having a pair of "U"-shaped planar electrodes disposed on the periphery of the recessed cavity. Other variations may include a pair of spaced apart parallel plate electrodes while other variations may include a plurality of needle electrodes which are mounted at the base of a back plate to control the penetration depth of the tissue as it it> sucked into the recessed cavity.

[0011] The back plate of each recessed cavity may have multiple apertures, such as on the order of 100 [mu]m in diameter. An air pump, e.g., an oscillating diaphragm air pump or other suction source, is then coupled to the support member on the side of the base w all support opposite the recessed cavity and is used to generate a mild suction that pulls the tissue to be treated into the cup-like volume.

[0012] In use, the support member may Miction or draw in tissue to be treated from various regions of the body into the recessed cavity into contact or proximity to the electrodes. Drawing in the tissue may further facilitate tissue treatment by clearly defining the treatment area to be treated for the operator. When nsPEF is applied to a tissue region such as a tumor, if a [iota]arge resistance between the electrode and the tumor restricts current flow (such as the presence of the stratum corneitm in skin), the field may not pass into the tumor effectively. Thus it may be desirable to apply, in one example, a minimum current of 20 A (although lower currents may be applied if so desired) that may pass through the tumor during nsPEF application to have a desired effect of triggering tumor apoptosis. In order to prevent damage to tissues surrounding the tumor, the nsPEF therapy may be applied at a pulse frequency that will not heat the tissue above, e.g.. 40 <0>C (the minimum temperature for hyperthermia effects). Therapy \\ ith nsPEF treatment is thus able to initiate apoptosis within the tumor cells without raising the temperature more than a few degrees so as to prevent harm to surrounding tissues from heat transfer. In one example, if 100 ns pulses were applied, the frequency of the applied pulses is desirably 7 pulses per second (Hz) or lower to prevent damage to surrounding tissues.

[0013] With the electrode assemblies described herein, treatment of tissue regions such as skin tumors may be effected by applying nsPEF while specifying various parameters. For instance, one or all of the following parameters may be adjusted to provide optimal treatment of tissue to effect tumor apoptosis: (1) pulse amplitude (kV'cm); (2) pulse duration (ns); (3) pulse application frequency (Hz); and/or (4) pulse number applied.

[0014] Because the value of these parameters may vary widely over a number of ranges, it has been determined that particular ranges may be applied for effecting optimal tissue treatment which may effect tumor apoptosis in as few as a single treatment. In varying pulse amplitude, an applied amplitude as low as, e.g., 20 kV/<'>cm, may be sufficient for initiating an apoptotic response in the treated tissue. The pulse amplitude may, of course, be increased from 20 kV/cm, e.g., up to 40 kV/'cm or greater. However, an applied amplitude of at least, e.g., 30 kV/cm or greater, may be applied for optimal response in the treated tissue, In varying pulse duration, durations in the range of. e.g., 50-900 ns, may be highly effective although shorter durations may be applied if the number of pulses is increased exponentially. In varying pulse application frequency, frequencies up to 7 Hz may be applied with 100 ns pulses without heating surrounding tissues to hyperthermic levels. Because tissue heating may be dependent on pulse width multiplied by the frequency of application, shorter pulses may be applied at proportionately higher frequencies with similar heat generation. In varying the number of pulses applied, the pulse number determines the total energy applied to the tissue region. Generally, applying a minimum pulse number of 600 pulses may result in

complete remission of tumors. In one example, nsPEF therapy having a pulse duration of 100 ns may be applied over a range of, e.g., 1000-2000 pulses, to effectively treat the tissue region.

[0015] Given the range of parameters, a relationship between these parameters has been correlated to determine a minimum number of electrical pulses which may effectively treat a tissue region, e.g., a tumor, with a single treatment of nsPEF therapy to cause complete apoptosis in the tumor tissue. Generally, the number of electrical pulses increases exponentially as the pulse duration is shortened. The correlation for a given pulse duration or width and number of pulses, N, to effectuate complete tumor remission after a single treatment may be described in the following equation:
$N = 28.714\ e^{-0026f}$
where, N = minimum number of pulses to cause tumor apoptosis with a single treatment t = pulse duration (in nanoseconds)

[0016] This non-linear dependence of pulse number on pulse width suggests that the effectiveness of the nsPEF therapy described herein is not simply due to energy delivery to the tumor as that is linearly proportional to N times t given a constant voltage and current.

[0017] In one particular variation, an elongate instrument which may be delivered via or through an endoscopic device may utilize any one or more of the nsPEF parameters described herein for tumor treatment. The endoscopic device, particularly an EUS device. may be used to image or locate a tissue region to be treated. Ultrasound imaging may be particularly useful in locating one or more tumors for treatment although conventional endoscopic imaging may also be utilized. With the targeted tissue region located within the body, the nsPEF instrument may be positioned or advanced within one or more working channels in the endoscope until a tapered piercing end of the nsPEF instrument is projected from a distal end of the endoscope.

[0018] The nsPEF instrument may also have an expandable or re configurable bipolar electrode assembly that may extend or reposition itself into a deployed profile. With the electrode assembly deployed, the piercing tip may be penetrated into or through the tissue to be treated (such as the tumor) while under the guidance of ultrasound imaging from the endoscope for desirably positioning the instrument. The outer reference electrodes may be actuated by the operator to reconfigure the electrodes for pulse delivery and/or to retract them for insertion and/ or withdrawal from the patient. Moreover, the electrodes may be coupled via an electrical cable having at least two conductors which may also extend through the endoscope or electrode assembly to conduct the pulsed electric fields to the distal end effector.

[0019] The electrode assembly may be advanced into the tissue to be treated until the deployed outer electrodes are positioned on or against a surface of the tumor or tissue region. The deployed outer electrodes may be spread or reconfigured into a variety of shapes, e.g.. hemi -circular plate configuration. Moreover, to facilitate contact between the electrode assembly and the tissue surface of the targeted tumor, suction may be applied through, e.g., a working channel either through the endoscope, electrode assembly, or both for drawing the tumor into apposition against the electrode assembly. Other mechanisms such as tissue graspers may also be used.

[0020] In other variations, the electrode assembly may be configured to project distally from the shaft of the nsPEF instrument to surround the tissue to be treated, such as a tumor. Various numbers of conductive needles may be utilized such that the tumor to be treated is surrounded by the needle array and the electric field created between the needles may be

uniformly applied to the tumor. These instruments may utilize any of the nsPEF parameters as described herein to effectively treat the tissue with, e.g., a single treatment of nsPEF therapy.

[0021] Follow ing nanosecond pulse application, the treatment instrument may be withdrawn from the tumor and the outer electrodes may be reconfigured back to their original low profile configuration for retraction back into the endoscope...

US2014106430
ACTIVATION AND AGGREGATION OF HUMAN PLATELETS AND FORMATION OF PLATELET GELS BY NANOSECOND PULSED ELECTRIC FIELDS

[Excerpt]

Also published as: WO2010057021 // JP2012508771 // EP2364155

Methods for forming activated platelet gels using nsPEF's and applying the activated gels to wounds, such as heart tissue after myocardial infarction. The platelets are activated by applying at least one nsPEF with a duration between about 10 picoseconds to 1 microsecond and electrical field strengths between about 10 kV/cm and 350 kV/cm.

BACKGROUND OF THE INVENTION

[0002] Electric fields can be used to manipulate cell function in a variety of ways. One specific cell mechanism that can be affected by electric fields is calcium mobilization within a cell. Calcium signaling, an important cell function, is responsible for a variety of cellular responses and actions. The release of internally stored calcium can stimulate responses to agonists, activate growth and respiration, cause the secretion of neurotransmitters, activate transcription mechanisms, cause the release of a variety of hormones, produce muscle contractions, and initiate release of key factors in the apoptosis pathway (Berridge, M. J., Bootman, M. D., Lipp, P. (1998) Nature. 395, 645-648). This calcium mobilization also triggers the influx of calcium from the external medium into the cell as a means of further propagating calcium signals and also replenishing depleted pools of calcium. Electric fields can be used to manipulate the movement of ions, such as calcium, in order to study calcium signaling.

[0003] One application of this calcium increase is to activate platelets and cause them to aggregate in vitro and in vivo. Platelet activation/aggregation is important for preventing blood loss during traumatic injury or surgery by forming a hemostatic plug at the site of injury. At present, treatment with thrombin, known to increase intracellular calcium in human platelets, is used to control slow bleeding at sites of injury. Thrombin treatment includes the topical application of bovine or recombinant thrombin, or the use of platelet gels in which autologous platelets are treated with bovine thrombin and added to the surgical site (Brissett and Hom (2003) Curr. Opin. Otolaryngol. Head Neck Surgery 11, 245-250; Man et al., (2001) Plast. Reconstr. Surg. 107, 229-237; Saltz (2001) Plast. Reconstr. Surg. 107, 238-239; Bhanot and Alex (2002) Facial Plast. Surg. 18, 27-33). However, the use of animal products could cause allergic reactions or cause possible contamination of platelet rich plasma (PRP) with infectious agents. The use of recombinant thrombin or a peptide that mimics thrombin action could be used as an alternative to animal-derived thrombin; however, this type of treatment is expensive and could also give rise to allergic reactions.

[0004] Since calcium signaling plays such an important role in so many cellular functions, there remains a need to further examine this signaling mechanism and explore ways to manipulate calcium signaling pathways for therapeutic purposes. For example, there is a need to develop methods of activating calcium-mediated cell functions, including aggregation of human platelets, for therapeutic purposes, such as wound healing. These and various other needs are addressed, at least in part, by one or more embodiments of the present invention.

SUMMARY OF THE INVENTION

[0005] One or more aspects of the invention provide a method for inducing calcium mobilization in a cell. The method comprises applying at least one electrical pulse to one or more cells, whereby calcium is mobilized in the cells. According to at least one embodiment, the electrical pulse comprises at least one nanosecond pulsed electric field (nsPEF). The at least one nsPEF has a pulse duration of at least about 100 picoseconds and no more than about 1 microsecond and an electric field strength of at least about 10 kV/cm and no more than about 350 kV/cm. In one or more embodiments of the invention, calcium influx into the cells occurs.

[0006] In one or more aspects of the invention, the cells are human platelets, whereby activation and aggregation of the platelets is induced.

[0007] The invention also provides a method for increasing intracellular calcium in cells comprising applying at least one nsPEF to the cells, whereby intracellular calcium in the cells is increased. The at least one nsPEF has a pulse duration of at least about 100 picoseconds and no more than about 1 microsecond and an electric field strength of at least about 10 kV/cm and no more than about 350 kV/cm. In one or more embodiments, the cells are human platelets, whereby activation and aggregation of the platelets is induced.

[0008] Also provided in the invention is a method for activating and aggregating human platelets comprising applying at least one nsPEF to the platelets, whereby the platelets are activated and induced to form aggregates. The at least one nsPEF has a pulse duration of at least about 100 picoseconds and no more than about 1 microsecond and an electric field strength of at least about 10 kV/cm and no more than about 350 kV/cm. In one aspect, the at least one nsPEF has a pulse duration of about 10 nanoseconds and an electric field strength of about 125 kV/cm. In another aspect, the at least one nsPEF has a pulse duration of about 60 nanoseconds and an electric field strength of about 30 kV/cm. In another embodiment, the at least one nsPEF has a pulse duration of 300 nanoseconds and an electric field strength of 30 kV/cm. The platelets may be suspended in a medium or included in a tissue or in a natural or synthetic tissue repair matrix, such as but not limited to bioresorbable collagen scaffold or matrix, or incorporated into bandage or wound closure devices. In other embodiments, activated platelets are applied or incorporated into bandages or sutures that may be applied to a wound.

[0009] The invention also provides a method of treating an injury, trauma, or the loss of blood in a subject, comprising applying at least one nsPEF to autologous platelets, whereby the platelets are activated and induced to form aggregates. The activated and aggregated platelets are then applied to the site of injury, trauma, or blood loss. The at least one nsPEF has a pulse duration of at least about 100 picoseconds and no more than about 1 microsecond and an electric field strength of at least about 10 kV/cm and no more than about 350 kV/cm. The blood loss in a subject may be related to a bleeding disorder resulting from inactive platelets or low platelet counts. The blood loss may also be related to a platelet disorder such

as congenital afibrinogenemia, Glanzmann's thrombasthenia, gray platelet syndrome, and Hermansky-Pudlak syndrome.

[0010] As a further embodiment for the preparation of activated platelet aggregations, at least another aspect of the invention provides a method for preparing platelet gels comprising human platelets comprising applying at least one nsPEF to the platelets, whereby the platelets are activated. The at least one nsPEF has a pulse duration of at least about 100 picoseconds and no more than about 1 microsecond and an electric field strength of at least about 10 kV/cm and no more than about 350 kV/cm. In one aspect, the at least one nsPEF has a pulse duration of about 10 nanoseconds and an electric field strength of about 125 kV/cm. In another aspect, the at least one nsPEF has a pulse duration of about 60 nanoseconds and an electric field strength of about 30 kV/cm. In another embodiment, the at least one nsPEF has a pulse duration of 300 nanoseconds and an electric field strength of 30 kV/cm. The platelets may be suspended in a medium or included in a tissue or in a natural or synthetic tissue repair matrix, such as but not limited to bioresorbable collagen scaffold or matrix, or incorporated into a bandage or wound closure devices.

[0011] At least another aspect of the invention provides a method for treating an injury, trauma, or the loss of blood in a subject, comprising applying platelets at or near the site of injury, trauma, or blood loss, whereby the platelets are activated and induced to form gels through application of at least one nsPEF. The at least one nsPEF has a pulse duration of at least about 100 picoseconds and no more than about 1 microsecond and an electric field strength of at least about 10 kV/cm and no more than about 350 kV/cm.

[0012] At least another aspect of the invention provides a method for treating and/or preventing infection at the site of an injury, trauma, or the loss of blood in a subject, comprising applying platelets at the site of injury, trauma, or blood loss, whereby the platelets are activated and induced to form gels through application of at least one nsPEF. The at least one nsPEF has a pulse duration of at least about 100 picoseconds and no more than about 1 microsecond and an electric field strength of at least about 10 kV/cm and no more than about 350 kV/cm. In another embodiment, the at least one nsPEF has a pulse duration of 300 nanoseconds and an electric field strength of 30 kV/cm.

[0013] At least another aspect of the invention provides a method for altering the acute changes in systolic and diastolic pressures in the left ventricle of the heart after an ischemic event, such as ischemia-reperfusion, whereby the platelets are activated and induced to form gels through application of at least one nsPEF and injected into the myocardial tissue. The at least one nsPEF has a pulse duration of at least about 100 picoseconds and no more than about 1 microsecond and an electric field strength of at least about 10 kV/cm and no more than about 350 kV/cm. In another embodiment, the at least one nsPEF has a pulse duration of 300 nanoseconds and an electric field strength of 30 kV/cm.

[0014] At least another aspect of the invention envisions the application of activated platelets to the surface of the heart, whereby the platelets are activated and induced to form gels through application of at least one nsPEF. The at least one nsPEF has a pulse duration of at least about 100 picoseconds and no more than about 1 microsecond and an electric field strength of at least about 10 kV/cm and no more than about 350 kV/cm. In another embodiment, the at least one nsPEF has a pulse duration of 300 nanoseconds and an electric field strength of 30 kV/cm.

[0015] At least another aspect of the present invention provides a bandage or wound closure device, such as a suture, containing an application or suspension of activated platelet gel,

whereby the platelets are activated and induced to form gels through application of at least one nsPEF. Various embodiments envision activation of platelets before and after application of the platelet gel to the bandage, where the at least one nsPEF has a pulse duration of at least about 100 picoseconds and no more than about 1 microsecond and an electric field strength of at least about 10 kV/cm and no more than about 350 kV/cm...
