Pulsed Electric Fields for Burn Wound Disinfection in a Murine Model

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Emerging bacterial resistance renders many antibiotics ineffective, making alternative strategies of wound disinfection important. Here the authors report on a new, physical burn wound disinfection method: pulsed electric fields (PEFs). High voltage, short PEFs create nonthermal, permanent damage to cell membranes, possibly by irreversible electroporation. In medicine, PEF technology has recently been used for nonthermal ablation of solid tumors. The authors have expanded the spectrum of PEF applications in medicine to burn wound disinfection. A third-degree burn was induced on the dorsal skin of C57BL/6 mice. Immediately after the injury, the burn wound was infected with *Acinetobacter baumannii* expressing the *luxCDABE* operon. Thirty minutes after infection, the infected areas were treated with 80 pulses delivered at 500 V/mm, 70 μs, 1 Hz. The authors used bioluminescence to quantify bacteria on skin. Three animals were used for each experimental condition. PEFs were effective in the disinfection of infected burned murine skin. The bacterial load reduction correlated with the number of delivered pulses. Forty pulses of 500 V/mm led to a 2.04 ± 0.29 Log10 reduction in bacterial load; 80 pulses led to the immediate 5.53 ± 0.30 Log10 reduction. Three hours after PEF, the bacterial reduction of the skin treated with 500 V/mm, 80 pulses was 4.91 ± 0.71 Log10. The authors introduce a new method of wound disinfection using high voltage, short PEFs. They believe that PEF technology may represent an important alternative to antibiotics in addressing bacterial contamination of wounds, particularly those contaminated with multidrug-resistant bacteria. (J Burn Care Res 2015;36:7–13)

In the United States in 2010, a fire injury occurred every 30 minutes leading to 3120 deaths and 17,720 injuries. Fire and burn injuries represent 1% of total injuries and cost $7.5 billion in total treatment and rehabilitation expenses each year.[1] Until wounds are closed, burn patients are subject to multiple septic complications.[2] Wound sepsis is the major cause of death in those who succumb to their injuries.[2,3] Bacteria, fungi, and viral infections are the reported culprits.[2,3] The most difficult to manage are those wound infections involving multidrug-resistant bacteria.[2]

Pathogens that infect burn wounds are primarily *Acinetobacter baumannii,* methicillin-resistant *Staphylococcus aureus, Pseudomonas,* and *Klebsiella*.[4] Factors that lead to improved clinical outcomes include early surgical debridement and skin grafting, topical and prophylactic antibiotics, as well as other general methods of infection control.[5] One of the serious challenges for burn patients is emerging antimicrobial resistance and nosocomial outbreaks.[3,6–8] A recent 2013 study, which reviewed 36 studies involving 2117 participants, concluded that there is no clear evidence of the efficacy of antibiotic treatment in wounds. Moreover, one of the

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widely used antibiotics—silver sulfadiazine—applied directly to the burn actually increased the rate of infection.[9] Clearly, new methods are needed for disinfection of burn wounds contaminated with multidrug-resistant bacteria.[4] In this paper, we report on the pulsed electric field (PEF) method for burn wound disinfection.

PEF technology emerged in the food processing industry as a method of bacterial decontamination. Centralized food production and preservation, which began in 10 to 11 BC, has been essential to the development of complex societies.[10] The search for new technologies has emphasized the development of chemical-free nonthermal methods for bacterial disinfection. Among these methods are PEF, high pressure, and pulsed light.[11] High voltage, short PEFs induce nonthermal permanent damage to cell membranes, presumably through irreversible electroporation.[12,13] Over the past four decades, hundreds of publications have demonstrated the efficacy of PEF technology for disinfection of food and water contaminated with different types of bacteria.[11] Both homogeneous and heterogeneous bacterial communities were inactivated by PEFs for food decontamination.[14,15] However, PEF technology has yet to be evaluated for disinfection in medical applications. Previously, we developed procedures for the long-term control of bacteria in pharmaceuticals and food by applying PEFs intermittently.[16–19] We showed the application of PEF for bacterial inactivation on gel surfaces, which serve as skin models.[20] The current work was predicated on the notion that the set of tools previously developed for food and pharmaceutical applications would be ideal for difficult cases of wound contamination.

In this proof-of-concept report, we demonstrate the effectiveness of PEFs in disinfecting murine third-degree burn wound infections contaminated with antibiotic-resistant *A. baumannii*. This gram-negative pathogen has been increasingly recognized for its ability to cause hospital-associated outbreaks involving multidrug-resistant strains.[21,22] In addition, *A. baumannii* has been reported to have caused intractable infections in traumatic wounds and burns suffered by military personnel injured in the Middle Eastern conflicts.[23,24] In this study, we demonstrate that direct application of PEF onto the infected wound reduces the bacterial load at the treated site by more than four orders of magnitude.

METHODS

Animal Research

The protocol was approved by the Institutional Subcommittee on Research Animal Care. The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. C57BL/6 4-month-old, female mice (~30 g) were purchased from Charles River Laboratories (Wilmington, MA). The animals were housed in cages, five animals per cage, with access to food and water ad libitum, and were maintained on a 12-hour light/dark cycle in a temperature-controlled room. All surgery was performed under ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthesia, and all efforts were made to minimize suffering.

Bacterial Culture

The bioluminescent pathogenic *A. baumannii* ATCC BAA 747 (ATCC, Manassas, VA) gram-negative bacterial strain was used. The bioluminescence genes (*luxCDABE* operon), originally cloned from *Photorhabdus luminescens*,[25] contained the *luxAB* genes that encode the luciferase enzyme, which catalyzes the light-emitting reaction, and the *luxCDE* genes that encode an enzyme complex, which synthesizes the luciferase substrate. The *luxCDABE* operon contained in plasmid pMF 385, a stable genetic reporter in the gram-negative organisms,[26] was introduced into the clinical *A. baumannii* strain by following standard molecular cloning protocols.[27] Bacterial cells were grown overnight in brain heart infusion at 37 °C with 100 rpm orbital shaking. The optical density at 600 nm was measured by a spectrophotometer (Thermo Scientific, Waltham, MA), OD600 = 0.8, corresponding to 10^8 colony forming units (CFU) ml^{-1}. The cells were washed and resuspended in phosphate-buffered saline (Dulbecco) and used at a density of 10^8 CFU ml^{-1} for the in vivo experiments.

Burn Injury

Before the creation of third-degree burns, the animals were anesthetized with ketamine /xylazine and their fur was clipped along the dorsal surface. Burns were produced by dorsal skin surface contact for 10 seconds with brass blocks (surface area 1 cm^2) preheated to 95°C, resulting in a nonlethal 1-cm^2, full-thickness burn.[28] One burn was created per animal. Immediately after the creation of the burns, the mice were resuscitated with intraperitoneal injections of 0.5 ml sterile saline (Phoenix Scientific Inc., St. Joseph, MO) to prevent dehydration.

Burn Infection Model

Bacterial infection was described by Ha and Jin.[29] The burns were allowed to cool for 5 minutes. Subsequently, a 40 μl suspension of *A. baumannii*,

ATCC BAA 747 including the *luxCDABE* operon,[30] in sterile phosphate-buffered saline containing 10^8 cells, was inoculated onto the surface of each burn with a pipette tip. The drop was then spread onto the burn surface with an inoculating loop. The mice were imaged with the luminescence camera, as described in the following section, immediately after application of the bacteria, and 30 minutes after the infection to ensure that the bacterial inoculum applied to each burn remained consistent.

Pulsed Electric Fields Disinfection

A designated area was subjected to treatment with PEFs using contact electrodes with a surface area of 1 cm^2. One square centimeter of burned and infected mice skin was treated in each animal. Pulses were delivered using a BTX 830 pulse generator (Harvard Apparatus Inc., Holliston, MA). Currents were measured in vivo using a PicoScope 4224 Oscilloscope with a Pico Current Clamp (60 A AC/DC) and analyzed with Pico Scope 6 software (Pico Technologies Inc., UK). The following PEF settings were used: 2 mm gap between electrodes; applied voltage of 1000 V; 70 μs pulse duration; 1 Hz pulse frequency. The pulses were delivered in two groups of 40 pulses with a 5-minute interval between groups to allow bioluminescence imaging for each dose of 40 pulses. In total, 80 pulses were delivered. The total treatment time in this protocol was 80 seconds (40 seconds per treatment, 2 treatments, separated by a 5-minute pause for imaging). Three animals were used for each experimental group.

Bioluminescent Imaging of Bacterial Load

The bioluminescent imaging system (Hamamatsu Photonics KK, Bridgewater, NJ) has been described in detail by Hamblin et al.[31] Briefly, it consists of an intensified charge-coupled-device camera mounted in a light-tight specimen chamber fitted with a light-emitting diode—a setup that allowed a background grayscale image of the entire mouse to be captured. Through the use of ARGUS software (Hamamatsu), the luminescent image was presented as a false-color image superimposed on the grayscale reference image. The image-processing component of the software calculated the total pixel values (in relative light units [RLU]) from the luminescent images of the infected wound area. Previously, we correlated the luminescence readout of *A. baumannii*-contaminated burns with CFU isolated from homogenized tissue extracts.[32] Imaging was performed immediately after the injury, 30 minutes after the infection, after 40 pulses, after 80 pulses, and 3 hours after PEF treatment.

Statistical Analysis

Statistical analysis was performed with Toolbox in MATLAB, R2009b (MathWorks, Natick, MA). The error bars show the standard deviation of the mean.

RESULTS

The experiment is described in Figure 1. Animals were subjected to third-degree burns (Figure 1A), which were infected with bioluminescent *A. baumannii* after 5 minutes (Figure 1B), and then treated twice with PEF (Figure 1C). Bioluminescent imaging was used throughout the experiment for infection monitoring (Figure 1D). The third-degree burn created a clear demarcation in the skin (Figure 1A). A representative bioluminescent signal captured from the *A. baumannii*-infected area is shown in Figure 1D.

The electrode application on the skin is shown in Figure 1C. Two plate electrodes were positioned on either side of the infected area for pulse delivery. The maximum current delivered with 1000 V (500 V/mm electric field strength) was 6.4 ± 0.7 A. To calculate the delivered energy, we used the following equation:

$$E = V_{\mathrm{RMS}} I_{\mathrm{RMS}} T \qquad (1)$$

where E (J) is the total delivered energy, I_{RMS} (A) is the root mean square of the current, and T (seconds) is the total application time of all pulses (combined duration of all pulses). Therefore, 80 pulses with 500 V mm^{-1} at 1 Hz deliver ~36 J.

Figure 2 describes the effect of number of pulses on *A. baumannii* survival immediately after treatment. The bacterial load reduction, R, was calculated using the following equation:

$$R = \mathrm{Log}_{10} \frac{\mathrm{RLU}_{\mathrm{bt}}}{\mathrm{RLU}_{\mathrm{at}}} \qquad (2)$$

where R is the Log10 reduction of bacterial load, $\mathrm{RLU}_{\mathrm{bt}}$ is the RLU measurement of the infected skin before treatment with PEF, and $\mathrm{RLU}_{\mathrm{at}}$ is the RLU measurement of the infected skin after PEF treatments.

Figure 2 shows that the application of 40 pulses at 1000 V (500 V/mm electric field strength) reduced the bioluminescent signal by 2.04 ± 0.29 Log10. The application of 80 pulses at 1000 V (500 V/mm electric field strength) reduced the bioluminescent signal by 5.53 ± 0.30 Log10 immediately after treatment.

Figure 3 shows the effect of electric field strength on bacteria survival 3 hours after PEF treatment. In the control, PEF untreated, burned and infected

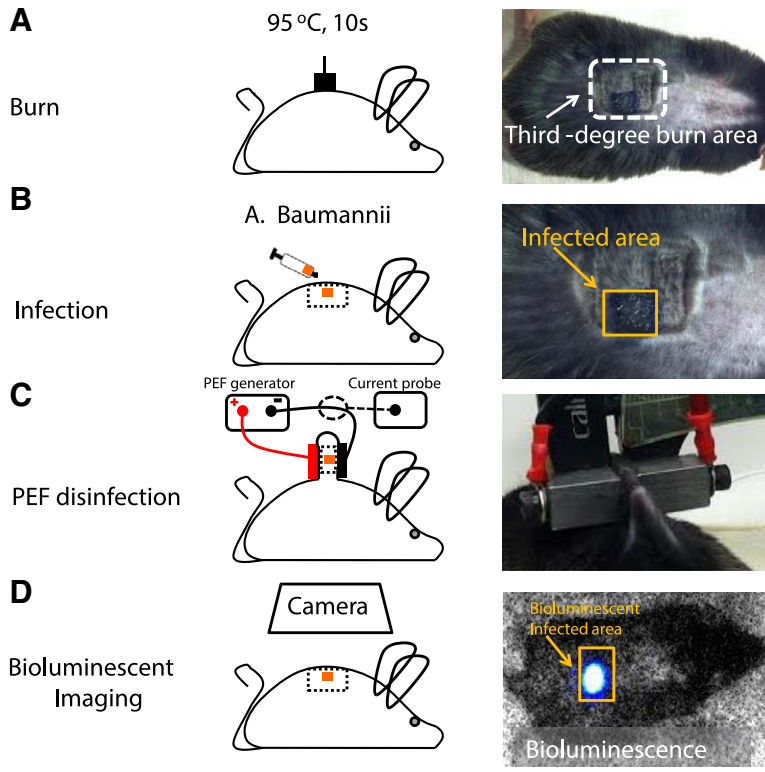

Figure 1. *Acinetobacter baumannii* disinfection by pulsed electric fields. Full-thickness burn C57BL/6 black mice model. A. The 1-cm^2 full-thickness burn. The burned area is shown inside the white frame. B. *A. baumannii* infection. The infected area is shown inside the orange frame. C. Pulsed electric field treatment. D. Bioluminescent imaging. The orange frame shows the infected area as detected by a strong bioluminescent signal emitted from bacteria. PEF, pulsed electric field.

skin, the 1 Log10 reduction is most likely due to the penetration of bacteria into the deep tissue and natural death of a portion of the bacterial population. The application of 80 pulses at 1000 V led to 5.53 ± 0.30 Log10 immediately after PEF treatment; 3 hours after this treatment, the total reduction was still 4.91 ± 0.71 Log10 in comparison with initial bacterial load.

DISCUSSION

In this work, we demonstrate the use of PEF in effective eradication of *A. baumannii* from the infected burn wounds. The bacterial load reduction positively correlated with the number of applied pulses (Figure 2). Using 80 pulses of 500 V/mm, we achieved stable disinfection with 4.91 ± 0.71 Log10 reduction of *A. baumannii,* up to 3 hours after treatment (Figure 3). Although we followed the disinfected wounds for 3 hours, this time is sufficient to show that the bacterial death is attributed to the therapy. Longer observations on the PEF-treated wounds are required before translation to clinics. Incomplete disinfection is usually observed in all types of disinfection technologies. In the case of PEF, previous studies in the food industry showed that the bacterial survival fraction follows Weibull or Fermi distributions as a function of electric field strength and pulse number.[11] Thus, the regrowth of bacteria in the wound is possibly secondary to incomplete disinfection and/or recontamination. To address the possibility of recontamination, we have previously developed the intermittently delivered pulsed electric field process (IDPEF).[16,17] In IDPEF, electric fields are applied intermittently on the target area for an indefinite period of time to prevent recontamination. To prevent the regrowth of *Listeria monocytogenes* in milk, the PEF treatment frequency correlated with the bacterial generation time (1.5 hours for *L. monocytogenes* in milk at 32 °C); therefore, the milk was treated every 1.5 hours.[16] The future translation of PEF to clinics will require the development of clinical protocols based on the IDPEF methods, adapted for each individual patient.

The exact mechanism for bacterial death after exposure to PEF is still not clear. Necrosis due to cell leakage, apoptosis due to calcium influx, cell membrane irreversible electroporation, oxidative damage to the membrane, local pH changes, reactive oxygen

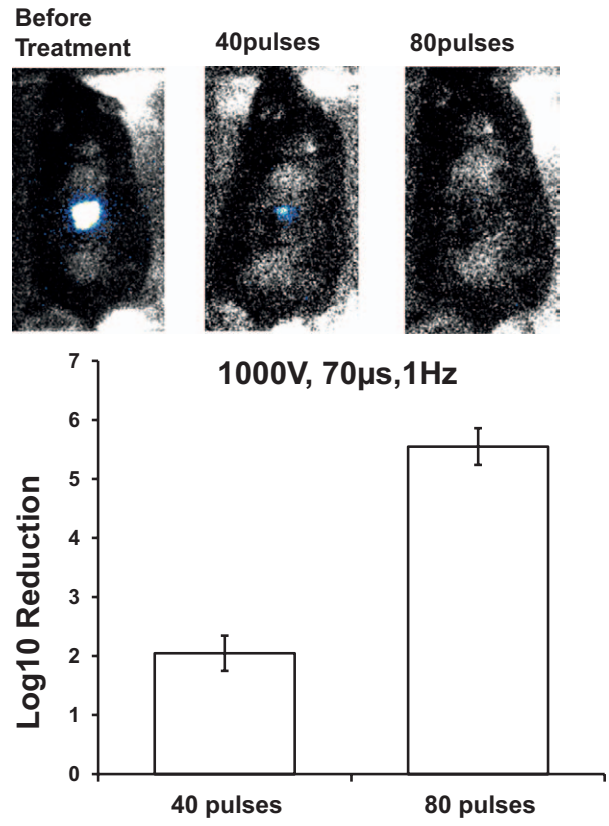

Figure 2. Effect of number of pulses on *Acinetobacter baumannii* survival in vivo immediately after the treatment. The top panel shows images taken inside the bioluminescent detection device. The bottom panel shows the Log10 reduction in the relative light units of *A. baumannii* as detected by the top panel images. Error bar ± standard deviation of the mean. Three mice were used for each experimental condition.

species (ROS) changes, and others have all been proposed in the past four decades and are currently under scrutiny.[13,33–37]

Larger cells are usually more vulnerable to PEF applied in the irreversible-electroporation mode as compared with smaller cells. In addition, cell vulnerability to PEF is predicated by its surface charge, membrane composition, environmental temperature, and pH.[11,19] Therefore, PEFs that destroy bacteria most likely affect the host cells that survived the burn injury. This nonselectivity of the PEF method may be a concern when treating infection in healthy uninjured tissue. To address the effects of PEF on nontarget tissue, we previously investigated the healing process of normal rat skin ablated by PEF.[38] In this study, we showed that PEF-ablated normal skin regenerated rapidly without scars.[38] Our results show that PEFs preserve the extracellular matrix and the microvasculature of the ablated area—properties that most likely promote scarless skin regeneration.[38]

Minimizing host cell injury is an essential concern and will likely require the design of a special contact pad, taking advantage of the superficial nature of most contamination and the absence of viable host cells in eschar. Recent theoretical advances in nanoscale PEF devices[39] imply the possibility of constructing an electrode array that will deliver very high PEF to localized areas of tissue, therefore preventing damage to the majority of host cells. We have recently developed a methodology for electrode array design in a way that focuses the electric fields on the area of interest and minimizes the exposure of the nontargeted tissue.[40]

PEFs that induce irreversible electroporation have recently emerged as a treatment alternative for inaccessible solid-organ malignancies.[41,42] Clinical trials have demonstrated that the procedure is generally safe for tumor ablation.[41,43–46] However, PEFs used for tumor ablation require lower electric field strengths than those used in this study regarding burn wound disinfection. Energy levels used in this study should be tested before clinical application can be considered. Alternative contact pad design and IDPEF strategies may allow for significant energy reduction.

An additional limitation of this study is the use of a single strain of bioluminescent bacteria *A.*

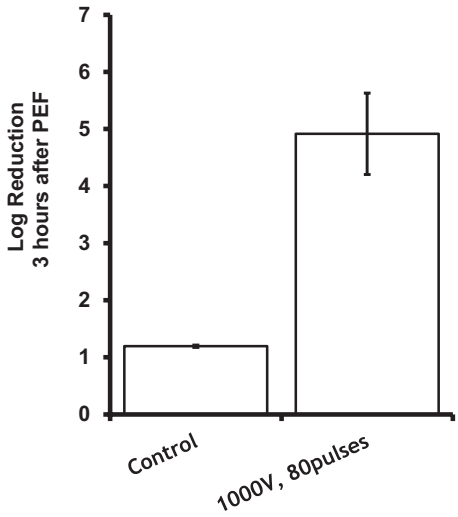

Figure 3. Recovery of *Acinetobacter baumannii* 3 hours after pulsed electric field. Control (untreated), burned, and infected burn wound. Error bar ± standard deviation of the mean.

baumannii. Burns and other wound types can be contaminated by multiple types of microorganisms, and resistance to antibacterial therapies may increase in heterogeneous communities. Future studies should evaluate the effects of PEF on polymicrobial wound infections.

Lastly, for rapid translation to the clinic setting, we believe additional studies should address the potential combination of PEF technology with existing systemic antibiotic regimens. It is likely that PEF will not only increase drug penetration into bacterial cells, but it will also induce increased drug diffusion into biofilms.[47,48] We believe that the application of PEF in combination with currently used drugs will bring the largest benefit to burn patients. To further translate this novel method of burn wound disinfection to patients, a new medical device is needed. The critical component of this device would be the treatment electrodes, which will need to cover large areas of damaged skin. These electrode arrays will be different from the currently used needle electrodes for solid tumor ablation. Multiple electrodes can be positioned close to each other[39] and thus decrease the input voltage required to maintain large electric fields, which are needed for bacterial inactivation. Treatment with PEF would be incorporated with systemic antibiotics and wound care. The infected wound will receive direct PEF treatment. The frequency of treatment will depend on the virulence and resistance of the bacteria to systemic treatment. The IDPEF approach will be tailored for each case individually. An infection that is susceptible to antibiotics will most likely require fewer treatments than a multidrug-resistant infection associated with bacteremia and sepsis. The indicated treatment frequency can be tailored to fit each individual patient's need.

To summarize, in this work we introduced a new chemical-free method of burn wound disinfection using high voltage, short PEFs. PEF antiseptic therapy in combination with systemic antibiotics will synergistically eradicate multidrug-resistant infection in burn wounds. PEF, a novel wound-healing strategy, will significantly improve the clinical management of burn patients. The optimized healing and recovery secondary to PEF could lead to enhanced rehabilitation, a higher quality of life, and overall improved patient outcomes.

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