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Porcine intact and wounded skin responses to atmospheric nonthermal plasma

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ABSTRACT

Thermal plasma is a valued tool in surgery for its coagulative and ablative properties. We suggested through *in vitro* studies that nonthermal plasma can sterilize tissues, inactive pathogens, promote coagulation, and potentiate wound healing. The present research was undertaken to study acute toxicity in porcine skin tissues. We demonstrate that floating electrode-discharge barrier discharge (FE-DBD) nonthermal plasma is electrically safe to apply to living organisms for short periods. We investigated the effects of FE-DBD plasma on Yorkshire pigs on intact and wounded skin immediately after treatment or 24 h posttreatment. Macroscopic or microscopic histological changes were identified using histological and immunohistochemical techniques. The changes were classified into four groups for intact skin: normal features, minimal changes or congestive changes, epidermal layer damage, and full burn and into three groups for wounded skin: normal, clot or scab, and full burn-like features. Immunohistochemical staining for laminin layer integrity showed compromise over time. A marker for double-stranded DNA breaks, γ-H2AX, increased over plasma-exposure time. These findings identified a threshold for plasma exposure of up to 900 s at low power and <120 s at high power. Nonthermal FE-DBD plasma can be considered safe for future studies of external use under these threshold conditions for evaluation of sterilization, coagulation, and wound healing.

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1. Introduction

Thermal plasma, administered in the form of bovie cautery and the argon beam coagulator, has become a standard tool in surgery. These devices rely on their extreme heat and energy for tissue coagulation and ablation. Electrical plasma is widely used in technologies ranging from computer printers to televisions and has many potential applications in medicine. The ability of plasma to modify chemical bonds means that it can produce active species in air or water that can be used to kill bacteria or modify organic materials. It has many potential applications, including direct sterilization of tissues *in vivo* [1–3], promotion of coagulation [2,4], potentiation of wound healing [1–6], and possibly production of an anticancer effect through apoptosis [2,7]. The thermal plasmas that are currently used cause severe physical damage and burns to living tissues at the site of application [3]. Conversely, nonthermal plasma can be used selectively for treatment because one can avoid burning healthy tissue. Recently, our laboratories have successfully generated normal atmospheric nonthermal plasma and applied it to tissues and organisms using our floating electrode-dielectric barrier discharge (FE-DBD) plasma technique. This plasma has been shown to be completely safe from the electric perspective and non-damaging to animal and human skin and soft tissues [1,2]. Because the plasma that is generated is cold (temperature does not exceed 25°C–27°C), it does not cause tissue damage or burns (unlike traditional thermal plasma and electrocautery) [1,2,8,9]. The plasma-generating probe is well characterized and is being evaluated for multiple biological and medical applications for sterilizing surfaces and wound tissues [4,5,8,10–12]. The device is easy to operate in outpatient clinics and can be carried in the field as well.

FE-DBD nonthermal plasma is a recent concept in which an object with a high capacity for charge storage replaces one of the electrodes of the FE-DBD plasma probe. Living cells or tissues with water content and a relatively high dielectric constant have the required high capacity for charge storage [1,2,11,12]. In FE-DBD, plasma is created in the gap between the living cells or tissues (which act as the second electrode) and the other insulated electrode (primary electrode). Although the current in the gaseous discharge gap is mainly because of the motion of charge carriers (electrons and ions), it continues mostly in the form of displacement current through the cell or tissue. No special gaseous or air currents are used in this technique, and it uses normal atmospheric plasma (i.e., room air).

The effectiveness of this plasma in biological and medical applications, such as sterilization of living tissue without damage, sterilization of heat-labile articles, disinfection of fruit surfaces, and control of superficial bleeding and blood coagulation phenomena, is under investigation [1,2,13]. Despite the tremendous clinical interest in this application, how FE-DBD plasma interacts with living tissues *in vivo* in terms of local toxicity is unknown. Therefore we decided to study acute local toxicity. Our earliest experiments, conducted on rats, showed no major toxicity to the animal [11]. However, because rat skin was too thin to be a good model, we decided to evaluate the acute toxicity of FE-DBD plasma in the pig; pig skin is physiologically close to human skin and therefore suitable for skin toxicity studies [14–16]. Our goal was to identify the boundaries of skin acute toxicity after treatment of intact and wounded skin with the novel FE-DBD nonthermal plasma technique.

2. Materials and methods

2.1. Animals

We evaluated the potential toxic effects of FE-DBD plasma on intact and wounded porcine skin in seven adult female (40–45 kg) Yorkshire pigs (John Meck Farm, Lancaster, PA). The pigs were allowed to acclimate for about a week before the initiation of the experiments and housed in smooth-walled stainless steel cages to minimize wound trauma and disruption of wound dressings, with free access to food and water. All the procedures and protocols were approved by the Institutional Animal Care and Use Committee of Drexel University College of Medicine and strictly followed. The animals were premedicated with an intramuscular injection of tiletamine/zolazepam (Fort Dodge Laboratories, Madison, NJ), 5 mg/kg. Anesthesia was induced with propofol (Abbott Laboratories, Abbott Park, IL), 2–4 mg/kg, and fentanyl, 10 mcg/kg, administrated intravenously. Animals were intubated and maintained with isoflurane (Vedco, Saint Joseph, MO) and oxygen inhalational anesthetic. Analgesia included meloxicam (Boehringer Ingelheim, Ridgefield, CT), 0.3 mg/kg, intramuscularly and buprenorphine (Abbott Laboratories), 0.015 mg/kg, intramuscularly peri- and postoperatively. Parenteral/intravenous fluid therapy included lactated Ringer's solution (Baxter, Deerfield, IL), 10 mL/kg/h. On completion of the terminal surgical procedures, the animals were euthanized by intravenous injection of phenobarbital/phenytoin (Virbac Corporation, Fort Worth, TX), 150 mg/kg. All procedures were performed in the large-animal surgery suite using aseptic techniques.

2.2. Treatment with nonthermal plasma

Nonthermal atmospheric pressure FE-DBD plasma was produced in all experiments as previously described [5,13] using the FE-DBD technique. The discharge gap between the bottom quartz surface and the skin or wound surface was about 1.5–2 mm using a special electrode holder (Fig. 1A) or a modified planar electrode (Fig. 1B).

2.3. Study design

The pigs were anesthetized. The dorsum of each pig was shaved and cleaned with povidone iodine followed by removal of iodine and cleaning with chlorhexidine solution. The back of the animal was marked in a grid with a sterile skin-marking pen to designate treatment areas. To study intact skin, we placed the plasma probe ~1.5–2 mm above the skin and tested variable treatment times at different powers of plasma. To study wounded skin, we used an electric dermatome to create a skin abrasion of 20 mm^2 of the epidermis and dermis

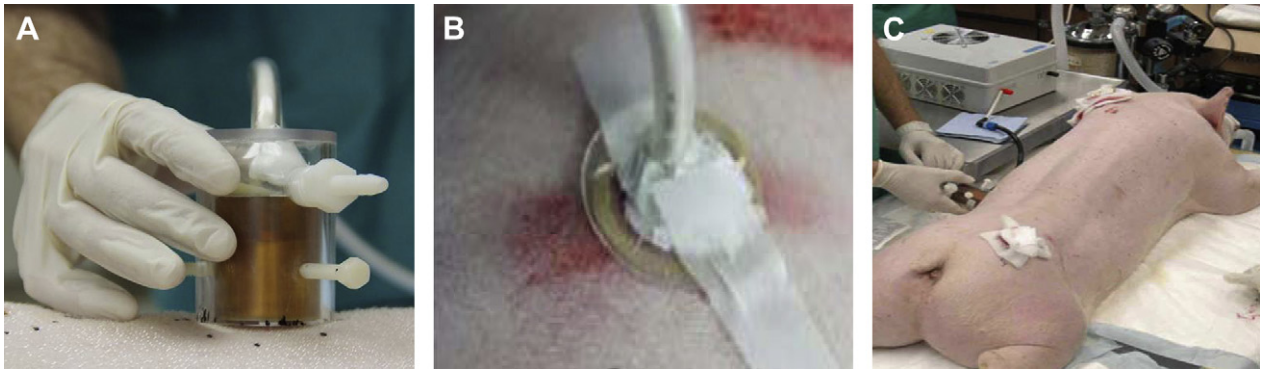


Fig. 1 – The nonthermal dielectric barrier discharge plasma device. Setup shows the method used to treat intact skin (A) and the technique used to treat wounded skin (B). The dorsum of the pigs was separated into treatment areas for both intact and wounded skin specimens as shown in (C).

(approximately 1 mm deep). We applied pressure immediately to stop bleeding if any and applied the plasma treatment immediately after stopping the bleeding. The pigs were then humanely killed immediately after the surgical procedure or after 24 h. The wounds were dressed aseptically with sterile gauze followed by an adhesive polyurethane dressing sleeve to keep the dressings in place. Tissue specimens from each treatment area were harvested by punch biopsies from the marginal regions of treated and normal skin, examined for macroscopic and microscopic histological changes and analyzed using immunohistochemical techniques.

For the intact skin model (3 pigs), we had a total of 42 treatment areas on the three pigs that were harvested 24 h after treatment (Table 1). One area of intact skin ($n = 1$) was treated with an electrocautery burn (positive control), and one area of intact skin ($n = 1$) was untreated (negative control). The remaining 40 areas were treated with plasma at one of four power settings: highest power, 0.31 W/cm^2 (12 specimens); 0.17 W/cm^2 (12 specimens); 0.15 W/cm^2 (12 specimens); and lowest power, 0.13 W/cm^2 (4 specimens). For 0.31 W/cm^2 and 0.17 W/cm^2, three samples ($n = 3$) were treated with plasma for each of the following times: 30 s, 1 min, 2 min, and 3 min. For 0.15 W/cm^2, three samples ($n = 3$) were treated with plasma for each of the following times: 1 min, 2 min, 5 min, and 15 min. For 0.13 W/cm^2, one sample ($n = 1$) was treated with plasma for each of the following times: 1 min, 2 min, 5 min, and 15 min (Table 1).

For the wounded skin model (4 pigs), we had a total of 42 treatment areas on two pigs harvested immediately after surgery and 42 treatment areas on two pigs harvested 24 h after plasma treatment. In the nonsurvival pigs, 21 specimens were treated with low power (0.13 W/cm^2) and 21 specimens were treated with high power (0.31 W/cm^2). In the 24-h survival pigs, 21 specimens were treated with low power (0.13 W/cm^2) and 21 specimens were treated with high power (0.31 W/cm^2). Each group of 21 specimens was divided into three samples ($n = 3$) treated for the following times: no treatment (negative control), bovie electrocautery (positive control), and 30 s, 1 min, 3 min, 5 min, and 15 min (Table 2).

2.4. *Clinical observations and macroscopic findings*

The animals were observed during each procedure and throughout the study. The macroscopic changes in skin (if any) were noted after each plasma treatment, immediately as well as before harvesting of the tissues.

2.5. *Histological analysis*

In addition to recording all gross observations of the specimens, the formalin-fixed specimens were analyzed using microscopic histological techniques. Specimens were biopsied as mentioned above, and the sections were processed for hematoxylin and eosin staining using the standard protocol. Our pathologists were blinded to all group designations throughout the study; they categorized each specimen on the basis of levels of cellular/tissue injury within two grading systems: one for intact and one for wounded skin. The

Table 1 – Correlation of plasma power and skin treatment times to the amount of plasma energy deposited at the site of application.

Frequency (Hz)	Power density (W/cm^2)	Time of plasma exposure (min)						
		0.5	1.0	2.0	3.0	5.0	10.0	15.0
500	0.13	3.9	7.8	15.6	23.4	39	78	117
800	0.15	4.5	9	18	27	45	90	135
1,000	0.17	5.1	10.2	20.4	30.6	51	102	153
1,500	0.31	9.3	18.6	37.2	55.8	93	186	279
		Dose of plasma treatment (J/cm^2)						

Table 2 – Intact skin treatment regimes at four different power settings and amount of plasma energy deposited at the site of application.

Time (min)	No damage	Damage	Burn	Total samples
(a) Power: 0.15 W/cm^2				$n = 12$
1	3			3
2	3			3
5	3			3
15	3			3
(b) Power: 0.17 W/cm^2				$n = 12$
0.5	3			3
1	3			3
2	3			3
3	1	2		3
(c) Power: 0.31 W/cm^2				$n = 12$
0.5	3			3
1	2	1		3
2		1	2	3
3		1	2	3

microscopic histological changes for intact skin specimens were classified as normal and minimal change (collectively, "no damage") and epidermal damage ("damage") or full burn through the dermis (Fig. 2). Similarly, for wounded skin, the specimens were classified as normal, having a clot or scab, or having full burn through the dermis (Fig. 3).

2.6. *Immunohistochemical staining*

Slides prepared in triplicate were used for immunohistochemical staining to analyze basement membrane integrity (laminin) and demonstrate DNA double-stranded breaks (DNA damage) using γ-H2AX as a marker in our intact skin samples. Anti-laminin mouse monoclonal (Sigma-Aldrich, St. Louis, MO) or anti-γ-H2AX mouse monoclonal antibodies (Abcam, Cambridge, MA) were used. The slides were first warmed at 60°C to melt the paraffin wax and then hydrated through a graded ethanol series. Heat-induced target retrieval was performed by heating the slides to 95°C in a modified citrate buffer (Dako North America, Carpentaria, CA) to improve antibody binding. To block nonspecific binding sites, the tissue sections were incubated with heat-inactivated goat serum for 1 h at room temperature. Slides were then incubated with the primary antibody overnight at 4°C. The next day, the slides were washed three times with phosphate-buffered saline (PBS) to remove excess primary antibodies and then incubated with a rhodamine-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature and protected from light. Three more washes with PBS were performed to remove excess secondary antibody; then the slides were incubated for 3 min with a nuclear stain, Hoechst 33258 (0.05%) (Sigma-Aldrich, St. Louis, MO). The slides were washed another three times with PBS and then mounted with Citifluor antifading mounting medium (Electron Microscopy Sciences, Hatfield, PA) and examined for fluorescence microscopy using a Leica DMRX fluorescence microscope with attached Leica DG300FX digital camera system, using the appropriate band-pass filter (for corresponding blue and red dyes). The images were captured from five randomly selected areas for three different sets of experiments, saved as tagged image file format (TIFF) files, and edited using Adobe Photoshop CS3.

2.7. *Data analysis*

All the treatment conditions had built-in negative (no treatment/normal tissues) and positive (treatment with electrocautery to create burn) controls. The sample numbers are indicated in Tables 2 and 3. A minimum of three samples per condition were selected for analysis, unless specifically stated otherwise. The groups were compared with untreated controls; Welch's t-test was performed with Welch corrections. The data were analyzed using the SPSS statistics program (San Diego, CA). A P value <0.05 was considered statistically significant.

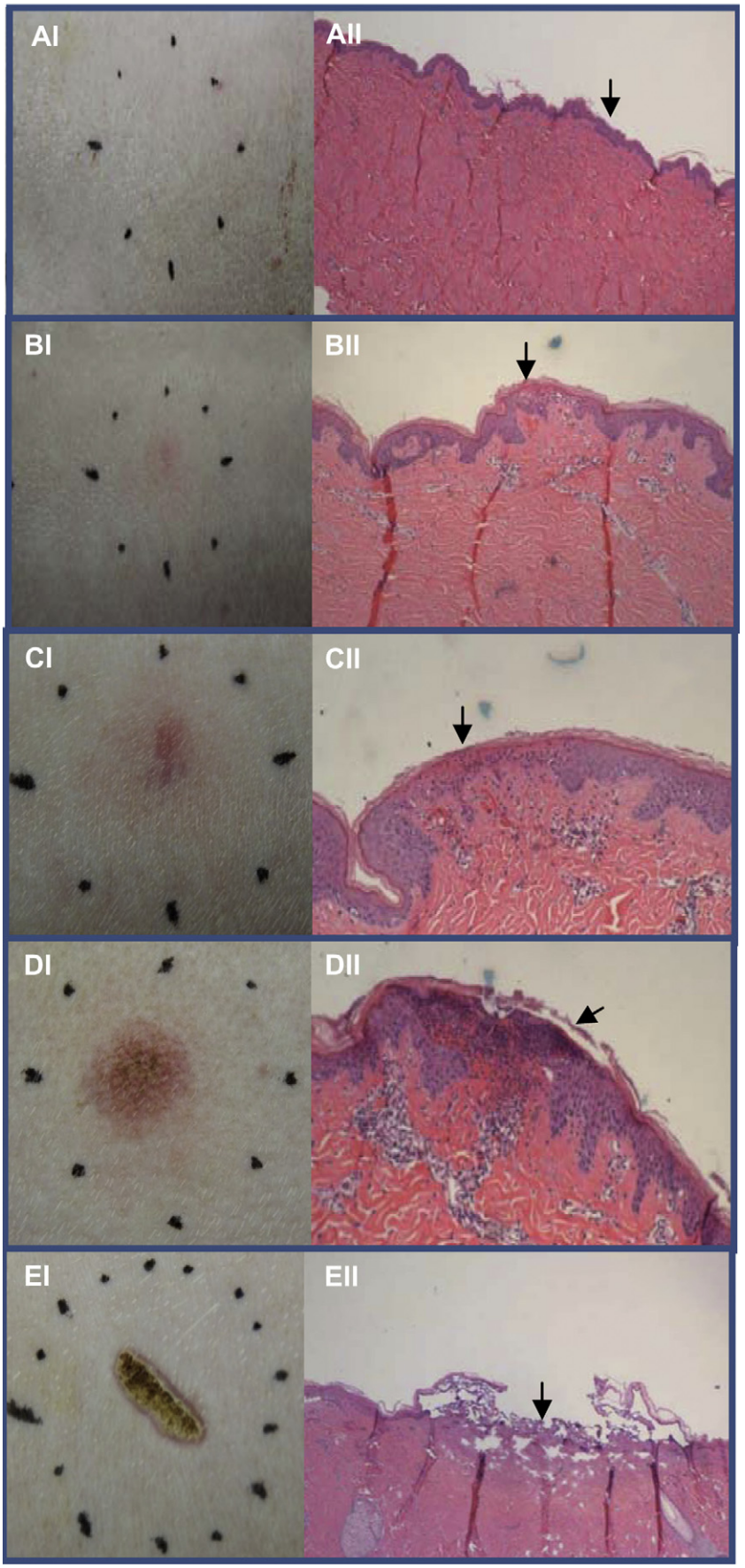
3. Results

Fig. 1 shows the plasma-generating probe (Fig. 1A), a modified nonthermal FE-DBD plasma treatment planar electrode for treatment of wounded tissue (Fig. 1B), and the plasma being applied to the dorsum of the pig (Fig. 1C). A gap (distance) of ~1.5–2 mm can be noted between the surfaces of plasma probe and the skin (Fig. 1A). Table 1 shows the correlation between the plasma power settings with exposure (treatment) times and the amount of energy deposited over the area of application.

3.1. *Intact skin*

Fig. 2 shows the representative macroscopic and microscopic intact skin changes that were used for categorization and burn grading of tissue injuries. Fig. 2 shows that, with normal histological skin samples, the skin appeared normal grossly (Fig 2A); with minimal change, there was a small area of erythema on the skin (Fig. 2B); with epidermal damage, there was mild erythema that usually resolved within 30 min (Fig. 2C); with full burn through the dermis, there was diffuse erythema surrounding the burn that remained until the time of harvest (Fig. 2D). Fig. 2 also demonstrates the corresponding histological changes associated with plasma-induced skin responses in the intact skin model. The minimal changes seen in Fig. 2BII appeared very close to normal skin (Fig. 2AII), and the architecture of the skin tissue was well preserved; therefore, for convenience, these two were categorized together as "no damage"; mild epidermal damage is categorized as "damage" (Table 2). Burn-like changes caused by high-power plasma (Fig. 2D), although less severe than those in the positive control (bovie), were similar macroscopically and microscopically (Fig. 2E).

Table 2 shows the total number of intact skin samples with their gradings. At 0.13 W/cm^2 (low power), only four specimens from four experimental conditions were studied as observational trials (data not shown). In subsequent trials, we took more precautions and tried to remove operator errors. (The gap [in mm] may change when the operator is holding the plasma probe in his hand and applying it to the skin


AI
AII
BI
BII
CI
CII
DI
DII
EI
EII

surface. This situation is in part because of the amount of pressure applied by the operator against the surface of the skin, despite the spacer.) At the next higher level of power, 0.15 W/cm^2 (12 specimens), three of three specimens treated with plasma for 1, 2, 5, and 15 min were classified as normal and "no damage" (Table 2a). Together these findings indicated a safe margin for use, and erythema was resolved in all cases. There was no significant damage ($P > 0.05$ compared with positive bovie control) observed after plasma exposure at 0.15 W/cm^2 for up to 15 min. Table 2b shows the analysis of treatment conditions at the next power setting (0.17 W/cm^2) (12 specimens); three of three specimens treated with plasma for 30 s and three of three treated for 1 min were classified as normal skin. In the samples treated for 2 min, two of three samples showed minimal change and one of three had normal skin and therefore categorized as "no damage." In the group treated with plasma for 3 min, two or three samples showed epidermal damage and one of three samples showed minimal change, all samples still falling within a safe margin for use under these circumstances, as per the pathologist. At high power (0.31 W/cm^2) (12 specimens), three of three specimens treated with plasma for 30 s were classified as normal skin. In the samples treated for 1 min, one of three samples showed minimal change; one of three had normal skin (thus 2/3, "no damage"); and one of three had skin with epidermal damage ("damage"). In the groups treated with plasma for 2 and 3 min, two of three samples showed full-thickness burns and one of three had epidermal damage. Table 2c indicates that the time limit for high-powered plasma is between 30 s and 1 min.

3.2. *Wounded skin*

The plasma device also was tested on four pigs with wounded skin with either low- (0.15 W/cm^2) or high- (0.31 W/cm^2) power settings. The samples from two pigs were processed immediately after the plasma procedure; samples from the remaining two were processed 24 h after the plasma treatments. Fig. 3 shows the macroscopic and microscopic histological criteria used to classify the responses of the samples of wounded skin. Untreated wounded skin is shown in Fig. 3A, where Fig. 3AI shows a fresh normal wound injury and Fig. 3AII shows the corresponding microscopic changes. A small, thin clot covering the wounded area developed in some of the plasma-treated wounds (depicted in Fig. 3 as gross changes) (Fig. 3BI) with corresponding microscopic scab-like features (Fig. 3BII). There was minimal damage to the underlying tissue layer, and the scab/clot here likely acted as a protective element. In pigs harvested at 24 h, scabs were more common than clots on gross examination (a natural defense mechanism to minimize injury). Wounded skin exposed to high-power plasma developed a burn (Fig. 3CI and II); a peculiar dark brown discoloration and histological changes consistent with a superficial burn could be seen (please refer the online version of figures for colors). A positive control bovie burn is seen in the panel of Fig. 2EI and II for comparison, demonstrating complete destruction from epidermis to dermis and extending down the hair follicle.

Table 3 shows an analysis of all wounded skin responses to different power levels of plasma. Of the wounds treated with low power (0.13 W/cm^2) (21 specimens) and harvested immediately, three samples with 0.5-min (30 s) treatment exposure showed a clot on histological examination. With groups treated for 1, 3, and 5 min, two of three showed a clot and one of three was normal in each group. With the group treated for 15 min, all three samples showed a clot (Table 3a). For the pig treated with high power (0.31 W/cm^2) (21 specimens) and harvested immediately, all specimens treated for 30 s and 1 and 3 min showed a clot. With the groups treated with plasma for 5 and 15 min, two of three showed a clot and one of three was normal in each group (Table 3b). In both cases (for immediate harvest), all samples fell within a safety margin (significantly normal, $P < 0.05$ compared with normal skin samples) of up to 15 min with minimal or no changes in the histological appearance. For the pig treated with low power (0.13 W/cm^2) (21 specimens) harvested 24 h after treatment, at 0.5 min (30 s) of treatment, two of three samples showed a scab and one of three had normal tissue. With 1, 3, and 5 min of treatment, all three samples in each group showed a scab. With 15 min of treatment, two of three showed a burn and one of three was normal (Table 3c). For the pig treated with high power (0.31 W/cm^2) (21 specimens harvested after 24 h), of the samples treated for 30 s, two of three showed a scab and one of three had normal tissue. With 1 and 3 min of treatment, one of three in each group showed a burn and two of three had normal tissue. With 5 min of treatment, all three samples showed a burn. With 15 min of treatment, all three samples showed a full-thickness burn (Table 3d). Thus, at 24 h posttreatment, samples treated with low-power (0.13 W/cm^2) plasma for up to 15 min showed minimal histological changes, whereas samples treated for up to 1 min with high power showed marginal damage (with minimal tissue damage); treatment of all other wounded tissue for >1 min led to full-thickness burn lesions (significant damage, $P < 0.05$ compared with bovie-treated wounded skin). Therefore, exposure to high-power plasma for more than 1 min is likely detrimental to wounded skin.

3.3. *Immunohistochemical analysis*

To complement our gross and histological findings, we performed immunohistochemical studies to look at the architecture of the laminin layers and nuclear changes in the form of DNA double-stranded breaks. These studies were performed on intact samples of skin. Integrity of laminin is essential for healthy skin tissues; any breach or dissociation of the laminin layer indicates damage to skin epidermis and underlying structures [10,13]. Fig. 4 demonstrates laminin changes associated with the application of plasma. Normal skin showed integrity of the laminin layer (untreated). Application of high heat with electrocautery caused burns,

Fig. 2 – Representative gross findings (I) with associated histological classification (II) of intact skin. Representative photographs of the criteria used in classification: (A) normal skin (negative control); (B) minimal changes in skin; (C) epidermal damage; (D) burn; and (E) bovie electrocautery (positive control). Arrows indicate the corresponding changes.

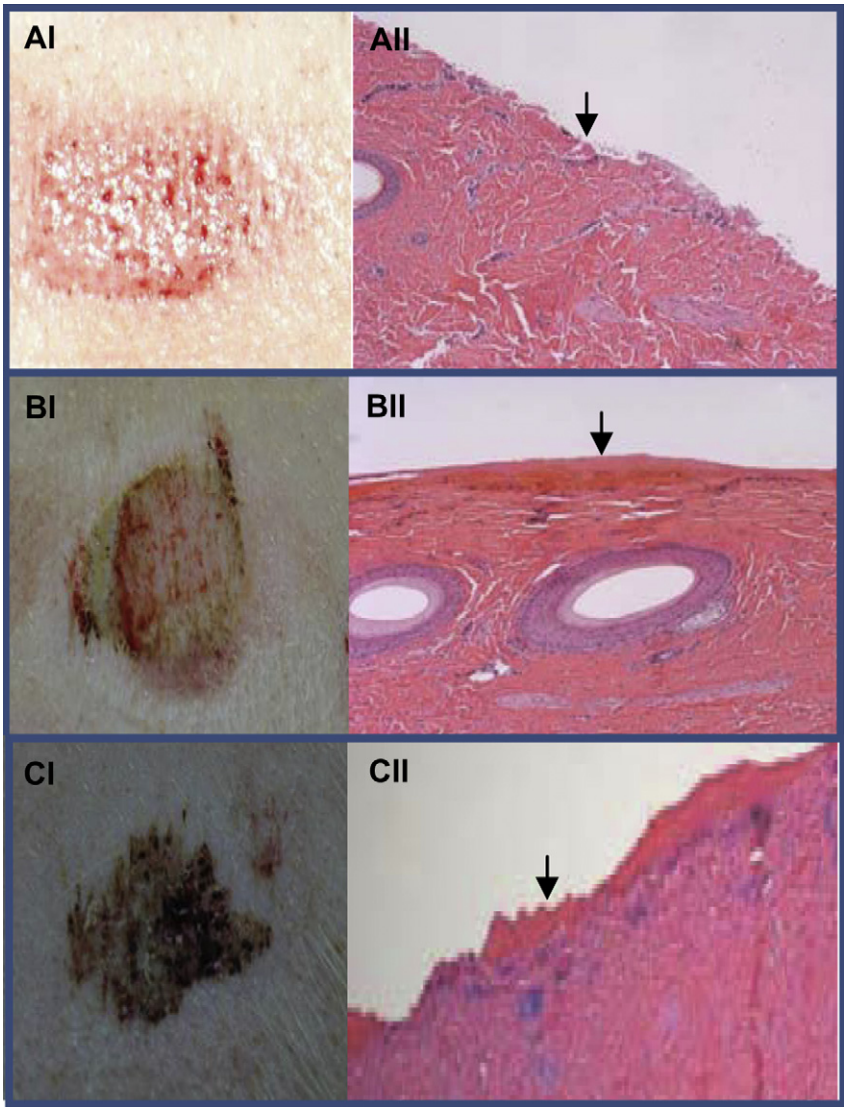


Fig. 3 – Representative photographs showing gross findings (I) and associated histological features (II) used in classification of wounded skin: (A) normal wounded skin; (B) scab or clot; and (C) burn. Arrows indicate the corresponding changes.

demonstrating classical disintegration of the laminin layer. The untreated skin and the skin treated with electrocautery served as negative and positive controls, respectively. On comparison with controls, we observed that nonthermal FE-DBD plasma (0.17 W/cm^2) is safe to apply for up to 5 min (minimal laminin disintegration), after which laminin damage is observed. These findings are in line with the hematoxylin and eosin findings.

To examine the changes at the subcellular level, we conducted γ-H2AX immunostaining. After treatments with plasma, we compared the immunostained intact skin tissue sections for accumulation of γ-H2AX in the nuclear region. An accumulation of γ-H2AX was observed in skin exposed for 5 min or more (Fig. 5). We did not see any noticeable changes in skin exposed for shorter periods. Fig. 5 shows the representative micrographs, including positive and negative controls for double-stranded breaks with two different plasma-exposure times. When we considered both immunohistochemical findings together, it appeared that treatment with low-power plasma (0.17 W/cm^2) for up to 5 min did not cause cellular injury to intact skin *in vivo*. Fig. 6 is a schematic diagram showing safe margins for proposed treatments with nonthermal plasma.

4. Discussion

To the best of our knowledge, this study was the first done *in vivo* to assess the boundaries of toxicity of nonthermal plasma on living tissue using the FE-DBD plasma technique. To evaluate the acute response to plasma treatments, we selected the pig as a model for intact and wounded skin. The pig skin model is widely used in medicine and in the pharmaceutical industry to assess the bioefficacy of agents for

Table 3 – Analysis of nonthermal plasma treatment on wounded skin at low- and high-power settings, harvested either immediately or 24 h after exposure to plasma.

Time (min)	Normal	Clot	Burn	Total samples
(a) Low power: 0.13 W/cm^2 (nonsurvival)				$n = 12$
Control (+ve)			3	3
Control (−ve)	3			3
0.5		3		3
1	1	2		3
3	1	2		3
5	1	2		3
15		3		3
(b) High power: 0.31 W/cm^2 (nonsurvival)				$n = 12$
Control (+ve)			3	3
Control (−ve)	3			3
0.5		3		3
1		3		3
3		3		3
5	1	2		3
15	1	2		3
(c) Low power: 0.13 W/cm^2 (survival)				$n = 12$
Control (+ve)			3	3
Control (−ve)	3			3
0.5	1	2		3
1		3		3
3		3		3
5		3		3
15	1	2		3
(d) High power: 0.31 W/cm^2 (survival)				$n = 12$
Control (+ve)			3	3
Control (−ve)	3			3
0.5	1	2		3
1	2		1	3
3	2		1	3
5			3	3
15			3	3

+ve = positive control; −ve = negative control.

local applications and wound studies [15,16]. Because the field of nonthermal plasma is rapidly moving toward direct tissue applications in medicine, and on the basis of our *in vitro* data, we anticipate the use of FE-DBD plasma on human skin for disinfection, sterilization, coagulation, and possibly cancer therapy or wound healing. These studies were undertaken to evaluate the safety margins of plasma in terms of power and exposure time. The plasma settings used in these studies were based on our previous findings, are relevant to clinical use, and are therefore appropriate for *in vivo* acute toxicity studies. We reported previously that <120 s is sufficient for complete inactivation of bacterial pathogens (at 7 log CFU), including those in biofilm forms [11,12].

When we treated intact skin with nonthermal plasma, we found that 2 min was the threshold for signs of histological tissue damage at all power settings except for high power (0.31 W/cm^2). With high-power plasma, signs of minimal change, epidermal damage, and full-thickness burn were seen as early as 1 min after treatment. Although the gross changes appeared similar to normal skin, the presence of microscopic changes led to an additional grading of "minimal change," defined by the pathologists as the presence of epidermal and dermal cellular changes in the size of the cell and in the nucleus. Looking at the similarity of the minimal change to normal, we categorized this change as "no damage" (Table 2). Epidermal damage showed a notable increase in vascular congestion and cellular disorganization.

When wounded skin was treated with plasma, we observed the formation of clots on all wounds, which probably protected the underlying skin from plasma-treatment damage. In nonsurvival pigs, a clot was seen on the wounded skin; in 24-h survival pigs, a scab was seen on the wounded skin. All time points (0.5, 1, 3, 5, and 15 min) showed some type of clot or scab formation without disruption or damage to the underlying skin except for the subgroup whose wounded skin was treated with high power for 5 min and 15 min (3 and 2 specimens showed full-thickness burn, respectively). It has been reported that treatment with plasma for as little as 30 s may sterilize tissue [2,4,5], but the degree of sterilization depends largely on the power (or energy) of plasma used. The use of plasma has been reported to enhance blood coagulation [2,4,5]. Therefore, using plasma is likely to promote the protection of underlying wound tissues on injury. Clot formation is an inevitable step of wound healing. Our earlier *in vitro* data also suggested that the application of plasma using the FE-DBD technique promoted endothelial cell proliferation and enhanced growth-promoting factors [2,6]. Therefore, the enhancement of clot formation by plasma can play a role in the wound healing process in two different ways: (1) enhancing rapid clot formation and (2) protecting underneath soft tissues required for wound healing and promoting cell proliferation by appropriate stimulation with low-energy plasma. It was recently demonstrated that plasma produces different physical and chemical species, which mount variable reactive oxygen species responses required for cell signaling and stress-induced wound healing processes [2,12,17,18]. Exposure to plasma for 2 min at 0.13 W/cm^2 is sufficient for sterilization of bacterial skin flora and pathogens, such as *Staphylococcus aureus*, *Escherichia coli*, and methicillin-resistant *S. aureus* (strains of USA300 and USA400) by both direct and indirect (fluid-mediated) treatment with plasma. Therefore, the plasma settings and exposure times are highly relevant [11,12]. In either case, the results of *in vivo* wound healing studies are of interest. The present safety levels for plasma energies can be used as guidelines for such studies.

Continuous application of plasma for more than 5 min caused changes in skin tissue, such as dissociation of the laminin layer and changes in the nuclear DNA (double-stranded breaks) in our intact skin specimens. If one harvests skin tissue 24 h after treatment with plasma, it is unlikely that tissues would have had an adequate opportunity to undergo normal cellular repair and compensate for acute transient hyperemic change. Under normal physiological conditions, injuries to cells and tissues occur every day and the body's repair and defense mechanisms heal them completely [19]. Our findings favor the observations that a plasma-exposure time of <5 min in intact skin is safe, does not cause acute toxicity in skin tissues, and does not lead to significant loss of architecture. Laminin and γ-H2AX are good indicators of disruption of membrane layers, severity of skin tissue injury,

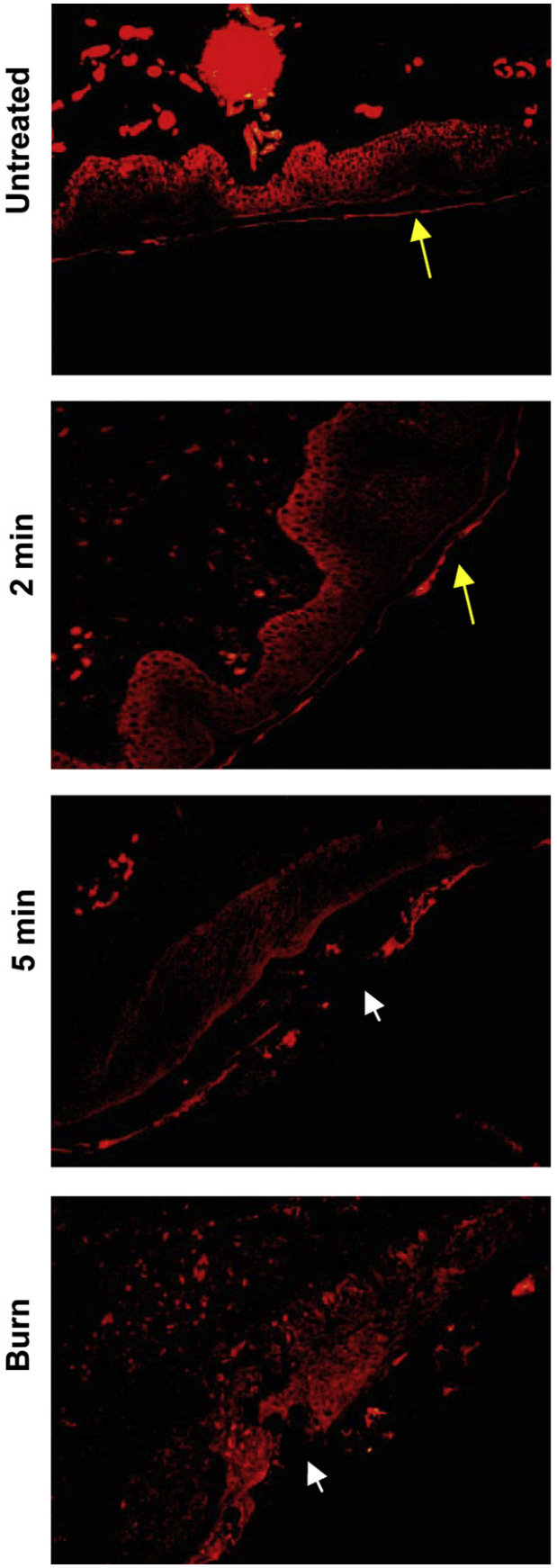


Fig. 4 – Representative microphotographs of immunohistochemical staining of intact skin section, showing positively stained laminin layers. Damage to the laminin layer appears at around 5 min of treatment with plasma (0.17 W/cm^2 power setting). Yellow arrows indicate normal whereas white arrowheads indicate dissociated laminin layers (please refer the online version of figures for colors).

and DNA double-stranded breaks respectively, and thus of cellular insults [10,20,21]. We have looked for changes in the laminin and in the γ-H2AX in intact skin. Studies of the relationship between γ-H2AX and laminin in wounded skin are currently under way.

As a pilot study, our experiments were more qualitative than quantitative and demonstrated a highly variable threshold for toxicity under some of the conditions used. We were limited by groups with small numbers, which made the statistical comparisons challenging. For all sample groups, three specimens were studied; the one exception was one specimen per group for the low-power treatment of intact skin. The latter was the first subgroup to be tested, and one sample for each time point was performed to determine feasibility. Subsequently three samples were tested in each group for each time point. In addition, intact skin on nonsurvival pigs treated with plasma was not tested. Our initial study design was to assess the presence of tissue damage and toxicity from treatment with plasma only after 24 h of survival. As we finished our pig studies of intact skin and proceeded with the wounded skin model, there was concern that the invasiveness of the procedure of creating more than 20 separate 20-mm^2 wounds on the dorsum of the pig would complicate postoperative recovery and care for 24 h. We subsequently altered our design of study groups to include two pigs with wounded skin treated with plasma that would be harvested immediately. Fortunately, our 24-h survival pigs in the wounded model provided useful information in terms of safety margins.

One variable that must be accounted for is the use of the porcine model. Some of the trends from our results are unexpected; intuitively, the higher the power and the longer the time of plasma treatment, the greater the risk of burn. Several specimens did not exhibit this result. For example, in intact skin treated with 0.15 W/cm^2, three skin specimens treated for 15 min yielded one sample with a full-thickness burn and two with normal skin; in contrast, the specimens treated for 5 min yielded two with a full-thickness burn and one with epidermal damage. Much of this variability is because of the anatomy of the pig. Because the dorsum is divided into plasma treatment areas, the surface of the skin that is exposed to plasma is not flat. Treatment of plasma skin samples closer to the spine of the pig was more likely to cause greater erythema relative to treated skin samples lateral to the spine because of the increased thickness of the skin and the greater amount of subcutaneous tissue. In addition, the experimenter (operator) had to hold the plasma device for the appropriate period of time for each subgroup 3 mm above the intact skin. Human error likely played a part in exposing the plasma device at the incorrect distance for each period of time. We minimized this error by placing a 3-mm spacer on the tip of our plasma probe, which resulted in less variability

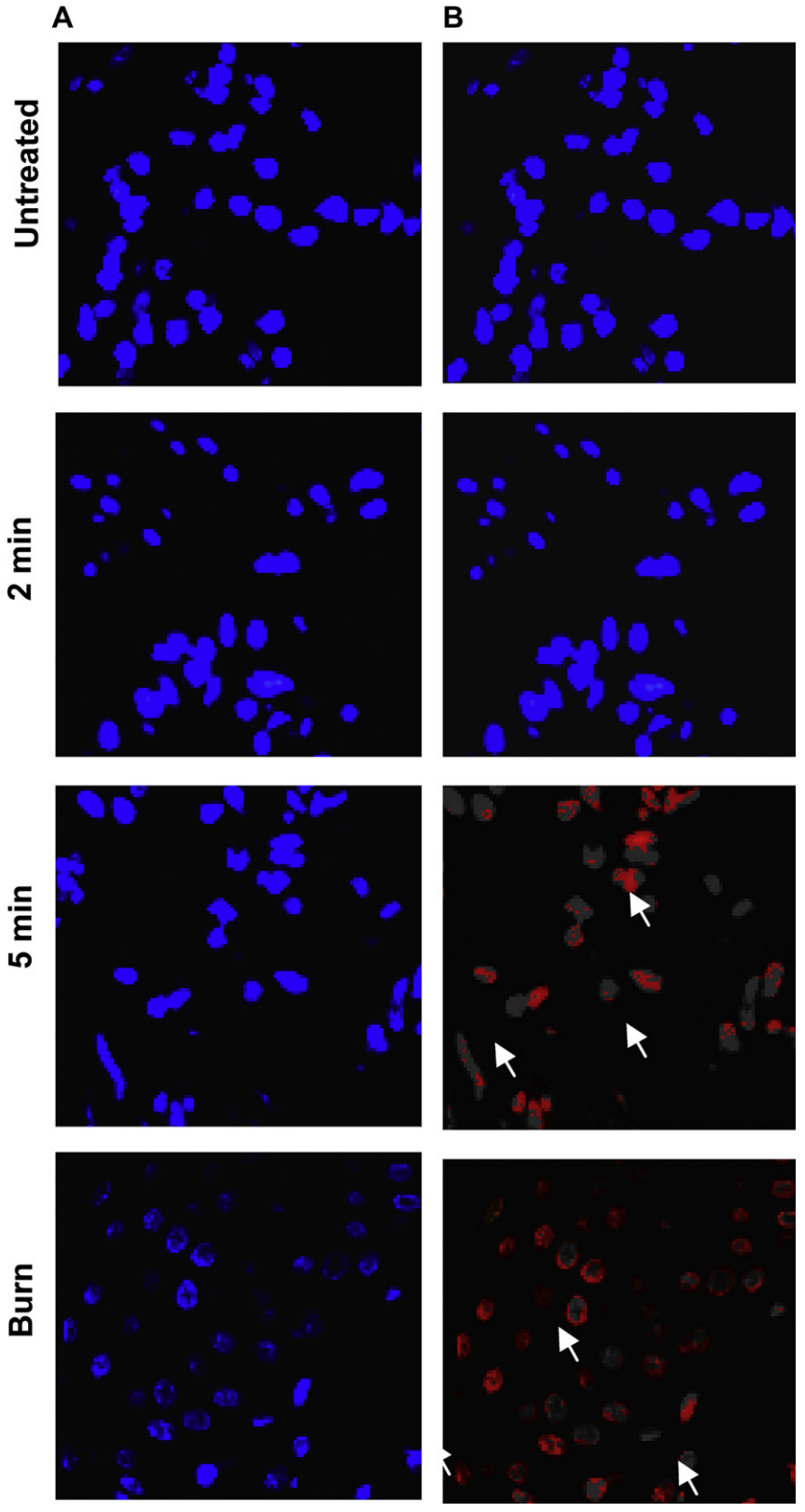


Fig. 5 – Representative microphotographs of immunohistological staining of γ-H2AX proteins of intact skin sections that were dually stained with Hoechst dye (A) for nuclear staining. An overlay generated (B) of these stained images showed γ-H2AX-positve nuclei (arrows). Damage occurred at about 5 min of plasma treatment. (0.17 W/cm^2 power setting; the specimens correspond to those in Fig. 4.)

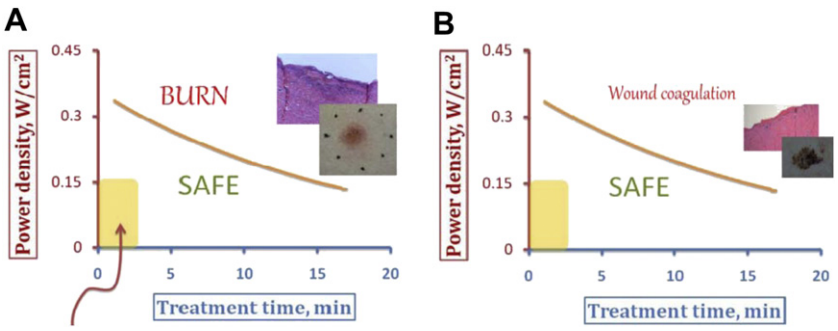


Fig. 6 – Schematic diagrams showing safe regimes of treatment with nonthermal plasma for porcine intact skin (A) and wounded skin (B) tissues. (A) Exposure to doses of plasma for more than five times longer than that is required for complete sterilization is safe at low power (0.13 W/cm^2). We noted classical burn of intact skin only after 5 min or more of a continuous high-power (0.31 W/cm^2) dose of plasma (inset: gross and microscopic samples). (B) Plasma starts coagulating an open wound after 3 min, which protects the underlying wound from further damage by plasma (inset: the gross and microscopic samples of a coagulated wound exposed to high-power [0.31 W/cm^2] dose of plasma for 5 min). Shaded area is the safest plasma-exposure time at given plasma power.

in our data for the wounded skin. Because both the energy and the power and frequency of the plasma matter, at lower levels of plasma power and frequency, we can use longer exposure (treatment) times without causing tissue damage, whereas high-power plasma causes damage at much lower doses of energy (Fig. 6). Further studies for longer than 24 h and sublethal exposures are required to evaluate subacute and chronic toxicity. Such studies are in progress.

5. Conclusion

We concluded from these studies that FE-DBD nonthermal plasma can safely be applied to intact skin for up to 2 min at a plasma power of 0.17 W/cm^2 (20.4 J/cm^2) without inducing any microscopic tissue damage. When this treatment is applied to wounded skin, its blood coagulation properties help induce an early clot, which probably protects against any underlying tissue damage for at least 5 min at a plasma power of 0.13 W/cm^2 (39 J/cm^2) ($P < 0.05$ compared with untreated samples) and possibly more but at least up to 15 min. This pilot study has established thresholds to guide further investigations into medical applications of nonthermal plasma.

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