Toxicity of Direct Non-Thermal Atmospheric Pressure Plasma Treatment of Living Tissue

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Abstract

Non-thermal atmospheric pressure dielectric barrier discharge plasma applied directly to living tissues is now being widely considered for various clinical applications. One of the key questions that arise in this type of topical treatment is if the skin remains undamaged after non-thermal plasma treatment.

The results from the previous rodent model provided strong evidence for the ability of non-thermal plasma to sterilize the surface of the tissue without any thermal damage to the tissue. It is well established that porcine (pig) skin closely resembles human skin; hence we evaluated the potential toxic effects of non-thermal plasma treatment on underlying skin cells and tissue on porcine skin. In a Yorkshire pig model, the intact skin and wounded tissue treatment was carried out at varying doses to locate the damaging power/time (dose) combination and the resulting skin damage was analyzed.

In this paper we study the possible short term and long term toxic effects of the non-thermal plasma treatment on intact living tissue. Non-thermal plasma has been shown to sterilize intact tissue without visible or microscopic damage, and our goal was to identify the boundaries of skin toxicity after treatment.

I. INTRODUCTION

Over the past few years non-thermal atmospheric pressure plasma has emerged as a new promising tool in medicine. Non-thermal atmospheric pressure plasma is generated through direct contact with living tissue. Compared to the effects of the more conventional thermal plasma [1], non-thermal plasma can be selective in its treatment because of the ability to avoid burning healthy tissue (Figure 1.) Medical applications of non-thermal plasma include sterilization of living tissue without damage [2], blood coagulation [2, 3], modulation of cell attachment [4-6], increase transfection efficiency [7, 8] and surface sterilization [9-12]. Our goal was to identify the boundaries of skin toxicity after treatment of intact and wounded tissue with non-thermal plasma.

The principle of operation of the discharge used in this work is similar to the Dielectric Barrier Discharges (DBD) introduced by Siemens in the middle of 19th century [13]. DBD occurs at atmospheric pressure in air or other gases when sufficiently high voltage of sinusoidal waveform or pulses of short duration are applied between two electrodes, when at least one of them is insulated.

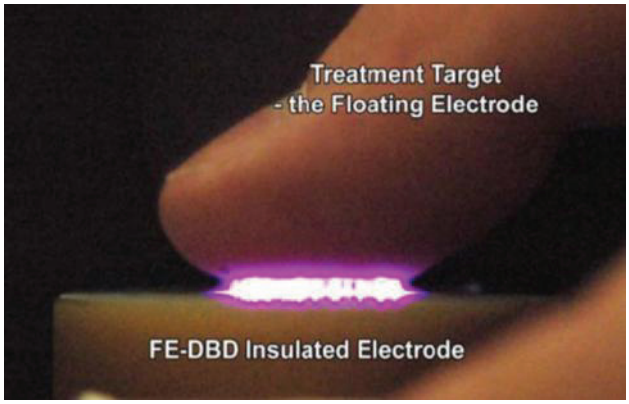


Figure 1. Non-Thermal Atmospheric Pressure Dielectric barrier discharge plasma can be safely applied on living tissue without thermal damage.

Because the presence of an insulator between the electrodes prevents the build-up of high current, the discharge creates electrical-plasma, not to be confused with blood plasma, without substantial heating of the gas (Figure 1). This approach allows for treatment of biological samples without thermal damage while biological processes are initiated and/or catalyzed with the help of electrical charges[8]. The human body, with its high capacity for charge storage on account of its high water content and a relatively high dielectric constant, can function as the second electrode. Thus, the generation of charged particles, radicals, electrically excited molecules, and atoms makes DBD plasma a potentially active yet safe medium.

II. METHODS

A. Plasma Treatment

Non-thermal atmospheric pressure dielectric barrier discharge plasma was produced using an experimental setup similar to one previously described []. The non-thermal plasma was generated by applying alternating polarity pulsed (500 Hz – 1.5 kHz) voltage of ~20 kV magnitude (peak to peak), 1.65 μs pulse width and a rise

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time of 5 V/ns between the insulated high voltage electrode and the sample undergoing treatment using a variable voltage and variable frequency power supply (Quinta, Russia). 1 mm thick, polished clear fused quartz was used as an insulating dielectric barrier covering the 1 inch diameter copper electrode. The discharge gap between the bottom of the quartz and the treated sample surface was fixed at 3 mm (Figure 2*a*). Discharge power density was measured to be 0.13 W/cm^2 (at 500Hz) and 0.31 W/cm^2 (at 1.5 kHz) using both electrical characterization and a specially designed calorimetric system.



Figure 2. (a) Non-thermal plasma treatment of intact skin at low power (b) Electro cauterization of intact skin using high frequency "*Bovie knife*" as a positive control for thermal tissue damage.

B. Study Design

We evaluated the potential toxic effects of the DBD plasma on both intact and wounded porcine skin in 7 Yorkshire pigs. Standard operative procedure included the following: The pig was anesthetized and the dorsum of the pig marked and divided for treatment areas. When intact skin was studied, the plasma probe was placed 3 mm above the skin, varying the power and time of treatment (Figure 3*a*). When wounded tissue was studied, a dermatome was used to create a skin abrasion, removing 1 square inch of the epidermis and dermis (approximately 3 mm deep), followed by the treatment of the plasma one hour after the wounds were created. The plasma probe was also placed 3 mm above the skin (Figure 3*b*), varying the power and time of treatment. Pigs were then sacrificed immediately after the surgical procedure or after 24 hours. Tissue specimens from each treatment area were harvested and sent for histological analysis.

For the intact skin model (3 pigs), we had a total of 42 treatment areas on the three pigs that were harvested 24 hours after surgery. 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 1 of 4 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 (12 specimens). For 0.31 W/cm^2 and 0.17 W/cm^2, there were 3 samples (n=3) treated with plasma for each of the following time points: 30 seconds, 1 minute, 2 minutes, and 3 minutes. For 0.15 W/cm^2 and 0.13 W/cm^2, there were 3 samples (n=3) treated with plasma for each of the following time points: 1 minute, 2 minutes, 5 minutes, and 15 minutes.

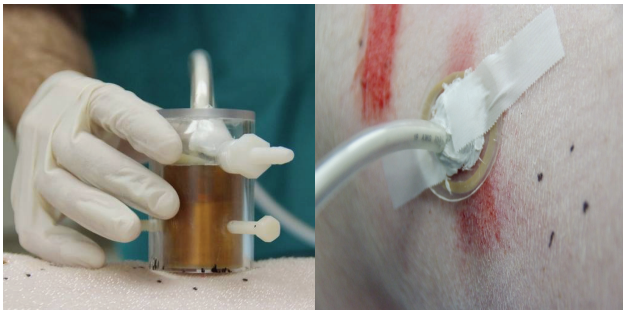


Figure 3. Non-thermal plasma treatment setup (a) Plasma treatment of intact skin and (b) Plasma treatment of wounded tissue.

For the wounded tissue model (4 pigs), we had a total of 42 treatment areas on 2 pigs harvested immediately after surgery and 42 treatment areas on 2 pigs harvested 24 hours after surgery. In the non-survival pigs there were 21 specimens treated with low power (0.13 W/cm^2) and 21 specimens treated with high power (0.31 W/cm^2). In the 24 hour survival pigs, there were also 21 specimens treated with low power (0.13 W/cm^2) and 21 specimens treated with high power (0.31 W/cm^2). Each group of 21 specimens had a breakdown of 3 samples (n=3) treated for the following time points: no treatment (negative control), electrocautery bovie (Figure 2*b*, positive control), 30 seconds, 1 minute, 3 minutes, 5 minutes, and 15 minutes.

C. Histological Analysis

In addition to recording all gross observations of the specimens, all specimens were analyzed for microscopic histological analysis. Specimens were longitudinally sectioned (Figure 4.) and fixed for 24 hours in formalin. Sections for histology were processed in a standard fashion and stained with hematoxylin-eosin.

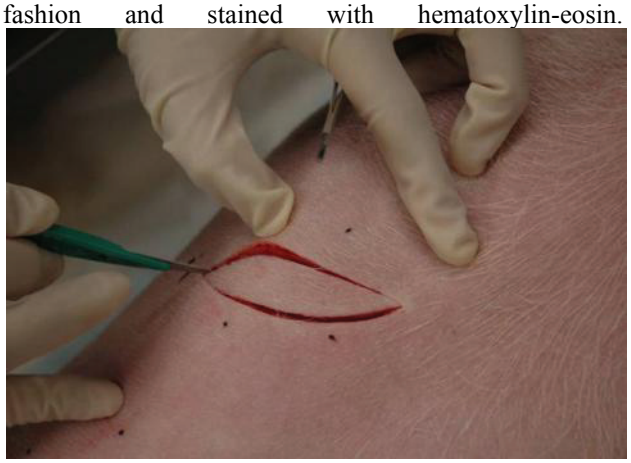


Figure 4. Harvesting of tissue sample after plasma treatment of intact pig skin for histological analysis.

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Our pathologists were blinded to all specimens and categorized each specimen into a burn grading system for the intact and wounded tissue data analysis. For intact skin, the specimens were either classified as normal, minimal change, epidermal damage, or full burn through the dermis. For wounded tissue, the specimens were either classified as normal, presence of a clot or scab, and full burn through the dermis.

III. RESULTS

Endpoints for toxicity analysis consisted of recording both gross and histological examination of the intact skin and wounded tissue specimens. Gross observation correlated with the histologic grading system as mentioned previously.

A. Intact Skin

The plasma device was tested on 3 pigs with intact skin at 4 different power settings, all harvested 24 hours after the procedure. With normal histologic skin samples, the skin appeared normal grossly (Figure 5*a*). With minimal change, there was a small area of erythema on the skin. With epidermal damage, there was mild erythema that resolved usually within 20 minutes. With full burn through the dermis, there was diffuse erythema that remained until time of harvest.

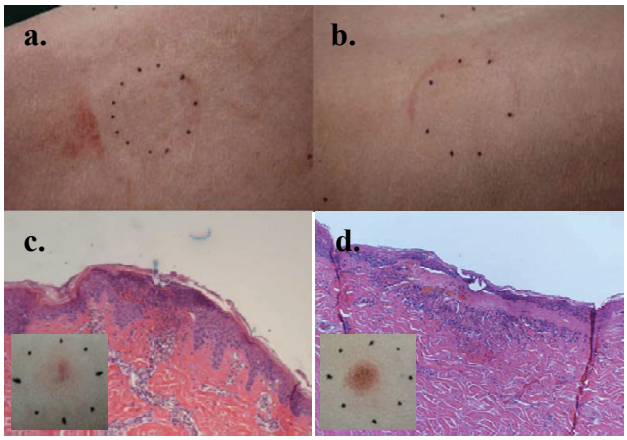


Figure 5. (a) Intact skin before plasma treatment (b) Intact skin after plasma treatment at a dose of 0.13 W/cm^2 for 5 min (c) histological analysis of intact skin after plasma treatment at 0.15 W/cm^2 for 5 min (inset: gross observation of treated skin shows mild erythema which disappears after 20 min) and (d) histological analysis after plasma treatment at a dose of 0.31 W/cm^2 for 3 min (inset: gross observation of skin after plasma treatment shows a full thickness burn)

At low power 0.13 W/cm^2 (4 specimens), 1 specimen treated with plasma for 1 minute was classified as normal skin. The remaining 3 specimens treated with plasma at 2, 5, and 15 minutes all showed epidermal damage on histology and mild erythema on gross examination (Figure 5*b*)

At the next higher level of power 0.15 W/cm^2 (12 specimens), 3 specimens treated with plasma for 1 minute were classified as normal skin. In the samples treated for both 2 and 5 minutes, 2 samples showed epidermal damage and 1 with normal skin. In the 15 minute plasma treatment group, 1 sample showed epidermal damage and 2 with normal skin. (Figure 5*c*)

At power setting 0.17 W/cm^2 (12 specimens), 3 specimens treated with plasma for 30 seconds and 3 treated for 1 minute were classified as normal skin. In the samples treated for 2 minutes, 2 samples showed minimal change and 1 with normal skin. In the 3 minute plasma treatment group, 2 samples showed epidermal damage and 1 sample showed minimal change.

At high power 0.31 W/cm^2 (12 specimens), 3 specimens treated with plasma for 30 seconds were classified as normal skin. In the samples treated for 1 minute, 1 sample showed minimal change, 1 with epidermal damage and 1 with normal skin. In the 2 and 3 minute plasma treatment groups, 2 samples showed full thickness burn and 1 with epidermal damage each (Figure 5*d*)

For our controls, one specimen (n=1) served as our positive control, that of an electrocautery burn which yielded a full thickness burn. Another specimen (n=1) was intact skin without any plasma treatment, serving as our negative control.

Finally (Figure 6.) summarizes all the findings from plasma treatment of intact skin and identifies different regimes of non-thermal plasma treatment

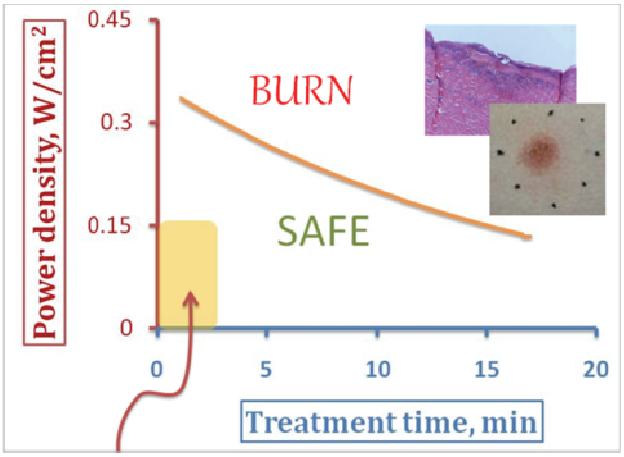


Figure 6. Schematic showing safe regimes of plasma treatment of intact tissue. Plasma treatment at normal operating parameters does not cause tissue damage: Plasma doses up to 10 times longer than required for complete sterilization are safe. (Inset: We see a burn formation only after 10 min of continuous application of high power plasma dose).

B. Wounded tissue

The plasma device was tested on 4 pigs with wounded tissue with either low (0.13 W/cm^2) or high

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(0.31 W/cm^2) power settings. Two pigs were harvested immediately after the procedure, and two pigs were harvested 24 hours after the procedure. With normal histologic skin samples, the skin appeared normal grossly. With the presence of a clot seen on histology, the clot was visible on gross examination in pigs harvested after surgery. In pigs harvested at 24 hours, a scab was seen on gross examination instead of a clot. With full burn through the dermis seen on histology, there was burned tissue seen grossly.

For the pig treated with low power 0.13 W/cm^2 (21 specimens) harvested immediately, 3 samples of our untreated wounds (negative control) and 3 samples of wounds with an electrocautery burn (positive control) showed normal tissue and full thickness burn respectively. 3 samples with 30 seconds treatment showed a clot on histology. With 1, 3, and 5 minute treatment groups, 2 showed a clot and 1 was normal in each group (Figure 7*a*). With 15 minute treatment, all 3 samples showed a clot.

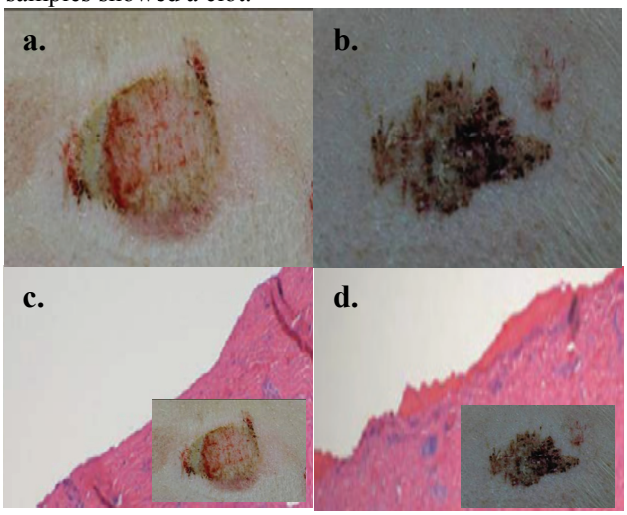


Figure 7. (a) Wounded tissue after plasma treatment at a dose of 0.13 W/cm^2 for 1 min (b) wounded tissue after plasma treatment at a dose of 0.31 W/cm^2 for 15 min (c) histological analysis of wounded tissue after plasma treatment at 0.13 W/cm^2 for 1 min (inset: gross observation of wounded tissue shows normal wound without burn) and (d) histological analysis after plasma treatment at a dose of 0.31 W/cm^2 for 15 min (inset: gross observation of wounded tissue after plasma treatment shows scab formation due to blood coagulation)

For the pig treated with high power 0.31 W/cm^2 (21 specimens) harvested immediately, 3 samples of our untreated wounds (negative control) and 3 samples of wounds with an electrocautery burn (positive control) showed normal tissue and full thickness burn respectively. With 30 seconds, 1 and 3 minute treatment groups, all three specimens showed a clot. With 5 and 15 minutes of plasma treatment, 2 showed a clot and 1 was normal in each group (Figure 7*b*)

For the pig treated with low power 0.13 W/cm^2 (21 specimens) harvested 24 hours after, 3 samples of our untreated wounds (negative control) and 3 samples of wounds with an electrocautery burn (positive control) showed normal tissue and full thickness burn respectively. With 30 seconds of treatment, 2 samples showed a burned scab and 1 with normal tissue. With 1, 3, and 5 minutes of treatment, all 3 samples in each group showed a burned scab. With 15 minutes of treatment, 2 showed a burned scab and 1 was normal (Figure 7*c*)

For the pig treated with high power 0.31 W/cm^2 (21 specimens harvested 24 hours after, 3 samples of our untreated wounds (negative control) and 3 samples of wounds with an electrocautery burn (positive control) showed normal tissue and full thickness burn respectively. With 30 seconds of treatment, 2 samples showed a burned scab and 1 with normal tissue. With 1 and 3 minutes of treatment, 1 sample in each group showed a burned scab and 2 with normal tissue. With 5 minutes of treatment all 3 samples showed a burned scab. With 15 minutes of treatment 2 samples showed a full thickness burn and 1 with a burned scab (Figure 7*d*).

Finally (Figure 8.) summarizes all the findings from plasma treatment of wounded tissues and identifies different regimes of non-thermal plasma treatment

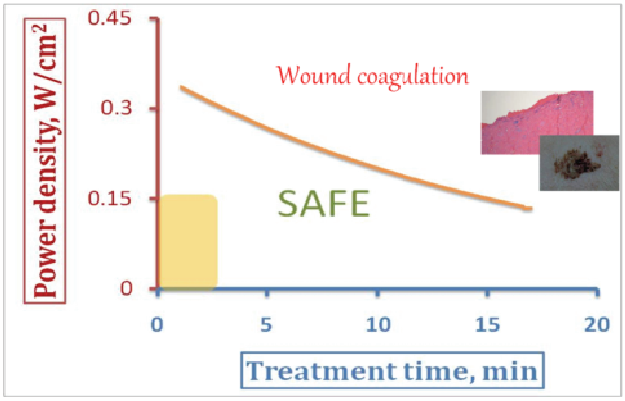


Figure 8. Schematic showing safe regimes of plasma treatment of wounded tissue. Plasma treatment at normal operating parameters does not cause tissue damage in wounds. Non-thermal Plasma starts coagulating an open wound after 3 min which protects the underlying wound from further damage by the plasma. (Inset: We see coagulated wounds at higher doses of 0.31 W/cm^2 or longer treatment times beyond 5 min)

IV. DISCUSSION

This animal study was the first in vivo study to assess the boundaries of non-thermal plasma toxicity on living tissue. From data of plasma treatment of intact skin, we can conclude that 2 minutes is the observed threshold for any signs of histological tissue damage created from non-thermal plasma treatment at all power settings except for high power (0.13, 0.15, 0.17 W/cm^2) treatment. When high power plasma was used, signs of minimal change,

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epidermal damage and full thickness burn were all seen beginning as early as 1 minute. The pathologists defined "minimal change" as the presence of epidermal and dermal cellular changes in both size of the cell and nucleus. Although almost appearing similar to normal skin, it was determined that due to the presence of some microscopic changes that an additional grading of "minimal change" be provided. With epidermal damage, there was notable increased vascular congestion and cellular disorganization.

From data of plasma treatment of wounded tissue, we can conclude that the presence of a wound induced clot formation on all wounds which was protective to the underlying skin from plasma treatment damage. In non-survival pigs, a clot was seen on the wounded tissue, and in 24 hour survival pigs, a scab was seen on the wounded tissue. All time points of 30 seconds, 1, 3, 5, and 15 minutes showed some type of clot or scab formation without disruption and/or damage to the underlying skin except for only one subgroup: high power treatment of wounded tissue for 15 minutes (2 specimens showed full thickness burn). It has been reported in the literature that plasma treatment of tissue may sterilize tissue with as little as 30 seconds of treatment[2]. Since tissue coagulation is a property of non-thermal plasma[2], we can see that the ability to sterilize wounded tissue while stopping bleeding and inducing the formation of a protective clot may be of a clinical benefit in the field of medicine.

Drawbacks to our experimental study are threefold. The first is that our representative sample specimens for each type of skin (intact or wounded) treated for a particular time point and power were not even. For almost all sample groups, 3 specimens (n=3) were studied, except for the low power treatment of intact skin in which we had 1 specimen per group. This was attributed to the novelty of the experiment in which low power treatment of intact skin was the first subgroup to be tested and 1 sample for each time point was performed for feasibility purposes. Subsequently 3 samples were tested in each group for each time point.

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 burn. There were several specimens which did not show this. For example, in our intact skin treated with 0.15 W/cm^2, 3 skin specimens treated for 15 minutes yielded one sample with a full thickness burn and 2 with normal skin whereas the specimens treated for 5 minutes yielded 2 with a full thickness burn and 1 with epidermal damage. The reason for much of this variability rests on 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. Plasma treatment of skin samples closer to the spine of the pig were more likely to have greater erythema relative to treated skin samples lateral to the spine due to increased thickness of the skin and greater subcutaneous tissue. In addition, the experimenter had the task of holding the plasma device for the appropriate period of time of each subgroup 5mm above the intact skin. We minimized this error by placing a 3 mm spacer on the tip of our plasma probe which we began using on our wounded tissue specimens. There is less variability in our data for the wounded tissue.

V. CONCLUSIONS

Non-thermal plasma has only recently been applied within the field of medicine. It has been shown to induce apoptosis in malignant tissue and provide a means for tissue coagulation and sterilization while limiting damage to healthy tissue both grossly and microscopically. From our pig studies, we have shown that non-thermal plasma treatment of tissue can safely be applied to intact skin for up to 2 minutes without inducing any microscopic tissue damage. When applied to wounded tissue, its coagulative properties helps induce a clot which is protective of any underlying tissue damage for at least 5 minutes and possibly as high as 15 minutes. Once application of non-thermal plasma can be safely shown to prevent and/or limit toxicity in the animal model, its use may be studied and applied in humans for the medical treatment of diseases, wound healing, and trauma.

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