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A new flexible DBD device for treating infected wounds: *in vitro* and *ex vivo* evaluation and comparison with a RF argon plasma jet

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Abstract

Cold plasma has been shown to provide a promising alternative antimicrobial treatment for wound healing. We developed and tested a flexible surface dielectric barrier discharge (DBD) and compared it to an argon gas based plasma jet operated remotely with a distance between plasma plume and sample of 8 mm. Tests were conducted using different models: on cultured cells, on *ex vivo* human skin and on bacteria (*Pseudomonas aeruginosa*) (on agar, in suspension, in collagen/elastin matrix or on *ex vivo* human skin), allowing us to directly compare bactericidal with safety aspects under identical conditions.

Both plasma devices were highly efficient when used on bacteria in non-buffered solutions, but DBD was faster in reaching the maximum bacterial reduction. Treatment of bacteria on intact skin with DBD resulted in up to 6 log reductions in 3 min. The jet was far less efficient on intact skin. Even after 8 min treatment no more than 2 log reductions were obtained with the jet. Treatment of bacteria in burn wound models with DBD for 6 min resulted in a 4.5 log reduction. Even when using DBD for 6 min on infected burn wound models with colonizing or biofilm phase bacteria, the log reductions were 3.8 or 3.2 respectively.

DBD plasma treatment for 6 min did not affect fibroblast viability, whereas a treatment for 8 min was detrimental. Similarly, treatment with DBD or plasma jet for 6 min did also not affect the metabolic activity of skin biopsies. After treatment for 8 min with DBD or plasma jet, 78% or 60% of activity in skin biopsies remained, respectively. Multiple treatments of *in vitro* burn wound models with surface DBD for 6 min or with plasma jet for 8 min did not affect re-epithelialization.

With the flexible surface DBD plasma strip we were able to quickly inactivate large numbers of bacteria on and in skin. Under the same conditions, viability of skin cells or re-epithelialization was not affected. The DBD source has potential for treating larger wound areas.

Keywords: dielectric barrier discharge, human burn wound model, pseudomonas aeruginosa, bacterial reduction, radio frequency argon jet, wound healing

(Some figures may appear in colour only in the online journal)

1. Introduction

Patients with extensive burns are susceptible to opportunistic pathogens, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* [1, 2], due to their large open wounds and compromised immune system [3]. Treatment with topical as well as systemic antimicrobials is hampered by poor penetration into the burn eschar, while systemic treatment is also problematic due to development of bacterial resistance to antibiotics. To combat burn wound infections, different antimicrobials are available but many have detrimental effects on the healing process [4]. Cold atmospheric plasma might provide an alternative treatment to reduce the bacterial load in burn wounds. Using the same conditions, bacteria can be inactivated with an argon radiofrequency plasma jet with no impact on the cell viability of fibroblasts or keratinocytes [5]. Plasma might provide a disinfection technology that is not constrained by bacterial resistance [6]. Cold atmospheric plasma devices have been applied to chronic wounds to reduce the bacterial load *in vivo* [7–10] and have been tested for safety on donor site healing [11]. Safety aspects such as pain and trans epidermal water loss have been investigated on patients and volunteers [8–10, 12]. Acidification of the skin, NO penetration and enhanced microcirculation was shown for a DBD device [13]. However, due to low levels of bacterial reduction, repeated treatments are required for infection control. Therefore increased (antimicrobial) efficacy of plasma treatment for *in vivo* relevant conditions without inducing negative effects would be an improvement.

In our search for an optimal antibacterial plasma treatment without reducing the wound healing potential, we investigated a new flexible, surface Dielectric Barrier Discharge (DBD) strip and compared it to a remote argon radiofrequency (RF) plasma jet, which has been studied extensively [5, 14]. The RF plasma jet consists of a concentric powered needle electrode surrounded by a glass tube and a grounded ring electrode positioned at the end of the glass tube [14]. The general design of a DBD device consists of a powered electrode (a copper plate) and a ground electrode of stainless steel wire mesh. A dielectric plate, often quartz, separates the powered electrode from the ground electrode. Application of a high voltage on the powered electrode results in plasma at the ground electrode. An advantage of such a design is that DBD's can be sustained in a variety of gases—including air at atmospheric pressure—at reasonable voltages. The antibacterial effects of DBD's have been shown in several studies [15, 16]. Effective reduction of bacteria on skin has been demonstrated for DBD although non-biological surfaces were easier to disinfect [17, 18]. Safety settings have been explored for DBD treatments on pig revealing maximum treatment times of 2 min at high power (0.3 W cm^{-2}) and 15 min at low power (0.13 W cm^{-2}) [19].

The general design of DBD's limits their use to straight surfaces. Because the curvature of the human body has to be taken into account as well, the applicability of a more flexible DBD was studied in the present paper. In a similar approach, application of flexible direct DBD showed no damage in human skin biopsies [20] and treatment with a hand-held DBD resulted in reduction of bacterial load and reduction of ulcer size [8].

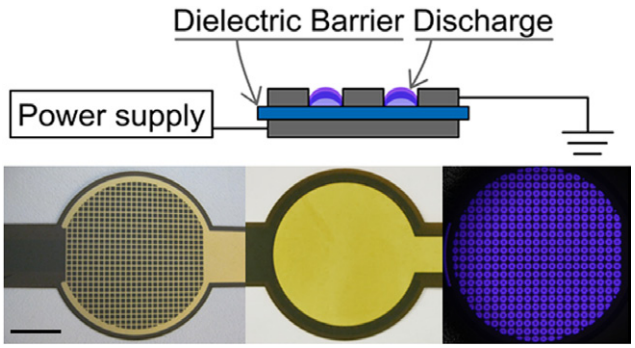

Figure 1. (Top) Side-view schematic representation of the surface dielectric barrier discharge strip; (left) top-view photograph of the grounded electrode of the plasma strip; (middle) bottom-view photograph of the charged electrode of the plasma strip; (right) top-view photograph while plasma is on. Scale bar is 1 cm.

Typically, inactivation by plasma has been tested using bacteria on agar plates, in distilled water or (buffered) saline solution. Conditions mimicking or approaching the *in vivo* situation have been investigated as well [18, 19, 21–28] but most studies focused on either bacteriology or safety only. Test conditions are highly relevant because the plasma efficacy (for safety and bacterial inactivation) is greatly influenced by the composition of the media particularly its buffering capacity [29, 30]. We therefore performed antibacterial tests on agar plates, in saline solution, on a 3D-collagen/elastin matrix, on intact human skin and in an *ex vivo* human burn wound model. Safety tests were performed on primary human dermal fibroblasts, skin biopsies and re-epithelialization in the burn wound model. This allowed us to directly compare bactericidal aspects to safety aspects under identical conditions.

2. Materials and methods

2.1. Plasma sources

2.1.1. Structured surface dielectric barrier discharge (plasma strip). A dedicated structured surface dielectric barrier discharge (DBD plasma strip) has been constructed for wide area measurements. The schematics can be seen in figure 1. The device was made using the printed circuit board technology. It consists of a dielectric barrier (polyimide, 100 μm thick) with a uniform powered electrode on one side and a meshed grounded electrode on the other side of the barrier. The mesh lines are 30 μm thick, 0.25 mm wide with 0.7 mm spacing between them. The strip measured 2.5 cm in diameter but can be produced in larger formats. The advantage of such a surface DBD device is that a counter electrode is not required to generate plasma and no current is flowing through the treated sample. In addition, gas flow is not required for DBD, which simplifies the handling and reduces operating costs. The plasma strip was ac-driven at 6.6 kHz, 3.5 kV (peak-to-peak) in air. Power dissipated in the plasma in these conditions was 0.7 ± 0.22 W, the plasma surface power density was 0.14 W cm^{-2}. The plasma strip was operated for up to 8 min at the above

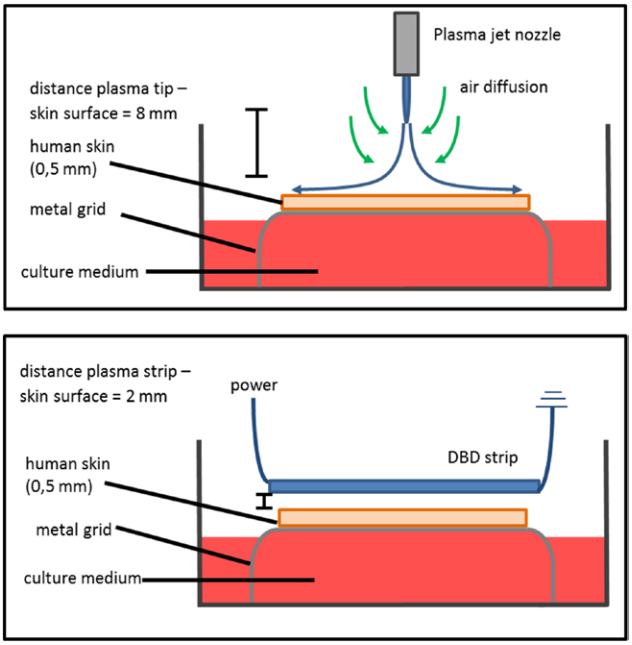

Figure 2. Side-view schematic representation of treatments of burn wound models with the plasma jet (top) or DBD strip (bottom). *Ex vivo* human skin is placed on stainless steel grids in 6 well plates.

given conditions. The surface of the suspension was always set at 2–3 mm from the visible tip of the plasma effluent for the reported plasma settings (figure 2).

2.1.2. Plasma jet. A kHz modulated cold atmospheric RF plasma jet with a frequency of 13.56 MHz was used. The plasma jet is as described in detail in [14]. The jet is made in a coaxial geometry, with a pin as a powered electrode housed in a quartz capillary. The grounded ring electrode is present at the outer side of the capillary, coinciding with the end of the powered inner needle electrode. Treatments with the jet were performed for 30 s up to 8 min with a gas flow of 1.5 slm of argon (purity 5.0). To reduce the gas temperature of the plasma, the RF voltage signal was modulated with a 20 kHz TTL pulse with a duty cycle of 20% or 50%. A continuously driven jet was tested for comparison. The average power, which was set by adjusting the input voltage, was 1.7 ± 0.1 W for both continuous and time modulated plasma and has been obtained as described in detail in [31]. Because pulsing did not result in major differences in outcomes, only the results for the 50% duty cycle are reported. The surface of the suspension was always set at 8 mm from the visible tip of the plasma effluent for the reported plasma settings (figure 2). It needs to be stated that distance variations greatly affect the reactivity of the plasma [32–34].

We previously showed that the plasma jet in the remote treatment, as used in the present work, exerts its bactericidal effect through plasma induced fluid chemistry and that (V)UV, electric fields, ions or heat flux do not play an important role [14].

2.2. Bacterial culture

P. aeruginosa (strain PAO1) and *S. aureus* (burn wound isolate 06050415283) were routinely cultured on Luria Broth (LB, Invitrogen, Paisley, UK) agar at 37 °C. Bacteria from a proliferating/logarithmic culture in 5 ml LB were diluted in LB, 0.85% NaCl, phosphate buffered saline (PBS, Invitrogen), or DMEM culture medium (Invitrogen) to approximately 10^7 colony forming units (CFU)/ml, based on OD_{600}. To quantify the CFU/ml before and after treatment, bacterial suspensions were serially diluted and plated on LB agar plates. The antibacterial effect of the treatment was calculated by the log reduction $\log(N_T\text{-}N_C)$, where N_T is the number of viable cells after treatment and N_C the number of viable cells in control samples.

To determine the sensitivity of PAO1 for different combinations of H_2O_2, $NaNO_2$ and pH, cross Minimum Inhibitory Concentration/Minimum Bactericidal Concentration (MIC/MBC) assays were performed. In a standard MIC assay, a 2-fold dilution range of an antibacterial component is added to bacterial suspensions in LB. After overnight incubation, bacterial survival is apparent by the turbidity of the medium. Bacterial death in clear medium can be confirmed by plating on LB agar. The lowest concentration at which bacteria are inhibited in their growth is the MIC. MBC is the lowest concentration at which bacteria are killed. Separate 2-fold dilutions were made starting at 0.012% (H_2O_2) or 240 mM ($NaNO_2$) in LB pH 5, 6 or 7. Fifty μl of each of these dilutions were combined with 100 μl of PAO1 suspension (5×10^4 CFU ml^{-1}) in LB with corresponding pH, yielding 144 different combinations. After overnight incubation at 37 °C, bacterial growth was scored based on turbidity. Killing was confirmed by plating 100 μl on LB agar plates.

2.3. Cell culture

Human dermal fibroblasts were isolated from the dermis as described in [35] and cultured in fibroblast culture medium (DMEM, supplemented with 10% fetal calf serum, 1 mM L-glutamine (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Fibroblasts were seeded in 96 well plates at a density of 4000–8000 cells/well (96 wells) or 40.000 (6 wells) and cultured until 80% confluence was reached.

The temperature of the DBD surface was measured by using a hand-held infrared thermometer (Fluke 61) with an accuracy of 2% at close to room temperature conditions. The ozone production was measured by using UV absorption spectroscopy. A UV LED source (UVTOP 250-HL-TO39) emitting at 255 nm was directed parallel to the meshed electrode on the grounded side of the plasma strip to be absorbed by the plasma forming in the gaps of the grounded mesh. Avantes Avaspec-2048-USB2 spectrometer was used as a detector. The measurements were done in a semi-enclosed volume comparable in size to a well in a 6 well plate but without liquid.

To estimate the number of viable eukaryotic cells after treatment, MTT assays [36] were performed. Immediately after plasma treatment, cells were washed and fibroblast culture medium containing 2 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added. Note that there is no direct plasma induced liquid chemistry effect on the MTT assay possible. Cells were incubated with MTT medium for 2h at 37°C, 5% CO_2. After discarding the medium,

formazan in the cells was dissolved in DMSO and quantified in a spectrophotometer (OD_{560}–OD_{650}).

2.4. Burn wound model

To determine the antibacterial effect of plasma in a wound environment, an *ex vivo* human skin model for infected burn wounds was used [37]. The effect of plasma treatment on wound healing was determined using the same model without bacteria.

Human skin was obtained from healthy donors undergoing dermolipectomy, or from deceased donors via the Euro Tissue Bank, after obtaining consent according to institutional guidelines. Split-thickness skin grafts (0.7 mm thickness) were harvested using a dermatome and were cut into 1 cm^2 pieces. With a copper device (2 × 10 mm) attached to a soldering iron, burn wounds were created (95 °C, applied for 10 s). This would correspond to a full thickness burn. The burned skin samples were placed epidermis up on stainless steel grids and were cultured air exposed at 37 °C with 5% CO_2 in burn wound medium [36], with 0.25% penicillin/streptomycin.

For the infection model, 10 μl of a bacterial suspension containing approximately 10^5 CFU was placed in the burn area. Antibacterial treatments were applied after 45–60 min (figure 2). Controls consisted of untreated, infected skin samples. Bacteria were dislodged from the skin in 1 ml PBS by using a TissueLyser LT (Qiagen, Venlo, The Netherlands) during 4 min at 45 Hz. Bacterial survival was determined by plating serial dilutions.

For the wound healing model, skin samples without bacteria were kept in culture for up to three weeks.

2.5. Plasma treatment methods and analysis

During treatment, the surface to be treated was always set at 2–3 mm for the DBD and at 8 mm from the tip of the plasma jet. Bacterial suspensions were treated in 6 well plates, in a 3D-collagen-elastin matrix (15 mm in diameter, 1 mm thickness) or intact skin in petridishes. Immediately after treatment, bacterial survival was determined by plating serial dilutions. Bacteria in matrices or on skin were dislodged by using a TissueLyser as described above under section 2.4.

The pH was measured over time with a pH meter (VWR Symphony SB70P) in 2 ml of solution in 6 well plates. The DBD device fits in the wells of 6 well plates. As the size of the pH probe does not allow measurements of small volumes, volumes of 2 ml were treated. In this way, pH can directly be related to the bacterial treatments in 6 well plates.

Fibroblasts in 6 well plates were washed twice with 0.85% NaCl and were treated in 2 ml 0.85% NaCl. Immediately after treatment, cells were washed twice with culture medium to remove any active compound and fresh medium was added. In this way, the cells were removed from the treated solution within 10 s after treatment, similar as for the bacteria. Similarly, skin biopsies (6 mm in diameter, 0.5 mm in thickness) were treated in petridishes with plasma and stained for activity with MTT as described in section 2.3. Formazan was dissolved from skin in 1 ml DMSO by using a TissueLyser during 4 min at 45 Hz.

Table 1. Plasma parameters of DBD and the RF jet.

	DBD	Jet
Driving frequency	13.56 MHz	6.6 kHz
Distance to treated surface	8 mm	3 mm
Power (plasma dissipated)	0.7 W	1.7 W
Power surface density	0.14 Wcm^{-2}	0.45 Wcm^{-2} (non-homogeneous)
Modulation	No	50%
Gases	Air	Ar with small amount of air
Ozone density	3000 ppm	~2 ppm

2.6. Histology

Burn wound samples were processed for paraffin embedding. Sections (5 μm) were deparaffinised and rehydrated for haematoxylin and eosin (H&E) staining, using standard techniques. The newly formed epidermis was measured with digital image analysis (NIS Elements Ar software, Nikon, Amsterdam, The Netherlands).

2.7. Statistics

Results of MTT assays were expressed relative to their respective untreated controls before averaging to circumvent donor variations. Statistical analysis was performed with SPSS (Version 16.0 for MS Windows, SPSS Inc, Chicago, IL). The Mann-Whitney U (MWU) test was used to determine significant differences. Wilcoxon matched-pairs signed rank test (WMP) was used for paired samples (wound healing).

3. Results

We developed a flexible DBD plasma strip (figure 1) to enable treatment of larger, curved surfaces like skin. Safety aspects as well as bactericidal effects have been investigated. Where possible, a comparison is made with the previously described argon plasma jet. Major parameters of both plasma devices are listed in table 1.

3.1. Bactericidal effect

Inactivation of bacteria in suspension by various plasma devices has been studied extensively. The inactivation mechanism is often driven by acidification of the sample and production of ROS and RNS (e.g. H_2O_2 and NO_2^-). Initially we focused on determining the most optimal settings for the argon plasma jet by varying time modulation (duty cycle), power and distance. Optimal bactericidal effect was observed for the jet between 7 and 12 mm distance between tip of the plasma plume and the treated surface. Time modulation of the RF jet at the same average plasma power resulted in similar log reductions of bacteria. Therefore only the results with the plasma jet at 50% duty cycle, which has an optimal performance at 8 mm, are reported here. Note that optimization has

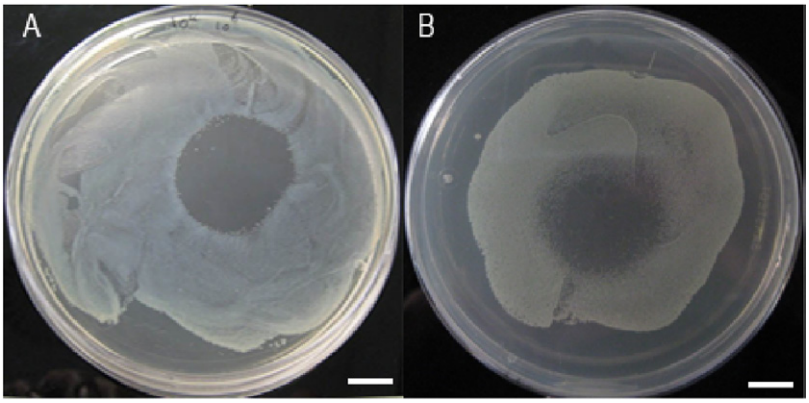

Figure 3. Example of plasma treated *P. aeruginosa* on agar plate. Ten million CFU were spread on LB agar plates, treated with DBD for 10 s (A) or with plasma jet for 2 min (B) and incubated overnight. Effective treatment resulted in a clear halo. Scale bar is 1 cm.

been performed at fixed power (1.7 W), flow rate and gas composition (Ar).

Because the area that can be treated with a plasma jet is limited in size, we developed and tested a DBD strip. Initial tests with bacteria spread on agar plates showed that the DBD strip inactivated bacteria efficiently for treatment times starting from 10 s (figure 3(A)). Similar results have been shown for example by Heinlin and co-workers [38]. To inactivate bacteria in a similar area with the jet, a treatment time of at least 2 min was needed (figure 3(B)). Treatments of small spots of 50 μl of different amounts of bacteria also demonstrated that up to 8 log reductions on agar could be achieved in 1 min with DBD.

For a better quantification of the bactericidal effect, the bacteria were treated in saline solution. A DBD treatment for 8 min was required to obtain a maximal log reduction of 5.6 in 2 ml saline solution (figure 4(A)). This proved to be slightly faster compared to the plasma jet, which resulted in a 2.8 log reduction after 8 min. When treating smaller volumes of 100 μl with the jet, maximal log reduction can be obtained within 2 min [5].

Acidification of the solution was evident for the DBD device similar to the jet, but appeared to progress faster and to a lower pH value for the DBD (figure 4(B)). A low pH did not coincide with a high log reduction indicating that other components such as H_2O_2 are involved as well. The ozone measurements on DBD determined a steady production of ozone in the gas volume reaching the level of 3500 ppm after 3 min of operation. The presence of increased water vapour present for the treatment conditions will most likely reduce this value. Although the DBD slowly decreased the pH of PBS from 7.3 to 6.3 in 6 min, this did not result in bacterial inactivation.

The measured temperature of the DBD on the surface of the strip was between 51 and 56 °C. When 2 ml saline in 6 well plates were treated with the DBD plasma for 6 min, the temperature of the liquid increased from 21.9 °C to 32.3 °C (figure 4(C)). Although the gas temperature near the plasma tip has been shown to be approximately 80 °C [14], the temperature of the liquid did not exceed 30 °C after 6 min of treatment. This is ascribed to flow enhanced evaporation effects in the case of the jet. To thermally inactivate bacteria, incubation at 60 °C for 30 min is required [39].

In summary, the DBD plasma induces a fast acidification of the solution. The net result is that the log reduction by the DBD and the jet follow a similar pattern but the effect with DBD is faster (figure 4(A))

The dependency of bacterial killing on pH, NO_2^- and H_2O_2 was further illustrated by a cross MIC/MBC assay. We tested different combinations of H_2O_2 and $NaNO_2$ in LB pH 7, 6 or 5 (figure 5). *P. aeruginosa* was much more sensitive for H_2O_2 than for $NaNO_2$ at pH below 6. Clearly, pH has a larger impact on the bactericidal effect of H_2O_2 than on that of $NaNO_2$. At pH 5, the lowest bactericidal combination was 1.88 mM $NaNO_2$ and 1–4 μM H_2O_2.

To simulate/approach the wound environment, we tested the plasma strip on bacteria in a 3D-collagen/elastin matrix (CEM). Collagen and elastin are major components of the dermis. Treatment of bacteria in CEM with DBD resulted in 5 log reductions in 1.5–3 min when saline was used. When bacteria were applied to CEM in a buffered solution (PBS or DMEM), the log reduction by the DBD was strongly reduced (data not shown). The buffering capacity of the used solution plays a dominant role in this model as well.

Following, bacteria on the surface of intact skin were treated with both plasma devices (figure 6(A)). These tests revealed that relatively short treatment times (3 min) were required for the DBD plasma to obtain 6 log reductions. In contrast, treatments of up to 6 min with the plasma jet did not yield more than 2 log reductions for bacteria on intact skin (figure 6(A)).

Because good results were obtained with the DBD strip when treating bacteria on intact skin, we proceeded with treating infected burn wound models. Small burn wounds (2×10 mm) were created in *ex vivo* skin, bacteria were applied and treated with the DBD strip. Up to 4.6 log reductions were obtained when treating bacteria in burn wound models for 6 min (figure 6(A)). The longer treatment times required for maximal reduction in the burn wound model, compared to bacteria on intact skin, are probably related to bacteria present underneath the (burned) epidermis [37]. The DBD was similarly effective against *S. aureus* in burn wound models (data not shown). The DBD was even effective against *P. aeruginosa* in these burn wound models after 3 h of

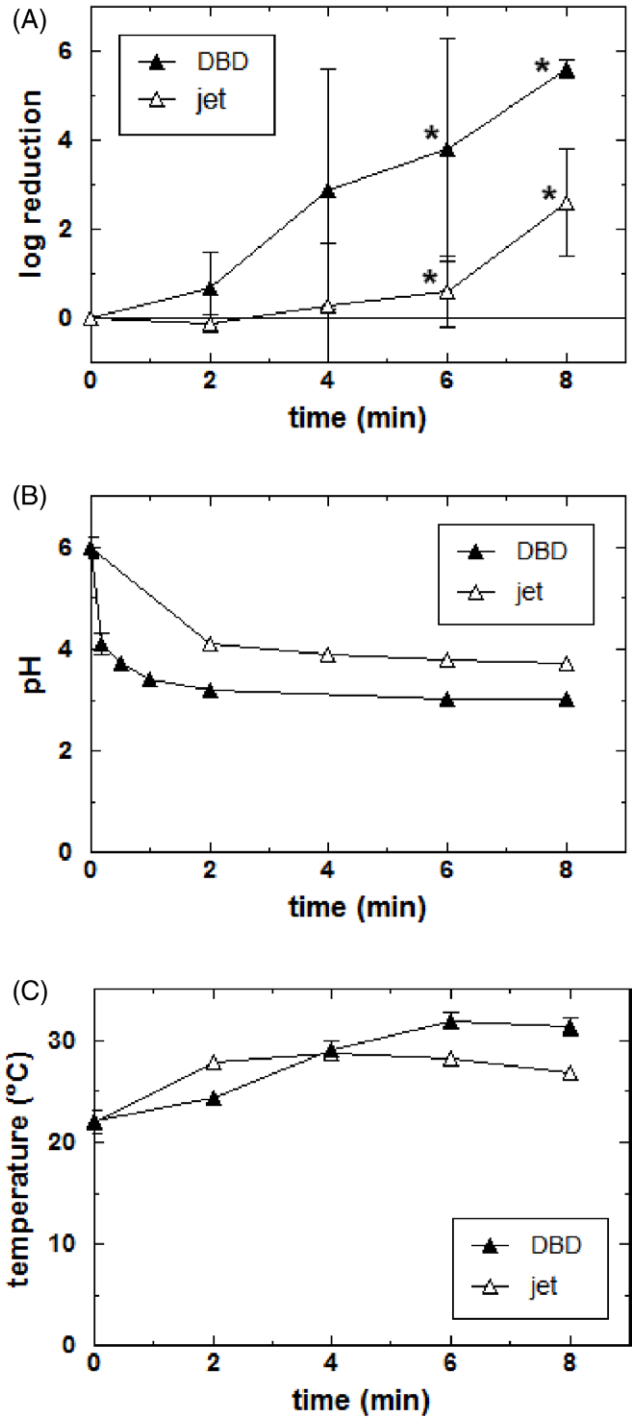

Figure 4. (A) Log reduction after plasma treatment of *P. aeruginosa* in 2 ml saline solution in 6 well plates as a function of treatment time. Bacteria were treated with DBD or with jet. Mean values and standard deviation of at least 3 experiments are shown. Significant differences are indicated from untreated control (*, MWU, $P < 0.05$). (B) The effect of plasma treatment on pH. (C) The effect of plasma treatment on temperature of the solution. Two ml of saline solution in 6 well plates were treated with DBD or with jet in all cases. Mean values and standard deviation of at least 3 experiments are shown.

adherence ($P < 0.05$) or when present in the form of a biofilm after 24 h (although the latter observation should be indicated as a trend, $P > 0.05$) (figure 6(B)).

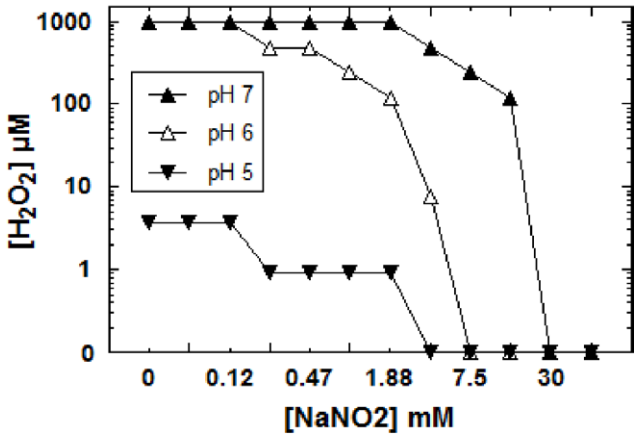

Figure 5. Representation of the cross-MBC results for *P. aeruginosa* in LB at different pH's. Shown are the MBC values for the different combinations. On the *x*-axis, the concentration of $NaNO_2$ is depicted. On the *y*-axis, the concentration of H_2O_2 is shown on a log-scale. Data points indicate the concentration at which bacteria are killed. Conditions under the curve do not kill or inhibit bacterial growth. A shift to the left (from pH 7 to pH 6), indicates increased sensitivity for $NaNO_2$. A shift down (from pH 6 to pH 5), indicates increased sensitivity for H_2O_2.

3.2. Safety aspects

The safety of the DBD was tested on near confluent layers of human dermal fibroblasts (from 4 donors) with 2 ml of saline present in 6 well plates, similar to the tests for log reduction and pH. Immediately after treatment samples were washed and incubated with MTT containing medium. Up to 6 min of treatment with the DBD did not affect fibroblast viability. These conditions were sufficient to obtain 4 log reduction when treating bacteria in saline solution (figure 4(A)). Treatment for 8 min was detrimental for fibroblasts and reduced activity to 3% ± 0% (WMP, $P < 0.05$), which is most likely related to the relatively long incubation at pH 3.

The effect of plasma on the metabolic activity of human skin samples was tested by treating the epidermal side of punch biopsies (4 mm in diameter, 0.5 mm in thickness, 3 donors) in plastic petridishes. Immediately after treatment samples were washed and incubated with MTT containing medium. Treatment with DBD plasma up to 6 min did not affect the activity of these biopsies, while a 3 min treatment was sufficient for a 6 log reduction on intact skin (figure 4(A)). Similarly, treatment with the plasma jet for 6 min did also not affect the activity of skin samples. During this treatment, the temperature of the skin increased to 30 °C (DBD) or 35 °C (jet). Treatment for 8 min with DBD or jet did reduce activity in skin samples to 78% ± 5% or 60% ± 26%, respectively (WMP, $P < 0.05$).

To determine the effect of plasma on wound healing, burn wound models were treated with the DBD plasma for 4 or 6 min or with the plasma jet for 8 min. These treatment times were based on the results presented in figure 6(A), to gain a maximal antibacterial effect. Treatment was applied every other day, 5 times in the first 10 d of the experiment. Silver sulphadiazine crème (Flammazine), which is the standard of care in many burn centres, was included

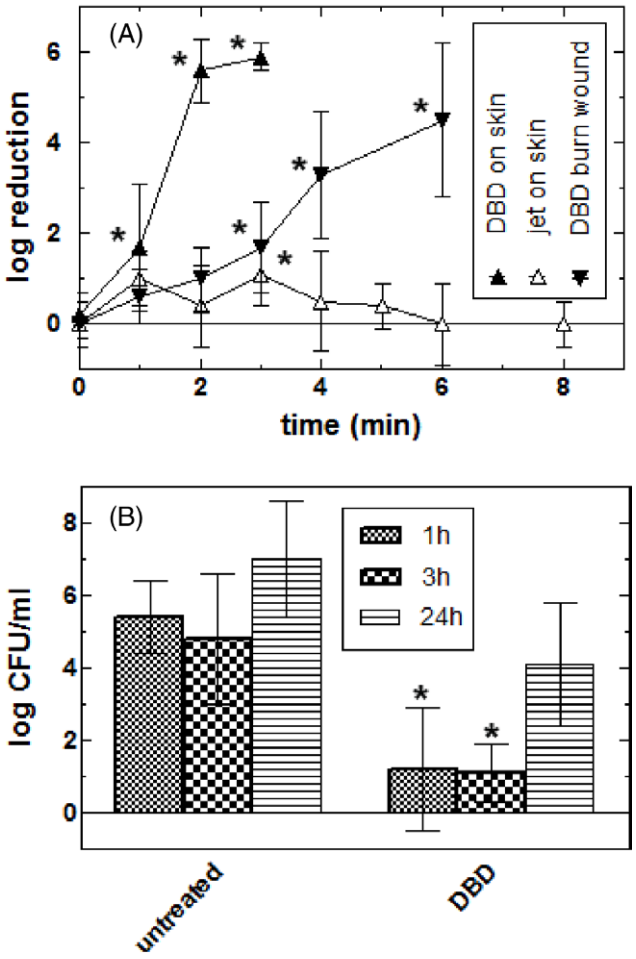

Figure 6. (A) Log reduction of *P. aeruginosa* present on intact skin or in burn wound model after treatment with DBD or with jet. One million CFU (in 10 μl) were applied on human skin (0.7 mm thickness, 1 cm^2) or in the burn wound (2 $\times$ 10 mm) and left to dry for 45–60 min. Immediately after treatment with plasma, bacteria were dislodged with the Tissuelyser and plated on LB agar after serial dilution to determine the surviving CFU/ml. Shown are mean values and standard deviation of at least 3 experiments. (B) Log reduction of *P. aeruginosa* in burn wound model after extended incubation. Plasma DBD was applied for 6 min after 1 h, 3 h or 24 h of infection. Immediately after plasma treatment bacteria were quantified. Note that the mean number of bacteria and standard deviation is depicted (in log CFU/ml) to show the increase in bacteria after 24 h of incubation (Untreated). The differences between untreated and DBD (log reductions) were 3,8 $\pm$ 2,2 (after 3 h) and 3,2 $\pm$ 1,7 (after 24 h). Significant differences from untreated control are indicated (*, MWU, $P < 0.05$).

for comparison as in [37]. After a total culture period of 3 weeks, the re-epithelialization was measured microscopically in histological sections (figure 7). There was no positive or negative effect on wound healing in the burn wound model by treatment with the DBD or plasma jet (table 2). In contrast, Flammazine treatment greatly reduced re-epithelialization or even led to detachment of the epidermis in this study. Although products with high silver concentrations can be very effective, resulting in more than 3-log reductions [37, 40], they can also be cytotoxic and detrimental for wound healing [4, 37].

4. Discussion

This study was not performed with the intention to elucidate the mechanism by which different plasma sources exert their effect, but rather to improve existing or develop new plasma modalities for treating infected (burn) wounds. Nevertheless, some key factors in the plasma mediated bactericidal effect can be discussed.

In an effort to find the most optimal settings for the argon plasma jet, time modulation (duty cycle), power and distance were varied. Although 6 log reductions could be achieved with bacteria in non-buffered suspension in min, bacterial reduction on intact skin was limited with the jet for the same conditions. Varying degrees of bacterial reduction on skin or skin equivalents have also been found by others using a jet [12, 21, 27, 41], good results have been achieved on chronic wounds [7]. Due to the design of the jet, the surface area that can be treated is small and the location of the jet was kept fixed in the middle of the wound in our experiments. The entire infected wound area (2 $\times$ 10 mm) was covered by both tested devices; the jet reaches as similar sized area as the DBD (see figure 3). With DBD devices, larger surface areas can be more easily treated. However, also with different DBD devices reported in literature, varying degrees of bacterial reduction on skin or skin equivalents were found [8–10, 18, 21, 41].

The bacterial load after 24 h in the used burn wound models (10^7 CFU) was very high compared to the clinical situation. 10^5 CFU would be more common for an infected wound. However, lower amounts of bacteria could not be achieved with this model after an extended incubation time of 24 h without treatment. A reduction by plasma treatment from 10^7 to 10^4 CFU is still a huge difference in absolute numbers. Together with the body's own response, this plasma treatment will therefore have a significant impact on infection control.

Treatments with different types of DBD have been performed on cells or skin in *in vitro* [13, 18, 20, 26] or *in vivo* tests [19, 42] and the results on cellular activity, inflammation or DNA damage were found to be within safe limits. We have shown here that DBD treatment of infected wounds is effective and safe. The flexible DBD plasma strip will allow treatment of curved body parts. A flexible DBD device (PlasmaDerm®) is commercially available but only limited test data is available in literature.

A direct effect of VUV on bacterial reduction is not likely because it does not penetrate the liquid. The amount of produced UV radiation >200 nm for the jet was significantly below the biological active threshold [14]. As Ar excimer emission (125 nm) is responsible for the VUV emission in the jet and excimer radiation is typically much stronger than VUV emission from atomic N and O, the same conclusion is valid for the air DBD. Moreover, VUV produced by DBD is very low and therefore does not play a role in the skin experiments.

We assume that plasma exerts its effects through induced liquid chemistry mainly by (neutral) reactive species [14]. For some plasma sources, NO related chemistry is thought to be the key component [30, 43, 44], while reactive oxygen species have been reported for other specific conditions [16, 45, 46]. Because previous work indicated a major influence of

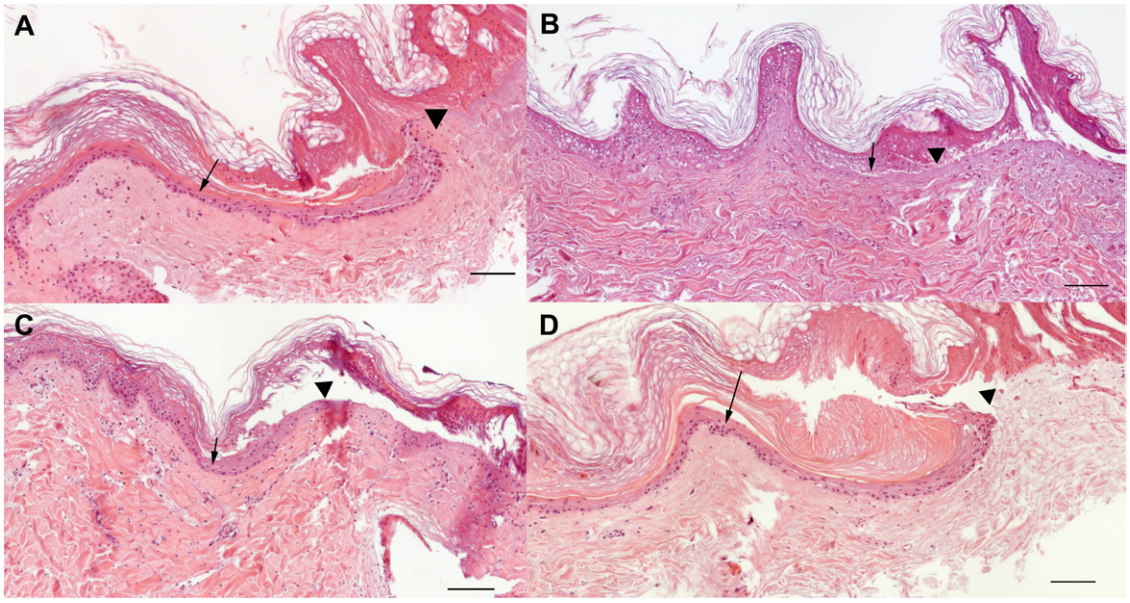

Figure 7. Re-epithelialization in H&E stained sections of burn wound models after 3 weeks of culture. Shown are examples without treatment (A), or after treatment with Flammazine (B), plasma jet (C) or DBD plasma (D). Arrows mark the border of the burned epidermis, triangles mark the tip of the newly formed epidermis. Scale bars are 100 μm.

Table 2. Re-epithelialization in the burn wound model after multiple topical treatments with plasma. Silver sulfadiazine (Flammazine) is a standard of care for treatment of many burn wounds in dedicated centers and was used for comparison. Shown are the mean values and standard deviations.

Treatment	Re-epithelialization (μm)			Number of donors
Negative control	657	±	155	8
Flammazine	9	±	2[a]	4
DBD 4 min	714	±	64	3
DBD 6 min	587	±	196	3
Jet 8 min	641	±	141	5

[a] WMP, $P < 0.05$.

buffer in the medium on bacterial killing by using a plasma jet [14], a non-buffered saline solution was used. As shown in figure 5, pH greatly determines sensitivity of bacteria to H_2O_2 and/or NO_2^-. We used 2 ml of saline solution in 6 well plates to be able to directly relate the bactericidal effect to changes in pH. Acidification is a key aspect for killing bacteria as shown here and in other studies, but acidification alone is not sufficient [29, 30, 47, 48]. Mixtures of NO_2^- and H_2O_2 at reduced pH have been shown to lead to the production of peroxynitrous acid, which is bactericidal [49–52]. Assuming that NO_x^- are the predominant negative ions introduced in the liquid by the plasma, the pH is greatly determined by the NO_x^- concentration [47]. DBD plasma treatment resulted in a faster acidification of the solution compared to the jet (figure 4(B)). The cross MIC/MBC demonstrated a greater effect of both H_2O_2 and NO_2^- on *P. aeruginosa* at low pH. Synergistic effects of H_2O_2, pH and NO_2^- have been reported [29, 32, 48, 53]. A buffering capacity of the wound could potentially contribute to the difference between the treatments in saline solution and the wound model.

Ozone has been reported to correlate with bacterial killing by a DBD device [16]. Despite high levels of ozone production by the flexible surface DBD, bacterial reduction in a buffered medium (PBS) was not observed. This might suggest that O_3 was not the key active species in that particular test. Ozone might even reduce the bactericidal effect as it does reduce H_2O_2 production in the liquid [32]. When mixing was applied, treatment with ozone for 15 min at 10 mg l^{-1} resulted in a log reduction of 3.5, while 5 mg ml^{-1} was not effective at all [54]. In our experiments, 3500 ppm (or 7.5 mg l^{-1}) of ozone by diffusion was not sufficient to inactivate bacteria in a buffered solution. Transfer of O_3 from gas to liquid is slow due to the small Henry`s constants and can be increased by mixing or convection, which is not the case for the DBD device. High power density and absence of convection will favor an NO_x^- based mechanism in the case of DBD [55]. Ozone is not a key component in the jet based treatments because of the low production levels (≈2 ppm) [56, 57]. Although skin activity and wound healing were not affected, the high concentration of ozone is a potential safety issue for the DBD. Active coal might be added in a layer at the edge of the device to act as an O3 absorber.

The treatment of tissue or wounds is significantly different from treatment of a (bulk) solution. Reactive species can accumulate in a liquid while significantly more reactive species will be lost in the gas phase, particularly for the jet with the convective flow, when treating a surface. This is exemplified by the high bacterial inactivation of *S. aureus* on a reconstructed human skin model that was only observed when using plasma discharge in liquid and not when using plasma discharge in gas [27]. Liquid is not present on the burn wound models, except for moisture in the skin. The different geometry and gas transport conditions above the tissue and wound will also lead to significant different species densities. The plasma jet continuously blows away the reactive species

in the effluent (figure 2). Therefore the build-up of species above the wound might be smaller as in the case of the DBD. The DBD geometry is larger and covers the wound. In this case, the reactive species are mainly lost to the wound. This geometrical effect could increase the flux of reactive species to the wound compared to the jet significantly.

The effluent of the jet causes a convective flow in the liquid, which enhances the mass transfer. When no solution is present, this could cause a significant reduction in species transfer to the bacteria. These flow effects could thus explain the observed differences between the jet and the DBD on bactericidal efficacy on skin or in the wound model. It should be noted that efficient disinfection of wounds/tissue with a plasma jet have been performed in touching conditions and not remotely as is the case in this work. Touching conditions have a significant higher efficiency of species transfer to the sample [58].

Measurements on [H_2O_2], [NO_x] and VUV have not been performed in the case of the DBD. However, a similar relation between bactericidal action and reduction in pH has been found as for the RF argon jet suggesting a similar working mechanism. Note that the amount of air in the argon jet in this study is determined by flow mixing. This physically given amount can be controlled better when actively admixing air [33]. In addition, the inactivation efficiency of the RF argon jet could be further optimized by mixing the argon gas with N_2 or O_2 and increasing the power [32, 34]. The jet treatment is intrinsically inhomogeneous, which might be improved with a jet array.

5. Conclusion

In this study, we tested a newly developed flexible surface DBD and compared it to a remotely operated argon gas based plasma jet. Tests were conducted on bacteria (*P. aeruginosa*), on cultured cells and on *ex vivo* human skin allowing us to directly compare bactericidal with safety aspects under identical conditions.

Both plasma devices were highly efficient when used on bacteria in non-buffered solutions, but the DBD was faster in reaching the maximum bacterial reduction. A strong correlation between pH reduction and bactericidal effect was found for both sources.

In the present study it was found that the DBD was much more effective in the disinfection of skin or the burn wound model compared to the jet. This difference is attributed to differences in efficiency of species delivery to the sample between the DBD and jet.

The effect of plasma on the metabolic activity of skin samples was tested. Treatment with DBD plasma up to 6 min did not affect the activity of these biopsies, while a 3 min treatment was sufficient for 6 log reduction on intact skin. Similarly, treatment with the plasma jet for 6 min did also not affect the activity of skin samples. Repeated treatments of burn wound models with either the DBD or plasma jet did not affect re-epithelialization.

The DBD strip has excellent bactericidal properties and does not negatively affect wound healing. It makes it an effective plasma device for treating infected or contaminated wounds.

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