In vitro analysis of nonthermal plasma as a disinfecting agent

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Objective—To determine the effect of nonthermal plasma on *Staphylococcus aureus*, fibroblasts in monolayer culture, and clean and contaminated skin explants.

Sample Population—Normal skin from euthanized horses.

Procedures—*S aureus* organisms were plated and treated with nonthermal plasma followed by bacterial culture to assess viability. Fibroblasts in monolayer culture and the epidermal and dermal surfaces of clean and *S aureus*–contaminated skin explants were treated. The effects of distance and duration on the response to treatment were compared.

Results—Compared with controls, treatment with nonthermal plasma resulted in significantly decreased bacterial growth and significantly inhibited survival of fibroblasts in monolayer culture. When epidermal and dermal surfaces of skin explants were treated, there was no effect on production of normal fibroblasts during explant culture, except when extended exposure times of ≥ 2 minutes were used. Treatment with nonthermal plasma resulted in significantly lower bacterial counts after 24 hours of culture of *S aureus*–contaminated epidermis but not of dermis.

Conclusions and Clinical Relevance—Nonthermal plasma resulted in bacterial decontamination of agar and epithelium; negative effects on fibroblasts in monolayer; and no negative effects on skin explants, except at long exposure times. Use of nonthermal plasma appears safe for treatment of epithelialized surfaces, may be safe for granulating wounds, and results in decontamination of *S aureus*. Investigations on the effects that nonthermal plasma may have on patient tissues are indicated with a clinically applicable delivery device. (*Am J Vet Res* 2006;67:2030–2035)

Abbreviation

DMEM Dulbecco modified Eagle medium

In today's medical practice, there are a number of decontamination techniques for accidental and surgical wounds. These techniques consist of preoperative surgical scrub, intraoperative lavage with and without antimicrobials or antiseptics, systemic and local administration of antimicrobials, local antimicrobial depots, aseptic technique, and postoperative continuous–vacuum-suction drains. Despite use of these and good operative techniques, the surgical site infection rate is 3% to 5.8% in all procedures in dogs and cats, 2% to 6% in human elective orthopedic patients, 8.1% in clean orthopedic procedures in horses, and 0% to 50% in human open orthopedic wounds.[1-4] Surgical site infection increases the length of hospitalization by a mean of 7.5 days and costs up to an extra $26,019/patient in human hospitals and is associated with high morbidity rates and costs in veterinary patients.[5,6] These infections are often caused by hospital-acquired organisms, which are increasingly multidrug resistant. Antimicrobial resistance is an ever-growing concern in human and veterinary patients, with methicillin-resistant *Staphlycoccus aureus* and multi–drug-resistant gram-negative organisms becoming an increasing problem, especially in surgical site infections.[4,7] For these reasons, it is important to continue development of better methods to increase bacterial killing and control contamination without development of bacterial resistance or suppression of the immune response of the surgical site.

Nonthermal plasma is produced by electric discharge in liquid or air and is referred to as the fourth state of matter.[8,9] Commonly encountered plasmas are found in fluorescent light, electric welders, and lightning.[9] The generation of plasma produces reactive media, ozone and radicals via excitation, dissociation and ionization of any gaseous or vaporous substance, and a visible glow.[9]

It has been known for the past century that the application of plasma produced in air by electric discharge can be used for sterilization.[10] One theory of its action is that plasma kills cells by disruption of the cellular membrane or wall (ie, its etching mechanism).[10-12] This is specific to nonthermal plasma disinfection. Bacteria are also vulnerable to the destructive reactions of toxic species in nonthermal plasma, such as UV radiation, energetic ions, and cytotoxic free radicals, as well as the electrostatic stress of charged particles.[10,11,13,14,a] To date, there is no widely accepted mechanistic explanation of bacterial killing by nonthermal plasma.[15]

Generation of nonthermal plasmas in a vacuum chamber at subatmospheric pressure has been used for more than 40 years as an alternative to ethylene oxide gas sterilization.[b,c] This system allows rapid, reliable sterilization of low-pressure–tolerant materials and does not pose any environmental risk or leave any

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toxic residues.[16] Gas plasma appears to be the safest, easiest, and most efficacious method of sterilization available.[6] The use of plasma at atmospheric pressure was limited, until the development of pulsed discharges, because of the high temperature caused by continuous plasma generation. With pulsed-discharge delivery, it is possible to generate plasma for a short time and create the necessary active molecular species at ambient pressure and temperature.[10] Nonthermal atmospheric plasma would be inexpensive and convenient and could be used on materials that are not amenable to subatmospheric pressure, such as certain polymers, foodstuffs, and living tissues.[17]

Recently, nonthermal plasma has been investigated for its biomanipulation effect on cells. Living cells have several beneficial responses to plasma treatment, and short-term plasma treatment does not cause accidental cell death, which would exacerbate inflammation and tissue damage.[13,18] One report[19] suggested that the lethal dose of nonthermal plasma for bacteria is very low, compared with doses needed to harm living eukaryotic cells. Therefore, nonthermal plasma has been suggested as a possible decontaminant for infected wounds.[19]

The purpose of the study reported here was to determine the effect of nonthermal plasma on *S aureus*, fibroblasts in monolayer culture, and clean and contaminated skin explants. Our hypothesis was that the plasma-generating device would inhibit bacterial growth and have minimal negative effects on cultured fibroblasts and dermal explants.

Materials and Methods

Nonthermal plasma-generating device—The plasma in the device was produced in an air stream with infused gases by pulsed discharges powered by a standard microwave oven magnetron. In the device, the ionized species decayed in nanoseconds, leaving the excited oxidative species such as hydroxyl radicals and excited species of molecular oxygen, which decayed in seconds.[20] The nonthermal plasma device used a metal tube that delivered the plasma at 39° to 40°C (temperature was measured 1 or 2 inches from the metal tip). Plasma was allowed to flow at approximately 1 m/sec from the probe in a downward direction. The stage on which samples were placed was moveable and could be raised or lowered to maintain the desired distance of the sample to the device tip. The device tip was 6 mm in diameter.

Bacterial exposure—The plasma-generating device was first tested for its ability to inhibit growth of a virulent strain of *S aureus*. The *S aureus* organisms were isolated from clinical cases of pyoderma and frozen and stored at –70°C. Prior to use, the culture was thawed and inoculated onto 5% sheep blood agar plates. A suspension matching a 0.5 McFarland turbidity standard was used to create a broth of 10^5 organisms/mL. One milliliter of this suspension was used to create serial dilutions of 10^4 and 10^3 organisms/mL. Each broth was plated on fourteen 100-mm^2 round plates covered with trypticase soy agar and assigned a treatment group. Each treatment group of 10^3 (group 1), 10^4 (group 2), and 10^5 (group 3) organisms/mL consisted of 6 plates treated at a 1-inch distance (subgroup A) from the plasma device tip, 6 plates treated at a 2-inch distance (subgroup B) from the plasma device tip, and 2 untreated controls. All groups (except controls) were exposed to the plasma device for 30 seconds. The plasma plume was delivered only at the center of the plate and allowed to flow peripherally during treatment. Following exposure to the device, each group was incubated at 37°C and 5% CO_2 for 48 hours and the colonies on each plate were manually counted.

Fibroblast exposure—The plasma-generating device was tested on fibroblast monolayer cultures of type B equine synoviocytes, which are fibroblastlike. Equine synoviocytes[d] that had been frozen and stored at –70°C were thawed, and 500,000 cells in 2 mL of DMEM containing 20% fetal calf serum, fungicide, streptomycin, penicillin, and kanamycin[e] were placed in each well of four 6-well plates. The cells were allowed to attach for 24 hours at 37°C and with 5% CO_2. Following incubation, the bulk of the medium was aspirated from all wells on a plate. Each well on the plate was treated with nonthermal plasma for 30 seconds at a 2-inch distance from the device tip, followed by the addition of 2 mL of fresh medium to each well. For the control plate (plate 1), medium was withdrawn and fresh medium was replaced at the same intervals as the treated plates. Following treatment, plates were either immediately analyzed or incubated at 37°C and 5% CO_2. For analysis, the wells were observed by use of an inverted microscope at 100× lens objective. Live-dead cell counts were performed immediately and 24 hours after treatment on 2 control wells and all 6 wells of 1 treated plate for each time point (0 and 24 hours after treatment). For the live-dead ratios, the plates were treated with trypsin, which allowed attached cells to be removed from the plate without harming them, and cells were stained with erythrocin B and manually counted by use of a hemacytometer. Cells were counted as live if they excluded the dye and remained clear and dead if they were stained pink.[21]

Short-duration skin explant exposure—Full-thickness skin explants were harvested from the ventral portion of the abdomen of a 2-year-old Thoroughbred filly euthanized for problems unrelated to skin. The hair was clipped and shaved, and the skin was prepared via standard aseptic technique. A 7 × 10-cm area of full-thickness skin was removed and placed in a sterile container of PBS solution[e] containing fungicide. While being bathed in PBS solution, the skin was minced with a scalpel blade into 4- to 6-mm square sections. Three sections of full-thickness skin were placed in each well of a 6-well plate. In three 6-well plates (1 control and 2 experimental), skin explants were placed with the dermis oriented upward, and in three 6-well plates (1 control and 2 experimental), skin explants were placed with the epidermis oriented upward. Two milliliters of DMEM was added to each well; this amount of medium allowed the explants to be completely covered but not floating. The medium was aspirated from all wells on the plate, and each well was exposed to the nonthermal plasma individually for 30 seconds with the device tip 2 inches above the well floor. Following exposure of all wells on a plate, 2 mL of fresh DMEM was replaced in each well. Medium was aspirated from the control plates, and the plates were left for the same duration of exposure as a treated plate before fresh medium was replaced. Following treatment, the plates were incubated at 37°C and 5% CO_2. One half milliliter of medium was exchanged every 48 hours, and the plates were examined with a 40× lens on a reverse-viewing microscope daily and subjectively graded for presence of fibroblasts and degree of confluence. On day 7, following exposure to the plasma, the explants were removed and the medium was exchanged every 24 hours. The times when fibroblasts were first observed and when fibroblasts reached 90% to 100% confluence were recorded. Cells were graded as confluent when < 10% of the plate was devoid of cells and cells were touching but not yet overlapping.

Long-duration skin explant exposure—The effect of nonthermal plasma was tested on skin explants with longer

exposure times, and each group was assigned directly exposed epidermis or dermis. Skin explants were harvested from the ventral portion of the abdomen of a 10-month-old Clydesdale colt euthanized for problems unrelated to the skin. The hair was clipped and shaved, and the skin was prepared by use of standard aseptic technique. Six-millimeter punch biopsy specimens of full-thickness skin with subcutaneous tissues and fat removed were used. The explants were washed in sterile PBS solution with fungicide. One biopsy specimen was placed in each well of a 6-well plate. Plate 1 contained nontreated controls, plate 2 contained explants placed epidermis upward, and plate 3 contained explants placed dermis upward. Each well on plates 2 and 3 was exposed to the plasma for 2 minutes. Plate 4 contained explants placed epidermis upward, and plate 5 contained explants placed dermis upward, each for a 5 minute exposure, and plate 6 contained explants placed dermis up for a 1-minute exposure to the nonthermal plasma. The unbalanced design of only dermis upward in the 1-minute exposure group was used because of the assumption that the dermis was more susceptible to nonthermal plasma exposure and the previous experiment in which the explants were exposed for 30 seconds with no apparent effect. Two millimeters of DMEM was added to each well. Prior to plasma exposure, medium was aspirated from all wells on each plate. Every well was exposed to the plasma individually with the device tip 2 inches above the well floor. Following exposure of all wells on a plate, 2 mL of DMEM was replaced in each well. The control plates had medium aspirated for the longest duration of exposure (5 minutes) for a treated plate before the medium was replaced. Following treatment, the plates were incubated at 37°C and 5% CO_2 with all explants positioned dermis downward. One-half milliliter of medium was exchanged every 48 hours, and the plates were examined with a 40× lens on a reverse-viewing microscope daily and subjectively graded for fibroblasts and degree of confluence. On day 7, following exposure to the plasma, the explants were removed and fibroblast cultures continued. The times when fibroblasts were first observed and when fibroblasts reached 90% to 100% confluence were recorded. On day 12, the wells were trypsinized to remove attached cells, the cells were stained with erythrocin B, and total cell number and cell viability were determined with a hemacytometer.[21]

Dermal explant with bacterial contamination exposure—The effect of the plasma was tested on skin explants contaminated with *S aureus*. Skin explants were harvested from horses euthanized for reasons unrelated to the skin (a 12-year-old Thoroughbred mare and a 5-month-old Standardbred colt). The skin was harvested and prepared as described except that 3-cm square areas of skin were obtained. A full-thickness 3-cm square section of skin was placed in a dry 100-mm^2 petri dish, epidermis upwards (groups 1, 2, and controls) or dermis upwards (group 3). Each group consisted of 6 skin pieces. The skin was inoculated with 0.1 mL of 10^5 organisms/mL of *S aureus* (prepared as described) dropped into the center of the skin piece, followed by exposure to the plasma for 30 seconds (group 1 and 3) or 60 seconds (group 2) with the device tip 2 inches above the explant surface. After exposure, a 6-mm punch biopsy specimen was obtained from the center of the skin piece. This was placed in a tube with 1 mL of PBS solution and vortexed for 10 seconds. The PBS solution was aspirated, and 0.1 mL of the aspirate was plated on 100 mm^2 trypticase soy agar plates that were incubated for 48 hours at 37°C and 5% CO_2. Each biopsy specimen was placed epidermis upwards in a well of a 6-well plate. Two milliliters of DMEM was placed into each well. Following treatment, the bacterial plates and explant plates were incubated at 37°C and 5% CO_2. The agar plates were inspected for bacterial colonies, and the colony number was manually counted at 24 and 48 hours after plating. The explant plates were examined daily with a 40× lens on a reverse-viewing microscope for fibroblastic cells, and 0.5 mL of medium was exchanged every 48 hours. On day 7, following exposure to the plasma, the explants were removed. The times when fibroblasts were first observed and when fibroblasts reached 90% to 100% confluence were recorded.

Statistical analysis—Because the data were approximately normally distributed and generally balanced, statistical analyses were performed via 1- or 2-way ANOVA with post hoc testing via the Tukey procedure. Differences were considered significant at $P < 0.05$.

Results

Throughout the experiment, the plasma-generating device functioned reliably and was easily operated. On a few occasions during testing, the device ceased to generate plasma as indicated by lack of a blue-purple light in the viewing window. This was easily fixed but required the operator to regularly check for appropriate light in the viewing window, which could be missed if consistent evaluation was not performed.

Bacteria exposure—Nonthermal plasma effectively killed bacteria, and following exposure, there was no visual evidence of dessication of the agar plates. The mean ± SD number of colonies present after 48 hours was determined: group 1A had 4.3 ± 3.98 colonies/plate, group 1B had 0.33 ± 0.52 colonies/plate, group 2A had 6.67 ± 3.50 colonies/plate, group 2B had 1.67 ± 1.63 colonies/plate, group 3A had 59.33 ± 33.54 colonies/plate, and group 3B had 31.33 ± 17.84 colonies/plate. Groups 1 and 2 were not significantly different but were different in comparison to group 3 at both exposure times. There was no significant difference between exposure times. All colonies on the treated plates grew at the periphery of the agar plates. All control plates were completely and evenly covered with bacterial colonies.

Fibroblast exposure—Immediately after exposure, few recognizable fibroblasts were observed at 100× magnification. Abundant cellular debris was accompanied by moderately rounded, crenated fibroblasts along the periphery of the plate. All wells appeared grossly desiccated at the center following treatment. At 24 and 48 hours after exposure, there were fewer recognizable cells. Controls had coalescing groups of normal fibroblasts that were advancing toward confluence at 24 and 48 hours after exposure. Plate 2 (plasma treated and counted immediately after exposure) had a mean of 3×10^4 viable fibroblasts/well with a mean viability of 43%, whereas corresponding control wells had a mean of 11×10^4 fibroblasts/well and mean viability of 67%. The control group had an obvious outlier, and if not included, the viability was 97.7% and 16.9×10^4 viable fibroblasts/well. Plate 3 (plasma treated and counted after 24 hours of culture) had a mean of 0.67×10^4 viable fibroblasts/well with a mean viability of 9%, whereas its corresponding control well was clearly different with a mean of 39×10^4 fibroblasts/well and mean viability of 92%. Statistical analysis was not performed because of the obvious dif-

ference between plasma-treated and nontreated controls.

Short-duration skin explant exposure—All wells (controls and explants exposed to nonthermal plasma) had fibroblasts by day 5 and reached 90% to 100% confluence on day 11 after exposure and culture. Wells were not trypsinized, and total cell counts were not performed because of the overwhelming appearance of a rapidly expanding monolayer of fibroblasts that indicated that short-term exposure to plasma had no detrimental effect on explant viability.

Long-duration skin explant exposure—Skin explants were exposed to plasma to determine whether the side of the skin (epidermis or dermis) exposed to the nonthermal plasma affected the ability of fibroblasts to migrate from the explants. Fibroblasts were first recognized on the plates on day 6 after exposure and culture. Via 10X objective viewing, plate 1 (control) had a range of 10 to 30 fibroblasts/field, plate 2 (2-minute exposure of epidermis to plasma) had a range of 5 fibroblasts/field, plate 3 (2-minute exposure of dermis to plasma) had occasional fibroblasts, plate 4 (5-minute exposure of epidermis to plasma) had a range of 1 to 3 fibroblasts/field, plate 5 (5-minute exposure of dermis to plasma) had occasional fibroblasts per field, and plate 6 (1-minute exposure of dermis to plasma) had a range of 5 to 20 fibroblasts/field.

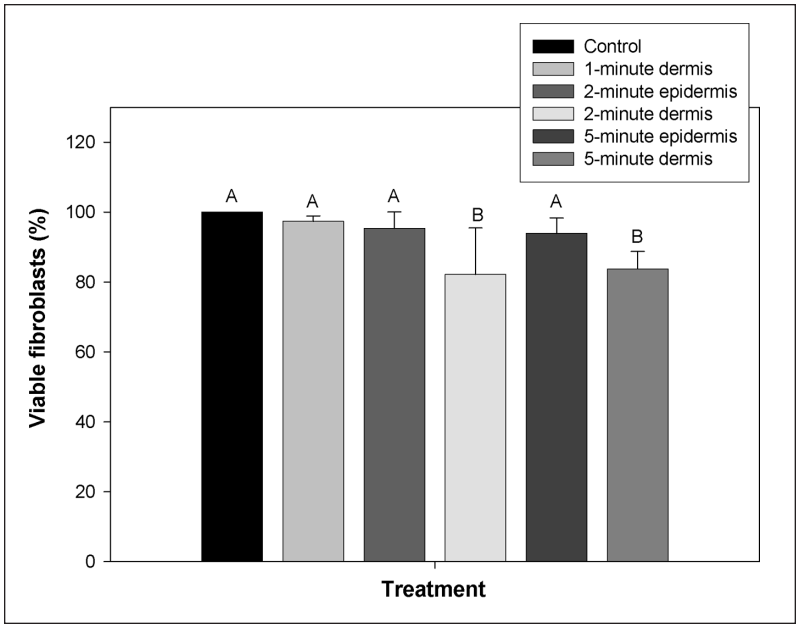

Figure 1—Mean ± SD percentages of viable fibroblasts cultured from 6-mm equine skin explants treated for various times with exposure of either the epidermal or dermal surface to nonthermal plasma. [A,B]Values with different letters are significantly ($P < 0.05$) different.

On day 13 following exposure, plate 1 (control) had a mean ± SD of $15.2 \pm 10.4 \times 10^4$ viable cells, plate 2 (2-minute exposure to epidermis) had $10.2 \pm 11.2 \times 10^4$ viable cells, plate 3 (2-minute exposure to dermis) had $3.0 \pm 2.2 \times 10^4$ viable cells, plate 4 (5-minute exposure to epidermis) had $3.7 \pm 4.1 \times 10^4$ viable cells, plate 5 (5-minute exposure to dermis) had $2.0 \pm 2.4 \times 10^4$ viable cells, and plate 6 (1-minute exposure to dermis) had $21.4 \pm 5.7 \times 10^4$ viable cells. Values for control and 1-minute dermis samples were not significantly different. Values for 2-minute epidermis samples were significantly less than for 1-minute dermis samples but not different from controls. Values for 2-minute dermis and 5-minute epidermis and dermis samples were significantly less than for control, 2-minute epidermis, and 1-minute dermis samples but not different from each other. The percentage of fibroblasts after 13 days of culture that were viable was also determined (**Figure 1**).

Exposure of dermal explants contaminated with *S aureus*—Dermal explants were contaminated with *S aureus* to determine whether the nonthermal plasma could kill bacteria on a biological surface that might impede the dispersion or flow of the nonthermal plasma plume. Control plates and group 3 (dermal contamination and 30-second exposure) were completely and evenly covered with bacterial colonies after 24 hours of culture. Colony numbers after 48 hours of culture were determined. Bacterial cultures for group 1 (epidermal contamination and 30-second exposure) had significantly fewer colonies per plate, with a mean ± SD of 9.6 ± 6 colonies/well, and group 2 (epidermal contamination and 60-second exposure), with 11.3 ± 7.6 colonies/well, had significantly fewer colonies per plate, compared with controls, but no difference compared with group 1. Fibroblast cultures all grew well and had fibroblasts by day 5 and reached 90% to 100% confluence on day 11.

Discussion

Although the molecular species in the nonthermal plasma were not measured or identified, use of the nonthermal plasma device resulted in death of bacteria and isolated fibroblasts. The few colonies and fibroblasts that did grow following treatment were located along the periphery of the plate, which suggested that this was attributable to the stationary plume being delivered at the center of the plate. It is likely, although it was not measured, that the same mechanism was responsible for the killing of bacteria and fibroblasts. Interestingly, there was a lasting negative effect on remaining fibroblasts because the total fibroblast number and percentage viability of remaining cells continued to decrease for 24 hours after exposure. This may have been caused by the long-term effects of irreparable damage to the cellular membranes, which caused delayed cell destruction, or because the molecular species in the plasma persisted and caused continued cell destruction.

The peripheral location of surviving bacterial colonies and fibroblasts also suggested that, when applied to a surface, the plasma spread peripherally. This movement could be affected by surface irregularities. The irregular dermal surface of the skin explants

may account for the plasma not causing significant bacterial killing on those explants.

Because free radicals in the nonthermal plasma attack the cell wall of microorganisms, inactivation should be equally effective on all types of microorganisms (ie, vegetative bacteria [gram-negative and gram-positive], spores, viruses, and fungi). Thus, the same method of disinfection could potentially be used on all pathogens. Moreover, the killing mechanism is such that microorganisms are unable to develop any resistance to this treatment.

Compared with its effects on isolated fibroblasts, the nonthermal plasma had no substantial detrimental effect on skin exposed for a short time (30 or 60 seconds) via either the dermal or epidermal surface. This may have been because of a protective extracellular matrix and subsequent cell survival. It may also have been attributable to the abundance of cells available for migration and expansion from the explant surface, compared with a single monolayer of cells, or if the cellular killing was confined to the superficial layers and cells were not damaged to the point of cell lysis, there may not have been a measurable difference in fibroblast growth. This may indicate the usefulness of nonthermal plasma as a mechanical wound debridement tool because it kills surface contaminants and debrides their associated host cells without inciting inflammation and tissue reaction.

When explants were exposed for longer periods of 2 to 5 minutes, fibroblast production was affected. Despite the presence of a protective, completely keratinized stratum corneum composed of thick, tightly packed, dead cornified cells, the epidermal exposures resulted in similar decreased fibroblast production. Although it is unlikely that the nonthermal plasma was able to travel under the explant for contact with the dermis when the epidermis was exposed, it is possible that the negative effects during prolonged epidermal treatments were caused by an effect on the deeper layers of the epidermis and dermis along the cut surface of the punch biopsy specimen. This may be further studied by treating larger skin explants for 2 and 5 minutes prior to obtaining the specimens. Fibroblast production could have been altered by lysis of the cellular membranes and resultant decreased neighboring-cell survival, greater depth of penetration of nonthermal plasma during longer exposures, or the drying effect of the 40°C plume. Although not tested during this experiment, it is unlikely that exposure to these temperatures alone would cause lasting effects, especially of the epidermis.

This difference in fibroblast migration from the explants exposed for longer duration was not limited to total number of fibroblasts but also seen in the percentage of total fibroblasts that were viable. This suggested that not only were the explants negatively affected in their ability to produce fibroblasts but that the fibroblasts they produced had an altered life span. This is not explainable as simply an effect of cellular membrane damage or cell death but suggests that there may be lasting DNA damage to the cells that do survive and divide.

These results suggest that nonthermal atmospheric plasma is safe during 30-second exposures and may be useful for disinfection of dry epithelial surfaces and infected wounds. Because the flow of the plasma may be seriously inhibited by small surface irregularities, an easily moveable plasma source would be necessary. Further investigations on the effects of nonthermal plasma on patient tissues are indicated.

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Correction: In "Evaluation of the effects of short-chain fatty acids and extracellular pH on bovine neutrophil function in vitro," published November 2006 (*Am J Vet Res* 2006;67:1901-1907), reference 33 should read as follows:

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