*Plasma Medicine,* 5(2–4): 109–123 (2015)

# *In Vitro* and *In Vivo* Analysis of Hydrogen Peroxide–Enhanced Plasma-Induced Effluent for Infection and Contamination Mitigation at Research and Medical Facilities

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**ABSTRACT:** The growing prevalence of multidrug-resistant bacteria poses a unique challenge to animal and human health. The threat of nosocomial infections in hospitals and infectious outbreaks in vivaria through fomites requires novel technological solutions. We describe the use of a compact device capable of producing a disinfecting air stream based on electrical plasma-induced chemistry and hydrogen peroxide additives. We show that this device can deactivate strains of the bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa* in vitro on a potential fomite in medical and research facilities. Deactivation takes less than a minute and does not require high temperatures. Exposure of human epidermal keratinocytes (HEKa) and human dermal fibroblasts (HDFa) in isolated cultures show that human skin cells are much less affected by the treatment than bacteria. In addition, an in vivo acute exposure of shaved CD-1 mice and subsequent histology shows no adverse effects on the skin as compared to alcohol-based hand sanitizers and Silvadene. The results suggest that the technology is suitable as a general disinfection procedure for heat-sensitive inanimate objects in a short exposure time. In addition, it is not a danger to live tissue when exposed acutely, suggesting potential use as a regular disinfection procedure.

**KEY WORDS:** plasma medicine, nonthermal plasma, disinfection, nosocomial infections, ozone therapy

## I. INTRODUCTION

Infections result in hundreds of thousands of patient deaths every year in the United States, and infectious outbreaks in animal research vivaria are a routine hazard that can incur significant financial losses. There is no doubt among experts that the number of deaths and associated expenditures could very easily be reduced with improved practice and decontamination procedures of both living skin and inanimate objects in hospitals, clinics, and research facilities.[1-7] Some infections result from a lack of methods to simply and quickly disinfect hands and fomites, such as identification badges, pens, keys, and other equipment that are easily carried into laboratories, hospitals, and vivaria.[8,9]

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This was recently demonstrated in a public health case reported in June 2012 at Clark College in Vancouver, Washington, where a roommate of a laboratory student working with salmonella was sickened. The laboratory student is believed to have carried home the salmonella on items used in the lab, such as pens and pencils that were subsequently used by the individual that became sick.[9] This type of situation is also a high risk for animals housed under specific pathogen free conditions in a vivarium, because it is common practice for research staff to carry supplies into and out of an animal facility.[8–10]

Currently, there is no commercially available technology that can be used effectively at room temperature to quickly and safely treat contaminated devices or objects, such as personal electronics, that cannot be subjected to conventional heat or chemical sterilization.[1,13] Processes that use ethylene oxide, glutaraldehyde, formalin gas, chlorine dioxide gas, and vaporized hydrogen peroxide can be applied, but most are toxic, all require many hours to ensure deactivation, and all require highly specialized and expensive equipment. They may also require specialized training to use, making them impractical for routine disinfection of fomites carried into the facility.[2,3] Therefore, many institutions that restrict research staff from bringing in outside equipment must have increased storage in the facility for research supplies that have been decontaminated prior to vivarium entry. In some cases, research staff are required to duplicate equipment to ensure that contaminants are not inadvertently brought into the facility from laboratory spaces. However, despite these controls, smaller fomites such as keys, pens, cell phones, and identification badges are still a concern. These items must be routinely transported into and out of facilities, are rarely effectively cleaned on a frequent basis, and are handled both outside and within the facility.[9]

Growing multidrug-resistant bacteria populations greatly reduce the efficacy of antibiotics,[3] making outbreaks and infections more difficult to treat in both humans and animals. Antibacterial hand soaps containing triclosan[11] have been shown to increase resistance of bacteria, and frequent hand washing can cause skin to dry out. In addition, hand washing sinks are not always located in convenient locations, or it may not be possible to install them in locations where they were not originally intended. Options in these areas are generally limited to hand sanitizers, which can be more gentle to the skin than hand washing,[12] but frequent monitoring is required in highly trafficked areas to ensure sufficient supplies. Therefore, a method to sanitize hands without a ready water supply or the need to stock supplies is also needed.

Nonthermal plasmas offer a potential solution to these problems. Nonthermal plasmas are known to induce chemical processes normally associated with extremely high temperatures at room temperature, thus making nonthermal plasma processes attractive as alternatives to conventional chemical or heat-based disinfection methods.[13–15] In the last decade, nonthermal plasma processes for biomedical applications have received increased attention. In addition to sterilization and decontamination, nonthermal plasmas are also being explored as treatments for wounds, dermatological conditions, and even cancers.[13–25] The primary driver of nonthermal plasma biomedical properties is the generation of reactive oxygen and nitrogen species in addition to the plasma ions

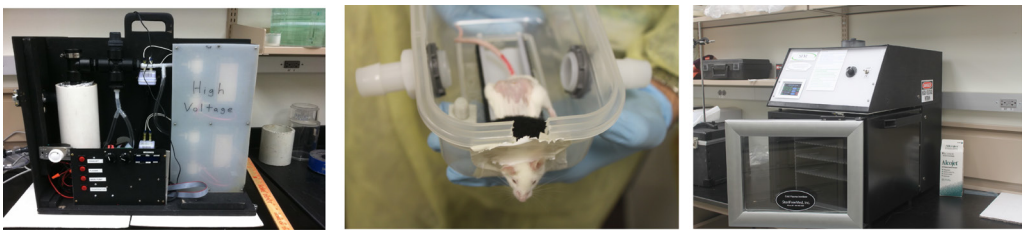


**FIG. 1:** Device used for *in vivo* and *in vitro* tests (left). Sedated mouse with shaved back in special disinfection chamber that allows exposure of the body to the disinfecting effluent (middle). For the exposure, the animals were deeply sedated with isoflurane, and a lid was placed over the top to seal the chamber. Implementation of device technology to benchtop equipment sterilizer (right).

and electrons themselves.[20,21] Nevertheless, the exact mechanisms of nonthermal plasma pathogen inactivation and optimal technological solutions remain topics of active research.[23–25] Membrane lysing, lipid peroxidation, DNA damage, and cell signaling all have been shown to play potential roles.[21,23]

Here we report on the use of a nonthermal plasma device that uses hydrogen peroxide additives to generate a disinfecting air stream. We perform in vitro and in vivo tests to explore the viability and practicality of using this technology to disinfect fomites and live tissue.

## II. MATERIALS AND METHODS

### A. Plasma Device

The plasma device used in this work, shown in the left panel of Figure 1, was constructed by Super Pulse (now SteriFre Inc.) in Ithaca, New York, and it has been described by Golkowski et al.[22] and Plimpton et al.[23] The device has several distinguishing features when compared to other nonthermal plasma devices. The dielectric barrier discharge and plasma produced does not make direct contact with the sample to be disinfected. The setup does not involve any pressurized gases, and hydrogen peroxide additives are used to enhance the bactericidal efficacy. The system employs a closed loop flow with a variable size sterilization chamber. Detailed optical spectroscopy measurements show that the device is able to produce a copious stream of gaseous reactive species including ozone ($O_3$, ~350 ppm), hydrogen peroxide ($H_2O_2$, ~400 ppm), nitrous oxide ($N_2O$, ~10 ppm), and nitrogen dioxide ($NO_2$, ~10 ppm).[22] Subsequent electron paramagnetic spin resonance analysis of the plasma effluent performed by Plimpton et al.[23] showed evidence of hydroxyl radical production in secondary chemical reactions. Hydrogen peroxide is introduced to the closed loop air flow via a special bubbler filled with 50% hydrogen peroxide solution by mass. The bubbler can be filled with up to 200 mL of solution. Operation of the device for 30 seconds uses about 0.5–1 mL of the hydrogen

peroxide solution. The temperature of the air stream is 25°C. The device has low capital costs (~$2,000) and does not require the proximity of high voltage electrodes or a water supply. The right panel of Figure 1 shows an implementation of the same technology as a desktop equipment sterilizer.

### B. *In Vitro* Experiment

*Staphylococcus aureus* and *Pseudomonas aeruginosa* were inoculated on plastic identification badges of the type often worn by medical personnel. The badges were chosen as an inoculation medium because such objects are known to be a major vector for pathogens within laboratories and vivaria and are currently not subject to any satisfactory disinfection or sterilization procedures. Each badge was inoculated with four spots of $10^6$ colony-forming units (CFU) of *S. aureus* and *P. aeruginosa*. The badges were placed in the plasma sterilization chamber for 15, 30, or 60 sec. The bacteria were removed with dampened cotton swabs and placed in 2 mL lysogeny broth (LB) medium. The cultures were serially diluted in phosphate buffered saline, spotted on LB agar plates, and the viable bacteria enumerated after overnight incubation at 37°C. A sample size of three for each condition and each bacteria strain was used. Mean and standard deviation of percent bacteria recovered were calculated relative to an unexposed control. Statistical significance of the results was quantified using a two-way analysis of variance (ANOVA) of transformed data with Bonferroni's post test.

A second in vitro experiment involved human epidermal keratinocytes (HEKa) and human dermal fibroblasts (HDFa) in isolated cultures. HEKa and HDFa cells were exposed for 30 sec, 1 min, 3 min, 5 min, or 10 min to the plasma effluent. Toxicity was measured by trypan blue cell count 24 hours after treatment.

### C. *In Vivo* Analysis

In vivo analysis was performed to determine if the plasma effluent treatment is suitable for live tissue and does not create any adverse effects after several days of repeated use. The effect of the plasma treatment for 30 sec was compared to that of Silvadene and Purell hand sanitizer, which are both commonly used standard antimicrobial agents. Silvadene (silver sulfadiazine) is a topical cream that is often used to treat infected burn wounds. Purell hand sanitizer is composed of 62% ethyl alcohol and less than 5% isopropanol, and it is used regularly by clinicians and researchers. Exposure to the plasma for 30 seconds was chosen because it was the shortest duration that provided deactivation of over 99.9% of pathogens while leaving more than 90% of the cultured human cells viable in the in vitro analysis.

Forty-eight specific pathogen free CD-1 mice (age 4–8 weeks) that were offspring of an in-house breeding colony were housed in static microisolator cages, with 2–5 mice per cage. Mice were housed in autoclaved cages on aspen chip bedding with a nestlet, provided autoclaved reverse osmosis hyperchlorinated water via water bottle, and provided ad lib irradiated diet (Harlan 2918). Sentinel testing was performed quarterly by

**TABLE 1:** Description of experiment groups for *in vivo* testing on CD-1 mice.

| Group | Treatment | Duration/Dose |
|---|---|---|
| 1 | none | N/A |
| 2 | plasma | 30 sec, 1 time/day for 3 days |
| 3 | plasma | 30 sec, 3 times/day for 3 days |
| 4 | plasma | 30 sec, 5 times/day for 3 days |
| 5 | Silvadene | 3 times/day for 3 days |
| 6 | Purell | 3 times/day for 3 days |

Charles River Laboratories. Excluded pathogens are Sendai virus, pneumonia virus of mice, *Mycoplasma pulmonis*, mouse hepatitis virus, Reo-3 virus, mouse parvovirus, epizootic diarrhea of infant mice virus, GDVII, and NS-1. Yearly testing is performed for ectromelia virus, lymphocytic choriomeningitis virus, Theiler's mouse encephalomyelitis virus, minute virus of mice, polyomavirus, and mouse adenovirus. *Helicobacter* and mouse norovirus are present in the facility and not excluded at this time. Mice were housed at 72°F ± 2°F with a minimum of 10–15 air changes per hour and 30%–70% humidity.

Both male and female mice were divided into five groups as shown in Table 1. Three days before the start of the experiment, all mice had their backs closely clipped and then shaved with a razor under isoflurane sedation. Shaving allowed for maximum exposure of the skin to the effluent. A special sterilization chamber was designed to allow exposure of the body of the mice but prevent exposure of the head so that the mice would not inhale the plasma effluent (Figure 1). The Silvadene and Purell were applied as lotions on a 1 cm × 2 cm area on the back. Each of these agents was applied in a volume of 0.1 mL with no dilution of the product. Animals that received more than one treatment in a day had the treatments separated by 1 hour. Mice that were exposed to the plasma were briefly sedated using 5% isoflurane delivered via a precision vaporizer into an induction chamber. The mice were then placed into the special sterilization chamber. The mice were exposed to the plasma-induced effluent for 30 sec, immediately removed from the chamber, and allowed to recover from the sedation within their home cage.

After the last exposure or treatment, the animals were euthanized by exposure to carbon dioxide followed by cervical dislocation. A full-thickness skin sample was collected and placed in 10% formalin fixative and then sent to Charles River Research and Animal Diagnostic Services for processing and histopathologic analysis to reveal any evidence of skin damage and acute inflammation. The tissues were stained with hematoxylin and eosin and evaluated by light microscopy.

All studies were performed under a protocol approved by the University of Colorado Institutional Animal Care and Use Committee. The University of Colorado Denver is accredited by the Association for the Assessment of Laboratory Animal Care, International and follows all applicable regulations and policies.

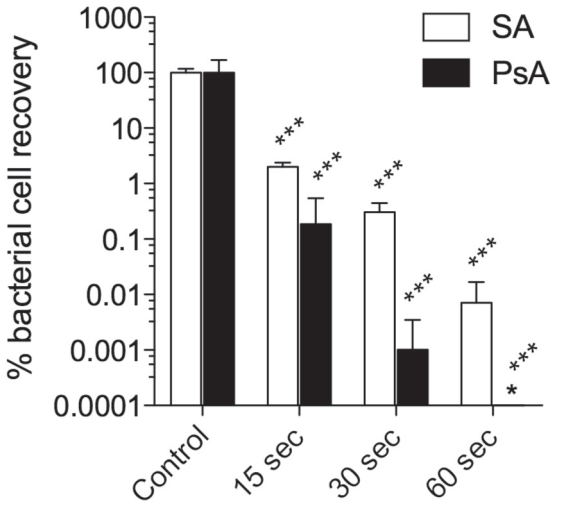


**FIG. 2:** Survival of *Staphylococcus aureus* (SA) and *Pseudomonas aeruginosa* (PsA) on plastic badges after exposure to plasma for various times. Data are presented as % bacterial cell recovery ± standard deviation. ***($p$ <0.001 compared to SA or PsA controls by two-way ANOVA of transformed data with Bonferroni's post test).

## III. RESULTS

### A. *In Vitro* Results

Figure 2 shows the results of the bacterial in vitro tests with data presented as percent of bacterial cell recovery in reference to the unexposed control group. Data are presented as % bacterial cell recovery ± standard deviation. A 30-sec exposure results in a 3 log reduction in culturable bacteria (0.1% recovery) for *S. aureus* and a 5 log reduction (0.001% recovery) for *P. aeruginosa*. A 60-sec exposure led to 4 log and 6 log reduction for for *S. aureus* and *P. aeruginosa*, respectively.

Figure 3 shows the resulting viability of HEKa and HDFa cell types as a function of treatment time. Exposures of less than 1 min left more than 90% of the cells still viable. Even after a 10-min treatment, about 50% of the cells were found to still be viable.

### B. *In Vivo* Results

A summary of the skin pathology microscope findings for all animals exposed to the plasma treatment is shown in Table 2. Table 3 shows the results for the control group and Silvadene and Purell treatments. All observed changes were subjectively graded as: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, or 5 = severe. In this subjective scale, a 1 corresponds to barely detectable and 5 to as extensive and intensive as possible. The pathology report also noted that all of the observed changes, except the fasciitis, could

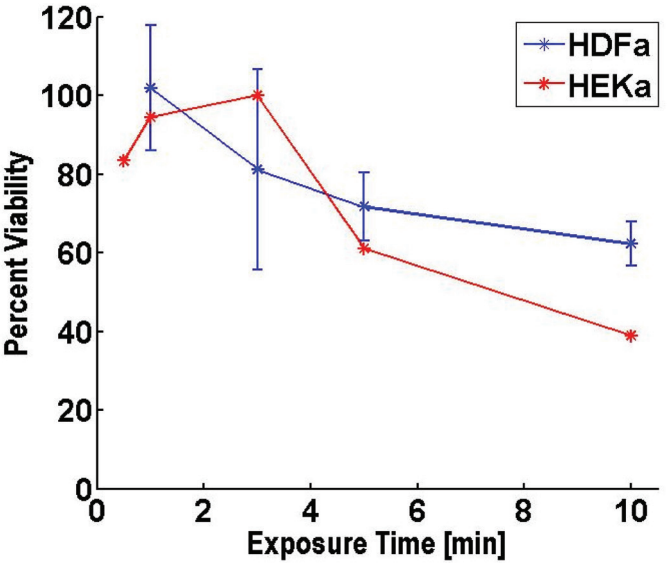


**FIG. 3:** Cell toxicity after 24 hours for human epidermal keratinocytes (HEKa) and human dermal fibroblasts (HDFa) as a function of treatment time with the plasma effluent.

**TABLE 2:** Microscope findings for animals exposed to plasma treatment (groups 2–4 of Table 1). All observed changes were subjectively graded as: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, or 5 = severe. In this subjective scale, a 1 corresponds to barely detectable and 5 to as extensive and intensive as possible.

| | Specimen Group Number | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Skin Finding | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Within normal limits | X | X | | | | | X | | X | | | | | | | | X | | | | X | |
| Superficial serocellular crust | | | | | | 2 | | | | | | | | | 1 | | | 1 | | | 1 | 1 |
| Fasciitis, subacute | | | 1 | | | 1 | | | | | 1 | | | | | | | 1 | | | | |
| Acanthosis, focal or multifocal | | | 1 | | | | | | | | | | 1 | | | | | | | | | |
| Acanthosis with fibrosis, focal or multifocal | | | | 1 | 1 | 2 | | | | 1 | | | | | | 2 | | 2 | 2 | 2 | 2 | 1 |
| Dermatitis | | | 1 | | | | | 1 | | | 1 | 1 | 1 | 1 | | 1 | | 1 | 1 | 1 | | 1 |

**TABLE 3:** Microscope findings for control group and animals treated with Silvadene and Purell (groups 1, 5–6 of Table 1). All observed changes were subjectively graded as: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, or 5 = severe. In this subjective scale, a 1 corresponds to barely detectable and 5 to essentially as extensive and intensive as possible.

| | Specimen Group Number | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Skin Finding | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| Within normal limits | | | X | X | | | X | | | | | | | | | | | X | X | X | X | X | | X |
| Superficial serocellular crust | 1 | | | | | | | | | | | | | | | | | | | | | | | |
| Fasciitis, subacute | | 2 | | | 1 | | | | 1 | | | | 1 | | | | 1 | | | | | | | |
| Acanthosis, focal or multi-focal | | | | | 1 | | | | | | | | | | | | | | | | | | | |
| Acanthosis with fibrosis, focal or multi-focal | | | | | | 1 | | 2 | 3 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | | | | | 1 | | |
| Dermatitis | | | | | | | | | 2 | | | | | | | | 1 | | | | | | | |

result from mild surface trauma. There was minimal fasciitis across all groups, which suggests subcutaneous penetration (incision, implantation, injection, etc.). Changes graded as minimal (grade = 1) were so mild that they may not have been recorded in a routine diagnostic case, but were noted here as part of the stringent evaluation requested.

Overall, there are very minimal differences in the findings across all animal groups. We note that the group treated with Silvadene exhibit the most pronounced acanthosis with fibrosis, although the findings are still classified as mild to moderate. At the same time, the group treated with Purell is almost all within normal limits. Gross and histologic photos of groups 1–6 are presented in Figs. 4–9 showing the most severely affected samples from each of the six groups.

## IV. DISCUSSION

The U.S. Food and Drug Administration defines high level disinfection to be a 6 log reduction in viable pathogens.[2] Our finding that a plasma effluent treatment of approximately 1 min can achieve this level of disinfection level means that the technology is suitable for implementation in routine disinfection procedures in hospitals, laboratories, and animal research facilities. The low temperature and lack of liquids makes possible the disinfection of plastics and portable electronics. Our results can be compared to a

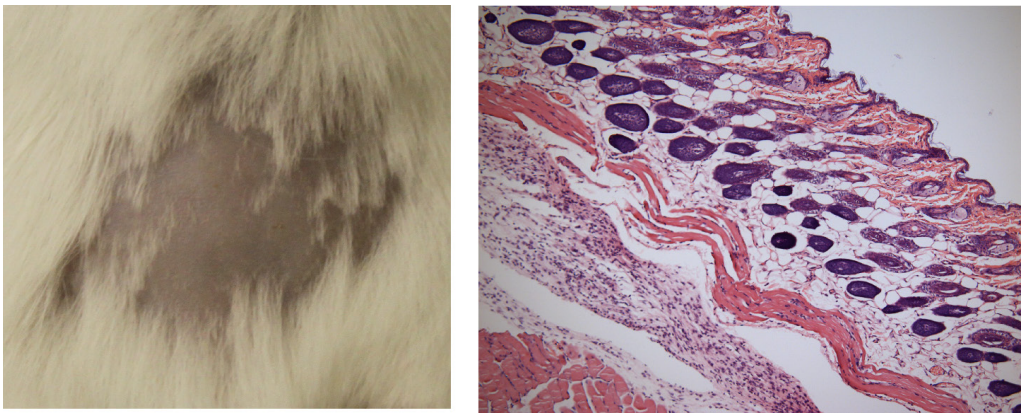

**FIG. 4:** Gross pathology (left) and histopathology (right) of sample number two of the control group (group 1) showing subacute fasciitis. Hematoxylin and eosin stain, original magnification ×10.

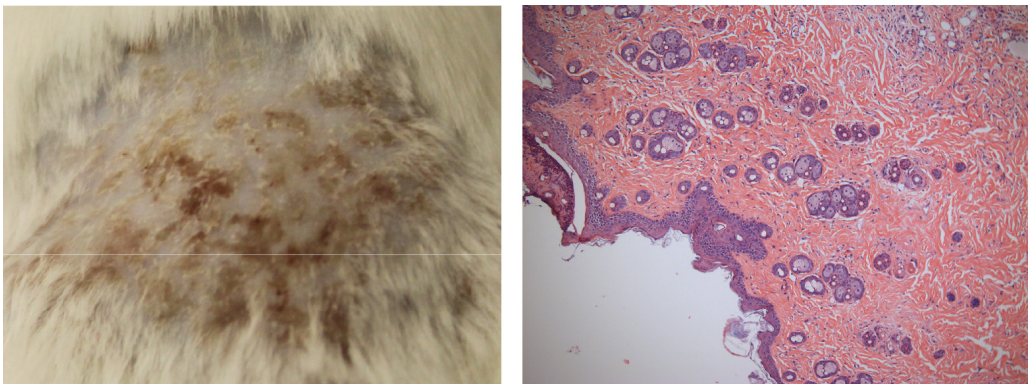

**FIG. 5:** Gross pathology (left) and histopathology (right) of sample number six of the one time per day plasma exposure group (group 2) showing a superficial serocellular crust. The healing wounds on the skin were present prior to exposure and were a result of shaving wounds. Hematoxylin and eosin stain, original magnification ×10.

study performed by Burts et al.,[26] which used a different indirect exposure nonthermal plasma technology to treat electronic pagers. In their work, exposures of 10 min were required to achieve 4-5 log kill rates.[26] Direct exposure techniques, of which the floating electrode dielectric barrier discharge (FE-DBD) is the most commonly cited example, achieve even faster kill rates but require the electrode to be close to the treated sample.[17] The desktop sterilizer implementation of the plasma effluent technology considered here, shown in the right panel of Figure 1, can be operated like a microwave oven. Objects can be placed inside freely (no special mounts or adapters) and removed after a short (<5 min) sterilization cycle. The goal of the skin culture exposures and in vivo tests was twofold: to test if objects treated with this technology could be readily

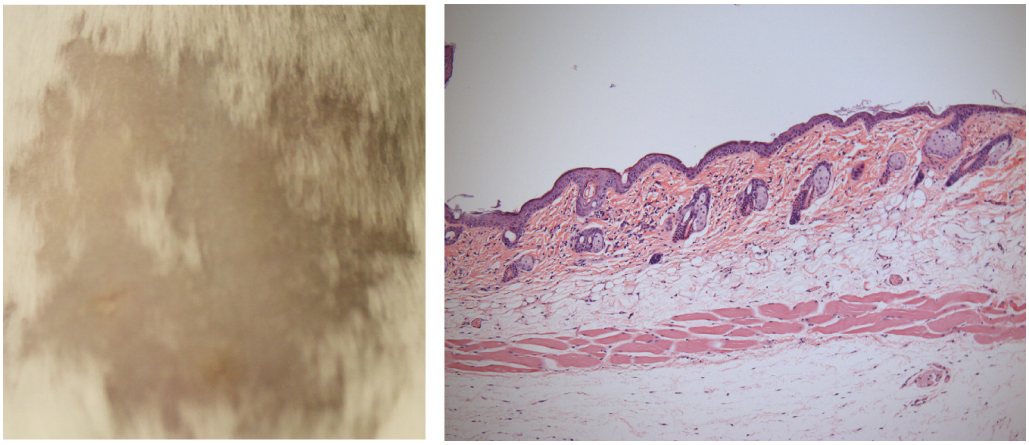

**FIG. 6:** Gross pathology (left) and histopathology (right) of sample number five of the three times per day plasma exposure group (group 3) showing acanthosis and dermatitis. Hematoxylin and eosin stain, original magnification ×10.

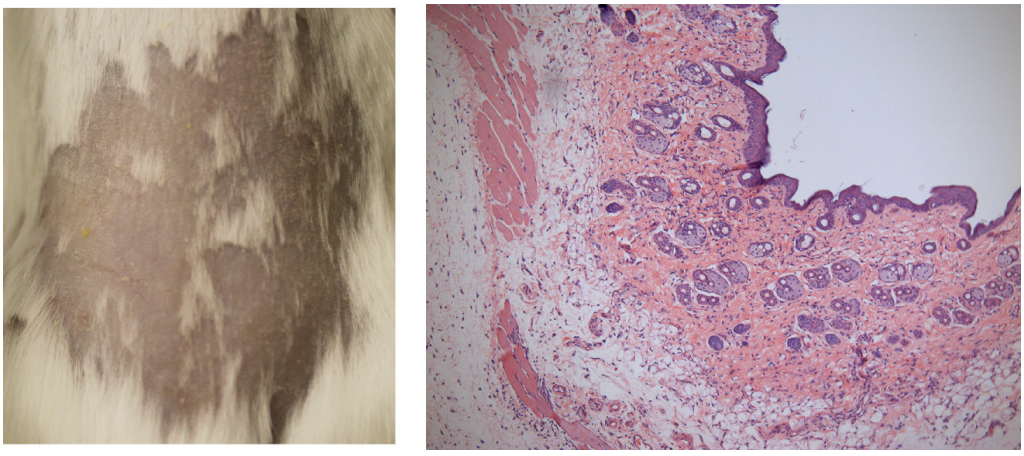

**FIG. 7:** Gross pathology (left) and histopathology (right) of sample number three of the five times per day plasma exposure group (group 4) with superficial serocellular crust, subacute fasciitis, acanthosis with fibrosis, and dermatitis. Hematoxylin and eosin stain, original magnification ×10.

handled without gloves and if the technology could be used directly on bare tissue as a supplement or alternative to hand washing. The human fibroblasts and keratinocytes experienced less than 1 log reduction after 10 minutes of exposure. Thus, they were much less affected than the bacteria cells, which were exposed for only tens of seconds. In this context, it is important to note that the human fibroblasts and keratinocytes tested in isolated cultures lack survival signals from the surrounding microenvironment. They are

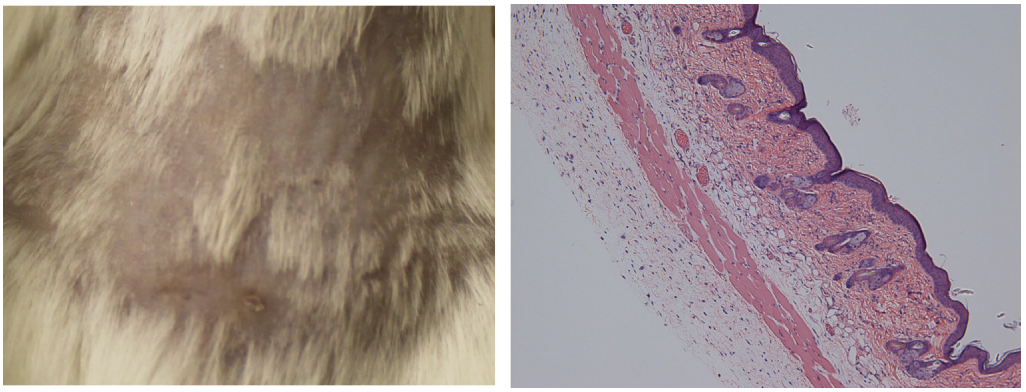

**FIG. 8:** Gross pathology (left) and histopathology (right) of sample number one of the Silvadene group (group 5), with subacute fasciitis, acanthosis with fibrosis, and dermatitis. Minor skin trauma was present prior to exposure to the Silvadene. Hematoxylin and eosin stain, original magnification ×10.

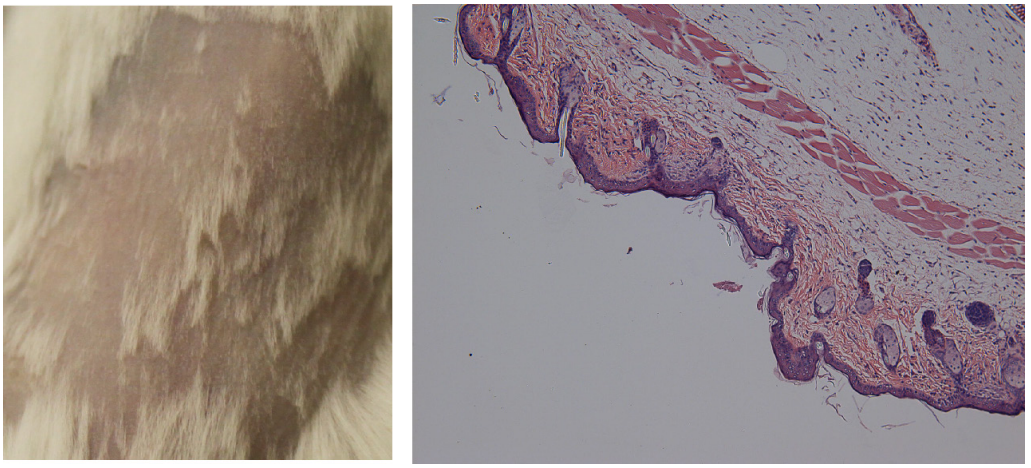

**FIG. 9:** Gross pathology (left) and histopathology (right) of sample number one of the Purell group (group 6), with subacute fasciitis, acanthosis with fibrosis, and dermatitis. Hematoxylin and eosin stain, original magnification ×10.

more proliferative and less differentiated cells, thus they are significantly more sensitive to various triggers in isolated cultures than they would be in a true skin enviroment. The general resistance of skin cells to the plasma treatment is in agreement with other works that show nonthermal plasma has a degree of kill selectivity for prokaryotic cells. The greater resistance of eukaryotic cells is believed to result from a larger and more complicated cell structure as well as mechanisms for mitigating oxidative stress.[24]

The skin pathology results show that the plasma treatment did not cause significant

adverse affects after acute exposure of only a few days and performed well against Purell and Silvadene. Although Silvadene is not used as a rapid hand sanitizer, it is a routinely used antimicrobial topical agent with documented side effects.[27,28] Our results confirm other recent work showing that nonthermal plasma treatments can be safe for intact tissue. For example, Wu et al.[29] performed a study on porcine skin using the FE-DBD and found that 2 min was the threshold for tissue damage. As mentioned previously, the FE-DBD technique involves a plasma discharge directly on the treatment site, unlike the indirect exposure via a plasma-induced air stream used here.

We note that the shaving of the animals before the experiment created several nicks and minor cuts on the skin, and therefore the minor histologic changes seen in this study could be consistent with shaving-related superficial trauma. While fibroblasts could be activated during this 3-day exposure, most likely the fibrosis is not attributable to the treatments provided. This, along with the dermatitis (increased leukocytes within the dermis) and deeper inflammation (fasciitis) observed in some animals, will need to be explored more completely in future studies of a longer duration. Although there appeared to be a trend for a dose-related increased incidence of dermatitis in the plasma-treated group, it was minimal and would need to be further evaluated over a longer time period to fully ensure the safety of this device on living tissue. In additional studies, alternative methods of hair removal will be tested to decrease the confounding factor of shaving on the skin.

The time points were chosen to ensure that an acute exposure of skin to the plasma, which might occur when removing an inanimate object, would not cause significant adverse pathology such as chemical burns. Although more comprehensive studies are needed, this technology has potential as an alternative or supplement to hand washing, which requires time scales and frequency that are similar to the exposures in these experiments. Hand washing and many chemical disinfectants generally require access to water to be effective. This device is portable and could be set up anywhere near an electrical outlet, including the entrance to a vivarium or laboratory. The device would only require periodic refill of the hydrogen peroxide solution, about 1 L every 1,000 treatments.

Additional uses of this device on skin include surgical preparation, burn disinfection, or wound therapy. In all of these cases the device may be used frequently over a small period of time, similar to the 30-sec exposures of the experiment. For the cases on nonintact tissue, pathology will need to be explored on a more complete scale to ensure that adverse effects such as inflammation, ulceration, and chemical burns do not develop. Recent work on nonthermal plasma wound treatment suggests that this device can also yield promising results.[29–31] Likewise, a comprehensive literature search revealed that ozone was used as an effective treatment for combat wounds and mustard gas burns in World Wars I and II.[32–35] This ozone treatment was later discontinued, most likely because of hardware limitations of ozone production at the time. As discussed earlier, ozone is one of the main active species in the plasma effluent of the tested device. The inclusion of hydrogen peroxide additives yields an improved bacteriacidal cocktail

including hydroxyl radicals.[23]

Overall, this technology has the potential to mitigate multiple problems related to the disinfection of inanimate objects and skin sanitation in the laboratory and vivarium environments. We have successfully shown that this device is able to quickly and significantly reduce bacterial loads on plastic identification badges typically used by medical and research personnel. The use of the device on murine skin and comparison with standard disinfection treatments shows that the plasma effluent treatment did not produce any more acute pathologic affects and may be suitable as an alternative for disinfecting live tissue without harsh chemicals or the availability of a water source. Future studies include in vitro testing of more complex inanimate objects such as keys, pens, microtubes, and small electronics (e.g., cell phones) to determine if the plasma will effectively penetrate them. For in vivo research, we plan to test the device on skin for a longer, chronic, time period and to investigate methods of disinfecting wounds and burns that will promote healing and possibly decrease scarring.

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