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Original article

In vitro antibacterial effects of non-thermal atmospheric plasma irradiation on *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa*

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Abstract

In the last decade, atmospheric plasma has been used to treating bacterial infections in humans due to its bactericidal effects; however, its efficacy in dogs is unclear. This study evaluated the in vitro bactericidal efficacy of atmospheric plasma on *Staphylococcus pseudintermedius* and *Pseudomonas aeruginosa*, two of the most important bacterial agents isolated from canine pyodermas. Three isolates each of *S. pseudintermedius* and *P. aeruginosa* obtained from dogs with pyoderma were subjected to atmospheric plasma. The isolates from the control group were not exposed to plasma, while those from the treatment groups were exposed to plasma for 15 (7.5 J/cm$^{2)}$, 30 (15 J/cm^2), 60 (30 J/cm^2), or 90 (45 J/cm^2) seconds. After each treatment, a reduction in colony formation was observed. Bacterial viability was evaluated using the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit. The antibacterial effects were evaluated with Image J software and significance was assessed statistically in comparison to the control group. The bactericidal effect of atmospheric plasma against both bacteria increased significantly in a time-dependent manner. These results demonstrate the bactericidal capacity of atmospheric plasma, and suggest that it could serve as an alternative treatment method for canine pyoderma. Further studies are needed to evaluate the safety and efficacy of atmospheric plasma in dogs.

Key words: antibacterial effects, cold-atmospheric plasma, dog, *Staphylococcus pseudintermedius*, *Pseudomonas aeruginosa*

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Introduction

Staphylococcus pseudintermedius and *Pseudomonas aeruginosa* are two of the most important pathogens isolated from the skin surface of dogs with pyoderma (Hillier et al. 2006, Weese 2013). Clinicians usually turn to antimicrobials to treat bacterial skin infections (Miller et al. 2013). However, the prevalence of multidrug resistant bacteria has gradually increased (Jones et al. 2007, Rubin et al. 2008, Ruscher et al. 2010), and in some cases, such as in neonates, pregnant or lactating patients, antimicrobials may be contraindicated (Miller et al. 2013). Thus, there is a need to develop innovative alternative therapeutic strategies for treating bacterial infections.

Plasma is considered the fourth state of matter, the others being solid, liquid, and gas (Fridman and Kennedy 2004). As energy level increases, the state of matter progresses from the solid to the liquid phase, then to the gas phase, and finally, to the plasma phase (Fridman and Kennedy 2004). Over the last few decades, many studies have revealed that plasma could inactivate microbes through several mechanisms (Moisan et al. 2001, Joshi et al. 2010, Xiaohu et al. 2013, Liao et al. 2017, Nishime et al. 2017). The antimicrobial effects of plasma on many microorganisms have since been demonstrated, leading to the introduction of plasma as a novel treatment for bacterial infections in human medicine (Daeschlein et al. 2012, Julák and Scholtz 2013, Švarcová et al. 2014). However, there is still a lack of data on the use of plasma in veterinary medicine.

The aim of this study is to evaluate the in vitro antibacterial effects of plasma against *S. pseudintermedius* and *P. aeruginosa* isolated from canine pyoderma.

Materials and Methods

Bacterial isolates

Three isolates each of *S. pseudintermedius* and *P. aeruginosa* were obtained from skin pustule lesions of six dogs. Intact pustules of dogs with superficial skin infection were lanced using a 25-gauge needle and the contents were transferred onto sterile swabs. The samples were cultured aerobically on tryptic soy agar (TSA, Difco, Detroit, MI, USA) at 37°C for 18-24 h. Based on colony morphology, *S. pseudintermedius* and *P. aeruginosa* as suspected and colonies were isolated.

In this study, two methods were used for identification of bacteria.

First, DNA extracted from isolates was identified through 16S rRNA gene sequencing using 27F and 1492R primer. The National Center for Biotechnology Information (NCBI) basic local alignment search tool was used to search the NCBI database for bacterial DNA nucleotide sequences matching those of the microorganisms isolated.

Second, conventional the Vitek system using the card (bioMerieus) were adjusted to identify the bacteria. For the Vitek System, fresh colonies were used to prepare an inoculum in 0.5% sterile saline equivalent to a specified turbidity standard. Test cards were inoculated according to protocol and transferred to the Reader/Incubator. All procedures were performed in accordance with manufacturers' product recommendation.

According to these two methods, *P. aeruginosa* was identified definitely; however, *S. pseudintermedius* was not distinguished from *S. aureus* clearly, Thus, we cultured the isolated bacteria aerobically on sheep blood agar (Difco, Detroit, MI, USA) at 37°C for 18-24 h; and confirmed that their colony color was white.

Plasma generator

Figure 1 shows the plasma generator (Plapet; PLABIO, Seoul, Korea) used in the experiments. The generator has a comb-like design with two sites for plasma generation: the surfaces of the comb, and a square electrode on the side measuring 0.3 × 3.0 cm. For this study, we used the side square electrode. The current and voltage of the plasma device were 1.5 A and 5.0 V, respectively. The amount of plasma energy was 0.5 J/cm^2 and the energy levels (J/cm^2) used in this study were 0 (0 s), 7.5 (15 s), 15 (30 s), 30 (60 s), and 45 (90 s) (Sanaei et al. 2015). No elevation of the surface temperature by more than 1°C was observed during plasma application.

Evaluation by plate method

Each bacterial isolate was cultured in tryptic soy broth (Difco) at 37°C for 18-24 h. After incubation, bacterial broth concentration was adjusted to 10^7 colony forming units (CFU)/mL, and 10 µL of the diluted bacterial solution was cultured on TSA. After culturing, the TSA plates with each bacterial isolate were divided into four groups: Control (no treatment, Group A), or treatment with plasma for 15 s (Group B), 30 s (Group C), 60 s (Group D), or 90 s (Group E). Plasma was applied to the selected area (3.0 × 2.0 cm) on each plate under the respective conditions, and the plates were incubated at 37°C for 24 h. For each group, the experiments were repeated in duplicate. After incubation, the colony area on the plasma-treated surface was calculated using Image J (NIH freeware, http://rsb.info.nih.gov/ij/download.html) and compared to the results on the control plate.

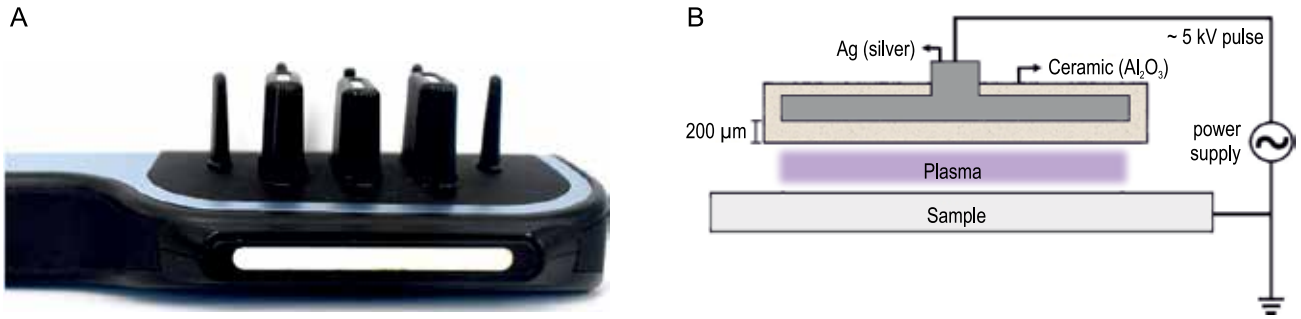

Fig. 1. The plasma device used for present study (A) and schematic of apparatus (B).

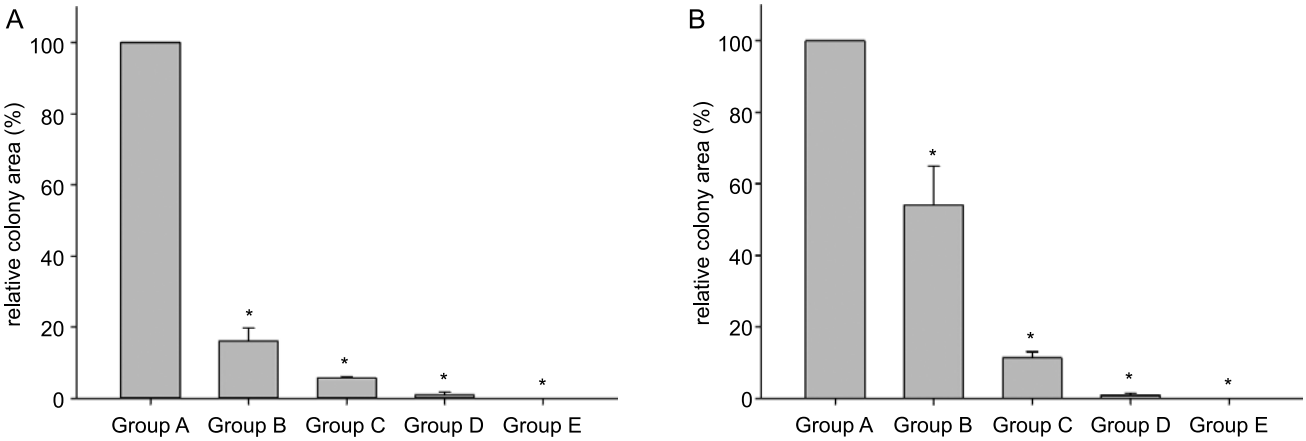

Fig. 2. The changes of colony area for *S. pseudintermedius* (A) and *P. aeruginosa* (B) after each treatment condition. * $p<0.05$ compared with the non-treatment group at each dose. Group A, no treatment; Group B, 15 s irradiation; Group C, 30 s irradiation; Group D, 60 s irradiation; Group E, 90 s irradiation.

Evaluation by slide method

Before the experiment, each isolate was cultured on TSA at 37°C for 18-24 h to obtain a single colony. A swab from a single colony of each of the isolates was applied to a slide glass. The slide glass samples were divided into the same five test groups specified above, and each slide was treated with plasma according to the condition for each group. After plasma application, the LIVE/DEAD Bacterial Viability kit (L-7007; Invitrogen, Carlsbad, CA, USA) was used to analyze the samples to evaluate the direct bactericidal effect of plasma. The green SYTO9 stain and red propidium iodine stain differ in their abilities to penetrate normal bacterial cells. Live bacteria with intact membranes fluoresce green, while dead bacteria with damaged membranes fluoresce red. After plasma irradiation, 20 µL of the stain mixture from the viability kit was added to each slide, which was covered with an 18 × 18 mm^2 glass slip and incubated for 15 min in the dark at room temperature. The excitation/emission maxima were approximately 450/490 nm for the live and dead cells and 510/560 nm for only dead cells. Fluorescence microscopy images were obtained using the Eclipse 80i fluorescence microscope (Nikon Instech Co., Ltd., Tokyo, Japan) with an oil immersion lens at 40 X magnification.

The fluorescence intensities of the images were quantified using the Image J software as described previously. Percentage survival was calculated as the value of green fluorescence based on the total fluorescence values (red + green fluorescence).

Statistical analyses

Statistical analyses were conducted using SigmaPlot (Windows version 12.0; Systat Software, Inc., San Jose, CA, USA). Percentage survival between the treatments was evaluated by repeated-measures analysis of variance. Dunnett's test was performed to compare the results of the treatment groups with those of the control group. $p<0.05$ was considered statistically significant. Quantification of fluorescence was subjected to the paired Student's t-test.

Results

Evaluation using plate method

Colony formation of both bacteria was inhibited by plasma application. Figure 2 shows the colony area reduction rate compared to that in the untreated group. When the colony area of the control group was set

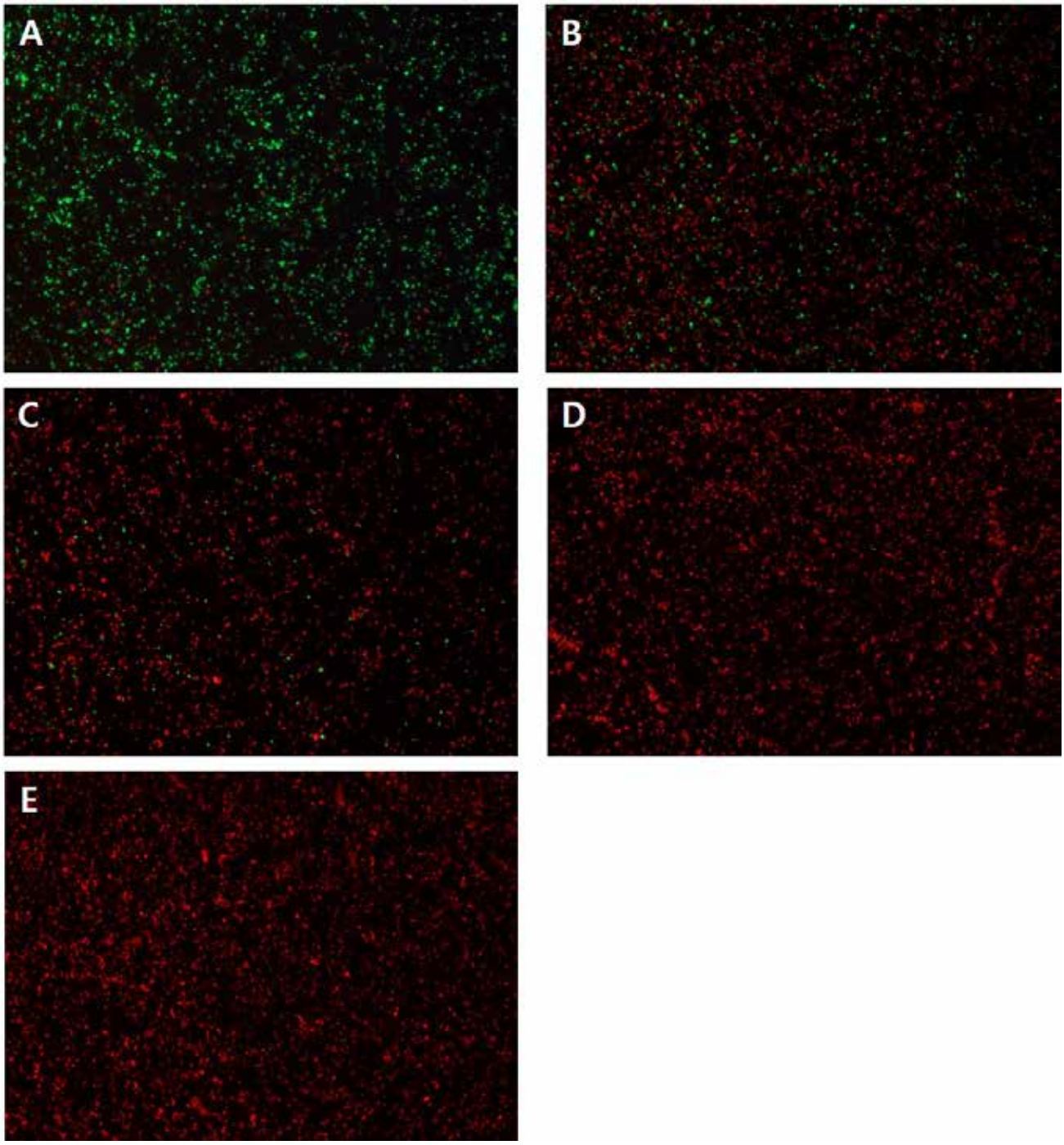

Fig. 3. Fluorescence microscopic images of *S. pseudintermedius*; ×40. *S. pseudintermedius* cells in non-treated group (A), after plasma irradiation for 15 s (B), after plasma irradiation for 30 s (C), and after plasma irradiation for 60 s (D).

as 100%, the relative colony areas of *S. pseudintermedius* were 16.17%, 5.76%, 1.01 %, and 0 % after 15, 30, 60, and 90 s of plasma application, respectively ($p<0.05$). The relative colony areas of *P. aeruginosa* were 53.99%, 11.34%, 0.86%, and 0% after 15, 30, 60, and 90 s of plasma application, respectively ($p<0.05$). The colony area decreased dose-dependently after plasma application for all time periods. Compared with *P. aeruginosa*, the growth of *S. pseudintermedius* was inhibited in a short time after plasma application. However, after 90 s of plasma application, both bacteria showed no growth.

Evaluation using slide method

Epifluorescence staining using the by LIVE/DEAD Bacterial Viability assay revealed decreased bacterial viability after plasma application (Figs. 3 and 4). In the untreated group, most cells were alive and it was difficult to detect dead cells. After plasma application,

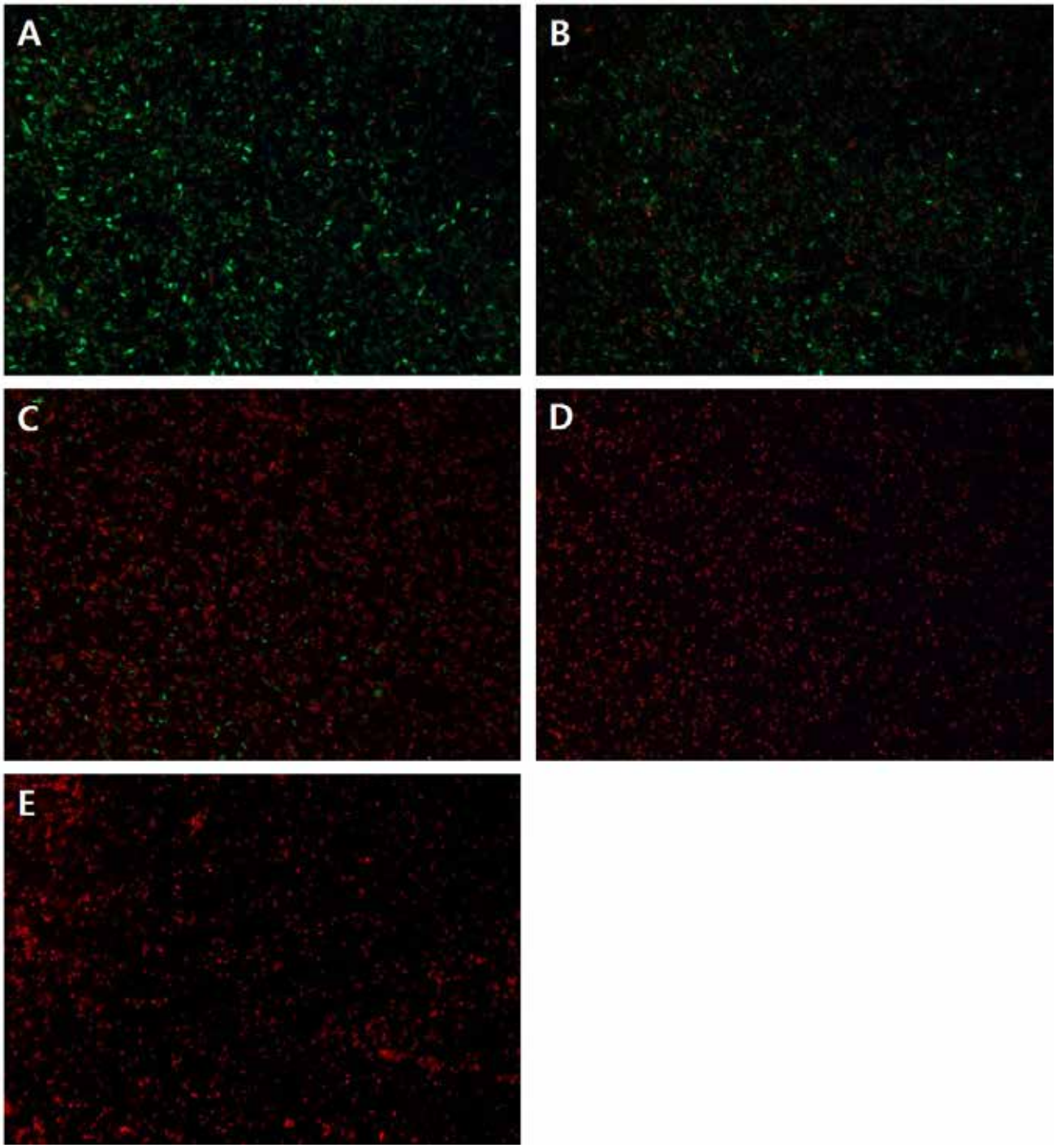

Fig. 4. Fluorescence microscopic images of *P. aeruginosa*; ×40. *P. aeruginosa* cells in non-treated group (A), plasma irradiation for 15 s (B), plasma irradiation for 30 s (C), plasma irradiation for 60 s (D), plasma irradiation for 90 s (E).

the number of dead cells increased compared to the control group. The proportion of dead cells increased after plasma application in a time-dependent manner. Figure 5 presents the percent viability of both bacteria following each treatment. The viability of *S. pseudintermedius* and *P. aeruginosa* exposed to plasma for 60 s were 0.01% and 0.03% respectively ($p<0.01$). After 90 s of plasma application, the viability of both bacteria was 0% ($p<0.01$)

Discussion

Plasma is a state associated with high-energy physics and is generated by different methods, including gas discharge, photo ionization, and radio frequencies (Fridman and Kennedy 2004). Plasma can be divided into high-temperature and low-temperature plasma based on the temperature of electrons (Fridman et al. 2005). High temperature plasma application could

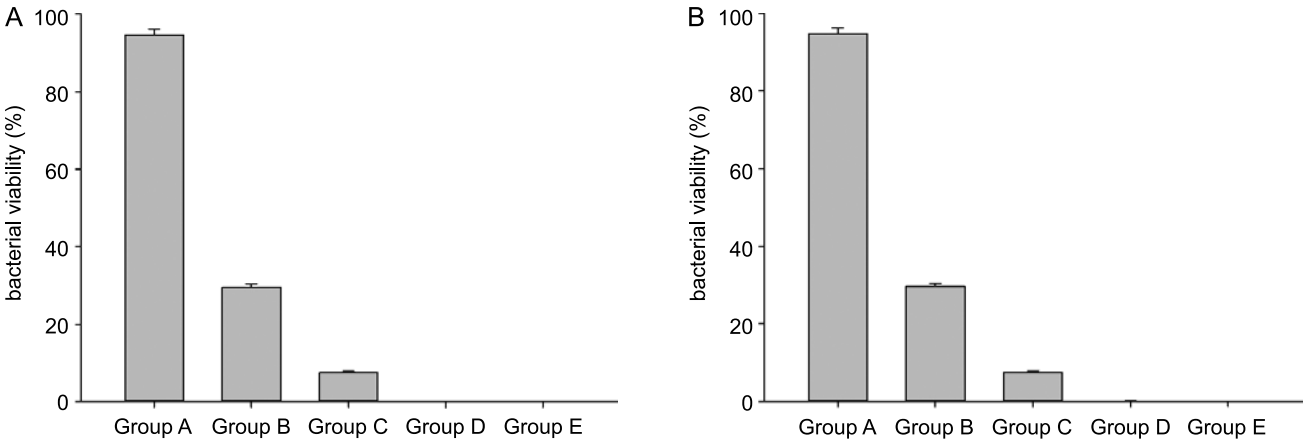

Fig. 5. The survival rate of *S. pseudintermedius* (A) and *P. aeruginosa* (B) under each treatment condition. ** p<0.01 indicating a significant difference compared with the non-treatment group at each dose. Group A, no treatment; Group B, 15 s irradiation; Group C, 30 s irradiation; Group D, 60 s irradiation.

be painful or lead to significant thermal tissue damage. Thus, non-thermal plasma has emerged as an option for medical applications (Scholtz et al. 2015). In our *in vitro* study, we used a non-thermal atmospheric plasma generator as a plasma source and evaluated the antimicrobial effects of the generated plasma.

The results reveal the antibacterial effects of non-thermal atmospheric plasma against *S. pseudintermedius* and *P. aeruginosa* isolated from canine skin infection samples. This antibacterial effect was plasma dose-dependent and was stronger for *S. pseudintermedius* than for *P. aeruginosa*.

Plasma reduced the colony formation ability of both bacteria at all doses, compared to the untreated control. The LIVE/DEAD Bacterial viability kit was also used to monitor bacterial viability. Fluorescence microscopic images of the bacteria showed that the number of viable microorganisms decreased compared to the number observed before plasma application. After 90 s of irradiation, all the bacteria had died.

Pyoderma is one of the most common skin diseases in dogs (Miller et al. 2013). *S. pseudintermedius* is the primary pathogen found on the canine skin surface with or without pyoderma (Weese 2013). *P. aeruginosa* is infrequently responsible for canine skin infections but is considered the major pathogen associate with chronic or deep pyoderma (Petersen et al. 2002, Hillier et al. 2006). Thus, controlling both these bacteria is essential to prevent and treat canine pyoderma. Numerous studies have revealed the inactivation of microbes by non-thermal plasma (Moisan et al. 2001, Joshi et al. 2010, Xiaohu et al. 2013, Liao et al. 2017, Nishime et al. 2017). Some reports have also shown that plasma has the ability to control biofilm formation (Joshi et al. 2010). The present results provide the first direct data for the efficacy of plasma as an alternative treatment to kill canine skin pathogens.

Since *S. pseudintermedius* is one of the normal components of the skin microflora in dogs, it typically does not pose a threat to healthy dogs (Weese 2013). However, when the dog has an underlying metabolic or immunological abnormality, *S. pseudintermedius* can increase in number and cause pyoderma (Miller et al. 2013). The present results indicate the potential for the plasma generator as an alternative therapy and as a prophylactic treatment for canine superficial pyoderma. In the present study, application of plasma for only 60 s was almost completely lethal for both bacteria and after 90 s of plasma irradiation, all bacteria had died. This device generates the plasma through radio frequency-based methods. Thus, it is very light and does not need to be recharged the gas. Therefore, owners can comfortably and easily treat their pet's skin surface with plasma.

In conclusion, plasma from an atmosphere plasma generator kills *S. pseudintermedius* and *P. aeruginosa* in a plasma dose-dependent manner. Before clinical applications can be contemplated, *in vivo* studies will be needed to validate the clinical effects and safety of plasma treatment. Based on the present findings, we think that plasma could be applied onto the skin of canine patients with skin infections or be utilized as a prophylactic treatment.

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