A THESIS FOR THE DEGREE OF MASTER

In vitro antibacterial and antibiofilm effects of cold atmospheric microwave plasma against *Pseudomonas aeruginosa* causing canine skin and ear infections

개의 피부 및 귀의 감염증을 일으키는 녹농균 및 바이오필름에 대한 Cold atmospheric microwave plasma의 항균 효과

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수의학과 임상수의학(피부과학)

김 은 주

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지도교수 황 철 용

이 논문을 수의학석사 학위논문으로 제출함

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In vitro antibacterial and antibiofilm effects of cold atmospheric microwave plasma against *Pseudomonas aeruginosa* causing canine skin and ear infections

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Abstract

Pseudomonas aeruginosa is an opportunist pathogen that causes purulent inflammation in the skin and in the ears of dogs. Among the various virulence factors of *P. aeruginosa*, biofilms have been reported to result in antibiotic resistance, leading to therapeutic limitations. Cold atmospheric microwave plasma (CAMP) is known to have a high antimicrobial effect, which causes physical cell wall rupture and DNA damage.

The objective of this study was to evaluate the antibacterial and antibiofilm effects of cold atmospheric microwave plasma (CAMP) against *P. aeruginosa.*

The antibacterial effect of CAMP against *P. aeruginosa* ATCC strain and clinical isolates (n = 30) was evaluated using the colony count method. This study also assessed the effect of CAMP on biofilm by colony count method, water-soluble tetrazolium salt (WST) assay, and confocal laser scanning microscopy (CLSM).

The complete eradication of *P. aeruginosa* (ATCC strain and clinical isolates) was achieved within 120 seconds at 50 watts (W), and clinical isolates required shorter time than the ATCC strain for complete eradication. In the biofilms, almost 3 $\log_{10}$ (99.9%) reduction in the number of viable cells was achieved within 4 minutes at 50 W, while with 30 W of plasma exposure no significant reduction in biofilms occurs.

CAMP was effective against both planktonic bacteria and also against the biofilm formed by *P. aeruginosa.* However, further studies are needed about the safety of

CAMP to canine skin and ears to fully validate its potential in the clinical application.

Key words: antibacterial effect, antibiofilm effect, canine, cold atmospheric microwave plasma, *P. aeruginosa*

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1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative rod-shaped bacterium ubiquitous in the environment. This bacterium is the most common problematic pathogen in dogs with chronic or recurrent bacterial otitis externa and occasionally found in cases of deep pyoderma [1,2]. *P. aeruginosa* is an opportunistic pathogen with a high prevalence of antibiotic resistance, which is partly due to its potent ability to form biofilms [3,4].

The bacterial biofilms are a sessile community of bacteria attached to the surface that produce a matrix formed by an extrapolymeric substance (EPS) of carbohydrates, proteins and DNA, and then develop irreversibly into monolayers of planktonic bacterial cells [5]. Due to the physical protection conferred by EPS, metabolism alterations and several other changes in each individual bacterium and the bacterial community, the bacteria within the biofilm can markedly evade the host immune response, have desiccation tolerance, and resist the antimicrobial therapy [6].

In human medicine, the biofilms are estimated to represent approximately 80% of all the chronic human infections, being important mediators of healthcare-associated infections [7,8]. The biofilms can be formed in the lungs of patients with cystic fibrosis, within wounds, on surgical implants and within the middle ear [9-11]. The bacteria within the biofilms are generally more resistant to antibacterial challenge therapy and to the host immune system than planktonic bacteria of the same species [12-16]. The resistance of the antibiotic challenge may explain the reason why biofilm-mediated infections often fail to respond to conventional antibiotic

treatments [15]. Biofilms have introduced a limitation to existing antibiotic treatments by acquiring antibiotic resistance in veterinary medicine [17]. In addition, the biofilm-forming bacteria can potentially lack a response to the treatment in dogs due to the minimum inhibitory concentration for biofilm-embedded bacteria, which is different from planktonic counterparts [18]. In comparison to their counterpart planktonic form, understanding the antibacterial resistance of bacteria within biofilms is important for developing the most appropriate treatment regimens for canine patients.

The application of physical treatments can be an alternative method when the effects of chemicals are weak due to biofilm resistance and in this context nonthermal plasma may be an option. The plasma is a neutral ionized gas, consisting of photons, electrons, charged ions, atoms, free radicals, and excited and nonexcited molecules produced under the application of an electric field [19]. The antibacterial effect of plasma is mainly attributed to the synergies of the reactive particles in torch and ultraviolet–visible that cause oxidative stress and deoxyribonucleic acid (DNA) damage [20,21]. Two categories of plasma exist, namely thermal and nonthermal, being defined according to the conditions in which they are generated. Thus, nonthermal plasma is obtained at lower pressure and uses a lower power18. This effective equipment for eradicating microorganisms is also known by atmospheric plasma (CAP) [22-24]. CAP has been evaluated for a significant number of biomedical applications in the human dermatology, including wound healing, skin regeneration and also the healing of infected areas [25-28]. However, in veterinary medicine, studies on CAP applications are lacking. In this study, microwave discharge plasma torch, called cold atmospheric microwave plasma (CAMP) was

used regarding its advantage in the medical field application, allowing to maintain the surface temperature below 40°C [29].

The object of the present study is to investigate the effectiveness of CAMP for the in vitro eradication of the clinically significant *P. aeruginosa* biofilms and planktonic bacterial cells. Several experimental methods have been applied to evaluate the bacterial cell viability after plasma application, such as colony count method, water-soluble tetrazolium salt (WST) assay and Live/Dead staining followed by confocal laser scanning microscopy (CLSM).

2. Materials and Methods

2.1. Bacterial strains

Bactericidal effects of CAMP were evaluated against all clinically isolated and stocked strains of *P. aeruginosa* from dogs with pyoderma or otitis at the Veterinary Medical Teaching Hospital of Seoul National University (n = 30) between 2017 and 2021. The isolates obtained from 12 dogs with pyoderma and 18 dogs with otitis were identified using the Vitek 2 system (BioMerieux; Hazelwood, MO, USA). The dogs had never been in contact with each other.

As a standard strain, *P. aeruginosa* (obtained from American Type Culture Collection, number 10145) was used in this study. The strains were stored in tryptic soy broth (TSB, Oxoid; Basingstoke, UK) with 50% glycerol at the temperature of -80°C before further examinations, being the bacteria routinely cultured on blood agar plates at 37°C in an aerobic condition.

2.2. Cold atmospheric microwave plasma

All the experiments were performed with a microwave plasma pen (IonMedical inc., Seongnam, Korea). When the microwave energy [30–50 watts (W), 2450 MHz] entered to the tube and the argon plasma came into contact with the air, the reactive species were produced, and all the plasma products were then delivered to the object by the gas flow. The plasma was then exposed at a distance as far as the end of the

jet, which of the length was in the range of 3 to 15 mm. CAMP was set at gas flow 15 L/min and the power consumption was 30 W or 50 W. The surface temperature of the plasma was maintained between 37°C–40°C.

2.3. Plasma inactivation of planktonic cells

P. aeruginosa (ATCC10145) and clinical isolates (n = 30) were used to determine the bactericidal effect. A single colony from the agar plate was inoculated in Luria-Bertani broth (LB) and incubated overnight at 37°C, while shaking at 120 rotations per minute (rpm), for 24 hours and then diluted with 0.5 Mcfarland using a turbidimeter (Densichek McFarland Densitometer, Biomerieux; Lyon, France). The final inoculum concentration was adjusted to approximately 5.0×10^4 colony-forming units (CFU) per mL by broth dilution, and 10 µl of bacterial suspension was spotted on the surface of the Mueller–Hinton agar (MHA, BD Diagnostic Systems; Sparks, MD, USA) plate. The plates were exposed to the plasma for 10, 30, 60, 120, and 240 seconds as described above. For the control conditions, nonionized argon gas and unexposed plasma were used. Each plate was left in the incubator at 37°C for 24 hours, and the number of colonies was counted, and the number of surviving cells was calculated as colony forming units per peg (CFU/peg). All the experiments were performed in triplicate.

2.4. Biofilm growth

P. aeruginosa (ATCC10145) was used to grow biofilms. The biofilms were grown on the peg lid of Calgary Biofilm Device (MBEC AssayTM for Physiology & Genetics (P & G), Innovotech Inc.; Edmonton, Alberta, Canada) [30]. As previously described, a single colony from the agar plate was inoculated in LB and incubated overnight at 37°C in an aerobic condition, while shaking at 120 rpm during 24-hours, and diluted to a concentration equivalent of 0.5 McFarland turbidimeters. The final inoculum concentration was adjusted to about 5.0×10^6 CFU/mL by broth dilution [31]. The standardized bacterial suspension was inoculated in the Calgary Biofilm Device (with a volume of 150 µl in each well) and then incubated at 37°C for 48 hours in an aerobic incubator. Every 24 hours, each well was replaced by fresh LB. The peg lids were replaced in a 96-well microtiter plate that contained fresh LB in each well. At the end of the 48 hours, which is the time of the final incubation, each peg was rinsed with for 1 min in a plate containing 200 µl of phosphate- buffered saline (PBS, Biosesang; Gyeonggi, Korea). This procedure allows to remove any planktonic or loosely adhered bacteria prior to plasma exposure.

2.5. Plasma inactivation of biofilms

The biofilm treatment methods used in this work were modified from a previous study [32]. Each peg was exposed to plasma at different time points (0, 15, 30, 45, 60, 120, and 240 seconds) [33]. The pegs exposed to gas only were also examined and used as a negative control. All the experiments were performed independently three times. After the plasma exposure, the pegs were placed in the wells of a 96-

well microtiter plate containing 200 µl of PBS in each well and sonicated for 10 min to dislodge the biofilm cells and the surviving bacteria were re-suspended in PBS. The pegs were discarded, being the resultant bacterial suspensions used to determine the viability of surviving bacterial cells.

The standard colony count method and WST assay were used to determine the viability of surviving cells. In the standard colony count method, the recovered bacterial suspensions were serially diluted and plated on MHA plate for viable cell counting. After incubating for 24 hours at 37°C, the number of surviving cells was calculated as described above. The results were calculated as a percentage of cell survival for normally cultured cells without exposure.

The cell viability was also determined using the EZ-Cytox kit (DoGen; Seoul, Republic of Korea) based on the WST reagent, according to the manufacturer's instructions. Briefly, 50-µl aliquots of LB for growth media were transferred into the wells of a 96-well microtiter plate. After plasma exposure, 50 µl aliquots of the recovered bacterial suspensions were then transferred into each well and 10 µl of the WST reagent was added to each well. The microtiter plate was incubated at 37°C for 1-hour and the absorbance was measured at 450 nm using a plate reader (Versamax Microplate Reader, Molecular devices; California, USA). The intensity of the WST metabolic product is proportional to the number of viable cells. The rate of cell reduction at each point was calculated by comparing the absorbance of the samples at each time point with the corresponding samples of the plasma-untreated samples.

2.6. Confocal Laser Scanning Microscopy (CLSM)

For microscopic examination, the biofilms were grown on four-well chamber slides (Nunc NuncTM Lab-TekTM, Thermo Fisher Scientific; Waltham, USA). To maintain biofilm viability, the medium was changed every 24 hours. After 48 hours of incubation, the chambers were gently washed thrice with PBS. The biofilm grown in the chamber slides was exposed to the plasma for 0, 60, 120, and 240 seconds, respectively and were stained with Live/Dead Baclight™ viability kit (Molecular Probes, Life Technologies; Oregon, USA). The viability kit includes two nucleic acid stains, SYTO 9 and propidium iodide (PI). The live bacterial cells were stained by SYTO 9, which appears green-fluorescent. The dead cells were stained by PI, which infiltrates within the damaged cells, resulting in red-fluorescent staining. Each sample was examined by confocal laser scanning microscope (LSM 710, Carl Zeiss; Jena, Germany).

2.7. Statistics

All the experiments were performed using triplicate samples. Statistical Package for the Social Sciences version 20.0 (IBM Corp., Armonk, New York, USA) and R software version 4.0.2 (The R Foundation for Statistical Computing; Vienna, Austria) were used to all the statistical analyses. To determine the bactericidal effect of CAMP for each plasma exposure time, statistical significance was analyzed by T-test and analysis of variance. The *P*-value less than 0.5 ($P \leq 0.05$) was considered statistically significant.

3. Results

3.1. *In vitro* study of the bactericidal effect of CAMP

The effectiveness of CAMP against standard bacterial strain and clinical isolates (n = 30) of *P. aeruginosa* is shown in Figure 1. The bactericidal effect was determined as the percentage of CFU after the exposure of plasma compared in comparison with the untreated control. For *P. aeruginosa* ATCC10145, the survival rates irradiated for 10, 30, 60,120, and 240 seconds were, respectively, 30%, 6%, 5%, 3%, and 0% at 30 W. At 50 W, the survival rate of the standard strain of *P. aeruginosa* was reduced to 8% with 30 seconds of irradiation and completely eradicated after 120 seconds. For the 30 clinical isolates, the survival rates irradiated for 10, 30, 60, and 120 seconds were, respectively, 25%, 10%, 1%, and 0%. At 50 W, the survival rate of clinical isolates of *P. aeruginosa* was reduced to 8% with only 10 seconds of irradiation and completely eradicated after 60 seconds. In the standard ATCC strain and clinical isolates of *P. aeruginosa*, there was a significant difference in the viability of bacteria by plasma power ($P \leq 0.05$) and in the viability of bacteria by plasma exposure time ($P \leq 0.001$). In addition, there was no significant difference in nonionized gas flow as negative control with nonplasma in all strains.

3.2. Bactericidal effect of CAMP on *P. aeruginosa* ATCC10145 in biofilms

Surviving curve of biofilms of *P. aeruginosa* ATCC10145 treated with 30 W plasma, along with the curve with 50 W plasma, is shown in Figure 2. Almost 3 $\log_{10}$ (99.9%) reduction in the number of viable cells of *P. aeruginosa* ATCC10145 biofilms were presented within 240 seconds of 50 W plasma exposure. The error bar was obtained using the average calculated before the log was taken since there was data with the number of cells 0. With 30 W plasma exposure, there was no significant reduction in biofilms.

WST assay was used for evaluate the viability of *P. aeruginosa* biofilms treated with 30 W and 50 W plasma. After the plasma exposure as described previously, absorbance was measured at 450 nm for 0, 10, 30, 45, 60, 120, and 240 seconds. There was no significant reduction in biofilms with 30 W plasma (data not given). With 50 W plasma exposure, the absorbance ratio continuously decreases with plasma exposure time (Figure 3).

Based on Colony count method and WST assay, the percentage of cell reduction values were calculated (Figure 4). The cell reductions of colony count method were 24%, 39.4%, 52.5%, 59.5%, 90.7%, and 99.8% at 50 W, irradiated respectively 10, 30, 45, 60,120, and 240 seconds. The cell reductions of WST assay were 4.1%, 15.4%, 17.7%, 27.4%, 54.9%, and 82.5% at 50 W, irradiated respectively for 10, 30, 45, 60,120, and 240 seconds. The colony count method presents generally higher cell reduction value in comparison with WST assay. In the preliminary experiments, no significant differences were found between nonsonicated and sonicated experiments (data not shown).

3.3. Live/Dead Staining and CLSM

To visualize the viable and nonviable cells, Live/Dead BacLight bacterial viability staining kit was used for staining the *P. aeruginosa* ATCC10145 biofilms. The stained biofilms were then examined by CLSM. The confocal images of the plasma exposed to the biofilms are presented in Figure 5. From Figure 5, it can be observed the differences between viable and nonviable cells in order distinguish within a population of cells. The biofilms that were not exposed to plasma generally showed green color (viable), and red color (nonviable) became dominant as the plasma exposure time increased.

4. Discussion

The aim of the present study was to evaluate the efficacy of CAMP as an antibacterial / antibiofilm. In comparison with other devices, CAMP has the advantage of maintaining the surface temperature below 40°C and lowering power consumption in comparison with the other commercial microwave discharge plasma machines [29,34]. Due to these advantages, we investigated the effectiveness of CAMP as a tool to prevent and control the infections caused by *P. aeruginosa* in dogs.

In this study, CAMP showed significant bactericidal effect against *P. aeruginosa*. The *P. aeruginosa* ATCC10145 was completely eradicated within 240 seconds at 30 W and 120 seconds at 50 W, while clinical isolates achieved complete eradication within 120 seconds at 30 W and 60 seconds at 50 W. This demonstrate that a longer plasma exposure time was required to eradicate the standard ATCC strain compared with clinical isolates of *P. aeruginosa*. A previous study showed that the difference of EPS leads to the different susceptibilities of biofilms [35]. This may explain the strain-specific susceptibility profiles to the nonthermal plasma exposure. In standard strain and clinical isolates of *P. aeruginosa*, there was a significant difference in the viability of bacteria by plasma power ($P \leq 0.05$). In addition, a significant difference was found in the viability of bacteria according to plasma exposure time ($P \leq 0.001$). This indicates that the bactericidal effect of CAMP is dose- and time-dependent.

In the biofilms, a time-dependent reduction in the number of viable cells of *P. aeruginosa* biofilms was achieved within 4 min of 50 W plasma exposure compared with the negative control. With 30 W plasma exposure, there was no significant

reduction in the biofilms. This indicates that more than a certain power of CAMP is required for the eradication of *P. aeruginosa* biofilms. A previous study [35] identified that resistance to the treatment with cold atmospheric plasma (CAP) occurs in *P. aeruginosa* biofilms. Thus, an effective plasma dose should be previously established for the treatment in clinical medicine. The cell viability was also determined by WST assay, which was performed alongside with standard colony count enumeration post-exposure. The survival fractions of the standard colony count at shorter exposure times are greater than those reported by the WST assay. Despite low colony counts, the WST assay revealed some residual metabolic activity after the CAMP exposure. This finding probably indicates that bacterial cells might enter a viable-but-nonculturable (VBNC) stage, which was also studied in a previous study [37]. The VBNC state is a survival mechanism of the bacteria against the environmental stress conditions [38, 39]. The Live/Dead BacLight bacterial viability staining kit was used to visualize and evaluate the bactericidal effect of *P. aeruginosa* biofilms and to demonstrate the biofilm cells rendered nonviable by CAMP. The confocal microscopy examination supported the evidence of the time-dependent bactericidal effect by CAMP.

The CAMP showed remarkable bactericidal effects against biofilm and planktonic phenotypes of *P. aeruginosa*. The CAMP susceptibilities were identified in different phenotypes (planktonic / biofilm), and the bacterial eradication was related to the exposure time. Generally, longer plasma exposure times were required to eradicate biofilms compared with planktonic bacteria of *P. aeruginosa*. This difference in the eradication time indicates the difference in susceptibility to plasma exposure

between planktonic and biofilm bacteria, which is in agreement with a previous study [36]. Moreover, another study [40] supports that the biofilm associates phenotypic resistance to nonthermal argon plasma.

In general, nonthermal argon plasma is more effective against Gram-negative than Gram-positive bacteria [39]. Therefore, plasma treatment may be especially useful for the treatment of the bacteria *P. aeruginosa* investigated in this study, which is an important Gram-negative bacterium that causes bacterial otitis externa and pyoderma in dogs. The CAMP used in this study maintains the surface temperature low and the device has a pencil-like shape and a comfortable size to grip. This is especially useful for the application in veterinary medicine, particularly for exposed skin. CAMP also could be used in cases of otitis externa in dogs. Since there are concerns about the safety of the tympanic membrane due to the gas pressure and the noise, further experiments are required for clinical application in veterinary medicine.

A limitation of this study is that Gram-negative bacteria other than *P. aeruginosa* that can cause pathogenic clinical causes of otitis externa and pyoderma in dogs were not evaluated. Owing to the experimental limitations, only the biofilm of the standard strain was tested and that of the clinical strains was not evaluated. In the examination by CLSM, the intensity of fluorescence was not measured, and it was purely additional data for visualizing the cells. Further large-scale studies on cytotoxicity or biofilm assays using clinical *Pseudomonas* isolates are required. Also, the mechanisms of bacterial eradication by plasma, their interactions with the biological matter and its individual biomacromolecules related to these processes are not identified in this study. The biocompatibility and safety issues in the skin barrier will

be necessary before the successful application of this approach for the bacterial eradication in the *in vivo* studies. However, despite of these limitations, this study provides the basis for the future clinical examinations of CAMP in veterinary medicine.

5. Conclusions

In conclusion, the present study describes that CAMP has antibacterial / antibiofilm effects in vitro against *P. aeruginosa*. The clinical studies may be required to confirm whether the *in vitro* results are applicable to actual clinical situations and the safety of the *in vivo* efficacy for the real clinical cases in veterinary medicine.

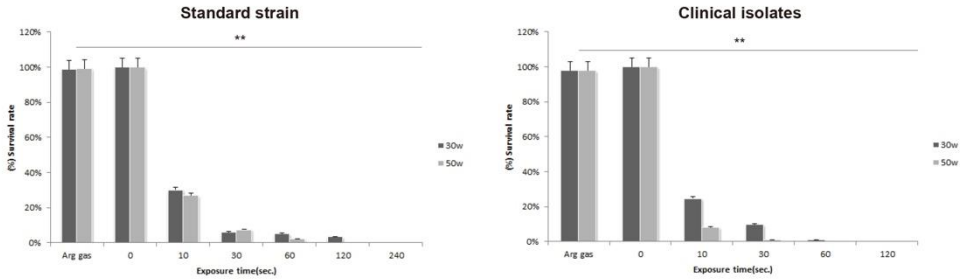

Figure 1. Survival rates of the CAMP treated standard strain and clinical isolates of *P. aeruginosa*.

The percentage of colonies formed after 30 W and 50 W plasma treatment relative to untreated control plates is shown as percentage survival. Argon gas flow for 60 seconds was used to control.

**: $P \leq 0.001$, error bar: $\pm$ SD (Standard deviation) for triplicate analysis.

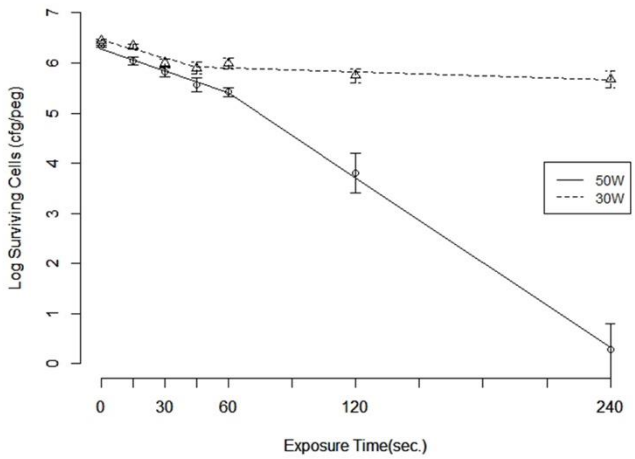

Figure 2. Survival curves of biofilms treated with 30 W and 50 W plasma.

P. aeruginosa biofilms grown on CBD were exposed to both 30 W and 50 W plasma for 10, 30, 45, 60, 120, 240 seconds. The number of biofilms surviving cells in each sample was calculated by colony count method.

Error bar: ± SD (Standard deviation) for triplicate analysis.

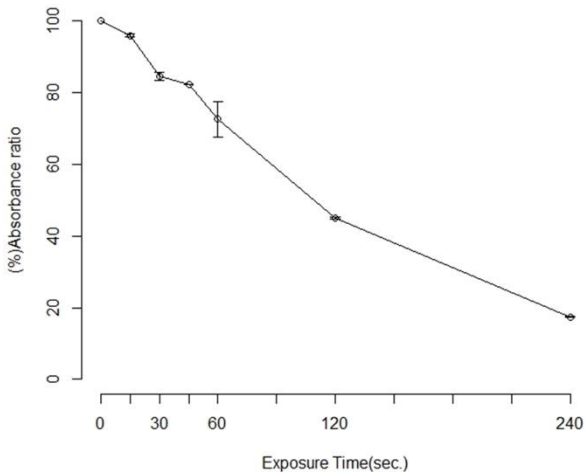

Figure 3. Absorbance ratio of WST assay product treated with 50 W plasma.

P. aeruginosa biofilms grown on CBD were exposed to 50 W plasma for 10, 30, 45, 60, 120, 240 seconds. After plasma exposure, the recovered bacterial suspensions were then transferred into each well and the WST reagent was added to each well. The microtiter plate was incubated and the absorbance was measured at 450 nm. The intensity of the WST metabolic product is proportional to the number of viable cells.

Error bar: ± SD (Standard deviation) for triplicate analysis.

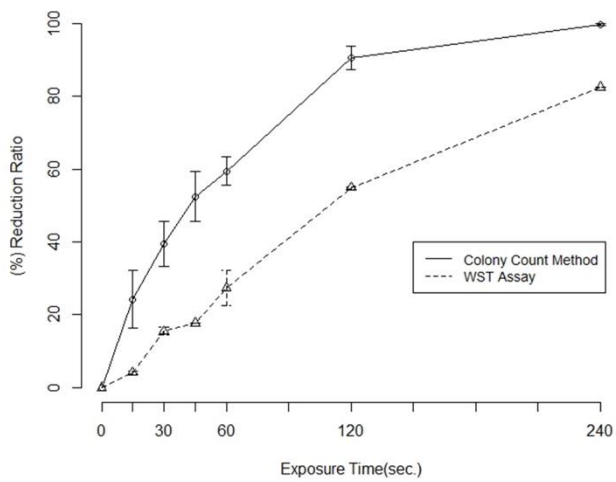

Figure 4. Percentage of cell reduction curves of colony count method and WST assay at 50 W plasma exposure.

Percentage of cell reduction curves of *P. aeruginosa* biofilms were calculated at 50 W plasma exposure. The solid line is based on the colony count method and the dotted line is based on the WST assay.

Error bar: ± SD (Standard deviation) for triplicate analysis.

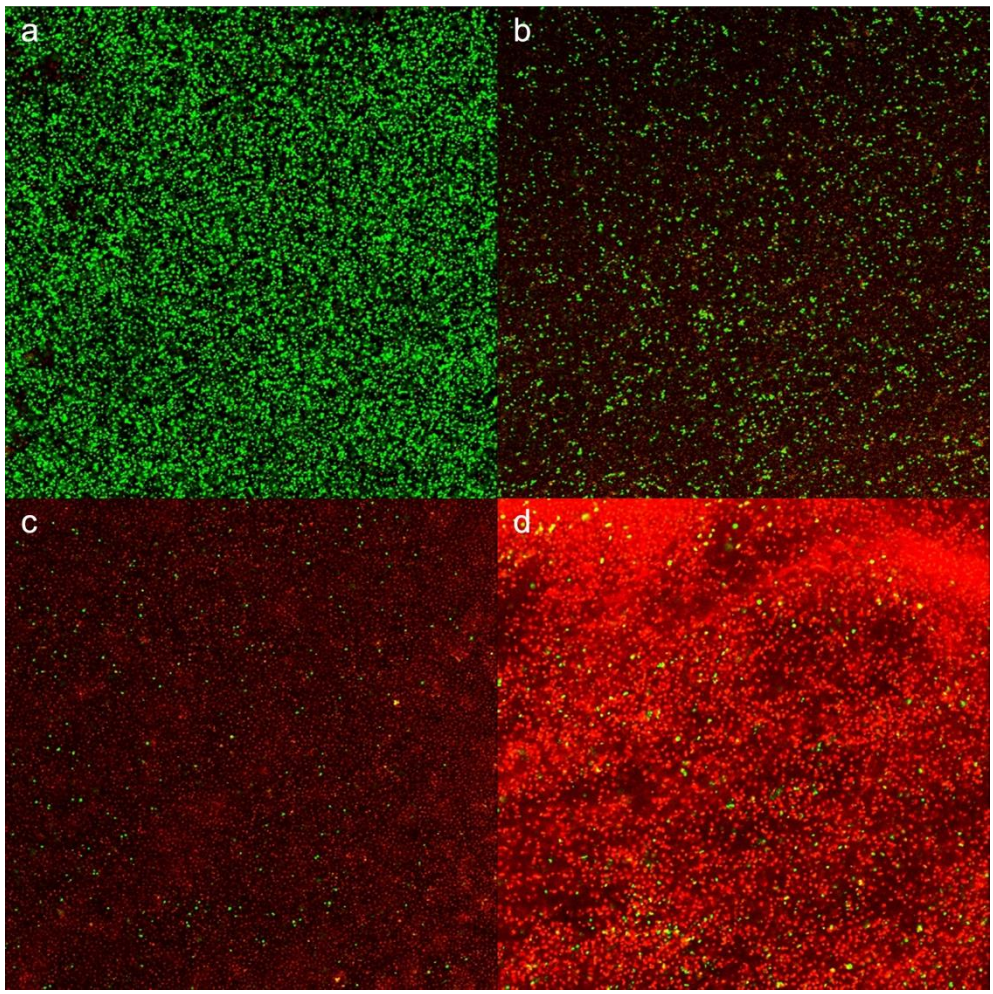

Figure 5. CLSM images of the CAMP treated biofilms.

Confocal laser scanning micrographs of *P. aeruginosa* biofilms grown on four-well chamber slides, exposed to the 50 W plasma for 0 second (a), 60 seconds (b), 120 seconds (c), and 240 seconds (d). After plasma exposure, the biofilms were stained with Live/Dead Baclight™ viability kit. Green color means surviving cells while red color means dead cells. Magnification power is 400x.

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국문초록

개의 피부 및 귀의 감염증을 일으키는 녹농균 및 바이오필름에 대한 Cold atmospheric microwave plasma의 항균 효과

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녹농균은 개의 피부와 귀에서 만성적인 염증을 일으키는 기회감염균이다. 항생제 처치에 대한 효능에 있어서 다양한 방해 요소들이 있으며, 바이오필름의 형성으로 인하여 항생제 내성이 야기될 수 있고 따라서 새로운 치료법이 필요하다. Cold atmospheric microwave plasma (CAMP)는 물리적으로 세포벽을 파괴하고 DNA에 손상을 야기해 높은 항균 효과를 가진 것으로 알려져있다.

이 논문은 CAMP의 녹농균과 바이오필름에 대한 항균 효과를 평가하였다.

CAMP의 녹농균에 대한 항균 효과는 표준 균주와 임상 균주 (n = 30)를 대상으로 시험되었고, colony count method를 사용하여 평가하였다. 이 논문은 또한 colony count method, water-soluble tetrazolium salt (WST) assay, confocal laser scanning microscopy (CLSM)를 이용하여 바이오필름에 대한 CAMP의 효능을 평가하였다.

녹농균 (표준 균주와 임상 균주)은 50 watts (W)에서 120초안에 완전히 사멸되었고, 임상 균주는 표준 균주보다 완벽한 사멸에 더 짧은 시간이 소요되었다. 바이오필름의 경우, 살아있는 세포는 50 W에서 4분 안에 거의 3 $\log_{10}$ (99.9%) 감소하였으며, 30 W에서는 유의적인 감소가 확인되지 않았다.

결론적으로, CAMP는 녹농균과 바이오필름에 대해 항균 효과가 있다는 것을 시사한다. 하지만, 임상적인 적용을 위해서는 개의 피부와 귀에서 CAMP 적용 시에 안정성에 대해 추가적인 연구가 필요하다.

주요어: 개, 녹농균, 마이크로웨이브 플라즈마, 바이오필름, 항균효과

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