A THESIS FOR THE DEGREE OF MASTER

Antimicrobial effect of cold atmospheric microwave plasma on bacteria causing canine skin and ear infections

Cold atmospheric microwave plasma의 개의 피부 및 귀의 감염증을 일으키는 세균에 대한 항균효과

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진 희 정

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Antimicrobial effect of cold atmospheric microwave plasma on bacteria causing canine skin and ear infections

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Abstract

Cold atmospheric plasma (CAP) is a new generation medical therapeutic option for bacterial infections. CAP causes physical cell wall rupture and DNA damage, therefore making it highly useful in the treatment of various conditions such as skin infections. CAP is divided into several types according to the production method. Among them, microwave discharge plasma, or also called cold atmospheric microwave plasma (CAMP), is reasonable for generating plasma than other types of plasma device. The purpose of this study was to evaluate antimicrobial activity of CAMP against major strains in canine skin infections and the difference in antimicrobial activity between antibiotic resistant and antibiotic susceptible strains of *Staphylococcus pseudintermedius* was evaluated. American Type Culture Collection (ATCC) strains (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*) and clinical isolates identified as methicillin-resistant *S. pseudintermedius* (MRSP, n = 27) and methicillin-susceptible *S. pseudintermedius* (MSSP, n = 13) were exposed to the CAMP for 10, 30, and 60 seconds. Afterwards, bacterial survival rates were confirmed. Gram-negative bacteria (*P. aeruginosa* and *E. coli*) were more susceptible than gram-positive bacteria (*S. aureus* and *S. pseudintermedius*) at the same time of CAMP exposure. Despite only 60 seconds of exposure, the gram-negative bacteria were completely killed. In *S. pseudintermedius* isolates, CAMP exposure had similar antibacterial effects regardless of antibiotic resistance.

CAMP has sufficient antimicrobial activity against major bacterial strains that cause pyoderma and otitis externa in dogs, and may be an alternative therapeutic option for *S. pseudintermedius* skin infections, for which antibiotics are often ineffective due to its antimicrobial resistance in clinical veterinary medicine.

Key words : CAMP, antimicrobial activity, antibiotic-resistant, S.pseudintermedius, canine, pyoderma, otitis externa

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1. Introduction

The major bacteria that causes canine pyoderma and otitis are *Staphylococcus spp.* (Gram-positive bacteria) and *Escherichia coli*, *Pseudomonas aeruginosa*, and *Proteus spp.* (Gram-negative bacteria) [1, 2]. Of these, *Staphylococcus pseudintermedius* causes the most skin infections in dogs; in particular, methicillin-resistant *S. pseudintermedius* (MRSP) causes difficulties in using appropriate antibiotics in the treatment of infectious diseases [3, 4]. Due to these restrictions, alternative therapeutic options have been explored, particularly cold atmospheric plasma (CAP) [5 - 8].

Plasma is a substance generated when gas flow, such as compressed air, argon, helium, or nitrogen, passes through an electric field and becomes ionized. Non-thermal plasma, which is generated at room temperature, is composed of radicals, and charged particles, except for neutral electrons that are generated at high temperatures, and contain ultraviolet photon [9, 10]. Non-thermal plasma, produced at low pressures, is convenient, and economically effective. This is used in human medicine and is called CAP. It is effective in removing microorganisms [10 - 12]. Through the action of reactive oxygen species and reactive nitrogen species released during plasma generation, CAP damages the bacterial cell wall which causes high oxidative stress and cell membrane rupture. Its reactions to bacterial DNA cause bacterial death [5, 11]. In addition, CAP stimulates several cytokine factors

to promote anti-inflammatory action, tissue healing, and microcirculation stimulation [13, 14]. In human medicine, various dermatological applications are being studied using CAP [13, 15, 16]. CAP is divided into several types according to the production method. Among them, microwave discharge plasma, or cold atmospheric microwave plasma (CAMP), is capable of generating plasma at a lower temperatures and at a more reasonable price [5, 8, 10].

This study aimed to evaluate the *in vitro* bactericidal activity of a CAMP type device (IonMedical inc., Seongnam, Republic of Korea) developed for use in veterinary medicine and the degree of efficacy with regards to methicillin-resistance status of *S. pseudintermedius*.

2. Materials and Methods

2.1. Bacterial strains

Field strains of *S. pseudintermedius* were obtained from the skin of 24 dogs with pyoderma and ears of 16 dogs with otitis externa at the Veterinary Medical Teaching Hospital of Seoul National University in Korea. Using the Vitek 2 system (bioMérieux; Hazelwood, MO, USA), we identified the isolates as *S. pseudintermedius*. The strains were stored in Tryptic Soy Broth (Thermo Fisher Scientific Oxoid Ltd., Basingstoke, UK) with 50% glycerol at −70°C for further examination. We defined methicillin resistance in *S. pseudintermedius* using the 1 μg oxacillin disk (Thermo Fisher Scientific Oxoid Ltd., Basingstoke, UK) agar diffusion method [17]. After 24 hours of incubation at 37°C, the inhibition zones were determined and methicillin resistance was confirmed according to the Clinical Laboratory Standards Institute (CLSI) guidelines for animals (VET08) [18]. For confirming the identification of methicillin resistance, polymerase chain reaction (PCR) using the targeting *mecA* gene was performed for molecular identification of *S. pseudintermedius* isolates [19]. As standard strains, this study also used other pathogenic bacterial strains purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA): *P. aeruginosa* (ATCC 1750), *E. coli* (ATCC 29513), and *S. aureus* (ATCC 29154).

2.2. Antimicrobial susceptibility testing

To determine the resistance of MRSP and methicillin-susceptible *S. pseudintermedius* (MSSP) strains to antimicrobial agents, each strain was subjected to an antibiotic susceptibility test with the agar disk diffusion method on Mueller–Hinton Agar (MHA; BD Diagnostic Systems, Sparks, MD, USA). The antimicrobial agents used (Thermo Fisher Scientific Oxoid Ltd., Basingstoke, UK) were (in μg/disk): penicillin (10); amoxicillin–clavulanic acid combination (30); amikacin (30); chloramphenicol (30); ciprofloxacin (5); clindamycin (2); erythromycin (15); gentamicin (10); norfloxacin (10); tetracycline (30); minocycline (30); trimethoprim-sulfamethoxazole (25); and rifampicin (5). After 24 hours of incubation at 37°C, the diameters of the inhibition zones around the discs was measured and antibiotic resistance was interpreted according to the VET08 [18]. And isolated strains were divided into sensitive or resistant to the drug. Intermediate susceptibility was regarded as resistant.

2.3. Cold atmospheric microwave plasma

In all experiments, CAMP was used with non-ionized argon gas. The plasma

used in the experiment was generated in the device shown in Figure 1. After microwave energy (30 - 50W, 2450 MHz) entered the tube, the argon gas existing as a carrier gas was ionized in the tube. As a result, plasma was generated and emitted through the nozzle. The rate of argon gas flow into the tube could be adjusted 10 - 20 L/min, and the microwave energy could be adjusted to power of 30 or 50 W. The amount of plasma emitted changed according to the power consumed and gas velocity. The length of the plasma jet emitted was also affected, and was in the range of 3 - 15 mm. The surface temperature of plasma was maintained below 40°C.

2.4. Plasma treatment and determination of antimicrobial effect using colony forming units

Each isolate and standard bacteria strain in Mueller–Hinton Broth (MHB; BD Diagnostic Systems, Sparks, MD, USA) was grown in a shaking incubator at 37°C for 24 hours at 120 rpm, and then it was diluted to 0.5 Mcfarland. The final inoculum concentration was $1 - 5 \times 10^4$ colony-forming units (CFU)/mL and 10 µL of bacterial suspension was inoculated into MHA made in six-well cell culture plates (each area, 9.6 cm^2). After inoculation, the inoculum was streaked on the plate with an inoculation loop and needle. The rate of gas flow out of the CAMP was set at 15 L/min, the microwave energy at 2450 MHz, 3.5 kV, and power consumption at 30 or 50

W. The distance from the plasma surface to the plate was the length of the jet, and the plasma took 10 seconds to pass each agar plate surface. The plates were exposed to the plasma for 10, 30, and 60 seconds. Flow of non-ionized argon gas and unexposed plasma were used for control condition. After exposure of CAMP, an additional 24 - 48 hours of growth at 37°C, the bacterial survival rate was measured after confirming the number of CFU. This study conducted a minimum of three independent tests to measure the survival rates.

2.5. Statistical methods

GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical comparisons in this study. Comparison of differences in the survival rates according to plasma exposure time, one-way ANOVA was performed. P-values of less than 0.05 were considered significant.

3. Results

3.1. Bacterial isolates and antimicrobial susceptibility tests

Antimicrobial susceptibility tests were conducted with 40 clinical *S. pseudintermedius* isolates. According to the presence of the *mecA* gene and oxacillin resistance, 27 (67.5%) of the strains were MRSP and 13 (32.5%) were MSSP. The agar disk diffusion method was used to characterize the antimicrobial resistance patterns of the isolates. The resistance profiles of MRSP and MSSP strains to the antimicrobial agents are shown in Table 1. Three isolates (7.5%) were susceptible to all antibiotics, and one isolate (2.5%) was resistant to only one antibiotic. All 36 other isolates (90%) were confirmed to have at least three antibiotic multidrug resistance classes. Multidrug resistance was detected in 22 (100%) MRSP strains and 13 (76.5%) MSSP strains, and high resistance to penicillin and tetracycline was identified among both strains.

3.2. Bactericidal effect of cold atmospheric microwave plasma *in vitro*

First, standard bacterial strains were evaluated for the effectiveness of CAMP (Figure 2). The plasma intensity was tested at two levels, 30 and 50 W. The

negative control without plasma exposure was assumed to have a survival rate of 100%, for convenience. At 30 W, the survival rates of *P. aeruginosa* when irradiated for 10, 30, and 60 seconds, were 22.9%, 8.1%, and 0.7%, respectively, and those of *E.coli* were 30.1%, 10%, and 0.8%, respectively; However, the survival rates of *S. aureus* were 65%, 28.1%, and 9.9%, respectively, which were similar to those of the methicillin-resistant standard strain. At 50 W, the survival rate of *P. aeruginosa* was reduced to 5.7% with only 10 seconds of irradiation, and the organism was completely killed after 60 seconds. All *E. coli* organisms were also confirmed to have been completely killed after irradiation of 60 seconds. In the case of *S. aureus*, the survival rate was 50.3% when exposed for 10 seconds, and the survival rate was 2.8% after 60 seconds of irradiation; thus, it was not completely killed. When additional 120 seconds were given, the bacteria was confirmed to have completely died. In all strains, there was no significant difference in non-ionized gas flow as positive control with non-plasma.

Figure 3 shows the comparison of the antimicrobial activity between MRSP and MSSP, which were clinical isolates. For both MRSP and MSSP, the survival rates decreased as plasma exposure time increased. Among the MRSP isolates, survival rates were confirmed to be 51.96% ± 37.37% after 10 seconds of exposure, 21.40% ± 16.10% after 30 seconds of exposure, and 4.79% ± 4.79% after 60 seconds of exposure. In MSSP isolates, the survival rates were confirmed to be 44.59% ± 11.26% after 10 seconds of exposure,

20.33% ± 6.10% after 30 seconds of exposure, and 6.08% ± 2.64% after 60 seconds of exposure ($P < 0.005$). CAMP exposure thus had a bactericidal effect, which occurred regardless of antimicrobial resistance.

4. Discussion

Among the mechanical methods that generate plasma, those most currently commercialized in the medical industry are the dielectric barrier discharge (DBD) system, in which gas flows between two electrodes and produces voltage to instantly generate plasma. Once generated the temperature of the gas is instantaneously raised to 150°C. Also, the power consumption is high because of the high voltage required [8, 20]. The microwave discharge system that generates CAP, which is called CAMP, has an advantage of being easy to use because no electrode is necessary to generate plasma and particles are formed in a like torch manner [7, 8, 10]. Moreover, it promotes bacterial death more quickly because the plasma exposes on bacterial cell wall surface directly. The device used in this study has the maximum efficiency in generating plasma with a surface temperature below 40°C and its power consumption (only 50 W) is lower than that of other commercial microwave discharge plasma machines [8].

Previous studies confirmed that gram-negative bacteria are more susceptible to CAP than gram-positive bacteria [6, 8, 12]. CAMP generated in this study exhibited the same antibacterial effect. In this study, when exposed to CAMP for 1 minute, gram-negative bacteria strains (*P. aeruginosa* and *E.coli*) were killed even at a low power of 30 W, but the gram-positive bacteria (*S. aureus* and *S. pseudintermedius*) showed survival

rates of 9.05% at 30 W and 3.44% at 50 W. This result was attributable to the difference in thickness of the bacterial cell wall, as observed in previous studies [12, 21]. The peptidoglycan in the Gram-positive bacteria was approximately 15–80 nm thickness, whereas that in the Gram-negative bacteria was 1.5–15 nm thickness [22]. Considering the varied thickness of these peptidoglycans, the degree of cell lysis caused by plasma exposure also varied, indicating the result of differences in depolarization of the cell membrane and integrity [12].

In the case of *S. pseudintermedius*, the experiment was performed by dividing it into methicillin-resistant and methicillin-susceptible strains. When exposed to CAMP, the two types of strains showed no significant differences. It showed the average of survival rate on both strains; 47.12% when treated for 10 seconds, 18.61% when treated for 30 seconds, and 4.71% when treated for 60 seconds. And also both of MRSP and MSSP strains did not show a significant difference compared with the untreated group which treated with argon gas only for 60 seconds. The bactericidal effect is not attributable to temperature and argon gas was also considered to have no antibacterial effect, because the temperature of the surface generated during plasma exposure did not exceed 40°C. Plasma physically causes bacterial cell lysis; therefore, it could be believed that the plasma shows the same antibacterial effect even in highly antibiotic-resistant strains.

This study is different from other studies showing decontamination or antibacterial effect of previous plasma because the study evaluated the antibiotic-resistant strain of *S. pseudintermedius* that was clinically isolated in dogs' skin infections (pyoderma and otitis externa). In terms of the energy use and exposure time, the CAMP used in this study was more effective than that used for evaluating the results of the antimicrobial effect on *Staphylococcus spp.* in other types of plasma machines. Moreover, when compared to other microwave type plasma machines, the energy use is generally > 80 W; however, it is a very efficient method because it has similar antimicrobial effects even at 30 W [8, 21, 23]. The CAMP used in this study, the surface temperature is low and the device where plasma comes out is pen type; therefore, it might be more applicable to veterinary medicine, especially exposed skin. However, in the case of otitis externa, the gas velocity required when CAMP is generated creates pressure and some noise. Therefore, otologic safety test is necessary because there is a possibility of damage to the tympanic membrane or other adverse effects on the auditory system. Additional experiments are necessary to determine its suitability for clinical use. When applied to tissues, it not only is antiseptic but also enables tissue regeneration. Therefore, the effectiveness of the plasma–cell and plasma–tissue interactions must be evaluated [24]. Furthermore, for actual clinical application, an effective plasma dose should be established, skin irritation should be assessed, as well as skin hydration,

trans-epidermal water loss should be evaluated as to whether it affects the skin barrier function *in vivo*.

There are limitations of the current study that various bacterial agents other than *S. pseudintermedius* can cause skin diseases in dogs, but clinical samples of other bacteria were not evaluated. Furthermore, the clonal lineage in the isolates of *S. pseudintermedius* has not been confirmed; therefore, additional experiments to confirm genotyping are necessary to determine whether a specific lineage is more susceptible. And fungi such as dermatophytes and *Malassezia spp.*, which could also cause skin infections, were not identified. Despite these limitations, this study provides basic study confirming the antimicrobial activity of CAMP against clinical isolates in veterinary medicine. Therefore, based on this experiments, further trials are expected on other bacterial skin infections or other skin infections.

5. Conclusion

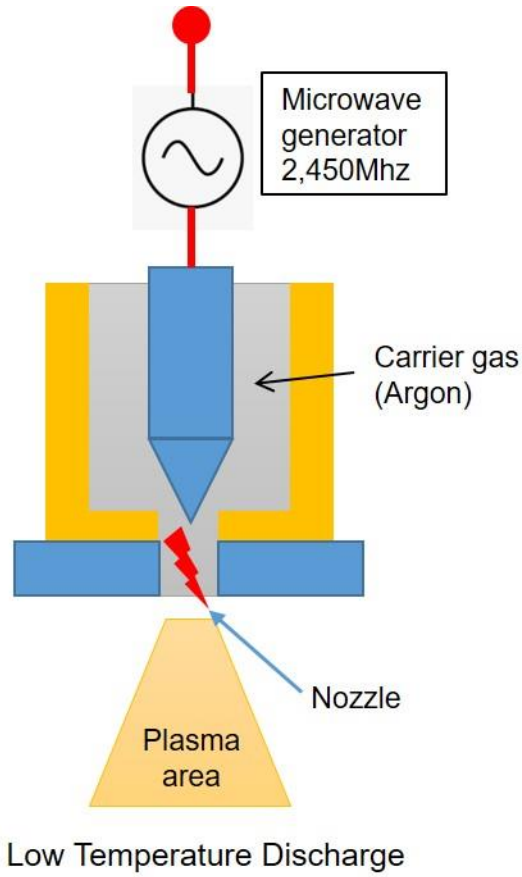
In conclusion, the present study has demonstrated that CAMP has antibacterial effects *in vitro* against antimicrobial-resistant strains of *S. pseudintermedius*. Clinical studies are necessary to assess the safety and efficacy of *in vivo* using CAMP against various infections in veterinary medicine.

Table 1. Antimicrobial resistance profiles of MRSP and MSSP isolates from dogs with canine pyoderma and otitis externa.

		Number of resistant strains (%)								
Isolates	*n*	P	OX	GEN	ERY	CLI	TET	SXT	CHL	RI
MRSP	27	27 (100)	27 (100)	24 (88.9)	22 (81.5)	23 (85.2)	25 (92.6)	20 (74.1)	15 (55.6)	2 (7.4)
MSSP	13	9 (69.2)	0	8 (61.5)	8 (61.5)	8 (61.5)	9 (69.2)	4 (30.8)	6 (46.2)	0

Abbreviation : P, penicillin; OX, oxacillin; GEN, gentamicin; ERY, erythromycin; DA, clindamycin; TE, tetracycline; SXT, trimethoprim-sulfamexothazole; CHL, chloramphenicol; and RI, rifampicin.

Figure 1. Device schematic diagram that generates cold atmospheric microwave plasma (CAMP).



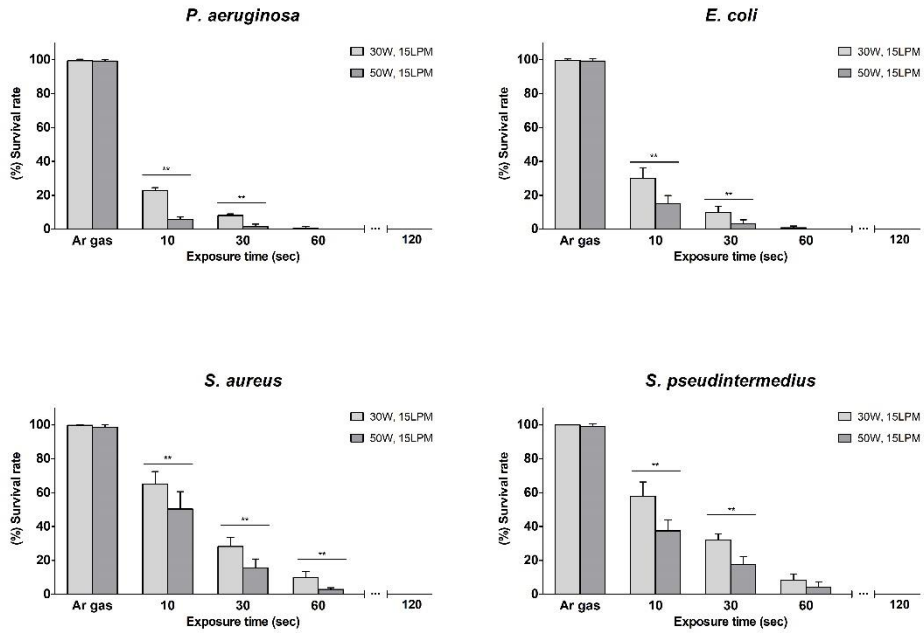


Figure 2. CAMP treatment of bacterial strains.

Each bacteria strains grown on MHA for 24~48h and CAMP exposed for 10, 30, and 60 seconds with argon feeding gas at 15 L/min and it was treated with 30 W and 50W power, respectively. Argon gas flow for 60 seconds was used to positive control group. P. aeruginosa was ATCC1750, E. coli was ATCC 29513, S. aureus was ATCC 29154, and S. pseudintermedius was one of clinical isolation at MSSP strains.

**: $P < 0.005$, error bar: ± SD (Standard deviation) for triplicate analysis

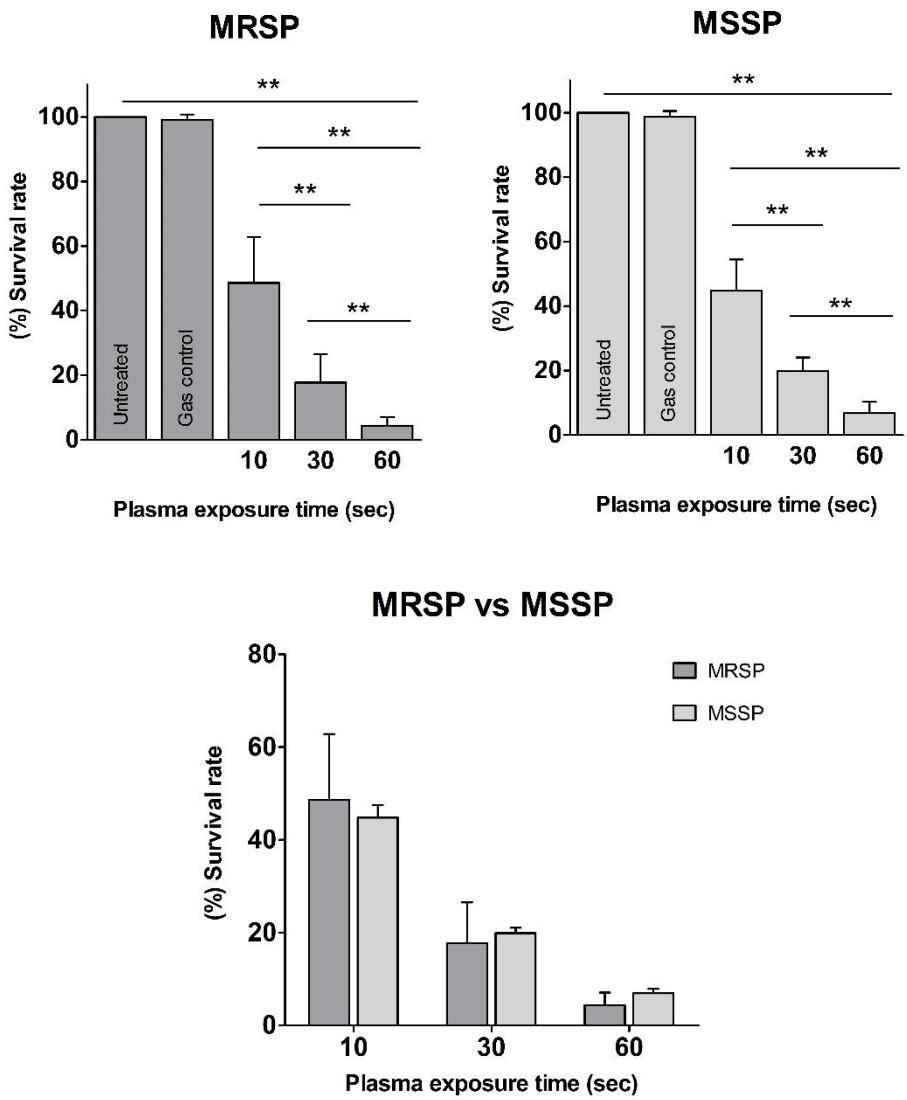


Figure 3. CAMP exposure to MRSP and MSSP.

MRSP and MSSP were grown on MHA for 24~48h and CAMP exposed for 10, 30, 60 seconds with argon feeding gas at 15 L/min, and 50 W power consumption. Untreated control group set to 100% and just argon gas flow for 60 seconds was used to positive control group.

**: $P < 0.005$, error bar: ± SD (Standard deviation) for triplicate analysis

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국문초록

Cold atmospheric microwave plasma의 개의 피부 및 귀의 감염증을 일으키는 세균에 대한 항균효과

지도교수: 황철용

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Cold atmospheric plasma (CAP)는 인의에서 세균 감염 등에서 치료적 방법으로 채택되고 있는 새로운 세대의 기술이다. 플라즈마는 물리적으로 세포벽 및 세포막의 파열과 DNA 손상을 입혀 피부 감염과 같은 분야에서 효율적인 치료 대안이 될 수 있다. 플라즈마를 생성하는 방식에 따라 구분이 되게 되는데 이 중 cold atmospheric microwave plasma (CAMP)는 경제적이어서 효율적인 방법으로 고려된다. 임상 수의학 분야에서 다수의

항생제에 내성을 가지는 다재내성균에 대한 보고가 늘어나고 있으며 다재내성균들은 항생제를 이용한 치료에 큰 걸림돌이다. 본 학위논문의 목적은 개의 피부에서 감염증을 일으키는 균주와 실제 환견에서 분리된 40개의 *Staphylococcus pseudintermedius* 의 내성도에 따른 CAMP의 항균효과를 검증하는데 있다. 실험에는 개의 피부 감염증을 일으키는 균주로 American Type Culture Collection (ATCC) 표준균주 중 *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*를 이용하였으며 플라즈마 생성시 소비되는 에너지 크기(30 W, 50 W)와 노출 시간(10, 30, 60초) 에 따라 항균효과를 평가하였다. 또한 농피증과 외이염이 있는 개에서 분리된 *S. pseudintermedius* 균주들을 대상으로 디스크 확산법 (Disc diffusion test) 과 중합효소 연쇄 반응(Polymerase chain reaction)를 통해서 내성균임을 확인한 뒤, 노출 시간(10, 30, 60초)에 따른 CAMP의 항균효과를 생존률에 따라 평가하였다.

그람 음성균에서 그람 양성균에 비해 CAMP의 같은 노출시간을 가졌을 때 항균효과가 더 좋았다. 60초 간 노출시켰을 때에 소비 에너지 크기에 상관없이 그람 음성균은 생존률이 0% 임을 확인할 수 있었으며 그람 양성균의 경우 120초 이상 노출해야 생존률이

0%에 유사하게 측정되었다. 전체 40개의 *S. pseudintermedius* 분리주에 대해서는 27개 (67.5%) 메치실린 내성이었으며 총 36개 (90%)는 다재내성균주로 확인되었다. 균의 항생제 내성과 관계없이 CAMP의 노출시간에 따른 항균 효과는 유사한 정도로 확인되었다.

이 결과는 CAMP가 개의 농피증, 외이염을 일으키는 주요 세균에 대해 충분한 항균효과를 가지는 것으로 평가되어진다. 특히 임상 수의학 분야에서 항생제 사용이 효과적이지 못한 항생제 내성균에 의한 피부 감염증 치료에 새로운 치료 대안으로 사용할 수 있음을 시사한다.

주요어: 개; 항생제 내성; 마이크로웨이브 플라즈마; 항균효과; 포도상 구균

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