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In vitro antifungal activity of cold atmospheric microwave plasma and synergistic activity against *Malassezia pachydermatis* when combined with chlorhexidine gluconate

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Abstract

Background: The antifungal efficacy of cold atmospheric microwave plasma (CAMP) against *Malassezia pachydermatis* has not been to be evaluated.

Objective: To examine the antifungal effects of CAMP against *M. pachydermatis* and its synergistic effects with chlorhexidine gluconate (CHX).

Methods: A *M. pachydermatis* isolate was collected from a dog with otitis externa and *Malassezia* dermatitis at the Seoul National University Veterinary Medical Teaching Hospital. The antifungal effect was determined by applying CAMP to a *M. pachydermatis* isolate that was incubated for 3 days at 37°C. After 1, 2, 3 and 5 min of application, the efficacy of the plasma treatment was determined according to the number of colony forming units (CFUs). A mixture consisting of inoculum and CHX was applied to evaluate the synergistic effect of the plasma treatment in the same way.

Results: The application of CAMP showed significant antifungal effects against *M. pachydermatis*. The antifungal effect of CAMP was enhanced by an increased exposure time and output power. The application of CAMP with 0.02% and 0.2% CHX resulted in lower survival rates against *M. pachydermatis* when compared with its sole application at 1 or 2 min.

Conclusions: The study findings demonstrate that CAMP has a potential as a new antifungal option for *M. pachydermatis* and has synergistic antifungal effects with CHX in vitro. Clinical applications for CAMP are necessary to assess the antifungal efficacy for patients.

KEYWORDS
antifungal effects, chlorhexidine gluconate, cold atmospheric microwave plasma, *Malassezia pachydermatis*, synergism

1 | INTRODUCTION

Malassezia pachydermatis is classified as a lipophilic, less lipid dependent, nonmycelial saprophytic yeast that is commonly found on the skin, in ear canals and on mucosal surfaces including the lips, chin, anal sacs in the rectum and vagina (Bond et al., 2020; Chen et al., 2002). *M. pachydermatis* is a commensal yeast that commonly colonizes the superficial layers of the epidermis; it is the predominant fungi in canine skin (Guillot & Bond, 1999). *M. pachydermatis* is one of the most frequently isolated fungi that causes skin disease and otitis externa in dogs (Cafarchia et al., 2012; Chiavassa et al., 2014). The most common clinical sign of *Malassezia* dermatitis is erythema, commonly with seborrhoea, hyperkeratinization, pruritus, hyperpigmentation, lichenification, malodor and otitis externa (Bond et al., 2020). *Malassezia* dermatitis is common in atopic dogs and this can worsen clinical signs (Chen et al., 2002). *M. pachydermatis* has a thick (up to 0.25 μm) and multilamellar cell wall, similar to other *Malassezia* yeasts (Guillot & Bond, 1999). Although the components of *M. pachydermatis* have been poorly studied, polysaccharides were found to contribute to the rigidity of the cell wall in *Malassezia* spp. (Stalhberger et al., 2014). *M. pachydermatis* has crossed electron-translucent bands with helicoidal ridges which withstands cell changes, osmotic pressure, and other environmental changes (Stalhberger et al., 2014).

Plasmas represent the fourth state of matter (the first three being solid, liquid and gas) and is generated through ionized gas by electric fields (Cvelbar et al., 2006; Daeschlein et al., 2012; Morfill et al., 2009). In general, heat is generated by plasma at high temperatures. However, cold atmospheric microwave plasma (CAMP) generates heat below 40°C and devices using CAMP are applicable in medical practice/setting (Lackmann et al., 2013). One of the main purposes of these applications is to kill parasites, bacteria, fungi and viruses; other purposes include blood coagulation, sterilization and wound healing (Cvelbar et al., 2006; Daeschlein et al., 2011; Weltmann et al., 2010). Several studies have assessed the bactericidal and fungicidal efficacies of various types of plasma (Cvelbar et al., 2006; Daeschlein et al., 2011; Weltmann et al., 2010). One reasonable hypothesis states that plasma can destroy the membrane that derives its tensile strength from electrostatic forces that increase as charge accumulates (Daeschlein et al., 2012; Laroussi et al., 2003). CAMP also decreases bacterial and fungal growth in a previous study (Daeschlein et al., 2011). Therefore, CAMP has potential as a new bactericidal and fungicidal agent. Unlike studies on the soil-borne fungal pathogen *Fusarium oxysporum* and the normal human fungus *Candida albicans*, no in vitro studies have yet been conducted related to any plasma treatment against *M. pachydermatis* (Alonso-Monge et al., 2009; Panngom et al., 2014). Chlorhexidine gluconate (CHX), a bisbiguanide antiseptic, has shown antimicrobial effect against many fungi and enveloped viruses and remarkable antifungal effects in shampoo with antifungals such as miconazole (Bond et al., 1995; Lloyd & Lamport, 1999; Oh et al., 2012).

The purpose of this study was to evaluate the antifungal potential of CAMP against *M. pachydermatis* at each output power and length of time. We also evaluated synergism with CHX at three concentrations. It is hypothesized that there will be a synergistic effect of CAMP and CHX.

2 | MATERIALS AND METHODS

2.1 | malassezia pachydermatis isolate

A *M. pachydermatis* isolate was collected from a dog with otitis externa and *Malassezia* dermatitis at the XXXXX. Ear discharge was collected using a sterile cotton swab and transported by a transport medium to the laboratory. The isolate has been identified as *M. pachydermatis* in a previous molecular study using a sequence analysis of the internal transcribed spacer 1 (ITS-1) and the intergenic spacer 1(IGS-1) of rDNA (Han et al., 2013). The *M. pachydermatis* isolate was kept in deep freezer (−80°C) until it was used for the experiment. The isolate was cultured on sabouraud dextrose agar (SDA; Sigma-Aldrich; Milan, Italy) and incubated for 3 days at 37°C (Banovic et al., 2013; Chiavassa et al., 2014; Mason et al., 1996).

2.2 | In vitro antifungal susceptibility testing for cold atmospheric microwave plasma

We used CAMP (IonMedical Inc.; Seongnam, Korea), which consists of a handpiece jet pen in which the plasma is generated by atmospheric pressure, output power (W), and a gas flow. Argon gas was applied in this device, and the parameters were set to a temperature below 40°C, 10–15 LPM (litres per min), and 3–50 W with 2450 MHz, 3.5 kV.

Stock inoculum suspensions were prepared using a 3-day-old colony cultured on SDA. The colony was suspended in 3 ml of sabouraud dextrose broth (SDB; Sigma-Aldrich; Milan, Italy) until reaching an optical density of 0.5 McFarland using a turbidimeter (Densichek McFarland Densitometer, Biomerieux, Lyon, France) (equivalent to 7–9 $\times$ 10^5 colony forming units (CFUs)/ml as validated by quantitative plate counts of CFUs in SDA) (Banovic et al., 2013; Cafarchia et al., 2012). The inoculum was diluted 100-fold with SDB. Ten microliters of the inoculum was plated onto the SDA, and each agar was treated for 1, 2, 3 and 5 min with 10 or 15 LPM and 30 or 50 W, respectively. After plasma treatment, each plate was incubated for 3 days at 37°C, and the total number of CFUs was determined. Plasma application without ignition (argon gas only) was used as a negative control. Three independent tests were conducted for the evaluation of CFU.

2.3 | In vitro antifungal susceptibility testing for combination with cold atmospheric microwave plasma and chlorhexidine gluconate

A 5% aqueous solution of CHX (Greenpharmaceutical; Chungchungbuk-do, Korea) was used. The CHX was diluted to 0.02%, 0.2% and 2% with distilled water. The in vitro antifungal activity

methods were modified from a previous study (Banovic et al., 2013; Oh et al., 2012). The inoculum (equivalent to 7–9 × 10^5 CFU/ml as validated by quantitative plate counts of CFUs in SDA) and each concentration of CHX was added to each conical tube (SPL; Gyeonggi-do, Korea) in equal quantities (1.5 ml). Each tube was left at room temperature for 1, 2, 3 and 5 min. After 1, 2, 3 and 5 min, the mixtures were transferred to SDB, and the mixtures were finally diluted so that the inoculum was diluted 100-fold with SDB. Ten microliters of the inoculum was plated onto SDA and incubated for 3 days at 37°C, after which the total number of CFUs was determined. All tests were performed at least triplicate for the evaluation of CFU.

The combination methods were modified from a previous study (Koban et al., 2011) that had determined the CFUs by treating the plasma in broth medium followed by inoculation on an agar plate (Koban et al., 2011). Similar to testing for antiseptics, each final diluted mixture that was added to the inoculum and each concentration of CHX (i.e., 0.02%, 0.2% and 2%) was placed in the wells of a 24-well sterile cell culture plate (Falcon, Corning, USA). Each mixture in the wells was applied to the plasma for 1, 2, 3 and 5 min. After 1, 2, 3 and 5 min, 10 μl of the inoculum was plated onto SDA and incubated for 3 days at 37°C, after which the total number of CFUs was determined. CAMP was applied at 50 W with 15 LPM. The combination tests were conducted in triplicate for the evaluation of CFU.

2.4 | Statistical analysis

SPSS Statistics 23 (IBM Corporation, Armonk, NY, USA) was used to perform all statistical comparisons. To determine the antifungal effect of CAMP and to evaluate the synergistic effect between CAMP and CHX for each trial time, the Kruskal–Wallis test was used. If the antifungal effect and the synergistic effect were confirmed, the Mann–Whitney *U* test was used to compare each condition for each trial time. Statistical significance was defined as $p < 0.05$.

3 | RESULTS

3.1 | Determination of the antifungal effect of cold atmospheric microwave plasma against *M. pachydermatis*

The CFU values are described in Table 1 as the survival rate that was compared with the negative control without plasma exposure at 100%. The survival rates were significantly reduced in all conditions of CAMP (30 W with 10 LPM, 30 W with 15 LPM, 50 W with 10 LPM and 50 W with 15 LPM) for 3 and 5 min ($p < 0.05$). No significant difference was observed in the efficacy of CAMP for 1 or 2 min compared to each trial time ($p > 0.05$).

For 3 min CAMP application, all conditions except 30 W with 15 LPM demonstrated better antifungal effects than argon gas alone ($p < 0.05$). CAMP which was applied at 50 W with 10 LPM had the lowest survival rate followed by 50 W with 15 LPM. This was significantly less survival rate when compared with those of other conditions of CAMP in which the same time application ($p < 0.05$) except the condition in which CAMP was applied at 50 W with 10 LPM for 3 min application ($p > 0.05$). The CFU values in these two conditions (50 W with 15 LPM and 50 W with 10 LPM) did not differ significantly ($p > 0.05$).

In all conditions in which CAMP was applied for 5 min, the survival rates were greatly reduced compared with those applied for 1, 2 and 3 min. When CAMP was applied at 30 W for 5 min, the survival rates were 17 ± 7.55% in 10 LPM, 13.67 ± 5.77% in 15 LPM. At 50 W for 5 min, the survival rates were also 3.67 ± 4.62% in 10 LPM, 5 ± 4.58% in 15 LPM. CAMP showed the significant antifungal effects at 5 min regardless of watt and litres per minute. There were shown better antifungal effects of plasma with ignition than the condition in which plasma without ignition ($p < 0.05$).

3.2 | Evaluation of the synergistic antifungal effect of cold atmospheric microwave plasma and chlorhexidine gluconate against *M. pachydermatis*

The survival rates were significantly reduced by CHX and CHX with CAMP for 1, 2, 3 and 5 min ($p < 0.05$) (Table 2). The antifungal effects of CHX increased with increasing concentration of CHX (i.e., 0.02%, 0.2%, and 2%) for 1 and 2 min ($p < 0.05$).

Because no *M. pachydermatis* colony growth was observed either at 2% CHX or 2% CHX with CAMP, other concentrations of CHX (i.e., 0.02% and 0.2%), both alone and with CAMP, were evaluated for synergistic antifungal effects against *M. pachydermatis*. For 1 min, when CAMP was combined with 0.02% CHX, the survival rate was 15.33 ± 11.59% that was compared with 39.67 ± 13.01% at the application of 0.02% CHX only. Also, when CAMP was combined with 0.2% CHX, the survival rate was 1 ± 1.73% that was compared with 5.67 ± 3.79% at the application of 0.2% CHX only ($p < 0.05$). The application of 0.02% CHX with CAMP reduced survival rates more significantly than the application of 0.02% CHX alone ($p < 0.05$). Except at a concentration of 0.02%, CHX alone reduced CFU values more significantly than CAMP alone ($p < 0.05$).

For 2 min, when CAMP was combined with 0.02% CHX, the survival rate was 19 ± 6% that was compared with 28.67 ± 5.03% at the application of 0.02% CHX only ($p < 0.05$). Although CAMP combined with 0.2% CHX decreased more CFU than 0.2% CHX only, there was no significant difference between 0.2% CHX alone and 0.2% CHX with CAMP ($p > 0.05$).

For 3 and 5 min, no significant difference was observed in the synergistic antifungal effect between CHX and CAMP ($p > 0.05$). The application of CAMP alone showed more antifungal efficacy than 0.02% CHX alone and less antifungal efficacy than both 2% CHX alone and 2% CHX with CAMP ($p < 0.05$).

In addition, except application for 1 min, the effects of 0.2% CHX alone differed significantly from those of 0.02% CHX with CAMP ($p < 0.05$).

TABLE 1 Survival rates of *Malassezia pachydermatis* colony forming units (CFU) after cold atmospheric microwave plasma (CAMP)

	LPM (liters per min)_W				
time	10 LPM_30 W	10 LPM_50 W	15 LPM_30 W	15 LPM_50 W	Ar gas only
1 minute	63 ± 16.64	50.33 ± 28.01	76.33 ± 12.59	36 ± 10	49.66 ± 19.73
2 minutes	46 ± 19.47	31 ± 19.08	61 ± 23.64	30.67 ± 9.81	51 ± 12
3 minutes*	26 ± 11.36[a]	13.67 ± 7.37[b]	48 ± 13.75[a,b,c]	10.33 ± 3.51[a,c,d]	47 ± 10.58[a,b,d]
5 minutes*	17 ± 7.55[e]	3.67 ± 4.62[e]	13.67 ± 5.77[e]	5 ± 4.58[e]	69.67 ± 2.31[e]

The CFU values are described the survival rates that were compared with the negative control at 100%.
*Denotes significant difference ($p < 0.05$) about the antifungal effect of CAMP for each trial time, the Kruscal–Wallis test.
[a,b,c,d and e]Denote significant difference ($p < 0.05$) about comparison of the antifungal effect of CAMP for each condition, the Mann-Whitney *U* test.
Different letters mean statistically significant differences ($p < 0.05$).
Data are presented as mean standard deviation.

TABLE 2 Survival rates of *Malassezia pachydermatis* colony forming units (CFU) after cold atmospheric microwave plasma (CAMP) and chlorhexidine gluconate (CHX)

	Treatment						
time	0.02% CHX	0.2% CHX	2% CHX	0.02% CHX+ Plasma	0.2% CHX+ Plasma	2% CHX+ Plasma	Plasma
1 minute*	39.67 ± 13.01[a]	5.67 ± 3.79[a,b]	0[a,b,c]	15.33 ± 11.59[a,c,d]	1 ± 1.73[a,b,d,e]	0[a,b,c]	36 ± 10[b,c,d,e]
2 minutes*	28.67 ± 5.03[a]	5 ± 4.58[a,b]	0[a,b,c]	19 ± 6[a,b,c,d]	4.67 ± 4.73[a,d,e]	0[a,b,c]	30 ± 9.81[b,c,d,e]
3 minutes*	27.33 ± 10.69[a]	4.67 ± 4.04[a,b]	0[a,c]	19.33 ± 6[b,c,d]	1.33 ± 2.31[a,d,e]	0[a,c]	10.33 ± 3.51[a,c,d,e]
5 minutes*	23.67 ± 13.45[a]	3.67 ± 3.51[a,b]	0[a,c]	14.67 ± 9.29[b,c,d]	1.33 ± 1.53[a,d]	0[a,c]	5 ± 4.58[a,c]

The CFU values are described the survival rates that were compared with the negative control at 100%.
*Denotes significant difference ($p < 0.05$) about the antifungal effect of CAMP for each trial time, the Kruscal–Wallis test.
[a, b, c, d and e]Denote significant difference ($p < 0.05$) about comparison of the antifungal effect of CAMP for each condition, the Mann-Whitney *U* test.
Different letters mean statistically significant differences ($p < 0.05$).
Data are presented as mean standard deviation.

4 | DISCUSSION

Cold atmospheric microwave plasma has recently been considered an important application for disinfection in medicine (Fridman et al., 2007). Previous studies indicated the bacterial inactivation mechanism by which plasma-emitted particles can destroy the cell envelope (Lackmann et al., 2013). Compared to bacteria, fungi have a different cell wall structure, membrane and complex defence mechanisms against various pressures (Alonso-Mongo et al., 2009). *M. pachydermatis* is the predominant skin microbiota fungus in dogs and is thought to exacerbate the skin of patients with atopic dermatitis. No in vitro studies have yet been conducted to evaluate plasma treatment against *M. pachydermatis* in veterinary medicine. This study was worthwhile in evaluating the in vitro activity of plasma against *M. pachydermatis* that has a thick and multilamellar cell wall (Guillot & Bond, 1999).

In our study, CAMP showed a significant antifungal effect against *M. pachydermatis* (Table 1). We confirmed that CAMP requires the ignition process for effective antifungal activity against *M. pachydermatis*. A previous study had indicated that there was a difference in antiseptic efficacy depending on the presence of ignition (Koban et al., 2011). Similarly, our study showed that the ignition process should be included in plasma application for consistent and effective antifungal activity.

We confirmed that CAMP requires longer application times to achieve greater antifungal efficacy against *M. pachydermatis*. In our study, CAMP applied for over 3 min produced statistically significant antifungal effects against *M. pachydermatis*. The lack of statistical significance for the 5 min application suggests that the antifungal effect was efficacious for all conditions of CAMP at 5 min. In this study, complete decontamination was not achieved within 5 min. Similar to a previous study on human pathogenic dermatophytes, there was significant decontamination of *Trichophyton rubrum*, *Trichophyton interdigitale*, *Arthroderma benhamiae* and *Microsporum gypseum* after a few minutes of plasma application, but complete decontamination was not achieved in *M. gypseum* (Scholtz et al., 2015). Previous studies demonstrated that the required time of plasma application for significant antifungal effect was longer than that for antibacterial effect. (Daeschlein et al., 2012; Fridman et al., 2007; Nishime et al., 2017). These results imply that fungi have a complex structure including a very thick and rigid cell wall, which was reported in a previous study on *C. albicans* (Nishime et al., 2017). The difference in plasma devices and application methods can also influence these results. Furthermore, our study found that a stronger output power for CAMP can lead to more effective antifungal activity against *M. pachydermatis*.

This study also reported a synergistic antifungal effect of CAMP and CHX against *M. pachydermatis* (Table 2). The combination with 0.02% or 0.2% CHX and CAMP produced better antifungal effects than the single use of 0.02% or 0.2% CHX, respectively. A previous study also showed that two different plasma treatments were significantly more

antiseptic effects against *Streptococcus mutans* compared with 0.1% CHX after 10 min applications (Koban et al., 2011). This finding implies that if the plasma application time is much longer, the decontamination level can be similar to that achieved with 0.2% and 2% CHX. Various combinations and options for topical treatment are necessary for continuous lifelong care against *Malassezia* overgrowth that is often accompanied by atopic dermatitis. As CHX is a broad-spectrum antiseptic for microorganisms such as bacteria and fungi, CHX was used in this study to determine its synergistic effect with CAMP. Additionally, it is necessary to determine the synergistic effect of CAMP with a specific antifungal agent against *M. pachydermatis* such as miconazole in future studies.

This study had the limitation that one strain of *M. pachydermatis* was used. A large number of *M. pachydermatis* clinical isolates and standard strains of *M. pachydermatis* are required for further study. However, because there are no other plasma studies related to *M. pachydermatis* in veterinary medicine, this study has great significance in showing its antifungal efficacy against *M. pachydermatis*. Therefore, despite the small sample size, this study highlights a new potential antifungal agent. Further studies are necessary to investigate the physical mechanism of *M. pachydermatis* destruction and to assess its efficacy in vivo.

In conclusion, the present study has demonstrated that CAMP shows significant antifungal effect against *M. pachydermatis* under the conditions of 50 W and 5 min application in vitro. This study also confirmed that cold atmospheric microwave plasma has synergistic antifungal effects in vitro against *M. pachydermatis* when combined with CHX.

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CONFLICT OF INTEREST

Cheol-Yong Hwang is a founder and chief clinical officer, and Seung-Joon Baek is a founder and chief medical officer at IonMedical Inc.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal noted on the journal's author guidelines have been adhered to.

AUTHOR CONTRIBUTION

Tae-Hyun Lee: conceptualization; data curation; investigation; methodology; writing – original draft. Jae-Eun Hyun: data curation; formal analysis; methodology; writing – review and editing. Yeong-Hun Kang: resources; software; writing – review and editing. Seung-Joon Baek: conceptualization; software; project administration; writing – review and editing. Cheol-Yong Hwang: conceptualization; project administration; supervision; writing – review and editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

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