WALAILAK JOURNAL
http://wjst.wu.ac.th Engineering and Physical Sciences

Inhibitory Activity of Cold Atmospheric Plasma on *Candida albicans*[†]

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Received: 18 December 2017, Revised: 15 January 2019, Accepted: 30 January 2019

Abstract

Cold Atmospheric Plasma (CAP) application of dental treatment technologies has recently gained much interest. However, information regarding safety, applications, and how they work is limited. In this study, we examined the inhibitory activity of CAP against *Candida albican*. The Ar gas has been discharged by CAP of 100 KHz and has been produced inside 4 L/min. The system was operated with the HVRF power input 20 W and treatments of 0, 5, 10, 15 min for samples. The *Candida albican* standard strain used in this experiment was ATCC 10231. In order to assess the inhibitory activity of CAP against *Candida albican*, we employed 2 methods; the colony number count and MTT colorimetric assay. The colonies of *Candida albican* standard strain ATCC 10231 were significantly at 5, 10 and 15 min exposure of CAP ($p < 0.05$) as compared to un-treatment. According to the result of MTT colorimetric assay, we found that cell viability was significant after 10 min exposure as compared to un-treatment ($p < 0.05$). In summary, the overall results stated that CAP possesses the inhibitory activity against *Candida albican* standard strain ATCC 10231 at 100 KHz, 20 W after 5 min.

Keywords: MTT assay, *Candida albican*, Cold Atmospheric Plasma

Introduction

Candida albican is a pleomorphic micro-organism demonstrating different growth forms such as germ tubes, yeasts, pseudo- and true hyphae, and chlamydospores [1]. It has been proposed that undergoes reversible morphogenic transitions between budding yeast, pseudohyphal, and hyphal growth forms are important for pathogenicity mechanism [2]. This property is a major virulent attribute of its ability to adhere to host cell or all most surfaces and form surface attached microbial communities know as biofilms. Additionally, the expression of adhesins and invasions on the cell surface, thigmotropism, and the formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes are considered virulence factors. Biofilm development can be divided in 4 sequential steps: 1) adhesion of microorganisms to a surface; 2) discrete colony formation and organization of cells; 3) secretion of extracellular polysaccharide and maturation into a three-dimensional structure; and 4) dissemination of progeny biofilm cells [3]. Therefore, biofilm production is also related to a high level of their inherent resistance to commonly used antifungal agents [4]. Chandra and colleagues showed that *C. albican* biofilms exhibited resistance to amphotericin B, nystatin, chlorhexidine, and fluconazole, which had been to evaluate the antifungal susceptibility of Candida biofilms formed on denture acrylic *in vitro* [5,6].

[†]Presented at the 10th International Conference on Plasma Science and Applications 2017: October 10th - 11th , 2017

Candidiasis is an infection involving every part of the body and the most common cause is *C. albicans* overgrowth [7,8]. It can be a superficial, mucocutaneous, or systemic mycosis. Infection by any of the species of Candida is nearly always preceded by a compromise of the host defense mechanisms [8,9]. It appeared through various clinical forms, involving one or more oral sites, up to affect the whole oral cavity and to disseminate into invasive forms. Its primary location is the posterior tongue and other oral sites as the mucosa, while the film that covers the dental surfaces is colonized secondarily [10]. Since 1936, candida-associated denture stomatitis has been termed and some 65 per cent of denture wearers were the commonest form of oral candidiasis [11,12]. The study showed that *C. albicans* was isolated more frequently from the denture fitting surface than from the corresponding mucosa and was correlated with the severity of the denture stomatitis lesions [13-15].

Therefore, the treatment should be strategized the use of topical and systemic antifungal drugs, the use of preservatives and disinfectants, and the scrupulous removal and control of Candida species present on the denture and on the oral mucosa [16,17]. Risk of stomatitis should be decreased which used antimicrobial coating of dentures, but cannot prevent biofilm development. Dentures can be cleaned by mechanical methods, chemical methods or a combination of both. Cleansing with a brush and an abrasive is the most popular mechanical method widely used [18,19]. Denture cleansers are the most preferred chemical cleansing methods, which have been considered to be the most effective for inhibiting *C. albicans* infection and denture biofilm formation. The highest efficacy for the removal of *C. albicans* biofilms was identified for sodium hypochlorite, but can cause discoloring of the metal components such as cobalt chromium alloys used for partial dentures and bleaching of the acrylic resin. Chlorhexidine gluconate in a concentration of 0.2% was found to be effective on plaque formed on denture surfaces, but the use causes heavy discoloration on routine soaking and cannot be used on a daily basis [20,21]. The use of chemical denture cleansers may lead to color changes of the denture base or increase the roughness, which increase the ability for microorganisms to adhere and form biofilm.

An alternative to regular irradiation with Cold Atmospheric Plasma (CAP) has been proposing to the denture disinfection [22]. Plasma is the fourth state of matter apart from liquid, gas, and solid. Plasma is a partially ionized gas with ions, electrons, and uncharged particles. This is critical for application which the effectiveness of non-thermal argon plasma treatments can be eliminating pathogenic bacteria from biofilms and wound surfaces [23]. Furthermore, there was study which showed inactivation of Candida biofilms using different plasma devices [24]. The various studies have been performed to investigate the application of CAP on *C. albicans*. The bacterial killing efficacies were dependent on the plasma exposure time, which increased with increasing treatment time [25-27]. This plasma discharge is generated by applying high voltage to accelerate electrons to ionize the surrounding air molecules which in turn increases the antimicrobial effect.

In this experiment the main purpose of this work was to feasibility study of the inhibitory of cold atmospheric pressure plasma against *C. albicans*. Consequently, it should be encouraged the usage of plasma as effective antifungal devices to promote dental hygiene.

Materials and methods

Preparation of *C. albicans* strains suspension

C. albicans ATCC 10231 was obtained from the Department of Medical Science, Ministry of Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand, and was subcultured from vial stocks onto Sabouraud dextrose agar under aerobic conditions at 37 °C. Yeast inocula were cultured on Sabouraud dextrose broth (SDB) (Merck KGaA, Darmstadt, Germany) at 37 °C overnight. The turbidity of cell suspensions was measured in a spectrophotometer at 540 nm in order to obtain suspensions with an optical density according to a 1.0 McFarland standard tube [28].

CAP device

CAP unit was used in this study as shown in **Figure 1**. A high voltage RF power supply was connected to two electrodes which has an 80 mm length of jet plasma generating. The device contained gas spacing between 2 glass barriers was fixed at 10 mm. The power of the device was fixed at 100 KHz

and was operated with the HVRF power input 20 W. To generate plasma, argon (Ar) gas was injected into the device with a 4 liter per minute flow rate.

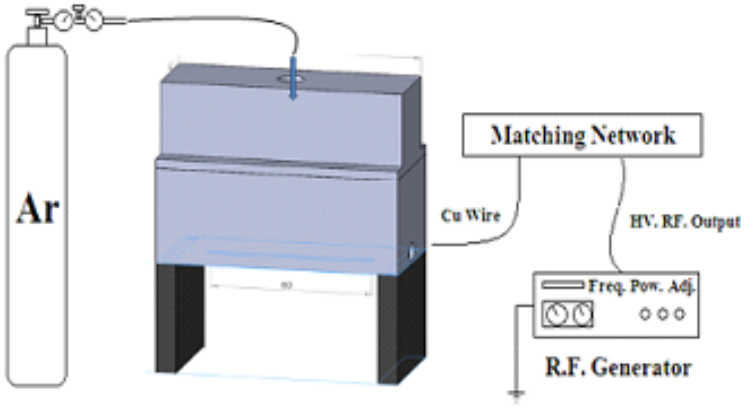

Figure 1 Schematic diagram of CAP device.

Measurement of cell survival

About 3×10^3 of *C. albicans* cells seeded in 100 µl of Sabouraud dextrose broth were placed on Sabouraud dextrose agar (SDA) (Merck KGaA, Darmstadt, Germany) plate. The cells were spread on the plate by using a glass spreader, and were then exposed to CAP for 5, 10 and 15 min. Plates were incubated at 37 °C for 1 day. After 1-day incubation, a number of colonies on the plate are counted. Colony numbers were counted in triplicate.

MTT colorimetric assay

For Ar plasma treatment, 3×10^4 of *C. albicans* cells were seeded in a 200 µl volume of SDB on a polystyrene plate. Cells were then exposed to the CAP discharge at a 2-mm distance for 5, 10 and 15 min. Cells without CAP treatment were used as a control sample. After exposure period by the CAP discharge, the 100 µl of the suspensions per well was transferred to the 96 microtiter plate (Corning Incorporated, Corning, NY, USA). Then, 15 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma-Aldrich Co., USA) (5 mg/ml water) was added in each well, and the plate was incubated at room temperature for 3 h. The content in each well was transferred to a 1.5 ml microcentrifuge tube, and the tubes were centrifuged at 10,000 rpm for 2 min. After the liquid was removed, cells were washed with 100 µl of water once, and 70 µl of DMSO was added in each tube. The tubes were incubated at room temperature for 3 min; then, the content in each tube was transferred to a new 96-well plate. The plate was read at 540 nm by using a Tecan's Sunrise absorbance microplate reader (T ecan Group Ltd., Männedorf, Switzerland).

Statistical analysis

All measurements were performed in triplicate. In order to make analytical comparison with different exposure time of the CAP treatment, a one-way ANOVA statistic test was implemented and according to these results, there was statistically significant difference between exposure time of Ar plasma discharge ($p < 0.05$).

Results and discussion

The CAP generated from Ar identified various reactive species in the optical emission spectroscopy analysis. The spectrum data were analyzed using the SpectraSuit software. The optical emission spectra analysis identified several reactive oxygen species of biological importance including the hydroxyl (OH) radical (309 nm) and singlet oxygen (778 nm) in Ar plasma. Peaks between 690 and 900 nm (Ar plasma) are excited by high energy and are involved in generating hydroxyl radical and singlet oxygen by exciting molecules as show in **Figure 2**.

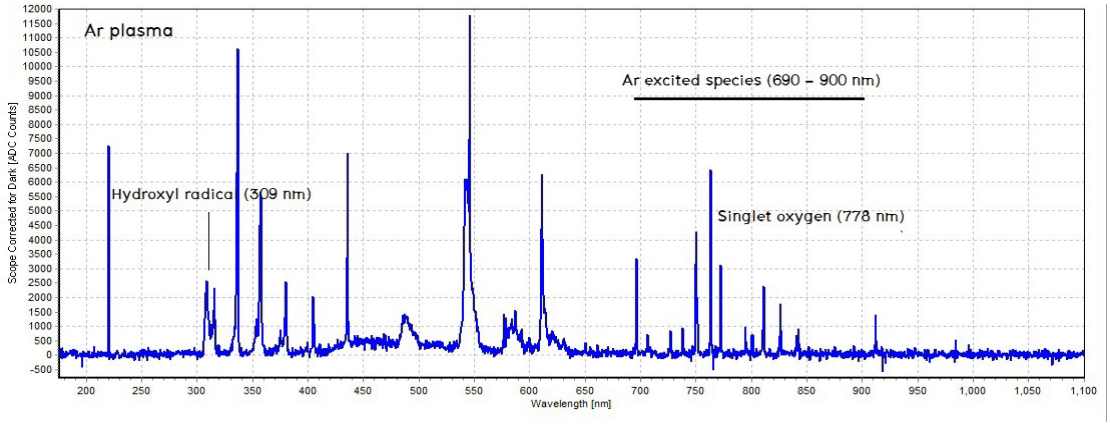

Figure 2 Optical emission spectroscopy of plasma generated using Ar gas.

The effects of CAP treatment on the growth and survival of *C. albicans* cells were evaluated by the colony formation measurement and mitochondrial activity was also determined by the standard MTT assay [29]. First, the susceptibility of survival to the plasma device was determined by applying plasma to Candida cells plated on Sabouraud dextrose agar plates and are exposed to CAP keeping distance to the Petri dish fixed at 10 mm. After incubation for 2 days, the result presented in **Figure 3** showed that the number of colonies grown on the plate decreased upon increasing exposure time. The number of colonies was significantly reduced after an exposure of 10 min and, the majority of cells are dead after exposure for 10 min as shown in **Figure 4**. Several *in vitro* studies have support this find. Mattes and colleagues reported that yeast cells are exposed to Ar plasma alone, the number of colonies grown on the plate decreases with increasing exposure time, and after 10 min of exposure [24,30,31]. They also suggested that the plasma treatment reduced the CFU significantly compared to chemical disinfectants. The use of plasma alone and in combination with antiseptics was promising anticandidal regimens for daily use on dentures when biofilms were not older than 2 days [32]. Secondary, cytotoxicity assay of *C. albicans* cells to the argon plasma treatment was determined by measuring the mitochondrial dehydrogenase activity. Mitochondrial dehydrogenase in viable cells was able to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, yellow color) to MTT formazan (purple color), and this enzyme was inactive in dead cells. Thus, cell viability can be measured by using the absorbance value at 540 nm of product color. The correlation between amounts of formazan generated and a decrease in 3×10^4 CFU/ml of living cells numbers with increasing plasma exposure time was shown in **Figure 5.** The figure was indicated that survival of *C. albicans* value decreased after an exposure of 10 min. In these tests, a significant difference ($p < 0.05$) can observed during irradiation of plasma in 10 and 15 min. It was based on the fact that mitochondrial respiratory activity can be quantified with a simple colorimetric procedure and that reduction in mitochondrial activity usually will correlate with the number of viable and metabolically active fungal cells [33,34].

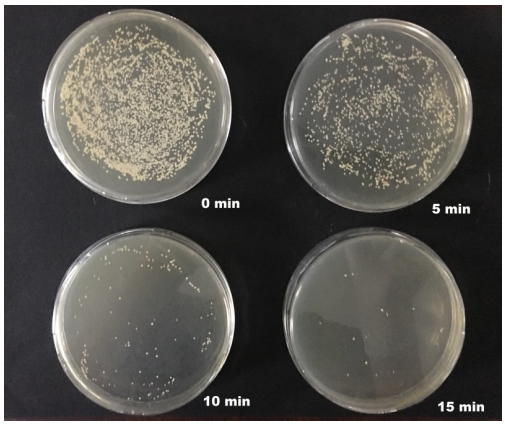

Figure 3 Colony morphological figures of *C. albicans* cells after treatment with CAP discharge for 0 (Control), 5, 10, and 15 min, followed by incubation at 37 °C for 48 h.

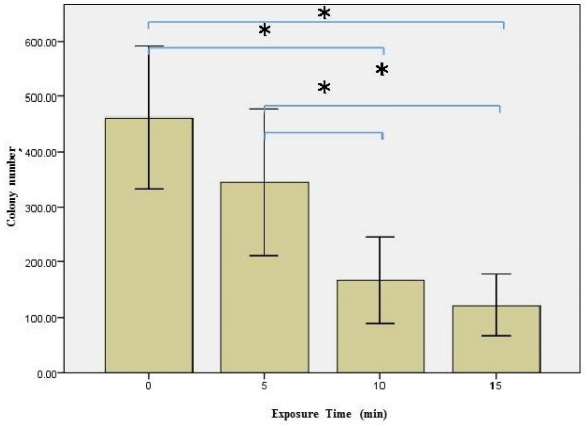

Figure 4 Colony numbers count of *C. albicans* cells exposed to CAP for 5, 10, and 15 min in comparison with the non-treated control; *Value significantly different at $p < 0.05$.

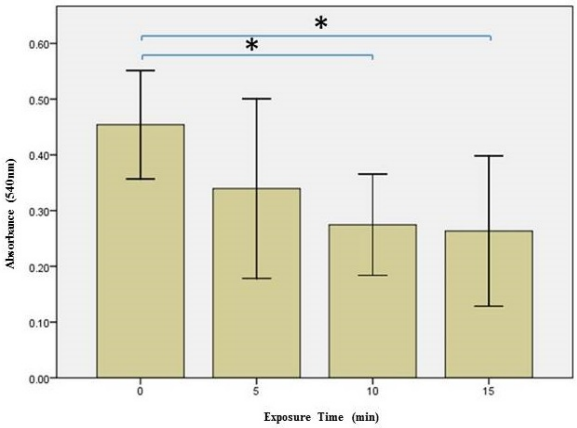

Figure 5 MTT colorimetric assay of *C. albicans* cells exposed to CAP for 5 min, 10 min, and 15 min in comparison with the non-treated control; * Value significantly different at $p < 0.05$.

In the current study, we tested the inhibitory of cold atmospheric pressure plasma against *C. albicans*, and demonstrated significant a decrease in growth and survival number of Candida cells with increasing plasma exposure time. However, the results of colony count in **Figure 4** and the results of MTT colorimetric assay in **Figure 5** were not totally compatible. The limitation of this study is that the data may be due to the process of exposure and the non-homogeneous intensity of plasma emission of the current first-generation of CAP device. In further studies, we should use the second generation of the device should take this into account.

Conclusion

The inhibitory activity of CAP on *C. albicans* cells was investigated in this study. The study has shown that antifungal activity could be scientific evidence with both colony formation and mitochondrial activity test after 10 minutes of CAP treatment. This study will help in the development preventing biofilm associated Candida form on denture surfaces.

Acknowledgements

The first author thanks the Department of Physics, Faculty of Science, Maejo University Phrae Campus, Maejo University and the Department of Physics, School of Science, Walailak University, Thailand for help with CAP. Faculty of Dentistry, Chiang Mai University, Thailand was also acknowledged.

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