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Cold atmospheric plasma inhibits the growth of *Candida albicans* by affecting ergosterol biosynthesis and suppresses the fungal virulence factors *in vitro*

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ABSTRACT

Background: The pathogenic yeast *Candida albicans* is the most common opportunistic fungal pathogen that is responsible for a wide array of infections in susceptible individuals. Despite recent progress in developing novel antifungal drugs which combat *Candida*-related disorders, this fungus is still a major cause of life-threatening infections all over the world. In the present study, the effect of cold atmospheric plasma (CAP) was evaluated on the growth of *C. albicans* with special attention to the ability of the CAP-treated fungus for biofilm formation, ergosterol biosynthesis and phospholipase and proteinase secretory production.
Methods: *C. albicans* cell suspensions were irradiated over time-scales ranging of 90, 120, 150 and 180 s under cold atmospheric plasma contained He/O_2 (2%). Treated and untreated yeast cells were analyzed for the growth, biofilm formation, ergosterol content, and activities of phospholipase and proteinase.
Results: Our results showed that CAP remarkably suppressed the growth of *C. albicans* by 31–82% at the given times. Likewise, CAP strongly inhibited the ergosterol biosynthesis by the fungus in the range of 40–91%, biofilm formation by 43–57% and the activities of phospholipase and proteinase enzymes by 4–45%, dose-dependently.
Conclusion: CAP strongly inhibits the growth and virulence factors of *C. albicans* and thus, it could be a potential candidate to treat *Candida*-related superficial and cutaneous infections in practice.
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1. Introduction

In recent years, the incidence of fungal infections specially Candida-related disorders termed generally as "candidiasis" has increased dramatically in many countries. Due to the emergence of resistance to antifungal agents, determining an effective strategy plan is a very important issue for the treatment of fungal diseases in clinical mycology. *Candida albicans* is a diploid yeast fungus, resided as a normal flora in mucus membranes of the mouth, vagina and intestinal tract of human and animals. The fungus is responsible for a wide array of fungal infections from superficial types such as thrush and vulvovaginitis to life-threatening systemic infections in immune-compromised patients and organ transplants. The imbalance of environmental microbial flora of the body, long-lasting antibiotic therapy, immunodeficiency and organ transplantation are considered as the most predisposing factors which facilitate onset of candidiasis [1–4].

Traditionally, different antifungal drugs including miconazole, fluconazole, clotrimazole, ketoconazole, and nystatin are used in the form of topical creams or vaginal gels for treatment of cutaneous and mucosal candidiasis. Apart from the efficacy of these drugs there are many harmful side effects such as anemia, decrease in blood potassium, fever, and nausea. Furthermore, the time required for the treatment of fungal diseases could be very long-lasting [5].

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Nowadays due to the special useful features of plasma, its utilization in medicine and industry is world known. In general, the plasma is a state of matter that occurs at very high temperatures where molecular structures lose their ability to function properly. Plasma is often called the fourth state of matter after solid, liquid and gas. The present study was carried out in cold atmospheric plasma (CAP) [6–10], which has been highly regarded in different fields of biomedicine, such as sterilization, blood clotting and tissue healing. It should be noted that numerous studies have been performed to investigate the effects of CAP on the fungi *C. albicans* and dermatophytes. The results of the studied have illustrated that fungal growth stops after plasma treatment [11–15].

In the present study, the *in vitro* effect of CAP was examined on the growth of the pathogenic yeast fungus *C. albicans*. Furthermore, the effect of CAP was investigated on ergosterol biosynthesis, biofilm formation and phospholipase and proteinase production by the fungus. Special attention was made on the mechanism of action of CAP on inhibiting the growth of *C. albicans* yeast cells *in vitro*.

2. Materials and methods

2.1. Plasma device

The device consisted of helium and oxygen gas mixture (volume ratio: 98% He and 2% O_2) and a power supply that worked by 15 MHz radio frequency, 10 V voltage, 10 W output power and earth was the external electrode. A transistor regulated the voltage to 10 kV (peak to peak). There was a manometer (a very low pressure gauge) in order to check gas output pressure. Irradiation was carried out at a distance of 2 cm between plasma jet nozzle and sample surface.

2.2. Fungal strain and culture conditions

C. albicans ATCC 10231 (PFCC 50271) was prepared from pathogenic Fungi Culture Collection of the Pasteur Institute of Iran. The fungus was cultured on Sabouraud dextrose agar contained chloramphenicol (0.005%) and incubated at 37 °C for 48 h. After harvesting, the yeast cells were diluted with sterile normal saline to produce 10^6–10^8 colony-forming units (CFUs)/mL.

2.3. Effect of CAP on C. albicans growth

The *C. albicans* cell suspension with the concentration of 10^6 CFU was used. An amount of 30 μL of cell suspension was poured into selected alternate wells of 96-well microtiter plates. Each sample was treated by plasma in different durations of 0, 90, 120, 150 and 180 s. For obtaining the growth of *C. albicans*, 10 μL of untreated and treated suspensions were cultured on separate plates containing dextrose agar medium. Eventually, to verify colony counting, the plates were incubated at 28 °C for 24 h.

2.4. Effect of CAP on biofilm formation

A 18 h culture of *C. albicans* was prepared in YPD (yeast peptone dextrose) liquid medium by inoculating a loop full colony of *C. albicans* into 25 mL of YPD in an orbital shaker incubator (180 rpm) at 30 °C Cultures were centrifuged at 3000 rpm for 5–10 min, washed twice with sterile PBS, and resuspended in 25 mL of RPMI 1640 medium buffered with 0.165 M morpholine propanesulfonic acid (MOPS) at pH 7.0. In order to prepare a suspension of cells at the final density of (10^6 cells mL^{-1}) in RMPI 1640, the sample were measured spectrophotometrically at a wavelength of 520 nm and adjusted to an optical density (OD) of 0.38. In the next stage, a 100 μL of *C. albicans* suspension was added into selected alternate wells of the 96-well microtiter plate. Some samples were each treated with cold atmospheric plasma in each well of the plate for 0, 90, 120, 150 and 180 s. The plates were covered by their lids, sealed by parafilm and incubated for 24–48 h at 37 °C and shaken at a rotational speed of 75 rpm for attachment of the cells on the bottom of the wells. To verify the extent of biofilm formation, the ready plates were processed using the colorimetric method. For this purpose, the XTT/menadione reagent was added. The XTT (a tetrazolium salt) was prepared as a saturated solution (0.5 g L^{-1}) in sterile PBS and then filter-sterilized using a 0.22 μm filter. Menadione was prepared as a 10 mM stock solution in 100% acetone and stored at −70 °C. The XTT/menadione solution was prepared just prior to use, by adding 1 μL of the stock solution of menadione to a tube containing 10 mL of the thawed XTT solution. This solution was added to the wells of microplate and the plates were incubated in the dark for 2 h at 37 °C. About 80 μL of the resulting colored supernatant from each well was transferred into the corresponding wells of a new microtiter plate. The plates were read in a microtiter plate reader at 490 nm. After that the optical density values were used to determine the percentage of growth inhibition [16].

2.5. Effect of CAP on ergosterol biosynthesis

A single *C. albicans* colony from an overnight Sabouraud dextrose agar plate culture was used to prepare a suspension of cells at density of 2 McFarland. Then, 100 μL of the *C. albicans* suspension was added into selected alternate wells of 96-well microtiter plate and treated with CAP during different time intervals as follows: 0, 90, 120, 150 and 180 s. After that 100 μL of untreated and treated suspensions were poured in 30 mL of Sabouraud dextrose broth. The cultures were incubated for 48–72 h at 30 °C and harvested by centrifugation at 2700 rpm for 5 min. The net weight of the cell pellet was determined. A 3 mL of 25% alcoholic potassium hydroxide solution was added to each pellet and vortex mixed for 1 min. The cell suspensions were transferred to sterile borosilicate glass screw-cap tubes and were incubated for 1 h in a water bath of 80 °C. Following incubation, the tubes were allowed to cool. Sterol was extracted by addition of a mixture of 1 mL of sterile distilled water and 3 mL of hexane followed by vigorous vortex mixing for 3 min. The hexane layer was transferred to a clean borosilicate glass screw-cap tube and stored at −20 °C. An amount of 0.6 mL aliquot of sterol extract was diluted by 5 fold in 100% ethanol and scanned spectrophotometrically between 240 and 300 nm. Finally, the amount of ergosterol in each control and treated tubes was obtained using the following equation:

$$\%\ \text{Ergosterol} = \left[\frac{(A_{281.5}/290 \times F)}{\text{sample weight}}\right] - \left[\frac{A_{230}/518 \times F}{\text{sample weight}}\right] \quad (1)$$

where F is the factor for dilution in ethanol at 290 and 518 cm^{-1} wavelengths [17].

2.6. Assessment of CAP on C. albicans virulence factors

2.6.1. Phospholipase assay

In order to measure the phospholipase activity, a single colony from an overnight culture of *C. albicans* on Sabouraud dextrose agar plate culture was used to prepare a cell suspension at the density of 10^6 cells mL^{-1}. Phospholipase production was measured in egg-yolk medium which consisted of 13 g Sabouraud dextrose agar (SDA), 11.7 g NaCl, 0.111 g CaCl2 and 10% sterile egg yolk. Then, 40 μL of the *C. albicans* suspension was added into selected alternate wells of 96-well microtiter plate and treated by CAP for different time intervals as follows: 0, 90, 120, 150 and 180 s. In the next step, a volume of 10 μL of untreated and each treated suspension were poured into the wells punched onto the surface of the egg-yolk medium. The diameter of the precipitation zone around the well was measured after incubation at 37 °C for 48 h.

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