Archives of Oral Biology 73 (2017) 21–24

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/aob

Candida tropicalis biofilm inhibition by ZnO nanoparticles and EDTA

Vinoth Jothiprakasam*, Murugan Sambantham, Stalin Chinnathambi, Singaravel Vijayaboopathi

CAS in Marine Biology, Annamalai University, Parangipettai-608 502, Tamil Nadu, India

ARTICLE INFO

Article history:
Received 2 August 2014
Received in revised form 16 December 2015
Accepted 13 September 2016

Keywords:
Candida tropicalis
Biofilm
Antifungal agent
Nanoparticles
ZnO

ABSTRACT

Objective: Biofilm of *Candida tropicalis* denote as a complex cellular congregation with major implication in pathogenesis. This lifestyle of fungus as a biofilm can inhibit immune system and antifungal therapy in treatment of infectious disease especially medical device associated chronic disease. In this study effects of Zinc Oxide (ZnO) nanoparticles and EDTA were evaluated on *C. tropicalis* biofilm by using different techniques. ZnO nanoparticles were synthesized from Egg albumin.

Design: To assay the formation of biofilm of yeast cells like Fluconazole-susceptible *C. tropicalis* (ATCC 13,803) and fluconazole-resistant standard strains of *C. tropicalis* (ATCC 750) were grown in 24 well plates and antifungal effect of ZnO and EDTA were evaluated on *C. tropicalis* biofilm using ATP bioluminescence and tetrasodium salt (XTT) reduction assays.

Results: Synthesized ZnO NPs and EDTA had effective antifungal properties at the concentration of 5.2, 8.6 μg/ml for Fluconazole susceptible strain and 5.42, 10.8 μg/ml Fluconazole resistant strains of *C. tropicalis* biofilms compared to fluconazole drug.

Conclusion: In present study we conclude, ZnO considered as a new agent in field of prevention *C. tropicalis* biofilms especially biofilms formed surface of medical device.

1. Introduction

Biofilms- are cell–cell and solid attached microbes by self produced matrix of extracellular polymeric substances (EPS). Ecologically biofilm formation is a part of several microorganisms in disease especially fungi like the genus *Candida* (Stoodley, Costerton, & Stoodley, 2004). *Candida tropicalis* is one of the more common opportunistic human diseases in tropical countries. Taxonomically *C. tropicalis* is closely by resembled to *C. albicans*. *C. tropicalis* is mainly virulent in neutropenic hosts commonly with hematogenous seeding to peripheral organs. *C. tropicalis* fungemia was common in patients with leukemia in bone marrow transplant (Kontoyiannis et al., 2001). This fungus has proper potential for biofilms formation (Kojic and Darouiche, 2004). Cells in the biofilms exhibit an increase resistance to the antifungal drugs. Biofilm on dentures is an important medical problem as well on catheters which provides a common infection for hospital patients (Kojic and Darouiche, 2004; Rizk, Falkler, & Meiller, 2004). Several recent reports have indicated nanoparticles may be effective anti biofilm agents against Gram positive and Gram negative bacteria as well as fungi like *C. albicans* (Percival et al., 2005). Zinc oxide (ZnO) nanoparticles (NPs) are being widely used in health care commercial products due to their unique properties such as UV light absorption, and being catalytic, semi-conducting, magnetic and antimicrobial (Li et al., 2011; Rizk et al., 2004). Several studies proposed that ZnO nanoparticles with different sizes and shapes have different degrees of antimicrobial activities (Rizk et al., 2004). Consequently, ZnO NPs are considered being non-toxic, bio-safe and have been used as drug carrier cosmetics and fillings in medical materials (Shoeb et al., 2013). In present study, synthesized ZnO NPs are using egg albumen and explore the anti-candidal biofilm inhibition against fluconazole resistant *C. tropicalis*.

2. Materials and methods

2.1. Synthesis of ZnO NPs

The synthesis of ZnO NPs using egg albumin as a bio-template was performed. In brief, freshly extracted 20 ml of egg albumin ($5\ mg/ml^{-1}$) was dissolved in mixed deionized water and mixed drop-wise into 80 ml of aqueous zinc acetate solution. The mixture was stirred for 10 min at room temperature to form the colloidal solution. The colloidal solution was precipitated by the addition of

* Corresponding author at: CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai-608 502, Tamil Nadu, India.
E-mail addresses: vinodbio89@gmail.com, microking15@gmail.com (V. Jothiprakasam).

http://dx.doi.org/10.1016/j.archoralbio.2016.09.003
0003-9969/

ammonia solution (NH_3) at ~pH 7.0 and centrifuged at 5000 rpm for 10 min and twice washed with the deionized water. The washed material was collected and dried in the vacuum oven and grounded into a fine powder. The obtained dried powder was subjected to sintering at 500 °C for 3 h in ambient atmosphere and synthesized ZnO NPs were stored in a clean dry and dark place until further use.

2.2. Characterization of zinc oxide nanoparticles

The progress of ZnO nanoparticles formation during the biosynthesis was concluded based on surface Plasmon vibrations in a UV–vis spectrophotometer at 300–900 nm. To determine the size of the ZnO NPs, The morphological analysis of the particle was done with transmission electron microscopy (TEM). TEM images were recorded on a FEG-TEM (Phillips CM 200 field emission gun). Samples were prepared by spin coating of the precursor solutions on carbon coated copper grids using a microprocessor controlled spin coater (Model GP3-8 Spin coat, PI-KEM Ltd., UK) and dried under air at room temperature before transferring to the electron microscope, which was operated at an accelerated voltage of 120 kV.

2.3. Preparation of standard fungal cell suspension

Fluconazole-susceptible *C. tropicalis* (ATCC 13,803) and fluconazole-resistant standard strains of *C. tropicalis* (ATCC 750) were generous gift from Mahatma Gandhi Medical College and Research Centre (MGMCRC), Pondicherry, India, which were used for biofilm formation and the antifungal activity of mentioned agents. The two strains were grown on Sabouraud dextrose agar medium (Himedia Lab, Mumbai) at 37 °C for 24 h. Then the colonies are enriched into yeast nitrogen base medium (Himedia Lab, Mumbai) containing 50 mM glucose and incubated at 37 °C for 24 h. After that a few colonies were transferred into sterile PBS (pH 7.2). The turbidity was compared to 0.5 McFarland standards for estimation of cell density and finally cells were counted and adjusted at 1×10^6 cells/ml by Neubauer counting chamber (Rex et al., 2008; Tudela et al., 2008).

2.4. Biofilm formation

24 well plates were used to grow *Candida tropicalis* biofilms. Initially 100 μl YNB was enriched with 50 mM glucose then 10 μl of C. tropicalis cells was added into each well. Cells were allowed to adhere into bottom of the plates for 90 min at 37 °C. Subsequently, each well was gently submerged in 100 μl PBS and then added 50 mM containing 200 μl YNB medium. The plates were incubated for 48 h at 37 °C (Ernst & Rogers, 2005; Kavanagh, 2007). Later, the YNB medium was removed and wells were washed by PBS and different concentration of ZnO (4–8 μg/ml) and EDTA (Ethylene Diamine Tetra acetic Acid) (6.5/15 μg/ml) added in to each well. Finally the well volume was reached in to 100 μl with RPMI-1640 (Gibco).

2.5. XTT assay

XTT [2,3-bis (2-methoxy-4-nitro-5- sulfophenyl)-5- (phenyl amino) carbonyl) - 2*H*-tetrazolium hydroxide], as colorimetric method was carried out to assay antifungal effect of the ZnO nanoparticles. This method is based on determining the viability of collected cells. Plates were incubated with 50 mM glucose containing 50 μl of the YNB medium, 50 μl coenzyme Q0 (Sigma) and 100 μl XTT (Himedia). Plates were incubated at 37 °C for 3 h. Optical absorbance was measured at the wavelength of 492 nm by an ELISA reader (Awareness Technology Co) (Ernst & Rogers, 2005).

2.6. ATPase assay

ATP's for marker of cell viability. Because of its presence in all metabolically live cells, the concentration declines very rapidly when the cells undergo the biocides. This assay based on production of light at 560 nm through the reaction with addition of luciferase and D-luciferin (Case, 2001). After the biofilm formation and treatment of the cells by nanoparticles, ATPase assay was used to confirm the result. In this assay, 10 μl of susceptible and resistant *C. tropicalis* biofilms and 10 μl complex reagent containing luciferin, luciferase, and Mg^{2+} mixed in each well of Luminescence micro plates. Then the optical density was measured by illuminometer at 560 nm (Berthold Co).

2.7. Statistical analysis

Data analysis was performed by t-test method with SPSS Statistics version 17. The level of statistical significance was set at $P < 0.05$.

3. Results

Clear zinc acetate solution was changed into colloidal nature white colour substrate, due to the excitation of surface Plasmon resonance confirms the generation of ZnO NPs. In this same condition, no change was noted for without egg albumin in zinc acetate. This colour change is indicated to the ZnO NPs. The UV–vis spectroscopy absorption band optimized at 265, 350, 360 and 370 nm (Singla, Shafeeq, & Kumar, 2009). Clearly indicating the formation of zinc oxide nanoparticles Transmission Electron Microscopy (TEM) analysis revealed the size and shape of nanoparticles, sizes ranging from 5 nm to 50 nm with spherical in shape and agglomeration free particles were also observed (Fig. 1).

In this present study, ZnO nanoparticles at the concentration of 5.2 μg/ml and EDTA at the concentration of 8.6 μg/ml inhibited biofilms of fluconazole-susceptible strain of *C. tropicalis* which was shown by using XTT and ATPase assays. ZnO at 5.42 μg/ml and EDTA at 10.8 μg/ml inhibited growth of resistant-fluconazole strain of *C. tropicalis*. Fluconazole is one of the most effective drugs that inhibited *C. tropicalis* growth used as a control in this study. Results showed that at the concentration of 4 and 8 μg/ml, fluconazole stopped *C. tropicalis* biofilm for susceptible and resistant strains, respectively (Fig. 2). ATPase assay results showed that then concentration of the declared antimicrobial agent against biofilms was according to XTT assay. Synthesized ZnO nanoparticles and EDTA suppressed *C. tropicalis* biofilms at the concentration of 5.2 μg/ml, 8.6 μg/ml for fluconazole susceptible strain and 5.42, 10.8 μg/ml for fluconazole resistant strain (Fig. 3).

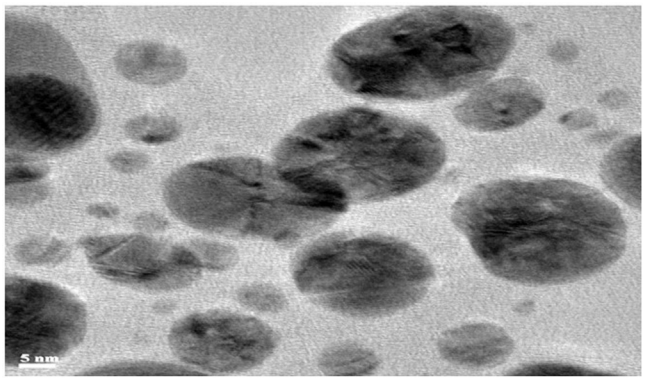

Fig. 1. TEM image of ZnO nano particle.