Research Article

Effect of Hypoxia on *In Vitro* Adhesion, Biofilm Formation and Antifungal Susceptibility of *Candida albicans*

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ABSTRACT

Candida albicans is a well known pathogen in nosocomial infections. Adhesion, biofilm and resistance to antifungal are main virulence properties. Pathogen faces hypoxic microenvironment in tissues which affects pathogenesis. We set our experiments to investigate directly the effect of hypoxia on growth, *in vitro* adhesion, biofilm formation and antifungal susceptibility of pathogen. Effect of hypoxia on growth was checked by spotting assays. *In vitro* adhesion, biofilm development and susceptibility of biofilm to amphotericin B were performed on polystyrene plates and quantified by XTT assay in RPMI 1640 and YNB media. Experiments were performed in triplicates and Student's t-test was used for statistical analysis. Cells grew well under hypoxia. Adhesion was reduced upon exposure to hypoxia in YNB medium but remains unchanged in RPMI-1640. Hypoxia increased biofilm activity to 287.24 ± 22.05% in RPMI 1640 and 130.02 ± 2.99% in YNB. Biofilm susceptibility to amphotericin B was reduced significantly under hypoxia in RPMI. In conclusion, hypoxia increased biofilm formation and reduced susceptibility to amphotericin B of *C. albicans* in RPMI 1640.

Keywords: Hypoxia, *Candida albicans,* biofilm, adhesion, XTT, RPMI-1640

INTRODUCTION

Candida albicans is the most common opportunistic fungal pathogen responsible for candidiasis, a life threatening infections ranging from superficial to systemic, particularly in the patients with compromised immunity[1,2]. Candidiasis stands fourth among the nosocomial ailments, causing a mortality rate ranging 15% to 35%[3]. Antibiotic resistance of *C. albicans* is marked in the recent report of year 2013 from Center for Disease Control and Prevention, U. S. Department of Health and Human Services[4].

Yeast to hyphal transition, secretion of hydrolytic enzymes, adherence to host tissue and biofilm formation are the major virulence factors of *C. albicans*[5,6]. Biofilm formation is a one of the major factors that determines drug resistance by *C. albicans*[7,8]. Tissue hypoxia, a state of low oxygen microenvironment (2.5-9 % in normal tissues and approximately 1% in damaged tissues) of host tissue is an important factor which regulates biofilm formation by pathogen[9,10]. Few studies has demonstrated that anaerobic conditions modulate biofilm differently in different strains and isolates; but in most of the studies anaerobic conditions do not support the growth of the biofilm[11,12]. Hypoxia mediated transcriptional response has been investigated well in *C. albicans* and other fungi[13-15]. Hypoxia upregulated the genes of biosynthesis of sterol, lipids, heme along with glycolytic pathways. Sterol regulatory element-binding proteins (SREBPs) regulate hypoxic responses in mammals[16]. But in *C. albicans,* the role of SREBPs is replaced by different regulator Upc2 and Ecm22 which binds sterol regulatory element in the promoters of target genes[17]. In *C. albicans*, Upc2 and Efg1 (transcription factors) regulate genes of ergosterol and fatty acid biosynthesis pathways respectively under hypoxic conditions while Tye7 and Gal4 induce genes of glycolytic pathway in hypoxia[1,18,19]. In *C. parapsilosis,* similar kind of transcriptional responses were reported during biofilm formation and hypoxia, suggesting the interrelationship of biofilm development with hypoxia[20]. In a similar study on *C. glabrata*, hypoxia is reported to enhance the biofilm formation *in vitro*, approximately by 2.5 times[21].

To the best of our knowledge, there is no direct study indicating the role of hypoxia in the virulence of *C. albicans*. Therefore, in this study, we planned to study the direct effect of hypoxia (1% Oxygen) upon key virulence factors like growth, *in vitro* adhesion, biofilm formation and susceptibility to drug in two growth media, RPMI and YNB.

MATERIALS AND METHODS

Cultures, Culture condition and Chemicals

C. albicans (SC5314) strain was provided by K. Ganeshan from Imtech Chandidgarh, Punjab, India. Strain was routinely cultivated at 37°C in YPD medium (1% yeast extract, 2% Bacto-peptone and 2% dextrose; BD) in orbital shaker at 120 rpm. Biofilm and adhesion assays were performed in RPMI 1640 (HiMedia) medium supplemented with 50 mM HEPES and L-Glutamine, pH 7.0 (referred as to RPMI 1640 henceforth) and YNB medium (0.67% yeast nitrogen base w/o amino acids with ammonium sulfate supplemented with 2% dextrose; BD; pH 7.0) on the surface of pre-sterilized, polystyrene, flat bottomed 96-well microtiter plates (HiMedia)[22,23]. All other routine chemicals of molecular grade were procured from Merck and plastic-wares from Tarson.

Amphotericin B (amp B), Menadione and 2,3-bis (2-methoxy-4-nitro-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) were purchased from HiMedia. For exposure to hypoxic conditions, plates were incubated in a hypoxic chamber (Multi-gas incubator, CO_2/O_2 incubator Model GA156, LEEC, UK) with 1% O_2, 5% CO_2 and 94% N_2. Normoxic exposures were given by incubating the plates in normal incubator shaker and the data is considered as control.

Effect of Hypoxia on Growth

Growth of cells under hypoxia and normoxia was analyzed by plate spotting assay[24]. Log phase cells of *C. albicans* (OD_{600nm}: 0.6) were spotted in 10 fold serial dilutions (10^3, 10^2, 10^1, 10^0 cells) onto YPD and YNB agar plates. For normoxic plates were incubated at 37°C for 18 hours (h) while for hypoxia, plates were incubated in hypoxia chamber at 37°C for 18 h. Experiments were done in triplicates.

Effect on Adhesion

The *in vitro* effect of hypoxia on adherence of *C. albicans* was studied on polystyrene surface using flat bottomed 96-well microtiter plate in YNB and RPMI-1640 medium[21]. 100 µl of cell suspension (10^7 cells/ml) in both medium was added to the wells and plates were incubated at 37°C for 90 minutes (min) with shaking on normoxic and hypoxic conditions, separate plates were used. After incubation, unadhered cells were removed by washing wells twice with 200 µl of PBS.

The adhered cells were quantified in each well using XTT-reduction assay. Absorbance was measured at 450 nm using ELISA Reader (Molecular Devices, SPECTRA max M2). Values are represented in terms of relative metabolic activity (RMA) in percentage, relative to the adhesion of respective normoxia control (Mean of metabolic activities of normoxia control is taken as 100%).

Effect on Biofilm Formation

C. albicans in vitro biofilm was developed on the surface of pre-sterilized, polystyrene, 96-well microtiter plate[25]. Briefly, 100 µl of cell suspension, prepared in PBS at a concentration of 10^7 cells/ml was added to each well. Plates were incubated for 90 min at 37°C on normoxic condition for adhesion phase. After incubation, wells were washed twice with 200 µl of PBS and 200 µl of culture media was added to the wells and plates were incubated at 37°C on normoxia and hypoxia for 60 h with shaking. Afterwards, wells were again washed twice with 200 µl of PBS and biofilm developed was quantified by XTT reduction assay[7] at 450 nm using ELISA Reader (Molecular Devices, SPECTRA max M2).

Biofilm developed is represented in terms of relative metabolic activity (RMA) in percentage, relative to the biofilm of respective normoxia control (Mean of metabolic activities of normoxia control is taken as 100%).

Effect on Biofilm Antifungal Drug Susceptibility

Effect of hypoxia upon susceptibility of biofilm development to antifungal drug was analyzed in the presence of different concentrations of amphotericin B (0, 0.0625, 0.125, 0.25 µg/ml) following the methodology as mentioned above by XTT reduction assay[7,21]. We investigated the susceptibility of *C. albicans* biofilm development to amphotericin B in both the media, YNB and RPMI 1640 upon exposure to hypoxia in comparison to normoxia controls.

Statistical Analysis

All experiments were performed in triplicates and values presented are the mean with standard deviation, obtained from three different observations for XTT assays. Student's t-test was used for statistical analysis and a value of $p < 0.05$ was considered statistically significant (*) and $p < 0.001$ as highly significant (**) for comparisons[16].

RESULTS

Hypoxia did not affect the growth of *C. albicans*

For determining the effect of hypoxia on growth of *C. albicans*, we performed growth assay where the cell containing plates were incubated under hypoxic and normoxic conditions. Cells grew well under both the conditions (Figure 1).

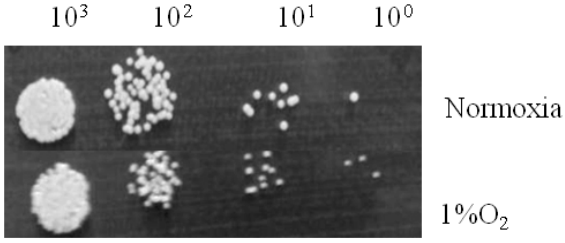

Figure 1: Growth and cell viability of *C. albicans* in spotting assays onto YPD agar plates upon exposure to normoxia and hypoxia.

C. albicans Adhesion was not increased under Hypoxia

In vitro adherence of *C. albicans* cells, under hypoxia and normoxia was investigated in 96-well flat bottom polystyrene plates.

Under hypoxic condition, the adhesion potential of the cells was found to be reduced significantly in YNB medium (RMA: 73.24 ± 4.4756%; $P < 0.05$) while remain unchanged in RPMI-1640 (101.35 ± 2.645%; $P > 0.05$) compared to that of normoxia control (Figure 2A).

Hypoxia increased Biofilm formation by *C. albicans*

The biofilm was formed in polystyrene plates in both media under both the conditions. The biofilm formation, under hypoxia was strikingly increased in RPMI-1640 (RMA: 287.24 ± 8.26%; $P < 0.001$), while in YNB, hypoxia increased it significantly as compared to control (RMA: 130.02 ± 1.2%; $P < 0.05$) (Figure 2B).

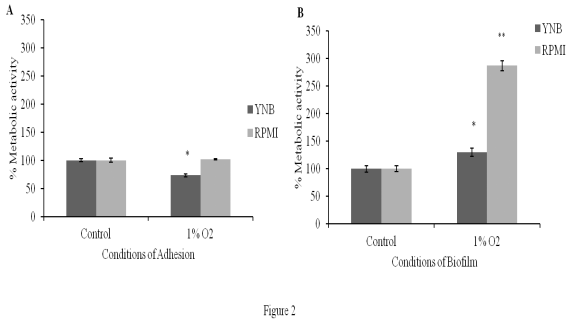

Figure 2

Figure 2: *In vitro* adhesion and biofilm formation by *C. albicans*. **A:** Relative metabolic activities (%) of adhesion of *C. albicans* cells under different conditions in different media. **B:** Relative metabolic activities (%) of biofilm formation by *C. albicans* cells under different conditions in different media. Mean values of RMA ± SD of three independent experiments are shown; *P<0.05* is considered statistically significant and *P < 0.001* highly significant and represented as (*) and (**) respectively.

Hypoxia reduced Biofilm Antifungal Susceptibility of *C. albicans*

Biofilm was developed in the presence of different concentrations of amphotericin B (as shown in Figure 3) in polystyrene 96-well plate. In RPMI media, in the presence of different concentrations of amphotericin B, biofilm activities were found significantly higher under hypoxic conditions than that of normoxic controls. While in YNB, in the presence of different concentrations of drug, biofilm activities under hypoxia were not found higher to that normoxia (Figure 3A, 3B).

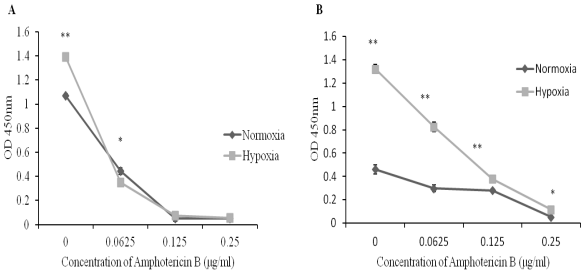

Figure 3: *In vitro* biofilm susceptibility to amphotericin B by *C. albicans*. **A**: Susceptibility of biofilm development in YNB medium. **B**: Susceptibility of biofilm development in RPMI-1640 medium. Results were shown here as mean ± SD of *A*450 plotted on Y-axis against concentration of amphotericin B on X- axis. Statistically significant differences in biofilm activities in presence of drug under normoxia and hypoxia are shown as (*) if *P<0.05* and (**) if *P<0.001*.

DISCUSSION

Hypoxia is an important host factor which favours the virulence of *C. albicans* by different means. Hypoxia is reported to induce distinct transcriptional response in *C. parapsilopsis, C. albicans,* and *S. cerevisiae*[19,20,26]. It upregulates the expression of the genes of sterol and glycolytic pathways for implementing the hypoxic adaptations[19]. Our results indicated that *C. albicans* has required adaptations to grow well under hypoxic conditions (Figure 1). It is reported earlier that adherence is higher in RPMI than in YNB under normoxia at pH 5.6 and pH 7.0[27].

We found that hypoxia did not enhance adhesion in both the media (Figure 2A), probably due to the effect of hypoxia on cell metabolism[28] and requirement of more time for transcriptional responses. Upregulation of *C. albicans* adhesins, Als1, Als3 and Eap1 is required for adhesion, a step prior to the biofilm development[29,30].

Yeater (2007) demonstrated that Als1 expression increases at least after 6 hours of incubation but we performed adhesion experiment for 90 minutes only. It is also noteworthy that under hypoxia, two transcription factors, Efg1 and Ace2 repress the filamentous growth in *C. albicans*[14].

Our results of increased biofilm under hypoxia can be explained by the homology of *C. albicans* Rfg1 with *Saccharomyces cerevisiae* Rox1, a key regulator of hypoxic genes in baker's yeast. Hypoxic regulator function of Rfg1 of *C. albicans* is lost for the virulence regulator function due to evolutionary pressure for surviving as human pathogen.

Under normal conditions, *C. albicans* Rfg1 is a transcription repressor of several genes related to virulence, particularly *HWP1*, *RBT1* and *ALS*[32] but under hypoxia, these virulence determinants are derepressed, increasing the biofilm formation. It has also been shown earlier that biofilm activity is media dependent and higher in RPMI than in YNB media[27]. RPMI has composition similar to human fluids[33] therefore experiments done in RPMI media might have direct clinical significance.

Since hypoxia increased biofilm, we further investigated the effect of hypoxia on biofilm development in the presence of amphotericin B or drug susceptibility of biofilm. Hypoxia did not affect susceptibility remarkably in YNB media because of being less nutrient rich. But hypoxia reduced susceptibility of biofilm to amphotericn B in RPMI (Figure 3B), probably due to the hypoxia dependent modulation of different transcription factors (like *RFG1*, *UPC2*, *EFG1* and *TYE7*) and virulence determinants (like *HWP1*, *RBT1*, *ALS1, ALS3* and *EAP1*) which result in increased yeast to hyphal transition, adhesion and biofilm formation. Earlier studies have proven that biofilm form of *C. albicans* is more resistant to antifungals[34,35].

Amphotericin B is a polyene which target ergosterol of the fungal cell membrane to generate pores[36,37] and hypoxia reduces ergosterol level in yeast by affecting different steps of ergosterol biosynthesis involving oxygen dependent enzymes[28]. It has been demonstrated in previous studies that polyene resistance in *Candida* species results when ergosterol content lowers[38,39]. Therefore, in the present study, increased resistance of

biofilm to the antifungal drug might be the manifestations of increased biofilm and reduced ergosterol level under hypoxic condition.

To our knowledge, this is the first direct study on *in vitro* virulence properties of *C. albicans* under hypoxia in RPMI and YNB media. Further studies are being undertaken to find out the molecular targets, effective under hypoxic conditions to develop effective anti-*Candida* drugs.

CONCLUSION

Findings of present study clearly indicate that *C. albicans* has adaptations for hypoxic mode of life style, as present inside host tissues. Hypoxia enhanced the biofilm formation in the growth media similar to body fluid i.e. RPMI. Hypoxia also reduced the susceptibility of biofilm against antifungal drug. Hence, hypoxic adaptations in *C. albicans* support its pathogenic mode of survival inside the host.

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Disclosure Statement

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