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Morphological changes and induction of antifungal resistance in *Aspergillus fumigatus* due to different CO_2 levels

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A B S T R A C T

Background and Purpose: Aspergillosis is one of the most common opportunistic fungal infections in immunocompromised and neutropenic patients. *Aspergillus fumigatus* (*A. fumigatus*) is the most common causative agent of this infection. Due to variable CO_2 concentrations that pathogens are exposed to during the infection process and to understand the role of CO_2, we examined the effects of various CO_2 concentrations as one of the environmental factors on morphological changes and induction of antifungal resistance in *A. fumigatus*.

Materials and Methods: *A. fumigatus* strains were cultured and incubated under 1%, 3%, 5%, and 12% CO_2 atmospheres, each time for one, two, and four weeks. The control culture was maintained for one week without CO_2 atmosphere. Morphological changes were investigated and antifungal susceptibility test was performed according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) M38-A2 document. The results of different CO_2 atmospheres were compared with that of the control sample.

Results: We found that 1%, 3%, 5%, and 12% CO_2 atmospheres were associated with morphological colony changes. Macroscopically, the colonies were shallow dark green, smooth, crisp to powdery with reduced growth; microscopic examination revealed the absence of conidiation. The induction of antifungal resistance in the susceptible strains to itraconazole, voriconazole, and amphotericin B increased after exposure to 12% CO_2 atmosphere and four weeks of incubation. The MIC values for itraconazole, voriconazole, and amphotericin B were 16 µg/ml, 1 µg/ml, and 16 µg/ml, respectively. These values for the control group were 0.125 µg/ml, 0.125 µg/ml, and 2 µg/ml, respectively.

Conclusion: Exposure to different CO_2 atmospheres induced morphological changes in *A. fumigatus*, it seems to increase the MIC values, as well. In parallel, resistance to both itraconazole and voriconazole was also observed.

Keywords: *Aspergillus fumigatus*, Carbon dioxide, Itraconazole, Voriconazole

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Introduction

Aspergillus species are widely distributed in the environment and have a wide range of manifestations from asymptomatic colonization or allergic reaction to invasive infection depending on host immunity [1, 2]. Invasive aspergillosis is the most severe manifestation with high mortality rate in immunocompromised patients [3, 4]. Amphotericin B (a member of the polyene antibiotics) and several azole-based medicines such as itraconazole, voriconazole, posaconazole, and isavuconazole are used for the treatment and prophylaxis of aspergillosis [5-7]. Recent reports from different European countries, the United States, South America, China, Japan, Iran, and India showed an increase in the frequency distribution of *A. fumigatus* isolates, indicating phenotypic resistance to itraconazole, voriconazole, and posaconazole [8-13].

Besides, specific point mutations in the *cyp51A* gene in combination with tandem repeats in the *cyp51A* promoter region are indicated as major environmentally derived mutations among azole-resistant *A. fumigatus* isolates. Additionally, *cyp51A*-

independent resistance mechanisms, such as the upregulation of drug efflux transporters, have been recognized [14, 15].

Acquired resistance in *A. fumigatus* may be caused by long-term treatment and exposure of the fungal cells to azole fungicides used in agriculture. Along with these factors, environmental changes including the nutrient sources, humidity, temperature, and air pollution could potentially lead to physiological changes in microorganisms [16]. Atmospheric CO_2 is considered an important agent for the biosphere homeostasis, which may cause changes in the physiological and morphological characteristics and the allergenic properties of saprophytic and pathogenic fungi. Such changes have been shown in *Cryptococcus neoformans* and *Candida albicans* during colonization and infection [17]. In addition, the effect of CO_2 pressure on growth and aflatoxin production in *Aspergillus flavus* was previously reported [18-21]. Other studies revealed that increasing CO_2 atmosphere in the culture medium could influence the pathogenicity of *A. fumigatus* [22, 23]. Due to the effectiveness of CO_2 pressure as one of the environmental factors, we examined the effects of different CO_2 concentrations on morphology and induction of antifungal resistance in *A. fumigatus*.

Materials and Methods

A. fumigatus strain

The wild-type strain of *A. fumigatus* (ATCC 1028) was cultured on Potato Dextrose Agar (PDA; Merck, Germany) and incubated at 35°C for 5-7 days. The suspension was prepared according to Clinical And Laboratory Standard Institute (CLSI M38-A2) protocol [24]. Briefly, fungal spores were harvested from 3 to 7 day-old cultures (in logarithmic growth phase) and mixed with distilled water containing 0.05% Tween 40; the mixture was adjusted at the final concentration of 1×10^6 CFU/ml by spectrophotometer (530 nm) [24]. Afterwards, 200 µl of the obtained suspension was added to PDA culture medium plates [25]. The plates were incubated at 35°C for 1, 2, and 4 weeks under 1%, 3%, 5%, and 12% CO_2 atmospheres separately.

CO_2 atmosphere was provided by using a 20-L cell culture CO_2 incubator (SLS, USA). The desired CO_2 injection was controlled by the calibrated automatic control panel in this incubator. The control culture was prepared under the same conditions without CO_2 pressure.

Macroscopic and microscopic examinations

Macroscopic and microscopic criteria were investigated during culture under various CO_2 atmospheres. The color and texture features of the colony (i.e., flat, granular, downy to powdery, and radial grooves) were studied during the incubation time. By using Lactophenol Cotton Blue, the microscopic criteria were considered to assess the morphological changes [26, 27]. All the cultures revealing morphological changes under CO_2 atmospheres were inoculated in the Tryptic Soy Broth (TSB) medium (Merck, Germany) and stored at -20°C. After three weeks, the stocked cultures in TSB medium were sub-cultured in PDA medium and incubated at 35°C without CO_2 atmosphere, and their morphological aspects were checked for reversibility evaluation.

Antifungal susceptibility test

The minimum inhibitory concentrations (MICs) of three antifungal agents were evaluated according to CLSI (M38-A2) protocol [24, 28]. Itraconazole (Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer, NY, USA), and amphotericin B (Bristol-Myers-Squibb, Woerden, the Netherlands) were obtained as reagent-grade powders dissolved in dimethyl sulfoxide (DMSO) and were diluted in standard RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) buffered to pH 7.0 with 0.165 $mol.L^{-1}$ morpholinepropanesulfonic acid buffer with L-glutamine without bicarbonate (MOPS, Sigma-Aldrich, St. Louis, MO, USA). The final concentrations of itraconazole, voriconazole, and amphotericin B ranged from 0.016 to 16 µg/ml. The plates were stored at -20°C prior to use [29]. All the cultured fungal cells under CO_2 pressure were scraped after sporulation and the obtained spores were suspended in distilled water containing 0.05% Tween-40. The optical density (OD) of the supernatant was adjusted spectrophotometrically at 530 nm. The final concentrations of the stock inoculum suspensions were within the range of $0.5\text{-}4 \times 10^4$ CFU/ml. Microdilution plates were incubated at 35°C and examined visually for MIC determination within 48 h of incubation. The MICs for itraconazole, voriconazole, and amphotericin B were defined as the concentration that fully inhibits fungal growth. The strain *Candida parapsilosis* ATCC 22019 was used as the negative quality control. All the tests were performed in duplicate.

This study was approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran (code: IR.TUMS.SPH.REC.1396.2289).

Results

We observed significant changes in the macroscopic and microscopic characteristics of *A. fumigatus* wild-type in different situations (CO_2 atmosphere and incubation duration). The initial macro- and micro-colony changes occurred in the presence of low CO_2 pressure (1%). However, the minimum of such changes were observed under 3% and 5% CO_2 atmospheres after one, two, and four weeks, while maximum changes happened under 12% CO_2 atmosphere after four weeks. The major macroscopic changes appeared in the culture media that included shallow colonies with dark green color, smooth and crisp to powdery appearance, and reduced growth. However, the major microscopic changes appeared in exposed fungal cells with short

conidiophores, deformed vesicles, and declined conidia (Figure 1). These results were obtained by comparison with the control colonies, which were blue-green, fast-growing, fluffy to powdery colonies with normal conidiophores, arranged vesicles, phialides, metulae, and smooth hyaline conidia (Figure 1). The reversibility of morphological changes was reviewed through culture of the samples after four weeks in the absence of CO_2 atmosphere. The obtained results indicated that *A. fumigatus* could maintain the described changes even in the absence of CO_2 atmosphere.

The MIC values for itraconazole, voriconazole, and amphotericin B under various CO_2 atmospheres and incubation times are listed in Table 1. The MIC values after 48 h of incubation increased with elevated CO_2 pressure and prolonged incubation time. Under 12% CO_2 atmosphere and after four weeks of

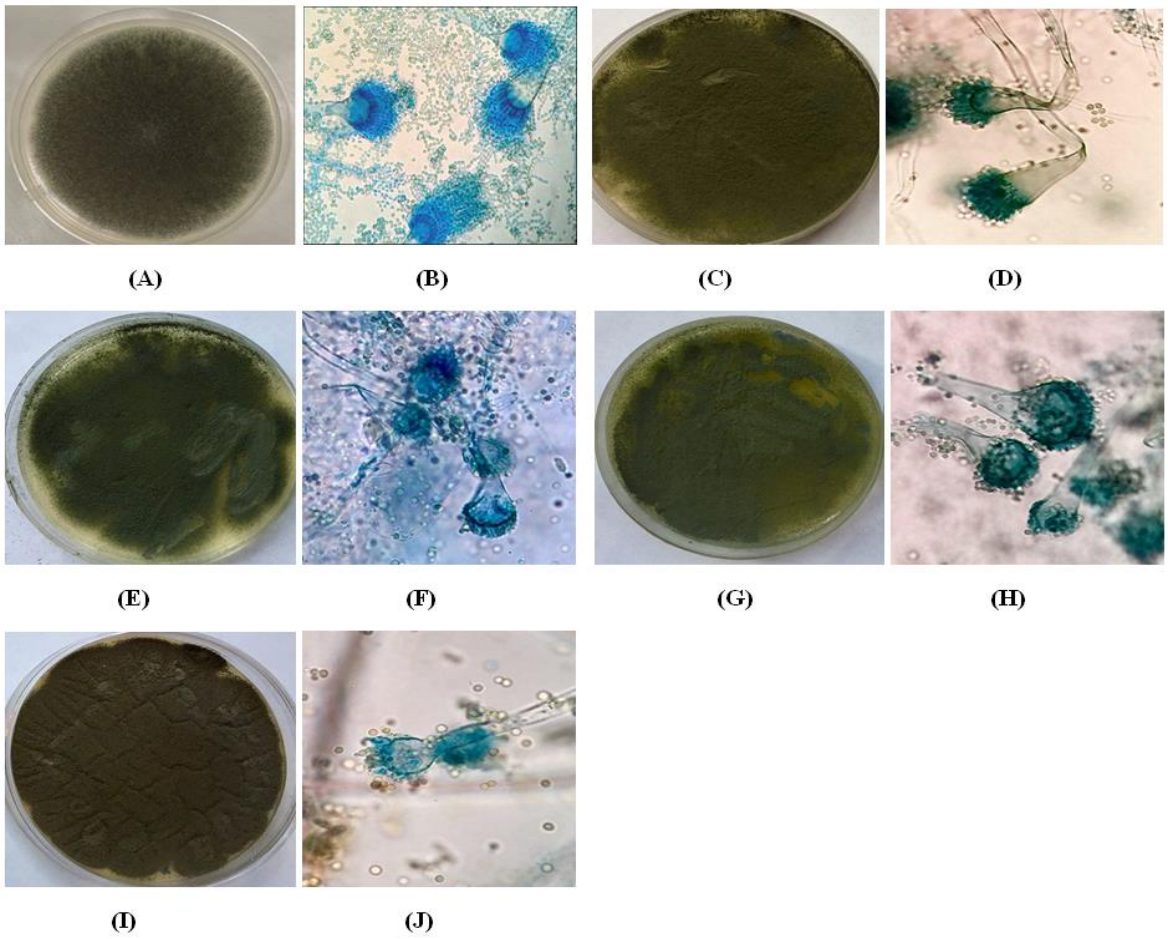

Figure 1. Photographs of macroscopic morphological changes of *A. fumigatus* following exposure to different levels of CO_2 after four weeks. (A) Macroscopic examination with the original colonie; (B) microscopic examination with the normal features of conidiation; (C, D) inception of changes were observed under 1% CO_2 atmosphere, (E, F) 3% CO_2 atmosphere, (G, H) 5% CO_2 atmosphere, and (I, J) the most changes occurred under 12% CO_2 atmosphere.

Table 1. In vitro susceptibility of *A. fumigatus* to itraconazole, voriconazole, and amphotericin B under different CO_2 atmospheres

Strain	CO_2 level	Time (week)	Minimum inhibitory concentration (µg/ml)		
			ITR	VOR	AMB
A. fumigatus (ATCC 1028)	1% CO_2	1	0.125	0.5	4
		2	0.5	0.125	4
		4	1	0.125	8
	3% CO_2	1	1	0.5	8
		2	1	0.125	8
		4	1	0.5	8
	5% CO_2	1	0.5	0.5	8
		2	2	0.5	8
		4	8	0.5	16
	12% CO_2	1	1	0.5	8
		2	2	0.5	8
		4	16	1	16
	Control cultures		0.125	0.125	1

Abbreviations: ITR: itraconazole; VOR: voriconazole; AMB: amphotericin B

incubation, the MIC values for itraconazole, voriconazole, and amphotericin B were 16 μg/ml, 1 μg/ml, and 16 μg/ml, respectively. However, these values were 0.125 μg/ml, 0.125 μg/ml, and 1 μg/ml, respectively, in the control group (Table 1).

Discussion

A. fumigatus is one of the most important fungal pathogens and the main causative agent of invasive aspergillosis. This species is affected by environmental conditions such as CO_2 [23, 30]. Studies showed that CO_2 could significantly affect fungal physiology and morphology [31]. Previous studies on the effects of CO_2 atmosphere on pathogenesis and metabolism of *C. albicans* and *Cryptococcus neoformans* revealed the induction of invasion to blood in *C. albicans* cells under 5% CO_2 atmosphere. Besides, filamentation and pseudohyphal formation were reported to be induced in such cells [25, 32, 33]. Capsular polysaccharide synthesis of *C. neoformans* was stimulated after exposure to 5% CO_2 atmosphere [23, 34-36]. Another study indicated the occurrence of arthroconidium formation, a resistance factor in *T. rubrum*, after 10 days of incubation on Sabouraud Dextrose Agar medium at 37°C under 10% CO_2 atmosphere [37]. Some investigations have considered the effect of CO_2 on fungal growth and found a general inhibitory effect under CO_2 atmospheres [31]. Coelho et al. reported that high CO_2 pressure could have detrimental effects on growth and metabolism of yeasts, and therefore, could contribute to the inactivation of *Saccharomyces cerevisiae* cells [38, 39]. Due to its antimicrobial activity, CO_2 can be particularly used for storing food commodities through the inactivation of the microorganisms [40]. Changed CO_2 atmosphere, carbonic anhydrases (CAs), and C: N ratio have been reported to affect growth by influencing the production of proteins and the content of fungal spores [41, 42]. The effects of CO_2 on morphology, growth, and citric acid production in *A. niger* were also reported by McIntyre and McNeil [31].

Recent investigations indicated that *A. fumigatus* and *A. flavus* spores grown under varying CO_2 atmosphere showed higher allergenicity [22, 32]. In line with the previous investigations, we appraised the effect of CO_2 on morphological changes in *A. fumigatus*. The obtained results indicated that CO_2 atmosphere could enhance some morphological (macroscopic and microscopic) changes such as sporulation, vesicle deformation, and the development of *Chlamydia* spores in *A. fumigatus* strains. The majority of the mentioned changes were also indicated to occur under 12% CO_2 atmosphere and after four weeks of incubation. Previous reports revealed the capability of pathogenic *Aspergillus* species in growth under CO_2 atmosphere; they also showed that this capacity might affect the pathogenicity and antifungal activity of these species [20]. Our findings may help with deeper understanding of this process. Besides, variation in CO_2 atmosphere in environments where *A. fumigatus* is able to grow reveals the importance of effective factor and CO_2 absorb regulator genes in these fungi.

Our results manifested that MICs of itraconazole, voriconazole, and amphotericin B against *A. fumigatus* increased near high CO_2 environments. However, the mechanism of resistance induction by CO_2 remains inconspicuous. Some medicines and environmental factors have been used for the induction of drug resistance in *A. fumigatus* [43]. In some studies, different types of resistance mechanisms, including changes in the target lanosterol-14-demethylase and induction of high-capacity drug efflux pumps, have been shown to promote drug resistance following UV-induced mutations [44-46]. In the study of Escribano et al., the MICs of itraconazole, voriconazole, and posaconazole against *A. fumigatus* cells were determined before and after exposure of the fungal cells to itraconazole. The obtained results indicated that the final MICs were substantially higher than the initial ones [47]. Faria-Ramos et al. investigated cross-resistance in *A. fumigatus* to clinical azoles after the exposure of fungal cells to prochloraz (an agronomical azole) and found that resistance to prochloraz increased after the initial exposure [48]. In addition, prochloraz exposure caused some morphological changes in fungal colony such as changing the appearance to white-colored colonies, losing the typical pigmentation, and absence of conidiation [48]. Our results also indicated the macroscopic and microscopic morphological changes in *A. fumigatus* due to CO_2 pressure. However, these morphological changes were not similar to the alterations described by Faria-Ramos et al.

The results of these studies indicate that *A. fumigatus* can be resistant to antifungal compounds due to antifungal agents in medicine, fungicides used in agriculture, and environmental factors. The results of this study also indicated that high concentrations of CO_2 could be considered as an environmental factor affecting the occurrence of drug resistance in fungi.

Conclusion

Different levels of CO_2 exposure induced morphological changes in *A. fumigatus*, an evident increase in MIC values, and the development of cross-resistance to itraconazole and voriconazole. Our next step is assessing the underlying molecular resistance mechanisms in these induced resistant strains, as well as in isolates with naturally high MIC values in different levels of CO_2 and evaluating resistance to antifungal drugs without any prior in vitro induction.

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Author's contribution

S. R., S.J. H., and S. K. designed and managed the project. S. D. performed the tests and wrote the first draft of the manuscript. S. R. edited the final manuscript. S. S., M. K., M. A.D., and F.A. were the project partner.

Conflicts of interest

None declared.

Financial disclosure

No pecuniary interest related to the material of this writing has been declared.

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