Antifungal Activity of Silver Nanoparticles on Fungal Isolates from Patients of Suspected Mucormycosis

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Abstract

Introduction: The present study aimed to investigate the antifungal activity of Silver Nanoparticles (SNPs) against agents of suspected rhino orbital mucormycosis.

Methods: Thirty-two strains were isolated from Endoscopy guided nasal swab and/ or tissue biopsy after debridement/surgery on Sabouraud dextrose agar without cyclohexamide. Antifungal activity was conducted according to Clinical and Laboratory Standards Institute's (CLSI) guidelines, document M38-A2. The average size of silver nanoparticle was less than 10 nm.

Results: Minimum Inhibitory Concentration (MIC) of nanoparticles of all strains was in the concentration range of 1 *µg/ml* -64 *µg/ml* and minimal fungicidal concentration (MFC) at 16 *µg/ml* -512 *µg/ml*.

Conclusion: The SNPs revealed significant antifungal activity against agents of mucormycosis.

Introduction

As the pandemic of SARS Co-V-2 (COVID-19) continues to be a significant problem worldwide, fungal infections emerged as a new complication.[1] Most common presentations of invasive fungal infection post COVID is rhino orbito cerebral (ROCM) and pulmonary.[2] Mucormycosis is an opportunistic infection affecting patients especially with poorly controlled diabetes whereas aspergillosis can develop in immunocompetent patients as well.[3] Mucormycosis and invasive Aspergillosis both are fulminant diseases as the fungi are angioinvasive, and rapidly destructive. Mucormycosis and Aspergillosis coinfection in the same host is also a known entity. The most frequently isolated species causing rhino-orbital and rhino-orbital-cerebral mucormycosis and aspergillosis are *Rhizopus arrhizus* and *Aspergillus flavus* respectively. High-dose liposomal amphotericin B along with surgical debridement is strongly recommended as first-line treatment with

Nanomaterials with unique physical and chemical properties, including a small size and a high surface area-to-volume ratio, allow for an increased ability to surpass most physiological barriers to therapeutic targets and to interact with pathogen membranes and cell walls.[4] Several nanomaterials have shown anti-microbial activity against *E.coli, P.aeruginosa, B. subtilis, S.aureus*, and *C.albicans* through multiple mechanisms including the interruption of transmembrane electron transfer, disruption of cell envelop oxidization of cell components, or production of reactive oxygen species (ROS).[5]

Silver as a nanomaterial has been used for application with antifungal and antimicrobial agents. Silver nanoparticles are well-known for their biocidal properties, including antibacterial, antifungal, antiviral, and anticancer activities.[5] As nanosilver has shown potential antifungal effect, it may be evaluated for its role in the treatment of mucormycosis.[6]

The objectives of the present study were to evaluate the antifungal activity of Silver Nanoparticles (SNPs) against agents of mucormycosis, and aspergillosis isolated from patients of rhino orbital mucormycosis during the second wave of COVID-19.

Material And Methods

Study design:

This prospective observational study was conducted for a period of 6 months on all isolates from confirmed patients of rhino orbital mucormycosis presenting to a tertiary care hospital in Faridabad, India from May 2021 to October 2021 during the second wave of Covid 19. The study was conducted at the Department of Microbiology, ESIC Medical College and Hospital, Faridabad.

Clinical Specimen:

Endoscopy guided nasal swab and/ or tissue biopsy after debridement/surgery were collected from patients suspected of having rhino orbital mucormycosis were sent to microbiology laboratory for direct KOH mount and culture.

Specimen processing:

Tissue specimens were cultured on Sabouraud dextrose agar without cyclohexamide at 37°C and 25°C for 3 weeks. Fungal growth was identified on the basis of colony morphology and microscopic appearance on lactophenol stain by slide culture technique.

Quality control:

Aspergillus flavus ATCC 204304 and *Aspergillus fumigatus* ATCC 204305 were used as quality control strains. Antifungal activity of Ag-NPs was investigated according to Clinical and Laboratory Standards Institute's (CLSI) guidelines mentioned in the document M38-A2. [7]

Preparation of culture media for microbroth dilution:

RPMI powder (HiMedia, Mumbai) was dissolved in water and sodium bicarbonate (2 g/l) and added to the medium. Then the medium was filtered and distributed and transferred to tubes and stored at 4˚C.Before using the medium, 1 ml glutamine was added to 100 ml medium.

Preparation of suspension of fungi:

Fungal isolates were checked for purity and identified to the species level by studying detailed morphology by slide culture technique. Isolates which were not in their pure form were excluded from the study. *Aspergillus* & *Rhizopus* conidial inoculum suspensions were prepared from well-sporulated cultures (typically 3 days old) grown on potato dextrose agar and adjusted spectrophotometrically to a turbidity that ranged from 0.4 to 0.7 McFarland standards at 530 nm. For the M38-A2 method, the suspension was then diluted with RPMI 1640 broth to twice the density needed for the final inoculum density (0.4 x 10^4 to 5x10^4 CFU/ml), as demonstrated by quantitative colony counts.

Silver nanoparticles:

The average size of silver nanoparticle was less than 10 nm. The SNPs were prepared using Green nanotechnology.[8]

Preparation of SNP serial dilutions:

The silver nanoparticles (SNPs) were procured from Jagsonpal pharmaceuticals in a concentration of 1000 ppm. 1ppm of SNP equals 1 µg/mL using which following dilutions were made: 8 µg/mL, 16 µg/mL, 32 µg/mL ,64 µg/mL, 128 µg/mL,256 µg/mL, 512 µg/mL.[9]

Determination of MIC:

Growth (SNP-free) and fungus-free controls were included along with afore mentioned dilutions of SNP in microtitre plates. The plates were incubated at 35°C and examined for the MICs after 48 h. MIC endpoints were defined as 50% reduction in growth compared to the drug-free wells. MICs were recorded for each isolate.

Determination of MFC:

The minimum fungicidal concentration was determined according to the protocol described by Espinel-Ingroff et al.[9] 20 µL of each well with complete inhibition of fungal growth was withdrawn and cultured in plates with Sabouraud Dextrose Agar for 72 h at 30°C. The MFC was defined as the lowest drug dilution that yielded fewer than three colonies or complete absence of growth.

Statistical analysis

Microsoft excel was used to enter data and perform statistical analysis wherever applicable.

Results

A total of 48 patients were diagnosed with rhinoorbital mucormycosis during 6 months period. Patients were diagnosed on the basis of history, clinical examination, radiological investigation, histopathology, direct KOH mount examination and fungal culture. 32 fungal isolates were retrieved from the confirmed cases. *Rhizopus arrhizus* was isolated alone from 5 patients and *Rhizopus* and *Aspergillus* spp as coinfection infection in 7 cases. However, *Aspergillus* alone was isolated from 12 patients. *Fusarium* spp. was isolated from 1 patient of suspected invasive rhino orbital sinusitis. In 16 cases fungal pathogen could not be isolated on culture.

Minimum inhibitory concentration of SNP

Table I shows antifungal activity of SNPs was evaluated on 32 isolates of *Rhizopus arrhizus, A. niger, A. fumigatus, A. flavus* and *F.oxysporum*. After 48 hrs of incubation, *R. arrhizus* had MIC range from <8 µg /ml -64 µg /ml, *A. flavus* had MIC range from <8 µg /ml -64 µg /ml, *A. fumigatus* had MIC range from 8 µg /ml -32 µg /ml, *A.niger* MIC of < 8 µg /ml and *F.oxysporum* MIC as 32 µg /ml.MIC 50 & MIC 90 of *Aspergillus* spp was 16 µg/ml & 64 µg/ml respectively whereas MIC 50 & MIC 90 of *Rhizopus* spp was 16 µg/ml each.

The minimum fungicidal concentration

R. arrhizus had MFC range from 16µg /ml -512µg /ml, *A. flavus* had MIC range from 32µg /ml -256µg /ml, *A. fumigatus* had MFC range from 64 µg /ml -256 µg /ml, *A.niger* MFC of 32 µg /ml-256 µg /ml and *F.oxysporum* MFC as 64 µg /ml.

Discussion

Liposomal Amphotericin B in initial dose of 5mg/kg body weight (10 mg/kg body wt in case of CNS involvement) is the treatment of choice for invasive mucormycosis. Amphotericin B is associated with renal insufficiency, hypokalemia, hypomagnesemia, hypocalcemia, and hypophosphatemia. With limited armour of antifungals available, the need for alternate agents is on the rise. SNP have been shown to be anti-mycotic activity against pathogenic yeast and dermatophytes and non-toxic to human keratinocytes.[10]

Earlier studies on SNPs have revealed significant antifungal activity against amphotericin B-resistant *C. glabrata* strains. Results of antifungal activity against resistant *C. glabrata* strains after exposure to Ag-NPs with inhibitory action at a 0.125–0.5 µg/ml concentration .[11]

The mechanism of inhibitory effect of SNP on microorganisms is that they are capable of changing membrane and cell wall structure in the resistant strains, possibly through pore formation on the membrane and cell wall. [12] Some studies have reported that the positive charge on the Ag^+ ion is crucial for its antimicrobial activity through the electrostatic attractions between the negatively charged cell membrane of microorganisms and the positively charged nanoparticles.[13] SNPs have shown to have antifungal therapeutic potential in various studies.[14]

Several applications of SNPs are established in literature. Silver nanoparticles (AgNPs), are utilized in the dental prosthesis matrixes in the field of dental medicine at low concentrations, have been able to selectively destroy cellular membranes. [15]Administration of topical formulations of nanosilver particles in combination with current drugs has been used for treating vaginal candidiasis and preventing the disease recurrence.[14]

However, this is the first study of SNP on agents of mucormycosis and other invasive rhinoorbital mycosis. In a study on toxigenic species of *Aspergillus* species, the MIC 50 of AgNPs against *Aspergillus flavus*, has shown to be 8 µg/mL. [16] SNPs were evaluated for anti-fungal activity against agents of mucormycosis in the current study which showed that they prevented visible growth (MIC) at concentration range <8 µg/ml -64 µg/ml and inhibited fungal growth at 16 µg/ml -512 µg/ml (Table 1). Aspergillus spp. showed MIC at concentration range <8 µg/ml -128 µg/ml and inhibited fungal growth at 32 µg/ml -256 µg/ml (Table 1). Single isolate of fusarium showed MIC & MFC of 32 & 64 µg/ml respectively. The MIC 50 & MIC 90 of *Aspergillus* spp was 16 µg/ml & 64 µg/ml respectively whereas MIC 50 & MIC 90 of *Rhizopus* spp 16 µg/ml each. Other study showing filamentous fungi susceptible to Ag-NPs were clinical isolates of *Aspergillus*, *Alternaria*, and *Fusarium* isolated from fungal keratitis. In this study, MIC90 values of approximately 1 µg/ mL were measured.[17]

Table 1
MIC of SNP against 32 strains of *Rhizopus, Aspergillus* and *Fusarium*

Strains	<8µg/ml		8µg/ml		16µg/ml		32µg/ml		64µg/ml		128µg/ml		256µg/ml		512µg/ml	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Aspergillus sp n=19	1	0	6	0	5	0	5	2	1	3	1	6	0	6	0	0
Rhizopus sp n=12	2	0	3	0	6	1	0	4	1	2	0	4	0	0	0	1
Fusarium sp n=1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0

Conclusions

ROCM is an acute, invasive and often fatal mycosis despite aggressive treatment with systemic antifungal therapy and surgical debridement. Successful outcome of the cases depend on the early diagnosis and prompt surgical intervention. SNPs exhibits potent *in vitro* antifungal activity against agents of rhino orbital mucormycosis. This may be exploited in the treatment of fungal infections by local instillation by nasal spray when used within therapeutic limits at early stage of the disease. Further studies may be needed to confirm the

antimycotic role of SNPs against agents of mucormycosis as it works regardless of their antifungal resistance mechanisms and taking into consideration the risk of toxicity.

Declarations

- ETHICS APPROVAL AND CONSENT TO PARTICIPATE

"This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of ESIC Medical College & Hospital Faridabad, India (IEC no.134 X/11/13/2021*)."*

- CONSENT FOR PUBLICATION

Not applicable

- AVAILABILITY OF DATA AND MATERIAL

"Data sharing not applicable to this article as no datasets were generated or analyzed during the current study."

- COMPETING INTERESTS

"The authors declare that they have no competing interests."

- FUNDING

Not applicable

- AUTHORS' CONTRIBUTIONS

"All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Dr Kuhu Chatterjee], [Dr Juhi Taneja], [Dr Shilpa Khullar] and [Dr Anil Kumar Pandey]. The first draft of the manuscript was written by [Dr Juhi Taneja] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript."

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- AUTHORS' INFORMATION

Authors are members of **SIHAM (Society for Indian Human and Animal Mycologists)**

And the laboratory participates in External Quality Assurance Scheme for Mycology.

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