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Susceptibility Evaluation of *Aspergillus fumigatus* to Silver Nanoparticles Compared with Voriconazole

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Background: This study was performed to determine antifungal activity of silver nanoparticles (nano-Ag) compared to voriconazole on clinical and standard strains of *Aspergillus fumigatus*.
Materials and Methods: Inhibitory potency of nano-Ag was determined using microtiter broth dilution method. Susceptibility tests were performed against *A. fumigatus* isolated from BAL (bronchoalveolar lavage) of patients who suffered from respiratory problems and compared with the strain (ATCC: 204305) by broth dilution antifungal susceptibility test of filamentous fungi approved by the Clinical and Laboratory Standards Institute M38-A. In addition, cytotoxicity effect of silver nanoparticles was studied on epithelial cell line by MTT assay.
Results: From 60 BAL samples the following strains were isolated; *A. flavus* (n=21), *A. niger* (n=3), and *A. fumigatus* (n=1). The minimum inhibitory concentration (MIC_{90}) values of nano-Ag were 0.25 and 0.5 $\mu g.mL^{-1}$ for standard strain and clinical isolates respectively. The Minimum Fungicidal Concentration (MFC) values of nano-Ag were 0.5 and 1 $\mu g.mL^{-1}$for standard strain and clinical isolates respectively. MIC90 values of voriconazole were 0.125 and 0.25 $\mu g.mL^{-1}$ for standard strain and clinical isolate respectively. The MFC values of voriconazole were 0.25 and 0 $\mu g.mL^{-1}$ for standard strain and clinical isolates respectively. Silver nanoparticles exhibited low cytotoxicity in 0.25 $\mu g.mL^{-1}$ concentration.
Conclusion: Our results showed high antifungal activity of silver nanoparticles against *Aspergillus* isolates. Furthermore, the availability of a wide form of nano-Ag structures can be considered as novel agents to decrease fungal burden in medical application.

Keywords: Aspergillus fumigatus, Silver nanoparticles, Cytotoxicity

1. Background

Aspergillus spp. conidia are widespread in outdoor and indoor environment, which are breathed every day by humans (1). *Aspergillus* is a saprophyte fungus that plays a significant role as the most common etiologic agent in aspergillosis infections (2). Aspergillosis is associated with a variety of diseases from pneumonia, sinusitis, and allergies to invasive and systemic aspergillosis (IA). The prevalence of IA is between 1-15%, which leads to the 80-90% mortality (3, 4). However, it can be the cause of serious problem in high risk people such as asthmatic and diabetic patients, hospitalized population, and steroid users. Fungus free air doesn't occur, even in well managed environments. Thus, It seems some strategies including control of contamination of hospital indoor space and use of proper prophylaxis decrease the Aspergillus infections.

An increase in these infections occurs due to azole-resistant *Aspergillus fumigatus.* From the health point of view, reduction of fungal burden in the environment with alternative agents can be useful for prevention. They can also be used for coating of devices, filters, and surface of medical tools. Antimicrobial potency of some nanoparticles has been proven; nanosilver is one of the most investigated agents because of its antifungal properties.

2. Objectives

Nanoparticles have a high area for surface atoms; thus, they have great sites for interaction with other agents, thus, the present study aimed to evaluate antifungal property of AgO against the most frequent etiologic agent of aspergillosis (*Aspergillus fumigatus*).

3. Materials and Methods

3.1. Collection of BAL samples

Bronchoalveolar lavage (BAL) fluid samples were collected from 60 patients with respiratory problems from bronchoscopy Center, Shariati Hospital in Tehran during 8 months, from May 22, 2013 to January 20, 2014.

BAL Samples were transported to the laboratory immediately after bronchoscopy and centrifuged at 3,000 RPM for 20 minutes at room temperature (RT) then supernatant was discarded, and pellets were resuspended in the small amount of supernatant, and direct microscopy and culture were performed. For culture, we used Czapek-Dox agar medium (Merck), and then plates incubated at 32 °C for 48-72 hours. Then the positive cultures were examined for macroscopic and microscopic features, and then the slide culture was performed to exactly determine the species. 22219165

3.2. Fungal suspension

Aspergillus fumigatus (ATCC 204305) and clinical isolates were cultured in Czapek-Dox agar medium (Merck) then cultures were incubated at 32 °C for 48 hours. Then Conidia were collected by Phosphate-buffered saline (PBS) and counted by Neobar chamber, and suspension was prepared at concentration of 1×10^3 ($CFU.mL^{-1}$) conidia.

3.3. Preparation of silver nanoparticles solution

A stock solution of nanosilver with the size of 10-20 nm was prepared from a liquid (L)-form of a nanosilver colloid product (Nano pishgaman Co., Mashhad, Iran). The stock solution was then used to prepare the subsequent dilutions;

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100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19 μg.mL^{-1}, using serial two-fold dilutions.

3.4. Characterization of silver nanoparticles

Scanning Electron Microscopy (SEM) of silver nanoparticles was carried out by standard techniques.

3.5. Preparation of antifungal (voriconazole) solution

Voriconazole powder standard for use as positive control was purchased from Sigma-Aldrich Company.

For preparing a stock solution of (1600 μg.mL^{-1}) voriconazole, 4 mg of the drug was dissolved in 2.5 ml Dimethyl sulfoxide (DMSO). The stock solution was then used to prepare the subsequent dilutions.

3.6. Broth microdilution test

Broth microdilution test was performed according NCCLS recommendation. briefly, one row of a 96-well microplate was marked for each *A. fumigatus* 1 × 10^3 cells inoculation. Serial dilutions of silver nanoparticles was prepared in 10 dilutions in sterile 96-well microtitre plates, 100 μL to each well, so that the first well had the highest concentration (100 μg.mL^{-1}) and the tenth well contained the lowest concentration (0.19 μg.mL^{-1}) of nanoparticles (5). Following this step, 100 μL of conidia suspension was added to each well. The eleventh well was used as the growth control (positive) to be compared with the growth of the other wells, containing 100 μL of conidia suspension and without the silver nanoparticle. The twelfth well acted as sterile control (negative), containing 200 μL DMEM medium (Gibco). The microplate was then incubated at 32 °C for 48 hours.

Like silver nanoparticles, Serial dilution of voriconazole was prepared as following, the first well had the highest concentration (16 μg.mL^{-1}) and the tenth well contained the lowest concentration (0.03125 μg.mL^{-1}) of the drug. Following this step, 100 μL of conidia suspension was added to each well. The eleventh well as the growth control (positive) was used to compare the growth of the other wells, and contained 100 μL of conidia suspension and without the drug. The twelfth well was used as sterile control (negative), containing 200 μL DMEM medium. The microplate was incubated at 32 °C for 48 hours. Then to interpret the results, from each well 10 μl suspension was cultured and incubated at 32 °C for 48 hours. After that, minimum inhibitory concentration at which 90 and 100% of the fungal growth is inhibited, was considered as MIC_{90} and MFC respectively.

3.7. Cell culture

Epithelial cells were purchased from Pasteur Institute of Iran and were cultured in 75 ml flask containing DMEM, FCS 5%, L-Glutamine, and Pen/Strep antibiotic then incubated at 37 °C and 5% CO_2; When the cells formed monolayer, they were trypsinized, and 1×10^3 cells were used for MTT assay.

3.8. MTT assay

Silver nanoparticles were prepared in dilution of 0.5 and 0.25 μg.mL^{-1}. MTT (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) reduction assay is widely used to evaluate cell viability. MTT reduction is interpreted to be indicative of cellular metabolic activity. The MTT (Merk) was dissolved in PBS, filtered, and stored at -20 °C until used. The MTT solution was added to each well at one tenth of its volume. Briefly, 20 μl of MTT (5 mg.mL^{-1} in PBS) was added to wells, and each plate was incubated for 4 hr. Then the supernatants were gently removed, and 10 μl Dimethyl sulfoxide (DMSO) was added in order to dissolve the formazan crystals generated with MTT reduction by the living cells. The plates were incubated for 20 min at 37 °C, and the absorbance was read at 540 nm on a microplate LabSystem Multiskan MS reader. The result of the test was expressed as a Stimulation Index (SI).

4. Results

4.1. Fungal isolates

From 60 BAL samples the following strains were isolated; *A. flavus* (n=21), *A. niger* (n=3), and *A. fumigatus* (n=1) (Figure 1).

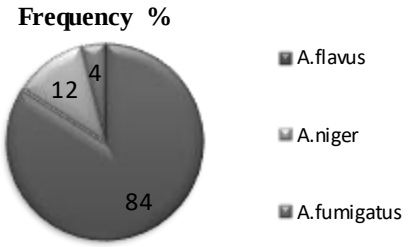

Figure 1. Frequency of *Aspergillus* species isolated from BAL samples.

4.2. Characterization of silver nanoparticles

For determination of antifungal assay of silver nanoparticles, characterization was carried out by scanning electron microscope as shown in Figure 2.

SEM micrograph showed spherical particles of 10-20 nm.

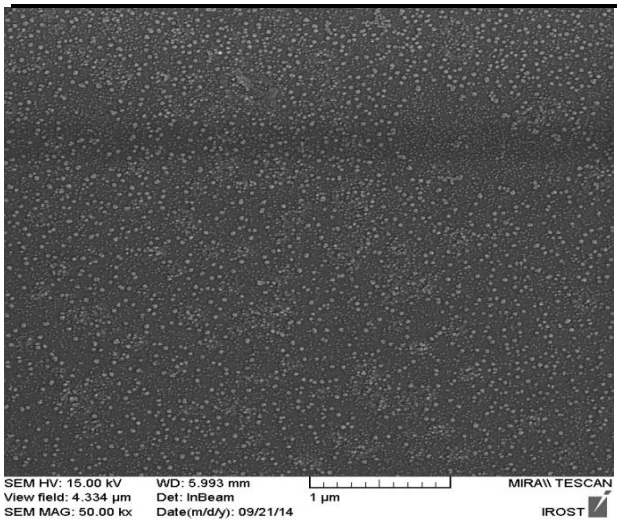

Figure 2. Scanning Electron Microscopy (SEM) of silver nanoparticles

4.3. Antifungal susceptibility

Microtiter assay was conducted according to National Committee for Clinical Laboratory Standards (NCCLS) guideline (5), and the MIC_{90} and MFC values of silver nanoparticles were compared with selected antifungal drug (voriconazole) against standard and clinical isolates of *A. fumigatus* as shown in the Table 1 and 2.

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Table 1. MIC_{90} value and MFC value of nano-Ag

organism	Total spore inoculum	MIC90 ($\mu g.mL^{-1}$)	MFC ($\mu g.mL^{-1}$)
A .fumigatus (ATCC204305)	1×10^3	0.25	0.5
A. fumigatus (Clinical isolate)	1×10^3	0.5	1

Table 2. MIC_{90} value and MFC value of voriconazole

Organism	Total spore inoculum	MIC90 ($\mu g.mL^{-1}$)	MFC ($\mu g.mL^{-1}$)
A. fumigatus (ATCC204305)	1×10^3	0.125	0.25
A. fumigatus (Clinical isolate)	1×10^3	0.25	0.5

Results showed that both were sensitive to voriconazole, and silver nanoparticles inhibited the fungal growth by two-fold MIC in comparison with voriconazole.

Microdilution method has been accepted for the detection of antifungal activity according to CLSI guideline (5)

4.4. Cytotoxicity of nano-Ag

Silver nanoparticles presented higher cytotoxicity to the epithelial cells at the concentration of 0.5 $\mu g.mL^{-1}$ than at 0.25 $\mu g.mL^{-1}$.

5. Discussion

Infection due to *Aspergillus* spp. is one of the most common fungal diseases in human and animals. *A. fumigatus* is also responsible for acute and chronic pulmonary and rhinosinusitis diseases, and microscopic findings of this study showed that unlike in many countries, *A. flavus* has higher frequency in Iran. However, *A. fumigatus* is the most common etiological agent of aspergillosis because of its multiple virulence factors. Overall, *A. fumigatus* is responsible for various forms of aspergillosis.

Since 1920s, many studies have been conducted on antimicrobial activity of silver. In ancient Romans, silver was used for the treatment of some diseases. In 1920s, drug administration approved colloidal silver for wound healing (6).

The use of silver as an antimicrobial agent has been accepted in many societies as a belief. On the other hand, it is generally accepted that silver nanoparticles have better properties as nanostructures. The current study used nano-Ag with 10-20 nm diameters (Fig. 1), and the MIC results (Table 1 and 2) showed that it has antifungal potency, especially in comparison with voriconazole (a known drug). The MIC of nano-Ag was twofold higher than the MIC of voriconazole.

Previous studies have shown that Nano-Ag particles have antifungal activity in *Candida* spp. (7). Silver nanoparticles exhibit antibacterial activity against both gram-positive and gram-negative bacteria (8, 9).

It is accepted that antimicrobial action of nanoparticles increases with decreasing particle size. It can be concluded that silver nanoparticles have great potency of antifungal activity. It was found that it correlated with the small size of silver nanoparticles and their higher reactivity (10). Several studies have been carried out on synthesis procedure, concentration of nanoparticles, and the presence or absence of capping agents that determine function and toxicity of particles (11). In our study, investigation of viability of cells by the MTT assay showed that toxicity of nano-Ag with this size was low and reduced to 0.25 ($\mu g.mL^{-1}$) concentrations.

It is proposed that nano-Ag disrupts fungal cell wall and increase permeability of its wall.

Voriconazole remains a clinically important agent on fungal diseases; however, as other azoles, it can lead to development of resistance after long-term use. Dose-limiting toxicity, drug resistance, and spread of *Aspergillus* spores in our environment require introduction of other agents. Nanoparticles such as Ago have highly potent antifungal activity (12, 13).

Azoles are the drugs of choise for therapy in the management of fungal infections, including aspergillosis (14, 15). the antifungal susceptibility results showed that the MIC_{90} nano-Ag for *clinical isolate of A. fumigatus* and *A .fumigatus* (ATCC204305) was 0.5 $\mu g.mL^{-1}$ and 0.25 $\mu g.mL^{-1}$ respectively. The MFC values of nano-Ag were 0.5 and 1 $\mu g.mL^{-1}$for the standard species and clinical isolates respectively.

SEM micrograph revealed that the particles were found almost spherical and were not aggregated.

We assayed the level of toxicity of nano-Ag on epithelial cells which showed lower toxicity at the concentration of 0.25 ($\mu g.mL^{-1}$) than 0.5 ($\mu g.mL^{-1}$). Nowadays fungal infections have significantly increased in human society for many reasons. Aspergillosis (especially invasive and allergic aspergillosis) has emerged as a complicated problem in several patient populations. The increasing rates of immunocompromised status in host, drug resistance, the presence of *Aspergillus* spores worldwide on many surfaces such as medical devices are enough reasons for aspergillosis infections.

An increase in these infections occurs due to azole-resistant *A. fumigatus.* From the health point of view, reduction of fungal burden on the environment with alternative agents can be useful in hospital environment, on devices, filters, and surface of medical tools.

Voriconazole resistance is problematic for immunecompromised patients (16); thus, alternative agent must be introduced to decrease the fungus burden. The availability of a wide range of nanostructures can provide suitable alternatives.

6. Conclusion

In this research, our data support the high antifungal activity of silver nanoparticles against *Aspergillus* isolates compared to voriconazole and this nanoparticle can be used as antifungal drug however more studies are required in the future.

Conflict of Interests

The authors declare they have no conflict of interest.

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Authors' Contribution

All of authors contribute to this study.

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