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Comparison of Difference Between Fluconazole and Silver Nanoparticles in Antimicrobial Effect on Fluconazole-Resistant *Candida Albicans* Strains

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Background: Opportunistic fungi cause fungal infections. Whereas some microorganisms are resistant to chemical drugs, scientists are looking for new natural and inorganic antimicrobial agents. The recent research on metal nanoparticles showed that silver nanoparticles (nanosilver) exhibits lower toxicity to mammalian cells and higher toxicity to microorganisms.
Objectives: This study aimed to compare the difference between antimicrobial effect of nanosilver and some antibiotic agents on *Candida albicans*.
Materials and Methods: We studied effect of fluconazole, nanosilver, and their combination on 20 fluconazole-resistant *C. albicans* from two centers and one standard sample (ATCC10261) by minimal inhibitory concentration (MIC) method.
Results: Result of fungi static and fungicidal activities of nanosilver plus fluconazole on fluconazole-resistant *C. albicans* showed better inhibitory effect on the growth of standard *C. albicans* when MIC of fluconazole (8 μg/mL) combined with MIC of Nanosilver (0.0625 μg/mL).
Conclusions: Totally, our results showed nanosilver caused an increase of at least nine-fold in inhibitory effect of fluconazole.

Keywords: Fluconazole; Silver; Nanoparticles; *Candida albicans*

1. Background

In recent years, morbidity and mortality are increased significantly by severe fungal infections (1). *Candida* species have been one of the most common pathogens responsible for fungal infections, which cause hospital-acquired sepsis with annually mortality rate of up to 40% (2). Opportunistic fungi cause fungal infections, especially in vulnerable people with special conditions such as pregnancy or HIV-positive and immune-compromised patients who need intensive treatment with broad-spectrum antibiotics (3-5). Nowadays most of the available effective antifungal agents are based on polyenes (amphotericin B), echinocandins (caspofungin, micafungin, and anidulafungin) and triazoles (fluconazole, itraconazole, voriconazole, and posaconazole) (6, 7). However, scientists are looking for new natural and inorganic antimicrobial agents (8, 9). The recent research on metal nanoparticles showed that silver nanoparticles (nanosilvers) have received special attention as a possible antimicrobial agent (10-16). Since ancient times, silver has been used widely to treat infections and has strong inhibitory effects as well as a broad spectrum of antimicrobial activities against microorganisms, which has been thoroughly investigated (1, 9, 15, 17). This toxicity effect on bacteria has been investigated for more than 60 years (16) and in comparison to other metals, silver exhibits lower toxicity to mammalian cells and higher toxicity to microorganisms (18). Nanosilver exerts antimicrobial effects through interacting with main components of microorganisms including DNA (19), microbial proteins (20), and cell wall (20, 21); moreover, nanosilver produces reactive oxygen species (ROS) (21). The accumulation of intracellular ROS is as an important regulator for starting early apoptosis phase (22). Subsequently, increasing level of intracellular ROS lead to initiation of mitochondrial fragmentation (23).

2. Objectives

Regarding comparison of difference between antimicrobial effect of nanosilver and some antibiotic agents on *Candida albicans*, we compared the effect of nanosilver with fluconazole and their combination on collected fluconazole-resistant and fluconazole-sensitive *C. albicans*.

3. Materials and Methods

In this experimental study, 18 fluconazole-resistant *C. albicans* isolated from the patient's blood at the Shiraz University of Medical Sciences, Shiraz, Iran, two fluconazole-resistant *C. albicans* isolated from the patient's blood at the Mycology Laboratory of Tehran University, Tehran, Iran, and one standard sample (ATCC10261) were used. We studied effect of fluconazole, nanosilver, and their combination on these *C. albicans* samples. The yeasts were identified using conventional mycological procedures.

In first stage, to identifying the fluconazole minimal inhibitory concentration (MIC) we used Clinical and Laboratory Standards Institute (CLSI) (M27-A3) protocol "Broth micro dilution", which included:

1. Preparation of fluconazole stock solution (2048 μg/mL; potency, 999 μg/mL) (Pars Daru, 99.95% potency): Briefly, 21.5 mg of fluconazole was dissolved in 500 μL of absolute ethanol, and 10 mL of distilled water was added. The stocks were stored in -20°C.

2. Preparation of RPMI 1640 Medium: Briefly, 1.73 g MOPS [3- (N-morpholino propane sulfonic acid)] was dissolved in 50 mL of RPMI1640 Medium after filtration and was stored in 4°C.

3. Preparation some yeast suspension: After 24 hours incubation at 35°C, five *C. albicans* colonies in PDA (Potato Dextrose Agar) medium were mixed in 5 mL of physiology serum. After 15 second of vortex, they were diluted by RPMI-1640 medium to 1/100 and after that 1/20; therefore, this suspension had 0.5×10^3 to 2.5×10^3 cells.

4. Preparation of serial dilution of fluconazole: Chosen plate had 12 wells; we added 1 mL of RPMI to each one and then added 1 mL of fluconazole stock (2048 μg/mL) to first well. After turning up and down, we infused 1 mL of this well to second well and went on to tenth well. Therefore, tenth well had 0.5 μg/mL of fluconazole stock. Eleventh well had just 1 mL of RPMI (Negative control) and twelfth well as a positive control had 1 mL of RPMI and 100 μL of fungi suspension.

5. Identification of drug sensitivity tests: We added 100 μL of yeast suspension to each well except negative control, and incubated them at 35°C for 48 hours.

In second stage, we used this protocol to identify the nanosilver's MIC:

1. Preparation of Nanosilver stock solution (100 μg/mL): *Klebsiella pneumoniae* was injected in Muller Hinton, incubated in 37°C for 24 hours, and then centrifuged at 12000 rpm for five minutes. Then 1 mL of floating liquid was added to 100 mL of silver nitrate (AgNO3, 1 mM) and after five minutes, existence of nanosilver was shown by color changing to light brown. Spectrum analysis was performed by spectrophotometer UV-Vis (Model Sessile 9200; resolution, 1 nm; Japan). Specification of nanosilver was illustrated by transmission electron microscope (model EM Philips, Eindhoven, the Netherlands) and energy-dispersive spectroscopy.

2. Preparation of RPMI 1640 Medium: Briefly, 1.73 g of MOPS [3- (N-morpholino propane sulfonic acid)] was dissolved in 50 mL of RPMI1640 medium after filtration and was stored at 4°C.

3. Preparation of some yeast suspension: After 24 hours of incubation in 35°C, five *C. albicans* colonies in PDA medium (diameter, 1 mm) were mixed in 5 mL of physiology serum. After 15 second of vortex, they were diluted by RPMI medium to 1/100 and after that 1/20. Therefore, this suspension had 0.5×10^3 to 2.5×10^3 cells.

4. Preparation serial dilution of Nanosilver: Selected plate had nine wells. We added 1 mL of RPMI to each on and then added 1 mL of nanosilver stock solution (64 μg/mL) to first well. After turning it up and down, we infused 1 mL of this well to second well and went on to seventh well. Therefore, seventh well had 0.5 μg/mL of Nanosilver stock solution. Eighth well had just 1 mL of RPMI (Negative control) and ninth well, as a positive control, had 1 mL of RPMI and 100 μL of fungi suspension.

5. Identification of drug sensitivity tests: We added 100 μL of yeast suspension to each well except negative control, and incubated them at 35°C for 48 hours.

In third stage, to identifying the MIC of nanosilver plus fluconazole was performed as follows:

1. Preparation of Fluconazole plus Nanosilver stock solution Fluconazole (2048 μg/mL) diluted by RPMI medium solution to 16 μg/mL, and Nanosilver stock solution (100 μg/mL) diluted by distilled water to 4 μg/Ml.

2. Preparation of RPMI 1640 Medium: Briefly, 1.73 g of MOPS [3- (N-morpholino propane sulfonic acid)] was dissolved in 50 mL of RPMI1640 medium and after filtration, was stored at 4°C.

3. Preparation of yeast suspension: After 24 hours incubation at 35°C, five *C. albicans* colonies in PDA medium (diameter, 1mm) were mixed in 5 mL of physiology serum. After 15 seconds of vortex, they were diluted by RPMI medium to 1/100 and after that 1/20. Therefore, this suspension had 0.5×10^3 to 2.5×10^3 cells.

4. Preparation of serial dilution of fluconazole plus nanosilver: chosen plate had 12 wells. We added 1 mL of RPMI to each one and then added 1 mL of nanosilver stock solution (4 μg/mL) to first well; After turning up and down, we infused 1 mL of this well to second well and went on to sixth well. Therefore, sixth well had 0.0625 μg/mL of Nanosilver stock. Seventh well had just 1 mL of RPMI (Negative control) and eighth well, as a positive control, had 1 mL of RPMI and 100 μL of fungi suspension. Then we added 1 mL of fluconazole (16 μg/mL) to each one except seventh and eighth wells.

5. Drug sensitivity tests: we added 100 μL of yeast suspension to each well except negative control, and incubated them at 35°C for 48 hours.

4. Results

Result of fungi static and fungicidal activities of fluconazole against *C. albicans* showed: 1) The MIC of fluconazole concentration for standard sample was 16 μg/mL (Table 1).

2) The growth of 20 fluconazole-resistant *C. albicans* was inhibited at MICs > 512 μg/mL (Table 1).

Result of fungi static and fungicidal activities of nanosilver against *C. albicans* showed: 1) The MIC of nanosilver for standard sample was 4 μg/mL. (Table 2). 2) The MICs of nanosilver for 20 resistant *C. albicans* were 2 μg/mL (58%) and 4 μg/mL (42%).

Result of fungi static and fungicidal activities of nanosilver plus fluconazole (8 μg/mL) on *C. albicans* showed: 1) the combination had better inhibitory effect on the growth of standard *C. albicans* when MIC of fluconazole (8 μg/mL) was combined with MIC of Nanosilver (0.0625 μg/mL) (Table 3). 2) Results on 20 resistant *C. albicans* showed there are several MIC of nanosilver: 40% of resistant *C. albicans* samples grew on 0.25 μg/mL, 11% on 0.0625 μg/mL, 22% on 0.03125 μg/mL, and 27% had no growth on 0.03125 (Table 3).

5. Discussion

Between azoles, Fluconazole has excellent in vitro activity against *C. albicans* at a wide range of body sites and tissues (24, 25). In addition, fluconazole is effective against some other *Candida* species, including *Candida tropicalis*, *Candida parapsilosis,* and *Candida glabrata* (25, 26). While the most important problem in treatment by the chemical antimicrobial agents is multidrug resistance, silver, as an antimicrobial agent in various fields, has brought some hope (17). It is well known that inorganic drug such as silver ions and its compounds have strong antimicrobial effects (27). In our study, the MICs of fluconazole and nanosilver against Standard *C. albicans* were 8 μg/mL and 2 μg/mL, respectively. Investigation of our finding shows the followings:

1) The study of results in first stage showed that MIC for standard and drug resistant *C. albicans* were 256 to 512 μg/mL and ≥ 64 μg/mL, respectively. Moreover, these results were similar to another researches such as a study by Pfaller et al. that reported MIC ≥ 64 μg/mL (28), or study by Enwuru et al. on HIV-positive patients that showed fluconazole's MIC of 64 μg/mL against *C. albicans* (29).

2) In second stage, comparison between MIC of nanosilver and fluconazole showed that nanosilver inhibited *C. albicans* growth seven-fold to nine-fold more than fluconazole did.

3) In third stage, the MIC analysis showed that nanosilver combined with fluconazole had the most effective activity against *C. albicans*. Another study by Kim et al. in 2008 showed that amphotericin plus nanosilver and fluconazole plus nanosilver were the most effective combinations against trichophyton/mentagrophytes and *C. albicans,* respectively (30).

According to our study, fluconazole with insignificant amount of nanosilver exhibit higher antifungal activity against pathogenic *C. albicans* compare with fluconazole and nanosilver alone. The nanosilver inhibits growth of these fungi at very low concentrations that are

Table 1. The Growths of Standard and Fluconazole-Resistant *Candida albicans* on Difference Fluconazole Concentrations

Variables	Fluconazole Concentrations, μg/mL										
	0.5	1	2	4	8	16	32	64	128	256	512
Resistant *Candida albicans* samples	+	+	+	+	+	+	+	+	+	+	+
Standard sample	+	+	+	+	+	-	-	-	-	-	-

Table 2. The Growths of Standard Sample and Fluconazole-Resistant *Candida albicans* on Difference Nanosilver Concentrations

Variables	Nanosilver Concentrations, μg/mL							
	0.5	1	2	4	8	16	32	64
58% of resistant *Candida albicans* samples	+	-	-	-	-	-	-	-
42% of resistant *Candida albicans* samples	+	+	-	-	-	-	-	-
Standard sample	+	+	+	-	-	-	-	-

Table 3. The Growth of Standard and Fluconazole-resistant *Candida albicans* on Difference Nanosilver Concentrations Combined with 8 μg/mL Fluconazole

Variables	Nanosilver Concentrations (0.5-0.0625 μg/mL) Plus 8 μg/mL of Fluconazole				
	0.015625	0.03125	0.0625	0.125	0.25
22% of resistant *Candida albicans* samples	+	+	-	-	-
11% of resistant *Candidaalbicans* samples	+	+	+	-	-
40% of resistant *Candida albicans* samples	+	+	+	+	+
27% of resistant *Candida albicans* samples	+	-	-	-	-
Standard sample	+	-	-	-	-

comparable to those of current antifungal drugs. Although the nanosilver exhibit no significant cytotoxic effects on human fibroblasts in these concentrations (31), clinically prescription of these particles needs more clinical trial studies.

Authors' Contributions

Shadi Alimehr: study concept and design, acquisition of data, and drafting the manuscript. Sassan Rezaie: study concept and design and data acquisition. Ahmadreza Shahverdi: administrative, technical, and material support. Jamal Hashemi: study supervision. Kamyar Zomorodian: administrative, technical, and material support. Maryam Moazeni and Sahar Vosoghian: data acquisition, analysis, and interpretation. Hamide Shekari Ebrahim Abad: critical revision of the manuscript for important intellectual content.

References

1. Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev.* 2007;**20**(1):133–63.
2. Patterson TF. Focus on candidemia. New York: Applied Clinical Education; 2007. Treatment and prevention of fungal infections.
3. Nasrollahi A, Pourshamsian K, Mansourkiaee P. Antifungal activity of silver nanoparticles on some of fungi. *Int J Nano Dim.* 2011;**1**(3):233–9.
4. Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med.* 2003;**348**(16):1546–54.
5. Pappas PG, Rex JH, Lee J, Hamill RJ, Larsen RA, Powderly W, et al. A prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. *Clin Infect Dis.* 2003;**37**(5):634–43.
6. Levin MD, den Hollander JG, van der Holt B, Rijnders BJ, van Vliet M, Sonneveld P, et al. Hepatotoxicity of oral and intravenous voriconazole in relation to cytochrome P450 polymorphisms. *J Antimicrob Chemother.* 2007;**60**(5):1104–7.
7. Venkatakrishnan K, von Moltke LL, Greenblatt DJ. Effects of the antifungal agents on oxidative drug metabolism: clinical relevance. *Clin Pharmacokinet.* 2000;**38**(2):111–80.
8. Kim TN, Feng QL, Kim JO, Wu J, Wang H, Chen GC, et al. Antimicrobial effects of metal ions (Ag+, Cu2+, Zn2+) in hydroxyapatite. *J Mater Sci Mater Med.* 1998;**9**(3):129–34.
9. Cho KH, Park JE, Osaka T, Park SG. The study of antimicrobial activity and preservative effects of nanosilver ingredient. *Electrochimica Acta.* 2005;**51**(5):956–60.
10. Silver S. Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. *FEMS Microbiol Rev.* 2003;**27**(2-3):341–53.
11. Baker C, Pradhan A, Pakstis L, Pochan DJ, Shah SI. Synthesis and antibacterial properties of silver nanoparticles. *J Nanosci Nanotechnol.* 2005;**5**(2):244–9.
12. Lee BU, Yun SH, Ji JH, Bae GN. Inactivation of S. epidermidis, B. subtilis, and E. coli bacteria bioaerosols deposited on a filter utilizing airborne silver nanoparticles. *J Microbiol Biotechnol.* 2008; **18**(1):176–82.
13. Melaiye A, Sun Z, Hindi K, Milsted A, Ely D, Reneker DH, et al. Silver(I)-imidazole cyclophane gem-diol complexes encapsulated by electrospun tecophilic nanofibers: formation of nanosilver particles and antimicrobial activity. *J Am Chem Soc.* 2005;**127**(7):2285–91.
14. Sondi I, Salopek-Sondi B. Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria. *J Colloid Interface Sci.* 2004;**275**(1):177–82.
15. Lok CN, Ho CM, Chen R, He QY, Yu WY, Sun H, et al. Proteomic analysis of the mode of antibacterial action of silver nanoparticles. *J Proteome Res.* 2006;**5**(4):916–24.
16. Franke S, Grass G, Nies DH. The product of the ybdE gene of the Escherichia coli chromosome is involved in detoxification of silver ions. *Microbiology.* 2001;**147**(Pt 4):965–72.
17. Kim JS, Kuk E, Yu KN, Kim JH, Park SJ, Lee HJ, et al. Antimicrobial effects of silver nanoparticles. *Nanomedicine.* 2007;**3**(1):95–101.
18. Zhao G, Stevens SJ. Multiple parameters for the comprehensive evaluation of the susceptibility of Escherichia coli to the silver ion. *Biometals.* 1998;**11**(1):27–32.
19. Yang W, Shen C, Ji Q, An H, Wang J, Liu Q, et al. Food storage material silver nanoparticles interfere with DNA replication fidelity and bind with DNA. *Nanotechnology.* 2009;**20**(8):085102.
20. Yamanaka M, Hara K, Kudo J. Bactericidal actions of a silver ion solution on Escherichia coli, studied by energy-filtering transmission electron microscopy and proteomic analysis. *Appl Environ Microbiol.* 2005;**71**(11):7589–93.
21. Park HJ, Kim JY, Kim J, Lee JH, Hahn JS, Gu MB, et al. Silver-ion-mediated reactive oxygen species generation affecting bactericidal activity. *Water Res.* 2009;**43**(4):1027–32.
22. Benaroudj N, Lee DH, Goldberg AL. Trehalose accumulation during cellular stress protects cells and cellular proteins from damage by oxygen radicals. *J Biol Chem.* 2001;**276**(26):24261–7.
23. Pozniakovsky AI, Knorre DA, Markova OV, Hyman AA, Skulachev VP, Severin FF. Role of mitochondria in the pheromone- and amiodarone-induced programmed death of yeast. *J Cell Biol.* 2005;**168**(2):257–69.
24. Goa KL, Barradell LB. Fluconazole. An update of its pharmacodynamic and pharmacokinetic properties and therapeutic use in major superficial and systemic mycoses in immunocompromised patients. *Drugs.* 1995;**50**(4):658–90.
25. Arevalo MP, Arias A, Andreu A, Rodriguez C, Sierra A. Fluconazole, itraconazole and ketoconazole in vitro activity against Candida spp. *J Chemother.* 1994;**6**(4):226–9.
26. Van't Wout JW. Fluconazole treatment of candidal infections caused by non-albicans Candida Species. *Eur J Clin Microbiol.* 1996;**15**:228–42.
27. Furno F, Morley KS, Wong B, Sharp BL, Arnold PL, Howdle SM, et al. Silver nanoparticles and polymeric medical devices: a new approach to prevention of infection? *J Antimicrob Chemother.* 2004;**54**(6):1019–24.
28. Pfaller MA, Diekema DJ, Sheehan DJ. Interpretive breakpoints for fluconazole and Candida revisited: a blueprint for the future of antifungal susceptibility testing. *Clin Microbiol Rev.* 2006;**19**(2):435–47.
29. Enwuru CA, Ogunledun A, Idika N, Enwuru NV, Ogbonna F, Aniedobe M, et al. Fluconazole resistant opportunistic oro-pharyngeal Candida and non-Candida yeast-like isolates from HIV infected patients attending ARV clinics in Lagos, Nigeria. *Afr Health Sci.* 2008;**8**(3):142–8.
30. Kim KJ, Sung WS, Moon SK, Choi JS, Kim JG, Lee DG. Antifungal effect of silver nanoparticles on dermatophytes. *J Microbiol Biotechnol.* 2008;**18**(8):1482–4.
31. Kvitek L, Panacek A, Prucek R, Soukupova J, Vanickova M, Kolar M, et al. Antibacterial activity and toxicity of silver – nanosilver versus ionic silver. *J Phys: Conference Series.* 2011;**304**:012029.