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Antifungal Activity of Selenium Nanoparticles and Selenium Disulfide against Two *Malassezia* Species

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Abstract: Fungi in the genus *Malassezia* are involved in skin disorders, including dandruff and seborrheic dermatitis, two commonly recurring scalp conditions that are often treated with selenium-containing materials. The antifungal effects of SeS_2 and SeNPs against *M. sympodialis* and *M. furfur* were compared. The results showed that SeNPs had higher antifungal activity than SeS_2 against both species. Both preparations were more effective against *M. sympodialis* than against *M. furfur.* Post-antifungal effects were not observed for *M. furfur* after short exposure to either SeNPs or SeS_2. However, cultures treated for a short time with the MIC or lower than the MIC of SeNPs or SeS_2 showed better growth than untreated cultures following incubation with 1/10 MIC or less of either of the compounds. Brief exposure of *Malassezia* spp. to less than the MFC of SeNPs or SeS_2 may result in the proliferation of fungal cells that are less sensitive to selenium and may contribute to the recurrence of infection following treatment.

Keywords: Selenium nanoparticles, *Malassezia*, antifungal activity, post-antifungal effect

Introduction

Yeasts of the genus *Malassezia* are lipophilic fungi of the normal skin flora of humans and other warm-blooded animals, but they may also be the cause of skin disorders, including dandruff and seborrhoeic dermatitis [Aggarwal ,et al.,2003; Anwar et al.,2016; Roques ,et al.,2006; Rudramurthy et al.,2014]. Seborrhoeic dermatitis (SD), covering areas of the scalp, face and trunk, is a superficial inflammatory skin disorder, which affects 1%-10% of the population[Roques et al., 2006]. These diseases are treated with antimycotic shampoos containing various antifungal agents, including selenium disulfide (SeS_2), which has antifungal activity against a variety of fungi, including *Malassezia*spp[Aggarwal ,et al., 2003; Chu et al., 1984; McGinley et al., 1982; Van Cutsem et al.,1990]. A low concentration of selenium is essential to living organisms [Kitajima et al., 2013]. As a trace element, it is incorporated in the amino acids of selenoproteins and antioxidant enzymes and it protects cells from free radicals. However, in higher concentration, it inhibits fungal growth[Keiliszek et al., 2015; Kitajima et al., 2013] The reason for the antifungal activity of selenium in*Malassezia* has not been studied but Wu et al [Wu et al., 2014] found that a high concentration of selenium damages the cellular oxygen-eliminating system of*Penicilliumexpansum,* which leads to an increase in the production of intracellular ROS. In *P. expansum,* the inhibitory effect is related to the selenium concentration used[Wu et al.,2014]. SeS_2 has been shown to have antifungal activity against *Malassezia*[Aggarwal ,et al., 2003,McGinley et al., 1982]. However, after treatment with antifungal agents, including SeS_2, SD tends to relapse. Aggarwal et al treated 20 SD patients once a week for three weeks with 2.5% SeS_2 shampoo and one month after therapy found that three of the patients had mild or considerable residual disease. There is a need for finding more effective compounds for the treatment of S.D. and investigating the reason for the recurrence of infection after SeS_2 treatment.

In recent years, interest in the biological activities of metal nanoparticles has increased and the use of metal nanoparticles as antimicrobial agents has been investigated [Anwar et al., 2016; Ormland et al., 2004; Ren et al., 2009; Sadiq et al., 2009; Tan et al., 2009; Wang et al. 2005;Wang et al. 2007; Gijjar et al., 2009]. SeNPs have been reported to have antioxidant activity [Ormland et al., 2004; Tan et al., 2009; Wang et al. 2005; Zeng et al., 2008; Zhang et al., 2001]. We previously have shown that SeNPs biosynthesized by *Klebsiella pneumoniae* have antifungal activities against several fungi, including *Malassezia*spp[Shahverdi et al., 2010].

In this study, we compared the antifungal effect of SeS_2 and SeNPs for *M. furfur* and *M. sympodialis* and determined the MFCthat results in eradication of these species. Since scalp SD is treated with a selenium-containing shampoo, we also investigated the post-antifungal effect of short exposures of *M. furfur* to SeS_2 and SeNPs on the subsequent growth of this fungus.

Materials and Methods

Synthesis of SeNPs

Biogenic SeNPs were freshly prepared and purified using a method previously described [Shahverdi et al., 2010,Fesharaki et al., 2010]. Briefly, an inoculum of *Klebsiellapneumoniae*[Fesharaki et al., 2010] was prepared by transferring a single colony from a tryptic soy agar plate to TSB (Merck,Darmstadt, Germany) and growing the culture at 37°C to an OD_{600} of 1.0. Fresh TSB, pH 7.2, supplemented with 200mg/l Se^{4+} (equal to 559.19 mg of selenium chloride) was inoculated with 1% (v/v) of a *K. pneumoniae* culture and incubated at 37°C for 24 h. *K. pneumoniae* cells containing red selenium particles were disrupted by autoclaving for 20 min at 121°C and 1.2 kg/cm^2 pressure. The released SeNPs were centrifuged at 25,000 x *g* for 15 min and washed three times with distilled water. The washed sample and SeS_2 were sonicated for 10 min (Tecna6, Tecno-Gaz Industries, Parma, Italy). The sizes of the SeNPs and SeS_2 used during this study were less than 250 nm, as determined by TEM[Shahverdi et al., 2010,Fesharaki et al., 2010].

Comparison of antifungal activity of SeNPs and SeS_2 for Malassezia spp.

Clinical strains of *M. sympodialis* and *M. furfur* were obtained from the Culture Collection of the Department of Medical Mycology and Parasitology, School of Public Health, Tehran University of Medical Sciences, Tehran (Iran). The effects of biogenic SeNPs and SeS_2 (Sigma-Aldrich, Germany), on the survival of *M. sympodialis* and *M. furfur* were separately tested in LNB supplemented with different concentrations (0-300 µg/ml) of SeNPs or SeS_2, according to the antifungal susceptibility testing recommendations of the CLSI. The broth cultures were inoculated with 10^6 CFU/ml of each fungal species and incubated for 120 h at 30°C. The viability of cells of each strain remaining in each culture was determined by plating the culture on LNA and counting the resulting colonies.

MIC and MFC Determination

A conventional serial dilution method was used to determine MIC of both selenium preparations for *M. sympodialis* and *M. furfur* in LNB according to the CLSI. The media contained either SeNPs or SeS_2, in a concentration range of 0 to 300µg/ml, and were inoculated with approximately 10^6 CFU/ml of each species. The minimum concentrations of SeNPs and SeS_2 capable of inhibiting visible growth of the fungal strains during 96-120 h of incubation at 35°C were determined and reported as MICs. The MFC of SeNPs and SeS_2 was measured by plating the cultures used for MIC determinations onto selenium-free LNA plates. After incubation of plates at 35°C for 96-120 h, the lowest concentration of SeNPs or SeS_2 that killed 99.0-99.5% of the cells and resulted either in no growth or fewer than three colonies on the plates, was considered the MFC [Espinol et al., 2002].

Post-antifungal effect (PAFE) of SeNPs and SeS_2

The effect of pre-exposure of *M. furfur* to (50µg/ml) SeNPs and SeS_2 on its growth after removal of the drug was determined by a modification of the Odenholt-Tornqvist method [Espinol et al., 2002]. as follows: SDB medium (Merck,Darmstadt, Germany) containing 50µg/ml of SeNPs and SeS_2 was inoculated with 5 ×10^6 cells/ ml of *M. furfur* separately and incubated for 2 h at 35°C. For controls, cultures of *M. furfur* were grown in SDB medium without selenium. The cultures were diluted with pre-warmed SDB at a ratio of 1/10 and incubated at 35°C for 120 h. Then, the viability of *M. furfur* in cultures with and without selenium compounds was determined by plating the samples on LNA medium and counting the CFUs. All samples were homogenized before CFU determination. The experiments were repeated three times.

Results and Discussion

In this study, we compared the *in vitro* antifungal activity and post-antifungal effect of SeNPs and SeS_2. We found higher antifungal potency of SeNPs for *M. furfur* and *M. sympodialis*in comparison with SeS_2 and showed that neither of the compounds had a PAFE for *M. furfur.* To compare the antifungal activities of biogenic SeNPs and SeS_2, the viability of *M. sympodialis* and *M. furfur* after incubation with different concentrations of these compounds was determined (Figure 1, Figure 2).

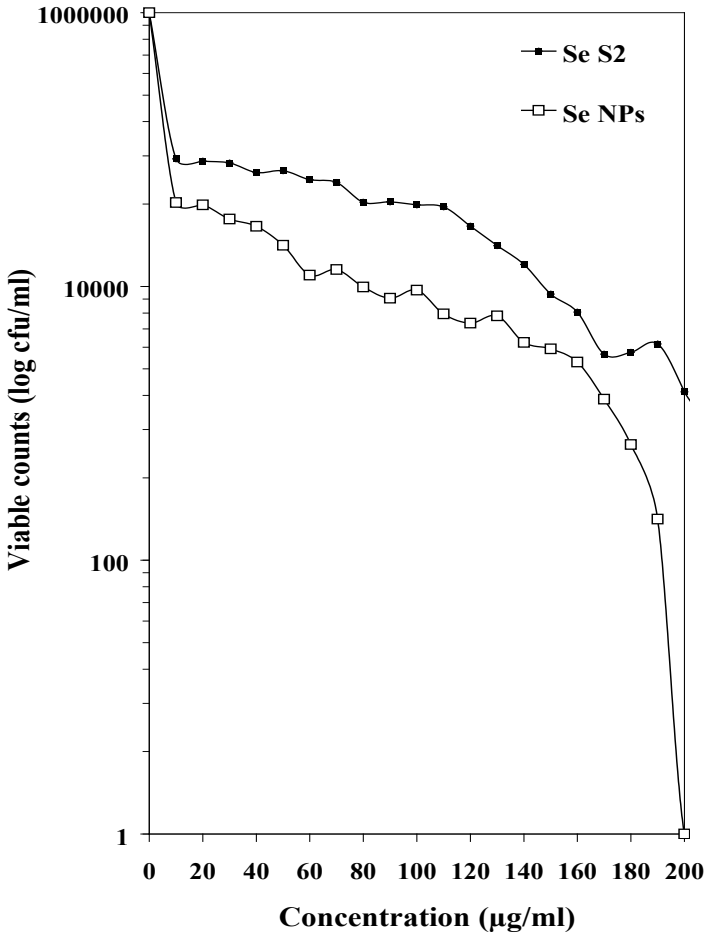

Fig1. *The viability of M. sympodialis cells after growth with different concentrations of biogenic SeNPs and SeS_2 as determined by CFU.*

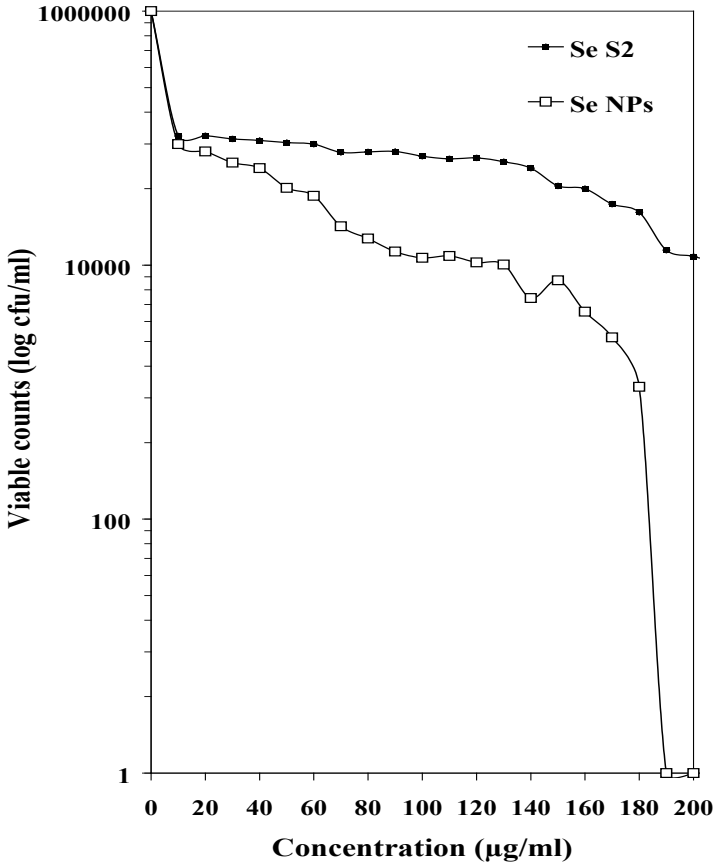

Fig2. *The viability of M. furfur cells after growth with different concentrations of biogenic SeNPs and SeS_2 as determined by CFU.*

The addition of as little as 10µg/ml of SeNPs or SeS_2 to the cultures resulted in a decrease in the number of viable cells. As the concentrations of the compounds increased from 10µg/ml to 200µg/ml, the number of surviving CFUs of both fungal species decreased proportionally, and the viable count obtained in cultures treated with each concentration of SeNPs was lower than that obtained in the cultures treated with SeS_2 ($p<0.05$). Whereas a low number of cells from both fungal species survived in the cultures containing200µg/mlof SeS_2, no viable cells of these fungi were found in the cultures grown with the same concentration of SeNPs (Figure 1, Figure 2), indicating the higher antifungal potency of SeNPs.

The MICs and MFCs of SeNPs and SeS_2 were also calculated and compared (Table 1). The MIC of SeNPs was three times lower than that of SeS_2 for *M. furfur* and eight times lower than that of SeS_2 for *M. sympodialis* (Table 1). The MFCs of SeS_2 for *M. sympodialis* and *M. furfur* were 220 and 260µg/ml, respectively, and higher than the MFC of SeNPs for eliminating these fungi (Table 1). So, our data show that the fungicidal effect of SeNPs was higher than that of SeS_2. The MFCs of both compounds were slightly higher for *M. furfur* than for *M. sympodialis.*

Table1. *The MIC and MFC of SeNPs and SeS_2 against M. sympodialis and M. furfur.*

MFC (µg/ml)		MIC (µg/ml)		Test strain
SeS_2	Se NPs	SeS_2	Se NPs	
260	190	150	50	*M. furfur*
220	180	80	10	*M. sympodialis*

To investigate if exposure to SeNPsand SeS_2 resulted in the suppression of later fungal growth, the growth of *M. furfur* treated with 50µg/ml of SeS_2 (0.33 x MIC) and SeNPs (1 x MIC) was compared with the growth of *M. furfur* in the cultures grown without selenium.

In our experiments, exposure of *M. furfur* to 50µg/ml of either SeS_2 or SeNPs for 2 hours did not suppress growth in cultures with 5µg/ml of either SeS_2 or SeNPs. After 120 hours of incubation, the viable count of untreated *M. furfur* grown in media without selenium was 12 x 10^6 CFU, but for those treated cells grown with 5µg/ml of either SeS_2 or SeNPs, the counts were 18 x 10^6 and 19 x 10^6 cells of *M. furfur,* respectively, indicating that neither of the preparations had a PAFE for this fungus (Figure 3).

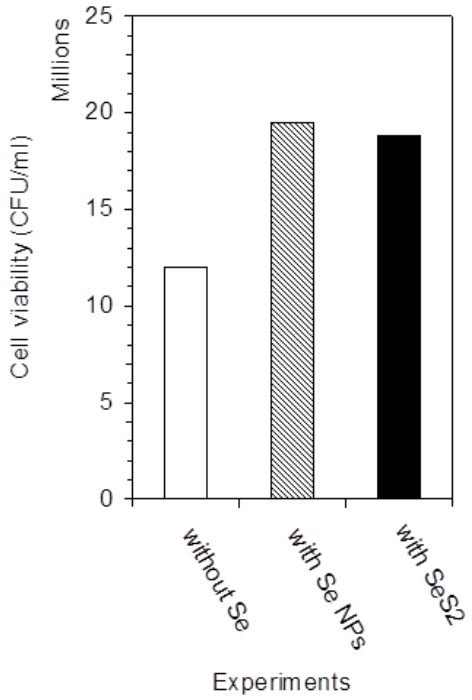

Fig3. *The effect of 2 h pre-exposure of M. furfur to 50µg/ml of biogenic SeNPs and SeS_2 on its regrowth with 5µg/ml of the compound, as compared with control cultures grown in medium without selenium. All flasks were inoculated with 5 × 10^6 cells/ml. (Data of each group are presented as mean ±SD (P = <0.001).*

Drug PAFE, a suppression of fungal growth after limited drug exposure, has been observed for various compounds in some fungi [Vitale et al.,2003]. Vitale et al showed that the PAFE of amphotericin B for several fungi is dependent on concentration; the effect is not seen for all fungi. Moreover, in our experience for *M. furfur* neither of the preparations had a PAFE for this fungus (Figure 3).

Figure 2 shows that a large number of *M. furfur* cells survived after 120 h incubation following a pre-treatment for 2 hours with 50µg/ml of either SeS_2 or SeNPs. It is possible that the original inoculum contained a population of cells with different degrees of sensitivity to selenium. The surviving cells were either resistant to 50µg/ml of selenium originally or became resistant to selenium during incubation. In either case, they may have been metabolically different from the rest of the cells in the population. The better growth observed for cells of *M. furfur* exposed to 50µg/ml of SeS_2 and SeNPs and then grownin the presence of 5 µg/ml of SeS_2 and SeNPs (Figure 3) could be the result of the elimination of less tolerant cells and growth of more resistant cells that could proliferate more rapidly. Development of resistance to selenium has been shown in other fungi [Buxton et al., 1989]. Selenate resistance was found in an *Aspergillus* species mutant that lacked ATP sulfurylase and was

not able to use sulfate as a sole sulfur source [Buxton et al., 1989] Selenium-resistant strains of yeast also have been found [Kitajima et al., 2013; Berdicevsky et al., 1993].

SeS_2 is commercially available as an antifungal agent in over-the-counter shampoos for the treatment of dandruff and seborrhoeic dermatitis. To avoid skin irritation, users are recommended to limit their exposure time to SeS_2[Chu et al., 1984; McGinley et al., 1982; Van Cutsem et al.,1990; Hersle et al., 1971]. The proliferation of *Malassezia* spp. after short exposure to selenium in our study should be considered in regard to the treatment of fungal infection with selenium shampoos. The survival and the growth of viable *Malassezia*spp. cells after short treatment may be the cause of recurrent infection.

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