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 SeS₂ and SeNPs against *M. sympodialis* and *M. furfur* were compared. The results showed that SeNPs had higher antifungal activity than SeS₂ 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 SeS₂. However, cultures treated for a short time with the MIC or lower than the MIC of SeNPs or SeS₂ 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 SeS₂ 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 seborrheic dermatitis [Aggarwal et al., 2003; Anwar et al., 2016; Roques et al., 2006; Rudramurthy et al., 2014]. Seborrheic 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 antifungal shampoos containing various antifungal agents, including selenium disulfide (SeS₂), which has antifungal activity against a variety of fungi, including *Malasseziaspp*[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 [Keilisz 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 *Penicillium expansum*, 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]. SeS₂ has been shown to have antifungal activity against *Malassezia* [Aggarwal et al., 2003, McGinley et al., 1982]. However, after treatment with antifungal agents, including SeS₂, SD tends to relapse. Aggarwal et al treated 20 SD patients once a week for three weeks with 2.5% SeS₂ 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 SeS₂ treatment.
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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 MFC that 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 Klebsiella pneumoniae [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 200 mg/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, Tecnoc-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 300 µg/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 Se\(\text{S}_2\)

The effect of pre-exposure of *M. furfur* to (50µg/ml) SeNPs and Se\(\text{S}_2\) 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 Se\(\text{S}_2\) was inoculated with 5 \(\times\) 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 Se\(\text{S}_2\). We found higher antifungal potency of SeNPs for *M. furfur* and *M. sympodialis* in comparison with Se\(\text{S}_2\) and showed that neither of the compounds had a PAFE for *M. furfur*. To compare the antifungal activities of biogenic SeNPs and Se\(\text{S}_2\), the viability of *M. sympodialis* and *M. furfur* after incubation with different concentrations of these compounds was determined (Figure 1, Figure 2).

![Graph](image-url)

**Fig1.** The viability of *M. sympodialis* cells after growth with different concentrations of biogenic SeNPs and Se\(\text{S}_2\) as determined by CFU.
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 containing 200µg/ml of 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.

**Table 1.** The MIC and MFC of SeNPs and SeS$_2$ against M. sympodialis and M. furfur.

| Test strain | SeS$_2$ (µg/ml) | Se NPs (µg/ml) | SeS$_2$ (µg/ml) | Se NPs (µg/ml) |
|-------------|-----------------|----------------|-----------------|----------------|
| M. furfur   | 260             | 190            | 150             | 50             |
| M. sympodialis | 220           | 180            | 80              | 10             |
To investigate if exposure to SeNPs and SeS\textsubscript{2} resulted in the suppression of later fungal growth, the growth of \textit{M. furfur} treated with 50μg/ml of SeS\textsubscript{2} (0.33 x MIC) and SeNPs (1 x MIC) was compared with the growth of \textit{M. furfur} in the cultures grown without selenium.

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

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 \textit{M. furfur} neither of the preparations had a PAFE for this fungus (Figure 3).

Figure 2 shows that a large number of \textit{M. furfur} cells survived after 120 h incubation following a pre-treatment for 2 hours with 50μg/ml of either SeS\textsubscript{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 \textit{M. furfur} exposed to 50μg/ml of SeS\textsubscript{2} and SeNPs and then grown in the presence of 5 μg/ml of SeS\textsubscript{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 \textit{Aspergillus} species mutant that lacked ATP sulfurylase and was
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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 *Malassezias*pp. cells after short treatment may be the cause of recurrent infection.

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