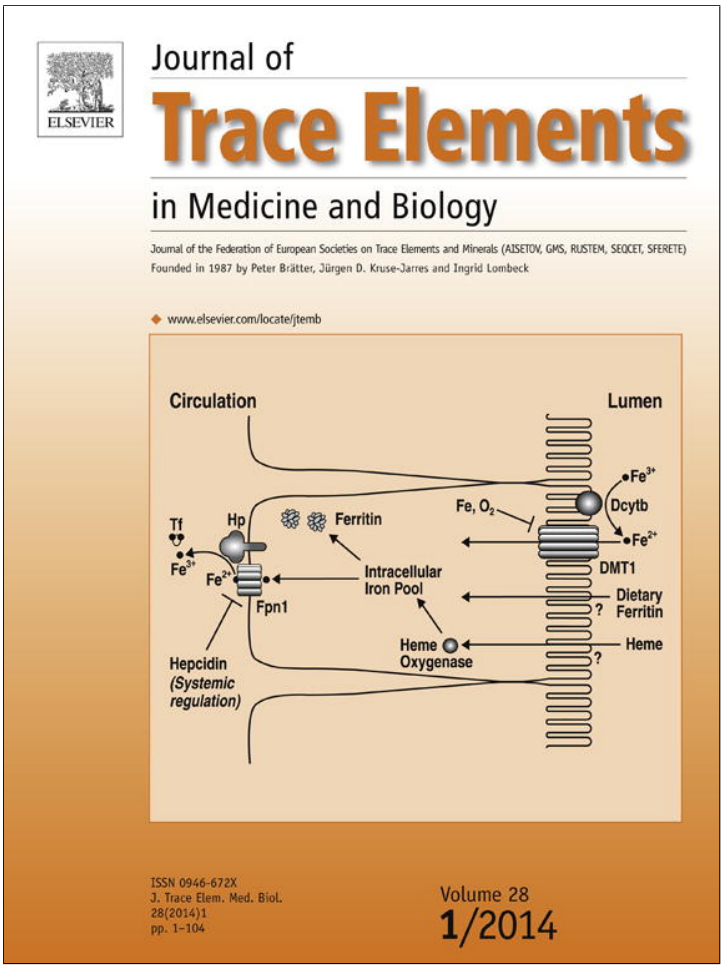

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PHARMACOLOGY

Antioxidant and cytotoxic effect of biologically synthesized selenium nanoparticles in comparison to selenium dioxide

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ABSTRACT

The present study was designed to evaluate antioxidant and cytotoxic effect of selenium nanoparticles (Se NPs) biosynthesized by a newly isolated marine bacterial strain *Bacillus* sp. MSh-1. An organic–aqueous partitioning system was applied for purification of the biogenic Se NPs and the purified Se NPs were then investigated for antioxidant activity using DPPH scavenging activity and reducing power assay. Cytotoxic effect of the biogenic Se NPs and selenium dioxide (SeO_2) on MCF-7 cell line was assesed by MTT assay. Tranmission electron micrograph (TEM) of the purified Se NPs showed individual and spherical nanostructure in size range of about 80–220 nm. The obtained results showed that, at the same concentration of 200 μg/mL, Se NPs and SeO_2 represented scavenging activity of 23.1 ± 3.4% and 13.2 ± 3.1%, respectively. However, the data obtained from reducing power assay revealed higher electron-donating activity of SeO_2 compared to Se NPs. Higher IC_{50} of the Se NPs (41.5 ± 0.9 μg/mL) compared to SeO_2 (6.7 ± 0.8 μg/mL) confirmed lower cytotoxicity of the biogenic Se NPs on MCF-7 cell line.

Introduction

Oxidative stress and uncontrolled production of reactive oxygen species (ROS) have been identified as one of the most important reasons in pathological progression of many diseases and several kinds of cancers [1,2]. Different mechanisms such as enzymatic conversion of free radicals and detoxification using antioxidants have been developed by living cells to defend against dangerous effect of the mentioned radical intermediates [2,3]. So, introducing novel, efficient and cost effective antioxidants with lower toxicity has been the aim of many investigations [4,5].

Selenium (Se) is an essential dietary trace element, the antioxidant activity of which has been supported by a large number of clinical and epidemiological studies [2,6]. This micronutrient metalloid is the main component of selenoenzymes which are found to protect animal cells from oxidative damage [6,7]. Nutritional deficiency of Se is the main reason of Keshan disease, age-related degenerative diseases and muscular dystrophy in humans [7,8]. Stimulation of immune responses [8] and reduction of overall cancer mortality [2,9] are among other advantages of Se intake determined by recent studies. However, dose and chemical form of selenium derivatives play an important role in both their bioavailability and biological activities [1,6]. Excellent biological properties of selenium nanoparticles (Se NPs) together with their lower toxicity have introduced them as a good candidate for replacing other forms of selenium in nutritional supplements [8,9].

There are several reports on the ability of bacterial strains like *Bacillus megaterium* [10], *Pseudomonas alcaliphila* [11] and *Enterobacter cloacae* SLD1a-1 [12] for producing Se NPs. An efficient Se NPs producer bacterial strain was recently isolated from the Caspian Sea and identified as *Bacillus* sp. MSh-1 [13]. In the present study, the biologically synthesized Se NPs was purified from the whole-cell lysate of *Bacillus* sp. MSh-1 and the antioxidant and cytotoxic activities of the purified Se NPs were evaluated compared with selenium dioxide. To the best of our knowledge and according to the literature review, limited works have been conducted to assay both antioxidant and cytotoxic properties of the biogenic Se NPs.

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Materials and methods

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Selenium dioxide (SeO_2) and butylated hydroxytoluene (BHT) were provided by Merck Chemicals (Darmstadt, Germany). Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM) and antibiotics were supplied by Gibco (Life Sciences Inc., USA). All other chemicals and solvents were of analytical grade.

Bacterial strain, biosynthesis and purification of the Se NPs

The bacterial strain used for biosynthesis of Se NPs in the present study was previously isolated from the Caspian Sea and identified as *Bacillus* sp. MSh-1 based on 16S rDNA gene analysis [13]. Se NPs were prepared according to a recent method described by Shakibaie et al. [13]. Briefly, sterile nutrient broth (NB) medium containing selenium dioxide (SeO_2, final concentration 1.26 mM) was inoculated with 1 mL of the fresh inoculums (OD_{600}, 0.1) of *Bacillus* sp. MSh-1 and incubated in a shaker incubator (150 rpm) at 30 °C for 14 h. The bacterial cells were then harvested by centrifugation at 4000 × *g* for 10 min. After washing the obtained pellets with sterile solution of NaCl (0.9%) for three times, the bacterial cells were disrupted by grinding the frozen cells in liquid nitrogen using a mortar and pestle. The resulting slurry was then ultrasonicated at 100 W for 5 min and washed three times by sequential centrifugation (14,000 × *g*, 5 min) with a 1.5 M Tris–HCl buffer (pH 8.3) containing 1% SDS and deionized water, respectively. Subsequently, Se NPs were extracted and purified by organic–aqueous partitioning system (*n*-octanol–water), as previously described [13]. Micrographs of the prepared biogenic Se NPs were obtained using a TEM apparatus (Zeiss 902A) operated at accelerating voltage of 80 kV. The related size distribution pattern of Se NPs was plotted by manual counting of 400 individual particles from different TEM images.

DPPH scavenging activity of the purified Se NPs

Purple DPPH radicals changes into a yellow stable compound in presence of an antioxidant and the extent of the reaction depends on hydrogen donating ability of the antioxidant [14]. DPPH scavenging activity of the biogenic Se NPs was determined according to the method described by Turlo et al. [4] with some modifications. One mL of Se NPs or SeO_2 solution (20–200 μg/mL) was mixed with 1 mL of the freshly prepared DPPH solution in methanol (0.15 mM). After addition of methanol (3 mL), the mixture was incubated in dark at room temperature for 30 min and absorbance of the mixture was then recorded at 517 nm using a UV–vis spectrophotometer (UVD-2950; Labomed). The negative control was designed by replacing Se NPs or SeO_2 stocks with deionized water. Scavenging percentage of DPPH was calculated as follows:

$$\text{DPPH radical scavenging ability } (\%) = \left[1 - \frac{(A_a - A_b)}{A_c}\right] \times 100$$

where A_a is absorbance of the sample mixed with DPPH solution, A_b is absorbance of the sample without DPPH solution and A_c is absorbance of the control solution. Required concentration of the test samples for inhibiting 50% of DPPH (IC_{50}) was calculated by linear regression. The same experiment was performed for BHT as positive control at similar concentration. All the experiments were carried out in triplicate and mean of the obtained results was reported.

Reducing power assay

Reducing power of Se NPs and SeO_2 was determined by modified protocol of Oyaizu et al. [4,15]. This assay is based on reduction of Fe^{3+} to Fe^{2+} in the presence of antioxidants. Briefly, 1 mL from different concentrations of Se NPs or SeO_2 (20–200 μg/mL) was mixed with 0.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 0.5 mL of potassium ferricyanide (30 mM). The mixture was then incubated at 50 °C and 100 rpm for 20 min. Thereafter, 2 mL of trichloroacetic acid (TCA, 0.6 M) was added to the above mixture followed by centrifugation at 3000 rpm for 10 min. The obtained supernatant (0.5 mL) was mixed with deionized water (0.5 mL) and 0.1 mL of the $FeCl_3$ solution (6 mM) and the absorbance was measured at 700 nm. The negative control was designed by incubating the mentioned reaction mixture in absence of Se. The same experiment was also repeated for BHT as a reference compound at similar concentration. These procedures were replicated three times on different days, and mean of the absorbencies was used to draw a suitable curve.

Cell culture and cytotoxicity assay

MCF-7 cell lines were obtained from the National Cell Bank of Iran, Pasteur Institute of Iran (Tehran, Iran). The cells were maintained in DMEM medium supplemented with FBS (10%, v/v) and antibiotics [penicillin (100 units/mL) and streptomycin (100 μg/mL)] at 37 °C in a CO_2 incubator (5% CO_2 and 95% relative humidity). In order to evaluate cytotoxic effect of the Se NPs and SeO_2, MCF-7 cells were harvested in the exponential phase of growth, seeded into 96-well tissue culture plates (15,000 per well) and allowed to adhere for 24 h. Thereafter, Se NPs and SeO_2 were added to the desired wells to reach final concentration of 0–200 μg/mL. After 24 h of incubation, 20 μL of DMEM medium containing MTT (5 mg/mL) was added to each well and incubated for 4 h. Consequently, the medium was replaced with 100 μL of DMSO, and optical densities were determined at 570 nm. MTT assay was performed in three replicates for each experiment.

Statistical analysis

Each value was expressed as mean ± SD. SPSS software 15 for windows (SPSS Inc., Chicago) was used for statistical analysis. Differences between the groups were determined using one-way analysis of variance (ANOVA) and *p*-values less than 0.05 were considered significant.

Results

Biosynthesis of Se NPs

The obtained results confirmed that bacterial strain of *Bacillus* sp. MSh-1 effectively reduced Se^{4+} to elemental Se which was evident from the color change of cultivation medium to orange-red due to generation of Se(0) during the exponential growth phase (the data were not shown). The biologically synthesized Se NPs was successfully purified using *n*-octanol–water partitioning system. According to Fig. 1 which shows TEM image of the purified nanostructures, Se NPs represented spherical shape and most of them were in the size range of 80–220 nm. TEM image clearly illustrated individual Se NPs with a small amount of aggregation. Size distribution pattern (Fig. 2), measured from manual counting of 400 individual particles from different TEM images, revealed that the most frequent particles were in size range of 125–150 nm.

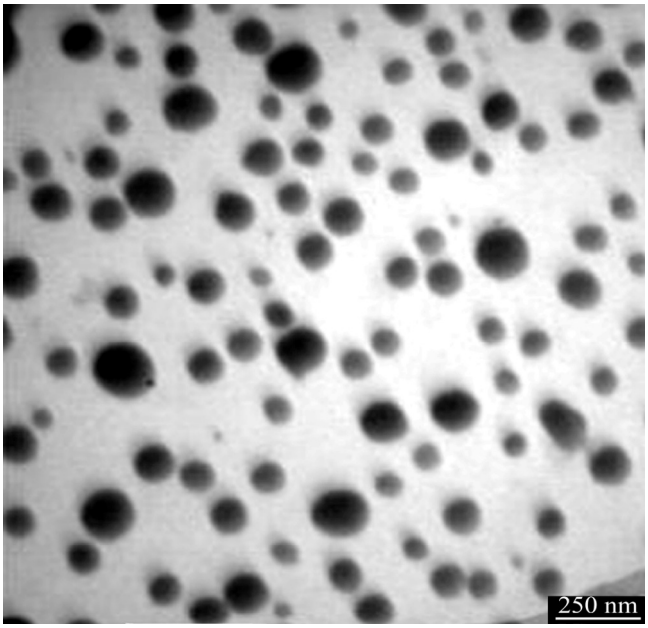

Fig. 1. Transmission electron microscopy (TEM) image of Se NPs synthesized by *Bacillus* sp. MSh-1 and purified using *n*-octyl alcohol/water extraction system.

Antioxidant activity of biogenic nanoselenium

The antioxidant activity of Se NPs and SeO_2 solution was measured using two methods of DPPH and reducing power assay. As shown in Fig. 3a, BHT was the strongest compound in DPPH scavenging activity while Se NPs and SeO_2 only represented moderate effects. The biogenic Se NPs and SeO_2 solution showed $23.1 \pm 3.4\%$ and $13.2 \pm 3.1\%$ scavenging effect at the same concentration of 200 μg/mL, respectively (Fig. 3a). Increasing Se NPs concentration from 200 to 800 μg/mL did not significantly enhance scavenging effect ($p > 0.05$, the data not shown). Reducing power of Se NPs and SeO_2 exhibited a dose-dependent manner in concentration range of 0–200 μg/mL (Fig. 3b). At all concentrations, reducing power of Se NPs and SeO_2 were significantly lower than BHT ($p < 0.05$). Moreover, reducing power of SeO_2 at the same concentration of above 80 μg/mL was significantly higher than that of Se NPs ($p < 0.05$).

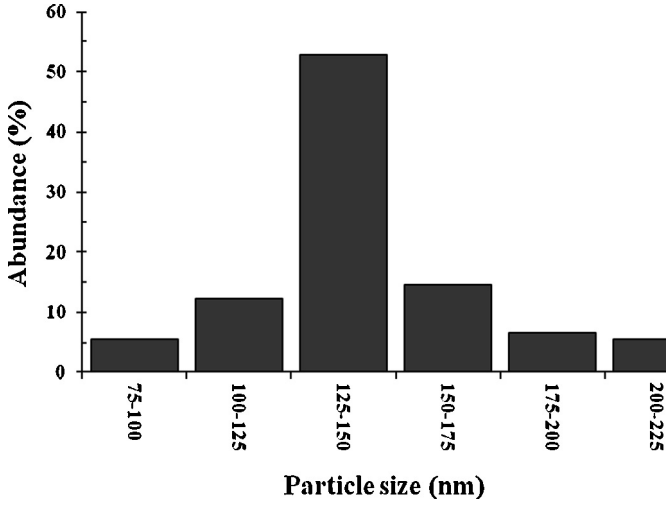

Fig. 2. Particle size distribution histogram of the biogenic Se NPs obtained by manual counting of 400 individual particles from different TEM images.

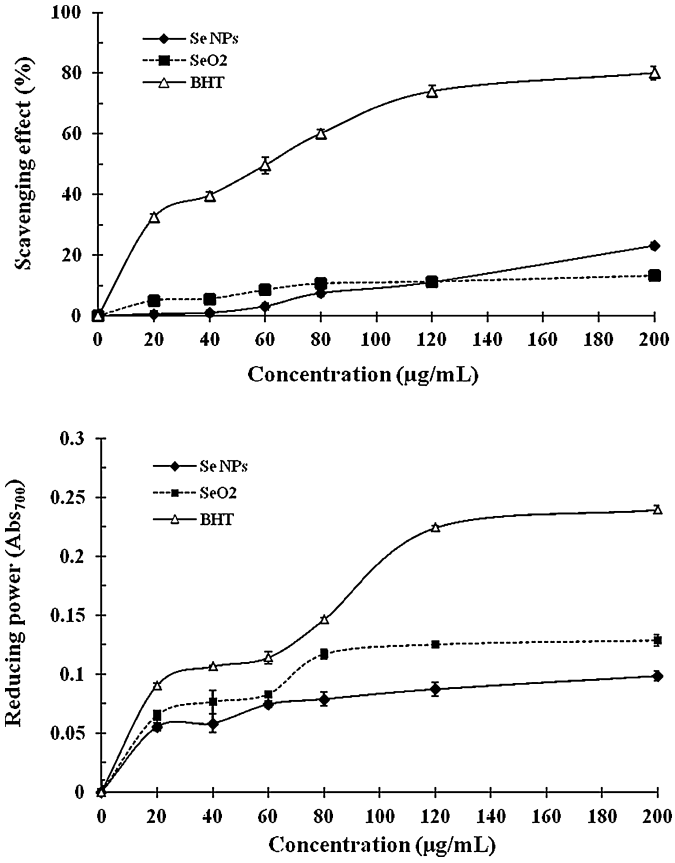

Fig. 3. (a) Scavenging effects of biogenic Se NPs and SeO_2 on DPPH free radicals and (b) reducing power of the biogenic Se NPs and SeO_2 compared to BHT as a standard control. Data are mean of triplicate experiments.

Cytotoxicity assay

MTT assay was applied in order to investigate cytotoxic activity of the biogenic Se NPs and SeO_2 solution. As represented in Fig. 4, the concentration necessary for causing 50% cell death (IC_{50}) was 41.5 ± 0.9 μg/mL in the cells treated with Se NPs. However, SeO_2 produced the same effect (i.e. 50% cell death) at very low concentration of 6.7 ± 0.8 μg/mL. In other words, SeO_2 at all concentration was more toxic than Se NPs ($p < 0.05$).

Discussion

Due to higher biological activity and lower toxicity of Se NPs compared to those of Se ions, trend of research is now being directed towards synthesis and application of Se NPs [16]. Both physicochemical [17] and biological [9,13] methods have been used for fabrication of Se NPs. In the present study, red elemental Se NPs was successfully synthesized by marine strain of *Bacillus* sp. MSh-1 after 14 h of incubation. In a study by Yazdi et al. [9] Se NPs with average size below 250 nm was biologically synthesized after 72 h of incubation of SeO_2 containing medium in the presence of *Lactobacillus plantarum*. Reduction of selenite ion in the presence of halotolerant strain of *B. megaterium* led to formation of Se NPs with average size of 200 nm [10].

Antioxidant activity of selenocompounds has been confirmed by both *in vivo* and *in vitro* experiments [18,19]. In a study by Haung et al. [20] large size Se NPs (80–200 nm), prepared by bovine serum albumin (BSA) as reducing agent, exhibited $41.5 \pm 2.7\%$ of scavenging effect at concentration of 197.7 μg/mL. In the present study, the obtained data of antioxidant activity of biologically synthesized

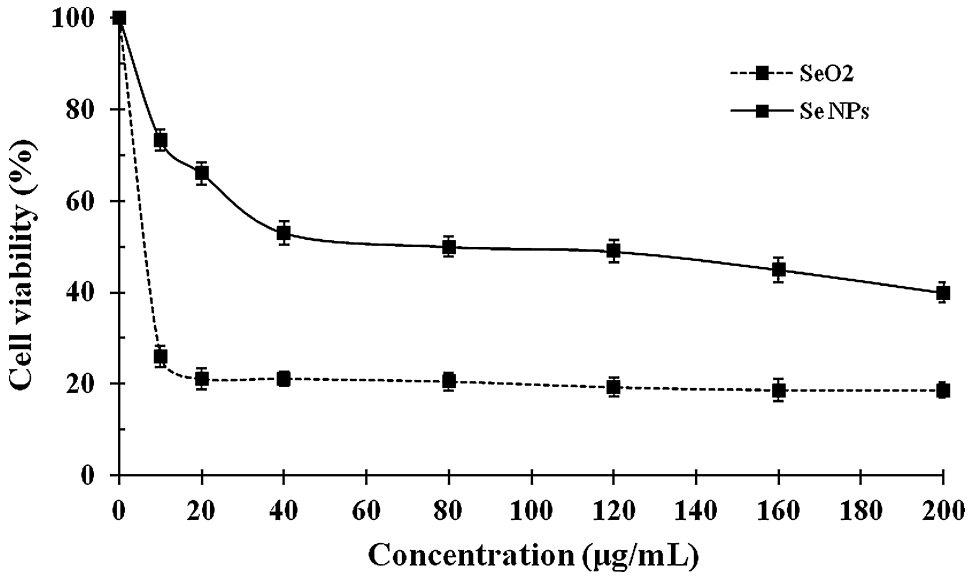

Fig. 4. Effect of biogenic Se NPs and SeO_2 on cell viability of MCF-7 cell line determined by MTT assay. Each value is represented as mean ± SD of three independent experiments.

Se NPs (determined by DPPH scavenging activity) revealed moderate antioxidant activity compared to BHT as standard compound. Reduction of Fe (III) ions has been frequently used as a marker of electron-donating activity [14]. Higher absorbance of the reaction mixture (at 700 nm) indicates a higher reducing power [4,14]. In general, reducing SeO_2 solution by biological methods leads to formation of insoluble elemental selenium [10,21]. Such observation might be the possible reason for higher reducing power activity of soluble SeO_2 compared to the biogenic Se NPs with lower solubility in the present study.

Cytotoxicity analysis of the samples in the present work showed direct dose–response relationship, in which cell viability decreased at higher concentrations (Fig. 4). Such pattern was obtained in our previous investigation in which cytotoxic effect of biogenic Se NPs was assessed on human fibrosarcoma cell line (HT-1080) [13]. Biogenic Se NPs represented lower cytotoxicity compared to SeO_2 in the present work. These results were in contrast to those of Chen et al. [22] indicating higher cytotoxicity for Se NPs, fabricated by *Undaria pinnatifida* polysaccharide solution, on cancer cell lines (Hep G2 and MCF-7) compared to Se^{4+} ions. However, their work revealed that mentioned Se NPs was less toxic than SeO_2 on the normal cell line of Hs68 [22]. Lower toxicity of Se NPs compared to selenite ion has been also reported in previous *in vivo* studies [23–25]. Comparing the measured IC_{50} for SeO_2 and biogenic Se NPs revealed that the *Bacillus* sp. MSh-1 converted the soluble SeO_2 into insoluble Se particles at nano size which was approximately 6-fold less toxic for MCF-7 cell line.

Conclusion

In conclusion, the obtained data of DPPH radical scavenging showed that antioxidant activity of the biogenic Se NPs (synthesized by *Bacillus* sp. MSh-1) and SeO_2 seems to be identical. However, reducing power of SeO_2 solution was somehow higher than biologically synthesized Se NPs. On the other hand, Se NPs represented lower toxicity on MCF-7 cell line compared to Se^{4+} ions. Nevertheless, more studies should be conducted to investigate action mechanisms of the produced Se NPs.

Conflict of interest

The authors certify that there is no conflict of interest.

Acknowledgments

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