

Biogenic selenium nanoparticles target chronic toxoplasmosis with minimal cytotoxicity in a mouse model

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Abstract

Introduction. Nanoparticles (NPs) have numerous biological benefits due to their large surface-volume ratio and convenient entry into cells compared to other particles. Previous research has shown the antimicrobial properties of biogenic selenium NPs (SeNPs) and their effects on cellular immunomodulatory cytokines that play a key role in controlling infections.

Aim. This study aimed to evaluate the therapeutic effects of SeNPs against chronic toxoplasmosis in mice.

Methodology. Infected mice with *Toxoplasma gondii* (Tehran strain) were orally treated with SeNPs at doses of 2.5, 5 and 10 mg kg⁻¹ once a day for 14 days. On the fifteenth day, the mean number of brain-tissue cysts and the mRNA levels of TNF- α , IL-12, IL-10, IFN- γ and inducible nitric oxide synthase (iNOS) in the mice of each group were recorded. Moreover, serum clinical chemistry factors in the treated mice were examined to determine the safety of SeNPs.

Results. The mean number of tissue cysts was significantly ($P < 0.001$) decreased in mice treated with SeNPs in a dose-dependent manner compared with the control group. The mRNA levels of inflammatory cytokines were significantly increased in mice treated with SeNPs at a dose of 10 mg kg⁻¹ compared with the control subgroup ($P < 0.05$). No significant variation ($P > 0.05$) observed in clinical chemistry parameters among the mice in the control subgroup compared with those treated with SeNPs.

Conclusion. The findings demonstrated the therapeutic effects of SeNPs with no considerable toxicity against latent toxoplasmosis in the mouse model. Nevertheless, further studies are obligatory to reveal the exact anti-Toxoplasma mechanisms of SeNPs.

INTRODUCTION

Toxoplasmosis is a parasitic disease caused by *Toxoplasma gondii* that infects humans and many types of mammals and birds. This parasite has a sexual life cycle, which takes place in the small intestine of cats, and an asexual cycle that takes place in warmed animals and humans [1, 2]. Humans become infected by eating raw and undercooked meat and also through the ingestion of contaminated vegetables and fruits. The other possible routes of transmission can be from the placenta to the embryo during pregnancy in humans, which can cause serious health problems [3, 4]. The symptoms

of toxoplasmosis are diverse, although immunocompetent patients may have light symptoms such as fever, headache and muscle pain or be asymptomatic. However, serious clinical manifestations such as central nervous system involvement may also be observed in immunocompromised patients [2, 5–8].

At present, no suitable vaccine is available for the treatment of toxoplasmosis in humans and animals [9]. The prescribing concurrently of pyrimethamine and sulfadiazine is the preferred treatment for toxoplasmosis. Recent studies have indicated adverse side-effects such as osteoporosis and

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Abbreviations: ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; BUN, Blood urea nitrogen; Cr, Creatinine; SeNPs, Selenium nanoparticles; TB, Total bilirubin.

teratogenic effects, mostly in immunocompromised individuals, when treated with this combination [5, 8, 10]. Therefore, there is a need for further research aiming to discover new agents from natural products or biosynthetic agents with fewer side-effects and higher efficacy for the treatment of toxoplasmosis.

Selenium (Se) is an essential element for human health. In the case of deficiency of this element in the human body, some severe complications, such as deficiency and impairment of the immune system, can occur [11]. This element also exists in some beneficial proteins that have anti-cancer, anti-oxidants and anti-microbial properties [12, 13]. Moreover, nanoparticles (NPs) have numerous biological benefits due to having a large surface-volume ratio. One such biological benefit is their convenient entry into the cell compared to other particles [14]. Recent studies have demonstrated that SeNPs have the ability to prevent the growth of a number of pathogenic bacteria, including *Staphylococcus aureus*, *Escherichia coli* and *Leishmania* spp. [15, 16]. Although the precise antimicrobial mechanisms of this element have not yet been determined, recent studies have reported that the inorganic types of this element are able to affect membrane peroxidases to produce oxygen-free radicals, including superoxide anion [17]. Nevertheless, other studies have revealed that SeNPs can stimulate apoptosis in various eukaryotic cells [18, 19].

Previous studies have demonstrated that innate and adaptive immune responses against bacterial and parasitic infections rely on selenium for killing these microbes, hence selenium deficiency was revealed to damage innate and acquired immunity and encourage susceptibility to a wide range of parasitic (*Trypanosoma cruzi*, *Nippostrongylus brasiliensis*, *Heligmosomoides bakeri* infection), bacterial (*Listeria monocytogenes*, *Mycobacterium tuberculosis* infection) and viral (hepatitis C virus and HIV infection) infections. Recent studies have shown that treatment with SeNPs increases the cellular immunomodulatory cytokines such as IL-12, IFN- γ , TNF- α , IL-2 and some inflammatory mediators such as nitric oxide (NO) [20].

The Th1 immune response, explained by the production of IL-12 and IFN- γ , is typical of infections with many intracellular microbes, such as *T. gondii*, and mice that are deficient in either IL-12 or IFN- γ cannot control the *T. gondii* infection [21]. Based on this knowledge and the documented antimicrobial and immune-modulating effect of selenium, we hypothesize that SeNPs could be a potent agent to combat *T. gondii* infections. In the present study, we determine the effect of SeNPs on latent toxoplasmosis in a mouse model.

METHODS

Biosynthesis and characterization of SeNPs

Biosynthesis of SeNPs was performed according to the method explained elsewhere [11]. In the first step, a sterile nutrient broth (NB) medium supplemented with the Se +4 ions (100 mg l⁻¹; equal to 1.26 mM SeO₂ solution) and 100 ml of this medium was moving to a 0.5 liter Erlenmeyer

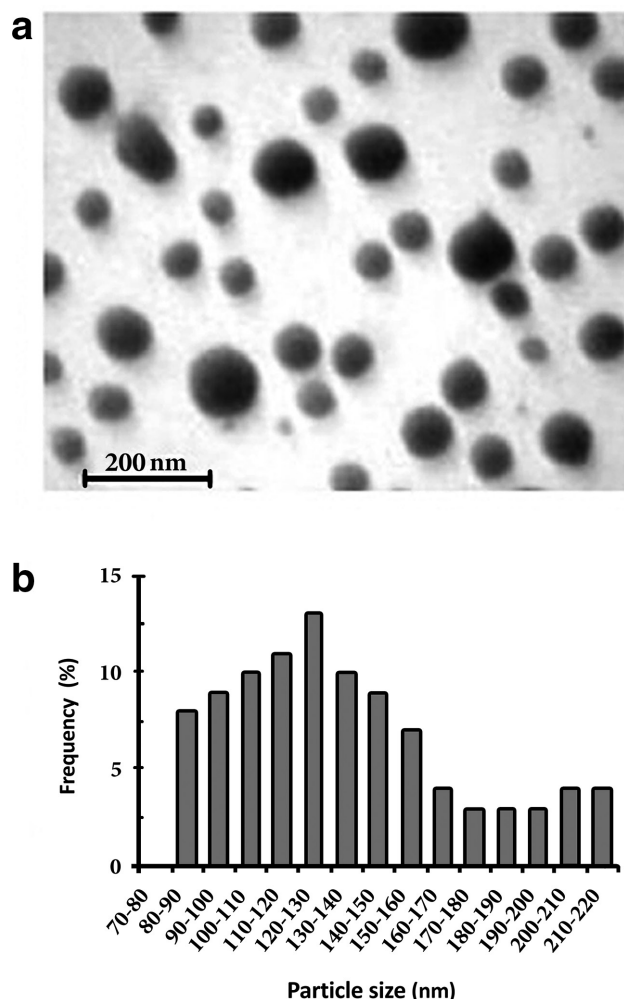


Fig. 1. (a) TEM of biogenic SeNPs prepared by *Bacillus* sp. MSh-1 and (b) particle size distribution pattern of SeNPs measured from manual counting 300 individual NPs from different TEM images.

flask and inoculated with 1 ml of the fresh inoculums (OD600, 0.1) of *Bacillus* sp. MSh-1 aerobically at 30 °C in a shaker incubator (150 r.p.m.).

In the next step, the bacterial cells and SeNPs were removed by centrifugation at 4000 g (10 min). The pellets were washed with 0.9% NaCl solution using centrifugation and were frozen by adding liquid nitrogen. The obtained slurry was ultrasonicated at 100 W for 5 min and washed three times by sequential centrifugation (10000 g, 5 min), with a 1.5 M Tris-HCl buffer (pH 8.3) containing 1% SDS and deionized water.

The SeNPs were extracted and purified using an organic-aqueous partitioning system (n-octyl alcohol-water). After preparation, for characterization of the SeNPs by transmission electron microscopy (TEM), an aqueous suspension comprising the SeNPs disseminated ultrasonically, and a portion of the solution poured into the carbon-coated copper TEM grids and dehydrated below an IR lamp (Fig. 1). Finally, the structure of crystalline of the SeNPs

assessed by means of the x-ray diffraction (XRD) method by an x-ray diffractometer (Philips PW1710) with Cu K α radiation ($\lambda=1.5405 \text{ \AA}$) over a scanning variety of Bragg angles from 20–80°C.

Experimental animals

In total, 56 male BALB/c mice aged 6 to 8 weeks old and weighting from 20 to 25 g were applied to establish the animal model of toxoplasmosis and safety of SeNPs. Mice were prepared from the animal house of Faculty of Medicine (Kerman, Iran) and were kept in a colony room with a 12:12 h light/dark cycle at $21\pm 2^\circ\text{C}$.

Parasite

The *T. gondii* Tehran strain as representative of type II [22] was kindly obtained by Professor Hossein Keshavarz of Tehran University of Medical Sciences (Tehran, Iran). This strain kept by intraperitoneal injection of 15 to 20 brain-tissue cysts of infected mice each 12 weeks.

Animal model of chronic toxoplasmosis

Chronic toxoplasmosis in mice was arisen based on the process explained by Mahmoudvand *et al.* [23]. At first, a solution of homogenized brain in normal saline obtained from the infected mice with toxoplasmosis. In the next step, 500 μl of brain suspension containing at least 25–30 tissue cysts plus antibiotics (penicillin and streptomycin) were administered intraperitoneally to BALB/c mice in each test group.

Serological tests

To approve the toxoplasmosis in mice, the obtained serum specimens from the tested mice after 45 days post-infection were examined for anti-*T. gondii* IgG antibody through a modified agglutination test (MAT) kit (Toxoscreen DA, Biomérieux, Lyon, France) in agreement with manufacturer protocol; so that agglutination titres of $\geq 1/20$ were considered as a positive cut-off.

Experimental design

Firstly, groups of mice ($n=48$) inoculated with 25–30 cysts 45 days previously (except non-treated non-infected mice in the control group) were examined for MAT and for initial treatment in day 60 post-infection were divided into two main groups. Control group (C) contained 24 mice in three subgroups (eight mice in each group); C1 (non-treated non-infected mice), C2 (infected mice treated with normal saline), C3 (infected mice treated with Atovaquone 100 mg kg^{-1} once a day for 2 weeks). Experimental group (E) contained 32 mice in four subgroups; E1 (orally treated with Se NPs 2.5 mg kg^{-1} once a day for 2 weeks), E2 (orally treated with Se NPs 5 mg kg^{-1} once a day for 2 weeks), E3 (orally treated with Se NPs 10 mg/ kg^{-1} once a day for 2 weeks), E4 (orally treated with Se NPs 10 mg kg^{-1} +Atovaquone 100 mg kg^{-1} once a day for 2 weeks).

Sample collection

Inoculated mice in all tested groups with *Toxoplasma* cyst 75 days previously were deeply anesthetized using injection of ketamine and xylazine (100 mg kg^{-1} +10 mg kg^{-1}) (10:1) as intraperitoneally. To obtain the blood samples the abdomen of each mice opened and blood samples were collected from the heart. Subsequently, after decapitation total brain tissues were quickly harvested and divided into along the middle.

Calculating tissue cysts

The right hemisphere was used to study the parasitological study. Unstained-smears were prepared from the whole brains and the numbers of tissue cysts were counted at two magnifications of $\times 100$ and $\times 400$ using light microscopy (Olympus Microscopes, Japan) [24, 25].

Safety evaluation by assessing the liver and kidney serum enzymes

In order to evaluate the serum clinical chemistry factors, the collected blood specimens of mice in the subgroups C1, C2, as well as E1–E4 were put in glass tubes with no anticoagulant to allow the clot, and serum obtained by means of centrifugation at 5000 g for 5 min. In this study some clinical chemistry parameters such as creatinine (Cr), blood urea nitrogen (BUN), total bilirubin (TB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), as well as alkaline phosphatase (ALP) were determined using the commercial kits (Roche, Germany) based on the manufacturer protocols [24, 26].

Cytokine expression by real-time PCR

Since SeNPs influence on the immune system particularly cellular immunity, the mRNA levels of IFN- γ , IL10, IL12, TNF- α and inducible nitric oxide synthase (iNOs) as the main effector mechanisms for control and resistance to *T. gondii* during infection were examined in the tested groups by quantitative real-time PCR.

The left hemisphere was also kept in pre-cooling preservation tubes and stored in -80°C to assess the cytokine expression level. In the first step, RNeasy kits (Qiagen, Hilden, Germany) were used to extract the total brain RNA; in the next step, all isolated RNAs were reverse transcribed based on the manufacturer's instrument to complementary DNA (cDNA) (Qiagen, Hilden, Germany) for conventional PCR amplification or real-time PCR. In this study, the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) applied to carry out real-time PCR; whereas we used the SYBR green for identification amplification yields [27].

The real-time PCR conditions in this survey were

- (1) Early denaturation at 95°C for 10 min, 40 amplification cycles.
- (2) Denaturation at 94°C for 10 s.
- (3) Annealing at 55°C for 30 s.
- (4) Elongation at 73°C for 30 s, followed by one cycle at 72°C for 5 min.

Table 1. Sequences of primers used for real-time PCR

Amplicon	Primers	Sequence (5'–3')
IL12	F	ACGACATTCGTCAACTGCAA
	R	TAAATTGGCACCCGTAGGC
IFN- γ	F	GATCGTGTGTCGTCACCAGAAAGG
	R	TGCCTGGTAACGAGTTGTCC
NO	F	CGTGAAAGGATCCAGAAAGG
	R	TCCACGATGCCTGGTAGTTG
TNF- α	F	ATTCTCACGCCAGGATTTG
	R	GATCGGCAAAGGTTAGGTCA
IL10	F	ACGACATTCGTCAACTGCAA
	R	TAAATTGGCACCCGTAGGC

NO, Nitric oxid.

Finally, the collected data were analysed by means of iQTM5 optical system software. β -actin as a housekeeping gene considered as a standardization control. The sequences of applied primer for IFN- γ TNF- α , IL10, IL-12, iNO and β -actin are revealed in Table 1.

Statistical analysis

In this investigation, the obtained data were analysed after being entered into the SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA). Furthermore, to evaluate the differences among the tested groups, one-way ANOVA with Tukey's *post hoc* test were used. $P < 0.05$ was also considered statistically significant.

RESULTS

Parasitological study

As shown in Table 2, the mean number of brain-tissue cysts significantly decreased in mice in experimental subgroups

Table 2. Mean numbers of brain-tissue cysts in mice of each tested group with mice infected with 25–30 cysts of the Tehran strain of *T. gondii* 2 months previously, with SeNPs and atovaquone

Groups	Mean no. of tissue cysts ($\pm$ SD)
Control	672 $\pm$ 8
SeNPs (2.5 mg kg ⁻¹)	68 $\pm$ 4*
SeNPs (5 mg kg ⁻¹)	14 $\pm$ 2*
SeNPs (10 mg kg ⁻¹)	2 $\pm$ 1*
Ato 100 mg kg ⁻¹	0
SeNPs (10 mg kg ⁻¹) + Ato 100 mg kg ⁻¹	0

* $P < 0.05$, significantly different when compared with control.
Ato, Atovaquone.

E1 ($P < 0.001$), E2 ($P < 0.001$) and particularly E3 ($P < 0.001$) via a dose-dependent response in comparison with control group C2. In the experimental subgroup E4 (SeNPs 10 mg kg⁻¹+atovaquone 100 mg kg⁻¹), similar to control group C3 (atovaquone 100 mg kg⁻¹), no brain-tissue cyst was observed in the tested mice.

Safety evaluation by assessing the liver and kidney serum enzymes

Several key clinical chemistry factors were tested in the mice of subgroups E1–E4 compared with those in control group C2 after 14 days after the administration of SeNPs. Results indicated that there was no mortality found in mice in any tested subgroup after 2 weeks. Table 3 shows the mean Cr, BUN, bilirubin (total), AST, ALT and ALP in tested mice. No significant change ($P > 0.05$) was observed in clinical chemistry among the mice in the control subgroups compared with those in experimental subgroups E1–E4.

mRNA expression level of cytokines

The mRNA levels of IFN- γ , TNF- α , IL-12 and iNO in mice in the tested groups are depicted in Fig. 2. The findings revealed that, although mRNA levels of IFN- γ , TNF- α , IL-12 and iNO increased in mice in subgroups E1 and E2, there was no significant difference in comparison with control subgroups. However, the mRNA levels of IFN- γ , TNF- α , IL-12 and iNO were significantly increased in mice in the subgroups of E3 and E4 compared with control subgroups. The expression level of IL10 was significantly decreased in mice in subgroups of E3 and E4 compared with control subgroups, while no significant difference was observed in the expression level of IL10 in E1 and E2 subgroups in comparison with control groups.

DISCUSSION

Today, the emergence of unpleasant side-effects in the treatment of toxoplasmosis has prompted researchers to find an alternative agent with a higher efficacy and lower toxicity. [5] NPs as components of nanoscale sizes are well-known as essential agents in modern medicine, with numerous therapeutic properties such as drug and gene delivery, tissue engineering, cancer therapy and antimicrobial effects [16, 28]. Studies have indicated that such remedial characteristics are related to small nanoscale sizes as well as high surface-to-volume ratios of NPs which produce extra active places for interrelating with biological molecules such as microbial pathogens [14, 29].

Between various NPs, inorganic NPs of metals including Ag, Fe, Zn, Cu and Se, represent a considerable biological activity in medicine [30]. As an essential element, Se has demonstrated numerous medicinal characteristics, including anti-cancer, anti-oxidant and anti-microbial effects [15, 16]. Several studies have reported the unique biomedical applications of SeNPs such as anti-oxidant, anti-cancer and anti-microbial effects, which are attributable generally to its immune modulatory possessions [30]. To the best of our knowledge and according to a review of the literature, the effect of biogenic

Table 3. Results of clinical biochemistry parameters among SeNP-treated groups, at doses of 2.5, 5 and 10 mg kg⁻¹ body weight/day and control mice

Clinical biochemistry parameters	BUN (mg dl ⁻¹)	Cr (mg dl ⁻¹)	AST (U l ⁻¹)	ALT (U l ⁻¹)	ALP (U l ⁻¹)	TB
Control	33.1±4.2	0.21±0.03	127.3±18.6	38.2±6.3	134.7±14.1	0.17±0.03
SeNPs (2.5 mg kg ⁻¹)	35.1±4.2	0.26±0.03	133.1±20.7	39.4±9.2	136.6±23.4	0.18±0.02
SeNPs (5 mg kg ⁻¹)	35.5±4.4	0.26±0.04	131.8±14.4	43.2±4.6	138.1±16.7	0.18±0.03
SeNPs (10 mg kg ⁻¹)	35.6±4.6	0.30±0.04	138.4±18.1	44.7±7.1	138.4±19.3	0.2±0.03
Ato 100 mg kg ⁻¹	39.4±5.1	0.34±0.06	144.2±23.7	56.3±9.2	142.8±24.1	0.24±0.03
SeNPs (10 mg kg ⁻¹)+Ato 100 mg kg ⁻¹	39.3±5.1	0.35±0.07	145.6±24.2*	50.4±4.4*	143.4±15.7	0.28±0.04

**P* < 0.05, significantly different when compared with control. All data are given as mean±SD, as compared with the controls.

BUN, blood urea nitrogen; Cr, creatinine; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; TB, total bilirubin

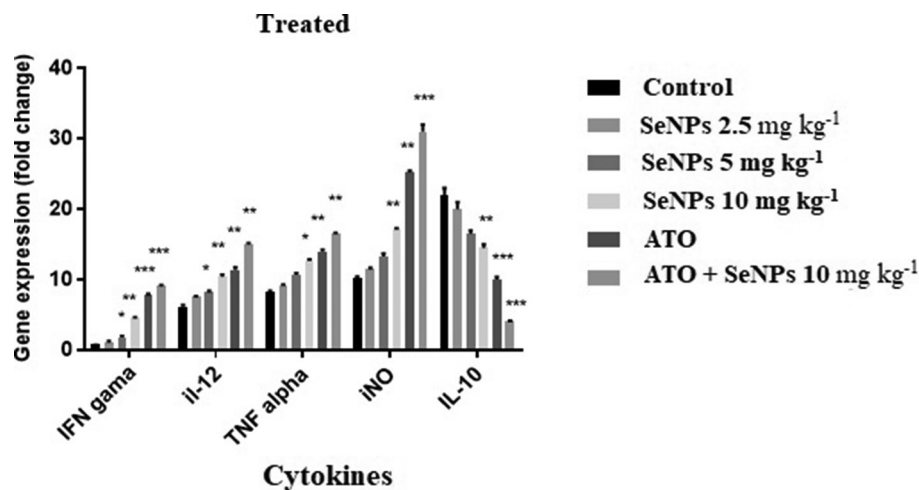
SeNPs on chronic toxoplasmosis remains generally unknown. This study for the first time aims to assess the therapeutic effects of biogenic SeNPs against chronic toxoplasmosis induced by Tehran strain of *T. gondii* in mice.

The results revealed that the mean number of brain-tissue cysts significantly decreased in mice in experimental subgroups E₁, E₂ and E₃ in a dose-dependent manner in comparison with control group C2. In addition, no brain-tissue cysts were observed in the mice of experimental subgroup E₄ (SeNPs 10 mg kg⁻¹+atovaquone 100 mg kg⁻¹) similar to control group C₃ (atovaquone 100 mg kg⁻¹). Previous *in vivo* studies demonstrated the increasing effect of SeNPs on the expression of different genes such as transferrin and its receptor genes in sheep [15]. Studies also showed that Se improves the immune system response, principally cellular immune system, through several pathways.

Moreover, treatment with SeNPs significantly reduced the brain-cyst burden upon increasing the continuous doses of

drug alone, and administration of SeNPs in combination with atovaquone resulted in the absence of cysts compared to atovaquone alone. This may reflect the unknown effect of each drug on *T. gondii* treatment or their synergistic effect, which needs clarification with further studies [31–34].

In the studies conducted by Yazdi *et al.* [20, 35], it has been reported that the level of cellular immunomodulatory cytokines such as granzyme B, IL-12, IFN-γ and IL-2 was significantly enhanced (*P*<0.05) in mice treated with SeNPs. In line with these findings, our results revealed that the expression level of IFN-γ, TNF-α, IL-12 and iNO was significantly increased in infected mice treated with SeNPs compared with untreated BALB/c mice [9]. These results indicate that the reduction in parasite load in the infected mice treated with SeNPs may be linked to the strengthening of the immune system, mainly the cellular immune system, of the animal which led to the control of toxoplasmosis.

**Fig. 2.** Results of the study of the expression of genes producing IL10, TNF-α, IL12, IFN-γ and iNO cytokines in mice treated with SeNPs. (**P*<0.05, ***P*<0.01 and ****P*<0.001 and *****P*<0.0001 were considered significantly different when compared with the control.)

Considering anti-inflammatory activity of SeNPs, El-Ghazaly *et al.* [36] have demonstrated that SeNPs at the dose of 2.55 mg kg⁻¹ had significant anti-inflammatory activity in reducing the paw volume in non-irradiated and irradiated rats but it did not alter the nociceptive threshold. In another study, Miroliaee *et al.* [37] showed that SeNPs along with silymarin (120 mg kg⁻¹ silymarin + 2 µg kg⁻¹ SeNPs) exhibited significant reduction in experimental trinitro benzene sulphonic acid (TNBS)-induced colitis in rats; they also concluded that SeNPs had potent anti-inflammatory activity through inhibiting the (Mitogen activated protein kinase) MAP kinase, and nuclear factor kappa-light-chain-enhancer of activated B-cell (NFκB) levels.

Regarding the antimicrobial effects of SeNPs, Beheshti *et al.* [19] have shown that SeNPs at doses of 5 or 10 mg kg⁻¹ significantly improved the localized lesions of cutaneous leishmaniasis in BALB/c mice after 2 weeks. In addition, Mahmoudvand *et al.* have demonstrated that SeNPs, mainly in combination with meglumine antimoniate, can significantly decrease the growth rate of promastigote and amastigote forms of sensitive and glucantime-resistant *Leishmania tropica* *in vitro* [8]. Furthermore, previous studies demonstrated that SeNPs have effective protoscolicidal properties on *Echinococcus granulosus* protoscolices, so that it can be considered as a protoscolicidal drug during hydatid cyst surgery [38]. Considering the antibacterial effects of SeNPs, studies conducted by Tran *et al.* [16] and Yang *et al.* [17] proved that SeNPs can significantly inhibit the growth of a number of pathogenic bacterial strains such as *S. aureus* and *E. coli* as some common bacterial pathogens.

Based on previous studies, measurement of liver and renal enzyme activities such as ALT, AST, ALP, bilirubin (total, direct), Cr and BUN is among the most reliable methods to assess the toxicity of some agents in animal models. The findings of the present study showed that there is no significant difference in liver and renal enzyme activities among mice treated with SeNPs and those in the control group. Thus, based on the toxicity classification, SeNPs had no significant toxicity against male BALB/c mice [39] at the concentrations of 2.5, 5 and 10 mg kg⁻¹ after 14 days after the administration.

In previous decades, the majority of studies on *Toxoplasma* focused on treatment targeting the proliferative stage of the parasite in reactivated toxoplasmosis [40, 41]. In recent years, however, different projects concentrated on the use of atovaquone as an effective drug for the treatment of the chronic form that may potentially change to the acute phase in immunocompromised patients.

Conclusion

In line with previous research, we showed that SeNPs reduced the number of brain cysts in murine models. Thus, the low toxicity, improved cellular immunity, and excellent anti-*Toxoplasma* properties of SeNPs shows promise for future therapeutic use. We demonstrate that the therapeutic effect of SeNPs is likely to occur primarily by stimulating the immune system by increasing inflammatory cytokines and immune

mediators such as IFN-γ, TNF-α, IL-12 and iNO. Since SeNPs and Se supplementation are currently used widely to treat a wide range of diseases, we suggest that future research aimed at identifying an effective dose of selenium with low toxicity in high-risk individuals will be an important next step towards the control of toxoplasmosis.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

The procedure of the current work approved by the Ethical Committee of the Kerman University of Medical Science (Permit Number: 94/679).

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