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ORIGINAL PAPER

The High Potency of Green Synthesized Copper Nanoparticles to Prevent the *Toxoplasma gondii* Infection in Mice

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Abstract

Purpose Nowadays, due to the lack of an effective vaccine to prevent the toxoplasmosis, chemotherapy with the combination of pyrimethamine and sulfadiazine is considered as the "gold standard" treatment for toxoplasmosis. Recent reports have exhibited that these synthesized chemical drugs are associated with some serious side effects. The present study aims to evaluate the prophylactic effects of copper nanoparticles (CuNPs) green synthesized by *Capparis spinosa* fruit methanolic extract alone and combined with atovaquone against chronic toxoplasmosis induced by the Tehran strain of *Toxoplasma gondii* in mice

Methods Mice were then orally administrated with CuNPs at the doses of 2 and 4 mg/kg/day and in combined with atovaquone 50 mg/kg for 14 days. Male BALB/c mice were divided into two seven groups include C1 (non-treated non-infected); C2 (treated with normal saline); C3 (Infected mice treated with atovaquone 100 mg/kg/day); Ex1 (treated with CuNPs 2 mg/kg/day); Ex2 (treated with CuNPs 4 mg/kg/day); Ex3 (treated with CuNPs 2 mg/kg/day + atovaquone 50 mg/kg/day); Ex3 (treated with CuNPs 4 mg/kg/day + atovaquone 50 mg/kg/day). On the 15th day, the mice were infected with the intraperitoneal inoculation of 20–25 tissue cysts from the Tehran strain of *T. gondii*. The mean numbers of brain tissue cysts and the mRNA levels of IL-12, IFN-γ, and inducible nitric oxide synthase (iNOS) in mice of each tested group were measured.

Results CuNPs were green synthesized by *C. spinosa* methanolic extract. Scanning electron microscopy showed that the particle size of CuNPs was 17 and 41 nm with maximum peak at the wavelength of 414 nm. The mean number of *T. gondii* tissue cysts in mice of tested groups of Ex1, Ex2, Ex3, and Ex4, significantly decreased as a dose-dependent response compared with control group. Moreover, in similar to the control group C3, no *T. gondii* tissue cysts was observed in mice of experimental group Ex3 and Ex4. The mRNA levels of IFN-γ, IL-12, and iNO was measured in mice of all tested groups. The mRNA levels of IFN-γ, IL-12, and iNO was increased in all mice of experimental groups in comparison with the control group C2; however, a significant enhancement was detected in mRNA level of IFN-γ, IL-12, and iNO in the tested groups of Ex3 and Ex4 when compared with control group C3.

Conclusion The obtained results revealed the high potency of CuNPs alone and combined with atovaquone to prevent toxoplasmosis in mice. Although, the prophylactic effects of CuNPs and other properties, such as improved cellular immunity and low toxicity, are positive topics; however, more studies are required to approve these findings especially in clinical settings.

Keywords Chronic toxoplasmosis · Nanomedicine · *Toxoplasma gondii* · Mice · mRNA · *In vivo*

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Introduction

Toxoplasmosis, which caused by the intracellular parasite *Toxoplasma gondii* is well known as one of the most important parasitic diseases which has infected more than one-third of the world's population [1]. Since humans are described intermediate hosts, they are normally infected with the parasite by (i) eating of undercooked or uncooked meat infected with tissue cysts of *T. gondii*, (ii) consumption of water and food contaminated with sporulated oocysts excreted in feces of cat as definitive host (iii) congenital infection, when the mother becomes infected during pregnancy by one of the two previous methods [2, 3].

Toxoplasmosis is routinely asymptomatic with no specific symptoms in most immunocompetent people; however, severe consequences and even deadlier form can occur in immunocompromised peoples (e.g., patients with human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS), and patients with organ transplantation) [4]. In pregnant women, the infection also can result in abortion, abnormality as well as fetal death, especially in women who have no earlier exposure or immunity to parasites [5].

Nowadays, due to the lack of an effective vaccine to prevent the disease, chemotherapy with the combination of pyrimethamine and sulfadiazine, trimethoprim-sulfamethoxazole, as well as azithromycin, clindamycin, and atovaquone is considered as the "gold standard" treatment for toxoplasmosis [6, 7]. Recent reports have exhibited that these synthesized chemical drugs are associated with some serious side effects such as osteoporosis, and teratogenic effects common in immune-compromised patients [8]. Therefore, prophylaxis, especially in susceptible and high risk individuals such as organ transplant recipients, patients with malignancies, patients with immunodeficiency, and pregnant women without a history of infection can be considered as the best way to control and prevent toxoplasmosis [9, 10].

In recent decades, the use of nanotechnology in medicine to treat diseases, especially infectious diseases, is rapidly expanding [11]. It has been proven that development and advance of the drug delivery systems are considered the most common applications of nanotechnology in medicine [11]. Drug delivery system is based on the use of various drug-loaded nanocarrier systems, such as organic (Chitosan, polymeric) and inorganic nanoparticles (metals, metal oxide, metal salts), nanoemulsions, and nanostructured lipid carriers, which assist targeted delivery, improved bioavailability, and reducing toxicity of drugs to treat infectious diseases [12]. Although various chemical and physical techniques are used to synthesize nanoparticles; however, "green synthesis" of nanoparticles by means of plant products is well-known as one of the best methods for the synthesis of nanoparticles [13].

Copper (Cu) is well-known as one of the most beneficial elements available, which has a broad range of medicinal and pharmacological benefits of pharmacological such boosting immune system, anti-tumor, anti-inflammatory, analgesic, microbial infection therapy, etc. [14, 15]. Recent studies have revealed that Cu nanoparticles (CuNPs) because of their high surface-to-volume ratio, which enables them to react well with other particles have different pharmacological and therapeutic properties [16]. To the best of our knowledge, there is no study about the anti-parasitic effects of CuNPs against *T. gondii* infection; therefore, the present study aims to evaluate the prophylactic effects of CuNP green synthesized by *Capparis spinosa* fruit methanolic extract alone and combined with atovaquone against chronic toxoplasmosis induced by the Tehran strain of *T. gondi*i in mice.

Materials and Methods

Green Synthesis and Characterization of CuNPs

Dried fruits of *C. spinosa* that were collected from rural areas of the Kuhdasht city (Lorestan Province, Iran) were used for extraction through percolation method using of 80% methanol for 3 days at room temperature. The green synthesis of the CuNPs was performed based on the previously described protocols [17]. After adding the extract (75 ml) to 100 ml copper sulfate solution (0.01 M); the solution was stirred at 60 °C for 1 day and then was centrifuged two times at the 12,000 rpm for 15 min. Finally, the synthesis of nanoparticles was confirmed by changing the color of the solution from green to yellow. Finally, the obtained nanoparticles were dried at 60 °C for 120 min for the further testing. Finally, the obtained nanoparticles were dried at 60 °C for 2 h and stored until the desired tests were performed.

In this study, the evaluation of transformation of the copper ions into copper nanoparticles was performed based on the determination of surface plasmon resonance (SPR) by means of ultraviolet–visible (UV–Vis) spectrophotometer (JENWAY 6405). For this purpose, 300 µl of a solution of nanoparticles diluted with normal saline was evaluated using UV–Vis spectrum analysis by means of a spectrophotometer apparatus ranged from 300 to 700 nm. To do that Fourier transform infrared spectroscopy (FTIR) (model Nicolet32) analysis with ranging from 400 to 4000 and the resolution of 10–40 mm was performed on the solution of the obtained nanoparticles combined with the potassium bromide (KBr) granules (1/100 ratio) after taking the tablets. The characterization and specifications (e.g., size and morphology) of the obtained nanoparticles were evaluated by scanning electron microscope (SEM) (Mira3, Made in Czech) with 15 kv, magnification of 10x, and resolution of 1 nm.

Parasitological Study

Inducement of Chronic Toxoplasmosis in Mice

Forty-eight male BALB/c mice (6–8 weeks old) weighing from 20 to 25 g were used to establish the chronic toxoplasmosis. Mice were kept in a colony room with a 12:12 h light/dark cycle at 21 ± 2 °C and handled based on the standard protocols for the use of laboratory animals [18]. The strain used in the present study to induce chronic toxoplasmosis was the Tehran strain of *T. gondii* (type II) which kindly gained from Prof. Hossein Keshavarz (Tehran University of Medical Sciences, Tehran, Iran). The parasite was prepared by intraperitoneal inoculation of tissue cysts of *T. gondii* (15–20 cysts) from brain tissue of infected BALB/c mice every 3 months into new mice based on the technique explained by Elfadaly *et al.* [19]. The mice model of latent toxoplasmosis was established according to the process reported by Mahmoudvand *et al.* [20]. For this purpose, a homogenized suspension (0.5 ml) from the brains of infected mice containing 25–30 *T. gondii* tissue cysts with penicillin and streptomycin antibiotics was injected intraperitoneally into mice of any test group.

Study Design

Considering the experimental design of current investigation, as shown in Fig. 1, male BALB/c mice were divided into two main groups include control (C) and experimental group (Ex) with seven sub-groups include: C1 (non-treated non-infected); C2 (treated with normal saline); C3 (infected mice treated with atovaquone 100 mg/kg/day); Ex1 (treated with CuNPs 2 mg/kg/day); Ex2 (treated with CuNPs 4 mg/kg/day); Ex3 (treated with CuNPs 2 mg/kg/day + atovaquone 50 mg/kg/day); Ex3 (treated with CuNPs 4 mg/kg/day + atovaquone 50 mg/kg/day). After 21 days of oral administration, the mice in all groups except C_1 group were infected with the Tehran strain of *T. gondii.* Confirmation of toxoplasmosis was performed by the evaluation of anti-*T. gondii* IgG antibody in the serum samples of tested mice using a modified agglutination test (MAT) kit (Toxo screen DA, Biomérieux, Lyon, France), the formalized killed whole tachyzoites of *T. gondii* was prepared and procedures were carried out according to the method described by Shaapan *et al.* [21]. The agglutination titer of 1/20 ≤ were positive and were end-titrated by twofold dilutions.

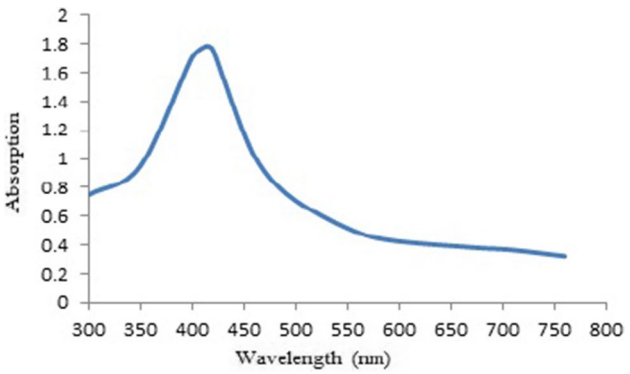

Fig. 1 The absorption spectrum of synthesized copper nanoparticles

Brain Tissues Collection

Initially, mice were totally anesthetized using the intraperitoneal administration of the ketamine (150 mg/kg) and xylazine (10 mg/kg). After decapitation of mice, whole brain tissues of mice were aseptically collected. The parasitological studies were performed on the right brain hemisphere; but the left hemisphere was stored in −80 ºC to use for the molecular tests.

Calculating *T. gondii* Tissue Cysts

The effects of CuNPs on *T. gondii* tissue cysts were determined by evaluation of the unstained-smears provided from the right brain hemisphere of each mouse. For this purpose, the numbers of tissue cysts were calculated at two magnifications of 100 × and 400 × by means of light microscopy [21].

Cytokine Expression by Real-Time PCR

The mRNA levels of some cytokines and immune mediators (IFN-γ, IL-12, and inducible nitric oxide synthase (iNOs) involve in control of toxoplasmosis were assessed in all tested mice using quantitative real-time PCR. The total brain RNA was extracted by means of the RNA-easy kits (Qiagen, Hilden, Germany); whereas all isolated RNAs were reverse transcribed according to the manufacture's protocols. Consequently, the collected complementary DNA (cDNA) was applied to conventional PCR amplification or real-time PCR. To perform the real-time PCR we used the iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). All amplification products were determined by SYBR green [22]. The reaction conditions of real-time PCR were include initial denaturation at 95 °C for 10 min, 40 amplification cycles [denaturation at 95 °C for 10 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s], followed by one cycle at 72 °C for 5 min. The iQ^{TM}5 optical system software (Bio-Rad) was used to data analysis. Here, β-actin which is well-known as a house keeping gene was considered as a normalization control. Oligonucleotide primers used for real-time RT-PCR analysis are shown in Table 1.

Table 1 Sequences of primers used for real-time PCR

Amplicon	Primers	Sequence (5′–3′)
IL-12	F	ACGACATTCGTCAACTGCAA
	R	TAAATTGGCACCCTGTAGGC
IFN-γ	F	GATCGTGTCGTCACCAGAAAGG
	R	TGCCTGGTAACGAGTTGTCC
iNOs	F	CGTGAAAGGATCCAGAAAGG
	R	TCCACGATGCCTGGTAGTTG

Statistical Analysis

Data analysis was carried out using SPSS statistical package version 17.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA with Turkey's *post hoc* test was used to assess differences between experimental groups. In addition, $p < 0.05$ was considered statistically significant.

Results

Characterization of CuNPs

Figure 1 exhibits the maximum peak (414 nm) of the green synthesized CuNPs. The presence of metallic copper was confirmed by EDX analysis. The copper nanoparticles at 1 keV exhibited the maximum peak; indicating the presence of the metallic CuNPs. The normal resonance band of the surface plasmon was also of CuNPs observed at the wavelength of 414 nm. Figure 2 demonstrates that the biomolecules in the extract may reduce the copper sulfate solution; indicated that these biomolecules may be applied as coatings for nanoparticles. Based on the results the maximum band was 3380 for O–H stretching of alcohol and phenol, followed by 2928 for C–H stretching of aliphatic group, and 1741 for C = O stretching of ester carbonyl. The results of SEM showed that the morphology of green synthesized CuNPs was spherical; whereas the particle size was detected among 17 to 41 nm (Fig. 3).

Parasitological Study

As shown in Fig. 4, the mean number of *T. gondii* tissue cysts in mice of tested groups of Ex1 ($n = 179.6$) and Ex2 ($n = 119.3$) significantly ($p < 0.001$) decreased at a dose-dependent response compared with C2 ($N = 262.6$). Moreover, in similar to the control group C3 ($n = 0$), no *T. gondii* tissue cysts were observed in mice of experimental group Ex3 ($n = 0$) and Ex4 ($n = 0$).

Cytokine Expression by Real-Time PCR

The mRNA levels of IFN-γ, IL-12, and iNO were measured in mice of all tested groups. As shown in Fig. 5, the mRNA levels of IFN-γ, IL-12, and iNO were increased in all mice of the experimental groups in comparison with the control group C2; however, a significant enhancement was detected in mRNA level of IFN-γ, IL-12, and iNO in the tested groups of Ex3 and Ex4 when compared with control group C3.

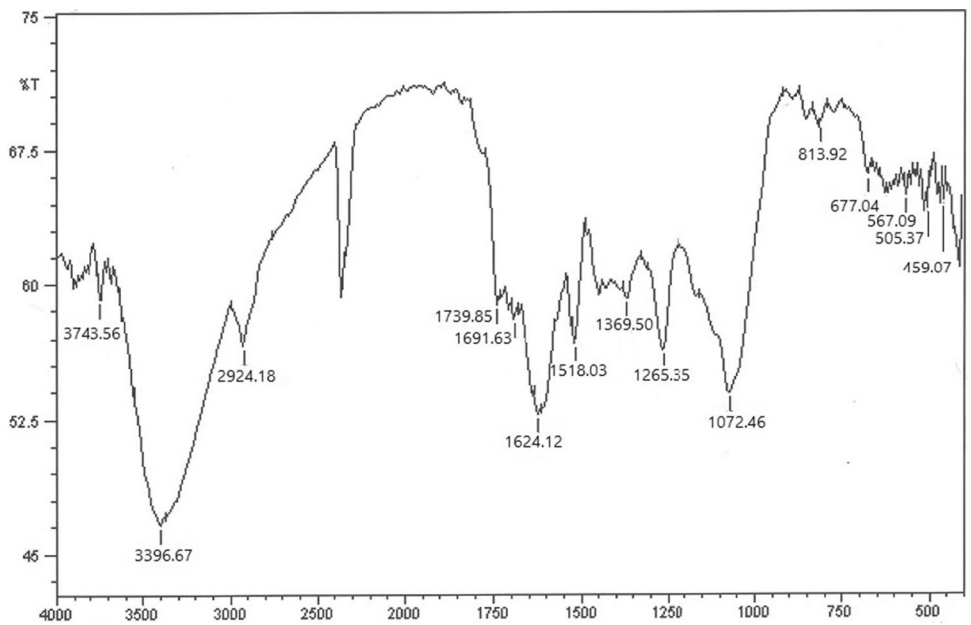

Fig. 2 The FTIR spectrum of synthesized copper nanoparticles

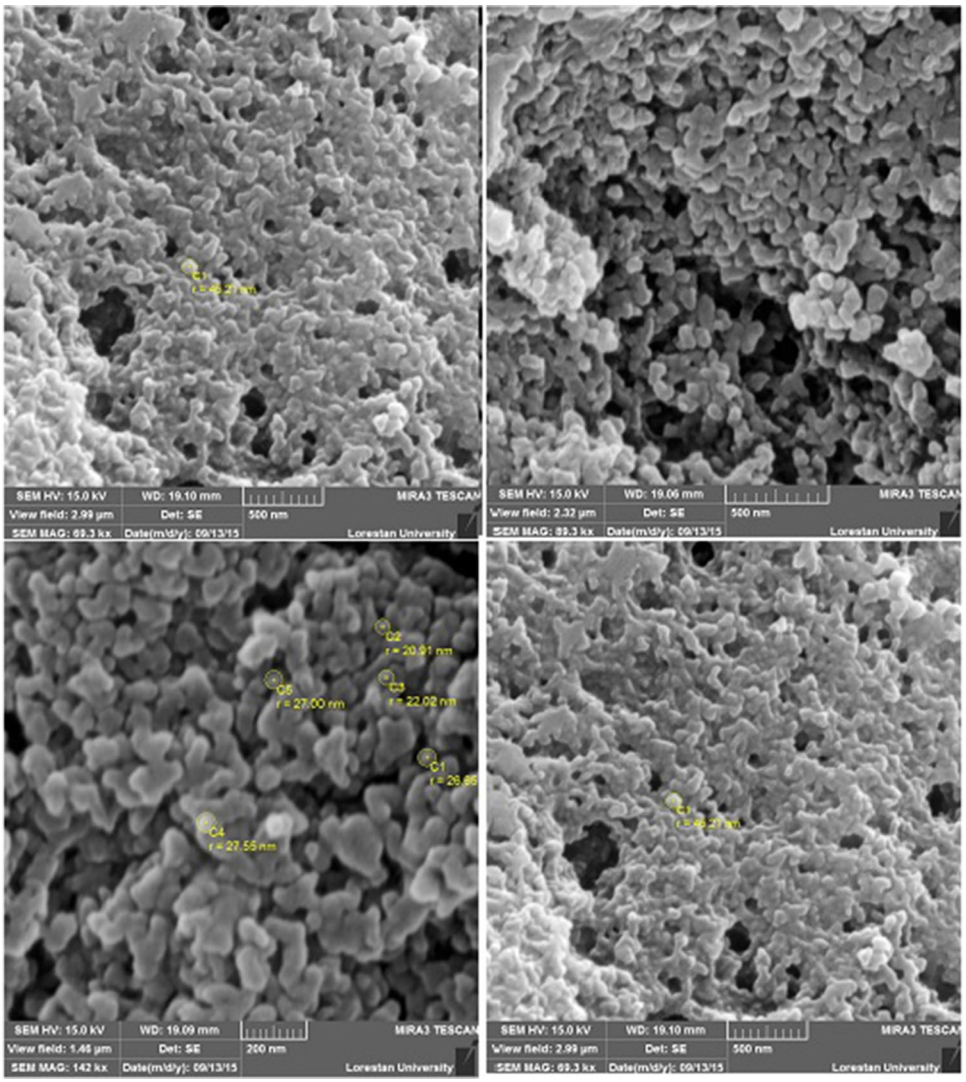

Fig. 3 Scanning electron microscope of copper nanoparticles synthesized using methanolic extract of *Capparis spinosa* fruit

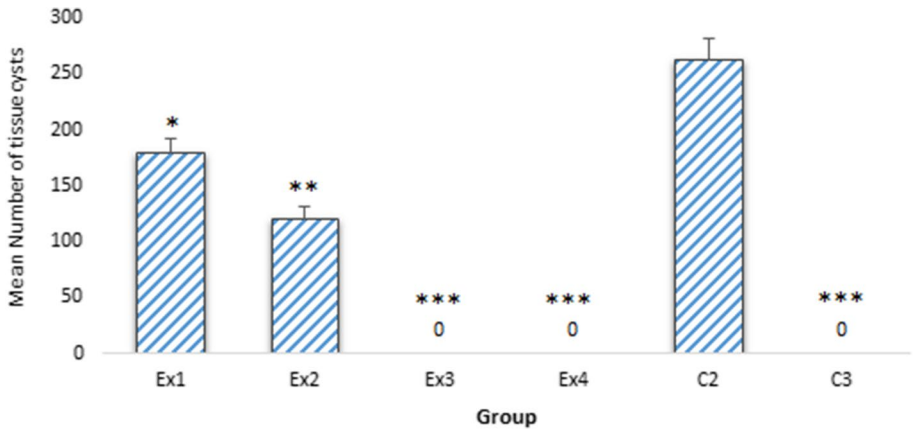

Fig. 4 The mean numbers of brain tissue cysts in mice of tested group. $*p<0.05$, $**p<0.01$, and $***p<0.001$ the difference was statistically significant compared with C2. C1 (non-treated non-infected); C2 (treated with normal saline); C3 (infected mice treated with atovaquone 100 mg/kg/day); Ex1 (treated with CuNPs 2 mg/kg/day); Ex2 (treated with CuNPs 4 mg/kg/day); Ex3 (treated with CuNPs 2 mg/kg/day + atovaquone 50 mg/kg/day); Ex3 (treated with CuNPs 4 mg/kg/day + atovaquone 50 mg/kg/day)

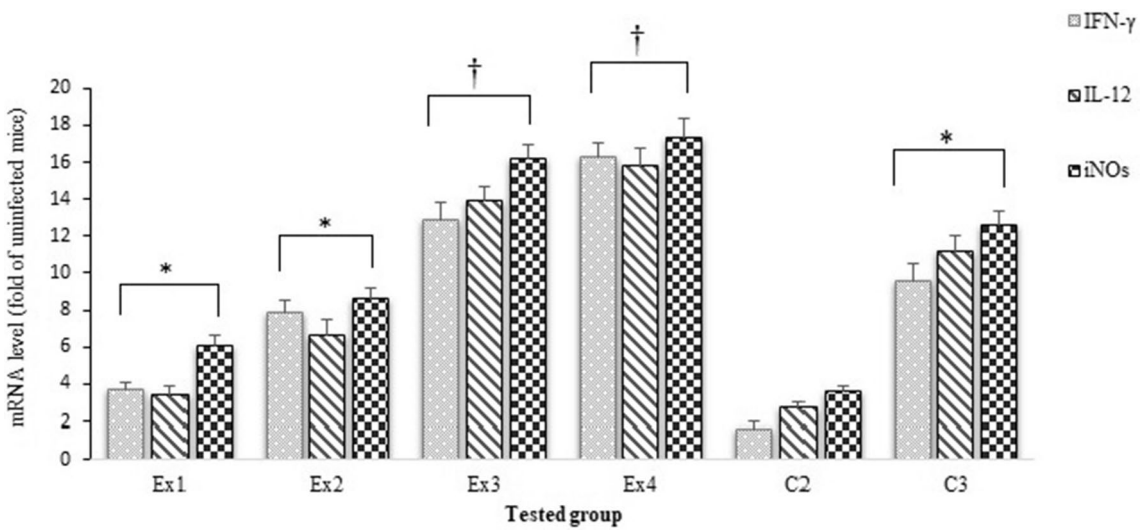

Fig. 5 The expression level of genes producing IL10, TNF-α, IL-12, IFN-γ and iNO cytokines in mice treated with CuNPs. *$p < 0.001$ the difference was statistically significant compared with control group C2. †$p < 0.01$ the difference was statistically significant compared with control group C3. C1 (non-treated non-infected); C2 (treated with normal saline); C3 (infected mice treated with atovaquone 100 mg/kg/day); Ex1 (treated with CuNPs 2 mg/kg/day); Ex2 (treated with CuNPs 4 mg/kg/day); Ex3 (treated with CuNPs 2 mg/kg/day + atovaquone 50 mg/kg/day); Ex3 (treated with CuNPs 4 mg/kg/day + atovaquone 50 mg/kg/day)

Discussion

From years ago until now, the "gold standard" treatment for *T. gondii* infection is the combination of sulfonamide and pyrimethamine [23]. However, based on the recent reports, this chemotherapy is associated with various serious side effects and responses such as gastrointestinal and hematological disorders, teratogenic effects, myelosuppression, etc. [6, 7]. These reasons encourage researchers to explore alternative drugs with low toxicities that can be used alone or in combination with conventional drugs.

Today, nanotechnology as a multidisciplinary scientific field has a wide and diverse range of applications in the development of novel and effective medical treatments which also called "Nanomedicine". In recent years, the application of nanoparticles is considered as one of the most promising plans to treat the microbial infections [23]. Considering the synthesis of nanoparticles, various physical and chemical approaches have been introduced to synthesize the nanoparticles with specific size, lower toxicity, and diverse biological activity [11]. Among these approaches, green synthesis is considered as one of the most standard, cost effective, reliable, sustainable, and synthesis methods to prepare safe and high performance nanoparticles [11]. In recent years, green synthesis of metallic nanoparticles through medicinal herbs is a preferred option rather than other methods that use bacteria and/or fungi to treat microbial pathogens, alone and combined with the exist/conventional antimicrobial drugs [12, 13]. Today, studies have demonstrated that CuNPs because having the large surface area to volume ratio have various biomedical applications such as anti-parasitic, antimycotic, antibacterial, antiviral, anti-cancer, anti-inflammatory, anti-diabetic, antioxidant, and immunomodulatory activities [24–26]. The present investigation aimed to evaluate the prophylactic effects of CuNPs alone and combined atovaquone against latent toxoplasmosis induced by the Tehran strain of *T. gondii* in mice. The mean number of *T. gondii* tissue cysts in mice of tested groups of Ex1, Ex2, Ex3, and Ex4, significantly decreased as a dose-dependent response compared with control group (C2). Moreover, in similar to the control group C3, no *T. gondii* tissue cysts was observed in mice of experimental group Ex3.

Regarding the antimicrobial activities of CuNPs, several studies have exhibited the potent antimicrobial effects of these nanoparticles against some bacterial and fungal pathogenic strains such as *Staphylococcus aureus*, *Salmonella enteric*, *Campylobacter jejuni*, *Escherichia coli, Listeria monocytogenes, Aspergillus niger*, etc [27–29]. In addition, Saad *et al.* [30] have reported that copper oxide nanoparticles have considerable anti-parasitic effects with IC_{50} values of 0.13 and 0.72 mg/l for *Entamoeba histolytica* and *Cryptosporidium parvum,* respectively; indicating that these nanoparticles could be introduced as a new nanoform drug to treat of *E. histolytica* and *C. parvum* infections. In another study carried out by Malekifard *et al.* [31], the findings demonstrated that copper oxide nanoparticles, especially at the dose of 0.6 mg/ml reduced the viability *Giardia lamblia* cysts by 97% after 3 h exposure similar to the effect of metronidazole. Recently, Albalawi have demonstrated

that CuNPs particularly along with glucantime, significantly reduced the growth of *Leishmania major* amastigotes as well as the mean diameter of the lesions in mice infected by cutaneous leishmaniasis [32].

By antimicrobial action mechanisms of CuNPs, although these mechanisms are not yet fully understood, however, in a study conducted by Mahmoodi *et al.* [33] for this purpose, the results showed that copper through the interaction with sulfhydryl groups(–SH) can result in to denaturation of protein in bacteria. Moreover, the results of the study of Chatterjee *et al.* [34] revealed that CuNPs may cause disruption of cell membrane and also multiple toxic effects such as destruction of DNA in bacteria cells, oxidation of proteins, peroxidation of lipids, and reactive oxygen production.

It has been previously proven that cytokines play a key role in the control of *T. gondii* replication in the central nervous system [35]. Reviews have demonstrated that IFN-γ, IL-12, IL-1, and IL-6 are able to control the growth of *T. gondii* in the brain through activation of microglia [35, 36]. On the other hand, *in vivo* investigations exhibited that cytokines promote anti-*Toxoplasma* activity in microglia through NO-mediated mechanism [36]. In the present investigation, we have evaluated the mRNA levels of some cellular immunity mediators such as IFN-γ, IL-12, and iNOs by quantitative real-time PCR. The mRNA levels of IFN-γ, IL-12, and iNO was increased in all mice of experimental groups in comparison with the control group C2; however, a significant enhancement was detected in mRNA level of IFN-γ, IL-12, and iNO in the tested groups of Ex3 and Ex4 when compared with control group C3. In line with our results, Douglass *et al.* [37] demonstrated that the CuNPs improved the NO release, and also the antimicrobial activity against both Gram-positive and Gram-negative bacteria via the oligodynamic effect of Cu. Our findings revealed that the decrease in parasite load in the infected mice treated with CuNPs may be linked to the reinforcement of the immune system, principally the cellular immune system, of the tested mice that result in the control of *T. gondii* infection.

With respect to the toxicity of CuNPs, Khatami *et al.* [17] have demonstrated that there was no significant toxicity in the liver enzymes (e.g., Aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase) and hematological parameters (e.g., hemoglobin, hematocrit, and platelet) after 14 days' oral administration of CuNPs at the doses of 1, 2, and 5 mg/kg.

Conclusion

The findings of the present study demonstrated the potent prophylactic effects of CuNPs especially in combination with atovaquone in infected mice with *T. gondii*. So that oral administration of CuNPs in doses of 0.2 and 0.3 ml/kg and in combined with atovaquone (100 mg/kg) for 14 days was able to prevent severe symptoms of the toxoplasmosis in mice model. The findings of the present investigation are the initial step towards a new potential treatment possibility of these nanoparticles. Although, the prophylactic effects of CuNPs and other properties, such as improved cellular immunity and low toxicity are positive topics; however, more studies are required to approve these findings especially in clinical settings.

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Availability of Data and Materials All data generated or analyzed during this study are included in this published article.

Declarations

Conflicts of Interest The authors declare that they have no conflict of interests.

Ethics Approval All required ethical licenses were obtained from the Ethical committee of the Lorestan University of Medical Science (LUMS.REC.1395.178).

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