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Evaluation of the effectiveness of Catechin-Loaded Silver Nanoparticles on Chronic Toxoplasmosis in Mice: Targeting Brain Cyst Count

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Abstract:

***Background*: Toxoplasmosis is a zoonotic disease caused by the protozoan *Toxoplasma gondii*, which is an obligate intracellular parasite. *Aim:* The current study was designed to evaluate the efficacy of (+)-Catechin loaded silver nanoparticles in reducing brain cyst count in chronic toxoplasmosis with the purpose of overcoming the limitations of conventional treatments. *Methods:* The experimental design included seven groups: Group 1 included uninfected, untreated mice. Group 2 included infected, untreated mice. Group 3 included infected mice treated with spiramycin. Groups 4 and 5 involved infected mice treated with (+)-Catechin-loaded silver nanoparticles (30 mg and 60 mg, respectively). Group 6 included infected mice treated with silver nanoparticles alone, and Group 7 involved infected mice treated solely with (+)-Catechin. Treatment effectiveness was detected by assessing *Toxoplasma* brain cyst count. *Results:* Mice treated with 30 mg and 60 mg of catechin-loaded silver nanoparticles showed the lowest mean cyst counts, establishing the highest percentage reductions of 64.63% and 58.94%, respectively with a significant statistical difference when comparing with group 2. *Conclusion:* In this experimental model, the study performed demonstrates that (+)-Catechin-loaded silver nanoparticles exhibit significant anti-parasitic activity against chronic toxoplasmosis.**

Keywords: (+)-catechin; Cat-AgNPs; Brain cyst count; *Toxoplasma gondii*.

Introduction

Toxoplasmosis is a parasitic protozoan infection caused by *Toxoplasma gondii* (*T. gondii*), an obligate intracellular parasite with the capacity to infect humans and all warm-blooded animals, such as mammals and birds [(1)]. About one-third of the world population is infected with *T. gondii* [(2)].

Clinical toxoplasmosis may be acute or chronic; reactivation can occur at any age [(3)]. The chronic form is usually asymptomatic, but it has been known to reactivate later in life in immunocompromised patients; this often leads to high mortality rates, particularly in HIV-positive patients [(4)].

Resistance to *T. gondii* is mainly mediated by a Th1-type cell-mediated immune response, which depends on the production of interleukin-12 (IL-12) and interferon-γ (IFN-γ) [(5; 6)]. IFN-γ is the main cytokine that controls both the acute and chronic phases of the infection [(5; 6)]. On the other hand, interleukin-10 (IL-10) plays an anti-inflammatory role during the course of the chronic phase of *T. gondii* infection by

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helping to down-regulate Th1 lymphocyte activity [7].

Current treatments in use against toxoplasmosis include pyrimethamine, sulfadiazine, and clindamycin; these agents suppress active infections. However, there are no current therapies that effectively target the dormant cyst stages of infection [8]. Spiramycin and atovaquone have small efficacies against *T. gondii* cysts in chronic toxoplasmosis [9].

Catechins are natural polyphenolic compounds of the flavanol subclass of the flavonoid family. They include the precursor isomers (+)-catechin and (-)-epicatechin as well as their gallate ester derivatives [10]. Catechins have been attributed to antioxidative, anti-inflammatory, and anti-infective effects [11].

These catechins are characterized by poor stability and, as a result, very low bioavailability. However, nanoparticles as carriers could enhance their stability and maintain it [12]. In recent years, silver nanoparticles (AgNPs) have been of interest for medical and chemical purposes due to their extraordinary characteristics like antibacterial activity, excellent oxidation resistance, excellent thermal conductivity and can be used as carriers to deliver drug molecules to target sites and hence increase their therapeutical efficacy [13].

The current study was designed to investigate the efficacy of (+)-catechin loaded onto silver nanoparticles for treating chronic toxoplasmosis, considering the shortcomings of conventional therapies.

Materials and methods

This was an experimental study. The study was conducted on seven groups, each consisting of five male Swiss albino mice: G1 included uninfected, untreated mice. G2 included infected, untreated mice. G3 included infected and treated mice with spiramycin. G4 included infected and treated mice with (+)-Catechin-loaded silver nanoparticles (Cat-AgNPs 30 mg). G5 included infected and treated mice with (Cat-AgNPs 60 mg). G6 included infected and treated mice with silver nanoparticles (AgNPs), while G7 included infected and treated mice with catechin.

Experimental infection: Swiss albino mice were inoculated orally through an esophageal tube with 100 µl of brain homogenate containing 10 cysts per mouse of the *T. gondii* ME49 strain, in an attempt to develop chronic infection. The experiments were conducted at the Theodor Bilharz Research Institute, a medical research facility affiliated with the Egyptian Ministry of Higher Education and Scientific Research. The study was approved by the Medical Ethics Committee, Faculty of Medicine, Suez Canal University.

Euthanization: All mice groups were sacrificed on the 75th day post-infection.

Evaluation of the therapeutic effect: Each mouse was assessed through the brain homogenate after sacrifice, where the count of *T. gondii* brain cysts was measured.

Experimental infection:

Materials for experimental infection:

Experimental animals: In this study, thirty-five Swiss albino mice were taken from the animal house of the Theodor Bilharz Research Institute. These mice were between 8 and 10 weeks old, with weights ranging between approximately 20 and 25 grams. They were housed under pathogen-free conditions in front-opening cages and were fed a standard diet. Food and water were given freely. Brain cysts of the ME49

strain of *T. gondii* were kindly provided by the Theodor Bilharz Research Institute, Egypt.

Drugs:

Spiramycin was bought as 3 M.I.U. (1 gm) tablet form from Pharaonia Pharmaceuticals. Then, these tablets were crushed into powder. The powder was then mixed with water and given orally to the mice by esophageal tube at a dose of 200 mg/kg/day [13].

A suspension of (+)-catechin was prepared by dissolving 150 mg of (+)-catechin hydrate powder in 30 ml of distilled water to which 0.3% w/v carboxymethyl cellulose had been added. This gave a concentration of 5 mg/ml.

AgNPs were prepared by Nanotech for Photo Electronics Company using a chemical reduction method [14]. In brief, reductant triethylamine reduces Ag+ ions to metallic silver (Ag0) atoms that agglomerate to oligomeric clusters that eventually form metallic colloidal silver particles. The AgNPs were prepared at a concentration of 40 ppm (0.04 mg/ml) [15].

Cat-AgNPs were synthesized by Nanotech for Photo Electronics Company following the method outlined by **Ikram et al.** [16]. In this procedure, a suspension of (+)-catechin was formulated in a methanol-water mixture with a ratio of 2:8, while a silver nitrate solution was created utilizing deionized water. When 100 µl of equimolar solutions of (+)-catechin mixed with 100 µl of $AgNO_3$ were mixed in a 1:5 ratio, adding 4-5 drops of triethylamine as a reducing agent, the color of the mixture almost immediately turned from colorless to yellow. This color change indicates the successful synthesis of Cat-AgNPs and optimal reduction of silver ions. The obtained suspensions of Cat-AgNPs were centrifuged at 12,000 rpm for 12 min and concentrated to 1.5 and 3 mg/ml.

Groups: Mice in G1 were used as a negative control, while mice in G2 acted as the positive control. Mice in G3 were treated with spiramycin (200 mg/kg/day, p.o.) from the 60th day post-infection (p.i.) onwards for 14 days [13]. Mice in both groups G4 and G5 were treated with Cat-AgNPs at 30 mg/kg/day and 60 mg/kg/day, respectively from the 60th day p.i. onward for 14 days [17]. Treatment of mice of G6 with AgNPs alone at a dose of 0.72mg/kg/day, was given to infected mice on day 60 p.i for 14 days [13]. Mice of G7 received treatment with catechin at a dose 60 mg/kg/day on day 60 p.i for 14 days [17].

Mice from all groups were sacrificed by rapid decapitation on 75th day p.i.

Evaluation of the therapeutic effect: The therapeutic effect was assayed in the treated groups compared with the normal control group. Assessment was made on each mouse's brain homogenate after sacrifice.

Estimation of *T. gondii* cyst counts in the brain homogenates of mice: The mice were sacrificed by cervical dislocation. The head was disinfected with 70% ethanol, the skin incised to expose the skull that was opened with a pair of small curved scissors, and the brain was removed with forceps into a mortar. It was then crushed therein with a pestle, after which 1 ml of phosphate-buffered saline was added. The suspension of the brain was homogenized by aspirating and then ejecting it repeatedly from a 1- or 3-ml syringe fitted with a 22-G needle. To enumerate the tissue cysts, three samples of 20 µl of the homogenate of each mouse brain were transferred onto microscope slides and overlaid with coverslips of 22 × 22 mm for counting an accurate mean. The

cysts in each sample were counted under the microscope at a magnification of 400× and the mean of these three counts was calculated. Then, the brain cyst burden of each mouse was calculated by using the following formula: brain cyst burden = mean × 50 [18].

Data management and statistical analysis: Data was analyzed by writing in Microsoft Excel 2019 from the Microsoft Office Bundle by Microsoft Corporation, USA, and analyzed by SPSS version 26, Statistical Package for Social Sciences, IBM Corporation, USA. The distribution of data was checked by Shapiro-Wilk test, which revealed normally distributed data regarding specific gravity and proteinuria while other variables were not normally distributed. Quantitative data were presented as mean ± SD, while qualitative data were represented in terms of frequencies and percentages.
The one-way ANOVA tested the differences of variance for parametric data among the seven groups, while the Kruskal-Wallis test was applied for non-parametric data and this test was selected because of sample size less than 50. Further testing was done by post-hoc testing by pairwise comparisons of the different mean values.
The Spearman correlation coefficient was used to perform a correlation matrix where p-values were calculated. The p-value refers to the measure of significance and was considered < 0.05 to be significant.
Results were presented in tables, figures, and graphs.

Ethical consideration
The present study was approved by the Medical Ethics Committee of the Faculty of Medicine at Suez Canal University in July 2021. Animal experiments were performed under the Guide for the Care and Use of Laboratory Animals of the National Research Council [19].
Care was taken to:
1. Animal species and quality appropriate to the study proposed and used in minimal numbers requisite to obtain valid data.
2. The pain and discomfort of the animal were kept at a minimum.
3. Whenever necessary, animals were humanely restrained and transported.
4. Husbandry, if applicable, was attended to properly.
5. No animal was used in more than one research study, and reutilization of the same animal in the same study was avoided.
6. Oral medication was administered with care to the animals.
7. The carcass of the animals was finally disposed of accordingly.

Results

***T. gondii* cyst count in mice brains**
Examination of the brain homogenates of infected mice revealed Spherical-shaped *T. gondii* cysts, each bearing hundreds of bradyzoites in their cyst walls (Figure 1).

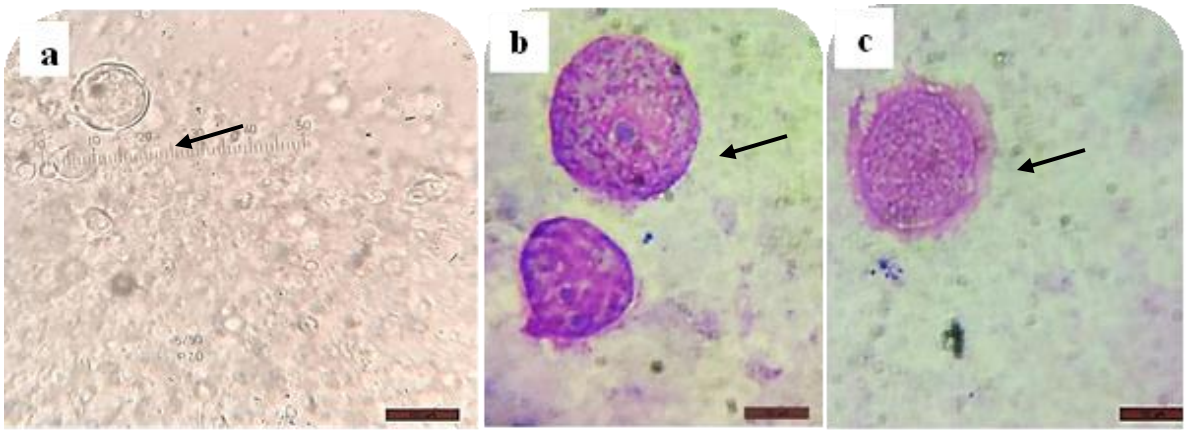

Figure 1: Photomicrographs of brain homogenates from chronically infected mice showing *T. gondii* cysts (arrows); **a)** Unstained, **b), c)** Giemsa stained (×1000).

Table 1: *T. gondii* brain cyst count among infected treated and untreated mice groups

Mice group	Cyst count (Mean ± SD)	Reduction%	p-value	
Infected non-treated (G2)	1417.33 ± 6.57	--------		--------
Spiramycin treated (G3)	772.67 ± 8.89	45.48%		--------
Cat-AgNPs 30 mgtreated (G4)	501.33 ± 17.23 [a, c, d]	64.63%		< 0.001[a] 0.002 [c] 0.02 [d]
Cat-AgNPs 60 mgtreated (G5)	582.00 ± 20.00 [a, b, c]	58.94%	< 0.001*	0.002 [a] 0.02 [c]
AgNPs treated (G6)	1071.20 ± 9.65	24.42%		--------
Catechin treated (G7)	685.53 ± 14.07 [a, b]	51.63%		0.02 [a]

***** Statistically significant at $p < 0.05$; Kruskal-Wallis test, $H = 33.427$
a Significantly different from the infected untreated group
b No significant difference with the Cat-AgNPs 30 mg treated group
c Significantly different from AgNPs treated group
d Significant difference from the spiramycin-treated group.

The mean count of cysts in the brains of infected, untreated mice was 1417.33 ± 6.57. In all infected treated groups of mice, the brain cysts were reduced after treatment. The rates of reduction in cysts counts in the treated mice ranged from 24.42% to 64.63%, as compared with the infected and untreated group, were significantly statistical different ($P < 0.001$) (Table 1 and Figure 2). Among them, Cat-AgNPs 30 mg and 60 mg groups exhibited the highest reduction percentage, 64.63% and 58.94%, respectively, with their mean cyst counts being 501.33 ± 17.23 and 582.00 ± 20.00. There is a statistical difference between G2 versus G4 ($P < 0.001$) and G5 ($P = 0.002$). However, no significant difference was observed between G4 and G5 ($P = 0.44$).

There is also a significant difference between G3 and G4 (P = 0.020). The marked reduction of cysts by 51.63% was seen in the catechin-treated group, having a mean cyst count of 685.53 ± 14.07, wherein it is significantly different from G2 (P = 0.02). However, no significance was observed between G7 and G3, (P = 0.44); G4, (P = 0.122); and G5, (P = 0.44). AgNPs-treated group resulted in the minimum percentage of cyst reduction as 24.42% with a mean cyst count of 1071.20 ± 9.65. While comparing G6 with G4 (P = 0.002) and G5 (P = 0.020), significant differences were observed.

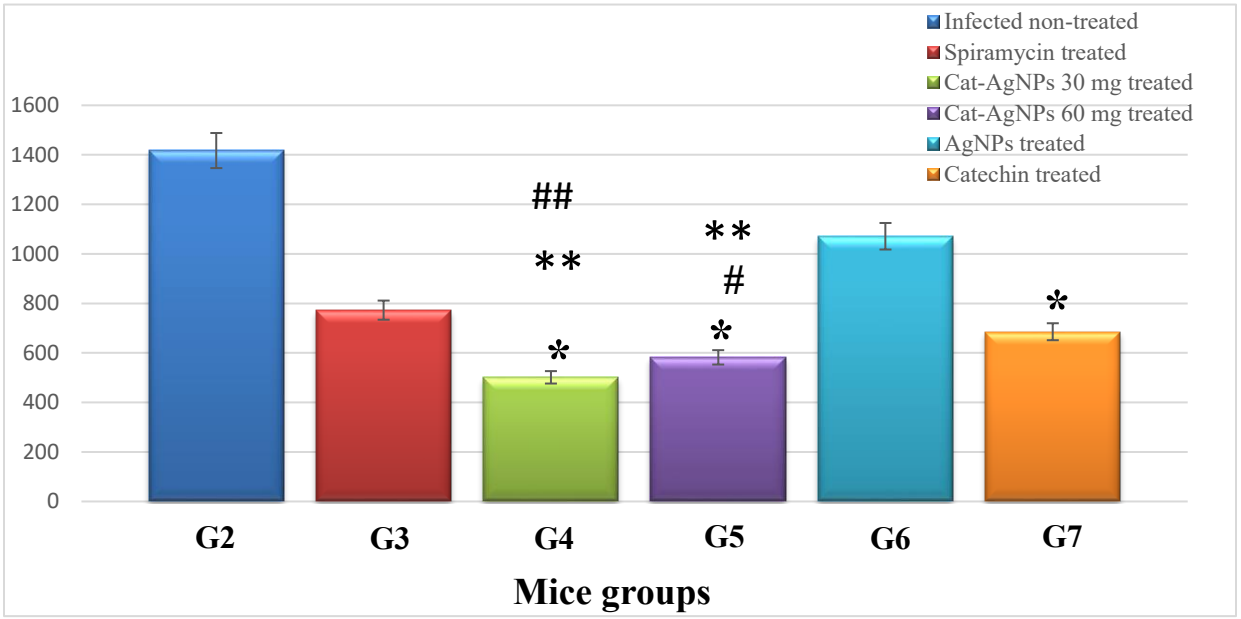

Figure 2: Brain cyst count in different mice groups
* Significantly different from infected untreated group
# Not statistically significant from Cat-AgNPs 30 mg/kg treated group
** significantly different from AgNPs-treated group
Significant difference from the spiramycin treated group.

Discussion

T. gondii chronically infects an estimated 2 billion people worldwide and is associated with life-threatening complications, particularly in immunocompromised individuals and pregnant patients [20]. In chronic toxoplasmosis treatment, it is tough due to the presence of a blood-brain barrier which restricts the drug concentration in the brain, leading to inadequate delivery of the therapeutic agent [21].

The currently available treatment of choice against toxoplasmosis is the first-line administration of pyrimethamine in combination with sulfadiazine and folinic acid, where the active stage of the disease is targeted to restrict the multiplication of the parasite during early infection [22]. However, this combination is not a valid therapeutic approach to chronic cerebral toxoplasmosis. Treatment usually consists of several weeks, even months, or even more than a year. It can be associated with adverse effects, which include leukopenia, neutropenia,

thrombocytopenia, increased serum creatinine and liver enzymes, and hypersensitivity reactions [23].

Recently, natural products have emerged as a quite promising source of lead drugs against an enormous variety of diseases and pathological conditions [24]. Catechins, naturally occurring flavan-3-ols, are one of the numerous potential therapeutic agents from nature. Among these, (+)-catechin constitutes one of the most important flavan-3-ol monomers studied [25].

Extremes of pH in the stomach and intestinal tract, along with associated digestive enzymes, have been seen to affect catechin stability. This, therefore, increases the bioavailability of the catechins through drug delivery systems based on nanostructures [26]. Relating to this, one of the metallic nanoparticles commonly used is silver nanoparticles due to its antimicrobial and anti-inflammatory properties [27]. Relating to this, one of the metallic nanoparticles commonly used is silver nanoparticles due to its antimicrobial and anti-inflammatory properties. The green synthesis of silver nanoparticles using green tea extract, and two of its components, that is, (+)-catechin and (-)-epigallocatechin gallate as capping and stabilizing agents, was elaborated by Das et al. [28] and the obtained material demonstrated antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* as well as anticancer properties. Besides, in the case of catechin-silver nanocomposites, as presented by Yayan et al., [29] catechin played the role of a reducing agent and also an agent providing protection; it may serve as long-acting antibacterial agents.

T. gondii tissue cysts count in infected mice brain homogenates from different groups of treated and infected versus non-treated infected group was studied. The cysts' count in brain homogenates was significantly reduced in all the treated and infected groups than the non-treated infected group. Spiramycin has also been illustrated by Chew et al., [30] as anti-toxoplasmic in nature since it has successfully reduced the numbers of brain cysts in murine models of chronic toxoplasmosis. Besides that, Edmundson et al., [27] discussed how silver nanoparticles displayed both antimicrobial and anti-inflammatory activity.

Catechin, Cat-AgNPs 30 mg and 60 mg treated groups showed the highest reductions in the brain cysts among all the treated groups. The complete eradication of the cysts was not observed in any group.

Ma et al., [31] reported that at high concentration, catechin might exert an anti-microbial effect. Besides, (-) epicatechin and (+) catechin derived from the root of *Geranium mexicanum* and the aerial parts of *Rubus coriifolius* Focke, respectively, exhibited antiparasitic activities against *Giardia duodenalis* and *Entamoeba histolytica* [32]. Abdulah et al., [33] reported that the (+)-catechin isolated from *Garcinia celebica* leaves showed significant inhibition on both in vitro growth of the trophozoites and schizonts of *Plasmodium falciparum*. From their studies, it was suggested that one possible mode of antiplasmodial action that can be ascribed to (+)-catechin may be attributed to its induction of oxidative stress, thus upsets redox balance and affects the survival of *Plasmodium* in intraerythrocytic stages. Moreover, Kemal et al., [34] stated that the antiplasmodial action of (−)-catechin is attributed to its antioxidant property, facilitated through the phenolic OH groups.

Argüello-García and Quiñonez-Bastidas [11] added that in parasites, catechins interact with various protein molecules. In

Plasmodium falciparum, erythrocyte membrane protein 1 has been identified as the target for catechins, particularly (–)-epigallocatechin gallate. This interaction inhibits the binding of *P. falciparum* infected erythrocytes to human intercellular adhesion molecules exposed on the surface of endothelial cells, hence affecting malaria pathogenesis. We hereby suggest that one target of (+)-catechin may be the *Toxoplasma* cyst wall proteins, including the cyst wall glycoprotein, CST1, in an attempt to affect the integrity of the cyst wall, its size, and the overall count of the cysts. This is in agreement with the fact that Tomita et al., [35] demonstrated that CST1 plays a very important role in maintaining the structural integrity and longevity of the brain cysts. Their results indicated that parasites lacking the gene for CST1 produce fewer brain cysts and form much weaker cysts.

Loading of (+)-catechin onto silver nanoparticles increased its effectiveness and considerably reduced the number of cysts in the brain tissue, an improvement in the bioavailability and release of (+)-catechin from loading into the tissue. This was agreed upon by Khalil et al., [36] stating that nanoparticles work as drug carriers, which ensure modification in pharmacokinetics, increase bioavailability, and also allow targeted release with reduced toxicities.

This agrees with the work of Molina-Hernández et al., [37] who reported the antifungal action of silver nanoparticles biosynthesized using catechin from ***** *Aspergillus niger*. The authors said that AgNPs and catechin each trigger various stress compensation pathways which affect growth, development, and survival in *A. niger*. The antifungal effect was attributed to the synergistic interaction between the antimicrobial activity of AgNPs and catechin, besides the overproduction of reactive oxygen species resulting from exposure to nanomaterials. As expected, this redox homeostasis imbalance, together with osmotic disequilibrium, finally provokes membrane damage.

Conclusion:

The current study demonstrated that Catechin-loaded silver nanoparticles exhibit superior anti-toxoplasmic effects compared to Catechin alone, highlighting their potential as a novel therapeutic approach. The anti-toxoplasmic effect was manifested as a significant reduction in parasitic cyst counts in the brain.

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