Original Article

Assessment of the therapeutic efficacy of zinc oxide nanoparticles on chronic toxoplasmosis in murine model

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ABSTRACT

Background: Toxoplasmosis remains a major global health problem with limited therapeutic options for chronic cases associated with immunodeficiency. These highlight the need for effective alternative therapies.

Objective: To assess the anti-*Toxoplasma* effects of zinc oxide nanoparticles (ZnO NPs) and spiramycin, individually and in combination.

Material and Methods: Experimental Swiss albino mice were divided into 5 groups: G1, non-infected control; G2, infected control; G3, infected and spiramycin treated; G4, infected and treated with ZnO NPs; G5, infected and treated with spiramycin and ZnO NPs. The treatment effects were evaluated by assessing their impact on the parasite burden and histopathological changes in the brain and liver, as well as their immunomodulatory and antioxidant effects.

Results: Oral ZnO NPs and spiramycin administration each induced a significant reduction of brain cyst counts, and the highest impact was noticed in the combination therapy group. Their administration also showed immunomodulatory effects by modulating TNF-α and IL-1β expression in brain and liver tissues. Moreover, treatments enhanced the oxidative stress and histopathological alterations in these tissues. The combination therapy achieved the best therapeutic impacts in all studied parameters.

Conclusion: Our results revealed that ZnO NPs were effective against *T. gondii*, offering a promising antioxidant and anti-inflammatory adjunct therapy in parasitic diseases. Moreover, the highest therapeutic impact was observed with combined treatment indicating ameliorative synergistic effects.

Keywords: chronic toxoplasmosis; IL-1β; SOD; Spiramycin; TNF-α; ZnO NPs.

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INTRODUCTION

Toxoplasmosis is one of the most prevalent zoonoses, caused by an obligatory intracellular protozoan, *T. gondii* that can invade and multiply inside all types of nucleated cells of warm-blooded mammals. One-third of people worldwide are infected with *T. gondii*[1]. Toxoplasmosis can be contracted through oocyst ingestion in contaminated water or food or by tissue cysts ingestion in raw or undercooked meat. Routes of transmission include congenital, blood transfusions, or organ transplantation[2]. In immunocompetent individuals, the infection is usually asymptomatic and can remain latent for life. However, it can lead to serious consequences in immune-suppressed patients, as those with AIDS[3]. Chronic toxoplasmosis may be the cause of some mental and behavior disorders, including schizophrenia, Alzheimer's disease, and manic-depressive disorder[4].

Infection by *T. gondii* is known to modulate the immune response through stimulation or suppression of cytokine production. During infection establishment, the brain is infiltrated by inflammatory monocytes, which trigger nuclear factor kappa B pathway (NF-kB) and activate proinflammatory cytokines release such as TNF-α, IFN-γ, IL-1β, and IL-6[5]. These cytokines induce inflammation and brain infiltration by immune cells. Disruption of microglial functions, along with persistent neuro-inflammation and elevated inflammatory cytokines, can result in development of neuropsychiatric disorders[6].

Currently existing medications for toxoplasmosis are more efficacious in treating acute infection, but are unable to eliminate the tissue cysts[7]. Hence, the cysts persist in the brain through the duration of the host's life with persistent neuro-inflammation[8]. Consequently, the search for novel treatment approaches to treat toxoplasmosis is necessary. Nanomaterials offer potential treatments for diseases affecting human and animal health, particularly in addressing resistance to conventional drugs[9]. Interestingly, ZnO NPs have gained great attention because of their special characteristics. They exhibit superior biocompatibility, bioavailability, lesser toxicity, and a high absorption rate[10]. They exhibited diverse biological and therapeutic activities, including antibacterial, antiprotozoal, antioxidant, and anticancer properties[11,12]. Prolonged exposure to these NPs has the additional potential to enhance immunity. Immunomodulation is beneficial for

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disease prevention and treatment[13]. Therefore, the current study aimed to assess the therapeutic impact and immunomodulatory effect of spiramycin, and ZnO NPs individually, and their combination on *T. gondii* (Me 49 strain) infected mice.

MATERIAL AND METHODS

This randomized control experimental study was conducted at the Medical Parasitology and Medical Biochemistry Departments, Faculty of Medicine, Zagazig University, during the period from January 2024 to September 2024.

Study design: Mice were infected with *T. gondii* (ME49 strain). Six weeks post-infection (PI), treatment was started daily for ten days. All groups were euthanized for evaluation of drug effects 9 weeks PI; half of the mice from each group were used for counting brain cysts, while the other half was used for biochemical and histopathological assessment.

Experimental animals and study groups: Swiss albino male outbred mice, 6–7 w, in age and 20–25 g in weight, were provided by the biological supply center of Theodore Bilharz Research Institute (TBRI), Egypt. Mice were maintained under standard laboratory breeding conditions throughout the experiment. Mice were randomly assigned to five groups (n = 12 mice per group) as follows: G1, non-infected control; G2, infected control; G3, infected and spiramycin treated; G4, infected and ZnO NPs treated; and G5, infected and treated with spiramycin and ZnO NPs.

Parasite maintenance and mice infection: *T. gondii* (ME49 strain), obtained from TBRI, was used for induction of chronic toxoplasmosis. It was maintained by sub-passage in mice with 0.1 ml of brain homogenate containing ~$1x10^2$ tissue cysts/ml every eight weeks given via oral route[14]. Mice were infected using the same dose and route.

Drugs: Spiramycin tablets, supplied by Paranoia Pharmaceuticals, were given orally at a dose of 100 mg/kg/d [15]. The ZnO-NPs from Nano Gate Company, Cairo, Egypt, were suspended in sterile distilled water and given orally at a dose of 10 mg/kg/d[16]. Transmission electron microscopy (TEM) and X-ray diffraction (XRD) were employed for NPs characterization.

Parasitological treatment evaluation: Brains of infected mice were removed and homogenized by adding 1 mL of saline. For brain cyst counting, 25 µl of brain homogenate were examined microscopically (X40). The count detected in 4 drops was then multiplied by 10 to estimate the number of tissue cysts per brain[17].

Gene expression assessment for IL1-β, TNF-α, and superoxide dismutase (SOD 2) enzyme

Total RNA extraction from tissue[18]: Total RNA was extracted from homogenized brain and liver tissues by Trizol (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturing instructions. To assess the RNA quality, the A260/A280 ratio was evaluated by the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies; Wilmington, Delaware, United States). The estimated purity used for any given RNA was between 1.8 and 2.0. High-Capacity cDNA Reverse Transcription Kit cDNA Kit (Applied Biosystems™, USA) was used for formation of cDNA that was kept at -20°C till it was used in the following PCR step.

Real-time quantitative PCR (qRT-PCR) analysis[19]: According to the manufacturer's instructions, the real-time PCR was performed in a Mx3005P Real-Time PCR System (Agilent Stratagene, USA) using TOPrealTM qPCR 2X PreMIX (SYBR Green with low ROX) (Cat.# P725 or P750) (Enzynomics, Korea). In brief, the 20-µl reaction volume included 10 µl TOPreal SyberGreen (Enzynomics, Korea), 1 µl each of forward and reverse primer, 1 µl of cDNA, and nuclease-free water up to 20 µl. Of note, primers were selected using Primer-BLAST program (NCBI/primer-BLAST)[20] (Table 1). The reaction was at 95 °C for 2 min followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec. Expression was measured as fold change relative to Gapdh reference gene compared to the control group *via* the $2^{-\Delta\Delta CT}$ method[21].

Table 1. Primers used in qRT-PCR.

	Primer (5′–3′)	Accession No.	Product length
IL-1β			
Forward	GCA ACTGTTCCTGAACTCAACT	NM_008361.4	81
Reverse	GGGTCCGTCAACTTCAAAGA		
TNF-α			
Forward	TGAACTTCGGGGTGATCGGT	NM_001278601.1	99
Reverse	GGTGGTTTGTGAGTGTGAGGG		
SOD2			
Forward	GCCCAAACCTATCGTGTCCA	NM_013671.3	70
Reverse	AGGGAACCCTAAATGCTGCC		
Gapdh			
Forward	AGGTCGGTGTGAACGGATTTG	NM_001289726.1	123
Reverse	TGTAGACCATGTAGTTGAGGTCA		

Histopathological evaluation: Brain and liver tissue specimens were collected from each group, processed, and stained by H & E technique[22].

Statistical analysis: Data were statistically analyzed by SPSS version 25 (IBM, NY, USA). Mean ± SD was used for presenting data. ANOVA (*F*) test was employed to compare multiple groups. Then LSD analysis was conducted for pairwise comparison between groups. A statistically significant difference is considered at $P<0.05$.

Ethical consideration: All procedures followed guidelines for using laboratory animals. Approval was granted by ZU-IACUC committee of Zagazig University (Approval No.: ZU-IACUC/3/F/288/2023).

RESULTS

Characterization of NPs: The TEM image revealed the hexagonal shape of NPs with a size of about 25-35 nm. For XRD results, the standard card number of ZnO NPs was 01-075-1526; the patterns at 2θ = 31.9°, 34.46°, 36.44°, 47.71°, 56.57°, 63.04°, 66.83°, 68.3°, and 69.54° were attributed to ZnO crystal phases of 100, 002, 101, 102, 110, 103, 200, 112, and 201, respectively. This speculation confirmed preservation of the NPs and their chemical structure (Fig. 1).

Impact of therapy on brain cyst counts: Administration of spiramycin and ZnO NPs induced a significant ($P<0.001$) statistical decrease in the mean number of brain cyst counts, with the least mean value in the combination group compared to the positive control. Combination therapy showed the greatest therapeutic outcome with the highest reduction percent (85.56%) compared to spiramycin alone (63.80%) (Table 2).

Gene expression of IL-1β, TNF-α, and SOD2 in brain and liver tissues (Tables 3 and 4): IL-1β and TNF-α gene expression in the infected control group (G2) significantly increased in relation to all other groups. There was a significant decrease in all treated groups compared to G2 groups with no significant difference between G3 and G4 in TNF-α expression levels in brain tissue. Combination therapy induced the maximum reduction of IL-1β and TNF-α gene expression in tissues.

As an antioxidant enzyme, SOD2 results demonstrated a significant down-regulation of gene expression levels in the infected control (G2) when compared to negative controls. However, there was a significant up-regulation in SOD2 levels in all treated groups. Combination therapy (G5) induced the maximum improvement of SOD2 expression levels.

Histopathological findings: Regarding pathological examination of the brain, infected control mice (G2) showed *Toxoplasma* cysts and encephalitis with

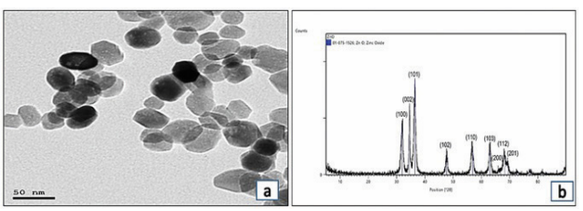

Fig. 1. NPs characterization: **a)** TEM image, **b)** XRD pattern.

Table 2. *T. gondii* brain cyst counts in different studied groups.

Groups	Mean ± SD	Range	Reduction %	Statistical analysis
G2	796.67 ± 78.15 ^{a,b,c}	710–900		
G3	288.33 ± 62.42 ^{a,d,e}	200–380	63.80%	$F = 110.869$
G4	496.67 ± 86.41^{b,d,f}	370–600	37.65%	$P < 0.001$*
G5	115.00 ± 33.91^{c,e,f}	70–160	85.56%	

Superscripts indicate significant difference between groups; **a:** G2 vs G3, **b:** G2 vs G4, **c:** G2 vs G5, **d:** G3 vs G4, **e:** G3 vs G5, **f:** G4 vs G5; ***:** Significant ($P<0.05$).

Table 3. Gene expression levels of IL-1β, TNF-α, and SOD2 in brain tissue.

	Mean ± SD of IL-1β (Range)	Mean ± SD of TNF-α (Range)	Mean ± SD of SOD2 (Range)
G1	1.01 ± 0.01^{a,b,c} (0.99– 1.03)	1.01 ± 0.01^{a,b,c} (0.99 – 1.02)	1.00 ± 0.01^{a,b,c} (0.99 – 1.01)
G2	2.12 ± 0.23 ^{a,d,e,f} (0.82 – 2.40)	1.75 ± 0.11^{a,d,e,f} (1.60 – 1.90)	0.20 ± 0.06^{a,d,e,f} (0.13 – 0.30)
G3	1.61 ± 0.13 ^{b,d,g,h} (1.45 – 1.80)	1.38 ± 0.09^{b,d,h} (1.25 – 1.50)	0.48 ± 0.08^{b,d,g,h} (0.40 – 0.60)
G4	1.34 ± 0.14^{c,e,g,i} (1.15 – 1.50)	1.43 ± 0.08^{c,e,i} (1.30 – 1.52)	0.72 ± 0.08^{c,e,g,i} (0.60 – 0.82)
G5	1.02 ± 0.01^{f,h,i} (1.00 – 1.03)	1.01 ± 0.01^{f,h,i} (1.00 – 1.03)	1.00 ± 0.01^{f,h,i} (0.98 – 1.01)
Statistical analysis	$F = 73.186$, $P < 0.001$*	$F = 104.519$, $P < 0.001$*	$F = 196.324$, $P < 0.001$*

Superscripts indicate significant difference between groups; **a:** G1 vs G2; **b:** G1 vs G3; **c:** G1 vs G4, **d:** G2 vs G3, **e:** G2 vs G4, **f:** G2 vs G5, g: G3 vs G4, h: G3 vs G5, i: G4 vs G5;***:** Significant ($P<0.05$).

infiltration by lymphocytes, gliosis, and edema. Treatments improved the brain histopathological picture and reduced inflammatory infiltration. Combined treatment presented the best improvement with minimal inflammatory infiltration and unremarkable pathological changes (Fig. 2).

Concerning liver pathological changes, infected control mice (G2) showed hepatocyte degeneration with lobular aggregates of inflammatory cells and giant cells, sinusoidal dilation and vascular congestion. The G3 exhibited focal lobular inflammatory cellular infiltration and moderate sinusoidal dilatation, while Zno NPs treatment (G4) displayed reduced pathological alteration with hydropic degenerative changes and reduced inflammatory infiltration. The combined treatment (G5) revealed few inflammatory lymphocytes and unremarkable pathological alterations (Fig. 3).

Table 4. Gene expression levels of IL-1β, TNF-α and SOD2 in liver tissue.

	Mean ± SD of IL-1β (Range)	Mean ± SD of TNF-α (Range)	Mean ± SD of SOD2 (Range)
G1	$1.01 \pm 0.02^{a,b,c}$ (0.98 - 1.04)	$1.00 \pm 0.01^{a,b,c}$ (0.99 - 1.02)	$1.01 \pm 0.01^{a,b,c}$ (1.00 - 1.02)
G2	$2.49 \pm 0.18^{a,d,e,f}$ (2.20 - 2.70)	$2.06 \pm 0.15^{a,d,e,f}$ (1.88 - 2.25)	$0.33 \pm 0.06^{a,d,e,f}$ (0.26 - 0.40)
G3	$1.79 \pm 0.23^{b,d,g,h}$ (1.50 - 2.10)	$1.6 \pm 0.12^{b,d,g,h}$ (1.50 - 1.82)	$0.63 \pm 0.13^{b,d,g,h}$ (0.45 - 0.78)
G4	$1.60 \pm 0.13^{c,e,g,i}$ (1.40 - 1.76)	$1.44 \pm 0.14^{c,e,g,i}$ (1.27 - 1.60)	$0.81 \pm 0.08^{c,e,g,i}$ (0.70 - 0.90)
G5	$1.02 \pm 0.01^{f,h,i}$ (1.01 - 1.04)	$1.01 \pm 0.01^{f,h,i}$ (1.00 - 1.02)	$1.00 \pm 0.01^{f,h,i}$ (0.98 - 1.02)
Statistical analysis	$F = 108.576$, $P < 0.001$*	$F = 102.337$, $P < 0.001$*	$F = 89.028$, $P < 0.001$*

Superscripts indicate significant difference between groups; **a:** G1 vs G2; **b:** G1 vs G3; **c:** G1 vs G4, **d:** G2 vs G3, **e:** G2 vs G4, **f:** G2 vs G5, g: G3 vs G4, h: G3 vs G5, i: G4 vs G5;***:** Significant ($P<0.05$).

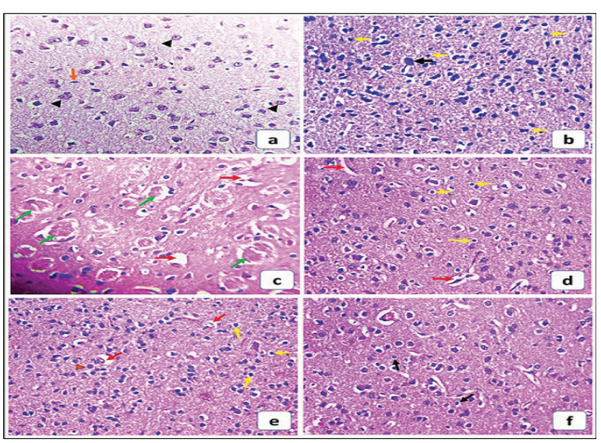

Fig. 2. Brain sections from different studied groups. **a)** G1 showing normal histological architecture of the brain with normal neuronal cells (arrowheads) and microglial cells (orange arrow). **b)** G2 showing infiltration by lymphocytes (yellow arrows) and *Toxoplasma* cyst (black arrow). **c)** G2 showing extensive gliosis (green arrows) and brain oedema (red arrows). **d)** G3 showing few inflammatory cells (yellow arrows) and reduced interstitial oedema (red arrows). **e)** G4 showing lymphocytic infiltration (yellow arrows) and perivascular oedema (red arrows) and degenerated cyst (orange arrowhead). **f)** G5 showing minimal infiltration by lymphocytes (black arrows) and unremarkable pathological changes (H&E X 400).

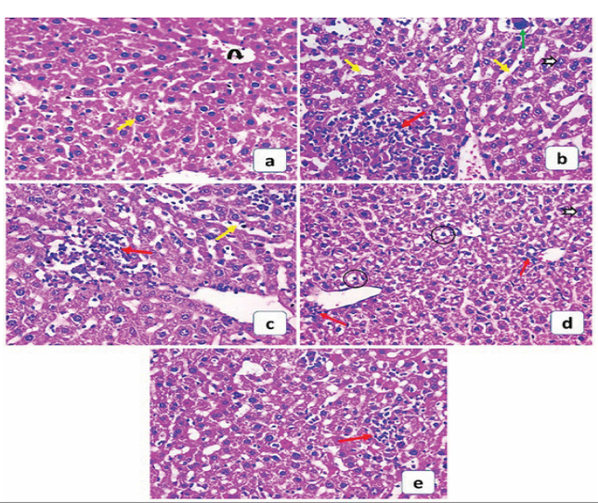

Fig. 3. Liver Sections from different studied groups. **a)** G1 showing the normal histological architecture of the liver with normal hepatocytes (yellow arrow) and central vein (curved arrow). **b)** G2 showing lobular aggregate of inflammatory cells (red arrow), a giant cell (green arrow), dilated sinusoids (yellow arrows), and binucleated hepatocytes (notched white arrow). **c)** G3 showing focal lobular inflammatory cellular infiltration (red arrow) and less sinusoidal dilatation (yellow arrow). **d)** G4 showing few inflammatory infiltrations (red arrows), binucleated cells (notched white arrow), and hydropic degenerative changes (circles). **e)** G5 showing few inflammatory cells (arrows), and unremarkable pathological changes (H&E X 400).

DISCUSSION

Toxoplasmosis can cause severe and fatal infection in immunocompromised individuals, necessitating the need to find novel therapeutics, as the present available therapeutics are not adequately effective due to *T. gondii* resistance[7]. Zinc is an important trace element, necessary for human enzymatic functions. It plays an important role in several biological activities, including phagocytosis, antioxidant defense, replication, and cytokines and immunoglobulin synthesis[23]. The ZnO NPs are biocompatible, cheap, and less toxic and absorbed easily by the body compared to other metal oxide NPs. This makes them ideal for use in the biomedical fields[10].

Regarding the parasitological results of our research, treatment with spiramycin resulted in significant decrease of the mean brain cyst counts. This agreed with other studies[15,24]. In the current study, ZnO NPs were able to significantly decrease the brain cyst counts in comparison with the infected control. Moreover, its combination with spiramycin showed a synergistic effect and the highest percentage of reduction. This aligns with Sarhan *et al*.[25] who found that ZnO NPs caused a significant decrease in *Toxoplasma* cyst number in the brain by 29.30%. Additionally, Saadatmand *et al*.[26] conveyed that ZnO NPs oral administration can induce a significant prophylactic effect against chronic *T. gondii* infection. Furthermore, in consensus, treatment of chronically infected mice with spiramycin-loaded chitosan NPs significantly decreased the parasite burden[15].

In acute toxoplasmosis, ZnO NPs successfully reduced *Toxoplasma* Rh strain tachyzoite counts in the liver and peritoneal fluid compared to infected control and spiramycin treatment[12]. Similarly, Cheraghipour *et al*.[27] found that ZnO NPs combination with eugenol could be considered for the treatment of acute toxoplasmosis. Additionally, ZnO NPs showed anti-parasitic effects against *L. tropica* and *L. donovani*[28] and *T. spiralis* infections[29]. This cytotoxic effect of NPs may be related to reactive oxygen species (ROS) generation and zinc ions release that increase cell permeability with subsequent toxic effects on pathogens[28].

Intracellular parasites, including *T. gondii*, have been known to induce pro-inflammatory cytokine release[30]. In the current study, the assessment of pro-inflammatory biomarkers showed a significant increase in IL-1β and TNF-α gene expression levels in brain and liver tissues of chronic toxoplasmosis in mice. These results are in confirmation with two previous studies[31,32] that detected significant increase of TNF-α, IL-6, and IL-1β genes expression levels in toxoplasmosis-infected controls in comparison to the normal group. These cytokines have been implicated in developing hyperalgesia and neuro-inflammation in the brain tissue of infected mice. Moreover, toxoplasmosis was found to significantly increase IL-1β production in macrophages and upregulate expression of inflammasome sensors[33].

In the present study, there was a significant reduction in IL-1β and TNF-α gene expression levels in all treated groups when compared to the infected non treated, with the maximum reduction attained in combination therapy. This is in agreement with Kang *et al*.[34] who stated that spiramycin significantly decreased nitric oxide (NO), IL-1, and IL-6 levels in RAW 264.7 cells (model of macrophages). Apparently, it suppressed NF-kappa B and MAPK signaling pathways, with subsequent reduced production of these cytokines. Other studies[35,36] also observed lower TNF-α levels in the spiramycin treatment group than *T. gondii*-infected control one.

Our study confirmed the anti-inflammatory effects of ZnO NPs observed as a significant decrease in IL-1β and TNF-α gene expression levels when compared to positive controls, and with best results in the combined therapy groups. Similarly, Goma *et al*.[37] detected a significant decrease in the IL-6 and TNF-α in rat brain tissues that received ZnO NPs. In addition, Nagajyothi *et al*.[38] reported that ZnO NPs inhibited expressions of several inflammatory markers, as iNOS, IL-6, TNF-α, IL-1β, and COX-2, indicating their potent anti-inflammatory properties.

Oxidative stress significantly affects the development and progression of parasitic infection, affecting both the host and the parasite as it struggles to persist[39]. In the current study, a significant decrease in the relative expression level of the antioxidant SOD2 was detected in brain and liver tissues of infected controls. Treatment resulted in enhanced SOD2 expression, with the highest levels induced by combination therapy.

Alajmi *et al*.[40] stated that toxoplasmosis leads to a significant reduction of antioxidant enzyme activities, resulting in more superoxide radical accumulation and lipid peroxidation. Similarly, Nazarlu *et al*.[41] found that toxoplasmosis (RH strain) induced oxidative stress in tissues, evidenced by significant decrease in SOD, catalase (CAT) activity, glutathione, and total antioxidant capacity with an increased malondialdehyde (MDA) level. Antioxidants suppression following toxoplasmosis is most likely the result of protein inactivation by ROS, since oxidative damage frequently results in loss of protein functions[42]. The significant ZnO NPs enhancement of SOD2 expression and special enhancement when combined with spiramycin, aligns with El-Kady *et al*.[12]. The researchers conveyed that ZnO NPs treatment of RH toxoplasmosis exhibited significant NO inhibition and increased CAT activity. Additionally, Goma *et al*.[37] found that ZnO NPs obviously increased SOD, CAT, and glutathione peroxidase (GPx) antioxidants and decreased MDA in brain tissues, suggesting its beneficial effects. This

agreed with Alanazi *et al*.[43], who stated that treating toxoplasmosis by combining the main drug with copper NPs reduced MDA levels but significantly raised antioxidants levels. ZnO NPs were also found effective in improving oxidative stress caused by various other parasites as *C. parvum*[44] and *Parascaris equorum*[45].

Examination of histopathological tissue samples revealed significant improvement following treatment, especially with the combination of spiramycin and ZnO NPs compared to the infected control or spiramycin treatment alone. This was consistent with Sarhan *et al*.[25] who revealed that ZnO NPs and magnesium-doped ZnO NPs markedly improved the brain and liver histopathological pictures. Abdel-Wahab *et al*.[24] also mentioned that spiramycin-loaded maltodextrin NPs produced more improvement of histopathological changes than single spiramycin therapy, and NPs could be used as adjuvants for treatment of chronic toxoplasmosis.

In conclusion, ZnO NPs possess attractive anti-parasitic effects against *T. gondii* mediated by their ability to reduce inflammation and oxidative stress via modulating IL-1β and TNF-α and SOD2 expression in tissues. In addition, ZnO NPs combined with spiramycin showed more ameliorative effects on chronic toxoplasmosis.

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