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Assessment of The Efficacy of Mefloquine on Murine Chronic Toxoplasmosis

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Abstract

Background: Toxoplasmosis is a serious parasitic infection caused by an obligatory intracellular parasite called *Toxoplasma gondii*. There is a demand for alternative anti-toxoplasmosis drugs because the available treatments for eradicating toxoplasmosis are limited. **Aim of the work:** This study aimed to determine the therapeutic effects of a single dose of mefloquine (MQ) on chronic toxoplasmosis, to meet the need for new anti-toxoplasmosis treatments. **Materials and methods:** Chronic toxoplasmosis was induced in mice under experimental conditions by the coccidian parasite *T. gondii*'s cystogenic strain (ME49). The evaluation was based on several factors including survival rate, parasitological assessment, histopathological examination, as well as immunohistochemical evaluation of Ki-67 and NF-κB immunoreactivity. **Results:** MQ significantly increased the SR compared to the infected untreated group. Moreover, MQ significantly reduced the number of brain cysts ($P < 0.0001$) by 42.6% compared to the infected-untreated group. After treatment with MQ, fewer histopathological abnormalities were observed in the brain, liver, and spleen. Additionally, MQ significantly reduced ($P < 0.05$) the inflammatory score in different tissues. The administration of MQ significantly increased ($P < 0.05$) the Ki-67 expression in brain sections of infected mice treated with the pyrimethamine and sulfadiazine combination or MQ. Although the NF-κB expression was significantly reduced in liver sections after treatment with the pyrimethamine and sulfadiazine combination, treatment with MQ showed moderate expression in the infected-untreated tissue. **Conclusion:** This work demonstrated the remarkable efficacy of MQ on mice's toxoplasmosis. So, it might be used as a promising repurposed therapeutic anti-toxoplasmosis drug.

Keywords: *Toxoplasma gondii*, Mefloquine, Brain cysts, Liver, Ki-67, NF-κB

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I. Introduction

Toxoplasmosis is a widely distributed parasitic infection caused by an obligatory intracellular apicomplexan parasite, *Toxoplasma* (*T.*) *gondii* that infects approximately 30% of the worldwide population **(Greigert *et al.*, 2020; Shammaa *et al.*, 2021)**. *T. gondii* can remarkably invade, live, and multiply almost in all mammalian cells **(Lima and Lodoen, 2019)**. Toxoplasmosis is known to spread in humans through undercooked meat infected with latent cysts, polluted water and food with sporulated oocysts **(Zarean *et al.*, 2017)**. Additional ways for *T. gondii* spread among human hosts include congenital transmission of tachyzoites from mother to fetus through the placenta, semen transmission in the case of a male secondary host, blood transfusion, and organ transplantation **(Saadatnia and Golkar, 2012; Oz, 2014; McAuley, 2014)**.

In individuals with a healthy immune system, toxoplasmosis is asymptomatic and often results in self-limiting adenopathy. On the other hand, infections in immunocompromised individuals (with AIDS/HIV, cancer, or undergoing immune-suppressive treatments) can be deadly and may manifest as toxoplasmic encephalitis **(Shojaee *et al.*, 2018)**. First-class medications for the treatment of toxoplasmosis include common medications (pyrimethamine with sulfadiazine). This combination's frequent side effects include hematologic toxicity, teratogenicity, and renal problems **(Azami et al., 2018; Teimouri *et al.*, 2018)**. Thus, it would appear that the development of efficient medications with few adverse effects is imperative.

Quinoline is a heterocyclic aromatic chemical that is generated when the rings of pyridine and benzene fuse together. Quinoline-based treatments were the mainstay of malaria therapy for many years and are still available as a therapeutic option for infections with *Plasmodium* spp. **(WHO, 2015)**. Furthermore, the quinoline chemotype provides efficacious treatment options for a wide variety of diseases, including infections caused by bacteria, viruses, fungi, parasites, and, in certain instances, non-infectious disorders **(Horta *et al.*, 2018)**. Since *T. gondii* and *Plasmodium* spp. share many morphological and biochemical similarities, it has been demonstrated that quinolines are effective against *T. gondii* infections in both the acute and chronic stages **(Alday *et al.*, 2017; McConnell *et al.*, 2018; Elgawad *et al.*, 2019)**.

One of the synthetic analogs of quinine is mefloquine (MQ), or 4-quinolone methanol. MQ was created as an antimalarial drug in 1971 **(Kumar *et al.*, 2011).** In recent years, MQ has received a lot of attention as an anticancer medication **(Liu *et al.*, 2016),** besides its antibacterial effect against some Gram-positive bacteria and multidrug-resistant tuberculosis **(Krieger *et al.*, 2015)**. Moreover, MQ has proved to possess satisfactory anti-parasitic activities, especially against apicomplexan parasitic infections such as babesiosis **(El-Bahnasawy *et al.*, 2011; Munkhjargal *et al.*, 2012)**, cryptosporidiosis **(Aly *et al.*, 2017; El-Wakil *et al.*, 2020)**, and acute and chronic toxoplasmosis **(Holfels *et al.*, 1994; Zhang *et al.*, 2017; El Sharazly *et al.*, 2023).** Regarding helminthology, MQ has been shown to have anti-trematode action against *Schistosoma* **(Xiao *et al.*, 2013; Abou-Shady *et al.*, 2016)**, *Fasciola hepatica* and *Clonorchis sinensis* **(Keiser *et al.*, 2010)**, *Fasciola gigantica* **(Shalaby *et al.*, 2016)**, and *Paragonimus westermani* **(Xiao *et al.*, 2010)**. Also, the anthelminthic activity of MQ was reported against *Echinococcus multilocularis* **(Küster *et al.*, 2015; Rufener *et al.*, 2018)**.

Many cellular pathways are affected by infection with *T. gondii*. One of these pathways is proliferation. The Ki-67 biomarker is widely used for recognizing the nuclear protein associated with cell proliferation **(Gerdes *et al.*, 1991)**. So, the Ki-67 biomarker can be useful in identifying tissues that exhibit atypical levels of growth and proliferation. Ki-67 expression varies over the cell cycle and peaks during mitosis **(Schulzen and Herdes, 2000)**. Recently, **Pires *et al.* (2023)** have concluded that the infection with *T. gondii* reduced cell proliferation, as shown by the immunoreactivity between the tissue and the Ki-67 antibody.

Signals resulting from infection and inflammation are transcription factors belonging to the nuclear factor kappa B (NF-κB) family that are involved in the regulation of immune responses associated with resistance to infection and the inflammatory process **(Napetschnig and Wu, 2013)**. Consequently, cells undergo reprogramming in response to *T. gondii* cellular invasion, which has an impact on a large number of transcription factors, including most members of the NF-κB family **(Hakimi and Bougdour, 2015)**. Recently, **Liempia *et al.* (2019)** have concluded that *T. gondii* inhibits the non-canonical NF-κB pathway but is ineffective on the canonical pathway.

This study aimed to determine the therapeutic effects of a single dose of MQ on chronic toxoplasmosis, to meet the need for new anti-toxoplasmosis treatments. Chronic toxoplasmosis was induced in mice under experimental conditions by the coccidian parasite *T. gondii*'s cystogenic strain (ME49). The evaluation was based on several factors including survival rate, parasitological assessment, histopathological examination, as well as immunohistochemical evaluation of Ki-67 and NF-κB immunoreactivity.

II. Materials and Methods

Ethics Proclamation

The IACUC of Zagazig University has authorized the current mouse research procedures following international animal care standards, including therapy and euthanasia. The study approval number is ZU-IACUC/1/F/78/2022.

Parasite Strain and Infection

The brain suspension containing tissue cysts, cystogenic ME49 strain, of *T. gondii* was donated by Theodor Bilharz Research Institute (TBRI). Then, the brain suspension was diluted by sterile Phosphate-Buffered Saline (PBS) (0.1 m, pH 7.4) to a concentration of 200 cysts per milliliter (ml). Finally, according to earlier research, 0.1 ml of this diluted suspension, which contained 20 cysts, was used to infect each mouse **(El-Shafey *et al.*, 2020)**.

Animal Model

This experimental case-control study was conducted at the Medical Parasitology Department lab of the Faculty of Medicine and Zoology Department lab, Faculty of Science, Zagazig University in Egypt. We used forty male Swiss albino lab mice aged between six to seven weeks (weighing: 20-25 g) for our experiment. All

of the test mice were housed in well-ventilated cages with access to water and daily grain feeding. The laboratory's temperature was maintained at 25 ±2 °C, and the lighting was monitored for 12 hours of light and 12 hours of darkness.

Drugs

Pyrimethamine and sulfadiazine were purchased from Sigma-Aldrich and Alfa-Aesar, respectively. The pyrimethamine and sulfadiazine combination was prepared as previously described by **El Sharazly *et al.* (2023)**. Each 1 ml of this combination contains 12.5 mg of pyrimethamine and 200 mg of sulfadiazine.

Experimental Design

Four experimental groups, each with ten mice, were created by randomly selecting all of the animals. The mice in the first group (GI: negative control group) were healthy and free of infection, while those in the other three groups received 0.1 ml of brain suspension orally to establish chronic toxoplasmosis. Two infected groups began receiving treatment after six weeks (42 days) post-infection **(El-Shafey *et al.*, 2020)** as follows:

- GII: infected without any treatment group.
- GIII: orally received 1 ml of the pyrimethamine and sulfadiazine combination per day for 10 days.
- GIV: orally received a single dose of mefloquine (400 mg/kg).

In accordance with international guidelines for animal care, all mice were sacrificed five days after the experiment concluded (10 days).

Collecting Samples

After sacrificing the mice, we removed their left hemisphere, liver, and spleen and stored them in separate labeled containers filled with 10% formalin for preservation. This process will allow us to carry out additional histopathological and immunohistochemical evaluations. The right hemisphere of each mouse's brain was then examined to determine the parasitic load (parasitological assessment).

Survival Rate (SR)

Survival rate (SR) has been calculated according to **El-Zawawy *et al.* (2015)** using the below-mentioned equation:

$$SR = \frac{ND}{NT} \times 100$$

Where NA is the number of alive mice at the end of the experiment and NT is the total number of mice at the beginning of the experiment.

Parasitic Burden Assessment

The right brain hemisphere of each mouse was processed using a tissue homogenizer in PBS for five minutes to create a brain suspension. A drop of 10 µl of the brain homogenate was placed on a clean microscopic glass slide and examined under a light microscope at x400 magnification to measure the tissue cyst burden **(Saraei *et al.*, 2014)**. In the end, each mouse's total number of brain cysts was determined.

Histopathological Assessment

Brains, livers, and spleens were chosen to evaluate the histopathological changes among different groups of the study as these are the most influenced organs by toxoplasmosis. Specimens from the brain, liver, and spleen were taken from all the groups and fixed in 10% formalin. They were dehydrated in an ascending series of ethyl alcohol [70%, 80%, 95% and 100%] then cleared in xylene. The samples were embedded in molten paraffin at 60°C for 1–2 hrs to form paraffin blocks. Block sections were cut using microtome. Each section was 4 µm thick. Two sections were prepared from the different organs of each mouse, they were mounted on slides, then left in the oven at 40 °C to dry and fix on the slides **(Suvarna et al., 2018)**. Slides were deparaffinized by dipping them in xylene followed by passing them in descending series of ethyl alcohol [100%, 95%, 80% and 70%] then rinsed with water for rehydration. Slides were stained with haematoxylin, rinsed under running water then counterstained with eosin. They were dehydrated using an ascending series of ethyl alcohol and then mounted in Canada balsam. Conventional light microscopy has been used for histopathological investigation.

Immunohistochemistry Assessment

Using a microtome, 4 µm-thick slices of paraffin-embedded brain and liver from various animal groups were generated for IHC assessment. The sections were then put on saline-coated microscopic glass slides and incubated at room temperature for the whole night. The sections that had been paraffinized were dewaxed in xylene and then rehydrated using ethanol at varying concentrations in water. After removing the endogenous peroxidase activity in methanol with H_2O_2, slices were microwave-pretreated in sodium citrate buffer. After letting the slides cool for fifteen minutes, they were cleaned three times in Tris-buffered saline **(Salem *et al.*, 2016)**. The brain and liver pre-prepared slides were treated for one night at 37 °C with anti-Ki-67 (1:8000; Santa Cruz Biotechnology) and anti-NF-κB (1:100; Novus) antibodies, respectively. The next day, each part was treated with secondary and tertiary antibodies for ninety minutes each. After treating each slice with the chromogen diaminobenzidine tetrachloride (DAB), hematoxylin was used as a counterstain, and the sections were then dried, cleaned, and mounted. Lastly, IHC slices were inspected at different magnifications using a light microscope.

Inflammatory and Immunohistochemical Scoring

The inflammatory score was calculated as previously described by **Yazdi *et al.* (2015)** and modified. In essence, tissues were examined using a 40x objective in a blinded way and rated as follows: 0 = no inflammation, 1 = a few inflammatory cells, 2 = perivascular inflammation infiltration, and 3 = perivascular cuffing intensity spread to surrounding tissues. Two researchers performed the score evaluation. The immunohistochemical score (IHS) of Ki-67 and NF-κB is classified as follows: negative immunoreactivity (0) = 0, weak (1-4) = 1, moderate (5-8) = 2, strong (9-12) = 3.

Analytical Statistics

All data is presented as the mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) test and statistical analysis was performed using IBM-SPSS version 23. Tukey's post hoc tests were utilized for different comparison analyses across research groups. Histopathological and immunohistochemical grading was done using the pairwise comparison Kruskal-Wallis (*H*) test, which was then followed by Dunn's post hoc test. Using the Kaplan-Meier test, survival curves were calculated. According to **Feeney (2016)**, differences between groups were considered statistically significant when *P* values were less than 0.05.

III. Results

Survival Rate (SR)

Although the lowest survival rate was observed in the infected untreated group (GII) (50%), the highest survival rate among the infected mice was observed in the group treated with MQ (GIV) (90%), followed by the group treated with pyrimethamine and sulfadiazine (GIII) (70%). The Kaplan-Meier survival curves of groups throughout the study time (57 days) are shown in **Figure 1**. There is no significant difference between groups ($P = 0.08$).

Parasite Burden Assessment

The potential load of chronic toxoplasmosis was calculated by counting the total number of tissue cysts detected in 10 µl of brain homogenate from each mouse in all infected groups. The main tissue cyst numbers are summarized in **Table 1**. As illustrated, the pyrimethamine and sulfadiazine combination and MQ significantly reduced the number of brain cysts ($P < 0.0001$) by 70.9% and 42.6%, respectively, compared to the infected untreated group.

Histopathological Assessment

For the assessment of the therapeutic effects of MQ on chronic toxoplasmosis, histopathological sections of the brain, liver, and spleen from the various experimental groups were examined.

- **Brain**

Histological analysis was performed on brain sections from all experimental groups to evaluate the pathological changes in the brain tissue. The brain cortex of the healthy control group (GI) was found to have a typical morphology made up of a combination of pyramidal cells and neuroglial cells **(Fig. 2a)**.

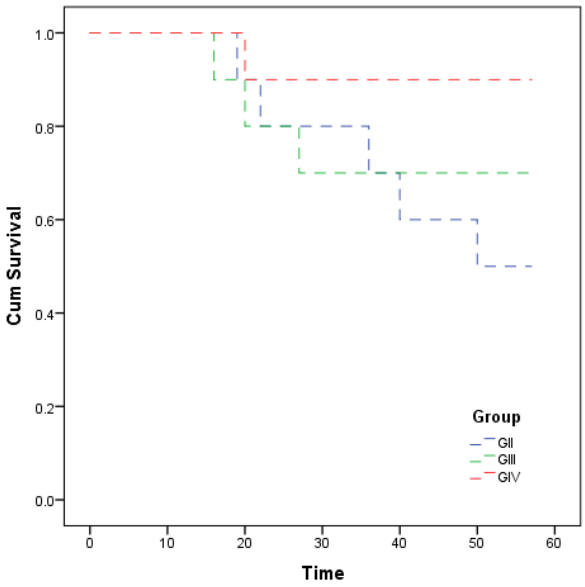

Fig.1: Kaplan Meier survival curves of *T. gondii* (ME49 strain) infected groups throughout the study time (42 days for infection period + 10 days for treatment + 5 days after treatment). GII: infected untreated group, GIII: pyrimethamine and sulfadiazine combination-treated group, and GIV: MQ-treated group.

Table 1. The main tissue cysts count in 1µl of brain suspension of different treated groups compared to the infected untreated (positive control) group.

Experimental group	Treatment	Mean ± SD	Std. Error	Reduction (%)	*F*- value	*P*- value
GII	Positive Control	4215.5 ± 159.9	50.6	-	1348.2	< 0.0001*
GIII	Pyr. + Sulf.	1227.0 ± 143.1	45.2	70.9		
GIV	MQ	2418.0 ± 65.8	20.8	42.6		

All means are statistically different from each other ($P < 0.05$).
*Statistically significant at *P*-values < 0.05.

Furthermore, capillaries appeared completely normal. In contrast, brain sections of infected mice (GII) clearly showed severe gliosis, degenerated cerebral cells, and tissue cysts of *T. gondii* **(Fig. 2b)**. On the other hand, brain sections of infected mice treated with pyrimethamine and sulfadiazine (GIII) showed brain edema in many sites **(Fig. 2c)**. After treatment with MQ (GIV), the histopathological examination of brain sections manifests a kind of normal histological tissue with degenerated tissue cysts and congested blood vessels **(Fig. 2d)**. However, the combination of pyrimethamine and sulfadiazine did not result in a substantial change in the inflammation severity of infected brain tissue; the single dose of MQ significantly reduced ($P < 0.05$) the inflammatory score (IS) by 84% **(Tab. 2)**.

- **Liver**

High magnifications of different fields of liver sections from uninfected mice (GI) showed uniform hepatocytes with eosinophilic cytoplasm and rounded, prominent vesicular nuclei arranged around the central vein and normal sinusoids between the hepatic cellular cords **(Fig. 3a)**. Inversely, hepatocytes in liver sections of GII demonstrated severe histopathological necrotic changes such as hydropic degeneration, binucleated hepatocytes, pyknotic nuclei, karyorrhexis, vascular congestion with marked periportal lymphocytic inflammatory cellular infiltration, and sinusoidal dilation **(Fig. 3b)**. Moreover, the infected group treated with the pyrimethamine and sulfadiazine combination (GIII) exhibited the same histopathological necrotic abnormalities observed in the untreated infected group with more severe hydropic degeneration **(Fig. 3c)**. For GIV treated with MQ, there were fewer histopathological abnormalities, such as few inflammatory cells, binucleated hepatocytes, pyknotic nuclei, karyorrhexis, congested central veins, and sinusoidal dilation **(Fig. 3d)**. Moreover, as shown in **Table 3**, the administration of MQ significantly reduced ($P < 0.05$) the IS in liver tissue by 89.3%.

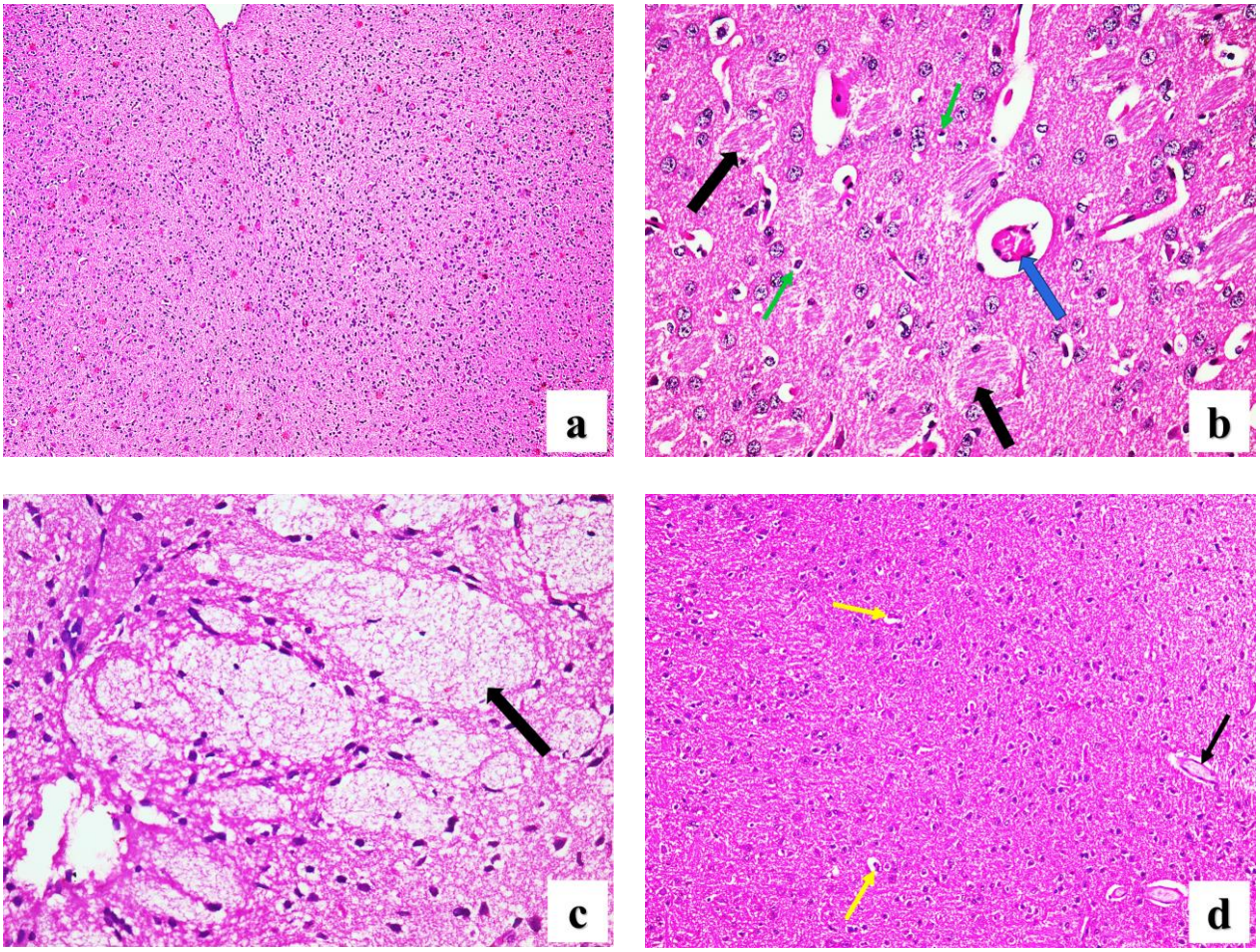

Fig.2: Microscopic histopathological representative images showing the histopathological changes in brain tissue of all groups in this experimental study. **a.** GI showing normal brain architecture (H&E x100)**. b.** in GII showing *T. gondii* tissue cyst (blue arrow), severe gliosis (black arrows), degenerated neural cells (green arrows) (H&E x400). **c.** GIII showing severe brain edema (black arrows) (H&E x400). **d.** GIV showing degenerated tissue cysts (yellow arrows) and congested blood vessels (black arrow) (H&E x200).

Table 2. The mean inflammation score (IS) in brain sections of experimental groups.

Experimental groups	IS in brain (mean ± SD)	Reduction (%)
GI	0.00 ± 0.00	
GII	2.50 ± 0.71	
GIII	2.60 ± 0.67	**- 4%**
GIV	0.40 [a] ± 0.70	**84%**
H	31.54	
***P*-value**	< 0.0001*	

H is the Kruskal-Wallis test statistic. Dunn's post hoc test was used to perform multiple comparisons between groups.
*Statistically significant at *P*-values ≤ 0.05.
a: Significant with GII (Infected non-treated group).

- **Spleen**

H&E-stained spleen sections for the negative control group (GI) showed normal histomorphology of the splenic pulps, white and red pulps, and the capsule. While the white pulps have been distinguished from the red pulps by the germinal center and follicular arterioles, the red pulps are composed of splenic cords and venous sinuses, which are detected by the presence of red blood cells **(Fig. 4a)**. On the contrary, lymphocytic depletion of splenic lymphoid follicles was noted in splenic sections of infected mice (GII), accompanied by numerous macrophages that were invaded by *T. gondii*, plasma cells, a thin capsule, fibrinoid material in splenic

trabeculae, extravasation of blood, and moderate deposition of hemosiderin pigment **(Fig. 4b)**. Furthermore, splenic sections

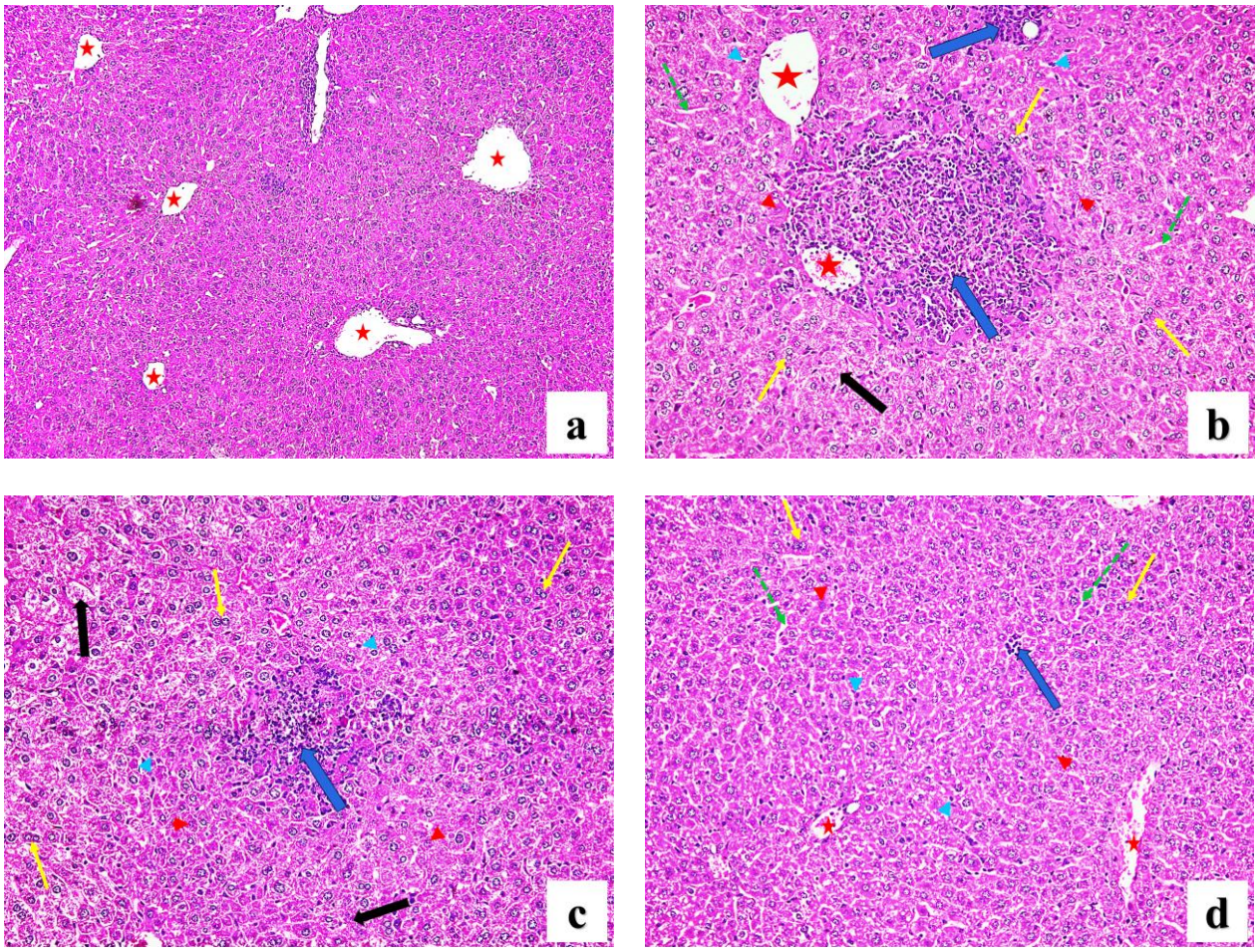

Fig.3: Microscopic histopathological representative images showing the histopathological changes in liver tissue of all groups in this experimental study. **a.** GI showing uniform hepatocytes with eosinophilic cytoplasm around the central veins (red stars) and normal sinusoids (H&E x100). **b.** GII showing severe periportal inflammatory cellular infiltration (blue arrows), hydropic degeneration (black arrow), binucleated hepatocytes (yellow arrows), pyknotic nuclei (blue arrowheads), karyorrhexis (red arrowheads), vascular congestion (red stars), and sinusoidal dilation (green arrows) (H&E x200). **c.** GIII showing inflammatory cellular infiltration (blue arrow), hydropic degeneration (black arrows), binucleated hepatocytes (yellow arrows), pyknotic nuclei (blue arrowheads), and karyorrhexis (red arrowheads) (H&E x200). **d.** GIV showing few lymphatic inflammatory cells (blue arrow), binucleated hepatocytes (yellow arrows), pyknotic nuclei (blue arrowheads), and karyorrhexis (red arrowheads) congested veins (red stars), and sinusoidal dilation (green arrows) (H&E x200).

Table 3. The mean inflammation score (IS) in liver sections of experimental groups.

Experimental groups	IS in liver (mean ± SD)	Reduction (%)
GI	0.00 ± 0.00	
GII	2.80 ± 0.42	
GIII	2.70 ± 0.48	**3.6%**
GIV	0.30 [a] ± 0.48	**89.3%**
H	34.11	
***P*-value**	< 0.0001*	

H is the Kruskal-Wallis test statistic. Dunn's post hoc test was used to perform multiple comparisons between groups.
*Statistically significant at *P*-values ≤ 0.05.
a: Significant with GII (Infected non-treated group).

of GIII demonstrated the same histopathological changes with more deposited pigment **(Fig. 4c)**. The splenic tissue of infected mice treated with MQ (GIV) showed normal histological splenic architecture

(Fig. 4d). A significant reduction of IS in splenic tissue was noticed after treatment with the single dose of MQ **(Tab. 4)**.

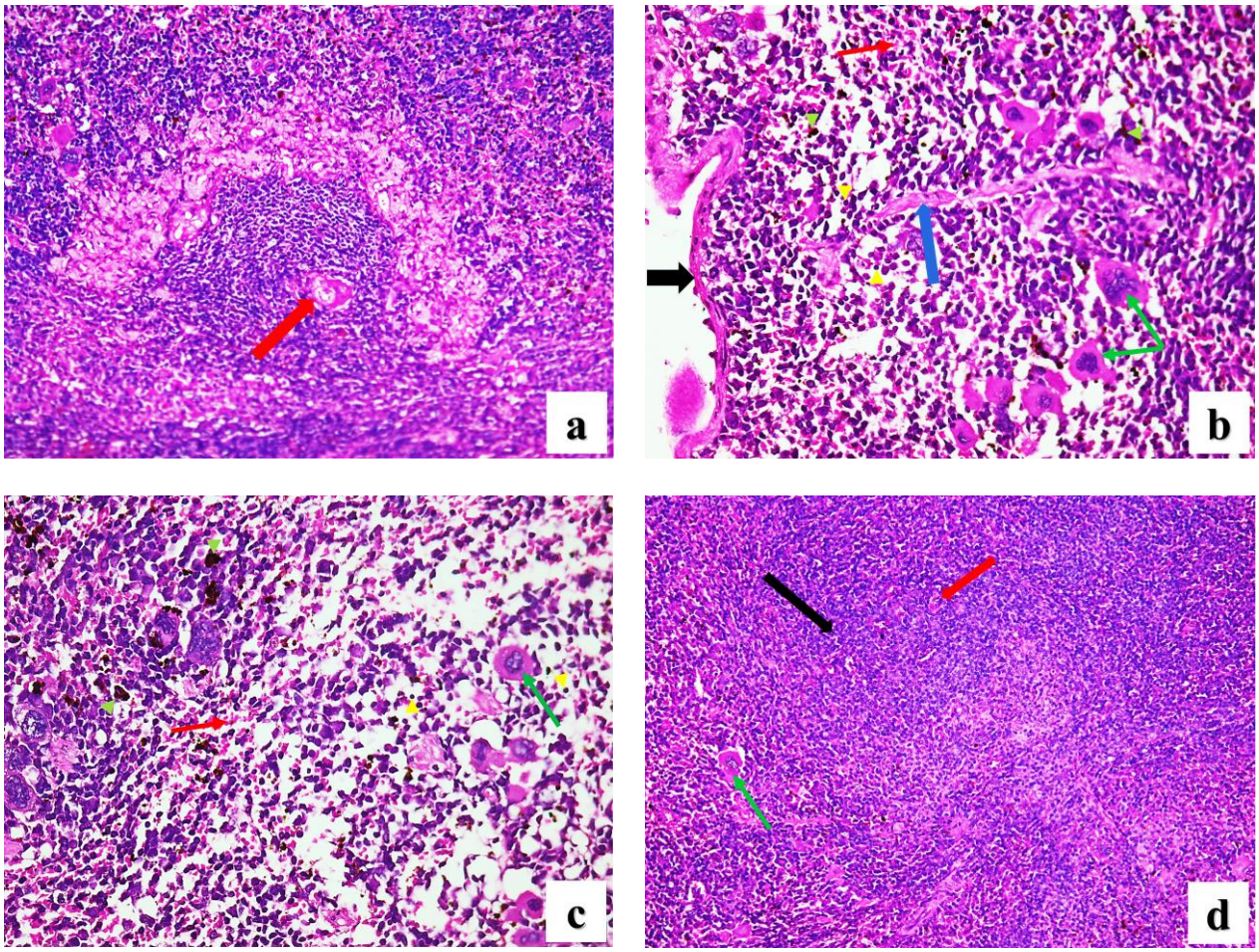

Fig.4: Microscopic histopathological representative images showing the histopathological changes in spleen tissue of all groups in this experimental study. **a.** GI showing normal splenic tissue of red pulp and white pulp with follicular arteriole (red arrow) (H&E x200). **b.** GII demonstrating splenic architecture loss, macrophages parasitized by *T. gondii* (green arrows), thin capsule (black arrow), fibrinoid material in splenic trabeculae (blue arrow), plasma cells (yellow arrowheads), extravasation of blood (red arrow), and deposition of hemosiderin pigment (green arrowheads) (H&E x400). **c.** GIII showing macrophages parasitized by *T. gondii* (green arrow), plasma cells (yellow arrowheads), extravasation of blood (red arrow), and deposition of hemosiderin pigment (green arrowheads) (H&E x400). **d.** GIV showing normal splenic architecture: white pulp (black arrow) with follicular arteriole (red arrow) and macrophages parasitized by *T. gondii* (green arrow) (H&E x200).

Table 4. The mean inflammation score (IS) in spleen sections of experimental groups.

Experimental groups	IS in spleen (mean ± SD)	Reduction (%)
GI	0.00 ± 0.00	
GII	2.60 ± 0.67	
GIII	2.70 ± 0.68	**- 3.8%**
GIV	0.20 [a] ± 0.42	**92.3%**
H	33.78	
***P*-value**	< 0.0001*	

H is the Kruskal-Wallis test statistic. Dunn's post hoc test was used to perform multiple comparisons between groups.
*Statistically significant at *P*-values ≤ 0.05.
a: Significant with GII (Infected non-treated group).

Immunohistochemical Assessment

For IHC evaluation, the immunohistochemical biomarker Ki-67 was used as a proliferation indicator in the cerebral tissues of all groups in this experimental study. The healthy group showed positive immunoreactivity **(Fig. 5a)**, while the cerebral sections of infected mice showed mild Ki-67 expression **(Fig. 5b)**. The Ki-67 expression was significantly increased ($P < 0.05$) **(Tab. 5)** in brain sections of infected mice treated with the pyrimethamine and sulfadiazine combination **(Fig. 5c)** or MQ **(Fig. 5d)**.

In addition, the immunohistochemical biomarker NF-κB was used as an indicator of the inflammatory response in the hepatic tissues of all groups in this experimental study. The healthy mice showed negative NF-κB reactivity with liver tissue **(Fig. 6a)**. Liver sections of infected mice exhibit moderate NF-κB expression in both hepatocytes and liver sinusoidal endothelial cells (LSECs) **(Fig. 6b)**. In comparison to the NF-κB level of infected mice, infected mice administered the pyrimethamine and sulfadiazine combination showed very mild expression **(Fig. 6c)**, while infected mice treated with MQ showed moderate expression only in LSECs **(Fig. 6d)**. The reduction of NF-κB expression was significant after the treatment with the pyrimethamine and sulfadiazine combination **(Tab. 5)**.

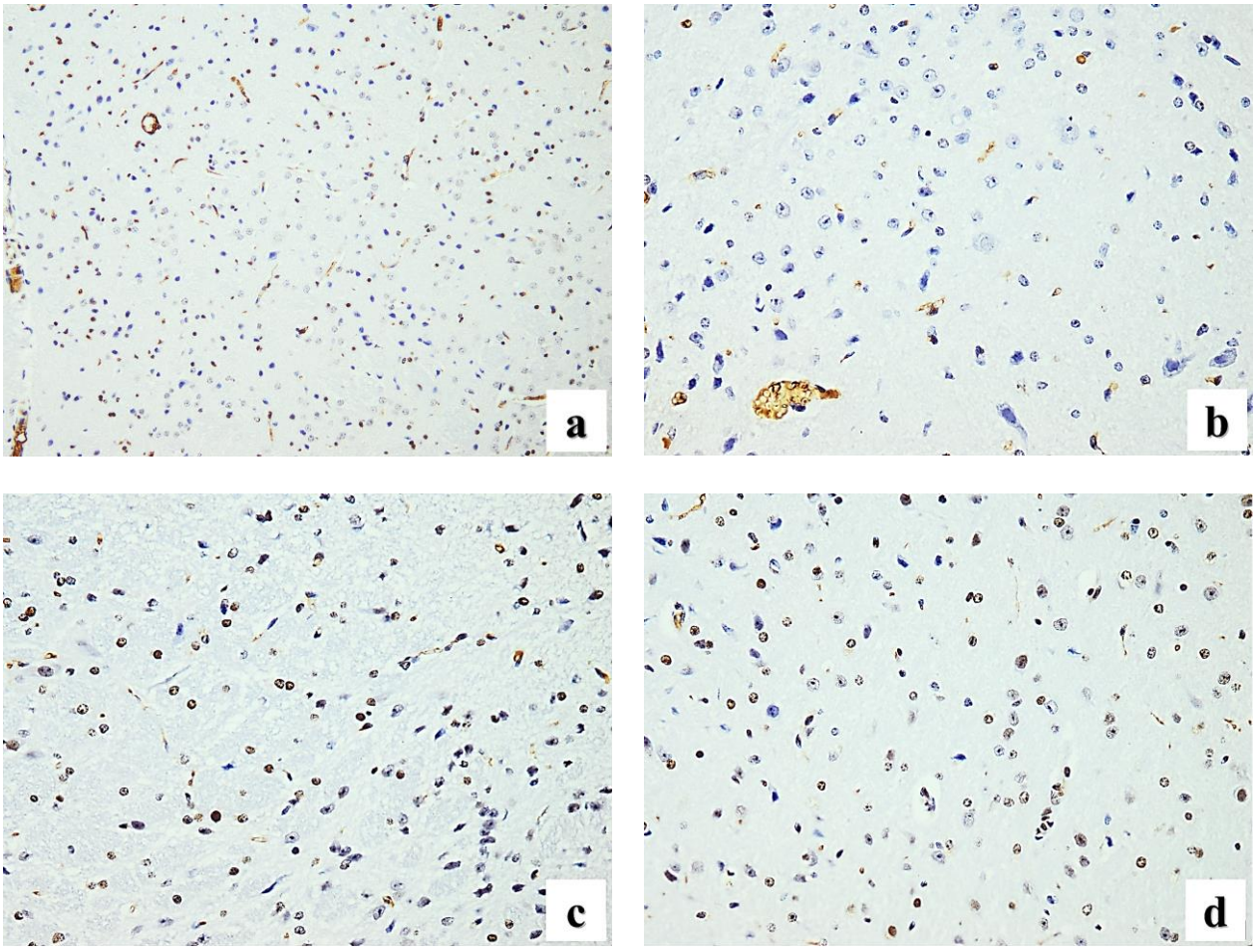

Fig. 5: Microscopic immunohistochemical representative images showing Ki-67 expression in brain sections of different study groups. **a**. GI showing positive Ki-67 expression (x200). **b.** GII showing mild expression of Ki-67 (x400). **c.** GIII showing strong expression (x400). **d.** GIV showing strong expression (x400).

Discussion

T. gondii is a worldwide-distributed apicomplexan that causes the severe infectious illness toxoplasmosis. Owing to pharmaceuticals' incapacity to cross the blood-brain barrier, whereas *T. gondii* can, therapy for chronic toxoplasmosis does not eliminate tissue cysts which raises the risk of reactivation in individuals with impaired immune systems **(Lai *et al.*, 2012)**. Additionally, pyrimethamine-based therapy is

linked to side effects and additional limitations since it acts on the folate biosynthesis pathway, reducing the activity of dihydrofolate reductase and finally stopping the synthesis of nucleic acids **(Elsheikha *et al.*, 2020)**. Thus, creating novel, secure and effective treatments for chronic toxoplasmosis is one of the most fascinating scientific topics of our day. Therefore, it was clearly shown what potential benefits there might be from using MQ to treat chronic toxoplasmosis in mice.

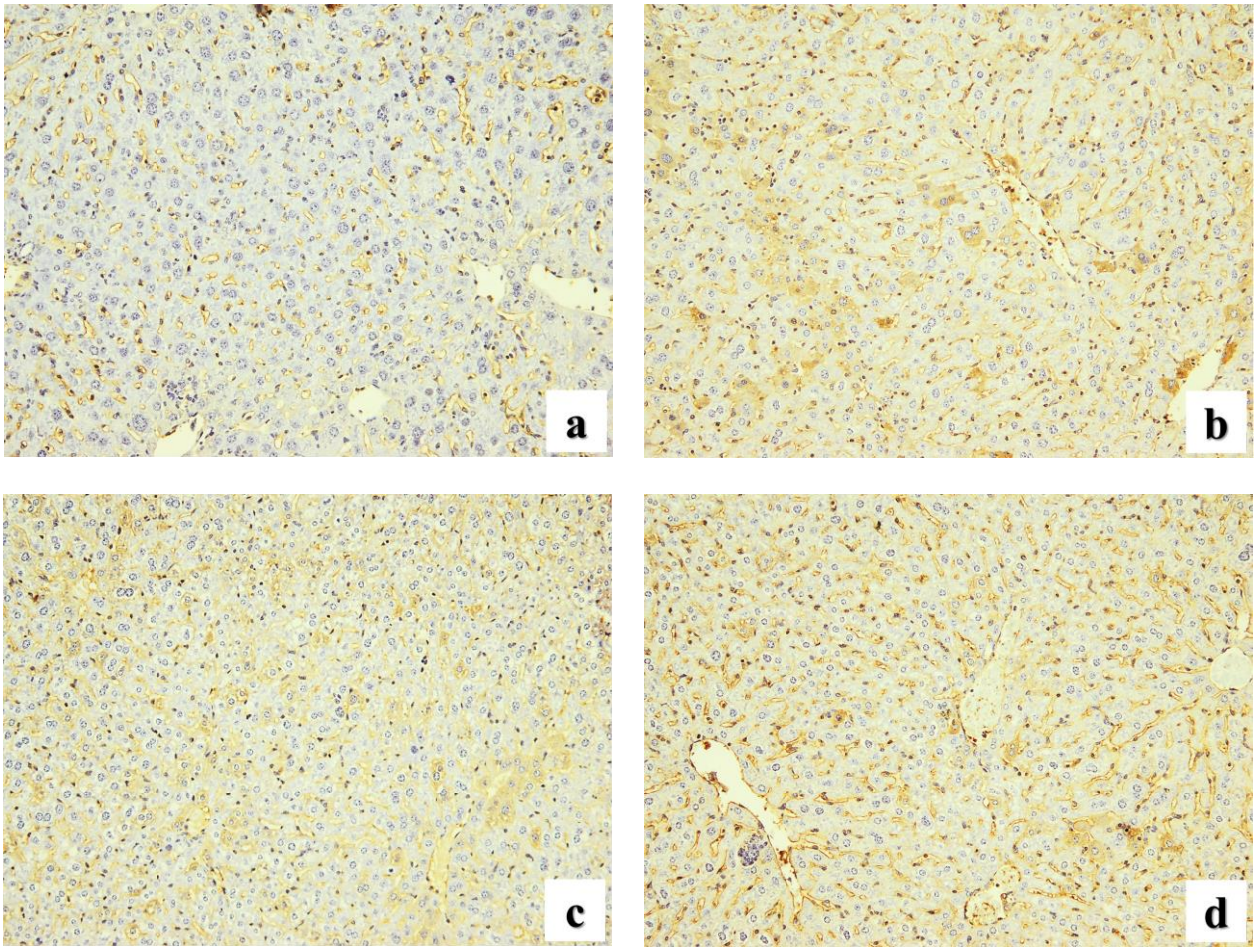

Fig. 6: Microscopic immunohistochemical representative images showing NF-κB expression in liver sections of different study groups. **a**. GI showing negative NF-κB reactivity with liver tissue. **b.** GII showed moderate NF-κB expression in both hepatocytes and liver sinusoidal endothelial cells (LSECs). **c.** GIII showed very mild expression of NF-κB. **d.** GIV showed moderate expression of NF-κB only in LSECs (x400).

Table 5. The mean immunohistochemical score (IHS) of Ki-67 and NF-κB in brains and livers of experimental groups, respectively.

Experimental groups	GI	GII	GIII	GIV	*H*	*P*-value
IHS of Ki-67 in brain tissue (mean ± SD)	3.00 ± 0.00	0.60 ± 0.52	2.40 [a] ± 0.67	2.30 [a] ± 0.82	26.23	< 0.0001*
IHS of NF-κB in liver tissue (mean ± SD)	0.00 ± 0.00	2.70 ± 0.48	1.10 [a] ± 0.88	1.70 ± 0.68	29.14	< 0.0001*

H is the Kruskal-Wallis test statistic. Dunn's post hoc test was used to perform multiple comparisons between groups.
*Statistically significant at *P*-values ≤ 0.05.
a: Significant with GII (Infected non-treated group).

In this study, the chronic infection was induced in thirty mice using the cystogenic ME49 strain of *T. gondii*. After six weeks, the chronically infected mice were subdivided into three groups, each of ten mice, to start the treatment schedule. GIII was orally given a combination of pyrimethamine (12,5 mg/kg/day) and sulfadiazine (200 mg/kg/day) for 10 days **(El-Shafey *et al.*, 2020)**, whereas GIV was orally administered a

single dose of MQ (400 mg/kg) and left alive for 10 days. To evaluate the drugs' efficacy, treated mice were scarified after 5 days from the end of the treatment schedule. The best treatment for toxoplasmosis is generally thought to involve pyrimethamine and sulfadiazine, while spiramycin is commonly administered during pregnancy to reduce the teratogenic effects of pyrimethamine **(Tari *et al.*, 2022)**. To rule out any possible external influences on the evaluation, GIII did not get any folinic acid supplements during the current study.

Regarding the SR, the highest survival rate among the infected mice was observed in the group treated with MQ (90%), followed by the group treated with pyrimethamine and sulfadiazine (70%) compared to the infected untreated group. Recent investigation reported that the treatment with MQ reduced the mortality rate by 25% **(El Sharazly *et al.*, 2023)**. In the current study, 50% of infected untreated mice died, which corresponds with earlier studies carried out by **Rayan *et al.* (2011)** and ***Mohammad et al.* (2023)** that recorded 50% and 66.7%, respectively. On the other hand, other investigations involving various strains of *T. gondii* reported the death of all infected untreated mice **(Mady *et al.*, 2016; Gomaa and Sheta, 2022)**, which has been interpreted as the capability of *T. gondii* to rapidly penetrate the host cells and distribute among different organs.

Regarding the parasite burden, the administration with a single dose of MQ statistically caused a significant reduction ($P < 0.05$) in the tissue cyst numbers (-42.6%), while the pyrimethamine and sulfadiazine combination was more effective and showed a significant reduction ($P < 0.05$) (-70.9%) compared to the chronically infected group. Similarly, **El Sharazly *et al.* (2023)** documented that the administration of MQ solution (50 mg/kg/day) for six consecutive days through the chronic phase of toxoplasmosis resulted in a significant reduction (-51.5%) in the parasitic load in the brain compared to the infected untreated group ($P < 0.001$). However, this previous study has represented the same efficacy of MQ and pyrimethamine and sulfadiazine treatment; the pyrimethamine and sulfadiazine treatment was more effective in the current investigation. Moreover, a similar effect has been documented for atovaquone, azithromycin, and spiramycin **(Chew *et al.*, 2012)**.

In the same manner, treatment with a single dose of MQ (400 mg/kg) showed 100% clearance of oocysts of *C. parvum* in the stool of infected immunosuppressed murine models after 14 days **(El-Wakil *et al.*, 2020)**. Moreover, treatment with a single dose of MQ (400 mg/kg) induced a moderate improvement in the ileocecal pathological changes induced by *C. parvum* in immunosuppressed mice. Moreover, a previous study concluded that treatment with a single dose of mefloquine (150 mg/kg) was significantly effective in significantly reducing egg production of *Schistosoma mansoni,* but no significant differences in worm burden were observed between treated and untreated infected mice **(Van Nassauw *et al.*, 2008)**.

Other derivatives of quinolones have shown very promising results against infections caused by *T. gondii* in both acute and chronic phases. They reduced the overall parasitic burden and could cross the blood-brain barrier, promoting the disintegration and reduction of *T. gondii* cysts containing bradyzoites in mice **(Elgawad *et al.*, 2018)**. As reported by **Bermudez *et al.* (2012)**, MQ has a long half-life and can reach concentrations in tissues that are 80 times higher than those in serum. These pharmacological properties are likely what give it its powerful therapeutic action against intracellular infections.

All experimental groups' brain, liver, and spleen tissue sections were analyzed for a more thorough assessment. The histological findings in the brain of the chronically infected mice (GII) matched many previous reported studies **(Rayan *et al.*, 2011; Etewa *et al.*, 2018; Farag *et al.*, 2019; Omar *et al.*, 2021; El-Hamed *et al.*, 2022; El Naggar *et al.*, 2023; Mohammad *et al.*, 2023)**. When MQ was administered, tissue cysts and abnormalities such as encephalitis, gliosis, brain edema, neurodegeneration, and infiltration of inflammatory cells were significantly reduced, according to the histological analysis of brain tissue sections. Severe histological necrotic alterations, including hydropic degeneration, ballooning hepatocytes, and mononuclear inflammatory cells, were also observed in the liver sections of persistently infected mice. Following MQ therapy, these tissue abnormalities were considerably reduced.

Many histopathological abnormalities were observed in the spleen tissue of infected untreated mice, such as depletion of splenic lymphoid follicles, in agreement with the previous studies **(GabAllah *et al.*, 2021; Yahia *et al.*, 2022; Zoghroban *et al.*, 2023)**. The lymphocytic depletion of splenic lymphoid follicles and the frequent deposition of hemosiderin pigment were observed in the spleen tissue of the infected group. According to **Emam *et al.* (2023)**, the presence of hemosiderin pigment may indicate hemolytic anemia and chronic congestion brought on by macrophages consuming red blood cells. When MQ was administered to infected

mice, their splenic tissue had a typical histological architecture with few hemosiderin pigment depositions and clogged blood vessels. Treatment with a single dose of MQ significantly reduced ($P < 0.05$) the inflammatory score, while the combination of pyrimethamine and sulfadiazine did not result in a substantial change in the inflammation severity of infected brain tissue. These findings are consistent with a prior investigation that found that administration of MQ solution or MQ-niosomes considerably reduced inflammation in the brain caused by a chronic toxoplasmosis **(El Sharazly *et al.*, 2023)**.

Moreover, *Toxoplasma* infection regulates many cellular pathways to support its survival and proliferation. Proliferation is among these paths. For the identification of nuclear proteins linked to cell proliferation, the Ki-67 biomarker is frequently employed **(Gerdes *et al.*, 1991)**. The Ki-67 expression was mild in the brain tissue of chronically infected mice. In the same manner, a recent study concluded that infection with *T. gondii* reduced Ki-67 immunoreactivity **(Pires *et al.*, 2023)**. On the other hand, the brain tissues of treated mice, even with MQ or pyrimethamine and sulfadiazine combination, significantly increased Ki-67 expression. Furthermore, the expression of NF-κB that regulates different proinflammatory and profibrotic cytokines was moderate in liver sections of chronically infected mice, which agrees with a previous study conducted by **Liempia *et al.* (2019)**. The NF-κB expression was significantly reduced only after treatment with the pyrimethamine and sulfadiazine combination.

Conclusion and Recommendation

In this experimental study, the findings revealed that treatment with MQ statistically reduced tissue cyst numbers and significantly improved *T. gondii*-induced adverse repercussions in the brain, liver, and spleen. Additionally, MQ could activate tissue proliferation and NF-κB, which regulates different proinflammatory and profibrotic cytokines. In addition, further clinical investigations are needed to explore the effect of MQ on infected pregnant women and its involvement in chemoprophylaxis against recurrent toxoplasmosis. Furthermore, in vivo and in vitro studies should be done to explore the efficacy of MQ against *T. gondii* on a more substantial scale, to properly characterize its action as anti-*T. gondii* medicine and to determine what the needs will be for a standardized human dose.

Author Contributions

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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