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ORIGINAL ARTICLE

Histopathological Evaluation of Melatonin's Role in The Treatment of Experimental Toxoplasmosis

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ABSTRACT

Background: *Toxoplasma gondii (T.gondii)* is an important pathogen that can infect almost all warm-blooded animals. It has a considerable health risk, particularly in pregnant women and those with compromised immune system. Existing treatments are constrained by adverse effects. This research assessed the therapeutic efficacy of spiramycin and melatonin either individually or combined with each other.

Methods: The study was performed on 55 male rats of matched age (6 weeks) and weight (200 gm) which were infected with avirulent ME49 pleural of *T.gondii*. Rats were divided into five equal groups: **(GI):** Normal healthy group. **(GII):** Infected control group. **(GIII):** Infected and received spiramycin. **(GIV):** infected and administered melatonin. **(GV):** Combined spiramycin and melatonin treated group. The assessment was evaluated through parasitological and histopathological studies.

Results: Our study showed that spiramycin and melatonin combination (GV) induced a highly significant decrease in the mean brain cyst counts 88.4%, followed by (GIII) and (GIV) with percentages 64.8% and 36.3% respectively. The treatment with melatonin by itself did not lead to a significant decrease in the parasite load, although there was a notable enhancement in the inflammatory infiltrates.

Conclusion: Melatonin can be used with spiramycin as a synergistic agent in the treatment of toxoplasmosis

Keywords: Spiramycin; Melatonin ; Toxoplasmosis and *Toxoplasma gondii*.

INTRODUCTION

Toxoplasma gondii (T.gondii) is an intracellular protozoan that can infect virtually mammals including humans [1]. Toxoplasmosis is one of the most widespread parasitic infections in humans. The disease affects approximately 30% to 50% of the global human population [2]. Despite being generally benign disease in people with adequate immunity, it is serious in immunodeficient individuals and in congenital infection [3]. In spite of ongoing and effective work to enhance diagnostic techniques, treatment approaches have not changed for many years [4].

Spiramycin is a macrolide antibiotic with anti-parasitic activity. This medication lowers the chances of congenital infection and transmission from mother to child [5]. Although it penetrates tissues well, it has limited ability to cross the blood-brain barrier. [6]. Because the available drugs are limited by several side effects, they also fail to completely eradicate parasites [7]. Hence, there is an urgent requirement for new drugs that are both safe and effective, utilizing novel mechanisms of action [8].

Melatonin (N-acetyl-5-methoxytryptamine) is a circadian neurohormone synthesized by the pineal gland and other tissues in vertebrates. It is derived from tryptophan and plays a role in numerous biological processes [9]. It exhibits antioxidant, immunomodulatory, and antitumoral properties. Various studies on different parasites such as *Trypanosoma cruzi* [10] and *Leishmania spp*. [11;12] observed that Melatonin has the ability to influence immune responses and the development of parasites. In addition, melatonin enhances the immune

response of the vertebrate host when infected with *T. gondii* [13].Thus, Melatonin is closely related to the biology of the parasite–host relationship [14]**.**

In our study, we tried to investigate the efficacy of melatonin both individually as well as in conjugation with spiramycin in rats infected with *T.gondii*.

METHODS

Parasite and infection: Avirulent ME49 *T.gondii* strain was got from Theodor Bilharz Research Institute, Giza, Egypt. To create the brain suspension, infected rats were euthanized 6 weeks post infection. Brain tissues were removed and homogenized in 1ml saline (0.9% NaCl). For counting the cysts, 0.1 ml of the brain suspension was put on a slide and examined under a microscope with × 40 lens. A concentration about 100 cysts / ml was then achieved by diluting the suspension [15]. Infection with tissue suspension containing *T.gondii* cyst was administered orally using a stomach tube (20 cysts / rat) [16].

Experimental animals and study groups

Fifty-five male rats that aging 6 weeks at the beginning of the study, bred in laboratory, free of parasites, about 200 gm in weight were used. Rats were cared for in accordance with national and institutional guidelines and housed in five groups, each consisting of eleven rats, as the following: (GI): Normal healthy group. (GII): Infected control group. (GIII): Spiramycin treated group (GIV): Melatonin treated group. (GV): Combined spiramycin and melatonin treated group. Treatment started six weeks after infection and lasted for three weeks. All rats were euthanized by submandibular decapitation preceded by intraperitoneal thiopental sodium administration two weeks after the end of treatment. The brain was extracted by opening the head. The abdominal skin was sterilized and incised for obtaining parts of the liver. The brain and liver tissues were used for histopathological assessment.

Drugs

Spiramycin: Rovac®, Delta Pharma, Egypt was available as a film-coated tablet. After crushing the tablet into a powder, weighing it, and calculating the amount of active component per rat per dosage, the tablets were suspended in 100µl of PBS and given orally by gavage 200 mg/kg/day for 3 weeks [17].

Melatonin: 40 mg of melatonin (M-5250, Sigma, Egypt) was dissolved in 3 ml of ethanol to yield a stock solution and moved into tubes that were then wrapped with aluminum foil and kept in the dark at -20°C until needed. 0.1 ml of this solution was mixed with 0.9 ml of saline at the time of use, and the resultant solution was administered intraperitoneally at a dose of 3 mg/kg/day for 3 weeks [18].

Parasitological assessment

Counting of *Toxoplasma* cysts in the brain (parasitic load) was performed. After mice were sacrificed; their brain tissues were removed and one hemisphere of the brain from all groups was homogenized with 1ml of 0.9% NaCl saline. On a microscopic slide, 0.1ml of the homogenized brain was put and examined using ×40 lens. The following equation was used for calculation of the mean cyst count per 1ml: Mean cyst number = cyst count in 100µl×10 [19].

Histopathological assessment

10% buffered formalin solution was used to fix the brain and liver samples. After 24 hrs, Following a 12-hour water wash, tissues were dehydrated using increasing alcohol grades, cleaned in xylene, and embedded in paraffin blocks that were microtome-sectioned to a thickness of 3 to 5 µm. Sections of paraffin were stained using hematoxylin and eosin stain [20]. Stained sections were searched for inflammation, degenerations, necrosis, and any other pathological changes. A swift microscope connected to a swift digital camera was the tool employed to capture all photographs. The histopathological grading was determined by semi-quantitative method as follows: “0 = No, 1 = Mild, 2 = Moderate, and 3 = Severe alterations” [21]. Two examiners, who were blind to the groups throughout the entire histological study executed within this research, inspected the sections for histopathology.

Ethical aspect

The National Institutes of Health's rules for animal experimentation were followed in the maintenance of the rats (Zu-IACUC/3/F/120/2023).

Statistical analysis

SPSS (Statistical Package for Social Science) version 27 (IBM, Armonk, NY, USA) was used to tabulate and calculate the data. The ANOVA test was performed for the probable differences among the studied groups. The P-value < 0.05 is considered significant, while a P-value <0.001 is for highly significant results.

RESULTS

I. Parasitological results

All treated groups showed a decrease in the mean brain cysts number of *T.gondii*. The least mean brain cyst count was obtained in the targeted group given combined spiramycin and melatonin (GV) with a percent reduction (88.4%) followed by spiramycin treated group (GIII) with a percent reduction 64.8%. In contrast, GIV exhibited the highest mean number of *T.gondii* brain cysts with a percent reduction (36.3%). The difference between these groups and the control infected group was statistically significant (p<0.001). Additionally, there was a highly statistical significant difference between all groups (Table 1, Fig.1).

II. Histopathological results

Regarding the inflammatory cell infiltrates in brain and liver tissues, mild to normal infiltrates were observed in GV. In contrast, GIII and GIV revealed mild to moderate inflammatory cell infiltrates with a high statistically significant difference (P <0.001) compared to the infected control group. Moreover, there was a high statistically significant difference between all treated groups (Table 2 & 3, Fig.4).

Brain

1. Samples of the normal group (GI) exhibited normal histological structures of neuronal cell bodies,neuropil(network of interwoven nerve fibers and their branches and synapses, together with glial filaments), and normal distributed glial cells (Fig. 2a). Brain tissue sections of the infected non treated rats (GII) showed parasitic cyst surrounded by clusters of glia cells and mononuclear cells particularly at the cerebral cortex (Fig. 2b). There is a gradual improvement in brain tissues at GIII, GIV, GV. Spiramycin treated group (GIII) showed a moderate improvement in the pathological changes in the form of moderate glial cell proliferation and pyknotic neurons (Fig.2c). Moreover, a moderate number of pyknotic neurons with pyknotic nuclei were noticed in melatonin treated group (GIV) (Fig.2d). Brain sections of the combined spiramycin and melatonin treated group (GV) revealed apparently preserved architecture with mildly dilated cerebral blood vessels and some pyknotic neurons primarily at hippocampal regions (Fig. 2e).

Liver

GI demonstrated normal morphology of hepatic cords and vascular tissues (Fig. 3a). Some examined sections of (GII) showed parasitic cyst surrounded by granulomatous reaction “neutrophils, macrophages, and lymphocytes” at the perivascular area (Fig. 3b). The infected and spiramycin treated group (GIII) exhibited a moderate improvement in the pathological changes, moderate vacuolated hepatocytes and focal areas of inflammatory cell aggregates (Fig. 3c). Moreover, perivascular focal necrotic area of hepatocytes beside inflammatory and hyperactive kupffur cells were observed in melatonin treated group (GIV) (Fig.3d). Liver sections of our targeted group (combined spiramycin and melatonin) (GV) revealed apparent normal hepatic parenchyma with minute foci of round cell aggregates (Fig.3e).

Table (1): The mean number of *T. gondii* brain cysts among different groups

Groups		Mean ± SD	Percentage of reduction
GII	**n=11**	82.5±2.94 [a]	-
GIII	**n=11**	29±2.08 [c]	64.8%
GIV	**n=11**	52.5±2.16 [b]	36.3%
GV	**n=11**	9.5±2.22 [d]	88.4%
F		554	-
P		**<0.001****	

a, b, c & d: There is no significant difference (P>0.05) between any two groups, within the same column have the same superscript letter **F**: ANOVA test, **P:** Probability, ****:** Highly significant difference

Table (2): Inflammatory cell infiltrates in brain tissue of *T.gondii* infected rats

Group	Inflammatory cell infiltrates in brain tissue								χ^2	P
	0		1		2		3			
	N	%	N	%	N	%	N	%		
GII	0	0.0%	0	0.0%	3	20.0%	8	66.7%		
G III	0	0.0%	7	20.0%	4	26.7%	0	0.0%	15.14	<0.001**
G IV	0	0.0%	8	22.9%	3	20.0%	0	0.0%	16.00	<0.001**
G V	3	20.0%	8	22.9%	0	0.0%	0	0.0%	22.00	<0.001**

χ^2: Chi square test**, P:** P-value for comparison between GII (Control +ve) and corresponding group, ****:** Highly significant difference 0: normal 1: mild 2: moderate 3: severe

Table (3): Inflammatory cell infiltrates in liver tissue of *T. gondii-infected* rats

Group	Inflammatory cell infiltrates in liver tissue								χ^2	P
	0		1		2		3			
	N	%	N	%	N	%	N	%		
GII	0	0.0%	0	0.0%	2	12.5%	9	64.3%		
G III	0	0.0%	6	18.2%	5	31.3%	0	0.0%	16.29	<0.001**
G IV	0	0.0%	7	21.2%	4	25.0%	0	0.0%	16.67	<0.001**
G V	3	21.4%	8	24.2%	0	0.0%	0	0.0%	22.00	<0.001**

χ^2: Chi square test**, P:** P-value for comparison between GII (Control +ve) and corresponding group, ****:** Highly significant difference 0: normal 1: mild 2: moderate 3: severe

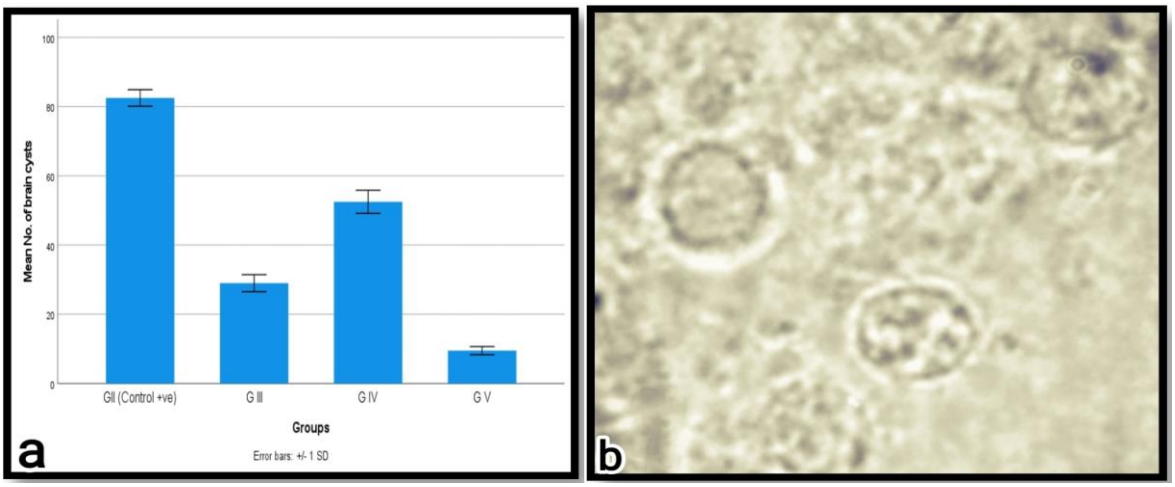


Figure (1.a): Brain cysts mean number of the different studied groups. (**b)** *T.gondii* cysts in brain suspension **(×1000)**

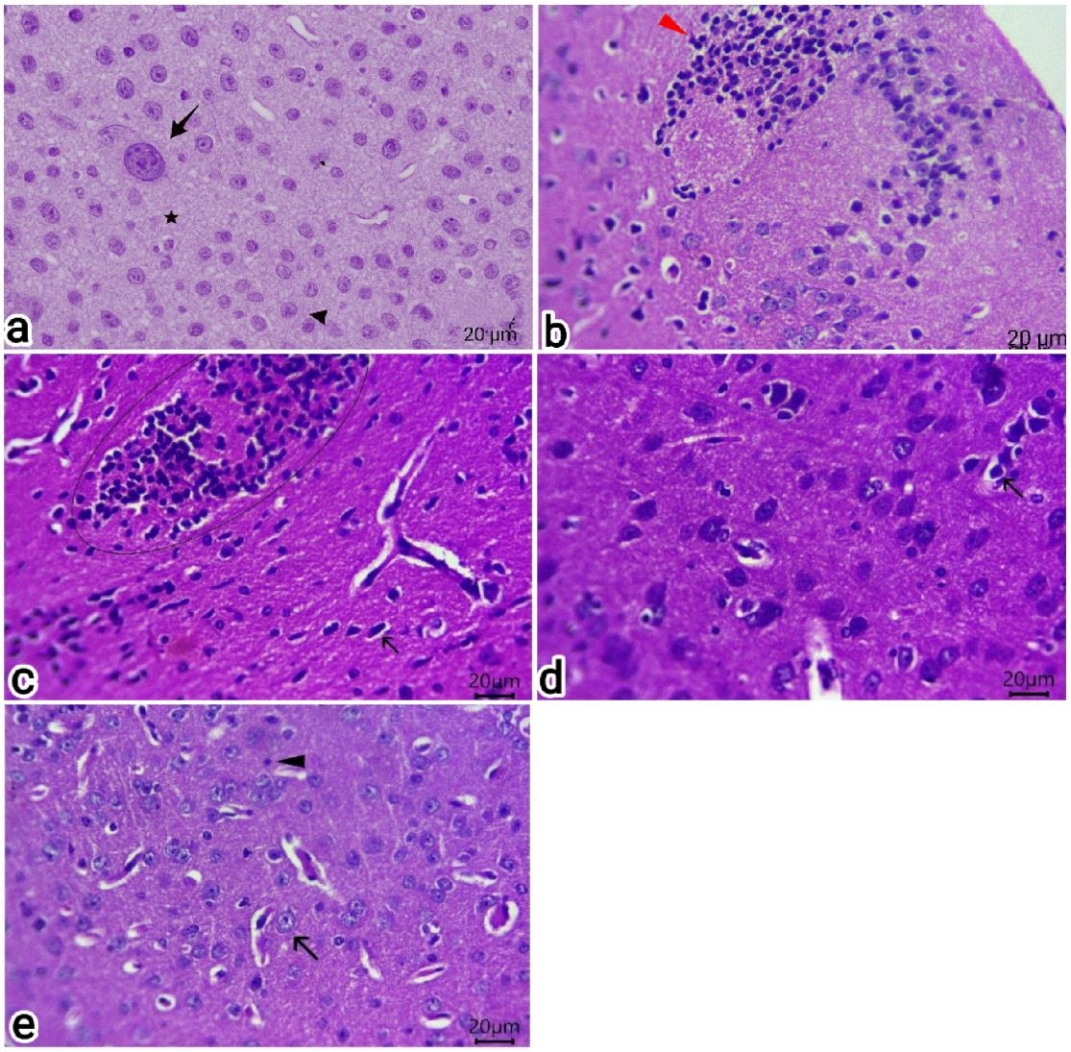
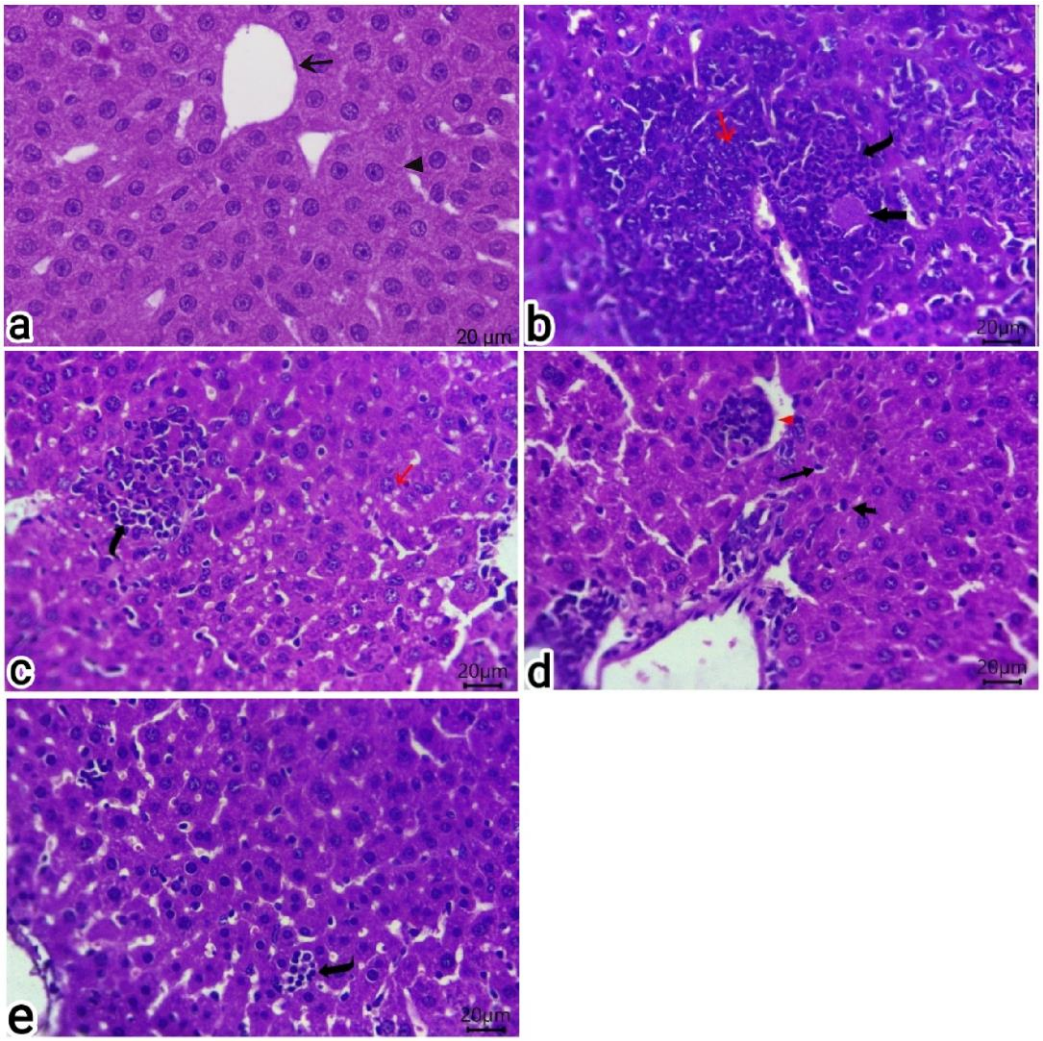
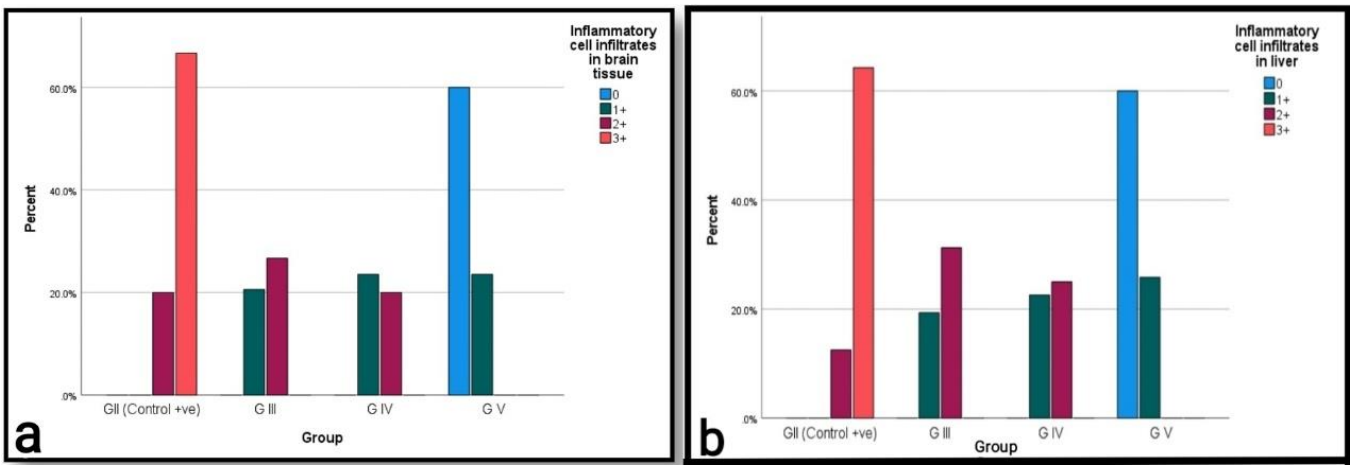


Figure (2): Histopathological brain sections of all tested groups a) Brain sections of the negative healthy group (GI) revealed normal histological structures of neuronal cell bodies, neuropil, and normal distributed glial cells. (b) GII showed clusters of glia cells (red arrow head) particularly at cerebral cortex. (c) GIII showed moderate glial cell proliferation (circle), and pyknotic neurons (arrow). (d) GIV revealed a moderate amount of pyknotic neurons and pyknotic nuclei (arrow). (e) GV revealed apparently preserved architecture of most neurons (arrow), glial cells (arrow head) with mildly dilated cerebral blood vessels primarily at hippocampal regions (Scale bar: 20 µm) (× 400).



Figure(3): Histopathological liver sections of all tested groups (a) Liver sections of GI revealed the typical morphology of hepatic cords and vascular tissues. (b) GII revealed parasitic cyst (thick arrow) surrounded by pyogranulomatous reaction "neutrophils (curved arrow), macrophages, and lymphocytes (red arrow) at perivascular area. (c) GIII exhibited moderate areas of vacuolated hepatocytes (red arrow) and focal areas of inflammatory cell aggregates (curved arrow). (d) GIV showed perivascular focal necrotic area of hepatocytes admixed with inflammatory cells (red arrow head) beside hyperactive kupffur cells (black arrows). (e) Liver sections of combined spiramycin and melatonin treated group (GV) revealed apparent normal hepatic parenchyma with minute foci of round cells (curved arrow) (Scale bar: 20 µm) (× 400).



Figure(4a): Inflammatory cell infiltrates in brain tissues of all treated groups. **(b)** Inflammatory cell infiltrates in liver tissues of all treated groups.

DISCUSSION

Human Toxoplasmosis is still challenging to treat [22]**.** Melatonin has drawn a lot of attention as a natural material with established antioxidant abilities beside its immunomodulatory actions [23]. Thus, the present study assessed the potential role of melatonin individually and in combination with spiramycin against experimental chronic *T.gondii* infection. In this study, a significant decrease in the parasite burden was observed in all treated groups relative to the infected control group. We observed that spiramycin combined with melatonin (GV) presented the highest significant decrease in the number of brain cyst (88.4%). The group that only received melatonin (GIV) experienced the lowest percent reduction (36.3%). Spiramycin produced a significant reduction in brain cysts (64.8%) compared to the infected group (GII). This was consistent with Allam et al. [6], who observed a considerable decrease in parasite count in infected mice received spiramycin, but not overall parasite eradication. Also, Omar et al. [24] found that spiramycin significantly reduced the number of brain cysts compared to the infected control animals. Spiramycin's anti-inflammatory actions are explained by its regulation of the inflammatory process [25]. Furthermore, it suppresses protein synthesis and, subsequently, cell development [26]**.** In our study, rats received melatonin reduced brain cyst count, but it was the least one compared to other treated groups (36.3%)**.** Our result agreed with Machado et al. [13] who found that melatonin suppresses *T.gondii* development in a monkey's renal cell line culture whereas preserving host survival. Melatonin was found to inhibit parasite growth via altering energy metabolism, resulting in both apoptosis and necrosis [13]**.** An opposite finding was reported in a human colon adenocarcinoma cell line; melatonin was identified to enhance parasite replication. It has also been claimed that *Toxoplasma* parasites breakdown indoleamine 2, 3-dioxygenase 1 (IDO1) and convert tryptophan to melatonin, which reduces reactive oxygen species (ROS) formation and promotes their proliferation. IFNγ inhibits IDO1 degradation and catabolizes tryptophan into kynurenine, causing cell death [27].

However, in melatonin and spiramycin-treated group (GV), the highest significant reduction of brain cysts count was observed compared to GII. This was consistent with Avunduk et al. [28] who found that melatonin in combination with zinc supplementation resulted in the greatest infiltration by CD_{3+}, CD_{4+}, and CD_{8+} lymphocytes in *T. gondii*-infected rats presented with retinochoroiditis. Also, Parvez et al. [29] found that melatonin combined with amphotericin B has considerably reduced the intracellular parasite burden in liver tissues of *L. donovani-infected* mice (98.89%). This result could be attributed to the fact that

melatonin was more effective when combined with other medications. These findings imply that melatonin in combination with antibiotics or antiviral medications may be an extremely efficient method to treat infections. Melatonin could boost the therapeutic index of medications and reduce their toxicity because of its anti-inflammatory and immune-balancing effects [30]. Melatonin's immune-boosting properties make it a common hormone that could be used as an adjunct treatment for *T. gondii* infections, particularly in elderly or immunocompromised individuals [28].

Regarding the histopathological results, the greatest improvement was exhibited in brain and liver tissues of combined spiramycin and melatonin treated group (GV). This can be explained that spiramycin exhibit a synergistic effect when combined with melatonin leading to increased treatment outcome. Mondal et al. [31] found the highest liver architecture recovery in the groups treated with melatonin and metronidazole benzoate for *Diplomonad* parasite infections in *Anabas testudineus* fish.

Sections of the group that received spiramycin treatment (GIII), showed a moderate improvement in the level of inflammatory infiltration in brain and liver tissues when compared to the control group (GII). These results agreed with Etewa et al. [32], who revealed that spiramycin reduce hepatic lesion in chronic toxoplasmic mice. Also, Ibrahim et al. [33] demonstrated that spiramycin causes an apparent decrease in tissue cysts and a moderate decrease in inflammatory infiltrates in the brain compared to control group.

In (GIV), after administration of melatonin, high improvement was demonstrated in brain and liver tissues. Melatonin's ability to reduce oxidative stress and guard cells from free-radical damage, is the primary factor contributing to the apparent improvement in inflammatory alterations. The primary mechanism underlying the pathophysiology of *Toxoplasma* encephalitis (TE) and apoptosis is thought to be infection-induced oxidative stress, which is caused by ROS generated by activated macrophages and microglia at high levels [34]. França-Botelho et al. [35] reported that hamsters infected with *E. histolytica* that received melatonin treatment showed a substantial decrease in hepatic necrosis in comparison to the control group.

CONCLUSION

From the previous findings, we can conclude that melatonin used alone did not considerably lower the parasite load. However, combination of spiramycin and melatonin has a notable parasiticidal and anti-inflammatory effects against chronic murine toxoplasmosis. Therefore, melatonin could be a valuable drug as an adjunct to spiramycin for *T.gondii* infection.

Conflict of interest: None.

Financial Disclosures: None

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