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ASSESMENT OF THE THERAPUTIC EFECT OF LATE AZITHROMYCIN TREATMENT ON CHRONIC MURINE TOXOPLASMOSIS

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Abstract

Toxoplasmosis is a widespread, neglected disease with significant morbidity and mortality. The current study aimed to investigate the potential effectiveness of azithromycin against the cystogenic Me 49 nonvirulent strain of *Toxoplasma gondii* after the 6th week post-infection (PI) in chronically infected mice. Fifty laboratory-bred female Swiss albino mice were divided into five groups: G1: Non-Infected, G2: Infected non-treated, G3: Infected and treated by azithromycin 200 mg/kg after the 6th week PI for 3 days, G4: Infected and treated by azithromycin 200 mg/kg after the 6th week PI for 10 days and G5: Infected and treated by spiramycin 200 mg/kg after the 6th week PI for 2 weeks, brain cysts number, size and histopathological changes were evaluated after 2 months.

The results showed that treating mice after the 6th week post-infection with azithromycin for 10 days reduced number and size of brain cysts by 64% & 30%, respectively, but without significant difference as compared with spiramycin-treated ones. Azithromycin treatment for 3 days achieved a greater reduction (34%) in brain cyst size than the group treated with azithromycin for 10 days. But, it caused the least reduction in brain cyst number (10%). Azithromycin treatment for 10 days improved the histopathological changes in cerebral toxoplasmosis.

Keywords: Mice; Azithromycin; Spiramycin; *Toxoplasma gondii*; Treatment

Introduction

Toxoplasmosis is an infection with an intracellular parasite called *Toxoplasma gondii* that affects about one-third of the world population with cats and other felidae serve as the definite host (CDC, 2023). In Egypt, toxoplasmosis and *T. gondi* were reported man, domestic and wild animals as well as birds (Abbass *et al,* 2020) and even in childbearing age females (Saleh *et al,* 2014). *Toxoplasma gondii* exists in nature as oocysts, bradyzoites (in latent tissue cysts) and replicating tachyzoites, which represents the hallmark of active disease (Duffy *et al*, 2019). Infection occurs by many routes include ingestion of cyst contaminated with fresh vegetables & fruits or water (main route), contact with oocysts cat litter, blood transfusion, and organ/tissue transplantation (Marín-García *et al,* 2022) as well as congenitally from an infected mother to fetus by placenta (Morsy *et al,* 2022). Nosocomial infection by needle-stick injury was reported (Abdel Motagally *et al,* 2017). Toxoplasmosis disrupts fetal brain risky development affects linked to many beh avioral and neurological disorders (Abo-Al-Ela, 2020). Al Malki *et al.* (2021) suggested that a maternal *T. gondii* infection may have a role in the childhood autism development linked to mtDNA and nDNA impairment. All these highlight the toxoplasmosis importance to be in differential clinical diagnosis by pediatricians for early management of the congenital and neurodevelopmental disorders (El-Beshbishi *et al,* 2020). Hence, the *T. gondii* unique pathogenesis also presents challenges for drug therapy, in contrast to other apicomplexans, penetrating the blood-brain barrier and forming drug resistant bradyzoites (Alday and Doggett, 2017).

Treatment guidelines for toxoplasmic encephalitis recommend either pyrimethamine® and sulfadiazine or pyrimethamine and clindamycin; trimethoprim/sulfamethoxazoleole is also known to possess comparable potency (Hernandez *et al,* 2017). Meanwhile, py-

rimethamine and sulfadiazine were linked to rare severe responses that may be fatal, such as agranulocytosis, Stevens-Johnson syndrome, toxic epidermal necrolysis or hepatic necrosis (Harr and French, 2010). The development of anti-*T. gondii* treatment with activity against cysts during the latent infection stage is greatly indicated to diminish the cyst load within the host and may eliminate the parasite from the host to achieve sterile immunity (Dunay *et al,*2018). The primary factor influencing the ongoing interest in azithromycin® is its unique pharmacokinetic characters. In short, azithromycin is considerably stable at low pH, a varied antimicrobial spectrum, a long half-life, well tissue penetration and extensive distribution with great concentrations within cells, including phagocytes (McMullan and Mostaghim, 2015). Besides, azithromycin in bacteria, it binds to the RNA of the 50S ribosomal subunit and acts synergistically to prevent protein synthesis (Beckers *et al,* 1995). Azithromycin also destroys tissue cysts in addition to its anti-replication effect on *T. gondii* tachyzoites; it has good intracellular penetration and can affect intracellular tachyzoites (Bosch-Driessen *et al,* 2002). Azithromycin has anti-inflammatoory and immunomodulatory activities (Kanoh and Rubin, 2010), exhibits bacteriostatic capabilities against a wide range of gram-positive and negative bacteria, and even atypical bacteria (McMullan and Mostaghim, 2015). Moreover, it has anti-parasitic activity against cryptosporidiosis (Kadappu *et al,* 2002), *Plasmodium* species (Srivastava *et al,* 2012), and *Trypanosoma brucei brucei* (Molefe *et al,* 2019), as well as was used to treat COVID-19 (Kamel *et al,* 2022). Also, Ahmed *et al.* (2023) reported that by adding antenatal azithromycin to women underwent the cerclage to prolong pregnancy and to reduce the risk of preterm birth, with a slight increase in birth-weight

The current study aimed to investigate the possible therapeutic effect of azithromycin against the cystogenic Me-49 non-virulent strain of *Toxoplasma gondii* in chronically infected mice.

Materials and Methods

Parasite: *T. gondii* Me 49 non-virulent strain was kindly provided by Zoonosis Department, National Research Center, and regularly maintained by sub passage in Swiss Alb ino mice with 0.1 ml of brain homogenate at $1x10^2$ tissue cysts/ml every 8 weeks to develop chronic toxoplasmosis. Brains were ground with sterile pestle in a clean mortar and diluted $1x10^2$ cysts/ml brain cyst suspension.

Drugs: Two drugs were used.1-Spiramycin® (Spirex) and 3 MIU (704mg) was purchased from Medical Union Pharmaceuticals, Egypt. Tablets were crushed and dissolved in distilled water for oral suspension at a dose of 200 mg/kg/day (Grujić*et al,* 2005).

2- Azithromycin (Zithromax, Pfizer Egypt) was prepared daily as a liquid suspension. After sonication, the homogenized suspensions were given orally to mice by stomach tube at a dose of 200mg/kg day. Oral dose was 0.1 ml/mouse.

Experimental infection: Mice were inoculated orally with 0.1ml of brain cysts suspensioncontained 10 cysts for every mouse.

Experimental Design: Fifty clean laboratory-bred female Swiss Albino mice, 10 weeks old and weighed 20-25gm were classified into five groups of 10 mice in each: G1: Non-infected (control negative), G2: Infected non-treated (control positive), G3: Infected and treated with azithromycin 200 mg/kg after the 6^{th} week post-infection (PI) for 3 days, G4: Infected and treated with azithromycin 200 mg/kg after 6^{th}week PI for 10 days, G5: Infected and treated with spiramycin 200 mg\kg after 6^{th} week PI for 2 weeks.

Assessment of anti-*Toxoplasma* action: After 8 weeks, all mice were sacrificed and their brains were removed. Each brain was separated into two sections, one for counting the cysts and the other was fixed in 10% formalin for the histopathological studies.

Brains were harvested, rinsed in sterile normal saline solution, weighed, and 1ml of st-

erile saline was added, followed by homogenization (Omni TH-220) for 5 minutes.

Histopathological examination for cysts: Brain specimens were fixed in 10% neutral buffered formalin, and processed for paraffin sections of 5µm thickness, stained with haematoxylin and eosin (H & E) and microscopically examined (Gad *et al*, 2022). Also, homogenate brain (0.1ml) smear was spread on a clean slide, fixed in methanol; air dried and stained in Giemsa stain (Merck, Germany) for 30-45 minutes, rinsed with water, dried, and microscopically examined to count *T. gondii* cysts. The following equation was adopted: Mean cyst number = cyst count in 100µl×10×2. The ocular and stage micrometers were used to count and to measure the cysts' size.

Ethical consideration: The Research Ethics Committee, Benha Faculty of Medicine, revised and approved the protocol (Approval No. Ms14-11-2018), and the Albino mice experimented with was done following the internationally standards of Helsinki declaration (2008).

Statistical analysis: Data were coded, tabulated and analyzed using ANOVA procedure and the post hoc test (Feldman *et al,* 2003).

Results

Both azithromycin and spiramycin significantly reduced the number of *Toxoplasma* brain cysts when compared with the control infected non treated group except the group treated with azithromycin for only 3 days. Mice group treated with azithromycin for 10 days caused more reduction in cyst number than those treated for 3 days ($P<0.05$). The number of *Toxoplasma* brain cysts achieved by the group treated with azithromycin for 3 days was much lower than those treated with spiramycin for 2 weeks ($P<0.05$). There was no difference in the outcome of treatment with the group of azithromycin treated for 10 days when compared with the group of spiramycin ($P>0.05$).

All azithromycin and spiramycin-treated mice exhibited significant reduction in the size of brain cysts compared with the control positive group. Azithromycin treatment for 10 days resulted in a smaller reduction in the size of brain cysts than spiramycin treatment ($P<0.05$). When mice treated with azithromycin for three days was compared to those treated with spiramycin, no significant reduction in brain cysts size ($P>0.05$). There was no statistically significant reduction in the size of brain cysts when the two azithromycin groups were compared ($P>0.05$).

Histopathological results: Brains of control negative mice showed normal histological structures. But, brain sections from positive control showed severe histopathological changes. Examined sections showed noticeable severe necrosis of neurons and neuronophagia with the presence of neurofibrillary tangles and numerous large *Toxoplasma* cysts distributed throughout the brain parenchyma. Noticeable vasculitis and perivasculitis as well as numerous focal necrosis associated with glial cell infiltration and severe perivascular cuffing with mononuclear inflammatory cells were also detected. Microscopically, examined brain sections from mice treated with azithromycin for 3 days after 6^{th} week showed moderate to severe necrosis of neurons, neuronophagia, neurofibrillary tangles, marked perivascular cuffing with mononuclear cells, moderate meningitis (inflammatory infiltrate in meninges) associated with *Toxoplasma* cysts and several different *Toxoplasma* developmental stages in cerebral cortex**.** Also, examination of brain sections from azithromycin-treated mice for 10 days showed several alterations such as necrosis of neurons with neurofibrillary tangles appearance and neuronophagia as well as glial nodules (focal gliosis). Few *Toxoplasma* cysts and intravascular bradyzoites were also detected, without meningitisbut mild perivascular cuffing with mononuclear cells. Brains of mice treated with spiramycin for 2 weeks PI showed focal infiltration with inflammatory cells and marked necrosis of some neurons.

Details were given in table (1) and figure (1).

Table 1: Effect of Azithromycin on number and size of *T. gondii* brain cyst in chronic infected mice

Group	Drugs	Dose/day (mg/kg)	Treatment duration (d)	Dose (mg/kg)	No of *T. gondii* cyst			size of *T. gondii* cyst		
					M±SD	Reduct %	95% CI	M±SD	Reduct%	95% CI
G2:	N/D	N/D	N/D	N/D	526±40	N/D	N/D	24±4	N/D	20-27
G3:	Azithr.	200	3	600	482±69	10	433-531	16±2	34	14-16
G4:	Azithr.	200	10	2000	192±30	64	170-214	17±2	30	16-19
G5:	Spira.	200	14	2800	148±48	72	113-183	14±2	42	13-15

Azithro. = azithromycin Spira.= Spiramycin Reduct + Reduction

Discussion

In the present study**,** treatment regimen 200 mg per kg per day azithromycin for 10 days showed an obvious therapeutic effect in treating the chronic toxoplasmosis infection among the studied groups, as the brain cyst number reduction rate was 64%. There was no significant difference with spiramycin-treated group (72% reduction rate). Conversely, the group treated with 200mg/kg/day azithromycinforonly3days had the lowest rate of brain cyst reduction among all groups (10%). Araujo *et al.* (1988) evaluated azithromycin treatment with a dose of 200mg/kg/day for 10 days in mice infected with strain C56 of *T. gondii*, treatment entirely protected mice from death induced by *T. gondii* intraperitoneal infection. Also, Araujo *et al.* (1991) reported that in toxoplasmosis, the tissue concentrations of azithromycin in the brain were ten-fold greater than the concentrations in the serum after treatment with 200mg/kg/day for 10 days (i.e., a dose regimen comparable to the present study used). Azithromycin monotherapy at a dose of 500mg/day was shown to be effective and well-tolerated for the treatment of active, non-vision-threatening toxoplasmic retinochoroiditis, also, duration of treatment was clinically longer for the azithromycin group (Balaskas *et al,* 2012). This agreed with the present data that the concentrations in infected mouse brains were approximately twofold greater than those in the brains of non-infected mice.

The present data also agreed with Lescano *et al.* (2004) investigated azithromycin treatment at a dose of 100mg/kg/day orally 20 days after infection and continued for 120 days. Number of brain cysts in mice infected intraperitoneally with a cystogenic *T. gondii* strain was reduced by 45% as compared to positive control group. Besides, Montaya and Remington (2008), who reported that azithromycin, especially at 200mg/ kg/day, was effective in both prophylaxis and treatment of toxoplasmosis due to its ability to reach significant concentrations in the CNS. Previously, mice given azithromycin and IFN survived at a rate of <40%; but, azithromycin alone protected less than 10% of mice, and IFN alone didn't protect against a lethal dosage of *T. gondii* (Araujo and Remington, 1991). Besides, Dumas *et al.* (1994) treated mice with azithromycin at a dose of 100mg/kg /day for 100 days beginning 4 weeks after infection didn't find significant change in the brain cysts number compared to the positive control group. The reduced severity of the retinal lesions and improvement vision by azithromycin was found to be a satisfactory as an alternative regimen to trimethoprim/sulfam-ethoxazole (Lashay *et al,* 2016).

The current study showed that the Albino mice treated with spiramycin for 2 weeks had the greatest reduction in the size of *Toxoplasma* brain cysts (42%), followed by mice treated with azithromycin for 3 days (34%), and the group treated with azithromycin for 10 days had the least reduction. However, many macrolides inhibited *T. gondii* intracellular growth; azithromycin was the only macrolide that inhibited intracellular tachyzoite multiplication (Chamberland *et al,* 1991).

Dumas *et al.* (1994) and Derouin (1995) reported that azithromycin is also effective *in vitro*, however the long-term administration of the drug to chronically infected mice failed to reduce the average number of cysts in the brain, but reduced the inflammatory response was noticed in the brains of infected and treated animals. This could be due to azithromycin's anti-replication effect on *T. gon-*

dii tachyzoites, which even destroyed tissue cysts. Azithromycin also possesses good intracellular penetration and may directly affect the intracellular tachyzoites (Bosch-Driessen *et al,* 2002). Also, Degerli *et al.* (2003) reported that the azithromycin acted against the *T. gondii* tachyzoites and cysts as well as bradyzoites

The fluctuation in size of *Toxoplasma* cysts discovered in the current study revealed that chronic infection resulted in the continued creation of new or second-generation tissue cysts in the brain. This agreed with Dzierszinski *et al.* (2004), who reported that the intracellular bradyzoites were motile in the host cells, with the ability to attack surrounding cells and initiate new cysts and that cysts can proliferate by fission, revealing that bradyzoites and cysts are highly dynamic denoting the mechanism of parasite spreading during chronic infection. Franco *et al.* (2019) reported that azithromycin treatment was as successful as conventional treatment in woman placental villi infected*;* it considerably reduced *T. gondii* intracellular proliferation.

In the current work, the histopathological examination of mouse brains was used to evaluate parameter for investigating the azithromycin efficacy of. The histological examin ation of brain sections of mice from the control positive mice revealed extensive and severe necrosis of neurons and neuronophagia along with the formation of neurofibrillary tangles and numerous large *Toxoplasma* cysts distributed in the brain parenchyma. There is also major vasculitis and perivasculitis, as well as various localized necrosis along with glial cell infiltration and perivascular cuffing with mononuclear inflammatory cells. This agreed with Waree (2008), who reported that the histopathological hallmarks in the brains of mice with chronic toxoplasmosis were meningeal congestion, with multiple mononuclear cells attacking the meninges and some cuffing of mononuclear cells, around vessels. These changes are the result of malfunction within the blood-brain barrier produced by parasite chemicals that increase the permeability of barrier and enable parasite establishment (Strunk *et al,* 2014). Astrocytes and glial cells play a dynamic role in the protection against infection by secreting cytokines such as IL-1, IL-6, GM-CSF, IL-10, IFN-g; and chemotactic cytokines (IP-10 & MCP-1) preventing lesions and decreasing its replication (Wilson and Hunter 2004). When the parasite infected a large number of cells, its protective action was reduced or abolished, which aggravates tissue damage and inflammatory process, favored the creation of tissue cysts. Spiramycin-treated mice had the fewest histopathological lesions. The most successful azithromycin regimen was achieved in the 10-day treated mice, which showed mild to severe neuropathological abnormalities such as neurofibrillary tangles, neuronophagia of degenerated neurons, cerebral blood vessel congestion, and glial cell proliferation. The present mice treated for only three days had the least effect in terms of severe neuron necrosis, neuronophagia, neurofibrillary tangles, perivascular cuffing with mononuclear cells, meningitis (inflammatory infiltrate in the meninges) associated with the presence of cysts and different *Toxoplasma* developmental stages in the cerebral cortex. Azithromycin inhibited *T. gondii* infection and replication in human trophoblastic cells. Franco *et al.* (2011) reported und that the anti-inflammatory response and enhanced MIF production were important to establish and maintain a healthy in *T. gondii* infected pregnant mother

Conclusion

Azithromycin treatment for 10 days after the 6^{th} week post-infection in chronic toxoplasmosis proved to be as effective as spiramycin.

Azithromycin reduced the number and size of cysts in brains of infected mice and alleviated the pathological alterations caused by *Toxoplasma gondii* in infected mice.

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oritical and practical studies of the paper and approved the final form of the manuscript.

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Explanation of figures

Fig. 1: Mice brain to assess azithromycin treating cerebral toxoplasmosis (Me 49 non-virulent strain) lesions (H & E stain). A- Infected non-treated showed necrosis of neurons (short arrow) with appearance of neurofibrillary tangles (long arrow). b-c Infected non-treated showed *Toxoplasma* cysts (arrows) and marked vasculitis and perivasculitis(arrows), respectively. d-f- Azithromycin treated for 3 days showed necrosis of neurons and neuronophagia (arrow), necrosis of neurons (short arrow) and perivascular cuffing with mononuclear cells (long arrow), and meningitis, respectively, with inflammatory infiltrate in meninges. g-i- Azithromycin treated for 10 days showed necrosis of neurons (short arrow) with appearance of neurofibrillary tangles (long arrow), necrosis of neurons (short arrow) and neuronophagia (long arrow), focal gliosis (arrow), respectively. J- Spiramycin treated (after 6thweek PI for 2 weeks) showed degeneration and necrosis of some neurons (arrow) (scale bar 25um).