

IN VIVO ASSESSMENT OF THE EFFECTS OF METHOTREXATE ON LATENT TOXOPLASMOSIS

By

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Abstract

Toxoplasma gondii (*T. gondii*) has been shown to result in life-threatening encephalitis in immunocompromised patients after reactivation of dormant parasites. The present work aimed to assess the effects of methotrexate on reactivation of latent toxoplasmosis alone and combined with a therapeutic combination of spiramycin, pyrimethamine and folinic acid on the progress of the disease. Forty-four laboratory-bred Swiss albino male mice were infected with Me49 non-virulent strain of *T. gondii* and equally divided into four groups: infected untreated control (I); infected and treated with combination of spiramycin, pyrimethamine and folinic acid (II); Infected and received methotrexate (III); and infected and received both methotrexate, spiramycin, pyrimethamine and folinic acid (IV). The mean number and size of tissue cysts in brain smears of mice of each group were determined, serum levels of anti-*Toxoplasma* IgM were assessed in different study groups by an enzyme linked immunosorbent assay and histopathological examination of brain and liver were done. The results showed that the mean serum IgM level was significantly different in the treated groups compared to that in infected control group. The highest level of serum IgM was found in the infected and received methotrexate group. After treatment with spiramycin, pyrimethamine and folinic acid, it was significantly decreased. In this study, reactivation of latent toxoplasmosis was observed by a significant increase in the mean number and sizes of *Toxoplasma* tissue cysts in brains of mice with established chronic toxoplasmosis after treatment with methotrexate alone or combined with spiramycin, pyrimethamine compared to both untreated chronically infected controls and infected mice treated with spiramycin, pyrimethamine and folinic acid. Moreover, considerable pathological lesions in the brain and liver was observed in groups received methotrexate alone or combined with spiramycin, pyrimethamine compared to both untreated chronically infected controls and infected mice treated with spiramycin, pyrimethamine and folinic acid.

Keywords: *Toxoplasma gondii*, Methotrexate, Spiramycin, Pyrimethamine, IgM, Histopathology.

Introduction

Toxoplasmosis is an opportunistic disease affects humans and warm-blooded animals caused by *Toxoplasma gondii* (Halonen and Weiss, 2013). Transmission to humans occurs either through ingestion of *T. gondii* oocysts shed by cats into the environment, eating the undercooked meat of infected animals, blood transfusion, congenitally across the placenta and organ transplantation (Yazar *et al*, 2006). After the acute stage, the parasite forms cysts in variety of organs, including the brain, heart, and skeletal muscle, thereby establishing a

chronic infection (El-Sayed *et al*, 2012). Antibodies play a minor role but remain the

essential means for diagnosing toxoplasmosis in humans (Stray-Pedersen, 1997). The antibodies act on the extracellular tachyzoites released following lysis of infected cells (Xu *et al*, 2005).

Toxoplasma IgM is the first antibody to appear in infected patients, detected at the surface of the parasite, from the second day following infection. However, it can be persistent and depending on the tests used, it can be detected for shorter or longer periods of time making the interpretation of serological results sometimes difficult (Roberts *et*

al., 2001). These immunoglobulins are the best activators of the complement system. In addition, due to its structure, it enables excellent agglutination and has a high level of cytotoxicity. This phenomenon is used especially in serological diagnostic techniques (Huang *et al.*, 2012).

T. gondii infection in immunocompetent individuals is mostly asymptomatic or can be manifested as lymphadenopathy and is usually followed by a lifelong latent infection that may be reactivated as a result of immune disorders inducing serious complications (Meers, 2010). This reactivation of latent infection may occur due to acquired immunodeficiency syndrome (AIDS), organ transplantation, hematopoietic stem cell transplantation, or cancer chemo-therapy as methotrexate (MTx) (Halonen and Weiss, 2013). Cerebral toxoplasmosis in patients receiving multiple immunosuppressive medications, including methotrexate, cortisone infliximab and leflunomide for rheumatoid arthritis has been reported (Young and McGwire, 2005).

Methotrexate (MTx) is an antimetabolite chemotherapeutic agent that binds to the enzyme dihydrofolate reductase, which is involved in the synthesis of purine nucleotides. This interferes with DNA synthesis and disrupts cell multiplication (Wessels *et al.*, 2008).

MTx appears to have multiple sites of action within the immune system. They include stimulation of interleukin-2 production, antiproliferative effects on B and T cells (Quéméneur *et al.*, 2003), inhibition of histamine release from basophils, and inhibition of immunoglobulin production (Montesinos *et al.*, 2003; Bunescu *et al.*, 2004). Reactivation of latent murine *T. gondii* was induced by long administration of immunosuppressive drugs as dexamethasone (DXM) and associated with increased brain cysts burden and marked decrease in spleen size and cellularity (Sumyuen *et al.*, 1996).

Treatment of toxoplasmosis patients includes a variety of therapeutic protocols

which comprises sulphadiazine and pyrimethamine with vitamin supplements continued for 6 weeks (Holliman, 2009). A potent antitoxoplasmosis like spiramycin, a macrolide antibiotic produces high tissue concentration, especially in the placenta, but does not cross the placental barrier, and has been given to pregnant women who were primarily infected with *Toxoplasma* during this pregnancy without teratogenic effects to the fetus as it is relatively nontoxic to the mother and the fetus (Vaz *et al.*, 2011).

The aim of this study was to assess *in vivo* the effects of methotrexate in reactivation of latent toxoplasmosis alone and with a therapeutic combination of spiramycin, pyrimethamine and folic acid on the progress of the disease.

Materials and Methods

An experimental study was conducted at Medical Parasitology Department (Post-Graduate Research Laboratory), Faculty of Medicine, Zagazig University, Sharkia Governorate, Egypt.

Brain cysts of the Me49 non-virulent strain of *T. gondii* were kindly provided by Department of Medical Parasitology, Faculty of Medicine, Alex, Egypt. The parasite strain was regularly maintained by repeated inoculation of Swiss albino mice every 8 weeks with 0.1 ml of brain homogenate of previously infected mice containing, approximately, 1×10^2 cysts/ml to establish chronic toxoplasmosis (El-Sayed and Aly, 2014).

Experimental design: Laboratory-bred male Swiss albino mice, 10 weeks-old, each weighing 20-25 g were selected from the animal house of Faculty of Medicine, Zagazig University. They were housed in plastic cages with white wood chips for bedding, fed by commercial complete food mixture and tap water for drinking, and maintained under controlled conditions of lighting (12 h light/12 h dark cycle) and temperature ($25 \pm 2^\circ\text{C}$). Mice were inoculated intraperitoneally with 0.1 ml of the brain cysts suspension, which containing 1×10^2 cysts/ml. Four weeks after the infection, the efficiency of

the experimental *Toxoplasma* infection in mice was confirmed by the complement fixation test for detection of specific anti-*Toxoplasma*-IgM (Ondriska *et al*, 2003) and mice with undetected or very low concentrations of specific antibodies were excluded from the experiment. The final set of experimental animals included 44 mice. Animals were divided into four groups of 11 mice each: Group I (GI) infected untreated control group, Group II (GII) infected and treated with combination of spiramycin at a dose of 100 mg/kg/day (Filice and Pomeroy, 1991), pyrimethamine at a dose of 12.5mg/kg/day and folinic acid at a dose of 10-25 mg orally daily (Romand *et al*, 1993). The drugs were provided in powder form and prepared daily as liquid suspensions; after brief sonication, the homogenized suspensions were administered orally to mice via tube feeding. Six weeks post-infection; treatments were administered daily at a fixed hour for 10 days, Group III (GIII) Infected and received methotrexate (MTX). Four weeks post-infection; infected mice were injected intraperitoneally by MTX at a dose of 5 mg/kg every 48 hours for 3 doses, Group IV (GIV) *T. gondii* infected mice and received both MTX as GIII and combined treatment as GII. At the end of the experiment (6 weeks post the last dose of the administered drugs), the blood was collected from the mouse's orbital sinus (Hoff and Rlagt, 2000). The sera were separated and stored at -20°C until the determination of anti-*Toxoplasma* antibody (IgM) serum levels. Then all mice were sacrificed by cervical dislocation. The effect of the used drugs was evaluated by:

Immunological study: Determination of anti-*Toxoplasma* IgM in sera samples using commercially available ELISA kit (Catalogue No. 201-11-1845 Egypt). The kit uses a double-antigen sandwich enzyme-linked immunosorbent assay (ELISA). Serum samples are added to enzyme well which is pre-coated with mice anti-*Toxoplasma* IgM antibody (anti-toxIgM) antigen, then incubation.

Anti-*Toxoplasma* IgM antibody (anti-tox IgM) antigen is added labeled with biotin, and combined with Streptavidin-HRP to form immune complex; then incubation and washing are carried again to remove the enzyme-linked immunosorbent assay uncombined enzyme. Chromogen Solution A, B was added; the color of the liquid changes into the blue, and at the effect of acid, the color finally becomes yellow. The chroma of color and the concentration of the mice anti-*Toxoplasma* IgM antibody (anti-tox IgM) of sample are positively correlated. The optical density (OD) was measured at 450nm. The sensitivity cut off was equal the average value of negative control well + 0.15.

Parasitological study: Counting the brain cysts number and measuring of their size. The brains of the sacrificed mice were grinded individually in a mortar. Then, 5 ml of normal saline were added to the grinded brain tissue of each mouse obtaining brain emulsion homogenates. The total number of cysts per mouse brain was then estimated from four separate drops that were placed on glass slides and examined by bright-field microscope at 10 magnification (Djurkovic'-Djakovic'*et al*, 2002). Then, the mean cysts number in each group was calculated.

After that, the slides were placed in room temperature to dry completely, and were then fixed using absolute methanol. Fix smears were stained with 20% Giemsa solution (Merck, Darmstadt, Germany) in pH 6.8 of phosphate buffer for 20 min. Measuring brain cysts size of each group was done by computerized image analysis and accordingly the mean size in each group was calculated.

Histopathological study: Specimens from brain and liver were collected and fixed in 10% formadehyde solution for 24-48 hours. The tissue was dehydrated in an ethyl alcohol series, cleared in xylene and embedded in paraffin and further histological processing were performed. Then microtomy was performed to obtain two histological slices for each tissue, 5µ thick, which were

extended on clean slides, stained by hematoxylin and eosin (H&E) and mounted with the aid of Canada balsam and coverslips (Abdel Wahab *et al*, 1989).

Ethical consideration: All procedures in the present study met the International Guiding Principles for Biomedical Research Involving Animals, as issued by the International Organizations of Medical Sciences (National Research Council, 2006) and approved by Ethics Committee of the Faculty of Medicine, Zagazig University.

Statistical analysis: Data were analyzed using the Statistical Package for Social Science (SPSS) for windows version 11.0 with student (t) test and ANOVA test (analysis of variance) to evaluate the possible differences between the study groups. P values of less

than 0.05, 0.01 or 0.001 were used to indicate statistical significance.

Results

There was highly significant increase in serum IgM level in groups treated with methotrexate alone (GIII) compared to GI (P < 0.01). Significant increase in serum IgM level in methotrexate, spiramycin, pyrimethamine and folic acid GIV compared to GI (P<0.05). It was observed non-significant difference between methotrexate GIII & methotrexate, spiramycin, pyrimethamine and folic acid GIV (P>0.05). But, there was significant decrease in serum IgM level in spiramycin and pyrimethamine GII compared to GI (P<0.05).

Table 1: Comparison among mean values of ELISA(IgM) in studied groups using ANOVA test.

ELISA	GI: Infected control (N= 11)	GII: Spiramycin, Pyrimethamine (N=11)	GIII: Methotrexate (N= 11)	GIV: Methotrexate, spiramycin, pyrimethamine & folic acid (N=11)	F	P
<i>M ± SD</i>	0.32± 0.06	0.28± 0.08	0.40± 0.05	0.37 ±0.04	8.82	<0.001***
<i>Range</i>	0.28 – 0.37	0.23 – 0.36	0.38 – 0.47	0.35 – 0.41		

SD: Standard Deviation. ***: very highly significant (p<0.001).

Statistical analysis showed highly significant difference in tissue cysts size among some groups (P < 0.001). There was highly significant increase in tissue cysts size in GIII compared to infected GI (P < 0.01). There was significant increase in tissue cyst size in methotrexate, spiramycin, pyrimethamine and folic acid GIV compared

to infected GI (P < 0.05). It was noticed non-significant difference between methotrexate GIII and methotrexate, spiramycin, pyrimethamine and folic acid treated GIV (P>0.05). But, there was very high significant decrease in tissue cyst size in spiramycin and pyrimethamine treated group (GII) compared to infected GI (P<0.001).

Table 2: Comparison among mean values of cyst size in studied groups using ANOVA test

	GI	GII	GIII	GIV	F	P
Size: <i>M ±SD</i>	28.4 ±3.67	14.4 ± 1.27	41.29±5.51	36.46 ±10.73	39.79	<0.001***
<i>Range</i>	17.5 – 33	13.3 – 17.5	33 – 50	23 – 60		

SD: Standard Deviation. ***: very highly significant (p<0.001).

There was very high significant increase in number of tissue cysts in methotrexate GIII compared to GI (P<0.001). Significant increase in number of cysts was in methotrexate, spiramycin, pyrimethamine and folic acid GIV compared to GI (P<0.05). Non-significant difference between methotrexate

GIII and methotrexate, spiramycin, pyrimethamine and folic acid GIV (P>0.05) was noticed. Also, there was significant decrease in number of cysts in spiramycin and pyrimethamine GII compared to GI (P < 0.05).

Table 3: Comparison among mean values of cysts number in studied group using ANOVA test:

item	GI	GII	GIII	GIV	F	P
<i>M ± SD</i>	635.45 ±144.86	544.55 ±129.53	904.09 ±175.66	785 ±129.83		<0.001***
<i>Range</i>	480 - 810	380 - 660	800 - 1010	640 - 930	13.04	1***

SD: Standard Deviation. ***: very highly significant (p<0.001).

Histopathology of brain sections stained by H & E showed moderate lymphocytic in-

flammatory infiltrate in GI (Fig. 8), mild inflammatory infiltrate in spiramycin and

pyrimethamine GII (Fig. 9), but, diffuse lymphocytic inflammatory infiltrate in both methotrexate GIII (Fig. 10) and methotrexate, spiramycin and pyrimethamine GIV (Fig. 11). Histopathology of liver section stained by H&E stain showed liver necrosis and inflammation in GI (Fig. 12). Spiramycin and pyrimethamine GII revealed reduction in extent and severity of the hepatic lobular and portal inflammatory response, with reduced inflammatory cell infiltrates (Fig. 13). Methotrexate GIII revealed with extensive liver fibrosis and lytic necrosis (Fig. 14). Methotrexate, spiramycin and pyrimethamine GIV revealed variable hepatocyte ballooning and hydropic degeneration (Fig. 15).

Discussion

Toxoplasmosis is an important opportunistic infection in immunocompromised patients (Munoz *et al*, 2011). It is an obligate intracellular replication parasite (Ferreira da Silva *et al*, 2008), releasing more tachyzoites, which may form cysts containing bradyzoites especially in the brain that evade the immune system and remain in tissues (Skariah *et al*, 2010). The cysts remain in the host tissues for years without causing any local inflammatory reaction which is controlled mainly by cellular immune mechanisms (Hunter *et al*, 1996). However, impairment of the balance between the host immune response and the parasite evasion of the immune response causes cyst rupture and renewed parasite proliferation and this may lead to clinical reactivation (Sullivan *et al*, 2009).

The advent of AIDS and use of intensive immunosuppressive drugs for the treatment of malignant and systemic diseases, or pre/post organ transplantation, have established a population of immunocompromised individuals prone to reactivation of opportunistic pathogens, including *T. gondii*, particularly in geographic areas with a high exposure to this protozoan (Bernsteen *et al*, 1999). So, reactivation of toxoplasmosis was considered a serious complication in patients

given methotrexate (Pulivarthi *et al*, 2015).

Diagnosis of reactivated chronic toxoplasmosis is confirmed by detection of specific antibodies in serum as IgM antibody using ELISA technique which is more sensitive and specific than IgM-IFA and can detect IgM antibodies for a longer time (Pinon *et al*, 2001).

In the present study, there was significant increase in *Toxoplasma* IgM level in serum samples of infected mice treated with methotrexate alone or combined with spiramycin, pyrimethamine and folic acid compared to control group. These results agree with Kuba *et al*. (2014) who found the seroprevalence of *T. gondii* IgM was highly significant increase in rheumatoid arthritis patients receiving methotrexate in comparison to patients without treatment (control group) and the presence of *Toxoplasma* IgM antibodies in 20.4% of *T. gondii* IgG positive patients indicated the reactivation of *T. gondii* infection in them. In contrast, Kang *et al*. (2006) found that after dexamethasone treatment (immunosuppressive drug) of experimental mice infected with toxoplasma, serum IgM titer was depressed significantly. Moreover, the decrease in IgM level in group treated with spiramycin and pyrimethamine in our study may be attributed to the effective role of a combination of spiramycin and pyrimethamine in parasite killing and limiting the infection.

During the chronic stage of *T. gondii* infection, bradyzoites slowly multiply within the cysts and cyst sizes increase respectively (Dubey *et al*, 1998). In the present study, reactivation of latent toxoplasmosis was observed by highly significant increase in the mean size and number of *Toxoplasma* tissue cysts in brains of mice with chronic toxoplasmosis after treatment with methotrexate alone or combined with spiramycin, pyrimethamine and folic acid compared to both chronically infected controls and infected mice treated with spiramycin, pyrimethamine and folic acid. These results agree with El-Sayed *et al.*, (2016) who

found a significant increase in the mean number and sizes of *Toxoplasma* tissue cysts in brains of mice with established chronic toxoplasmosis after reactivation of latent toxoplasmosis by treatment with etanercept alone or combined with conventional treatment compared to both untreated chronically infected controls and infected mice treated with sulfadiazine and pyrimethamine. The present study found high significant decrease in the mean size and number of *Toxoplasma* cysts in brains of mice treated with spiramycin, pyrimethamine compared to chronically infected controls. This was due to the fact that pyrimethamine has a synergistic action by inhibiting *T. gondii* folic acid synthesis, essential for parasite survival and replication (Doliwa *et al.*, 2013).

The histopathological examination of brain tissue of infected control mice showed presence of inflammatory infiltrate reaction reflected mainly by proliferation of lymphocytes. In the present study, histopathological examination of brain tissue of mice treated with spiramycin, pyrimethamine and folic acid showed mild inflammatory infiltrate reaction compared to methotrexate treated group and infected control group. The repair of the lesions by treatment leads to reduce the number of damaged cells. Prompt treatment may achieve rapid resolution, decrease inflammatory damage, prevent more tissue destruction and decrease the chances of the parasite dissemination. Those agree with Soheilian *et al.* (2011) who found that classic therapy (pyrimethamine, sulfadiazine) for *Toxoplasma* retinochoroiditis can reduce lesion size and vitreal inflammation as well as improve visual acuity.

In the present study, histopathological examination of brain tissue of mice treated with methotrexate showed massive increase in number of tissue cysts and presence of diffuse lymphocytic inflammatory infiltrate reaction compared to infected control group and spiramycin, pyrimethamine treated group. These data agreed with Djurk-

ović-Djaković and Milenković (2001) who observed that high mortality of mice treated with dexamethasone (DXM) and cortisone acetate (CA) was associated with increased brain cyst burdens compared to both healthy and infected untreated mice and presence of perivascular and meningeal infiltration consistent with the diagnosis of meningoencephalitis, despite the anti-inflammatory activity of corticoids due to reactivation of previously latent murine *T. gondii* infection.

In the present study, histopathological examination of liver tissue in infected control mice showed presence of liver inflammation and areas of necrosis. These findings are in agreement with Rifaat *et al.* (1981) who found that chronic infection of *Toxoplasma gondii* in liver of rat cause vascular congestion with localized areas of haemorrhage and liver cells around haemorrhagic areas were necrosed and brown pigments were seen. Mady *et al.* (2016) found also that the hepatic lobules reveal moderate inflammatory cell infiltrate with frequent spotty necrosis as well as focal confluent necrosis. We found that treatment by methotrexate alone or combined with spiramycin, pyrimethamine and folic acid causes extensive liver fibrosis, hepatocytes ballooning and hydropic degeneration. This finding approved the predominance of the immunosuppressive effect of methotrexate and its ability to reactivate *Toxoplasma gondii*. Marked reduction in the pathological changes observed in liver sections in spiramycin, pyrimethamine treated group as compared to infected control group. This revealed remarkable therapeutic effects of a combination of spiramycin and pyrimethamine against chronic toxoplasmosis.

Conclusion

The outcome data showed that methotrexate as an immunosuppressive drug have a role in reactivation of latent toxoplasmosis in murine models evidenced by increasing size and number of brain tissue cysts. Its immunosuppressive effect predominates the therapeutic effects of a potent antitoxoplas-

mosis combination even if combined with it. Serological screening for cases offers a valuable aid for patients who must be treated with methotrexate.

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Explanation of figures

- Fig. 1: Comparison among mean values of ELISA (IgM) in different groups.
- Fig. 2: Comparison among mean values of cyst size in different groups.
- Fig. 3: Comparison among mean values of cysts number in different groups.
- Fig. 4: Tissue cysts (arrows) in brain tissue in GI (Giemsa stain) x100.
- Fig. 5: Tissue cysts (arrows) in brain tissue of spiramycin and pyrimethamine GII (Giemsa Stain) x100.
- Fig. 6: Tissue cysts (arrows) in brain tissue of methotrexate GIII (Giemsa Stain) x100.
- Fig. 7: Tissue cysts (arrows) in brain tissue of methotrexate & spiramycin and pyrimethamine GIV (Giemsa Stain) x100.
- Fig. 8: Moderate lymphocytic inflammatory infiltrate (arrows) in brain section of GI (H&E stain) x400.
- Fig. 9: Mild inflammatory infiltrate (arrows) in brain section of spiramycin and pyrimethamine GII (H&E stain) x400.
- Fig. 10: Diffuse lymphocytic infiltration (arrows) in brain section of methotrexate GIII (H&E stain) x400.
- Fig. 11: Diffuse lymphocytic inflammatory infiltrate (arrows) in brain section of methotrexate & Spiramycin and pyrimethamine GIV (H&E stain) x400.
- Fig. 12: Liver necrosis and inflammation in liver section of GI (H&E stain) x400.
- Fig. 13: Mild inflammatory cell infiltrate in liver section of spiramycin and pyrimethamine GII (H&E stain) x400.

Fig. 14: Extensive liver fibrosis and lytic necrosis in liver section of methotrexate GIII (H&E stain) x400.

Fig. 15: Variable hepatocytes ballooning (arrows) and hydropic degeneration in liver section of methotrexate & spiramycin and pyrimethamine GIV (H&E stain) x400.



