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Effect of the anticancer drug tamoxifen on chronic toxoplasmosis in experimentally infected rats

Rabab Fawzy Selem[1,2], Gehan Abdel Rahman Rashed[1], Ashraf Mohamed Abdel Khalek Barakat[3], Hasan Mohamed Ali Elfadaly[3], Boshra EL-sayed Talha Hussien[4], Hemat Salah Mohamed[1], Marwa Mohamed Nageeb[1] and Ahlam Farag Moharm[1]*

[1]Parasitology Department, Faculty of Medicine, Benha University, Egypt.
[2]Taif Faculty of Medicine, Kingdom of Saudia Arabia.
[3]Department of Zoonotic Diseases, Veterinary Research Division, National Research Center, Egypt.
[4]Tropical Medicine Department, Faculty of Medicine, Tanta University, Egypt.

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***Toxoplasma gondii* is an opportunistic parasite that can cause severe disorders in infants and pregnant women and can also be lethal in immunologically compromised individuals. During unfit host immune conditions, and as a consequence to latent stage opportunity, the protozoan stimulates serious infection, and signifies higher morbidity and mortality including humans with Acquired Immuno-Deficiency Syndrome (AIDS) or those receiving corticosteroids and cancer chemotherapy. Tamoxifen drug (TAM) is a selective estrogen receptor modulator (SERM), which is commonly used for treatment of breast cancer; it has a known immunomodulatory effects on the patient, especially if administered for a long time as happens in cases of post breast cancer surgery and anti-recurrence prophylactic measures where women might persist to take TAM for years. The research question here was: Can TAM reactivates latent toxoplasmosis? To assess the possible stressful effect of TAM, rats were experimentally infected by *T. gondii* (RH strain). Three months later, they were treated by oral administration of TAM (10 mg/kg body weight/day) for 7, 14, 21 and 28 days. Tamoxifen effect on toxoplasmosis dynamics was estimated by counting *Toxoplasma* brain cysts and serological detection of anti-parasitic IgM and IgG all through the experiment time. The results showed an initial insignificant decrease in parasitic burden in groups treated for one week followed by a significant increase in groups treated for 14, 21 and 28 days. There was also a significant decrease in IgM titers in groups treated for one and two weeks while there was a significant increase in IgM titers in groups treated for three and four weeks. There was a significant increase in IgG titrers in groups treated for 14 and 21 days and a border line significant increase in the 1st week while there was non-significant increase in 4th week.**

Key words: Toxoplasmosis, tamoxifen, breast cancer, serological, Toxoplasma brain cysts, immunomodulatory.

INTRODUCTION

Toxoplasma gondii (*T. gondii*) is the causal agent of toxoplasmosis and one-third of the world population had

*Corresponding author. E-mail: drahlammoharm@yahoo.com. Tel: 00201098053493.

been affected by this parasite (El-On and Peiser, 2003).In immune suppressed individuals, such as those undergoing chemotherapy, organ transplantation or in AIDS patients, reactivation of a latent *T. gondii* infection is often fatal. *T. gondii* has been identified as an important opportunistic infection in HIV/AIDS patients and a major contributor to death of AIDS patients in the developing world (Carruthers and Suzuki, 2007). Host affection was confirmed a sequel to reactivation of primary infection (Saadatnia and Golkar, 2012). Breast cancer is the most common malignancy in women around the world. Information on the incidence and mortality due to breast cancer is essential for planning preventive health measures (Ghoncheh et al., 2016). Treatment with TAM lowers the risk of breast cancer recurrence and also lowers the risk of death from breast cancer (Early Breast Cancer Trialists' Collaborative Group, 2011). Since both toxoplasmosis and breast cancer are widely distributed globally, the research question was: Could tamoxifen treatment lead to reactivation of latent primary toxoplasmosis? For that purpose, we treated toxoplasmosis experimentally infected rats with TAM for different periods and observed the possible stressful effects of it on *Toxoplasma* parasitosis.

MATERIALS AND METHODS

Study site

This study was conducted in National Research Center; NRC (Cairo, Giza).

Ethical considerations

The study was approved by the Parasitology Department Research Committee and the Ethical Committee at the Faculty of Medicine, Benha University.

Parasites

Toxoplasma gondii (RH strain) was obtained from Zoonotic Diseases Department, National Research Center, Egypt. Tachyzoites of *T. gondii* (RH) strain maintained through serial intra-peritoneal (i.p.) passage were used for experimental infection. Tachyzoites were collected from mouse peritoneal cavity 72 h post infection (p.i.), the parasites were counted and adjusted to 10^3/ ml in saline. Each 1 ml solution was inoculated subcutaneously into each experimental rat

Drugs

Tamoxifen(nolvadex)(Sigma-Aldrich) 10 mg tablets was orally administered to the rats at a dose of 10 mg/Kg body weight daily (Perumal et al., 2005), via oral gavage 90 days post infection for 1, 2, 3 and 4 weeks. Tablets were dissolved in sunflower oil (Sigma-Aldrich) and the dose was adjusted for each rat according to its weight.

Animals, infection and treatment schedule

To test the efficacy of tamoxifen in a chronic model of experimental toxoplasmosis, a total of 45 laboratory-bred male rats were used (10 weeks old, weighing ~250 g). Animals were housed and maintained in a suitable rearing environment with free access to food and water throughout the experiment. Infected rats were divided into four groups consisting of 10 to 11 rats each group (7 infected and treated + 3-4 rats served as positive control; infected non treated) in addition to 3 healthy non infected- non treated; negative control rats. The first group was treated by tamoxifen for 7 days, the second group treated for 14 days, the third one treated for 21 and fourth group treated for 28 days. At the end of each group treatment time, rats were sacrificed, their brains were dissected and examined for immediate direct parasitological assessment and blood samples were collected individually, sera were separated and kept at -20ºC for later serological evaluation.

Evaluation of tamoxifen efficacy

Parasitological assessment

To prepare the brain suspension, rats were sacrificed, brains were removed and prepared in a tissue homogenizer (Wheaton USA) with 1 ml saline each. For cyst enumeration, 0.1 ml of the brain suspension was placed on a slide. The number of *Toxoplasma* cysts was counted in ten high power fields (HPF) and then the mean number was determined for each rat followed by calculation of the mean numbers of cysts in each infected group (Djakovic and Milenkovic, 2001).

Serological assay

Serum samples were serologically assayed by ELISA to detect IgM & IgG titer according to procedures described by Lind et al. (1997).

Statistical analysis

Gathered data were tabulated and analyzed using SPSS statistical software (IBM Corp., Armonk, NY, USA). Data were expressed as mean ±SD. Analysis of variance between groups was done using t test. P value<0.05 was considered statistically significant.

RESULTS

The results showed that there was a decrease in average brain parasitic load (ABPL) in TAM infected and treated (IT) group for one week as compared to the infected untreated (IU) control group (3.4%), however the difference was statistically non-significant (p = 0.448). Inversely, in the other IT groups which were treated for 14, 21 and 28 days, there were a statistically significant (p = 0.0001 - 0.0045) increases in ABPL (by 11.4, 30.3 and 48.7%, respectively) as shown in Table 1. In the serological study, there was a statistically significant decrease (p = 0.0001 - 0.027) in Anti *Toxoplasma* IgM titers in IT groups treated for 7 and 14 days, inversely, in IT groups treated for 21 and 28 days, a statistically significant increase (p = 0.0001 - 0.0012) was found in anti-*Toxoplasma* IgM titers as shown in Table 2. Concerning anti *Toxoplasma* IgG titers, throughout the

Table 1. Average brain parasite load (ABPL) of Tamoxifen treated rats as compared with untreated rat at different time points.

DPI / DPT	Group/average brain parasite load (ABPL/10 mg/brain)			p value	*T*
	(I U) BPL	(I T)BPL	AGD (NO.) (%)		
90 (IPL)	26553.67±1031.253	-			
97(7DPT)	24206±1024.24	23364.86±1665.88	-841.14/ ↓3.4%	0.448	0.79
104(14DPT)	21141.25±1151.39	23861.71±1159.16	+2720.46 ↑ 11.4 %	0.0045*	3.75
111(21DPT)	19836.75±1239.87	28443.14±1843.32	8606.39 ↑30.2%	0.0001*	8.23
118(28DPT)	16335.67±875.22	31922.71±1884.4	+15587.05 ↑ 48.7%	0.0001*	13.36

ABPL: Average brain parasite load; IPL: Initial parasite load; IU: Infected untreated; IT: Infected treated; DPT: Day post treatment; DPI: Day post infection; ADG: Average difference between treated and untreated groups.

Experiment duration, the antibody generally increasedstarting from the first week and continued increasing till the fourth week, however those rises in IgG titers were statistically significant (p = 0.0062 - 0.0315) in groups treated for 14 and 21 days, borderline significant (p = 0.0579) in animals treated for 1 week and insignificant (p = 0.188) in groups treated for 28 days as shown in Table 3.

DISCUSSION

T. gondii is an obligate intracellular, parasitic protozoan. It is the etiologic agent for toxoplasmosis. About 30 to 50% of the world population is infected with the parasite, and it is the most prevalent infection among humans (Tenter et al., 2000; Flegr et al., 2014). Cluster of differentiation ($CD4^+$) and ($CD8^+$)T cells are highly activated during infection and are essential for adaptive immunity. As such, patients with defects in T cell-mediated immune responses (for example, patients with AIDS) are at risk for reactivation of latent *T. gondii* infections. $CD4^+$ and $CD8^+$ T cells act synergistically to prevent cyst reactivation during chronic latent *T. gondii* infection. $CD8^+$ T cells mediate protection against toxoplasmosis primarily through the generation of interferon- gamma (IFN-γ). Interleukin-12(IL-12) drives the generation of terminally differentiated $CD8^+$ effector T cells (Aliberti, 2005). $CD4^+$ T cells are critical for avoiding reactivation of latent toxoplasmosis, as the emergence of severe toxoplasmosis is concomitant with the decline in T cell numbers in patients infected with HIV (Luft et al., 1984; Israelski and Remington, 1988) and in mouse models, the lack of $CD4^+$ T cells is associated with increased susceptibility of reactivation during the chronic stage of infection (Johnson and Sayles, 2002). $CD8^+$ T cell responses to *T. gondii* are influenced by good functioning provided by $CD4^+$ T cells (Lutjen et al., 2006). $CD4^+$ T cells are necessary for the maintenance of $CD8^+$ T cell effector functions during the chronic stage of infection, and this help must be provided during the acute stage of infection (Lutjen et al., 2006). Tamoxifen (TAM) is a broadly known anti-estrogen, which has been used in adjuvant treatment of early stage, estrogen-sensitive breast cancer for over 20 years, especially for women who still have significant ovarian estrogenic activity which could not be controlled by aromatase inhibitors. Five years of adjuvant tamoxifen safely reduced 15-year risks of breast cancer recurrence and death (Behjati1 and Frank, 2009; Early Breast Cancer Trialists' Collaborative Group, 2011). It has also immunmodulatory effects. Tamoxifen is capable of inducing a shift from cellular (T-helper 1) to humoral (T-helper 2) immunity. Interestingly, the immune modulatory effects of tamoxifen appear to be independent of the estrogen-receptor and may be mediated through the multi-drug resistance gene product (Behjati1 and Frank, 2009). Robinson et al. (1993) studied the effects of tamoxifen on immunity in patients with bilateral breast cancer who were in remission and had completed radiotherapy and chemotherapy at least one year prior to the study. They observed that the relative proportion and absolute number of $CD4^+$ lymphocytes was reduced in tamoxifen treated patients, compared to untreated breast cancer patients and to healthy controls. Moreover, *in vitro* proliferation of lymphocytes derived from tamoxifen treated patients was decreased. Since $CD4^+$cells have the main rule in immunity against *T. gondii* whether on their own or by promotion of $CD8^+$ cells as mentioned above, so theoretically, letting down $CD4^+$ cells number or activity - as recorded before for TAM - can impair host immunity against toxoplasmosis. A community where both

Table 2. Optical density (ODs) of anti-toxoplasma IgM ELISA titers in Tamoxifen treated rats as compared with control groups.

Result	Group									
	Uninfected control ODs	**Infected control ODs**	**1 week ODs**		**2 weeks ODs**		**3 weeks ODs**		**4 weeks ODs**	
		90 DPI	**97 DPI**	**7 DPT**	**104 DPI**	**14 DPT**	**111DPI**	**21 DPT**	**118DPI**	**28 DPT**
	0.179	0.678	0.623	0.535	0.524	0.363	0.346	0.416	0.246	0.638
	0.151	0.708	0.564	0.496	0.502	0.389	0.279	0.375	0.313	0.626
	0.209	0.734	0.617	0.521	0.539	0.417	0.352	0.513	0.258	0.574
				0.611	0.521	0.354	0.293	0.439		0.583
				0.515		0.403		0.507		0.562
				0.489		0.342		0.488		0.546
				0.541		0.406		0.528		0.713
Mean OD	0.179±0.029	0.706±0.028	0.601±0.032	0.529±0.04	0.521±0.015	0.382±0.028	0.317±0.036	0.466±0.057	0.272±0.035	0.606±0.057
P-value between uninfected control and other groups	-	0.0001* *t*=22.63	0.0001* *t*=16.77	0.0001* *t* = 13.36	0.0001* t=20.53	0.0001* *t*=10.11	0.003* t=5.31	0.0001* *t*=8.037	0.025 t = 3.48	0.0001* *t*=11.87
P-value between each treated and untreated groups at the same time point			0.027* t=2.68		0.0001* t=8.82		0.0012 t= 4.62		0.0001* t=9.11	

toxoplasmosis and breast cancer are commonly recorded was the targeted one by this research, the assumption here was that TAM can lead to reactivation of latent toxoplasmosis through its immune modulatory effect mentioned above, consequently lead to unrecognizable exacerbated *T. parasitosis* with its possible serious sequalae on patients who are already devastated by the primary oncogenic condition. In this study, parasitologically, it was observed that the average brain parasitic load (ABPL) in infected treated (IT) rat group after one week decreased but the difference was statistically non-significant (3.4%). Inversely, there was a statistically significant increase in (ABPL) in other (IT) rat groups treated for 14, 21 and 28 days by 11.4, 30.3 and 48.7.1%, respectively. In the same line were the results of serological study of anti-*Toxoplasma* IgM antibodies; there was a significant decrease in Anti *Toxoplasma* IgM optical density in (IT) rat groups treated for 7 and 14 days followed by a significant increase in (IT) rat groups treated for 21 and 28 days.Assessing Anti *Toxoplasma* IgG antibodies showed that they generally increased throughout the experimental period with no initial decrease as happened with ABPL & IgM titers, which may be explained by the fact that IgG antibodies persist for a longer period after the primary infection in the infected host than IgM,so the general rise of IgG titers as compared to IgM may be attributed to the persisting IgG antibodies with the primary infection plus those generated as a result of infection reactivation. The initial decrease in both ABPL and anti-*Toxoplasma* IgM titers could be explained by the lethal effect of TAM on *Toxoplasma* parasites observed earlier by Dittmar et al. (2016) who explained that by the fact that estrogen was previously shown to increase the numbers of *Toxoplasma* tissue cysts in the brains of parasite-infected mice. Since TAM is the best-haracterized antiestrogen inhibitor, it has anti *Toxoplasma* effects (Pung and Luster,

Table 3. Optical density (ODs) of Anti *Toxoplasma* IgG ELISA titers in Tamoxifen treated rats as compared with control groups.

Result	Group									
	Uninfected control ODs	**Infected control ODs**	**1 week ODs**		**2 weeks ODs**		**3 weeks ODs**		**4 weeks ODs**	
			97 DPI	**7 DPT**	**104 DPI**	**14 DPT**	**111 DPI**	**21 DPT**	**118 DPI**	28 DPT
	0.227	1.254	1.306	1.331	1.514	1.487	1. 577	1.612	1. 703	1.647
	0.154	1.312	1.342	1.396	1.472	1.582	1. 624	1.718	1. 682	1.749
	0.186	1.276	1.351	1.378	1.456	1.616	1. 587	1.631	1. 674	1.725
				1.365	1.383	1.553	1. 492	1.655		1.684
				1.414		1.644		1.587		1.745
				1.362		1.639		1.646		1.808
				1.363		**1.547**		**1.711**		1.783
Mean OD	0.189± 0.036	1.280± 0.029	1.333±0.0238	1.372± 0.0267	1.456± 0.054	1.581± 0.056	1.570± 0.055	1.651± 0.048	1.686± 0.014	1.734±0.055
P-value between uninfected control and other groups	-	0.0001* t=40.34	0.0001* t=45.38	0.0001* t=58.15	0.0001* t=34.4	0.0001* t=38.45	0.0001* t=36.88	0.0001* t=46.21	0.0001* t=65.59	0.0001* t=43.65
P-value between each treated and untreated groups at the same time point			**0.0579? t = 2.211**		**0.0062* t= 3.55**		**0.0315* t=2.52**		**0.188 t = 1.437**	

1986).Dittmar et al.(2016) also indicated that TAM inhibited *Toxoplasma* replication via a mechanism independent of its ability to antagonize estrogen receptor (ER) signaling even though they found that *Toxoplasma* activates ER-dependent transcription. In addition, they showed that tamoxifen reduced the overall number of parasite vacuoles and also induced the accumulation of LC3-green fluorescent protein (GFP) on the parasitophorous vacuole membrane (PVM). These data point to a mechanism by which tamoxifen kills *Toxoplasma* by inducing xenophagy. Xenophagy is now a well-recognized mechanism used by IFN-γ and $CD40^{+}$to control *Toxoplasma* replication (Choi et al., 2014; Andrade et al., 2006). However, with continuing TAM administration to rats for another two weeks, it was observed that ABPL, IgM and IgG titers were vividly increased denoting that TAM induced a concrete reactivation of latent toxoplasmosis in subjected rats as proven comparing their ABPLs, IgM, IgG titers with those belonging to the control groups. It was assumed that with continuation of TAM treatment, its immune modulatory effects mediated through shifting from TH1 to TH2 cells and decreasing CD4 numbers as reported previously (Rotstein et al., 1988; Robinson et al., 1993; Behjati1 and Frank, 2009), contradicted and predominated its anti toxoplasmic effects reported before *by* Dittmar et al. (2016) yielding a flare up of infection as estimated by both parasitological and serological parameters.This study concluded that TAM treatment in chronically infected mice with *T. gondii* protozoon parasite resulted in initial control of infection then flare up and exacerbation of infection.Thus more studies was recommended on wider scale and for longer periods for assessment of the cost/benefit and medical rationale of screening patients of breast cancer on adjuvant TAM treatment for *Toxoplasma* infection before the start of and during the course of treatment so as to detect early any incoming reactivation of chronic infection by observation of the rising titers of anti-*Toxoplasma I*gM and IgG to guard against the fatal risk and complication of

toxoplasmosis in such immunocompromised patients.

There was a significant decrease in anti-*Toxoplasma* IgG optical density in (IT) group treated for 7, 14 and 21 days, and there was rising in anti-Toxoplasma IgG optical density in groups treated for 28 days but with no significant difference between (IU) and (IT) groups. These results confirmed the previous findings of ABPL and anti-toxoplasma IgM titre (Dittmar et al., 2016). The initial decrease of ABPL, IgM and IgG titers, might be due to the anti-*Toxoplasma* effects of tamoxifen reported previously by Dittmar et al. (2016) who explained that by the fact that estrogen was previously shown to increase numbers of *Toxoplasma* tissue cysts in the brains of parasite-infected mice. Since TAM is the best-characterized antiestrogen inhibitor, it has anti *Toxoplasma* effects (Pung and Luster, 1986). Dittmar et al. (2016) also indicated that TAM inhibited *Toxoplasma* replication via a mechanism independent of its ability to antagonize estrogen receptor (ER) signaling even though they found that *Toxoplasma* activates ER-dependent transcription. In addition, they showed that tamoxifen reduced the overall number of parasite vacuoles and also induced the accumulation of LC3-green fluorescent protein (GFP) on the parasitophorous vacuole membrane (PVM). Together, these data point to a mechanism by which tamoxifen kills *Toxoplasma* by inducing xenophagy. Xenophagy is now a well-recognized mechanism used by IFN-γ and $CD40^{+}$ to control *Toxoplasma* replication (Choi et al., 2014; Andrade et al., 2006). However, with continuing TAM administration to rats for another two weeks, it was observed that ABPL, IgM and IgG titers were vividly increased denoting that TAM induced a concrete reactivation of latent toxoplasmosis in subjected rats as proven by comparing their ABPLs, IgM and IgG titers with those belonging to the control groups. It was assumed that with continuation of TAM treatment, its immune modulatory effects mediated through shifting from TH1 to TH2 cells and decreasing $CD4^{+}$ numbers as reported before (Rotstein et al., 1988; Robinson et al., 1993; Behjati1 and Frank, 2009), contradicted and predominated its anti toxoplasmic effects yielding a flare up of infection as estimated by both parasitological and serological parameters. This study concluded that TAM treatment in chronically infected mice with *T. gondii* protozoon parasite resulted in initial control of infection then flare up and exacerbation of infection. Thus it was recommended that screening of *Toxoplasma* infection in patients of breast cancer on adjuvant TAM treatment before the start of and during the course of treatment so as to early detect any incoming reactivation of chronic infection by rising titer of anti-*Toxoplasma I*gM and IgG to guard against the fatal risk and complication of toxoplasmosis in such immunocompromised patients. More studies on TAM and toxoplasmosis in humans should be done.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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