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Artesunate effect on RH virulent and ME49 non-virulent strains of *Toxoplasma gondii: in vitro* and *in vivo* experimental studies

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Abstract. Toxoplasmosis, most commonly treated by combination of pyrimethamine and sulfadiazine, this combination is associated with non-tolerable adverse reactions, thus, the development of new therapy is mandatory. Our aim was to evaluate the lethal effect of artesunate on RH and ME49 strains of *Toxoplasma gondii* in *vitro* and *in vivo* respectively. *In vitro* experiments were performed on VERO cells line infected with virulent RH strain of *T. gondii*; the tested artesunate concentrations were 0.1, 0.5, 1 and 2 μg/ml. The lowest percentage of viable tachyzoites was obtained by 0.1μg/ml of 16.67% at 48hours. Evidence of possible apoptotic effects was observed by TEM of treated tachyzoites, in the form of vacuolization of the cytoplasm, tachyzoite shrinkage and membrane alteration. *In vivo* the effect of artesunate on ME49 non-virulent strain was observed by significantly lowering brain cyst count in chronic toxoplasmosis mice (48%) and (41%) in reactivated toxoplasmosis mice as compared to corresponding control groups after eight days of artesunate administration. Artesunate treated toxoplasmosis reactivated group showed the highest mean of survival (19.3 days) in comparison to untreated toxoplasmosis reactivated control (4.5 days), the data obtained in the present study proposed that artesunate could be a useful substitute to antifolates in the treatment of toxoplasmosis.

Keywords: *Toxoplasma gondii;* Artesunate; Pyrimethamine and sulfadiazine; Virulent RH strain; ME49 non-virulent strain.

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Introduction

Toxoplasma gondii: is an apicomplexan parasite with worldwide distribution (Ferguson and Dubremetz, 2007). It is anticipated that up to one third of the human population are infected (Tenter et al., 2000).

The acute phase of toxoplasmosis produces mild symptoms in immunocompetent adults and is readily controlled by the immune response of the patient; they rarely experience acute symptoms beyond fever, malaise, and adenopathy (Petersen and Liesenfeld, 2008). The dormant stage of infection is described by

the presence of parasites within tissue cysts that form in the skeletal muscle and central nervous system (Montoya and Liesenfeld, 2004).

Individuals with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS), cancer chemotherapy patients or those with otherwise compromised immune systems can suffer from neurologic, ocular or widespread systemic toxoplasmosis (WHO, 2010), in immunocompromised patients and in those taking immunosuppressive therapy, revitalized and untreated toxoplasmosis has a high mortality rate (Jones et al., 2009).

The standard therapy for human toxoplasmosis is the synergistic combination of pyrimethamine and sulfadiazine, which inhibits the production of folic acid in the tachyzoites, although effective, this treatment is usually associated with many side effects, especially in AIDS patients, including hematological toxicity due to pyrimethamine and hypersensitivity due to sulfadiazine (Montoya and Liesenfeld, 2004).

Better approaches are needed for treating toxoplasmosis because no currently used medicine eradicates this slowly growing lifelong infection (Boyer and McLeod, 2002).

Artemisinin is extracted from *Artemisia annua*, an annual herb that has been used in folk Chinese medicine for more than 2,000 years (Van Agtmael et al., 1999). Artemisinin derivatives have been used to heal *Plasmodium* infections and have not been accompanied with any significant toxicity (Taylor and White, 2004).

Artemisinin compounds are effective *in vitro* against *Trypanosoma cruzi* and *Trypanosoma brucei* parasites (Mishina et al., 2007). Oral artemether has been known to possess activity against immature worms of *Schistosoma japonicum* and *Schistosoma mansoni* and has proved to be an efficient chemoprophylactic agent against both infections (Utzinger et al., 2000; Xiao et al., 2000).

In the last decade, the 1, 2, 4-trioxane artemisinin and artemisinin derivatives such as artemether were found to have the ability to inhibit *T. gondii* replication *in vitro* (Sarciron et al., 2000). Artesunate contains an endo-peroxide bridge. The peroxide moiety has been demonstrated to be responsible for the antimalarial activity of these compounds and presumably for antitoxoplasmal activity. Since these drugs cross the blood-brain barrier, they have been tested as a treatment for toxoplasmosis (Sarciron et al., 2000). Artesunate, an intravenous drug for the management of deteriorated cases malaria, has been used in malaria endemic areas. Clinical data proposes the drug is both safe and highly effective (Li et al., 2006). Thus the aim of the present work is to study the possible effect of artesunate at a concentrations of (0.1, 0.5,1 and 2 µg/ml) on *T. gondii* RH (type I) strain – *in vitro* and on ME49 (type II) strain at a dose of 10 mg/kg/day, on chronic and reactivated forms of toxoplasmosis – *in vivo*.

Materials and methods

In vitro study

The *in vitro* study was performed at the Tissue Culture Unit in Medical Research Center, Ain-Shams University.

Tachyzoites of the highly virulent RH strain of *T. gondii* (type I) were maintained in our laboratory by repeated intraperitoneal inoculation of Swiss albino mice every 3-4 days according to Wu et al. (2008). The mice were sacrificed after 3-4 days by ether inhalation. *Toxoplasma* tachyzoites were harvested under sterile condition. The peritoneal aspirate was washed three times with RPMI 1640 containing 1% penicillin and streptomycin. The tachyzoites were counted and adjusted by dilution in RPMI 1640 to 10^6 tachyzoites/ml (Suresh et al., 1991).

VERO cells were distributed in 24-well tissue culture plates and grown to confluence in a humidified incubator at 37°C with 5% CO_2. *T. gondii* tachyzoites were inoculated in a concentration of $5x10^5$ (Phelan, 2007), following inoculation by 3 hours, 50 µl of medium was aspirated and replaced by the same volume of medium containing the drugs at final concentrations of 0.1, 0.5,1 and 2 µg/ml

for artesunate and 0.5 µg/ml for Pyrimethamine and Sulfadiazine. drugs were added in three replicate wells for each concentration. The *T. gondii* tachyzoites inoculated VERO cells plates were then incubated in a humidified incubator at 37°C with 5% CO_2 and examined microscopically for cytopathic effect after 24, 48 and 72 hours. Parasite and drug free controls were included. Intracellular parasites were harvested by trypsinization of infected VERO cells, followed by repeated passages through a 25-gauge needle with proper mixing, in order to disperse parasite aggregates and liberate intracellular tachyzoites (Leepin et al., 2008). At 24, 48 and 72 hours intervals, after repeated passage, a drop of the Trypan blue was added to a drop of the tachyzoites on the hemocytometer and left for one minute to allow penetration of the dye. Viable and non-viable tachyzoites were counted using a hemocytometer, the count was performed in triplicates, non-viable tachyzoites were stained and the viable tachyzoites were not stained.

Transmission Electron Microscopy (TEM)

TEM was performed according to Stadtländer (2007) to evaluate the *in vitro* effect of artesunate on the morphology of *T. gondii* tachyzoites in cell culture. Briefly for ultrastructural analyses, VERO cells were placed in culture flasks 75 cm^2 and infected with parasites as described before. Drug was added to the culture flasks at concentrations of 0.1, 0.5, 1 and 2 µg/ml artesunate. Transmission electron microscopy was performed after 24 hours interval, the culture samples were fixed in 2.5% glutaraldehyde examined with a JEOL–JEM-1010 transmission electron microscopy.

In vivo study

The *in vivo* study was performed at the Diagnostic and Research Laboratories, Parasitology Department, Faculty of Medicine, Ain-Shams University and Animal house in National Research Center, Cairo, Egypt.

The ME49 strain of *T. gondii* was maintained in our laboratory by repeated inoculation of Swiss albino mouse (Djurković-Djaković et al., 2002). Female Swiss albino mice 8 to 10 weeks old and of average weight of 25 to 30 grams were offered drinking water and regular mouse feed *ad libitum*.

Mice were sacrificed 8 weeks post infection by ether inhalation. Brain tissue was removed, suspended in 2 ml of 0.9% NaCl and grounded with a mortar and pestle. Further homogenization by passage through a needle and syringe. The concentration of cysts was adjusted to a final concentration of 20 cysts/0.2 ml/mouse (Sarciron et al., 2000). All the studied groups were infected with brain cysts (concentration of 20 cysts/0.2 ml/mouse) using nasogastric feeding tube. Chronic toxoplasmosis infected mice were divided into 6 groups of 12 mice each, as follows:

- group 1 chronic toxoplasmosis artesunate treated; group 2 reactivated toxoplasmosis artesunate treated; group 3 chronic toxoplasmosis Pyrimethamine/ Sulfadiazine treated; group 4 reactivated toxoplasmosis Pyrimethamine/ Sulfadiazine treated; group 5 chronic toxoplasmosis control; group 6 reactivated toxoplasmosis control.

Artesunate (Sigma-Aldrich, Germany) was administrated intraperitoneally at a dose of 10 mg/kg/day for 8 days to groups 1 and 2.

Pyrimethamine (Sigma-Aldrich, Germany) (25 mg/kg/day) and sulfadiazine (Sigma-Aldrich, Germany) (100 mg/kg/day) were administrated in combination by nasogastric feeding tube for 3 days to groups 3 and 4.

For induction of reactivated toxoplasmosis, each chronically infected mouse of groups 2, 4 and 6 received endoxane (Baxter oncology, Germany) dosage of 1mg/kg/day intramuscularly for 13 days starting 2 months post infection (Nishida and Mine, 1985), followed by administration of aresunate and Pyrimethamine/sulfadiazine for groups 2 and for respectively, at the end of the drugs course, 6 mice/group were monitored daily and deaths were recorded, and the remaining 6 mice/group were sacrificed and examined by counting brain cysts burden, by bright field microscopy, cysts were counted from 4

separate 25 µl aliquots of brain homogenate. The number of cysts per brain was calculated by multiplying the counted number by 20, counting was done in duplicate.

Infectivity of recovered cysts from brain of treated mice was tested by oral administration of brain homogenates to fresh mice. Mice were sacrificed after 8 weeks and brain tissue examined for the presence of brain cysts (Djurković-Djaković et al., 2002).

Ethical consideration all animal studies presented here has been approved by the local government (IRB 00006379) based on national regulations for animal experimentation.

Statistical analysis

SPSS software package version 19.0 was used for data analysis. Quantitative data was expressed using mean and standard deviation. Differences were considered significant if P values were equal to or less than 0.05 and highly significant when the P value was <0.001, by student "t" test.

Results

The results of the present study are shown in tables 1 and 2 and figures 1 and 2.

In vitro study

The percentage of viable tachyzoites of 0.1 µg/ml artesunate, after 24 hours was 41.67%. That decreased after 48 hours to 16.67% which was statistically significant ($p<0.05$), then the percentage of viable tachyzoite rise to 25% after 72 hours.

Table 1. Effect of artesunate and pyrimethamine/sulfadiazine on the viability of *Toxoplasma gondii* tachyzoites cultured on VERO cells

	24 hours (Mean ± SD)			48 hours (Mean ± SD)			72 hours (Mean ± SD)		
	V.T.	N.V.T.	V.P.	V.T.	N.V.T.	V.P.	V.T.	N.V.T.	V.P.
Artesunate 0.1 µg/ml	$5x\ 10^4$ ± 1.732	$7 x\ 10^4$ ±2.000	41.67%	$1x\ 10^4$± 0.000	$5x\ 10^4$ *±1.000	16.67%	$1x\ 10^4$± 0.000	$3x\ 10^4$ ±1.000	25%
Artesunate 0.5 µg/ml	$21 x\ 10^4$ *±3.606	$9x\ 10^4$ ±1.000	70%	$10 x\ 10^4$ ±2.000	$10 x\ 10^4$ ± 3.606	50%	$2 x\ 10^4$ ±1.000	$1x\ 10^4$ ± 0.000	66.67%
Artesunate 1 µg/ml	$5x\ 10^4$ *±1.000	$2x\ 10^4$ ± 0.000	71.43%	$3x\ 10^4$± 1.000	$2x\ 10^4$ ± 0.000	60%	$1x\ 10^4$± 0.000	$2x\ 10^4$ ± 1.000	33.33%
Artesunate 2 µg/ml	$5x\ 10^4$ ±1.000	$2 x\ 10^4$ ± 1.000	71.43%	$4x\ 10^4$± 1.000	$3 x\ 10^4$ ± 1.000	57%	$3x\ 10^4$± 1.000	$4 x\ 10^4$ ± 1.000	42.86%
pyrimethamine/ sulfadiazine 0.5 µg/ml	$12 x\ 10^4$* ± 2.000	$2x\ 10^4$ ± 1.000	85.7%	$6x\ 10^4$*± 1.732	$3x\ 10^4$ ± 1.000	66.67%	$2 x\ 10^4$± 1.000	$4x\ 10^4$ ± 1.000	33.33
Parasite control	$173.3 x\ 10^4$* ±25.1	$2 x\ 10^4$ ±1.000	98.8%	$448.3 x\ 10^4$* ±50.08	$4.3 x\ 10^4$ ±3.2	99.05%	$1016.6 x\ 10^4$* ±76.37	$17.3 x\ 10^4$ ±2.5	98.32%

* Significant difference between viable and non-viable tachyzoites count;
SD: standard deviation; VT: viable tachyzoites; NVT: non-viable tachyzoites; VP: viability percentage.

Table 2. Mean *Toxoplasma* cysts count in artesunate treated groups compared to pyrimethamine/sulfadiazine and untreated control groups

Groups	Chronic infection (Mean ± SD)	Reactivated infection (Mean ± SD)
Artesunate	1580* ± 311.126	2080** ± 452.548
Pyrimethamine/Sulfadiazine	2100* ± 424.264	2820 ± 424.264
Control	2920 ± 395.979	3600 ± 226.274

* Significant difference as compared with G5 (P ≤0. 05); ** Significant difference as compared with G4 (P ≤0. 05);
SD: standard deviation.

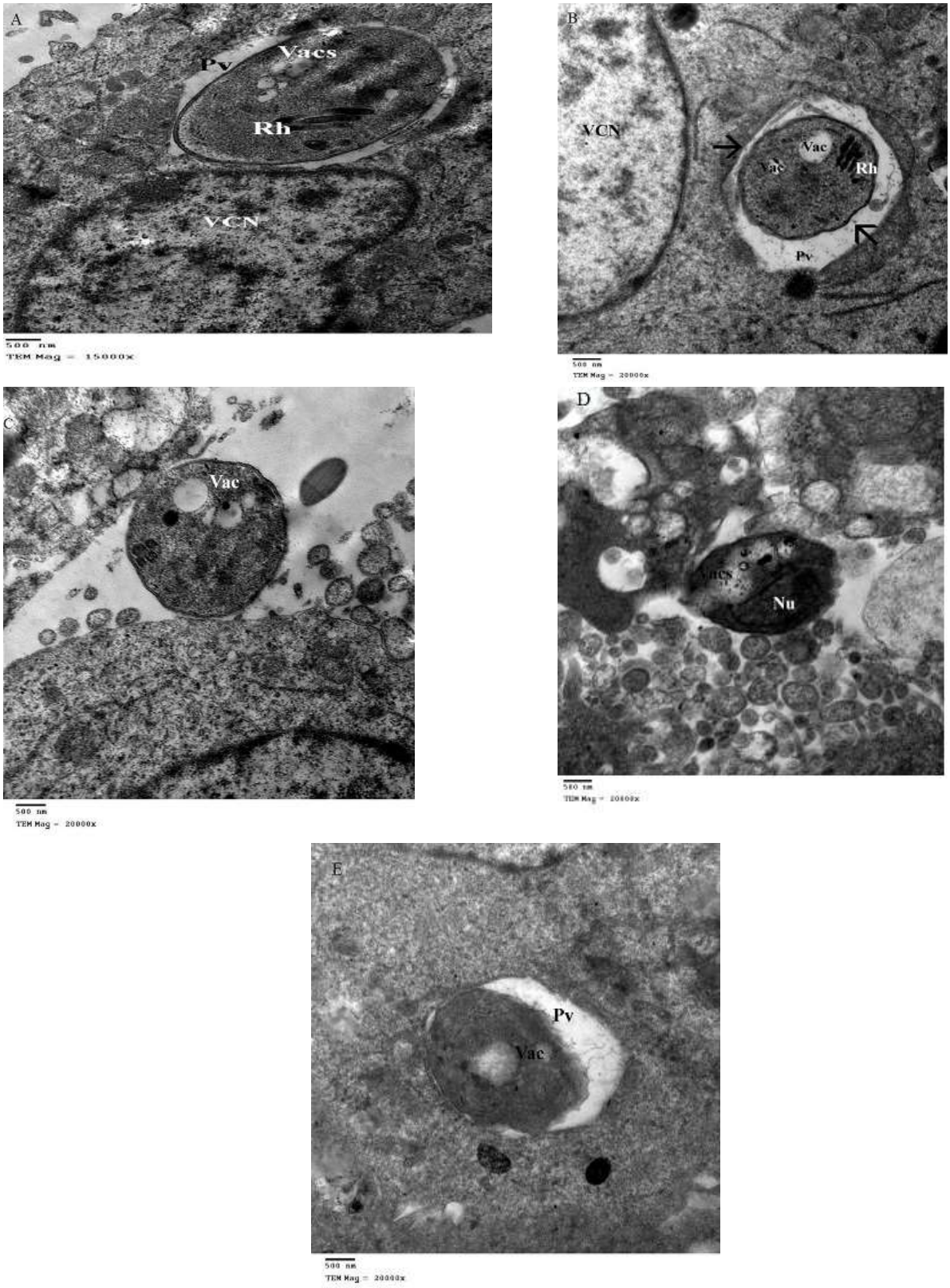

Figure 1. TEM of *T. gondii* tachyzoite treated with different doses of artesunate, **(A)** 0.1 µg/ml artesunate showing multiple vacuoles (Vacs), (Pv) parasitophorous vacuole, (Rh) rhoptry, (VCN) VERO cell nucleus (Bars = 500 nm, x15000), **(B)** 0.5 µg/ml artesunate showing vacuoles (vac), with alterations of the parasite membrane (arrow). (PV) parasitophorous vacuole, (VCN) VERO cell nucleus (Bars= 500 nm, x20000), **(C)** 1 µg/ml artesunate showed multiple vacuoles (Vac) (Bars = 500 nm, x20000), **(D)** 2 µg/ml artesunate showing multiple vacuoles (Vacs) and alterations of the parasite membrane, nucleus (Nu) (Bars = 500 nm, x20000), **(E)** 0.1 µg/ml artesunate showing large vacuoles (Vac) with shrinkage and alterations of the parasite membrane, (PV) parasitophorous vacuole (Bars = 500 nm, x20000).

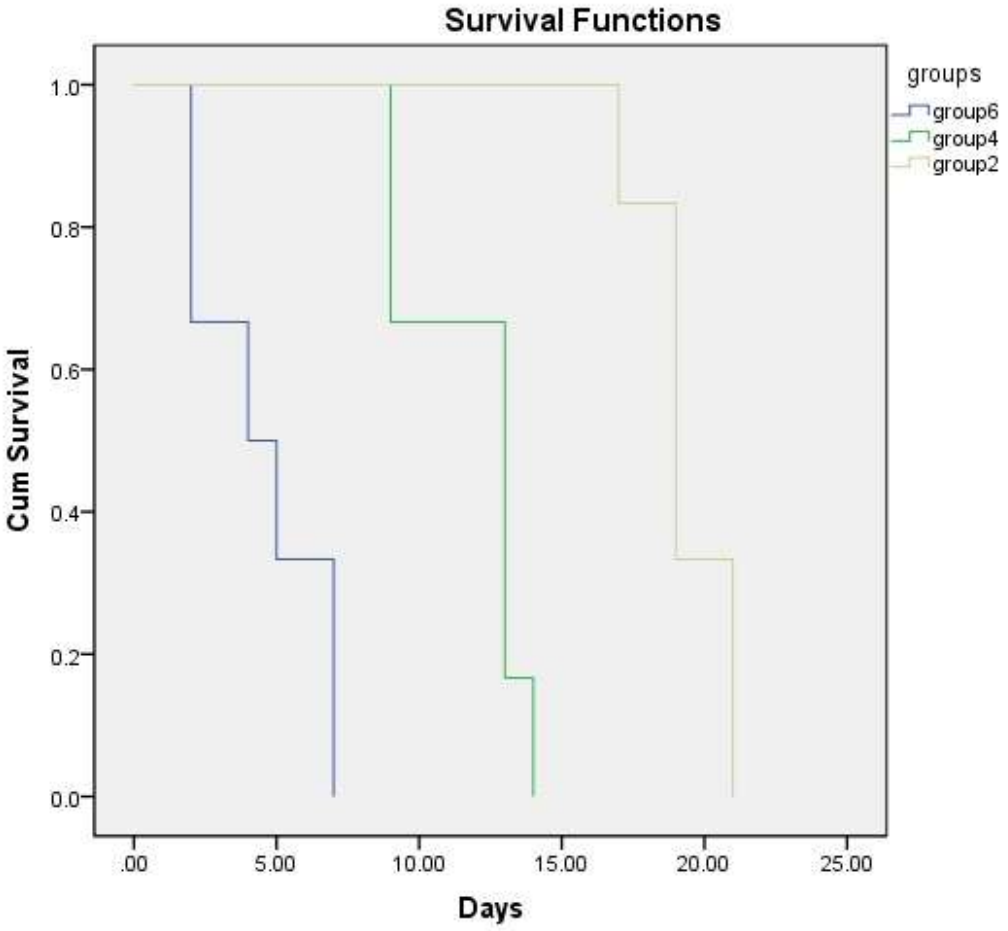

Figure 2. Kaplan-Meier curve for survival in mice of group 2, group 4 and group 6.

At a concentration of 0.5 µg/ml artesunate, after 24 hours the percentage of viable tachyzoite was 70%, then the percentage declined to 50% after 48 hours, after 72 hours the percentage was to 66.67%, no significant difference between viable and non-viable tachyzoite count. At a concentration of 1 µg/ml artesunate, after 24 hours the percentage of viable tachyzoite was 71.43%, decreased to 60% after 48 hours then to 33.33% after 72 hours, this showed insignificant difference between viable and nonviable tachyzoites. At a concentration of 2 µg/ml artesunate, after 24 hours, the percentage of viable tachyzoite was 71.43%, after 48 hours the percentage decreased to 57%, the percentage decreased to 42.86% after 72 hours, no significant difference was found between viable and non-viable tachyzoites count. Pyrimethamine and sulfadiazine 0.5 µg/ml, after 24 hours the percentage of viable tachyzoite was 85.7%. The difference between viable and nonviable tachyzoites was statistically significant ($p<0.05$), decreased to 66.67% after 48 hours, and declined to 33.33% after 72 hours.

TEM

TEM result showed alterations in the ultrastructure of tachyzoites treated with all used artesunate concentrations (figure 1.A-E). The main alteration observed in this study was the vacuolization of the parasite's cytoplasm with all used concentrations (figure 1.A-E) after 24 hours of treatment, as well as, shrinkage of the parasite with the concentrations of 0.5 and 0.1 µg/ml (figure 1.B, E) and membrane alteration was noted with the concentration of 0.5, 2 and 0.1µg/ml (figure 1.B, D, E).

In vivo study

Along the study course no death was reported among the immunocompetent groups, although, among the immunocompromised groups, group 2 showed the highest mean of survival rate 19.3 days, with high significant difference from both of group 4 (11.8 days) and group 6 (4.5 days).

Concerning the mean *Toxoplasma* cysts count and percent of reduction in chronic mice groups, group1 (artesunate treated group), showed the lowest mean cysts count (1580 ± 311.126) which was significantly lower than that of group5(control group), followed by group 3 (Pyrimethamine and Sulfadiazine treated group) (2100 ± 424.264) which was significantly lower than that of group 5 (control group). The reduction percentage in group 1 was of 24.7% when compared to group 3 and 45.89% when compared to group 5.

Concerning the mean *Toxoplasma* cysts count and percent of reduction in reactivated mice groups, group 2 (artesunate treated group), showed the lowest mean cysts count (2080 ± 452.548), which was significantly lower than that of group 4 (Pyrimethamine and Sulfadiazine treated group) (2820 ± 424.264), with reduction percentage in group 1 of 26.24% when compared to group 4 and 42.2% when compared to group 6. Cysts recovered from brains of group 2, was found to be infective to fresh mice.

Discussion

The treatment of toxoplasmosis relay on combination of pyrimethamine and sulfadiazine, co-application folic acid is also required in order to minimize the toxic effects of pyrimethamine in patients (Martins-Duarte et al., 2013). Adverse effects are more pronounced in AIDS patients, including hematological toxicity due to pyrimethamine and hypersensitivity to sulfadiazine (Meneceur et al., 2008). Side effects in most cases lead to the interruption of treatment and cause a relapse of the toxoplasmosis (Katlama et al., 1996). Moreover, the available drugs nowadays have no effect on tissue cysts, leading to the possibility of relapse after treatment cessation. Thus, the search for new drugs to treat toxoplasmosis is important (Martins-Duarte et al., 2010).

Artemisinin, derived from the Chinese herb *Artemisia annua L*. is a sesquiterpene lactone that possesses a 1, 2, 4 trioxane moiety. The artemisinin class of drugs has shown promising antiparasitic effects *in vitro* against *Plasmodium*, *Leishmania, Schistosoma, Trypanosoma* and *T. gondii* (Hencken et al., 2009), as well as aginst mixed coccidiosis in chicken (Pop et al., 2015).

In the present study, the effectiveness of artesunate for the treatment of toxoplasmosis *in vitro* and *in vivo* was evaluated and compared to that of pyrimethamine and sulfadiazine combination. Notable reduction in the percentages of viable tachyzoites were observed with all doses of artesunate among the duration of the study, the lowest percentage of viable tachyzoites (16.67%) was observed with 0.1 µg/ml artesunate at 48 hours with significantly lower difference from non-viable tachyzoites. in contrast to Sarciron et al. (2000) who observed maximum inhibition by artesunate at 24 hours followed by subsequent decrease in inhibition at 96 hours. Pyrimethamine and sulfonamide gave significantly higher viable tachyzoites count at 24 and 48 hours, and the highest percentage (85.7%) of viable tachyzoites at 24 hours, going in accordance with the results of Gomes et al. (2012) who reported that the efficacy of artesunate on *Toxoplasma* tachyzoites was highest among the studied compounds, followed by pyrimethamine. Sarciron et al. (2000) reported 60% viability with the concentration of 0.5 µg/ml artesunate after 96 hours, almost comparable to the results of our study where the concentration of 0.5 µg/ml artesunate gave viability percentage of (70%, 50%, 66.67% at 24, 48, 72 hours respectively), although the viability with pyrimethamine and sulfadiazine did not decline to 20% as reported by Sarciron et al. (2000). Difference in percentage of inhibition from our study could be attributed to longer study duration, possibly different strain of *Toxoplasma* and different cell line used. It is noteworthy that there was a decrease in the total tachyzoites count (viable and nonviable) in all studied drugs

concentrations along the duration of the study and reversal of the count from higher viable tachyzoites count at 24 hours to higher non-viable tachyzoites count at 72 hours.

Effective action of artesunate against tachyzoites was observed in several studies (El Zawawy, 2008; D'Angelo et al., 2009), who studied the *in vitro* action of artesunate against RH strain of *T. gondii* and observed a significant reduction in the viability of tachyzoites exposed to drugs compared with a non-treated control. Other components of artemesia, like artemisinin and artemether were reported to inhibit plaque formation by *T. gondii* in tissue culture (Ou-Yang et al., 1990; Jones-Brando et al., 2006).

In order to demonstrate the possible apoptotic effects of artesunate on *T. gondii* tachyzoites, electron microscopy was performed. In the present study TEM demonstrated comparable alterations in the ultrastructure of tachyzoites treated with different concentration of artesunate. Vacuolization, cell shrinkage and membrane alteration characterize the apoptotic changes in protozoan parasites (Jiménez-Ruiz et al., 2010). The main alteration observed in this study was the vacuolization of the parasite's cytoplasm after 24 hour of treatment. The vacuolization process was an outcome of the arrest of the cell cycle in the parasites (Carvalho and De Melo, 2010). As well as shrinkage of the parasite and membrane alteration was noted.

In the present study, the investigated activity of artesunate against ME49 strain of *T. gondii,* showed significantly lower mean in brain cyst count was found in artesunate treated chronic group as compared to corresponding control group with reduction in cysts count by 45.89%, as well as reduction in cysts count by 24.7% when compared to pyrimethamine/ sulfadiazine treated chronic group. Moreover, significantly lower mean of brain cysts count was found between reactivated toxoplasmosis artesunate treated group and reactivated toxoplasmosis pyrimethamine/sulfadiazine treated group, with reduction percentage of cysts count by 26.24% These results are going in accordance with that of Sarciron et al. (2000), who reported that the reduction in mean number of *T. gondii* cysts was 59% when comparing mice treated by artesunate to control, using low virulent DUR strain of *T. gondii*.

No mice died along the course of treatment in the chronic groups, the mean survival for group 2 was 19.3 days, significantly higher than the mean survival of group 4 (11.8 days) and group 6 (4.5 days). These results are going in accordance with the results of Dunay et al. (2009), who tested artemiside and artemisone using *in vivo* models for acute, chronic and reactivated toxoplasmosis and reported prolongation in the survival rate of treated mice as compared to control group. Other studies reported more protection in infected mice when treated with artemisinin derivatives as artemether when compared to roxithromycin (Chang and Pechére, 1988; Brun-Pascaud et al., 1996).

In this study total eradication of brain cysts was not achieved by either artesunate or pyremethamine/sulfadiazine, Similarly, Dunay et al. (2004) reported that atovaquone does not completely eradicate bradyzoites although was capable to controls replication of tachyzoites, that might be attributed to the lack of efficiency against bradyzoites within tissue cyst.

In the present work, the recovered cysts from brain of artesunate and pyremethamine/ sulfadiazine treated mice was tested, and found to be infective to fresh mice. Although existing therapy is available for toxoplasmosis, it suffers from problems of intolerance to sulfa drugs combined with a necessity for continuing maintenance, due to a lack of effectiveness against bradyzoites within tissue cysts (McCabe, 2001). Artemisinin derivatives do not directly address this deficiency; however they may offer some potential for covering the range of therapies available for toxoplasmosis. The competence to control tachyzoite replication may have value for treatment of either primary acute toxoplasmosis or reactivation of chronic infection (Jones et al., 2006).

The efficacy of artesunate against *T. gondii* virulent and non-virulent was demonstrated *in vitro* and *in vivo*. A small dose of artesunate for

a short period of time induced reduction in the *T. gondii* tachyzoite viability and also affected its ultrastructure; the difference in the lethal potential of each used dose highlighted a guide for the best concentration. Also the anti-*T.gondii* activity of artesunate in murine infection, in terms of significantly reduced brain cyst burdens as compared to control as well as prolongation of mice survival rate. The data obtained in the present study propose that artesunate could be a useful substitute to antifolates in the treatment of toxoplasmosis.

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