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Original Article

Treatment of Murine Toxoplasmosis with Oral and Parenteral Artemether and Following by Detection of *B1* Gene by Quantitative Real-Time PCR (qPCR) for Evaluating Parasite Density

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Received 16 Apr 2021
Accepted 11 Jul 2021

Keywords:
Toxoplasma gondii;
Artemether;
Oral;
Parenteral;
BALB/c mice;
Polymerase chain reaction

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Abstract

Background: *Toxoplasma gondii* is an obligate intracellular parasite that can infect humans and animals. As the choice drug have shown side effects, development a new drug with low toxicity will be necessary.

Methods: BALB/c mice were infected with tachyzoiets of *T. gondii*. After treatment by oral and parenteral artemether (250 µg/mice) and sulfadiazine (50 µg/mice), we evaluated the rates of survival in treated and control mice. The fold change of B1 gene (target gene) expression in liver and brain of mice treated with parenteral artemether (i.p.), oral artemether (via gavage) and sulfadiazine, were detected by using the Real-Time quantitative PCR.

Results: Both treatment with sulfadiazine and artemether showed significant prolongation in time to death of the infected mice compared to the control group. Median survival days for parenteral artemether, oral artemether, sulfadiazine and control group were 8, 11, 12 and 6 d respectively. Expression of B1 gene in liver and brain of mice after treatment with artemether and sulfadiazine were reduced in comparison to housekeeping gene (β-tubulin gene). The fold change (comparing to control group) for parenteral artemether, oral artemether, sulfadiazine is 0.034, 0.027 and 0.111 for liver and 0.220, 0.425 and 0.366 for brain respectively.

Conclusion: Artemether is effective to control the tachyzoites of *T. godii* in vivo conditions and oral treatment is more effective than parenteral treatment. Due to its low cytotoxicity and its high effective action against the tachyzoietes of *T. godii* in susceptible animals.

Introduction

Toxoplasma gondii is an obligate intracellular Apicomplexan in warm-blooded animals and humans that can infect approximately one-third of the whole human population (1). Acute infection in intermediate hosts happens by consuming food and drink contaminated to oocysts. Hence, tachyzoites rapidly grow and switch to bradyzoites form tissue cysts in host cells (2). Infection with *T. gondii* in immunocompetent human causes nonspecific symptoms or, are asymptomatic, but the infection in immunocompromised patients and foetus acquired during pregnancy can be lead to serious problems such as mental retardation, miscarriage, neurologic damage and retinochoroiditis (3,4).

The gold-standard treatment for toxoplasmosis are the combination of pyrimethamine and sulfadiazine, but both of them cannot fully eliminate the parasites in the cysts. These medicines have high hematologic toxicity and other side effects that often are not well tolerated by patients (5,6). Therefore, research on compounds with less-side effects and removing all cycle stages of the parasites in human is essential.

The Qing Hao (Artemisia annua L.) and its derivatives have shown the effectiveness of antimalarial (7). These compounds have a low toxicity for the patients and act fast that it is an advantage for them (8). Artemether is a derivatives of artemisinin that in our study and other research have been shown strong effects against *T. gondii* in vitro conditions (9,10).

Diverse studies in the recent years have shown that real-time PCR is an improved and accelerate assays for diagnosis of *T. gondii* from tissue biopsies, fluid and other samples of patients' body (11-13). Most, the 35-fold-repeated B1 gene was used for identifying of *Toxoplasma gondii* by real-time PCR. This gene is conserved in genome of different parasite strain and it has special sensitivity and specificity for molecular targets (11, 12).

In the previous paper, we examined artemether and sulfadiazine effects on the *T. gondii* tachyzoites in vitro term (14). We aimed to evaluate the comparison effect of artemether and in comparison with sulfadizine on the parasite in vivo conditions and detecting amount of live toxoplasma tachyzoites in the liver and brain tissue of infected mice after treatment by quantitative real-time PCR detection method.

Materials and Methods

Ethical Statement

The Ethical Committee of Tarbiat Modares University, Faculty of Medical Sciences approved this experiment with permit number: D52/3525.

Parasite

We used the tachyzoites of the virulent Rh strain of *T. gondii* maintained in Parasitology Department. Parasites were suspended in RPMI-1640 (Gibco) medium and the number of viable tachyzoites was determined by Trypan blue exclusion in hemocytometric chamber (15). The tachyzoites were used for in vivo experiments.

Effect of drugs on T .gondii infection in vivo

Initially, 40 female BALB/c mice (6-8 wk and 25 g) were infected intraperitoneally (i.p) with 10^3 tachyzoites of *T. gondii* RH strain (that obtained from the Parasitology Department of Tarbiat Modares University) and distributed into four groups with 10 animals (16,17). Three groups were treated 4 h after infection, during 10 d at regular 12-h intervals as follows (according to the results of in vitro tests) (14): (I) 250µg/mice/day of artemether orally via gavage; (II) 250µg/mice/day of artemether intraperitoneally (i.p.); (III)

50µg/mice/day of sulfadiazine orally via gavage; and (IV) artemether solvent (200 µl, gavage) as control group.

At the end of treatment period, three mice of each group were killed and peritoneal fluid was collected for detection of *T. gondii*. The tissues (liver and brain) of mice were collected separately for identity of *T. gondii* by Quantitative Real-Time PCR and expression amount of B1 gene. Daily mortality rates were examined for other mice.

Real-time PCR for detection of T. gondii RNA in tissues

DNA Extraction

Tachyzoites were harvested from the peritoneal in PBS. The tachyzoites number were determined using a counting chamber (Neubaur) and washed twice, with centrifugation at 3000 rpm for 10 min. The parasite pellets were used for DNA extraction. For preparation of positive control DNA, extraction of toxoplasma DNA was performed by DynaBio™ blood/Tissue DNA Extraction Mini Kit following the instruction of the manufacturer and concentration of purified DNA determined by UV spectrophotometry (13).

B1 gene target

The primer pairs used for RT- PCR were forward primer:5'-ACCCGGACCGTTTAGCAG-3', (GenBank accession no.AF 17981.1) was located at position 1841-1824 (sense), and Reverse primer, 5'-GGACTGGCAACCTGGTGTC-3', was located at position 1726-1744 (antisense) which amplified a 116bp sequence from a specific repetitive region of the B1gene (13).

β-tubulin gene (Houskeeping gene)

Primer pairs were designed to amplify a 129bp sequence of *T.gondii* β-tubulin gene as housekeeping gene for RT-PCR. Forward primer: 5'-CCAAGTTCTTCCGTCATTTC-3' was located at position 698 (sense) (Genbank accession no.M20025). The Reverse primer: 5'-CCTCATTGTAGAACACATTGAT-3' was located at position 806 (antisense) (18).

Amplification DNA procedures to set the Real-time PCR annealing temperature

PCR amplification for B1 and β-tubulin genes of *T. gondii* were performed with 25µl total reaction volume containing the following: 12.5 µl of 0.08 unit/µl Taq DNA polymerase in reaction buffer, 3Mm $MgCl_2$, 0.4 Mm each dATP, dGTP, dCTP and dTTP (CinnaGen PCR Master mix (2X) CAT.NO.:PR8252C), 5µl the extracted template DNA, 1µl Forward B1 or β-tubulin genes primer 20pmol, 1µl Reverse B1 or β-tubulin genes primer 20pmol of *T. gondii* respectively. The thermal cycling was the following conditions: initial denaturation of DNA at 95 °C for 5 min, 40 cycles run, as follows: 95 °C for 30 sec, annealing at 55 °C for 30 sec, and 72 °C for 1 min. The final extension step continued for 10 min at 72 °C. The annealing temperature obtained in real-time PCR thermocycler was used (13, 18).

RNA extraction and reverse transcription

To investigate the therapeutic effects artemether and sulfadiazine on *T. gondii* tachyzoites in various organs, brain and liver tissues of treated mice in all four groups were isolated under sterile condition. The tachyzoites grown and maintained in mice were used as controls. Then the total RNA extraction was performed by Guanidine/Phenol solution (RNX-Plus Solution (Cat. No.: RN7713C) following the instruction of the manufacturer. Absorbance of the samples was measured at 260/280. Total RNA was poured into RNase and DNase-free microtubes and immediately reverse transcription took to cDNA synthesize kit (Qiagen). In summary, 5 µl of Template RNA, 1 µl of Primer (Oligo (dt) 16), 4 µl of DEPC-treated water in the total volume of 10 µl of was incubated at 65 °C for 5 minutes. Then 10 µl of RT primer (2x) was added to mixture and incubated 10 min at 25 °C and 60 min at

50 °C. Finally, reaction was inactivated by heating at 70 °C for 10 min (19).

Real-time PCR: SYBR green detection

Real-Time PCR was performed using light cycler DNA master SYBR Green I kit (Roche) in a final volume of 20 µl in triplicate to detection of fluorescence on LightCycler system (Roche). For the test, each reaction mix contained 4 µl of SYBR Green PCR Master Mix, 1 µl of each primer 20 pmol/µl, 5 µl of cDNA and 9 µl of sterile distilled water. The Capillaries tube of reaction was into the system. The cycling was carried out with 10-min initial denaturation at 95 °C, 45 cycles of amplification at 95 °C for 10 sec, 55 °C for 10 sec and 72 °C for 25 sec, a final extension Continued for 1 min at 70 °C. Gene expression analysis for Real-Time PCR data was conducted using REST (Relative Expression Software Tool) 2009 software 2.0.13 (Qiagen) (20).

Statistical analysis

The log-rank (Mantel-Cox) test was utilized to compare the survival rates and curves of the studied groups. All graphs were plotted using GraphPad prism (version 5.04). Values of $P<0.05$ were considered significant the statistics. Data were analyzed using the $2^{-\Delta\Delta C_T}$ method for quantitative real-time PCR assay.

Results

Effect of artemether on T. gondii infection in vivo

According to the in vitro experiments, artemether and sulfadiazine dose administration for treatment of mice were determined 10µg/gr/day and 2µg/gr/day respectively. Untreated mice infected with the RH strain of *T. gondii* (as a control group) all died by 7 d after infection (median survival of 6 d) ($P=.56$). Comparing the treatments with artemether (orally) in dose of 250 µg/mice/day and sulfadiazine in dose of 50 µg/mice/day indicated similar results (Fig. 1A). For mice treated with artemether (orally) Survival rate of 31.25% and median survival of 11 d were obtained ($P=0.0013$) and for the group treated with sulfadiazine Survival rate of 38% and median survival of 12 days found out ($P=0.0026$). Both treatment with sulfadiazine and artemether (orally) showed significant prolongation in time to death of the infected mice compared to the control group. Moreover, according to the control group, the treatment with artemether by i.p. injection in dose of 250 µg/mice/day represented a prolongation in time of death (Survival rate of 28.5% and median survival of 8 days) ($P=0.0113$). However, compared with treatment of sulfadiazine and artemether by orally administration, therapeutic effect of artemether injection significantly was less (Fig. 1A).

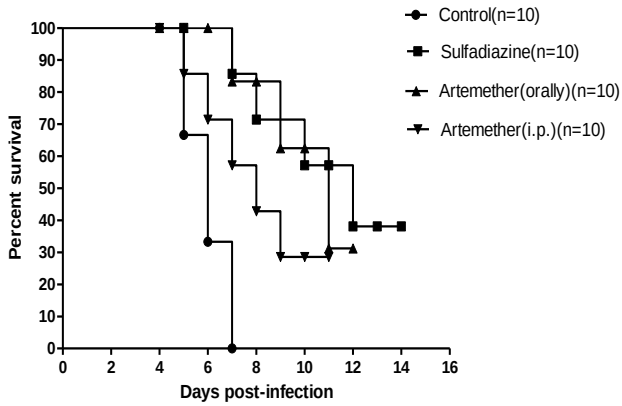


Fig. 1: A: Survival curve results for RH *T.gondii* – infected mice after treatment with artemether and sulfadiazine administration in vivo at 250 µg/mice/day and 50 µg/mice/day concentration , respectively (n=10 for every group). Log-rank (Mantel-Cox) test was used for comparison of survival curve

Clinical symptoms were investigated including hunching, reluctance to move, tousle, head move up and down, decreased appetite and body weight loss. All of symptoms as explained above were seen for the control group and animals treated with sulfadiazine and artemether orally via gavage but mice treated with artemether by intraperitoneal injection

did not show any clinical signs listed to the end experiments (data not shown). Early clinical signs were observed in the control group and all the symptoms were occurred with greater intensity. Evaluation of body weight for all groups during the first 10 d of treatment revealed that the animals treated with sulfadiazine and artemether edible and untreated group significantly decreased. The differences of mice weight 10 d after treatment for all treated group in comparison to control group were significantly reduced (P<0.05). However, in the group treated with i.p. injection artemether little change in the weight of the animals was observed during the treatment period. The stress caused at the time of drug injection in mice reduced the body weight of them (Fig. 1B).

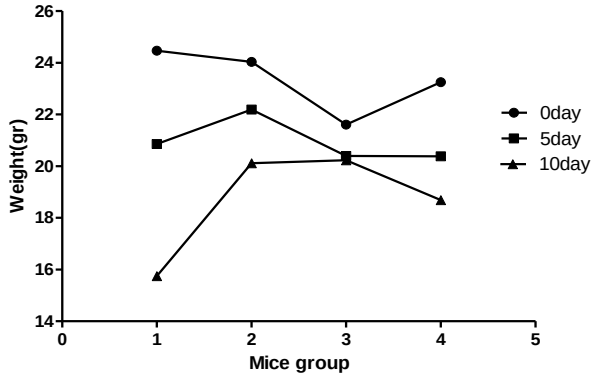


Fig. 1: B: Average weight of mice infected with toxoplasma before and after treatment with sulfadiazine and artemether**. 1.** Untreated group (control) (n=10). **2.** Group treated with artemether orally via gavage(n=10). **3.** Group treated with artemether by intraperitoneal injection(n=10). **4.** group treated with sulfadiazine orally via gavage (n=10). (Mean ± SD). The differences of mice weight 10 days after treatment for all treated group in comparison to control group were significantly reduced (P<0.05)

Real-time PCR for detection of T.gondii RNA in tissues

To evaluate the effects of drugs (artemether and sulfadiazine) in preventing the invasion of toxoplasma gondii tachyzoites in tissues after treatment, surviving mice in the treatment group were killed by dreaking the neck, then brain and liver tissues of mice treatment isolated and RNA of them extracted (Fig. 2). Invasion amounts of tachyzoites were detected based on change in the B1 gene expression (target gene) relative to reference gene by Real Time quantitative PCR (Fig. 3A). A 129 bp β-tubulin gene of *T. gondii* was selected as an internal control (IC) (Fig. 3B). In other to calculation the PCR efficiencies were used from the standard curves by serial cDNA dilutions (Fig. 4).

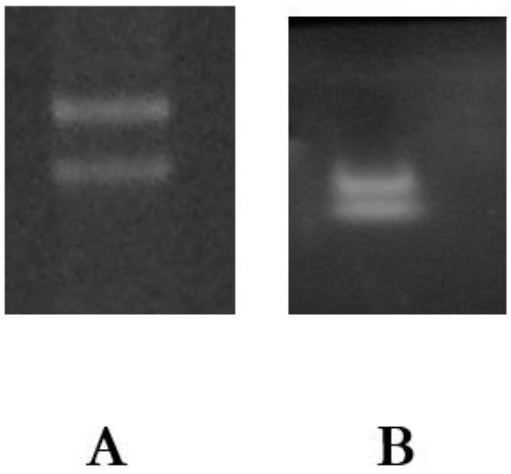
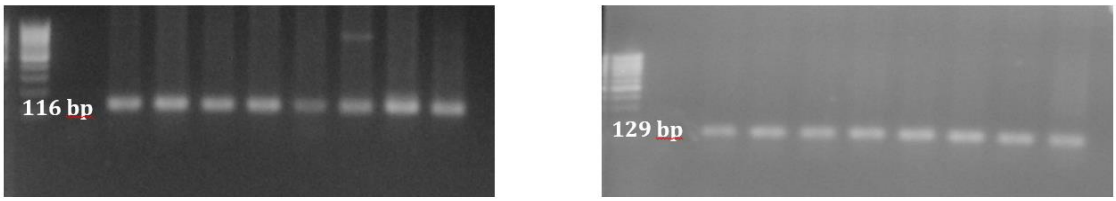


Fig. 2. Agarose gel electrophoresis of Total RNA extraction. **A.** Total RNA extraction of tachyzoites grown in the peritoneal cavity of mice (positive control). **B.** Total RNA extraction from 30-50 mg of brain and liver tissues isolated from the mice treated with artemether and sulfadiazine



A. **B.**

Fig. 3: Agarose gel electroghoresis of PCR products from b1 gene (specific primer) (A) and β-Tubulin gene (internal control primers) (B) in tachyzoites of *Toxoplasma gondii* harvested from peritoneal mice

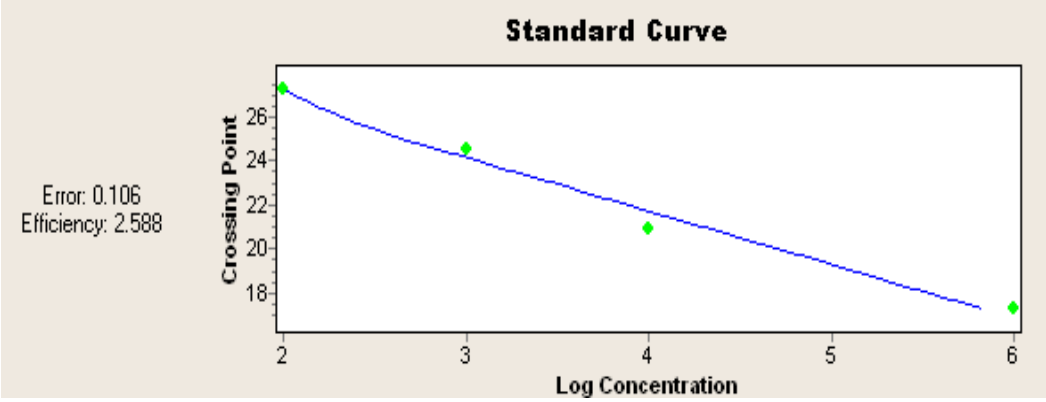


Fig. 4: The efficiency of amplification of the b1 (target gene) and β-Tubulin gene (reference gene) was assayed by real- time quantitative PCR and SYBR green detection (N= 3)

In the liver samples isolated from mice treated with artemether by intraperitoneal injection (i.p.), oral artemether (gavage) and sulfadiazine, B1 gene levels expression had significant reduction in the all three treatment groups compared to untreated control group. There was no change in the reference gene expression level (β-tubulin) (Table 1).

Table 1: Data analysis of the liver of mice treated with artemether and sulfadiazine using the change parameter in real time method the fold.Three replicates of each reaction were done

Position	***Symbol***	***Fold change (comparing to control group)***					
		Artemether (i.p.)*		Artemether (oral)*		Sulfadiazine*	
		Fold change	*P*-value	Fold change	*P*-value	Fold change	*P*-value
Experiment	B1	0.0341	0.0021	0.0216	0.0066	0.111	0.0198
Control		1		1		1	

*There is statistical differences with control group

Similar results were obtained in the study of disported brain tissues from treated mice compared to untreated control mice. Treatment with artemether either injection intraperitoneal or via gavage and treatment with sulfadiazine reduced the B1 gene expression in contrast to the untreated mice (Table 2).

Table 2: Data analysis of the brain of mice treated with artemether and sulfadiazine using the. change parameter in real time method the fold. Three replicates of each reaction were done

Position	*Symbol*	*Fold change (comparing to control group)*					
		Artemether (i.p.)*		Artemether (oral)		Sulfadiazine	
		Fold change	*P*-value	Fold change	*P*-value	Fold change	*P*-value
Experiment	B1	0.220	0.04827	0.425	0.2748	0.366	0.1017
Control		1		1		1	

*There is statistical differences with control group

Discussion

In vivo conditions, we used from RH strain of *T. gondii* for our experiments that is highly virulent infective for all mammalian. Although sulfadiazine and pyrimethamine are choice drugs for treatment of toxoplasmosis but there is no compound controller for complete elimination of infection. In our experiments, artemether as sulfadiazine were shown survival rates very higher than the untreated control group (survival rates range from 31.25% to 38% respectively) (14). In addition, prolongation in time of death were observed for mice treated with sulfadiazine and artemether related to untreated mice and the severity of symptoms was less compared to control group. Maybe artemether combination with the choice drugs or other investigated compounds is more effective in limiting the parasite invasion. Spiramycin effects on *T. gondii* tachyzoites was tested and survival rates of animals treated with spiramycin had no significant difference with the untreated control group (16). In other study, PHNQ6 alone and in combination with sulfadiazine were used against of RH strain of *Toxoplasma*. PHNQ6 in combination with sulfadiazine did not show prolongation in time to death but PHNQ6 alone was effectual in prolonging the survival time (17). Two groups of C57BL/6 mice infected with *Toxoplasma* and treated with enrofloxacin or sulfadiazine did not demonstrate different in susceptibility rates. Increase in survival was not observed in any of the groups (3). Combination drugs were used for the prevention of death in the acute phase of mice infected with parasite that did not present better results than medication alone. Another article artemisinin had similar effect with sulfadiazine on the control of parasite in infected mice (15).

The Real-Time PCR have been used for detection of B1 gene of toxoplasma gondii in biological and patient samples that this method had good sensitivity for observation of positive samples (13, 21). However, there is no information about discovery of the effects of plant compounds on *T. gondii* tachyzoites using the Real-Time PCR in tissues of experimental groups after treatment in vivo conditions. In the present study, livers and brains of four groups of mice treated with artemether (i.p.), artemether and sulfadiazine (via gavage) and untreated groups were used for assessment the rate effects of both drugs based on change expression of B1 gene of this parasite. When the Real-Time PCR was performed on cDNA synthesized, data analyzing shown that the expression levels of B1 gene reduced in all three treatment groups compared to β-tubulin gene (housekeeping) (19). In the other study from PCR was used to confirmation of presence or absence of *Toxoplasma gondii* DNA in brain tissues of mice infected with the RH strain and treated with sulfadiazine and *A. annua* infusion that its result demonstrated the present of parasite (15).

Artemisinin and its derivatives is not the only a potent new compound of antimalarials. Accordingly, other research show that artemisinin and its derivatives are able to control various strains of *T. gondii* in vitro and in vivo

terms (10, 22). Our experiments demonstrated anti-*T. gondii* therapy for artemether and this compound as a nature drug can inhibit intracellular replication of RH strain *T. gondii* tachyzoites and it was not toxic to cells tested. In addition, artemether as a semisynthetic anti- malarial drug (23) could increase the survival rate of infected mice and reduce the expression B1 gene in tissues.

Conclusion

The data that we have shown here suggest that it may be useful against the *T. gondii*. Therefore, there is a necessity to further research on artemether as a promising effective drug for treating toxoplasmosis.

Acknowledgements

This project was financial supported by Tarbiat Modares University (TMU), Tehran, Iran. The authors like to gratitude the stuffs in parasitology department for the helpful advice.

Conflict of interest

The authors declare that there is no conflict of interest.

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