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Evaluating the Inhibitory Effects of Colchicine and Propranolol on *Toxoplasma gondii* Entrance into Host Cells *in vitro* and *in vivo*

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Authors
Ahmad Daryani, *PhD*[1]
Mohammad Ali Ebrahimzadeh, *PhD*[2]
Abdol Sattar Pagheh, *PhD*[3]
Mahdi Sharif, *PhD*[1]
Shahabeddin Sarvi, *PhD*[1]
Ehsan Ahmadpour, *PhD*[4]
Sargis Aghayan, *PhD*[5]
Fatemeh Rezaei, *PhD*[6*]

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[1] Toxoplasmosis Research Center, Mazandaran University of Medical Sciences, Sari, Iran
[2] Pharmaceutical Sciences Research Center, School of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran
[3] Infectious Diseases Research Center, Birjand University of Medical Sciences, Birjand, Iran
[4] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[5] Laboratory of Zoology, Research Institute of Biology, Yerevan State University, Yerevan, Republic of Armenia.
[6] Islamic Azad University of Chalous Branch, Chalous, Iran

**** Correspondence***
Islamic Azad University of Chalous Branch, Chalous, Iran, PC 48168-95475, Sari, Iran.
Email: rezaei63@iauc.ac.ir

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ABSTRACT

Backgrounds: *Toxoplasma gondii* is a zoonotic parasite of increasing concern to humans and animals. Considering the side effects of drugs used to treat toxoplasmosis, it is essential to find alternative drugs.

Materials & Methods: In this study, colchicine and propranolol at four concentrations (1, 5, 10, and 15 µg/mL) were added to the RPMI medium containing peritoneal macrophages and incubated for 60 min, Then tachyzoites were added to the medium, and the efficacy rates of colchicine and propranolol in inhibiting tachyzoites entry into macrophages were evaluated after 30 and 60 min. For *in vivo* assay, one group received no drugs, and the second group was treated with colchicine and propranolol at different concentrations for different durations.

Findings: The *in vitro* experiment showed that treatment with 15 mg/mL of colchicine and propranolol for 60 min following tachyzoites addition was the most efficient method to inhibit tachyzoites penetration, indicating the efficacy rates of 80.20%±1.20 and 89.97%±1.30, respectively ($p< .05$). Based on the *in vivo* test, pretreatment with 2 mg/kg of colchicine one hour before tachyzoites injection had the best inhibitory effect (70.32%±4.07). Also, pretreatment with 2 mg/kg of propranolol 90 min before tachyzoites injection (78.54%±1.99) induced the best inhibitory effect ($p< .05$).

Conclusion: According to the results, colchicine and propranolol could inhibit tachyzoites entrance into nucleated cells *in vitro* and *in vivo*. In this study, the most efficient concentrations and times for using these substances were determined.

Keywords: *Toxoplasma gondii*, Colchicine, Propranolol, *In vitro, In vivo*

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Introduction

Toxoplasma gondii is an intracellular protozoan parasite and ubiquitous pathogen that could infect warm-blooded animals including humans [1]. Toxoplasmosis is usually asymptomatic in healthy individuals but may lead to chronic infection. The disease could be complicated and cause serious illness in immunocompromised patients [2, 3], leading to high morbidity and mortality rates. *T. gondii* could be transmitted from one host to another through food and water contaminated with oocysts, consumption of contaminated raw or undercooked meat, placenta, organ transplantation, and blood transfusion [4, 5].

Among the extensive research conducted in the field of prevention and treatment of toxoplasmosis, few studies have evaluated the efficacy of drugs. Moreover, it should be noted that existing drugs may have serious side effects [6, 7]. For instance, the use of pyrimethamine and sulfonamides in the treatment of clinical toxoplasmosis has been shown to block folate metabolism with a combination of antifolates. However, the use of pyrimethamine alone is not sufficient to prevent and inhibit dihydrofolate reductase, while it could have a suppressive effect on the bone marrow [8, 9] and often cause allergic reactions to sulfonamides; these effects limit the continuous prescription of these drugs [8, 10]. Although there are other drugs such as clindamycin, azithromycin, and atovaquone for the treatment of clinical toxoplasmosis [8, 9], they could not eliminate intracellular parasites and tissue cysts. Also, the treatment of toxoplasmosis in immunocompromised patients and infants is difficult because of serious side effects and recurrent infections [9, 11].

Despite the increasing knowledge about the biology of toxoplasmosis, its treatment is limited to the use of only a few drugs, which are often not tolerated or induce significant side effects [12, 13]. Thus, it is essential to find alternative anti-*Toxoplasma* strategies. *T. gondii* is highly motile and actively invades host cells. Its cytoskeleton plays an important role in motility, invasion, and replication. Thus, interference with any of these functions might kill or inhibit the parasite. *T. gondii* enters host cells via endocytosis after binding to cell surface molecules [14].

Cell membrane-stabilizing drugs have been found to change the cell membrane resistance by blocking the actin gel and interfering with microfilament functions. Colchicine and propranolol are two drugs that stabilize the cell membrane.

Previous studies have demonstrated that treatment of *T. gondii* is still a major problem in the human population. Moreover, although our knowledge about *T. gondii* biology is increasing, its treatment is limited to a few therapeutic options. Thus, it is essential to investigate alternative anti-*T. gondii* strategies. The idea of stabilizing the cell membrane to prevent parasites from entering the cells is a novel subject that could help find new methods to treat toxoplasmosis.

Objectives: This study aimed to investigate whether cell membrane-stabilizing drugs, such as colchicine and propranolol, could inhibit the entrance of *T. gondii* into host nucleated cells *in vitro* and *in vivo.*

Materials and Methods

Animals: For this experiment, six-week-old inbred female Balb/c mice weighing 20-25 g were used. All experiments complied with local animal welfare laws and policies.

Tachyzoites preparation: Tachyzoites of the virulent RH strain of *T. gondii* were obtained routinely by intraperitoneal (IP) passage in Swiss-Webster female mice. Using phosphate-buffered saline (PBS) supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL), tachyzoites

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were harvested after three to four days from the peritoneal cavities of infected mice.

Macrophage monolayer: To prepare peritoneal macrophages, the sterile PBS (pH=7.2) was injected into the peritoneal cavity of mice and then aspirated. Next, the harvested macrophages (1×10^5 /mL) were seeded in 16-well plates containing a glass coverslip. Non-adherent cells and supernatants were removed after plating and incubation at 37 °C for 60 min. Then the macrophages were incubated in RPMI-1640 culture medium at 37 °C in a 5% CO_2 atmosphere for up to 24 hrs [15].

Counting of intracellular tachyzoites: To determine the average number of parasites in macrophages, parasites in 100 macrophages were counted under a light microscope after fixation and staining with Giemsa [16].

***In vitro* experimental design and groups**: Colchicine and propranolol at different concentrations were dissolved in sterile PBS and used. In the experimental group, colchicine and propranolol at concentrations of 1, 5, 10, and 15 µg/mL were prepared and added to the RPMI medium containing peritoneal macrophages and incubated for 60 min. Next, 4×10^5 tachyzoites were added to the medium and incubated at 37 °C for 30 and 60 min. Meanwhile, the control group received only PBS and RPMI. To evaluate the efficacy of drugs in inhibiting the entrance of *T. gondii* tachyzoites into macrophages, the number of tachyzoites in macrophages was counted by Giemsa staining under a light microscope after 30 and 60 min. Data were obtained after triplicate experiments for each concentration and expressed as percentage using the formula proposed by Ryning and Remington (1978) as follows:

Inhibition rate = [Number of *T. gondii* entered into treated host cells / Number of *T. gondii* entered into untreated host cells] ×100

Finally, the results were compared with those of the control group.

***In vivo* experimental design and groups**: The activity of colchicine and propranolol against the entry of tachyzoites into nucleated cells was evaluated by intraperitoneal (IP) inoculation of these drugs into female BALB/c mice (age: six weeks; weight: 20-25 g). Eight mice were used to evaluate the efficacy of both drugs. Four mice were pretreated with IP injection of colchicine (1 and 2 mg/kg) 2 and 1 hrs prior to exposure to tachyzoites, and the other four mice were pretreated with IP injection of propranolol (1 and 2 mg/kg) 3 and 1.5 hrs prior to exposure to tachyzoites. Then 4×10^5 tachyzoites were injected IP. After 60 min, the results were evaluated by Giemsa staining under a light microscope. The control group received only tachyzoites in PBS.

Statistical analysis: For statistical analysis, all data were analyzed using IBM Statistical Package for the Social Sciences (SPSS Inc. Chicago, IL, USA) Version 20.0.0. Mann-Whitney U test and Fisher's exact test were used for comparisons. A *p*-value of < .05 was considered as statistically significant.

Findings

***In vitro* experiment:** According to the results, by increasing the concentration of colchicine and propranolol, the average number of macrophages infected with *T. gondii* tachyzoites was reduced compared to the control group (Table 1). After 30 min of treatment with colchicine following exposure to tachyzoites, a higher inhibition rate was recorded for colchicine concentrations of 10 and 15 µg/mL with an average inhibition rate of 78.20%±0.9 and 79.79%±1.03, respectively. The lower drug concentration (10 µg/mL) was considered more appropriate due to the proximity effect of the mentioned doses and a statistically insignificant difference (p> .05). Also, after 60 min of treatment with 10 and 15 µg/mL of colchicine following tachyzoites

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addition, the cell entry inhibition rates of *T. gondii* tachyzoites were 79.28%±1.30 and 80.20%±1.20, respectively. Therefore, the most successful inhibition rate using colchicine occurred at a concentration of 15 µg/mL after 60 min of tachyzoites addition (80.20%±1.20) (p< .05; Figure 1-A).

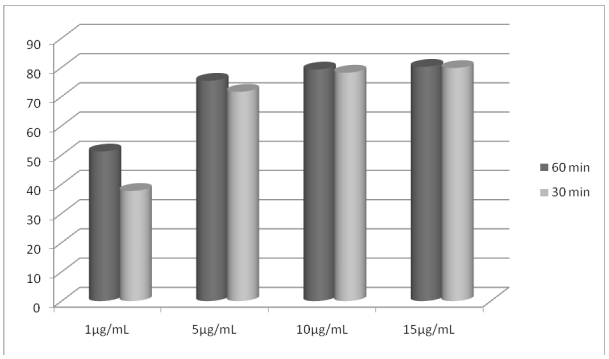

Figure 1-A) The inhibitory effect of colchicine on the entry of *T. gondii* tachyzoites into host cells in vitro

Regarding the inhibitory effect of propranolol, it was found that its inhibitory effect increased with increasing concentration both after 30 and 60 min (Figure 1-B).

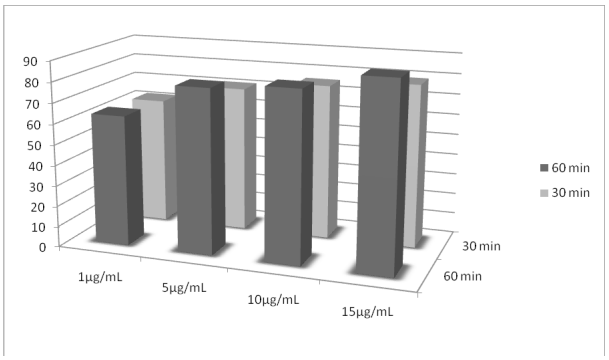

Figure 1-B) The inhibitory effect of propranolol on the entry of *T. gondii* tachyzoites into host cells in vitro

After 30 min of treatment with propranolol following tachyzoites addition, the inhibitory effect of propranolol was higher at concentrations of 10 and 15 µg/mL with an average inhibition rate of 75.51%±1.68 and 79.08%±1.03, respectively. In addition, there was no significant difference between these concentrations (p > .05). Also, after 60 min of treatment following tachyzoites addition, the inhibitory effect of propranolol was higher at concentrations of 10 and 15 µg/mL with an average inhibition rate of 82.38%±1.05 and 89.97%±1.30, respectively. Therefore, the most successful inhibition rate using propranolol occurred at a concentration of 15 µg/mL after 60 min of tachyzoites addition (Figure 1-B). Comparing the concentrations of colchicine and propranolol at different times indicated that the highest inhibition rate occurred using 15 µg/mL of propranolol for 60 min following tachyzoites addition (89.97%±1.30) (p< .05).

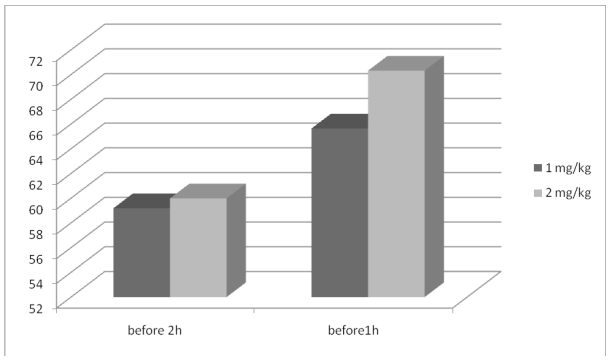

Figure 2-A) The inhibitory effect of colchicine on the entry of *T. gondii* tachyzoites into host cells *in vivo*

***In vivo* experiments**: In the control group, 96% of nucleated cells were infected after 10 min of tachyzoites injection. Also, the number of intracellular tachyzoites in the groups treated with colchicine and propranolol significantly decreased compared to the control group (p< .05).

According to the results, the number of intracellular tachyzoites decreased by increasing the concentration of colchicine (p< .05). In the case of pretreatment with 1 mg/kg of colchicine 2 and 1 hrs before tachyzoites injection, the inhibition rates were 59.19%±1.78 and 59.99%±1.39, respectively. Meanwhile, in the case of pretreatment with 2 mg/kg of colchicine 2 and 1 hrs before tachyzoites injection, the inhibition rates were 65.64%±2.43 and 70.32%±4.07, respectively (Figure 2-A). It was found that the inhibitory effect

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Table 1) Proportion of infected macrophages with *T. gondii* tachyzoites

	30 min Control PBS (%)	60 min Control PBS (%)	30 min Colchicine (%)	60 min Colchicine (%)	30 min Propranolol (%)	60 min Propranolol (%)
1 µg/mL	94	97	45	56	36	46
5 µg/mL			25	30	24	29
10 µg/mL			20	24	16	26
15 µg/mL			21	23	16	26

was dose-dependent. The inhibition rates induced using 1 mg/kg of propranolol 3 and 1.5 hrs before tachyzoites injection were 71.60%±1.39 and 72.57%±1.23, respectively. Meanwhile, the inhibition rates induced using 2 mg/kg of propranolol 3 and 1.5 hrs before tachyzoites injection were 74.67%±1.52 and 78.54%±1.99, respectively (Figure 2-B). Totally, the highest inhibition rate of *T. gondii* tachyzoites was obtained using 2 mg/kg of propranolol 1.5 hrs before tachyzoites injection ($p< .05$).

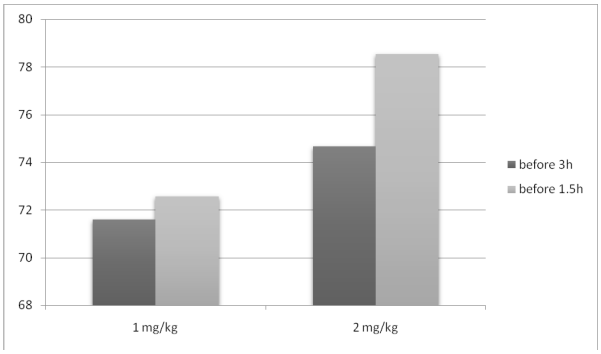

Figure 2-B) The inhibitory effect of propranolol on the entry of *T. gondii* tachyzoites into host cells *in vivo*

Discussion

In this experimental study, the effect of two cell membrane-stabilizing drugs (colchicine and propranolol) on inhibiting *T. gondii* entry into the cells *in vitro* and *in vivo* was investigated. The results showed that both drugs inhibited the parasites from entering the cell by stabilizing the cell membrane without noticeably affecting the host cells.

When *T. gondii* enters the cells, micro-pseudopods are frequently observed partially enveloping this organism as it gains an intracellular position [17]. After being released from *T. gondii* tachyzoites rhoptry, penetration enhancing factor (PEF) attaches to the surface of nucleated cells receptors, called actin, laminin, and collagen. *T. gondii* enters host cells via endocytosis after binding to cell surface molecules. In most cells, the microfilament structure is formed by actin and myosin polymers and plays an important role in bringing micro-pseudopods. Cell membrane-stabilizing drugs have been found to change cell membrane resistance by blocking the actin gel and interfering with microfilament functions. The importance of contractile proteins in the formation of these pseudopods and the interaction of their subunits results in actin filament gelation, which could be prevented by colchicine and propranolol. This supports the hypothesis that microfilaments are the most likely subcellular sites of colchicine and propranolol action in preventing *T. gondii* entry. Colchicine and propranolol might induce cell membrane resistant to parasites' efforts to gain an intracellular position. Their cytoskeleton is suspected to play an important role in motility, invasion, and endodyogeny. Thus, disruption of the mentioned functions may be effective in inhibiting parasites [16].

In this study, it was observed that colchicine

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and propranolol inhibited *T. gondii* entry into cells by interfering with this mechanism. Cytoskeleton plays an important role in motility, invasion, and endodyogeny [18]. In this regard, Rezaei et al. (2016) showed that ketotifen and cromolyn sodium drugs stabilized the cell membrane and inhibited the entry of *T. gondii* tachyzoites into nucleated cells; therefore, they could be introduced as effective agents to inhibit the entry and invasion of *T. gondii* tachyzoites into the cells [16].

D'angelo et al. (2008) showed that artemisinin derivatives were effective in inhibiting *T. gondii*, and the treatment of extracellular tachyzoites with these derivatives inhibited parasite invasion. This inhibition could occur at more than one stage in the *T. gondii* lytic cycle [19].

In another study, Ohnishi et al. (1989) investigated the effects of several membrane-acting drugs on malaria and sickle cell anemia and demonstrated that propranolol could inhibit the growth of *Plasmodium falciparum* and *P. vinckei* [20]. Cytochalasin D has also been shown to inhibit the entry of *T. gondii* into peritoneal macrophages and bladder tumor cells. Evidently, actin and myosin polymers and other sub-cellular structures appear to be the major binding sites for cytochalasin D [21].

Stokkermans et al. (1996) showed that dinitroaniline herbicides interfered with tubulin polymerization in plants [22]. They described that *T. gondii* sub-pellicular microtubules interacted with dinitroaniline [25]. *Leishmania* tubulin [23] and *Plasmodium* [24] has been shown to inhibit *T. gondii* replication. Trifluralin binds to *Leishmania* tubulin and inhibits polymerization of microtubules [23]. In *Plasmodium*, trifluralins bind to microtubules of gametocytes, inhibiting erythrocytic stages and exflagellation of gametocytes [24, 26].

Cortez et al. (2009) showed that statins could act as a tool to interfere with the intracellular cycle of the parasite and inhibit *T. gondii* multiplication [15]. Thus, it seems that cell membrane-stabilizing drugs could inhibit *T. gondii* entry into host nucleated cells. To determine whether one or both mechanisms are involved, new methods should be developed to differentiate between the effects of colchicine and propranolol on the host cell membrane and on *T. gondii* itself. Finally, considering the inhibitory effects of colchicine and propranolol on the entrance of *T. gondii* tachyzoites into macrophages and other nucleated cells, these drugs could be introduced as effective agents to inhibit the entry and invasion of *T. gondii* tachyzoites into cells. Nonetheless, the drugs used in this study, especially colchicine, have side effects that could limit their usage. Thus, drugs with fewer side effects are required. If the effectiveness of cell membrane-stabilizing drugs is proven, other studies might be conducted on this type of drugs with fewer side effects.

Conclusion

In this study, the inhibitory effects of colchicine and propranolol on *T. gondii* tachyzoites entry into macrophage and nucleated cells were shown. As a result, these drugs could be introduced as effective agents to inhibit the entry and invasion of *T. gondii* tachyzoites into cells.

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Ethical Permissions: The study was conducted in accordance with the ethical standards of the Institutional Ethics Committee of Mazandaran University of Medical Sciences, Iran (no. 805).

Conflicts of Interests: None declared.

Authors' Contribution: Conceptualization:

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FR, MAE, and AD; laboratory analysis: FR, EA, SS, and ASP; interpretation of the results: SA, MAE, and MS; writing of the original draft: FR and AD; and writing, reviewing, and editing: FR, AD, and ASP. All authors read and approved the manuscript.

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