Artesunate showed a mean tachyzoite inhibitory concentration (IC50) of 0.075µM. LLC-MK2 cells were analyzed using nonlinear regression analysis with Prism 3.0 software. Pyrimethamine, quinine, and trimethoprim. The effects of these drugs on tachyzoites and strain were used. The following drugs were tested: artesunate, cotrimoxazole, pentamidine, pyrimethamine, quinine, and trimethoprim. The effects of these drugs on tachyzoites and LLC-MK2 cells were analyzed using nonlinear regression analysis with Prism 3.0 software.

Results: Artesunate showed a mean tachyzoite inhibitory concentration (IC50) of 0.075µM and an LLC MK2 toxicity of 2.003µM. Pyrimethamine was effective at an IC50 of 0.482µM and a toxicity of 11.178µM. Trimethoprim alone was effective against the in vitro parasite. Cotrimoxazole also was effective against the parasite but at higher concentrations than those observed for artesunate and pyrimethamine. Pentamidine and quinine had no inhibitory effect over tachyzoites. Conclusions: Artesunate is proven in vitro to be a useful alternative for the treatment of toxoplasmosis, implying a subsequent in vivo effect and suggesting the mechanism of this drug against the parasite.

Keywords: Artesunate. Treatment. Anti-Toxoplasma activity. Toxicity.

INTRODUCTION

Toxoplasmosis is a highly prevalent cosmopolitan infection, but the disease occurs in only a fraction of infected people, mainly as a nonspecific immune activation syndrome, chronically in ocular forms as chorioretinitis. The main problems are congenital disease and the infection of immunocompromised people, especially those with acquired immunodeficiency syndrome (AIDS) or those undergoing chemotherapy for cancer or transplant rejection.

The etiological agent of toxoplasmosis is Toxoplasma gondii, the development of which has many forms. Tachyzoites are found in the acute phase of the disease and are responsible for clinical manifestations. They are susceptible to the immune response of the host and to drug action. Cysts are the resistant form of the parasite, persisting for the host's entire life. Cyst walls are resistant to both drugs and the immune system.

Felids are the definitive hosts for the parasite, with other mammals and birds acting as intermediate hosts. Humans can be infected either congenitally or through ingestion of raw or undercooked meat; manipulation of infected meat containing tissue cysts; or consumption of water, fresh vegetables, or other food contaminated by oocysts eliminated in cat feces.

The most effective treatment against toxoplasmosis is a combination of the drugs sulfadiazine and pyrimethamine, which can cause hematological effects that are controlled with the administration of folic acid. An association of great interest is the one between trimethoprim and sulfamethoxazole. Known as cotrimoxazole, its active compounds act synergistically, inhibiting two consecutive steps of folic acid biosynthesis in a manner similar to that observed for pyrimethamine-sulfadoxine. Cotrimoxazole is well tolerated and less toxic to hematopoiesis. Human immunodeficiency virus–acquired immunodeficiency syndrome (HIV-AIDS) patients taking cotrimoxazole show a high incidence of adverse effects, and its use is discouraged in pregnant women because it crosses the placental barrier.
Although antifolate compounds, such as pyrimethamine, exhibit good anti-*Toxoplasma* activity, their toxicity limits widespread use, particularly for extended treatment periods. The discovery of viable low-toxicity compounds capable of preventing and treating *T. gondii* would represent a great advance in the treatment of infections in immunocompromised patients. Some compounds that are effective against species of *Plasmodium* could be effective against *T. gondii*. Those agents were selected for further testing in the present study because malaria and toxoplasmosis are caused by protozoans belonging to the phylum Apicoplasta, and antimicrobial agents that have been effective for the treatment of malaria, such as artesimisin and its derivatives, also have been effective for the treatment of toxoplasmosis. Artesimisin (*qinghaosu*) is a product extracted from the plant *Artemisia annua* L. Despite the fact that artesimisin has produced teratogenic effects in laboratory animals, precluding its use in pregnant women, few adverse effects have been observed in humans.4,5

In the present study, the toxicity of artesunate and its effectiveness for the treatment of toxoplasmosis were studied *in vitro* and compared with the actions of three drugs: pyrimethamine, trimethoprim, and cotrimoxazole, which are currently in use against toxoplasmosis. Pentamidine and quinine, used for the treatment of other protozoans, also were evaluated and compared. Pentamidine is an organic compound and derivative of guanidine that has shown activity against *Leishmania sp.*, African trypanosomiasis, and pneumonia caused by *Pneumocystis carinii* (*jiroveci*). Quinine is an alkaloid extracted from species of the genus *Cinchona*, the application of which is limited to cases of malaria caused by *Plasmodium falciparum*.

### METHODS

#### Parasites

Tachyzoites of the type I RH strain of *T. gondii* were routinely maintained by intraperitoneal passage in BALB/c mice.

#### Drugs

All drugs were obtained from commercial sources (Sigma, USA) or as human use drugs, supplied by the pharmacy of the Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HC-FMUSP). Artesunate and quinine dichlorhydrate were supplied by Cipla Medpro (Belville, SA), pentamidine was supplied by Itaca Labs (Rio de Janeiro, BR), and cotrimoxazole was supplied by Ducto (Anapolis, BR).

#### Cell culture

The epithelial cell line LLC-MK2, derived from rhesus monkey (*Macaca mulatta*) kidneys, was used. Cells were cultivated in RPMI medium with the addition of 10% inactivated bovine fetal serum and gentamicin. Cultures were kept in disposable plastic bottles and incubated at 36°C with 5% CO₂.

**In vitro** assays of drug effectiveness and toxicity

The assays were conducted in four stages, the first three on consecutive days. On the first day, LLC-MK2 cells were extracted from a plastic bottle with ATV enzyme and counted in a Neubauer counting chamber. Cells were diluted in RPMI medium containing 10% inactivated bovine fetal serum until a concentration of 1 × 10⁶ was obtained. One hundred microliters of the mixture was added to each well of a 96-well plate and placed inside the CO₂ incubator. On the second day, the supernatant from all the wells was aspirated. Tachyzoites from the peritoneal fluid of BALB/c mice were extracted with a syringe and diluted in complete RPMI medium containing 10% bovine fetal serum until a concentration of 1 × 10⁴ was obtained. One hundred microliters of the mixture was added to the wells in rows A to E and columns 1 to 12 of the plate and put into a CO₂ incubator. The remaining wells were filled with complete RPMI containing 10% bovine fetal serum. On the third day, the plate was washed with complete RPMI medium containing 10% inactivated bovine fetal serum. A mother solution of each drug was prepared by diluting each one in a solution of complete RPMI medium containing 10% inactivated bovine fetal serum until a 200µL/mL concentration was obtained. Two-fold serial dilutions of all compounds were performed, starting with initial concentrations of 100µg/mL. Therefore, the concentrations used were as follows: 100, 50, 25, 12.5, 6.25, 3.1, 1.6, 0.8, 0.4, 0.2, and 0.1µg/mL. For pyrimethamine and trimethoprim, the first dilution was made in dimethyl sulfoxide, and the remaining dilutions were made in RPMI because of the low solubility of these compounds in water. On another 96-well plate, 200µL of each drug solution was pipetted into wells in column 1, whereas 100µL of RPMI medium containing 10% inactivated bovine fetal serum solution was pipetted into the wells in columns 2 to 12. A two-fold serial dilution was performed by transferring 100µL from column 1 to those in column 2 and repeating this procedure until column 11. The contents of the second plate were transferred to the first plate and placed inside the CO₂ incubator. The reaction was interrupted when the tachyzoites had destroyed all the cells from the positive control wells. After that, the supernatant was moved, and the plate was washed with PBS, fixed with methanol, and stained with 1% aqueous crystal violet solution. After being washed, the plate was dried, and 200µL of methanol was added to dissolve the stain. The A₆₂₀ was measured with an ELISA microplate reader. Adherent live cells were stained. The A₆₂₀ is proportional to the number of viable cells. This allowed both the measurement of *T. gondii* cell destruction, or cell toxicity, and detection of infected or non-infected cell layers.

#### Statistical analysis

Drug effects on both tachyzoites *in vitro* and LLC-MK2 cells were analyzed using nonlinear regression analysis with Prism 3.0 software, yielding mean inhibitory concentrations (IC₅₀) for the studied compounds.

### RESULTS

Artesunate at a concentration of 100µg/mL killed all cells in the culture. Therefore, the experiment was performed again with a maximum concentration of 10µg/mL. It was observed that artesunate was effective against tachyzoites at an IC₅₀ of 0.075µM, resulting in preservation of the cell line (*Figure 1A* and *1B*). The mean toxicity of the drug was 2.003µM (*Figure 2A* and *2B*).

Cell death also was observed with pyrimethamine at 100µg/mL in the cell culture so that experiment also was performed again with a maximum concentration of 10µg/mL. It was observed that pyrimethamine was effective against tachyzoites at an IC₅₀ of 0.482µM, resulting in the preservation of the cell line. The mean toxicity of the drug was 11.178µM (*Figure 3A* and *3B*). Cotrimoxazole proved to be effective against tachyzoites at an IC₅₀ of 11.884µM, whereas trimethoprim was effective against
FIGURE 1 – Morphology of LLC-MK2 cells after (A) *Toxoplasma gondii* infection and (B) artesunate treatment following parasite challenge.

Digital images are from inverted microscope phase-contrast microscopy with 20 x objective.

FIGURE 2 - A) Artesunate action in cell culture infected with *Toxoplasma gondii* at concentrations of 10 to 0.01µg/mL. B) Artesunate toxicity in cell culture, at concentrations of 10 to 0.01µg/mL.

FIGURE 3 - A) Pyrimethamine action in cell culture infected with *Toxoplasma gondii* at concentrations of 10 to 0.01µg/mL. B) Pyrimethamine toxicity in cell culture at concentrations of 10 to 0.01µg/mL.
**DISCUSSION**

An *in vitro* effect of artesunate against *T. gondii* was found. Previous reports had demonstrated that this compound showed action against other parasites, such as *Plasmodium* and *Fasciola*. Although inhibitory concentrations were higher than those used against the parasites that cause malaria, artemisinin and many of its derivatives were effective against *T. gondii*. In this experiment, artesunate exhibited a strong effect against *T. gondii* tachyzoites in *in vitro* assays. This work represents an initial step toward future studies of the *in vivo* action of this compound and its effect on the cystic form of the parasite. Artesunate could be an alternative to the standard pyrimethamine-sulfonamide treatment.

Effective action of artesunate against tachyzoites was observed, and it was the highest among all studied compounds. Several studies demonstrating the action of artemisinin derivatives have been conducted, including Ke Ou-Yang et al., D’Angelo et al., and Sarciron et al. El Zawary, in 2008, studied the *in vitro* action of artesunate against RH strain *Toxoplasma* and observed a significant reduction in the viability and effect of tachyzoites exposed to drugs compared with a no-treatment control. Although the efficacy of artesunate was demonstrated both here and in El Zawary, the inhibitory concentration values for artesunate found by El Zawary were higher than those observed in the present work. However, because there is no standard methodology for drug testing in cell lines, different outcomes could result.

Clark et al. observed that artesunate is toxic to the embryos of mice, rabbits, and nonhuman primates, causing cardiovascular and skeletal problems, even death, when given at higher doses and over longer periods than recommended for the treatment of malaria.
At this time, no adverse effects related to the drug have been reported in pregnant women treated with artemisinin, including artesunate. Although the number of pregnant women exposed to artemisinin during the first trimester is considered too small to demonstrate safety, the absence of any adverse effects to the babies in these limited published clinical studies is encouraging.

In this work, cotrimoxazole was effective against *T. gondii* and not toxic to the cell culture at tested concentrations. The synergistic *in vitro* effect between trimethoprim and sulfamethoxazole was demonstrated many decades ago, by Grossman and Remington\(^2\) and Derouin and Chastang\(^3\). More recent studies such as those by Dumas \(^4\) and Soheilian et al.\(^5\) support the use of this combination to prevent cerebral and ocular toxoplasmosis\(^4, 5\).

Lindsay et al.\(^7\) examined the activity of pentamidine and nine of its analogs to inhibit the replication of RH strain *T. gondii* in Vero cell cultures. In that study, pentamidine at 25 and 10µg/mL was shown to have significant effects over tachyzoite replication. Lindsay et al.\(^7\) obtained a different result from that in the present work, where no anti-*Toxoplasma* activity of pentamidine was observed at tested concentrations. The conflicting results between these studies may be due to the different cell lines used or differences in methodology because there is no standard model among the authors who perform assays with drugs\(^7\).

Pyrimethamine is the main drug of choice for the treatment of toxoplasmosis. It is well known that this drug exhibits *in vitro* activity against the parasite, as demonstrated by studies such as Derouin and Chastang\(^3\), Cantin and Chamberland\(^3\), Ven et al.\(^7\), and Meneceur et al.\(^4\). The findings of the present study were very similar to those previously mentioned, all of them showing effective action at concentrations between 0.05 and 0.24µg/mL on RH strain. When compared with artesunate, pyrimethamine has been shown to be less effective but also less toxic. The selectivity index obtained for these drugs were similar; therefore, artesunate was shown to be a promising option for the treatment of toxoplasmosis\(^14, 20\).

No anti-*Toxoplasma* activity was observed for quinine at tested concentrations. These results are compatible with the *in vitro* experiments from Holfels, which tested quinine sulfate on RH strain *T. gondii* at 2, 10, and 20µg/mL and observed no inhibitory effects on intracellular tachyzoites.

Since the 1970s, several works have demonstrated the effectiveness of trimethoprim against *T. gondii* *in vitro*, among them, Grossman and Remington, Derouin and Chastang, Ven et al.\(^7\), and D’Angelo et al.\(^8\). Most of these works used RH strain, and all of them found IC\(^{50}\) values between 2 and 10µg/mL. The authors of these studies point out that a murine model is inadequate to evaluate trimethoprim’s efficacy because of the difference in the drug’s half-life in human and rat sera with a mean toxicity of 60µg/mL, and more human *in vitro* studies are therefore needed. Because of the short half-life of trimethoprim, a significant inhibitory concentration may not be sustained in human sera, which could explain the poor efficacy of this agent alone. Therefore, besides its low toxicity, trimethoprim on its own is not considered an alternative to pyrimethamine for the treatment of toxoplasmosis\(^8, 14, 16, 20\).

In this study, artesunate showed the highest efficacy among the compounds studied, followed by pyrimethamine. Along with their higher efficacy, however, these drugs showed higher toxicity in cell culture. Trimethoprim has shown both efficacy and low toxicity, but treatment with this drug alone is not effective. It is combined with sulfamethoxazole to form cotrimoxazole, which also has been tested and shown to be effective and nontoxic at administered concentrations, lending support to the use of this drug as an alternative treatment to toxoplasmosis.

The data obtained in the present study suggest that artesunate could be a useful alternative to antifolates in the treatment of toxoplasmosis. Further study of artesunate is still required, specifically into its action against *T. gondii in vivo* and its efficacy against tissue cysts.

The possible toxic effects of artesunate on pregnant women who are being treated for malaria should continue to be investigated, keeping in mind that the dose necessary to kill *T. gondii* is higher than that for *Plasmodium* sp.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

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