Enrofloxacin is able to control *Toxoplasma gondii* infection in both *in vitro* and *in vivo* experimental models

Bellisa Freitas Barbosa\(^a\), Angelica Oliveira Gomes\(^a\), Eloisa Amália Vieira Ferro\(^a\), Danielle Reis Napolitano\(^b\), José Roberto Mineo\(^c\), Neide Maria Silva\(^d,\ast\)

\(^a\) Laboratory of Histology and Embriology, Institute of Biomedical Sciences, Federal University of Uberlândia, 38405-320 Uberlândia, MG, Brazil

\(^b\) Institute of Biomedical Sciences, Federal University of Uberlândia, 38400-902 Uberlândia, MG, Brazil

\(^c\) Laboratory of Immunoparasitology, Institute of Biomedical Sciences, Federal University of Uberlândia, 38400-902 Uberlândia, MG, Brazil

\(^d\) Laboratory of Immunopathology, Institute of Biomedical Sciences, Federal University of Uberlândia, 38400-902 Uberlândia, MG, Brazil

**A B S T R A C T**

Currently, toxoplasmosis is treated with sulfadiazine and pyrimethamine. However, this treatment presents several adverse side effects; thus, there is a critical need for the development and evaluation of new drugs, which do not present the same problems of the standard therapy. Enrofloxacin is a fluoroquinolone antibiotic known to control infection against several bacteria in veterinary medicine. Recently, this drug has demonstrated protective effects against protozoan parasites such as *Neospora caninum*. The present study aimed to determine the effect of enrofloxacin in the control of *Toxoplasma gondii* infection. For this purpose, human foreskin fibroblast (HFF) cells were infected with *T. gondii* RH strain and treated with sulfadiazine, penicillin/streptomycin, pyrimethamine, or enrofloxacin. Following treatment, we analyzed the infection index, parasite intracellular proliferation and the number of plaques. Additionally, tissue parasitism and histological changes were investigated in the brain of *Calomys callosus* that were infected with *T. gondii* (ME49 strain) and treated with either sulfadiazine or enrofloxacin. Enrofloxacin was able to reduce the infection index, intracellular proliferation and the number of plaques in HFF cells infected by *T. gondii* in comparison with untreated or penicillin/streptomycin-treated ones. Enrofloxacin was more protective against *T. gondii* in HFF infected cells than sulfadiazine treatment (*P<0.001*). In addition, pyrimethamine, enrofloxacin or the associations of sulfadiazine plus pyrimethamine, enrofloxacin plus sulfadiazine or enrofloxacin plus pyrimethamine-treatments were able to reduce the plaque numbers in HFF cells infected by *T. gondii* when compared to medium, penicillin/streptomycin or sulfadiazine alone. *In vivo* experiments demonstrated that enrofloxacin diminished significantly the tissue parasitism as well as the inflammatory alterations in the brain of *C. callosus* infected with *T. gondii* when compared with untreated animals. Based on our findings, it can be concluded that enrofloxacin is a potential alternative drug for the treatment of toxoplasmosis.

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1. **Introduction**

*Toxoplasma gondii* is an intracellular protozoan parasite able to infect any cell of warm-blooded vertebrates (Montoya and Liesenfeld, 2004). In immunocompetent hosts, *T. gondii* infection results in mild or nonspecific symptoms (Montoya and Liesenfeld, 2004; Lang et al.,...
2007). However, the infection of immunocompromised patients by this parasite represents serious problems for public health (Kim and Weiss, 2004). In these patients, upon reactivation of a latent infection, this parasite can cause retinochoroiditis or lesions in the central nervous system (CNS) (Ferreira and Borges, 2002; Lang et al., 2007). Additionally, newborns from women that acquire the parasite during pregnancy can develop similar symptoms as those described for immunocompromised patients and, women infected with T. gondii may be at an increased risk of miscarriage (Jones et al., 2001).

The immune response against T. gondii is typically cellular. The major contributors of the immune response against T. gondii are macrophages, dendritic cells and lymphocytes (Filisetti and Candolfi, 2004). IFN-γ is an important cytokine involved in the control of T. gondii replication. IFN-γ triggers several anti-parasitic mechanisms in macrophages and natural killer cells, including the production of reactive oxygen intermediates, nitric oxide and the induction of tryptophan starvation (Dauben et al., 1996; Lang et al., 2007). This immune response results in the control of infection by promoting the conversion of the fast-replicating tachyzoite to the slow-replicating bradyzoite located in tissue cysts (Bohne et al., 1999). However, this immune response is not able to clear the infection. In this sense, the use of drugs to control the infection by T. gondii is very important, considering that the immune response cannot be sufficient in some circumstances, especially in immunocompromised patients and congenitally infected children.

Currently, toxoplasmosis is being treated with a combination of sulfadiazine and pyrimethamine, as both drugs are able to inhibit crucial enzymes involved in the biosynthesis of T. gondii pyrimidines (Anderson, 2005). However, numerous toxicity problems have been described with the use of these drugs, including suppression of the bone marrow and teratogenic effects in the first trimester of pregnancy (Degerli et al., 2003; Schmidt et al., 2006). Thus, the study of new therapeutic strategies for toxoplasmosis treatment is necessary to find active and less toxic drugs.

Enrofloxacin is a fluoroquinolone antibiotic that exhibits increased antibacterial activity against Enterobacteriaceae, Gram-negative bacteria such as Pseudomonas aeruginosa and some Gram-positive cocci (Martínez et al., 2006). Fluoroquinolones inhibit the DNA gyrase activity, an enzyme found in all bacteria, preventing the formation of replication forks and transcription (Willmott et al., 1994). These antibiotics including enrofloxacin are currently being used in clinical veterinary medicine (Martínez et al., 2006). In this context, enrofloxacin is an important antibiotic to control the rapidly growing mycobacteria isolated from cats and dogs (Govendir et al., 2011) and in addition, it has been used as an alternative drug treatment for endometritis in susceptible mares (González et al., 2010). Additionally, enrofloxacin was shown to control Bartonella henselae infection in both cats and humans (Biswas et al., 2010) and infectious disease caused by sensitive pathogens in Angora rabbits (Elmas et al., 2007). Despite not as efficient as toltrazuril, enrofloxacin was able to reduce infection with Neospora caninum, an apicomplexan parasite, in the brains of pregnant mice (Gottstein et al., 2005).

However, there are no current studies that have analyzed the effect of enrofloxacin in the control of T. gondii replication in vitro or in vivo. Hence, in the present study, we aimed to determine the effect of enrofloxacin to control T. gondii infection. It was set up two experimental conditions to demonstrate the effects of enrofloxacin, as follows: an in vitro experimental model using human foreskin fibroblasts (HFF) as host cells and an in vivo model developed in Calomys callosus rodents, which is an appropriate host to study toxoplasmosis, according to our previous studies (Ferro et al., 2002; Barbosa et al., 2007; Costa et al., 2009; Franco et al., 2011) and C57BL/c mice.

2. Materials and methods

2.1. Parasite strains

Tachyzoites from RH strain of T. gondii were maintained by serial passages in HFF cells in order to obtain in vitro parasites for further in vitro infection experiments, whereas T. gondii from ME-49 strain was maintained in chronically infected C. callosus, as previously described (Barbosa et al., 2007), and used in in vivo experiments. Experimental infections with cysts of ME-49 strain were performed as follows: brains were collected from Calomys reservoirs 30–45 days after infection and homogenized in 1 ml PBS. Cyst counts were determined by microscopic analysis in 10 μl aliquots of brain homogenates, in duplicates. Inocula were adjusted to 20 cysts per 100 μl and administered orally (Resende et al., 2008).

2.2. Cell cultures and maintenance of the T. gondii RH strain

HFF cells were obtained from the American Type Culture Collection (ATCC, Manassas, USA). Cells were cultured in 25 cm² culture flasks in RPMI 1640 medium (Cultilab, Campinas, Brazil) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, USA) and 10% heat-inactivated fetal bovine serum (FBS) (Cultilab) and maintained in a humidified incubator at 37 °C and 5% CO₂. In parallel, T. gondii tachyzoites (RH strain) were maintained in HFF cells cultured in RPMI 1640 medium supplemented with 2% FBS, by serial passage at regular 48 h-intervals.

2.3. Cytotoxicity assay

In order to verify if the treatments with the antibiotics could be toxic to the host cells, and to determine which concentration of the antibiotics could be used without decreasing the viability of the cells, the tetrazolium salt colorimetric (MTT) assay was conducted after treatments, as previously described (Mosmann, 1983). For this purpose, HFF cells were cultured in 96-well plates (3 × 10⁴ cells/well/200 μl) for 24 h in RPMI medium with 10% FBS at 37 °C and 5% CO₂. Next, cells were treated for 72 h with sulfadiazine (Catarinense S.A. Laboratory, Joinville, Brazil), a drug that may control T. gondii in HFF cells (positive control) (Anderson, 2005; Oliveira et al., 2009), or enrofloxacin (Bayer Healthcare, São Paulo, Brazil),
both at the following concentrations in medium with 5% FBS: 1.56, 3.125, 6.25, 12.5, 25, 50, 100, or 200 μg/ml. As control, cells were treated with medium plus 5% FBS only or 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co.). After treatments, the supernatants were removed, and the cell cultures were washed and pulsed with 10 μl of MTT (Sigma Chemical Co.) at 5 mg/ml in 90 μl of medium with 10% FBS for 4 h at the same culture conditions. The tetrazolium salt MTT, a pale yellow substrate, was then reduced by living cells to dark-blue formazan particles, that were solubilized in 10% sodium dodecyl sulfate (SDS, Sigma Chemical Co.) and 50% N,N-dimethyl formamide (Sigma Chemical Co.). The optical density was measured after 30 min at 570 nm in a plate reader (TP Reader, Thermo Plate).

2.4. Effect of enrofloxacin on the intracellular proliferation and invasion of T. gondii in HFF cells

HFF cells were cultured on 13-mm round glass cover-slips in 24-well plates (3 × 10^4 cells in a volume of 200 μl per well) for 24 h in RPMI medium with 10% FBS at 37 °C and 5% CO₂. Cells were infected with tachyzoites of the T. gondii RH strain at a ratio of 5 parasites per cell (5:1). After 3 h of incubation, cells were treated as follows: (i) medium (control without antibiotics), (ii) 100 U/ml penicillin/100 μg/ml streptomycin (negative control), (iii) 100 μg/ml (400 μM) sulfadiazine (positive control), or (iv) 25 μg/ml (69.5 μM) enrofloxacin in medium with 5% FBS at 37 °C and 5% CO₂. The effect of penicillin and streptomycin was analyzed because these drugs were used to obtain the parasite to infect cells in the present study. Additionally, they have no effect on the growth of T. gondii in vitro. After 72 h, the cells were washed in cold phosphate-buffered saline (PBS), fixed in 10% buffered formalin for 24 h, stained with 1% toluidine blue (Sigma Chemical Co.) for 5 s and mounted on glass slides. The cells were analyzed by light microscopy to verify the T. gondii infection index (number of infected cell per 200 examined cells) and parasite intracellular proliferation (total number of parasite per 200 examined cells). Next, the inhibition percentages of the infection index by T. gondii and the inhibition of intracellular proliferation in the presence of treatments were calculated as follows: the average infection index or intracellular proliferation analyzed in untreated cells corresponded to 100% on the infection index or intracellular proliferation, respectively. The inhibition percentages of these parameters under antibiotic treatments were calculated by subtracting the percentage values obtained in cells treated with each antibiotic from those obtained with untreated cells.

2.5. Effect of enrofloxacin in T. gondii plaque assay

In addition, the effects of antibiotics alone as well as the association between them in the control of T. gondii replication were analyzed by plaque assay, and pyrimethamine was included in these experiments, because the association of pyrimethamine plus sulfadiazine constitutes the standard treatment against T. gondii. At first, we performed experiments to verify the effect of pyrimethamine alone in cell viability, infection index and T. gondii intracellular proliferation using different concentrations of the drug. It was observed that the use of 5000 μg/ml of pyrimethamine do not interfere in the cell viability (data not shown); and 200 μg/ml was the better concentration that was able to control the parasite in HFF cells (data not shown).

For plaque assay analysis, HFF cells were grown into 24-well plates (1 × 10^5 cells/well/1000 μl) in RPMI medium with 10% FBS at 37 °C and 5% CO₂ for 72 h until reach confluence for plaque assays. Cells were infected by T. gondii tachyzoites (5 × 10^5 cells/well/100 μl), and after 3 h of incubation, they were treated or not with different antibiotics: (a) sulfadiazine (positive control, 100 μg/ml – 400 μM), (b) pyrimethamine (positive control, 200 μg/ml – 804 μM), (c) sulfadiazine (100 μg/ml) plus pyrimethamine (200 μg/ml), (d) enrofloxacin (25 μg/ml – 69.5 μM), (e) enrofloxacin (25 μg/ml) plus sulfadiazine (100 μg/ml), or (f) enrofloxacin (25 μg/ml) plus pyrimethamine (200 μg/ml), in medium with 5% FBS at 37 °C and 5% CO₂. After five-day cultures, cells were washed, fixed in 10% buffered formalin for 24 h and stained with 1% toluidine blue. The stained cells were analyzed under light microscope and images of microscopic fields were captured (Software HL Image Model 97++) to quantify the number of plaques (number of areas with lysed cells) and measure the plaque area (dimension of the areas with lysed cells).

2.6. Experimental animals

The effects of enrofloxacin were also verified in experimental models utilizing T. gondii infection with the ME49 and RH strains. For this purpose, C. callosus (Canabrava strain) or C57BL/6 mice were maintained in the Animal Experimentation Center, Biomedical Sciences Institute, Federal University of Uberlândia, Brazil. All experimental procedures were conducted according to institutional guidelines for animal ethics.

2.7. Effect of enrofloxacin on the infection of T. gondii in C. callosus

Fifteen females of C. callosus were infected orally with 20 cysts of the T. gondii ME49 strain. After 5 days of infection, the females were divided into 3 treatment groups of 5 animals: sulfadiazine (group 1), enrofloxacin (group 2), and the control group or PBS treatment (group 3). In group 1, the animals were treated orally with sulfadiazine (500 μg/ml) diluted in filtered drinking water for the course of 25 days. In group 2, the animals were subcutaneously treated with 3 mg/kg enrofloxacin diluted in PBS for three consecutive days. Following the initial treatment, they received enrofloxacin treatment twice a week for the duration of the 25-day treatment. Group 3 received a similar treatment protocol as group 2; however, instead of enrofloxacin, they received PBS alone. Five days following the treatments (35 days of infection), the animals were intraperitoneally injected with anesthetics ketamine (Syntec Brasil Ltda, São Paulo, Brazil) and xylazine (Schering-Plough Coopers, São Paulo, Brazil) and were sacrificed by cervical dislocation. The brains of the animals were collected, fixed in 10% buffered formalin, and processed for paraffin embedding.
and sectioning. The brain tissue sections were submitted for histological analysis to detect inflammatory changes and perform an immunohistochemical assay for the detection of *T. gondii*.

### 2.8. Quantification of tissue parasitism by immunohistochemistry

Tissue parasitism was evaluated in the organs by immunohistochemistry as previously described (Silva et al., 2010). To detect specific parasite antigens, deparaffinized sections were incubated at room temperature with PBS plus 3% (w/v) nonfat milk (Nestlé Brasil Ltda., São Paulo, Brazil) to reduce nonspecific binding and then incubated at 4 °C overnight in polyclonal mouse anti-*T. gondii* serum (produced by our laboratory by infecting a Swiss mouse with the ME-49 strain of *T. gondii*) diluted in 0.01% saponin. After incubation with biotinylated goat anti-mouse antibodies (Sigma Chemical Co.), we utilized the avidin–biotin complex (ABC kit, PK-4000; Vector Laboratories, Inc., Burlingame, USA) to improve sensitivity. The reaction was visualized by incubating the section with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co.) for 5 min. Control slides were incubated with mouse serum from non-infected mice. The sections were counterstained with Harris’s hematoxylin and examined by light microscopy using a 40× objective. The numbers of cyst-like structures and parasitophorous vacuoles were quantified per brain tissue section.

### 2.9. Histological analysis

The histological alterations seen in the brain were analyzed from tissue sections that were stained by hematoxylin and eosin (HE). The inflammatory score in the brain was determined in two non-contiguous sections (40 μm distance between them) and from five animals per group, as previously described (Silva et al., 2010). The total numbers of focal (glial nodules) or diffuse inflammatory infiltrates were counted in a sagittal section. The cuffing of blood vessels and inflammatory cell infiltrates in the meninges were also analyzed. The inflammatory score was represented as arbitrary units: 0–2, mild; 2.1–4, moderate; 4.1–6, severe; and above 6, very severe. All analyses were done using a 40× objective in a blind manner by two different researchers.

### 2.10. Effect of enrofloxacin in the infection of C57Bl/6 mice by *T. gondii* RH strain

Eighteen females of C57BL/6 mice were infected intraperitoneally with 100 tachyzoites RH strain. One day after infection, the females were divided into 3 groups of 6 animals: sulfadiazine (group 1), enrofloxacin (group 2), and control group or PBS treatment (group 3). In group 1, the animals were treated orally with sulfadiazine (500 μg/ml) diluted in filtered drinking water. In group 2 and group 3, the animals were treated with enrofloxacin 3 mg/kg diluted in PBS or PBS only, respectively, by subcutaneous injection during three days consecutively; and after, twice a week. The animals were observed daily to monitor mortality.

#### 2.11. Statistical analysis

The data were analyzed using GraphPad Prism 4 software package (GraphPad Software Inc., San Diego, USA). Data were expressed as mean ± standard error mean (SEM) of experimental groups. The comparison of data obtained with cells treated with different drugs and, tissue parasitism enumeration and inflammatory score in animals treated with different drugs was analyzed by ANOVA (one way) and post-test of multiple comparison of Bonferroni. The Kaplan–Meier method was used to compare the survival rates of the sulfadiazine, enrofloxacin and PBS-treated groups infected with RH strain of *T. gondii* and the survival curves were compared using logrank and chi square tests that generate a two-tail *P* value, in order to test the null hypothesis that they were identical in the overall groups of animals. Differences were considered statistically significant when *P* < 0.05.

### 3. Results

#### 3.1. Cellular viability is not decreased by enrofloxacin treatment

To determine whether enrofloxacin treatment was toxic to the cells, we investigated cell viability using the MTT assay following treatments with penicillin/streptomycin, enrofloxacin or sulfadiazine in HFF cells. The treatments with sulfadiazine or enrofloxacin at several concentrations (ranging from 1.56 to 200 μg/ml) did not reduce cell viability in HFF cells in relation to cells treated with medium alone (Fig. 1A and B). Additionally, the penicillin/streptomycin treatment did not reduce cell viability (data not shown).

#### 3.2. *T. gondii* infection in HFF cells is reduced by enrofloxacin treatment

Different concentrations of enrofloxacin (ranging from 1.56 to 200 μg/ml) were used in experiments of HFF cell culture and infection with *T. gondii* to determine the doses that was able to control parasite proliferation in vitro, and 25 μg/ml was defined as the minimal concentration to be used (data not shown). Related to sulfadiazine, it was previously shown that 100 μg/ml of sulfadiazine was efficient to control *T. gondii* proliferation in HFF cells (Oliveira et al., 2009). Thus, the concentrations used in subsequent experiments were 100 μg/ml for sulfadiazine and 25 μg/ml for enrofloxacin. Next, we investigated the effect of enrofloxacin on the control of parasite proliferation in HFF cells in comparison with another drugs. Cells were infected by *T. gondii* and treated with penicillin/streptomycin, sulfadiazine, or enrofloxacin for 72 h and the infection index and parasite intracellular proliferation were quantified in 200 examined cells.

The penicillin/streptomycin treatment was not able to control parasite proliferation according to both the infection index (133.2 ± 5.43) and parasite intracellular
Table 1
Summary of the data obtained for infection index (cellular invasion) and parasite intracellular proliferation in HFF cells, evidencing total number (mean ± SEM) as well as inhibition (%) of both parameters after each treatment (100 U/ml penicillin/100 μg/ml streptomycin, 100 μg/ml sulfadiazine, or 25 μg/ml enrofloxacin).

<table>
<thead>
<tr>
<th>Treatments (antibiotics)</th>
<th>Infection index (mean ± SEM)</th>
<th>Inhibition of infection index by T. gondii (%)</th>
<th>Parasite proliferation (mean ± SEM)</th>
<th>Inhibition of T. gondii intracellular proliferation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium (control)</td>
<td>142.3 ± 19.55</td>
<td>-</td>
<td>1273.6 ± 133</td>
<td>-</td>
</tr>
<tr>
<td>100 U/ml penicillin</td>
<td>133.2 ± 5.43</td>
<td>6.4%</td>
<td>1174 ± 91.82</td>
<td>7.83%</td>
</tr>
<tr>
<td>100 μg/ml streptomycin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfadiazine (100 μg/ml)</td>
<td>104.5 ± 11.03**</td>
<td>26.57%**</td>
<td>707.7 ± 77.45***</td>
<td>44.44%***</td>
</tr>
<tr>
<td>Enrofloxacin (25 μg/ml)</td>
<td>58.75 ± 8.34***</td>
<td>58.72%***</td>
<td>510.5 ± 66.64***</td>
<td>60%***</td>
</tr>
</tbody>
</table>

HFF cells were cultured on 13-mm round glass coverslips (3 × 10^4 cells/well/200 μl), infected with T. gondii tachyzoites RH strain at a proportion of 5 parasites per cell and treated with penicillin/streptomycin, sulfadiazine, or enrofloxacin.

a Number of infected cells per 200 examined cells.

b Parasite intracellular proliferation: total number of parasite per 200 examined cells.

c The average of total number of cellular invasion or intracellular proliferation detected in cells non-treated with antibiotic corresponded to 100% of infection index or intracellular proliferation, respectively; the percentages of reduction of the infection index and intracellular proliferation under antibiotics treatments were calculated by subtracting the values obtained in cells treated with each antibiotic from those obtained by non-treated cells.

**P < 0.01 or ***P < 0.001 in comparison with medium (control) or penicillin and streptomycin, and *P < 0.001 in comparison with sulfadiazine treatment.

Fig. 1. Cellular viability verified by MTT assay in HFF cells treated with sulfadiazine (A) or enrofloxacin (B) in several concentrations ranging from 1.56 to 200 μg/ml. The cells were cultured in RPMI 1640 medium with 5% FBS for 72 h at 37°C and 5% CO2.

proliferation (1174 ± 91.82) in relation to untreated cells (142.3 ± 19.55 and 1273.6 ± 133, respectively) (Table 1). However, sulfadiazine treatment significantly reduced both the infection index (104.5 ± 11.03) and intracellular proliferation (707.7 ± 77.45) when compared to the untreated cells (P < 0.01 and P < 0.001, respectively) or the penicillin/streptomycin-treated cells (P < 0.05 and P < 0.001, respectively) (Table 1). Similar to the sulfadiazine treatment, enrofloxacin was able to reduce the infection index (58.75 ± 8.34) and intracellular proliferation (510.5 ± 66.64) in relation to the untreated cells (P < 0.001) or the penicillin/streptomycin-treated cells (P < 0.001) (Table 1). Interestingly, enrofloxacin treatment was more effective at decreasing the infection index of T. gondii in HFF cells than sulfadiazine treatment (58.75 ± 8.34 versus 104.5 ± 11.03, P < 0.001) (Table 1). It was also observed that enrofloxacin and sulfadiazine resulted in a significant inhibition of the percentage of infected cells by the parasite (58.72% and 26.57%, respectively) (Table 1) relative to untreated cells. Additionally, sulfadiazine treatment induced a significant reduction in the percentage of intracellular replication (44.44%); however, enrofloxacin was more efficient, reducing parasite proliferation by 60% (Table 1).

As pyrimethamine plus sulfadiazine is the standard toxoplasmosis treatment, we use the plaque assay to observe the efficacies of enrofloxacin alone compared with pyrimethamine alone or with the associations of sulfadiazine plus pyrimethamine, enrofloxacin plus sulfadiazine or enrofloxacin plus pyrimethamine treatments in HFF infected cells. It was verified that penicillin/streptomycin treatment was not able to reduce the number of plaques in relation to untreated cells (medium) (Fig. 2A). Smaller plaque numbers were detected when sulfadiazine was added to the infected cell culture compared to untreated or penicillin/streptomycin-treated cells (P < 0.001, P < 0.05, respectively) (Fig. 2A). Each pyrimethamine or enrofloxacin treatment alone or the associations of sulfadiazine plus pyrimethamine or enrofloxacin plus sulfadiazine or enrofloxacin plus pyrimethamine treatments reduced significantly the number of plaques when compared
to untreated or penicillin/streptomycin or sulfadiazine treated cells (P < 0.001, P < 0.05, P < 0.001, respectively) (Fig. 2A). In addition, the plaque areas were reduced when sulfadiazine (P < 0.05), pyrimethamine (P < 0.01) or enrofloxacin (P < 0.01) were added in the infected cells in comparison with untreated or penicillin/streptomycin treated cells (P < 0.01) (Fig. 2B). These data show that either sulfadiazine or pyrimethamine or enrofloxacin treatments are able to control T. gondii in HFF cells infected by RH strain; in addition, the effect of enrofloxacin or pyrimethamine are more pronounced to control the parasite replication in vitro.

3.3. Enrofloxacin reduces the T. gondii parasite load in the brain of C. callosus

In order to observe the effect of drugs in the control of ME-49 strain of T. gondii, which cause a chronic infection, C. callosus were infected orally with 20 cysts. It was observed that tissue parasitism, as detected by immunohistochemistry (cyst-like structures plus parasitophorous vacuoles), was significantly reduced in the brains of Calomys treated with sulfadiazine (11.18 ± 3.26, P < 0.05) or enrofloxacin (17.56 ± 10.82, P < 0.05) in relation to PBS-treated animals (54.4 ± 10.80) (Fig. 3).

3.4. Enrofloxacin treatment decreased inflammation in the brain of T. gondii-infected C. callosus

On day 35 of T. gondii infection, the brains from infected animals presented lesions that were characterized by glial nodules, vascular cuffing by lymphocytes and focal mononucleated cell infiltrates in the meninges (Fig. 4A–C). Diffuse infiltrates of mononuclear cells were also found. The inflammatory changes were intense in the brain of C. callosus PBS-treated (Fig. 4A and D). Interestingly, both the sulfadiazine and enrofloxacin treatments decreased inflammation in the organ of T. gondii-infected animals in comparison with PBS-treated Calomys (P < 0.05) (Fig. 4B–D).

3.5. C57BL/6 mice presented highly susceptible to infection with RH strain of T. gondii independently of enrofloxacin or sulfadiazine treatments

In order to verify the effect of enrofloxacin in infection with the highly virulent RH strain of T. gondii, using a highly susceptible lineage of mice, C57BL/6 mice were infected
with 100 tachyzoites of the parasite. The enrofloxacin or sulfadiazine treatments were not able to control the parasite infection, as the animals did not present a significant difference in terms of the susceptibility when compared with PBS-treated mice (Fig. 5), despite antibiotics-treated mice showed a trend to survive later.

4. Discussion

As current toxoplasmosis treatments present numerous problems with toxicity (Degerli et al., 2003; Schmidt et al., 2006), in the present study, we aimed to determine if enrofloxacin was able to control T. gondii infection in experimental models, as this antibiotic has been successfully used in veterinary medicine.

First, we determined the toxicity of enrofloxacin treatment in HFF cells. It was observed that enrofloxacin or sulfadiazine in doses used in this experimental work did not result in cell cytotoxicity. After ensuring the experimental doses given were not toxic, we observed...
that sulfadiazine or enrofloxacin treatment significantly reduced the number of infected cells, parasite intracellular proliferation, and the number of plaques in comparison with untreated cells. Also, pyrimethamine, pyrimethamine plus sulfadiazine or pyrimethamine plus enrofloxacin diminished significantly the number of plaques in relation to untreated cells. In addition, enrofloxacin showed to be more effective against *T. gondii* infection in our *in vitro* experimental model than sulfadiazine treatment; and the combination of enrofloxacin plus sulfadiazine improved the effect of sulfadiazine. This is the first study to demonstrate the effect of enrofloxacin against *T. gondii* infection in vitro.

In our experimental protocol we treated cells after 3 h of *T. gondii* infection, and enrofloxacin was able to inhibit either number of infected cells or intracellular proliferation. We also carried out experiments treating the parasites before infection the host cells, and enrofloxacin was not able to control parasite proliferation (*data not shown*). This is interesting because, in a natural infection, treatment normally starts after the infection of host cells by the parasite.

Additional alternative drugs have shown useful effects against *T. gondii in vitro*. Azasterols, developed as inhibitors of sterol biosynthesis, demonstrated an ability to inhibit *T. gondii* proliferation (*Dantas-Leite et al., 2004; Martins-Duarte et al., 2011*). The azasterols reduced *T. gondii* proliferation in LLC-MK2 cells by acting against the mitochondria of the parasite (*Dantas-Leite et al., 2004*). In addition, the azasterols were able to induce structures similar to amyolectin granules in the tachyzoites of the RH strain cultured in LLC-MK2 cells. This suggests that azasterols promote the differentiation of tachyzoites to bradyzoites (*Martins-Duarte et al., 2011*).

In the present study, it was also analyzed the effect of enrofloxacin in *in vivo* in chronic *T. gondii* infection using the rodent *C. callosus*. Similar to sulfadiazine, we found that enrofloxacin significantly diminished the number of parasites of the ME-49 strain in the brains of infected animals. However, sulfadiazine was more effective than enrofloxacin in controlling infection in *C. callosus*, as seen with an inhibition of 79.45% of parasite detection in the brain versus 67.73% for enrofloxacin treatment. The effect of enrofloxacin in *in vivo* was also analyzed in acute phase of infection with a highly virulent strain of the parasite, RH, in C57BL/6, a mouse lineage that are highly susceptible to toxoplasmosis. It was observed that the C57BL/6 enrofloxacin or sulfadiazine-treated mice presented very susceptible to infection with RH strain of *T. gondii*. Swiss Webster mice treated with sulfadiazine were more resistant to the infection with RH strain compared to non-treated mice (*Alves and Vitor, 2005; Ferreira et al., 2006*), thus, the different results obtained with our experiments is probably due to the C57BL/6 mouse lineage used. In contrast to our *in vivo* studies, enrofloxacin was more effective than sulfadiazine against the RH strain of *T. gondii in vitro*. Therefore, it is plausible to speculate that enrofloxacin may be more active against infection in human cells (HFF) or that the efficacy of this drug differs either based on the strain of the parasite or the animal experimental model used, since C57BL/6 is a susceptible lineage of mouse and the RH strain is a highly virulent strain of the parasite. This is the first study demonstrating the effect of enrofloxacin against *T. gondii* infection in *vivo*. Additional studies are necessary to verify the efficacy of enrofloxacin treatment using parasite strains that are genetically different and other animal experimental models.

Previous studies have shown the effects of other drugs against *T. gondii* infection in *vivo* (*Costa et al., 2009; Oliveira et al., 2009*). *A. annua* infusion treatment was able to decrease and abolish the mortality of C57BL/6 mice infected with the RH and ME-49 strains of *T. gondii*, respectively (*Oliveira et al., 2009*). *A. annua* infusion and azithromycin reduced and impaired the vertical transmission of the *T. gondii* ME49 strain in *C. callosus*, respectively (*Costa et al., 2009*). Previous studies have demonstrated the effect of enrofloxacin against protozoan parasites (*Gottstein et al., 2005; Delepaux et al., 2010*). The combined treatment of isometamidium chloride and enrofloxacin was crucial to control infection by *Trypanosoma congolense* in cattle. All treated animals had a significantly longer prepatent period than animals treated only with isometamidium chloride (*Delepaux et al., 2010*). Enrofloxacin partially reduced parasitism in the brains of mice infected with *N. caninum*, however, the drug was ineffective in controlling tissue inflammation in these animals (*Gottstein et al., 2005*). It was previously reported that the CNS of C57BL/6 mice undergoes an intense and progressive inflammatory reaction that is insufficient to control *T. gondii* replication and may be detrimental to the host (*Silva et al., 2010*). Therefore, significant immunoregulation is necessary to avoid immunopathology (*Gazzinelli et al., 1996; Neyer et al., 1997; Suzuki et al., 2000*). Our present investigation demonstrates that enrofloxacin significantly diminished the parasite load and brain inflammation in *C. callosus* infected by *T. gondii*. In addition to the diminished parasite load in the brain, the lower inflammatory changes in the organ observed in animals enrofloxacin-treated is important because intense inflammation in the brain is detrimental to the host.

In conclusion, it is critical to find out new therapeutic strategies for toxoplasmosis treatment that minimizes the toxic effects to the host and increases the treatment efficacy against the parasite. Thus, our findings demonstrate that enrofloxacin is a potential alternative drug for toxoplasmosis treatment, as demonstrated with the observed significant effects against *T. gondii* infection in both *in vitro* and *in vivo* experimental models.

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