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PROTOZOOLOGY - ORIGINAL PAPER

Development of an in vitro system to study the developmental stages of *Toxoplasma gondii* using a genetically modified strain expressing markers for tachyzoites and bradyzoites

J.A. Portes[1,2,3] · W. De Souza[1,2,3]

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Abstract

Toxoplasma gondii, the agent of toxoplasmosis, is an intracellular parasite that can infect a wide range of vertebrate hosts. Toxoplasmosis causes severe damage to immunocompromised hosts and its treatment is mainly based on the combination of pyrimethamine and sulfadiazine, which causes relevant side effects primarily observed in AIDS patients, including bone marrow suppression and hematological toxicity (pyrimethamine) and/or hypersensitivity and allergic skin reactions (sulfadiazine). Thus, it is important to investigate new compounds against *T. gondii*, particularly those that may act on bradyzoites, which are present in cysts during the chronic disease phase. We propose an in vitro model to simultaneously study new candidate compounds against the two main causative stages of *Toxoplasma* infection in humans, using the EGS-DC strain that was modified from a type I/III strain (EGS), isolated from a case of human congenital toxoplasmosis in Brazil and engineered to express markers for both stages of development. One feature of this strain is that it presents tachyzoite and bradyzoite in the same culture system and in the same host cell under normal culture conditions. Additionally, this strain presents stage-specific fluorescent protein expression, allowing for easy identification of both stages, thus making this strain useful in different studies. HFF cells were infected and after 4 and 7 days post infection the cells were treated with 10 μM of pyrimethamine or atovaquone, for 48 or 72 h. We used high-throughput screening to quantify the extent of parasite infection. Despite a reduction in tachyzoite infection caused by both treatments, the atovaquone treatment reduced the bradyzoite infection while the pyrimethamine one increased it. Ultrastructural analysis showed that after treatment with both drugs, parasites displayed altered mitochondria. Fluorescence microscopy of cells labeled with MitoTracker CMXRos showed that the cysts present inside the cells lost their mitochondrial membrane potential. Our results indicate that this experimental model is adequate to simultaneously analyze new active compounds against tachyzoite and bradyzoite forms.

Keywords *Toxoplasma gondii* · Bradyzoites · Tachyzoites · EGS strain · Atovaquone · Pyrimethamine

Introduction

Toxoplasma gondii is an intracellular protozoan parasite with worldwide distribution and it is the causative agent of toxoplasmosis, which occurs in warm-blooded animals, including humans (Levine et al. 1980; Lyons and Johnson 1995). The parasite presents three infective forms: sporozoites released from the oocysts produced during the sexual cycle that occurs exclusively in the felines' gut, the definitive hosts of the parasite; tachyzoites, intracellular stages found in the toxoplasmosis' acute phase in the hosts; and bradyzoites, which are present inside tissue cysts and commonly found in the brain and skeletal muscle in the chronic phase of the infection (Hill et al. 2005). Although only occurring in felines, a recent work has shown that the sexual cycle can also be reproduced in vitro

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✉ W. De Souza
wsouza@biof.ufrj.br

[1] Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, UFRJ, Av. Carlos Chagas Filho 373, Ilha do Fundão, Rio de Janeiro, RJ, Brazil

[2] Centro de Ciências da Saúde-UFRJ, Av. Carlos Chagas Filho 373, Ilha do Fundão, Rio de Janeiro, RJ, Brazil

[3] Instituto Nacional de Ciência e Tecnologia em Biologia Estrutural e Bioimagens, UFRJ, Av. Carlos Chagas Filho s/n, Ilha do Fundão, Rio de Janeiro, RJ, Brazil

and in vivo in mice after supplemented with linoleic acid and/or inhibition of the enzyme delta-6-desaturase, which is required for linoleic acid metabolism. This enzyme is absent in felines, resulting in systemic excess of linoleic acid, which probably is a crucial factor to the oocysts production and explain the exclusivity to the microenvironment of the felines' organism (Di Genova et al. 2019). When the hosts are infected for the first time by tissue cysts or oocyst ingestion, bradyzoites or sporozoites, respectively, infect and pass through enterocytes where are converted into tachyzoites that multiply rapidly inside the host cells, causing the lytic cycle, which occurs in the acute phase of the infection (Sullivan Jr et al. 2009; Speer and Dubey 1998). In the intermediate hosts, under pressure from the host's immune system, tachyzoites may transform within the cells into bradyzoites that divide more slowly and produce a cyst wall, forming the cyst (Denkers and Gazzinelli 1998). The influence of the host on *Toxoplasma* stage differentiation has been studied (Lüder and Rahman 2017) and a lot of factors can trigger the conversion from tachyzoites to bradyzoites and the cysts generation in in vitro systems, such as follows: cell type (skeletal muscle cells and neurons) (Luder et al. 1999; Ferreira-da-Silva et al. 2008, 2009a); modulators molecules like nitric oxide, produced by IFN-γ stimulated macrophages (Bohne et al. 1994); starvation and absence of molecules which are essential to the parasite (Eaton et al. 2006; Fox et al. 2004); pH and temperature variation; and treatments with drugs (Soete et al. 1994; Weiss et al. 1995; Soete and Dubremetz 1996). Reactivation of the acute infection may occur when the immune response decreases and bradyzoites from cysts convert into tachyzoites that are able to proliferate. Primary or recurrent infections in immunocompromised patients produce significant morbidity, including toxoplasmic encephalitis and ocular toxoplasmosis (Sullivan Jr et al. 2009; Fergurson et al. 1989). The susceptibility of fetus in formation also gives an important place to the congenital transmission. In Brazil, there is a high prevalence of congenital infections, mainly in Minas Gerais State, with one case of the disease for every 770 live births, and 79.8% develop retinochoroiditis (Vasconcelos-Santos et al. 2009). Toxoplasmosis is considered to be a neglected disease especially because it affects people of low socioeconomic status (Carellos et al. 2014).

T. gondii infection has recently been associated with the development of neuropsychiatric disorders such as schizophrenia and depression (Halonen and Weiss 2013; Prandota 2014). It is consistent with its particular tropism to the brain (Elsheikha et al. 2016). Recently, *Toxoplasma* has emerged as a threat in South America and Africa because of the recognition of some strains that are highly virulent. Besides the three main lineages from North America and Europe, which are designated types I, II, and III (Boothroyd and Grigg 2002), strains that do not belong to these main lineages have been isolated in other continents (Lehmann et al. 2006). Type I strains are more virulent and lethal, and types II and III are considered avirulent and are associated with chronic infections (Howe and Sibley 1995). The newly isolated strains are generally more pathogenic and even lethal to immunocompetent individuals, as seen in French Guiana and Suriname (Dardé 2008; Carme et al. 2009). These strains are also responsible for severe eye diseases and blindness (De Moura et al. 2006). The mechanisms of replication, migration, virulence, and differentiation to bradyzoites may vary according to the parasite strain: parasites of the type I RH strain, for example, differentiate spontaneously into bradyzoites, but at a low rate (3%) when infecting primary culture from muscle tissue (Ferreira-da-Silva et al. 2009a, b); the type I strain named GT-1, an isolate from goat skeletal muscle, is able to generate cysts in culture using either sporozoites, tachyzoites, or bradyzoites as inoculum (Lindsay et al. 1991); other strains, such as the Brazilian EGS strain, a recombinant from type I/III isolated from human amniotic fluid, is a virulent and cystogenic strain when it infects mice and has a high rate of tachyzoite-bradyzoite conversion with formation of cysts in cell cultures. Therefore, this strain is an excellent tool to study *Toxoplasma* development (Paredes-Santos et al. 2013). To make this tool more functional, Paredes-Santos et al. developed parasites from the EGS strain that were genetically modified and that combine the double expression of stage-specific fluorescent proteins, allowing the identification of tachyzoites and bradyzoites during culture and monitoring of cystogenesis in in vitro and in vivo models (Paredes-Santos et al. 2016).

Although *T. gondii* is a model organism for intracellular Apicomplexa parasites and there is reasonable knowledge about its biology (Hakimi and Bougdour 2015; Coffey et al. 2016; Francia et al. 2016), toxoplasmosis treatment is limited to the use of compounds that present some toxicity to the host. Currently, the most effective and used therapy for toxoplasmosis is based on the simultaneous use of two antifolate compounds: sulfadiazine and pyrimethamine (Montoya and Liesenfeld 2004). These compounds act synergistically, blocking the metabolic pathway that drives the synthesis of folic and folinic acid, respectively (Georgiev 1994), thereby affecting the production of thymidine and parasite replication. Although effective, this therapy is often associated with the occurrence of several side effects, which are mainly observed in patients with AIDS, such as suppression of bone marrow and hematological toxicity (pyrimethamine) and/or hypersensitivity and allergic skin reaction (sulfadiazine) (Haverkos 1987; Leport et al. 1988; Georgiev 1994). Other medications such as clindamycin, which targets the parasite's protein translation, could be used in cases of allergy to sulfa drugs, but this alternative is less effective and also has toxicity (Katlama et al. 1996). Atovaquone or azithromycin may also be used in combination with pyrimethamine or sulfadiazine for the treatment or prophylaxis against toxoplasmosis when the standard therapy is contraindicated (Torres et al. 1997; Jacobson et al.

2001; Chirgwin et al. 2002). Additionally, these compounds are mainly active against tachyzoites, with little to no activity against the bradyzoites in the cysts (Halonen and Weiss 2013), such as atovaquone that has shown to reduce cyst burden in experimentally infected mice (Araujo et al. 1992; Doggett et al. 2012).

Most of the work related to experimental chemotherapy against toxoplasmosis uses tachyzoites as the main target because of the ease of testing using cell cultures and their sensitivity to the compounds. There is little research in vitro against bradyzoites. As mentioned above, the EGS strain allows simultaneous observation of tachyzoites and bradyzoites in the same cellular system. Therefore, we analyzed the strain, in its form that expresses the stage-specific fluorescent proteins, named EGS DC, as a model to analyze the effects of compounds against both forms of *T. gondii*. Here, we describe experiments to validate the model using compounds that were previously shown to be as active as pyrimethamine and atovaquone against the parasite. To accelerate the process for screening many compounds in the future, we used automatized and sensitive methods such as high-throughput screening (HTS) to facilitate the data acquisition process and to make the data more robust (Freitas-Junior et al. 2012).

Materials and methods

Toxoplasma gondii

The parasites used belong to an atypical strain, type I/III, that show infection with bradyzoites, could be presenting cysts, and also rosettes, as a result of tachyzoite cell division. This strain was originally isolated from human amniotic fluid with congenital toxoplasmosis (Vidigal et al. 2002). A stage-specific reporter version of the *T. gondii* EGS strain was developed by Paredes-Santos et al. (2016). It introduced the fluorescent proteins mCherry and GFP, whose expression is driven by the tachyzoite- and bradyzoite-specific promoters Sag1 and Bag1, respectively. These parasites were maintained by in vitro passages in human foreskin fibroblast (HFF; kindly donated by Sheila Nardelli—ICC/FIOCRUZ-BR) cell culture and parasites from the supernatant were collected and centrifuged at 1000×*g* for 10 min before use. These parasites with dual expression were called EGS DC and the wild type was called EGS WT.

Host cell

HFF cells used for the assays were cultured in 25 cm^2 culture flasks (SPL Life Sciences) with DMEM high glucose medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% antibiotic antimycotic solution (penicillin, streptomycin, and amphotericin B; Sigma Aldrich, St. Louis, MO, USA) at 37 °C in a 5% CO_2 atmosphere. Parasites infected cells in confluent cultures in flasks, over coverslips in 24-well tissue culture plates (SPL Life Sciences), or in cells in 96-well tissue culture plates (Corning® 96-Well Black Polystyrene Microplate).

Atovaquone and pyrimethamine

For in vitro studies, these drugs were dissolved in dimethylsulfoxide (DMSO) and then stored at − 22 °C. At the concentrations used, did not exceed 0.05% (v/v), DMSO did not interfere with the host cell's and parasite's viability (Da Silva et al. 2015).

Parasite infection assay

HFF cells were seeded over coverslips in a 24-well plate or in a 96-well plate 4 days before the assay. Cells in confluence were infected with parasites using 1×10^5 parasites per well for experiments in 24-well plates or 5×10^4 parasites per well in 96-well plates. Parasites were grown in the host cells for 7 days for experiments in 24-well plates to study the long-term infection profile, and after this period, the cells were treated with atovaquone or pyrimethamine or left untreated and maintained in the absence of these compounds. Parasites also were grown in the host cells for 4 days for experiments in 96-well plates to perform studies that were analyzed using a high-throughput method, and after this period, the cells were treated as described above. The drugs were used at 10 μM, a value which is higher than the IC50 of both drugs against *T. gondii* (RH strain). IC50 of atovaquone is 138 nM (Doggett et al. 2012), and that of pyrimethamine is approximately 3 μM (Abugri et al. 2017). We supposed that the treatment with a higher concentration could present a more marked effect on the bradyzoite forms. The treatment lasted up to 72 h and then the infection/rate of the parasite growth was analyzed by counting cells in 96-well plates using automatized fluorescence microscopy. At least three independent experiments were carried out. Statistical analysis was made using the Student's *t* test.

Automatized microscopy

To evaluate parasite growth inside HFF cells, samples were prepared as described above to 96-well plates, and after different time points of growth and treatment, they were incubated with Hoechst 33342 (Trihydrochloride, Trihydrate; Thermo Fisher) (2 μg/mL) in RPMI 1640 (Sigma Aldrich) phenol red free supplemented with 10% FBS, 1% L-glutamine, and 1% antibiotic antimycotic solution (Sigma Aldrich) at 37 °C in a 5% CO_2 atmosphere for at least 30 min before observation. Cells were maintained alive throughout the experiment and the fluorescence images (4

images per well) were acquired from each assay well using the fluorescence automatized light microscope ImageXpress Micro (Molecular Devices) with a ×20 objective lens magnification. The acquired images were analyzed with MetaXpress® High-Content Image Acquisition and Analysis software. The software accessed the image acquired and created a flow of images, which were sequentially analyzed by algorithms developed in the software. The parameters recognized by the software to quantify the burden of parasite infection were the specific fluorescence emitted and its intensity in relation to the background (mCherry to tachyzoites and GFP to bradyzoites), and the size of the structure to be quantified. Tachyzoites are easily individually detected with the size 2 to 7 μm set, while bradyzoite, as it is usually grouped in cystic structures, is difficult to detect each individual organism; the detection included structures from 1 to 50 μm. When *Toxoplasma* undergoes successive replication cycles (both tachyzoite or bradyzoite stages), the detection of individual parasites is impaired. In this case, the formed parasite clusters are eventually detected as a unit by the software. If the structure on the image had the specific fluorescence intensity and the size that were set by the operator, the software will quantify it as a unit and an Excel table with the data will be generated. The term "parasite growth" was used to express the rate of infection in cells by tachyzoites and bradyzoites, so the system could detect fluorescent spots, which are representative of parasite vacuoles or cysts, and also individual parasites. The image acquisition process and analysis from microscope ImageXpress Micro (Molecular Devices) system are similar to the system used by Siqueira-Neto et al. (2012).

Fluorescence microscopy

HFF cells grown over coverslips in 24-well plates were infected with the *T. gondii* EGS DC strain for 7 days and were then treated with atovaquone or pyrimethamine at 10 μM for another 48 h, as described above. Samples were fixed with 4% freshly prepared formaldehyde in PBS, pH 7.2, washed with PBS, and the cells were mounted in ProLong Gold with or without 4′,6-diamidino-2-phenylindole (DAPI). Samples were observed using a Zeiss LSM-710 confocal laser scanning microscope.

Mitochondrial membrane potential analysis

To evaluate the mitochondrial membrane potential of the parasites in cysts (indirect viability assay), HFF cells grown over coverslips in 24-well plate were infected with EGS WT parasites and treated to undergo "Automatized Microscopy." Cells were incubated with MitoTracker CMXRos at 0.1 μM for 45 min at 37 °C and were protected from light (adapted from Lin et al. 2009). Samples were washed with PBS and fixed with 4% using 1% Triton X-100 in PBS for 10 min, incubated with 100 mM NH_4Cl for 30 min, and then incubated with PBS containing 3% bovine serum albumin (PBS-BSA) for 30 min at room temperature. Cells were incubated for 1 h in the presence of *Dolichos biflorus* lectin conjugated to fluorescein isothiocyanate (DBA-FITC) at 10 μg/mL (Sigma Aldrich Co.). The cells were washed with PBS, and coverslips with cells were mounted in ProLong Gold with or without DAPI. The percentage of cysts (DBA-FITC positive) that were labeled or not with MitoTracker CMX-Ros was determined using a fluorescence microscope (Zeiss Axio Observer), with a ×100 objective and the filters FITC (excitation 490; emission 525) for DBA-FITC detection and rhodamine (excitation 551; emission 573) for MitoTracker detection. A total of 50 DBA positive cysts were analyzed in each coverslip. This quantitative data represents the mean of the values of one experiment did in triplicate for each condition.

Transmission electron microscopy

For transmission electron microscopy, cells cultivated in 25 cm^2 flasks were infected with 1.5×10^6 parasites from EGS DC for 7 days and treated with atovaquone or pyrimethamine at 10 μM for another 48 h. After treatment, cells were fixed for 1 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Cells were scraped off the flasks with a rubber scraper, washed with sodium cacodylate buffer, and post-fixed for 1 h in the dark with a solution containing 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Cells were washed, dehydrated in acetone, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a FEI Tecnai Spirit transmission electron microscope.

Results

The genetically modified EGS DC strain is a useful tool to determine the effect of new compounds on *T. gondii* bradyzoite and tachyzoite growth. After an initial infection of HFF cells with tachyzoites of the EGS DC strain, this developmental stage predominated for 7 days of infection (Fig. 1a, b). They divide by endodiogeny, forming grouped parasites expressing the protein SAG1-mCherry. Bradyzoites expressing BAG1-GFP also appear indicating the possibility of cyst development (Fig. 1a, b). These tachyzoites and bradyzoites from the EGS DC strain grown in HFF cells for 7 days were, since this time, treated with atovaquone or pyrimethamine at 10 μmol L^{-1} for another 48 h, or left untreated. Many cells infected by parasites of this strain may present both parasite stages, as shown in an untreated cell (Fig. 1a, b). The effect of any compound on tachyzoites and bradyzoites can be evaluated using the same model of study, because here it was checked after atovaquone (Fig. 1c–f) and

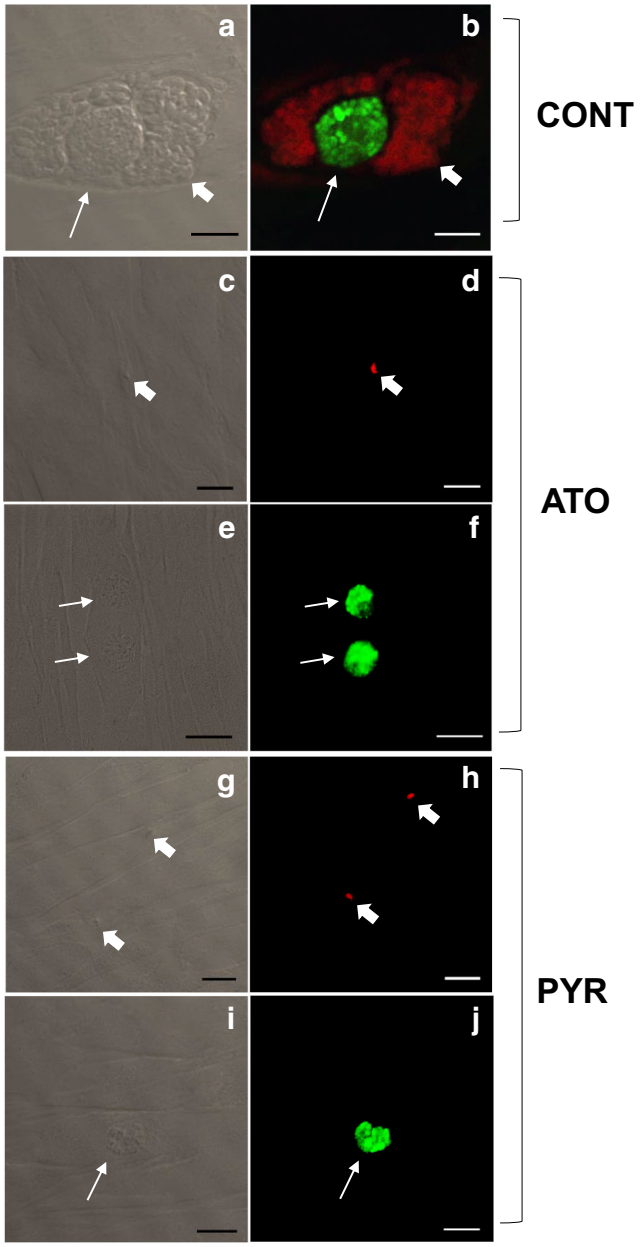

Fig. 1 Confocal laser scanning microscopy images showing tachyzoites and bradyzoites from the EGS strain, which were grown inside HFF cells for 7 days after infection and were then treated with atovaquone (**c**–**f**) or pyrimethamine (**g**–**j**) at 10 μmol L^{-1} for another 48 h or left untreated (**a**, **b**). **b**, **d**, **f**, **h**, **j** Fluorescence of tachyzoites (red fluorescence: mCherry) or bradyzoites (green fluorescence: GFP). **a**, **c**, **e**, **g**, **i** Differential interference contrast microscopy images. Bradyzoites grouped in cysts (thin arrows: **b**, **f**, **j**) and tachyzoites (thick arrows: **b**, **d**, **h**). Bars, 10 μm

pyrimethamine (Fig. 1g–j) treatment, which are standard drugs.

To perform a quantitative analysis using HTS, several images have to be obtained using a fluorescence microscope, and the acquired images after 96 h of infection showed fluorescent tachyzoites (Fig. 2a) and fluorescent bradyzoites (Fig. 2c). Then, these images were analyzed using specific software MetaXpress® High-Content Image Acquisition and Analysis, where parameters as size and fluorescence intensity were set to identify and quantify the parasite infection in the samples. According to these parameters, the software generates "masks" (marked in a different color on the image analyzed) that correspond to those spots that will be counted (Fig. 2b, d).

HFF cells were infected and treated with atovaquone or pyrimethamine to use the system of infection in 96-well plate in order to be analyzed by HTS. We found the same results that the qualitative assay described above: atovaquone and pyrimethamine controlled tachyzoite growth, with 85 and 70% reduction in the infection, respectively, and for bradyzoites, atovaquone reduced the growth by 50%, while pyrimethamine-treated cultures presented a much higher rate of bradyzoite infection compared to untreated cells after 24 and 48 h of treatment (Fig. 3a), presenting a *P* value smaller than or equal to 0.0001.

Analyzing the effect of these compounds in the parasite ultrastructure, we observed, as expected, some structural markers of the bradyzoite stage such as amylopectin-like granules (Fig. 4b–d) and some parasitophorous vacuoles, which presented a slight thickening of its membrane. This may represent cystic wall formation in the untreated cells and many parasites inside the parasitophorous vacuole (Fig. 4a) with their typical organelles preserved, such as mitochondria. Treatment with atovaquone (Fig. 4e, f) or pyrimethamine (Fig. 4g, h) for 48 h affected the integrity of parasites' mitochondria, which appeared to show a degraded content.

The mitochondrial integrity of the parasites inside cysts that were resistant to treatment with both compounds was evaluated using labeling with a MitoTracker, which is an indicator of mitochondrial membrane potential. Besides the MitoTracker, cells were labeled with DBA lectin, which is a cystic wall marker. Cysts inside untreated cells showed parasites with a normal mitochondrial membrane potential (Fig. 5a, b). This mitochondrial viability profile was found in around 68% (Fig. 5g) of the cysts that were present in untreated cells while cysts from cells that were treated with atovaquone (Fig. 5c, d) and pyrimethamine (Fig. 5e, f) showed 7 and 36% (Fig. 5g) of the cysts with parasites having active mitochondria inside them, respectively.

Discussion

It is known that *T. gondii* is a protozoan parasite that infects 30% of humans, mainly in the chronic stage, which is the parasite's most resistant stage. This resistant form is unaffected by current drugs that are used to treat toxoplasmosis, which are pyrimethamine and sulfadiazine; these drugs control the parasite's acute stage, tachyzoites (McAuley et al. 1994; Montoya and Liesenfeld 2004). Although these compounds are most often used to treat toxoplasmosis, they cause severe side effects (Haverkos 1987; Leport et al. 1988; Georgiev

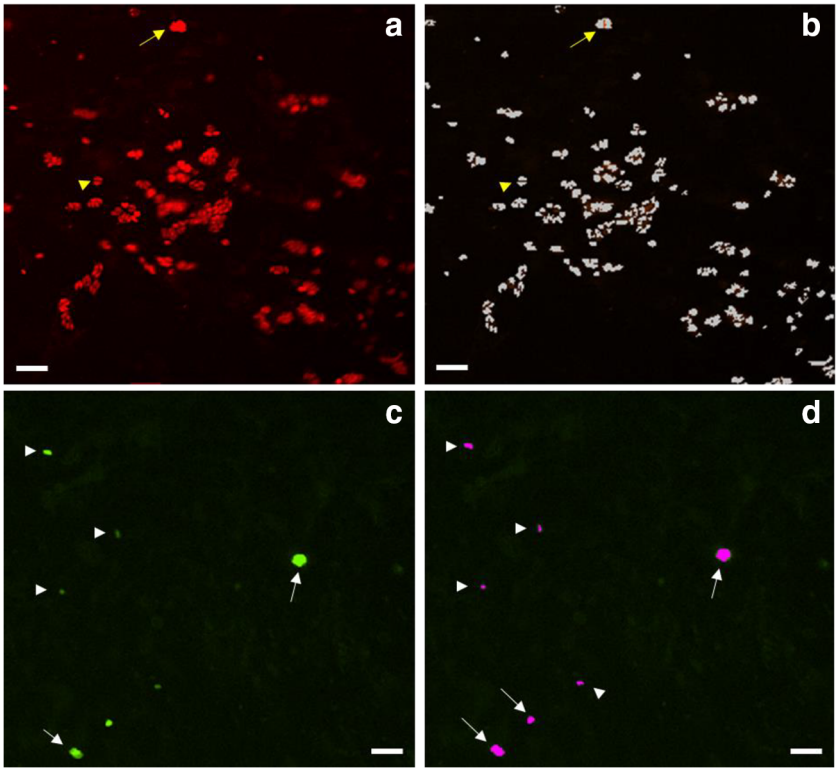

Fig. 2 Representative images of the *Toxoplasma gondii* EGS strain growing in HFF cells. *T. gondii* tachyzoites or bradyzoites can be identified using fluorescence microscopy ImageXpress Micro (Molecular Devices). Cells were infected, and 4 days after infection, representative images were obtained (**a**–**d**). The EGS strain showed tachyzoites (**a**) and bradyzoites (**c**). The mask used for detection and counting of tachyzoites, in gray (**b**), or bradyzoites, in magenta (**d**), is presented in the respective obtained images. Arrows—grouped parasites; arrow heads—individual parasites; bars, 20 μm

1994), and they are not active against the bradyzoites found in the cyst (Dunay et al. 2018).

Some studies have pointed out to the need to establish new in vitro and in vivo experimental models to test new compounds against *T. gondii* tachyzoites and bradyzoites. McPhillie et al. (2016) reported that the EGS strain, isolated in 1994 from amniotic fluid of a congenitally infected Brazilian fetus (Vidigal et al. 2002; Paredes-Santos et al. 2013), spontaneously forms cyst-like structures in vitro and therefore may constitute a good model for experimental chemotherapy in vitro. This strain belongs to the group of *T. gondii* atypical strains and it is a recombinant type I/III, which is virulent and cystogenic in mice, and it presents a high level of conversion to cysts (Paredes-Santos et al. 2013). These atypical strains are related to the most severe clinical symptoms compared with the classical symptoms (Montazeri et al. 2018). Our data corroborate the idea that the EGS strain, especially the genetically modified type of EGS strain that

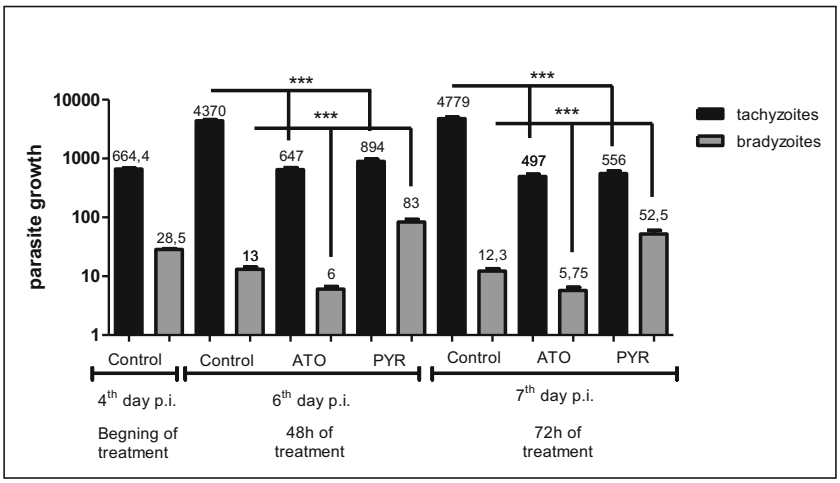

Fig. 3 *Toxoplasma gondii* EGS DC strain grown in HFF cells after treatment with atovaquone, pyrimethamine, or no treatment. Proliferation was measured by automatic counting using high-throughput screening. Cells were previously infected, and after 4 days, treatment started and lasted for 72 h (**a**). Atovaquone (ATO) and pyrimethamine (PYR) were both used at 10 μM and untreated cells (Control) were maintained in the absence of any compound. At least three independent experiments were performed. Significant difference compared to control (***$P \leq 0.0001$) by the Student's *t* test

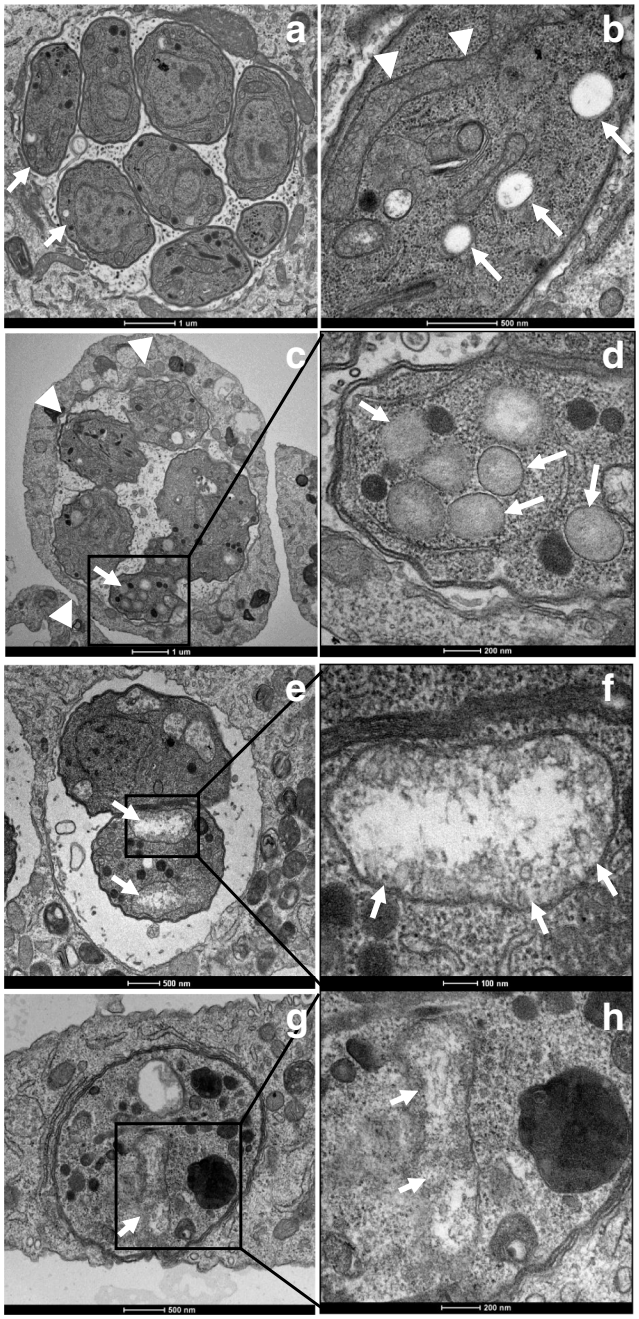

Fig. 4 Transmission electron microscopy images showing HFF cells infected with *T. gondii* EGS strain after 7 days, which were treated with atovaquone or pyrimethamine at 10 μM for another 48 h. (**a**–**d**) *T. gondii* within untreated HFF cells 9 days after infection. There were many parasites inside the parasitophorous vacuole (arrows; **a**) with its typical structures preserved, such as mitochondria (arrowheads; **b**) and granules similar to amylopectin granules of bradyzoites (arrows; **b**–**d**). Some parasitophorous vacuoles showed a slight thickening of its membrane (arrowhead; **c**). Treatment with atovaquone (**e**, **f**) or pyrimethamine (**g**, **h**) affected the integrity of parasites' mitochondria, which appeared to contain degraded content (arrows)

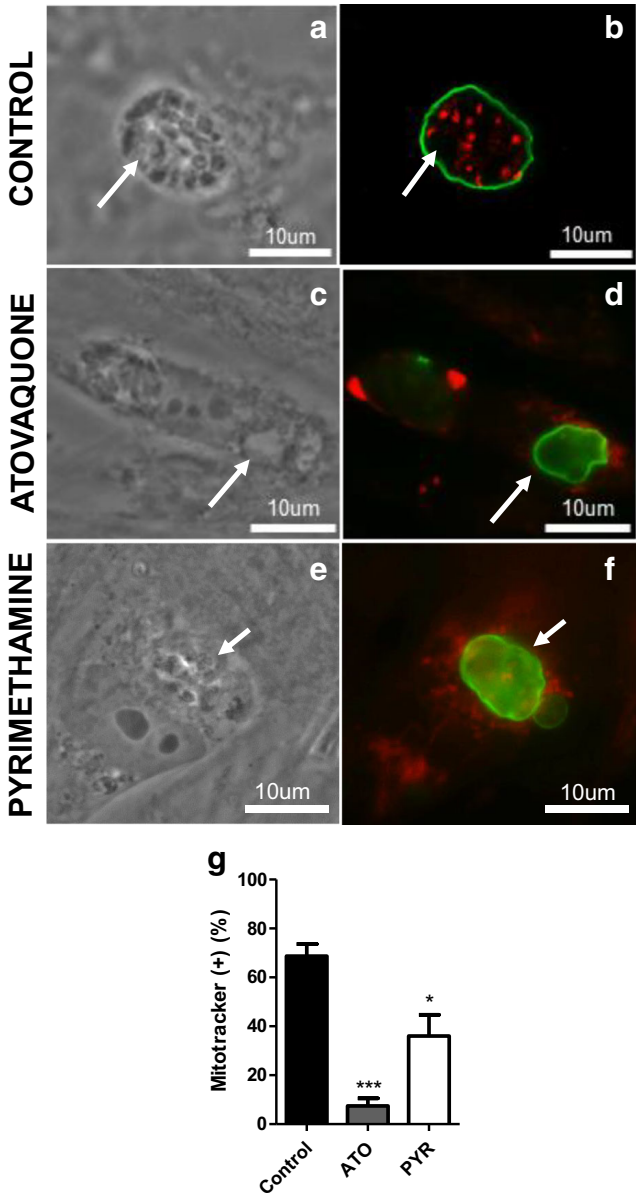

Fig. 5 Fluorescence microscopy images showing HFF cells infected with *T. gondii* EGS WT strain after 7 days, which were treated with atovaquone or pyrimethamine at 10 μM for another 48 h. After treatment, cells were incubated with MitoTracker CMXRos and also labeled with *Dolichos biflorus* lectin conjugated to fluorescein isothiocyanate (10 μg mL^{-1}). Images of infected cells that were treated with atovaquone (**c**, **d**) or pyrimethamine (**e**, **f**) or left untreated (**a**, **b**). **b**, **d**, **f** Fluorescence of mitochondrial potential (red fluorescence: MitoTracker) and cystic wall (DBA-FITC). **a**, **c**, **e** Phase contrast microscopy images. Labeled parasites were present inside cysts in untreated cells (arrows; **a**, **b**) and parasites with a light or absent mitochondrial potential labeling were present in infected cells that were treated with both drugs (**d** or **f**). The proportion of cysts that were positive for MitoTracker was counted and expressed as a percent (**g**). Representative of one experiment that was performed in triplicate of each condition. Significant difference compared to control (*P = 0.01 and ***$P \leq 0.0001$) by the Student's t test. Bars, 10 μm

expresses specific-stage fluorescent proteins (Paredes-Santos et al. 2016), named here as EGS DC, is a powerful tool to study *T. gondii* biology. Our results show that the EGS DC strain is a useful model for studying cysts in vitro, as previously reported by McPhillie et al. (2016), and it is an excellent

model to simultaneously study the two main stages of *T. gondii*, tachyzoites and bradyzoites (with formation of cysts), in vitro. Therefore, this in vitro system may constitute a potential model to investigate the effect of new drugs against *T. gondii* tachyzoites and bradyzoites.

We used the EGS DC strain that allows simultaneous detection of tachyzoites and bradyzoites during infection through the automatic fluorescence microscopy method, HTS. HTS is useful for quantifying several cellular events at the same time (Gaji et al. 2013; Mital et al. 2006), including the amount of *T. gondii* cysts inside brain tissue (Aldebert et al. 2011). HTS is particularly important for identifying active compounds from hundreds of candidate compounds when researching drug development and when optimizing research, costs, and time (Freitas-Junior et al. 2012). Here, we showed that this method can be used to quantify *T. gondii* in its tachyzoite and bradyzoite forms, which was previously used to verify the effects of other inhibitors such as cytochalasin D, pyrimethamine, and clindamycin against *T. gondii* tachyzoites (Gubbels et al. 2003) and to develop new therapies against other parasites such as *Leishmania* (Freitas-Junior et al. 2012) and *Plasmodium* (Spangenberg et al. 2013). Thus, this kind of automatic approach, in addition to being a fast way to quantify the parasite infection rate, allows us to follow and analyze several steps of the experiment without interfering with the process or stopping it by fixation, for example.

To validate the experimental model, we initially decided to analyze the effect of two compounds that were previously shown to be active against the protozoan: pyrimethamine and atovaquone (Montazeri et al. 2018). We observed, as expected, that both inhibited the tachyzoite proliferation, but only atovaquone inhibited bradyzoites to some extent. However, for pyrimethamine, we observed an increase in the bradyzoites infection rate, the stage able to form cysts. We hypothesized that after treatment for 48 h with pyrimethamine, the tachyzoites that were not killed could transform into viable bradyzoites that form cysts, thus explaining the significant increase in the bradyzoites, generally presents in cysts, in cultures that were incubated in the presence of this compound. Some previous studies have shown that cyst induction is a common phenomenon. *T. gondii* tachyzoites that belong to the virulent strain RH, which does not usually generate tissue cysts in mice (Howe and Sibley 1994, 1995; Mavin et al. 2004), form cysts in vitro when primary embryonic muscle cells are infected in culture (Ferreira-da-Silva et al. 2009a, b). In addition, treatment with chemical compounds such as atovaquone and pyrrolidine dithiocarbamate (Djurkovic-Djakovic et al. 2005) or sulfadiazine (Villard et al. 1997) also induces the conversion of tachyzoites from RH strain to bradyzoites in mice infected with *T. gondii*. Other compounds, such as an inhibitor of cyclic-GMP-dependent protein kinase (Gurnett et al. 2002; Nare et al. 2002), and an iron(III) compound used in an in vitro study, induced an increase of reactive oxygen species production resulting in this type of conversion (Portes et al. 2015).

We used electron microscopy to analyze the effect of pyrimethamine and atovaquone on *T. gondii.* Some ultrastructural alterations were observed. The infected cells that were not treated showed, as expected, some parasites with features from both tachyzoites and bradyzoites because of the presence of a large amount of typical amylopectin-like granules in the bradyzoite cytoplasm. The parasites that were treated with both atovaquone and with pyrimethamine presented as damaged between other changes, the structure of mitochondria was affected. We subsequently found that these drugs really collaborate to the arrest of the mitochondrial viability, because we observed a loss of mitochondrial potential when we used a MitoTracker to investigate their viability. Charvat and Arrizabalaga (2016) showed that treatment with the ionophore monensin generates oxidative stress that caused depolarization of *T. gondii*'s mitochondrial membrane potential, which was also detected through MitoTracker labeling. Treatment with the quinolone HDQ also caused depolarization of the mitochondrial membrane potential in *T. gondii* tachyzoites and a reduction in the intracellular ATP level of nearly 70%, which affected maintenance of *T. gondii*'s energy metabolism (Lin et al. 2009). Lin et al. (2009) also reported that differentiation from tachyzoites to bradyzoites could be associated with a reduction in mitochondrial membrane potential, which is related to the tachyzoites' mitochondrial activity. Although bradyzoites showed reduced mitochondrial activity, we found significant differences between the potential shown by cysts from untreated samples and cysts from samples treated with pyrimethamine or atovaquone, and a larger reduction was observed with atovaquone treatment. Atovaquone's capacity to control growth of part of the bradyzoite's population may result from cellular differences between tachyzoites and bradyzoites, including metabolic differences (Dzierszinski and Knoll 2007), because atovaquone has as molecular target the cytochrome bc1, a protein that participates of the mitochondrial electron transport chain and that has increased expression in EGS strain bradyzoites (McPhillie et al. 2016). Atovaquone could control the cyst burden in mice (Araujo et al. 1992), but resistance develops with clinical use (Torres et al. 1997; Chirgwin et al. 2002; Djurković-Djaković et al. 2002; Meneceur et al. 2008; Winterhalter et al. 2010).

Our findings show that these drugs affect mitochondrial viability in the cysts, which is consistent with results from previous studies on the profile of *T. gondii* mitochondrial activity in bradyzoites. Thus, the work reported herein provides another perspective for *T. gondii* studies by investigating a new target or compound that is effective against the parasite or studying the parasite's biology and development because it is easier and more complete to study in the same model cells that were infected with rosettes and also cysts and bradyzoites.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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