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PROTOZOOLOGY - ORIGINAL PAPER

Susceptibility of human villous (BeWo) and extravillous (HTR-8/SVneo) trophoblast cells to *Toxoplasma gondii* infection is modulated by intracellular iron availability

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Abstract

Congenital toxoplasmosis is a serious health problem that can lead to miscarriage. HTR-8/SVneo is a first trimester extravillous trophoblast, while BeWo is a choriocarcinoma with properties of villous trophoblast cells. In the placenta, iron is taken up from Fe-transferrin through the transferrin receptor being the ion an important nutrient during pregnancy and also for *Toxoplasma gondii* proliferation. The aim of this study was to evaluate the role of iron in *T. gondii* proliferation in BeWo and HTR-8/SVneo cells and in human chorionic villous explants. The cells were infected with *T. gondii*, iron supplemented or deprived by holo-transferrin or deferoxamine, respectively, and parasite proliferation and genes related to iron balance were analyzed. It was verified that the addition of holo-transferrin increased, and DFO decreased the parasite multiplication in both trophoblastic cells, however, in a more expressive manner in HTR-8/SVneo, indicating that the parasite depends on iron storage in trophoblastic cells for its growth. Also, tachyzoites pretread with DFO proliferate normally in trophoblastic cells demonstrating that DFO itself does not interfere with parasite proliferation. Additionally, *T. gondii* infection induced enhancement in transferrin receptor mRNA expression levels in trophoblastic cells, and the expression was higher in HTR-8/SVneo compared with BeWo. Finally, DFO-treatment was able to reduce the parasite replication in villous explants. Thus, the iron supplementation can be a double-edged sword; in one hand, it could improve the supplement of an essential ion to embryo/fetus development, and on the other hand, could improve the parasite proliferation enhancing the risk of congenital infection.

Keywords *Toxoplasma gondii* · BeWo villous trophoblast · HTR-8/SVneo extravillous trophoblast · Iron metabolism · Holo-transferrin · Deferoxamine

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Introduction

During pregnancy, the physiological iron demands increase to support fetoplacental development and maternal adaptation. Additionally, the iron requirements are higher in the third trimester due to the increase of maternal red blood cells mass and the acceleration of placental and fetal growth, reviewed by Fisher and Nemeth (2017). The iron transfer from the maternal to the fetal organism is considered as unidirectional and it is mediated by a transport against the concentration gradient, in an attempt to maintain the physiological needs of the concept (Brown et al. 1980; Starreveld et al. 1993).

In agreement with the progress of human pregnancy, the trophoblast assimilates and directs relevant amounts of iron conjugated with transferrin (holo-transferrin/Tf) to the embryo/fetus (Kennedy et al. 1992; Starreveld et al. 1993), through the interaction with its specific receptor (TfR) identified in populations of trophoblastic cells (Starreveld et al. 1993; Kroos et al. 1996). The acquirement and transfer of iron during pregnancy occur in the form of transferrin coupled to two iron atoms, also known as diferric transferrin (McArdle et al. 1984). The blastocystic-trophoblast cells of embryonic origin derive cytotrofoblastic cells that derive syncytiotrophoblast cell mass forming the placenta along with the maternal decidual cells (Hamilton and Boyd 1960; Gude et al. 2004). The TfR is present on the apical side of syncytiotrophoblast of the first trimester and term placenta, whereas ferrous iron exits the cell through ferroportin (FPN), a membrane transporter that is present on the basolateral (fetal) side of the cells (Bastin et al. 2006). The control of iron assimilation is mediated by the hepcidin, encoded by *HAMP* gene, which acts to induce cellular internalization and subsequent ferroportin degradation (Nemeth et al. 2004).

Toxoplasma gondii is an obligate intracellular protozoan parasite that is able to infect distinct types of nucleated cells of most warm-blooded vertebrates (Hill and Dubey 2002). In congenital toxoplasmosis that is characterized by the transplacental transmission of the parasite during pregnancy, the complications are associated with pregnancy loss (miscarriage or stillbirth) or severe diseases in neonates with visual injuries to malformations and neurological disorders, as reviewed by Arora et al. (2017). IFN-γ is the major regulator of cell-mediated immunity to *T. gondii* in both mouse and human cells (Nathan et al. 1983; Suzuki et al. 1988). It was previously shown in rat enterocytes that IFN-γ induces inhibition of *T. gondii* replication by limiting the availability of intracellular iron to the parasite, as iron chelator deferoxamine completely inhibits parasite growth, whose effect was reversed by ferrous sulfate or holo-transferrin addition (Dimier and Bout 1998). Moreover, the treatment of human foreskin fibroblasts (HFF) with deferoxamine prevented *T. gondii* replication in a dose-dependent manner, and holo-transferrin iron supplementation restored the parasite growth and induced transferrin receptor (TfR) expression (Gail et al. 2004).

In vitro studies with human villous cytotrophoblastic cells, such as the BeWo choriocarcinoma cell model (Pattillo and Gey 1968), and with extravillous cytotrophoblast, such as HTR-8/SVneo immortalized cell line (Graham et al. 1993), were performed to better understand the mechanisms of trophoblast response to *T. gondii* infection (Oliveira et al. 2006; Castro et al. 2013; Barbosa et al. 2014; Guirelli et al. 2015). BeWo cells have morphological and functional properties common to normal trophoblasts. BeWo cells present mRNA expression of IL-6, IL-10, IFN-α, IFN-β, and granulocyte macrophage colony stimulating factor (GM-CSF) as do normal purified human trophoblast obtained from term placenta (Bennett et al. 1996), Also, these cells produce IL-10 (Bennett et al. 1997) that synthesizes human chorionic gonadotropin (hCG) that represents a fundamental property of the trophoblast (Pattillo and Gey 1968) and other placental hormones including estrogenic and progestational steroids (Jeschke et al. 2007). HTR8/SVneo cells that were obtained from explant cultures of human first trimester placenta (8–10 weeks of gestation) are an established human trophoblast cells with prolonged in vitro life spans by transfecting normal first trimester trophoblast cells with a plasmid containing the gene for the simian virus 40 (SV40) large T antigen (Tag). Apart from their ability to proliferate indefinitely, these transfected cells retained many of the phenotypic features of the nontransfected parental cells, such as morphology, positivity for cytokeratin, indicating retention of their epithelial phenotype, proliferation inhibition by TGF-β, similar gelatinases expression, and production of hCG (Graham et al. 1993). Additionally, these cells are non-tumourigenic and nonmetastatic (Graham et al. 1993).

Recently, it was demonstrated that different trophoblast cells present variable susceptibility to *T. gondii* since syncytiotrophoblast, derived of primary human trophoblast (PHT), is more resistant to *T. gondii* infection than cytotrophoblast (Ander et al. 2018). Moreover, previous studies demonstrated that BeWo cells are a suitable model for understanding the pathways of iron acquisition and use by placental cells (Van Der Ende et al. 1987; Danzeisen and McArdle 1998; Morris Buus and Boockfor 2004; Heaton et al. 2008; Li et al. 2012a, b), whereas to date, knowledge about the metabolism of this nutrient by HTR-8/SVneo cells is unknown. In the BeWo trophoblast cell line, the addition of iron sulfate did not alter the parasite growth (Pfaff et al. 2005), however, the iron acquisition depends on transferrin receptor and iron starvation was not investigated yet in this cell phenotype.

As the iron availability is essential for pregnancy through its acquisition by trophoblastic cells and the nutrient promotes *T. gondii* proliferation in other non-placental cell models, the present study aimed to investigate the role of iron in the susceptibility of two distinct populations of human trophoblastic cells, the villous cytotrophoblast (BeWo) and the extravillous cytotrophoblast (HTR-8/SVneo) cells, and human chorionic villous explants from healthy third-trimester human placenta to *T. gondii* infection.

Materials and methods

Culture of BeWo and HTR-8/SVneo cells

The human choriocarcinoma (BeWo) and human cervix adenocarcinoma (HeLa) cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The human immortalized extravillous trophoblast (HTR-8/SVneo) cell line was kindly provided by Dr. Estela Bevilacqua (University of São Paulo, SP, Brazil). All cell lines were cultured in 25- or 75-cm^2 culture flasks in Roswell Park Memorial Institute (RPMI)-1640 medium (Cultilab, Campinas, SP, Brazil) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 10% heat-inactivated fetal bovine serum (FBS) (Cultilab) (complete medium) in humidified incubator at 37 °C and 5% CO_2 (Barbosa et al. 2015; Guirelli et al. 2015). According to protocol N° 13/2012, the Ethics Committee of the Federal University of Uberlândia, MG, Brazil, determines that commercially acquired cell lines do not require ethical approval.

Parasites

Tachyzoites of *T. gondii* 2F1 clone (RH strain), which constitutively express the cytoplasmic beta-galactosidase enzyme (β-Gal) (Seeber and Boothroyd 1996), were provided by Dr. Vern B. Carruthers (Medical School of Michigan University, USA). The parasites were maintained by passage every 2 days in monolayers of HeLa cells cultured in RPMI supplemented with 2% FBS (Guirelli et al. 2015).

Iron addition or deprivation and cellular viability assay

In order to add or decrease the availability of iron in the cell cultures, it used human plasma-derived holo-transferrin (R&D Systems, Minneapolis, MN, USA) or the iron chelator deferoxamine mesylate salt (DFO) (Sigma-Aldrich), respectively. To analyze the holo-transferrin or DFO effects on viability of BeWo, HTR-8/SVneo, and HeLa cells, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed, as described (Mosmann 1983). For this purpose, BeWo (3×10^4/200 μL/well), HTR-8/SVneo (1×10^4/200 μL/well), and HeLa (3×10^4/200 μL/well) cells were cultured in 96-well plate in complete medium for 24 h at 37 °C and 5% CO_2. As the extravillous trophoblast is larger than cytotrophoblastic cells (Kurman et al. 1984; Vićovac and Aplin 1996), it used 1×10^4/well of HTR-8/SVneo cells to obtain similar cellular confluence in 24 h incubation as do BeWo and HeLa cells. Thereafter, all cell types were treated with increasing concentrations of holo-transferrin (20, 100, 250, 500, 1000, or 2000 μg/mL) or DFO (62.5, 125, 250, 500 or 1000 μM) or with complete medium (control) during 24 h. Next, the supernatants were discarded and the cells were incubated with 10 μL of MTT reagent (Sigma) diluted in phosphate-buffered saline (PBS) at 5 mg/mL plus 90 μL of complete medium for 4 h at 37 °C. Next, the supernatants were removed and the cells were homogenized at room temperature with 100 μL of 10% sodium dodecyl sulfate (SDS) and 50% dimethylformamide (Sigma) to completely dissolve the formazan crystal. The absorbance was measured in a microplate reader (Versa Max ELISA Microplate Reader, Molecular Devices, Sunnyvale, CA, USA) at 570 nm and the data were expressed as the percentage of viable cells in relation to the control (untreated cells—100% viability).

T. gondii proliferation by β-galactosidase assay in cells treated with holo-transferrin or DFO

In order to evaluate the effect of cell treatment with holo-transferrin or DFO on *T. gondii* proliferation, the β-galactosidase assay was performed. For this purpose, BeWo (3×10^4/200 μL/well), HTR-8/SVneo (1×10^4/200 μL/well), or HeLa (3×10^4/200 μL/well) cells were cultured in 96-well plates in complete medium for 24 h at 37 °C and 5% CO_2. Then, the cells were infected with *T. gondii* at a proportion of one parasite per cell (1:1), in medium with 2% FBS and incubated for 3 h. Next, the plates were washed with medium without FBS to remove non-internalized parasites treated with human holo-transferrin (20, 100, 250, 500, 1000, or 2000 μg/mL), or with DFO (62.5, 125, 250, 500, or 1000 μM), or with complete medium (control), and incubated for additional 24 h. The parasite quantification was performed using chlorophenol red–β-D-galactopyranoside (CPRG; Roche, Mannheim, Germany) as described (Teo et al. 2007). *T. gondii* intracellular proliferation ratio was calculated in relation to the reference curve of 2F1 tachyzoites ranging from 1×10^6 to 15.625×10^3 total parasites. Finally, the results obtained from this assay were used to calculate the percentage rates of *T. gondii* proliferation under the influence of holo-transferrin or DFO treatments. Data were obtained from three independent experiments performed in eight replicates. The median inhibitory concentration (IC_{50}) of DFO treatment was calculated by extrapolation of the corresponding dose-response curve on a log-linear plot employing the portions of the curve that transected the 50% response point (Jones-Brando et al. 2006).

The viability assay of trophoblast and HeLa cells supplemented or deprived of iron after *T. gondii* infection

BeWo (3×10^4/200 μL/well), HTR-8/SVneo (1×10^4/200 μL/well), or HeLa (3×10^4/200 μL/well) cells were cultured for 24 h, infected with *T. gondii,* treated with human holo-

transferrin (250 μg/mL) or with DFO (the median inhibitory concentration (IC_{50}) specific for each cell line) or with complete medium (control), and incubated for additional 24 h. Next, the MTT analyses were performed as described above.

T. gondii proliferation in cells infected with DFO-pretreated tachyzoites

In order to evaluate the effect of DFO on *T. gondii* proliferation, the β-galactosidase assay was performed. For this purpose, parasites were treated for 1 h at 37 °C and 5% CO_2 before infection with different concentrations of DFO (62.5, 125, 250, 500, or 1000 μM) or with medium alone (control). Then, BeWo or HTR-8/SVneo cells were infected with pretreated *T. gondii* tachyzoites at a proportion of one parasite per cell (1:1) and incubated for additional 24 h as previously described. The parasite quantification was performed using chlorophenol red–β-D-galactopyranoside (CPRG; Roche, Mannheim, Germany) as described above.

Analysis of mRNA expression for iron regulatory factors by quantitative PCR

In order to investigate the influence of *T. gondii* infection on mRNA expression of proteins involved in iron metabolism, such as transferrin receptor (*TfR*), lactoferrin receptor (*LtfR*), hepcidin (*Hamp*), and ferroportin in the studied cell lines, a quantitative PCR (qPCR) assay was performed. For this purpose, BeWo (2×10^5/500 μL/well), HTR-8 (6×10^4/500 μL/well), and HeLa (2×10^5/500 μL/well) cells were cultured in 24-well plates in complete medium for 24 h at 37 °C and 5% CO_2. Then, the cells were infected or not (control) with *T. gondii* at a proportion of one parasite per cell (1:1), in medium with 2% FBS and incubated for 3 h in culture conditions. Next, cell lineages were washed with incomplete medium and cultured for additional 24 h in complete medium. The total RNA was extracted from the cells samples with TRIzol® reagent following manufacturer's instructions (Ambion®, Life Technologies, Carlsbad, CA, USA). After extraction, the RNA quantity, purity, and quality were determined by spectrophotometer apparatus (GeneQuant 1300, GE Healthcare) and complementary DNA (cDNA) of each sample was synthesized using 1 μg of RNA previously treated with DNase I Amplification Grade (Invitrogen, Thermo Fisher Scientific) in a thermocycler, Arktik Thermal Cycler (Thermo Fisher Scientific), by reverse transcription reaction using the ImProm-II™ Reverse Transcriptase enzyme in accordance with the manufacturer (Promega, Madison, USA). Finally, the quantitative PCR (qPCR) assay was performed using SYBR® Green PCR Master Mix (Applied Biosystems, Life Technologies) in Applied Biosystems (ABI) 7500 Real Time PCR System (Life Technologies). The reaction was constituted of 40 cycles with melting temperature (Tm) at 60 °C for 30 s. Samples Ct data (cycle threshold) were normalized to the expression of reference gene control (Gapdh) and the relative expression of each studied gene was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The sequences of the analyzed genes were: *TfR*: FW: 5′-TCCTATTGAAACTTGCCCAGATG-3′, RV: 5′-CATTCAGTGGCACCAACCG-3′; *LtfR*: FW: 5′-TGGTGGAGGAGGATACTTTCCA-3′, RV: 5′-TCCACTCCAATCAAAACCAGAA-3′; *Hamp*: FW: 5′-CTCCTGCTCCTCCTCCTCCT-3′, RV: 5′-AAGTTGTCCCGTCTGTTGTGG-3′; *Ferroportin*: FW: 5′-GAGCAGCAGCAGCGATAG-3′, RV: 5′-AGAATGACCAAGGTAGAGAAGG-3′ and *Gapdh*: FW: 5′-ATGCCATCACTGCCACCC-3′, RV: 5′-ACCTTGCCCACAGCCTTG-3′.

Intracellular iron measurement assay

BeWo (3×10^4/200 μL/well) and HTR-8/SVneo (1×10^4/200 μL/well) cells were cultured in 96-well plates for 24 h, infected or not with *T. gondii* (1:1), treated with DFO (250 μM) or with complete medium (control), and incubated for additional 24 h. Next, the culture supernatants were discarded and the iron levels in cells were measured by spectrophotometric analysis using a commercial kit according to manufacturer's instructions (Labtest Diagnóstica S.A., Lagoa Santa, MG, Brazil).

T. gondii proliferation assay in culture of human chorionic villous explants

Villous explants from healthy third-trimester human placenta (36–40 weeks of pregnancy) were obtained from Clinics Hospital of the Federal University of Uberlândia (HC-UFU) (Uberlândia, MG, Brazil) in accordance with Human Research Ethics Committee of the Federal University of Uberlândia (approval number: 2.360.812). The chorionic villous explants were only collected from the placenta with no evidence of infectious disease and other pathologies which could interfere with the results of this study. Placental tissues were washed in ice-cold sterile PBS to remove excess blood and aseptically dissected to discard fetal membranes and endometrial tissues up to 1 h after obtention of the samples. Next, floating terminal chorionic villi were extracted and transferred to 96-well plates (one per well) and cultured in complete medium (200 μL/well) for 24 h at 37 °C and 5% CO_2. The volume of the villous explants was approximately 10 mm^3 (Gomes et al. 2011; Silva et al. 2017). After, the villi were washed with medium without FBS to remove excess of remaining erythrocytes and infected with *T. gondii* in a proportion of 1×10^6 tachyzoites per well in complete medium (200 μL/well) for 24 h at 37 °C and 5% CO_2. Then, the villi were washed again to remove non-internalized parasites and treated with DFO (250 μM) or with complete medium

(control) and incubated for additional 24 h in culture conditions. The *T. gondii* intracellular proliferation in villous explants samples was performed by adding 150 μL RIPA lysis buffer [0.05 M Tris-HCl, 0.15 M NaCl, 1% Triton X-100, 1% (*w*/*v*) sodium deoxycholate, and 0.1% (w/v) sodium dodecyl sulfate, pH 7.5] supplemented with protease inhibitor cocktail (Complete®, Roche Diagnostic, Mannheim, Germany) and phosphatase inhibitors (0.001 M Na_3VO_4 and 0.025 M NaF) (Sigma-Aldrich) to each villous and homogenizing the samples in ice for protein extraction. The homogenates were centrifuged at 15000×*g* for 30 min at 4 °C and the supernatants obtained were collected to measure the total protein concentrations (μg/mL) using the Bradford assay (Bradford 1976). In parallel, aliquots of 20 μL of total protein from villi were used to determine *T. gondii* intracellular proliferation by β-galactosidase assay, as described above. Finally, the data of number of tachyzoites were normalized according to the total protein concentration of each villous.

Statistical analysis

All data obtained are expressed as mean and standard error of mean (± SEM). Differences among groups were assessed by one-way ANOVA with Bonferroni's multiple comparison post-test or Kruskal Wallis with Dunn's multiple comparison post-test, for parametric or non-parametric data, respectively. When appropriate, the differences were also assessed by Student's *t* test. Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA) and the differences were considered as statistically significant when $P < 0.05$.

Results

Holo-transferrin or DFO treatments change slightly the cellular viability

Firstly, it was evaluated the effect of human holo-transferrin or DFO treatment in different concentrations in the cellular viability of BeWo, HTR-8/SVneo, and HeLa cells by MTT colorimetric assay (Fig. 1). It was observed that the concentrations of 20 to 1000 μg/mL of holo-transferrin did not interfere in BeWo or HeLa cellular viability, and only the highest concentration of holo-transferrin, 2000 μg/mL, decreased the viability of these cell lines around 16% (Fig. 1a) ($P < 0.05$) and 23% (Fig. 1c) ($P < 0.05$), respectively, in comparison with untreated cells (medium). Interestingly, in HTR-8/SVneo cells, the concentrations of 100 and 250 μg/mL of holo-transferrin (Fig. 1b) ($P < 0.05$) were able to increase the proliferation of cells, and doses of 500 to 2000 did not alter the viability of this cell line compared with respective control (medium). In relation to DFO treatment, the concentration of 250 μM decreased the cellular viability at 13.1% in BeWo (Fig. 1b) ($P < 0.05$), the concentrations of 62.5, 125, 250, 500, and 1000 μM decreased at 8.1, 14.4, 15.2, 14, and 13.8%, respectively, the cellular viability in HTR-8/SVneo (Fig. 1e) ($P < 0.05$) and 1000 μM decreased at 11.6% ($P < 0.05$) the cellular viability in HeLa cells (Fig. 1f) in comparison with untreated cells of the same lineage.

Holo-transferrin treatment increases *T. gondii* proliferation in HTR-8/SVneo extravillous trophoblast cells

To verify the effect of iron supplementation with holo-transferrin in *T. gondii* intracellular proliferation in BeWo villous and HTR-8/SVneo extravillous cytotrophoblast cells, as well as in HeLa cervical cells, we measured the parasite growth using a highly virulent RH strain (2F1 clone) by the β-galactosidase colorimetric assay. It was observed that BeWo cells presented a slight increase in *T. gondii* proliferation when cells were supplemented with 250 μg/mL holo-transferrin in comparison to untreated cells (medium) ($P < 0.05$) (Fig. 2a). The addition of holo-transferrin at doses of 20 to 2000 μg/mL was able to increase the parasite proliferation in HTR-8/SVneo cells if compared to medium ($P < 0.05$) (Fig. 2b). In HeLa cells, the holo-transferrin treatment was not able to alter the parasite growth in any experimental condition in relation to the medium (Fig. 2c). In parallel, the percentage of parasite proliferation enhancement was evaluated when cells were supplemented with holo-transferrin. The concentration of 250 μg/mL of holo-transferrin was able to induce the *T. gondii* proliferation by 17.70% in BeWo cells (Table 1). In relation to HTR-8/SVneo, the iron supplementation augmented the parasite proliferation rates in all of holo-transferrin concentrations by 37.91, 32.35, 31.79, 34.37, 34.42, and 31.34%, when cells were infected and treated with doses of 20, 100, 250, 500, 1000, and 2000 μg/mL, respectively (Table 1). These results showed that the extravillous cytotrophoblast HTR-8/SVneo are more susceptible to *T. gondii* proliferation than villous trophoblastic BeWo and HeLa cervical cells under iron supplementation.

The iron chelator, deferoxamine, controls *T. gondii* proliferation in HTR-8/SVneo and BeWo trophoblast cells

It was previously shown that DFO at a concentration of 250 μM was able to control *T. gondii* proliferation in rat primary enterocytes (Dimier and Bout 1998). In BeWo trophoblast and HeLa cells, it was observed that DFO-treatment showed a dose-response inhibitory effect in parasite proliferation from 250 up to 1000 μM ($P < 0.05$) (Fig. 3a, c). Related to HTR-8/SVneo cells, DFO-treatment was able to decrease parasite proliferation in all of the doses tested and also in a

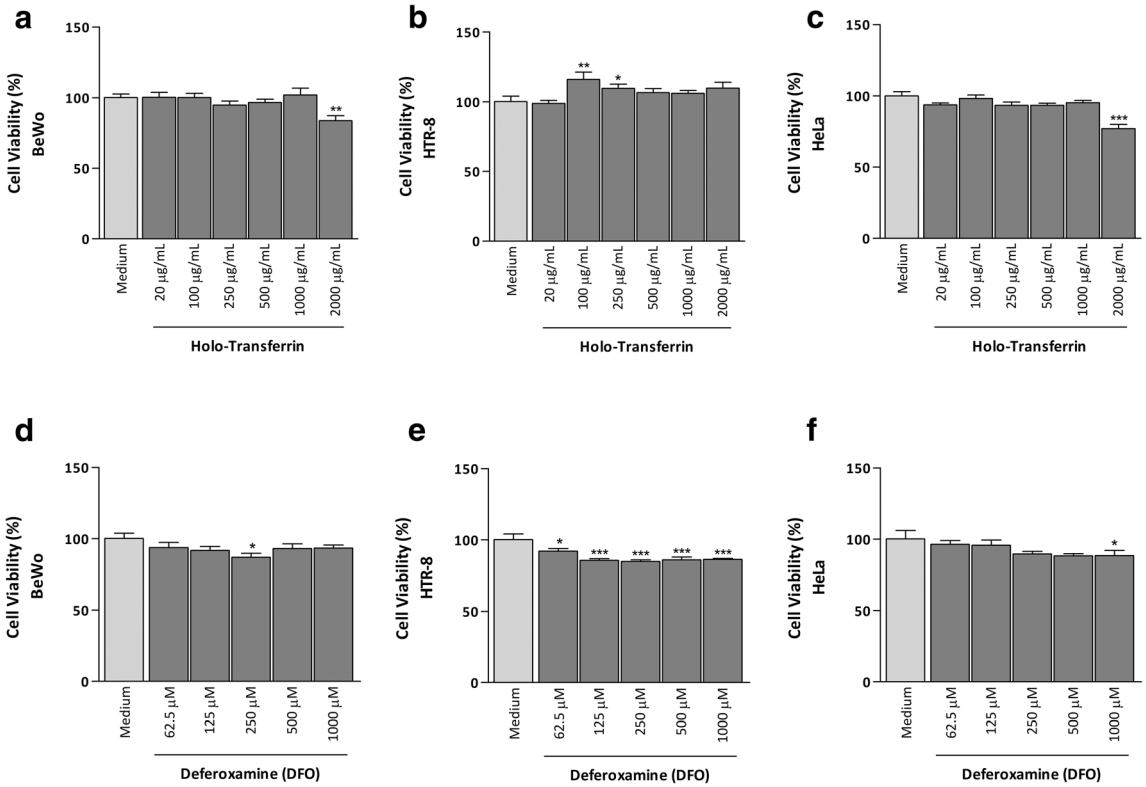

Fig. 1 Viability of cells treated with holo-transferrin or DFO. BeWo (3×10^4 cells/200 µL/well), HTR-8/SVneo (1×10^4 cells/200 µL/well), and HeLa (3×10^4 cells/200 µL/well) cells were cultured in 96-well plate during 24 h, treated with human holo-transferrin (20, 100, 250, 500, 1000, or 2000 µg/mL), DFO (62.5, 125, 250, 500, or 1000 µM) or incubated with complete medium (medium) only for additional 24 h and submitted to MTT assay. The data were shown as the percentage of viable cells (cell viability) for **a**, **d** BeWo, **b**, **e** HTR-8/SVneo, and **c**, **f** HeLa in relation to untreated cells (100% of cell viability) and expressed as mean ± SEM from three independent experiments in triplicate. *Significant differences in relation to untreated cells. (*) $P < 0.05$. One-way ANOVA with Bonferroni's Multiple Comparison Test (**c**, **e**, and **f**), Kruskal Wallis with Dunn's Multiple Comparison Test (**a**, **b**, and **d**)

dose-dependent manner ($P < 0.05$) (Fig. 3b). In parallel, DFO-treatment interfered minimally in the cellular viability at a maximum of 15.2% in HTR-8/SVneo cells (Fig. 1e).

Additionally, the percentages of parasite proliferation inhibition by DFO treatment were calculated (Table 2). The treatment of BeWo with DFO at 250, 500, and 1000 µM inhibited *T. gondii* proliferation at 42.81, 54.64, and 60.40%, respectively, in relation to untreated cells. The parasite proliferation inhibition was more pronounced in HTR-8/SVneo cells reaching more than 70% inhibition in relation to

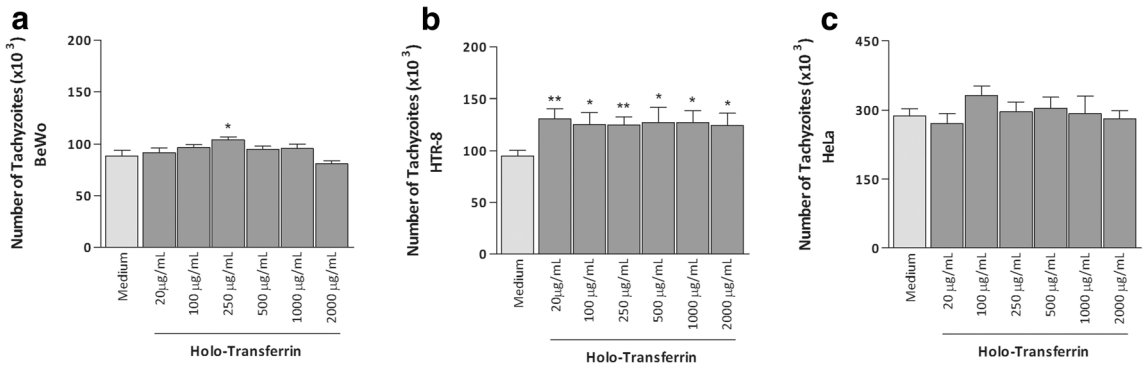

Fig. 2 Role of holo-transferrin treatment on *T. gondii* intracellular proliferation in the cell lines. BeWo (3×10^4 cells/200 µL/well), HTR-8/SVneo (1×10^4 cells/200 µL/well), and HeLa (3×10^4 cells/200 µL/well) cells were cultured in 96-well plate during 24 h, infected with *T. gondii* (1:1) for 3 h, treated with human holo-transferrin (20, 100, 250, 500, 1000, or 2000 µg/mL) or incubated with only medium (medium) for additional 24 h, and analyzed for *T. gondii* intracellular proliferation by β-galactosidase assay. The data were demonstrated as number of tachyzoites for **a** BeWo, **b** HTR-8/SVneo, and **c** HeLa cells in relation to untreated cells using a standard curve of free tachyzoites and expressed as mean ± SEM from three independent experiments in triplicate. *Significant differences in relation to untreated cells. (*) $P < 0.05$. One-way ANOVA with Bonferroni's Multiple Comparison Test (**a** and **c**), Kruskal Wallis with Dunn's Multiple Comparison Test (**b**)

Table 1 Role of human holo-transferrin treatments in the susceptibility to *T. gondii* infection of BeWo, HTR-8/SVneo, and HeLa cells. The parasite intracellular proliferation data were obtained and the parasite proliferation enhancement calculated in percentage values, which are presented below

Host cells	Treatment conditions	Infection rates	
		T. gondii intracellular proliferation ($\times 10^3$) (mean ± SEM)[a]	Induction of *T. gondii* intracellular proliferation (%)[b]
BeWo	Medium	88.28 ± 5.610	–
	Holo-transferrin 20 μg/mL	91.68 ± 4.186	3.86%
	Holo-transferrin 100 μg/mL	96.42 ± 2.673	9.22%
	Holo-transferrin 250 μg/mL	103.9 ± 2.689*	17.70%*
	Holo-transferrin 500 μg/mL	94.78 ± 3.117	7.36%
	Holo-transferrin 1000 μg/mL	95.67 ± 3.894	8.37%
	Holo-transferrin 2000 μg/mL	80.68 ± 2.838	− 8.61%
HTR-8/SVneo	Medium	94.51 ± 5.830	–
	Holo-transferrin 20 μg/mL	130.3 ± 9.905*	37.91%*
	Holo-transferrin 100 μg/mL	125.1 ± 11.74*	32.35%*
	Holo-transferrin 250 μg/mL	124.6 ± 7.674*	31.79%*
	Holo-transferrin 500 μg/mL	127.0 ± 14.84*	34.37%*
	Holo-transferrin 1000 μg/mL	127.0 ± 11.21*	34.42%*
	Holo-transferrin 2000 μg/mL	124.1 ± 11.85*	31.34%*
HeLa	Medium	287.5 ± 14.62	–
	Holo-transferrin 20 μg/mL	270.4 ± 21.84	− 5.97%
	Holo-transferrin 100 μg/mL	330.1 ± 21.20	14.80%
	Holo-transferrin 250 μg/mL	295.8 ± 21.37	2.88%
	Holo-transferrin 500 μg/mL	304.1 ± 23.04	5.75%
	Holo-transferrin 1000 μg/mL	292.7 ± 37.25	1.78%
	Holo-transferrin 2000 μg/mL	281.0 ± 17.46	− 2.26%

All cell lines were cultured in 96-well plate for 24 h, infected with *T. gondii* (1:1) for 3 h, treated with human holo-transferrin (20, 100, 250, 500, 1000, or 2000 μg/mL) or only medium (medium) for additional 24 h, and submitted for *T. gondii* intracellular proliferation using the β-galactosidase assay

[a] Number of total parasites (tachyzoites) quantified using a standard curve of free tachyzoites ranging from 1×10^6 to 15.625×10^3 total parasites in the β-galactosidase assay

[b] Percentages of *T. gondii* intracellular proliferation with human holo-transferrin treatments in relation to cells incubated with only medium (untreated). The data were expressed as percentage (%) of *T. gondii* proliferation: the mean number of tachyzoites from medium (untreated and infected cells) corresponded to 100% of parasite proliferation, then the number of tachyzoites from each treatment condition was transformed in percentage according to 100% of parasite proliferation from medium. Next, the data were calculated by subtracting the values of cells treated with holo-transferrin from those obtained in untreated cells. *Significant differences in relation to untreated cells (medium) (*) $P < 0.05$. One-way ANOVA with Bonferroni's Multiple Comparison Test for BeWo and HeLa cells, and Kruskal Wallis with Dunn's Multiple Comparison Test for HTR-8/SVneo cells

untreated cells at doses of 250 μM and higher. HeLa cells presented lower percentage of *T. gondii* proliferation inhibition that was 47.41% at dose of 1000 μM DFO. In accordance with higher *T. gondii* proliferation induced by holo-transferrin treatment, the iron chelator by DFO-treatment inhibited more efficiently the parasite proliferation in HTR-8/SVneo compared with BeWo or HeLa cells.

When cells were *T. gondii*-infected and posteriorly iron chelator-treated, DFO showed an IC_{50} for *T. gondii* proliferation inhibition of 56.61 μM for HTR-8/SVneo cells, 121.5 μM for BeWo cells, and 323.1 μM for HeLa cells (Fig. 3d).

In addition, the concentration of the iron was measured in infected and non-infected trophoblastic BeWo and HTR-8/SVneo cell lines. It was observed that the ion concentration was similar between infected and non-infected BeWo or HTR-8/SVneo cells. Of note was that DFO-treatment decreased the iron concentration in non-infected and infected cells, despite not statistically significant (Supplementary Fig. 1).

To verify whether lower parasitism observed in DFO-treated cells was due to the decrease in the cellular viability by the parasite, infected cells were treated with DFO using the median inhibitory concentration (IC_{50}) calculated for each cell line. It was observed that treatment with DFO did not alter the viability of the cells indicating that the control of *T. gondii* multiplication was due to the decrease of the iron availability to the parasite (Supplementary Fig. 2). Similarly, holo-

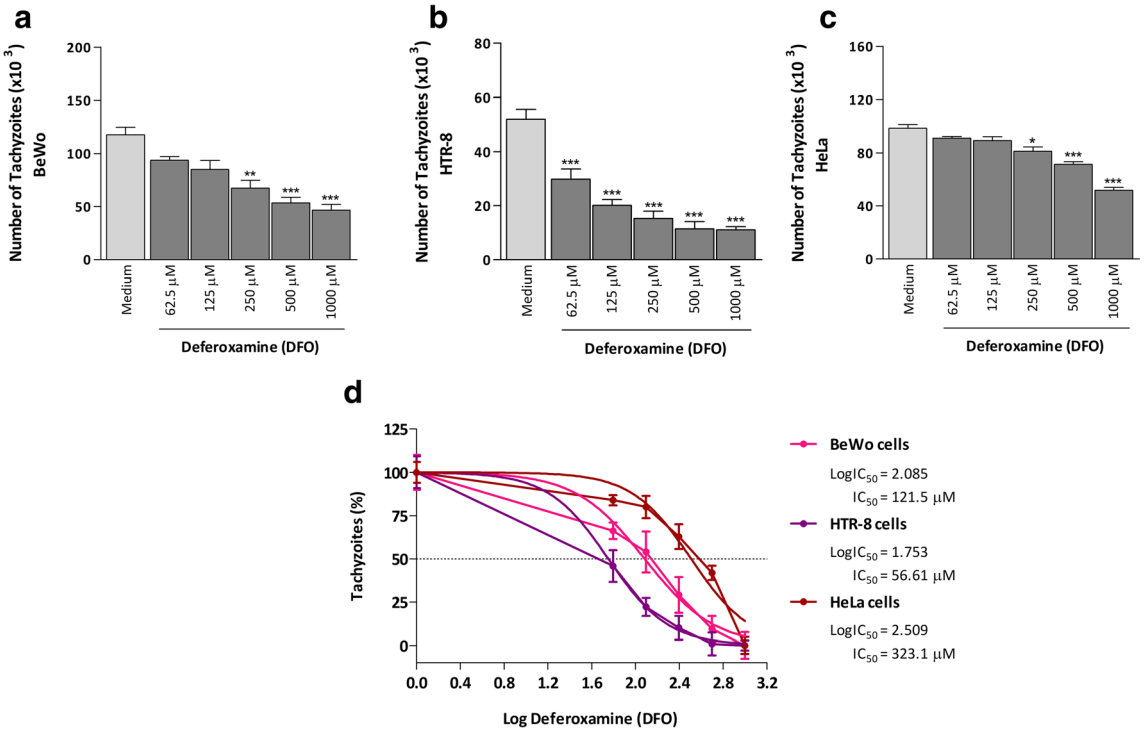

Fig. 3 Role of DFO treatment on *T. gondii* intracellular proliferation in the cell lines. BeWo (3×10^4 cells/200 µL/well), HTR-8/SVneo (1×10^4 cells/200 µL/well), and HeLa (3×10^4 cells/200 µL/well) cells were cultured in 96-well plate during 24 h, infected with *T. gondii* (1:1) for 3 h, treated with DFO (250 µM) or incubated with only medium (medium) for additional 24 h, and analyzed for *T. gondii* intracellular proliferation by β-galactosidase assay. The data were demonstrated as number of tachyzoites for **a** BeWo, **b** HTR-8/SVneo, and **c** HeLa cells in relation to untreated cells using a standard curve of free tachyzoites, and expressed as mean ± SEM from three independent experiments in triplicate. *Significant differences in relation to untreated cells. (*) $P < 0.05$. One-way ANOVA with Bonferroni's Multiple Comparison Test (**b**) or Kruskal Wallis with Dunn's Multiple Comparison Test (**a** and **c**). (**d**) Percentage (%) of tachyzoites in infected BeWo, HTR-8/SVneo, and HeLa cells treated with increasing concentration of DFO represented by median inhibitory concentration (IC_{50}) of DFO-treatment. The IC_{50} was calculated by extrapolation of the corresponding dose-response curve on a log-linear plot employing the portions of the curve that transected the 50% response point

transferrin treatment did not alter the cellular viability of the infected cells (Supplementary Fig. 2).

Pretreatment of parasites with DFO does not interfere with their ability to proliferate inside trophoblastic cells

As iron deprivation was able to diminish the parasite growth in BeWo, HTR-8/SVneo, and HeLa cells, it was verified whether DFO was able to act directly on the parasite and, consequently, interfere on the parasite growth. It was observed that even using crescent doses of DFO to treat *T. gondii* before infection, it did not affect the parasite multiplication inside trophoblast cells (Fig. 4a, b).

T. gondii infection increases the transferrin receptor mRNA expression in BeWo and HTR-8/SVneo cells

Considering that holo-transferrin treatment increased the parasite growth in trophoblastic villous (BeWo) and extravillous (HTR-8/SVneo) cells, the *TfR*, *LtfR*, *Hamp*, and *Ferroportin* mRNA expression levels were measured in infected cells. Despite not statistically significant, it was verified that the infection with *T. gondii* induced higher TfR expression levels in BeWo cells, and significantly augmented the Tf receptor mRNA levels in HTR-8/SVneo cells compared with uninfected cells ($P < 0.05$) (Fig. 5a). In addition, the TfR expression was higher in infected HTR-8/SVneo compared with infected-BeWo ($P < 0.05$) or HeLa cells ($P < 0.05$) (Fig. 5a). It any statistical difference was not observed in *LtfR*, *Hamp*, and *Ferroportin* mRNA expression levels among BeWo, HTR-8/SVneo, and HeLa cells, regardless of *T. gondii* infection (Fig. 5b–d).

The iron starvation by DFO-treatment is able to control *T. gondii* proliferation in human chorionic villous explants

As previously described, DFO was able to reduce *T. gondii* proliferation in trophoblastic BeWo and HTR-8/SVneo, and also in HeLa cervical cells. In order to verify whether the iron deprivation could control *T. gondii* proliferation in a primary

Table 2 Role of DFO treatments in the BeWo, HTR-8/SVneo, and HeLa cells susceptibility to *T. gondii* infection. The parasite intracellular proliferation data were obtained and inhibition of parasitism calculated in percentage values, which are presented below

Host cells	Treatment conditions	Infection rates	
		T. gondii intracellular proliferation ($\times 10^3$) (mean ± SEM)[a]	Inhibition of *T. gondii* intracellular proliferation (%)[b]
BeWo	Medium	117.70 ± 7.11	–
	DFO 62.5 μM	93.67 ± 3.37	20.42%
	DFO 125 μM	85.02 ± 8.41	27.77%
	DFO 250 μM	67.32 ± 7.29*	42.81%*
	DFO 500 μM	53.38 ± 5.35*	54.64%*
	DFO 1000 μM	46.61 ± 5.47*	60.40%*
HTR-8/SVneo	Medium	51.92 ± 3.71	–
	DFO 62.5 μM	29.82 ± 3.77*	42.56%*
	DFO 125 μM	20.15 ± 2.14*	61.19%*
	DFO 250 μM	15.22 ± 2.77*	70.68%*
	DFO 500 μM	11.44 ± 2.70*	77.97%*
	DFO 1000 μM	11.08 ± 1.18*	78.66%*
HeLa	Medium	98.39 ± 2.82	–
	DFO 62.5 μM	90.90 ± 1.40	7.61%
	DFO 125 μM	89.06 ± 3.01	9.48%
	DFO 250 μM	81.06 ± 3.33*	17.61%*
	DFO 500 μM	71.31 ± 1.95*	27.52%*
	DFO 1000 μM	51.74 ± 2.28*	47.41%*

All cell lines were cultured in 96-well plate for 24 h, infected with *T. gondii* (1:1) for 3 h, treated with DFO (62.5, 125, 250, 500, and 1000 μM) or only medium (medium) for additional 24 h, and submitted for *T. gondii* intracellular proliferation using the β-galactosidase assay

[a] Number of total parasites (tachyzoites) quantified using a standard curve of free tachyzoites ranging from 1×10^6 to $15{,}625 \times 10^3$ total parasites in the β-galactosidase assay

[b] Percentages of *T. gondii* intracellular proliferation inhibition with DFO treatment in relation to cells incubated with only medium (untreated). The data were calculated from β-galactosidase assay performed by subtracting the values obtained in cells treated with DFO from those obtained by untreated cells, which showed 100% of infection (−); *Significant differences in relation to untreated cells (medium) (*) $P < 0.05$. One-way ANOVA with Bonferroni's Multiple Comparison Test for HTR-8 cells, and Kruskal Wallis with Dunn's Multiple Comparison Test for BeWo and HeLa cells

experimental model of human maternal-fetal interface, we performed experiments with villous explants from human third-trimester placenta. For this purpose, we treated the infected villous explants with the concentration of DFO (250 μM) that was able to reduce the parasitism in the three cell lines used in this study. It was verified that DFO significantly reduced the parasite replication in the primary human placental tissues ($P = 0.0158$) (Fig. 6).

Discussion

In the early stage of human placental development, extravillous cytotrophoblastic cells detach from cytotrophoblast columns and invade the decidual microenvironment with the purpose of remodeling maternal arteries (Pijnenborg et al. 1980). These extravillous cytotrophoblast are larger than villous cytotrophoblastic cells and undergoes an epithelial-mesenchymal transition (Kurman et al. 1984; Vićovac and Aplin 1996). HTR-8/SVneo cells are a cell model of extravillous cytotrophoblasts (evCTB) that were derived from human first trimester placenta (Graham et al. 1993). Villous cytotrophoblasts (vCTB) are required for nutrient and waste exchange between the mother and fetus, as reviewed by Gude et al. (2004). BeWo cell line is a culture model of vCTB (Pattillo and Gey 1968). In the present study, we were interested in knowing the role of iron availability in *T. gondii* proliferation in trophoblastic cell phenotypes, BeWo, and HTR-8/SVneo, which present distinct functions in the placental development.

It was previously shown that holo-transferrin iron supplementation in HFF cells favors *T. gondii* replication (Gail et al. 2004), and deprivation of iron by deferoxamine, an iron chelator, was able to control the parasite in rat enterocytes and HFF cells (Dimier and Bout 1998; Gail et al. 2004); however, only one study has been done related to the parasite

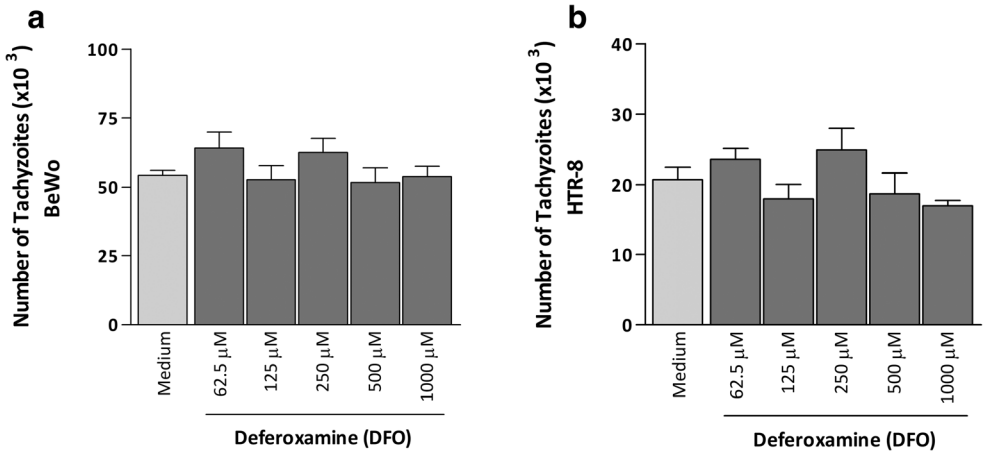

Fig. 4 Role of DFO treatment on *T. gondii* proliferation in cells infected with pretreated tachyzoites. *T. gondii* tachyzoites were pretreated with different concentrations of DFO (62.5, 125, 250, 500, and 1000 μM), or with medium alone (control), for 1 h at 37 °C and 5% CO_2. Next, BeWo (3×10^4 cells/200 μL/well) and HTR-8/SVneo (1×10^4 cells/200 μL/well) cells were infected with pretreated tachyzoites (1:1) for additional 24 h, and analyzed for *T. gondii* intracellular proliferation by β-galactosidase assay. The data were demonstrated as number of tachyzoites for **a** BeWo and **b** HTR-8/SVneo cells in relation to control (medium—parasites treated with only medium) using a standard curve of free tachyzoites and expressed as mean ± SEM from two independent experiments in six-replicates

proliferation with BeWo trophoblast cells supplemented with iron (Pfaff et al. 2005). BeWo cells present transferrin and transferrin receptor expression (Morris Buus and Boockfor 2004; Li et al. 2012b) and transferrin is observed in vesicular

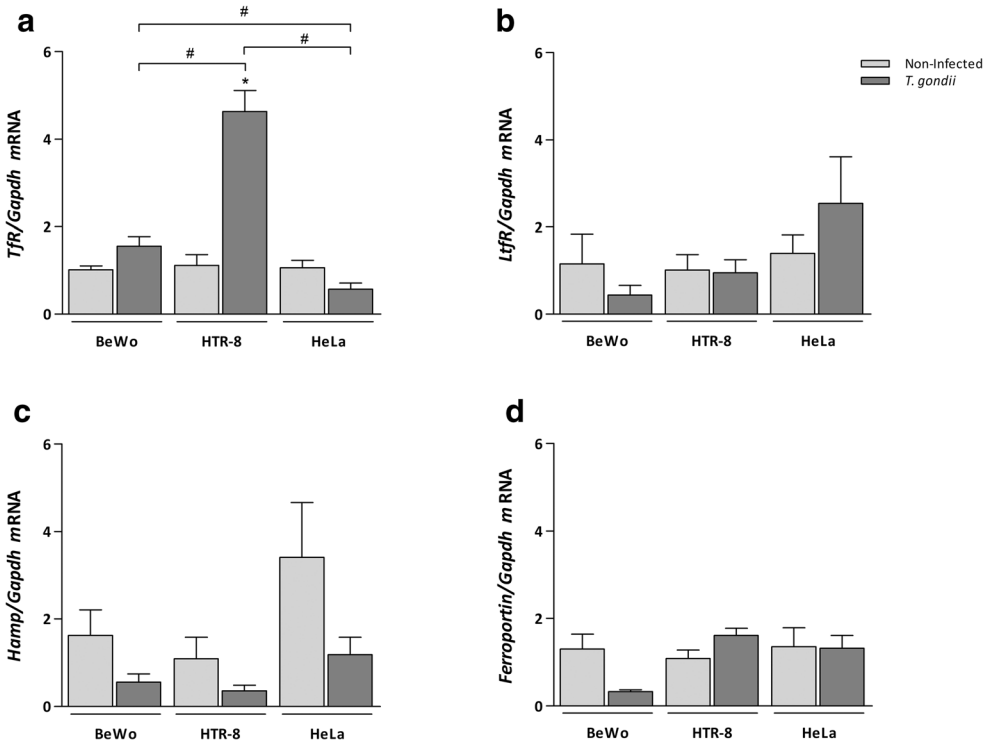

Fig. 5 Influence of *T. gondii* infection on mRNA expression of iron-regulatory factors in the cell lines. BeWo (2×10^5 cells/200 μL/well), HTR-8/SVneo (6×10^4 cells/200 μL/well), and HeLa (2×10^5 cells/200 μL/well) cells were cultured in 24-well plate during 24 h, infected or not with *T. gondii* (1:1) for 3 h, and incubated with only medium for additional 24 h. The total RNA was extracted and measured, where 1 μg was further treated with DNase and used as template to cDNA construction by reverse transcription reaction. To the end, the real time quantitative PCR (qPCR) assay was performed and the cycle threshold (Ct) data obtained was used for gene expression quantification of the studied targets, **a** *TfR*, **b** *LtfR*, **c** *Hamp*, and **d** *Ferroportin* in relation to endogenous control *Gapdh*. The data were expressed as mean ± SEM from one independent experiment in six-replicates. *Significant differences in relation to non-infected HTR-8/SVneo cells in comparison with *T. gondii*-infected HTR-8/SVneo cells. #Significant differences with respect to different infected trophoblast cell types. (*)(#) $P < 0.05$. One-way ANOVA with Bonferroni's Multiple Comparison Test

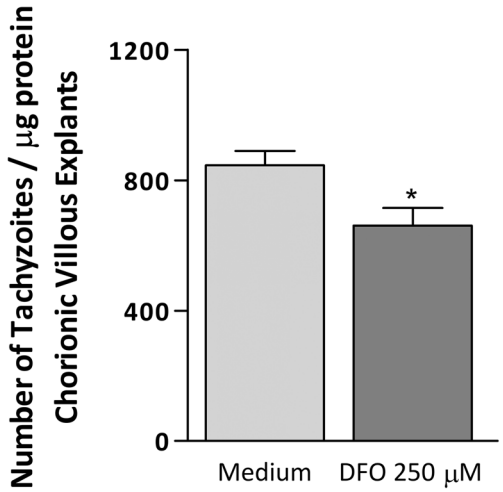

Fig. 6 Role of DFO treatment on *T. gondii* intracellular proliferation in human villous explants. Chorionic villous explants were cultured in 96-well plate (one per well/200 μL) during 24 h, infected with *T. gondii* (1×10^6 tachyzoites per well) for additional 24 h, treated with DFO (250 μM) or incubated with medium (medium) only for the additional 24 h and analyzed for parasite intracellular proliferation by β-galactosidase assay. The data were demonstrated as number of taczhyoites normalized per microgram of total proteins and expressed as mean ± SEM from one independent experiment in nine replicates. *Significant differences in relation to untreated villous explants. (*) $P < 0.05$. Student's *t* test

structures in HTR-8/SVneo cells treated with Nodal, a secreted growth factor of the transforming growth factor (TGF-β) superfamily (Law et al. 2014).

Thus, in the first set of experiments, it was verified the effect of holo-transferrin, an iron supplementation or deferoxamine, an iron deprivation, in different concentrations in BeWo and HTR-8/SVneo cellular viability. It was observed that holo-transferrin addition did not decrease the cellular viability of BeWo or HTR-8/SVneo cells, while DFO treatment affected the cellular viability slightly and in a similar way in these two trophoblastic cell phenotypes. It was verified that holo-transferrin increased *T. gondii* proliferation in low levels when used at a dose of 250 μg/mL in BeWo cells and in an expressive way in HTR-8/SVneo cells treated since 20 to 2000 μg/mL. It was previously shown that the addition of iron sulfate did not alter the parasite growth in BeWo cell line (Pfaff et al. 2005). The different result obtained with BeWo cells in our investigation is due to the manner which this cell phenotype was supplemented with iron since BeWo cells express the transferrin receptor, facilitating the holo-transferrin internalization and iron availability to the parasite. Interestingly, the holo-transferrin addition to the extravillous HTR-8/SVneo cells increased the parasite proliferation.

When cells were treated with DFO, it was observed that the iron chelator was able to diminish the parasite proliferation in the three cell phenotypes, inhibiting the parasite proliferation at a maximum of 60.4% in BeWo cells, 78.66% in HTR-8/SVneo cells, and 47.41% in HeLa cells when using the dose of 1000 μM. Thus, the HTR-8/SVneo cells were able to control more efficiently the parasite proliferation when treated with DFO. Thus, at the first time, it was demonstrated that iron chelating is an efficient mechanism to control *T. gondii* proliferation in trophoblast cells, mainly in extravillous trophoblast cells that in vivo are in contact with maternal blood vessels. In parallel, the cellular viability was determined in infected cells treated with DFO and all of the cell phenotypes presented high viability, showing that the lower parasite number was not due to cellular death. In addition, *T. gondii* tachyzoites were treated with crescent DFO concentrations before infection of BeWo and HTR-8/SVneo cells and *T. gondii* proliferation was evaluated. It was observed that pretreated parasites proliferate similarly to untreated parasites. Thus, these data indicate that the control of *T. gondii* growth by DFO is dependent of the iron pool availability rather than the direct effect of the iron chelator on the parasite.

In addition, previous studies of our group have demonstrated that chorionic villi explants from term placenta (third trimester pregnancy) are an important model of human maternal-fetal interface and are also responsive to *T. gondii* infection in different immunological contexts investigated (Gomes et al. 2011; Silva et al. 2017). In order to reinforce our findings with human BeWo and HTR-8/SVneo trophoblastic cells, the human chorionic villous explants that represent the human maternal-fetal interface were infected and treated with DFO. Interestingly, as observed with cell lines, the DFO-treatment was able to reduce *T. gondii* proliferation in the villous explants.

As the BeWo and mainly HTR-8/SVneo cells treated with holo-transferrin increased *T. gondii* proliferation, it was analyzed that the expression of genes is involved in the control of iron internalization by the cells, *TfR*, *LtfR*, and iron exiting, *Ferroportin* and *Hamp*, when cells are infected with *T. gondii*. It was verified that the infection with the parasite enhanced the transferrin receptor expression in HTR-8/SVneo cells and also BeWo cells, despite being not statistically significant, and the expression was higher in HTR-8/SVneo compared with BeWo and HeLa cells. This data is in accordance with higher parasite proliferation in HTR-8/SVneo treated with holo-transferrin compared with BeWo cells under the same treatment, and also HeLa cells, and as HeLa cells did not alter the organism growth under holo-transferrin treatment, the parasite did not interfere in the receptor expression in this cell line. The transferrin receptor (TfR) was specifically upregulated in *Toxoplasma*-infected HFF cells (Gail et al. 2001, 2004), and the binding activity of iron-responsive protein 1 (IRP1) to "iron responsive elements" (IRE) was increased 5.5-fold in *T. gondii*-infected fibroblasts compared to uninfected controls, indicating that the increase in IRP1 activity would stabilize the TfR mRNA increasing its expression (Gail et al. 2004). Interestingly, BeWo cells present high number of transferrin receptors, being that only 40% of these receptors are at the plasma membrane. During iron uptake by BeWo cells,

transferrin recycles every 19 min. The accumulated iron is released from the BeWo cells at the extracellular medium at a considerable rate, and this release process is cell line-specific as HeLa cells release very little if any iron (Van Der Ende et al. 1987). Thus, in our experimental investigation, despite increasing mRNA of *TfR* under infection which could reflex elevated transferrin receptor expression, elevated iron internalization and consequently, parasite growth, the holo-transferrin treatment was able to increase *T. gondii* intracellular proliferation only by 17.70% in BeWo cells which could be related to the high iron release to the extracellular medium by this cell line. Concerning HTR-8/SVneo cells, there is no information related to the iron release to the extracellular medium that could be even smaller than BeWo cells. Additional experiments are necessary to clarify iron internalization and release in HTR-8/SVneo cells.

Thus, in determined cell phenotypes, the parasite interferes in the iron transporters expression to improve the iron internalization favoring the parasite growth and related to the transferrin receptor, here it was demonstrated that extravillous HTR-8/SVneo is one of these cells. Lactoferrin receptor provides an alternative route of iron acquisition by biological systems and this receptor has been identified in BeWo cell line (Lopez et al. 2008). Additionally, it was previously identified that two rhoptry protein families (ROP2 and ROP4) in *T. gondii* are capable of binding to lactoferrin (Dziadek et al. 2005, 2007). However, in our experimental work, it was not verified any alteration in lactoferrin receptor expression levels in BeWo and HTR-8/SVneo cells under *T. gondii* infection, suggesting that at least in trophoblastic human cells, the *T. gondii* proliferation is influenced by iron internalization via transferrin-transferrin receptor transport.

The control of iron assimilation is mediated by the hepcidin, encoded by *HAMP* gene, which acts to induce cellular internalization and subsequent ferroportin degradation (Nemeth et al. 2004). It was previously observed that in the liver of pregnant rats, the iron content decreases, and in hepatic rat cells, the hepcidin expression declines and TfR1 augments with the progression of gestation (Millard et al. 2004) indicating an attempt to attend the fetal demand during development. The hepcidin can also be expressed in human syncytiotrophoblast cells and the expression is higher in placenta from mothers that deliver newborns with hemochromatosis (NH) (Shimono et al. 2016).

As ferroportin is expressed by trophoblast cells and it is important for the exit of iron to the fetus and hepcidin is involved with the control of iron levels during gestation, we measured the ferroportin and hepcidin expressions in BeWo and HTR-8/SVneo cells infected with *T. gondii*. It was verified that the infection with the parasite did not affect the ferroportin or hepcidin mRNA expression in these lineages of trophoblast cells.

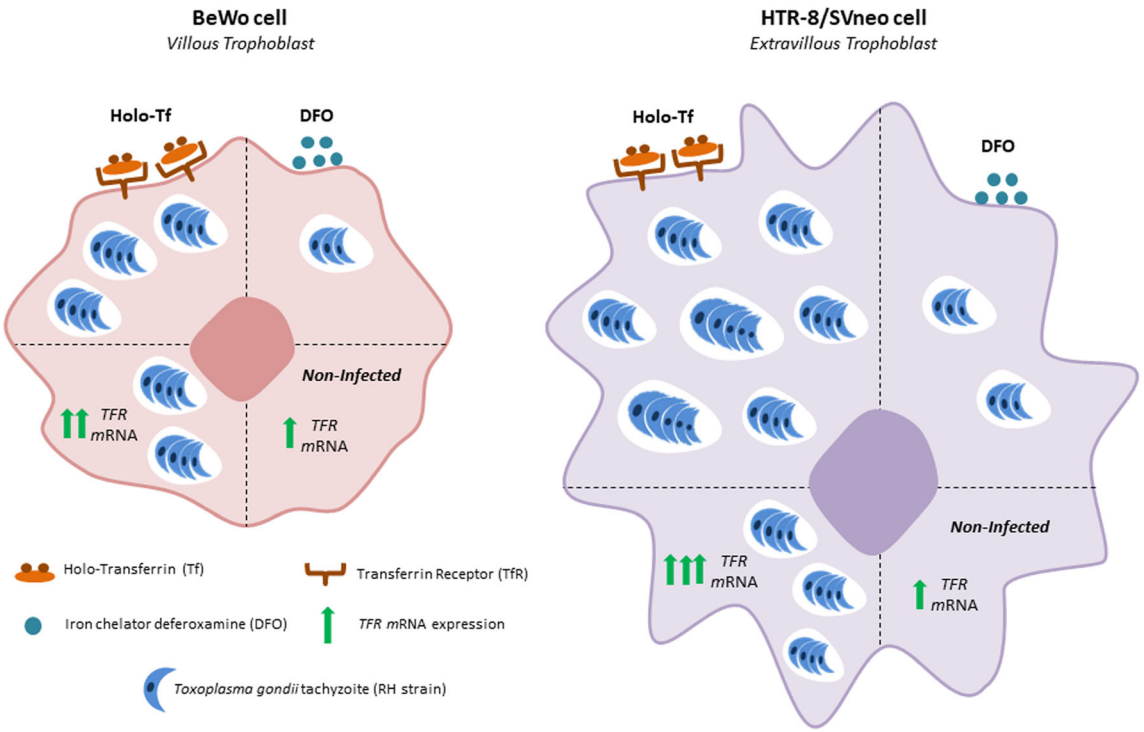

Fig. 7 Schematic model demonstrating the influence of intracellular iron availability on susceptibility of human villous (BeWo) and extravillous (HTR-8/SVneo) trophoblastic cells to *Toxoplasma gondii* infection. The *Toxoplasma gondii* proliferation in trophoblast cells depends on iron availability since holo-transferrin supplementation and deferoxamine-treatment enhanced and decreased the parasitism, respectively. The holo-transferrin supplemented extravillous HTR-8/SVneo cells were more susceptible to the parasite presenting higher *T. gondii* proliferation than villous cytotrophoblast BeWo cells in the same condition, and in parallel, higher TfR mRNA expression

In conclusion, the results of the present investigation show that *T. gondii* needs to acquire iron for its own metabolism from the placenta iron pool since the treatment of cells or chorionic villus explants with DFO decreases the parasite proliferation. In addition, the parasite affects the *TfR* mRNA levels and probably TfR protein expression levels mainly in HTR-8/SVneo cells, with the purpose to enhance the iron levels inside cells for its growth (Fig. 7).

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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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