Iron-modulated pseudocyst formation in *Tritrichomonas foetus*

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SUMMARY

Iron is an essential element for the survival of trichomonads during host–parasite interaction. The availability of this metal modulates several metabolic pathways of the parasites and regulates the expression of virulence factors such as adhesins and proteolytic enzymes. In this study, we investigated the effect of iron depletion on the morphology and life cycle of *Tritrichomonas foetus*. Scanning and transmission electron microscopy analyses revealed that depletion of iron from the culture medium (named TYM-DIP inducer medium) induces morphological transformation of typical pear-shaped trophozoites into spherical and non-motile pseudocysts. Remarkably, inoculation of pseudocysts into an iron-rich medium (standard TYM medium), or addition of $FeSO_4$ to a TYM-DIP inducer medium reverted the morphological transformation process and typical trophozoites were recovered. These results show that pseudocysts are viable forms of the parasite and highlight the role of iron as a modulator of the parasite phenotype. Although iron is required for the survival of *T. foetus*, iron depletion does not cause a cellular collapse of pseudocysts, but instead induces phenotypic alterations, probably in order to allow the parasite to survive conditions of nutritional stress. Together, these findings support previous studies that suggest pseudocysts are a resistance form in the life cycle of *T. foetus* and enable new approaches to understanding the multifactorial role of iron in the cell biology of this protozoan parasite.

Key words: *Tritrichomonas foetus*, pseudocyst, iron, morphological transformation, trophozoites.

INTRODUCTION

Tritrichomonas foetus is an extracellular protozoan parasite that colonizes the urogenital tract of cattle. This parasite is the etiological agent of bovine trichomoniasis, a venereal disease responsible for important production losses in livestock due to reproductive failures (BonDurant, 2005; Rae and Crews, 2006). Infected bulls are commonly asymptomatic, whereas cows present a complex symptomatology ranging from oligosymptomatic infections to severe complications including vaginitis, cervicitis, endometriosis, transient or permanent infertility and abortion (Parsonson *et al*. 1976; Rae and Crews, 2006).

During its life cycle, *T. foetus* possesses a trophozoite stage that is characterized by a pyriform body with three anterior flagella and one recurrent flagellum that is incorporated into an undulating membrane (Pereira-Neves *et al*. 2003; Honigberg and Brugerolle, 1990). Under unfavourable growth conditions the typical pear-shape trophozoites internalize their flagella and adopt a spherical or ellipsoid shape referred as the endoflagellar form (EFF) or pseudocyst, as it does not possess a true cyst wall (Granger *et al*. 2000; Pereira-Neves *et al*. 2003, 2012; Andrade Rosa *et al*. 2015). This form is also observed, although in low proportion, under *in vitro* axenic culture conditions (Pereira-Neves *et al*. 2011). Transformation of typical trophozoites to pseudocysts has also been observed in other species of Trichomonadidae such as *Trichomonas vaginalis*, a human pathogen of the urogenital tract (Pereira-Neves *et al*. 2003; Benchimol, 2004; De Jesus *et al*. 2007; Afzan and Suresh, 2012), *Tritrichomonas muris*, rodent gut colonizers (Lipman *et al*. 1999) and *Trichomonas tenax*, a commensal organism (Ribeiro *et al*. 2015).

Tritrichomonas foetus exhibits multiple granular organelles called hydrogenosomes that evolved from the same ancestral than mitochondria (Müller *et al*. 2012), and a complex cytoskeleton composed of a variety of structures such as a microtubular pelta-axostilar system and costa (Honigberg and Brugerolle, 1990; Müller, 1993; Benchimol, 2009). This trichomonad possesses the glucose metabolism compartmentalized in the cytoplasm and

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hydrogenosomes (Cerkasovova *et al*. 1984; Lindmark *et al*. 1989; Ali and Nozaki, 2007). Crucial steps of hydrogenosomal metabolism are mediated by iron–sulphur proteins (Müller, 1988; Ellis *et al*. 1993; Payne *et al*. 1993; Townson *et al*. 1994), which explains the high iron nutritional requirements of this parasite.

Iron is an essential element for *in vitro* and *in vivo* survival and proliferation of trichomonads. This metal plays important roles during host–parasite interaction, such as modulating the expression of crucial metabolic and proteolytic enzymes and regulating immunological processes mediated by adhesins and extracellular matrix proteins (Alderete *et al*. 1995; Crouch *et al*. 2001; Ryu *et al*. 2001; Melo-Braga *et al*. 2003). In addition, iron regulates the expression of several genes at transcriptional and post-transcriptional levels (Torres-Romero and Arroyo, 2009, Horváthová *et al*. 2012; Beltrán *et al*. 2013). To supply the high amount of iron required, *T. foetus* acquires it from different host sources such as lactoferrin, transferrin and haeme (Tachezy *et al*. 1996; Tachezy, 1998). The parasite may also obtain iron via endocytosis and pinocytosis of siderophores (Sutak *et al*. 2004).

This study investigates the role of iron in regulating the life cycle and ultrastructure of *T. foetus*. Using the iron chelator 2,2-dipyridyl it was demonstrated that iron depletion from culture medium (named TYM-DIP inducer medium) interrupts parasite proliferation and induces a morphological transformation from pyriform trophozoites to spherical, non-replicative and non-motile pseudocysts. Inoculation of pseudocysts into iron-rich medium (standard TYM medium), or addition of a high concentration of $FeSO_4$ to a TYM-DIP inducer medium, causes reversion of pseudocysts back to original pyriform morphology. These findings support previous studies that suggest pseudocysts are viable forms of the parasite and indicate that iron modulates the morphological transformation process.

MATERIALS AND METHODS

Chemicals

Trypticase peptone was purchased from Becton Dickinson (São Paulo, SP, Brazil), yeast extract, D-maltose, cysteine, potassium chloride, potassium phosphate monobasic, potassium phosphate dibasic and iron sulphate were purchased from Sigma (St. Louis, MO, USA); potassium carbonate and ascorbic acid were purchased from Merck (São Paulo, SP, Brazil). Milli-Q-purified water (Millipore Corp., Bedford, MA, USA) was used for all solutions. The iron chelator 2,2-dipyridyl is an organic, synthetic, membrane-permeable compound that associates with extracellular and intracellular iron (Breuer *et al*. 1995; Thompson and Carabero, 2011).

Parasite culture

Tritrichomonas foetus K strain (Silva-Filho *et al*. 1986) was used throughout. The parasites were axenically maintained at 37 °C in trypticase yeast extract maltose medium, (TYM: trypticase peptone 20 g L^{-1}, yeast extract 10 g L^{-1}, D-maltose 5 g L^{-1}, cysteine 1 g L^{-1}, potassium chloride 1 g L^{-1}, hydrogenated potassium carbonate 1 g L^{-1}, potassium phosphate monobasic 1 g L^{-1}, potassium phosphate dibasic 0·5 g L^{-1}, iron sulphate 0·1 g L^{-1}, ascorbic acid 0·2 g L^{-1}) pH 6·6 (Diamond, 1957), supplemented with 10% heat-inactivated bovine serum and 0·6 mM $FeSO_4$ (standard TYM medium). For pseudocyst induction, parasites were cultivated in the TYM medium, pH 6·6, supplemented with 10% heat-inactivated bovine serum plus 300 μM 2,2-dipyridyl (TYM-DIP inducer medium). In all assays, parasites viability was estimated by using the Trypan blue dye-exclusion test [0·4% in sterile phosphate-buffered saline (PBS)].

Effect of iron chelator on parasite proliferation and morphology

To evaluate the influence of iron chelation on parasite proliferation, 1×10^5 parasites were inoculated in a standard TYM medium and a TYM-DIP inducer medium and incubated at 37 °C for 72 h. Cellular density and morphotypes were evaluated daily by counting in a haemocytometer. *Tritrichomonas foetus* cultivated for 48 h at 37 °C in the TYM-DIP inducer medium were harvested by centrifugation and washed twice with PBS, pH 7·2. Parasite morphotypes were analysed by differential interference contrast microscopy [interference contrast microscopy (DIC)].

Analysis of phenotype reversibility and the effect of $FeSO_4$ on iron-depletion-induced pseudocysts

To evaluate whether iron-depletion-induced pseudocysts are viable and reversible forms, parasites cultivated in the TYM-DIP inducer medium at 37 °C for 48 h were collected by centrifugation at 2500 × *g* for 5 min, washed twice with PBS pH 7·2, resuspended in the standard TYM medium and incubated at 37 °C for 48 h.

Alternatively, to demonstrate that maintenance of the typical pear-shaped trophozoite morphology of *T. foetus* is dependent on iron, parasites were cultivated for 48 h in the TYM-DIP inducer medium and then 1·2 mM $FeSO_4$ was added and incubated for additional 24 h. Parasite morphology was examined by light microscopy and morphotypes were counted using a haemocytometer.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analyses

Parasite trophozoites (5×10^6 cells mL^{-1}) were cultivated in the standard TYM medium or the

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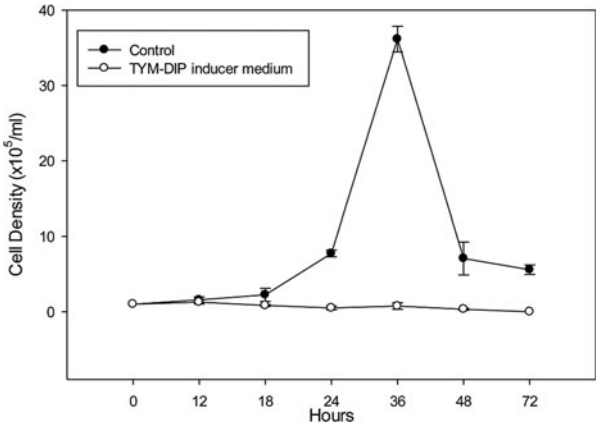

Fig. 1. Effect of iron depletion on *T. foetus* growth curve. Parasites were cultivated in the standard TYM medium (control – ●) or the TYM-DIP inducer medium (300 μM 2,2-dipyridyl – ○) for 72 h at 37 °C. Counting of parasites was performed in triplicate by using a haemocytometer. Bars represent means ± S.E. from three independent experiments.

TYM-DIP inducer medium for 48 h at 37 °C. The parasites were then fixed with 2·5% glutaraldehyde in 0·1 M Na-cacodylate buffer (pH 7·2) at room temperature for 1 h at 25 °C and post-fixed with a solution of 1% OsO_4, 0·8% potassium ferricyanide and 2·5 mM $CaCl_2$ in the same buffer for 1 h at 25 °C. The samples were dehydrated in an ascending acetone series, dried by the critical point method with CO_2, mounted on aluminium stubs, coated with a 20 nm thick gold layer and examined using a Jeol JSM6390LV scanning electron microscope (Tokyo, Japan). For TEM analysis, after the dehydration step, the samples were embedded in PolyBed 812 resin and the ultrathin sections obtained were stained with uranyl acetate and lead citrate. The examination was performed in a Jeol JEM1011 transmission electron microscope (Tokyo, Japan).

Statistical analysis

Growth assays were carried out in triplicate. Statistical analyses were performed with GraphPad Prism (GraphPad software, San Diego, CA). The Student *t* test was used to analyse differences between parasite morphotypes. The data are presented as means ± S.D.

RESULTS

Iron-depletion-inhibited T. foetus *growth and induced pseudocyst formation*

Parasites cultivated in a standard medium, rich in iron, reached the late logarithmic phase of growth at 36 h with a maximum cell density of ~$3{\cdot}8 \times 10^6$ parasites mL^{-1}. In contrast, parasites inoculated into the TYM-DIP inducer medium did not proliferate, indicating that iron depletion inhibits *T. foetus* proliferation (Fig. 1). Light microscopy analysis of parasites cultivated in the standard TYM medium showed that up to 24 h of culture all parasites were motile pear-shaped trophozoites, whereas from 36 to 72 h of culture, 5–8% of the parasites displayed a spherical form without external flagella, resembling pseudocysts (Fig. 2A and Fig. 3A, Supplementary material 1). Drastic effects were observed on the morphology of parasites cultivated in the TYM-DIP inducer medium. Between 12 and 36 h of culture, increasing non-motile pseudocyst formation was observed concomitant with a decreasing typical pear-shaped trophozoite population (Fig. 2B; Fig. 3B, Supplementary material 1). At 48 h of culture in the TYM-DIP inducer medium, all cells exhibited the pseudocyst form, while no viable parasites were observed at 72 h (Fig. 3B).

Iron-depletion-induced alteration of ultrastructure organization of T. foetus

SEM analysis of parasites cultivated for 48 h in the standard TYM medium showed that the population is mainly composed of typical pear-shaped trophozoites with evident axostyle at the posterior region of the body, three anterior flagella and one recurrent flagellum (Fig. 4A). In contrast, parasites cultivated for 48 h in the TYM-DIP inducer medium showed a population mainly composed of spherical pseudocysts with all flagella internalized (Fig. 4B) and some oval or spherical pseudocysts at different stages of flagella internalization (Fig. 4C and D).

TEM analysis of parasites cultivated in the standard TYM medium showed typical trophozoites with externalized flagellum, and common subcellular organization with one anterior nucleus and hydrogenosomes (Fig. 5A). On the other hand, analysis of *T. foetus* cultivated in the TYM-DIP inducer medium revealed parasites with internalized flagella, clearly visible in the cytoplasm, surrounded by a membrane (Fig. 5B).

Iron-depletion-induced pseudocysts are viable and reversible forms

To verify whether pseudocysts induced by iron depletion are viable forms and if this morphological change is reversible, parasites cultivated for 48 h in the TYM-DIP inducer medium were reinoculated into the standard TYM medium and cultivated for an additional 48 h. A progressive increase in the flagellated trophozoite population was observed over time, reaching a maximum density at 32 h concomitant with a drastic decrease in the number of pseudocysts. At 48 h, a drastic decrease in the trophozoite population was observed, whereas no pseudocyst forms were detected (Fig. 6).

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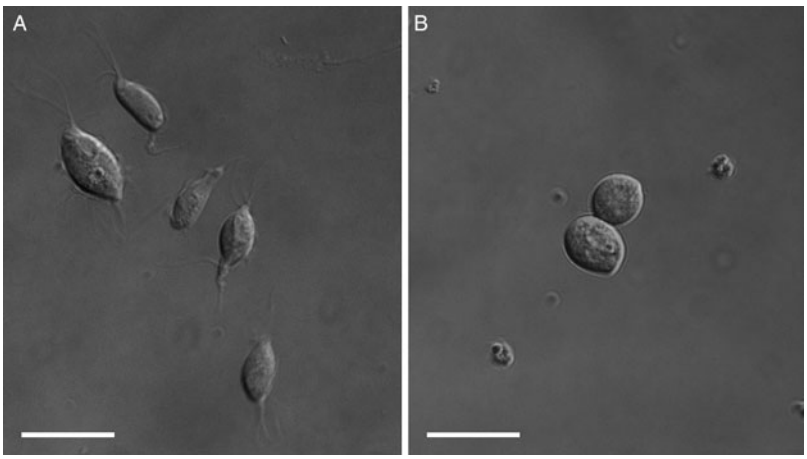

Fig. 2. Differential DIC of the effect of iron depletion on *T. foetus* morphology. Parasites were cultivated for 48 h in the standard TYM medium (A) or the TYM-DIP inducer medium (B). Typical pear-shaped trophozoites are observed in A, whereas pseudocysts are observed in B. Bar = 20 μm.

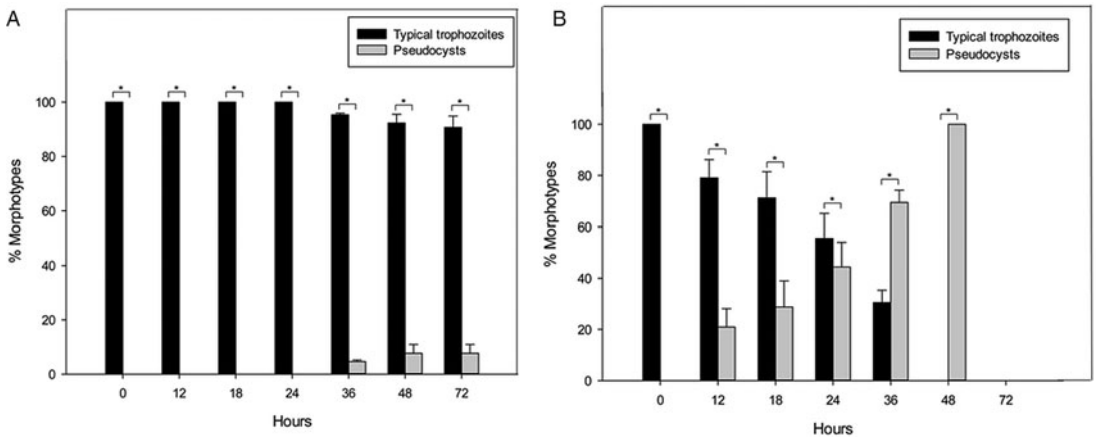

Fig. 3. Effect of iron depletion on *T. foetus* phenotypes. Parasites were cultivated for 72 h in the standard TYM medium (A) or the TYM-DIP inducer medium (B). Counting of parasites was performed in triplicates by using a haemocytometer. Results are presented as means and S.E. from three independent experiments. *Significant differences between morphotypes ($P < 0{\cdot}001$).

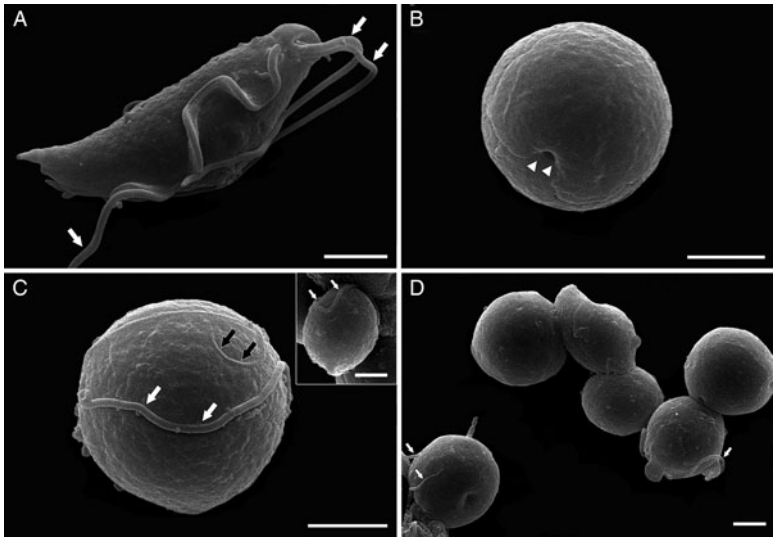

Fig. 4. SEM analysis of the effect of iron depletion on *T. foetus* morphology. *T. foetus* cultivated in the standard TYM medium (A) or the TYM-DIP inducer medium (B–D). Typical pear-shaped elongated trophozoites; arrows show multiple externalized flagella (A). Pseudocysts obtained by iron depletion show rounded forms with all flagella internalized (B, D) and membrane invaginations (arrowheads – B) or exhibiting distinct stages of flagella internalization (arrows – C, D). Bars = 2 μm.

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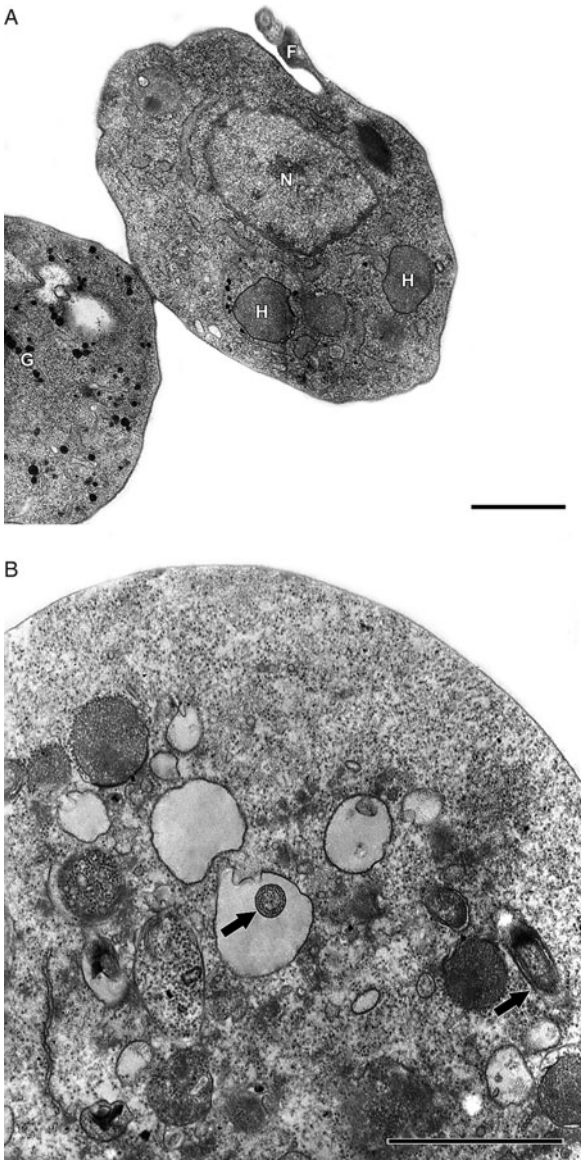

Fig. 5. TEM analysis of the effect of iron depletion on *T. foetus* ultrastructure. *T. foetus* cultivated in the standard TYM medium (A) or the TYM-DIP inducer medium (B). (A) Trophozoites with typical morphology, H, hydrogenosomes; G, granules of glycon; N, nucleus and F, flagellum. (B) Parasites cultivated in the presence of iron chelator exhibit internalized flagella (black arrows). Bars = 1 μm.

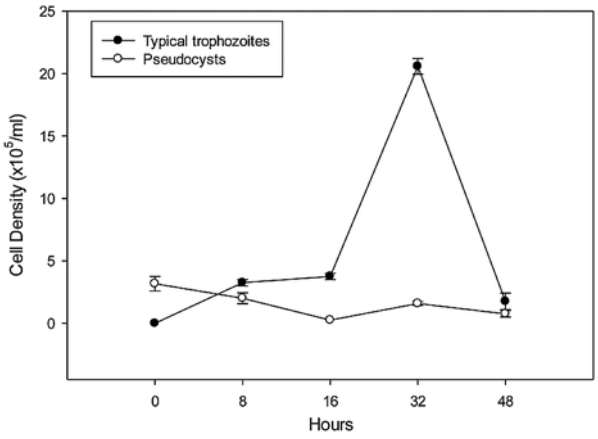

Fig. 6. Analysis of phenotype reversibility of iron-depletion-induced pseudocysts. Parasites maintained for 48 h in the TYM-DIP inducer medium were collected, washed in PBS, and transferred to a fresh standard TYM medium. Cell density and parasite morphotypes were monitored for 48 h by counting with a haematocytometer. Data obtained from three independent experiments are presented as mean ± S.E.

Addition of $FeSO_4$ to the TYM-DIP inducer medium reverted pseudocysts to trophozoites and recovered the parasites proliferation capability

To determine whether trophozoite transformation to pseudocysts is an iron-dependent phenomenon, TYM-DIP inducer medium containing fully transformed pseudocysts was supplemented with 1·2 mM $FeSO_4$. After 24 h of iron supplementation, ~90% of the pseudocysts recovered their typical pear-shape trophozoite form whereas ~10% remained in the pseudocyst form. In addition, pseudocysts maintained in the TYM-DIP inducer medium without $FeSO_4$ supplementation did not exhibit morphological transformation to trophozoites (Fig. 7).

DISCUSSION

The importance of iron to many cellular processes of parasitic protozoa has been extensively documented (Loo and Lalonde, 1984; Atkinson *et al*. 1991; Merschjohann and Steverding, 2006; De Jesus *et al*. 2007; Lee *et al*. 2008). In trichomonads, the presence of iron in the culture medium is essential for the cell metabolism, phosphohydrolase activities and hydrogenossomal metabolism as well as for the expression of crucial proteins involved in adhesion and cytotoxicity to host cells (Alderete *et al*. 1995; Vanacová *et al*. 2001; De Jesus *et al*. 2006; Hsu *et al*. 2009; Torres-Romero and Arroyo, 2009; Horváthová *et al*. 2012; Beltrán *et al*. 2013). We previously demonstrated that iron depletion alters global protein expression in *Tritrichomonas vaginalis* and induces morphological changes to the parasite resulting in cell forms similar to pseudocysts (De Jesus *et al*. 2007). Light microscopy revealed unusual morphological alterations in *T. foetus* cultivated in the absence of iron (Melo-Braga *et al*. 2003). In this study, iron depletion inhibited parasite proliferation and induced the transformation of pyrifom trophozoites into viable, non-replicative pseudocysts. Although the withdrawal of iron from the culture medium using 2,2-dipyridyl immediately inhibited parasite growth, the population remained viable for 48 h after treatment. These results are in agreement with previous reports that demonstrated that iron depletion affects the proliferation of other protozoan parasites including,

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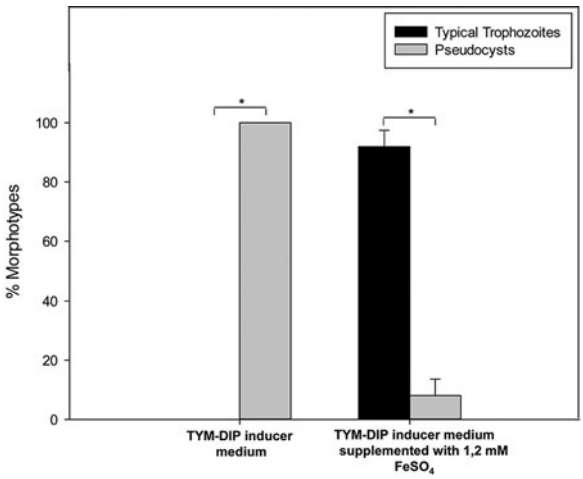

Fig. 7. Effect of $FeSO_4$ on iron-depletion-induced pseudocyst. Parasites were cultivated in in the TYM-DIP inducer medium for 48 h or the TYM-DIP inducer medium for 48 h, and then supplemented with 1·2 mM $FeSO_4$ and cultivated for an additional 24 h. Parasite morphotypes were estimated by counting in a haematocytometer. Data obtained from three independent experiments are presented as mean ± S.E. *Significant differences between morphotypes ($P < 0{\cdot}001$).

T. foetus, T. vaginalis, Leishmania braziliensis, Leishmania major, Leishmania infatum, Plasmodium falciparum and *Trypanosoma cruzi* (Lalonde and Holbein, 1984; Atkinson *et al*. 1991; Lehker and Alderete, 1992; Soteriadou *et al*. 1995; Melo-Braga *et al*. 2003; Mesquita-Rodrigues *et al*. 2013). These data suggest that the mechanisms underlying iron regulation of cell proliferation may be similar among the different species of pathogenic protozoa.

Inhibition of *T. foetus* proliferation by iron depletion could be a result of direct or indirect mechanisms of cell growth regulation mediated by the metal. Parasites require high iron concentrations for the correct synthesis and function of Fe–S enzymes involved in hydrogenosomal energy metabolism, such as pyruvate: ferredoxin oxidoreductase (PFO), ferredoxin and [FeFe]-hydrogenase. Consequently, the absence of iron would impede the production and function of these enzymes, resulting in inhibition of hydrogenosomal function followed by a decrease in ATP production (Vanacová *et al*. 2001). In *T. vaginalis*, iron depletion downregulated the expression of PFO and ferredoxin and induced parasite transformation to spherical forms (De Jesus *et al*. 2007). Downregulation of metabolic in *T. foetus* and *T. vaginalis* cultivated in iron depleted medium supports the theory that inhibition of parasite proliferation could occur by an uncharacterized, indirect mechanism such as negative feedback (Gorrell, 1985; Tachezy *et al*. 1996; Vanacová *et al*. 2001; De Jesus *et al*. 2007). Alteration of hydrogenosomal metabolism could signal a decrease in cell proliferation and induce a lethargic state, which would avoid the production and accumulation of potentially toxic secondary metabolites. On the other hand, iron could also regulate parasite proliferation by a direct mechanism mediated by iron-responsive elements (IREs), located in untranslated regions (UTRs) of target mRNAs that control gene expression at the post-transcriptional level (Ong *et al*. 2004; Solano-González *et al*. 2007; Torres-Romero and Arroyo, 2009). It was recently shown that iron regulates the expression of only some copies of paralogous genes in *T. vaginalis* whereas other copies are not regulated by iron, indicating that iron regulatory mechanisms are even more complex than previously thought (Beltrán *et al*. 2013).

Inhibition of *T. foetus* proliferation by iron depletion was followed by drastic morphologic changes. SEM analysis revealed that parasites cultivated for 48 h in iron-depleted medium acquired a spherical form with internalized flagella, resembling pseudocysts, also known as EFF. Intermediate forms exhibiting partial internalization of flagella were also observed. Flagella internalization in *T. foetus* pseudocysts induced by low temperature seems to follow a temporal dynamic in which the three anterior flagella are internalized together followed by the recurrent flagellum (Granger *et al*. 2000; Pereira-Neves *et al*. 2003). Flagella internalization seems to occur by processes similar to receptor-mediated endocytosis (Granger *et al*. 2000; De Jesus *et al*. 2007), as active endocytic machinery has been observed on the cell surface of trophozoites undergoing transformation to pseudocysts (Benchimol *et al*. 1990; Affonso *et al*. 1997). It was previously demonstrated that pseudocyst formation in *T. vaginalis* induced by iron depletion involves rearrangement of cytoskeletal structures along with the overexpression of actin genes, which may allow rapid morphological change to occur when exposed to different stimuli (De Jesus *et al*. 2007). *Tritrichomonas foetus* seems to respond in a similar way when exposed to drugs or drastic temperature variation, internalizing flagella and becoming spherical (Granger *et al*. 2000; Ribeiro *et al*. 2002; Pereira-Neves *et al*. 2003; Pereira-Neves and Benchimol, 2009; Pereira-Neves *et al*. 2015). TEM analyses corroborated the SEM results, revealing internalized flagella located in independent membrane invaginations, reinforcing that spherical forms induced by iron depletion are pseudocysts.

A recurrent question in the literature has surrounded the significance of pseudocysts of trichomonads: are these degenerative forms or are they viable intermediate forms resistant to variation in environmental conditions (Wenrich, 1939; Samuels, 1957; Honigberg and Brugerolle, 1990; Petrin *et al*. 1998; Mariante *et al*. 2004). This study demonstrated that the induction of pseudocysts by iron depletion is a reversible process such that spherical,

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non-replicative, non-motile forms recovered their typical pear-shaped and replicative trophozoite form after inoculation into the standard TYM medium. The growth of these trophozoites was similar to that observed in control parasites cultivated in the standard TYM medium. These results show that pseudocysts obtained by irondepletion are resistant, viable, non-replicative forms of the parasite, which revert back to the trophozoite form when conditions become favourable. Therefore, we postulate that formation of pseudocysts with such characteristics could confer to the parasite an additional capability for dissemination and host infection. In addition, pseudocysts maybe part of the normal *T. foetus* life cycle within the host, as small numbers of pseudocysts were observed in control cultures in this study as well as other studies (Pereira-Neves *et al*. 2011). The term 'pseudocyst' has been historically used to describe rounded forms of the parasite with internalized flagella with a regular cellular membrane, in contrast to true cysts, such as those from *Entamoeba* spp. and *Giardia* spp., which possess a true cyst wall (Chatterjee *et al*. 2015). Pioneering investigators considered pseudocysts as irreversible and degenerative forms of the parasite (Wenrich, 1939; Samuels, 1957). It has since been established that these forms are viable and reversible as demonstrated in this study and by other investigators (Pereira-Neves and Benchimol, 2009).

The existence of several parasitic or free-living trichomonads that possess pseudocysts such as *Trichomitus batrachorum, Trichomitus sanguisugae* and *Ditrichomonas honigbergii* (Mattern *et al*. 1973; Brugerolle, 1975; Farmer, 1993), reinforce the hypothesis that pseudocysts are intermediary forms that are resistant to variations in the environment. It was demonstrated that in preputial secretions from infected bulls, the pseudocyst form of *T. foetus* occurs more frequently than the pear-shaped parasites (Pereira-Neves *et al*. 2011). Additionally, pseudocysts of *T. foetus* are more cytotoxic to Madin-Darby Canine Kidney (MDCK) epithelial cells than are trophozoites (Pereira-Neves *et al*. 2012). Pseudocysts of *T. muris* released in hamster feces and pseudocysts of trichomonads from the intestinal tract of birds were described as viable and infective forms (Pereira and Almeida, 1940; Mattern and Daniel, 1980; Friedhoff *et al*. 1991; Lipman *et al*. 1999). It has been suggested that the ubiquitin–proteasome pathway, which is required for the cell cycle, may play an important role in the transformation of *T. foetus* trophozoites into pseudocysts (Pereira-Neves *et al*. 2015). Additionally, microtubules and calcium play important roles in the process of morphological reversibility of pseudocysts, participating in the mechanism of flagella externalization and restoration, resembling a process of exocytosis (Granger *et al*. 2000). In this study, iron depletion triggered the morphological transformation of *T. foetus* from replicating trophozoites to non-replicative pseudocysts, confirming, that iron also behaves as a modulator in this process.

While iron is necessary for *T. foetus* survival, it was observed in this study that short term iron depletion does not cause cell death in all parasites, but instead induces phenotypic alterations, probably in order to allow the parasite to survive conditions of nutritional stress. The precise mechanism by which iron modulates pseudocyst formation requires further exploration.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0031182016000573.

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CONFLICTS OF INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

CLFC and JBJ designed the study. CLFC, RFSMB, NSF, CB, LSV and GDL performed the experimental work. CLFC, RFSMB, PC and JBJ analyzed the data. CLFC, RFSMB, PC and JBJ prepared the manuscript. All authors read and approved the final manuscript.

REFERENCES

Affonso, A. L., de Almeida, J. C. and Benchimol, M. (1997). Partial characterization of cytoplasmatic compartments involved in the endocytic process of *Tritrichomonas foetus*. *European Journal of Cell Biology* **72**, 247–256.

Afzan, M. Y. and Suresh, K. (2012). Pseudocyst forms of *Trichomonas vaginalis* from cervical neoplasia. *Parasitology Research* **111**, 371–381.

Alderete, J. F., Provenzano, D. and Lehker, M. W. (1995). Iron mediates *Trichomonas vaginalis* resistance to complement lysis. *Microbial Pathogenesis* **19**, 93–103.

Ali, V. and Nozaki, T. (2007). Current therapeutics, their problems, and sulfur-containing-amino acid metabolism as a novel target against infections by "amitochondriate" protozoan parasites. *Clinical Microbiology Reviews* **20**, 164–187.

Andrade Rosa, I., De Souza, W. and Benchimol, M. (2015). Changes in the structural organization of the cytoskeleton of *Tritrichomonas foetus* during trophozoite-pseudocyst transformation. *Micron* **73**, 28–35.

Atkinson, C. T., Bayne, M. T., Gordeuk, V. R., Brittenham, G. M. and Aikawa, M. (1991). Stage-specific ultrastructural effects of

desferrioxamine on *Plasmodium falciparum in vitro*. *The American Journal of Tropical Medicine and Hygiene* **45**, 593–601.

Beltrán, N. C., Horváthová, L., Jedelský, P. L., Sedinová, M., Rada, P., Macinciková, M., Hrdý, I. and Tachezy, J. (2013). Iron-induced changes in the proteome of *Trichomonas vaginalis* hydrogenosomes. *PLoS ONE* **8**, e65148.

Benchimol, M. (2004). Trichomonads under microscopy. *Microscopy and Microanalysis* **10**, 528–550.

Benchimol, M. (2009). Hydrogenosomes under microscopy. *Tissue and Cell* **41**, 151–168.

Benchimol, M., Batista, C. and De Souza, W. (1990). Fibronectin- and laminin mediated endocytic activity in the parasitic protozoa *Trichomonas vaginalis* and *Tritrichomonas foetus*. *Journal of Submicroscopic Cytology and Pathology* **22**, 39–45.

Bondurant, R. H. (2005). Veneral diseases of cattle: natural history, diagnosis, and the role of vaccines in their control. *Veterinary Clinics Food Animal Practice* **21**, 383–408.

Breuer, W., Epsztejn, S. and Cabantchik, Z. I. (1995). Iron acquired from transferrin by K562 cells is delivered into a cytoplasmatic pool of chelatable iron (II). *The Journal of Biological Chemistry* **270**, 24209–24215.

Brugerolle, G. (1975). Étude de la cryptopleuromitose et de la morphogenése de divisíon chez plusiers genres de la trichomonadines primitive. *Protistologica* **4**, 457–468.

Cerkasovova, A., Cerkasov, J. and Kulda, J. (1984). Metabolic differences between metronidazole resistant and susceptible strains of *Tritrichomonas foetus*. *Molecular and Biochemical Parasitology* **11**, 105–118.

Chatterjee, A., Bandini, G., Motori, E. and Samuelson, J. (2015). Ethanol and isopropanol in concentrations presents in hand sanitizers sharply reduce excystation of *Giardia* and *Entamoeba* and eliminate oral infectivity of Giardia cysts in gerbils. *Antimicrobial Agents and Chemotherapy* **59**, 6749–6754.

Crouch, M. L., Benchimol, M. and Alderete, J. F. (2001). Binding of fibronectin by *Trichomonas vaginalis* is influenced by iron and calcium. *Microbial Pathogenesis* **31**, 131–144.

De Jesus, J. B., Ferreira, M. A., Cuervo, P., Britto, C., Silva-Filho, F. C. and Meyer-Fernandes, J. R. (2006). Iron modulates ecto-phosphohydrolases activities in pathogenic trichomonads. *Parasitology International* **55**, 285–290.

De Jesus, J. B., Cuervo, P., Junqueira, M., Britto, C., Silva-Filho, F. C. and Soares, M. J. (2007). A further proteomic study on the effect of iron in the human pathogen *Trichomonas vaginalis*. *Proteomics* **7**, 1961–1972.

Diamond, L. S. (1957). The establishment of various trichomonads of animals and man in axenic cultures. *The Journal of Parasitology* **43**, 488–490.

Ellis, J. E., Williams, R., Cole, D., Cammack, R. and Lloyd, D. (1993). Electron transport components of the parasitic protozoon *Giardia lamblia*. *Federation of European Biochemical Societies* **325**, 196–200.

Farmer, M. A. (1993). Ultrastructure of *Ditrichomonas honigbergii* n. g., n. sp. (Parabasalia) and its relation-ships to amitocondrial protists. *Journal of Eukaryotic Microbiology* **40**, 619–626.

Friedhoff, K. T., Kuhnigk, C. and Müller, I. (1991). Experimental infections in chicken with *Chilomastix gallinarium, Tetratrichomonas gallinarium*, and *Tritrichomonas eberthi*. *Parasitology Research* **77**, 329–334.

Gorrell, T. E. (1985). Effect of culture medium iron content on the biochemical composition and metabolism of *Trichomonas vaginalis*. *Journal of Bacteriology* **161**, 1228–1230.

Granger, B. L., Warwood, S. J., Benchimol, M. and De Souza, W. (2000). Transient invagination of flagella by *Tritrichomonas foetus*. *Parasitology Research* **86**, 699–709.

Honigberg, B. M. and Brugerolle, G. (1990). Structure in trichomonads parasitic in humans. In *Trichomonas Parasitic in Humans* (ed. Honigberg, B. M.), pp. 5–35. Springer-Verlag, New York.

Horváthová, L., Safaríková, L., Basler, M., Hrdý, I., Campo, N. B., Shin, J. W., Huang, K-Y., Huang, P-J., Lin, R., Tang, P. and Tachezy, J. (2012). Transcriptomic indetification of iron- regulated and iron-independent gene copies within the heavily duplicated *Trichomonas vaginalis* genome. *Genome Biology and Evolution* **4**, 1017–1029.

Hsu, H-M., Ong, S-J., Lee, M-C. and Tai, J-H. (2009). Transcriptional regulation of an iron inducible gene by differential and alternate promote entries of multiple Myb proteins in the protozoan parasite *Trichomonas vaginalis*. *Eucaryotic Cell* **8**, 362–372.

Lalonde, R. G. and Holbein, B. E. (1984). Role of iron in *Trypanosoma cruzi* infection of mice. *The Journal of Clinical Investigation* **73**, 470–476.

Lee, J., Park, S. J. and Yong, T. S. (2008). Effect of iron on adherence and citotoxicity of *Entamoeba histolytica* to CHO cell monolayers. *The Korean Journal Parasitology* **46**, 37–40.

Lehker, M. W. and Alderete, J. F. (1992). Iron regulates growth of *Trichomonas vaginalis* and the expression of immunogenic trichomonad proteins. *Molecular Microbiology* **6**, 23–132.

Lindmark, D. G., Eckenrode, B. L., Halberg, L. A. and Dinbergs, I. D. (1989). Carbohydrate energy and hydrogenosoma metabolismo of *Tritrichomonas foetus* and *Trichomonas vaginalis*. *The Journal of Protozoology* **36**, 214–216.

Lipman, N. S., Lampen, N. and Nguyen, H. T. (1999). Identification of pseudocysts of *Tritrichomonas muris* in Armenian hamsters and their transmission to mice. *American Association for Laboratory Animal Science* **49**, 313–315.

Loo, V. G. and Lalonde, R. G. (1984). Role of iron in intracellular growth of *Trypanosoma cruzi*. *Infection and Immunity* **45**, 726–730.

Mariante, R. M., Lopes, L. C. and Benchimol, M. (2004). *Tritrichomonas foetus* pseudocysts adhere to vaginal epithelial cells in a contact-dependent manner. *Parasitology Research* **92**, 303–312.

Mattern, C. F. and Daniel, W. A. (1980). *Tritrichomonas muris* in the hamster: pseudocysts and the infection of newborn. *The Journal of Protozoology* **27**, 435–439.

Mattern, C. F., Honigberg, B. M. and Daniel, W. A. (1973). Fine-structural changes associated with pseudocyst formation in *Trichomitus batrachorum*. *The Journal of Protozoology* **20**, 222–229.

Melo-Braga, M. B., Rocha-Azevedo, B. and Silva-Filho, F. C. (2003). *Tritrichomonas foetus*: the role played by iron during parasite interaction with epithelial cells. *Experimental Parasitology* **105**, 111–120.

Merschjohann, K. and Steverding, D. (2006). *In vitro* growth inhibition of bloodstream forms of *Trypanosoma brucei* and *Trypanosoma congolense* by iron chelators. *Kinetoplastid Biology and Disease* **5**, 1–5.

Mesquita-Rodrigues, C., Menna-Barreto, R. F. S., Sabóia-Vahia, L., Da Silva, S. A. G., De Souza, E. M., Waghabi, M. C., Cuervo, P. and De Jesus, J. B. (2013). Cellular growth and mitochondrial ultrastructure of *Leishmania* (*Viannia*) *braziliensis* promastigotes are affected by the iron chelator 2,2-dipyridyl. *PLoS Neglected Tropical Diseases* **7**, 10.

Müller, M. (1988). Energy metabolism of protozoa without mitochondria. *Annual Reviews in Microbiology* **42**, 465–488.

Müller, M. (1993). The hydrogenosome. *Journal of General Microbiology* **139**, 2879–2889.

Müller, M., Mentel, M., Van Hellemond, J. J., Henze, K., Woehle, C., Gould, S. B., YU, R.-Y., Giezen, M. V. G., Tielens, A. G. M. and Martin, W. F. (2012). Biochemistry and evolution of anaerobic energy metabolism in eukaryotes. *Microbiology and Molecular Biology Reviews* **76**, 444–495.

Ong, S-J., Huang, S-C., Líu, H-W. and Tai, J-H. (2004). Involvement of multiple DNA elements in iron-inducible transcription of the ap651 gene in the protozoan parasite *Trichomonas vaginalis*. *Molecular Microbiology* **52**, 1721–1730.

Parsonson, I. M., Clarck, B. L. and Dufty, J. H. (1976). Early pathogenesis and pathology of *Tritrichomonas foetus* infection in virgin heifers. *Journal of Comparative Pathology* **86**, 59–66.

Payne, M. J., Chapman, A. and Cammack, R. (1993). Evidence for an [Fe]-type hydrogenase in the parasitic protozoan *Trichomonas vaginalis*. *Federation of European Biochemical Societies* **317**, 101–104.

Pereira, C. and Almeida, W. F. (1940). Sôbre a verdadeira natureza das "formas ameboides", dos pretensos "cistos" e formas "degenerativas" no gênero "*Trichomonas Donné*", 1836. *Archivos Instituto de Biología Andina* **11**, 347–366.

Pereira-Neves, A. and Benchimol, M. (2009). *Tritrichomonas foetus*: budding from multinucleated Pseudocysts. *Protist* **154**, 313–329.

Pereira-Neves, A., Ribeiro, K. C. and Benchimol, M. (2003). Pseudocysts in trichomonads – new insights. *Protist* **154**, 313–329.

Pereira-Neves, A., Campero, C. M., Martínez, A. and Benchimol, M. (2011). Identification of *Tritrichomonas foetus* pseudocysts in fresh preputial secretion samples from bulls. *Veterinary Parasitology* **175**, 1–8.

Pereira-Neves, A., Nascimento, L. F. and Benchimol, M. (2012). Cytotoxic effects exerted by *Tritrichomonas foetus* pseudocysts. *Protist* **163**, 529–543.

Pereira-Neves, A., Gonzaga, L.; Menna-Barreto, R. F. S. and Benchimol, M. (2015). Characterization of 20S proteosome in *Tritrichomonas foetus* and its role during the cell cycle and transformation into endoflagellar form. *PLoS ONE* **10**, e0129165.

Petrin, D., Delgaty, K., Bhatt, R. and Garber, G. (1998). Clinical and microbiological aspects of *Trichomonas vaginalis*. *Clinical Microbiology Review* **11**, 300–317.

Rae, D. O. and Crews, J. E. (2006). *Tritrichomonas foetus*. *Veterinary Clinics Food Animal Practice* **22**, 595–611.

Ribeiro, K. C., Pereira-Neves, A. and Benchimol, M. (2002). The mitotic spindle and associated membranes in the closed mitosis of trichomonads. *The Biology of the Cell* **94**, 157–172.

Ribeiro, C. R., Santos, C. and Benchimol, M. (2015). Is *Trichomonas tenax* a parasite or a commensal? *Protist* **166**, 196–210.

Ryu, J. S., Choi, H. K., Min, D. Y., Ha, S. E. and Ahn, M. H. (2001). Effect of iron on the virulence of *Trichomonas vaginalis*. *The Journal of Parasitology* **87**, 457–460. doi: http://dx.doi.org/10.1645/0022-3395(2001)087[0457:EOIOTV]2.0.CO;2

Samuels, R. (1957). Studies on *Tritrichomonas batrachorum* (perty) 2. Normal mitosis and morphogenesis. *Transactions of the American Microscopical Society Journal* **76**, 295–307.

Silva-Filho, F. C., Elias, C. A. and De Sousa, W. (1986). Further studies on the surface charge of various strains of *Trichomonas vaginalis* and *Tritrichomonas foetus*. *Cell Biophysics* **8**, 161–176.

Solano-González, E., Burrola-Barraza, E., León-Sicairos, C., Avila González, L., Gutiérrez-Escolano, L., Ortega-López, J. and Arroyo, R. (2007). The trichomonad cysteine proteinase TVCP4 transcript contains an iron-responsive element. *Federation of European Biochemical Societies* **581**, 2919–2928.

Soteriadou, K., Papavassiliou, P., Voyiatzaki, C. and Boelaert, J. (1995). Effect of iron chelation on the in-vitro growth of *Leishmania* promastigotes. *Journal of Antimicrobial Chemotherapy* **35**, 23–29.

Sutak, R., Tachezy, J., Kulda, J. and Hrdý, I. (2004). Pyruvate decarboxylase, the target for omeprazole in metronidazole resistant and iron-restricted *Tritrichomonas foetus*. *Antimicrobial Agents and Chemotherapy* **48**, 2185–2189.

Tachezy, J. (1998). The host-protein-independent iron uptake by *Tritrichomonas foetus*. *Experimental Parasitology* **90**, 403–413.

Tachezy, J., Kulda, J., Bahniková, I., Suchan, P., Rázga, J. and Schrével, J. (1996). *Tritrichomonas foetus*: iron acquisition from lactoferrin and transferrin. *Experimental Parasitology* **83**, 216–228.

Thompson, C. C. and Carabero, R. A. (2011). An optimal method of iron starvation of the obligate intracellular pathogen, *Chlamydian trachomatis*. *Frontiers in Microbiology* **2**, 20.

Torres-Romero, J. C. and Arroyo, R. (2009). Responsiveness os *Trichomonas vaginalis* to iron concentrations: evidence for a post-transcriptional iron regulation by na IRE/IRP-like system. *Infection, Genetics and Evolution* **9**, 1065–1074.

Townson, S. M., Hanson, G. R., Upcroft, J. A., and Upcroft, P. (1994). Purified ferredoxin from *Giardia duodenalis*. *European Journal of Biochemistry* **220**, 439–446.

Vanacová, S., Rasoloson, D., Rázga, J., Hrdý, I., Kulda, J. and Tachezy, J. (2001). Iron-induced changes in pyruvate metabolism of *Tritrichomonas foetus* and involvement of iron in expression of hydrogenosomal proteins. *Microbiology* **147**, 53–62.

Wenrich, D. H. (1939). The morphology of *Trichomonas vaginalis*. *Jubilare Pro Professore Sadao Yoshida Osaka* **2**, 65–76.