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Iron modulates ecto-phosphohydrolase activities in pathogenic trichomonads

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Abstract

The presence of iron in the extracellular medium is essential for both *in vivo* and *in vitro* survival of pathogenic microorganisms, including *Trichomonas vaginalis* and *Tritrichomonas foetus*. In these parasites, iron is directly involved in the proliferation, protein expression and activation of critical enzymes. The purpose of this study was to investigate the role of iron in ecto-ATPase, ecto-phophatase and secreted phosphatase activities of these trichomonads. We observed that trichomonads grown in iron-depleted medium exhibited a remarkable decrease in both ecto-ATPase and ecto-phosphatase activities, when compared to those cultivated under control conditions (iron-rich medium). Furthermore, parasites grown in iron-depleted medium restored their enzyme activities when they were re-inoculated into fresh iron-rich medium. We demonstrated that modulation of ecto-phosphohydrolase activities is due neither to enzyme–iron nor to substrate–iron complex formation, since iron addition directly to the medium where the enzymatic reactions occurred did not alter their activities. Previously, we had reported that a fresh clinical isolate of *T. vaginalis* was much more cytotoxic to epithelial cell monolayers than a long-term cultured one. In this study we witnessed that the fresh isolate of *T. vaginalis* presented higher activities to all herein investigated enzymes than the long-term cultured one. Altogether, our data clearly point out that iron has a pivotal role in the expression of phosphohydrolases in both trichomonads.

Keywords: *Trichomonas vaginalis*; *Tritrichomonas foetus*; Iron; Ecto-ATPase; Ecto-phosphatase

1. Introduction

Both *Trichomonas vaginalis* and *Tritrichomonas foetus* are parasitic trichomonads usually harbored in the urogenital cavities of humans and cattle, respectively [1–3]. *T. vaginalis* is the etiologic agent of trichomoniasis which is in turn, one of the world's most widespread sexually transmitted diseases [1,4]. *T. foetus* has been frequently associated to the epithelium lining of the urogenital cavities in cattle [5]. In bulls the parasite can be detected in the preputial cavity and urethra as well as in the deeper parts of the urogenital tract [2], while in cows it inhabits the vagina and the uterus, leading to abortion [5,6].

The molecular mechanisms underlying *T. vaginalis* and *T. foetus* cytotoxicities are not yet fully understood. One of the features of both human and bovine trichomoniasis is the occurrence of an intense exfoliation of the host urogenital cavity epithelia lining [4–6]. It has already been described that both soluble and insoluble trichomonal proteases are directly implicated in the toxicity exerted by each one of these parasites on cultured mammalian cells [7,8]. Both ecto-phosphatase and ecto-ATPase trichomonal activities have been implicated in this phenomenon and suggested to be virulence factors of *T. vaginalis* and *T. foetus* [9–13]. While trichomonal ecto-ATPases have been found as surface-associated enzymes [14], phosphatases activities recently identified in *T. vaginalis* [11]

Abbreviations: TYM, trypticase yeast maltose; ATP, adenosine triphosphate; *p*-NPP, *p*-nitrophenylphosphate.

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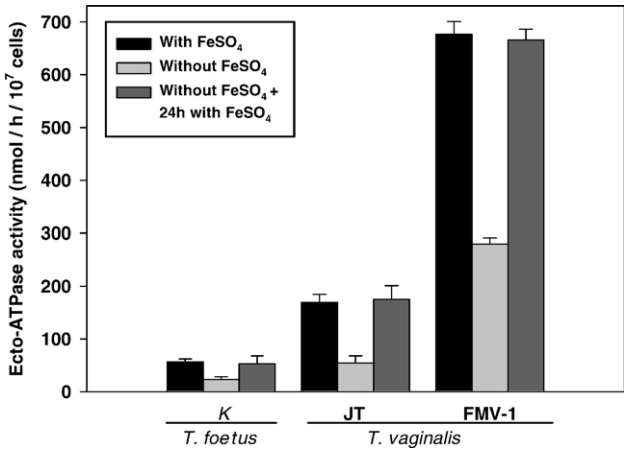

Fig. 1. Influence of iron on the ecto-ATPase activities of *T. foetus* and *T. vaginalis*. Trichomonads were cultivated during 24 h, at 37 °C, in iron-rich (black bars) and in iron-depleted TYM medium by addition of 180 μM iron chelator 2,2-dipyridyl (grey bars). Parasites grown for 24 h in iron chelator-containing TYM medium were transferred to a fresh iron-rich TYM medium and cultivated again for 24 h (dark grey bars). Values represent the average and standard error of three experiments, each one carried out in triplicate.

have been detected associated to the parasite's surface as well as recovered in abundance from parasite culture medium.

Iron plays a crucial role in the metabolism of parasitic protozoa [15], including pathogenic trichomonads [16,17]. Among trichomonads, iron has been considered as an important factor modulating the expression of cytoadhesins [16], immunogenic proteins [18], resistance to complement lysis [19] and proteases [20]. The crucial role played by iron in trichomonads is ascribed to the importance of [Fe–S] proteins in the carbohydrate metabolism of *T. vaginalis* and *T. foetus* [17,39]. Furthermore, iron has also been associated to regulation of gene transcription in *T. vaginalis*, and an iron-responsive promoter has been identified in this parasite [21]. The experiments herein were designed in order to detect and measure ecto-ATPase, ecto-phosphatase and secreted phosphatase activities in *T. foetus* and *T. vaginalis* which were cultured in the presence and absence of iron.

2. Material and methods

2.1. Chemicals

All reagents were purchased from Sigma (St. Louis, MO, USA) or Merck (São Paulo, Brazil). [γ-^{32}P]ATP was prepared as described by [22]. Distilled water for all solutions was deionized using a MilliQ system of resins (Millipore Corp., Bedford, MA, USA).

2.2. Microorganisms

Two strains of *Trichomonas vaginalis* (JT and FMV-1) and one of *Tritrichomonas foetus* (K strain) were analyzed throughout this study. The FMV-1 microorganisms [23] were recently collected from an asymptomatic patient, while the JT and K ones were long-term cultured microorganisms [24]. All the microorganisms were axenically maintained by weekly passages in 10% heat-inactivated bovine serum-supplemented TYM medium [25] incremented with 0.6 mM $FeSO_4$, pH 6.2 and 6.8 for *T. vaginalis* and *T. foetus*, respectively. Parasites from cultures which reached the logarithmic phase of growth were collected by centrifugation at 1400×*g* for 5 min at 4 °C, and rinsed twice with 0.01 M phosphate-buffered 0.145 M NaCl (PBS), pH 7.2. Immediately afterwards, parasites were inoculated into fresh TYM iron-supplemented medium (control condition) and TYM iron-depleted medium. Iron-depletion was carried out by the addition of 180 μM 2,2-dipyridyl to the TYM medium without 0.6 mM $FeSO_4$ supplementation, since this concentration of iron chelator does not induce cell death [17,20]. Parasites were counted in a hemocytometer, and their viabilities were estimated applying the erythrosine B dye-exclusion test [37]. The viability of all parasites was evaluated before and after each assay, and only samples with at least 95% viability were used.

2.3. Ecto-ATPase activities

Living parasites (10^7 cells) were incubated for 1 h at 37 °C in 0.5 ml of a mixture containing, unless otherwise specified, 50 mM HEPES–Tris buffer (pH 7.2), 116 mM NaCl, 5.4 mM KCl, 5.5 mM D-glucose and 5 mM ATP, with or without addition of 400 μM $FeSO_4$. The ATPase activity was determined by measuring the hydrolysis of [γ-^{32}P] ATP (10^4 Bq/nmol ATP). The experiments were initiated with the addition of living parasites to the last solution, and stopped by addition of 1 ml of a cold mixture containing 0.2 g charcoal in 1 M HCl. The resulting solution which contained the parasites was then submitted to centrifugation at 4000×*g* for 10 min at 4 °C. Aliquots (0.5 ml) of the supernatant containing the released ^{32}Pi were transferred to scintillation vials containing 9 ml of scintillation fluid (2 g

Table 1
Effect of iron on the ecto-ATPase activities of *T. foetus* and *T. vaginalis*

Specie	Enzymatic reaction medium	Parasites cultivated in iron-rich medium	Parasites cultivated in iron-depleted medium
T. foetus	With $FeSO_4$	58.0±6.3	25.7±3.0
	Without $FeSO_4$	55.3±4.2	28.0±1.7
T. vaginalis – JT strain	With $FeSO_4$	179.7±12.0	58.0±4.4
	Without $FeSO_4$	175.0±15.0	62.3±5.0
T. vaginalis – FMV-1 strain	With $FeSO_4$	665.7±25.0	271.0±15.0
	Without $FeSO_4$	655.0±20.0	266.7±12.0

Trichomonads were cultivated during 24 h, at 37 °C, in an iron-rich medium or in an iron-depleted medium, centrifuged and washed. The recovery parasites were used for ecto-ATPase activity determination in the presence or absence of 400 μM $FeSO_4$ in the enzymatic reaction medium. Values were expressed in nmol/h/10^7 parasites and represent average of three experiments done in triplicate, with standard error.

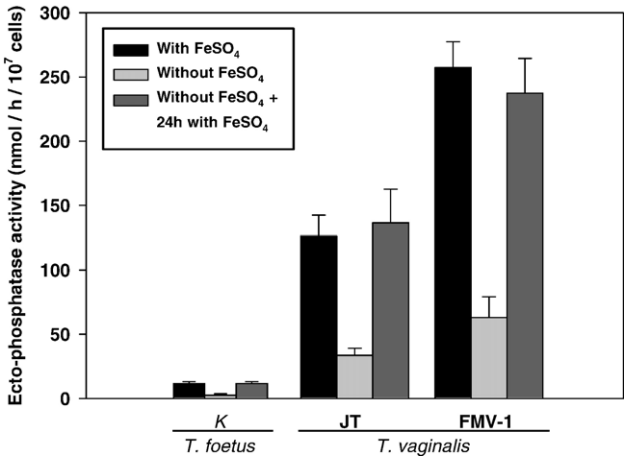

Fig. 2. Influence of iron on the ecto-phosphatase activities of *T. foetus* and *T. vaginalis*. Trichomonads were cultivated during 24 h, at 37 °C, in iron-rich (black bars) and in iron-depleted TYM medium by addition of 180 μM iron chelator 2,2-dipyridyl (grey bars). Parasites grown for 24 h in iron chelator-containing TYM medium were transferred to a fresh iron-rich TYM medium and cultivated again for 24 h (dark grey bars). Values represent the average and standard error of three experiments, each one carried out in triplicate.

POPOP in 1 L of toluene). The ATPase activity was calculated by subtracting the nonspecific ATP hydrolysis measured in the absence of parasites. The ATP hydrolysis had a positive correlation with respect to time under the assay conditions employed and was proportional to the cell number.

2.4. Ecto-phosphatase activities

The phosphatase activity was performed with measurements of the rate of inorganic phosphate production. Briefly, living parasites (10^7 cells) were incubated for 1 h at 37 °C in 0.5 ml of a reaction mixture containing 30 mM HEPES pH 7.2, 116 mM NaCl, 5.4 mM KCl, 5.5 mM glucose and 5 mM *p*-nitrophenylphosphate (*p*-NPP), with or without addition of 400 μM $FeSO_4$. Reactions were initiated with the addition of living parasites to the solution and stopped by addition of 2 ml 1 N NaOH. Controls where parasites were added after interruption of the reaction were used as blank. To determine the amount of released *p*-nitrophenol (*p*-NP), the parasites were submitted to centrifugation at 4000×*g* for 20 min, and the resulting supernatants were measured spectrophotometrically at 425 nm, using a *p*-NP curve as standard. The viability of all parasites was assessed before and after all assays.

2.5. Secreted phosphatase activities

10^7 parasites cultivated in either iron-rich or iron-depleted medium were centrifuged as previously described and assayed for phosphatase activity. Measurements of the parasite secreted phosphatase were carried out by incubation of the parasites in 1 ml of 30 mM HEPES pH 7.2, 116 mM NaCl, 5.4 mM KCl and 5.5 mM glucose for 2 h at 37 °C without addition of the substrate *p*-NPP. Immediately afterwards, the supernatants resulting from two steps of centrifugation (the first at 1400×*g* for 20 min at 4 °C in order to remove the cells without damaging them and the second at 16 500×*g* for 20 min at 4 °C to discard possible cell debris) were filtered by using 0.22 μm membranes. The reactions were initiated with the addition of substrate *p*-NPP to the filtered supernatants, and after 1 h of incubation, the reactions were stopped by addition of 2 ml 1 N NaOH. The phosphatase activities were measured as previously described. Reaction solution alone was used as blank.

2.6. Statistics

All experiments were performed in triplicate, each of which involved different independent cell suspensions. The resulting data were analyzed statistically by means of the Student's *t*-test. $P \leq 0.05$ were significant.

3. Results

3.1. Effects of iron in the ecto-ATPase activities

We performed assays to measure the ability of both *T. vaginalis* and *T. foetus* to hydrolyse extracellular ATP. The detected trichomonal ecto-ATPase activities are summarized in Fig. 1. It can be seen that the ecto-ATPase activity associated to FMV-1 parasites was about four times higher (676.7± 14.5 nmol Pi/h/10^7 parasites) than that found with the JT strain (169.3±5.2 nmol Pi/h/10^7 parasites) (Fig. 1, black bars). In addition, the *T. foetus* microorganisms did not hydrolyze ATP (56.3±2.7 nmol Pi/h/10^7 parasites) at the same rates exhibited by the JT and the FMV-1 strains (Fig. 1, black bars). Nevertheless, all

Table 2
Effect of iron on the ecto-phosphatase activities of *T. foetus* and *T. vaginalis*

Specie	Enzymatic reaction medium	Parasites cultivated in iron-rich medium	Parasites cultivated in iron-depleted medium
T. foetus	With $FeSO_4$	11.1±0.6	2.9±0.4
	Without $FeSO_4$	12.4±1.3	3.3±0.5
T. vaginalis – JT strain	With $FeSO_4$	128.0±12.0	40.0±5.0
	Without $FeSO_4$	130.0±18.0	33.6±8.0
T. vaginalis – FMV1 strain	With $FeSO_4$	259.3±20.0	55.5±7.0
	Without $FeSO_4$	271.7±23.0	58.5±8.0

Trichomonads were cultivated during 24 h, at 37 °C, in an iron-rich medium or in an iron-depleted medium, centrifuged and washed. The recovery parasites were used for ecto-phosphatase activity determination in the presence or absence of 400 μM $FeSO_4$ in the enzymatic reaction medium. Values are expressed in nmol/h/10^7 parasites, and represent average of three experiments done in triplicate, with standard error.

T. vaginalis and *T. foetus* displayed a remarkable inhibition in such enzyme activity when they were cultured in iron-depleted medium (Fig. 1, gray bars). The decreases in the ATPase activities were in the order of 59% for FMV-1, 68% for JT and 59% for *T. foetus* (Fig. 1, gray bars). Otherwise, all parasites which had been cultivated in iron-depleted medium restored their normal ecto-ATPase activities when they were reinoculated into fresh iron-rich culture medium and cultivated for up to 24 h (Fig. 1, dark gray bars). Additional assays were performed looking for the specificity concerning the effect of iron on such ecto-ATPase activities. Parasites cultured in either iron-rich or iron-depleted medium were collected, and their ecto-ATPase activities were evaluated when the parasites were introduced into solution containing or not 400 μM $FeSO_4$. The data presented in Table 1 clearly demonstrate that the addition of $FeSO_4$ to the reaction solution to detect ecto-ATPase activities in parasites cultivated in iron-rich or iron-depleted media did not result in alterations from the previously detected enzyme activities.

3.2. Effect of iron in the ecto-phosphatase activities

The effect of iron on the ecto-phosphatase activities of the herein studied trichomonads was also investigated. The results presented in Fig. 2 demonstrate that the ecto-phosphate activity of FMV-1 parasites grown in iron-rich medium was approximately two times higher (257.4±5 nmol Pi/h/10^7 parasites) than that observed for the JT strain (126.5±6.2 nmol Pi/h/10^7 parasites) (Fig. 2, black bars), while a low ecto-phosphatase activity was detected in *T. foetus* grown under the same conditions (11.7± 0.7 nmol Pi/h/10^7 parasites) (Fig. 2, black bars). Similar to that witnessed for ecto-ATPase activities, all parasites also exhibited strong decreases in their ecto-phosphatase activities when they were cultivated in iron-depleted medium. The decreases in enzyme activity were on the order of 75% for FMV-1 strain, 73% for JT and 78% for *T. foetus* (Fig. 2, gray bars). However, when these *T. vaginalis* and *T. foetus* microorganisms were reinoculated into iron-rich medium and cultivated for 24 h, their previous ecto-phosphate activities were restored (Fig. 2, dark-gray bars). Furthermore, neither parasites grown in iron-rich medium nor parasites grown in iron-depleted medium exhibited any detectable changes in the ecto-phosphatase activities when the reactions took place in reaction solutions with or without $FeSO_4$ (Table 2).

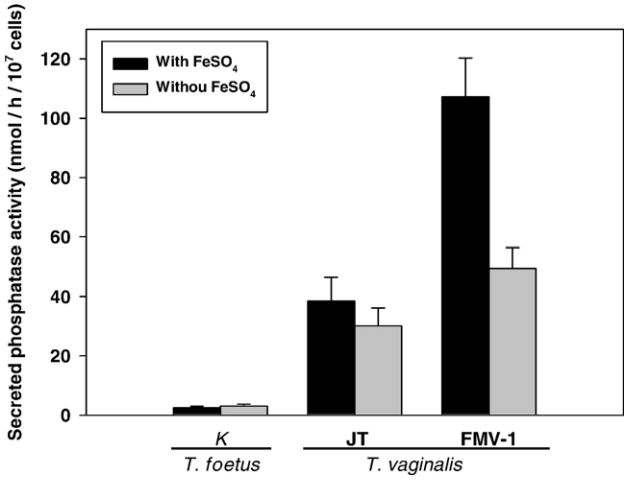

Fig. 3. Influence of iron on the secreted phosphatase activities of *T. foetus* and *T. vaginalis*. Trichomonads were cultivated during 24 h, at 37 °C, in iron-rich (black bars) and iron-depleted TYM medium by addition of 180 μM iron chelator 2,2-dipyridyl (grey bars). Values represent the average and standard error of three experiments which were carried out in triplicate.

3.3. Effect of iron on the secreted phosphatase activities

Parasites cultivated in either iron-rich medium or iron-depleted medium were submitted to secreted phosphatase activity assays. The data presented in Fig. 3 demonstrate that both *T. vaginalis* strains present higher secreted phosphate activities (107.3±3.2 nmol Pi/h/10^7 FMV-1 parasites, and 38.5± 1.9 nmol Pi/h/10^7 JT parasites) than observed for *T. foetus* (2.5±0.3 nmol Pi/h/10^7 parasites) (Fig. 3, black bars). Furthermore, FMV-1 parasites collected from cultivation carried out in iron-depleted medium exhibited a decrease of 54% in their secreted phosphatase activity (Fig. 3, gray bars) whereas the JT strain yielded a 21% decrease in the same enzyme activity. This tendency was not apparent when *T. foetus* parasites were assayed, as they displayed an increase of about 24% in the secreted phosphatase activity (Fig. 3, gray bars).

4. Discussion

Ecto-ATPases are enzymes whose active sites face the external medium rather than the cytoplasm. Plasma membrane ecto-ATPases are integral glycoproteins that are millimolar divalent cation-dependent, and low specificity enzymes that hydrolyze all nucleoside triphosphates [9,26]. The activities of these enzymes can be measured using living cells. In a previous cytochemical study describing the occurrence of nucleotidases on the plasma membrane of *T. foetus*, the presence of ATPase activity was demonstrated [14]. Furthermore, we have characterized ecto-ATPase activity on the external surface of both *T. foetus* and *T. vaginalis* [10,12], which has been confirmed by ultrastructural cytochemistry [13]. In addition, ecto-phosphatase activities in *T. vaginalis* have been detected at parasite surface as well as in both vacuoles and exocyted vesicles [11].

Herein, we demonstrated that trichomonads cultured in iron-depleted medium exhibit a remarkable reduction in both ecto-ATPase and ecto-phosphatase activities, when compared to parasites grown in control condition (iron-rich medium). Our results have pointed out that iron regulation of ecto-ATPase as well as ecto-phosphatase activities is due neither to iron-enzyme nor to substrate-iron complex formation, since the addition of iron to the medium where the enzymatic reactions occurred did not alter their activities. Such results also suggest that iron might exert a positive regulatory role in the expression of phosphohydrolases in both *T. foetus* and *T. vaginalis*. This hypothesis is reinforced by the fact that enzyme activities found in iron-depleted trichomonads were restored to the level of organisms grown under control condition when they were re-inoculated in a fresh iron-rich medium. Additionally, we also observed that phosphohydrolase activities are unvarying in different phases of

parasite growth (data not shown). In contrast, other authors have claimed that growth of *T. vaginalis* in high or low-iron-supplemented medium has no significant effect on extracellular ATP hydrolysis but seems to affect the AMP hydrolysis by parasites [27]. It is known that extracellular ATP is one of the key signaling molecules involved in several cellular responses including growth, differentiation and cell death [28]. Since this extracellular nucleotide mediated signaling is involved in crucial cellular functions, it is coherent to expect that it must be regulated. As reviewed elsewhere [29], ectonucleotidases interact with and regulate P2 receptor-mediated signaling processes. Thus, the data shown herein led us to speculate that iron regulation of ecto-ATPase activities may interfere in ATP-mediated signal transduction pathways. The interference in such pathways might have important consequences in the ability of trichomonads to interact and cause damage to host cells. This possibility may be supported by the observation that cytotoxicity of *Acanthamoeba* to the host cell is inhibited by the ecto-ATPase inhibitor suramin (P2 receptor antagonist) [30]. Moreover, our group also noted that ecto-ATPase activities of *T. foetus* were strongly abrogated by micromolar concentration of suramin [10]. However, studies must be conducted in order to ascertain the existence of ATP-induced signaling through P2 receptors in trichomonads and the role played by iron in such processes. Additionally, it is known that iron affects the activities of other crucial enzymes in trichomonads. Cultivation of *T. foetus* in an iron-depleted media induces a marked reduction in the activities of the hydrogenosomal enzymes, pyruvate–ferredoxin oxidoreductase (PFOR) and malic [17]. Such effects are detected in parasites displaying a decrease in the synthesis of mRNA which encode PFOR and the hydrogenosomal malic enzyme. In addition, it was also apparent that *T. vaginalis* grown in an iron-depleted medium present low levels of protein synthesis [18], including adhesins [16] and some proteases [31]. Concerning the effects of iron depletion on parasite growth, we observed that the addition of 180 μM of 2,2-dipyridyl to the culture medium inhibited trichomonad growth, but cell viability was maintained on the order of 95% (data not shown), similar to results previously reported by others [18,20,38].

Similar to that found for trypanosomatids [32,33], parasitic trichomonads secrete notable amounts of acid phosphatases into culture medium [11,34]. Furthermore, *T. vaginalis* has a lysosomal system implicated in the release of hydrolases which are able to induce damage to both vaginal and urethral epithelial cells *in vitro* [35]. With regard to secreted phosphatase we observed that *T. foetus* parasites as well as the JT strain of *T. vaginalis* collected from iron-depleted cultures displayed no noticeable changes in the enzyme activity. In contrast, the secreted phosphatase activity exhibited by FMV-1 parasites was further decreased, indicating that long-term cultured trichomonads are indeed very different from fresh isolates. Otherwise, it is possible that during the *in vitro* adaptation of trichomonads, some populations with different capabilities of accumulating phosphatase inside vacuoles or vesicles could be selected. In spite of the FMV-1 fresh isolate having presented a greater secreted phosphatase activity than the long-term cultured ones, the FMV-1 strain was more susceptible to iron depletion. Although such a difference in the enzymatic activity may have biochemical relevance for these parasites, further studies are essential to determine the biological implication of the differences in susceptibility to iron depletion observed between these isolates.

We clearly demonstrated that the fresh clinical FMV-1 strain of *T. vaginalis*, which is much more cytotoxic to epithelial cell monolayers than the JT strain [23], exhibited high activity in all investigated enzymes. In accordance with our observation regarding ecto-ATPase activity, it was described previously that some fresh isolates of *T. vaginalis* presented higher ATP and ADP hydrolysis than long-term-grown isolates [27]. Nevertheless, the last authors observed a difference of 1.4- to 2.4-fold in the ATP hydrolysis among the isolates, whereas we witnessed a difference of 4.0-fold when compared to the ATPase activity between the fresh isolate and the long-term cultured one. Concerning ecto-phosphatase activity, herein we demonstrated that the extracellular *p*-NPP substrate hydrolysis by *T. vaginalis* is greater in a fresh, as opposed to, a long-term grown isolate. These data strongly support the idea that as long as trichomonads have been adapted to *in vitro* growth conditions, they tend to lose some important biochemical features originally detected when they are encountered in their hosts, at least in the case of *T. vaginalis*, whose cytopathogenicity is strongly decreased after a long-term cultivation [36]. The distinct profile of ecto-phosphatase activity between the two *T. vaginalis* strains is another interesting instance of differential expression of a putative virulence factor among fresh versus culture adapted parasites. Nevertheless, we do not discard the possible existence of gene activation in both *T. vaginalis* and *T. foetus* during their adaptation to *in vitro* conditions.

Altogether, the herein reported data clearly point out that iron plays a pivotal role in the expression of phosphohydrolases in both *T. foetus* and *T. vaginalis*. Nevertheless, further studies are required to elucidate the exact mechanisms involved in such iron-regulated phenomena.

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