Iron from haemoglobin and haemin modulates nucleotide hydrolysis in *Trichomonas vaginalis*

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Extracellular ATP may act as a danger signalling molecule, inducing inflammation and immune responses in infection sites. The ectonucleotidases NTPDase and ecto-5'-nucleotidase are enzymes that modulate extracellular nucleotide levels; these enzymes have been previously characterised in Trichomonas vaginalis. *Iron plays an important role in the complex trichomonal pathogenesis. Herein, the effects of iron on growth, nucleotide hydrolysis and NTPDase gene expression in* T. vaginalis *isolates from female and male patients were evaluated. Iron from different sources sustained* T. vaginalis *growth. Importantly, iron from haemoglobin (HB) and haemin (HM) enhanced NTPDase activity in isolates from female patients and conversely reduced the enzyme activity in isolates from male patients. Iron treatments could not alter the NTPDase transcript levels in* T. vaginalis. *Furthermore, our results reveal a distinct ATP, ADP and AMP hydrolysis profile between isolates from female and male patients influenced by iron from HB and HM. Our data indicate the participation of NTPDase and ecto-5'-nucleotidase in the establishment of trichomonas infection through ATP degradation and adenosine production influenced by iron.*

Key words: *Trichomonas vaginalis* - iron - nucleoside triphosphate diphosphohydrolase - ecto-5'-nucleotidase - nucleotide hydrolysis

Trichomonosis caused by the flagellate protozoan *Trichomonas vaginalis* represents the most prevalent nonviral sexually transmitted disease worldwide (WHO-DRHR 2012). In women, the symptoms are cyclic and often worsen around the menstruation period. In men, trichomonosis is largely asymptomatic and these men are considered to be carriers of *T. vaginalis* (Petrin et al. 1998). This infection has been associated with birth outcomes (Klebanoff et al. 2001), infertility (Grodstein et al. 1993), cervical and prostate cancer (Viikki et al. 2000, Sutcliffe et al. 2012) and pelvic inflammatory disease (Cherpes et al. 2006). Importantly, *T. vaginalis* is a co-factor in human immunodeficiency virus transmission and acquisition (Sorvillo et al. 2001, Van Der Pol et al. 2008). Therefore, it is important to study the host-parasite relationship to understand *T. vaginalis* infection and pathogenesis. Colonisation of the mucosa by *T. vaginalis* is a complex multi-step process that involves distinct mechanisms (Alderete et al. 2004). The parasite interacts with mucin (Lehker & Sweeney 1999), adheres to vaginal epithelial cells (VECs) in a process mediated by adhesion proteins (AP120, AP65, AP51, AP33 and AP23) and undergoes dramatic morphological changes from a pyriform to an amoeboid form (Engbring & Alderete 1998, Kucknoor et al. 2005, Moreno-Brito et al. 2005). After adhesion to VECs, the synthesis and gene expression of adhesins are increased (Kucknoor et al. 2005). These mechanisms must be tightly regulated and iron plays a pivotal role in this regulation.

Iron is an essential element for all living organisms, from the most primitive to the most complex, as a component of haeme, iron-sulphur clusters and a variety of proteins. Iron is known to contribute to biological functions such as DNA and RNA synthesis, oxygen transport and metabolic reactions. *T. vaginalis* has developed multiple iron uptake systems such as receptors for hololactoferrin, haemoglobin (HB), haemin (HM) and haeme binding as well as adhesins to erythrocytes and epithelial cells (Moreno-Brito et al. 2005, Ardalan et al. 2009). Iron plays a crucial role in the pathogenesis of trichomonosis by increasing cytoadherence and modulating resistance to complement lyses, ligation to the extracellular matrix and the expression of proteases (Figueroa-Angulo et al. 2012). In agreement with this role, the symptoms of trichomonosis worsen after menstruation. In addition, iron also influences nucleotide hydrolysis in *T. vaginalis* (Tasca et al. 2005, de Jesus et al. 2006).

The extracellular concentrations of ATP and adenosine can markedly increase under several conditions such as inflammation and hypoxia as well as in the presence of pathogens (Robson et al. 2006, Sansom 2012). In the extracellular medium, these nucleotides can act as immunomodulators by triggering immunological effects.

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Extracellular ATP acts as a proinflammatory immune-mediator by triggering multiple immunological effects on cell types such as neutrophils, macrophages, dendritic cells and lymphocytes (Bours et al. 2006). In this sense, ATP and adenosine concentrations in the extracellular compartment are controlled by ectoenzymes, including those of the nucleoside triphosphate diphosphohydrolase (NTPDase) (EC: 3.1.4.1) family, which hydrolyze tri and diphosphates and ecto-5'-nucleotidase (EC: 3.1.3.5), which hydrolyses monophosphates (Zimmermann 2001). Considering that *de novo* nucleotide synthesis is absent in *T. vaginalis* (Heyworth et al. 1982, 1984), this enzyme cascade is important as a source of the precursor adenosine for purine synthesis in the parasite (Munagala & Wang 2003). Extracellular nucleotide metabolism has been characterised in several parasite species such as *Toxoplasma gondii, Schistosoma mansoni, Leishmania* spp, *Trypanosoma cruzi, Acanthamoeba, Entamoeba histolytica, Giardia lamblia* and fungi, *Saccharomyces cerevisiae, Cryptococcus neoformans, Candida parapsilosis* and *Candida albicans* (Sansom 2012). In *T. vaginalis*, NTPDase and ecto-5'-nucleotidase activities have been characterised and they are involved in host-parasite interactions by controlling ATP and adenosine levels (Matos et al. 2001, de Jesus et al. 2002, Tasca et al. 2003).

Considering that (i) iron plays a crucial role in the pathogenesis of trichomonosis, (ii) ATP exerts a proinflammatory effect in inflammation, (iii) adenosine is important to *T. vaginalis* growth and acts as an antiinflammatory factor (Frasson et al. 2012) and (iv) ectonucleotidases modulate the nucleotide levels at infection sites (such as those observed in trichomonosis), the aim of this study was to investigate the effect of iron on the extracellular nucleotide hydrolysis and gene expression of *T. vaginalis.*

SUBJECTS, MATERIALS AND METHODS

Parasite cultures - *T. vaginalis* isolates 30236 and 30238 from American Type Culture Collection, TV-LACM1 and TV-LACM2 (fresh clinical isolates from female patients) and TV-LACH1 and TV-LACH2 (fresh clinical isolates from male patients), all from Clinical and Toxicological Analysis Laboratory, Faculty of Pharmacy, Federal University of Rio Grande do Sul (UFRGS), Brazil (UFRGS Ethical Committee, project 18923) were used in this study. The parasites were cultured in trypticase-yeast extract-maltose (TYM) medium (Diamond 1957), pH 6.0, supplemented with 10% heat-inactivated bovine serum and incubated at 37ºC. Organisms in the logarithmic phase of growth and exhibiting motility and normal morphology were used in the experiments.

Iron treatment - Low-iron-grown trichomonads were obtained by cultivating *T. vaginalis* at a starting density of 2.0 x 10^5 trophozoites/mL in TYM-serum containing 50 µM of the iron chelator 2,2-bipyridyl (2,2-BP) at 37ºC for 24 h (Lehker et al. 1991). These organisms were then suspended in TYM-serum supplemented with 50 µM 2,2-DP for an additional 24 h before experiments. To obtain different iron-organism conditions, low-iron parasites were washed once and suspended in TYM-serum medium containing (i) 50 µM 2,2-BP, (ii) 25 µM protoporphyrin (PP), (iii) 100 µM ferrous sulphate (FS), (iv) 25 µM HB or (v) 25 µM HM and incubated for 24 h at 37ºC as the control (untreated organisms), representing 72 h of iron treatment. All experiments were performed at least three times in triplicate with different trophozoite suspensions. The iron chelator 2,2-BP, PP, FS, HB and HM were purchased from Sigma Chemical Co (USA).

Kinetic growth assay - Kinetic growth curves were performed with all isolates at an initial density of 2.0 x 10^5 trophozoites/mL in TYM medium supplemented with 2,2-BP, PP, FS, HB and HM, as previously described. The trophozoites were counted using a haemocytometer, considering motility and normal morphology and were expressed as 10^6 trophozoites/mL. Growth experiments were performed in triplicate on at least three different occasions.

Enzyme assays - After 72 h of iron treatment, parasites were immediately harvested and washed three times with a 0.9-0.2% NaCl-glucose solution. The reaction mixture contained 50 mM Tris buffer (pH 7.2) and 5.0 mM $CaCl_2$ for measuring ATP and ADP hydrolysis (Matos et al. 2001) and 50 mM Tris buffer (pH 7.5) and 3.0 mM $MgCl_2$ for quantifying AMP hydrolysis (Tasca et al. 2003). The intact trophozoites were added to the reaction mixture and the reaction was started by adding

TABLE

Polymerase chain reaction (PCR) primers design

Enzymes	Primer sequences	Anneling temperature (°C)	PCR product (bp)	GenBank accession (mRNA)
NTPDase A	F - TGAAGAAGAGTTGAAGGGCAAAG R - AATTCTTCGACAGGAGGCATTG	53	342	XM_001298945
NTPDase B	F - CGACTACATCATCTCTTGCCGATC R - GACTCTCTTATGTATCTTTGGGCAG	53	397	XM_001579653
α-tubulin	F - CCAACATGATGGTTAAGTGCGATCCAC R - CAGCTTCTTCCATACCCTCACCGACG	61	355	XM_001330630

NTPDase: nucleoside triphosphate diphosphohydrolase.

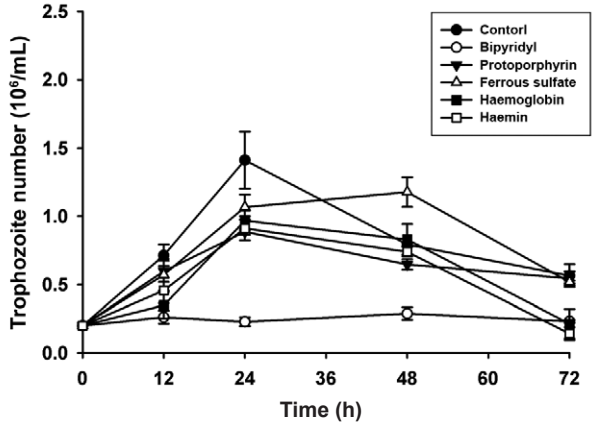

Fig. 1: *Trichomonas vaginalis* kinetic growth of a representative experiment performed with isolate 30236. Kinetic growth experiments with all *T. vaginalis* isolates were performed on three different occasions (different parasite suspensions), with similar results. Numbers of parasite densities were determined by a haemocytometer counting.

substrate (1.0 mM ATP or ADP or 3.0 mM AMP). ATP, ADP or AMP hydrolysis was stopped by adding 200 µL of 10% trichloroacetic acid and chilling on ice for 10 min before assaying the release of inorganic phosphate (Pi) (Chan et al. 1986). The incubation time and protein concentration were defined based on curves that were previously determined for linearity of the reactions for each isolate used (data not shown). Controls with intact organisms added to the reaction mixtures after the reaction was stopped were used to correct for non-enzymatic hydrolysis of the substrates. The specific activity was expressed as nmol Pi/min/mg protein. All samples were run in triplicate and similar results were achieved in at least three different parasite suspensions.

Protein measurement - Protein was measured according to the Coomassie Blue method (Bradford 1976) using bovine serum albumin as standard.

Analysis of gene expression by reverse transcriptase-polymerase chain reaction (RT-PCR) - Semiquantitative RT-PCR assay was performed with HB and HM treated-organisms. The NTPDase sequences were found using the BLAST function of the GenBank database and specific primers for NTPDase A and B were designed (Table) (Carlton et al. 2007). RNA extraction was performed using TRIzol reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. Afterwards, cDNA species were synthesised using the ImProm-II™ Reverse Transcription System (Promega®) following the supplier's instructions. All conditions for PCR were described in previous studies (Kucknoor et al. 2005, Giordani et al. 2010).

Statistical analysis - The results were expressed as the means ± standard deviations. Statistical analysis was conducted by ANOVA followed by Tukey's *post hoc* multiple comparisons and Student's *t* test. Statistical significance was considered at $p < 0.05$. All analyses were performed using Statistical Package for the Social Sciences (SPSS) software v.14.

RESULTS

Lack of iron hampers T. vaginalis growth - *T. vaginalis* kinetic growth was analysed for all of the isolates; however, taking into account that the growth rate and iron requirement were comparable, a representative experiment using the 30236 isolate was shown (Fig. 1). The organisms were first counted 12 h after beginning the incubation. Subsequently, counts were performed every 24 h and compared to untreated cells. The control exhibited the classical growth peak after 24 h of incubation and trophozoites treated with PP, FS, HB and HM demonstrated a similar growth peak. However, 2,2-BP-treated parasites, i.e., those in a low-iron condition, did not exhibit a growth peak (Fig. 1) and reduced viability was observed (data not shown). Thus, the lack of iron in the culture medium impaired *T. vaginalis* growth.

Iron from HB and HM modulates nucleotide hydrolysis - The effects of different iron treatments on ATP, ADP and AMP hydrolysis were evaluated in *T. vaginalis*. Cellular integrity was evaluated by assessing the motility and viability of the trophozoites by trypan blue exclusion before and after all enzyme assays. Trophozoite integrity was not affected by any iron treatment. Treatment with HB and HM induced a significant increase in ATP and ADP hydrolysis in 30236 and 30238 (long-term-growth isolates) and TV-LACM1 and TV-LACM2 (fresh clinical isolates) (Fig. 2A, D). However, the fresh clinical isolates, TV-LACH1 and TV-LACH2, treated with HB and HM exhibited significantly decreased ATP and ADP hydrolysis (Fig. 2E, F).

Moreover, the effect of different iron treatments on ecto-5'-nucleotidase activity was evaluated. Notably, AMP was not hydrolysed in the TV-LACM1 and TV-LACM2 isolates under control or treatment conditions. Nevertheless, in the 30236 and 30238 isolates, HB and HM promoted a significant reduction in AMP hydrolysis (Fig. 3A, B). The TV-LACH1 and TV-LACH2 isolates treated with low iron levels demonstrated significantly higher AMP hydrolysis (Fig. 3C, D). In this context, it is possible to suggest that extracellular nucleotide hydrolysis is heterogeneous among *T. vaginalis* isolates.

Iron did not affect NTPDase transcription - To assess the effect of HB and HM treatments on NTPDase gene expression, semiquantitative RT-PCR experiments were performed with all isolates. The different experimental conditions evaluated did not alter the NTPDase transcript levels in *T. vaginalis* (Fig. 4).

DISCUSSION

Iron is an essential cation for the growth and maintenance of *T. vaginalis* infection and plays a crucial role in the pathogenesis of trichomonosis (Figueroa-Angulo et al. 2012). Although the human host contains abundant iron, there is no free iron in the vagina or in the male urethra and *T. vaginalis* has developed multiple iron systems to acquire iron from iron-binding (lactoferrin) and iron-containing (HB) proteins (Moreno-Brito et al. 2005, Ardalan et al. 2009). In this report and in agreement with a previous study (Alderete et al. 2004), under conditions that

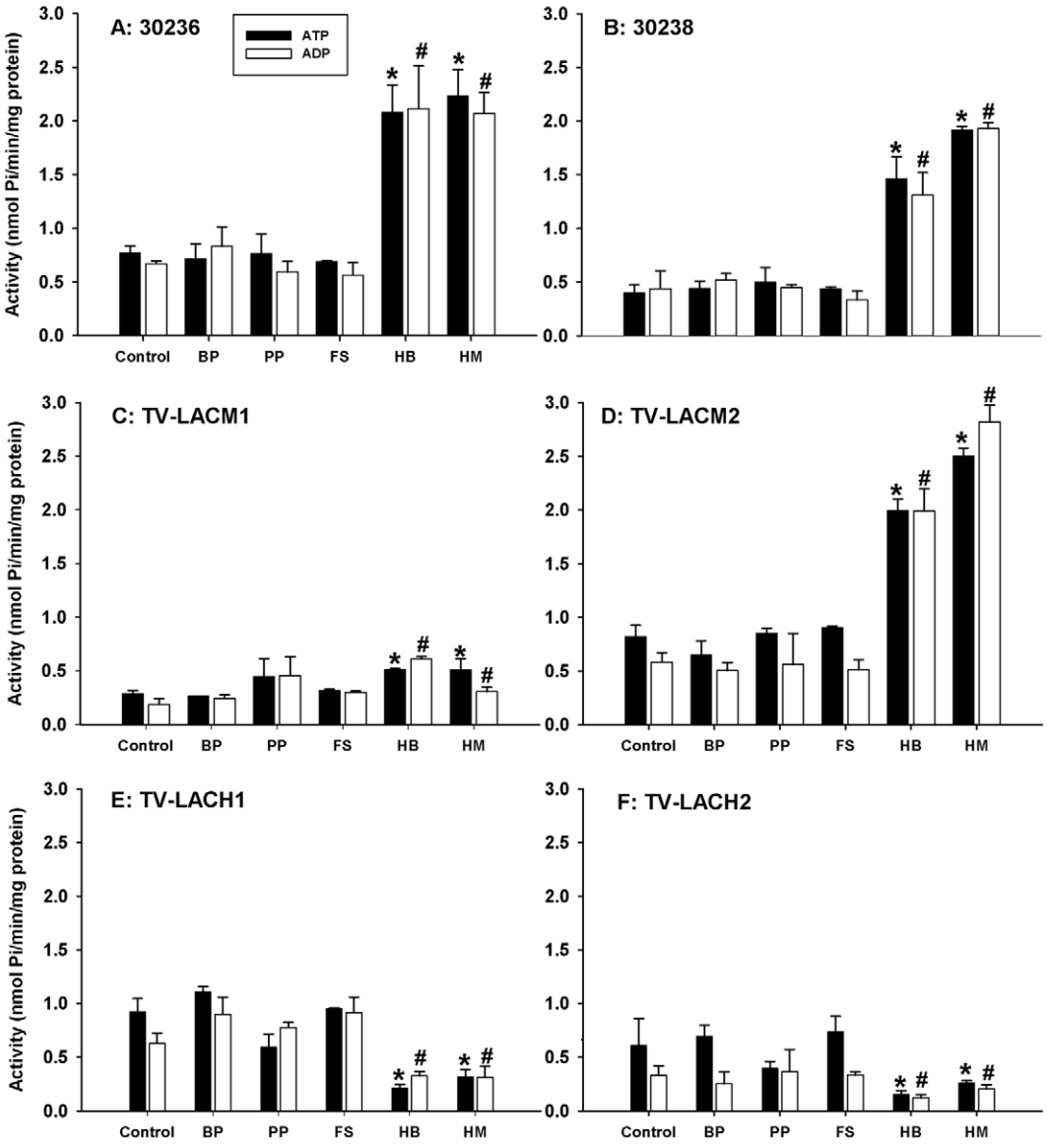

Fig. 2: effect of the different iron treatments on *Trichomonas vaginalis* nucleoside triphosphate diphosphohydrolase (NTPDase) activity. Bars represent the mean ± standard deviation of three different experiments (parasite suspensions) performed in triplicate. Data from haemoglobin (HB) and haemin (HM) treatments were analysed by Student *t* test ($p \leq 0.05$) (Statistical Package for the Social Sciences). *: ATP hydrolysis statistically different from control; #: ADP hydrolysis statistically different from control. BP: bipyridyl; FS: ferrous sulphate; PP: protoporphyrin; TV-LACH1 and TV-LACH2: fresh clinical isolates from male patients; TV-LACM1 and TV-LACM2: fresh clinical isolates from female patients.

maintain sufficient amounts of iron to meet the metabolic requirements (culture with PP, FS, HB and HM), the parasites demonstrated a similar growth peak to the control. Notably, the parasites adequately grew under PP treatment because although PP does not possess an iron moiety, the metal was not removed from the culture medium with a chelator; thus, this situation was similar to the control. As expected, trichomonads cultured in low-iron medium, i.e., TYM supplemented with the iron chelator bipyridyl, which removes iron from culture medium, exhibited reduced growth in comparison with the control, showing the importance of iron for trichomonad survival.

Although free haeme produces oxidative effects and inflammation (Dutra & Bozza 2014), these events were not significant enough to cause damage because the parasites were still viable after treatments with distinct iron sources, as observed in the kinetic growth assays. Although it is a critical component of the pathological processes and causes tissue damage, haeme is used by *T. vaginalis* to obtain iron through the iron-binding, multifunctional proteins AP51 and AP65 (Ardalan et al. 2009). Moreover, *T. vaginalis* is a microaerophilic protozoan that has fully developed mechanisms of adaptation to oxygen fluctuations in infection sites (Ellis et al. 1994).

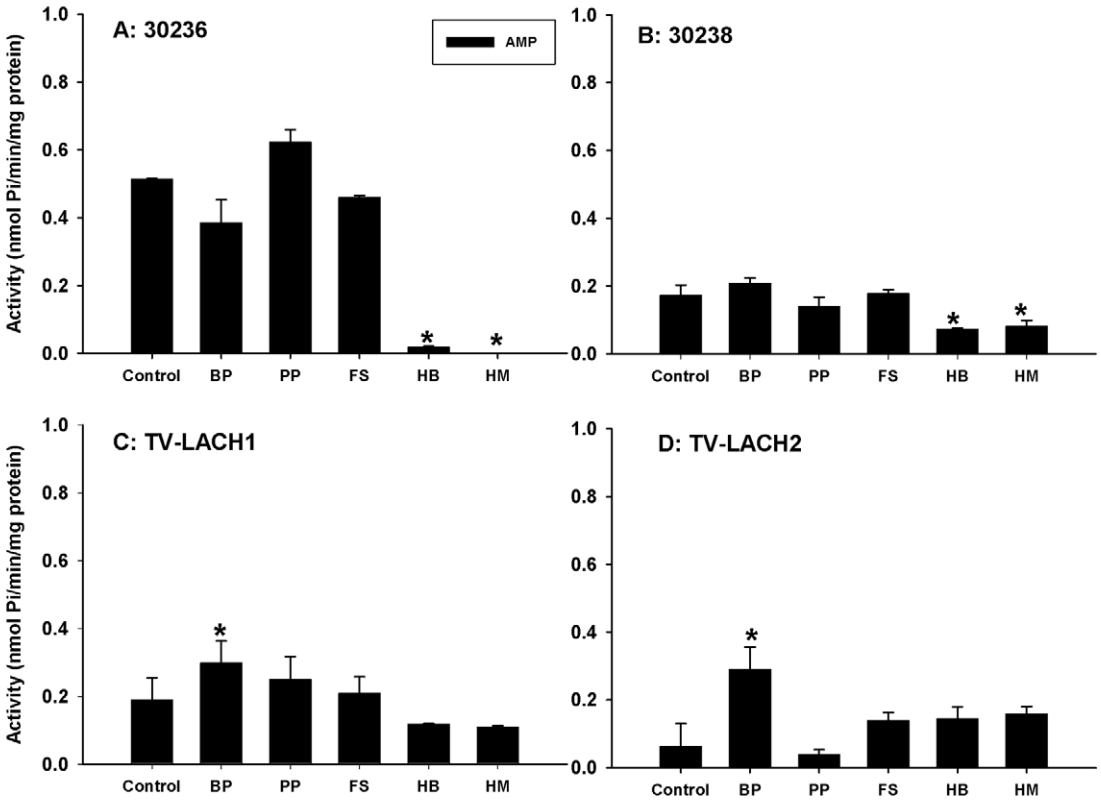

Fig. 3: effect of the different iron treatments on *Trichomonas vaginalis* ecto-5'-nucleotidase activity. Bars represent the mean ± standard deviation of three different experiments. Data were analysed by Student *t* test ($p \leq 0.05$) (Statistical Package for the Social Sciences). Asterisk means AMP hydrolysis statistically different from control. BP: bipyridyl; FS: ferrous sulphate; HM: haemin; HB: haemoglobin; Pi: inorganic phosphate; PP: protoporphyrin; TV-LACH1 and TV-LACH2: fresh clinical isolates from male patients.

Furthermore, apart from the importance of iron in *T. vaginalis* growth and multiplication, our results demonstrated that haeme-iron modulates extracellular ATP, ADP and AMP hydrolysis. These findings are sustained by testing PP as a control of the haeme ring for the effects of HB and HM because PP does not contain iron. Therefore, iron has an effect on NTPDase and ecto-5'-nucleotidase activities. Although de Jesus et al. (2004) have shown that well-established and fresh clinical isolates present distinct virulence against epithelial cells, our data revealed that both long-term-growth and fresh clinical isolates presented increased NTPDase and reduced ecto-5'-nucleotidase activities after the treatment of parasites with HB and HM.

With the aim to determine the importance of these effects of iron during infection, we discussed the role of this modulation considering the proinflammatory function of ATP vs. the antiinflammatory effect of adenosine. Considering that the enzyme cascade that hydrolyses extracellular ATP to adenosine is modulated by iron, it is conceivable that this purinergic signalling regulated by iron is involved in the immune response to trichomonosis. In addition, we observed that modulation of the iron from HB and HM on nucleotide hydrolysis is in agreement with clinical findings because women are symptomatic; in this study, *T. vaginalis* from female patients showed (i) higher NTPDase activity (the degradation of cytotoxic and proinflammatory ATP) and (ii) lower ecto-5'-nucleotidase activity (lower adenosine production, lessened antiinflammatory effects), which contribute to the well-known production of exacerbated symptoms, particularly shortly after menstruation (when high levels of HB are found) (Petrin et al. 1998).

In contrast, NTPDase activity decreased when the fresh clinical isolates from male patients were treated with HB and HM and the ecto-5'-nucleotidase activity was higher in iron chelator-treated isolates from male patients. This findings support our hypothesis because compared with isolates from female patients, the opposite effect was observed among *T. vaginalis* isolates from male patients: (i) lower NTPDase activity, ATP accumulation, a unfavourable conditions for parasitism because of the cytotoxic and proinflammatory features of ATP and (ii) treatment with the iron chelator generated low levels of iron, which caused increased ecto-5'-nucleotidase activity and adenosine accumulation. These effects led to two consequences: first, an antiinflammatory milieu to attenuate symptoms (men are asymptomatic) and, second, trichomonads took up the accumulated adenosine, an essential nucleoside for metabolism, under these conditions as a strategy to overcome the hostile environment produced by the high ATP levels. Therefore, adenosine

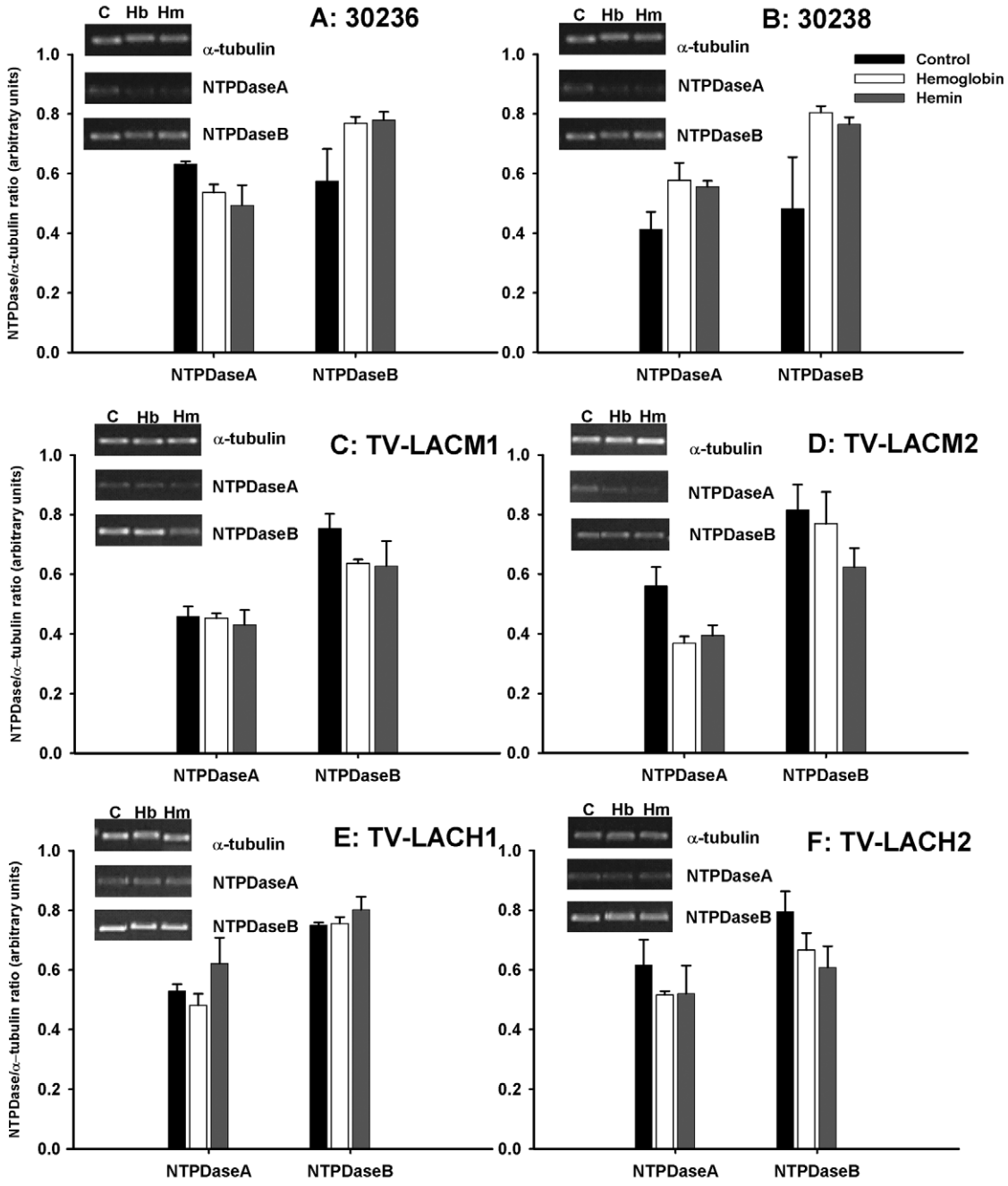

Fig. 4: gene expression patterns for nucleoside triphosphate diphosphohydrolase (NTPDase) A and B after haemoglobin (HB) and haemin (HM) treatments in *Trichomonas vaginalis* 30236 and 30238, fresh clinical isolates from female patients (TV-LACM1 and TV-LACM2) and fresh clinical isolates from male patients (TV-LACH1 and TV-LACH2). Bars represent the mean ± standard deviation of three different experiments. C: control.

contributes to parasite survival and persistence, leading to infection silencing at male sites associated with an antiinflammatory milieu. These data are in agreement with recent studies by Sutcliffe et al. (2012) that demonstrated the relationship between *T. vaginalis* and prostate cancer.

To investigate the possible role of transcription factors on NTPDase activity, RT-PCR was performed. In this assay, only organisms treated with HB and HM were used because these organisms present significantly different ATP and ADP hydrolysis. Although all of the isolates present comparable expression patterns, treatment with HB and HM did not affect NTPDase transcription. Conversely, previous research has shown that iron modulates the gene expression of adhesins and proteinases involved in trichomonosis pathogenesis (Lehker et al. 1991, Garcia et al. 2003, Alderete et al. 2004, Kucknoor et al. 2005, Alvarez-Sanchez et al. 2007). This was not the case in the present study; it can be suggested

that iron produced these effects through direct interactions with the enzymes. Moreover, these data reveal the promptness of the response of these isolates to microenvironment conditions because the effects of HB and HM were observed on NTPDase activity rather than at the transcriptional level. Further studies are necessary to explain the mechanism of iron modulation on *T. vaginalis* extracellular nucleotide hydrolysis.

Overall, the present study demonstrates the role of iron from HB and HM in *T. vaginalis* growth and extracellular nucleotide hydrolysis. Notably, our results suggest heterogeneity of the nucleotide hydrolysis profiles between isolates from female and male patients treated with HB and HM. These results contribute to the understanding of *T. vaginalis* infection through a fine regulation of NTPDase and ecto-5'-nucleotidase by iron, implicating purinergic signalling in the pathogenesis *via* antagonistic immune consequences produced by ATP and adenosine as well as a nucleoside-uptake strategy for survival.

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