The International Journal of Biochemistry & Cell Biology 40 (2008) 2442–2451

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Polyamine depletion down-regulates expression of the *Trichomonas vaginalis* cytotoxic CP65, a 65-kDa cysteine proteinase involved in cellular damage

María Elizbeth Alvarez-Sánchez[a], Bertha Isabel Carvajal-Gamez[a], Eduardo Solano-González[b], Máximo Martínez-Benitez[a], Ana F. Garcia[c], John F. Alderete[d], Rossana Arroyo[e,*]

[a] *Posgrado en Ciencias Genómicas, Universidad Autónoma de la Ciudad de México (UACM), San Lorenzo # 290, Col. Del Valle, CP 03100 Mexico City, Mexico*
[b] *Departamento de Biotecnología y Bioingeniería Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV-IPN), Mexico City, Mexico*
[c] *Department of Microbiology and Immunology, University of Texas Health Science Center, San Antonio, TX, USA*
[d] *School of Molecular Biosciences, Washington State University, Pullman, WA 99164-4660, USA*
[e] *Departamento de Patología Experimental, CINVESTAV-IPN, Av IPN 2508, Col. San Pedro Zacatenco, CP 07360 Mexico City, Mexico*

ARTICLE INFO

Article history:
Received 3 December 2007
Received in revised form 7 April 2008
Accepted 8 April 2008
Available online 24 May 2008

Keywords:
Trichomonas vaginalis
Cysteine proteinases
Cytotoxicity
Gene regulation by polyamines
Putrescine

ABSTRACT

Recently, we found that inhibition of putrescine synthesis by ornithine decarboxylase (ODC) significantly increased *Trichomonas vaginalis* adherence mediated by protein adhesins. Surprisingly and unexpectedly, trichomonal contact-dependent cytotoxicity was absent. Therefore, a role for polyamine depletion on regulation of *T. vaginalis* cytotoxicity mediated by the cysteine proteinase (CP) of 65-kDa, CP65, was investigated. We performed cytotoxicity and cell-binding assays followed by zymograms, as well as Western blot and indirect immunofluorescence assays using specific anti-CP65 antibodies to detect CP65. Trichomonads grown in the presence of the ODC inhibitor, 1-4-diamino-2-butanone (DAB) had lower levels of cytotoxicity that corresponded with diminished CP65 proteolytic activity when compared to untreated organisms handled identically. Likewise, semiquantitative and qRT-PCR as well as Western blot and immunofluorescence assays showed decreased amounts of *tvcp65* mRNA and CP65 protein in DAB-treated parasites. These effects were reversed by addition of exogenous putrescine. These data show a direct link between polyamine metabolism and expression of the cytotoxic CP65 proteinase involved in trichomonal host cellular damage.

Abbreviations: α-TUB, α-tubulin protein; *β-tub*, *β-tubulin* gene; B-TUB9, sense primer to amplify the *β-tubulin* gene; B-TUB2, antisense primer to amplify the *β-tubulin* gene; CPs, cysteine proteinases; CP65, the cytotoxic cysteine proteinase of 65-kDa with affinity to the surface of HeLa cells; CP39, cysteine proteinase of 39-kDa with affinity to the surface of HeLa cells; CP30, cysteine proteinase of 30-kDa with affinity to the surface of HeLa cells; CNCD, Centro Nacional de Clinica de Displasias; DAB, 1,4-diamino-2-butanone; DFMO, DL-α-difluoromethylornithine; DMEM, Dulbecco's Modified Eagle medium; HIV, human immunodeficience virus; IgG, immunoglobulin G; ODC, ornithine decarboxylase; NRS, preimmune normal rabbit serum; qRT-PCR, quantitative RT-PCR; S-65, antisense primer to amplify a fragment of the *tvcp65* gene; SSY65, sense primer to amplify a fragment of the *tvcp65* gene; STI, sexually transmitted infection; TCA, trichloroacetic acid; *tvcp65*, gene encoding the cytotoxic CP65 proteinase; TLR2, toll-like receptor 2; TYM, trypticase-yeast extract-maltose; TYM-serum, TYM medium supplemented with 10% heat-inactivated horse serum; VECs, vaginal epithelial cells.

* Corresponding author. Tel.: +52 55 5747 3800x3342; fax: +52 55 5747 3377.
E-mail address: rarroyo@cinvestav.mx (R. Arroyo).

1357-2725/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biocel.2008.04.023

1. Introduction

Trichomonas vaginalis is the human protozoan parasite responsible for trichomonosis, the most common sexually transmitted infection (STI). This STI caused by *T. vaginalis* is associated with adverse health consequences to women, which include infertility (El-Shazly et al., 2001), atypical pelvic inflammatory disease (Moodley et al., 2002), preterm delivery of low birth weight infants (Cotch et al., 1997), and cervical neoplasia (Viikki et al., 2000). Apart from urethritis in men, a relationship now has been established between serum antibodies to *T. vaginalis* and prostate cancer (Sutcliffe et al., 2006). This STI is more prevalent in disadvantaged communities (Sorvillo et al., 1998) and increases predisposition to human immunodeficience virus (HIV) seroconversion (Guenthner et al., 2005; Mason et al., 2005; Rughooputh and Greenwell, 2005). The high incidence and prevalence of this STI on the human population (Van Der Pol et al., 2005) make the study of *T. vaginalis* highly significant.

Successful host parasitism is achieved by parasite penetration of the mucus layer (Lehker and Sweeney, 1999) followed by adherence to vaginal epithelial cells (VECs) mediated by at least five protein adhesins (Arroyo et al., 1992; Moreno-Brito et al., 2005) and the proteolytic activity of at least two cysteine proteinases (CPs), CP30 and CP62, is needed (Arroyo and Alderete, 1989, 1995; Mendoza-López et al., 2000; Hernandez et al., 2004). Brief contact of *T. vaginalis* with VECs results in dramatic morphological changes from ellipsoid to amoeboid (Arroyo et al., 1993). Iron concentrations and cell contact upregulate adhesins expression in a coordinated fashion (Arroyo et al., 1993; García et al., 2003; Lehker et al., 1991). *T. vaginalis* cytoadherence modulates the expression of numerous parasite and host cell genes (Kucknoor et al., 2005a,b). Involvement of several CPs, some of which are directly implicated in contact-dependent cytolysis, contributes to *T. vaginalis* pathogenesis (Alvarez-Sánchez et al., 2000; Arroyo and Alderete, 1989, 1995; Hernández-Gutiérrez et al., 2003, 2004; Mendoza-López et al., 2000). The CPs of 65-kDa (CP65) (Alvarez-Sánchez et al., 2000, 2007; Solano-González et al., 2006) and 39-kDa (CP39) (Hernández-Gutiérrez et al., 2003, 2004) are involved in cytotoxicity. Both are surface-expressed and bind to the surface of human vaginal and cervical cells (Alvarez-Sánchez et al., 2000; Hernández-Gutiérrez et al., 2003, 2004). In particular, CP65 is active at pH and temperature of the infected vagina, degrades extracellular matrix proteins collagen IV and fibronectin, is immunogenic in patients with trichomonosis (Alvarez-Sánchez et al., 2000), and is down-regulated by iron, greatly reducing the trichomonal CP65-dependent cytotoxicity (Alvarez-Sánchez et al., 2007).

The polyamines putrescine, spermidine and spermine are abundant small cations found in all living species. These polycations are important multifunctional cellular components that are considered critical regulators of cell growth, division, differentiation and apoptosis. The intracellular concentration of polyamines is finely regulated by biosynthetic and metabolizing enzymes as well as by transport systems (Wallace et al., 2003).

The lead enzyme of polyamine biosynthesis of many cells is ornithine decarboxylase (ODC), which forms putrescine. *T. vaginalis* differs from other eukaryotes in several aspects of its polyamine metabolism. The putrescine formed from ornithine by ODC is not metabolized further but exported with the simultaneous uptake of spermine. Putrescine can be considered the end product of an energy-generating pathway (arginine dihydrolase pathway) and is found in large amounts in vaginal secretions of trichomonosis patients (Reis et al., 1999; Yarlett and Bacchi, 1988, 1994). *T. vaginalis* is unable to synthesize spermine; instead, spermine has to be obtained from the host through a putrescine/spermine antiporter system (Yarlett and Bacchi, 1994). Thus, putrescine is exchanged in a 2:1 molar ratio with the host-produced spermine, which is back-converted to spermidine by *T. vaginalis* (Yarlett and Bacchi, 1994; Yarlett et al., 2000). While inhibition of *Tritrichomonas foetus* ODC with 1,4-diamino-2-butanone (DAB) led to growth arrest, destruction of hydrogenosomes, and reduction of hydrogenosomal enzymes (Reis et al., 1999). For *T. vaginalis*, inhibition of ODC with DAB also resulted in growth arrest but there was no effect on the number and integrity of hydrogenosomes (García et al., 2005). Furthermore, no difference in the amount of adhesins (AP65, AP51, and AP33) between normal and DAB-treated organisms was observed, even though there was up to a 20-fold increase in the levels of cytoadherence (García et al., 2005). Remarkably and unexpectedly, the parasite contact-dependent cytotoxicity (Alderete and Pearlman, 1984; Krieger et al., 1985) was absent, a finding consistent with reduced trichomonal cytotoxicity due to DL-α-difluoromethylornithine (DFMO) treatment, another ODC inhibitor (Bremner et al., 1987).

The link between polyamine metabolism with *T. vaginalis* cytoadherence and cytotoxicity (García et al., 2005), together with the involvement of thiol proteinases in contact-dependent cytotoxicity (Alvarez-Sánchez et al., 2000, 2007; Arroyo and Alderete, 1989, 1995; Hernández-Gutiérrez et al., 2003, 2004; Solano-González et al., 2006), led us to hypothesize that polyamine depletion was linked to trichomonal cytotoxicity through the regulation of CP65 gene (*tvcp65*) expression, affecting the synthesis and proteolytic activity of CP65. Therefore, we tested whether polyamine depletion regulates CP65 expression and the CP65-dependent cellular damage inflicted by *T. vaginalis* to the host cells.

In this report we show that DAB treatment of *T. vaginalis* down-regulates CP65 expression concomitant with the reduction of the CP65-dependent host cytotoxicity. To our knowledge, this is the first report that shows a direct link between trichomonal polyamine depletion and the down-regulation of *tvcp65* expression, the gene encoding the cytotoxic CP65 proteinase.

2. Materials and methods

2.1. Culture and growth of T. vaginalis

The fresh clinical *T. vaginalis* isolate CNCD 147 was used in this study (Alvarez-Sánchez et al., 2000, 2007; Solano-González et al., 2006). Parasites were grown up to 3 weeks by daily passage in trypticase yeast extract maltose (TYM)

medium supplemented with 10% heat-inactivated horse serum (TYM-serum). For polyamine metabolism inhibition parasites were grown for 24 h at 37 °C in TYM-serum medium supplemented with 20 mM DAB (Sigma Chemical Co., St. Louis, MO), as before (García et al., 2005). For polyamine metabolism restoration, DAB-treated parasites were harvested after 18 h growth, washed with sterile PBS and transferred to TYM-serum medium supplemented with 40 mM putrescine (Sigma). Parasite viability after these treatments was checked by the trypan blue (Sigma) exclusion method. Control untreated and DAB-treated parasites showed between 95 and 97% viability in all experiments.

2.2. Cell-binding assay for proteinases

To detect proteinases with affinity to the surface of host cells, a cell-binding assay was performed as previously described (Alvarez-Sánchez et al., 2000, 2007; Solano-González et al., 2006). Briefly, a clarified detergent extract from 2×10^7 parasites was incubated for 18 h at 4 °C with 1×10^6 glutaraldehyde-fixed HeLa cells. Trichomonad proteinases bound to the surface of fixed-HeLa cells were eluted with Laemmli sample buffer for 20 min at 37 °C, and released proteinases were analyzed by substrate gel electrophoresis on 10% polyacrylamide gels copolymerized with 0.2% gelatin (Arroyo and Alderete, 1995).

2.3. Western blot assays

Parasites (2×10^7) grown in the absence or presence of DAB were precipitated with 10% trichloroacetic acid (TCA) to obtain total protein extracts (Alvarez-Sánchez et al., 2007). Solubilized proteins were boiled in sample buffer before loading the gel (SDS-PAGE in a 10% polyacrylamide gel) with an equivalent of 4×10^5 parasites per row. After electrophoresis, proteins were transferred onto nitrocellulose membranes, blocked with 0.5% skim milk in PBS–0.1% Tween 20 (PBS–Tween), for 1 h at 37 °C, incubated with the anti-CP65 rabbit serum (at 1:5000 dilution) previously obtained (Alvarez-Sánchez et al., 2000) for 18 h at 4 °C, washed five times with PBS–Tween, incubated with peroxidase-conjugated secondary antibody (at 1:3000 dilution) (Bio-Rad) for 2 h at 25 °C, and developed with 3-amino-9-ethyl carbazole (Sigma). Preimmune normal rabbit serum (NRS) was used as a negative control (at 1:5000 dilution). As a loading and internal control, an anti-α tubulin (α-TUB) monoclonal antibody (Zymed Laboratories, South San Francisco, CA) was also used (at 1:100 dilution).

2.4. Cytotoxicity assay

In this study we used confluent HeLa cell monolayers grown on 96-well microtiter plates (4×10^4 cells/well) obtained as described (Alvarez-Sánchez et al., 2000). Parasites (2×10^5) suspended in Dulbecco's Modified Eagle medium (DMEM) and TYM medium (DMEM-TYM at a 2:1 ratio) without horse serum (interaction media) were added to confluent HeLa cell monolayers at a 5:1 ratio (parasite:host cell) and incubated for 1 h at 37 °C in a 5% CO_2 atmosphere. HeLa cell monolayer destruction was assessed by a colorimetric method and quantitated spectrophotometrically at 570 nm. Each experiment was performed in triplicate and repeated at least three times.

2.5. RNA isolation and real-time RT-PCR analysis

Total RNAs from 1×10^7 parasites grown in the absence or presence of DAB were extracted by the TRIzol® reagent (Invitrogen, Life Technologies, Carlsbad, CA) as recommended by the manufacturer. For semiquantitative RT-PCR, total RNA was reverse-transcribed using the Superscript RNAse H Reverse Transcriptase Kit (Invitrogen) and an oligo (dT) primer. Then, a fragment of 370-bp of the *tvcp65* transcript was amplified by PCR using the sense primer SSY-65: 5′-ATAAGAGGAGCGTGATGGCACAT-3′ and the antisense primer S-65: 5′-ACGCGATTACATCTGGAGAACTC-3′ (Solano-González et al., 2006). As an internal control, a fragment of 112-bp of the *T. vaginalis β-tubulin* (*β-tub*) transcript was amplified using the BTUB9 sense primer (5′-CATTGATAACGAAGCTCCTTTACGAT-3′) and the BTUB2 antisense primer (5′-GCATGTTGTGCCGGACATAACCAT-3′) (León-Sicairos et al., 2004). Real-time quantitative RT-PCR (qRT-PCR) was performed using an Applied Biosystems instrument (Applied Biosystems, Foster City, CA) and the same specific primers described above. Briefly, 1 μg of total RNA sample from each condition was treated with RNAse-free DNAse according to the manufacturer instructions (Promega) and reverse-transcribed using oligo dT primers and Superscript II reverse transcriptase (Invitrogen) as above. RT-PCR amplification mixtures (25 μl) contained 1 μl (1 μg/μl) of templated cDNA, 300 nM specific primers for *tvcp65* and 2× SYBR Green I PCR Master Mix (12.5 μl) buffer (Applied Biosystems). Reactions were run on an ABI PRISM 7300 Sequence Detector (Applied Biosystems). The cycling conditions comprised 10 min polymerase activation at 95 °C and 40 cycles at 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s. Each assay (in triplicate) included: a standard curve of eight 1/10 serial dilution points of CP65 or tubulin cDNA, a no-template control, and 1 μl of each tested cDNA. All PCR efficiencies were above 95%. Sequence Detection Software (version 1.3) (Applied Biosystems) results were exported as tab-delimited text and imported into Microsoft Excel for further analysis. Detection of the fluorescent product was performed at the end of the extension period. To confirm amplification specificity, the PCR products were subjected to a standard curve analysis. The level of *tvcp65* mRNA was quantified by real-time PCR analysis using *β-tubulin* levels as normalizer with the Applied Biosystems analysis software.

2.6. Indirect immunofluorescence assays

For indirect immunofluorescence assays, parasites grown in the absence or presence of DAB were fixed with 4% paraformaldehyde for 1 h at 37 °C, washed with PBS and blocked with 0.2 M glycine for 1 h at 37 °C and with 0.2% foetal bovine serum for 15 min. Next, trichomonads were incubated with the anti-CP65 rabbit serum (Alvarez-Sánchez et al., 2000) or NRS (at 1:50 dilution) for 1 h at 4 °C and washed with PBS. Then, parasites were incubated

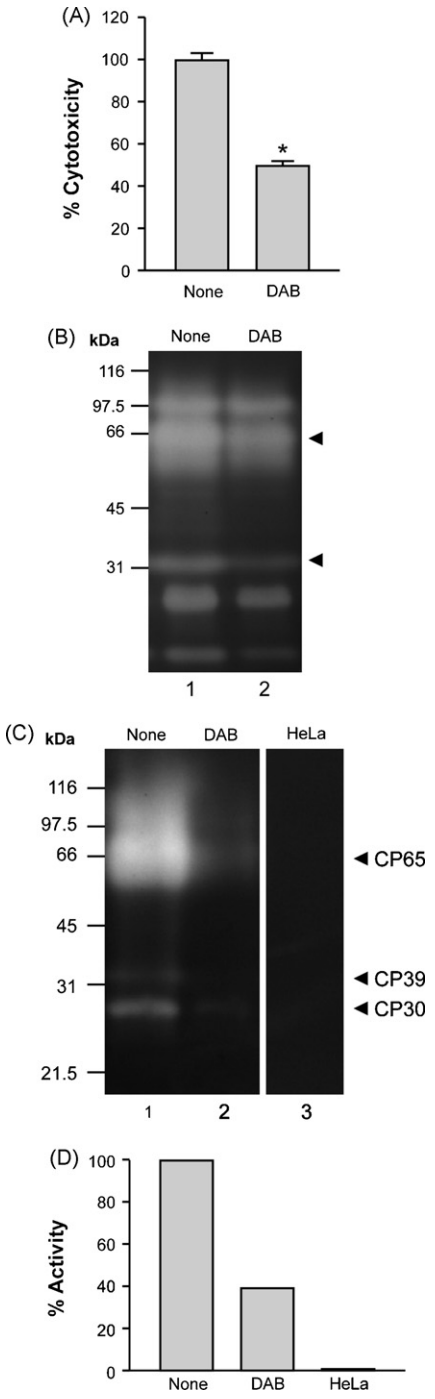

Fig. 1. Effect of DAB treatment over the trichomonal cytotoxicity and CP65 proteolytic activity. (A) Levels of cytotoxicity of control, untreated (none) and DAB-treated (DAB) organisms measured over HeLa cell monolayers using a colorimetric method. Results were normalized to 100% for the control organisms, and bars represent the standard error of triplicate samples. *$P < 0.05$ of the levels of cytotoxicity of DAB-treated parasites compared with control untreated parasites. (B) Zymograms of parasite lysates containing total proteinases in control (lane 1) and DAB-treated parasites (lane 2) obtained from a clarified detergent extract used for cell-binding assays. Arrowheads show the position of the two regions of proteinase activity where the CPs involved in cytotoxicity are found. (C) Zymograms to detect the CP65 proteolytic activity in control (lane 1) and DAB-treated (lane 2) parasites obtained after the cell-binding assay (Section 2). Lane 3 represents the mock experiment of fixed HeLa cells without parasite extracts (HeLa). Arrowheads show the CP65, CP39, and CP30 bands with proteolytic activity. (D) Densitometric scanning analysis of the cell-bound CP65 proteolytic activity from part (C) using the Quantity One Program (Bio-Rad). These experiments were performed at least three times with similar results.

with fluoresceine isothiocyanate-conjugated anti-rabbit immunoglobulins (Jackson ImmunoResearch, Laboratories Inc. West Grove, PA) (at 1:80 dilution) for 40 min at room temperature, washed, mounted with Vectashield mounting solution (Vector Lab., Burlingame, CA), and observed by confocal microscopy (Leica, DMLS).

2.7. Statistical analysis

All data are expressed as means ± S.E. from three samples. PCR experiments were repeated three times. The significance of the difference between means was determined by ANOVA and the Instat software. The level of significance was determined by Bonferroni Multiple Comparisons Test.

3. Results

3.1. DAB-treatment reduces growth rate of T. vaginalis

We first examined the effect of 20 mM DAB treatment over the CNCD 147 fresh *T. vaginalis* isolate growth. Addition of 20 mM DAB into the culture medium reduced growth of trichomonads as compared with the control untreated parasites handled identically. The generation time for DAB-treated parasites was two times greater than the control trichomonads (data not shown). This concentration of DAB did not affect overall trichomonal motility and viability (95–97% in all tested conditions) as before (García et al., 2005), using a different trichomonad isolate.

3.2. DAB treatment affects CP65 proteolytic activity and associated trichomonal cytotoxicity

Since we have previously established the major role of CP65 in contact-dependent cytotoxicity using the CNCD 147 trichomonad isolate and HeLa cell monolayers (Alvarez-Sánchez et al., 2000, 2007; Solano-González et al., 2006), we wanted to determine whether the reduced levels of cytotoxicity (50 ± 4.0%; $P < 0.05$) in DAB-treated parasites, as seen in Fig. 1A, are related with changes in the CP65 proteolytic activity. We first checked the total proteinase patterns of these parasites. Part B shows that no major changes were observed in the zymograms of total parasite lysates after DAB treatment (lane 2), as compared with untreated control parasites (lane 1), even in the regions where the CPs involved in cytotoxicity, CP65 and CP39, are located (arrowheads). These results are consistent with the previously reported (García et al., 2005).

We then performed a cell-binding assay between fixed HeLa cells and detergent extracts of control and DAB-treated trichomonads followed by substrate gel electrophoresis to analyze the proteolytic activity of the CP65

proteinase bound to HeLa cells. Part C shows a dramatic reduction in the cell-bound CP65 proteolytic activity after DAB-treatment (lane 2) as compared with control parasites (lane 1). The overall reduction in gelatin degradation was ~64% as determined by densitometric scanning of the zymograms when control protease activity was normalized to 100% (part D). These results suggest a direct link between polyamine depletion and the CP65-dependent cytotoxicity. Interestingly, the cell-bound CP39 and CP30 proteinases involved in cytotoxicity (Hernández-Gutiérrez et al., 2003, 2004) and cytoadherence (Mendoza-López et al., 2000), respectively, were also dramatically reduced after DAB treatment, as compared with untreated parasites (part C). However, since we have just shown that CP65 plays a major role in trichomonal cellular damage (Alvarez-Sánchez et al., 2007; Solano-González et al., 2006), we have focused this study in the effect of polyamine-depletion over the CP65 expression.

3.3. Exogenous putrescine restored cytotoxicity and rescued the CP65-proteolytic activity

Next, we wanted to determine whether the reduced levels of cytotoxicity in DAB-treated trichomonads (Fig. 2A, bar 2, $P < 0.05$) could be recovered to control levels (bar 1) by the presence of exogenous putrescine into the culture medium. Indeed, 15-min incubation of DAB-treated organisms transferred to medium containing 40 mM putrescine (bar 3, $P < 0.05$) restored the level of cytotoxicity to that of control, untreated parasites (bar 1). Only a slight increase in the levels of cytotoxicity was achieved by additional 15-min incubation with putrescine (bar 4, $P < 0.05$). As expected, DAB-treated trichomonads transferred to regular medium for 30 min did not restore cytotoxicity (bar 5) to control levels (bar 1). In addition, the levels of cytotoxicity of control untreated parasites transferred to medium containing putrescine for 30 min showed no significant changes (bar 6) as compared with the control level (bar 1).

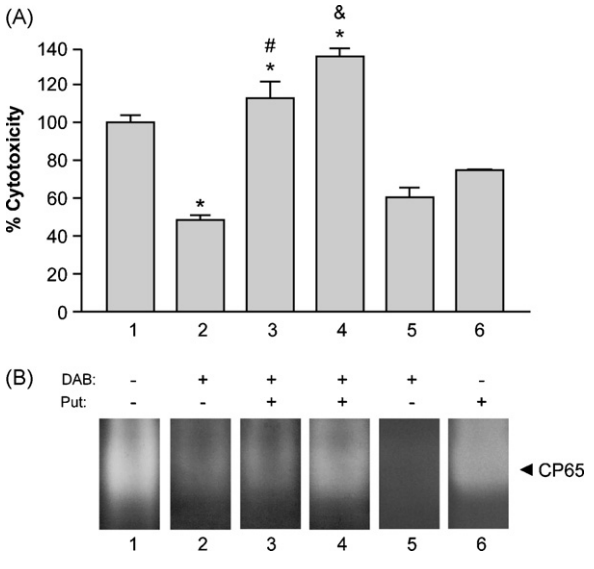

Fig. 2. Restoration of cytotoxicity and CP65 proteolytic activity rescue with exogenous putrescine. (A) Levels of cytotoxicity of control, untreated (lane 1) and DAB-treated (lane 2) organisms. DAB-treated parasites transferred to medium supplemented with 40 mM putrescine (Put) and incubated for 15 (lane 3) or 30 min (lane 4). DAB-treated parasites transferred to regular medium and analyzed after 30 min (lane 5). Control untreated parasites transferred to medium supplemented with 40 mM putrescine and incubated for 30 min (lane 6). Results were normalized to 100% for the control organisms, and bars represent the standard error of triplicate samples. Asterisk (*): $P < 0.05$ compared with controls and cells exposed to DAB. *#$P < 0.05$ recovery comparing DAB-treated parasites and DAB plus putrescine for 15 min, or *&$P < 0.05$ recovery after 30 min. (B) Zymograms to detect the CP65 proteolytic activity from parasites described in part (A). The arrowhead shows the position of the CP65 proteolytic activity. These experiments were performed at least three times with similar results.

When the proteolytic activity of the cell-bound CP65 proteinase from parasites used in part A was checked on the zymograms, a direct link between levels of cytotoxicity and extent of CP65 activity was observed. As compared to the control parasites with high CP65 proteolytic activity (part B, lane 1) and DAB-treated parasites with low CP65 activity (lane 2), DAB-treated parasites supplemented with exogenous putrescine showed partial recovery of the CP65 proteolytic activity after 15 min (lane 3) and almost complete recovery after 30 min (lane 4) incubation. As expected, DAB-treated parasites suspended in regular medium did not recuperate the CP65 proteolytic activity (lane 5), while the proteolytic activity of control untreated parasites transferred to medium with putrescine was not affected (lane 6), as compared with the control activity (lane 1). These data strongly suggest the idea that putrescine synthesis could be related to the *tvcp65* gene expression.

3.4. Expression of tvcp65 is related to the presence of putrescine

We then examined the effect of putrescine depletion over expression of *tvcp65* (Fig. 3) and CP65 protein synthesis (Fig. 4) under these experimental conditions. Semi-quantitative RT-PCR and qRT-PCR were performed using primers designed for *tvcp65* (Solano-González et al., 2006). Western blot assays were carried out using total protein extracts and anti-CP65 or anti-α-tubulin antibodies. Fig. 3 shows no detectable PCR product for RNA obtained from the DAB-treated parasites (part A, lane 2) when compared to the 370-bp *tvcp65* RT-PCR product of the control, untreated organisms (lane 1). Duplicate samples of DAB-treated trichomonads transferred to medium containing exogenous putrescine and incubated for 30 min (lane 3) gave visible *tvcp65* amplicon. Consistent with results of Fig. 2, DAB-treated organisms transferred to regular medium did not produce any detectable *tvcp65* RT-PCR product (lane 4). The 112-bp *β-tubulin* RT-PCR products were used as internal controls. As observed in part B, the *β-tubulin* transcript is not affected by the distinct treatments. These data indicate that the effect of DAB and putrescine are specific. Part C shows the results of the qRT-PCR and illustrates that depletion of cellular polyamines by DAB significantly inhibited *tvcp65* expression by ~50 ± 6.0% ($P < 0.05$), which was associated with a decrease in the CP65 protein (Fig. 4, lane 2). DAB-treated parasites in the presence of exogenous putrescine not only prevented the decreased levels of CP65 mRNA (Fig. 3) but also restored CP65 protein to near normal (Fig. 4, lane 3). Interestingly,

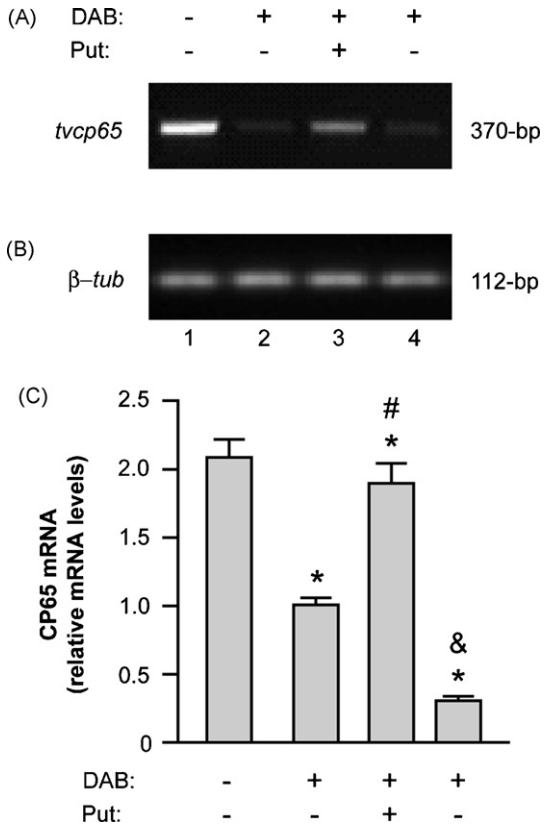

Fig. 3. Changes in expression of *tvcp65* after decreased or increased levels of cellular polyamines in trichomonads. (A) Semi-quantitative RT-PCR analysis to detect the *tvcp65* transcript separated on 2% agarose gel electrophoresis and ethidium bromide staining using total RNA from the same parasites described in Fig. 2; control, untreated (lane 1) and DAB-treated trichomonads (lane 2). DAB-treated parasites transferred to medium supplemented with 40 mM putrescine (Put) and incubated for 30 min (lane 3). DAB-treated parasites transferred to regular medium and analyzed after 30 min (lane 4). Arrowhead shows the 370-bp RT-PCR product for the *tvcp65* transcript. (B) RT-PCR analysis to detect the 112-bp *β-tubulin* transcript band (*β-tub*) from the same parasites described in part A as an internal control for quantity and specificity. Arrowhead shows the 112-bp RT-PCR product for the *β-tub* transcript. These experiments were performed at least three times with similar results. (C) qRT-PCR to quantify the differences in the level of *tvcp65* mRNA in trichomonads after inhibition of putrescine synthesis with DAB and after addition of exogenous putrescine to DAB-treated parasites, using total RNA from parasites described in part (A). Data were normalized to amount of β-tubulin (optical quantitative of TVCP65 mRNA/optical quantitative of β-tubulin mRNA). Values are means ± S.E. of data from three separate experiments. $^{*}P < 0.05$ comparing controls and cells exposed to DAB; $^{*\#}P < 0.05$ recovery comparing DAB-treated parasites and DAB plus putrescine for 30 min, or $^{*\&}P < 0.05$ absence of recovery of DAB-treated parasites transferred to normal medium without putrescine for 30 min.

an additional band was also observed in these parasites, which could correspond to the CP65 precursor (asterisk), and could explain the partial recovery of the CP65 proteolytic activity, by putrescine (Fig. 2B, lane 4). On the other hand, polyamine depletion did not alter expression of α- or β-tubulin used as controls. There were no significant differences in levels of β-tubulin mRNA (Fig. 3B) or α-tubulin protein (Fig. 4, bottom; α-TUB) between control cells and cells exposed to DAB alone or transferred to putrescine for 30 min. These results show that inhibition

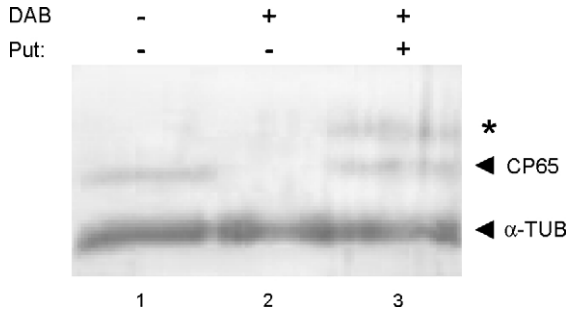

Fig. 4. DAB treatment reduced total amount of the CP65 protein. Western blot analysis on nitrocellulose membranes containing TCA-precipitated total trichomonad proteins of control untreated (lane 1), DAB-treated (lane 2) and DAB-treated and transferred to medium containing exogenous putrescine (Put) for 30 min (lane 3) of *T. vaginalis* organisms probed with: specific rabbit anti-CP65 serum (Alvarez-Sánchez et al., 2000) at 1:5000 dilution and control anti-α-tubulin antibody at 1:100 dilution as an internal control for quantity and specificity. Arrowheads show the position of the CP65 (CP65) and the α-tubulin (α-TUB) protein bands. The asterisk points to a higher sized band also recognized by the anti-CP65 antibody that could correspond to the CP65 precursor protein. These experiments were performed at least three times with identical results.

of putrescine synthesis is not affecting binding of CP65 to the surface of host cells, but CP65 protein levels. These data also indicate that decreased levels of cellular polyamines down-regulate expression of CP65 but not tubulin. Thus, these data reinforce the idea that putrescine synthesis is required for expression of the CP65 proteinase.

3.5. DAB treatment also decreased surface CP65 in *T. vaginalis*

Finally, to confirm that the extent of reduction on the CP65 proteolytic activity and amount of the CP65 transcript and protein affected expression of the surface CP65 proteinase, we performed indirect immunofluorescence assays using fixed and non-permeabilized parasites.

As expected, Fig. 5B shows reduction on the fluorescence intensity of the surface CP65 in DAB-treated trichomonads and partial recovery of fluorescence intensity in the presence of exogenous putrescine (panel C), when compared to the fluorescence of control untreated trichomonads (panel A). The presence of some fluorescence in DAB-treated parasites is consistent with the lower levels of the CP65-dependent cytotoxicity as shown in Fig. 1. The inset in panel B illustrates the extent of reduction on fluorescence intensity, ~80% in DAB-treated cells directly and only ~30% after addition of putrescine, as compared to control organisms in which the fluorescence intensity (*Y* axis) was normalized to 100%. No fluorescence was seen in trichomonads treated identically, but incubated with NRS as a negative control (panel D). Overall, these data strongly suggest that putrescine synthesis and/or polyamine depletion affect the amount and surface expression of CP65 due to the CP65 gene expression down-regulation by DAB, which resulted in a substantial reduction in CP65 protein levels such that the protein is undetectable in extracts and whole organisms of DAB-treated parasites, but is detected after addition of exogenous putrescine.

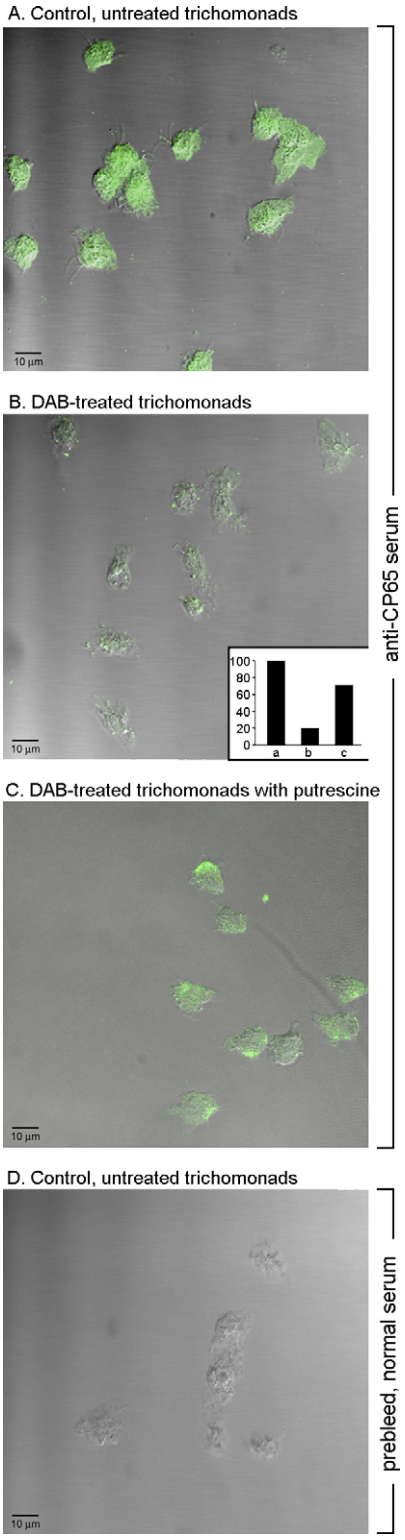

Fig. 5. DAB treatment reduces surface localization of CP65. (A–C) Immunofluorescence analysis of control untreated, DAB-treated, and DAB-treated organisms transferred to medium containing exogenous putrescine (Put) for 30 min, respectively. Parasites were fixed, non-permeabilized, and incubated with a polyclonal rabbit anti-CP65 serum (Alvarez-Sánchez et al., 2000) at 1:50 dilution followed by secondary anti-rabbit IgG conjugated with fluoresceine isothiocyanate (Jackson) at 1:80 dilution. (D) Immunofluorescence analysis of fixed, non-permeabilized control untreated parasites incubated with a preimmune, normal rabbit serum (NRS) at 1:50 dilution used as a negative control. The confocal microscopy images are the merge of bright field and the fluorescence channel in green. The inset in panel (B) shows the relative intensity of fluorescence in the *Y* axis between control, untreated trichomonads normalized to 100% (bar a) compared with decreased fluorescence of DAB-treated trichomonads (bar b), and DAB-treated and rescued by addition of exogenous putrescine for 30 min (bar c). These experiments were performed at least in three different occasions with identical results.

4. Discussion

Our studies extend the previous work on the polyamine metabolism of *T. vaginalis* particularly by showing that the synthesis of putrescine (Yarlett and Bacchi, 1994; Yarlett et al., 1996, 2000) is directly linked to the capacity of *T. vaginalis* to adhere and kill VECs. However, the increased levels of adherence did not result in new synthesis and/or changes in the amount of adhesins; while the mechanism to explain disappearance of contact-dependent cytotoxicity to VECs upon ornithine decarboxylase inhibition and thus putrescine synthesis (García et al., 2005) is still unknown. The implication of this finding was that, *in vivo*, adherence to VECs by *T. vaginalis* is not optimal, albeit there is cytolysis for nutrient acquisition (Peterson and Alderete, 1984a,b). Thus, synthesis and secretion of putrescine permits an intact polyamine metabolism in *T. vaginalis* due to an antiport uptake of host spermine, possibly through lysis of cells (Yarlett et al., 1996, 2000).

Accordingly, in this study we investigated the impact of putrescine synthesis (Yarlett and Bacchi, 1994; Yarlett et al., 1996, 2000) over expression of CP65 – at the transcript, protein and enzyme activity – and levels of cytotoxicity, using a different *T. vaginalis* isolate and HeLa cell monolayers as host cells. Our results, using cervical cells, show that in addition to decreased levels of cytotoxicity in DAB-treated parasites, a reduction in the cell-bound CP65 proteolytic activity occurred (Fig. 1); regardless of no apparent effect of ODC inhibition by DAB over the total trichomonad proteinase activity in parasites lysates. These results are consistent with the data previously reported (García et al., 2005). The reduced CP65 proteolytic activity partially restored by exogenous putrescine (Fig. 2), strongly suggest that either putrescine synthesis or the downstream pathways are important for CP65 expression and proteolytic activity. This work also shows that the polyamine metabolism affects levels of trichomonal host cytotoxicity by specifically regulating the amount of *tvcp65* transcript (Fig. 3) affecting the amount (Fig. 4), the proteolytic activity (Figs. 1 and 2) and the surface expression (Fig. 5) of the CP65 proteinase, as compared with the lack of effect of DAB treatment over the transcription and synthesis of the parasite tubulin (Fig. 3), and protein adhesins (García et al., 2005). However, these data do not rule out that additional molecules involved in cellular damage could also be modulated by the polyamine metabolism such as CP39 (Fig. 1) (Hernández-Gutiérrez et al., 2003, 2004) among others. Indeed, this possibility may explain the full recovery of cytotoxicity levels by exogenous putrescine (Fig. 2) with-

out complete synthesis and maturation of CP65 to control levels (Figs. 3 and 4).

To provide insights into the molecular basis for CP65 expression by polyamines, it is known that putrescine, spermine, and spermidine bind to nucleic acids and proteins affecting varied biological activities within cells. ODC and polyamines are essential for cell proliferation, cell death, and numerous cell responses such as modulation of signalling pathways and expression of specific genes (Bachrach et al., 2001; Childs et al., 2003; Pegg et al., 1995; Stefanelli et al., 2001, 2002; Thomas and Thomas, 2001), thus polyamines play a dramatic role in cellular functions. Therefore, it is not surprising that polyamine depletion is linked to inhibition of CP65 expression in *T. vaginalis* negatively affecting the ability of trichomonads for cellular damage.

A series of studies have demonstrated that polyamines are implicated in both transcription and post-transcription regulation of various genes encoding different cellular signalling proteins and adherent junction proteins, and that reduction in mRNA following polyamine depletion results predominantly from the inhibition of their gene transcription. For example, an increase in polyamines results in an increase in levels of c-*myc*, c-*jun*, E-cadherin, and toll-like receptor 2 (TLR2) mRNAs by stimulating mRNA synthesis without effect on their degradation, whereas polyamine depletion decreases c-*myc*, c-*jun*, E-cadherin, and TLR2 mRNAs by repressing their gene transcription but not by affecting their mRNA stability. In contrast, decreasing cellular polyamines increases levels of p53, TGF-β, nucleophosmin, and JunD through stabilization of their mRNAs without effect on their gene transcription (Chen et al., 2007; Patel and Wang, 1997; Rao et al., 2000; Wang, 2005, 2007).

Other studies also show that cellular polyamines are implicated in regulating expression of various intercellular junction proteins through distinct cellular signalling pathways in intestinal cells. Polyamine depletion decreases levels of tight junction proteins occluding, ZO-1, and ZO-2 without affecting their mRNAs but inhibits expression of both mRNAs and proteins of claudin-2 and claudin-3 (Wang, 2005).

Polyamines are able to influence gene expression not only through direct interaction with nucleic acids but also through protein modifications and apparent sequence-specific interaction with DNA (Childs et al., 2003). Although the exact mechanism by which polyamines regulate expression of CP65 in *T. vaginalis* remains unknown, the current study suggests that this stimulatory effect of polyamines on CP65 expression appears to occur at the transcript level as it does with the TLR2 expression in intestinal cells (Chen et al., 2007) and not at the proteolytic activity level as reported for some enzymes (Childs et al., 2003). Our data show that a decrease on the proteolytic activity of CP65 was a direct effect of polyamines on the amount of the CP65 transcript and protein, but not by inhibiting the CP65 enzyme activity. Reduction on CP65 mRNA level could be due to the alteration in its transcription rate or at the post-transcriptional level by modulating the *tvcp65* mRNA stability, or even can be an indirect effect by altering the expression of co-regulators of these proteins.

It is noteworthy to mention that we could not rule out that a post-transcriptional gene regulation mediated by polyamines may also be present in *T. vaginalis* based on our unpublished findings of regulatory sequences ERES-like in some mRNAs and the presence of genes encoding proteins required for a polyamine mediated eIF5A hypusination, an RNA-binding protein, which is one of the factors involved in mRNA stability mediated by polyamines (Childs et al., 2003). Thus, polyamine depletion negatively regulates the CP65-dependent cytotoxicity probably by altering the *tvcp65* transcript stability, as reported for the COX-2 expression in cancer cells (Parker and Gerner, 2002). Work is in progress to test whether polyamine depletion could affect the *tvcp65* transcript stability or its transcription rate.

The molecular process by which polyamine depletion of *T. vaginalis* exposed to DAB results in significant reduction in the level of *tvcp65* transcript remains to be elucidated. Several studies have suggested that changes in chromatin structure by polyamines may be correlated with levels of transcription of c-*myc* and other genes (Childs et al., 2003; Patel and Wang, 1997; Rao et al., 2000; Wang, 2005, 2007). Also polyamines have been shown to alter sequence-specific DNA-protein binding activities, affecting the regulation of initiation, elongation and termination during transcription. These could alter the binding or release of transcriptional regulatory factors by changing the intracellular ionic environment. Polyamines at physiological concentrations specifically enhance the binding of several proteins (USF, TFE3, Ig/EBP, YY1, etc.) to DNA, but inhibit others (Oct-1). Polyamines facilitate formation of complexes involving binding of more than one protein on a DNA fragment, but do not influence DNA–protein contact (Patel and Wang, 1997; Childs et al., 2003; Wang, 2007). Clearly, further studies are needed to define the molecular mechanism by which polyamines regulate expression of the CP65 gene in trichomonads. It would not be surprising, if shutdown of putrescine synthesis, with subsequent depletion of spermine and spermidine in trichomonads, is also affecting expression of other genes in addition to *tvcp65*. Our work at this time does not establish whether a particular polyamine or a combination of polyamines is affecting expression of CP65.

Interestingly, CP65 gene regulation by polyamines and its involvement in cytopathogenicity provides a link between iron and polyamine metabolism for regulation of trichomonal virulence during infection. Decreased levels of CP65 in polyamine-deficient trichomonads were associated with a significant reduction in levels of cytotoxicity over HeLa cell monolayers (Fig. 1). These findings are consistent with our recent observations (Alvarez-Sánchez et al., 2007), which demonstrated that iron also down-regulates expression of CP65 directly affecting the levels of trichomonal cytotoxicity. The implication of this finding is that trichomonads during infection are able to respond to both environmental signals to regulate levels of trichomonal cytotoxicity accordingly, giving the ability to the parasite to destroy cells for nutrient acquisition such as lipids, iron and spermine. Thus, our findings suggest that iron and polyamines function as the biological regulators for CP65 expression and are crucial for the maintenance of the chronic infection by *T. vaginalis*.

In conclusion, this work establishes yet another mechanism at work in *T. vaginalis* for control of gene expression, in addition to iron regulation (Tsai et al., 2002; Solano-González et al., 2007) and contact (Arroyo et al., 1993; García et al., 2003) for some virulence factors adhesins and proteinases (Alvarez-Sánchez et al., 2000, 2007; Hernández-Gutiérrez et al., 2003; Lehker et al., 1991; Arroyo et al., 1992; Moreno-Brito et al., 2005) among others. Undoubtedly, trichomonad intra- and extra-cellular polyamine levels are dependent on the multiple enzyme pathways that control polyamine synthesis, particularly in the back-conversion of spermine to spermidine in *T. vaginalis* (Yarlett et al., 2000). This illustrates once again the highly complex and orchestrated mechanisms that *T. vaginalis* uses to survive in the constantly changing host urogenital environment.

Acknowledgements

This work was supported by grants 33044-M, and 43763-Q from CONACYT Mexico (RA), by grant 060231 from CONACYT Mexico (M.E.A.S.), by a grant from the San Antonio Area Foundation and by Public Health Service grants AI 43940 and AI 45429 from the National Institutes of Health USA (JFA). Drs. Arroyo and Alderete would like to thank their respective members of their laboratories for their assistance and discussion throughout this work. We are grateful with Mr. Jaime Ortega-Arroyo for proofreading the manuscript and with Dr. Selene Zarate for her help with the statistical analysis. QFB Leticia Avila-González is acknowledged for her technical assistance. The excellent art work of Alfredo Padilla-Barberi is appreciated. Biol. Ivan Galván from the Microscopy Facility at CINVESTAV-IPN and M.Sc. Eduardo Carrillo from the Confocal Microscopy Facility at UACM are also acknowledged for their technical support with the confocal microscopy observations.

References

Alderete JF, Pearlman E. Pathogenic *Trichomonas vaginalis* cytotoxicity to cell culture monolayers. British Journal of Venereal Diseases 1984;60:99–105.

Alvarez-Sánchez ME, Avila-González L, Becerril-García C, Fattel-Facenda LV, Ortega-López J, Arroyo R. A novel cysteine proteinase (CP65) of *Trichomonas vaginalis* involved in cytotoxicity. Microbial Pathogenesis 2000;28:193–202.

Alvarez-Sánchez ME, Solano-González E, Yañez-Gómez C, Arroyo R. Negative iron regulation of the CP65 cysteine proteinase cytotoxicity in *Trichomonas vaginalis*. Microbes and Infection 2007;9:1597–605.

Arroyo R, Alderete JF. *Trichomonas vaginalis* surface proteinase activity is necessary for parasite adherence to epithelial cells. Infection and Immunity 1989;57:2991–7.

Arroyo R, Alderete JF. Two *Trichomonas vaginalis* surface proteinases bind to host epithelial cells and are related to levels of cytoadherence and cytotoxicity. Archives of Medical Research 1995;26:279–85.

Arroyo R, Engbring J, Alderete JF. Molecular basis of host epithelial cell recognition by *Trichomonas vaginalis*. Molecular Microbiology 1992;6:853–62.

Arroyo R, Gonzalez-Robles A, Martinez-Palomo A, Alderete JF. Signalling of *Trichomonas vaginalis* for amoeboid transformation and adhesion synthesis follows cytoadherence. Molecular Microbiology 1993;7:299–309.

Bachrach U, Wang YC, Tabib A. Polyamines: new cues in cellular signal transduction. News for Physiological Science 2001;16:106–9.

Bremner AF, Coombs GH, North MJ. Antitrichomonal activity of alpha-difluoromethylornithine. Journal of Antimicrobial Chemotherapy 1987;20:405–11.

Chen J, Rao JN, Zou T, Liu L, Marasa BS, Xiao L, et al. Polyamines are required for expression of Toll-like receptor 2 modulating intestinal epithelial barrier integrity. American Journal of Physiology and Gastrointestinal Liver Physiology 2007;293:G568–72.

Childs AC, Mehta DJ, Gerner EW. Polyamine-dependent gene expression. Cellular and Molecular Life Science 2003;60:1394–406.

Cotch MF, Pastorek II JG, Nugent RP, Hillier SL, Gibbs RS, Martin DH, et al. *Trichomonas vaginalis* associated with low-birth weight and preterm delivery. The Vaginal Infections and Prematurity Study Group. Sexually Transmitted Diseases 1997;24:353–60.

El-Shazly AM, El-Naggar HM, Soliman M, El-Negeri M, El-Nemr HE, Handousa AE, et al. A study on *Trichomoniasis vaginalis* and female infertility. Journal of the Egyptian Society of Parasitology 2001;31:545–53.

García AF, Chang TH, Benchimol M, Klumpp DJ, Lehker MW, Alderete JF. Iron and contact with host cells induce expression of adhesins on surface of *Trichomonas vaginalis*. Molecular Microbiology 2003;47:1207–24.

García AF, Benchimol M, Alderete JF. *Trichomonas vaginalis* polyamines metabolism is linked to host cell adherence and cytotoxicity. Infection and Immunity 2005;73:2602–10.

Guenthner PC, Secor WE, Dezzutti CS. *Trichomonas vaginalis*-induced epithelial monolayer disruption and human immunodeficiency virus type 1 (HIV-1) replication: implications for the sexual transmission of HIV-1. Infection and Immunity 2005;73:4155–60.

Hernandez H, Sariego I, Garber G, Delgado R, Lopez O, Sarracent J. Monoclonal antibodies against a 62 kDa proteinase of *Trichomonas vaginalis* decrease parasites cytoadherence to epithelial cells and confer protection to mice. Parasite Immunology 2004;26:119–25.

Hernández-Gutiérrez R, Ortega-Lopez J, Arroyo R. A 39-kDa cysteine proteinase CP39 from *Trichomonas vaginalis*, which is negatively affected by iron, may be involved in trichomonal cytotoxicity. Journal of Eukaryotic Microbiology 2003;50(Suppl.):696–8.

Hernández-Gutiérrez R, Avila-González L, Ortega-López J, Cruz-Talonia F, Gómez-Gutierrez G, Arroyo R. *Trichomonas vaginalis*: characterization of a 39-kDa cysteine proteinase found in patient vaginal secretions. Experimental Parasitology 2004;107:125–35.

Krieger JN, Ravdin JI, Rein MF. Contact-dependent cytopathogenic mechanisms of *Trichomonas vaginalis*. Infection and Immunity 1985;50:778–86.

Kucknoor AS, Mundodi V, Alderete JF. *Trichomonas vaginalis* adherence mediates differential gene expression in human vaginal epithelial cells. Cellular Microbiology 2005a;7:887–97.

Kucknoor AS, Mundodi V, Alderete JF. Adherence to human vaginal epithelial cells signals for increased expression of *Trichomonas vaginalis* genes. Infection and Immunity 2005b;73:6472–8.

Lehker MW, Sweeney D. Trichomonad invasion of the mucous layer requires adhesins, mucinases, and motility. Sexually Transmitted Infections 1999;75:231–8.

Lehker MW, Arroyo R, Alderete JF. The regulation by iron of the synthesis of adhesins and cytoadherence levels in the protozoan *Trichomonas vaginalis*. Journal of Experimental Medicine 1991;174:311–8.

León-Sicairos CL, León-Félix J, Arroyo R. *tvcp12*: a novel *Trichomonas vaginalis* cathepsin L-like cysteine proteinase-encoding gene. Microbiology 2004;150:1131–8.

Mason PR, Fiori PL, Cappuccinelli P, Rappelli P, Gregson S. Seroepidemiology of *Trichomonas vaginalis* in rural women in Zimbabwe and patterns of association with HIV infection. Epidemiology and Infection 2005;133:315–23.

Mendoza-López MR, Becerril-Garcia C, Fattel-Facenda LV, Avila-Gonzalez L, Ruíz-Tachiquín ME, Ortega-Lopez J, et al. CP30, a cysteine proteinase involved in *Trichomonas vaginalis* cytoadherence. Infection and Immunity 2000;68:4907–12.

Moodley P, Wilkinson D, Connolly C, Moodley J, Sturm AW. *Trichomonas vaginalis* is associated with pelvic inflammatory disease in women infected with human immunodeficiency virus. Clinical Infectious Diseases 2002;34:519–22.

Moreno-Brito V, Yáñez-Gómez C, Meza-Cervantez P, Avila-González L, Rodríguez MA, Ortega-López J, et al. A *Trichomonas vaginalis* 120 kDa protein with identity to hydrogenosome pyruvate:ferredoxin oxidoreductase is a surface adhesin induced by iron. Cellular Microbiology 2005;7:245–58.

Parker TM, Gerner EW. Polyamine-mediated post-transcriptional regulation of COX-2. Biochimie 2002;84:815–9.

Patel AR, Wang JY. Polyamines modulate transcription but not post-transcription of c-*myc* and c-*jun* in IEC-6 cells. American Journal of Physiology and Cellular Physiology 1997;273:C1020–9.

Pegg AE, Shantz LM, Coleman CS. Ornithine decarboxylase as a target for chemoprevention. Journal of Cellular Biochemistry 1995;22:132–8.

Peterson KM, Alderete JF. *Trichomonas vaginalis* is dependent on uptake and degradation of human low density lipoproteins. Journal of Experimental Medicine 1984a;160:1261–72.

Peterson KM, Alderete JF. Selective acquisition of plasma proteins by *Trichomonas vaginalis* and human lipoproteins as a growth requirement for this species. Molecular Biochemical Parasitology 1984b;12:37–48.

Rao JN, Li L, Bass BL, Wang JY. Expression of the TGF-beta receptor gene and sensitivity to growth inhibition following polyamine depletion. American Journal of Physiology and Cellular Physiology 2000;279:1034–44.

Reis AI, Martínez MP, Yarlett N, Johnson PJ, Silva-Filho FC, Vannier-Santos MA. Inhibition of polyamine synthesis arrests trichomonad growth and induces destruction of hydrogenosomes. Antimicrobial Agents Chemotherapy 1999;43:1919–23.

Rughooputh S, Greenwell P. *Trichomonas vaginalis*: paradigm of a successful sexually transmitted organism. British Journal of Biomedical Science 2005;62:193–200.

Solano-González E, Alvarez-Sánchez ME, Avila-González L, Rodriguéz-Vargas VH, Arroyo R, Ortega-Lopez J. Location of the cell-binding domain of CP65, a 65 kDa cysteine proteinase involved in *Trichomonas vaginalis* cytotoxicity. International Journal of Biochemistry and Cell Biology 2006;38:2114–27.

Solano-González E, Burrola-Barraza E, León-Sicairos CR, Avila-Gonzalez L, Gutiérrez-Escolano L, Ortega-López J, et al. The trichomonad cysteine proteinase TVCP4 transcript contains an iron-responsive element. FEBS Letters 2007;581:2919–28.

Sorvillo F, Kovacs A, Kerndt P, Stek A, Muderspach L, Sanchez-Keeland L. Risk factors for trichomoniasis among women with human immunodeficiency virus (HIV) infection at a public clinic in Los Angeles County, California: implications for HIV prevention. American Journal of Tropical Medicine and Hygiene 1998;58:495–500.

Stefanelli C, Pignatti C, Tantini B, Fattori M, Stanic I, Mackintosh CA, et al. Effect of polyamine depletion on caspase activation: a study with spermine synthase-deficient cells. Biochemical Journal 2001;355:199–206.

Stefanelli C, Tantini B, Fattori M, Stanic' I, Pignatti C, Clo C, et al. Caspase activation in etoposide-treated fibroblasts is correlated to ERK phosphorylation and both events are blocked by polyamine depletion. FEBS Letters 2002;527:223–8.

Sutcliffe S, Giovannucci E, Alderete JF, Chang TH, Gaydos CA, Zenilman JM, et al. Plasma antibodies against *Trichomonas vaginalis* and subsequent risk of prostate cancer. Cancer Epidemiological Biomarkers Prevention 2006;15:939–45.

Thomas T, Thomas TJ. Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. Cellular and Molecular Life Sciences 2001;58:244–58.

Tsai CD, Liu HW, Tai JH. Characterization of an iron-responsive promoter in the protozoan pathogen *Trichomonas vaginalis*. Journal of Biological Chemistry 2002;277:5153–62.

Van Der Pol B, Williams JA, Orr DP, Batteiger BE, Fortenberry JD. Prevalence, incidence, natural history, and response to treatment of *Trichomonas vaginalis* infection among adolescent women. Journal of Infectious Diseases 2005;192:2039–44.

Viikki M, Pukkala E, Nieminen P, Hakama M. Gynaecological infections as risk determinants of subsequent cervical neoplasia. Acta Oncologica 2000;39:71–5.

Wallace HM, Fraser AV, Hughes A. A perspective of polyamine metabolism. Biochemical Journal 2003;376:1–14.

Wang JY. Polyamines regulate expression of E-cadherin and play an important role in control of intestinal epithelial barrier function. Inflamopharmacology 2005;13:91–101.

Wang JY. Polyamines and mRNA stability in regulation of intestinal mucosal growth. Amino Acid 2007;33:241–52.

Yarlett N, Bacchi CJ. Effect of DL-alpha-difluoromethylornithine on polyamine synthesis and interconversion in *Trichomonas vaginalis* grown in a semi-defined medium. Molecular Biochemistry Parasitology 1988;31:1–9.

Yarlett N, Bacchi CJ. Parasite polyamine metabolism targets for chemotherapy. Biochemical Society Transactions 1994;22:875–9.

Yarlett N, Martínez MP, Moharrami MA, Tachezy J. The contribution of arginine dihydrolase pathway to energy metabolism by *Trichomonas vaginalis*. Molecular Biochemical Parasitology 1996;78:117–25.

Yarlett N, Martinez MP, Goldberg B, Kramer DL, Porter CW. Dependence of *Trichomonas vaginalis* upon polyamine backconversion. Microbiology 2000;146:2715–22.