Placenta 35 (2014) 1035–1042

Contents lists available at ScienceDirect

Placenta

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

Trypanosoma cruzi induces trophoblast differentiation: A potential local antiparasitic mechanism of the human placenta?

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ARTICLE INFO

Article history:
Accepted 24 September 2014

Keywords:
Trypanosoma cruzi
Human placenta
Trophoblast differentiation

ABSTRACT

Introduction: The congenital transmission of *Trypanosoma cruzi* (*T. cruzi*) is responsible for one-third of new Chagas disease cases each year. During congenital transmission, the parasite breaks down the placental barrier formed by the trophoblast, basal laminae and villous stroma. The observation that only 5% of infected mothers transmit the parasite to the fetus implies that the placenta may impair parasite transmission. The trophoblast undergoes continuous epithelial turnover, which is considered part of innate immunity. Therefore, we propose that *T. cruzi* induces differentiation in the trophoblast as part of a local antiparasitic mechanism of the placenta.
Methods: We analyzed β-human chorionic gonadotropin (β-hCG) and syncytin protein expression in HPCVE and BeWo cells using immunofluorescence and western blotting. Additionally, β-hCG secretion into the culture medium was measured by ELISA. We assessed the differentiation of trophoblastic cells in BeWo cells using the two-color fusion assay and by determining desmoplakin re-distribution.
Results: T. cruzi trypomastigotes induce β-hCG secretion and protein expression as well as syncyntin protein expression in HPCVE and BeWo cells. Addionally, the parasite induces the trophoblast fusion of BeWo cells.
Discussion: T. cruzi induces differentiation of the trophoblast, which may contribute to increase the trophoblast turnover. The turnover could be a component of local antiparasitic mechanisms in the human placenta.

1. Introduction

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*). This disease is endemic in Latin America, where ten million people are estimated to be infected [1,2]. In recent decades, Chagas disease has been increasingly detected in other non-endemic countries and continents, such as Canada, the United States of America, Australia and Japan, and in Europe. The global cost of Chagas disease is approximately $7 billion per year and $189 billion per lifetime. The economic burden of Chagas disease is similar to or exceeds that of other prominent diseases globally, such as rotavirus infection or cervical cancer [3].

Congenital *T. cruzi* infection is associated with premature labor, low birth weight, and stillbirth [4–6]. The serologic prevalence among pregnant women may reach 80%; however, the global congenital infection rate is estimated to be less than 5% [7]. WHO/PAHO has determined that approximately 1,809,000 woman of fertile age are infected and that 14,400 neonates are infected each year [8].

During congenital transmission, the parasite reaches the fetus by crossing the placental barrier [6,9,10]. The fact that only a percentage of infected mothers transmit the parasites to their fetuses implies that the placenta may impair parasite transmission. Congenital Chagas disease has been proposed to be a product of a complex interaction among the parasite, maternal and fetus/newborn immune responses and placental factors [6,11], with the placenta being the least studied component of this “trilogy”.

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http://dx.doi.org/10.1016/j.placenta.2014.09.017
0143-4004/

During congenital infection, the first fetal cells exposed to the parasite are those of the villous trophoblast, which is composed of two cellular layers: the superficial syncytiotrophoblast (STB) and the basal proliferative cytotrophoblast (CTB). The trophoblast is classified as renewing epithelia, which constitutes a physical barrier to pathogens [12]. The turnover of epithelia, particularly in the trophoblast, implies a precise orchestration of various cellular processes including CTB cell proliferation, differentiation (meaning syncytial fusion by incorporating CTB cells into a non-replicative STB) and cell death [12–14]. Epithelial maintenance is essential for adequate placental functioning because the trophoblast is involved in the exchange of gases, nutrients and waste products between the mother and the growing fetus and in the production of several pregnancy-associated hormones and growth factors [12,14].

Epithelial turnover is also considered part of the innate immune system [15] because prior to cell invasion, pathogens must attach to the surface of cells. As these cells are continuously eliminated, the attached pathogens are also removed. We have previously shown that *ex vivo* infection with a low concentration of parasites induces apoptosis in the trophoblast of human placental chorionic villi explants (HPCVE) [16].

Here, we propose that challenge with *T. cruzi* induces trophoblast differentiation, most likely as part of a local anti-parasitic response in the placenta. We analyzed the protein expression of β-human chorionic gonadotropin (β-hCG) and syncytin, major biochemical markers of trophoblast differentiation [12], in HPCVE and BeWo cells using immunofluorescence and western blotting. β-hCG secretion into the culture medium was also measured by ELISA. Additionally, we analyzed differentiation in BeWo cells by the two-color fusion assay and desmoplakin re-distribution. Our results clearly show that *T. cruzi* induces differentiation of the trophoblast.

2. Materials and methods

2.1. Cell cultures

VERO cells (ATCC® CCL-81) were grown in RPMI medium supplemented with 5% fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin) [17]. BeWo cells (ATCC CCL-98) were grown in DMEM-F12K medium supplemented with 10% FBS, L-glutamine and antibiotics (penicillin-streptomycin) [18]. Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2, with replacement of the culture medium every 24 h.

2.2. Production of the infective cellular form (trypomastigotes) of T. cruzi

After confluence, VERO cells were incubated with a culture of Ypsilon strain epimastigotes (non-infective cellular form of the parasite) in the late stationary phase, which increases the percentage of trypomastigotes to approximately 5% [19]. Trypomastigotes invade fibroblasts and replicate intracellularly as amastigotes. After 72 h, amastigotes transform back into trypomastigotes, which lyse the host cells. The parasites were recovered by low-speed centrifugation (500× *g*), which produces trypomastigotes in the supernatant and amastigotes in the sediment [17].

2.3. Infection of BeWo cells with T. cruzi trypomastigotes

BeWo cells were detached by trypsinization, sedimented and resuspended in 10% FBS-containing medium. Next, 2×10^5 cells were seeded in 6-well plates. The cells were allowed to adhere to the bottom for 3 h and then challenged with the parasite at a BeWo cell:parasite ratio of 1:1 or 1:0.1 or with 50 μM forskolin as a positive control [18] (data not shown). The cells were analyzed at 48 h post-infection. Parasites were identified by their different nuclear size and morphology.

2.4. HPCVE culture and infection with T. cruzi trypomastigotes

Ten human term placentas were obtained from uncomplicated pregnancies from vaginal or caesarean deliveries. Informed consent for the experimental use of the placenta was given by each patient as stipulated by the Code of Ethics of the Faculty of Medicine of the University of Chile. The exclusion criteria for the patients were the following: major fetal abnormalities, placental tumor, intrauterine infection, obstetric pathology, or any other maternal disease. The organs were collected in a cold sterile saline-buffered solution (PBS) and processed no more than 30 min after delivery. The maternal and fetal surfaces were discarded, and villous tissue was obtained from the central part of the cotyledons. The isolated chorionic villi were washed with PBS to remove blood, dissected into approximately 0.5-cm^3 fragments and co-cultured with *T. cruzi* trypomastigotes (1×10^5/ml and 1×10^6/ml) for 48 h in 1 ml of RPMI culture medium supplemented with inactivated FBS and antibiotics. *T. cruzi* infection was confirmed by parasite DNA detection using polymerase chain reaction as previously reported [20].

2.5. Immunofluorescence

HPCVE were fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h and then dehydrated in alcohol, clarified in xylene, embedded in paraffin, and sectioned at 5 μm. BeWo cells were fixed in cold 90% methanol. Standard immunofluorescence techniques [10] were utilized to show the distribution of different antigens in the tissue sections of HPCVE or BeWo cells. The cells or sections were incubated overnight at 4 °C with the following antibodies: syncytin (K-12), sc-30640 (Santa Cruz Biotechnology® [1:200 v/v]); desmoplakin, CBL173 (Millipore® [1:10 v/v]); or β-hCG, sc-51606 (Santa Cruz Biotechnology® [1:100 v/v]). The preparations were washed with PBS and incubated with fluorescein-conjugated anti-mouse IgG (ScyTek, ACA) in the presence of 1 μg/ml 4′6′-diamidino-2-phenylindole (DAPI). Then, the sections were mounted in Vectashield (ScyTek, ACA) and observed on an epifluorescence microscope (Motic BA310; Hong Kong, China).

At least ten fields were randomly selected for observation, and the signal intensity was scored as follows: +/, patchy; +, weak; ++, moderate; and +++, high [21].

2.6. Western blotting

HPCVE were homogenized in a lysis buffer (10 mM Tris pH 8.0, 1% w/v SDS and protease inhibitor cocktail [Complete Mini, Roche®]) at 4 °C using a Potter-Elvejem homogenizer. BeWo cells were incubated in RIPA buffer (Tris 50 mM,Nacl 150 mM, SDS 0.1%, Na Deoxycholate 0.5%, Triton X100 1%, 1 mM PMSF and protease inhibitor cocktail [Complete Mini, Roche®]) and sonicated for 1 min followed by incubation on ice for 20 min. The HPCVE and BeWo cell lysates were centrifuged at 15,000× *g* for 20 min to remove debris. The protein concentrations were measured using the Bradford assay, with Sigma bovine serum albumin as the protein standard. Then, 30 μg of protein was separated on a 10% sodium dodecyl sulfate polyacrylamide gel, blotted onto a nitrocellulose membrane, and probed with monoclonal antibodies against syncytin (1:600 v/v) or β-hCG (1:400 v/v). To correct for loading, the membranes were stripped and re-probed with an anti-human GAPDH (sc-51905 1:500 v/v) antibody. Immunoreactive proteins were detected using enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham Biosciences UK, Ltd.). The films were scanned, and NIH-Image v1.6 (NIH, Bethesda, MD) was used for the densitometric analysis of the bands.

2.7. Detection of β-hCG in the culture medium

The concentrations of β-hCG in the cell culture supernatants were determined using the β-hCG ELISA Kit (DRG®βHCG ELISA (EIA1911)) according to the manufacturer's instructions. The optical density (OD) was read at 450 nm using a microplate reader (Varioskan® Flash). Data were normalized with respect to the values obtained in control conditions. Intra-assay coefficients of variation were less than 10% in experiments with BeWo cells and HPCVE, inter-assay coefficients were 10.67–15.61% for BeWo cells and 11.67%–15.16% for HPCVE.

2.8. Two-color fluorescence BeWo cell fusion assay

The cells were detached by trypsinization, sedimented, resuspended in 5 ml of serum-free DMEM F12 medium and split into two fractions for staining with two different dyes, CellTracker Green CMFDA or CellTracker Orange CMTMR (Molecular Probes®) at a final concentration of 5 μM. Staining was performed for 30 min at 37 °C in the dark. The stained cell suspensions were centrifuged and re-suspended in 10% FBS-containing medium and counted. A total of 10^5 orange- and 10^5 green-labeled cells were seeded in 6-well plates, allowed to attach to the bottom for 3 h and then incubated [22] with *T. cruzi* trypomastigotes. After 48 h, the samples were observed on a Motic BA310 epifluorescence microscope, and images were obtained with a digital Moticam 5 camera and analyzed with Fiji/Image J. For quantitation of fusion events, at least twenty pictures were taken randomly from each sample. The figures from the different pictures were summed up to calculate the fusion index, which was defined as the average percentage of nuclei in double-fluorescent cell [22].

2.9. Statistics

All experiments were performed in triplicate using three different placentas. Results are expressed as means ± S.D and experimental data were normalized respect to control values. The significance of differences was evaluated using ANOVA followed by Dunnett's posttest.

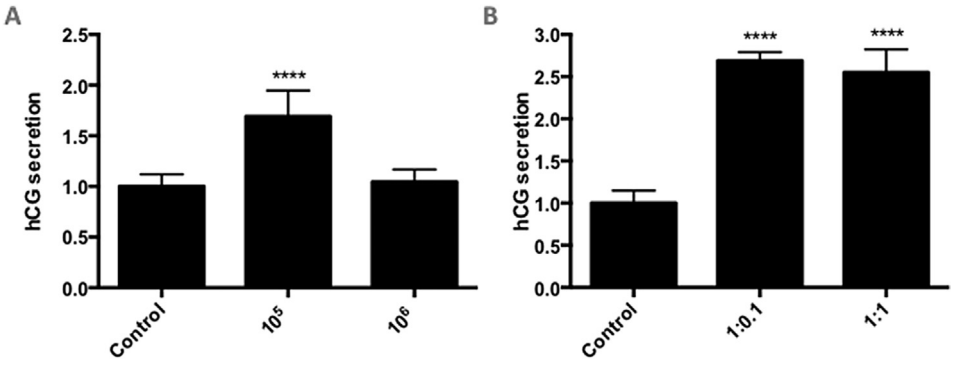

Fig. 1. *T. cruzi* trypomastigotes induce increased β–hCG secretion in HPCVE and BeWo cells: HPCVE were incubated with 10^5/ml or 10^6/ml *T. cruzi* Y strain trypomastigotes for 48 h (**A**). BeWo cells were incubated for 48 h with *T. cruzi* trypomastigotes at a cell:parasite ratio of 1:0.1 or 1:1 (**B**). β-hCG was measured by ELISA in the culture supernatant. Data were normalized with respect to control values and analyzed by ANOVA followed by Dunnett's post test. All values, given as the means ± S.D., correspond to at least 3 independent experiments performed in duplicate or triplicate. $^{****}p < 0.0001$.

3. Results

3.1. T. cruzi trypomastigotes induce increased β-hCG secretion in HPCVE and BeWo cells

The hormone β-hCG is synthesized by the trophoblast and is considered one of the major biochemical markers of trophoblast differentiation [12,23].

HPCVE were incubated with 10^5/ml or 10^6/ml *T. cruzi* Ypsilon (Y) strain trypomastigotes for 48 h. An inoculum of 10^5 trypomastigotes induced a significant increase in β-hCG in the culture medium (69.0 ± 2.6%; $p \leq 0.0001$, Fig 1A). A higher concentration of parasites (10^6/ml) did not induce a significant increase in the hormone (4.5 ± 1.2%, Fig 1A). BeWo cells were also incubated with *T. cruzi* trypomastigotes for 48 h at a cell:parasite ratio of 1:0.1 or 1:1. Both parasite concentrations induced a significant increase in β-hCG in the culture medium (268 ± 1.01% and 255 ± 2.73%; $p \leq 0.0001$, Fig. 1B).

3.2. Low concentrations of T. cruzi trypomastigotes induce increases in β-hCG protein expression in HPCVE and BeWo cells

HPCVE and BeWo cells were incubated with the parasite under the conditions described above. A low concentration of parasites produced a significant increase in β-hCG protein expression in HPCVE (248.9 ± 4.14%; $p \leq 0.0001$, Fig. 2A) and BeWo cells (225 ± 7.82%; $p \leq 0.05$, Fig. 3A), as determined by western blotting. In contrast, high concentrations of parasites do not alter β-hCG protein expression in HPCVE (Fig. 2A) and BeWo cells (Fig. 3A). However, a trend toward decreased hormone expression can be appreciated in both systems (Figs. 2A and 3A). The change in β-hCG protein expression in HPCVE and BeWo cells was confirmed by immunofluorescence. HPCVE trophoblasts challenged with a low parasite concentration showed strong (+++) immunoreactivity compared with control non-infected HPCVE (+), whereas a high concentration of parasites induced weak (+) immunoreactivity (Fig. 2B). Notably, a high

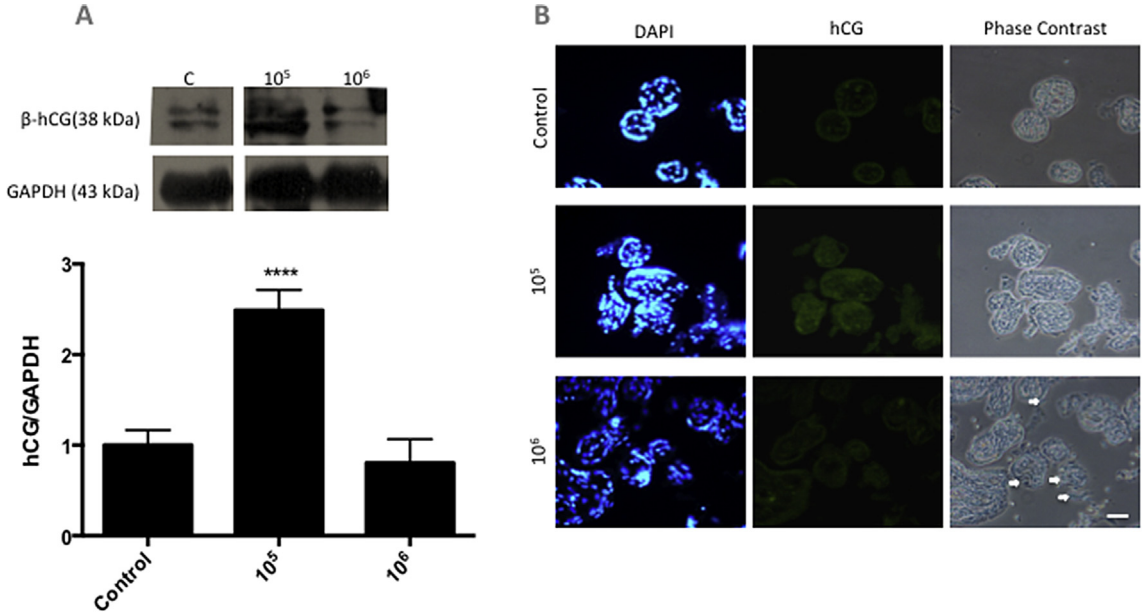

Fig. 2. Low concentrations of *T. cruzi* trypomastigotes induce increased β–hCG protein expression in HPCVE: HPCVE were incubated in the absence or presence of 10^5 or 10^6 Y strain trypomastigotes for 48 h. A (left panel), representative western blots for β-hCG and GAPDH from HPCVE infected with 10^5 or 10^6 *T. cruzi* trypomastigotes. The bar graph represents the ratios of β-hCG to GAPDH normalized with respect to the values obtained in control HPCVE under the different experimental conditions. All values are given as the means ± S.D. and correspond to at least 3 independent experiments performed in duplicate or triplicate. $^{****}p < 0.0001$. B, β-hCG immunoreactivity increases in HPCVE incubated with parasites. HPCVE challenged with 10^6 trypomastigotes show severe tissue damage as demonstrated by trophoblast detachment (arrows). Tissues were processed using routine immunohistochemistry methods. Bar scale: 25 μm.

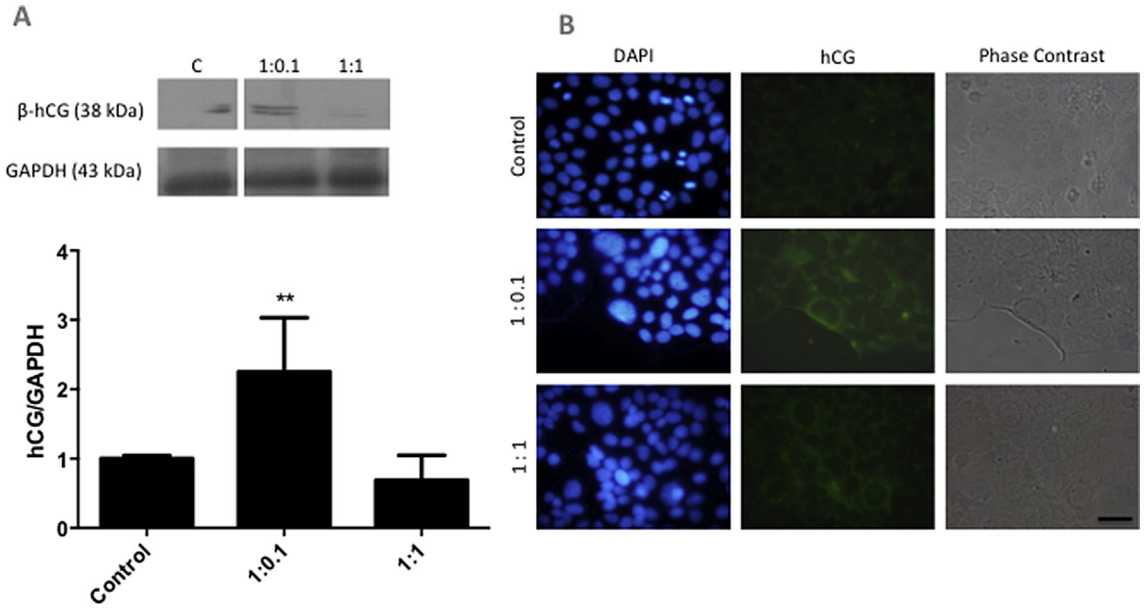

Fig. 3. Low concentrations of *T. cruzi* trypomastigotes induce increased β−hCG protein expression in BeWo cells: BeWo cells were incubated for 48 h with *T. cruzi* trypomastigotes at a cell:parasite ratio of 1:0.1 or 1:1. A (left panel), representative western blots for β-hCG and GAPDH from BeWo cells incubated at a cell:parasite ratio of 1:0.1 or 1:1. The bar graph represents the ratios of β-hCG to GAPDH normalized with respect to the values obtained in control BeWo cells under the different experimental conditions. All values are given as means ± S.D. and correspond to at least 3 independent experiments performed in duplicate or triplicate. $^{**}p < 0.01$. B, β-hCG immunoreactivity increases in BeWo cells incubated with parasites. The cells were processed using routine immunofluorescence methods. Bar scale: 10 μm.

concentration of trypomastigotes induced the destruction and detachment of the trophoblast, which has been described previously [10]. Similarly, BeWo cells showed an increase in β-hCG immunoreactivity (+++) when incubated with a low concentration of parasites and a similar immunoreactivity as control cells (+/−) when challenged with a high concentration of parasites (Fig. 3B).

3.3. *T. cruzi trypomastigotes induce increased syncytin protein expression in HPCVE and BeWo cells*

Syncytins are proteins encoded by the envelope genes of human endogenous retrovirus (HERV) and participate in the fusion of the villous trophoblast [12,24].

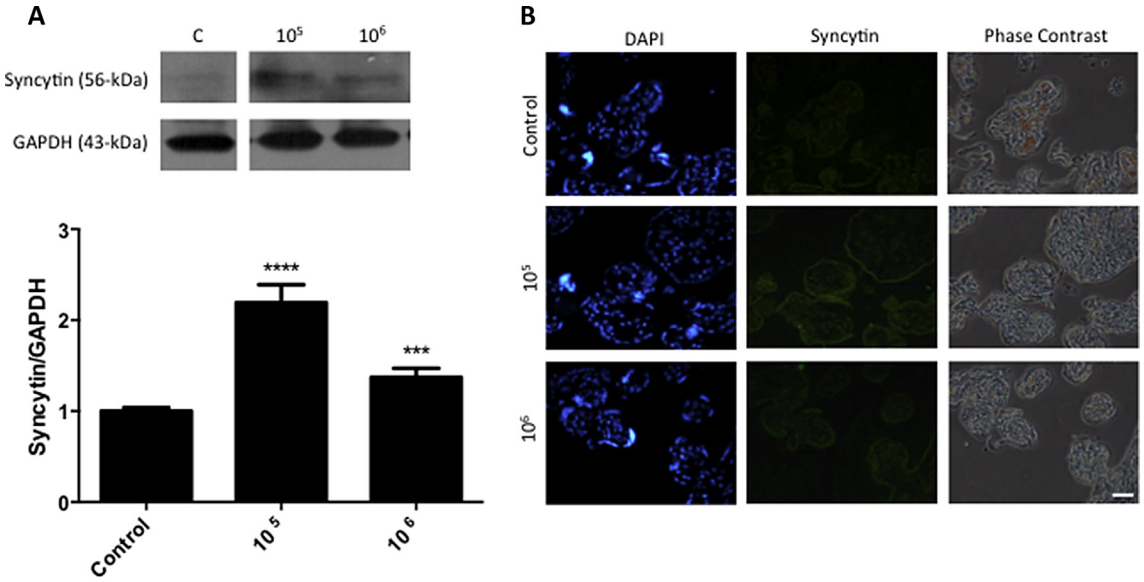

Fig. 4. *T. cruzi* trypomastigotes induce increased syncytin protein expression in HPCVE: HPCVE were incubated in the absence or presence of 10^5 or 10^6 Y strain trypomastigotes for 48 h. A (left panel), representative western blots for syncytin and GAPDH from HPCVE infected with 10^5 or 10^6 *T. cruzi* trypomastigotes. The bar graph represents the ratios of β-hCG to GAPDH normalized with respect to the values obtained in control HPCVE under the different experimental conditions. All values are given as the means ± S.D. and correspond to at least 3 independent experiments performed in duplicate or triplicate. $^{****}p < 0.0001$. B, β-hCG immunoreactivity increases in HPCVE incubated with parasites. The tissues were processed using routine immunofluorescence methods. Bar scale: 25 μm.

HPCVE and BeWo cells were incubated with the parasite under the conditions described above. Low concentrations of the parasite induced a significant increase in syncytin protein expression in HPCVE (219.4 ± 1.99%; $p \leq 0.0001$, Fig 4A) and BeWo cells (230.4 ± 1.53%; $p \leq 0.0001$, Fig 5A). High concentrations of the parasite also induced a significant, but lower, increase in syncytin protein expression in HPCVE (137.4 ± 1.02%; $p \leq 0.001$, Fig. 4A) and BeWo cells (153.6 ± 1.67%; $p \leq 0.0001$, Fig. 5A), as determined by western blotting. The change in syncytin protein expression in the HPCVE and BeWo cells was confirmed by immunofluorescence. HPCVE trophoblasts challenged with a low parasite concentration showed strong (+++) immunoreactivity compared with the control non-infected HPCVE (+), whereas a high concentration of parasites induced weak (+) immunoreactivity (Fig. 4B). BeWo cells also showed an increase in syncytin immunoreactivity (+++) when incubated with a low concentration of parasites and moderate immunoreactivity (++) when challenged with a high concentration of parasites (Fig. 5B). Interestingly, even infected BeWo cells showed strong immunoreactivity (Fig. 5B insets, white arrows indicate intracellular parasites).

3.4. *T. cruzi trypomastigotes induce the trophoblast fusion of BeWo cells*

BeWo cells were incubated with *T. cruzi* trypomastigotes for 48 h at a cell:parasite ratio of 1:0.1 or 1:1. A low concentration of parasites increased the percent fusion to 27.88 ± 5.30% ($p \leq 0.0001$, Fig 6) as determined by the two-color fusion assay [22]. A high concentration of trypomastigotes also increased the percent fusion (11.35 ± 4.51%; $p \leq 0.01$, Fig 6F). Additionally, we assessed trophoblast fusion by the morphological examination of intercellular junctions by determining the presence or absence of desmoplakin (Fig 7). Both low and high concentrations of trypomastigotes increased the percentage of fused BeWo cells to 21.65 ± 2.396% ($p \leq 0.0001$) and 18.62 ± 2.89% ($p \leq 0.01$), respectively (Fig. 7D). Notably, the intracellular replicative amastigote form of the parasite was observed in both fused and individual BeWo cells (Fig. 6A, D and E; Fig. 7A and C, white arrows indicate intracellular parasites, white circles indicate fused cells).

4. Discussion

The placenta generally forms a barrier against many pathogens. However, some bacteria, viruses and protozoa can be transmitted across the placenta [25].

The global congenital transmission rate of *T. cruzi* is low; in countries where the parasite is endemic, a higher rate of congenital transmission is present compared with countries where it is not endemic (5.0% versus 2.7%) [7]. Therefore, due to the low transmission rate, postulating that systemic and local antiparasitic factors, as shown in the present study, prevent the congenital transmission of *T. cruzi* is reasonable. Congenital transmission has been hypothesized to depend on the parasite characteristics, the maternal and fetus/newborn immune responses and placental factors [6,11].

T. cruzi demonstrates great biological, biochemical and genetic diversity; therefore, different strains of the parasite have been identified. However, no clear evidence exists that can establish a relationship between *T. cruzi* strains and congenital infection in humans [26,27].

A crucial factor in preventing, limiting, or permitting the fetal/neonatal infection is the capacity of the mother and fetus/newborn to mount innate and/or specific immune response(s) against pathogens [28]. The production of pro-inflammatory cytokines can be observed in uninfected babies born to infected mothers [11]. In contrast, the levels of inflammatory markers and activation of NK

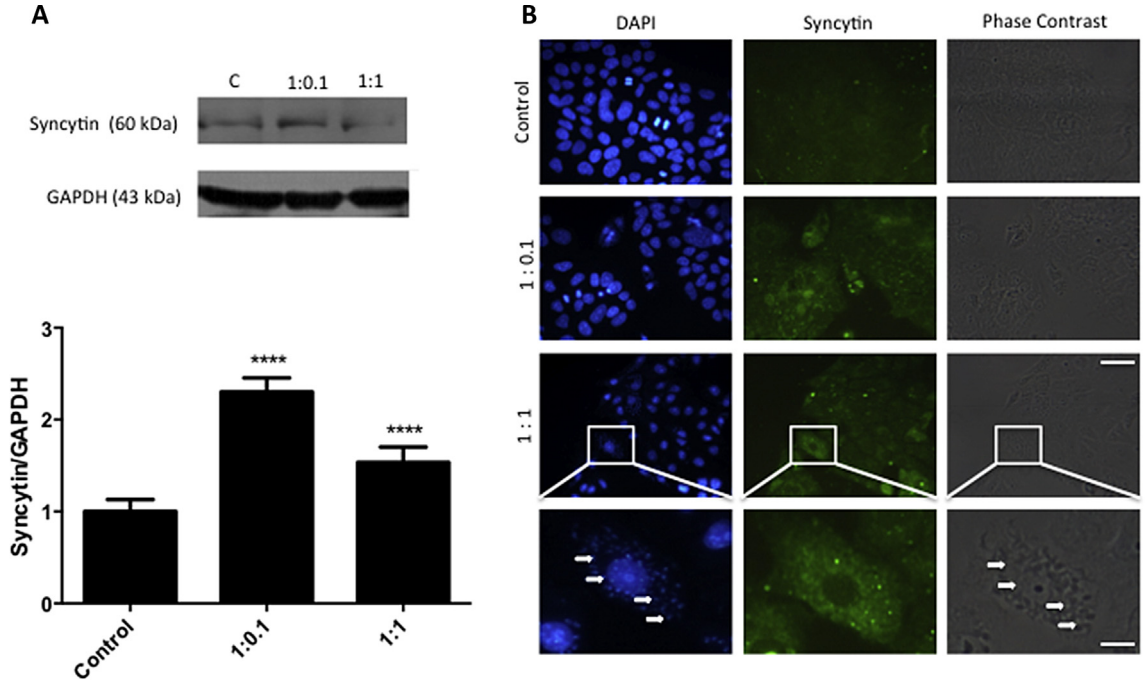

Fig. 5. *T. cruzi* trypomastigotes induce increased syncytin protein expression in BeWo cells: BeWo cells were incubated for 48 h with *T. cruzi* trypomastigotes at a cell:parasite ratio of 1:0.1 or 1:1. **A** (left panel), representative western blots for syncytin and GAPDH from BeWo cells incubated at a cell:parasite ratio of 1:0.1 or 1:1. The bar graph represents the ratios of syncytin to GAPDH normalized with respect to the values obtained in control BeWo cells under the different experimental conditions. All values are given as the means ± S.D. and correspond to at least 3 independent experiments performed in duplicate or triplicate. $^{****}p < 0.0001$. B, β-hCG immunoreactivity increases in BeWo cells incubated with parasites. The inset shows that even infected BeWo cells (white arrows) exhibit marked immunoreactivity. Intracellular parasites are particularly evident in the phase contrast image. The cells were processed using routine immunofluorescence methods. Bar scale: 25 μm; insets: 10 μm.

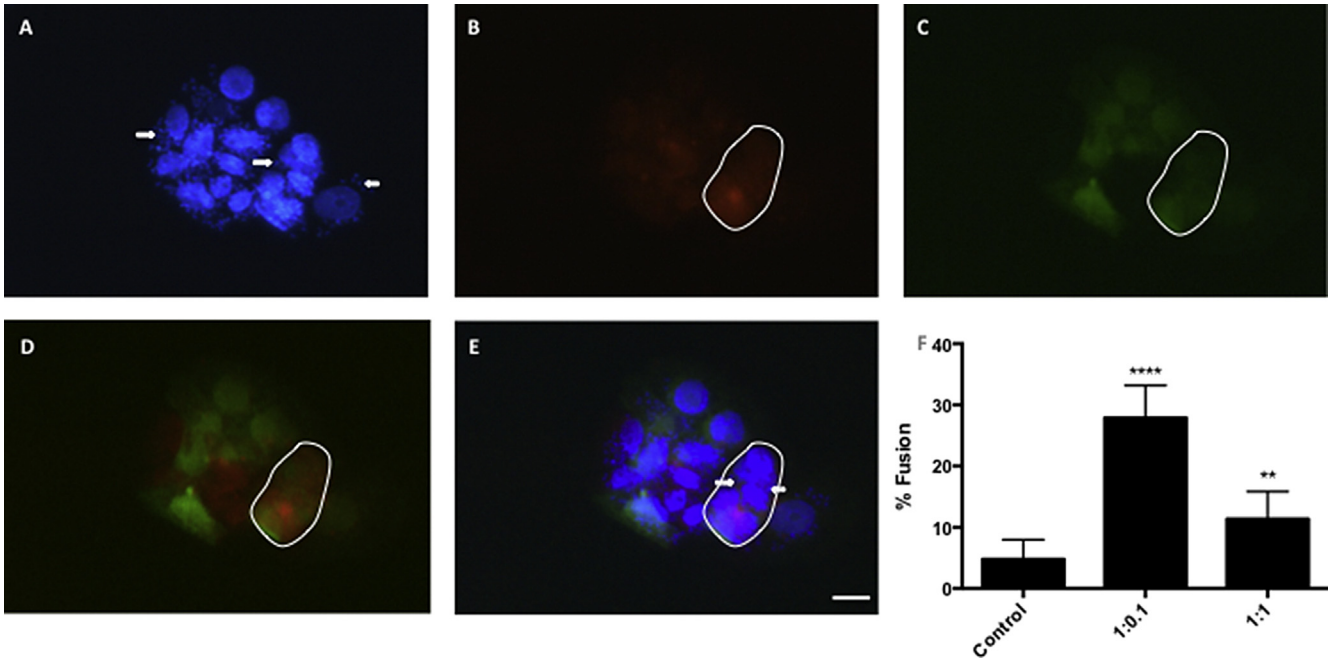

Fig. 6. *T. cruzi* trypomastigotes induce trophoblast fusion of BeWo cells: BeWo cells labeled with orange (B) or green (C) cell tracker were incubated with *T. cruzi* trypomastigotes at a cell:parasite ratio of 1:0.1 or 1:1 and incubated for 48 h. Nuclei were stained with DAPI (A, white arrows) to identify intracellular parasites. Cells were stained with orange cell tracker (B) or green cell tracker (C), (fused cells: white demarcation). D, digital superimposition of B and C images in which fused cells are evident (white demarcation). E, superimposition of A, B and C in which intracellular parasites (white arrows) are evident in fused cells (white demarcation). The cells were processed using routine fluorescence methods. Bar scale: 10 μm. The bar graph (F) represents the percentage of fused cells under the different experimental conditions. All values are given as the means ± S.D. and correspond to at least 3 independent experiments performed in duplicate or triplicate. $^{**}p < 0.01$; $^{****}p < 0.0001$.

cells are low in congenitally infected newborns [29]. These data strongly suggest a protective role for such innate defenses in uninfected newborns born to infected mothers.

The placenta is the least-studied among the possible antiparasitic mechanisms that impair the congenital transmission of *T. cruzi*. The trophoblast is the first tissue of the placental barrier to come in contact with circulating parasites from maternal blood [6,10,11], and as lining epithelia, this tissue forms an anatomical barrier and is part of the innate immune system [15,30]. There are three types of defense mechanisms in innate immunity: a) the above-mentioned anatomical barrier, the cells of which secrete various chemicals; b) cellular innate immune responses; and c) humoral innate immune responses. Once the invading pathogens breach the main component of the villous anatomical barrier, innate immune cells are activated and secrete cytokines and chemokines to control pathogen replication [30,31].

We have previously shown that *T. cruzi* induces parasite concentration-dependent tissue damage in HPCVE and that high concentrations of the parasite induce massive destruction and detachment of the trophoblast [10]. In contrast, low concentrations of the parasite induce minor tissue damage [10,32]. These histopathological changes can be related to the fact that high parasitemia, as in acute infection, and low parasitemia, as observed in chronically infected patients, are respectively associated with high (greater than 20%) and low (less than 2%) transmission rates [33,34]. Amastigote nests (the intracellular replicative form of the parasite) can only be visualized in the villous stroma after the trophoblast has been removed by trypsinization [35], which indicates the importance of the trophoblast as an anatomical barrier to pathogens.

Epithelial turnover is considered to be a protective mechanism against pathogens in other organs, such as the skin and the gastrointestinal and urogenital tracts [36,37]. There is a basal level of epithelial renewal, which can be accelerated or decreased in response to various stimuli. Infection usually accelerates epithelial turnover [37]. In the intestinal epithelium, the turnover is twice as fast in conventional mice as in germ-free mice [38]. Additionally, parasites such as nematodes have been shown to accelerate intestinal epithelial turnover [36]. In the urothelium, the basal level of tissue renewal is low (approximately 40 weeks in mice); however, during uropathogenic *Escherichia coli* infection, the turnover of these epithelia is rapidly accelerated and completed in 7 days [39].

The trophoblast is a unique bi-layered epithelium composed of proliferating CTB basal cells and a differentiated non-replicative superficial syncytium, the STB. The proliferation of CTB cells is followed by the differentiation of daughter cells that have exited the cell cycle. Differentiation is rapid and only requires 2–3 days; then, the syncytial fusion of highly differentiated CTB cells with the overlying STB occurs. Within the STB, further differentiation and maturation occur, leading to aging and late apoptosis of nuclei and other organelles at specific sites within the STB. This material is packed into protrusions of the apical membrane and extruded as membrane-enclosed apoptotic corpuscles (syncytial knots) into the maternal blood present in the intervillous space [40,41]. We previously showed that low concentrations of parasites induce apoptotic cell death in HPCVE, which is particularly increased in the trophoblast [16]. Therefore, the parasite, particularly at a low concentration as in chronic Chagas disease, likely induces epithelial turnover (meaning proliferation and differentiation) of the trophoblast. Studies of *T. cruzi*-induced proliferation in HPCVE and BeWo cells are in progress.

β-hCG is one of the main trophoblast differentiation markers; its secretion is augmented in differentiated trophoblast cells [42]. Challenge with a low concentration of parasites induces increased β-hCG secretion and protein expression in both HPCVE and BeWo cells. In contrast, high concentrations of parasites do not induce increased hormone secretion and expression in HPCVE (Figs. 1–3), only in BeWo cells an increase of the hormone can be detected in

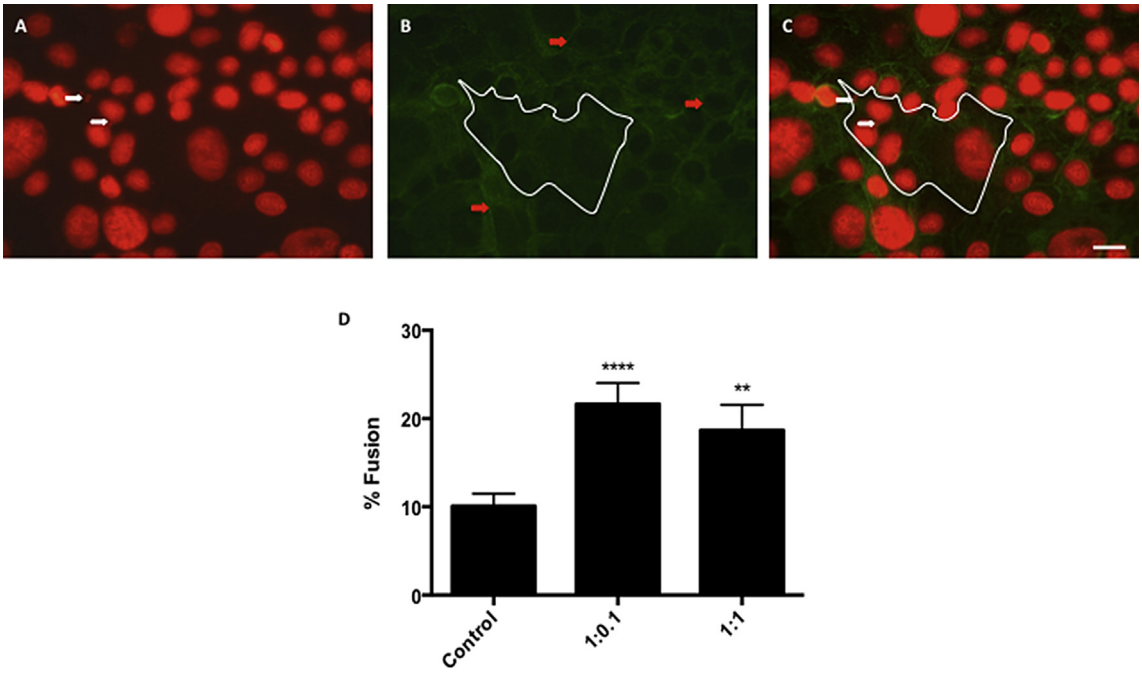

Fig. 7. *T. cruzi* trypomastigotes induce trophoblast fusion of BeWo cells: BeWo cells were incubated for 48 h with *T. cruzi* trypomastigotes at a cell:parasite ratio of 1:0.1 or 1:1. A, infected BeWo cells stained with propidium iodide, with the white arrows indicating nuclei with intracellular parasites. B, desmoplakin immunoreactivity (cell limits: red arrows; fused cells: white demarcation). C, merged images from A and B in which intracellular parasites are present in the cytoplasm of fused cells (parasite nuclei: white arrows; fused cells: white demarcation). The cells were processed using routine immunofluorescence methods. Bar scale: 10 μm. The bar graph (D) represents the percentage of fused cells under the different experimental conditions. All values are given as the means ± S.D. and correspond to at least 3 independent experiments performed in duplicate or triplicate. $^{**}p < 0.01$; $^{****}p < 0.0001$.

the culture media. The different response of HPCVE and BeWo cells could explained by the fact, that a challenge with high concentration of the parasite induce a severe tissue damage characterized by trophoblast destruction and detachment [10] and necrotic cell death [16]. Therefore the trophoblast in those HPCVE is significantly diminished and unable to express and secrete hCG. On the other hand, BeWo cells also present a decreased hCG protein expression, when incubated with the high parasite concentration. However, the damage induced by the trypomastigotes is less severe than in HPCVE and the cells are still able to respond, as is it evident in the syncyntin protein expression analysis (Fig. 5) and fusion assays (Figs. 6–7). Additionally, it should be considered that HPCVE and BeWo cells are two different experimental models. The trophoblast in the HPCVE is supported by the villous stroma; contrarily BeWo cells, as a cell line, are not in contact with other cells or tissue. Therefore, it is expectable that the response in both systems is not exactly the same.

Another trophoblast differentiation marker is syncytin, a human endogenous retrovirus (HERV) envelope gene. Both assayed parasite concentrations induced syncytin expression in HPCVE and BeWo cells, but low concentrations of the parasite were more effective in inducing this differentiation marker.

To further confirm trophoblast differentiation, we performed fusion assays in BeWo cells. The two-color fusion assay and analysis of desmoplakin re-distribution confirmed that the parasite induces trophoblast differentiation because the fusion index was increased. Similar to the observed increase in syncytin expression, challenge with a high concentration of parasites induced a lower fusion rate in BeWo cells. Notably, the intracellular replicative form of the parasite was observed in cells with marked syncytin immunoreactivity (Fig 5, insets) and in fused cells (white arrows in Figs. 6 and 7). The infective trypomastigotes expose on their cellular surface phosphatidylserine, which is implicated in the survival and multiplication of obligate intracelular protozoan by inducing the anti-inflammatory cytokine TGF-β [43]. On the other hand, TGF-β has been implicated in the inhibition as well as in the promotion of trophoblast differentiation. The induction of trophoblast differentiation has been related to MAPK signal transduction pathway activation [44]. We described previously, that ERK1/2 MAPK is regulated differentially, by low and high parasite concentration. 10^5 parasite activates the above mentioned signalling pathway and 10^6 parasites induce its inhibition [20]. This differential modulation of the MAPK signaling pathway could therefore be related to the here described results, which suggest that low concentration of *T. cruzi* induce trophoblast differentiation in contrast to high concentration, which induce a less significant differentiation. The latter might explain why high concentrations of the parasite overcome potential local defense mechanisms in the placenta.

Additionally, since the *T. cruzi* is present in fused cells (Figs. 6–7) and also exposes phosphatidylserine, the intracellular parasite may be a fusion-promoting signal per se.

The trophoblast expresses Toll-like receptors (TLRs), which recognize pathogens and an initiate an immune response. TLRs mediate the expression of defensins as well as several cytokines and chemokines [28]. Additionally, the TLRs link innate and adaptive immunity by up-regulating the ability of accessory cells to generate immune responses mediated by T and B lymphocytes [45]. *T. cruzi* is recognized by TLR-2, TLR-4 and TLR-9 [46], which are all expressed in the trophoblast. Therefore, changes in the expression of immune-modulating molecules may also contribute to potential antiparasitic factors in the placenta or facilitate transmission.

Conflict of interest

None.

Acknowledgments

This work was supported by "Fondo Nacional de Desarrollo Científico y Tecnológico" (FONDECYT) grants 1120230 (to UK), 1130189 (to JM) and 1130113 (to NG), Chile.

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