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Role of placental barrier integrity in infection by *Trypanosoma cruzi*

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ABSTRACT

American trypanosomiasis has long been a neglected disease endemic in LatinAmerica, but congenital transmission has now spread Chagas disease to cause a global health problem. As the early stages of the infection of placental tissue and the vertical transmission by *Trypanosoma cruzi* are still not well understood, it is important to investigate the relevance of the first structure of the placental barrier in chorionic villi infection by *T. cruzi* during the initial stage of the infection. Explants of human chorionic villi from healthy pregnant women at term were denuded of their syncytiotrophoblast and co-cultured for 3 h, 24 h and 96 h with 800,000 trypomastigotes (simulating acute infection). *T. cruzi* infected cells were identified by immunohistochemistry for cytokeratin-7 (+cytotrophoblast) and CD68 (+macrophages), and the infection was quantified. In placental tissue, the parasite load was analyzed by qPCR and microscopy, and the motile trypomastigotes were quantified in culture supernatant. In denuded chorionic villous, the total area occupied by the parasite (451.23 μm^2, 1.33%) and parasite load (RQ: 87) was significantly higher ($p < 0.05$) than in the entire villous (control) (5.98 μm^2, 0.016%) (RQ:1) and with smaller concentration of nitric oxide. Stromal non-macrophage cells were infected as well as cytotrophoblasts and some macrophages, but with significant differences being observed. The parasite quantity in the culture supernatant was significantly higher ($p < 0.05$) in denuded culture explants from 96 h of culture. Although the human complete chorionic villi limited the infection, the detachment of the first structure of the placenta barrier (syncytiotrophoblast) increased both the infection of the villous stroma and the living trypomastigotes in the culture supernatant. Therefore structural and functional alterations to chorionic villi placental barrier reduce placental defenses and may contribute to the vertical transmission of Chagas.

1. Introduction

The placenta connects the fetus with the mother, and all nutrients and waste products exchanged between them have to pass through the placental barrier. Moreover, *Plasmodium* sp., *Toxoplasma* sp., and some other unicellular parasites also employ this barrier to access the fetal blood (Souza et al., 2013; Robbins et al. 2012). The placental barrier is composed of the syncytiotrophoblast (STB) which is in contact with the maternal blood, the cytotrophoblast (CTB) (internal trophoblastic cells), and also the mesoderm and the fetal vascular system. Cytokine balance remains paramount during pregnancy, and an excess of inflammatory cytokines may jeopardize the pregnancy (Rusterholz et al., 2007). Moreover, the maternal immune system has to achieve conditions of immune tolerance to protect the fetus from immunological rejection, while also maintaining its anti-infectious capacity. Related to this, the placenta has the capacity to express both pro-inflammatory and anti-inflammatory cytokines in order to avoid fetus rejection while giving protection against infectious agents (Mor and Cardenas, 2010). Moreover, in this context of immunological tolerance, some pathogen agents that could be present in pregnant women are sometimes able to pass the placental barrier and infect the fetus (Mor and Cardenas, 2010; Zeldovich and Bakardjiev, 2012).

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Trypanosoma cruzi is an obligate intracellular parasite that can be transmitted from pregnant women to the fetuses, causing the congenital transmission of Chagas disease. American trypanosomiasis is a neglected disease that is endemic in LatinAmerica, but it has now spread outside this region to cause a global health problem (Schmunis and Yadon, 2010). The parasite is transmitted in different ways, with the most frequent being via a vectorial invertebrate triatomineo transmission, blood transfusion and vertical transplacental mother to fetus infection (Fretes and Kemmerling, 2012). The prevalence of Chagas in pregnant women in South America ranges from 4% to 52%, of whom only 0.1% to 5% transmit the infection to their fetuses (Carlier and Truyens, 2010; Howard et al., 2014) with Congenital transmission being considered to be one of the principal causes for spreading of the disease to non-endemic regions of the world (Carlier et al., 2015).

The most accepted via of vertical transmission is through the chorionic villi, which constitutes an area of 12 square meters of interaction between the mother's blood (where the *T. cruzi* may be present in chagasic pregnant women) and the syncytiotrophoblast. In addition, other ways of transmission have also been described, such as way the placental marginal sinus and the genital tract during the birth of the newborn (Schijman, 2007).

The infection of placental tissue and the vertical transmission of *T. cruzi* seem to depend on several factors, including the mother's parasitemia and the quantity of parasites in the placenta (Altemani et al., 2000; Bua et al., 2013), the effectiveness of the clearance of parasites in the intervillous space (Triquell et al., 2009) and the levels of nitric oxide (Díaz-Luján et al., 2012). Furthermore, the turnover of trophoblasts could be a local antiparasitic mechanism in the human placenta (Liempi et al., 2014).

Many reviews based mainly on microscopical studies of placental tissue have reported that *T. cruzi* multiply within mesodermal macrophages. In addition, a few cases of parasites in trophoblastic cells have also been observed (Bittencourt 1976; Moya et al., 1979; Bittencourt 1992; Moretti et al., 2005; Altemani et al., 2000). However, many of these descriptions only refer to placentas obtained at delivery when the process of the *T. cruzi* infection was ending, with the early stage of infection still not being fully understood.

Recently, by employing monolayers of isolated human placenta trophoblasts, it has been shown that the type of trophoblast cell in contact with the mother's blood may be another important aspect of placental infection (Díaz-Luján et al., 2012) with Liempi et al. (2014) having demonstrated the importance of the renewal of the syncytiotrophoblast in the infection of placental tissue by *T. cruzi*. Thus, it is important to explore the relevance of the integrity of the placental barrier in chorionic villi infection by *T. cruzi*, and to define which placental cells are infected when this parasite accesses the chorionic villi in the first stage of the infection.

2. Material and methods

2.1. Parasites

Bloodstream trypomastigotes from the Tulahuen strain DTU VI (Díaz-Luján et al., 2012) of *Trypanosoma cruzi* were isolated from C3H mice infected 10–14 days previously. Parasites were isolated by commonly used technique (Fretes and Fabro, 1995) and then propagated in confluent VERO cells. First, these cells were cultivated at 37 °C in a humidified 5% CO_2 atmosphere in cell culture flasks and passaged by trypsinization. Parasites were then grown in the VERO cells in MEM Eagle culture medium supplemented with 5% FCS and motile and infective trypomastigotes were collected from the culture supernatant, washed with PBS and used for the experiments.

2.2. Placental samples

Five term placentas were obtained from normal pregnancies immediately after scheduled caesareans from clinically and serologically healthy women without Chagas or any other infections including Chagas. All women had signed an informed consent and the project was approved by the National Committee of Ethics.

Villous tissue was obtained from the central part of the cotyledon, dissected into small fragments (30 ± 5 mg each explant) under sterile conditions and washed in Hank's Balanced Salt Solution. Two different in vitro models were employed: human chorionic villi explants (HCVE) with a complete placental barrier (HCVE-CV), and chorionic villi explants denuded of the syncytiotrophoblast (HCVE-DV).

2.3. Removal of the syncytiotrophoblast and culture of denuded and complete chorionic villi explants with T. cruzi

HCVE were denuded of their syncytiotrophoblasts according to Baczyc et al. (2006) with some modifications. Briefly, HCVE were treated with 0.125% of trypsin (Sigma, St. Louis) for 5′ at 37 °C with intermittent stirring. Then, 10% fetal bovine serum (FBS) was added, and the samples were washed with PBS (phosphate buffer solution) at pH 7.3. Detachment was assessed by immunohistochemistry for Placental Alkaline Phosphatase (PLAP) (Ab 228, BioGenex) at time 0 h and syncytin-1 (sc-130888 Santa Cruz Biotechnology) at 96 h. Furthermore, β-hCG and PLAP were quantified in the culture media at 72 h of culture, with β-hCG being measured by a quimioluminiscence immunometric assay (Abott β-hCG- 7k78-25) and the results expressed as mUI/m. PLAP activity was monitored in preheated culture media at 60° C for 15 min, before being measured at 640 nm in a spectrophometer employing *p*-nitrophenol phosphate as the substrate (Messer, 1967), with results being expressed as micromols of *p*-nitrophenol/min. The quantitation of total protein was carried out according to Lowry et al. (1951).

Three HCVE of each placenta (n = 5) were either treated with trypsin or left untreated, before being co-cultured with 800.000 trypomastigotes of the Tulahuen strain in 1 mL of DMEM (Sigma, St. Louis) in plates pH 7.35, 10% FBS (Natocor, Córdoba), and ATB at 5% CO_2 atmosphere at 37°C for 3 h or 24 h. This number of parasites mimics an acute infection of pregnant women in an ex vivo and in vitro model of explants of placenta-*T. cruzi* interaction (Fretes and Kemmerling, 2012). The 3 h group of placental explants *T. cruzi* co-cultures were collected and employed to evaluate invasion by qPCR. The media of the 24 h group of placental explants was replaced for one without parasites every 24 h until 96 h of culture. The culture supernatant was collected and centrifuged and the pellets were used to count the extracellular trypomastigotes, with the supernatants being stored at −20C° for measurement of β- hCG and PLAP. Placental explants were also harvested, fixed with buffered formalin pH7 and processed using histological techniques.

2.4. Measurement of nitric oxide in culture medium

The production of nitric oxide was measured as nitrites in the culture medium using the Green technique with Griess reagent (μM/ml). Results are presented as means ± SD.

2.5. Identification of chorionic villi cell types infected with T. cruzi in denuded chorionic villi

To identify the types of cells present in HCVE infected with *T. cruzi*, immunohistochemistry was employed to detect cytokeratin-7 (epithelial cells) and CD68 (macrophages) at three depths of the HCVE-DV explants. Unspecific antigens were blocked with FBS 20% in PBS, and then slices were incubated overnight with primary

anti-Citokeratin-7 mouse monoclonal IgG (DAKO, cytomation USA) or CD68 (DAKO, cytomation USA). Finally, slices were incubated with biotin conjugated Multilink secondary antibody, labelled with streptavidine-peroxidase (Genex), and revealed with DAB, H_2O_2 3%. The nuclei were counterstained with haematoxylin.

2.6. Stereological analysis of STB detachment and infection of placental tissue cultured with T. cruzi

A Zeiss Axioskop microscope was used at a magnification of 400× to measure the areas of enzymatic detachment of the STB and also to examine infection of the placental tissue by *T. cruzi.*

After fixing with formalin, the paraffin embedded placental tissue was cut every 60 μm and four sections of 4 μm were obtained every 60 μm, (Escolar Castellón, 1997). These slices were stained with H/E or immuno-histochemistry to detect Cytokeratin 7 and CD68 with measurements made on 12 random digital images per section by employing the quantitative image analysis system Axiovision 3.0.6 (Carl Zeiss Vision, Germany).

Explants of chorionic villi of three human placentas that were untreated or treated with trypsin previous to culture (time 0 h) were employed to quantify the area of remaining STB with respect to the total area of chorionic villi. These data are expressed as the percentage of the remaining STB.

Microscopical infection of HCVE-CV and HCVE-DV was quantified by measuring the area occupied by amastigote nests with respect to the total area of the chorionic villi at the three depths of each explant as well as by counting the number of nests of amastigotes per analyzed area and the number of proliferative amastigotes of *T. cruzi* per nest (Luján et al., 2004). To evaluate the statistical significance, the total area analyzed was compared to the area occupied by *T. cruzi* in HCVE-CV and HCVE-DV using the Student T-test.

The infection of Cytokeratin 7+ cells (cytotrophoblast), CD68+ cells (macrophages) and stromal cells (negative to these cells) was assessed by measuring the area occupied by the parasite in each of these cells with respect to the total area analyzed and the number of amastigotes per nest within cells was also counted. Results are expressed as means ± SD.

2.7. Parasite load in chorionic villi co-cultured with T. cruzi

Real time PCR (qualitative qPCR) was employed to quantify *T. cruzi* DNA load in placental explants at 3 h or 96 h of infection. This technique has a higher sensitivity to detect *T. cruzi* in tissues especially at 3 h when trypomastigotes are difficult to detect by microscopical methods (Castillo et al., 2013a,b; Liempi et al., 2016). Genomic DNA was extracted from the placental tissue with the Wizard Genomic DNA Purification Kit (Promega®,USA), according to the manufacturer's instructions, and quantified by the QUBIT Fluorometic System (Invitrogen, USA). For the amplification of human and parasite DNA, two specific pairs of primers were used. A 100 bp human GAPDH sequence as endogenous control was amplified using the primers hGAPDH-F (5′-TGATGCGTGTACAAGCGTTTT-3′) and hGAPDH-R (5′-ACATGGTATTCACCACCCCACTAT-3′), which were designed using Primer Express software (version 3.0; Applied Biosystems®).

For *T. cruzi* DNA detection, a 182 bp sequence of satellite DNA was amplified by using the TCZ primers TCZ-F 5′-GCTCTTGCCCACAMGGGTGC-3′ and TCZ-R 5′-CAAGCAGCGGATAGTTCAGG-3′ (Cummings and Tarleton, 2003). Each reaction mix contained 200 nM of each primer (forward and reverse), 1 ng of DNA from the samples, 12.5 μL of SensiMix®SYBR Green Master Mix (Bioline®, USA) and H_20 to complete 25 μL, with amplification being performed in an ABI Prism 7300 sequence detector (Applied Biosystems®, USA). The cycling program was as follows: A first step at 20°C for 2 min, a denaturation step at 95 °C for 10 min and 40 amplification cycles of: 95 °C (15 s), 60 °C (15 s) and 72 °C (30 s). Finally, a dissociation stage was added, ranging from 60 to 95 °C.

The relative quantification analysis of the results which calculates the ratio between the amount of target template and a reference template in the sample (GAPDH) was expressed as the RQ value by the comparative Control (ΔΔCt) method (Cummings and Tarleton, 2003; Pfaff, 2001; Castillo et al., 2013a,b). The threshold cycle value (Ct) ratio between experimental conditions is used to calculate parasite load based in the ddCt method (Pfaff, 2001), thus, relative quantity of parasite DNA between different samples can be obtained.

2.8. Quantification of extracellular trypomastigotes

We quantified the motile trypomastigotes in the culture supernatant (at 24 h, 72 h and 96 h). Culture media were collected at these times and centrifuged, with the pellets of trypomastigotes being resuspended and counted in a Neubauer chamber (Black and Berenbaum, 1964). Data was expressed as the number of parasites per ml of culture medium.

2.9. Statistical analysis

Results are presented as means ± SD. Statistical significance was established at $p < 0.05$. One- and two-way ANOVA analyse (with Tukey's or Bonferroniís post-test) were performed when required. All of the statistical analyses were carried out using GraphPad Prism (5.0) software.

3. Results

3.1. Assessment of removing STB from chorionic villi

The removal of the syncytiotrophoblast was demonstrated by biochemical, histological and immunohistochemical methods (Fig. 1). An absence of specific immunostaining for Alkaline phosphatise was shown in HCVE-DV, although some syncytium remnants can be seen at time 0 h (Fig. 1A,c). Also, there was a significant decreased area of the syncytiotrophoblast from the chorionic villi after the trypsin treatment technique at time 0 h of culture ($p < 0.05$) (Fig. 1B).

The reduced area of STB was maintained at 72 h in HCVE-DV, and resulted in decreased levels of hCG ($p < 0.05$) and PLAP activity ($p < 0.05$) in the culture media respect to HCVE-CV (Fig. 1B). At 96 h of culture, there was a partial recovery of STB in the HCVE-DV, as can be seen in Fig. 1A,i. The chorionic villi, stained with haematoxylin/eosin and IHQ to detect syncytin-1, which is specific to STB, revealed areas with and without STB in the same villi explants (Fig. 1A,f,i)

3.2. Productive infection increased in denuded explants cultured with T. cruzi

It was observed that the HCVE-DV had a much decreased area of STB with numerous cells infected with *T. cruzi* (Fig. 2A,c,d), especially cells next to areas without STB. This was in contrast with that observed in the HCVE-CV (controls), which had few parasites in the tissue (Fig. 2A,a,b). Cells could also be observed with amastigotes near the surface and within the chorionic villi, mainly in areas without STB in the trypsin treated explants (Fig. 2A,c). According to the H/E slices, these cells were compatible with cytotrophoblasts and stromal cells.

Infection was quantified in HCVE-DV and HCVE-CV at 96 h of culture HCVE-DV showed a significantly greater area (451.23 μm^{2*};

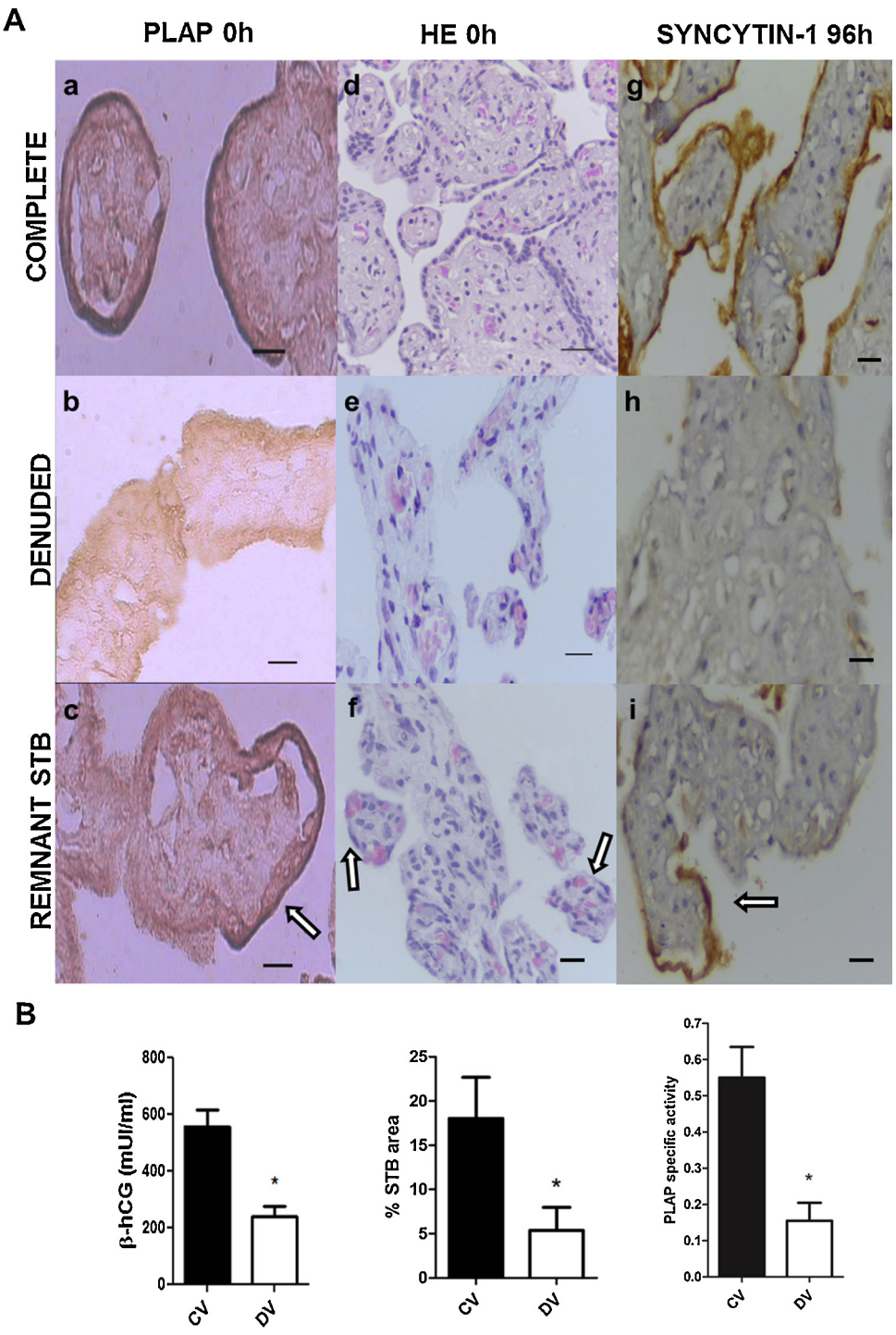

Fig. 1. (A) Immunohistochemistry to detect PLAP (Placental Alkaline Phosphatase) (a, b, c) and Syncytin-1 (g, h, i) which are specific markers for syncytiotrophoblast and H/E (d, e, f) in denuded chorionic villi (treated with trypsin) at time 0 h previous to culture or denuded and cultured for 96 h. (a, d, g): Complete chorionic villi with total placental barrier. (b,e,h): Denuded chorionic villi without its syncytiotrophoblast. (c, f, i): Areas with remnant STB (arrows). 400×.
(B) Activity of PLAP (placental alkaline phosphatase) and level of β-hCG (human chorionic gonadotrophin) in culture media at 72 h of culture. Percentage of STB (syncytiotrophoblast) area respect to total area of chorionic villi. VC: chorionic villi with all their placental barrier structures. VD: chorionic villi denuded of their syncytiotrophoblast. hCG: *$p < 0.05$ DV versus CV.PLAP: *$p < 0.05$ DV versus CV. Percentage area occupied by STB: *$p < 0.05$ DV versus CV.

mean ± SD: 37.60 ± 27.35 μm^2) and percentage of total area (1.33%) occupied by the parasite than HCVE-CV (5.98 μm^2; mean ± SD: 0.99 ± 0.42 μm^2; 0.016%) ($p < 0.05$). Moreover, the nests of amastigotes (Total: 31*, mean ± SD: 2.58 ± 1.62) and the number of amastigotes per nest in HCVE-DV (54.75 ± 43.98) revealed intense parasitism, which contrasted with the much smaller infection detected in HCVE-CV villi (Total nest: 6, mean ± SD: 5 ± 0.52; amastigotes per nest: 4.5 ± 2) (*$p < 0.05$).

The parasitic load in HCVE measured by qPCR (Fig. 2B) showed similar levels of infection at 3 h in complete and denuded explants ($p > 0.05$), which reflects a comparable ability of the parasite to enter in the two types of chorionic villi models. However, at 96 h of culture, there were significant differences between the HCVE-CV

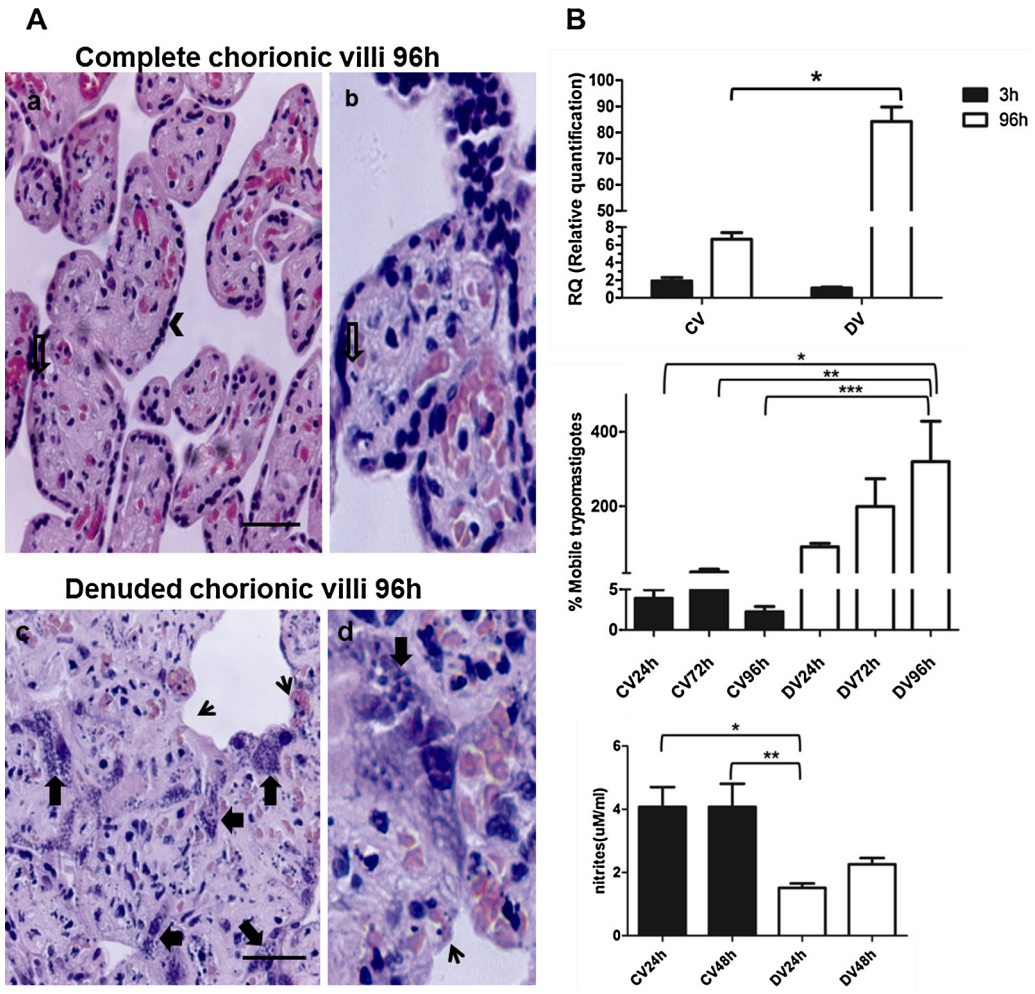

Fig. 2. (A) Chorionic villi of human term placentas infected with *T. cruzi* and cultured for 96 h. H/E. A: a, b: untreated chorionic villi with complete placenta barrier. Arrowheads indicate STB which is covering the chorionic villi. Translucent arrow shows the parasite in the villous stroma without forming nests of amastigotes. c, d: treated chorionic villi lacking syncytiotrophoblast. Thin arrows show areas of the villi without STB where cells located near the surface have been infected. Thick arrows indicate amastigote nests in the villous stroma cells. 400×. Original magnification. Bar 50 μm.
(B) Qualitative qPCR (RQ) in human placental chorionic villi, with a complete placental barrier (CV) or denuded of their syncytiotrophoblast (DV) and cultured for 3 h and 96 h with Tulahuen strain of trypomastigotes of *T. cruzi*. *$p < 0.05$ DV 96 h versus CV 96 h.
Motile trypomastigotes in the culture media of DV or CV placental chorionic villi co-cultured with *T. cruzi* for 24 h, 72 h and 96 h. *$p < 0.05$ CV 24 h versus DV 96 h. **$p < 0.05$ CV 72 h versus DV 96 h. ***$p < 0.05$ CV 96 h versus DV 96 h.
Nitrites in the culture media of CV and DV for 24 h and 48 h. *$p < 0.05$ CV 24 h versus DV 24 h. **$p < 0.05$ CV 48 h versus DV 24 h.

and HCVE-DV infections. The parasitic load measurement at 96 h represented a reproduction of amastigotes in the life cycle of the parasite in the denuded villi, with the significantly increased quantity ($p < 0.05$). Thus, explants without their syncytiotrophoblast had a more than ten-fold greater productive infection by *T. cruzi* than HCVE-CV.

3.3. Quantification of parasites in the culture media

Motile trypomastigotes were counted in the culture supernatant at 24 h, 72 h and 96 h of culture. At 24 h, there were similar numbers of parasites in complete and denuded chorionic villi. In contrast, there was a significantly increased number of parasites in the culture media from HCVE-DV respect to HCVE_CV at 72 h and also at 96 h ($p < 0.05$) (Fig. 2B) implying that new trypomastigotes had been released into the culture medium. The absence of STB permitted replication of *T. cruzi* in the chorionic villi, which is consistent with the more than ten-fold increased number of trypomatigotes observed in the culture medium.

3.4. Denuded placental explants had decreased nitric oxide in the culture media

Nitrites (indicating nitric oxide), were measured in the culture supernatant of the HCVE-DV and HCVE-CV cultures without *T. cruzi* to analyse the importance of this parasiticidal agent. Fig. 2B shows a decreased amount of nitrites in HCVE-DV respect to HCVE-CV ($p < 0.05$). Therefore, the value in the HCVE-DV did not reach the minimal concentration to be deleterious for *T. cruzi* (Villalta et al., 1998).

3.5. Identification of cells within chorionic villi infected by T. cruzi

It can be seen in Fig. 3 that most of the cells with amastigotes were epithelial cytokeratin-7(+) and stromal (cytokeratin-7(−) and CD68(−)) which were located near the surface or in the stroma of the chorionic villi respectively. CD68 positive Hofbauer cells were observed as internal cells of the villi and were scarcely infected.

The result of the quantification is shown in Table 1 with the most infected cells being cytotrophoblast cytokeratin-7(+) and the

Table 1
Typification of cells of the human denuded chorionic villi infected with *T. cruzi*.

Infected cells in chorionic villi without STB	Area analyzed Total and mean ± SD (μm^2)	Area of *T. cruzi* Mean ± SD (μm^2)	Amastigotes per cell Mean ±SD	Total number of amastigotes
CTB cells Citok-7 (+)	51412.78 1772.85 ± 2125.07	293.55 ± 24.46	15 ± 1.25	386.32
Stromal cells Citok-7 (−) and CD68 (−)	48587.62 799.54 ± 1655.56	157.68 ± 37.60	16 ± 5.26	271.22
Houfbauer cells CD68 (+)	53660.59 1676.89 ± 1927.88	73.43 ± 87.45*	11.27 ± 13.73	124*

Chorionic villi denuded of syncytium were co-cultured with *T. cruzi* for 96 h with trypomastigotes. The villous stroma cells susceptible to infection by *T cruzi* in the absence of STB were identified by immunohistochemistry. Cells with amastigotes in the cytoplasm were positive to CD68 (+) (Hofbauer) and cytokeratin-7 (cytotrophoblast) and other negative cells for these two markings were infected.

* $p < 0.05$.

stromal cells cytokeratin-7(−) CD68(−). Both these types of cells had the largest quantities of amastigotes in their cytoplasms within the placental chorionic villi with the least infected cell being the macrophage CD68(+). The cytotrophoblast revealed a greater area of parasites and a higher number of amastigotes compared with the Hofbauer cell ($p < 0.05$), indicating that the cytotrophoblast is a very susceptible cell for infection by *T. cruzi* in the initial cycles of reproduction of the parasite. Stromal cells which were negative for cytokeratin and CD68 were also more infected than CD68+ cells, and the former cells may correspond to fibroblast or mesenchymal cells.

4. Discussion

Even though congenital Chagas transmission is causing a worldwide public health problem (WHO, 2002; Carlier et al., 2015), little is known about the mechanisms of placental infection by *T. cruzi* in the initial stages (Fretes and Kemmerling, 2012). It was previously shown that the complete placental barrier can become infected by *T. cruzi* without a sustainable infection taking place (Luján et al., 2004), and recently, the important role played by the first structure of the placental barrier (Díaz-Luján et al., 2012) and the renovation of the trophoblast (Liempi et al., 2014) in the infection of the placenta by this parasite have been described. These studies indicate the possibility that discontinuities in the placental barrier might participate in chorionic villi infection by *T. cruzi*, with these discontinuities in the placental barrier having been previously described in normal pregnancies, as well as in complicated and chagasic ones (Altemani et al., 2000; Duaso et al., 2011).

The artificial detachment of the STB by the trypsin method (Baczyc et al., 2006) was shown to result in a considerable denuded area of the chorionic villi, according to the placental alkaline phosphatase and hCG measurements in the culture media and the detection of syncytin and alkaline phosphatase by IHQ (Fig. 1). Furthermore, analysis of the areas of the trophoblast in complete and denuded explants revealed that trypsin treated chorionic villi had significantly smaller areas of trophoblast than complete ones.

In the present investigation, the in vitro model we have utilized is similar to the real situation in the uterus and mimics a high parasitemia infection by *T. cruzi* (Fretes and Kemmerling, 2012). According to a previous study, a mean of 11 parasite cells/ml was found in the maternal blood of chronic chagasic pregnant women (Bua et al., 2013). However, in acutely infected mothers, the number of *T. cruzi* per ml of maternal blood was shown to be higher, with more than 100 parasites per ml (Torrico et al., 2005), and usually more than 500 trypomastigotes per ml of blood when detected by microstrout (Moretti E., personal communication). Here the infection was quantified by microscopical analysis (Luján et al., 2004; Liempi et al., 2015) and qPCR (Pfaff, 2001; Castillo et al., 2012, 2013a,b). According to the morphological study, there were significantly more nests of amastigotes and amastigotes per nest in chorionic villi without their syncytiotrophoblasts compared to complete (untreated) chorionic villi. Moreover, parasites were observed in areas of the chorionic villi which did not have the STB. These data suggest the importance of the integrity of the placental barrier, implying that when all structures of the barrier are present the infection is significantly lower than in the denuded chorionic villi, and therefore possibly easier to control. According to our results, the infection increased in the placental tissue when the first placental barrier structure was absent, which limited defenses and thereby favored a sustainable infection or proliferation of parasites.

Despite the enormous quantity of parasites that are able to reach the placenta in chronic chagasic pregnant women (Fretes and Kemmerling, 2012), the chorionic villi has been reported to be poorly infected with *T. cruzi*, which were difficult to find in the placental tissue (Moya et al., 1979; Altemani et al., 2000; Carlier and Truyens, 2010; Duaso et al., 2011). Although this phenomenon is possibly related to a low incidence of congenital transmission. In acutely infected pregnant women with high parasitemia the incidence of transmission is higher than in the chronic condition (Schijman, 2007) and thus there is a correlation between the degree of parasitemia and the congenital transmission of *T. cruzi* (Rendell et al., 2015; Bua et al., 2012). It was previously shown that high quantities of *T. cruzi* can alter the STB, and consequently the placental barrier, but this effect was either absent or insignificant for lower doses of parasites (Duaso et al., 2010), consequently, we wondered if the syncytiotrophoblast is the main structure of the placental barrier that acts against a challenge with high quantities of *T. cruzi* cells. In our experimental model, complete chorionic villi (employed as control) exposed to high quantities of *T. cruzi* resulted in a weaker detachment of the STB than an artificially induced detachment by trypsin, and a consequently smaller infection. In fact infection in HCVE-DV increased significantly, indicating that the infective form of *T. cruzi* requires large areas of chorionic villi without their STB in order to sustain a productive infection. These data seem to indicate a mainly defensive role of the first placental barrier structure.

Nitric oxide is one of the main deleterious agents against *T. cruzi* cells (Rivero, 2006; Vespa et al., 1994), which is produced in an important manner by syncytiotrophoblast by endothelial Nitric Oxide Synthase (Myatt et al., 1997). In this study, the culture medium from HCVE-DV showed non-deleterious concentrations of NO against *T. cruzi*, in agreement with Villalta et al. (1998). There may be an association between large areas of STB detachment with reduced concentrations of NO and an increased infection of the chorionic villi. Related to this, it has already been demonstrated that there is a correlation between NO and the viability of *T. cruzi* in the culture media (Díaz-Luján et al., 2012). However, although *T. cruzi* might alter the placental barrier, high parasitemias are not necessarily sufficient conditions to produce congenital transmission, as was previously confirmed in acutely infected pregnant women

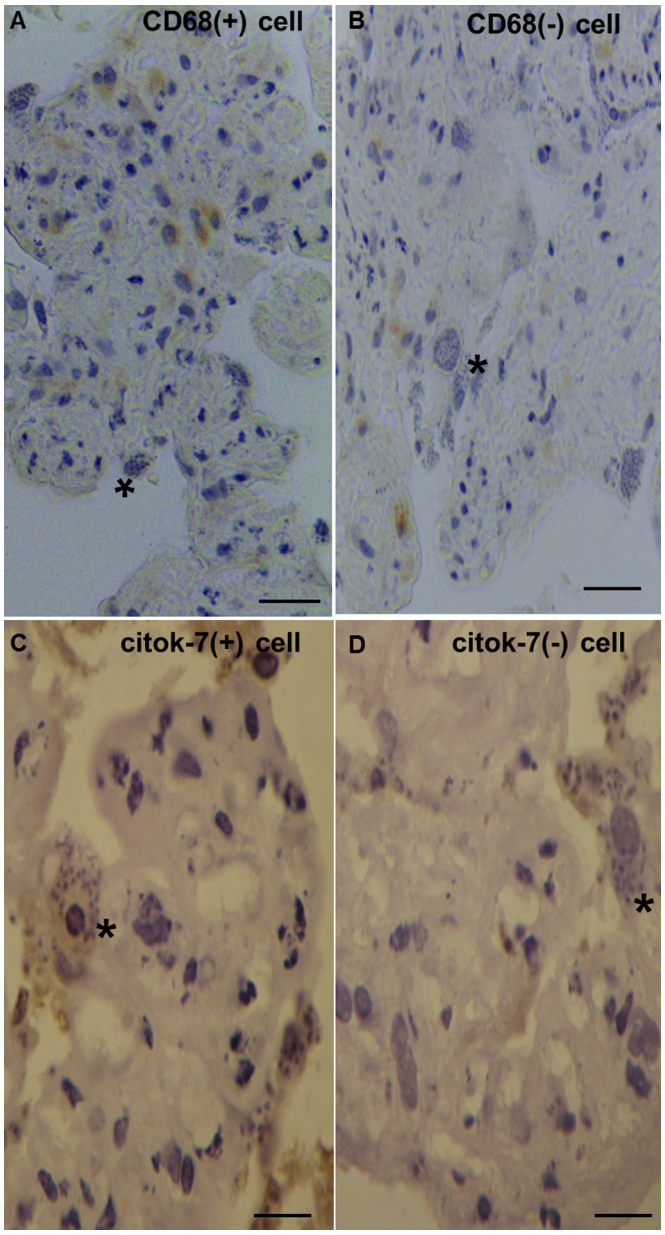

Fig. 3. Immunohistochemistry of cytokeratin-7 and CD68(+) positive cells in denuded human chorionic villi (without syncytiotrophoblast) cultured with *T. cruzi* for 96 h. A: *Cell positive for CD68(+) of villous stroma (Hofbauer cells) with amastigotes in the cytoplasm. B:*Cell villous stroma negative for CD68(−). C: *Cytotrophoblast Citokeratin-7 positive cells with amastigotes in the cytoplasm. D: Cell Citok-7 (−) negative with amastigotes in the cytoplasm. Photo representing a level. Original magnifications 400×. Bar 50 μm. Counterstained with hematoxylin.

who only transmitted the infection in one case out of three (Moretti et al., 2005). This finding might have been due to the deleterious effect that STB has on both the *T. cruzi* cells present in the intervillous space (clearence of the parasite) (Triquell et al., 2009) and on the reproduction of the parasite within the placental tissue (Díaz-Luján et al., 2012). Moreover, it is possible that renovation of the trophoblast may participate in the placental defenses (Liempi et al., 2014).

It has been previously described that trophoblast apoptosis can participate in placental infection by the causal agent of Chagas (Duaso et al., 2011), but this was observed at low doses of the infective forms of *T. cruzi*, and thus is probably more applicable to other clinical situations such as chronic chagasic pregnancies with low parasitemia. When chagasic pregnancies develop with high parasitemia, it seems probable that detachment of the first placental barrier occurs, thereby increasing the possibility of tissue parasitism. Nevertheless, according to our investigation, large areas of detachment of the STB were needed to obtain a sustainable infection of the chorionic villi. In a related study, Altemani et al. (2000) using placentas from chagasic mothers with a high number of parasites within their chorionic villi, reported simultaneously trophoblast destruction and an altered placental barrier.

Each human being lives in a sea of microbes with potentially infectious agents, and pregnant women are no exception. However, few pathogens are able to cross the placenta to produce a congenital infection (Robbins et al., 2012), with STB playing a key role in this defense, as previously reported for *Cytomegalovirus,* which was able to infect CTB but not STB; or in the case of the *Herpex simple* virus which could only infect chorionic villi when the STB was enzymatically damaged (Koi et al., 2002; Robbins et al., 2012). Thus, STB has an essential role in preventing placental infection from the infectious agents present in the intervillous space. Related to this, according to our results, although the chorionic villi with a complete barrier was able to be infected by *T. cruzi* as shown by qPCR at 3 h, but there was no sustainable infection at 96 h. This coincided with deleterious levels of nitric oxide at 3 h, and although infection of the STB was possible, there was no productive infection. Similar levels of infection in the placental tissue have also been found by other authors (Castillo et al., 2012).

Despite several reviews having described placental infection by *T. cruzi*, we were unable to find any investigation that reported which placental cells were infected at the initial stage of infection (Schijman, 2007; Carlier and Truyens, 2010; Bittencourt, 1988; Fretes and Kemmerlin, 2012). Therefore, we decided to try to identify epithelial and macrophage placental cells by immunohistochemistry, in order to quantify their respective infections during the first duplications of the parasite, but before new cycles occurred in other cells (De Souza, 2002). It was observed that there were significantly more cytotrophoblasts and stromal non-macrophage cells infected by *T. cruzi* than macrophages (Hofbauer cell), indicating that former types of cells were more susceptible to infection by *T. cruzi* after their invasion. However, the single trophoblastic cell was the most appropriate cell for parasitic proliferation in the first stages of placental infection, and therefore the cell most likely to maintain infection in the chorionic villi. This data is in agreement with a previous study using another in vitro system (Díaz-Luján et al., 2012).

The significantly high number of parasites obtained from the culture media of denuded chorionic villi compared with the complete one, corresponded with an increased level of infection in the former. The experimental design allowed trypomastigotes to invade the tissue and then the culture media was changed for a new one with no parasite. In this way, the *T. cruzi* obtained in the culture media at the end of culture represented only those coming from a new cycle of reproduction. Infection of the susceptible inner cells of the chorionic villi and the absence of the first placental barrier structure resulted in a multiplication of the parasite which may explain the higher number of parasites in the denuded placental chorionic villi.

STB, in common with other placental cells, produces nitric oxide (Myatt et al., 1997), which is a potent deleterious agent against *T. cruzi* (Díaz-Luján et al., 2012; Villalta et al., 1998). According to our results, in chorionic villi without the syncytiotrophoblast (the first structure of the placental barrier), the increased infection coincided with lower and non-deleterious levels of nitric oxide (Villalta et al., 1998). Therefore, the presence of STB may limit new cycles of *T. cruzi* with the participation of nitric oxide, and also might induce damage to the parasite cells in the intervillous space, as has been previously described (Triquell et al., 2009).

According to our results, the chorionic villi challenged with high quantity of trypomastigotes altered the placental barrier but they were not massively infected by *T. cruzi*, as they occurred when larger areas of STB were artificially detached and the levels of nitric oxide were low. Therefore high quantities of trypomastigotes are necessary, but not a sufficient condition to produce a sustainable infection of the placental tissue, as was shown by Moretti et al. (2005) in acutely infected pregnant women. In fact, the infection of chorionic villi by the causal agent of Chagas disease is favored by considerable damage to the first placental barrier structure, the syncytiotrophoblast, in situations with high parasitemia, which reduces the levels of nitric oxide and consequently the defenses of the placental tissue. In this condition, the inner cells of the chorionic villi are more susceptible to infection when exposed to *T. cruzi* and this permits the multiplication of the parasite with the possibility of obtaining a sustainable infection of the placental tissue.

In conclusion, we have shown that the syncytiotrophoblast is the main placental barrier structure that limits the chorionic villi infection by *T. cruzi*, thereby avoiding congenital transmission by this route which explains at least in part, the low incidence of vertical transmission of Chagas.

Declaration of interests

Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contribution

DLC, TMF, CC and HD performed experiments. Studies were designed by DLC and FRE. Manuscript was written by DLC and FRE. Revision of manuscript: FRE and UK. All authors read and approved the final manuscript.

Ethical statement

The project was approved by the Ethics Committee of the National Clinical Hospital (COEIS) of the Universidad Nacional de Córdoba, No. 120/12. Pregnant women whom placentas were obtained have written informed consent. Procedures agreed with the Helsinki Declaration of 1975 and revised in 1993.

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