Placenta 31 (2010) 705–711

Contents lists available at ScienceDirect

Placenta

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# *Trypanosoma cruzi* induces tissue disorganization and destruction of chorionic villi in an *ex vivo* infection model of human placenta

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ARTICLE INFO

*Article history:*
Accepted 4 May 2010

*Keywords:*
*Trypanosoma cruzi*
Placental tissue invasion

ABSTRACT

Congenital Chagas' disease, endemic in Latin America and also present with lower frequency in other countries, is associated with premature labor, miscarriage, and placentitis. The mechanism of tissue invasion and infection of human placenta by the parasite *Trypanosoma cruzi* (*T. cruzi*) remains unclear. In order to explore some morphological aspects of this infection in the placenta, we incubated chorionic villous explants from normal human placentae *ex vivo* with the parasite and studied the resulting effects by immunohistochemical and histochemical methods. Infection of the chorionic villi with the parasite was confirmed by immunofluoresence and PCR. *T. cruzi* induces syncytiotrophoblast destruction and detachment, selective disorganization of basal lamina and disorganization of collagen I in the connective tissue of villous stroma. These effects are a function of the number of parasites used for the infection. Our results suggest a participation of the proteolytic activity of the parasite on the placental basal lamina and connective tissue in the mechanism of infection of the fetus by *T. cruzi*.

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## 1. Introduction

Endemic diseases caused by parasites are one of the characteristic features of the developing countries. Chagas' disease, produced by the flagellated protozoan *Trypanosoma cruzi* (*T. cruzi*), is one of the most frequent endemic diseases in Latin America. At least twenty eight million people are at risk of exposure to infection, with an estimated total of fifteen million cases [1]. As a result of vector control (triatomids), the number of new cases per year has greatly decreased. Nonetheless, in the past few years congenital transmission of *T. cruzi* has increasingly become more important, and partly responsible for the "globalization" of Chagas' disease [2], constituting a public health problem of increasing relevance [3,4]. The vertical transmission of *T. cruzi* cannot be prevented, but early detection and treatment of congenital infection achieves cure rates close to 100% [5].

Fetal and maternal tissues are separated by a fetal epithelium (the trophoblast), the greatest area of which is in the villous placenta, the site of nutrient and gas exchange [6]. Within the villous placenta, a single multinucleated cell layer (syncytiotrophoblast) contacts maternal blood within the intervillous space. Beneath the syncytiotrophoblast reside replicating progenitors (cytotrophoblast) that are separated by a basal lamina from the connective tissue of villous stroma containing vascular endothelium, fibroblasts and macrophages. Damage to the villous placenta is almost always accompanied by inflammation, either in the intervillous space or within the fetal villi and, in severe cases, is accompanied by loss of the protective trophoblast. Extensive placental damage may lead to fetal loss or intrauterine growth retardation [6], clinical manifestations that can be observed in congenital Chagas' disease [7].

Parasite invasion in cell cultures has been studied in some depth. On the other hand, studies that analyze parasite invasion in tissues and organs are rare. *T. cruzi* penetration in host cells occurs through a complex multi-step process that includes both parasite and host cells molecules [8]. Metacyclic trypomastigotes adhere to specific receptors on the outer membrane of host cells as a prelude to intracellular invasion, causing calcium ion mobilization, rearrangement of host cell microfilaments, recruitment of lysosomes to the site of the parasite entry [9] and parasite internalization [9,10].

Interaction between *T. cruzi* and the chorionic villi tissue is fundamental for the invasion and infection of the parasite. The parasites have to cross the placental barrier that is formed by the trophoblast, fetal connective tissue, fetal endothelium and basal lamina. The basal lamina is present between all those structures. In addition, the parasite has to penetrate these structures. The parasite

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doi:10.1016/j.placenta.2010.05.007

secretes proteases (cruzipains) capable of degrading extracellular matrix (ECM) components such as collagen type I, IV and fibronectin [11,12].

*In vitro*, *T. cruzi* successfully invades and replicates in a wide variety of mammalian cells [8,13], including cells of placental chorionic villi [13,14].

In order to detect alteration of the chorionic villous tissue during parasite invasion of placental villi, we performed histopathological analysis of *ex vivo* infected human chorionic villi, immunohistochemical studies of syncytiotrophoblast and basal lamina, and histochemical studies of carbohydrate rich molecules and collagen I in villous stroma.

## 2. Material and methods

### 2.1. VERO cell culture

Green Monkey (*Cercopithecus aethiops*) renal fibroblast like cells (VERO cells (ATCC® CCL-81)) were grown in RPMI medium enriched with 5% fetal bovine (FBS) serum and antibiotics (penicillin–streptomycin). Cells were grown at 37 °C in a humid atmosphere at 5% $CO_2$ for 96 h, replacing the medium every 24 h [15].

### 2.2. Infection of VERO cells with T. cruzi and trypomastigote harvesting

After confluence, VERO cells were incubated with a culture of epimastigotes in late stationary phase, which increases the percentage of trypomastigotes to approximately 5% [16]. Trypomastigotes then invade fibroblasts and replicate intracellularly as amastigotes. After 72 h, amastigotes transform back to trypomastigotes that lyse host cells. Parasites were recovered by low speed centrifugation (500 × g), thus obtaining trypomastigotes in the supernatant and amastigotes in the sediment [17].

### 2.3. Placenta and chorionic villi culture

Human term placentas were obtained from uncomplicated pregnancies from vaginal or caesarean delivery. 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. Exclusion criteria for the patient were the following: major fetal abnormalities, placental tumor, intrauterine infection, obstetric pathology, and any other maternal disease. The organs were collected in cold sterile saline-buffered solution (PBS) and processed no more than 30 min after delivery. Their 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 in order to remove blood, and cut in approximately 0.5 $cm^3$ pieces. Placental villi were co-cultured under different concentrations ($1 \times 10^2$ to $2 \times 10^6$/ml) of trypomastigotes DM28c strain for 24 h in 1 ml of RPMI culture media supplemented with inactivated FBS and antibiotics. Controls and experimental conditions were the same except for the addition of parasites as indicated.

### 2.4. Histological and histochemical techniques

The placental villi were fixed in 10% formaldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h, then dehydrated in alcohol, clarified in xylene, embedded in paraffin, and sectioned at 5 μm. Paraffin histological sections were stained with hematoxylin–eosin for routine histological analysis, Picrosirius red–hematoxylin for collagen histochemistry [18], and periodic acid-Schiff (PAS) for carbohydrate containing tissue elements (Sigma–Aldrich kit 395B). As a control of PAS method, 5 μm-thick sections were incubated with a 4 μg/ml solution of α-amylase (Nutritional Biochemical Corporation) in PBS pH 6.0, for 30 min at 37 °C prior to the PAS-hematoxylin reaction. A decrease in the intensity of the PAS stain reaction after the enzyme treatment was considered as evidence of the presence of carbohydrates.

### 2.5. Immunohistochemistry

The placental chorionic villi were fixed in 10% formaldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h, embedded in paraffin wax and cut into 5 μm sections. Standard immunoperoxidase techniques were used to show human placental lactogen (Novocastra® NCL-PLp dilution 1:250 v/v), fibronectin (ABR® MA1-83176, dilution 1:50 v/v), laminin (ABR® MA1-21194, dilution 1:20 v/v), collagen IV (Novocastra® NCL-COLL-IV, dilution 1:100 v/v) and heparin sulphate (Novocastra® NCL-CD44-2, dilution 1:40 v/v). The primary antibodies were applied individually to each section for 30 min at 37 °C (anti-human placental lactogen and anti-cruzipain) or at 4 °C overnight (anti-laminin, anti-fibronectin, anti-collagen IV, anti-heparan sulphate). Immunostaining was performed using a horseradish peroxidase-labelled streptavidin biotin kit (RTU-Vectastain kit) following the manufacturer's directions using diaminobenzidine as the chromogen. Sections were counterstained with Mayer's hematoxylin (DAKO) and mounted with Entellan (Merck). Immunohistochemical controls were done by replacing the primary antibodies with phosphate buffered saline. All controls were negative. All sections were examined by light microscopy (Leitz Orthoplan) and images were captured with a Canon 1256 camera.

### 2.6. Immunofluorescence

The placental chorionic villi were fixed in 10% formaldehyde-0.1 M phosphate buffer (pH 7.3) for 24 h, embedded in paraffin wax and cut into 5 μm sections. A monoclonal antibody (mAb 25, dilution 1:100 v/v) specific to the *T. cruzi* flagellar calcium-binding protein (a gift from Dr. Schenkman, Universidade de Sao Paulo, Sao Paulo, Brasil) was applied to each section overnight at 4 °C. The preparations were washed with PBS and incubated with anti-mouse IgG conjugated with fluorescein (ScyTek, ACA) in the presence of 1 μg/ml of 4′6′diamidino-2-phenylindole (DAPI). Afterwards, sections were mounted in VectaShield (ScyTek, ACA) and observed in a Nikon Eclipse E400 microscope (Tokio, Japan).

### 2.7. Control of infection

*T. cruzi* invasion was tested by immunofluorescence and by parasite DNA detection using the polymerase chain reaction. For this purpose, genomic DNA was extracted from the placental tissue with the Wizard Genomic DNA Purifcation Kit (Promega) according manufacturer's instruction. A 984 base pair fragment of the *T cruzi* endonuclease (NL1Tc) gene was amplified. The sequence of the oligonucleotides is the following: forward: **5′-GGGGTACCATGGAGCCATTTACATGGCTG-3′** and backward: **5′- CCCAAGCTTTATAAGCGCTCTCGTGAAAGC-3′**. The PCR product was subjected to electrophoresis in 1.6% agarose gels and stained with ethidium bromide. PCR markers from Promega were employed as molecular weight standards.

## 3. Results

### 3.1. Ex vivo infection of human chorionic villi with trypomastigotes

After 24-h incubation of human chorionic villi with trypomastigotes from DM28c strain (obtained from infected VERO cell cultures), parasites were observed in the chorionic villi when $1 \times 10^5$ or more parasites/ml were used (Fig. 1E–H). In Fig. 1A–D control chorionic villi is shown.

Fig. 1I shows presence of the parasite DNA detected by PCR in homogenates of chorionic villi incubated with different trypomastigote concentrations ($1 \times 10^2$ to $1 \times 10^6$ parasites/ml). Parasitic DNA could be detected by PCR from chorionic villi incubated with $10^4$ parasites/ml or more.

### 3.2. Histopathological analysis of human chorionic villi incubated ex vivo with trypomastigotes

Fig. 2 shows the histopathological analysis of hematoxylin–eosin stained sections from human chorionic villi incubated with different concentration of the parasite ($1 \times 10^5$ to $2 \times 10^6$/ml). Chorionic villi incubated with $10^5$ or more parasites shows detachment and destruction of the syncytiotrophoblast (Fig. 2B–G). In samples incubated with $10^6$ parasites or more, destruction of villous stroma accompanied the damage of the syncytiotrophoblast. In the histological sections from chorionic villi incubated with parasite, possible nuclei from amastigotes are observed (Fig. 2B–H, yellow arrows). Considering, that the parasite is present in chorionic villi infected with $10^5$ or more trypomastigotes, and that histopathological damage is evident at those parasite concentrations, we performed immunohistochemical and histochemical analysis in these samples.

### 3.3. Ex vivo infection of human chorionic villi with T. cruzi induces disorganization and detachment of syncytiotophoblast

Fig. 3 shows additional evidence of the detachment and destruction of the syncytiotrophoblast induced by the parasite. Histological sections of chorionic villi incubated with or without $10^5$ and $10^6$ parasites/ml were immunostained with an anti human

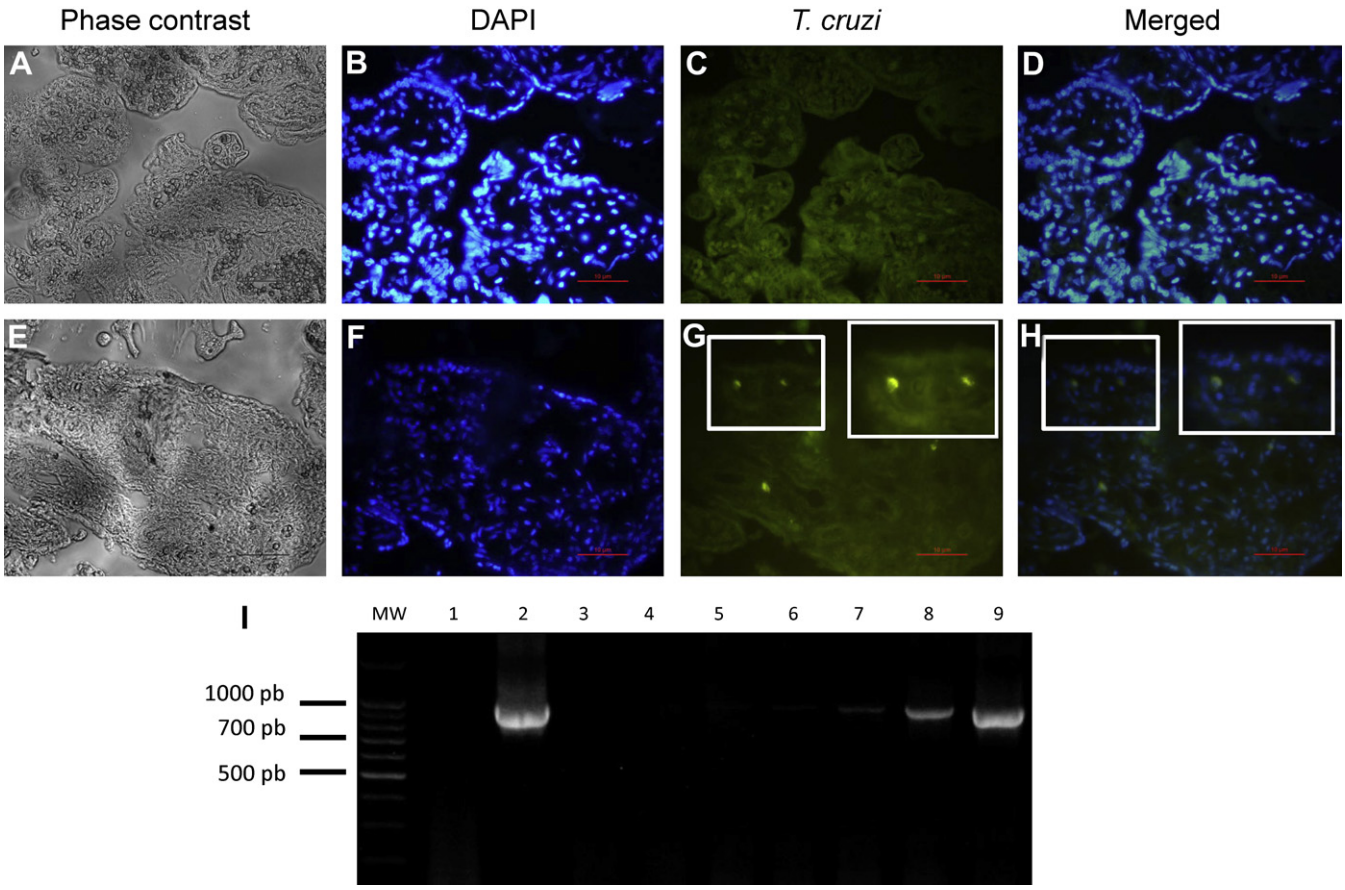


**Fig. 1.** *Detection of T. cruzi in ex vivo infected human chorionic villi*: Chorionic villi incubated with $1 \times 10^5$ (E–H) trypomastigotes DM28c strain for 24 h show the presence of parasite amastigotes in villous stroma by immunofluorescence as detected using mAb 25 antibody. In (A–D) control noninfected chorionic villi are shown. Panels A–D and E–H show images of the same fields. Panels A and D: phase contrast; B and F: nuclear staining with DAPI, C and G; detection of the parasite by immunofluorescence; D and E: merged images of B, C and F, G, respectively. Insets show an amplified region of the detection of the parasite by immunofluorescence (G, H). Bar scale: 10 μm. In I, detection of *T. cruzi* by PCR in *ex vivo* infected human chorionic villi is shown. A 984 base pair fragment of the *T cruzi* endonuclease NL1Tc gene was amplified from chorionic villi incubated with $1 \times 10^2$ to $1 \times 10^6$ trypomastigotes DM28c strain for 24 h. Parasite DNA can be clearly observed in tissues incubated with $1 \times 10^4$ or more trypomastigotes. MW: Molecular weight markers; lane 1: negative control without DNA; lane 2: DM28c epimastigote DNA; lane 3: not infected human blood DNA; lane 4: control (not infected) human chorionic villi DNA; lanes 5–9 human chorionic villi incubated with different concentrations of trypomastigotes DM28c strain (parasite/ml) (lane 5: $1 \times 10^2$; lane 6: $1 \times 10^3$; lane 7: $1 \times 10^4$; lane 8: $1 \times 10^5$; lane 9: $1 \times 10^6$).

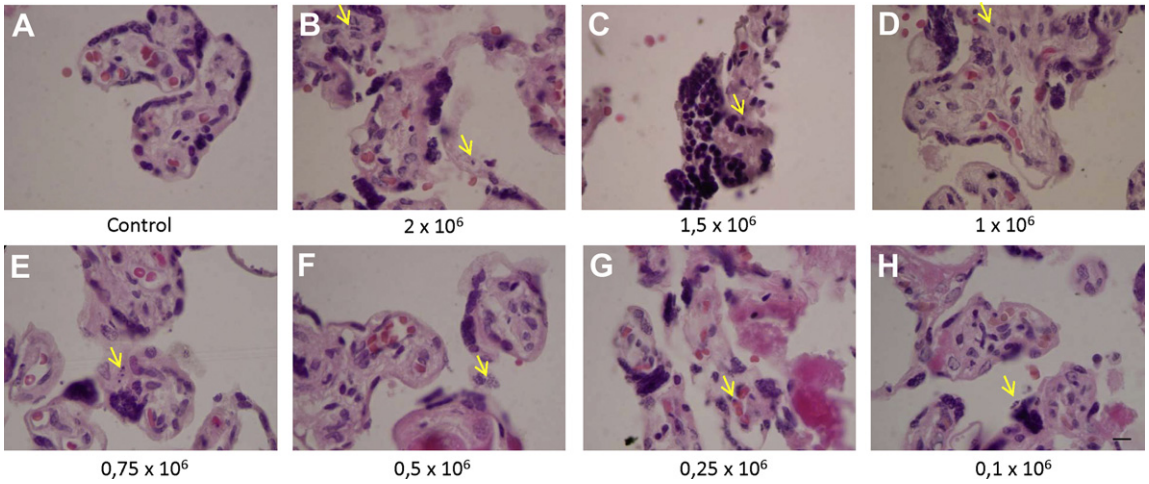


**Fig. 2.** *Histopathological analysis of ex vivo infected human chorionic villi with T. cruzi*: Chorionic villi incubated with $0.125 \times 10^6$ to $2 \times 10^6$ trypomastigotes DM28c strain for 24 h (B–H). Chorionic villi incubated with the parasite shows a parasite-concentration dependent damage of the tissue compared to control villi (A). Detachment and disorganization of syncytiotrophoblast as well as fetal connective tissue destruction of villous stroma are observed. Arrows indicate possible presence of the parasite in the human placental villi tissue. Chorionic villi were processed for routine histological techniques and stained with hematoxylin–eosin. Bar scale: 25 μm.

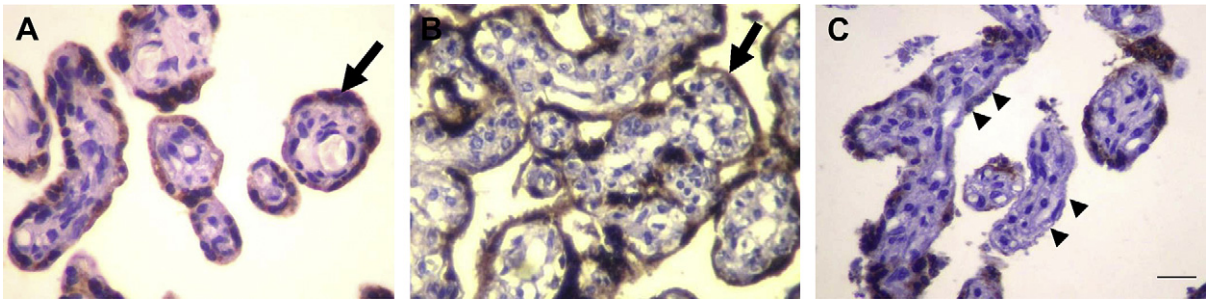


**Fig. 3.** *T. cruzi induces syncytiotrophoblast detachment and destruction*: Chorionic villi incubated with $10^5$ (B) or $10^6$ (C) trypomastigotes DM28c strain for 24 h were immunostained for human placental lactogen, a syncytiotrophoblast marker. Chorionic villi incubated with the parasites (B and C) show detachment and destruction of syncytiotrophoblast compared to control chorionic villi (A). Red arrows indicate the syncytiotrophoblast and black arrows areas of syncytiotrophoblast detachment. Chorionic villi were processed for routine immunohistological techniques and counterstained with Mayer's hematoxylin. Bar scale: 25 μm.

placental lactogen antibody. Placental lactogen is a member of an extensive growth hormon/prolactin family of peptides, which is exclusively secreted by the syncytiotrophoblast [19]. Chorionic villi incubated with $10^6$ trypomastigotes show a severe detachment and destruction of the syncytiotrophoblast (Fig. 3C, arrowheads), while incubation with $10^5$ trypomastigotes induces a minor damage.

### 3.4. *Ex vivo infection of human chorionic villi with T. cruzi induces a selective disorganization and destruction of basal lamina*

Histological sections of chorionic villi incubated with or without $10^5$ and $10^6$ parasites/ml respectively, were stained with the histochemical PAS method (Fig. 4). PAS reagent is mainly used for staining structures containing a high proportion of carbohydrate macromolecules (glycogen, glycoprotein, proteoglycans), typically found in connective tissues and basal lamina [20]. Basal lamina is part of the placental barrier, so the parasites have to cross this structure to invade the connective fetal tissue of villous stroma containing capillaries. In control chorionic villi (Fig. 4A, C) a strong PAS staining can be observed in the basal lamina between the trophoblast and the fetal connective tissue of villous stroma and around the fetal endothelium as well. As a control of the method, sections of chorionic villi were incubated with α-amylase previous to the PAS method; this treatment decreases the PAS staining (Fig. 4B). In chorionic villi infected *ex vivo* with the parasite (Fig. 4D, E) a general decrease of the PAS staining is observed, which is more evident in the basal lamina (arrows). In chorionic villi incubated with $1 \times 10^6$ trypomastigotes more severe damage to the tissue can be observed, evidenced by detachment of syncytiotrophoblast and a more homogenous PAS staining of the fetal connective tissue of villous stroma (Fig. 4E). During vertical transmission, pathogens must reach fetal capillaries, crossing the different basal lamina. It is highly probable that *T. cruzi* destroys these structures during tissue invasion.

*T. cruzi* presents surface molecules that can bind to laminin [21–23], fibronectin [21] and heparan sulphate [24]. On the other hand, the parasite secretes proteases as cruzipain which can degrade collagen I, IV and fibronectin [12]. Considering these data, we analyzed more specifically some of the basal lamina components, such as the glycoproteins laminin and fibronectin, the proteoglycan heparan sulphate and collagen IV.

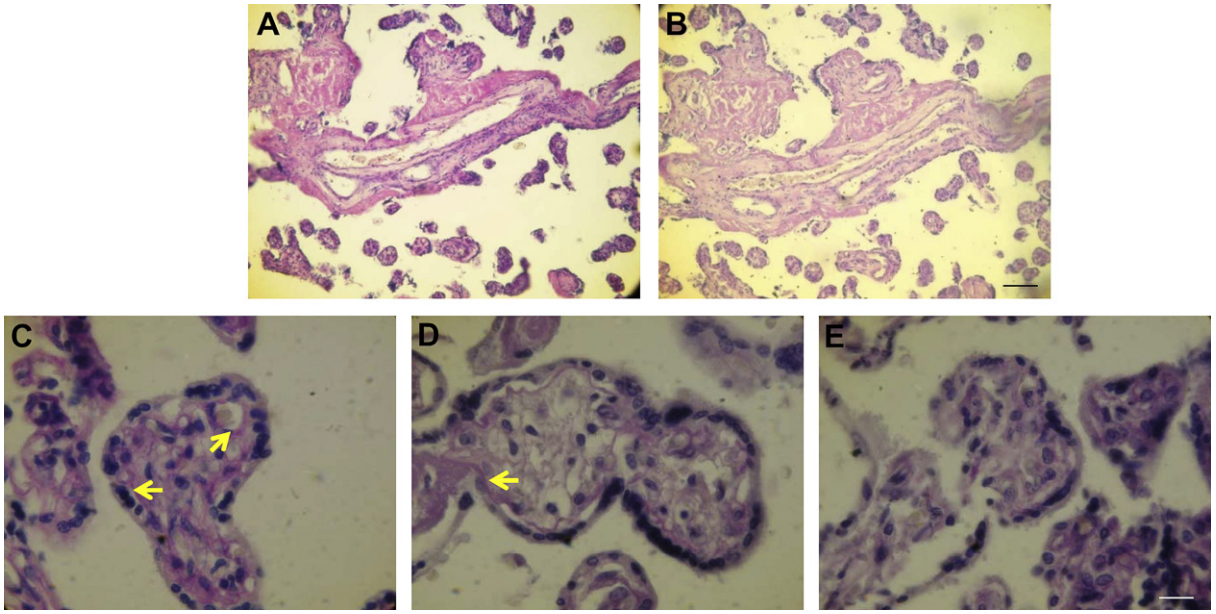


**Fig. 4.** *T. cruzi induces basal lamina disorganization in human chorionic villi*: Chorionic villi incubated with $10^5$ (D) or $10^6$ (E) trypomastigotes DM28c strain for 24 h were stained with the PAS method for the detection of glycosylated components. Chorionic villi incubated with the parasite show a severe parasite-concentration dependent damage of the tissue as compared to control villi (A, C) and less staining for glycosylated molecules (D, E). A and B shows control chorionic villi, where B was treated with α-amylase (4 μg/ml) for 30 min at 37 °C previous the PAS reaction. Arrows indicates basal lamina positive for PAS staining. Bar scales: 50 μm (A, B), 25 μm (C, D).

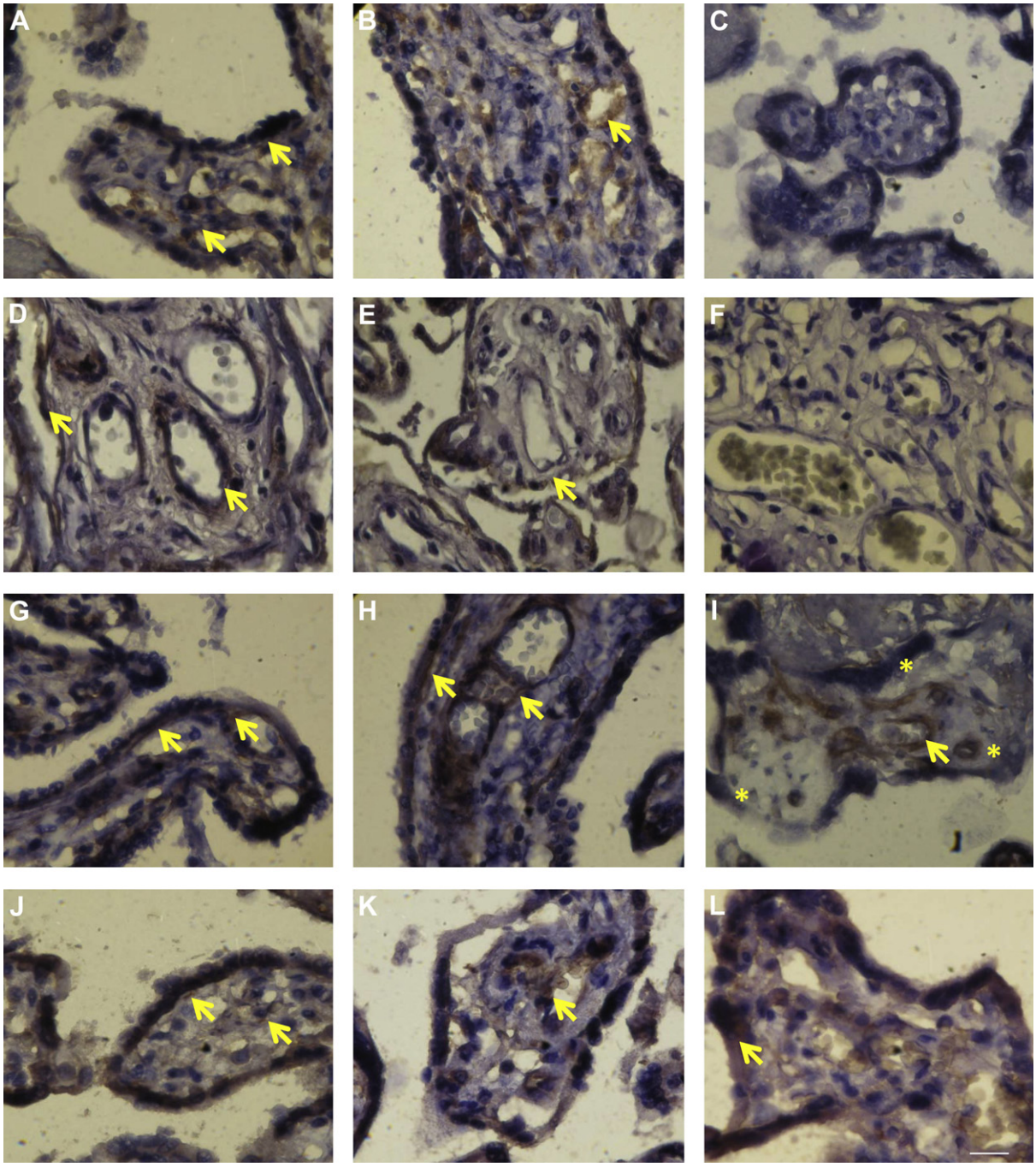


**Fig. 5.** *T. cruzi induces selective basal lamina disorganization in human chorionic villi*: Chorionic villi incubated with $10^5$ (B, E, H, K) or $10^6$ (C, F, I, L) trypomastigotes DM28c strain for 24 h were immunostained for heparan sulphate (A, B, C), laminin (D, E, F) collagen IV (G, H, I) and fibronectin (J, K, L). Yellow arrows indicate the immunodetection of the different antigens. Chorionic villi incubated with $10^5$ trypomastigotes show a decrease in immunoreactivity for heparan sulphate (B) and laminin (E), which is completely lost in the chorionic villi incubated with $10^6$ parasites (C, F). Chorionic villi incubated with $10^6$ trypomastigotes also show less immunoreactivity for collagen IV in the basal lamina between syncytiotrophoblast and fetal connective tissue, but not around fetal endothelium (yellow asterix). No difference in immunoreactivity for fibronectin is observed between control and infected chorionic villi (J, K, L). Chorionic villi were processed for routine immunohistological techniques and counterstained with Mayer's hematoxylin. Bar scale: 25 μm.

Histological sections of chorionic villi incubated with or without $1 \times 10^5$ and $1 \times 10^6$ parasites/ml were immunostained for heparan sulphate (Fig. 5B, C), laminin (Fig. 5E, F), collagen IV (Fig. 5H, I) and fibronectin (Fig. 5K, L). Fig. 5A, D, G and J are the corresponding controls. Chorionic villi incubated with $10^5$ trypomastigotes shows a decrease in immunoreactivity for heparan sulphate (Fig. 5B, arrows) and laminin (Fig. 5E, arrows), which is completely lost in the chorionic villi incubated with $10^6$ parasites (Fig. 5C, F). The immunoreactivity for collagen IV in the basal lamina located between the syncytiotrophoblast and the fetal connective tissue is also lost when the chorionic villi were incubated with $10^6$ trypomastigotes (Fig. 5I, asterix), but not arround fetal endothelium (Fig. 5I, arrow). Interestingly, no differences in immunoreactivity for fibronectin are observed between control and infected chorionic villi (Fig. 5J, K, L). Probably, during tissue invasion, *T. cruzi* first binds to molecules of the basal lamina, facilitating internalization to the different cells in the chorionic villi and particularly those of the connective tissue. Additionally, the parasite may secrete proteases

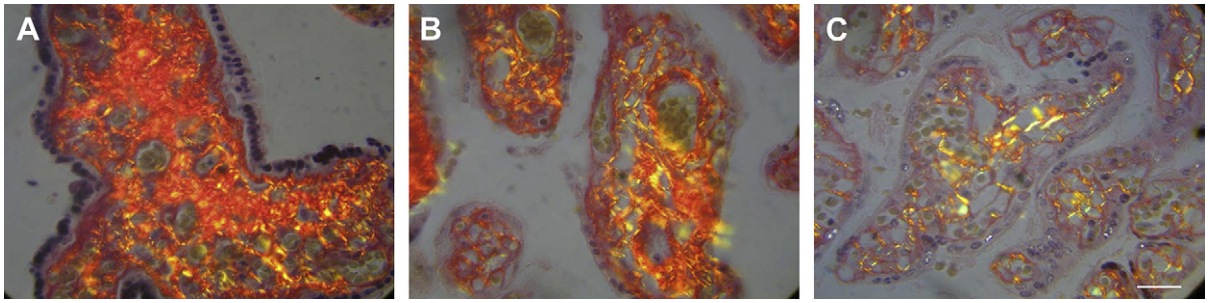


**Fig. 6.** *T. cruzi induces collagen I disorganization in chorionic villl connective tissue of villous stroma*: Chorionic villi incubated with $10^5$ (B) or $10^6$ (C) trypomastigotes DM28c strain for 24 h were stained with picro-sirius red. Chorionic villi incubated with the parasites (B and C) show less normal birefringency of collagen I fibers compared to control chorionic villi (A). Chorionic villi were processed for routine histological techniques and stained with picro-sirius red. Bar scale: 25 μm.

which can degrade the basal lamina and permit the spreading of the parasite into the tissue.

### 3.5. *Ex vivo T.cruzi infection of human chorionic villi induces disorganization of collagen I in connective tissue of villous stroma*

One of the principal fibrous components of chorionic villi connective tissue of villous stroma is collagen I [6]. It is known that the parasite secretes the protease cruzipain, which can degrade collagen I [12]. Histological sections of chorionic villi incubated with or without $10^5$ or $10^6$ parasites/ml were stained with the histochemical method picro-sirius red–hematoxylin to detect collagen I (Fig. 6). Chorionic villi incubated with the parasites show a severe disorganization of collagen I fibers in the connective tissue of villous stroma, evidenced by the loss of their normal birefringence with the picro-sirius red staining (Fig. 6B, C). The collagen disorganization and destruction is more severe in chorionic villi infected with $1 \times 10^6$ trypomastigotes (Fig. 6C).

## 4. Discussion

Congenital *T. cruzi* infection is associated with premature labor, low birth weight and stillbirth [7,25]. It has been suggested that the parasite reaches the fetus by crossing the placental barrier [3,7]. Nevertheless, knowledge of the cellular and molecular mechanisms of transplacental infection is scarce [1]. It is thought that congenital Chagas' disease is a product of a complex interaction between the maternal immune response, placental factors, and the characteristics of the parasite [3].

*Ex vivo* infection of human chorionic villous explants constitutes an adequate model to study the tissue damage caused by *T. cruzi* during vertical transmission [13,14,25]. These studies used parasite concentration ($1 \times 10^6$ parasites per ml), that are unlikely to be found even in the highest human parasitaemia. Therefore, as a first approach it was important to establish a more "physiological" condition for *ex vivo* infection of human chorionic villi by *T. cruzi*. Interestingly, PCR analysis showed presence of parasitic DNA in the chorionic villi when a concentration as low as $10^4$ trypomastigotes/ml was used for infection. However, damage to the tissue was evident when concentrations of $10^5$ trypomastigotes/ml or higher were used for *ex vivo* infection.

During congenital infection the first fetal cells exposed to the parasite are those of the syncytiotrophoblast. In human chorionic villi infected *ex vivo* the parasite induces detachment and destruction of this tissue layer (Figs. 4 and 5). Other pathogens like cytomegalovirus [26], *Plasmodium falciparum*[27] and *Toxoplasma gondii*[28] also induce syncytiotrophoblast damage. Probably, the detachment and destruction of the tissue in contact with the maternal blood, in which different pathogens circulate, is a defense mechanism to avoid the congenital infection. This could partially explain the low congenital transmission rate in Chagas' disease, that is in the order of 1–7% [1].

After invading trophoblast cells, the basal lamina, a specialized structure of ECM molecules located between trophoblast and the fetal connective tissue, is the next barrier for the parasite. *T. cruzi* presents surface molecules, such as gp85 [22] and gp83 [23] glycoproteins that bind to laminin and fibronectin [22,23] and to sulfated glycosamineglicans such as heparan sulphate [24]. Indeed, the parasite induces a decrease of glycosylated molecules of the basal lamina (Fig. 4), specifically laminin (Fig. 5E, F).

Collagen IV and heparan sulphate, other basal lamina components, are also destroyed by the parasite, as evidenced by the decrease in the immunoreactivity of these macromolecules. The decrease in immunoreactivity could also be explained by a change in the epitope as a result of this binding. Interestingly, fibronectin, another principal basal lamina component, is not altered during *ex vivo* infection. The selective destruction of the basal lamina could be part of the mechanism of connective tissue invasion, after an effective epithelial infection. Basal lamina is also present between the fetal endothelium and the connective tissue. This basal lamina is the last barrier that any pathogen should cross to reach fetal circulation. As expected, *T. cruzi* trypomastigotes induce a similar decrease of laminin and heparan sulphate in the basal lamina located around the fetal capillaries, as compared to that observed in the basal lamina beneath the trophoblast.

Collagen IV is an exception to this since no change is observed. Possibly, the destruction of collagen IV around fetal endothelium is not necessary for the parasite invasion of fetal capillaries or occurs at a later time. In other studies, an increase of laminin expression in cardiac tissue has been reported [12,22]. The increase of laminin expression could be induced by the parasite, which needs to attach to ECM molecules for cellular invasion [22]. The silencing of the laminin gene inhibits cell invasion of the *T. cruzi*[23]. The parasitic protease cruzipain degrades collagen IV and fibronectin, exposing epitopes to which *T. cruzi* binds [12], facilitating also the binding to laminin and consequently the cell invasion. On the other hand, the breakdown of the ECM facilitates the penetration of the parasite through basal lamina and connective tissue of villous stroma.

Between the trophoblast and fetal capillaries, the fetal connective tissue is another important barrier for the parasite. *Ex vivo* infection of human chorionic villi induces a severe collagen I disorganization as seen by Picrosirius red–hematoxylin staining. The collagen I degradation is probably due to the presence of cruzipain that degrades this type of ECM component [12]. In other tissue, specifically the lamina propria of seminiferous tubules in mice, *T. cruzi* infection also induces collagen I disorganization [29]. Other enzymes which may participate in collagen I destruction are the metalloproteinases MMP-2 and MMP-9. These proteases

are induced by *T. cruzi* in the myocardium of mice with acute Chagas' disease; and its inhibition reduces myocarditis and improves survival during the acute phase of infection [30]. Studies of the levels and activity of these enzymes and that of their inhibitors in the *T. cruzi* infected chorionic villi are in progress. Another important point is that collagen I constitutes a basic component of the tri-dimensional network of ECM, formed by different types of collagen and elastic fibers, proteoglycans and glycoproteíns. If the "basic skeleton" of the ECM is destroyed, the normal conformation of ECM is disorganized, a condition which may facilitate the mobilization of the parasite inside the tissue to its target. Additionally, it has been proposed that ECM alterations produced by the parasite's presence not only promote its motility in tissues and its entrance into cells, but also alter the presence of cytokines and chemokines, which in turn permits *T. cruzi* to modulate and escape both the inflammatory response and the immune response [22].

Our results confirm that the *ex vivo* infection of human chorionic villi with trypomastigotes is an excellent tool, not only for studying the mechanism of congenital infection, but also for understanding the invasion process in human tissue. In this paper we show for the first time tissue alterations, especially those of basal lamina and collagen I in villous stroma, during placental infection with *T. cruzi*. Additionally, a mechanism for the movement of *T. cruzi* trypomastigotes from maternal to fetal blood is proposed.

## Acknowledgements

This study was supported by grants 11080166 (to UK), 1090124 (to NG) and 1090078 (to JM) from FONDECYT and CONICYT-PBCT Anillo ACT 112, Chile. We are indebted to Dr. Catherine Connelly and to Dr. Henning Schneider for corrections and suggestions.

## References

[1] World Health Organization. Control of Chagas' disease. Report of the WHO Expert Committee. World Health Organ Tech Rep Series 2002;905.
[2] Schmunis GA. Epidemiology of Chagas disease in non-endemic countries: the role of international migration. Mem Inst Oswaldo Cruz 2007; 30(102 Suppl. 1):75–85.
[3] Carlier Y. Factors and mechanisms involved in the transmission and development of congenital infection with *Trypanosoma cruzi*. Rev Soc Bras Med Trop 2005;38(Suppl. 2):105–7.
[4] Lescure FX, Canestri A, Melliez H, Jauréguiberry S, Develoux M, Dorent R, et al. Chagas disease, France. Emerg Infect Dis 2008;14(4):644–6.
[5] Blanco SB, Segura EL, Cura EN, Chuit R, Tulian L, Flores I, et al. Congenital transmission of *Trypanosoma cruzi*: an operational outline for detecting and treating infected infants in northwestern Argentina. Trop Med Int Health 2000;5:293–301.
[6] Berniscke K, Kaufmann P, Baergen RN. Pathology of the human placenta. New York; 2006. 1069 pp.
[7] Bittencourt AL. Congenital Chagas' disease. Am J Dis Child 1976;130:97–103.
[8] Yoshida N. Molecular basis of mammalian cell invasion by *Trypanosoma cruzi*. An Acad Bras Cienc 2006;78(1):87–111.
[9] Andrews NW. Lysosomes and the plasma membrane: trypanosomes reveal a secret relationship. J Cell Biol 2002;158(3):389–94.
[10] Yoshida N. *Trypanosoma cruzi* infection by oral route how the interplay between parasite and host components modulates infectivity. Parasitol Int 2008;57(2):105–9.
[11] Santana JM, Grellier P, Schrével J, Teixeira AR. A *Trypanosoma cruzi*-secreted 80 kDa proteinase with specificity for human collagen types I and IV. Biochem J 1997;325(Pt 1):129–37.
[12] Scharfstein J, Morrot A. A role for extracellular amastigotes in the immunopathology of Chagas disease. Mem Inst Oswaldo Cruz 1999;94(Suppl. 1):51–63.
[13] Mezzano L, Sartori MJ, Lin S, Repossi G, de Fabro SP. Placental alkaline phosphatase (PLAP) study in diabetic human placental villi infected with *Trypanosoma cruzi*. Placenta 2005;26(1):85–92.
[14] Sartori MJ, Mezzano L, Lin S, Repossi G, Fabro SP. Cellular components and placental alkaline phosphatase in *Trypanosoma cruzi* infection. Rev Soc Bras Med Trop 2005;38(Suppl. 2):87–91.
[15] Salas C, Tapia RA, Ciudad K, Armstrong V, Orellana M, Kemmerling U, et al. *Trypanosoma cruzi*: activities of lapachol and alpha- and beta-lapachone derivatives against epimastigote and trypomastigote forms. Bioorg Med Chem 2008;16(2):668–74.
[16] Contreras VT, Salles JM, Thomas N, Morel CM, Goldenberg S. In vitro differentiation of *Trypasonosoma cruzi* under chemically defined conditions. Mol Biochem Parasitol 1985;16:315–27.
[17] Villalta F, Kierszenbaum F. Growth of isolated amastigotes of *Trypasonosoma cruzi* in cell-free medium. J Protozool 1982;29:570–6.
[18] Junqueira LC, Bignolas G, Brentani RR. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J 1979;11(4):447–55.
[19] Osafo J, Wei Y, Kenth G, Goodyer CG. Growth hormone during development. Endocr Metab Disord 2005;6(3):173–82.
[20] Burck HC. Histologische Technik: Leitfaden für de Herstellung mikroskopischer Präparate in Unterricht und Praxis. 5rd ed. Stuttgard-New York: Georg Thieme Verlag; 1982.
[21] Giordano R, Fouts Dl, Tewari D, Colli W, Manning JE, Alves MJM. Cloning of a surface membrane glycoprotein specific for the infective form of *Trypanosoma cruzi* having adhesive properties to laminin. J Biol Chem 1999;274:3461–8.
[22] Marino AP, Silva AA, Pinho RT, Lannes-Vieira J. *Trypanosoma cruzi* infection: a continuous invader–host cell cross talk with participation of extracellular matrix and adhesion and chemoattractant molecules. Braz J Med Biol Res 2003;36(8):1121–33.
[23] Nde PN, Simmons KJ, Kleshchenko YY, Pratap S, Lima MF, Villalta F. Silencing of the laminin gamma-1 gene blocks *Trypanosoma cruzi* infection. Infect Immun 2006;74(3):1643–8.
[24] Lima AP, Almeida PC, Tersariol IL, Schmitz V, Schmaier AH, Juliano L, et al. Heparan sulfate modulates kinin release by *Trypanosoma cruzi* through the activity of cruzipain. J Biol Chem 2002;277(8):5875–81.
[25] Shippey III SH, Zahn CM, Cisar MM, Wu TJ, Satin AJ. Use of the placental perfusion model to evaluate transplacental passage of *Trypanosoma cruzi*. Am J Obstet Gynecol 2005;192(2):586–91.
[26] Chan G, Guilbert LJ. Ultraviolet-inactivated human cytomegalovirus induces placental syncytiotrophoblast apoptosis in a Toll-like receptor-2 and tumour necrosis factor-alpha dependent manner. J Pathol 2006;210(1):111–20.
[27] Maubert B, Guilbert LJ, Deloron P. Cytoadherence of *Plasmodium falciparum* to intercellular adhesion molecule 1 and chondroitin-4-sulfate expressed by the syncytiotrophoblast in the human placenta. Infect Immun 1997; 65(4):1251–7.
[28] Abbasi M, Kowalewska-Grochowska K, Bahar MA, Kilani RT, Winkler-Lowen B, Guilbert LJ. Infection of placental trophoblasts by *Toxoplasma gondii*. J Infect Dis 2003;188(4):608–16.
[29] Pereira-Carvalho LO, Abreu-Silva AL, Hardoim Dde J, Tedesco RC, Mendes VG, da Costa SC, et al. *Trypanosoma cruzi* and myoid cells from seminiferous tubules: interaction and relation with fibrous components of extracellular matrix in experimental Chagas' disease. Int J Exp Pathol 2009;90(1):52–7.
[30] Gutierrez FR, Lalu MM, Mariano FS, Milanezi CM, Cena J, Gerlach RF, et al. Increased activities of cardiac matrix metalloproteinases matrix metalloproteinase (MMP)-2 and MMP-9 are associated with mortality during the acute phase of experimental *Trypanosoma cruzi* infection. J Infect Dis 2008;197(10):1468–76.