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Histological and morphometric investigations of changes induced by the RAL strain of *Trypanosoma cruzi* in the mouse placenta

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Pregnant Swiss mice (Mus musculus) were inoculated intraperitoneally with 2 x 10^5 trypomastigotes of the RAL strain of Trypanosoma cruzi on the 7^{th} day of pregnancy and sacrificed on the 19^{th} day of pregnancy. The placenta was sectioned for the assessment of histological and morphometric changes. The RAL strain showed intense tropism for the placenta, with parasitism reaching the three placental layers. There was involvement of the maternal and fetal portions of the placentas, and also of giant cells and spongioblasts. The placentas of infected animals presented sparse areas of degeneration and necrosis, with mild dystrophic calcification of the decidua. The inflammatory process consisted of plasmocytes and lymphocytes, revealing involvement of the decidua. Cytometric study of giant trophoblastic cells showed that the placentas of the infected group were seriously affected, also with respect to cell volume. The changes provoked by the RAL strain in the trophoblastic cells and the difference in behavior observed in the cell population of the various placental regions may affect intrauterine development, probably by a deficient production of hormones such as placental lactogen, which acts as a fetal growth hormone, or indirectly by deficient tissue invasion caused by inefficient utero-placental vascularization, thus impairing fetal nutrition.

Uniterms:
- *Trypanosoma cruzi*
- Mouse placenta
- Histopathology
- Morphometry

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INTRODUCTION

Infection with *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, is a serious public health problem in most Latin American countries. About five million people are estimated to be infected by *T. cruzi* in Brazil and 25 million live in risk areas (Dias, 1987).

Although campaigns for the control of triatomines has reduced the vectorial transmission of Chagas' disease in Brazil (Dias *et al.*, 2002), congenital transmission continues to occur. The rate of congenital transmission varies from country to country, but it is generally accepted that

2-3% of children born to infected mothers will also be infected (Dias, 1992).

Congenital transmission frequently occurs through colonization of the placenta by the parasite and its incidence varies according to geographic area and parasite strain. Experimental congenital transmission of Chagas' disease has been demonstrated in acutely infected animals such as guinea pigs, rabbits, dogs, rats, and mice.

A marked characteristic of *T. cruzi* is its biological heterogeneity (Andrade, 1982, 1999). Its biological and genetic traits show a marked polymorphism in natural populations, which differ in terms of their infecting behavior of vertebrate hosts.

In a study of the influence of different parasite populations on placental infection in mice, Andrade (1982) observed that placental parasitism was quite high for the Columbia strain and low for the Y strain, and also demonstrated that the strains of the parasite play an important role in congenital chagasic infection.

In view of the importance of transplacental Chagas' disease and of the different behavior of different parasite populations, the objective of the present study was to assess the morphological changes occurring in mouse placentas and to establish a quantitative profile of the lesions produced by the RAL strain of *T. cruzi*.

MATERIAL AND METHODS

Mice

Nulliparous 60-day-old Swiss mice (*Mus musculus*) weighing 30-35 g, kept in plastic boxes, were mated by placing one male and three females in each box. The animals had free access to standard ration and water. Ten animals were used for each experimental group. All animals were killed by cervical dislocation on the 19th day of pregnancy.

T. cruzi strain

The RAL strain of *T. cruzi*, isolated from *Triatoma infestans* in Brazil by Ribeiro *et al.* (1993), was kept by serial passages through Swiss mice at 12 day intervals. This strain proved to be highly pathogenic to mice and was characterized as lineage 1 (Dost *et al.*, 2002). Ten pregnant mice were inoculated intraperitoneally with 2 x 10^5 *T. cruzi* trypomastigotes on the 7th day of pregnancy.

DISSECTION

After a wide opening of the abdomen, the uterus with the placentas were collected and immersed in ALFAC fixative solution for 24 hours. Five placentas were collected at random from each experimental group.

Histology

After fixation, the placentas were cut lengthwise, dehydrated, cleared, and embedded in paraffin. Serial 6 mm sections were obtained and stained with hematoxylin and eosin.

Morphometry

The nuclei of the giant cells from the placentas were assessed morphometrically by measuring their larger (D) and smaller (d) diameters. The sections were examined with a Jenamed microscope fitted with a Jena light camera and 50 nuclei per animals were measured (Sala *et al.*, 1994).

Stereology

We used a Merz grid printed on paper which consists of a square limiting a test area and containing a system of points marked on sinuous lines formed by a succession of chained semicircles. The sections were examined under a Jenamed light microscope fitted with a Jenamed light camera. The relative volumes were determined by the number of points that fell on the histological structures in relation to the total number of points in the test area (Chalkley, 1943; Weibel, 1969).

Relative volume of the placental regions and of the giant cells

To assess the placental regions and giant cells on a percent basis we used the technique of Chalkley (1943) according to the following formula:

$$Vv = (Pn + Pct)/Pt$$

Where Pn are the points counted in the nucleus of the structure, Pct are the points counted in the cytoplasm, and Pt are the total points.

Absolute volume of the placental regions and of the giant cells

Absolute volume was calculated by the following formula:

$$V = \frac{Vv \,.\, W}{Wv}$$

Where Vv is the relative volume, W is the weight of the placenta, and Wv is the specific weight of the placental tissue.

Relative nuclear volume

Relative nuclear volume was calculated by the following formula:

$$Vvn = Pn/(Pn + Pct) \,.\, [2M/(2M + 3t)]$$

Where Pn and Pct are number of points falling on the cell nucleus and cytoplasm, respectively, M is the mean nuclear diameter, and t is the thickness of the histological section.

Relative cytoplasm volume

Relative cytoplasm volume was calculated by the following formula:

$$Vvct = 1 - Vvn$$

Where Vvn is the relative nuclear volume.

Nucleus/cytoplasm ratio

The nucleus/cytoplasm ratio was calculated by the following formula:

$$N/C = \frac{Vvn}{Vvcyt}$$

Cytoplasm volume and mean giant cell volume

The above parameters were calculated by the following formulas:

$$Vct = \frac{Vn}{N/C}$$

$$Vcell = Vn + Vct$$

Where Vn is the nuclear volume and Vct is the cytoplasm volume.

Numerical density of giant cells

The numerical density of giant cells was calculated by the following formula:

$$Nv = (Vv/Vcell) \,.\, 10^9$$

Number of giant cells per placenta

The number of giant cells per placenta was calculated by the following formula:

$$Np = (Nv \,.\, W/Wv)$$

STATISTICAL ANALYSIS

Several computer programs developed at the Dental School of Ribeirão Preto, USP, for the processing of experimental data were used for the mathematical calculations involved in the morphometric studies. Data were analyzed statistically by the nonparametric Wilcoxon-Mann-Whitney test (Conover, 1999).

RESULTS

Histopathology

Decidua

The decidua was found to be intensely parasitized, even in endothelial cells. A sparse inflammatory infiltrate was observed, consisting of plasmocytes and rare lymphocytes, with sparse areas of degeneration and coagulation necrosis. The endothelial cells of the vessels were increased in volume, with nuclei showing prominent nucleoli and homogeneous hyaline material marking and thickening the contour of their walls. The vessels were mildly ectatic and congested. Mild dystrophic calcification was observed in the areas of degeneration and necrosis. Figure 1A shows the general aspect of the decidua with areas of necrosis, and Figure 1B shows a decidual vessel with endothelial parasitism.

Spongy layer

The giant cells, mainly arranged between the decidua and the spongy layer, presented amastigote nests in their cytoplasm (Figure 1C). The spongy layer showed intense intracellular edema and a milder intercellular edema. The spongioblasts showed hyperchromatic nuclei and a more eosinophilic cytoplasm, especially in the more central area of the layer (degeneration). The vessels presented endothelial cells of increased volumes with clearly visible nucleoli, causing a thickening of the vessel contours with proteinaceous, homogeneous hyaline material and congestion of the lumens. Parasitism was mild, represented by small nests, with larger nests in the spongioblasts that invaginated into the labyrinth layer, at times containing large nests (Figure 1D).

Labyrinth layer

The labyrinth layer presented intense parasitism with large amastigote nests occupying the endothelial cells, without an inflammatory reaction. The capillaries were ectatic and congested, at times distorted, losing their

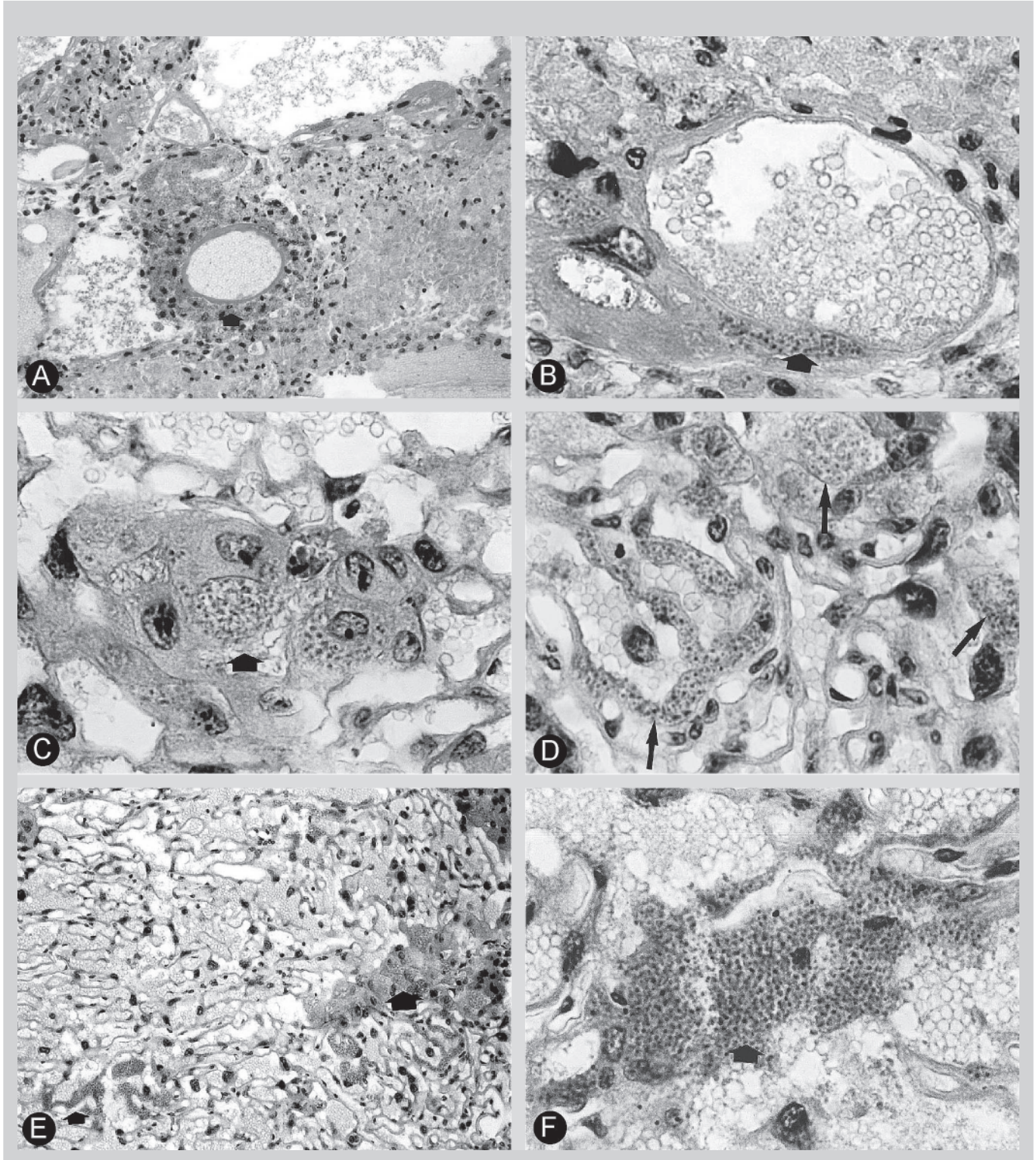

FIGURE 1 – Histological sections of the placenta of a mouse infected with the RAL strain of *T. cruzi*. Hematoxylin and Eosin. **A** – General aspect of the decidua with fibrinoid tissue on the vessel wall (arrow) and areas of necrosis (250 X); **B** – Decidual vessel presenting endothelial parasitism (arrow) (950 X); **C** – Giant cells with amastigote nests (arrow) (950 X); **D** – Spongy layer with amastigote nests (arrows) (950 X); **E**, **F** – Labyrinth layer with amastigote nests in the trophoblast (arrows) (250 X and 950 X, respectively).

normal stratification. The contours of the capillaries were well marked and thickened with proteinaceous, homogeneous hyaline material. The epithelium of the vitellin sac presented intracellular edema and ectatic and congested vessels. Figures 1E and 1F show the labyrinth layer with amastigote nests in the trophoblast.

Table I presents data about the inflammatory process in the placentas of control mice and of mice infected with the RAL strain of *T. cruzi*.

Stereologic results

The relative and absolute volumes of the placenta occupied by the decidua and spongy and labyrinth layers in the study groups are presented in Table II. Table III shows the stereologic parameters of giant cells.

TABLE I - Inflammatory process in the placentas of control mice and of mice infected with the RAL strain of *T. cruzi*

Structures	Control Group	Infected Group
Layers involved	Decidua	Decidua
Inflammatory infiltrate	Neutrophils, lymphocytes	Plasmocytes, lymphocytes
Edema in the layers	(Absent to mild) Decidua	Decidua, spongy and labyrinth
Degeneration, necrosis, calcification	(Absent to mild) Decidua	Decidua
Giant cells	Preserved	Mildly degenerated

TABLE II – Stereologic parameters of the placenta of control animals (C) and of animals infected with the RAL strain of *T. cruzi* (I). Nonparametric Wilcoxon-Mann-Whitney test

Parameters Studied	Control group	Infected group	Calculated U	p |U|
Relative decidua volume (%)	9.04	8.24	7^{ns}	0.155
Relative spongy layer volume (%)	29.64	26.69	4^{*}	0.048
Relative labyrinth layer volume (%)	61.32	65.08	6^{ns}	0.111
Absolute decidua volume (mm^3)	11.59	5.60	0^{**}	0.004
Absolute spongy layer volume (mm^3)	38.07	17.77	0^{**}	0.004
Absolute labyrinth layer volume (mm^3)	78.62	43.25	0^{**}	0.004

* $p < 0.05$; ** $p < 0.01$; ^{ns} nonsignificant

TABLE III - Stereologic parameters of the giant cells of placentas from control animals (C) and from animals infected with the RAL strain of *T. cruzi* (I). Nonparametric Wilcoxon-Mann-Whitney test

Parameters Studied	Control group	Infected group	Calculated U	p |U|
Relative nuclear volume (%)	16.39	16.83	11^{ns}	0.421
Relative cytoplasm volume (%)	83.61	83.17	11^{ns}	0.421
Nucleus/cytoplasm ratio	0.1964	0.2046	11^{ns}	0.421
Relative volume of giant cells (%)	1.78	0.49	0^{**}	0.004
Absolute volume of giant cells (mm^3)	2.27	0.32	0^{**}	0.004
Cytoplasm volume (mm^3)	18213.70	7596.30	0^{**}	0.004
Nuclear volume (μm^3)	3512.19	1467.65	0^{**}	0.004
Mean cell volume (μm^3)	21725.89	9063.96	0^{**}	0.004
Numerical density (n^o/mm^3)	833.83	580.96	4^{*}	0.048
Number of giant cells/placenta	105717.20	37649.85	0^{**}	0.004

* $p < 0.05$; ** $p < 0.01$; ^{ns} nonsignificant

DISCUSSION

Although Chagas' disease involves a low frequency of transmission from mother to fetus, *Trypanosoma cruzi* can invade placental tissue. The lesions may be mild (a sparse inflammatory infiltrate containing or not amastigotes) or serious (destruction of placental tissue. In human congenital Chagas' disease these lesions may be located in the decidua, amniochorionic plate, chorionic villi, and umbilical cord (Bittencourt *et al.*, 1991). In mice, these lesions may be located in the decidua, spongy and labyrinthic regions.

The body weight and length of the fetuses whose mothers were infected with the RAL strain of *T. cruzi* were reduced compared to control and the fetuses did not present tissue parasitism (Hermoso *et al.*, 2001). Retarded fetal growth associated or not with congenital infection has been reported to occur in chronically infected mice (Carlier *et al.*, 1987; Gonzalez *et al.*, 1999). Hermoso *et al.* (2001) also observed that placental diameter was reduced in animals infected with the RAL strain of *T. cruzi*.

Delgado and Santos-Buch (1978) reported the inherent ability of certain *T. cruzi* strains to infect the fetus inside the uterus. Transplacental transmission of the parasite depends on the tropism and pathogenicity of the strains. Data have indicated that placental phagocytosis is one of the factors that prevent the transmission of pathogenic strains to the fetus. The immunological competence of placental tissues is also an important factor in experimental congenital Chagas' disease.

In the present study, the RAL strain of *T. cruzi* (lineage 1) showed a high capacity of penetration of placental tissues, with intense parasitism reaching the three placental layers. There was involvement of the maternal and fetal portion of the placenta and of giant cells and spongioblasts.

Meneguette (1997) observed differences in the placental parasitism produced in mice by different *T. cruzi* strains during the acute phase of infection. Animals infected with the Bolivia (lineage 2) and RC (lineage 1) strains of *T. cruzi* presented high tropism for mouse placental cells, whereas animals inoculated with the Y strain (lineage 1) presented lower placental parasitism. These results indicate that the invasive ability of the different strains is not related to parasite lineage. It seems that several factors are involved in the biological behavior of different *T. cruzi* strains, e.g., the mainly predominant morphological form of the parasite isolates.

In a histological study of the placenta of mice infected with the Tehuantepec strain, Mjihdi *et al*. (2002) detected nests of amastigotes mainly located in the decidua, although some were also observed in the spongy area and, more rarely, in the junction between the labyrinthic and spongy regions. However, these investigators did not observe parasites in the fetal portion of the placenta.

The placentas of animals infected with the RAL strain of *T. cruzi* presented sparse areas of degeneration and necrosis, with mild dystrophic calcification in the decidua. The inflammatory process consisted of plasmocytes and lymphocytes, revealing involvement of the decidua. The giant cells of the placentas of animals infected with the RAL strain showed little degeneration.

Stereology showed a significant reduction in the relative volume occupied by the spongy region of the placentas of animals infected with the RAL strain of *T. cruzi*. In addition, the absolute volumes occupied by the three placental regions were significantly reduced in the infected group, probably due to the vascular alteration caused by parasitism of endothelial cells, leading to cell lysis.

Cytometry of the giant trophoblastic cells showed that the placentas from the infected group were more affected, also in terms of cell volume. Thus, the relative volume, absolute volume, numerical density and total number of giant cells were significantly lower in the placentas of this group than in control placentas.

In rodents, giant cells differentiate by endoreduplication and their functions are endocrine secretion and invasion of the maternal decidua. These cells are an exclusive source of placental lactogens I and II (PL-I and PL-II) and in the second half of pregnancy they also synthesize several prolactin-like proteins and a variant of placental lactogen I (PL-Iv) (Soares *et al.*, 1996). PL-I, PL-II and PL-Iv also act on the fetus. Faria *et al.* (1991) demonstrated the cellular origin of placental lactogen I and the PL-I to PL-II transition in the mouse placenta during pregnancy. PL-I was localized in trophoblastic giant cells up to the end of the first half of pregnancy, with these cells starting to express PL-II thereafter.

The changes provoked by the RAL strain in trophoblastic cells and the difference in behavior observed among the cell populations of different placental regions may affect intrauterine development, probably by deficient production of hormones such as placental lactogen, which acts as a fetal growth hormone.

Zybina and Zybina (1996) demonstrated that rat and mouse giant cells have 4c-8c ploidy on the 12th day of pregnancy (some of them 16c-32c), whereas on the 13th-14th day ploidy is 8c-16c (32c-64c in some cases). This increase in ploidy may be important for trophoblast differentiation, permitting invasion of the decidua.

Keighren and West (1993) did not observe higher order polyploidy in giant cells of the trophoblast of the mouse placenta, suggesting that these may be polytene and not polyploid cells. At a given stage of differentiation, giant cells divide into numerous nuclear fragments forming multinucleated cells that rapidly degenerate into nuclear fragments with 1 to 32c ploidy.

In the present study we demonstrated that the nuclear volume of giant cells was significantly smaller in the placentas of animals infected with the RAL strain of *T. cruzi*, possibly reflecting lower ploidy and consequently a change in cell differentiation that impairs invasion of the decidua.

Thus, the functional changes provoked by the RAL strain of *T. cruzi* in trophoblastic giant cells and in spongioblasts may act directly on fetal growth or indirectly by deficient tissue invasion due to inefficient utero-placental vascularization, thus impairing fetal nutrition.

RESUMO

Investigações histológicas e morfométricas das alterações da placenta do camundongo causadas pela cepa RAL de *Trypanosoma cruzi*

*Camundongos suíços prenhes (*Mus musculus*) foram inoculados, intraperitonealmente, com 2 x 10^5 tripomastigotas da cepa RAL de* Trypanosoma cruzi *no 7º dia da prenhez e sacrificados no 19º dia da prenhez. Foram realizados cortes histológicos para avaliar as alterações histológicas e morfométricas das placentas. A cepa RAL mostrou intenso tropismo pela placenta, com parasitismo atingindo as três camadas placentárias. Houve envolvimento da parte materna e fetal das placentas, bem como das células gigantes e dos espongioblastos. As placentas dos animais infectados apresentaram escassas áreas de degeneração e necrose, com calcificação distrófica exígua na decídua. O processo inflamatório era constituído de plasmócitos e linfócitos, revelando comprometimento da decídua. O estudo citométrico das células trofoblásticas gigantes mostrou que as placentas do grupo infectado foram bastante afetadas, inclusive no que se refere ao volume celular. As alterações provocadas pela cepa RAL sobre as células trofoblásticas e a diferença de comportamento observado nas populações celulares das diversas regiões placentárias podem afetar o desenvolvimento intra-uterino, provavelmente por uma produção deficiente de hormônios, tais como o lactogênio placentário, que atua como hormônio de crescimento fetal, ou indiretamente, mediante uma invasão decidual deficiente causada por uma vascularização útero-placentária ineficiente, prejudicando desta maneira a nutrição fetal.*

UNITERMOS: Trypanosoma cruzi. Placenta de camundongo. Histopatologia. Morfometria.

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