HISTOLOGIC AND HISTOMORPHOMETRIC STUDY OF BONE REPAIR UNDER ACUTE *TRYPANOSOMA CRUZI* INFECTION IN RATS

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ABSTRACT

Trypanosoma cruzi (T. cruzi) is an intracellular protozoan pathogen that causes American trypanosomiasis (Chagas disease). The aim of this study was to evaluate the histopathological effects of acute infection by T. cruzi on bone repair.

Wistar rats were used throughout. The animals were assigned to two groups: Control Group (CG n = 20) and Experimental Group (EG n = 20). All the animals were anesthetized, at t_0 the first lower right molar was extracted. The EG animals were inoculated subcutaneously at t_0 with 0.1 mL of 10^5 trypomastigotes of the virulent strain Tulahuen of T. cruzi. The CG animals were administered an equivalent volume of saline solution subcutaneously. The animals in both groups were euthanized at 15 days post-infection and tooth extraction. The mandibles were resected, fixed in formalin solution, radiographed, decalcified and embedded in paraffin. Bucco-lingually oriented sections were obtained at the level of the mesial tooth socket of the first lower molar, and stained with hematoxylin-eosin. Total alveolar volume (TV) and bone volume (TBV/TV) in the apical third of the tooth socket were evaluated histomorphometrically. The histological analysis revealed an alteration in post-extraction bone tissue repair in animals infected by T. cruzi. A reduction in osteogenic activity was observed concomitant with a rise in quiescent and eroded bone surfaces. Histomorphometric evaluation revealed a significant reduction (19%) in total alveolar volume (TV) and bone volume (TBV/TV) (24%) in the apical third of the tooth socket in animals infected with T. cruzi in comparison to non-infected animals ($p<0.05$). The results obtained using this experimental model showed decreased osteogenesis in bone tissue repair under acute Trypanosoma cruzi infection in rats.

Keywords: *alveolar ridge - wound healing - bone - osteogenesis - tooth socket - Trypanosoma cruzi*

ESTUDIO HISTOLÓGICO E HISTOMORFOMÉTRICO DEL EFECTO DE LA INFECCIÓN AGUDA POR *TRYPANOSOMA CRUZI* EN LA REPARACIÓN ÓSEA POST-EXODONCIA

RESUMEN

El Trypanosoma cruzi (T. cruzi) es un protozoario intracelular que causa Trypanosomoniasis Americana (Enfermedad de Chagas). El objetivo del presente trabajo fue el estudio histopatológico del efecto de la infección aguda por Trypanosoma cruzi sobre la reparación del tejido óseo.

Se utilizaron ratas Wistar macho que fueron asignadas a dos grupos: Grupo Control (GC n = 20) y Grupo Experimental (GE n = 20). Los animales de ambos grupos, bajo anestesia general intraperitoneal, fueron sometidos a t_0, a exodoncia del primer molar inferior derecho, en el GE fueron inoculados,a t_0 por vía subcutánea en la región inguinal izquierda con 0.1 mL de 10^5 tripomastigotes de la cepa virulenta Tulahuén de Trypanosoma cruzi. A los animales del GC se les administró el volumen equivalente de solución salina por vía subcutánea. A los animales de ambos grupos se les practicó la eutanasia a los 15 días. Se resecaron las mandíbulas, se fijaron en solución de formol al 10%, se radiografiaron, se descalcificaron y se incluyeron en parafina. Se obtuvieron cortes orientados en sentido vestíbulo-lingual a nivel del alvéolo mesial del primer molar inferior derecho y se colorearon con hematoxilina–eosina para su posterior estudio histológico e histomorfométrico.

Histológicamente se observó una menor actividad osteogénica a expensas de un incremento de las superficies quiescentes y de las superficies erosivas en el GE. En la evaluación histomorfométrica se detectó disminución estadíasticamente significativa del volumen óseo total (19%) y del volumen trabecular en el tercio apical del alvéolo (24%) en el GE con respecto al GC ($p<0.05$). Los resultados obtenidos en este modelo experimental evidencian una disminución de la osteogénesis en la reparación ósea en ratas con infección aguda por Trypanosoma cruzi.

Palabras clave*: reborde alveolar, reparación tisular, hueso, osteogénesis, alvéolo dentario, Trypanosoma cruzi.*

INTRODUCTION

Alveolar bone is a specialized part of the mandibular and maxillary bones that forms the primary support structure for the teeth. Alveolar bone is constantly renewed by modeling and remodeling mechanisms in response to functional demands and local and systemic factors [1,2]. Bone repair is a highly regulated process. All stages of the repair process are controlled by a wide variety of different growth factors and cytokines, and can be derailed by various endogenous and exogenous factors e.g. systemic infection[1-6].

Several species of kinetoplastid protozoa cause major human infectious diseases. *Trypanosoma cruzi* (*T. cruzi*) is an intracellular protozoan pathogen that causes American trypanosomiasis (Chagas disease), an endemic illness that affects several million people in Latin America[7-9]. *T. cruzi* is usually transmitted by infected triatomine vectors[8,10,11]. However, as *T. cruzi* develops a lifelong infection in humans, these people can serve as parasite reservoirs throughout their lifetime. Thus, the risk of congenital and/or horizontal transmission by infected blood transfusion or solid organ transplant may become a major problem in non-endemic regions, increased by the migration of people from endemic areas in South and Central America to developed countries[8,9,12,13].

Trypomastigotes, the mammalian infective forms of *T. cruzi*, are relatively large (~20 mm in length), motile organisms that have the capacity to infect most nucleate cell types. Non-dividing trypomastigotes must establish residence within the host cell cytoplasm and differentiate into amastigotes[9,10]. During the acute phase of the infection, the rupture of amastigote nests provokes destruction of the host cells and triggers inflammatory processes and intense immune responses[8,9,14-21]. Emerging evidence shows that the immune and skeletal systems share a number of regulatory molecules including cytokines, receptors, signaling molecules and transcription factors[22-25]. Therefore it has been suggested that the physiology and pathology of one system may affect the other.

In 2006, Morocoima A. et al. reported invasion in hyaline cartilage cells and bone cells including the marrow of laboratory mice infected with *T. cruzi* isolates from urban and rural areas of Venezuela[26].

There is no study to date on the effect of infection by *T. cruzi* on the bone repair response. Given that the alveolar bone healing after tooth extraction in rats provides a suitable experimental model for the study of bone formation and can be considered a sensitive indicator of bone damage under different experimental conditions[27-33], the aim of this study was to assess the effects of acute infection by *Trypanosoma cruzi* on alveolar bone healing in rats employing histological and histomorphometric evaluation.

MATERIALS AND METHODS

Animals

Forty male Wistar rats (International Laboratory Code Registry: Hsd:Wi-ffyb), 21-25 days old, were used throughout. The animals were not given a special diet. They were fed rat chow and given water *ad libitum,* housed in steel cages and maintained on a 12:12 hour light-dark cycle. All animal experiments were carried out according to the guidelines of the National Institutes of Health for the care and use of laboratory animals (NIH Publication Nº 85-23, Rev. 1985). The protocol was examined and approved by the institutional ethics committee at the School of Dentistry, University of Buenos Aires.

Experimental Procedure

Surgical procedure

The animals were assigned to two groups: Control Group (CG *n* =20) and Experimental Group (EG *n* =20).

All the animals were anesthetized by intraperitoneal administration of a 4:1 solution of ketamine/xylazine (ketamine chlorhydrate, 50 mg/mL, Ketamina 50® Holliday-Scott, Buenos Aires, Argentina) and xylazine, 20 mg/mL (Rompun® Bayer, Buenos Aires, Argentina) at a dose of 0.15 mL per 100 g body weight. At t_0 the first lower right molar was extracted according to the technique described by Guglielmotti et al.[27]

Experimental infection

The EG animals were inoculated subcutaneously (s.c.) in the left inguinal region at t_0, under ether anesthesia, with 0.1 mL of 10^5 trypomastigotes of the virulent strain Tulahuen of *Trypanosoma cruzi* kindly provided by the Institute of Experimental Pathology, School of Health Sciences, National University of Salta. The CG animals were administered an equivalent volume of saline solution subcutaneously.

Parasitaemia
Evaluation was performed by direct detection of parasitaemia (fresh blood observation) in blood samples extracted from the tail vein under anesthesia (10 µL with a heparinized capillary tube) at 7 and 15 days after the initial infection. The samples were placed between a glass slide and coverslip and examined by light microscopy. The number of parasites in 100 fields was counted with a x40 objective.

Haematological parameters
The values of haematocrit (Htc) and haemoglobinemia (Hb) were determined at baseline (t_0) and 7 and 15 days post-initial infection.
The animals in both groups were euthanized 15 days post-infection and tooth extraction. The mandibles were resected, fixed in 10% formalin solution and radiographed.

Histological processing
The mandibles were decalcified in 5% formic acid, embedded in paraffin, and semi-serially sectioned, at the level of the mesial tooth socket of the first lower right molar, in a frontal plane (bucco-lingual direction) at 10 mm thickness and stained with hematoxylin-eosin.

Histomorphometric Evaluation
Total Alveolar Volume
Total alveolar volume (TV) was considered as the bone tissue and its marrow spaces situated above line *a* drawn tangential to the upper cortical border of the mandibular canal and perpendicular to the external surface of the buccal plate[30].

Bone volume in the apical third of the tooth socket
Bone volume (TBV/TV) was considered as the ratio between the trabecular volume (TBV) and the total volume (TV), measured in the apical third of the socket as previously reported [30].
The following parameters were determined in the apical third of the tooth socket: percentage of osteoblast surface (Ob.S), eroded surface (ES), and quiescent surface (QS). Osteoblast surfaces are covered with osteoid seams and mature osteoblasts. Eroded surfaces are scalloped with Howship's lacunae with or without osteoclasts. Quiescent surfaces are covered with bone lining cells.[34]

Histomorphometric determinations were performed on sections using a light microscope (Zeiss Axioscop 2 MOP, Carl Zeiss, Jena, Germany), on line with an image analysis system (Kontron KS300 v. 2, Kontron Elektronik, Münich, Germany).

Statistical Analysis
Student's *t*-test was used for statistical analysis of the data ($p<0.05$). Data are presented as means ± SD.

RESULTS
No immediate or mediate post-operative complications were observed.

Parasitaemia
Parasitaemia (5 ± 2 /100 fields) was detected in the EG at 7 days post-infection. A tendency to negative parasitaemia values was observed at 15 days post-initial infection (1 ± 1 /100 fields).

Haematological Parameters
The EG exhibited an increase (10%) in haematocrit at 7 days post-infection compared to baseline values ($p<0.05$; Table 1). An increase above control values was observed in haematocrit (5%) and haemoglobinemia (33%) at 15 days post-infection ($p<0.05$) (Table 1).

Radiographic Study
The post-extraction tooth sockets of control and experimental animals were filled with radiopaque tissue.

Histological Study
Active osteogenesis evidenced by neoformed trabeculae filling almost the entire tooth socket was observed with light microscopy in control animals

Table 1: Haematological parameters.

		t_0	t_7	t_{15}
Control Group	Htc (%)	39±1	41±1	42±1
(n =20)	Hb (g/dL)	13.2±1	14±1	15±2
Experimental Group	Htc (%)	38±1	45±3*	40±2†
(n =20)	Hb (g/dL)	12.9±1	12.5±1	10±2†

Haematocrit (Htc) and haemoglobinemia (Hb).
Values are means ± SD;
*$p<0.05$ compared to baseline values (t_0),
†$p<0.05$ compared to the corresponding control values.
t_7 and t_{15}: 7 and 15 days post-initial infection, respectively.

15 days post-extraction (Fig. 1A). The woven bone tissue was lined with cuboidal osteoblasts. Full epithelialization of the alveolar ridge was observed.

The tooth socket of experimental animals was filled with woven bone tissue. Trabeculae lined with cuboidal osteoblasts and a predominance of bone lining cells, eroded surfaces and osteoclasts were observed (Fig. 1B). Noticeable presence of eosinophils, granuloma-like structures, with foam cells (macrophages) containing amastigotes were detected between the trabeculae (Fig. 2 A and B).

No difference in the healing of soft tissues lining the alveolar ridge was observed compared to control.

Histomorphometric evaluation

Total Alveolar Volume (TV, in mm²)

The EG exhibited a reduction in total alveolar volume ($1.4 \times 10^6 \pm 2 \times 10^5$) as compared to CG ($1.7 \times 10^6 \pm 2 \times 10^5$). Statistically significant differences were observed between the groups ($p < 0.05$).

Bone Volume in the Apical Third (TBV/TV, in %)

The EG exhibited reduced bone volume in the apical third of the tooth socket (44 ± 10) as compared to control values (58 ± 7). Statistically significant differences were found between the groups ($p < 0.05$).

The EG showed a statistically significant reduction (69%) ($p<0.05$) in the percentage of osteoblast surfaces concomitant with an increase in eroded and quiescent surfaces (Fig. 3).

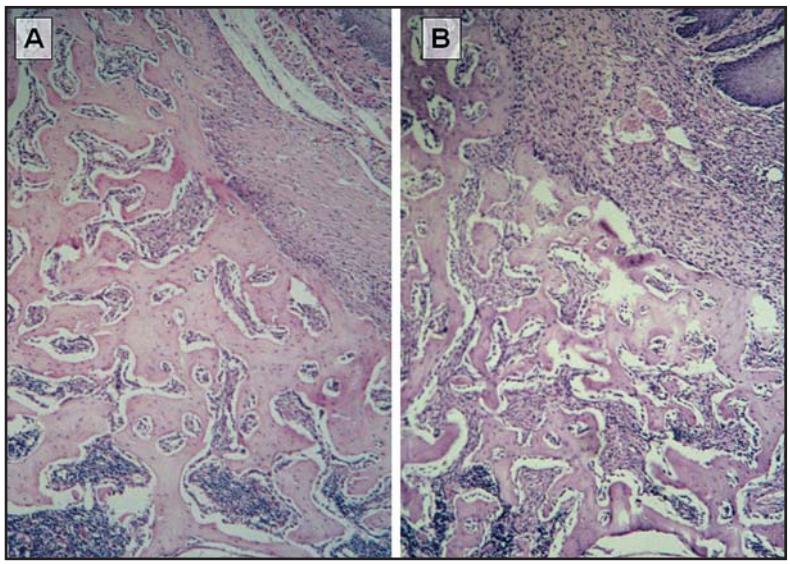

Fig. 1: Microphotograph of the bucco-lingual section of the mesial tooth socket of the first lower right molar 15 days post-extraction. (A) Note that the socket is almost completely filled with woven bone in a CG sample (hematoxylin and eosin; original magnification x100). (B) EG sample exhibiting a reduction in total alveolar volume and in bone volume in the apical third of the tooth socket (hematoxylin and eosin; original magnification x100).

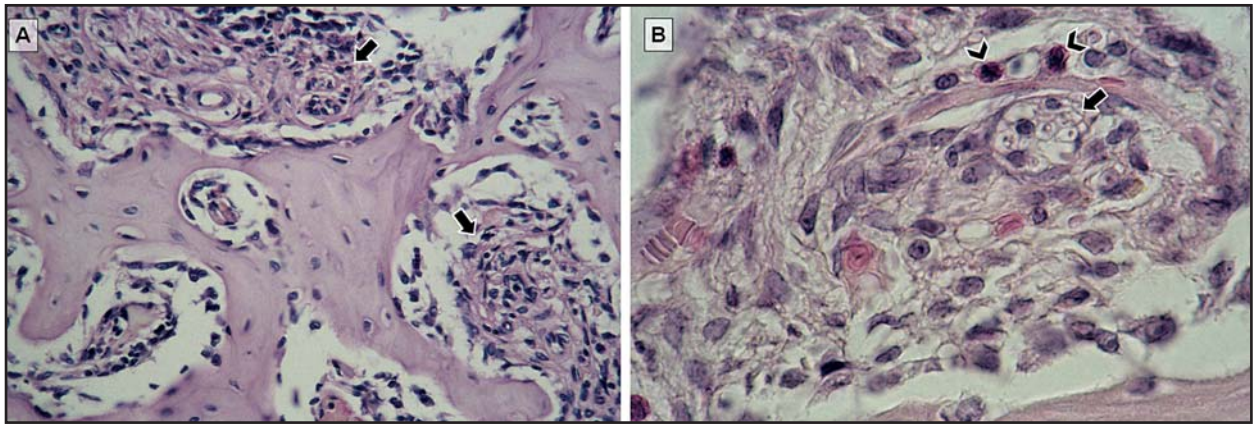

Fig. 2: (A) Note the granuloma-like structures between the trabeculae (arrows) (hematoxylin and eosin; original magnification x400) and (B) the presence of eosinophils (arrowhead) and foam cells containing amastigotes (arrow) (hematoxylin and eosin; original magnification x1000) in EG samples.

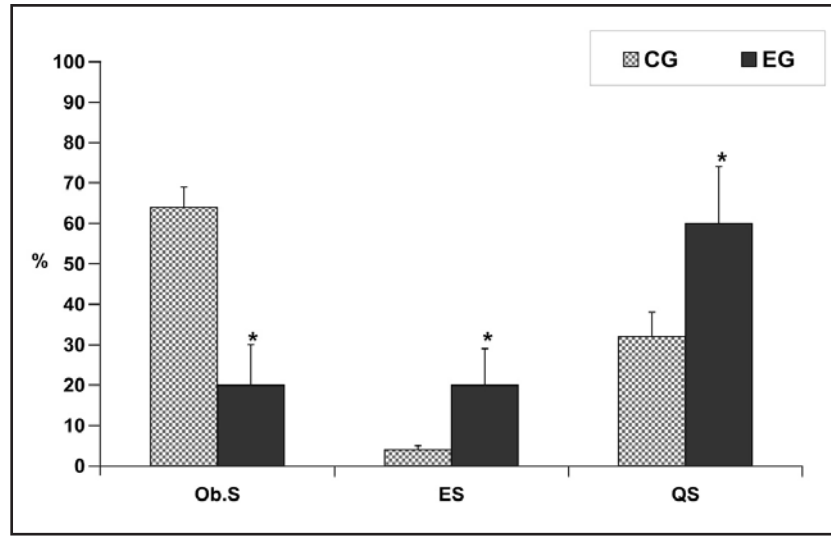

*Fig. 3: Histomorphometric evaluation showed a significant reduction in the percentage of osteoblast surfaces (Ob.S) and a significant increase in eroded (ES) and quiescent surfaces (QS) in experimental animals as compared to controls. Values are means ± SD (*p < 0.05).*

DISCUSSION

Morocoima A. et al. were the first to report the presence of *T. cruzi* stages in bone in a murine experimental parasitism model [26].

We describe in the experimental model used, for the first time, the bone tissue repair response to *in vivo* acute *T. cruzi* infection. Our results show that acute infection by the virulent strain Tulahuen of *T. cruzi* affects bone tissue repair in rats. The histological and histomorphometric analyses showed a decrease in osteogenic acitivity concomitant with an increase in quiescent and eroded bone surfaces. These alterations resulted in a reduction in alveolar total volume and bone volume in the apical third of the tooth socket in animals infected with *T. cruzi*.

In a previous study by our laboratory we described the chronology of socket healing after tooth extraction in the rat employing radiographic, histologic, and histomorphometric techniques[27].

The newly formed bone after tooth extraction undergoes intramembranous ossification[35-37]. Our previous studies using histomorphometric methods showed that under normal conditions maximum bone formation occurs on the fourteenth day after tooth extraction[27-33].

Various studies have demonstrated that experimental infection of rats with *T. cruzi* reproduces several aspects of the clinicopathological features of human chagasic infection[17,18,20,38,39]. In these studies, inoculation at weaning with living *T. cruzi* in rats resulted in a self-resolving acute infection characterized by marked parasitaemia and production of specific antibodies[17,18,20,21]. Briefly, parasites were evident microscopically by day 7 post-infection and declined gradually, as the adaptive immune response developed. In our study the parasitaemia reached its peak on day 7 post-infection. At this experimental time, an increase in haematocrit value was observed. Recently, Berra et al.[40] demonstrated increased plasma viscosity in experimentally *T. cruzi* – infected rats that was correlated with high blood parasite levels at 7 days post-infection. Marcondes et al.[41] reported that acute *T. cruzi* infection in mice results in alterations of the haematopoietic system associated with bone marrow suppression. The mechanisms involved in myelosuppression are not clear. The blood and bone marrow alterations may result from suppression of precursor cells via secreted cytokines or parasite or cell-dependent cytotoxicity. Host resistance to *T. cruzi* infection, both in humans and in experimental models, induces cells from the monocyte/macrophage lineage and other nonimmune cells to produce high levels of proinflammatory cytokines[15,16,19]. Various inhibitory cytokines exert profound inhibitory signals at critical stages of erythropoiesis, the production of erythropoietin and the maturation and differentiation of colony forming units-erythroid (CFU-E)[41]. These findings would explain the haematologic response observed 15 days post-infection in the present study.

Inflammatory reactions are among the first host responses to infection with *T. cruzi*[15,16,19]. Most inflammatory cells can interact with different life cycle stages of *T. cruzi*, causing parasite destruction extracellularly by antibody-dependent, cell-mediated cytotoxicity and intracellularly without antibody

requirement[15,16,18]. In the present study we observed the presence of foam cells (macrophages) containing amastigotes in the granulation tissue and eosinophils in bone marrow in agreement with the results informed by Morocoima , et al.[26] Rowland and Sibley-Phillips reported an increase in femoral bone marrow eosinophil levels during *T. cruzi* infection in mice with a peak coincident with that of parasitaemia[42].

Oba et al. showed that the eosinophil chemotactic factor- L (ECF-L), a previously described chemotactic factor for eosinophils, acts at the later stages of osteoclast formation[43]. In addition, cytokines play a critical role in the regulation of osteoclast differentiation and activation of initiation of bone resorption[44,45]. In the present study we demonstrated that acute infection by *T. cruzi* induces an increase in areas of bone resorption and quiescent bone and a concomitant reduction in bone formation surfaces. Recently, Unnikrishnan and Burleigh[46] reported that *T. cruzi* elicits the selective repression of basal connective tissue growth factor (CTGF) expression in fibroblasts, a TGF-b dependent cytokine normally up-regulated in tissue repair processes. CTGF is a member of the CCN (cyr61, ctgf, nov) proteins, which are an important family of matricellular regulatory factors involved in internal and external cell signaling. This family participates in angiogenesis, chondrogenesis, and osteogenesis, and they are probably involved in the control of cell proliferation and differentiation[47].

Kanyama et al. demonstrated that CTGF was expressed at an early stage of the rat tooth extraction wound healing process, and stated that CTGF may play an important role in angiogenesis and granulation tissue formation specifically at the early healing stage after tooth extraction to initiate alveolar bone repair[48]. In addition, Safadi et al. demonstrated that CTGF plays a role in osteoblast differentiation and function *in vitro* and elicits an osteogenic response *in vivo*[49].

Unnikrishnan and Burleigh indicated that the mechanisms of *T. cruzi* – mediated repression of CTGF are complex and involve targeted inhibition of the TGF-b-induced host signaling pathway followed by down-regulation of the extracellular matrix proteins, fibronectin, and collagen I a1 expression.[46] These mechanisms could be responsible for the deleterious effects of acute *T. cruzi* infection on bone tissue repair observed herein.

The results of our study reveal that acute infection by the virulent strain Tulahuen of *Trypanosoma cruzi* affects the bone tissue repair process in the post-extraction tooth socket in rats quantitatively and qualitatively.

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