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# Toxic and therapeutic effects of Nifurtimox and Benznidazol on *Trypanosoma cruzi ex vivo* infection of human placental chorionic villi explants

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ABSTRACT

Nifurtimox (Nfx) and Benznidazole (Bnz) are the only available drugs in use for the treatment of Chagas disease. These drugs are recommended but not fully validated in evidence-based medicine and reports about the differential toxicity of both drugs are controversial. Here, we evaluated the toxic and therapeutic effects of Nfx and Bnz on human placental chorionic villi explants (HPCVE) during *ex vivo* infection of *Trypanosoma cruzi*, performing histopathological, histochemical, immunohistochemical as well as immunofluorescence analysis of the tissue. Additionally, we determined the effect of both drugs on parasite load by real time PCR. Bnz prevents the parasite induced tissue damage in *ex vivo* infected HPCVE compared to Nfx, which is toxic *per se*. The presence of *T. cruzi* antigens and DNA in infected explants suggests that these drugs do not impair parasite invasion into the HPCVE. Additionally, our results confirm reports suggesting that Bnz is less toxic than Nfx and support the need for the development of more effective and better-tolerated drugs.



## 1. Introduction

Chagas disease or American Trypanosomiasis is caused by the flagellated protozoan parasite *Trypanosoma cruzi* (*T. cruzi*) and is recognized by the WHO as one of the world's 17 neglected tropical diseases (WHO Technical Report Series, N° 975, 2012). This illness has been a scourge to humanity since antiquity and continues to be a relevant social and economic problem in many Latin American countries(Mathers et al., 2007; Moncayo and Silveira, 2009; Rassi et al., 2010). Currently, around 10 million individuals are infected with *T. cruzi* in endemic areas while it has been estimated that there are around 325,000 cases in the USA and about 100,000 in Europe (Le Loup et al., 2011).

The predominant modes of transmission are vectorial, through infected dejections of triatomine bugs ('kissing bugs') and by blood transfusion. Additionally, congenital transmission and oral infection following ingestion of parasite-contaminated food are important (WHO, 2012).

Chagas disease occurs in two phases: acute and chronic, this last with two forms, indeterminate and symptomatic. After the acute phase, either asymptomatic or symptomatic with constitutional, cardiac, and neurologic symptoms, patients who have not been cured enter the chronic phase. Around two-thirds of patients will remain in the indeterminate stage, whereas the remaining one-third becomes symptomatic. Of these, two-thirds develop a cardiac form of the disease and one-third develops a gastrointestinal form. Progression from the indeterminate phase to a symptomatic form can take years or even decades (Lescure et al., 2010).

Present treatment of Chagas disease relies on two drugs, Nifurtimox [3-methyl-4 (nitrofurfurilideneamino) tetrahydro-4H-1,4-thiozine-1,1-dioxide] (Nfx, Lampit®) and Benznidazole [N-benzyl-2-nitroimidazole acetamide] (Bnz, Rochaga®, Roche, now produced by LAFEPE, Brazil) discovered empirically more than three decades ago (Cerecetto and Gonzalez, 2002; Boiani et al., 2010; Mejia et al., 2012; Hall and Wilkinson, 2012). Despite their long history in the treatment of Chagas disease, both compounds induce significant side effects. Moreover, treatment failure

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is known to occur even during acute infection, the stage in which anti-parasitic drug therapy is most effective (Machado et al., 2010). The main side effects of Bnz are characterized by hypersensitivity reactions at the beginning of treatment, medullar toxicity and peripheral neuropathies at the end of treatment. On the other hand, Nfx induces digestive symptoms: nausea, vomiting, diarrhea, anorexia and weight loss, and neuropsychiatric symptoms: irritability, sleep disorders, and peripheral neuropathies (Lescure et al., 2010). Whereas several studies have been performed about the mode of action of these drugs, knowledge on this subject is still incomplete (Maya et al., 2007; Boiani et al., 2010; Hall and Wilkinson, 2012).

It is important to point out that both drugs are not fully validated in evidence-based medicine; they are recommended but not clearly permitted by the US Food and Drug Administration or the European Medicines Agency (Rassi et al., 2010; Lescure et al., 2010).

Reports about the differential toxicity of both drugs are controversial, though it is proposed that Bnz is usually better tolerated than Nfx (Rassi et al., 2010; Bern, 2011; Le Loup et al., 2011). On the other hand, in large series of patients treated with these drugs, no significant problems of adverse drug effects have been found (Maya et al., 2007). Therefore, it is imperative to better clarify this controversy by applying appropriate experimental models.

The use of human placental chorionic villi explants (HPCVE) has been widespread for a long time in basic biomedical studies (Grimm, 1955; Seeho et al., 2008), including the effect of drugs (Gedeon and Koren, 2005) and mechanism of pathogen invasion (Halwachs-Baumann, 2006; Duaso et al., 2010; Fretes and Kemmerling, 2012). We have previously established the optimal conditions for *ex vivo* infection of HPCVE with the infective trypomastigote form of *T. cruzi* (Duaso et al., 2010). In addition, we have determined the parasite load by real time PCR in infected HPCVE in response to doxycycline, a metalloproteinase inhibitor (Castillo et al., 2012).

During *ex vivo* infection of the HPCVE, the parasite induces severe tissue damage in the placental barrier formed by the trophoblast (the first fetal tissue with which the parasite interacts), fetal connective tissue, fetal endothelium and basal lamina (Duaso et al., 2010).

In order to determine both the toxic and therapeutic effects of Nfx and Bnz on HPCVE during the *ex vivo* infection of *T. cruzi*, we performed histopathological, histochemical and immunohistochemical analysis of the tissue and determined the effect of both drugs on parasite infection by immunofluorescence and real time PCR.

## 2. Material and methods

### 2.1. T. cruzi trypomastigote culture and harvesting

Green Monkey (*Cercopithecus aethiops*) renal fibroblast like cells (VERO cells (ATCC® CCL-81)) were grown in RPMI medium enriched with 5% fetal bovine serum (FBS) 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. After confluence, VERO cells were incubated with a culture of epimastigotes, DM28c strain, in late stationary phase, which increases the percentage of trypomastigotes to approximately 5% (Contreras et al., 1985). Trypomastigotes then invade fibroblasts and replicate intracellularly as amastigotes. After 72 h, amastigotes transform back to trypomastigotes that lyses host cells. Parasites were recovered by low speed centrifugation (500 × *g*), thus obtaining trypomastigotes in the supernatant (Villalta and Kierszenbaum, 1982).

### 2.2. Placenta and placental chorionic villi explants 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, University of Chile. Exclusion criteria 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. Maternal and fetal surfaces of placenta were discarded, and the villous tissue was obtained from the central part of the cotyledons. The isolated chorionic villi were washed with PBS in order to remove blood, cut in approximately 0.5 $cm^3$ pieces and co-cultured with *T. cruzi* trypomastigotes DM28c strain ($1 \times 10^6$/ml) in the presence and absence of Nfx 1, 10 and 100 μM or Bnz 2, 20 and 200 μM for 72 h in 1 ml of RPMI culture media supplemented with inactivated FBS and antibiotics. 10 μM of Nfx and 20 μM of Bnz correspond to the respective $IC_{50}$ values (Faundez et al., 2005), therefore we used one order of magnitude below and above the $IC_{50}$. Final concentration of the drug s solvent, dimethyl sulfoxide (DMSO), was lower than 1%. DMSO alone did not induce tissue damage in the HPCVE nor inhibit the effect of the parasite (data not shown). *T. cruzi* infection was tested by immunofluorescence and real time PCR (see below). All the experiments were performed in triplicate in at least three different placentas.

### 2.3. Histological and histochemical techniques

The HPCVE 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 and with Picro Sirius red–hematoxylin for collagen histochemistry (Duaso et al., 2010).

### 2.4. Immunohistochemistry

The HPCVE 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 detect human placental lactogen (Novocastra® NCL-PLp dilution 1:250, v/v), a trophoblast marker. The primary antibody was applied individually to each section for 30 min at 37 °C. Immunostaining was performed using a horseradish peroxidase-labeled 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, performed by replacing the primary antibodies with PBS, as well as other controls used, were negative. All sections were examined by light microscopy (Motic BA310, China) and images were captured with a Motic 5 camera.

#### 2.4.1. 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. An anti-cruzipain antibody, dilution 1:2000 (v/v) (a gift from Dr. J.J. Cazzulo, Instituto de Investigaciones Biotecnológicas, Universidad Nacional de General San Martín, Buenos Aires, Argentina) 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 presence of propidium iodide (0.5 μg/ml). Afterwards, sections were mounted in VectaShield (ScyTek, ACA) and