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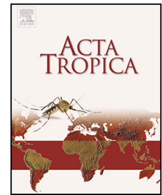

Toxic and therapeutic effects of Nifurtimox and Benznidazol on *Trypanosoma cruzi ex vivo* infection of human placental chorionic villi explants

Gemma Rojo[a], Christian Castillo[a], Juan Duaso[a], Ana Liempi[a], Daniel Droguett[a], Norbel Galanti[b], Juan Diego Maya[c], Rodrigo López-Muñoz[c], Ulrike Kemmerling[a,*]

[a] *Programa de Anatomía y Biología del Desarrollo, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Independencia 1027, Santiago de Chile 8380453, Chile*

[b] *Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Independencia 1027, Santiago de Chile 8380453, Chile*

[c] *Programa de Farmacología Molecular y Clínica, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Independencia 1027, Santiago de Chile 8380453, Chile*

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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.

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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

* Corresponding author. Tel.: +56 2 29786261; fax: +56 2 29786264.
E-mail addresses: ukemmerling@med.uchile.cl, ukemmerling@u.uchile.cl (U. Kemmerling).

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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 (1×10^6/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

observed in a Motic BA310 microscope and images were captured as described above. At least ten fields were selected randomly and the signal intensity was scored as follows: +/−, patchy; +, weak; ++, moderate; +++, high (Castillo et al., 2012).

2.5. DNA amplification by real time PCR (qPCR)

Genomic DNA was extracted from the placental tissue with the Wizard Genomic DNA Purification Kit (Promega®, USA) according manufacturer's instructions and quantified by μDrop Plate DNA quantification system in a Varioskan Flash Multimode Reader (Thermo Scientific, USA). For amplification of human and parasite DNA, two specific primer pairs were used. A 100 bp human GAPDH sequence was amplified using the primers hGDH-F (5′-TGATGCGTGTACAAGCGTTTT-3′) and hGDH-R (5′-ACATGGTATTCACCACCCCACTAT-3′), which were designed using the Primer Express software (version 3.0; Applied Biosystems®). For *T. cruzi* DNA detection, a 182 bp sequence of satellite DNA was amplified by using TCZ primers: TCZ-F 5′-GCTCTTGCCCACAMGGGTGC-3′ and TCZ-R 5′-CAAGCAGCGGATAGTTCAGG-3′ (Castillo et al., 2012; Cummings and Tarleton, 2003). Each reaction mix contains 200 nM of each primer (forward and reverse), 1 ng of DNA from samples, 12.5 μL of SensiMix® SYBR Green Master Mix (Bioline®, USA) and H20 to complete 25 μL. Amplification was 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. Relative quantification analysis of the results was expressed as RQ value by the comparative Control (ΔΔCt) method (Pfaffl, 2001; Castillo et al., 2012).

2.6. Statistics

Results are expressed as mean ± SD. The significance of differences was evaluated using ANOVA followed by Dunnett's post-test.

3. Results

3.1. Nfx and T. cruzi induce similar histopathological changes in HPCVE while Bnz prevents the parasite-induced damage

As previously reported (Duaso et al., 2010) HPCVE incubated during 24 h with 10^6 trypomastigotes from DM28c strain show severe histopathological changes in the infected tissue. In HPCVE incubated during 72 h, very similar destruction and detachment of the trophoblast (Fig. 1D, F arrows) as well as collagen I disorganization in the fetal connective tissue (Fig. 1E) are evident as compared to control, non-infected HPCVE (Fig. 1A–C). HPCVE incubated during 72 h with 10 μM Nfx (Fig. 1G–I) or with *T. cruzi* and 10 μM Nfx (Fig. 1J–L) shows similar tissue damage as the one induced by the parasite alone (Fig. 1D–F).

Contrarily, HPCVE incubated with 20 μM Bnz (Fig. 1M–O) or with the parasite and 20 μM Bnz (Fig. 1P–R) shows tissue features similar to control non-infected ones.

3.2. Nfx and Bnz do not impair the presence of T. cruzi antigens and DNA in ex vivo infected HPCVE

HPCVE were incubated in absence (Fig. 2A–C) or presence (Fig. 2D–F) of 1×10^6 trypomastigotes DM28c strain for 72 h, in the presence of 100 μM Nfx (Fig. 2G–I) or 200 μM Bnz (Fig. 2J–L). HPCVE incubated with the parasite (++ immunoreactivity, Fig. 2E) and in the presence each anti-chagasic drugs, evidenced the presence of parasite antigens in fetal connective tissue (−/+ immunoreactivity, Fig. 2H (Nfx), Fig. 2K (Bnz))) as shown by immunodetection with an anti-cruzipain antibody (parasite marker).

Additionally, we analyzed by qPCR whether Nfx (1, 10 or 100 μM) (Fig. 3A) or Bnz (2, 20 or 200 μM) (Fig. 3B) are able to diminish the parasite load in the infected HPCVE. None of the anti-chagasic drugs eliminated the amount of parasite DNA in the HPCVE. However, 20 μM and 200 μM Bnz as well as 10 μM and 100 μM Nfx decrease the parasite DNA in the HPCVE. The decrease of parasite DNA induced by 100 μM Nfx is statistically significant ($p \leq 0.001$) (Fig. 3A).

Though the presence of *T. cruzi* antigens or DNA does not permit to distinguish whether the HPCVE presents death or live parasites, importantly both markers are detectable. Therefore, our results strongly suggest that these drugs do not impair parasite invasion into the HPCVE.

4. Discussion

The aim of treatment is to cure the infection in acute Chagas disease, to prevent organ damage in chronic asymptomatic infection, and to limit incapacity and prevent morbidity and mortality once the disease is already clinically manifested (Rassi et al., 2009; Rassi et al., 2012). Nfx and Bnz are the only drugs recommended for the treatment of Chagas disease. Bnz has been more extensively investigated in clinical studies; considering that this drug is proposed to present a better safety and efficacy profile, it is usually selected for first-line treatment (Rassi et al., 2012). However, only two randomized controlled trials on anti-trypanosome treatment for Chagas disease has been published (de Andrade et al., 1996; Sosa-Estani et al., 1998) and only one is in progress (Marin-Neto et al., 2008). No studies have compared anti-chagasic treatment with Nfx and Bnz. Moreover reliable conclusions about the effectiveness of these drugs are not possible because of the heterogeneity of study population as well as the designs and treatments schedules, among others (Lescure et al., 2010). Here we have compared the effect of both drugs in an *ex vivo* infection model of HPCVE, clearly showing that: (1) none of them impairs the presence of a parasite antigen and of *T. cruzi* DNA in the infected tissue (Figs. 2 and 3), (2) Nfx *per se* induces important histological damage in the placental tissue, and (3) Bnz prevents the parasite-induced tissue damage (Fig. 1), confirming the clinical evidence that Bnz is better tolerated than Nfx (Rassi et al., 2012).

Previous reports have shown that Nfx induces severe biochemical and ultra-structural damage in heart tissue (Bartel et al., 2007) while Bnz does not induce observable ultra-structural alterations in the same organ (Mecca et al., 2008). However, Bnz induces esophageal cell injury after 1 and 3 h intra-gastric administration to rats. The esophageal tissues show detachment and conglomeration of polyribosomes, reduction in the number of desmosomes and in the amount of bacteria on its surface. The esophageal tissue damage may be due to the administration via, which does not correspond to the recommended one. Additionally, it is not clear whether the observed tissue damage is due to a toxic effect of Bnz or to the reduction of the normal digestive bacterial flora (de Castro et al., 2003). Other recent study, applying a whole organism toxicity screening in zebrafish embryos, shows similar results. In this model, Nfx induces weakened heartbeat, pericardial edema or death of the embryos; contrarily Bnz did not induce significant phenotype changes (Buchanan-Kilbey et al., 2013).

The inability of Nfx and Bnz to prevent the *ex vivo* infection of the HPCVE as well as the differences in toxicity among these drugs should be related to their mode of action. Nfx and Bnz are believed to exert their biological activity through bio reduction of the nitrogroup (Maya et al., 2007; Hall et al., 2011; Hall and Wilkinson, 2012; Boiani et al., 2010; Mejia et al., 2012). This process starts with the

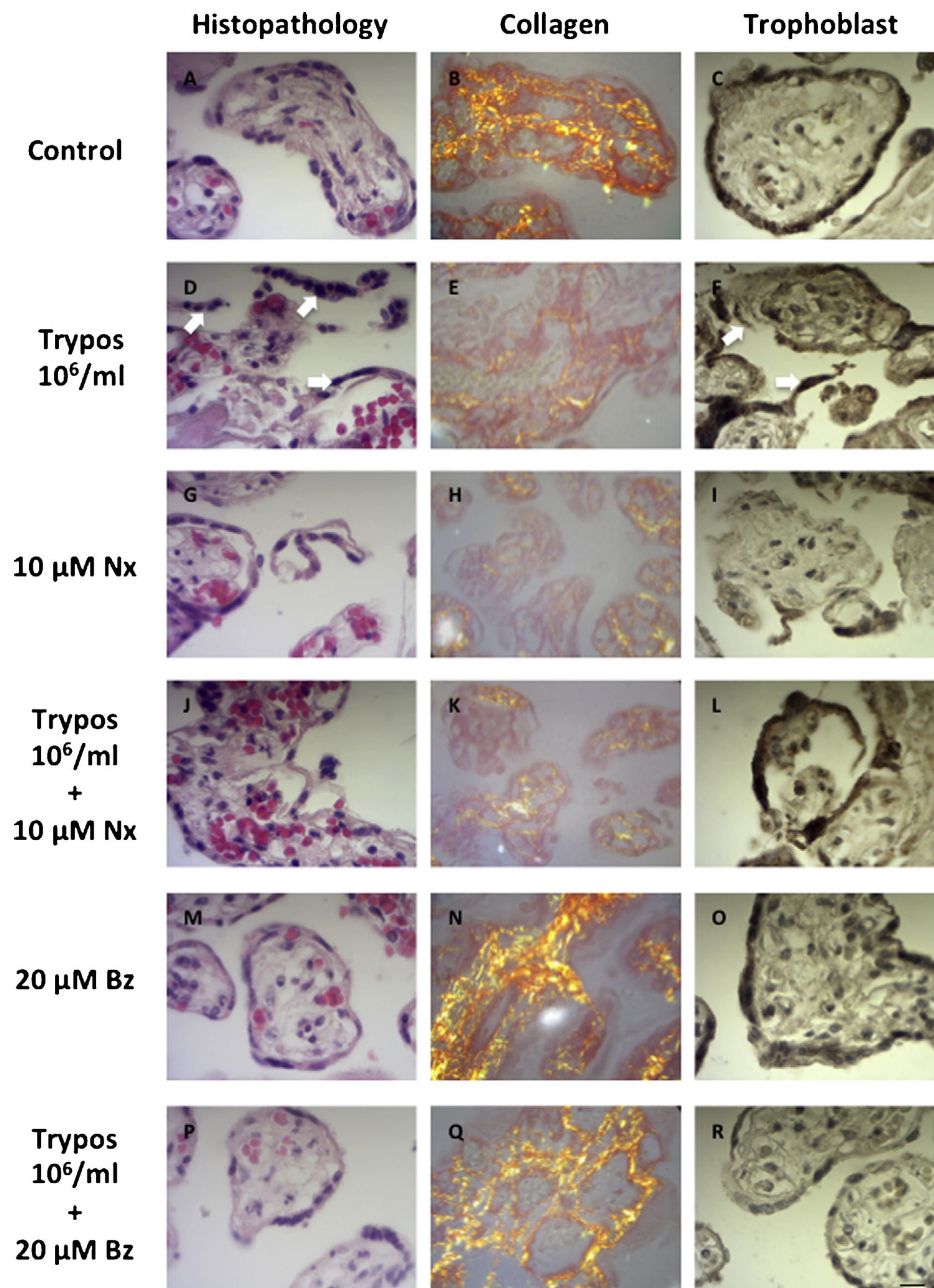

Fig. 1. Histopathological analysis of HPCVE challenged with *T. cruzi*, Nfx and Bnz. HPCVE were incubated in absence (A–C, G–I, M–O) or presence (D–F, J–L, P–R) of 1×10^6 trypomastigotes DM28c strain or for 72 h, in the presence of 10 µM Nfx (G–L) or 20 µM Bnz (M–R). HPCVE incubated with the parasite alone (D–F), with Nfx (G–H) or with both (J–L) show a severe tissue damage compared to control villi (A–C). Destruction and detachment of the trophoblast (D, F, arrows) as well as disorganization of collagen I in fetal connective (E) are observed. Contrarily, HPCVE incubated in the presence of Bnz (20 µM) (M–O) or Bnz and parasites (P–R) do not present any significant tissue damage. Tissues were processed for routine hematoxylin–eosin histology (A, D, G, J, M, P), Picro Sirius red collagen histochemistry (B, E, H, K, N, Q) or immunohistochemistry for placental lactogen (trophoblast marker) (C, F, I, L, O, R). Bar scale: 20 µm.

reduction of the nitro-group, which is attached to the aromatic ring, generating superoxide anions and nitro anion radicals. Two classes of enzyme, specifically type I and type II nitroreductases (NTRs), can catalyze this reaction. Both of them have been identified in trypanosomes (Hall and Wilkinson, 2012; Mejia et al., 2012). The type II NTRs are oxygen sensitive and contains FMN or FAD as co-factor, generating a nitro anion radical. In the presence of oxygen, this molecule is rapidly re-oxidized back to the parent compound with the concomitant production of superoxide anions. The resultant futile cycle can potentially cause oxidative cell damage (Hall and Wilkinson, 2012). It has been proposed, that Nfx mediates its activity through induction of oxidative stress in reactions catalyzed by type II NTR (Hall et al., 2011). However, to date there is insufficient functional evidence to suggest that this occurs *in vivo* since trypanosomes overexpressing trypanothione reductase display the same susceptibility to nifurtimox as control cells (Hall et al., 2011). On the other hand, the overexpression of peroxidases in *T. cruzi* does not protect the parasite from Nfx damage (Wilkinson et al., 2000; Boiani et al., 2010). In addition, the same pattern of redox cycling seen in *T. cruzi* is observed in mammalian cells (Hall et al., 2011). Probably, inducing also oxidative and nitrosative stress in mammalian cells and tissues.

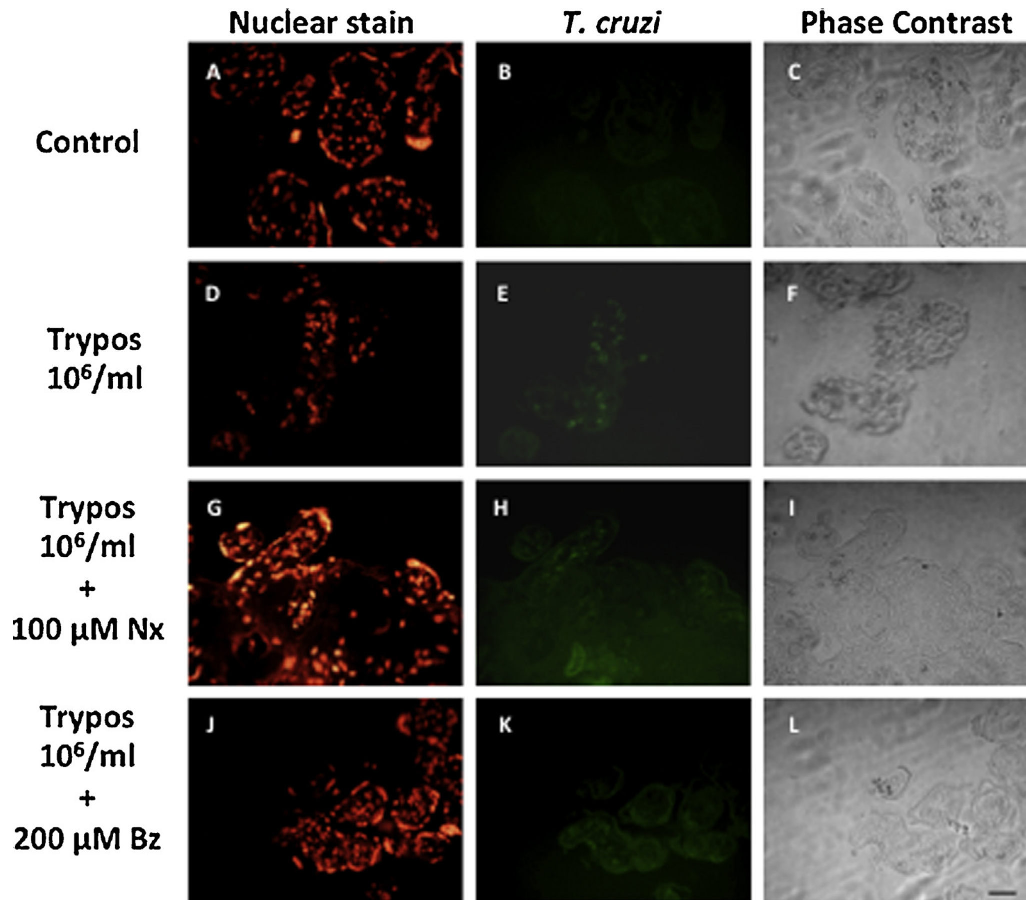

Fig. 2. Nfx and Bnz do not eliminate *T. cruzi* antigens from *ex vivo* infected HPCVE. HPCVE were incubated in the absence (A–C) or presence (D–F) of 1×10^6 trypomastigotes DM28c strain or for 72 h, in presence of 100 μM Nfx (G–I) or 200 μM Bnz (J–L). HPCVE incubated with the parasite in presence and absence of the anti-chagasic drugs show the presence of parasite amastigotes in fetal connective tissue (E, H, K) by immunodetection with an anti-cruzipain antibody (parasite marker). HPCVE were processed for routine immunofluorescence techniques and counterstained with propidium iodide. Bar scale: 20 μm.

Recently, catalysis by trypanosomal type I NTRs has been considered as an alternative activation mechanism of Nfx. Type I NTRs are flavin mononucleotide (FMN) binding, NAD(P)H-dependent proteins restricted largely to prokaryotes, generating a hydroxylamine. This derivative can interact directly with biological macromolecules, leading to cell damage, or undergo further processing to generate cytotoxic agents.

Therefore, it is highly probable that Nfx induces a strong oxidative and nitrosative stress in the HPCVE, being responsible for the severe tissue damage produced by this drug.

The trypanocidal effect of Bnz seems to be related to the covalent binding of the reduced metabolites of the drug to macromolecules, such as DNA, lipids and proteins (Maya et al., 2007) and by the interference of the host immune response. Bnz improves phagocytosis, increases trypanosomal death through interferon (IFN)-γ (Romanha et al., 2002), and inhibits *T. cruzi* NADH-fumarate reductase (Turrens et al., 1996). However, both parasite products and parasite-driven endogenous IFN-γ production are important factors responsible for eliciting chemokine synthesis and the ensuing tissue inflammation during infection with *T. cruzi* (Teixeira et al.,

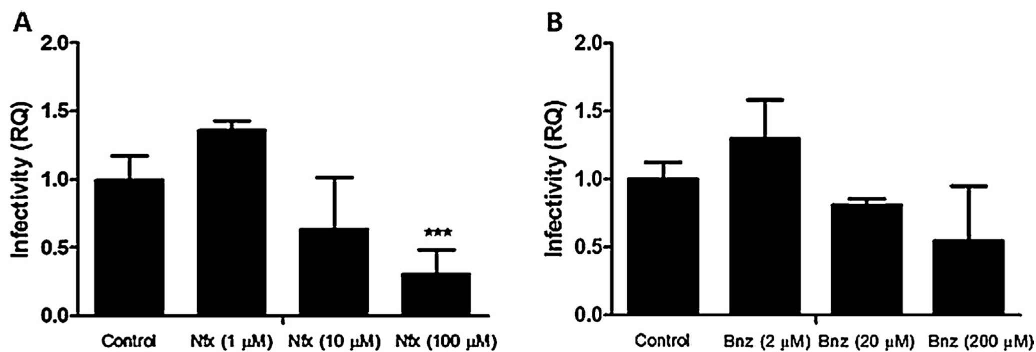

Fig. 3. Nfx and Bnz do not eliminate *T. cruzi* DNA from *ex vivo* infected HPCVE. HPCVE were incubated with 1×10^6 trypomastigotes DM28c strain (left column in both graphs) and Nfx (1, 10 and 100 μM) (A) or Bnz (2, 20 and 200 μM) (B). Data are a comparison of parasite DNA in 1 ng of total DNA isolated from infected HPCVE. Real-time quantification by qPCR was performed using ΔΔCt method. Data represents means ± SD of 3 independent experiments and were analyzed by ANOVA followed by Dunnett's post-test; $p \leq 0.001$.

2002) leading to a persistent infection (Boscardin et al., 2010) and tissue damage. On the other hand, Bnz is able to inhibit iNOS gene expression and consequently down regulate nitric oxide (NO) and NO-derived metabolites (Pascutti et al., 2004), which are responsible for maintaining a tissue inflammatory response and also for the parasite clearing in infected macrophages. It is highly probable, that the interference of Bnz in the parasite induced inflammatory response is responsible not only for the incapacity of parasite clearance in the HPCVE by the drug, but also for the preventive effect on the placental tissue damage.

Therefore it is of outstanding importance to search for effective and well-tolerated drugs. Since the parasite presents a variety of invasion and infection mechanisms and on the other hand, different tissues and organs have their own responses, treatment strategies should consider more than one therapeutic target. For example, we have previously shown in murine experimental Chagas disease, that the use of non-esteroidal anti-inflammatory drugs can prevent and treat acute and chronic chagasic cardiomyopathy (Molina-Berríos et al., 2013a,b).

Additionally, it should be relevant to study the effect of both drugs as well as different parasite strains on the *ex vivo* infection of HPCVE, particularly Nfx or Bnz resistant strains.

Probably, the future of treatment of parasitic diseases lays on the combination of "classic" anti-parasitic drugs (such as Nfx or Bnz in Chagas disease) with drugs, which, for example, modulates the immune or inflammatory response (Molina-Berríos et al., 2013a,b; Castillo et al., 2012) or the ECM remodeling (Castillo et al., 2013). In this way, with a multi-drug approach, more effective and better-tolerated treatments for neglected tropical diseases could be available.

5. Conclusion

Bnz is more recommendable to treat Chagas disease than Nfx, since it is able to prevent the parasite induced tissue damage in *ex vivo* infected HPCVE compared to Nfx, which is toxic *per se*. Additionally, our results suggest that none of both drugs impair parasite invasion into the HPCVE. Therefore the search for more effective and better-tolerated drugs is of outstanding importance for the treatment of Chagas disease.

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