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Different Therapeutic Outcomes of Benznidazole and VNI Treatments in Different Genders in Mouse Experimental Models of *Trypanosoma cruzi* Infection

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The lack of translation between preclinical assays and clinical trials for novel therapies for Chagas disease (CD) indicates a need for more feasible and standardized protocols and experimental models. Here, we investigated the effects of treatment with benznidazole (Bz) and with the potent experimental *T. cruzi* CYP51 inhibitor VNI in mouse models of Chagas disease by using different animal genders and parasite strains and employing distinct types of therapeutic schemes. Our findings confirm that female mice are less vulnerable to the infection than males, show that male models are less susceptible to treatment with both Bz and VNI, and thus suggest that male models are much more suitable for selection of the most promising antichagasic agents. Additionally, we have found that preventive protocols (compound given at 1 dpi) result in higher treatment success rates, which also should be avoided during advanced steps of *in vivo* trials of novel anti-*T. cruzi* drug candidates. Another consideration is the relevance of immunosuppression methods in order to verify the therapeutic profile of novel compounds, besides the usefulness of molecular diagnostic tools (quantitative PCR) to ascertain compound efficacy in experimental animals. Our study aims to contribute to the development of more reliable methods and decision gates for *in vivo* assays of novel antiparasitic compounds in order to move them from preclinical to clinical trials for CD.

Chagas disease (CD), discovered by Carlos Chagas (1), is a neglected pathology caused by the obligately intracellular protozoan parasite *Trypanosoma cruzi*. The disease is endemic in 21 countries of Central and South America, where about 8 million people are infected and more than 12,000 die annually (http://www.dndial.org). The treatment for CD is limited to the use of two nitroderivatives (benznidazole [Bz] and nifurtimox [Nf]), which are largely unsatisfactory, indicating a need for novel, safer, and more efficient therapies (2). Although a large number of *in vitro* and *in vivo* studies have been performed on experimental chemotherapy of novel drug candidates for CD, besides Bz and Nf, very few compounds have moved to clinical trials (3).

Recently, two antifungal drugs, posaconazole and E1224 (the prodrug of ravuconazole), which are inhibitors of fungal sterol 14a-demethylase (CYP51), were evaluated as potential antichagasic drugs on chronic patients, but unfortunately both displayed rather high (70 to 80%) rates of therapeutic failure (4, 5). It has been suggested that at least part of this unexpected failure could be due to the lack of translation from *in vitro* and *in vivo* models to the clinic and that a redesign of the current screening strategy during the drug discovery process should be considered (5). On the other hand, recent data demonstrated the potency and selectivity of a novel experimental inhibitor of *T. cruzi* CYP51, the VNI molecule, which yielded promising *in vivo* findings even with highly resistant *T. cruzi* strains (6). In this vein, we evaluated the effects and outcomes of the treatment with benznidazole and VNI using mouse models of *T. cruzi* infection assaying different animal genders and parasite strains and employing distinct types of drug administration schemes (preventive, i.e., starting therapy at 1 day postinfection [dpi], and therapeutic, i.e., starting at parasitemia onset, 5 to 6 dpi).

Our results confirm that female mice are less vulnerable to *T. cruzi* infection than males (7) and show that male mice are also less susceptible to the antiparasitic effects of Bz and VNI. We found that the use of preventive models may be less sensitive in detecting therapeutic efficiency and therefore should be avoided at the advanced stages of preclinical trials. Also, immunosuppression methods are highly recommended to assess the therapeutic success of novel compounds, since these protocols allow the parasite reactivation and easier detection through the analysis of parasitological parameters such as parasitemia relapse and blood and tissue parasite DNA detection by PCR.

MATERIALS AND METHODS

Drugs. Benznidazole (Bz) was purchased from Laboratório Farmacêutico do Estado de Pernambuco, Brazil. Bz was dissolved in distilled and sterile water supplemented with 3% Tween 80, which does not have any detectable effect on mice (8). VNI was synthesized as reported elsewhere (9), and the compound was suspended in 20% Trappsol (CTD, Inc., USA). Cyclophosphamide (Cy) (Genuxal) was purchased from Baxter Oncology (Frankfurt, Germany) and prepared in distilled and sterile water.

***In vivo* infection.** Male and female Swiss mice (18 to 20 g) were obtained from the animal facilities of the Oswaldo Cruz Foundation (CE-

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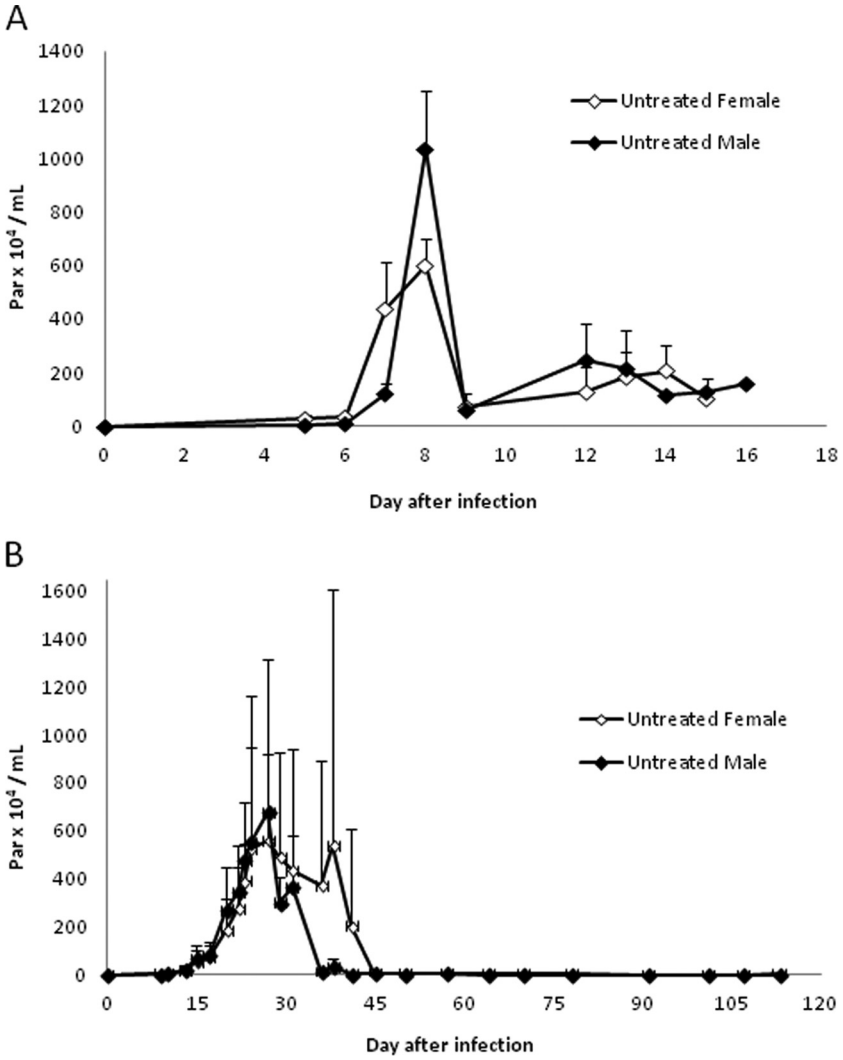

FIG 1 Parasitemia levels in experimental mouse models of *T. cruzi* infection from female and male groups infected with strains Y (A) and Colombiana (B). The high standard deviation in the female group with the Colombiana infection is due to one animal that exhibited high parasitemia.

CAL, Rio de Janeiro, Brazil). Mice were housed at a maximum of five per cage and were kept in a conventional room at 20 to 24°C under a 12-h/12-h light/dark cycle. The animals were provided with sterilized water and chow *ad libitum*. Infection was performed by intraperitoneal (i.p.) injection of 10^4 and 5×10^3 bloodstream trypomastigotes (Y and Colombiana strains, respectively). The animals were divided into the following groups: uninfected (noninfected and nontreated), untreated (infected with *T. cruzi* but treated only with vehicle), and treated orally (p.o.) with 100 mg/kg benznidazole once a day or 25 mg/kg VNI twice a day.

Treatment schemes. Bz therapy was started at 1 day postinfection (dpi) or at the onset of the parasitemia, which corresponds to 5 to 6 dpi for strain Y and 10 dpi for strain Colombiana. Bz was administered using daily consecutive doses (30 and 60 days for Y and Colombiana infections, respectively). VNI was administered for 30 days, with the treatment starting at 5 dpi. In all assays, only mice with positive parasitemia were used in the infected groups.

Parasitemia and mortality rates. The level of parasitemia was checked by the Pizzi-Brener method. Mice were individually checked by direct microscopic counting of parasites in 5 μl of blood. The mortality rates were checked daily and expressed as cumulative mortality (CM) (10).

Cure assessment. Mice that exhibited consistent negative parasitemia up to 30 days after treatment with Bz and VNI were subjected to three cycles of cyclophosphamide exposure (50 mg/kg/day), each with four consecutive days of administration (i.p.) and with 3 days between cycles (11). As reported previously (8), cure criteria were based on parasitemia negativation observed (i) by light microscopy and (ii) by quantitative real-time PCR (qPCR). Animals with negative results for all tests were considered cured. For qPCR, 500 μl blood was diluted 1:2 in a guanidine solution (6 M guanidine-HCl–0.2 M EDTA) and heated for 90 s in boiling water in order to promote the denaturation of the parasite kinetoplast DNA network-associated minicircles (12). Guanidine-EDTA blood samples (GEB) were processed using the QIAamp DNA minikit (Qiagen), as described by in reference 13. qPCR multiplex assays were performed targeting the *T. cruzi* satellite nuclear DNA and the internal amplification control (IAC) (plasmid pZErO-2 containing an insert from the *Arabidopsis thaliana* aquaporin gene), as described by Duffy et al. (14). The standard curves for the absolute quantification were constructed with serial 1/10 dilutions of total DNA obtained from a negative GEB sample spiked with 10^5 parasite equivalents per milliliter of blood.

Ethics. All procedures were carried out in accordance with the guidelines established by the FIOCRUZ Committee of Ethics for the Use of Animals (CEUA LW16/14).

TABLE 1 Analysis of biological parameters of male and female Swiss mice infected with *Trypanosoma cruzi* and treated with benznidazole or left untreated

Parasite strain	Experimental group	Highest parasitemia level (10^4 parasites/ml)[a]	Day of highest parasitemia
Y (TcII)	Untreated females	602 ± 99	8
	Bz-treated females	66 ± 146	7
	Untreated males	1,035 ± 215	8
	Bz-treated males	564 ± 241	8
Colombiana (TcI)	Untreated females	560 ± 357	27
	Bz-treated females	1.6 ± 1.3	10
	Untreated males	675 ± 642	27
	Bz-treated males	2 ± 1	10

[a] Values are means ± standard deviations.

TABLE 2 Cure assessment of benznidazole in a murine model of acute *T. cruzi* Y infection[a]

Gender	Treatment initiation (dpi)	Treatment	No. of animals with negative parasitemia/no. of surviving animals[b]	No. of qPCR-negative blood samples/no. of mice[b]
Female	1	Vehicle	0/4	ND
		Bz	4/5	3/5
	6	Bz	5/5	2/5
Male	1	Vehicle	0/5	ND
		Bz	4/4	1/4
	6	Bz	5/5	1/5

[a] Swiss male and female mice were inoculated with 10^4 bloodstream trypomastigotes (strain Y). Treatment was initiated at 1 and 6 dpi (parasitemia onset) and followed by 30 consecutive daily oral doses.
[b] Analysis was performed 30 days posttreatment after cyclophosphamide administration. ND, not determined (0% animal survival).

RESULTS

The analysis of parasitological parameters (parasitemia and mortality rates) showed that Swiss male mice are more vulnerable to strain Y (discrete typing unit [DTU] II) *T. cruzi* infection than age-matched female animals inoculated with the same parasite load. The light microscopy analysis indicated that although both genders exhibited similar prepatent periods with a parasitemia peak at 8 dpi, males displayed higher blood parasite loads (1.7-fold) than females (Fig. 1A; Table 1). Regarding cumulative mortality (CM), although both groups reached 0% survival, only 25% of the females died at 15 dpi, while in the male group, CM at the 15 dpi was 80% (data not shown). When animals were infected using the same inoculum with a well-known highly resistant strain of the parasite (Colombiana [DTU I]), again the males displayed higher parasitemia levels at the peak (at 29 dpi), as levels were about 20% lower for the female group than for the male group (Fig. 1B; Table 1), in addition to the higher mortality rates: 20 and 40% for females and males, respectively. The molecular analysis of the surviving animals by qPCR showed much lower (about 19-fold) blood parasite load in females than in males infected by strain Colombiana (27 ± 43 and 504 ± 693 parasite equivalents/ml of blood, respectively), confirming gender-related differences in the parasite infection vulnerability.

Next, potential gender-related differences during Bz therapy were evaluated using Y and Colombiana experimental models of *T. cruzi* acute infection. In these assays, the treatment was started at the onset of parasitemia. In the strain Y infection, the treatment began on day 6 and lasted for 30 consecutive days. Although no difference in survival rates (100% survival) was found between the sexes (data not shown), there was a huge suppression of the parasitemia in the female group (>90%), while the male group displayed only about a 50% decrease in parasitemia (Table 1). According to cure parameters, females also had higher cure rates (2 out of 5 mice) than the male group (1 out of 5 mice) (Table 2). Also, qPCR findings demonstrated lower parasite load in the blood of females than males (Fig. 2A). In the infection with Colombiana, the treatment was started on day 10 and lasted for 60 days. Although similar levels of parasitemia reduction (>99%) were observed in both treated animal groups, qPCR showed lower blood parasitism (1.9-fold for the mean values) in females (117 ± 60 parasite equivalents/ml) than in males (217 ± 289 parasite equivalents/ml) (Fig. 2B). When the treatment was finished, all Bz-treated mice from both genders relapsed, as assayed by light microscopy analysis (Table 3). However, one animal from the male Bz-treated group, although displaying positive relapse (only one parasite counted into 50 fields by light microscopy), had no detectable parasite DNA in the corresponding qPCR (Fig. 2B), possibly because the DNA amount was under the limit of detection of the qPCR assay (13). It is worth mentioning that despite the higher strain Y inoculum (10^4 parasites/mouse versus 5×10^3 parasites/mouse in the case of Colombiana) and shorter period of treatment (30 versus 60 days), animals treated with Bz (both male and female) exhibited lower parasite loads, as determined by qPCR, when strain Y was used, confirming the naturally resistant profile of the Colombiana strain (Fig. 2). Once more, qPCR confirmed that the drug efficacy is higher in females (0.066 and 117 parasite equivalents/ml for Y and Colombiana, respectively) than in males (0.278 and 217 parasite equivalents/ml for Y and Colombiana, respectively) (Fig. 2).

Next, in order to evaluate the impact of earlier drug administration on the parasite load, mice of both sexes were infected with *T. cruzi* strain Y and then treated with Bz starting at 1 dpi. In this preventive scheme, in both genders, Bz completely suppressed parasitemia (Fig. 3) and resulted in 100% animal survival (data not shown). The qPCR analysis also revealed the superior outcome when Bz was given earlier (at 1 dpi): both male and female groups displayed much lower parasite loads than with the treatment at 6 dpi, which corresponded to parasitemia onset (Table 4). As expected, when Bz therapy was started at 1 dpi, females still showed lower (3-fold) parasitemia (qPCR analysis) than males (Table 4), confirming that male mice are more vulnerable to *T. cruzi* infection and at the same time less sensitive to Bz therapy.

Finally, in order to ascertain if lower sensitivity to etiological treatment of male mice is also observed upon treatment with other trypanocidal compounds, we evaluated gender-related effects of the *T. cruzi* CYP51 inhibitor VNI. The activity of VNI was assayed in male and female mice infected with strain Y, and the treatment was started at 5 dpi (Fig. 3). The data show that at the peak of parasitemia, VNI induced an 86% reduction in the number of circulating bloodstream trypomastigotes in the male mice, while in the female group, the suppression of parasitemia reached 99.8%. Moreover, when qPCR analysis was performed at the endpoint (30 days after the end of therapy with VNI and followed by

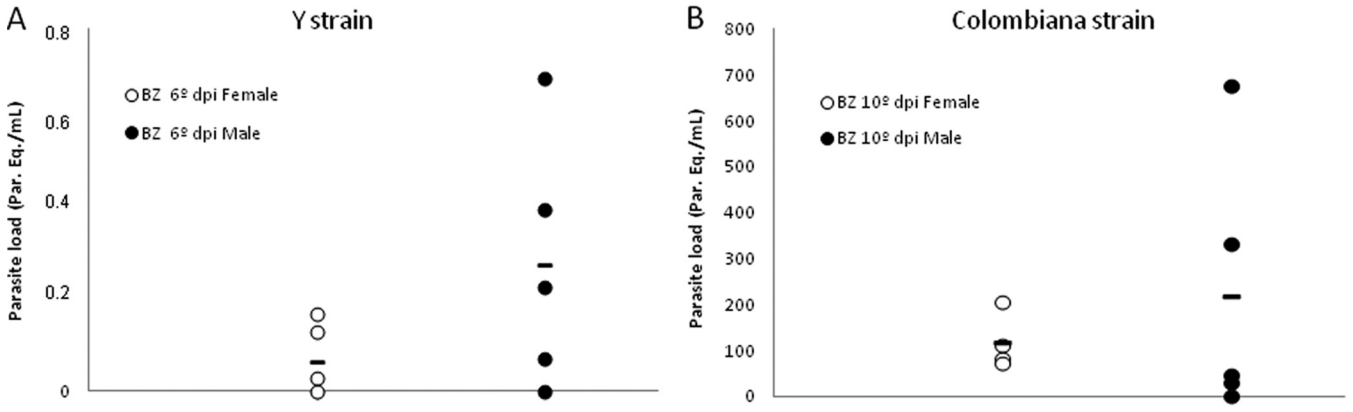

FIG 2 Parasite burden (parasite equivalents [Par. Eq.]/ml) in the blood samples of male and female mice infected with strains Y (A) and Colombiana (B) of *T. cruzi* and either left untreated or subjected to benznidazole therapy, starting at parasitemia onset (6 and 10 dpi, respectively) and lasting for 30 days and 60 days for Y and Colombiana infections, respectively.

three cycles of immunosuppression with cyclophosphamide), the findings were 151 ± 18 and 0.35 ± 0.5 parasite equivalents/ml for blood samples from male and female mice (Table 4), respectively, corroborating the data regarding gender drug susceptibility obtained during Bz treatment.

DISCUSSION

The therapy for Chagas disease (CD) remains unsatisfactory, with only two available options based on nitroderivative compounds (benznidazole [Bz] and nifurtimox) introduced into clinical use more than 4 decades ago. Limited efficiency and significant toxicity of these two drugs justifies the search for novel anti-*T. cruzi* candidates. However, despite the huge number of *in vitro* and *in vivo* assays performed on experimental chemotherapy for CD, very few compounds moved to clinical trials (15). Recent data demonstrated that although the antifungal azoles posaconazole and E1224 (the prodrug of ravuconazole) displayed very promising preclinical results, both displayed high rates of therapeutic clinical failure (4). The lack of translation from preclinical to clinical trials raised different points, including the need to use more stringent and standardized screening protocols and experimental models (5). In our opinion, the extremely high variability of *T. cruzi* populations (>70 genetically diverse strains are known) (2) represents another serious concern. Thus, a 30-day treatment was sufficient for VNI to cure, with 100% efficiency, the acute and chronic mouse models of CD caused by the Tulahuen strain of *T. cruzi* (BALB/c female mice) (16), but it failed to do so in the strain Y infection in both the male and female mouse models. One possible reason for this failure could be the lower susceptibility of strain Y CYP51 to inhibition, which may also be the case in other strains of the parasite due to high genetic variability of CYP51 across the *T. cruzi* population (17). Other possibilities would be strain-related differences in tissue distribution and the presence of possible dormant nonmultiplying forms of the parasite. Altogether, these observations call for thorough testing of new potential antichagasic drug candidates against various *T. cruzi* strains by using the most stringent animal model protocols before proceeding to clinical trials.

In this context, we evaluated the effects and outcomes of the treatment with Bz and VNI in mouse models of *T. cruzi* infection using different parasite strains and animal genders and employing different drug administration schemes (preventive and therapeutic).

Regarding animal gender, a previous study demonstrated that *T. cruzi*-infected BALB/c, C3H, and C57BL/6 female mice have a longer survival time than males (18). Those authors reported that as alterations in the humoral *T. cruzi*-specific response were ruled out, hormones like estradiol could play a relevant role on the higher vulnerability of male than female mice (19). Also, because *T. cruzi* infection in mammalian hosts leads to diverse clinical manifestations, and because this may be partly due to the occurrence of different parasite strains, experiments evaluating vaccination or chemotherapy should take into consideration the use of distinct DTUs relevant for human infection (20). Thus, in our study, we compared the outcome of parasite infection in female and male mice using two strains, the reticulotropic strain Y (DTU II) and the myotropic strain Colombiana (DTU I) (21). The present findings, obtained by using an outbred mouse lineage (Swiss), confirmed that female mice are less vulnerable to *T. cruzi* infection than males regardless of the parasite strain (Y or Colombiana). We found that the parasitemia levels (determined by light microscopy and qPCR measurements) were higher in males than females, and mortality was more prominent and/or earlier in the former group. In both males and females, qPCR analysis confirmed the natural Bz-resistant profile *in vivo* of Colombiana compared to Y (22, 23). Lower numbers of DNA parasite equivalents per milliliter were

TABLE 3 Cure assessment of benznidazole in murine model of acute *T. cruzi* Colombiana infection[a]

Gender	Treatment	No. of animals with negative parasitemia/no. of surviving animals[b]	No. of qPCR-negative blood samples/no. of mice[b]
Female	Vehicle	2/4	0/4
	Bz	0/5	0/4
Male	Vehicle	0/3	0/3
	Bz	0/5	1/5

[a] Swiss male and female mice were inoculated with 5×10^3 bloodstream trypomastigotes (strain Colombiana). Treatment was initiated at 10 dpi (parasitemia onset) and followed by 60 consecutive daily oral doses.
[b] Analysis was performed 30 days posttreatment after cyclophosphamide administration.

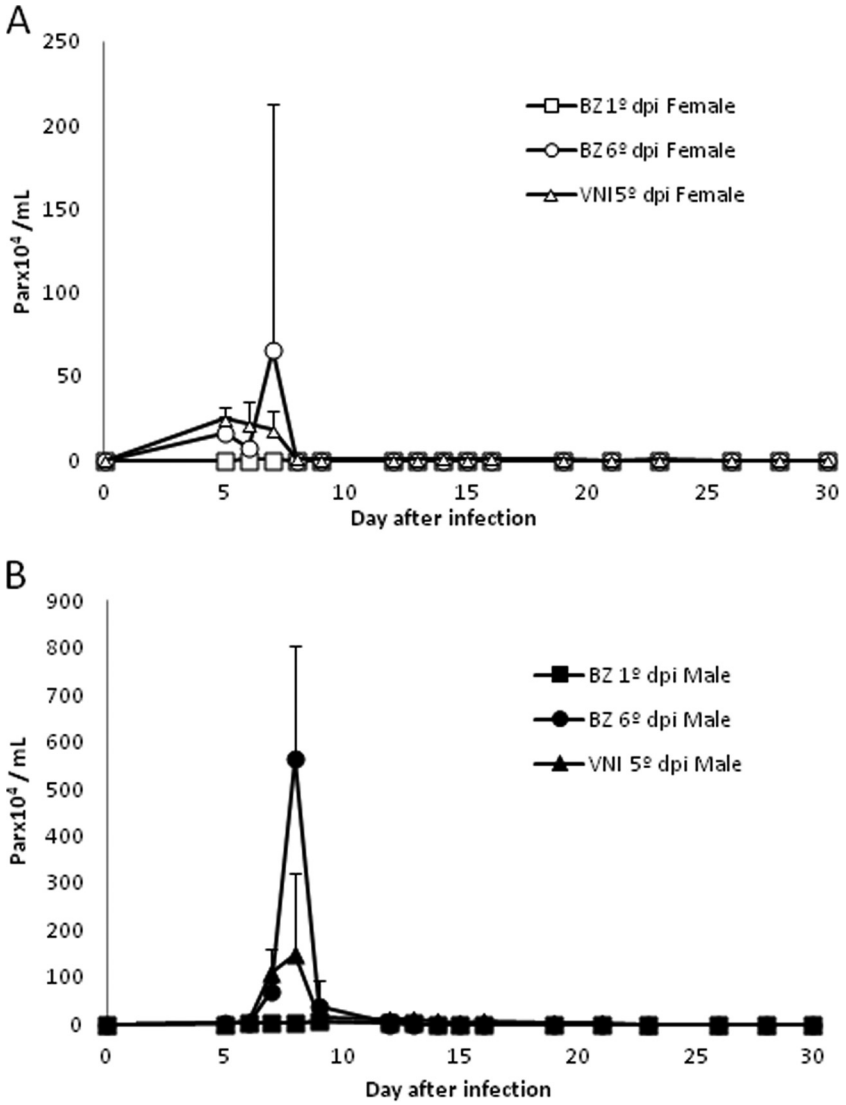

FIG 3 Parasitemia levels in experimental female (A) and male (B) mouse models of Y strain *T. cruzi* infection upon treatment with benznidazole or VNI for 30 days. The corresponding levels of parasitemia in untreated animals are shown in Fig. 1.

found when Bz was given to animals infected with Y than to animals infected with Colombiana, although twice the number of parasites were used to infect the mice (inocula of 10^4 and 5×10^3 bloodstream trypomastigotes per mouse, respectively) and longer periods of treatment (30 and 60 days, respectively) were employed.

TABLE 4 Parasite burden DNA from blood samples of *T. cruzi* (Y strain)-infected male and female mice subjected to benznidazole and VNI therapy for 30 consecutive days, starting at 1 dpi and at parasitemia onset

Compound	Gender	Burden (parasite equivalents/ml) when treatment started at[a]:	
		1 dpi	Parasitemia onset
BZ	Female	0.027 ± 0.05	0.066 ± 0.08
	Male	0.08 ± 0.08	0.278 ± 0.28
VNI	Female	ND	0.35 ± 0.5
	Male	ND	151 ± 18

[a] ND, not determined.

The animal gender must be considered when novel pharmacological entities are being assayed. For instance, data in the literature show that although both sexes most often respond similarly in acute toxicity studies, females are usually more sensitive when differences do exist (24, 25). This highlights the need to compare sexes in experimental chemotherapy studies to ascertain potential drug differences in sensitivity between the genders. In this vein, we tested the outcome of benznidazole and VNI therapy in mouse *T. cruzi* infection by performing a head-to-head comparison with female and male animals. In all assays, we found that males were less susceptible to the effect of Bz than females, with greater differences (parasitemia decreases of about 50 and >90% for males and females, respectively, with Bz and about 86 and 99.8% for males and females, respectively, with VNI) being observed when the Y strain was used and treatment was given for 30 consecutive days. The molecular analysis by qPCR confirmed that the blood

parasite load at the endpoint (30 days posttreatment) was significantly higher in males than females (about 4- and 431-fold for Bz and VNI, respectively).

Another interesting point is the choice of the therapeutic regimen, especially concerning the time when the treatment begins, i.e., at parasitemia onset or immediately after the parasite inoculation (starting at 1 dpi). Our data showed that in both male and females, Bz treatment that was started at 1 dpi resulted in undetectable parasitemia and lower parasite burden detected by qPCR than drug administration started at parasitemia onset, when the infection is already established. This, of course, supports the general knowledge that the earlier treatment begins, the better the outcomes that may be expected. However, in order to identify the most potent drug candidates, drug administration starting at parasitemia onset, when the pathogen has already invaded different tissues and organs, appears to be more sensitive and therefore preferable. The use of immunosuppression protocols (such as cyclophosphamide administration) is another relevant approach that is very helpful in enhancing the assay sensitivity (after treatment parasite detection), expanding the parasitism to detectable levels when adaptive immunity is compromised (8, 26, 27). As reported previously, the parameters for "cure" analysis *in vivo* may be influenced, depending on whether immunosuppression protocols are or are not included (25). As determined by parasitemia analysis, qPCR of blood from surviving male animals at 30 days posttreatment also showed differences in parasitism when animals were treated with Bz starting at 1 and at 6 days after infection with strain Y. Also, qPCR and parasitemia relapses after cyclophosphamide administration (at 30 days after the end of Bz therapy) revealed that, although 0% cure was found for both male and female groups infected with Colombiana, higher cure rates were found in females than males when strain Y was assayed, especially when a preventive scheme was employed (3 out of 5 and 1 out of 4 cured, respectively).

In summary, our findings confirmed that female mice are less vulnerable to *T. cruzi* infection than male animals and present additional evidence that male models are less susceptible to trypanocidal agents and therefore are highly preferable for the selection of more potent compounds. Additionally, we report that the use of preventive protocols in the *in vivo* assays is less sensitive and therefore should be avoided. Another useful consideration is to apply immunosuppression protocols to verify the therapeutic profile of novel compounds besides the use of molecular diagnostic tools (such as qPCR) to investigate compound efficacy in experimental animals. With these findings, we aim to contribute to the design of more reliable methods and decision gates for *in vivo* assays of novel antiparasitic compounds in order to move them from preclinical to clinical trials in CD.

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