1. Introduction

American trypanosomiasis also known as Chagas disease is a tropical neglected disease endemic in 21 Latin America countries. This disease afflicts more than 6 million people around the world[1]. In spite of the great effort in the identification of new drug targets and in the development of new active molecules against this parasite there are only two drugs available for its treatment – benznidazole and nifurtimox[2].

Benznidazole (N-benzyl-2-nitro-1-imidazole acetamide) is the main antiparasitic drug used against Trypanosoma cruzi (T. cruzi) as it shows less toxicity to adult patients than nifurtimox[2]. Similarly to other nitroimidazolic drugs, benznidazole has been chosen as an antiparasitic drug in spite of its undetermined mode of action[3]. Both T. cruzi epimastigotes and trypomastigotes are capable to biotransform this drug into its metabolites that covalently bind to DNA, proteins and lipids[4]. The catabolism of benznidazole within the parasite is linked to activities of reductases such as aldo-keto reductase which is an enzyme involved in detoxification of the drug within the cytoplasm[5]. The determination of the metabolic effect of benznidazole on T. cruzi may help to identify drug targets[6].

The glucose metabolism in trypanosomatids including T. cruzi has been described in its different evolutive forms such as trypomastigote, epimastigote and amastigote[7]. There are glucose transporters expressed in all its evolutive stages and the parasite may use both glucose and amino-acids such as L-proline as energy sources. After the glucose incorporation an aerobic fermentation is undertaken within the parasite and the main products of these metabolic pathways in epimastigotes are succinate and alanine[7,8]. Other products of Trypanosoma carbohydrate catabolism are acetate, lactate and CO₂. Acetate may also be originated from amino acids and fatty acids catabolism[9].

The metacyclogenesis is the epimastigote transformation into metacyclic trypomastigotes which are capable of infecting the vertebrate host. It has been described that T. cruzi spontaneously performs metacyclogenesis in vitro at the stationary phase, around 9–11 days of culture[10]. Although several aspects of
the metacyclogenesis have been described such as morphology, infectivity and gene expression[10-12], the differences in the energetic metabolism throughout the culture growth have not yet been explored. Shah-Simpson et al.[13] described the glucose metabolism in epimastigotes, metacyclic trypomastigotes and amastigotes determining the differences in excretion of pyruvate, acetate and alanine. The alternative energy production pathways such as proteins catabolism and fatty acids oxidation have not been described during this process.

In order to evaluate the biochemical effect of the most used antiparasitic drug against T. cruzi, the aim of this study was to determine the biochemical alterations of the energetic metabolism of T. cruzi epimastigotes in vitro exposed to different concentrations of benznidazole. Also this study evaluated the metabolic differences within different culture days of epimastigotes of T. cruzi during the metacyclogenesis.

2. Materials and methods

2.1. T. cruzi culture

Epimastigotes of T. cruzi, Y strain, were cultured in liver infusion tryptose (LIT) medium supplemented with 10% inactivated fetal bovine serum at 26 °C. Cultures were initiated by inoculating exponentially growing epimastigotes to a final concentration of 3.5 × 10⁶ parasites/mL. The parasites were followed up through counting in a Neubauer chamber. After 12 days of culture a concentration of 13 × 10⁶ parasites/mL was obtained.

Biochemical analyses were performed at 3, 6, 9 and 12 days of culture, corresponding to the log (3 and 6 days) and stationary (9 and 12 days) phases of the parasite’s growth in culture. At each experimental day, a culture tube was centrifuged and frozen in liquid nitrogen.

2.2. Benzinidazole exposure

At Day 0, a total of 3.5 × 10⁴ parasites/mL were inoculated in LIT culture medium and exposed to the following concentrations of benznidazole: 100 µmol/L, 50 µmol/L, 25 µmol/L, 12.5 µmol/L and 6.125 µmol/L. The drug was diluted in DMSO (4%). After 24 h the 100 µmol/L group was centrifuged and frozen in liquid nitrogen as described previously because the parasites were not seen in the Newbauer chamber follow up. The other experimental groups were frozen after 3 days (72 h) of exposure.

2.3. Biochemical analysis

The organic acids were extracted from the culture medium as described previously[14]. The organic acids were identified through high performance liquid chromatography (HPLC) according to the previously determined retention time and calibration. The organic acids analyzed were the ones indicating glycolysis (pyruvate, lactate), tricarboxylic acid cycle (citrate, alpha-ketoglutarate, succinate, fumarate, malate and oxaloacetate) and fatty acids oxidation (acetate, acetoacetate, beta-hydroxybutyrate and propionate). Glucose, urea and creatinine were quantified through spectrophotometric analysis in an Architect device 8000 according to the protocol of the commercial kits from Abbott®.

2.4. Statistical analysis

All experiments were repeated five times independently. The statistical analysis was performed using the Sigma Stat 3.5 software. Descriptive statistics were applied to determine the mean and standard deviation and to evaluate the differences between the groups analyzed. The variables were tested for normal distribution and homogeneous variance. As they presented normal distribution, variance analysis (ANOVA) was used followed by the Bonferroni post-hoc test. The comparison of two groups was performed through the student’s t-test. The differences were considered significant when P < 0.05.

3. Results

The in vitro growth of T. cruzi epimastigotes was followed through 12 days while the growth of the parasites under the influence of the different concentrations of benznidazole was followed through 3 days. There was a significant difference between the parasites concentrations when exposed to benznidazole at 25 µmol/L, 50 µmol/L and 100 µmol/L (P < 0.05) (Figure 1).
profile of the energetic metabolism of the parasite. The organic acids detected are detailed in Table 1.

Glucose was not used as an energy source for the epimastigotes form as its concentrations did not alter during the different culture days. The anaerobic metabolism was only observed at the 3rd and 12th days of culture with a significant difference between the concentrations of lactate detected (P < 0.05).

There was a consumption of citrate and succinate from the culture medium as there was a significant difference between the concentrations detected in the culture medium and in the analysis from the parasites at the 6th, 9th and 12th days of culture (P < 0.05). It was not possible to detect oxaloacetate and alpha-ketoglutarate. Also beta-hydroxybutyrate was only detected at the 12th day of culture.

The DMSO used to dilute the drugs did not influence the metabolic profile of the energetic metabolism of the parasite. The organic acids consumed for malate and fumarate production. The non-detection of oxaloacetate may indicate that it was not found in the literature when the parasite undergoes culture growth, and how different concentrations of benznidazole affect their metabolism is not found as well.

The non-detection of oxaloacetate may indicate that it was consumed for malate and fumarate production. The non-detection of oxaloacetate may indicate that it was consumed for malate and fumarate production. The non-detection of oxaloacetate may indicate that it was consumed for malate and fumarate production. The non-detection of oxaloacetate may indicate that it was consumed for malate and fumarate production. The non-detection of oxaloacetate may indicate that it was consumed for malate and fumarate production. The non-detection of oxaloacetate may indicate that it was consumed for malate and fumarate production.

### 4. Discussion

The *in vitro* growth of *T. cruzi* epimastigotes in LIT culture medium has been extensively described in the literature [15-17]. Our findings are in accordance with such descriptions. The concentrations of parasites found in our study after the exposure to different concentrations of benznidazole are in accordance with previous descriptions of *in vitro* survival of different strains of *T. cruzi* exposed to various concentrations of anti-parasitic drugs such as benznidazole, nifurtimox and miltefosine [18]. Also Moreno *et al.* [19] have shown the *in vitro* susceptibility and resistance of different *T. cruzi* strains to benznidazole. Other authors also used similar benznidazole concentrations to determine *in vitro* trypansomidal effect on epimastigote of *T. cruzi* [20].

It is interesting to highlight that all the descriptions of the metabolic pathways of *T. cruzi* are in accordance to the organic acids detected in the 3rd day of culture [7]. However, the metabolic differences were not found in the literature when the parasite undergoes culture growth, and how different concentrations of benznidazole affect their metabolism is not found as well.

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### Table 1

| Parameter | Parasites without drugs | 5 Days | 6 Days | 9 Days | 12 Days |
|-----------|-------------------------|--------|--------|--------|---------|
| Glucose (mg/dL) | 318.3 ± 42.61 | 322.83 ± 40.56 | 397.00 ± 44.90 | 356.33 ± 16.97 | 356.33 ± 4.93 |
| Pyruvate (µmol/L) | ND | ND | ND | 0.12 ± 0.03 | 0.14 ± 0.04 |
| Lactate (µmol/L) | ND | 123.60 ± 53.61 | ND | ND | ND |
| Citrate (µmol/L) | 26.98 ± 15.12 | 25.04 ± 18.42 | 1.38 ± 0.61 | 6.38 ± 4.79 | 3.82 ± 2.38 |
| Succinate (µmol/L) | 373.38 ± 116.26 | 227.92 ± 240.21 | 8.09 ± 2.86 | 12.40 ± 1.96 | 24.25 ± 9.36 |
| Malate (µmol/L) | 244.80 ± 159.96 | 915.10 ± 1193.96 | ND | ND | ND |
| Acetate (µmol/L) | 146.82 ± 22.69 | 45.58 ± 45.54 | 6.61 ± 3.84 | ND | ND |
| Acetoacetate (µmol/L) | 195.50 ± 110.55 | 90.73 ± 95.52 | ND | ND | ND |
| BHBT (µmol/L) | ND | ND | ND | 2.17 ± 1.71 | 2.17 ± 1.71 |
| Propionate (µmol/L) | 152.04 ± 80.72 | 41.40 ± 12.54 | ND | 3.56 ± 0.83 | 4.60 ± 1.83 |
| Fumarate (µmol/L) | 3.89 ± 1.28 | 2.15 ± 1.28 | 0.12 ± 0.05 | 0.15 ± 0.05 | 0.60 ± 0.87 |
| Urea (mg/dL) | 14.42 ± 1.24 | 13.50 ± 2.43 | 16.60 ± 0.54 | 17.40 ± 1.14 | 17.00 ± 0.81 |
| Creatinin (mg/dL) | 0.88 ± 0.05 | 0.14 ± 0.05 | 0.13 ± 0.03 | 0.17 ± 0.02 | 0.17 ± 0.03 |

Results are expressed as mean ± SD. CM: Culture medium; *: P < 0.05; ND: Non detected.

### Table 2

| Parameter | Parasites without drugs | DMSO control | Benznidazole |
|-----------|-------------------------|--------------|--------------|
| Glucose (mg/dL) | 318.3 ± 42.61 | 322.83 ± 40.56 | 397.00 ± 3.60 |
| Citrate (µmol/L) | 29.77 ± 29.13 | 34.10 ± 13.72 | 14.85 ± 8.30 |
| Malate (µmol/L) | 915.10 ± 1193.96 | 427.16 ± 467.58 | 250.07 ± 107.64 |
| Succinate (µmol/L) | 227.92 ± 240.21 | 395.85 ± 186.70 | 251.73 ± 117.77 |
| Lactate (µmol/L) | 123.60 ± 53.61 | 332.58 ± 190.50 | ND |
| Acetoacetate (µmol/L) | 90.73 ± 95.52 | 187.24 ± 60.04 | ND |
| Fumarate (µmol/L) | 2.15 ± 1.28 | 2.69 ± 1.24 | 1.36 ± 0.87 |
| Urea (mg/dL) | 13.5 ± 2.43 | 13.5 ± 2.43 | 15.00 ± 10.00 |
| Creatinin (mg/dL) | 0.88 ± 0.05 | 0.90 ± 0.14 | 1.13 ± 0.05 |

Results are expressed as mean ± SD; *: P < 0.05 in comparison with the control group; ND: Non detected.
alpha-ketoglutarate indicates the preferential use of the glycosomal metabolic pathways for energy production due to the consumption of succinate to produce pyruvate in the epimastigotes of *T. cruzi* [7,21]. The detection of citrate indicates the presence of the mitochondrial metabolic pathway of energy production [7]. However, the decrease in the citrate, succinate and fumarate concentrations throughout the experimental days indicates that these pathways are being suppressed while the epimastigotes tend to undergo metacyclogenesis in the culture medium [22]. Also the consumption of the energetic sources from the culture medium throughout the experimental days may be detected by the decrease in the metabolic rates observed since the 6th day of culture.

The detection of pyruvate only at the 9th and 12th days of culture indicates that its secretion occurs as the final product of the pentose phosphate pathway described previously in *T. brucei* [23]. Phosphoenolpyruvate is the precursor of both pyruvate and acetate [24], and acetate was not detected in the 9th and 12th culture days; it is possible to infer that it was used to produce pyruvate in the stationary phase of the culture and to produce acetate in the logarithmic one. It is interesting to highlight that when there was the detection of pyruvate, acetate was not detected, and vice-versa, which means that the precursor of both these organic acids is consumed in one metabolic pathway or the other [24].

It is interesting to highlight that at the 3rd day of culture, when all the metabolites in the culture medium are in optimum concentration for the parasitary growth, all the metabolic pathways are active and the parasites present all the described final products of its energetic metabolism [7], including the anaerobic metabolism marker lactate. However, the anaerobic metabolic pathway is not observed in the 6th and 9th days of culture. Also the anaerobic metabolism was increased in the 12th day of culture due to the detection of lactate only. The lactate production is well described as a final product of the methylglyoxal detoxification pathway [25].

There was an increase in the proteins catabolism in the epimastigotes since the 6th day of culture due to the maintenance of urea, creatinin and fumarate levels after the exposure to the drugs. It is important to highlight that other factors that interfere in the protein turnover or in the translation inhibition were not evaluated.

On the other hand, the mitochondrial metabolites such as citrate and acetooacetate and the glycosomal ones such as succinate and malate were not detected after the exposure to high concentrations of benznidazole. Considering that benznidazole is a pro-drug that undergoes activation within the parasite’s mitochondria, one or more of its metabolites may alter functions of these organelles or interfere within main enzymatic pathways [6,28]. Trochine *et al.* [6] described more than 14 metabolites in *T. cruzi* after benznidazole exposure.

As Chagas disease is still a chronic disease in which the treatment is not entirely effective, Bermudez *et al.* [2] pointed out the need for improvements in the current treatment. The detection of metabolic responses of the parasite when exposed to the drug and therefore the identification of possible resistance or susceptibility [5,28] may help the development of more effective substances.

Therefore, due to the non-detection of the final products from neither the glycosome nor the mitochondrion, it is possible to conclude that one of the benznidazole modes of action is to impair the aerobic metabolism of the parasite inducing the anaerobic one due to the detection of lactate when exposed to higher concentrations of benznidazole. The metabolic analysis shown in this study may help the understanding of benznidazole modes of action as well as to determine new drug targets.

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**Conflict of interest statement**

We declare that we have no conflict of interest.

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