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Nitazoxanide Induces in Vitro Partial Blockage of Glucose Uptake and Aerobic Preference in Taenia Crassiceps Cysticerci

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ABSTRACT

Nitazoxanide (NTZ) is a broad-spectrum anti-parasitic drug used against a wide variety of protozoans and helminths. This study aimed the evaluation of the in vitro metabolic impact of NTZ on Taenia crassiceps cysticerci, which were in vitro exposed to different concentrations of NTZ for 24h, 37ºC. Products of the metabolism were detected both in the culture medium (CM) and in the cysticerci (CY). The results were observed in the groups treated with the highest concentrations of NTZ. It was possible to observe an impairment of glucose uptake and the preference for the aerobic metabolism due to the high concentrations of glucose in CM, a decrease in the lactate concentrations in CY. The absence of acetate occurred due to its use to produce acetyl-coA. The blockage of the pyruvate ferredoxin oxidoreductase enzyme induced the fatty acids oxidation and protein catabolism. Therefore NTZ induced partial impairment of glucose uptake, a preference for aerobic energy production and enhanced alternative pathways for energy production.

KEYWORDS Nitazoxanide; Taenia crassiceps; glucose uptake, aerobic metabolism

1. Introduction

Nitazoxanide (NTZ) is a broad-spectrum anti-parasitic drug used against a wide variety of protozoans and helminths. It is the only drug effective against *Cryptosporidium* infections and one of the most effective against intestinal giardiasis [1]. It has been described as a effective treatment of cutaneous leishmaniasis by *Leishmania donovani* [2]. Also this drug has been indicated against intestinal and tissue helminth infection such as cystic echinococcosis [3], soil-transmitted helminths [4]; *Ancylostoma ceylanicum, Ascaris suum, Trichuris muris and Caenorhabditis elegans* [5], *Taenia saginata* and *Hymenolepis nana* [6]. Interestingly this drug has been successfully used against nosocomial pathogens [7], *Clostridium difficile* [8] and hepatitis C virus [9].

This drug has been indicated as an alternative treatment when the resistance to traditional anti-parasitic drugs is detected. For example, nitazoxanide has been described as effective against *T. saginata* niclosamide- and praziquantel-resistant [10].

It has been established that NTZ as well as its reduced form, tizoxanide, are effective against most target pathogens such as aerobic and anaerobic bacteria [11], *C. parvum* [12], canine influenza A (H3N8) virus [13]; *Trypanosoma cruzi* and *Leishmania mexicana* [14]; hepatitis B and C virus [15] and *Tania*

crassiceps [16].

In protozoans both NTZ and tizoxanide inhibit central physiological enzymes such as pyruvate ferredoxin oxidoreductase, nitroreductase 1, quinone reductase and induces lesions in the cell membrane and vacuolization [17]. While in *Neospora caninum* NTZ inhibited the protein disulfide isomerase [18]. In bacteria this drug inhibits the pilus biogenesis by the chaperone/usher pathway [19]. There are few studies determining the effect of this drug on helminths. Somvanshi et al. [4] using the *C. elegans* experimental model for soil-transmitted nematodes studies determined that NTZ acts on an alpha-type subunit of a glutamate-gated chloride ion channel which is also responsible for ivermectin susceptibility in this organism.

Taenia crassiceps is a cestode widely used as an experimental model for cestodes studies with emphasis in the cysticercosis caused by *T. solium* due to their antigenic similarity [20]. It has been used previously to determine the impact of anti-helminthics such as albendazole, praziquantel and a benzimidazole derivative in the metabolism of cestodes [21-23]. Therefore this study aimed the evaluation of the *in vitro* metabolic impact of NTZ on *T. crassiceps* cysticerci.

2. Material and Methods

2.1. Maintenance of T. crassiceps cysticerci

The maintenance of *T. crassiceps* cysticerci was performed according to the description by Fraga et al. [24]. Briefly, BALB/c female mice with 8 – 12 weeks old are intraperitoneally infected with 10 initial stage (no buds, translucent membrane and vesicular liquid) *T. crassiceps* cysticerci, maintained in cages with five animals maximum, received standard ration and water ad libitum. After 90 days of infection, the animals are euthanized, the cysticerci removed from the peritoneal cavity and inoculated into another mouse. The ethical principles of animal experimentation stipulated by the Brazilian Society of Laboratory Animals Science (SBCAL) were obeyed. This study was approved by the Ethics Committee in Animal Use from the Federal University of Goias (CEUA/UFG) (protocol number 050/2013)

2.2. Cysticerci culture and drugs exposure

A mouse with 30 days of intraperitoneal infection was euthanized within a laminar flow chamber and the cysticerci were removed and washed with sterile saline solution as to remove cells and any other contaminants [25]. Afterwards the larval stage cysticerci (with buds, vesicular membrane and fluid translucent) [26] were collected and cultured as described by Fraga et al. [23]. Briefly, 30 larval stage cysticerci were added into 5 mL of supplemented RPMI culture medium. The cysticerci were treated with 1.2; 0.6; 0.3 or 0.15 µg/mL of NTZ. The drug was diluted with DMSO (0.60%). The control groups were constituted by a group of cysticerci without NTZ and another one that received the concentration of DMSO used to dilute the drugs.

After 24 hours of culture the cysticerci were separated from their culture medium and frozen into liquid nitrogen as to stop the metabolic reactions [21,22].

2.3. Biochemical analysis

The organic acids secreted/excreted into the culture medium were extracted through an ionic exchange solid phase extraction column (Bond Elut® Agilent®), as described by Vinaud et al. [26].

After the liquid nitrogen metabolic stasis, the cysticerci were defrost and homogenized in 500 µL of tris-HCl 0.1 M buffer supplemented with a protease inhibitor (SIGMAFAST, protease inhibitor cocktail tablets, EDTA free, Sigma), pH 7.6 (Rendón *et al.* 2004, 2008). The extract obtained was centrifuged at 15,652g (10,000 rpm) per 10 minutes at 4ºC and then the organic acids were extracted through an ionic exchange solid phase extraction column (Bond Elut® Agilent®) [26].

The resulting samples were frozen at -20°C for posterior analysis in high performance liquid chromatography.

For the chromatographic analysis an exclusion BIORAD-Aminex HPX-87H column was used. The eluent was sulfuric acid 5 mM, 0.6 mL/min, with spectrophotometric reading of absorbance at 210 nm. The results were analyzed through the Star Chromatography Workstation software (Agilent®), previously calibrated for the following organic acids identification: pyruvate and lactate (anaerobic glycolisis); citrate, oxaloacetate, malate, fumarate and succinate (tricarboxilic acid cycle), acetate, acetoacetate, β-hydroxybutyrate and propionate (fatty acids oxidation) [26]. The analysis of the proteins catabolism was performed through the quantification of urea and creatinin through an Architec C8000 Plus device, using a commercial kit protocol with an enzymatic method [24].

2.4. Statistical analysis

All experiments were repeated five times independently. The statistical analysis was performed through the Sigma Stat 2.3 software. The descriptive analysis was performed as to determine the normal distribution and homogenous variation as well as mean and standard deviation. As the values presented normal distribution, the analysis of variation test (ANOVA) was performed followed by the Bonferrone post-test. The differences were considered significant when $p<0.05$.

3. Results

This study analyzed the *in vitro* effect of different concentrations of nitazoxanide in the energetic metabolism of *T. crassiceps* cysticerci. Figure 1 shows a schematic representation of the metabolic effect of NTZ on *T. crassiceps* cysticerci. The highest concentrations of NTZ (1.2 and 0.6 µg/mL) induced an impair in the glucose uptake by the cysticerci (Table 1) as the concentrations of glucose in the culture medium of the treated groups are significantly higher than the concentrations of glucose detected in the culture medium of the control groups ($p<0.05$). Consequently, the glucose concentrations in the cysticerci extract from the groups treated with 1.2 and 0.6 µg/mL of NTZ are significantly lower than the ones detected in the control groups ($p<0.05$).

The glucose seem to have been used in aerobic pathways since the lactate concentrations in the cysticerci extract from the groups treated with 1.2 and 0.6 µg/mL of NTZ are significantly lower than the ones found in the control groups ($p<0.05$) (Table 1). The pyruvate and oxaloacetate concentrations were not affected by the presence of the drug in any concentration neither in the culture medium nor in the cysticerci extract.

However the malate concentrations detected in the culture medium analysis from the groups treated with 1.2 and 0.6 µg/mL of NTZ were significantly higher than the ones found in the control group ($p<0.05$) (Table 1). In the control groups was possible to observe a significant difference between the malate concentrations found in the culture medium and in the cysticerci extract. This difference was not found in the treated groups.

There was a significant increase in the citrate concentration detected in the culture medium of the group treated with 1.2 µg/mL of NTZ when compared to the control group ($p<0.05$) (Table 1).

The TCA cycle was functional in its traditional pathway due to the detection of alpha-ketoglutarate and succinate which concentrations were not altered in the drug's presence (Table 1).

However, acetate was not detected in the culture medium nor in the cysticerci extract in the treated groups, confirming the described mode of action of NTZ which affects the fumarate oxidoreductase enzyme. The absence of acetate induced an increase in the fatty acids oxidation and a significant difference in the beta-hydroxybutare concentrations detected in the cysticerci extract from the groups treated with 1.2 and -.6 µg/mL of NTZ ($p<0.05$) (Table 2).

Regarding the proteins catabolism there was a decrease in the urea concentrations in the culture medium analysis from the treated groups when compared to the concentrations detected in the control group ($p<0.05$) (Table 2).

4. Discussion

NTZ is a drug that has been used against a wide spectrum of pathogenic agents such as helminths, protozoans, bacteria and virus [1-3,7-9]. Some of its mechanism of action has been described such as the effect on enzymes in protozoan [17] and secretory pathways in bacteria [19]. However the metabolic effect of this drug on parasites has not yet been described.

This study shows that at least one of the NTZ known mechanisms of action described, the effect on the pyruvate ferredoxin oxidoreductase enzyme, have influenced the cysticerci metabolism. The determination of the dosage used in this study (1.2; 0.6; 0.3 and 0.15 µg/mL of NTZ) occurred through the literature determination of NTZ IC50. Hu et al. [5] testing this drug against nematodes determined that it's IC50 ranged from 1.63 to 31.56 µg/mL. While Palomares-Alonso et al. [16] determined that NTZ IC50 against *T. crassiceps* in an *in vitro* evaluation was of 0.15 µg/mL. To determine the metabolic effect of this drug on *T. crassiceps* was necessary to use a dosage that would not kill the parasite within 24h but would induce a metabolic stress. Interestingly, as seen in tables 1 and 2 the metabolic effect was observed after 24h of exposure occurred under the higher dosages, 0.6 and 1.2 µg/mL, two and three fold the IC50 previously described against *T. crassiceps* cysticerci [16].

Previous studies reported that *T. crassiceps* cysticerci *in vitro* exposed to NTZ present structural damage to the parasite's tegumentary and subtegumentary layers [16, 27]. These structural damages may impair the glucose uptake which was observed in our results since *Taenia* sp adult and larval forms present glucose transporters (TGTP1 and TGTP2) in its tegumental surface [28]. Tegument damage caused by other anti-helminthic drugs such as praziquantel and mebendazole has also been reported to cause a decrease in glucose uptake by cestodes [29].

Under NTZ treatment, the preferential source of energy, glucose [28], is not abundantly available for the cysticerci to perform both aerobic and anaerobic energy production pathways, it was observed in our results that the cysticerci preferred the aerobic pathways due to the decrease in lactate concentrations detected in the cysticerci extract of the groups treated with 1.2 and 0.6 µg/mL of NTZ. As indicated by Venkatesh and Ramalingam [30] the lactate/pyruvate rate indicates the metabolic rate of the intermediary metabolism in tapeworms. In our study the concentrations of lactate and pyruvate detected in the cysticerci extract from the control group presented a lactate/pyruvate rate of 127.13, while in the treated groups this ratio decreased to 67.45 (1.2 µg/mL of NTZ treated group); 69.92 (0.6 µg/mL of NTZ treated group); 72.49 (0.3 µg/mL of NTZ treated group) and 81.83 (0.15 µg/mL of NTZ treated group). These data reflect the effect of the drug on the *in vitro* metabolic performance of the parasite.

It was possible to observe an increase in the malate concentrations in the culture medium analysis of the groups treated with 1.2 and 0.6 µg/mL of NTZ when compared to the concentrations detected in the control group. This may have happened due to the effect of the drug on the activity of the pyruvate ferredoxin oxidoreductase enzyme [17]. Malate is converted into pyruvate through the activity of the malic enzyme and then pyruvate is transported into the mithocondrion in which it will be converted into aceytil-coA through the activity of the pyruvate ferredoxin oxidoreductase [31]. As this last step is blocked by action of the drug it consequently generates an excess of malate which was observed in our results.

As described previously, *T. crassiceps* cysticerci are able to perform both aerobic and anaerobic pathways for energy production [31,32] in our study the TCA cycle was not influenced by the NTZ *in vitro* treatment since the detection of oxaloacetate, alpha-ketoglutarate and succinate were not altered. This was observed because its main substrate, acetyl-CoA, is being supplied by other sources such as fatty acids oxidation; ketonic bodies and amino acids [33].

The blockage of the enzyme pyruvate ferredoxin oxidoreductase [17] has influenced the acetate detection, which did not occur in any of the treated groups. Since the convertion of pyruvate into Acetyl-CoA is blocked the parasite used acetate as a source of Acetyl-CoA [34,35] as not to affect the citrate production and the TCA cycle. Other acetyl-CoA sources are fatty acids degradation via beta-oxidation, ketone bodies degradation and the catabolism of ketogenic amino acids such as leucine, isoleucine and lysine which are removed from the host [35].

The fatty acids oxidation was increased in the groups treated with 1.2 and 0.6 µg/mL due to the increase in the concentrations of beta-hydroxibutyrate detected in the cysticerci extract. The use of alternative energy sources was also observed in the protein catabolism detected through the alterations in urea concentrations in the cysticerci extract of the treated groups. These alternative pathways were increased as a source of acetyl-coA in order to maintain the energy production [35].

Kohler [36] has described that helminths who present multiple fermentative and anaerobic pathways also present greater versatility and metabolic flexibility to respond to different stressful conditions. In our study the environmental challenge to our experimental model was the NTZ exposure. It was possible to observe the enhance of adaptative pathways which ensured the parasite survival after a single non-lethal dose of the drug.

It is possible to conclude that NTZ induced an impairment in the glucose uptake due to the tegumental alterations. Also the metabolic effect of the NTZ mechanism of action previously described in *G. lamblia* was observed in *T. crassiceps* cysticerci which lead to the consumption of acetate for acetyl-coA synthesis. Also it was possible to observe that there was a dose dependent metabolic response in the cysticerci as the main alterations were seen in the groups treated with 1.2 and 0.6 µg/mL of NTZ.

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