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PARASITOLOGY

In vitro anthelmintic activity of nitazoxanide in comparison to praziquantel against *Eurytrema coelomaticum*

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ABSTRACT: *Eurytrema coelomaticum* are trematodes that parasitize cattle, buffaloes, goats, sheep, and camelids, and, accidentally, humans and cats. The affected animal usually has a subclinical pancreatic disease, but the damage caused to health and animal production is underestimated. Currently, praziquantel is the unique drug proven effective against this parasite, but no formulations containing this drug are available for ruminants in Brazilian market. The objective of the present study was to separately evaluate *in vitro* anthelmintic activity of praziquantel (PZQ) and nitazoxanide (NTZ) against *E. coelomaticum* by assessing motility after 3, 12, and, 15 hours of incubation and subsequent histopathological examination of the parasites. *E. coelomaticum* specimens were obtained from the pancreas of naturally infected cattle, collected from animals slaughtered in the city of Concordia-SC, Brazil. The specimens were incubated in plates with a culture medium (n = 60 per group), with 80µg/ml of PZQ (positive control group), or 61,5µg/ml of NTZ (treated group), or UNT (untreated control). After 12 hours of incubation all parasites of the NTZ and PZQ groups were motionless or dead, while in the negative control group, 91.67% (55/60) presented normal motility after 15 hours of incubation ($P < 0.001$). Histopathological examination showed severe damage in vitellogenic gland, intestine, parenchyma, integument, and testicle in both treated and positive control groups. It was concluded that PZQ and NTZ showed *in vitro* anthelmintic action against the parasite *Eurytrema coelomaticum*, as they caused significant lesions in the evaluated organs and reduced the parasite's motility.
Key words: Euritrematosis, histopathology, nitazoxanide, pâncreas, praziquantel.

Atividade anti-helmíntica *in vitro* de nitazoxanida em comparação com praziquantel contra *Eurytrema coelomaticum*

RESUMO: *Eurytrema coelomaticum* são trematódeos que parasitam bovinos, bubalinos, caprinos, ovinos e camelídeos, e de forma acidental, humanos e felinos. O animal acometido geralmente apresenta doença pancreática subclínica, mas os danos causados à saúde e produção animal são subestimados. Atualmente, o praziquantel é o único medicamento comprovadamente eficaz contra este parasita, mas não há formulações contendo esse medicamento disponíveis para ruminantes no mercado brasileiro. O objetivo deste trabalho foi avaliar separadamente a atividade anti-helmíntica *in vitro* de praziquantel (PZQ) e nitazoxanida (NTZ) frente ao *E. coelomaticum* pela avaliação de motilidade após três, 12 e 15 horas de incubação e posterior exame histopatológico dos parasitos. Exemplares de *E. coelomaticum* foram obtidos do pâncreas de bovinos naturalmente infectados, coletados em animais abatidos na cidade de Concórdia, SC, Brasil. Incubados em placas com meio de cultura (n = 60 por grupo), adicionados de 80µg/ml de PZQ (grupo controle positivo) ou 61,5µg/ml de NTZ (grupo tratado) ou UNT (grupo controle sem tratamento). Em 12h de incubação todos os parasitos dos grupos NTZ e PZQ estavam imóveis ou mortos, enquanto no grupo controle negativo 91,67% (55/60) apresentaram motilidade normal após 15h de incubação ($P < 0.001$). Por exame histopatológico observou-se danos graves em glândula vitelogênica, intestino, parênquima, tegumento e testículo em ambos os grupos tratado e controle positivo. Concluiu-se que PZQ e NTZ apresentaram ação anti-helmíntica *in vitro* contra o parasito *Eurytrema coelomaticum*, pois causaram lesões significativas nos órgãos avaliados e diminuíram a motilidade do parasito.
Palavras-chave: Euritrematose, histopatologia, nitazoxanida, pâncreas, praziquantel.

INTRODUCTION

Eurytrema spp. are digenetic trematodes that parasitize cattle, buffaloes, goats, sheep, and camelids, in addition to accidentally affecting humans and felines (BASSANI et al., 2007; ARMSTRONG & WILLIAMS, 2012; BALQIS, 2018; OGAWA et al., 2019). They are primarily

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reported in the pancreatic ducts of ruminants, but can also be observed in the small intestine and bile ducts (BASSANI et al., 2007; QUEVEDO et al., 2013).

Surian et al. (2022) demonstrated alterations in the exocrine pancreatic function of naturally parasitized cattle, depending on the degree of parasitemia of the animals. In Brazil, the disease stands out for its high rates observed in the pancreas of cattle during slaughter, being considered a neglected and emerging disease (SCHWERTZ et al., 2015). A ranging prevalence of *E. coelomaticum* in cattle has been observed in Brazilian states, probably related to different environmental conditions for the parasite biological cycle (AZEVEDO et al., 2004). Studies reported a prevalence of 20.7% in the southwestern region of the state of Minas Gerais (SILVA-JÚNIOR, 2017), and a prevalence of *Eurytrema* spp. ranging from 8.3% (AZEVEDO et al., 2004) to 72.9% (BASSANI et al., 2006) in cattle slaughtered in the state of Paraná. In the state of Rio Grande do Sul, the prevalence in abattoirs is unusual (TESSELE et al., 2013). In the state of Santa Catarina studies showed a prevalence of 68.99% in dairy herds, with 100% of the properties studied presenting at least one parasitized animal (LUCCA et al., 2015).

In vitro studies identified that PZQ, due to its high efficacy, can be used as a treatment of choice for the control of eurytrematosis (JIRAUNGKOORSKUL et al., 2005). PZQ action on parasites occurs by inhibiting the sodium and potassium pump, thus increasing the permeability of the membrane to mono and divalent cations such as calcium, which results in spastic paralysis of the parasite's muscle cells. Paralysis assists in the expulsion of the parasite from the pancreas and promotes an environment conducive to rapid vacuolization and disintegration of the integument (KEISER & UTZINGER, 2004; JIRAUNGKOORSKUL et al., 2005; TAYLOR et al., 2016).

NTZ acts by inhibiting enzymes essential to the anaerobic metabolism of the parasite, particularly pyruvate: ferredoxin oxidoreductase (SHAKYA et al., 2018). This drug has not yet been tested for *Eurytrema* spp. However, it has been widely used in human medicine for treatment of liver trematodes, mainly for *Fasciola hepatica* for which PZQ or triclabendazole are not proved to be effective (KEISER & UTZINGER, 2004; MASCOMA et al., 2019). Due to the morphophysiological similarity between the mentioned digenetic trematode species, nitazoxanide can also be an alternative in the control of Euritrematosis. In addition, anthelmintic resistance is widespread among parasites of livestock because of frequent drug administration of the same class of compounds over long periods (FAIRWEATHER et al., 2020).

Over the last 13 years, there have been no studies that evaluated the drugs already tested or that have sought new chemotherapy alternatives with anthelmintic action against the adult parasite of *E. coelomaticum*. Thus, the evaluation of new molecules in order to control this globally disseminated parasite with high prevalence in the southern region of Brazil is justified. The objective of the present study was to verify, separately, the *in vitro* anthelmintic activity of PZQ and NTZ on adult *E. coelomaticum*, including the histopathological lesions induced by these drugs to the parasites' organs.

MATERIALS AND METHODS

E. coelomaticum specimens were obtained from the pancreas of naturally infected cattle, collected from animals slaughtered in the city of Concordia-SC, Brazil. The pancreas were kept in saline solution, placed in a thermal box at a temperature of 37 ºC, and sent to the Laboratory of Animal Parasitology of the Federal Institute of Santa Catarina – *Campus* Concordia.

Parasites were carefully removed from the pancreatic ducts in order to preserve their anatomical integrity. Afterwards, they were washed in 0.9% saline solution to completely remove the blood from the host. The intact specimens were transferred to tissue culture plates using sheep serum (YAMAMURA et al., 1992).

A total of 180 parasites of uniform size were used, with average length from 8.5±1.0 mm and width from 5.0±0.5 mm, distributed in tissue culture plates with six wells each and ten helminths per well, forming three groups: UNT (untreated control) (n = 60); PZQ (positive control) (n = 60) treatment with 80µg/mL of PZQ (brand Farmabase Saúde Animal LTDA®, Jaguariúna, Brazil, lot number A20/0007), and NTZ (testing drug) (n=60) treatment with 61,5µg/mL of NTZ (Althaia S. A. Indústria Farmacêutica®, São Paulo, Brazil, lot number 20020091). The drugs were diluted in dimethylsulfoxide (DMSO), and separately added to the culture medium at a maximum concentration of 0.02% (v/v). In the untreated control group, sheep serum and DMSO were used at the maximum concentration of the treatment groups, but without the drugs. Parasites in the culture medium were kept in incubators at 37 ºC with 5% of CO^2, being evaluated for motility after 3, 12, and 15 hours of incubation (JIRAUNGKOORSKUL et al., 2005).

The dose of 61,5 µg/ml (200 µM) of NTZ was based on a pilot study carried out earlier in the same laboratory, testing the drug with the same dosages used as a nematicide in the research of Fonseca-Salamanca et al. (2003). Regarding the concentrations of 50 µM, 100 µM, and 200 µM, the latter was the most effective *in vitro* with 78% (63/80) of the parasites killed within 12 hours, while the doses of 50 µM and 100 µM had only 18% (14/80) of dead parasites after the same period.

The dose of 80 µg/mL of PZQ and the motility assessment time followed a previous study by Jiraungkoorskul et al. (2005). The motility was evaluated by individually observing each parasite in a Stereo microscope with a 20x magnification, using the following criteria: 3 (normal movement), 2 (slow movement), 1 (very slow movement), and 0 (no movement, dead) (TRITTEN et al., 2012).

After the incubation period of 15 hours, the parasites were placed in a 10% buffered formalin solution for a minimum of 24 hours and sent for histopathological analysis. The parasites were dehydrated in increasing alcohol solutions, added in paraffin blocks, microtomized (3µm), and stained by the hematoxylin and eosin (H&E) staining method. Under an optical microscope, histopathological alterations in the organs of *E. coelomaticum* were analyzed comparatively between the UNT (untreated control), NTZ, and PZQ in 100x and 400x increase.

Morphometric analysis by ImageJ® software was also performed on images of 50 testicles of parasites of each group (n = 150) in order to validate the reduction of the reproductive cells in the treated groups. The data was tested for normality of distribution and homogeneity of residuals, using Shapiro Wilk's and Levene's tests, respectively, with $P < 0.05$, then submitted to analysis of variance using the GLM procedure. The averages were compared using the Tukey's test. The analyses were performed in the Statistical Analysis System (SAS INSTITUTE INC., 2004) and significant statistical differences were considered when $P < 0.05$.

The motility degrees between the groups were analyzed using the SAS program (Analysis System Institute, Cary, NC, USA, version 9.1), submitted to the chi-square analysis (PROC FREQ). Significant statistical differences were considered when $P < 0.05$.

RESULTS

Both PZQ and NTZ were effective in the *in vitro* test performed, since they induced necrosis in different organs of the parasites and, consequently, their death.

The results regarding the parasite's motility are shown in table 1. After three hours of incubation, all parasites in the UNT showed normal motility (3). All parasites in the PZQ

Table 1 - Motility evaluation of *E. coelomaticum* treated in culture medium UNT (untreated control), with praziquantel (PZQ), and nitazoxanide (NTZ) at different post-incubation periods.

	Degree of motility*	UNT	PZQ	NTZ
After 3 hours of incubation	3	60 (100%)[a]		
	2			
	1			2 (3.33%)
	0		60 (100%)[a]	58 (96.67)[b]
After 12 hours of incubation	3	55 (91.67%)[a]		
	2	1 (1.67%)		
	1	2 (3.33%)		
	0	2 (3.33%)	60 (100%)[b]	60 (100%)[b]
After 15 hours of incubation	3	55 (91.67%)[a]		
	2	1 (1.67%)		
	1	2 (3.33%)		
	0	2 (3.33%)	60 (100%)[b]	60 (100%)[b]

Different letters indicate statistically significant differences between groups ($P < 0.0001$) by chi-square analysis.
*3, normal movements; 2 slow movements; 1 very slow movements; 0 still/dead.

were motionless or dead (0) and in the NTZ only two parasites showed very slow movement (1). In the 12 hour period, all specimens of the PZQ and NTZ groups were either motionless or dead. After 15 hours the motility parameters remained the same as in the previous evaluation.

As shown in the figure 1, histopathological examination revealed cytotoxic effects of both PZQ and NTZ on *E. coelomaticum* organs. In the reproductive tract, lesions were seen primarily in the testicules and the vitellogenic gland. Testicular lesions were characterized by irregular contour of the organ and the presence of invaginations in the PZQ group. In both treated groups there was a decrease in the number of testicular cells associated with a large amount of amorphous, slightly eosinophilic content. However, the lesions in the NTZ group were more intense (Figures 1D and 1G). The decrease of the area in pixels representative of reproductive cells in the testicle was confirmed after morphometric image analysis of 50 testicles of each group, analyzed by computer, and the program ImageJ® software was used for evaluation (Figure 2).

In the vitellogenic glands (Figures 1E and 1H) disorganization of the glandular parenchyma's acini was observed, some swollen cells and vacuolated nuclei, and other fragmented cells, which is suggestive of necrosis in the acinar cells. Additionally, glandular secretory content dispersed throughout the parasite's parenchyma was observed. Lesions were more pronounced in the NTZ group (Figure 1H) when compared to the PZQ group (Figure 1E). No lesions were identified in the specimens of the UNT (untreated control) (Figure 1B).

In the digestive tract, for both treated groups, the lesions were characterized by depression of the villi and a decrease in the basal cells lining the organ. Lesions were more pronounced in the PZQ group when compared to the NTZ group. No lesions were identified in the specimens of the UNT.

In the parenchyma of trematodes submitted to treatments with both PZQ and NTZ, there was an increase in parenchymal eosinophilia, with fragmentation of smooth muscle and vacuolated, sometimes fragmented, parenchymal cell nuclei. The integument (Figures 1C, 1F, and 1I)

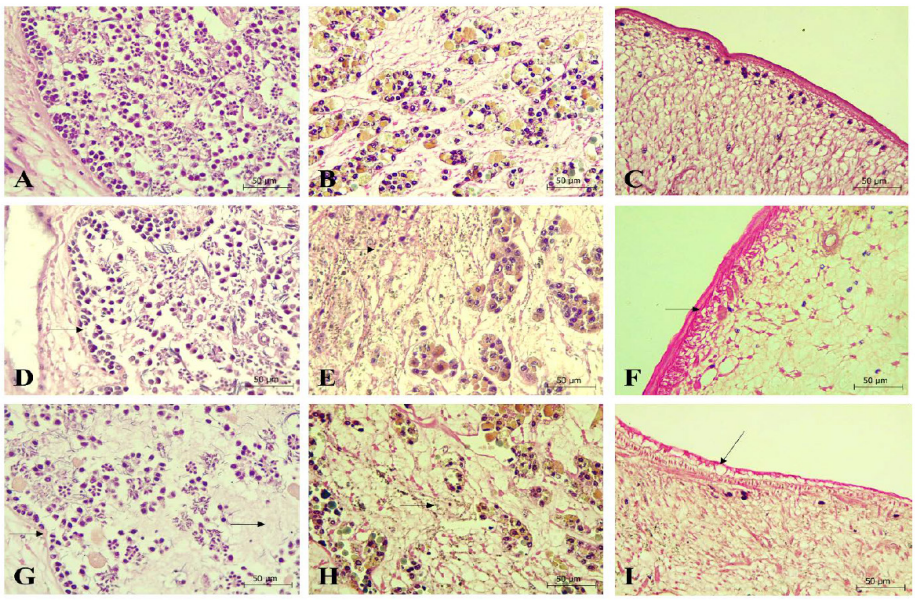

Figure 1 - *In vitro* effect of praziquantel and nitazoxanide on specimens of *E. coelomaticum*. In the UNT (untreated control) (A-C) the integrity of the testicles (A), vitellogenic gland (B), and integument (C) is noted. For both positive control group–treated with PZQ (D-F)– and for the NTZ group (G-I), lesions in the testicles (D and G) characterized by a decrease in the amount of testicular cells associated with the deposition of slightly eosinophilic content (arrow) are observed. In the vitellogenic gland (E and H), there is disorganization and necrosis (arrow) of acinar cells. In the integument (F and I), there is a decrease in eosinophilia, associated with basal lamina discontinuity and subtegumentary vacuolization (arrow).

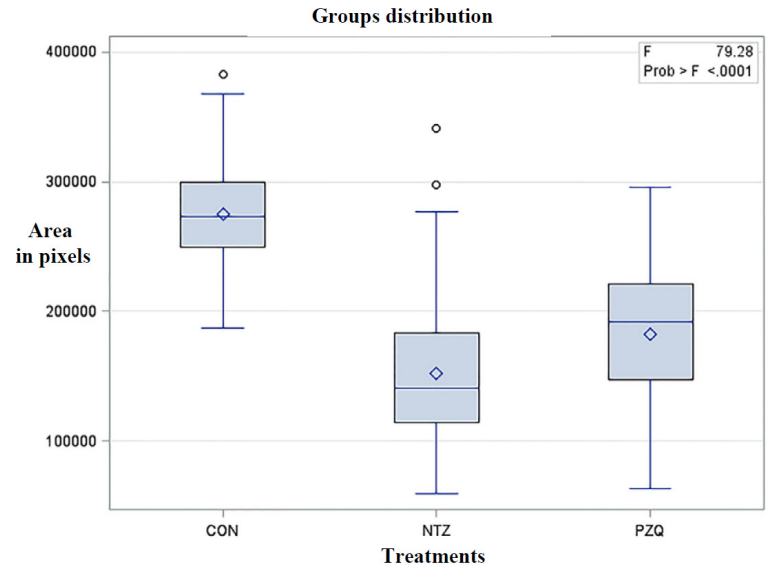

Figure 2 - Results distribution of the morphometric analysis of *E. coelomaticum* testicles incubated *in vitro* with PZQ (positive control group) or NTZ (treated group), compared to the CON (untreated control). The three groups evaluated differed statistically from each other ($P < 0.001$), demonstrating that the drugs used have a cytotoxic action on testicular cells of the parasite. Diamond – mean. Vertical lines – standard deviation (SD). Central horizontal lines – median. Circles – outliers.

showed a decrease in eosinophilia, associated with basal lamina discontinuity and sub-tegumentary vacuolization in both treated groups. Lesions in both structures were more pronounced in the NTZ group when compared to the PZQ group. No lesions were identified in the specimens of the negative control group. In the studied groups, no lesions were reported in the ovaries and suction cups.

DISCUSSION

The results of the present study demonstrated that both tested drugs were effective in reducing parasitic motility, reaching complete immobility up to 12 hours after the drug introduction to the parasitic specimens collected. This result was a consequence of important lesions in several organs, among which the integument, reproductive, and digestive tract stood out. This result was expected for PZQ, but for NTZ it is an unprecedented finding, since there are no previous studies on the *in vitro* action for the drug in *Eurytrema* spp.

PZQ has an inhibitory action on the sodium and potassium pump and consequent damage to the parasitic cell membrane–a lesion that was identified in the specimens evaluated in the present study–causing spastic paralysis in trematodes of several species, including individuals of the *Eurytrema* genus (KEISER & UTZINGER, 2004; JIRAUNGKOORSKUL et al., 2005; TAYLOR et al., 2016). This membrane lesion results in integumentary vesicles that appear in minutes or hours, depending on the PZQ dosage used (KEISER & UTZINGER, 2004; JIRAUNGKOORSKUL et al., 2005; XIAO et al., 2009; WANG et al., 2019).

Similarly, to the results described in the present study, previous *in vitro* studies evaluating the effectiveness of PZQ in the treatment of *E. pancreaticum* demonstrated the antiparasitic effect of this drug within three hours after exposure to the active ingredient. After 12 hours of incubation, the death of the parasites was induced by spastic paralysis, resulting from lesions in multiple organs such as the integument, musculature, and testicles. The study conducted by Jiraungkoorskul et al. (2005) showed lesions in the testicles and integument were similar to those found in the present study. However, the

vitellogenic glands remained intact, differing from the results found in the present study.

Unlike PZQ, NTZ promotes the inhibition of enzymes essential for parasitic metabolism, which results in the death of the parasites (SHAKYA et al., 2018). Although, it has been tested against other trematodes (KABIL et al., 2000; ATHERTON, 2004), in cestodes (WALKER et al., 2004), nematodes (TRITTEN et al., 2012), and protozoa (MEGEED et al., 2015).

The efficacy of NTZ in other trematodes such as the *Fasciola* genus is known, being a drug widely used in the control of fasciolosis in humans. It has been tested as an alternative to triclabendazole (TCBZ), to reduce dependence on a single drug, and to avoid the problems stemming from the development of resistance (FAIRWEATHER et al., 2020). For this parasite genus, *in vivo* NTZ is able to reduce the excretion of eggs in the feces, the serum eosinophil count, and, consequently, cause the death of the parasite (KABIL et al., 2000).

For parasites of the *Schistosoma* genus, NTZ results in rapid paralysis because of inhibit the binding of α-bungarotoxintonicotinicreceptors,inadditiontocausing important integumentary lesions (ATHERTON, 2004).

In cestodes, NTZ was tested *in vitro* in different species of the *Echinococcus* genus. The drug was able to induce microscopic lesions similar to those described in the present study for *E. coelomaticum*. The death of the cestodes was confirmed by verifying the loss of motility of the specimens (STETTLER et al., 2003; WALKER et al., 2004). Ultrastructurally, three hours after the parasites were exposed to NTZ, the drug was able to induce the formation of membranous vesicles on the tegument periphery, close to the laminated layer. Simultaneously, the undifferentiated cells (associated with the germ layer) produced large vacuoles filled with lipid-like and often electron-dense membranous segments. Stettler et al. (2003), who cited vacuolization of the germinal layer, accumulation of lipid droplets, and loss of mitochondria, also described these alterations. Although, ultrastructural analysis was not performed in the present study, due to the similarity of the histological lesions, it is believed that the lesions are similar.

The use of nitazoxanide to control diarrhea caused by *Cryptosporidium parvum* in young buffaloes–between one and two months old–at a dose of 500mg, orally, at every 12 hours for 3 days, reduced the number of oocysts eliminated via feces in the first week after administration, when compared to the UNT, promptly restoring the normal hematological parameters of the animals (MEGEED et al., 2015). The formulation used is the commercial drug for human use, which is two tablets of 500 mg (1000 mg nitazoxanide) per day, the maximum dose recommended in the medicine leaflet (ANITTA, 2016). There are no formulations for veterinary use available for cattle, so for the present study it was employed a dose based on human prescription. The NTZ is approved for veterinary use for the treatment of helminthic infections in cats and dogs in Switzerland and France (ZHAO et al., 2008). Food and Drug Administration (FDA) approved the use of NTZ in equine protozoal myeloencephalitis (1999).

NTZ is a molecule with great potential for application and repositioning, covering numerous possibilities. However, a more detailed investigation is required in order to perform the pharmacokinetic analysis of plasma metabolites and to clarify the pharmacological activity of these metabolites, allowing more relevant studies on the bioactivity and role of NTZ in the therapy of diseases in ruminants.

CONCLUSION

Praziquantel (PZQ) and nitazoxanide (NTZ) demonstrated *in vitro* effectiveness against trematode *E. coelomaticum*, confirmed by the total loss of motility after 12 hours of incubation and by the induction of pronounced histological lesions when compared to the untreated control. Severe damage in various organs such as the vitellogenic gland, intestine, parenchyma, integument, and testicle were observed after the test.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The methodology adopted was approved by the Animal Ethics Committee of the Instituto Federal de Santa Catarina, *Campus* Concordia, State of Santa Catarina, Brazil (Protocol No. 15/2020).

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