EFFICACY OF NITAZOXANIDE AGAINST *Toxocara canis*: LARVAL RECOVERY AND HUMORAL IMMUNE RESPONSE IN EXPERIMENTALLY INFECTED MICE

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SUMMARY

The efficacy of nitazoxanide (NTZ) against toxocariasis was investigated in an experimental murine model and results were compared to those obtained using mebendazole. Sixty male BALB/c mice, aged six to eight weeks-old, were divided into groups of 10 each; fifty were orally infected with 300 larvaed eggs of *T. canis* and grouped as follows, G I: infected untreated mice; G II: infected mice treated with MBZ (15 mg/kg/day) 10 days postinfection (dpi); G III: infected mice treated with NTZ (20 mg/kg/day) 10 dpi; G IV: infected mice treated with MBZ 60 dpi; G V: infected mice treated with NTZ 60 dpi; GVI: control group comprising uninfected mice. Mice were bled via retro-orbital plexus on four occasions between 30 and 120 dpi. Sera were processed using the ELISA technique to detect IgG anti-*Toxocara* antibodies. At 120 dpi, mice were sacrificed for larval recovery in the CNS, liver, lungs, kidneys, eyes and carcass. Results showed similar levels of anti-*Toxocara* IgG antibodies among mice infected but not submitted to treatment and groups treated with MBZ or NTZ, 10 and 60 dpi. Larval recovery showed similar values in groups treated with NTZ and MBZ 10 dpi. MBZ showed better efficacy 60 dpi, with a 72.6% reduction in the parasite load compared with NTZ, which showed only 46.5% reduction. We conclude that administration of these anthelmintics did not modify the humoral response in experimental infection by *T. canis*. No parasitological cure was observed with either drug; however, a greater reduction in parasite load was achieved following treatment with MBZ.

KEYWORDS: Experimental toxocariasis infection; Migrating larvae; Mebendazole (MBZ); Nitazoxanide (NTZ).

INTRODUCTION

Human infection by *Toxocara* larvae is considered an important and frequent zoonosis worldwide, and the main cause of visceral larva migrans (VLM) and other related syndromes. In humans, the main etiological agents for these syndromes are *Toxocara canis* and *T. cati,* respectively, ascarids of dogs and cats[5,11,16].

Several drugs are currently used to treat VLM in humans, and benzimidazole anthelmintics have shown moderate or good efficacy in the resolution of clinical signs or symptoms[16,28]. Experimentally, however, the various drugs tested in a murine model, which, as occurs in humans, are paratenic hosts species for *Toxocara* spp. larvae, showed a reduction of the parasite load that did not result in complete parasitological cure[1,6,22]. The same situation probably occurs in the treatment of infected humans.

In recent years, studies have demonstrated the efficacy of nitazoxanide in the treatment of infections caused by certain protozoa, helminths, anaerobic bacteria and hepatitis C virus[19], but few reports exist concerning its use in the treatment of infections caused by helminth larvae, particularly, roundworm larvae[15]. More recently, DELGADO *et al.*[9] reported an efficacy of 61.2% in the treatment of mice experimentally infected by *T. canis*.

The aim of this study was to evaluate the response of mice experimentally infected by *T. canis* following treatment with nitazoxanide on the tenth and sixtieth days after infection (dpi), regarding the reduction in parasitic load and the humoral response represented by anti-*Toxocara* IgG antibodies, compared to the response obtained in mice treated with mebendazole, previously considered a good schedule in the reduction of *T. canis* load in mice[21].

MATERIALS AND METHODS

Eggs: *T. canis* eggs used in this experiment were obtained from adult female worms, eliminated by naturally infected dogs following treatment with piperazine. After dissecting the worms, eggs were incubated in 2% formalin at 26 ºC for four to six weeks, and washed in filtered water to remove formalin before use.

Mice infection and treatment: Sixty male BALB/c mice, aged six to eight weeks-old, provided by the Faculty of Medicine of São Paulo

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University, were divided into groups of 10 mice each. Fifty mice were infected by stomach tube with 300 *T. canis* embryonated eggs in 200 µL of filtered water suspension, and ten mice were maintained as an uninfected control group, receiving filtered water alone, as indicated in Table 1. All the animals used in this study received water and a commercial diet *ad lib* and were treated in accordance to the ethical guidelines of the Institutional Experimental Guidelines for animal studies (CEUA) of the Institute of Tropical Medicine of São Paulo University (IMT/USP).

Table 1
Experimental infection and treatment of BALB/c mice with different doses of mebendazole and nitazoxanide

Group	Infection phase	Drug/ dose
I	Infected	No treatment
II	Acute phase	mebendazole (15 mg/kg/day)
III	Acute phase	nitazoxanide (20 mg/kg/day)
IV	Chronic phase	mebendazole (15 mg/kg/day)
V	Chronic phase	nitazoxanide (20 mg/kg/day)
Control	Noninfected	No treatment

At the acute phase of infection, 10 days postinfection (dpi), mice from group II were treated *per os* with mebendazole (15 mg/kg/day), and those from group III with nitazoxanide (20 mg/kg/day), for five consecutive days. Groups IV and V were treated with mebendazole and nitazoxanide respectively, using the same doses at 60 dpi (chronic phase of the infection) for the same period. Group I remained untreated throughout the experiment.

Sera collection: All groups were anesthetized with 100 mg/kg of xylazine and 10 mg/kg of ketamine, and bled by orbital plexus puncture at 30, 60, 90 and 120 dpi. The sera obtained were frozen and stored in aliquots in plastic micro tubes at -20 °C, for subsequent processing by ELISA.

Enzyme-linked immunosorbent assay (Ig ELISA): Serum samples collected from mice with different treatment schedules were examined by ELISA to detect IgG class anti-*Toxocara* antibodies; using excretion-secretion *T. canis* antigen (TES) prepared from the culture of infective larvae of this parasite in Eagle medium, according to a standardized technique described by DE SAVIGNY *et al*.[10] and modified in our laboratory. Different concentrations of ES antigen of *T. canis* were tested using positive and negative control sera and a protein concentration of 20 µg/mL (100 mL per well in plate) was determined to performe the tests. Mouse sera were diluted in PBS-T-Gelatin at 1:800. The enzyme conjugate anti-mouse IgG labeled with horseradish peroxidase (γ-chain specific, Sigma Immunochemicals) was used at 1:4,000 dilution. A mixture of H_2O_2 and O-phenylenediamine (1.2-Benzenediamine) (Sigma Chemical Co.) [$C_6H_8N_2$. 2HCl], diluted in citrate-phosphate buffer, was used as substrate.

The results were evaluated by spectrophotometric reading at a wavelength of 492 nm in Titertek Multiskan apparatus MCC/340 P version 2.20 (Lab-Systems, Finland). To calculate the threshold of reactivity ("cut-off") the mean optical density (OD) of readings of sera from control group mice was considered, plus two standard deviations. As a positive control, serum samples from three mice infected for 90 days were used[18] and, as a negative control, serum samples from mice bled before infection were used.

T. canis larval recovery in tissues: At the end of the experiment (120 dpi), mice from all the infected groups were sacrificed by cervical dislocation and larvae were recovered from their brains and carcasses, following digestion with 0.5% HCl for 24 h at 37 ºC. Sedimental liquid was centrifuged for two min at 1500 rpm, 2 mL of the sediment were collected, thoroughly mixed, and 0.1 mL samples were viewed under a light microscope to determine larval counts[33].

Statistics: Arithmetic mean and standard deviation were used to present data. One-way ANOVA and Tukey test for multiple comparisons were conducted with Optical Density (OD) readings of sera from infected and treated mice from different groups, and two-way ANOVA and Bonferroni post test were used to compare larval recovery in tissues using the Prisma program, version 5.0. Differences were considered to be significant when $p < 0.05$.

RESULTS AND DISCUSSION

An issue that has not been fully resolved is the way of assessing drugs used in the treatment of experimental toxocariasis, in which the murine model is the most frequently used. Numerous authors have used the recovery of *T. canis* larvae, following digestion of the carcass and some organs of experimentally infected mice[14,23,24,28,31]. Others, however, have employed immunological techniques[3,7]. In this study, we sought to determine the kinetics of IgG anti-*Toxocara* antibodies in mice, experimentally infected with larvae of this nematode and subsequently treated with mebendazole (MBZ) or nitazoxanide (NTZ), as well as observing larval recovery after acid digestion of different mouse organs and tissues.

The humoral antibody response of the IgG class anti-*Toxocara* (Fig. 1 and 2) was detected within the first 30 dpi, which remained positive up to 120 dpi in both groups treated with both anthelmintic drugs. Moreover, a marked increase in antibodies was observed between 90 and 120 dpi. This reactivity is probably due to the continuous release of *Toxocara* antigens even by dead larvae, from antigens stored in their cuticles. On the other hand, it is known that most anthelmintics do not reach all larvae present in the host[8,21]. No other significant differences were observed in the levels of anti-*Toxocara* antibodies among mice treated with nitazoxanide or mebendazole compared with the group infected, but not submitted to treatment.

Comparing the amount of *T. canis* larvae recovered in various organs of mice treated with mebendazole or nitazoxanide with the result in the group not submitted to treatment, differences were only observed when the treatment occurred after 60 days of infection (Table 2). Moreover, the difference was significantly more evident in the group treated with mebendazole.

Posttreatment larval recovery, in all the organs analyzed, revealed a decrease in the amount of larvae recovered in the central nervous system of the mice (Table 3).
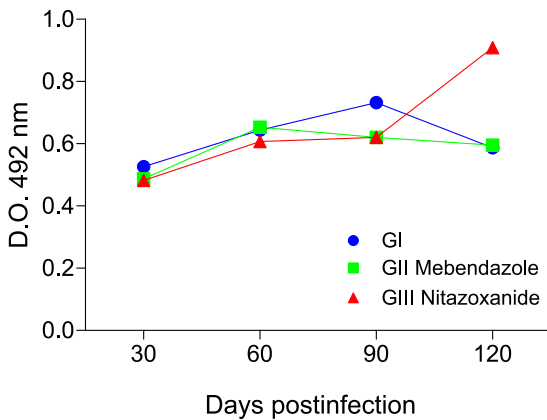
Fig. 1 - Dynamics of ELISA circulating anti-*T. canis* Ig G antibodies in BALB/c mice infected with *Toxocara canis* eggs and treated in the acute phase of infection (10dpi) with G II- mebendazole (■) or G III- nitazoxanide (▲), comparing with the G I (●) infected non-treated group.
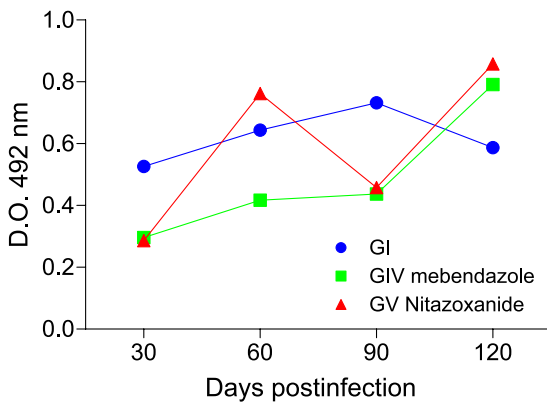

Fig. 2 - Dynamics of ELISA circulating anti-*T. canis* Ig G antibodies in BALB/c mice infected with *Toxocara canis* eggs and treated in the chronic phase of infection (60 dpi) with G IV-mebendazole (■) or G V-nitazoxanide (▲), comparing with the G I (●) infected non-treated group.

Previous studies indicated that mice and other murine behave like paratenic hosts when infected by larvae of *T. canis*[30] and are therefore suitable models for studying host-parasite relationships in the case of

Table 2
Toxocara canis larval recovery in BALB/c mice treated with MBZ or NTZ in the acute and chronic phases of experimental infection (mean and standard deviation).

Groups	Acute phase	Chronic phase
MBZ	84.4 ± 38.1	24.6 ± 23.9 *
NTZ	71.6 ± 24.3	48.3 ± 27.7
Infected untreated	89.9 ± 22.9	

MBZ = mebendazole; NTZ = nitazoxanide; * $p < 0.05$

human infection with this nematode[4,11]. In C57BL/6 and BALB/c mice infected with *T. canis* and untreated, the larvae can be found in the brain and carcass, even up to a year after inoculation, with a slight reduction in the total larval count[17,20]. Various anthelmintics have been used in the treatment of murine toxocariasis, in order to evaluate their efficacy: mebendazole, albendazole, fenbendazole and thiabendazole. The first three, and diethylcarbamazine, were recommended by the WHO in 1995 as effective drugs against toxocariasis[20]. According to SMITH *et al.*[29], mebendazole seems to be a good alternative for toxocariasis treatment in paratenic hosts. LESCANO *et al.*[21] also reported a significant decrease in the parasite load of mice experimentally infected by *T. canis* and treated with mebendazole, thiabendazole or ivermectin.

Nitazoxanide (NTZ) is a novel compound with broad-spectrum activity against several genera of parasites, including intestinal protozoa, helminths and anaerobic bacteria[13,32]. Its activity is associated with the inhibition of enzymatic activity of pyruvate ferredoxin oxidoreductase (PEOR) and against the disulfide isomerase protein (PDI), an essential enzyme for anaerobic metabolism. However, the mode of action of NTZ and its principal metabolite, tizoxanide, on nematodes is unknown[27]. Its use has been approved for treatment of humans with diseases caused by *Giardia intestinalis* and *Cryptosporidium*[2,25], and DELGADO *et al.*[9] reported some efficacy against *T. canis* larvae in experimental infections.

The results of this research suggest that the humoral immune response is not an appropriate parameter for evaluating drug efficacy in paratenic hosts experimentally infected by *T. canis*. However, at the end of the experiment, larval recovery was shown to be the most appropriate tool for evaluating drug efficacy in this experimental model.

Table 3
Mean number and standard deviation of *Toxocara canis* larvae recovered in organs and tissues of BALB/c mice infected and treated with MBZ or NTZ in the acute and chronic phases of infection

		Acute		Chronic	
	GI (Non treated)	GII-MBZ	GIII-NTZ	GIV-MBZ	GV-NTZ
Carcass	59 ± 11.59	45.4 ± 10.21	42.6 ± 16.94	16 ±14.54	38.6 ± 22.52
Liver	2.8 ± 2.16	2.8 ± 2.38	1.6 ± 1.67	0.4 ± 0.89	1.2 ± 1.6
Lungs	0.4 ± 0.89	0	0.2 ± 0.44	0	0
Kidneys	0	0	0	0	0
Brain	26.8 ± 16.11	35.8 ± 31.52	27 ± 14.37	8.2 ± 8.64	7.4 ± 3.43
Eyes	0.8 ± 1.30	0.4 ± 0.54	0.2 ± 0.44	0	0.8 ± 1.30

Considering the larval recovery results, the relative ineffectiveness of mebendazole or nitazoxanide administration in the treatment of *Toxocara* infections in the murine model is evident and this is probably true in the case of human infections by *T. canis*, which would result in visceral larva migrans syndrome. Despite these fairly moderate results, the greater reduction observed in the parasite load following mebendazole administration, compared with the results achieved when nitazoxanide was used, deserves some attention.

RESUMO

Eficácia da nitazoxanida contra *Toxocara canis*: recuperação larvária e resposta imune humoral em camundongos experimentalmente infectados

Foi investigada a eficácia da nitazoxanida (NTZ) na toxocaríase murina experimental e os resultados comparados com os obtidos usando mebendazol (MBZ). Sessenta camundongos BALB/c machos, com idade entre seis e oito semanas foram divididos em grupos de 10 cada, 50 foram infectados oralmente com 300 ovos larvados de *T. canis* e agrupados a seguir: GI: camundongos infectados não tratados; GII: camundongos infectados tratados com MBZ (15 mg/kg/dia) 10 dias pós-infecção (dpi); GIII: camundongos infectados tratados com NTZ (20 mg/kg/dia) 10 dpi, GIV: camundongos infectados tratados com MBZ 60 dpi; GV: camundongos infectados tratados com NTZ 60 dpi; GVI: controle não infectado. Os camundongos foram sangrados via plexo retro orbitário em quatro ocasiões entre o 30º e 120º dpi. Os soros foram processados pela técnica de ELISA para detecção de anticorpos IgG anti- *Toxocara.* Aos 120 dpi, os animais foram sacrificados para a recuperação larvária do SNC, fígado, pulmões, rins, olhos e carcaça. Os resultados mostraram níveis similares de anticorpos IgG anti-*Toxocara* entre os camundongos infectados mas não submetidos a tratamento e os grupos infectados e tratados com MBZ ou NTZ, aos 10 e 60 dpi. Os valores da recuperação larval foram similares nos grupos tratados com NTZ e MBZ 10 dpi. MBZ mostrou melhor eficácia aos 60 dpi, com redução de 72,6% da carga parasitária comparada com NTZ, que mostrou redução somente de 46,5%. Concluímos que a administração destes anti-helmínticos não modificou a resposta humoral na infecção experimental por *T. canis*. Não foi observada cura parasitológica com nenhuma das drogas; porém maior redução na carga parasitária foi obtida após o tratamento com MBZ.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

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