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Original Article

Cyclosporine A increases the intensity of *Toxocara canis* infection in swiss mice

Ciclosporina A aumenta a intensidade da infecção por *Toxocara canis* em camundongos suíços

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Abstract

Toxocariasis is a zoonotic disease of worldwide distribution. The connection between parasitic diseases and conditions that depress the immune system, such as the use of immunosuppressive drugs, has been studied. The purpose of this study was to evaluate the effect of Cyclosporine A (CsA) on the intensity of infection, humoral response and gene transcription of interleukins IL-4, IL-10 and IL-12 in mice experimentally infected with *Toxocara canis*. To this end, mice were divided into two groups treated with CsA (G1: 10 mg/Kg and G2: 50 mg/kg), the G3 and G4 group received PBS. After the last administration of the drug or PBS (orally every 48 hours for 15 days), groups G1, G2 and G3 were inoculated with 1200 eggs of *T. canis.* Was collected blood samples on days zero, 15 and 30 days post-inoculation (PI), for ELISA test and the mice were euthanized 30 days PI. The organs and striated muscle tissue were collected for the recovery of larvae. The splenocytes were analyzed by RT-PCR. The intensity of infection in the mice treated with 50 mg/kg of CsA was 65.5% higher than in the control group (p=0.001). An analysis of the kinetics of anti-*Toxocara* antibody revealed that the groups treated with CsA showed significantly higher mean levels of antibodies on day 15 PI. The transcription of the three tested interleukins showed no statistical difference between G2 and G3 (control). It was concluded that the immunosuppression triggered by CsA (50 mg/Kg) favored the establishment of a larger number of *T. canis* larvae without, however, altering immunoglobulin production and IL-4, IL-10 and IL-12 transcription on day 30 PI.

Keywords: toxocariasis, IL-4, IL-10, IL-12, IgG.

Resumo

A toxocaríase é uma zoonose de distribuição mundial. A conexão entre doenças parasitárias e condições que deprimem o sistema imunológico, como o uso de drogas imunossupressoras, tem sido estudada. O objetivo deste estudo foi avaliar o efeito da Ciclosporina A (CsA) na intensidade da infecção, resposta humoral e transcrição gênica das interleucinas IL-4, IL-10 e IL-12 em camundongos experimentalmente infectados com Toxocara canis. Para tanto, os camundongos foram divididos em dois grupos tratados com CsA (G1: 10 mg/Kg e G2: 50 mg/kg), os grupos G3 e G4 receberam PBS. Após a última administração da droga ou PBS (via oral a cada 48 horas por 15 dias), os grupos G1, G2 e G3 foram inoculados com 1200 ovos de T. canis. Foram coletadas amostras de sangue nos dias zero, 15 e 30 dias pós-inoculação (PI), para teste de ELISA e os camundongos foram eutanasiados 30 dias PI. Os órgãos e tecido muscular estriado foram coletados para a recuperação das larvas. Os esplenócitos foram analisados por RT-PCR. A intensidade da infecção nos camundongos tratados com 50 mg/kg de CsA foi 65,5% maior do que no grupo controle (p=0,001). Uma análise da cinética do anticorpo anti-Toxocara revelou que os grupos tratados com CsA apresentaram níveis médios de anticorpos significativamente maiores no dia 15 PI. A transcrição das três interleucinas testadas não apresentou diferença estatística entre G2 e G3 (controle). Concluiu-se que a imunossupressão desencadeada pela CsA (50 mg/Kg) favoreceu o estabelecimento de um maior número de larvas de T. canis sem, no entanto, alterar a produção de imunoglobulinas e a transcrição de IL-4, IL-10 e IL-12 no dia 30 PI.

Palavras-chave: toxocaríase, IL-4, IL-10, IL-12, IgG.

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1. Introduction

Toxocariasis is a widespread zoonotic disease, *Toxocara* spp eggs that are sources of infection for humans are often found contaminating the environment, the main zoonotic specie that cause infection are *Toxocara canis* (Leon et al., 2020, Fialho and Corrêa, 2016). *Toxocara canis* prevalence rates, in humans vary among different populations, ranging from 6.4% among pregnant women (Santos et al., 2015), to 20% among neonates (Santos et al., 2017) and from 43.9% to 50.6% among children (Araújo et al., 2020; Schoenardie et al., 2013).

The clinical manifestations of the disease can also vary (Fillaux and Magnaval, 2013). In the acute phase, there is an inflammatory response with an increase in eosinophils and neutrophils (Resende et al., 2015). In the chronic phase, the immune response is mediated by T-helper 2 cells and is related to the production of anti-*Toxocara canis* antibodies (Carvalho and Rocha, 2011).

Immunosuppressive drugs are necessary and widely used for treating various diseases (Bressan et al., 2010). However, if patients that use these medications are hosts of parasitic agents before they begin immunosuppressive treatment, or become infected during the treatment, they may develop a more severe condition (Braz et al., 2015). The reactivation of latent forms of infectious agents in individuals undergoing immunosuppressive therapy has been extensively reported (Santos-Neto et al., 2003; Fishman, 2011; Riganti et al., 2012; Rizo-Topete et al., 2015), most of them involving protozoan infections.

Individuals with impaired immune system are strongly affected by helminth infections, e.g., immunocompromised patients infected with *Strongyloides stercoralis* and *Schistosoma mansoni* (Crowe et al., 2019; Brandão et al., 2012). There is also evidence that *T. canis* infection in immunosuppressed mice causes an increase in the intensity of the infection and an increase in tissue damage (Eid et al., 2015; Avila et al., 2012).

Thus, given the prevalence of *T. canis* in different populations and the widespread use of immunosuppressive drugs, a better understanding of the dynamics of toxocariasis is needed, including an experimental model of immunodeficiency.

Thus, the objectives of this study were to verify the effect of Cyclosporine A on the intensity of infection in Swiss mice experimentally infected with *T. canis* and to evaluate possible mechanisms triggered by the host immune response.

2. Material and Methods

2.1 Animals

A total of 34 female Swiss mice were used in this experiment. The five to seven weeks old mice, which were supplied by the Central Vivarium of the Federal University of Pelotas (CEEA - 7921-2014), had unrestricted access to food and water and were kept in a 12/12 light cycle.

2.2. Experimental design

For the experiment, three groups of 10 mice each (G1, G2 and G3) and one group of four mice (G4) were formed. The mice in G1 and G2 received a dose of 10 mg/kg and 50 mg/kg, respectively, of Cyclosporine A (CsA – Sandimmun®). The CsA was administered orally on alternate days (every 48 h), for 15 days. The mice of G3 (infection control) were given PBS during the same period and by the same route. Twenty-four hours after the last administration of CsA, each animal in groups G1, G2 and G3 was infected with 1200 embryonated *T. canis* eggs administered by intragastric tube (Avila et al., 2012). Group G4 (negative control) did not receive CsA and was not infected, acting as a negative control for the evaluation of interleukin gene transcription.

2.3. Recovery of Toxocara canis larvae

At the end of the experimental period (30 days), the animals were euthanized under deep anesthesia (Thiopental by intraperitoneal injections 75 mg/kg). *Toxocara canis* larvae were then recovered by means of tissue digestion of organs (liver, lungs, brain, kidneys, heart and eyes) and skeletal muscle, using a solution of 1% pepsin and 1% hydrochloric acid (HCl) at 37ºC, which was kept under agitation for 24 hours (Xi and Jin, 1998).

2.4. Evaluation of the production kinetics of anti-Toxocara canis IgG

To study total anti-*Toxocara* antibodies, three blood samples were drawn on days zero, 15 and 30 post-inoculation (PI) to obtain serum. An indirect ELISA was performed using the excretion and secretion antigen of *T. canis* L3 larvae (TES) at a concentration of 1 µg/mL, and serum diluted 1:50 to examine total IgG (Avila et al., 2012). The samples were evaluated in duplicate and analyzed in an ELISA reader (ThermoPlate®) at a wavelength of 492 nm.

2.5. Evaluation of interleukins

The spleens of the mice of G2 and G3 were removed and the splenocytes were isolated and stored in TRIzol reagent (Life Technologies, Carlsbad, CA, USA). RNA was extracted according to the protocol recommended by the manufacturer of TRIzol® and was then quantified (GE Healthcare® NanoVue Plus). The cDNA was synthesized using 400 ng/µL of RNA. The reaction was performed following the instructions of the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The relative transcription of the interleukin genes IL-4, IL-10 and IL-12 was analyzed by real-time polymerase chain reaction (qPCR) in a Stratagene MX005P real-time PCR system (Agilent Technologies, Santa Clara, CA, USA), as described by Avila et al. (2016). β-actin and GAPDH genes were used as endogenous reference controls, and based on the M-values of 0.9 and 1.5 obtained, respectively, the β-actin gene was selected as the standard. The specific oligomer initiators for the IL-4, IL-10, IL-12, β-actin and GAPDH genes, as well as the conditions of the qPCR of the latter two, have been described previously (Cardona et al., 2003; Dummer et al., 2014). All the samples were analyzed in triplicate. The relative expressions of the genes were calculated from the threshold cycle (Ct) values obtained by comparison with the β-actin expression, according to the $2^{-\Delta\Delta C}{}_{T}$ method described by Livak and Schmittgen (2001).

2.6. Statistical analysis

A statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS ®), version 22 for Microsoft Windows®. The variables under study were characterized by means (M) and standard deviation (SD), considering a 5% level of significance.

The Kruskal-Wallis test (comparison of three independent groups), the Friedman test (comparison of the levels of anti-*T. canis* antibodies produced in the post-infection periods – repeated measures) and the Correlation Coefficient (correlation between the number of larvae and titration of antibodies) were applied. The Kruskal-Wallis and Friedman tests were considered significant when the p value was less than 0.05 ($p<0.05$).

An analysis of variance (one-way ANOVA), followed by Dunnett's test, were performed to determine whether there was a statistical difference in the levels of relative transcription of interleukin genes.

3. Results

3.1. Treatment with CsA increases the recovery of T. canis larvae

Table 1 describes the intensity of infection (M ± SD) in different organs and skeletal muscle tissue of the mice in the three groups under study. The total mean number of larvae recovered from the mice of G2 (CsA 50 mg/Kg) was significantly higher than the ones recovered from G1 (CsA 10 mg/Kg) and G3 (control), and the recovery of larvae from G2 was 65.5% higher than from G3. The mean recovery of larvae from liver, lungs, brain and skeletal muscle tissue (carcass) of G2 was also significantly higher than from the other groups. However, this parameter showed no significant difference between G1 and G3. All the animals treated with 50mg/kg of CsA showed splenomegaly.

3.2. Kinetics of anti-T. canis antibody production

Table 2 lists the average levels of anti-*Toxocara canis* IgG antibodies and the comparison between the three groups of mice in the post-inoculation (PI) periods. Antibody production in all the groups (G1, G2 and G3) showed a statistically significant difference between day zero and day 15 PI, but only the control group (PBS) showed an increase in antibody production ($p<0.05$) on day 15 and day 30 PI.

3.3. Correlation between the number of recovered larvae and the levels of antibodies

In this study, no correlation was found between the levels of antibodies and the number of recovered larvae. Table 3 describes the correlation coefficient between the total number of larvae and the levels of antibodies on days 15 and 30 PI in each experimental group.

Table 1. Number of *Toxocara canis* larvae (mean ± standard deviation) recovered from organs and skeletal muscle tissue of mice previously treated with Cyclosporine A (n = 10).

Variables	G1 (CsA 10 mg/Kg)	G2 (CsA 50 mg/Kg)	G3 Control (PBS)	Kruskal-Wallis test
Liver	10.50 ± 6.08 [a]	24.00 ± 9.68 [b]	12.70 ± 6.13 [a]	*p = 0.006*
Lungs	4.40 ± 2.41 [a]	9.70 ± 3.37 [b]	5.20 ± 3.39 [a]	*p = 0.005*
Brain	60.90 ± 36.23 [a]	122.50 ± 26.37 [b]	74.20 ± 24.27 [a]	*p = 0.002*
Kidneys	1.70 ± 0.48 [a]	1.00 ± 1.05 [a]	0.90 ± 0.99 [a]	*p = 0.067*
Heart	1.50 ± 1.43 [a]	2.10 ± 1.73 [a]	0.80 ± 0.79 [a]	*p = 0.141*
Muscle tissue	15.60 ± 4.79 [a]	33.70 ± 9.07 [b]	23.30 ± 13.23 [a]	*p = 0.001*
Eyes	0.30 ± 0.48 [a]	0.70 ± 0.67 [a]	0.40 ± 0.52 [a]	*p = 0.323*
Total	94.90 ± 42.50 [a]	193.70 ± 33.66 [b]	117.50 ± 37.02 [a]	*p = 0.001*

[a,b]There are no significant differences between groups with the same letter: $p > 0.05$ in Dunn's multiple comparisons test.

Table 2. Total anti-*Toxocara* antibody levels (mean ± standard deviation) in Swiss mice treated with Cyclosporine A and subsequently infected with *T. canis* eggs, on days zero, 15 and 30 days post-inoculation (n = 10).

Groups	Periods			Friedman test [(1)]	Multiple comparisons		
	Day zero	Day 15 PI	Day 30 PI		Day zero vs. 15 PI	Day zero vs. 30 PI	Day 15 vs. 30 PI
G1 - CsA 10 mg/Kg	-	0.33 ± 0.06	0.32 ± 0.05	p = 0.001	p = 0.002	p = 0.002	*p = 1.000*
G2 - CsA 50 mg/Kg	-	0.29 ± 0.06	0.28 ± 0.04	p = 0.001	p< 0.001	p = 0.001	*p = 0.655*
G3 - Controle	-	0.20 ± 0.07	0.33 ± 0.06	p< 0.001	p = 0.014	p< 0.001	*p = 0.034*
Kruskal-Wallis test [(2)]	-	*p = 0.004*	*p = 0.126*				

[(1)] Comparison between the periods in each group; [(2)] Comparison between the three groups in each period.

Table 3. Correlation coefficient between the number of *Toxocara canis* larvae recovered from organs and skeletal striated muscle tissue and levels of total anti-*Toxocara canis* antibodies produced in Swiss mice treated with Cyclosporine A (n = 10).

Larvae	Day 15 post-infection	Day 30 post-infection
G1CsA 10 mg/Kg	r=0.200(p=0.580)	r=0.018(p=0.960)
G2CsA 50 mg/Kg	r=-0.176(p=0.627)	r=-0.200(p=0.580)
G3Control	r=-0.571(p=0.084)	r=-0.286(p=0.424)

r – Correlation coefficient; p – level of significance.

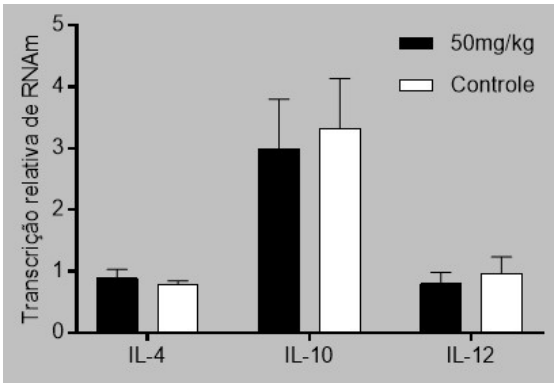

Figure 1. Relative mRNA transcription of interleukins IL-4, IL-10, IL-12 in splenocytes of Swiss mice treated with CsA (50 mg/Kg), infected with *Toxocara canis* eggs and euthanized 30 days post-inoculation.

3.4. mRNA transcription of interleukins IL-4, IL-10, and IL-12

The transcription of interleukins IL-4, IL-10 and IL-12 was evaluated only in the groups that showed a statistical difference in the number of recovered larvae (groups G2 and G3). G4 was not treated with CsA and was not infected, acting as negative control for the evaluation of interleukin gene transcription.

Splenocytes from the mice in G2 and G3 showed a similar mRNA transcription profile as the interleukins IL-4, IL-10, IL-12 when compared to each other, with no statistically significant difference (Figure 1).

4. Discussion

Two doses (10 mg/kg and 50 mg/kg) of CsA, administered to Swiss mice every 48 hours for 15 days prior to infection by *Toxocara canis*, were evaluated in this study. The sites with the largest number of recovered larvae were brain and skeletal muscle tissue, which is consistent with the chronic phase of the infection (Lescano et al. 2004; Avila et al., 2012). During acute infection the larvae are usually present in greater numbers in the lungs and liver (Moura et al., 2018).

All the mice treated with CsA 50 mg/kg showed splenomegaly, which may have been caused by the use of the immunosuppressant, since the control group (infected but not treated) did not present this clinical symptom, possibly due to the immunotoxic effect that it can trigger in murine models (Hussain et al., 2005). This was also reported in mice infected with the microsporidium *Encephalitozoon intestinalis* and treated with 50 mg/kg of CsA, although no increase or dissemination of the parasite was observed (Galván et al. 2006), unlike what was observed in this study when the same concentration of CsA was administered.

The animals in group G2 (CsA 50mg/Kg) showed a 65.5% higher number of *T. canis* larvae recovered than the control group. Lescano et al. (2004) reported similar results, stating that the same dosage of CsA increased the intensity of *T. canis* infection in BALB/c mice infected with 300 eggs. When less than 20 mg/kg of CsA was administered, mice infected with *T. cruzi* showed no increase in parasitemia or mortality (Andrade et al., 1997). A result similar to ours was also reported by Dias et al. (2013), who infected hamsters with *Ancylostoma ceylanicum* and treated them with 10 mg/kg of CsA, which did not interfere in the course of the infection.

The effect of other immunosuppressive drugs, such as cyclophosphamide, dexamethasone and betamethasone have also been evaluated in *T. canis* infections (Lescano et al., 2004; Eid et al., 2015; Avila et al., 2012). Like CsA, a dose of 50 mg/kg of cyclophosphamide had an immunosuppressive effect in Swiss mice infected with *T. canis*, leading to a 162.1% increase in the intensity of the infection compared to the control group (Avila et al., 2012). The results of these studies, like ours, suggest that the dose of the drug may be directly linked to the occurrence of immunosuppression, and hence, to the greater intensity of the infection.

Mice that received a dose of 50mg/kg of CsA showed an increase in the parasitic load in this study, but no statistically significant difference in the expression of IL-4, IL-10 and IL-12 was found between the treated group (50mg/kg) and the control. Other studies have reported that mice treated with 20 mg/kg of cyclophosphamide for 5 days showed intensified *T. canis* infection on day 15 PI, as well as significantly reduced levels of IL-5 (Eid et al., 2015), which is a pro-inflammatory interleukin that acts in the recruitment of eosinophils (Harish and Schwartz, 2020).

This study evaluated IL-12, an important pro-inflammatory interleukin produced by dendritic cells, macrophages and plasmocytes. IL-12 directs the immune system to a Th1-type response (Vignali and Kuchroo, 2012), which may be suppressed during *T. canis* infection (Kuroda et al., 2001; Avila et al., 2016; Moura et al., 2017), since the parasite modulates the host's immune system to a Th2 response (Resende et al., 2015). However, the suppression found in previous studies was not observed in the two groups evaluated here (G2 and G3), which kept the transcription of this interleukin at baseline levels. A similar result was observed in another study, in which IFN-γ was maintained at baseline during *T. canis* infection (Cadore et al., 2021), in that study IL-12 was not evaluated, but considering that IFN-γ production by natural killer cells is stimulated by IL-12 (Airoldi et al., 2002), probably both were at baseline levels.

Increased levels of anti-*Toxocara* antibodies have already been linked to higher intensity of infection (Fonseca et al., 2017; Novák et al., 2017). However, in this study, no correlation was found between the levels of antibodies

and the number of recovered larvae, which is similar to that reported by Avila et al. (2012).

Although other studies have described the antiparasitic effect of CsA against several protozoan and helminthic infections, this effect was not observed in this study, since there was no decrease in the number of larvae in the treated groups compared to the control. However, the lowest dose of CsA used in this study was 10mg/kg, and its antiparasitic effect was only detected when concentrations of less than 10mg/kg of the drug were used (Chappell and Wastling, 1992; Bell et al., 1996; Colebrook et al., 2002; Dzik et al., 2006).

The use of immunosuppressive drugs is on the rise; therefore, studies linking the effect of these drugs and their association with the occurrence or recrudescence of parasitic diseases are highly relevant. This study revealed the dynamics of toxocariasis in a *Mus musculus* (Swiss) experimental model, since no study had been carried out on this strain using CsA as an immunosuppressant during infection by *T. canis*. The introduction of immunosuppressive drugs in the treatment of autoimmune diseases, cancers and transplant recipients, among others, has led to the more frequent report of cases of parasitic diseases (Braz et al., 2015), especially of protozoan infections. Nevertheless, further studies are needed to clarify the effects of these drugs on helminths.

The action of 50 mg/kg of CsA increased the intensity of *T. canis* infection in mice. Therefore, it is recommended that patients using this drug be tested for parasitic infections, since different doses of the drug seem to trigger different reactions with respect to the intensity of these infections (Chappell and Wastling, 1992; Bell et al., 1996; Colebrook et al., 2002; Dzik et al., 2006; Dias et al., 2013).

5. Conclusions

It was concluded that the effect triggered by CsA (50 mg/Kg) in mice was to increase the number of *T. canis* larvae infesting them, albeit without altering the production of IgG immunoglobulins and the transcription of IL-4, IL -10 and IL-12 on day 30 after infection.

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