

Clinical and parasitological evaluation of dogs naturally infected by *Leishmania (Leishmania) chagasi* submitted to treatment with meglumine antimoniate and allopurinol

Avaliação clínica e parasitológica de cães naturalmente infectados por Leishmania (Leishmania) chagasi submetidos a tratamento com antimoniato de meglumina e alopurinol

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Abstract

Aiming to assess the efficacy of the treatment, to verify the occurrence of possible disease relapses and to search for the presence of parasites after the treatment, seven dogs naturally infected by *Leishmania sp.*, were submitted to a treatment with meglumine antimoniate and allopurinol. For this, lymph node and bone marrow aspiration biopsies were carried out at seven moments. After the end of the six-month observation period all dogs were submitted to euthanasia. Then, spleen and liver “imprints” and *in vitro* cultures were carried out to search for amastigote forms of the parasite. All animals presented remission of the symptoms and during all the observation period no dog presented relapse of the disease, although amastigote forms of the parasite were observed in two of the animals at the end of the experiment. Thus, it was possible to conclude that the treatment promotes clinical healing but it does not eliminate the parasites completely.

Keywords: Dogs. Visceral leishmaniasis. Treatment. Allopurinol. Meglumine antimoniate.

Resumo

Com objetivo de avaliar a eficácia do tratamento, verificar a ocorrência de possíveis recidivas da doença e pesquisar a presença de parasitas após a realização do tratamento, foram utilizados sete cães naturalmente infectados por *Leishmania sp.*, submetidos a tratamento com antimoniato de meglumina e alopurinol. Para tanto, foram realizadas punções biópsias aspirativas de linfonodos e de medula óssea em sete momentos. Após o término dos seis meses de observação, todos os cães foram submetidos à eutanásia e realizados “imprints” e cultivo *in vitro* do baço e fígado para a pesquisa de formas amastigotas. Todos os animais apresentaram remissão dos sintomas e durante todo o período de observação nenhum cão apresentou recidiva da doença apesar de ter sido observada a presença de formas amastigotas do parasita em dois animais, ao término do experimento. Desta forma, foi possível concluir que o tratamento promove a cura clínica, entretanto não elimina completamente os parasitas.

Palavras-chave: Cães. Leishmaniose visceral. Tratamento. Alopurinol. Antimoniato de meglumina.

Introduction

Visceral leishmaniasis, also known as Kalazar, is an anthrozoosis caused by a protozoan belonging to the order Kinetoplastida, family Trypanosomatidae and genus *Leishmania*, of which *Leishmania (Leishmania) chagasi* (Cunha and Chagas, 1937) is the

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species found in Brazil^{1,2,3,4}. *Leishmania (Leishmania) chagasi* has already been identified in humans, dogs, cats, wild canids, marsupials and rodents^{2,3}. In the domestic environment, the dog is considered the main epidemiological reservoir, which makes controlling the disease difficult. From the epidemiological point of view, canine leishmaniasis is considered more important than the human disease because besides being more prevalent, the canine zoonotic disease has preceded the occurrence of human cases, with a great contingent of infected animals with skin parasitism serving as source of infection for the vector insects^{2,5}.

The transmission among vertebrate hosts occurs through the bite of a hematophagous phlebotomine^{5,6}. *Leishmania (Leishmania) chagasi* may attack practically all body systems, leading to a great variety of unspecific symptoms in the dog^{1,7,8,9}.

The safest form of diagnosis of visceral leishmaniasis is the direct observation of amastigote forms of the parasite in lymph node and bone marrow smears, spleen aspirates, liver biopsies and blood smears^{1,7}. This is the simplest method and the one most used in veterinary clinics. The technique, if carried out appropriately, is quick and little traumatic⁷. The specificity of this method is virtually 100%, but depending on the time spent looking for the parasite the sensitivity becomes 80% at the most in symptomatic dogs and lower in asymptomatic dogs¹⁰. According to some authors, the sensitivity of the method varies, ranging from 50 to 83% for bone marrow samples, from 30 to 85% for lymph node cytology^{7,11}, and from 71% to 91% when both tissues are associated¹¹. The number of amastigotes detected in aspirates varies considerably, and few parasites are evidenced in treated animals¹.

Pentavalent antimonials, particularly meglumine antimoniate, are the medications of choice in the treatment of human visceral leishmaniasis, and have been used as treatment protocol for dogs in Europe^{12,13,14,15}. In Brazil prophylactic control program for visceral leishmaniasis includes the elimination of infected dogs⁵.

The mechanism of action of these medications have not been completely established, but it is known that they act on the amastigote forms of the parasite, blocking their metabolism by inhibiting the glycolytic activity and the oxidation pathway of fatty acids, being, thus, considered leishmanicidal^{5,12}. Pentavalent antimonials cause reduction of the symptoms and, in some cases, even clinical cure of the dogs^{13,15,16,17,18,19,20}. Nonetheless, there is evidence that they do not promote the complete elimination of the parasites, thus enabling treated dogs to remain as reservoir of the disease^{14,16,19,20}.

Another medication widely used in combination with pentavalent antimonials is allopurinol, a low cost drug to be administered orally with few side effects^{6,10,12,20}. It is believed that the mechanism of action of allopurinol is the formation of a highly toxic ATP analog, which is incorporated within the RNA of the parasite, thus inhibiting its protein synthesis¹¹. As it is a leishmanostatic agent, its action is greater when combined with the use of other medications. Thus, it has been used to maintain treatment after the use of meglumine antimoniate, with reduction of the rates of relapse of the disease^{17,18}.

There are few reports in the literature about the follow-up of animals infected by *Leishmania (Leishmania) chagasi* submitted to treatment. Hence, the present study aimed to assess the efficacy of the protocol in the treatment of canine visceral leishmaniasis, to verify the occurrence of relapses, and to evaluate the presence of parasites in the treated dogs to know whether they remained or not carriers of the disease.

Material and Method

Seven pet dogs naturally infected by *Leishmania sp.*, five males (71%) and two females (29%), of various breeds, with ages ranging from ten months to six years, were used in this study, conducted in 2003. Six animals were symptomatic and one asymp-

omatic (Table 1). The diagnosis of the disease was reached by the identification of amastigote forms of *Leishmania sp.* in the cytological examination of lymph node and bone marrow aspirates and confirmed by enzyme immune assay (ELISA) which cut-off was determined as 0.270. After the confirmation of the diagnosis, all animals were submitted to a complete clinical examination, and the data were collected in individual charts. During the whole experimental period the dogs were fed with balanced commercial dog food (Selection Special Croc Evolution® - Royal Canin), were given water *ad libitum*, wore deltamethrin antiparasitic collar (Scalibor® - Intervet Production S.A.), and were kept in a screened kennel to avoid reinfection.

All the animals were submitted to a treatment with 75 mg/kg subcutaneous meglumine antimoniate (Glucantime® - Aventis Pharma Ltda) every 12 hours for 21 days, combined with a 10 mg/kg oral dose of allopurinol (generic - Hexal Ltda) every 12 hours for 180 days and were followed-up through a period of 180 days to verify the occurrence of relapses. Food and water ingestion was evaluated and the animals were submitted daily to a physical examination, and monthly to urinalysis and haemathological analysis, which included a complete blood count (erythrogram, leukogram and qualitative platelet count).

Lymph node and bone marrow aspiration biopsies were carried out to search for the parasite in seven moments: M1- before the beginning of the treatment; M2- 30 days; M3- 60 days; M4- 90 days; M5- 120 days; M6- 150 days and M7- 180 days after the beginning of the treatment. The collection of bone marrow was done, under anesthesia, with a 40x16 mm needle for bone marrow aspiration biopsy, through a prick in the crest of the ilium of the animals, and the popliteal lymph node aspiration biopsy was performed with a 25x7 mm needle and a 10 ml syringe.

After lymph node and bone marrow aspiration biopsies, 180 days after the beginning of the treatment, all dogs were euthanized with 15 mg/kg intravenous sodium pentobarbital (Hypnol 3% - Fontoveter - Itapira, SP), followed by 10 ml potassium chloride (19.1% potassium chloride - Darrow - Rio de Janeiro, RJ). Spleen and liver imprints and *in vitro* culture were performed from tissues collected at necropsy. The slides were stained with rapid hematological staining and observed under optical microscope at a 100x magnification to search for amastigote forms of the parasite in the smear.

The samples obtained from the spleen and the liver were incubated in RPMI-1640 media (SIGMA) supplemented with 10% fetal bovine serum (v/v), antibiotics (100 IU / ml penicillin and 100 µg / ml streptomycin), hemin (0.01 g/l), folic acid (0.02 g/l) and 2% human male urine, at 26 °C for 30 days. The cultures were observed under optical microscope to search for promastigote forms of the parasite once a week for 30 days.

Results

All dogs had positive serology by enzyme immune assay (ELISA) with cut-off ranging from 0.410 to 0.886. The six symptomatic dogs presented: weight loss, lymphadenomegaly, splenomegaly, onychogryphosis, alopecia and skin lesions characterized basically by cutaneous ulcers. Some of them (42.9%) had proteinuria, granular casts and renal cells in urinary sediment, indicating renal lesions, and (42.9%) had anaemia, confirmed by the erythrogram (Table 1). The other one dog were asymptomatic, with normal urinalysis and haemathological analysis, although it had amastigote forms of *Leishmania* visualized through lymph node and bone marrow aspiration biopsies.

None of the dogs had side effects with the treatment. All six symptomatic dogs presented remis-

Table 1 - Breed, sex, age and clinical evaluation (physical examination, urinalysis and haemathological analysis) of dogs with visceral leishmaniasis before the beginning of the treatment (M0), 30 days (M1), 60 days (M2), 90 days (M3), 120 days (M4), 150 days (M5) and 180 days (M6) after the beginning of the treatment with meglumine antimoniate and allopurinol - Araçatuba - SP - 2009

Dog	Breed	Sex	Age	SYMPTOMS							
				M0	M1	M2	M3	M4	M5	M6	
1	Rottweiler	Male	2 years	Thin, limphadenomegaly, hyporexy, skin lesions, mioatrophy, proteinuria, renal cells, granular casts in urinary sediment and anaemia	Thin, limphadenomegaly, proteinuria, renal cells, granular casts in urinary sediment and anaemia	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation
2	Teckel	Female	2 years	Limphadenomegaly, splenomegaly, skin lesions, proteinuria and normal haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation
3	Mongrel	Female	2 years	Limphadenomegaly, splenomegaly with normal urinalysis and anaemia	Limphadenomegaly with normal urinalysis and anaemia	Limphadenomegaly with normal urinalysis and anaemia	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation
4	Mongrel	Female	10 months	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation
5	Doberman Pinscher	Female	5 years	Thin, skin lesions, onychogryphosis with normal urinalysis and haemathological evaluation	Skin lesions, onychogryphosis with normal urinalysis and haemathological evaluation	Skin lesions, onychogryphosis with normal urinalysis and haemathological evaluation	Onychogryphosis with normal urinalysis and haemathological evaluation	Onychogryphosis with normal urinalysis and haemathological evaluation	Onychogryphosis with normal urinalysis and haemathological evaluation	Onychogryphosis with normal urinalysis and haemathological evaluation	Onychogryphosis with normal urinalysis and haemathological evaluation
6	Mongrel	Male	6 years	Limphadenomegaly, splenomegaly, onychogryphosis, skin lesions, alopecia, normal urinalysis and anaemia	Limphadenomegaly, splenomegaly, onychogryphosis, skin lesions, normal urinalysis and anaemia	Limphadenomegaly, splenomegaly, onychogryphosis, skin lesions with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation
7	Mongrel	Female	1 year	Limphadenomegaly, splenomegaly, skin lesions, proteinuria, granular casts in urinary sediment with normal haemathological evaluation	Limphadenomegaly, splenomegaly, proteinuria, granular casts in urinary sediment with normal haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation	Asymptomatic with normal urinalysis and haemathological evaluation

sion of the symptoms within 60 days after the beginning of the treatment, accompanied by restoration to normal of urinary and haemathological profiles and all animals remained asymptomatic until the end of the experiment. During all the observation period amastigote forms of the parasite were not identified in any of the animals through bone marrow and lymph node aspiration biopsies. When spleen and liver “imprints” and *in vitro* cultures were carried out at the end of the experiment, the

presence of parasites was verified in two (28.5%) of the seven dogs (dogs 3 and 4). In dog 3, they were present in both “imprints” (spleen and liver), and in dog 4 only in the spleen (Table 2).

Discussion and Conclusion

All animals submitted to the treatment with meglumine antimoniate and allopurinol presented remission of the symptoms, which corroborates the

Table 2 - Clinical status and parasitological evaluation of the dogs with visceral leishmaniasis 180 days after the beginning of the treatment with meglumine antimoniate and allopurinol - Araçatuba – SP - 2009

Dogs	Pre-treatment	180 days after the beginning of the treatment (M6)				
		Clinical Status	Lymph nodes smears	Bone marrow smears	Spleen “imprint” and culture	Liver “imprint” and culture
1	Symptomatic	Asymptomatic	-	-	-	-
2	Symptomatic	Asymptomatic	-	-	-	-
3	Symptomatic	Asymptomatic	-	-	+	+
4	Asymptomatic	Asymptomatic	-	-	+	-
5	Symptomatic	Asymptomatic	-	-	-	-
6	Symptomatic	Asymptomatic	-	-	-	-
7	Symptomatic	Asymptomatic	-	-	-	-

findings of Alvar et al.¹⁶, Ginel et al.¹⁷, Denerolle and Bourdoiseau¹⁸, Riera et al.¹⁹ and Baneth and Shaw²⁰, who affirmed that the treatment with meglumine antimoniate promotes the clinical cure of the dogs. During the whole period of observation none of the animals presented relapses, in accordance with the findings of Ginel et al.¹⁷, who reported that the intermittent administration of allopurinol, after the initial treatment with meglumine antimoniate, is efficient to hinder the occurrence of clinical relapses.

During the whole period of 180 days the presence of amastigote forms of the parasite was not evidenced in none of the treated animals, giving the impression the treatment had provided a parasitological cure. However, spleen and liver “imprints” and *in vitro* culture, carried out at the end of the experiment, revealed the presence of parasites in two dogs. Thus, it is evidenced that lymph node and bone marrow aspiration biopsies are not a 100% efficient and safe method to confirm

parasitological cure in dogs submitted to treatment, as the identification of the parasite was only possible through the investigation of organs such as the liver and the spleen. These data disagree with the findings of Koutinas et al.¹¹, which affirmed that the sensitivity of the lymph node and bone marrow cytological examination varied from 71% to 91%. On the other hand, they confirm the reports of Kontos and Koutinas¹ and Slappendel and Ferrer¹⁰, who affirmed that the cytological examination presented low sensitivity and that, in asymptomatic animals, submitted to treatments, few parasites were identified. Roura, Sánchez and Ferrer²¹ verified that in 15 treated dogs no parasite was observed in the cytological examination of the bone marrow, while seven animals presented positivity when the PCR technique was carried out. Therefore, we cannot affirm that the agent was definitively eliminated in the five dogs that did not present parasites at the end of the experiment. In spite of the clinical improvement ob-

served in the treated animals, the combination of meglumine antimoniate and allopurinol was not able to eliminate the parasite from all dogs, which makes this treatment protocol not recommended, particularly in endemic areas, because the animals remain a source of infection for humans and for other dogs.

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