Clinical trial on the efficacy of the N-methyl glucamine associated to immunotherapy in dogs, experimentally infected with *Leishmania (Leishmania) chagasi*


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**SUMMARY**

The available drugs for treatment of human visceral leishmaniasis do not frequently promote cure of the dogs. Whereas new drugs are not available for use in dogs, it is necessary to test therapeutic trials using old drugs in association with other agents. The objective of this experiment was to test the therapeutic efficacy of the antimonial N-methyl glucamine in association with an antigenic extract of *Leishmania braziliensis* in asymptomatic experimentally infected dogs. Thirty-two laboratory reared mongrel dogs were infected with $1 \times 10^7$ amastigotes of *L. chagasi* by via intravenous. During 450 days, the follow up was done by specific antibody detection, cellular immune response, and detection of the parasite in the bone marrow and other tissues. After the confirmation of the infection by antibody or parasite detection, the dogs were randomly allocated in four groups of eight animals each : Group A received antigen (500 µg/day) ; Group B received Glucantime® (100 mg/Kg/day) ; Group C received the antigen plus Glucantime® and group D received no treatment. All dogs were submitted to necropsy 450 days after inoculation. They were all infected. After the treatment, parasites were found in 50 % of the dogs from Group A, 14.2 % in the Group B, 42.8 % in the Group C and 71.4 % in the Group D. Antibody levels dropped in B and C groups, but still detectable, not completely disappearing, remaining serologically positive during the whole period of observation.

**KEY-WORDS** : Canine visceral leishmaniasis - treatment - N-methyl glucamine - immunotherapy - *Leishmania chagasi*.

**RÉSUMÉ**


Les médicaments disponibles par le traitement de la leishmaniose viscérale humain n’entraînent pas fréquemment la guérison des chiens. Alors que les nouveaux médicaments ne sont pas disponibles pour leur usage, il est nécessaire de faire des essais thérapeutiques en utilisant des médicaments communs associés à d’autres agents. L’objectif de cette expérience a été de tester l’efficacité thérapeutique de l’antimonial N-méthyle glucamine en l’additionnant à un extrait antigénique de *Leishmania braziliensis* chez des chiens infectés de façon expérimentale. Trente-deux chiens croisés de laboratoire ont été infectés avec $1 \times 10^7$ amastigotes de *L. chagasi* par voie intraveineuse. Pendant 450 jours le suivi a été fait par une détection spécifique des anticorps, la réponse de l’immunité cellulaire, et la mise en évidence du parasite dans la moelle osseuse et dans d’autres tissus. Après la confirmation de l’infection par la détection d’antécors ou du parasite, les chiens ont été autopsiés 450 jours après l’expérimentation. Tous les chiens sont autopsiés 450 jours après l’expérimentation. Ils sont tous infectés. Après le traitement, les parasites ont été trouvés chez 50 % des chiens du Groupe A, 14,2 % du Groupe B, 42,8 % du Groupe C et 71,4 % du Groupe D. Le niveau d’antécors a diminué pour les groupes B et C, mais est toujours détectable, ne disparaissant pas complètement, restant sérologiquement positif pendant la période entière de l’observation.

**MOTS-CLÉS** : Leishmaniose viscérale canine - traitement - N-methyl glucamine - immunothérapie : *Leishmania chagasi*.

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1. Introduction

American visceral leishmaniasis (AVL), a zoonotic disease characteristic of rural areas, has quickly expanded in Brazil, in the last years, to urban and its neighborhood areas of the big cities [2, 15, 33]. The most highly endemic foci (70 % of cases) are in north-eastern Brazil in the states of Ceará, Piauí, Pernambuco and Bahia where there are semi-arid, dry poorly forest area with xerophilic vegetation [48].

From the epidemiological point of view, canids, mainly dogs, are important reservoirs of Leishmania (Leishmania) chagasi [11] and are also the responsible for spreading the disease by migrating with its owners to free areas, where the vector Lutzomyia longipalpis can be easily found due to its wide geographical distribution [32].

Visceral leishmaniasis control, in Brazil, is basically performed through three procedures: a) control of the vector by using residual action insecticide on residence walls and its annexes; b) kill of the infected dogs, detected though broad serum-epidemiological inquiries in endemic areas and c) treatment of human cases. Such measures, when vigorously employed throughout the years, are able to transmission [29] or drastically reduce it [20]. Additionally, the treatment of asymptomatic infected dogs can also reduce the disease incidence, as observed in Elba Island, Italy [22].

For human treatment, the antimonial N-methyl glucamine (Glucantime®) has been the chosen drug, in Brazil, with an excellent therapeutic response in the dose of 20 mg of anti-parasite [20]. Other drugs have been used with significant results such as allopurinol [27, 28]; aminosidine [46]; imidazoles [1]; association of Glucantime® and aminosidine [4] or allopurinol [12].

In Brazil, between 1983 and 1997, the canine serological inquiry magnitude has varied in 765.000-1.180.000 annually examined dogs. Approximately, 14.000 to 28.000 dogs were killed through the leishmaniasis control program of the Health Ministry [45]. Through such measure, several significant issues might be raised as economical value and affecting feelings of the animal. Many times, the owners deny the sacrifice of their dogs, which in several cases, is clinically asymptomatic, thus leading to a conflict with the local health agents, making the disease control very difficult resulting in its expansion. On the other hand, small animals clinicians anxiety in treating the infected dogs have led to a diversity of treatment protocols, selecting so the resistant parasites to the antimonials.

Hence, new therapeutic assays with old and/or new drugs are needed so that they promote the cure of the infected animals, indirectly contributing to the disease control.

The current work is aimed at testing different therapeutic protocols in asymptomatic experimentally infected dogs with L. chagasi, using antimonial N-methyl glucamine associated or not to the dead promastigote extracts of Leishmania as immunotherapy. The main purpose of the immunotherapy was to induce the cellular immune response against the parasite.

2. Materials and methods

1) ANIMALS

Thirty two mongrel dogs were used (15 males and 17 females), aging from 8 to 14 months. They were born and raised in the kennel of vaccine tests against leishmaniasis of the Leishmaniasis Laboratory of the Parasitology Department at Federal University of Minas Gerais, Belo Horizonte city, MG, Brazil. Before the beginning of the experiment, all the animals were treated with a large spectrum antihelminthic (Drontal-plus®, Bayer, Brazil) and vaccinated against infections by distemper virus, parvovirus, adenovirus type I, parainfluenza virus and leptospirosis (Masterguard-plus®, Solvay, Brazil), in accordance with the immunization protocol proposed by the manufacturer. The dogs were fed with antimonial after several cycles of treatment; after therapy the animals remain infecting for Phlebotomus perniciosus [23].

The mechanism of action of the antimonial products in dogs is very little known. [43], through the study on the pharmacokinetic of the Glucantime®, via intravenous, subcutaneous and intramuscularly, suggested that the subcutaneous via would be the most indicated one as it allows a greater presence and persistence of the drug in the blood. Serum concentrations of this antimonial was maintained at levels considered effective after 12 hours when the drug had been administered associated with aminosidine, via subcutaneous [4].

The infected dog develop emaciation, splenomegaly, lymphadenomegaly, skin ulcers, desquamations and eczema, onycogryphosis, nasal and intestinal bleeding, keratoconjunctivitis and paraplegia [33]. [25] described the major changes on the lymphoid organs consisted of a reduction in the lymphocyte population of the areas which are primarily T cell domains.

Several studies were undertaken on treatment of infected dogs using pentavalent antimonials or pentamidine [13, 21, 33, 40, 44], therefore, none of them provided parasitological cure to the animals. However, [30] treated naturally L. infantum infected dogs with N-methyl glucamine, in the dose of 100 mg SbV/Kg/day, in three cycles of ten days, followed by the same period of interval. They achieved the clinical signs recovery and a negative serology of 47.2 % in asymptomatic animals, 33.3 % in oligosymptomatic and 11.1 % in symptomatic animals, besides the prevention of the patent disease in 90 % of the asymptomatic and not recovered animals. In contrast, [37] observed 100 % of cure rate with chemotherapy, using N-methyl glucamine combined with immunotherapy.

The parasite resistance to such drug has enhanced in many countries [10]. It has been shown a decrease in the sensibility of isolated L. infantum, from naturally infected dogs, to the antimonial after several cycles of treatment; after therapy the animals remain infecting for Phlebotomus perniciosus [23].
commercial balanced ration (Kinus®, Brasvel, Brazil) and drinking water provided ad libitum.

The animals were managed according to the guidelines for animal experimentation of the USA National Institute of Health and the experiments were performed following the institutional guidelines.

2) EXPERIMENTAL INFECTION

Infection experimentation of the dogs was carried out via intravenous, injecting 1 x 10^7 amastigotes of L. chagasi (strain MHOM/BR/1972/BH46) obtained from infected hamster spleens. The viability was assessed through immediate inoculation of the same material into four hamsters, which were killed when they presented loss of weight and ascitis for the parasitological confirmation of the infection through spleen smears on slides that were examined after Giemsa staining.

3) THERAPEUTIC AGENTS

After confirmation of the dogs infection, through detection of specific antibodies by the indirect immunofluorescence assay (IFA) and/or parasitological exams of the bone marrow and biopsy of skin, monthly applied after the parasites inoculation, the animals were randomly separated into four groups of eight animals each. Such groups received the following therapeutic agents: a) 100 mg Sb/v/Kg/day of N-methyl glucamine (Glucantime®, Rhodia) used as chemotherapy agent; b) canine vaccinal antigen anti-visceral leishmaniasis, consisting of dead promastigote stage of the strain MCAN/BR/1972/C348 of Leishmania (Viannia) braziliensis, in the dose of 500 µg of protein/0.25 ml, used for immunotherapy. The strain of Leishmania (Viannia) braziliensis was tested in dogs as vaccine; it resulted in effective protection of 90 % [35].

The antigen used in immunotherapy was prepared as follows: promastigote stage was cultured for 7 days in LIT medium (Liver Infusion Triptose, DIFCO), threefold washed in phosphate buffer solution, pH 7.2, and submitted to ultrafiltration. The antigen was immersed in paraffin, cut measuring 5 mm of thickness and also through the culture of spleen fragment in NNN/LIT medium.

4) TREATMENT GROUPS

The following groups of dogs were treated in three cycles of 20 days with intervals of 10 days between each cycle: Group A, immunotherapy, using vaccine against canine visceral leishmaniasis; Group B, chemotherapy using Glucantime®; Group C, immunochemotherapy using Glucantime® and vaccine at the same dose and intervals between the series used for groups A and B; Group D, control group (not treated). Glucantime® and vaccine were administered through subcutaneous via in the region of the right and left hemitorax, respectively. The treatment of the dogs was initiated at the 150th day post inoculation (p.i.) of the parasites and confirmation of the infection, and it ended at day 240th, totaling 90 days of treatment.

5) FOLLOW UP

To assess the response of the animals during infection and treatment, some parameters were used: parasitological exam of the bone marrow and skin, detection of specific antibodies by the indirect immunofluorescence assay (IFA) and by the rapid test antibody Leishmania donovani (RTALd) [3], cutaneous test for delayed type cellular response evaluation (intradermal test) and electrophoresis of serum proteins.

It was monthly performed in order to detect amastigotes stages in bone marrow through smears of the marrow aspirate after Giemsa staining and culture. The culture media used were NNN [38] and LIT (Liver Infusion Tryptose) [9]. Both media were associated for the preparation of the medium NNN/LIT [9] to which the marrow aspirate was added. Bone marrow puncture was performed in the intercondyloid fossa of the tibia, previously sedated with sodic thiopental (Thionembutal®). The cultures obtained were examined through three successives passages every ten days. After finishing the experiment, 450 days p.i., the dogs were sedated and sacrificed with saturated solution of potassium chloride, via intravenous and then submitted to necropsy. The parasitism was assessed in bone marrow smears, popliteal and mesenteric lymph nodes, spleen, liver, skin (nasal and ear) and also through the culture of spleen fragment in NNN/LIT medium.

6) HISTOLOGICAL ASSAYS

During the performance of the necropsy, parts of spleen, liver, popliteal and mesenteric lymph nodes, kidneys, intestine and skin (nasal and ear) were removed. Such fragments were kept in buffered formol at 10 %, pH 7.2, dehydrated, immersed in paraffin, cut measuring 5 µm of thickness and then haematoxylin and eosin stained. The search for amastigote phase was also carried out through these histological cuts.

7) HUMORAL IMMUNE RESPONSE ASSESSMENT

It was evaluated through the specific antibodies detection by indirect immunofluorescence (IFAT) and the rapid test antibody Leishmania donovani (RTALd). Serological assays were monthly done, using plasma of the animals. IFAT was performed with serial dilutions of the plasma of 1/40 until the last dilution of the reagent, according to [8]; similar titration and/or over 1/40 were considered positive. The dog anti-IgG conjugate, marked with fluorescein was used (Biomanuniquinhos, FIOCRUZ, Rio de Janeiro). The second test used for antibody was the RTALd. This is a new test for visceral leishmaniasis. It is a rapid immunochromatography assay for the qualitative detection of antibodies to a recombinant antigen (rK39) specific for visceral leishmaniasis [6] caused by parasite members of the L. donovani complex. In the RTALd (Corixa Corp., Seattle, WA), rK39 antigen is attached to the test region on the membrane of the strip and goat anti-protein A is attached to the control region. During the assay the dog serum sample reacts with the conjugate (Protein A-colloidal gold) which is pre-dried on the assay strip. After applying 3-5 drops of wash buffer the mixture moves along the strip.
capillary action. If the sample contains antibodies to K39 a line with the K-39-anti-protein A-colloidal gold complex will form on the test region of the membrane. Absence of this line on the test region suggests a negative result. To serve as a procedural control, a line will always appear in the control region regardless of the presence of anti-leishmanial antibodies. The result should be read after 5-10 minutes. Depending on the concentration of anti-leishmanial antibodies in the serum, positive results may be observed in as little as 1 minute. However, to confirm negative results the complete reaction time of 10 minutes is required. This assay was available for the diagnosis for canine visceral leishmaniasis in Brazil [18].

8) CELLULAR IMMUNE RESPONSE EVALUATION

It was performed through the cutaneous test. The antigen, partly consisted of Leishmania promastigote extract and whole promastigote [36], was intradermally injected in the medial region of the leg in a volume of 0.1 ml, containing 200 μg of protein. Reading was done after 72 hours, being the induration areas twice measured and calculated the mean according to [17].

9) BIOCHEMICAL ASSAYS

Dosage assays of total protein, albumin and the fractions α-1, α-2, β and γ-globulin were undertaken through the electrophoresis of the serum plasma in cellulose acetate. Electrophoresis migration was done at 200 volts for 20 minutes. Streams were stained with Ponceau during 5 minutes and then turned transparent for 3 minutes. Drying was carried out in air incubator at 60°C during five minutes. Reading of the strains was performed in a densitometer, model Shimadzu Dual Wavelength Flying Spot Scanning Densitometer, and analysed by the software CS-9301 PC.

10) STATISTICAL ANALYSIS

The results are referred to the period of 450 days p.i.. For statistical analysis, data correspondent to the pre-injection period (0), during treatment (150, 180, 210 and 240 p.i.), and after treatment (30, 270, 420 and 450 days p.i.) were selected. Variance analysis and Student’s t-test were used by the GLM procedure of the SAS software (1995). Non parametric data were analyzed through Kruskal-Wallis test.

3. Results

The dogs were followed up during 450 days after parasites inoculation being submitted to necropsy. Confirmation of the infection was performed in all animals 150 days p.i. through serological and/or parasitological exams. During experimentation, three dogs accidentally died and were not considered in final analysis.

1) CLINICAL EVOLUTION OF THE INOCULATED DOGS

During the infection course, until 150 days p.i. when the dogs initiated treatment, all of them showed to be asymptomatic for the clinical signs of the AVL. Afterwards, during the course of the experiment, four dogs (12.5%) (one from the A group and three from B group) presented characteristic clinical signs of AVL such as loss of weight, onycogryphosis, symmetric bilateral alopecia with dorsal dry scaliness, periorbital alopecia, lesions in the tarsus articulation, carpal joint, coxofemoral and also femur, tibia and patellar lesions. Such lesions varied from a scab dermatitis to the presence of ulcers. Two of these animals (group A) presented infestation by Demodex canis, visualized through skin scrapings, and some of the cutaneous alterations were attributed to this parasite.

2) PARASITOLOGICAL EXAMINATION

Before the beginning of the treatment, at 150 days p.i., 15 animals (46.8%) presented bone marrow parasitism: 2 (25%) from A group, 5 (62.5%) from B group, 4 (50%) from C group and 4 (50%) from D group. After the second series of treatment (240 days p.i.) parasites were not detected in bone marrow of the dogs belonging to the B and C groups, contrasting to the A and D groups (Table I).

In a final analysis on the presence of parasites in the animals, after 450 days post infection (210 days after treatment) and considering parasitological and/or histological assays, parasites were found in: 50% (4/8) of the dogs from A group; 14.2% (1/7) from B group; 42.8% (3/7) from C group and 71.4% (5/7) from D group (Table I).

3) HUMORAL IMMUNE RESPONSE

Through IFA, specific antibodies were observed 60 days p.i., with titers of 1/40 in five animals. After 90 days, all the dogs presented to be positive with titration ranging from 1/320 to 1/5120. After the third cycle of treatment (240 days p.i.) it could be noticed a decreasing in antibody levels of the B and C groups, remaining at low levels until the end of the study. Statistical difference was observed (p < 0.05) 450 days p.i., among the groups A/C, B/D and C/D. Despite the drop in antibody titers, there was not a conversion to negativity till 210 days after treatment end (Figure 1, Table II).

Rapid test antibody Leishmania donovani (RTALd) presented a similar positivity to IFA, with all the animals being reactive from 90 days p.i.. From this period all dogs showed to be monthly positive, until the moment of necropsy, without difference among the groups submitted to treatment and control group. Figure 2 shows the RTALd reactivity in four dogs of different treatment groups, randomly chosen, during the entire follow up period.

Through electrophoresis analysis, albumin, alpha and beta globulin fractions remained at normal values in the comparison among groups and collections. Gammaglobulin fraction was enhanced in the comparison among groups and collections. Gammaglobulin fraction was enhanced in the A and D groups. During the experiment, the increasing was observed 150 and 180 days p.i. (reference value: 0.4 - 1.0 g/dL), in the four groups. In such groups gammaglobulin fractions were above normal level with 150 and 180 days p.i.; B and C
groups presented normal values from the second cycle of treatment; in the same period, there was inversion of the ratio albumin/globulin (A/G) only in B group. A/G inversion (reference value: 0.7 - 1.1) and the formation of the beta-gamma block were observed in isolated animals into the A, B and D groups, 450 days p.i. (data not shown).

4) CELLULAR IMMUNE RESPONSE

Cutaneous test was performed 72 hours before sacrificing the dogs. The response rate of dogs was 32.5% (5/8) in A group; 85.7% (6/7) in B group; 71.4% (5/7) in C group and 28.5% (2/7) in the D group (Table II).

4. Discussion

Canine visceral leishmaniasis is more resistant to treatment than the human, with unfavorable prognosis in dogs presenting renal deficiency [42]. Therapeutic results through treatments with pentavalent antimonial seems to basically depend on the evolution of the disease clinical picture in such animals [30] and the administered dose, inasmuch as the best results obtained were from asymptomatic animals, presumably with recent infections and treatment based on high dose antimonial, about 5 to 15 times the human dose. Unfortunately, antimonial action mechanism is not completely known, but these compounds probably destroy the parasites.

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Table II. — Immunological findings after 450 days post infection and 210 days after treatment of dogs with asymptomatic visceral leishmaniasis.

<table>
<thead>
<tr>
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<th>IFAT (reciprocal titers)</th>
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Figure 1. — Findings of indirect immunofluorescence assay (IFA) before, during and after treatment of dogs with asymptomatic visceral leishmaniasis. Day 0 = inoculation of the parasites on the dogs; days 0-150 = period pre treatment; dys 150-240 = period of the treatment; days 240-450 = period post treatment.
through the blockage of the GTP and ATP formation, inhibiting two enzymes of the parasite metabolism: phosphofructokinase and dehydrogenase pyruvate [1, 24]. The addition of immunotherapy seems to contribute for the increasing of the cure rate as observed by [37].

In our clinical assay, _L. chagasi_ experimentally infected dogs were used, clinically classified as asymptomatic and submitted to the treatment with (Glucantime®) in identical dose to that used by [30]. An immunotherapeutic agent, developed in our laboratory, was associated for immunoprophylactic control of AVL [35].

The addition of a vaccinal antigen in the therapeutic protocol was motivated by previous experiments in our laboratory in which phase II clinical trial for evaluation of immunogenicity and efficacy tests of an anti AVL vaccine, resulted in a 90 % protection. Among 10 vaccinated dogs, with three doses of the vaccine consisted of promastigote phase of _L. braziliensis_, strain MCAN/BR/1972/C348 (600 µg/dose) associated to the BCG (400 µg/dose) as adjuvant, challenged with promastigotes of the strain MHOM/BR/1972/BH46 of _L. chagasi_; only one animal infected itself. Among the nine non vaccinated controls, all were infected. The vaccine was also able to induce cellular immune response, detectable through lymphocyte proliferation test and intradermal test [35].

During treatment, the animals presented a good tolerance to the antimonial and the immunotherapy. In some of the dogs, during the period of drug administration only local oedema, on the site of injection of the antimonial, was observed with regression in approximately two days after substitution of the application site. The medicine tolerance was probably enabled by the good clinical conditions of the dogs during treatment, maybe allowing a reasonable elimination of the drug. No reactions were observed in the vaccinated dogs, confirming the safety of the vaccine as previously observed [35].

According to the analyzed parameters, B and C groups were the ones which best responded to the treatment, exactly those animals that received the antimonial, with a positivity rate of 14.2 % and 42.9 %, respectively, in the final assessment of parasitism. In the same way, these groups presented a significant enhance in gammaglobulin fractions, during the course of infection, and after the second cycle of treatment such levels returned to normal levels. It was followed by the reduction of IgG antibodies, but not converted to negativity, specifically against _Leishmania_, mainly anti rK39, detected by RTALd. Inversely, A group received only immunotherapy and D group, not treated control, presented higher positivity rate for parasitism of 50 % and 71.4 %, respectively. Also, the levels of gammaglobulin fractions and specific antibodies anti _Leishmania_ increased along the infection course in these groups.

Parasitism frequency in D group (71.4 %, controls) is slightly superior to those found by [19], who detected bone marrow parasitism in 59.6 % of the asymptomatic dogs, naturally infected in endemic area, Montes Claros city, Brazil. The groups, which received treatment, showed a lower parasitism frequency, particularly, the groups that received anti-

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### Table: Period of analysis of RTALD

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<th>Period of analysis of RTALD (days)</th>
<th>Group A, immunotherapy (dog 77)</th>
<th>Group B, chemotherapy (dog 90)</th>
<th>Group C, immunochemo (dog 40)</th>
<th>Group D, control (dog 93)</th>
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_Figure 2. Findings of immunochromatography assay (rapid test antibody Leishmania donovani - RTALd) before, during and after treatment of dogs with asymptomatic visceral leishmaniasis. Day 0 = inoculation of the parasites on the dogs; days 0-150 = period pre treatment; days 150-240 = period of the treatment; days 240-450 = period post treatment._
monial drugs. Therefore, we can infer that the antimonial was able to reduce the number of parasites in dog.

IFA was a test used as reference to determine the beginning of infection and evolution of treatment of the dogs for three reason: a) good performance of the test to detect antibodies anti- *Leishmania*, being used as diagnosis method in Brazil and also for dogs identification and their control; b) considering the long incubation and pre-patent periods of the disease, the animals might take months to present positivity in the bone marrow parasitological exam and c) the animals were raised in experimentation kennels and showed to be negative through this test before the beginning of the experiment, thus, serological conversion observed was considered as result of the experimental infection.

The finding of specific antibodies of the G class and rK39 detected by IFA and RTALd, respectively, suggest the presence of a latent parasitism, which was not detected through the parasitological test, but enough to keep the production of lower levels of antibodies. However, there are observations on human who presented high levels of IgG, detected until 8 months after the end of treatment with sodium stibogluconate [37]. One should consider the possibility of a serological scar due to the presence of such antibodies.

Our findings are similar to those obtained by [34] who used naturally infected dogs with *L. chagasi* and treated with Glucantime® in a dose of 60 mg/Kg/day associated with 600 µg of *L. (V.) braziliensis* protein (MCAN/BR/72/C348) and with 500 µg of BCG in two doses with 3-month intervals. The dogs presented, after one year of observation, decreasing, but not serological conversion to negativity, of the antibody level, negative skin and parasitological tests and normalization of the gammaglobulin fractions, disappearing the symptoms with gain of weight.

The results of the current work differ from those obtained by [30] who obtained cure of 47.2 % in asymptomatic dogs, treated with Glucantime® against *L. infantum* and also [37], who got serological and parasitological cure in dogs treated with Glucantime® associated with immunotherapy. In the latter work, the authors reported a 100 % parasitological cure in asymptomatic dogs treated with 20 applications of 300 mg/Kg of Glucantime®, intramuscularly injected in alternated days, associated with a vaccine consisting of purified antigen of *L. infantum* promastigote, named LiF2 (fraction of 92-94 kDa). The vaccine was administered 15 days after the end of chemotherapy, in three doses with 7-day intervals. On the other hand, the groups treated with Glucantime® or immunotherapy, showed a parasitological cure of 37.5 and 25 % respectively.

Observations on the probability of development of cellular immune response after natural infections in dogs have indicated that there is a spectrum in canine visceral leishmaniasis through the remarks on the human disease. [7] demonstrated the cellular proliferation of T cells in 20/41 asymptomatic dogs infected by *L. infantum*, however, [31] observed the lack of specific response of T cells to the *Leishmania* antigen in dogs with progressive disease by *L. infantum*. Positive delayed type hypersensitivity was also observed by [39] in asymptomatic dogs, and by [35] in protected dogs after vaccination.

In our study, cellular response analysis compared to the cutaneous test performed in the dogs, a few moments before sacrificing, showed that the groups which received treatment, associated or not, differed significantly from the non treated control group, with a low responsiveness rate (28.5 %). In A group, there were 62.5 % of responsive dogs due to the antigenic stimulation of the vaccine, even being serologically similar to the control group, contrasting with the B (85.7 %) and C (71.4 %) groups. This shows that, possibly, the treatment might have positively interfered in the development of cellular response of delayed type hypersensitivity, enabling the prognosis of the animals.

Decreasing in plasmatic albumin levels, increasing in beta and gammaglobulins and inversion in A/G rate were reported by [5, 13, 16, 21, 25, 40, 42] in canine visceral leishmaniasis, mainly in symptomatic animals. In the present work, there were not remarkable alterations in the A/G levels, probably due the fact the animals were asymptomatic and treatment was established at the beginning of infection or yet by the short time between infection and sacrifice of the animals, once this profile was also kept in A and D groups.

Finally, considering that the parasitological exam do not detect 100 % of the infected canine population [20], inferences among the infection rates post treatment, having the parasitological assay related to the D group, with 71.4 % (100 %) positive, will lead to an infection rate close to the actual of 70 % for the A group, 19.8 % for B group and 59.9 % for C group, during the studied period. At that rate, A group that received immunotherapy treatment, obtained a parasitological «cure» of 30 %; B group which was treated only with antimonial, 80.2 % and C group, that received treatment with immunochemothapy, got 40.1 % of parasitological «cure», out of the experimentally infected dogs. Such data indicate that the antimonial is really able to reduce parasitism in asymptomatic dogs and the association with immunotherapy used, did not show to be so efficient enough to increase potency of the treatment, very likely due to the absence of BCG as adjuvant, as previously shown [34, 35]. All the animals, supposedly cured, presented variable antibody levels, suggesting the permanence of antigenic stimulus, probably entire viable parasites not detected through the methodology. The positivity of the RTALd, during the post treatment period in the groups submitted to treatment with or without antimonial, suggest the remaining of specific humoral immune response to rK39, supporting the hypothesis of the possible presence of amastigote into the tissues, even in small quantities, but enough to keep the antigenic stimulus.

The main question remains: If dogs were followed up for a longer period wouldn’t the parasites be detected again? Which would be the minimal period for a follow up necessary to consider a dog cured? Did the antibodies detect in all animals post treatment mean active subpatent infection or a serological scar? At last, more sensitive techniques such as PCR may indicate the possibility or not of a parasitological cure of dogs when submitted to treatments.

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5. Conclusion

The antimonial was able to reduce the parasitism in asymptomatic dogs but the association with immunotherapy used, did not show to be so efficient enough to increase potency of the treatment, very likely due to the absence of adjuvant.

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7. References


