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Research paper

Therapeutic vaccine of killed *Leishmania amazonensis* plus saponin reduced parasite burden in dogs naturally infected with *Leishmania infantum*

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ABSTRACT

A key goal in the control of canine visceral leishmaniosis (CVL) has been the development of vaccines with a highly protective capability to interrupt the parasite transmission cycle. However, in addition to promising vaccine searches, researchers have sought to develop new drugs capable of eliminating parasites in humans and dogs. With that in mind, this study analyzed an immunotherapeutic approach in dogs naturally infected with *Leishmania infantum*. Fourteen dogs were divided into two groups and received a protocol of immunotherapeutic treatment with five doses of total antigens of *Leishmania amazonensis* or total antigens of *L. amazonensis* plus saponin (LaSap). All the animals were evaluated before and 90 and 180 days after treatment, hematology, liver and renal biochemical analyzes, serology, lymphoproliferation, and parasite load by qPCR. The results of immunotherapy with the LaSap vaccine were promising since it was able to preserve hematological and biochemical parameters, as well as improve the clinical status, reduce serum levels of IgG, induce a lymphoproliferative capacity against soluble antigens of *L. infantum,* and provide a marked reduction in the parasite load after LaSap immunotherapeutic treatment. The immunotherapy data demonstrated that LaSap offered the best formulation to induce clinical cure associated with a parasite load reduction in the skin. However, after 180 days of treatment, the animals again showed a slight increase in parasitism, indicating that immunotherapy does not promote sterilizing cure and a new immunotherapeutic intervention would be necessary to maintain low parasitism in dogs.

1. Introduction

Visceral leishmaniosis (VL) and canine visceral leishmaniosis (CVL) is an infection caused by intracellular protozoa that infect organs such as skin, liver, spleen, lymph nodes, and bone marrow (McElrath et al., 1988; Murray, 2000). Most cases of VL occur in six countries: Bangladesh, India, Nepal, Ethiopia, Sudan, and Brazil (Chappuis et al., 2007). In East Africa and the Indian subcontinent, the disease is caused by *Leishmania donovani*. In Europe, North Africa, and Latin America, the infection is predominantly caused by *Leishmania infantum* (Lukes et al., 2007).

A key goal in the control of CVL has been the development of vaccines with a ´highly protective capability to interrupt the parasite transmission cycle. However, in addition to promising vaccine searches, researchers have sought to develop new drugs capable of eliminating parasites in humans and dogs (Romero et al., 2017). In endemic regions, multiple anti-leishmanial agents are in use or have been tested, although treatment efficacy may vary by species and/or region of acquisition. In this approach, strategies involving immunotherapy and/or chemotherapy, initially used in cancer treatment, have been directed towards VL treatment (Miret et al., 2008; Trigo et al., 2010).

The importance of dogs as reservoirs in the VL epidemiological cycle has been demonstrated since the 1950s (Deane and Deane, 1962), as asymptomatic animals have full capacity to transmit the parasite to sandflies (Da Costa-Val et al., 2007; Molina et al., 1994). Furthermore, there is no treatment to date capable of eliminating the parasite from infected dogs (Noli and Auxilia, 2005). In CVL, the only drug approved for treatment in Brazil is miltefosine. However, although the drug

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temporarily improves the clinical signs in dogs, the drug cannot completely eliminate the parasite and new therapeutic interventions are necessary (Manna et al., 2008, 2015). Different studies have used therapeutic vaccines as strategies for the treatment of leishmaniosis. In this case, the tegumentary forms are the main targets of immunotherapy and immunochemotherapy. In addition to vaccines with applicability in the field of immunotherapy against leishmaniosis, other immunobiologicals have been used in the treatment of these diseases. Among them, pro-inflammatory cytokines such as IFN-γ, granulocyte-macrophage colony stimulating factor (GM-CSF), immunomodulators, and monoclonal antibodies against cytokines or their receptors may or may not be associated with conventional chemotherapy (Firooz et al., 2006; Murray et al., 2002). To date, few treatment studies for CVL have been applied. Thus, this study approached an immunotherapeutic treatment in naturally infected dogs with *L. infantum*.

2. Methods

2.1. Antigens production

Promastigotes of *L. amazonensis* (IFLA/BR/67/PH8) were maintained in *in vitro* culture in NNN/LIT media as previously described (Mayrink et al., 1996; Giunchetti et al., 2007). Parasites were harvested by centrifugation (2000 × *g*, 20 min, 4 °C) from 7-day-old cultures, washed three times in saline buffer, fully disrupted by ultrasound (Sonifier Cell Disruptor®-Brason Sonic Power Co., USA) treatment (40W, 1 min, 0 °C), separated into aliquots, and stored at −80 °C freezer (Form Scientific, USA) until required for use. Protein concentration was determined according to the method described by Lowry et al. (1951).

2.2. Animals and treatment protocol

Fourteen non-breed dogs, older than six months, symptomatic and naturally infected with *L. infantum* were included in this study. All dog owners who agreed to participate in the study signed an informed consent form.

No animal had ever been treated for CVL, which is a criterion for exclusion from the study. The animals included in the study were positive in the official serological tests established by the Brazilian Ministry of Health, being the Dual Path Platform (DPP®) and ELISA (EIE-Biomanguinhos). In addition, an ear skin biopsy was performed prior initiating the study to perform the qPCR (de Almeida et al., 2012; Reis et al., 2013) and all animals were positive, which was a criterion for inclusion in the study.

The animals were divided into two groups: Antigen (N = 6) animals received five doses with a seven-day interval between them each dose containing 300 μg of total antigen from *L. amazonensis*; (N = 8) animals were given five therapeutic vaccine antigens doses with a seven- day interval between them, each dose containing 300 μg of total antigens of *L. amazonensis* plus 300 μg of saponin (Sigma Aldrich Co., St Louis, MO, USA). All doses had their volumes adjusted to 1ml of total volume, which was supplemented with sterile 0.9% saline solution. The dogs received subcutaneous injections (sc), changing the injection sites each week, being the right posterior flank, posterior left flank, and the anterior and posterior dorsal region. All animals were monitored weekly for clinical and behavioral changes in relation to vaccine applications. After receiving the five therapeutic applications, all dogs underwent clinical and laboratorial analyses (hematology, biochemistry, serology, lymphoproliferation, and qPCR) at 90 days (T90) and 180 days (T180) after treatment. The study was approved by the ethics committee on animal research (CEUA Protocol no.277/16).

2.3. Biochemical and hematological evaluation

The hematological profile was analyzed by blood cell counts using an electronic haematology particle counter (BC2800Vet, Mindray, Hamburg, Germany). Differential leukocyte counting was performed on Giemsa stained blood smears, and a total of 100 cells were counted.

The biochemical evaluations consisted of the following tests: renal function (urea and creatinine) and hepatic function tests (alanine aminotransferase – ALT and aspartate aminotransferase – AST). For this analysis, the automated biochemical system (CELM SBA-200, Barueri, SP, Brazil) and Labtest® commercial kits (Labtest Diagnóstica S.A., Lagoa Santa, MG, Brazil) were used, following the method described by the manufacturer.

2.4. ELISAanti-Leishmania infantum

Immunogenicity was evaluated by induction of the determination of anti-*L. infantum* antibodies using a soluble lysate of *L. infantum* antigen (MHOM/BR/1972/BH46) (SLcA) according to conventional enzyme-linked immunosorbent assays (ELISA) (Reis et al., 2006a,b). Briefly, 96 well microtiter plates (Nalge Nunc Intl., Rochester, NY, USA) were coated overnight with 2 μg/mL of SLA from *L. infantum*. For IgG Total analysis, sera were added at a 1:100 dilution. Peroxidase-conjugated rabbit anti-dog IgG antibodies were added at a 1:10,000 dilution for 1 h. The reaction was developed using O-phenylenediamine and H_2O_2 (Sigma Aldrich Co., St Louis, MO, USA), and absorbance was measured on a Multiskan® MCC 340 (Labsystems, Helsinki, Finland) automatic microplate reader at 492 nm. Cut-off values were calculated using the mean OD of 10 negative samples plus 2 standard deviations.

2.5. Isolation of peripheral blood mononuclear cells (PBMC)

Blood samples (10 mL each) from the 14 dogs (Antigen – total antigens of *L. amazonensis*; and LaSap group – total antigens of *L. amazonensis* plus saponin) were collected in heparinized tubes intended for obtaining peripheral blood mononuclear cells (PBMC), as previously described (Viana et al., 2013, 2015). The whole blood volume collected was placed in a mixture of Ficoll-Hypaque (Sigma Chemical Co.; density: 1.119 g/mL) and Ficoll-Hypaque (Sigma Chemical Co.; density: 1.077 g/mL) at a 1:3 ratio (Ficoll/blood) in sterile polystyrene conical bottom tubes (Falcon, Corning, USA). All samples were centrifuged at 700× for 40 min at 22 °C. The ring of PBMC was collected at the Ficoll-Hypaque interface and transferred to another tube with 40 mL of Falcon sterile 1 × PBS containing 10% FBS. This tube was centrifuged two times at 400× for 10 min at 4 °C. After the supernatant was discarded, the cells were resuspended in 1 mL of cell culture medium RPMI 1640. Cells were counted in a Neubauer hemocytometer chamber to determine the numbers of monocytes or lymphocytes per milliliter.

2.6. Lymphoproliferation

After counting the cells in the Newbauer chamber, the amount of plated PBMC was 5×10^6 cells/well in 96-well plates (NUNC®) in RPMI/20% SFB/37 °C/5% of CO_2. After 24 h, 25 μg of soluble antigen of *L. infantum* (stimulated culture) was added to the wells and only the culture medium was changed (control) in the other wells. The wells were maintained for another 72 h in medium. The lymphoproliferation assay was based on the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, and made it possible to evaluate both cell proliferation and viability. Four hours after the end of the incubation period, 20 μL of a MTT solution (2.5 mg/mL) was added to each well (containing 200 μL final). After 4 h of incubation and formation of formazan crystals, the supernatant was carefully removed and 200 μL of a 0.04M HCl solution in isopropanol was added to each well. After solubilization of the forming crystals formed by the viable cells' metabolism of MTT, the plates were read in an ELISA reader at a wavelength of 595 nm.

2.7. Co-culture system

Monocytes were adjusted and plated at 5×10^5 monocytes/well using 24-well plates (NUNC, Thermo Fisher Scientific Inc., USA) on circular coverslips (15 mm; Glasscyto, Brazil), as previously described (Viana et al., 2013). Cultures were established using RPMI supplemented with 20% fetal calf serum and incubated at 37 °C/5% CO_2. Monocytes differentiating into macrophages were evaluated after 5 days of culture, as previously described (Viana et al., 2015). The cells were infected with 5×10^6 of *L. infantum* promastigotes in the stationary phase, using a 10:1 ratio (10 parasites per macrophage). Each well was washed gently 3 h after infection and the cultures were maintained in co-culture with total lymphocytes (1:1 ratio) simultaneously to assess microbicidal activity at 72 h after infection. To calculate the rate of parasitic infection, we counted the numbers of amastigotes in 200 macrophages. Thus, the total number of amastigotes was divided by the total number of infected macrophages to obtain the average number of amastigotes per macrophage (Viana et al., 2016).

2.8. qPCR and parasite burden

The parasite loads were calculated by real-time PCR according to a method described elsewhere (de Almeida et al., 2012; Reis et al., 2013). Dogs were anesthetized by intramuscular injection using the combination of 2 mg/kg of body weight of xylazine chloridrate (Calmium; União Química Farmacêutica, Brazil) and 11 mg/kg of body weight of ketamine chloridrate (Ketamina Agener; UniãoQuímica Farmacêutica, Brazil). Skin biopsy was performed in the middle region of the left ear. A fragment was removed with the aid of sterile biopsy punch, 5 mm in diameter (Punch for Biopsy®, Kolplast LTDA, Brazil) and stored in a freezer at −80 °C for further analysis of the parasite load. This material was collected before starting the immunotherapy in T0, T90, and T180 after the treatment. DNA was extracted from the skin using NucleoSpin® Tissue (Macherey-Nagel) according with manufactures' instructions. The parasite burdens were estimated using the following primers: Forward, 5′ TGTCGCTTGCAGACCAGATG 3′ and reverse, 5′ GCATCGC AGGTGTGAGCAC 3′. These primers amplified a 90 bp fragment of a single-copy-DNA polymerase gene (DNA pol α) from *L. infantum* (GenBank accession number AF009147). PCR was carried out in a final volume of 10 µL containing 2.0 pmol of each primer, 2X SYBR® Green PCR Master Mix (Applied Biosystems, USA), 4.0 µL of DNA with a final concentration of approximately 20 ng/mL and ultrapure water. Reactions were processed and analyzed in an ABI Prism 7500 Sequence Detection System (SDS Applied Biosystems, Foster City, CA, USA). The following steps were programmed: an initial denaturation at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Parasite quantification for each skin sample was calculated by interpolation from the standard curve included in the same run, performed in duplicate, and expressed as the number of *L. infantum* organisms per 20 ng of total DNA.

2.9. Statistical analysis

The statistical analysis was performed with the software GraphPad Prism 5.0 (Prism Software, CA, USA). Data normality was assessed using the Kolmogorov-Smirnov test. The analyses were performed using repeated measures ANOVA. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Clinical status after treatment

In general, all the animals from both experimental groups showed improvement in the clinical profile, with a reduction in vasculitis lesions in the region of the ear tip, and resolution of seborrheic dermatitis, mainly between the beginning of the treatment until T90. Likewise, all the animals presented weight gain. However, two animals from the group treated with the antigen only presented a worsening in their clinical status shortly before T180 and died. These dogs presented apathy, anorexia, dermatitis, and vasculitis.

Table 1
Leukogram of dogs before and after immunotherapy. Absolute values (mean ± SD) of the white blood cells (WBC) count of dogs naturally infected by *Leishmania infantum* before and after receiving immunotherapy with Antigen and LaSap vaccine. Significant differences ($P < 0.05$) are represented by "*" as compared to T0.

	WBC	T0 (mm^3)	T90 (mm^3)	T180 (mm^3)
Antigen	Leukocytes	8,407 ± 2,072	10,837 ± 2,633	9,439 ± 2841
	Neutrophils	4,170 ± 1,003	4,991 ± 1,184	4,064 ± 1,153
	Eosinophils	240 ± 149	164 ± 80*	299 ± 103
	Lymphocytes	2,668 ± 1,193	3,760 ± 1,143	2,899 ± 1,530
	Monocytes	218 ± 103	277 ± 132	253 ± 130
LaSap	Leukocytes	9,846 ± 2,843	11,412 ± 2,107	10,632 ± 1,956
	Neutrophils	4,780 ± 1,901	3,486 ± 1,164	3,961 ± 1,032
	Eosinophils	304 ± 115	212 ± 80*	288 ± 105
	Lymphocytes	3,106 ± 1,879	4,221 ± 783*	3639 ± 946
	Monocytes	371 ± 168	207 ± 56	286 ± 114

3.2. Hematologic and biochemical analysis

Compared with the reference values, there were no hematological alterations (total leukocytes, neutrophils, eosinophils, lymphocytes and monocytes) in dogs before and after immunotherapy with the antigen and LaSap. However, a reduction was observed in absolute values of eosinophils for the Antigen group at T90 when compared to T0 ($P < 0.05$). In addition, a reduction of this cell population was also observed in the LaSap group at T90 when compared to T0. Notably, there was also an increase in the population of lymphocytes at T90 in the LaSap group when compared to the time before treatment ($P < 0.05$) (Table 1).

Liver function tests indicated that, although the serum levels of AST (U/L) and ALT (U/L) in dogs at T0 were within normal values, there was a reduction ($P < 0.05$) at T90 in the mean levels of both enzymes of the Ag group (AST: 45.1 ± 3.2; ALT: 48.5 ± 3.7) and LaSap group (AST: 47.8 ± 6.2; ALT: 45.1 ± 3.2) (Fig. 1). Similar results were described at T180 with low ($P < 0.05$) mean levels of hepatic enzymes in the Ag group (AST: 51.6 ± 5.7; ALT: 48.5 ± 3.7) and LaSap group (AST: 51.0 ± 5.7; ALT: 50.2 ± 4.7) (Fig. 1). The results of the renal function analyses, based on the urea (mg/dL) dosage (Fig. 1), showed that there was a significant reduction in both experimental groups in urea dosages at T90 (Ag group: 43.3.1 ± 6.4; LaSap group: 31.3.1 ± 4.6) and T180 (Ag group: 41.8 ± 2.8; LaSap group: 35.8 ± 3.7). A similar reduction ($P < 0.05$) was also observed in mean values of creatinine levels (mg/dL) at T90 (Ag group: 1.16 ± 0.1; LaSap group: 0.90 ± 0.1) and T180 (Ag group: 1.18 ± 0.1; LaSap group: 1.06 ± 0.1) (Fig. 1). However, there was an increase ($P < 0.05$) in creatinine levels in the LaSap group at T180 when compared to T90 (Fig. 1).

3.3. Dogs treated with LaSap reduced serum IgG production

Data on the IgG dosage (Fig. 2) from the sera of dogs at T0, T90, and T180 days indicated that, after treatment, both groups had their mean values of antibody serum levels (optical density) production reduced ($P < 0.05$), as shown at T90 (0.59 ± 0.02) and T180 (0.52 ± 0.03) (Fig. 2A–C). There were no significant differences between the T90 (0.54 ± 0.04) and T180 (0.56 ± 0.09) days of the antigen group. In the LaSap group, there was a significant reduction between T180 and T90 (Fig. 2).

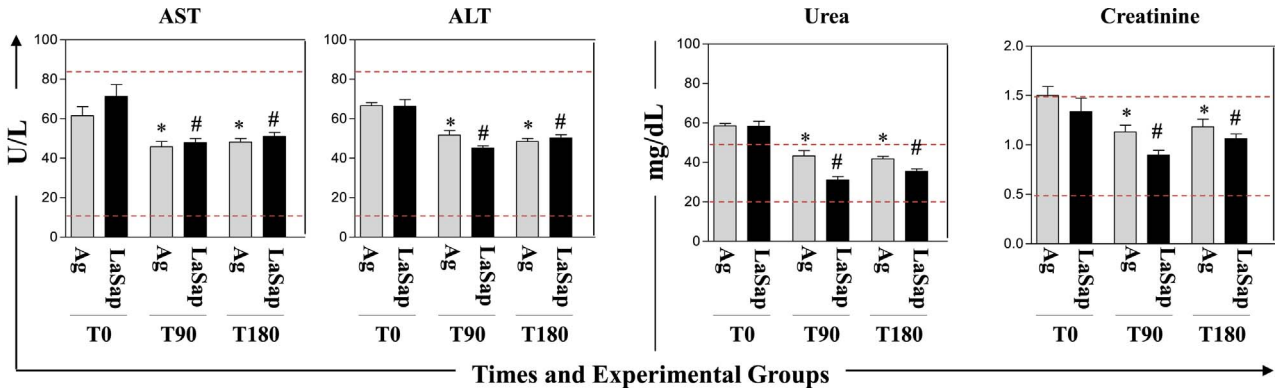

Fig. 1. Hepatic and renal function of dogs naturally infected by *L. infantum* before and after immunotherapeutic treatment.
(A) Hepatic function from the serum levels of the enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) at time zero (T0), 90 (T90) and 180 (T180) days after treatment with total antigens of *L. amazonensis* (Ag) and total antigens plus saponin (LaSap). (B) Renal function from the serum levels of urea and creatinine in the same times as above. Significant differences ($P < 0.05$) between T0 and T90 or T180 days are represented by symbols "*" or "#", related to Antigen or LaSap groups, respectively.

3.4. PBMC from dogs treated with LaSap proliferate after stimulation with soluble antigen of L. infantum

Lymphoproliferative analyses showed that, regardless of the treated group (Ag and LaSap), there was a significant increase in the mean values of optical density regarding total lymphocyte proliferation in cultures stimulated with *L. infantum* soluble antigen at T90 (Ag group: 0.71 ± 0.07; LaSap group: 0.80 ± 0.08) and at T180 (Ag group: 0.65 ± 0.05; LaSap group: 0.82 ± 0.05) (Fig. 3).

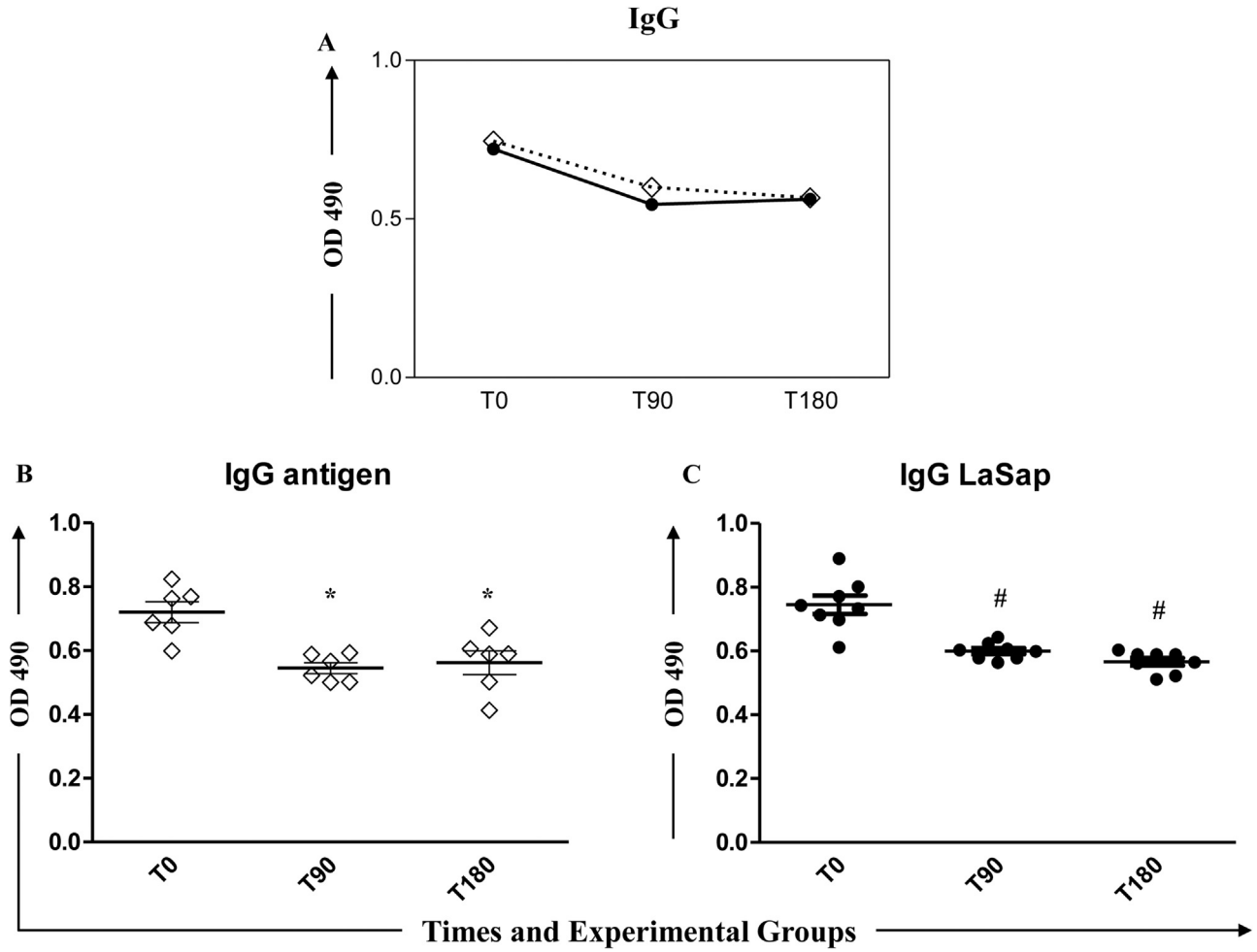

Fig. 2. Profile of the humoral immune response of dogs naturally infected by *Leishmania infantum* after immunotherapeutic treatment.
(A) Evaluation of total IgG levels at T0, T90, and T180 from ELISA absorbance values in the Antigen group (···◇···) and LaSap (—●—). (B) and (C) Dispersion of the absorbances obtained from the Antigen and LaSap groups. Significant differences ($P < 0.05$) between T0 and T90 or T180 are represented by symbols "*" or "#", related to Antigen or LaSap groups, respectively.

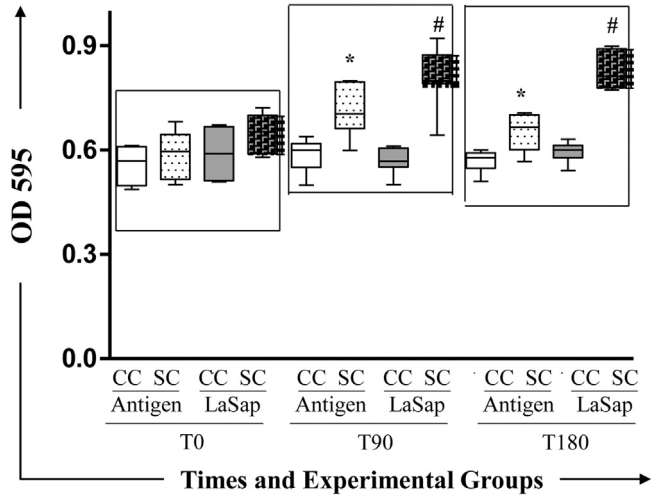

Fig. 3. Lymphoproliferation of PBMC from dogs naturally infected by*Leishmania infantum* after immunotherapy.
Groups of dogs treated with Antigen and LaSap at T0, T90, and T180, represented as control culture (without any stimulus; CC) and culture stimulated (CS) with soluble antigen of *L. infantum*. Significant differences ($P < 0.05$) between T0 and T90 or T180 days are represented by symbols "*" or "#", related to Antigen or LaSap groups, respectively.

3.5. Macrophages from treated dogs have better leishmanicidal capacity

Analysis of the co-culture system between macrophages infected with *L. infantum* and total lymphocytes showed that, after 72 h of infection, there was a significant reduction in the mean values of parasite counting in macrophages at T90 (Ag group: 3.8 ± 1.3; LaSap group: 3.0 ± 1.0) and at T180 (Ag group: 6.8 ± 1.1; LaSap group: 6.4 ± 1.3) (Fig. 4).

3.6. LaSap therapeutic vaccine induced a marked reduction in skin parasite load

Analyses of skin biopsies by qPCR indicated there was a significant reduction of parasitic load, especially in dogs treated with LaSap ($P < 0.05$) at T90 (313.1 ± 718.5) and T180 (348.9 ± 735.5), compared to T0 (10,470 ± 10,270). In the group treated only with the antigen, although the parasite load reduced at T90 (12,270 ± 16,990), there was a tendency to increase the parasite load at T180

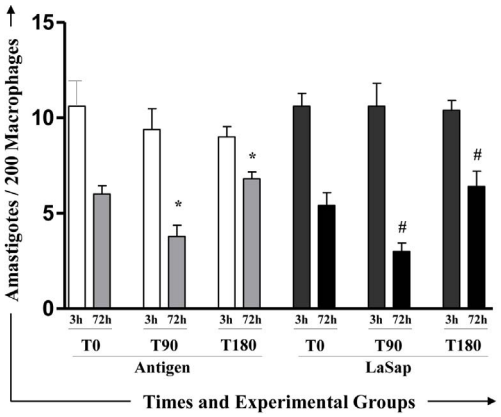

Fig. 4. Co-culture system using *Leishmania infantum* infected macrophages and total lymphocytes.
Number of amastigote forms by macrophages after 3 h or 72 h of *in vitro* infection with *L. infantum* (5×10^6 promastigotes; ratio 10 parasites:1 Macrophage) at T0, T90, and T180. The *x*-axis represents the Antigen group (Ag) and LaSap after 3 h or 72 h of co-cultures during the immunotherapeutic treatment. Significant differences ($P < 0.05$) between T0 and T90 or T180 days, after 72 h of co-culture, are represented by symbols "*" or "#", related to Antigen or LaSap groups, respectively.

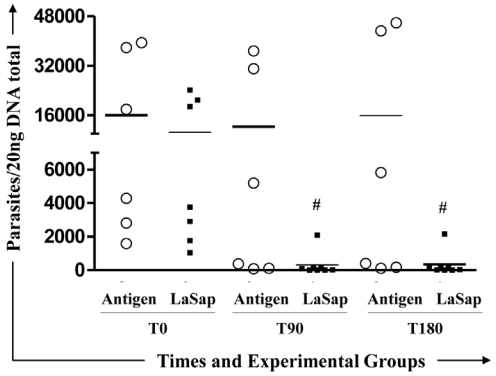

Fig. 5. Analysis of the parasite load in the skin of naturally infected dogs by*Leishmania infantum* after immunotherapy.
Groups of dogs before (T0) and after immunotherapeutic treatment with Antigen and LaSap at T0, T90 and T180 (x-axis). Detection of parasites (expressed as number of parasites/20 ng of total DNA) by qPCR (y-axis). Significant differences ($P < 0.05$) between T0 and T90 or T180 days are represented by symbols "*" or "#", related to Antigen or LaSap groups, respectively.

(15,950 ± 22,310). Two dogs from the antigen group continued to present a high parasite load, and soon after the analyses at T180, they died. No deaths occurred in the LaSap group until the end of the study (Fig. 5).

4. Discussion

In CVL, although there are therapeutic protocols capable of improving the clinical condition in dogs, the drugs are not able to eliminate the parasite in different organs. Moreover, the treatment protocol generate high costs for the owners. The improvement of immunogenicity in antigens used for vaccine or immunotherapy has been obtained using saponin an adjuvant, recognized for inducing strong cellular recruitment in the skin, plus inducing the synthesis of proinflammatory cytokines and Th1 response (Vitoriano-Souza et al., 2012). In this manner, saponin has been used for enhancing immunogenicity in immunobiological products against CVL (Giunchetti et al., 2007; Roatt et al., 2012; Aguiar-Soares et al., 2014; Martin et al., 2014; Viana et al., 2016). In this study, dogs naturally infected with *L. infantum* were treated with two immunotherapeutic formulations, one composed solely of total antigens of *L. amazonensis* (antigen group) and one with total antigens of *L. amazonensis* plus saponin (LaSap group).

Proposals for preventive immunizations with heterologous vaccines against CVL have already been used by different research groups, resulting in partial protection (Fujiwara et al., 2005; Giunchetti et al., 2007; Roatt et al., 2012; Aguiar-Soares et al., 2014). Our results indicated that, after treatment, there was an improvement in the clinical status of all the animals, mainly until T90. After that, two animals in the antigen treated group presented clinical worsening, leading to death soon after T180. These two animals also presented a high parasite load after T90, increasing even more at T180. It is important to emphasize that both dogs at the beginning of treatment had a clinical profile with advanced cachexia and apathy. In contrast, all dogs in the LaSap group did not present progressive disease evolution after T90 and T180. However, it was possible to notice that, although the hematological and biochemical profiles did not show great changes, there was an increase in the parasite load at T180, particularly in the antigen group.

There was a reduction in the amount of eosinophils in T90 in both treated groups, with an elevation of lymphocytes in the LaSap group (Table 1). Other studies reported in naturally infected dogs with *L. infantum* that animals with low parasitism showed an increase in the overall values of monocytes and lymphocytes when compared to the group of dogs with high parasitism (Reis et al., 2006a). In a study of dogs treated with encapsulated meglumine antimoniate, normalization

of the mean values in the hematologic parameters after treatment was verified (Da Silva et al., 2012). An increase in overall lymphocytes and monocytes was found in a recent study that evaluated the treatment of dogs naturally infected by *L. Infantum*, with a therapeutic vaccine consisting of *L. braziliensis* antigens (LB) associated with the Monophosphoril Lipid A (MPL) adjuvant, in addition to decreased eosinophil values (Roatt et al., 2017).

Renal and hepatic function tests showed that, although the enzyme levels were within normal values, these levels were normalized after treatment, especially at T90, with a slight increase at T180 (Fig. 1). In another study, no changes were found in relation to creatinine concentrations in the MPL and LBMPL groups when compared to the reference values. Conversely, serum urea analysis demonstrated that LBMPL therapeutic vaccine induced normalization of this parameter after treatment (Roatt et al., 2017). However, increased serum urea concentrations were observed in dogs treated with liposomal antimoniate (Da Silva et al., 2012). In another study, dogs were treated with either Glucantime or Glucantime plus Leish-110f plus MPL-SEAs. Evaluation of hematology, renal, and hepatic function, humoral and cellular immune response, in addition to analysis of bone marrow and skin parasitism, revealed improvement of the clinical parameters and parasite clearance in both chemotherapy alone and immunochemotherapy. However, the immunotherapy and immunochemotherapy had reduced number of deaths, higher survival probability, and specific cellular reactivity to leishmanial antigens (Miret et al., 2008).

It is recognized in the literature that symptomatic dogs with a high parasite load in the bone marrow and spleen present higher levels of IgG (Reis et al., 2006a,b, Da Silva et al., 2012; Roatt et al., 2017). We found a reduction in total IgG levels in the sera of dogs at T90, with a tendency for these levels to increase at T180 (Fig. 2). Although not statistically significant ($P > 0.05$), LaSap therapeutic vaccine induced lower levels of antibodies. Dogs treated with encapsulated meglumine antimoniate showed a reduction in antibody levels after treatment (Da Silva et al., 2012). In fact, it has been reported that the response to treatment is accompanied by a reduction in antibody titers (Alvar et al., 1994, 2004). The speed of this decrease, however, may not keep pace with clinical recovery. Clinically cured animals may remain seropositive for years during the treatment period (Reis et al., 2010).

The positive lymphoproliferative response to the *Leishmania* sp. antigen stimulus is a classic biomarker of resistance in canine disease (Day, 2004). Analyses have shown there were increases in total lymphocyte levels in cultures stimulated with soluble antigen of *L. infantum* at T90 and T180 after treatment with the LaSap therapeutic vaccine when compared to T0 (Fig. 3). It is known that asymptomatic dogs and naturally infected by *L. infantum* present high levels of $CD4^+$ and $CD8^+$ T-cells as resistance biomarkers, and reduction in T-cells as a marker of susceptibility (Reis et al., 2006a,b, 2010). Although the T and B lymphocyte subpopulations were not analyzed in this study, in an immunotherapeutic study with the LBMPL therapeutic vaccine, an increase in total T lymphocytes ($CD3^+$ T-cells) was observed, accompanied by an increase in the subpopulation of $CD4^+$ and $CD8^+$ T-cells 30 days after immunotherapy (Roatt et al., 2017). Likewise, similar results of lymphoproliferation were obtained in an immunotherapeutic study with the FML vaccine in dogs (Borja-Cabrera et al., 2004).

It is the first time that microbicidal analyses from the co-culture system between infected macrophages and lymphocytes are presented in dogs with visceral leishmaniosis and treated with therapeutic vaccine. Previous studies are related to the microbicidal activity of macrophages from vaccinated dogs. A study with the LiESP/QA-21 vaccine demonstrated that the vaccinated group inhibited twice the parasite replication in comparison to the control group (Martin et al., 2014). Co-cultures of macrophages and purified T-cells from dogs immunized with $LdCen^{-/-}$ and challenged with *L. infantum* were able to identify high microbicidal activity, especially in the co-culture using $CD4^+$ T-cells, as compared to the Leishmune® group (Viana et al., 2016). Although there was a parasite load reduction in the macrophages in all the analyses after 72 h of infection, it was not possible to establish that the cells of the dogs from the LaSap group presented a higher leishmanicidal capacity as compared to the group treated with the antigen using this method.

The evaluation of the skin parasitic load by the real-time PCR technique showed that all treated animals from both experimental groups underwent a reduction in parasitism, especially in the animals treated with the LaSap therapeutic vaccine at T90 and T180 after treatment. However, it was possible to notice that in the group treated only with the antigen, two animals failed to significantly reduce parasitism, dying soon after T180 (Fig. 4). This indicates that treatment through immunotherapy can reduce the parasitic load only for a short period, and a new therapeutic intervention is necessary when the parasite load begins to rise. In two other studies using dogs submitted to treatment, there was also a significant reduction in the parasite load on the skin, spleen, and bone marrow, but in some dogs, an increased parasite load was observed after treatment (Da Silva et al., 2012; Roatt et al., 2017).

5. Conclusions

In general terms, the results of immunotherapy with the LaSap presented as better option of therapeutic vaccine formulation since (i) it showed a capacity to preserve hematological and biochemical parameters; (ii) improved the clinical status; (iii) triggered a lymphoproliferative capacity against soluble antigens of *L. infantum,* (iv) induced *in vitro* leishmanicidal activity; and (v) presented a marked reduction in the skin parasite load.

Competing interests

The authors declared that they have no competing interests.

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