

Antileishmanial efficacy and tolerability of combined treatment with non-ionic surfactant vesicle formulations of sodium stibogluconate and paromomycin in dogs.

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Abstract

Infection with *Leishmania infantum* causes the disease visceral leishmaniasis (VL), which is a serious clinical and veterinary problem. The drugs used to treat canine leishmaniasis (CanL) do not cause complete parasite clearance; they can be toxic, and emerging drug resistance in parasite populations limits their clinical utility. Therefore, in this study we have evaluated the toxicity and efficacy of joint treatment with a 1:1 mixture of sodium stibogluconate-NIV (SSG-NIV, 10 mg Sb^v/day) and paromomycin-NIV (PMM-NIV, 10 mg PMM /kg/day), given intravenously daily for seven days from day 270 post-infection, to nine-month-old female beagle dogs (n=6) experimentally infected with *Leishmania infantum*. Treatment significantly improved the clinical symptoms of VL infection in all the treated dogs, reduced parasite burdens in lymph nodes and bone marrow, and all symptomatic treated dogs, were asymptomatic at 90 days post-treatment. Treatment was associated with a progressive and significant decrease in specific IgG anti-*Leishmania* antibodies using parasite soluble antigen ($p < 0.01$) or rK39 ($p < 0.01$) as the target antigen. In addition, all dogs were classified as parasite negative based on *Leishmania* nested PCR and quantitative real time PCR tests and as well as an inability to culture of promastigote parasites from lymph nodes and bone marrow tissue samples taken at day 90 post-treatment. However, treatment did not cure the dogs as parasites were detected at 10 months post-treatment, indicating that a different dosing regimen is required to cause long term cure or prevent relapse.

Keywords: *Leishmania infantum*, canine leishmaniasis, sodium stibogluconate, paromomycin, non-ionic surfactant vesicles formulations

1. Introduction

Visceral leishmaniasis (VL) caused by infection with *Leishmania donovani* or *L. infantum* is a serious health problem that can be fatal if untreated. In 2020, the World Health Organization estimated that there were 50.000 to 90.000 new cases a year and the disease was responsible for 20,000 death/year (<https://www.who.int/leishmaniasis/en/>). Most of the cases occur in a limited number of countries but epidemics occur in areas of civil unrest and drug resistance in the endemic parasite population can result in treatment failures (Ponte-Sucre et al., 2017). Reservoir hosts play an important role maintaining endemic parasite populations and the dog is an important reservoir host for *L. infantum* (Ribeiro et al., 2018) and can be responsible for transmission of VL into new areas where a permissive vector occurs (Maia and Cardoso, 2015). There are vaccines to prevent canine leishmaniasis (CanL) e.g. CaniLeish[®] and Leish-tec[®], but these are not 100% effective (Martin et al., 2014, Grimaldi et al., 2017). Therefore, treatment of active and relapsed cases is important in control of CanL. The main drugs used in canine treatment are as limited as those used in humans. The main drugs used are pentavalent antimonials (Sb^v), miltefosine, amphotericin B deoxycholate (AMB) or liposomal AMB formulations, paromomycin (PMM), or allopurinol in combination with pentavalent antimonials or miltefosine (Apostolopoulos et al., 2018). The choice of drug used depends on the owner of the dog, but relapse often occurs and treatment is importantly used to improve the quality of life of the dog (Ponte-Sucre et al., 2017). Parasites from drug treated dogs could be an important reservoir for drug resistant parasites as studies have shown that parasites isolated from drug treated dogs were more resistant to allopurinol (Yasur-Landau et al., 2016) or Sb^v (Eddaikra et al., 2018). It is now recognized that combination therapy, using drugs that act via different mechanisms, may provide a better therapeutic outcome and help reduce the emergence of drug resistant parasites.

In previous studies, we have shown that encapsulating antileishmanial drugs within non-ionic

surfactant vesicles (NIV) improved treatment efficacy in animal models of leishmaniasis by targeting the entrapped drug to phagocytic cells, which are the host cells for *Leishmania*, and favored delivery to organs rich in phagocytic cells e.g. spleen and liver, over renal excretion (Shaw and Carter, 2014). We have shown that NIV formulations of the antileishmanial drugs: sodium stibogluconate (SSG), PMM or AMB are more effective at reducing *L. donovani* parasite burdens than the corresponding drug solution in a murine model of VL (Mullen et al., 1998, Williams et al., 1998; Alsaadi et al., 2012). In addition, we have shown that treatment with SSG-NIV is well tolerated by healthy dogs (Nieto et al., 2003), but the efficacy of the formulation against CanL was not determined. As parasite antimony resistance is now an issue in some areas, we decided to compare the efficacy and toxicity profile of monotherapy with SSG-NIV with combination treatment with PMM-NIV in CanL to determine if joint treatment was more effective than treatment with SSG-NIV alone.

2. Materials and methods

2.1. Animals and parasites

Age-matched BALB/c mice (weight, 20 to 25 g; male or female mice inbred in-house) were used in this study. In canine studies healthy male and female beagles' dogs 10-24 months old obtained from breeder in Barcelona (Spain), were used. Commercially obtained Golden Syrian hamsters (*Mesocricetus auratus*; (Harlan Olac, Bicester, Oxon, United Kingdom) were used for maintenance of the *L. donovani* strain. *L. donovani* strain MHOM/ET/67:LV82 and *L. infantum* parasites strain MCAN/ES/98/LLM-724 were used in studies. Dogs were housed indoors in kennels at a suitable temperature and humidity and protected with mosquito netting to prevent natural infection. The dogs were fed with dry food once a day and given drinking water ad libitum. All dogs were pre-treated with an oral broad-spectrum anthelmintic drug combination (Drontal Plus®, Bayer, Sant Joan Despí, Spain) before use in the study and vaccinated against parvovirus, distemper virus, adenovirus Type 2, parainfluenza virus and leptospirosis (Vanguard®, Pfizer, Alcobendas, Spain). Each dog was also treated every month with 1 mL permethrin insecticide ampoule spot-on (Exspot®, Schering- Plough, Madrid, Spain) to give additional protection against sandflies.

2.2 Ethical approval

Murine and hamster studies were carried out in accordance with United Kingdom Home Office regulations and under ethical clearance from the University of Strathclyde's Ethical Committee. The procedures for this research meet the International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for the International Organizations of Medical Sciences and were approved by the Scientific and Ethical Committee of the Institute of Research in Health Sciences of the University National of Asuncion, Paraguay (Letter n° M01/2020).

2.3 Vesicle formulations

SGG was provided by Glaxo Wellcome Ltd. (31.1% wt/wt SbV, Ware, United Kingdom) or purchased from Unichem, Livingston, United Kingdom, (27% wt/wt SbV lot A3532A). The non-ionic surfactant tetraethylene glycol mono-n-hexadecylether was purchased from Chesham Chemicals Ltd. (Harrow, United Kingdom). Dicetyl phosphate, ash-free cholesterol and PMM were obtained from Sigma, and all other reagents were of analytical grade. Water for irrigation was obtained from Baxter Healthcare. Paromomycin sulfate and all other analytic grade reagents were obtained from Sigma-Aldrich (Poole, United Kingdom). SSG-NIV or PMM-NIV consisting of a 3:3:1 molar ratio of tetraethylene glycol mono-n-hexadecylether, cholesterol, and dicetyl phosphate, was prepared as described previously (Mullen et al., 1998). Briefly, 6.3 mmoles of total lipids were melted at 130°C for 5 min. The molten mixture was then cooled to 70°C, hydrated with 105 mL of preheated SSG (70°C, 62.6 mg of Sb^V/ml) or PMM (70°C, 62.6 mg/ml) solution, and homogenized at 9,000 ± 100 rpm for 15 min at 70°C with a Silverson mixer (model L4R SU; Silverson Machines, Chesham, United Kingdom), fitted with a 5/8" tubular work head. The resultant NIV formulations had a final lipid concentration of 60 mM. Using the particle analyzer Zetasizer 4 (Malven Instruments Ltd, Malvern, United Kingdom), SSG-NIV vesicles size was 425.3 ± 53.2nm, with a polydispersity index of 0.310±0.059 and a zeta potential of -75.9 ± 1.7mV. PPM-NIV vesicles size was 513.7 ± 78.2, with a polydispersity index of 0.325 ± 0.081 and a zeta potential of -12.8 ± 1.8mV.

2.4 In vivo murine studies

Mice (n = 4 or 5/treatment) were infected by intravenous injection (tail vein, no anesthetic) with 1×10^7 to 2×10^7 *L. donovani* strain MHOM/ET/67:LV82 amastigotes and then treated on day 7 post-infection by the intravenous route with 0.2 ml PBS pH 7.4 (control), SSG solution (300 mg Sb^v/kg), paromomycin solution (20 mg/kg), SSG-NIV (300 mg Sb^v/kg) or PMM-NIV (20 mg/kg). Mice were sacrificed on day 14 post-infection and parasite burdens in the spleen, liver and bone marrow determined by Leishman Donovan Unit (LDU) = amastigote number per 1000 host cell nuclei per organ weight (g), (Carter et al., 1988). The effect of drugs treatment on parasite burdens was determined as the mean reduction in parasite burdens compared to the mean control value.

2.5 Single doses canine toxicity studies

We calculated the most appropriate dosage regimen for this study based on our previous pharmacokinetics studies using NIV formulations (unpublished data), the knowledge of therapeutic peak levels of two drugs (Athanasίου et al., 2013; 14. Valladares et al., 1996; 97) and outputs from a pharmacokinetic simulation and dosage regimen calculations program (APIS[®]) using doses of 5, 10, 15 and 20 mg/kg for each drug formulation. We decided to use a dose of 10 mg/kg for SSG-NIV and PMM-NIV and there was sufficient time between dosing to ensure that the previous drug had been eliminated (Belloli et al. 1995). Five dogs dosed by the intravenous route on day 0 with SSG solution (10 mg Sb^v/Kg using a 100.07 Sb^v/mL stock solution prepared in 5% v/v dextrose water for injection), then on day 14 the dogs were dosed with PMM solution (10 mg PMM/kg). Finally, on day 21 the dogs were treated with a 1:1 mixture of SSG-NIV: PMM-NIV (10 mg Sb^v/kg: 10 mg PMM /kg). A complete serum biochemical analysis was performed before and at 24 hours and 7 days post-dosing and dogs were observed during the first 48 h after drug administrations to evaluate possible clinical or behavioral changes. Food and water intake, body temperature, heart and respiratory rate were also observed. Biochemical analysis of total plasma proteins, albumin, globulins, AST (aspartate transaminase), ALT (alanine transaminase), creatinine, BUN (blood urea nitrogen) and ALP (alkaline phosphatase), in the serum was determined before, 24 hours and 7 days after drug treatment using an automatic biochemistry analyzer (OPERA[®], Bayer).

2.6 Canine antileishmanial efficacy studies

Nine-month-old female Beagle dogs (n = 6) were kept in a pound for 8 weeks to ensure that dogs were not naturally infected with *L. infantum*. Three methods were used to confirm that dogs were not infected:

- 1) Absence of specific *L. infantum* serum antibodies as tested by immunofluorescent antibody test (IFAT) and ELISA with rK39 and soluble *Leishmania* (SLA) antigens (Guarga et al., 2002).
- 2) Absence of parasites by culturing in NNN (Novy-MacNeal-Nicolle medium) and Ln-PCR (*Leishmania* nested polymerase chain reaction), analysis of different biopsies (bone marrow and lymph node aspirates, skin and peripheral blood mononuclear cells (PBMC), (Cruz et al., 2002).
- 3) Absence of cell responses to soluble *Leishmania* antigen (SLA) tested using an *in vitro* lymphoproliferative assay (Nieto et al., 2003).

2.7 Experimental infection in dogs

Leishmania infantum parasites (MCAN/ES/98/LLM-724) isolated aseptically from the spleen of a dog infected by *L. infantum* with symptomatic disease, were grown as promastigotes by culturing at 27°C in a mixture of Novy- MacNeal-Nicolle (NNN) and RPMI-1640 medium pH 7.4 supplemented with 10% heat-inactivated fetal calf serum (hiFCS, Sigma-Aldrich, Tres Cantos, Spain), 20.000 UI/mL penicillin and 20.000 µg/mL streptomycin (Pen-Strep® Cambrex, Belgium). When the parasites had reached a suitable density they were passed in a mixture at pH 5.5 to stimulate the metacyclogenesis. Promastigotes harvested from cultures were pelleted and washed twice in phosphate buffered saline (PBS, pH 7.0) and resuspended in PBS to a density of 1×10^8 *L. infantum* promastigotes per mL. Dogs were infected by intravenous injection with 1×10^8 metacyclic promastigotes and followed up monthly, for up to 9 months post- infection. After this time dogs were treated at month 10 by intravenous bolus injection (± 3 min) with a 1:1 mixture of SSG-NIV and PMM-NIV (10 mg Sb^v/kg: 10 mg PMM/kg) every 24 hours for 7 days.

2.8 Clinical evaluation in dogs

Before starting treatment, dogs were examined by two veterinarians and allocated to one of the clinical groups that have been previously established (Alvar et al., 1994):

1. *Asymptomatic*, those dogs without clinical signs but with positive parasitology and/or serology.
2. *Symptomatic*, those dogs showing any clinical sign compatible with leishmaniasis.
3. *Oligosymptomatic*, when dermatitis and lymph node swelling are present.
4. *Polysymptomatic*, when additional signs like weight loss, onychogryphosis, hemorrhage, alopecia, etc. are present.

At 90, 180 and 300 days post-treatment, dogs were again reclassified into one of the groups according to their clinical response to treatment. Biochemical analysis of, albumin, globulins, total plasma proteins, AST, ALT, creatinine, BUN, erythrocytes, leucocytes, hemoglobin, and hematocrit in serum was determined before and 24 hours after each drug treatment (data not show) and every 30 days for the 10-month follow up period using an automatic analyzer (OPERA[®], Bayer). Clinical and behavioral changes, food and water intake, temperature, heart and respiratory rate were also observed.

2.9 Parasitological evaluation in dogs

Parasite levels were monitored every 30 days after experimental infection and during the 10-month follow-up period. Dogs were sedated with an intramuscular injection of 3 mg/kg xylacin hydrochloride 2% (Rompun[®], Bayer) and bone marrow aspirates were taken from the costochondral junction. In addition, lymph node biopsies were taken from the popliteal node and peripheral blood mononuclear cells samples (buffy coat) were separated from blood using Ficoll-hypaque gradient centrifugation as described by Chamizo et al., 2005. Ear skin biopsies were obtained using a biopsy punch (Stiefel[®], United Kingdom). Lymph node samples, bone marrow aspirates, skin biopsy and peripheral blood mononuclear cells were cultured in NNN medium and sub cultured every week for 4 weeks. Every week the samples were viewed by optical microscopy to determine if parasites were present. Qualitative *Leishmania* nested polymerase chain reaction *Leishmania* nested PCR (Ln-PCR) and quantitative real time PCR

(qPCR) were used to determine the parasite load in samples of bone marrow aspirate and lymph node before treatment and during monitoring of treatment of animals using methods described by Cruz et al., 2002, 2013. Specific *L. infantum* serum antibodies were tested before treatment and at 90, 180 and 300 days post-treatment by immunofluorescent antibody test (IFAT) and ELISA with soluble *Leishmania* (SLA) and rK39 antigens and by lymphoproliferative response to soluble *Leishmania* (SLA) antigens as described by Guarga *et al.* 2002.

On day 300 days post-treatment dogs were euthanized by intravenous treatment with a lethal dose of sodium pentobarbital (Dolethal[®], Vetoquinol, Spain). At necropsy, spleen samples were obtained aseptically for parasite detection.

2.10. Statistical analysis of results

Parasite data from murine experiments were analyzed using a one-way ANOVA using log₁₀ transformed parasite burden for the liver data. Differences between treatments were analyzed using a Fisher's PLSD test using the Statview[®] version 5.0.1 software package. Data from canine studies is presented as mean and standard deviation, after a descriptive analysis, the normality was assessment using a Shapiro–Wilk test. A Friedman non-parametric test was used to evaluate differences after single dose administration toxicity studies. GLM repeated measures analysis of variance were used to study the influence of the treatment with the vesicular formulation on the biochemical, hematological, and parasitological parameters; a simple contrast using a reference category of values obtained before treatment (9 first months) was applied. The validity of the sphericity assumption was assessed by the Mauchly test and the Greenhouse-Geisser correction was used for repeated-measures ANOVA when the assumption of sphericity was violated. The levels of the within-subjects factor were analyzed using a simple contrast, so that each level of the factor except the first was compared to the first level (before treatment). SPSS[®] version 22.0 software package was used for canine studies data. A p value of < 0.05 was considered significant.

2. Results

3.1. In vivo murine studies

Preliminary studies were carried out to determine the antiparasitic efficacy of combination treatment

with different PPM and SSG formulations against *L. donovani* in a murine model. Treatment with PPM alone had no significant effect on parasite burdens in any of site, whereas treatment with SSG solution caused a significant reduction in liver parasite burdens (Fig. 1). Joint treatment with SSG and PMM solution also resulted in a significant reduction in liver parasite burdens but paromomycin and SSG solution gave a similar efficacy as treatment with SSG alone. As expected from previous studies, treatment with SSG-NIV resulted in a significant reduction in parasite burdens in the spleen, liver and bone marrow . Combination treatment with SSG-NIV and PMM solution gave a similar activity as treatment with SSG-NIV alone (Fig.1). Treatment with PMM-NIV caused a significant reduction in splenic and liver parasite burdens compared to controls, but had no effect on bone marrow parasite burdens. As expected, treatment with PPM solution had no significant effect on parasite burdens in all three sites (Fig. 2). The high activity of SSG-NIV against parasite burdens in all three sites could indicate that joint treatment with SSG-NIV and PMM-NIV it could be more effective than treatment with SSG-NIV alone at the doses used and would circumvent resistance or relapse.

3.2. Comparison of the toxicity of different drug formulations in healthy dogs

In previous studies we have only determined the toxicity of SSG-NIV treatment in dogs. Therefore, it was important to demonstrate the PMM-NIV formulation was non-toxic to animals. Single-dose treatments with SSG solution, PMM solution or a 1:1 mixture of SSG-NIV: PMM-NIV did not cause obvious acute toxic side effects in dogs during or after treatment. The only significant differences observed were a reduction in albumin in dogs given SSG solution and after administration of SSG-NIV:PMM-NIV at 7 days post-treatment. A reduction of total proteins value after administration of SSG-NIV:PMM-NIV at 7 days post-treatment , a reduction of ALT and AST in dogs given SSG solution at 7 days post-treatment and reduction of ALT and ALP in dogs given SSG-NIV:PMM-NIV at 7 days post-treatment. However, none of the biochemical parameters studied was outside the physiological reference values at 24 hours and 7 days post-dosing compared to day 0 values (Table 1). Treatment was not associated with any changes in food or water intake, body temperature, heart

or respiratory rate.

3.3. Clinical and parasitological response to treatment in experimental infected dogs

Joint treatment with SSG-NIV: PMM-NIV formulations was not associated with any obvious signs of acute toxic side effects even though dogs were given 7 drug doses. Infection with *L. infantum* was associated with a significant reduction in serum albumin levels whereas the levels of total serum globulin, plasma proteins, alkaline phosphatase, AST, ALT and urea, were not significantly different from uninfected control levels. Treatment was associated with a significant increase in serum albumin levels whereas the levels of total serum globulin, plasma proteins, AST, ALT were not significantly different before treatment. At the end of the study BUN and creatinine levels were significantly lower in dogs compared to pre-infection levels but both parameters were within the physiological range (Table 2). Blood levels of leucocytes, erythrocytes, lymphocytes, monocytes, granulocytes, hemoglobin and hematocrit was unchanged after *L. infantum* infection (data not show) and they were not significantly altered after drug treatment of infected animals and only the hematological analysis showed a higher count of eosinophils both after infection and at the end of evaluation period (data not shown). Drug treatment was associated with a progressive and significant decrease of the specific IgG anti-*Leishmania* antibodies, using SLA or rK39 as the target antigen (Table 3). A 6 dogs had high anti-*Leishmania* antibody titres (1:640) using the IFAT assay at 90 and 180 days after infection. However, at the end of the follow-up at 300 days after treatment, 4 of the dogs had lower titres of 1:40 and the remaining two had titres of 1:320 and 1:640. The healthy status of all 6 *L. infantum* infected dogs improved after treatment, based on reduction in lymphadenopathy, splenomegaly, alopecia, onychogryphosis and skin ulcers. Before treatment 6/6 dogs were classified as symptomatic, 90 days post-treatment 6/6 dogs were classified as asymptomatic and 180 days post-treatment 2/6 dogs were classified as asymptomatic and 4/6 dogs as oligosymptomatic (with lymph node hypertrophy). At the end of the study, on day 300, 2/6 dogs were classified as asymptomatic, 3/6 as oligosymptomatic and only 1 dog was classified as symptomatic (Figure 3). Two months after treatment ended, PCR analysis showed a clear decrease in the levels of parasite DNA in all tested

tissues, and only one dog remained parasitological positive in a popliteal lymph node sample. Three months after treatment all the dogs remained parasitological negative based on PCR tests and culture of samples (Tables 4 and 5). However, all 6 dogs showed a gradual increase in symptoms of *L. infantum* infection and at 10 months post-treatment all 6 dogs were parasite positive based on NNN culture results and/or PCR studies using lymph node, spleen and/or bone marrow samples.

4. Discussion

In previous studies using a SSG-NIV-dextran formulation, where untrapped drug was removed from the formulation, so that the majority of the drug dose given was entrapped within the NIV significantly increased the distribution half-life and distribution volume values. This formulation gave an elimination half-life that was 4 times longer and a mean residence time that was twice that obtained after free SSG administration. However, the SSG-NIV-dextran formulation was toxic to dogs, causing chills, salivation, and respiratory distress, vomiting and passing of mucous diarrhea, which turned hemorrhagic (Nieto et al., 2003). A liposomal formulation of meglumine antimoniate also caused acute side effects in dogs 15 minutes following every administration shown by prostration, defecation, tachypnea and sialorrhea (Schettini et al., 2005). These side effects were also present in animals given empty liposomes, indicating that the effects could be caused by the carrier system and not drug related. In previous studies, lipid vesicle-induced acute adverse reaction was attributed to the activation of the complement system (Szebeni, 1988; Szebeni et al., 2000). In our study, joint treatment with a single dose of the SSG-NIV: PMM-NIV formulations was not associated with any obvious signs of acute toxic side effects and during the follow-up period, 7 days after treatment, all the hematological and biochemical parameters were within normal range. A reduction in albumin levels was observed at 9 months post-infection but after treatment the albumin levels were within normal ranges. Levels of BUN and creatinine, which are increased in VL infection, were significantly lower after treatment, which would indicate that the dogs were responding well to therapy (Solano-Gallego et al. 2011). Combination therapy with SSG-NIV and PMM-NIV formulations gave a better toxicity profile than treatment with the previous SSG-NIV formulation

that tested.

A previous study showed that intravenous infection with promastigotes of the same *L. infantum* strain (MON-1) is the most reliable model in dogs for establishing reliable and rapid infections in dogs (Poot et al., 2005). In our study, at day 270 post infection, all 6 infected dogs showed clinical symptoms of canine visceral leishmaniasis (CanL) and all 6 dogs were classified as symptomatic based on a physical examination, although clinical signs of disease were variable and probably reflects variability in the phase of disease, host immunity and variability in individual responses. An observation that is not unexpected for *Leishmania* infected dogs (Mancianti et al., 1988, Ciaramella et al., 1997). In this study, after treatment, we observed a gradual decrease in the total IgG anti-*Leishmania* antibody titers, with ELISA-SLA and ELISA-rK39. However, the animals did not give a negative specific antibody response at the end of the follow-up, at 300 days after treatment. Our results are consistent with those obtained by others authors who described a decrease in the levels of anti-*Leishmania* antibodies after treatments and dogs did not present negative titers at the end of treatment (Ribeiro et al., 2008; Manna et al., 2008; 2015; Andrade et al., 2011; Cantos-Barreda et al., 2018).

In this study work, a positive lymphoproliferative response to the soluble *Leishmania* antigen (SLA) was observed, 30 days post-infection with the virulent strain of *L. infantum*. However, the specific lymphoproliferative response was falling over time, and was mirrored by a decreased proliferative response to the both tested i.e. phytohemagglutinin and concanavalin A. This was not unexpected as suppression in lymphoproliferative immune response occurs in advanced CVL, a fact that had already been reported by other authors (Martínez Moreno et al., 1995; Moreno et al., 1999; Guarga et al., 2002). In contrast, after a positive lymphoproliferative immune response was observed on days 30, 150 and in the final evaluation at day 300 after drug treatment. A similar reactivation of host lymphoproliferative responses between 5-12 months after treatment has been reported for naturally infected dogs given sodium stibogluconate and allopurinol treatment (Fernández-Pérez et al., 2003). Combination treatment with SSG-NIV and PMM-NIV gave a gradual remission of the clinical signs in all the dogs. It also caused a reduction in parasite burdens based on a decrease in parasite DNA

load in all tested tissues at 2 months post-treatment, and only one dog remained parasitological positive. However, the 7-day drug regimen did not achieve parasite eradication as all 6 dogs gave a positive parasitological culture test and/or PCR test at 10 months post therapy for lymph node, spleen and/or bone marrow samples. This same inability to produce a complete parasitological cure is shown by other drugs and combinations of drugs evaluated so far (Schettini et al., 2005; Ribeiro et al., 2008; Vexenat et al., 1998; Athanasiou et al., 2013; 2014; Manna et al., 2008; 2015; Andrade et al., 2011; Dos Santos Nogueira et al., 2019). However, joint treatment was as effective or even better than other reported treatment protocols. This includes treatment with PMM solution (15-30 days, Vexenat et al., 1998; Athanasiou et al., 2013; 2014), antimonial drug solution (45 days, Poli et al., 1997), combination treatment with MA and allopurinol for 30 days (Manna et al., 2008; 2015), combination treatment with MA and PMM for 21 days (Oliva et al., 1998;), PMM and allopurinol for 28 days (Kasabalis et al., 2019; 2020), administration of liposomal MA (4 days, Schettini et al., 2005; Ribeiro et al., 2008), oral administration with miltefosine for 28-45 days (Andrade et al., 2011; Dos Santos Nogueira et al., 2019), combination treatment of miltefosine and alopurinol (30 days, Manna et al., 2015) or oral administration of a new alkylphosphocholine molecules (Hernández et al., 2014; Hernández Martínez, 2015). Therefore, increasing the dose drug administered or giving a second round of SSG-NIV: PMM-NIV treatment could result in parasite eradication or prevent relapse of the treated dogs.

Leishmaniasis, despite its global importance, has been relatively under-researched by pharmaceutical companies compared to other diseases, mainly due to the complexity of the parasitic infection and the lack of economic incentives. In such a scenario, drug delivery systems have a crucial role to play in the effective management of current and emerging anti-parasitic agents, enhancing their specificity, with a concomitant reduction in adverse effects associated therewith. In view of this, colloidal carriers such as liposomes have demonstrated good potential in improving the efficacy and tolerability of leishmanicidal agents because of their biodegradability, biocompatibility, nontoxic and nonimmunogenic nature, ease of administration and sustained drug release. Despite extensive research, therapy in humans with liposome-encapsulated leishmanicidal drugs is not a reality. So far,

the only successful commercialized liposomal formulation that has proved its usefulness is the expensive AmBisome®. Advances in liposome preparation technologies, as well as the use of adjuvants to improve liposome targeting (peptides, sugar and positively charged lipids) are leading to the development of better formulations for the treatment of leishmaniasis. (Ortega et al., 2017). Vesicular drug delivery systems are novel means to improve the bioavailability of the encapsulated drug along with numerous advantages over conventional drug delivery systems. Liposomes were first in such type of delivery systems but it was not so successful due to their numerous drawbacks, particularly cost and lipid physicochemical stability. Niosomes or non-ionic surfactant vesicles are formed from self-assembly of mixtures of hydrated surfactants incorporated in order to achieve colloiddally stable vesicular systems. The most striking feature of such delivery systems is that they can be used to encapsulate both hydrophobic or hydrophilic drugs. These vesicular drug delivery systems can locate to sites of high parasitic infection and provide targeted, slow, controlled, release of drug resulting in sustained activity, reduced toxicity, and favorable modification in drug distribution profile that enhances the bioavailability of encapsulated drug (Khan and Irchhaiya, 2016).

5. Conclusion

In summary, the co-administration of SSG-NIV (10 mg Sb^v/kg/day) and PMM-NIV (10 mg/kg PMM/day) for seven days to six polysymptomatic dogs experimentally infected with *L. infantum*, did not produce toxicity and was able to induce clinical improvement from day 30 post-administration. At the end of the study (day 300), 2 dogs were asymptomatic, 3 were oligosymptomatic and one was symptomatic. Therefore, despite the ability of co-treatment causing a significant reduction in parasitic load at day 90 post-treatment, the treatment was not able to cure all of the of dogs of their infection. Joint treatment was as however, as effective or even better than other reported treatment protocols and further studies are required in order to determine if it is possible to identify a treatment protocol than can cure all treated dogs or keep them symptom free.

CRedit author statement

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Conflict of interests

The authors declare that they do not have competing interests.

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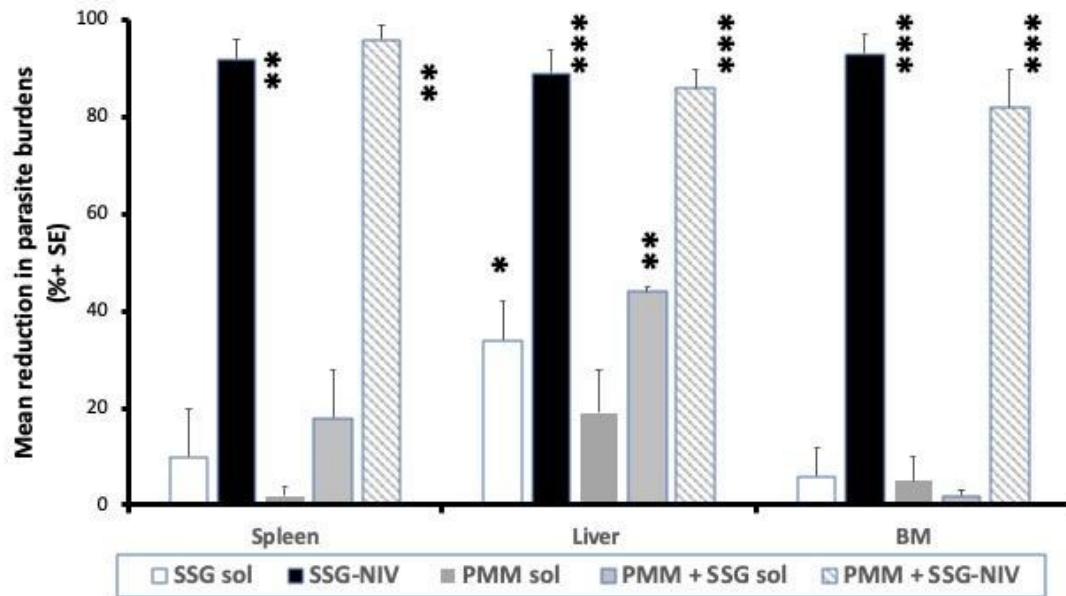


Figure 1. Mice, infected with *L. donovani* were treated on day 7 with PBS pH 7.4 (control), SSG solution alone (300 mg Sb^v/kg), paromomycin solution alone (20 mg/kg), SSG-NIV alone (300 mg Sb^v/kg) or PMM and SSG solution (1:1 mixture) or SSG-NIV and PMM-NIV (1:1 mixture). The controls had a n = 5, whereas all other treatments had a n = 4. The mixed formulations were prepared at twice the concentration so that they were at given at the same drug and lipid dose as the single dose treatments. Mice were sacrificed on day 14 post-infection and the effect of drug treatment on parasite burdens in spleen, liver and bone marrow (BM), determined as the mean reduction in parasite burdens compared to the mean control value. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the control value.

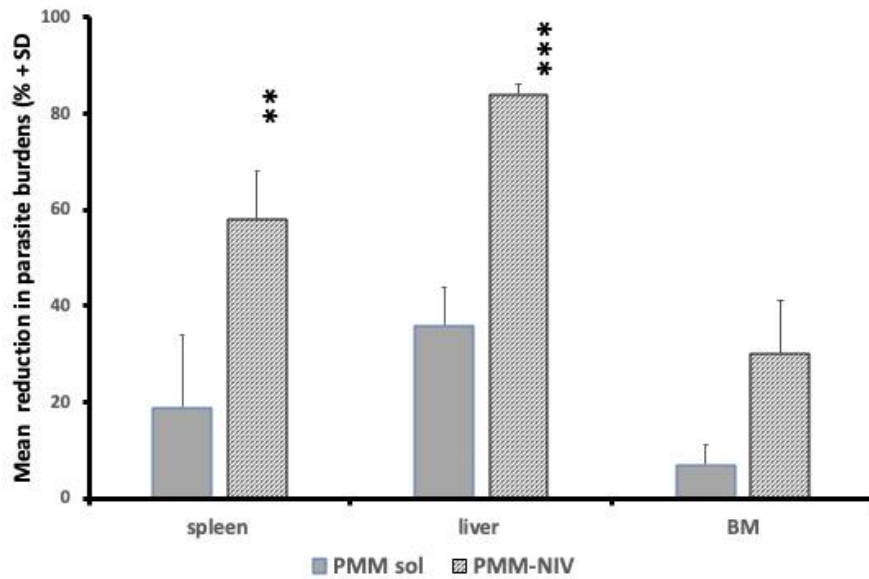


Figure 2. Mice (n = 4/treatment), infected with *L. donovani* were treated on day 7 with PBS pH 7.4 (control), paromomycin solution alone (20 mg/kg) or PMM-NIV alone (20 mg/kg). Mice were sacrificed on day 14 post-infection and the effect of drug treatment on parasite burdens determined as the mean reduction in parasite burdens compared to the mean control value. **p < 0.01, ***p < 0.001 compared to the control value.

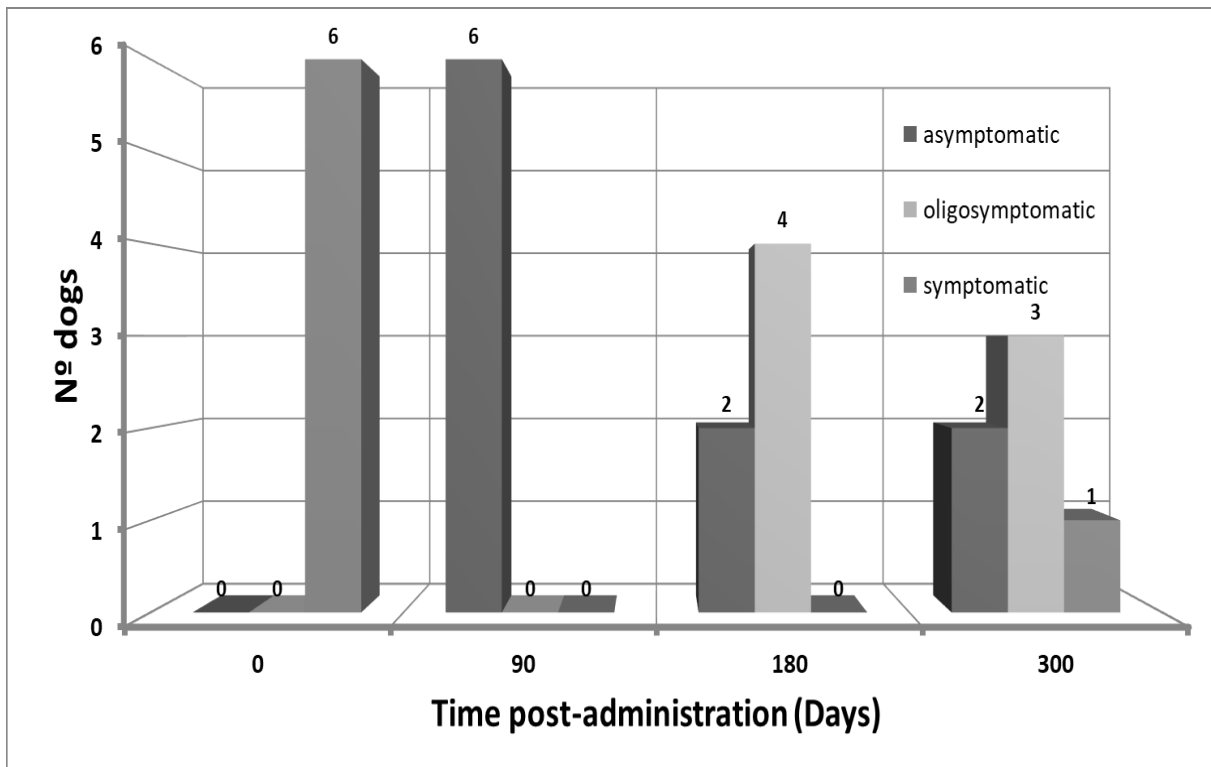


Figure 3. Clinical status of 6 dogs before (day 0) and after administration of SSG-NIV and PMM-NIV formulations at a daily dose of 10 mg/kg of sodium stibogluconate and 10 mg/kg of paromomycin with a non-ionic surfactant vehicle, for seven days to six dogs experimentally infected with *L. infantum*. At the end of the study (day 300), 2 dogs were classified as asymptomatic, 3 were oligosymptomatic and 1 symptomatic.

Table 1. The effect of an intravenously single dose of sodium stibogluconate solution (10 mg Sb^v/Kg). and paromomycin solution (10 mg PMM/kg) or a 1:1 mixture of sodium stibogluconate and paromomycin with a non-ionic surfactant vehicle (SSG-NIV: PMM-NIV) (10 mg Sb^v/kg: 10 mg PMM/kg) on selected biochemical parameters before and 24 hours and 7 days post-dosing.

| Biochemical parameter (Reference range in parentheses) | SSG | PMM | SSG-NIV:PMM-NIV |
|---|------------------|------------------|------------------------|
| Albumin (2.7-3.8g/dL) | mean ± SD | mean ± SD | mean ± SD |
| 0h | 3.34 ± 0.37 | 3.16 ± 0.35 | 3.02 ± 0.25 |
| 24h | 3.22 ± 0.40 | 2.94 ± 0.26 | 3.04 ± 0.20 |
| 7d | 2.92 ± 0.31 | 3.00 ± 0.18 | 2.74 ± 0.21 |
| Globulins (2.5-4.5g/dL) | | | |
| 0h | 3.32 ± 0.82 | 3.48 ± 0.32 | 3.48 ± 0.27 |
| 24h | 3.28 ± 0.34 | 3.42 ± 0.46 | 3.42 ± 0.35 |
| 7d | 3.12 ± 0.38 | 3.24 ± 0.32 | 3.48 ± 0.25 |
| Total proteins (5.2-8.2g/dL) | | | |
| 0h | 6.64 ± 0.77 | 6.62 ± 0.13 | 6.56 ± 0.32 |
| 24h | 6.48 ± 0.14 | 6.28 ± 0.17 | 6.48 ± 0.32 |
| 7d | 6.04 ± 0.23 | 6.22 ± 0.31 | 6.24 ± 0.32 |
| BUN (7-27 mg/dL) | | | |
| 0h | 16.2 ± 2.2 | 13.0 ± 2.0 | 17.6 ± 5.5 |
| 24h | 14.0 ± 2.6 | 14.0 ± 1.6 | 16.8 ± 3.4 |
| 7d | 13.8 ± 7.4 | 14.8 ± 1.1 | 19.6 ± 3.6 |
| Creatinine (0.5-1.8mg/dL) | | | |
| 0h | 0.9 ± 0.2 | 0.8 ± 0.2 | 1.0 ± 0.1 |
| 24h | 0.8 ± 0.1 | 0.9 ± 0.1 | 1.0 ± 0.1 |
| 7d | 0.9 ± 0.1 | 0.8 ± 0.1 | 1.0 ± 0.1 |
| ALT (10-100 U/L) | | | |
| 0h | 51.6±21.4 | 48.0 ± 13.8 | 46.4 ± 18.7 |
| 24h | 70.8±23.1 | 42.8 ± 22.4 | 73.2 ± 39.6 |
| 7d | 45.2±18.9 | 38.4 ± 24.5 | 44.6 ± 23.5 |
| AST (0-50 U/L) | | | |
| 0h | 30.8±21.6 | 27.2 ± 19.3 | 15.0 ± 8.23 |
| 24h | 54.6±39.2 | 24.6 ± 19.3 | 24.4 ± 18.8 |
| 7d | 13.6±16.2 | 15.8 ± 18.2 | 19.8 ± 6.7 |
| ALP (32-212 UI/L) | | | |
| 0h | 63.0±28.7 | 68.4 ± 24.7 | 72.8 ± 25.4 |
| 24h | 79.6±29.7 | 62.4 ± 21.8 | 89.0 ± 29.4 |
| 7d | 67.6±14.7 | 67.0 ± 21.4 | 76.4 ± 19.3 |

(Ref)= physiological values; OD= optical density; SSG=sodium stibogluconate; PMM= paromomycin; (SSG-NIV:PMM-NIV)= 1:1 mixture of sodium stibogluconate and paromomycin with a non-ionic surfactant vehicle); BUN= blood urea nitrogen; AST= aspartate aminotransferase; ALT= alanine transaminase; ALP= alkaline phosphatase; * there is not stats in this table, is that because there are not changes.

Table 2. The effect of 7 days/24 h intravenously dose of a 1:1 mixture of SSG-NIV (10 mg Sb^v/kg/day) and PMM-NIV (10 mg PMM /kg/day) on selected clinical parameters before and post-treatment.

| Clinical parameters (Ref.) | Time after drug administration (days) | | | | | | | | | | | | | | | | | |
|---|---------------------------------------|-----------------------------|-----------------------------|------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------|------------------|------------------|------------------|-----------------------------|------------------|-----------------------------|------------------------------|------------------------------|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 15 | 30 | 60 | 90 | 120 | 150 | 180 | 210 | 240 | 300 |
| Albumin ⁴⁴ (2.7-3.8 g/dL) | 2.50 (0.34) | 4.52 ^b (0.82) | 4.52 ^b (0.87) | 4.57 ^b (0.84) | 4.63 ^b (0.99) | 4.37 ^b (0.75) | 4.33 ^b (0.69) | 4.22 ^a (0.82) | 4.28 ^b (0.66) | 2.47 (0.27) | 2.45 (0.42) | 2.43 (0.23) | 2.52 (0.24) | 2.63 (0.34) | 2.67 (0.39) | 2.45 (0.33) | 2.57 (0.34) | 2.17 ^a (0.42) |
| Globulins ⁶ (2.5-4.5 g/dL) | 4.37 (0.82) | 4.52 ^a (0.82) | 4.52 (0.87) | 4.57 (0.84) | 4.63 (0.99) | 4.37 (0.75) | 4.33 (0.69) | 4.22 (0.82) | 4.28 (0.66) | 4.23 (0.55) | 4.15 (0.54) | 4.30 (0.79) | 4.12 (0.69) | 4.05 (0.49) | 4.12 (0.80) | 3.67 ^b (1.05) | 3.77 ^a (0.69) | 3.63 ^b (0.63) |
| Total protein ⁵ (5.2-8.2 g/dL) | 6.88 (0.66) | 7.05 (0.62) | 7.00 (0.70) | 7.07 ^a (0.64) | 7.10 (0.45) | 6.83 (0.55) | 6.75 (0.45) | 6.65 (0.62) | 6.78 (0.50) | 6.70 (0.46) | 6.62 (0.36) | 6.75 (0.64) | 6.62 (0.63) | 6.68 (0.58) | 6.77 (1.04) | 6.22 (1.22) | 6.37 ^a (0.60) | 5.85 ^b (0.54) |
| AST (0-50 U/L) | 27.83 (3.87) | 34.00 (8.53) | 39.50 (10.11) | 32.33 (14.81) | 25.67 (15.21) | 27.33 (16.08) | 21.17 (12.43) | 18.67 (9.61) | 22.50 (18.36) | 31.50 (4.42) | 30.83 (9.17) | 34.67 (8.80) | 24.00 (6.81) | 31.00 (9.03) | 29.00 (11.42) | 27.33 (11.00) | 22.83 (11.32) | 28.67 (8.89) |
| ALT (10-100 U/L) | 42.17 (10.98) | 51.00 (14.11) | 54.67 (17.17) | 55.83 (16.58) | 50.67 (16.97) | 50.67 (13.82) | 51.00 (16.32) | 46.67 (14.60) | 45.83 (16.65) | 46.83 (8.38) | 49.17 (20.76) | 42.83 (18.32) | 37.67 (10.76) | 51.50 (13.11) | 46.00 (16.55) | 62.83 (29.31) | 53.17 (46.01) | 54.67 (18.78) |
| BUN ⁵ (7-27 mg/dL) | 16.50 (5.32) | 14.17 (4.54) | 13.67 (3.93) | 14.83 ^a (4.54) | 14.50 (4.32) | 13.83 (4.79) | 11.50 (3.67) | 16.67 (5.75) | 13.00 (3.35) | 10.83 (2.40) | 11.17 (2.79) | 11.00 (2.45) | 9.67 (1.63) | 9.33 (1.37) | 10.00 (2.00) | 9.50 (2.43) | 10.17 ^a (4.54) | 11.83 ^b (7.49) |
| Creatinine ⁵ (0.5-1.8 mg/dL) | 0.98 (0.12) | 0.92 (0.12) | 0.90 ^a (0.13) | 1.03 (0.15) | 0.93 (0.14) | 0.95 (0.12) | 0.90 ^a (0.09) | 1.02 (0.12) | 0.93 (0.14) | 0.93 (0.10) | 1.00 (0.14) | 0.92 (0.08) | 0.93 (0.10) | 0.82 ^b (0.08) | 0.87 (0.12) | 0.78 ^b (0.08) | 0.83 ^b (0.08) | 0.90 (0.26) |

(Ref)= physiological values; SSG= sodium stibogluconate; PMM= paromomycin; (SSG-NIV:PMM-NIV)= 1:1 mixture of sodium stibogluconate and paromomycin with a non-ionic surfactant vehicle); BUN= blood urea nitrogen; AST= aspartate aminotransferase; ALT= alanine transaminase.

Within-subject statistical difference: □ p<0.05; □□ p<0.01; □□□ p<0.001. Statistical difference between each level of the factor compared to the first level (before treatment: time 0): ^a p<0.05, ^b p<0.01; ^c p<0.001

n.d.: no data

Table 3. The effect of 7 days/24 h intravenously dose of a 1:1 mixture of SSG-NIV (10 mg Sb^v/kg/day) and PMM-NIV (10 mg PMM /kg/day) on selected clinical parameters before and post-treatment..

| Clinical parameters | Time after drug administration (days) | | | | | | | | | | | | | | | | | | |
|---|---------------------------------------|--------|--------|--------|--------|--------|--------|--------|--------|-------------------|-------------------|-------------------|-------------------|-------------------|----------------------|-------------------|-------------------|-------------------|--|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 15 | 30 | 60 | 90 | 120 | 150 | 180 | 210 | 240 | 300 | |
| (Ref.) | | | | | | | | | | | | | | | | | | | |
| Erythrocytes | 6.52 | 6.30 | 6.17 | 6.37 | 6.50 | 6.16 | 6.15 | 6.31 | 6.36 | 6.54 | 6.47 | 6.30 | 5.79 | 6.61 | 6.65 | 6.55 | 6.44 | 5.93 | |
| (5.5-8.5. 10 ⁶ mm ³) | (1.46) | (0.90) | (0.84) | (0.98) | (1.10) | (0.88) | (0.95) | (1.02) | (0.89) | (0.84) | (0.94) | (1.20) | (0.43) | (0.39) | (0.48) | (0.64) | (0.59) | (0.61) | |
| Leucocytes | 13.30 | 11.25 | 10.50 | 9.62 | 9.55 | 9.97 | 9.03 | 11.33 | 13.00 | 10.07 | 9.63 | 10.37 | 11.38 | 9.50 | 8.88 | 10.33 | 12.20 | 9.77 | |
| (6-17. 10 ³ mm ³) | (5.10) | (2.32) | (1.85) | (2.48) | (2.75) | (2.43) | (1.57) | (1.99) | (3.77) | (2.61) | (1.82) | (2.05) | (3.34) | (2.06) | (1.61) | (2.27) | (3.60) | (3.42) | |
| Hemoglobin | 14.58 | 14.62 | 14.33 | 14.37 | 14.70 | 13.73 | 13.87 | 13.82 | 14.35 | 14.98 | 14.67 | 14.10 | 14.08 | 15.17 | 15.33 | 15.68 | 15.05 | 13.75 | |
| (12-18 g/dL.) | (3.32) | (2.45) | (2.31) | (2.49) | (2.88) | (2.16) | (2.37) | (2.45) | (2.16) | (2.14) | (2.21) | (1.65) | (1.26) | (1.15) | (1.24) | (1.83) | (1.17) | (1.30) | |
| Hematocrit | 44.10 | 42.72 | 41.67 | 43.18 | 44.15 | 41.68 | 41.52 | 42.57 | 43.20 | 43.80 | 43.02 | 40.23 | 39.27 | 44.08 | 44.25 | 44.07 | 42.97 | 39.73 | |
| (37-55%) | (10.26) | (6.60) | (6.23) | (7.28) | (8.13) | (6.57) | (7.06) | (7.43) | (6.24) | (5.81) | (6.01) | (4.45) | (3.08) | (3.44) | (3.48 ^v) | (4.92) | (3.42) | (3.77) | |
| IgG SLA ELISA^{OD} | 1.68 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 1.76 | 1.46 | 1.49 | 1.48 | 1.24 ^b | 1.39 ^b | 1.48 | 1.29 ^b | 1.20 ^b | |
| OD.1000 405nm | (0.54) | | | | | | | | | (0.48) | (0.60) | (0.63) | (0.62) | (0.61) | (0.64) | (0.45) | (0.61) | (0.68) | |
| IgG K39ELISA^{OD} | 1.21 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 1.05 ^a | 1.00 ^b | 0.97 ^b | 0.98 ^b | 0.87 ^c | 0.89 ^c | 0.87 ^c | 0.86 ^c | 0.89 ^c | |
| OD.1000 405nm | (0.79) | | | | | | | | | (0.84) | (0.79) | (0.75) | (0.79) | (0.83) | (0.84) | (0.83) | (0.84) | (0.84) | |
| Lymphoproliferative response to SLA | 0.19 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | 0.31 | 0.14 | 0.05 | 0.14 | 0.30 | 0.12 | 0.10 | 0.10 | 0.19 | |
| antigen OD 405 nm | (0.09) | | | | | | | | | (0.37) | (0.04) | (0.05) | (0.05) | (0.20) | (0.04) | (0.07) | (0.06) | (0.08) | |

(Ref)= physiological values; OD= optical density; SSG= sodium stibogluconate; PMM= paromomycin; (SSG-NIV: PMM-NIV) = 1:1 mixture of sodium stibogluconate and paromomycin with a non-ionic surfactant vehicle); IgG= immunoglobulin G. SLA= Soluble *Leishmania* antigen.

Within-subject statistical difference: [□] p<0.05; ^{□□}p<0.01; ^{□□□}p<0.001. Statistical difference between each level of the factor compared to the first level (before treatment: time 0): ^a p<0.05, ^b p<0.01; ^c p<0.001.

n.d.: no data.

Table 4. The parasitological effect of combination treatment with a 1:1 mixture of sodium stibogluconate and paromomycin with a non-ionic surfactant vehicle (SSG-NIV: PMM-NIV) (10 mg SbV/kg: 10 mg PMM/kg) every 24 hours for 7 days starting at month 10 of infection on *Leishmania* nested PCR and culture and the monthly follow up during 300 days post- treatment.

| Dog | Technique | Time after infection with virulent strain of <i>L. infantum</i> | | | Time after chemotherapy of infected animals (d) | | | | | | | |
|-----|-----------|---|-----|-----|---|----|----|-----|-----|-----|-----|-----|
| | | 0 | 180 | 270 | 30 | 60 | 90 | 120 | 150 | 180 | 240 | 300 |
| T20 | Ln-PCR | - | L | L | - | - | - | LS | L | - | L | - |
| | Culture | - | - | - | - | - | - | - | - | - | - | - |
| T21 | Ln-PCR | - | LS | L | - | - | - | - | - | - | - | - |
| | Culture | - | - | - | - | - | - | - | - | - | - | - |
| T22 | Ln-PCR | - | - | L | - | - | - | - | - | L | L | L |
| | Culture | - | - | - | - | - | - | - | - | - | L | L |
| T23 | Ln-PCR | - | S | L | - | - | - | L | b | - | L | - |
| | Culture | - | - | L | - | - | - | - | - | - | L | - |
| T24 | Ln-PCR | - | LB | LB | L. B | - | - | - | L | L | - | - |
| | Culture | - | LBS | LB | - | - | - | - | - | - | - | - |
| T26 | Ln-PCR | - | LB | LB | B | L | - | - | LB | L | LB | LB |
| | Culture | - | LB | LB | B | - | - | LB | LB | L | LB | L |

L= lymph node; B= bone marrow; S= skin; b= buffy coat; - = negative; Ln-PCR=*Leishmania* nested PCR; d= days; T20-T26: dog's number.

Table 5. The effect of combination treatment with a 1:1 mixture of SSG-NIV:PMM-NIV (10 mg Sb^v/kg: 10 mg PMM/kg) every 24 hours for 7 days starting at month 10 of infection on the number of *L. infantum* parasites in 50 ng of total DNA (qPCR).

| Dog | Tissue | Time in days | | | | | | | | | |
|-------------------------|--------|--------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | | 30 | 60 | 90 | 120 | 150 | 180 | 210 | 240 | 270 | 300 |
| Before treatment | | | | | | | | | | | |
| T20 | B | - | - | - | - | - | - | - | - | - | - |
| | L | - | - | - | - | - | - | - | - | - | - |
| T21 | B | - | - | - | - | - | - | - | - | - | - |
| | L | - | 226 | - | 115 | - | 14 | - | - | - | - |
| T22 | B | - | - | - | - | - | - | - | - | - | - |
| | L | - | - | - | - | - | - | - | 26 | - | - |
| T23 | B | - | - | - | - | - | - | - | - | - | - |
| | L | - | - | - | - | - | - | 27 | 59 | - | - |
| T24 | B | 557 | - | 702 | - | 484 | 502 | 80 | - | 40 | - |
| | L | - | 54 | 146 | 59 | 29 | 59 | 20 | - | 81 | - |
| T26 | B | - | - | - | - | 467 | 218 | 60 | - | 80 | - |
| | L | 291 | 68 | 56 | 61 | - | 18 | 41 | 88 | 168 | - |
| After treatment | | | | | | | | | | | |
| T20 | B | - | - | - | - | - | - | - | - | - | - |
| | L | - | - | - | 15 | - | - | - | 100 | - | - |
| | S | - | - | - | - | - | - | - | - | - | 84 |
| T21 | B | - | - | - | - | - | - | - | - | - | - |
| | L | - | - | - | - | - | - | - | - | - | - |
| | S | - | - | - | - | - | - | - | - | - | 17 |
| T22 | B | - | - | - | - | - | - | - | - | - | - |
| | L | - | - | - | - | - | 119 | 91 | 91 | - | 97 |
| | S | - | - | - | - | - | - | - | - | - | 146 |
| T23 | B | - | - | - | - | - | - | - | - | - | - |
| | L | - | - | - | - | - | - | - | 52 | - | - |
| | S | - | - | - | - | - | - | - | - | - | 29 |
| T24 | B | 262 | - | - | - | - | - | - | - | - | - |
| | L | 277 | - | - | - | - | 44 | - | - | - | - |
| | S | - | - | - | - | - | - | - | - | - | 85 |
| T26 | B | 209 | - | - | - | - | - | - | 209 | - | 599 |
| | L | - | 47 | - | - | - | 20 | 53 | 24 | - | 23 |
| | S | - | - | - | - | - | - | - | - | - | 98 |

B= bone marrow; L= popliteal lymph node; S= spleen; T20-T26: dog's number; qPCR= quantitative PCR.