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Short communication

Immunotherapy with the saponin enriched-Leishmune® vaccine *versus* immunochemotherapy in dogs with natural canine visceral leishmaniasis

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ABSTRACT

Leishmune®, the first licensed vaccine for prophylaxis against canine visceral leishmaniasis (CVL) and is also immunotherapeutic when used with double saponin adjuvant concentration. The Leishmune® therapeutic vaccine was assessed for immunotherapy (IT) in 31 infected dogs and for immunochemotherapy (ICT) in combination with allopurinol or amphotericinB/allopurinol, in 35 dogs. Compared to infected untreated control dogs, at month 3, both treatments increased the proportion of dogs showing intradermal response to *Leishmania* antigen to a similar extent (from 8 to 67%, in the IT and to 76%, in the ICT groups), and conversely reduced from 100 to 38% (IT) and to 18% (ICT) the proportion of symptomatic cases, from 54 to 12% (IT) and to 15% (ICT) the proportion of parasite evidence in lymph nodes and from 48 to 19% (IT) and 12% (ICT) the proportion of deaths, indicating that the immunotherapy with enriched-Leishmune® vaccine promotes the control of the clinical and parasitological signs of CVL rendering most dogs asymptomatic although PCR positive. By month 8, negative lymph node PCR results were obtained in 80% of the ICT-treated dogs, but only in 33% of the IT group ($p = 0.0253$), suggesting that the combination of additional chemotherapy with Leishmune®-enriched saponin vaccination abolished, not only the symptoms but also the latent infection condition, curing the dogs. The animals were followed up until 4.5 years after the beginning of the experiment and, compared to the untreated control group at month 3 (12/25 dogs; 48%), a decrease in the rate of CVL deaths was only seen after ICT treatment (7/35 dogs; 20%; 0.0273) but not after IT treatment (10/31 dogs; 32%; $p = 0.278$), pointing out an additional advantage of the ICT treatment with the enriched-Leishmune® in the control and cure of CVL.

1. Introduction

Leishmania (*L.*) *chagasi* and *Leishmania* (*L.*) *infantum* are the ethiological agents of human kala-azar in America, the Mediterranean basin, Middle East and Asian Countries. Kala-azar is a severe and frequently lethal disease if untreated after the onset of symptoms. In these regions it occurs as a canid zoonoses. The parasites are found in the skin of wild canids and dogs, and transmitted to humans through the bite of a sand fly. Zoonotic visceral leishmaniasis (ZVL) is thus a re-emergent canid zoonosis, the epidemiological control of which involves: the elimination of seropositive infected dogs, insecticide treatment within domestic and peri-domestic habitations and the systematic treatment of human cases [1]. Different form the dogs and wild canids, the human is not a dead-end host for ZVL, therefore the chemotherapy treatment is more related to the cure and survival of patients, decreasing the human incidence rather to the interruption of the transmission of the disease. Brazil is one of the four countries responsible for 90% of the total human cases (500,000 world wide) [1]. As a tool for epidemiological control, the killing of seropositive dogs is widely practiced in Brazil and China but unacceptable in Europe. Canine surveillance programs are very laborious, expensive and require continual vig-

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ilance [1] and sensitive serological diagnostic methods [2–4] to be effective. Furthermore, since many seropositive infected dogs are asymptomatic, owner compliance is complicated [1] even though the infectivity of asymptomatic dogs to sand flies has already been established [5].

Together with the insecticide treatment of residences, preventive and effective human and canine vaccines against visceral leishmaniasis are considered the best tools for eradication of the disease, diminishing both the number of human and canine cases [6]. Although several canine vaccines have been tested in kennel assays [7–18], few of them showed efficacies in field trials against natural challenge [19–23] and only the Leishmune® is licensed [21,24–26] Leishmune® is the commercial FML-saponin vaccine which previously induced 92–95% of protection in vaccinated dogs, as evidenced by early seroconversion to FML, intradermal response to leishmanial lysate, survival and absence of symptoms and developed 76–80% of vaccine efficacy [19,20]. Sustained intradermal response to leishmanial antigen, no parasites nor *Leishmania* DNA were found in vaccinated dogs after 3.5 years of exposure [20]. After 2 years of vaccination of a cohort of 550 Leishmune® vaccinated exposed dogs, only 1% of the animals died of CVL and 1.2% were symptomatic. Simultaneously, 39% of deaths and 20.6% of symptomatic cases were detected among untreated exposed control animals ($p < 0.005$) [21]. The Leishmune® vaccine is prophylactic against canine visceral leishmaniasis (CVL) and protects 98% of vaccinated dogs [21] which do not expose parasite to sand flies [24] The generated antibodies block the transmission of the disease by sand flies in the field [25] and even at a low vaccine coverage, the observed decrease in human and canine incidence of the disease is significantly correlated to the use of the vaccine in vaccinated towns of Brazil [26]. A total of 65,000 healthy dogs were vaccinated in Brazil up to October 2008. We also observed that Leishmune® formulated with double saponin adjuvant concentration has a therapeutic effect against naturally [27] or experimentally acquired CVL [8]. This immunotherapy with Leishmune® might represent a benefit in the potential treatment of dog disease, since infected dogs are sacrificed and chemotherapy treatment alone is not recommended due to its influence on the favourable selection of resistant parasites [1], and for its controversial efficacy [28]. Indeed, even if a temporary clinical remission is achieved in susceptible animals, a relapse is usual within weeks or years after drug withdrawal [28]. CVL is most frequently treated with meglumine antimoniate (Glucantime®), allopurinol, amphotericin B or a combination of meglumine antimoniate and allopurinol [28].

In the present work we compare the industrial to the laboratory-made formulation of the saponin enriched-Leishmune® vaccine and its therapeutic efficacy when administered alone (immunotherapy) or in combination with amphotericin B and allopurinol (immunochemotherapy) against CVL in dogs naturally infected with *Leishmania chagasi* of a Brazilian endemic area.

2. Material and methods

2.1. Animals and study design

In this study we aimed to analyze if the commercial vaccine (Fort Dodge Animal Heath, Campinas, SP, Brazil) [8,21,24–26] maintains the immunogenic and curative potential of the vaccine produced under laboratory conditions in the Laboratory of Biology and Biochemistry of *Leishmania* of the Universidad Federal do Rio de Janeiro, RJ, Brazil [27] and we also aimed to elucidate whether chemotherapy has any additive effect to immunotherapy with the Leishmune®-enriched vaccine (1.5 mg of the FML and 1 mg of Riedel de Haen saponin/dose) in the treatment of naturally acquired CVL. A total of 91 dogs with natural CVL from Araçatuba and Andradina, São Paulo State, Brazil, were included in this study. These towns show a recent epidemics of canine and human VL [26,27]. All dogs were seropositive to the FML antigen of *Leishmania* (*L.*) *donovani* on the FML-ELISA assay [29] and asymptomatic at the beginning of the assay. The clinical signs used for definition of symptomatic cases included: enlargement of lymph node diameters, loss of weight, alopecia, onycogryphosis, skin lesions conjunctivitis, anaemia, oedema, ictericia, cachexia, cough, asthenia, cataract or nasal purulent secretion and death caused by CVL. Among the 91 dogs, 25 remained as untreated controls, 31 received immunotherapy treatment (IT) and 35 immunochemotherapy treatment (ICT). The IT treatment was then performed on 31 regular patients of the clinics: "Clínica Auquemia" and "Clínica Doutores da Criação" of Araçatuba that received either the laboratory-made ($n = 16$) or the commercial ($n = 15$) formulation. The ICT treatment was done on 35 dogs of the "Clínica Veterinária Mundo Animal" from Andradina, SP, that received either the laboratory-made ($n = 14$) or the commercial ($n = 21$) formulation.

In order to avoid any bias and knowing that the increase in anti-FML serum antibodies of infected dogs is correlated with the increase in number of symptoms [24] and with the positive *Leishmania* DNA PCR results [24,30,31], we used a stratified randomization (random.org, random integer generator) according to the dog serum absorbency values in the FML-ELISA assay. In this way, following a double blind protocol, the groups treated with the commercial or the laboratory-made vaccine of each clinic, had an equal number of dogs of each one of the following anti-FML serum absorbency categories: 0.451–0.500; 0.501–0.600; 0.601–0.700; 0.701–0.800; 0.801–0.900; 0.901–1.000; 1.001–1.200; >1.201. The cut-off of the method is Abs 492ηm = 0.450 [29].

Three doses of either vaccine were injected through the *sc* route on the back of the animals, with 20–30 days interval between October and December 2004. The effect of IT with the enriched-Leishmune® only was compared to the effect of ICT using the enriched-Leishmune® vaccine in addition to either allopurinol (Zyloric®, Glaxo Smithkleine Aebe) alone (10 mg/kg each 12 h for 15 months in 24 dogs) or allopurinol in combination with amphotericin B (Fungizon®, Bristol-Myers Squibb) (0.5 mg/kg, each 3 days, 16 doses in 11 dogs) treatment against ZVL. Against secondary infections, Enrofloxacin® (Baytril®, Bayer) (5 mg/kg once a day during 10 days) was also used in a dog treated with allopurinol in combination with amphotericin, and cefalexin (Keflex®, Lilly) (30 mg/kg each 12 h for 7 days) was administered to another dog treated with allopurinol only. The IT group included 23 females and 8 males while the ICT group was composed of 16 females and 19 male dogs. Heterogeneity of age composition was found in the treated groups regarding their age at the beginning of the assay: <1 year ($n = 2$); 1 year ($n = 6$); 2 years ($n = 8$); 3 years ($n = 4$); 4 years ($n = 4$); 5 years ($n = 3$); 6 years ($n = 2$) and 7 years old dogs ($n = 2$) in the IT group and 2 years ($n = 2$); 3 years ($n = 3$); 4 years ($n = 5$); 5 years ($n = 5$); 6 years ($n = 8$); 7 years ($n = 3$); 8 years ($n = 7$); 9 years ($n = 1$) and 10 years old dogs ($n = 1$) in the ICT group.

The saponin enriched-Leishmune® formulation used in this investigation differs in adjuvant concentration from the prophylactic Leishmune® vaccine which contains only 0.5 mg of the saponin and is industrialized and registered in Brazil as a prophylactic vaccine against canine visceral leishmaniasis (Patent: INPI number: PI1100173-9 (18.3.97), Federal University of Rio de Janeiro, Brazil).

All dogs were monitored at 0, 3 and 8 months after vaccination for anti-FML IgG antibody levels and symptoms. The delayed type of hypersensitivity (DTH) was assayed on months 3 and 8. The presence of amastigotes was evaluated through microscopic evaluation of Giemsa stained smears of aspirates of popliteal lymph nodes obtained after fine needle biopsies [32] and by PCR analysis for *Leishmania* DNA [33] also on months 3 and 8 after vaccination. All the animals included in this investigation were treated follow-