

Short communication

Safety trial using the Leishmune[®] vaccine against canine visceral leishmaniasis in Brazil

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Abstract

A group of 600 healthy and asymptomatic dogs from Brazilian canine visceral leishmaniasis endemic areas was vaccinated with three sc doses of Leishmune[®] which is the industrialized formulation of the FML-saponin, recently licensed for commercialization in Brazil, which previously showed 76–80% vaccine efficacy against canine visceral leishmaniasis. Safety evaluation was performed for 14 days after each vaccine injection and disclosed transient reactions of local pain (40.87%), anorexia (20.48%), apathy (24.17%), local swelling reactions (15.90%), vomit (2.4%) and diarrhoea (1.5%). All effects showed significantly correlating declines, from the first to the third dose ($p < 0.0001$). Most of the noticed reactions of pain (73%), anorexia (79%) and local swelling (84.7%) were mild. No significant differences between puppies and adults dogs were found in the number of adverse reactions. Adult dogs developed however, 94.5% of the small swelling reactions (<3 cm), and indicating that they are more resistant to the inflammatory response promoted by the saponins. No dead by anaphylaxis occurred, and only two dogs (0.1%) showed allergic reactions (facial oedema and itching) after the third dose. Transient alopecia on injection site occurred in only five poodles (0.28%) with total recovery and no need of treatment. All the mild adverse events in response to Leishmune[®] injection were transient and disappeared before the injection of the following vaccine dose, confirming the tolerability of the vaccine. The Leishmune[®] preparation was less haemolytic ($HD_{50} = 180 \mu\text{g/ml}$) than expected for a QS21 saponin-containing vaccine, indicating that its formulation with the FML antigen diminished the potential *in vitro* toxicity.

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1. Introduction

Human visceral leishmaniasis (VL) is a canid zoonoses leading to 500,000 new cases/year [1], against which no vaccine is yet available and chemotherapy is highly toxic. Dogs, foxes and wild canids are the reservoir for the visceral leishmaniasis agents, *Leishmania chagasi*/*Leishmania infantum*,

in Europe, Asia, North Africa and South America. Domestic sand flies acquire the parasite by feeding on dogs and transmit it to humans (reviewed in [2]). Three prophylactic strategies, recommended by the WHO, are used in areas where the disease is endemic: (1) systematic treatment of human cases, (2) elimination of seropositive infected dogs and (3) residual insecticide treatment within domestic and peridomestic human and dog habitations [2]. Brazil is the only country with endemic VL that has conducted systematic epidemiological control programs since 1980 [3]. These programs, however, are very expensive and labour intensive. Their efficiencies and feasibilities have therefore been reviewed. Mathematical models and experimental results point out the use of a

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prophylactic human or dog vaccine as potential effective tools for control [4–6]. The development of a preventive dog vaccine would also reduce culling of infected dogs for sacrifice, being a more acceptable and humane method for the prevention of the disease [4]. A few Phase IIa studies dealt with experimental second and third generation-vaccines against canine visceral leishmaniasis [7–9]. Regarding the field Phase III assays, the failure [10,11] or low protection [12] achieved by first generation vaccines, was also reported.

In previous work, we reported the induction of 92–95% of prophylactic protection (76–80% of vaccine efficacy) in dog vaccination against canine visceral leishmaniasis with the second generation FML-saponin vaccine [4,5]. The vaccine was also immunotherapeutic for dogs, when formulated with a higher saponin concentration [13]. The FML-vaccine is formulated with Riedel de Haen saponin [5,14–16] which contains as active adjuvant compounds, the QS21 aldehyde-containing saponin (18.0%), and a mixture of two deacylated aldehyde containing saponins from *Quillaja saponaria* Molina (19.4%) [17,18]. The QS21 and the deacylated saponins both showed the typical aldehyde group in C-24, considered responsible for the interaction with the T helper lymphocyte surfaces and the induction of TH1 response [19]. On the other hand, while QS21 showed the normonoterpene moiety acylated in the triterpene C-28, the deacylsaponins lack the normonoterpene moiety which is related to the induction of CTL response and secondary toxicity [19].

Saponins of *Q. saponaria* Molina have been widely used since they behave as the best adjuvants in human and veterinary experimental vaccination [20–24]. Also, they are the adjuvant of several industrialized and licensed veterinary vaccines [25–28]. One of the main and rare properties of these saponins is the simultaneous induction of a CTL, TH1 lymphocyte and humoral immune response (reviewed in [19]) against the antigen, making them effective candidates for adjuvants of vaccines against Virus [22–27], cancer [21,29,30] and intracellular parasite infections [5,6,28,31].

Haemolysis by direct contact was described for saponins, being stronger in the steroidal than in triterpenoid ones [32,33]. *Q. saponaria* saponins, in spite of their triterpenoid structure are also haemolytic [34,35]. The strong haemolytic *in vitro* potential attributed to QS21 however [34,35], was never related to a proved decrease in animal haematocrit in *in vivo* assays. Local pain reactions and mild *in vivo* toxicity undesirable effects have also been reported to occur after injection of *Quillaja saponaria* saponins vaccines [34–37]. The low proportions of QS21 and deacylated saponins of *Q. saponaria* (18–19.4%), found in the Riedel de Haen saponin used as adjuvant of the FML-saponin vaccine [18], explain its described low toxicity in mice [35]. The recent launch of the industrialized licensed FML-vaccine formulation Leishmune® demanded a safety test in a large sample of target animals. In the present work we summarize the safety analysis of the Leishmune® vaccine performed in 600 dogs of canine visceral leishmaniasis endemic areas in Brazil.

2. Material and methods

2.1. Animals and study design

Six hundred dogs from the canine visceral leishmaniasis endemic (Araçatuba, Andradina, Valparaíso, Guararapes, Bauru, Belo Horizonte, Nova Lima, Sete Lagoas) and at risk areas (Viçosa, Marília, São José do Rio Preto) of the state of São Paulo and Minas Gerais, Brazil, were vaccinated with three doses of Leishmune®, in a 21 days interval, through the subcutaneous (sc) route. The first dose was injected on the flank, the second over the ribs and the third on the neck. Veterinarians and dog-owners recorded the potential vaccine adverse events: local pain, nodules, anorexia, apathy, vomit, diarrhoea, itching, dyspnoea, coughing, anaphylaxis and facial and forelimbs oedema. Pain reactions detected after touch, begun 8 h after injection. We discriminated them as: 0 for no pain; 1 for mild pain, observed when the dog stares at the injection site or a skin contraction occurs after local touch and 2 for severe pain, observed when the dog whines or tries to bite the hand touching the injection site. Also, three levels of anorexia were discriminated 8 h after injection: 0 for no reaction, 1 when the dog eats less than usual and 2 when the dog rejects the food. Local swelling at the injection site was detected by palpation. Its size variation (diameter in cm) was measured daily during 14 days after each injection. Swelling reactions were classified according their diameter scores in cm as: 0 for no swelling; 1 for 0.1–3.0 cm and 2 for more than 3.1 cm.

Each one of the 30 veterinarians participating of this assay vaccinated 20 dogs with three doses Leishmune®, accomplishing 1800 doses. The animals were observed for 40 min at each Veterinary Clinic after each vaccine dose injection (days 0, 21 and 42), for eventual anaphylaxis, and the owners observed their dogs daily during the following 14 days, for any adverse event and for its potential regression. It was possible hence, to obtain 27,000 independent observations, 25,200 of which were performed by the dog owners and 1800 by the veterinarians. Only dogs in good physical conditions were included in this study and were maintained at their dwelling with regular nutrition and sanitary care conditions during all the trial duration (56 days). For ethical reasons, veterinarians were not able to keep an untreated and exposed control dog population.

The cohort used in this study was composed of 511 animals (85%) from 61 different breeds and of 89 (15%) mongrel dogs. Animals' ages ranged from 4 months to 13 years. We considered puppies all participating dogs with ages ranging from 4 to 9 months. All animals completed previous vaccination program against distemper, parvovirus, Parainfluenza virus, leptospirosis, coronavirus, type 2 adenovirus and rabies. The 600 dogs were routinely monitored by veterinarians. At the beginning of the assay, all animals were healthy and seronegative, as disclosed by previous screening for anti-*Leishmania* antibodies with ELISA or immunofluorescence (RIFI) (reviewed in [38]). The FMLELISA assay [39] per-

formed on sera of the animals collected at day 0, disclosed however values above the cut-off in 50/600 dogs. All animals were monitored for safety analysis. In this investigation, all the manipulations performed on the animals were done, keeping the animal suffering the minimal as possible.

For determination of the haemolytic potential of the Leishmune[®] vaccine normal human red blood cell suspension (0.6 ml of 0.5%) was mixed with 0.6 ml diluent containing 20, 40, 80, 120, 160, 200, 400, 1000 or 2000 µg/ml of Leishmune[®] which represent, respectively, 5, 10, 20, 30, 40, 50, 100, 250 and 500 µg/ml concentrations of the Riedel de Haen saponin. Mixtures were incubated for 30 min at 37 °C and centrifuged at 70 × g for 10 min. Free hemoglobin in the supernates was measured by absorbance at 412 nm [32]. Saline and distilled water was included as minimal and maximal haemolytic controls. The haemolytic percent developed by the saline control was subtracted from all groups. The adjuvant concentration inducing 50% of the maximum hemolysis was considered the HD₅₀ (graphical interpolation). Each vaccine concentration was determined using five replicates. Human red blood cells for the haemolytic assay, were obtained from healthy adult blood bank donors (Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, RJ, Brazil) [32]. The red blood cell suspension was washed by centrifugation at 1180 × g and finally diluted to 0.5% in saline solution.

2.2. Vaccine preparation

Each Leishmune[®] vaccine dose [28] was composed of lyophilized FML antigen (1.5 mg) and saponin (0.5 mg) and was reconstituted in 1 ml NaCl 0.9% sterile saline solution and administered subcutaneously. The FML-vaccine,

Leishmune[®], is registered as a Patent: INPI number: PI1100173-9 (18.3.97). Federal University of Rio de Janeiro, Brazil.

2.3. Statistical analysis

Means were compared by ANOVA analysis, simple factorial test and by one-way ANOVA, Tukey's honestly significant difference method (SPSS for windows). Correlation coefficient analysis was determined on a Pearson bivariate, two-tailed test of significance (SPSS for windows).

3. Results

The incidence of adverse effects in Leishmune[®] vaccinated dogs was quantified as the percent of reactive animals, for each effect, among the total 600 dogs. This analysis disclosed: 40.87% of dogs showing pain reactions, 20.48% showing anorexia, 24.17% with apathy, 15.90% with local swelling reactions, 2.4% with vomit and 1.5% with diarrhoea.

In Table 1, the percent of observed pain, anorexia, apathy and local swelling events, recorded among the total 25,200 observations, performed by the dog owners along the 14 days after each dose, is summarized. All pain, anorexia, apathy and local swelling reactions showed significantly correlating declines in positive percents, from the first to the third dose ($p < 0.0001$, Pearson correlation coefficient). Also, significant variations of the reaction levels were detected between different doses, for pain reactions (ANOVA, $p < 0.0001$) that were equally high after the first and second dose and only diminished after the third vaccine application ($p < 0.05$; Tukey honestly significant difference-SPSS) and for the

Table 1
Percent of observations of pain, anorexia, apathy and node reactions and their quantitative classification, after Leishmune[®] vaccination

Dogs	Dose	Adverse effect	Number of reactions/total observations	% of positive observations	Reaction levels ^a	
					1	2
480	First	Pain	798/8400	9.50	577	221
478	Second		738/8372	8.82	543	195
473	Third		484/8229	5.88	362	122
480	First	Anorexia	349/8400	4.15	284	65
478	Second		314/8372	3.75	245	69
473	Third		300/8229	3.65	235	65
480	First	Apathy	318/8400	3.79	–	–
478	Second		375/8372	4.48	–	–
473	Third		329/8229	4.00	–	–
480	First	Local swelling	596/8400	7.10	496	100
478	Second		452/8372	5.70	370	74
473	Third		299/8229	4.00	268	31

^a Quantification of pain, anorexia and injection site local swelling reactions: (1) for mild pain and anorexia and for node diameters ranging from 0.1 to 3.0 cm, respectively and (2) for severe pain and anorexia and for node diameters above 3.1 cm. We did not qualify different degrees of apathy.

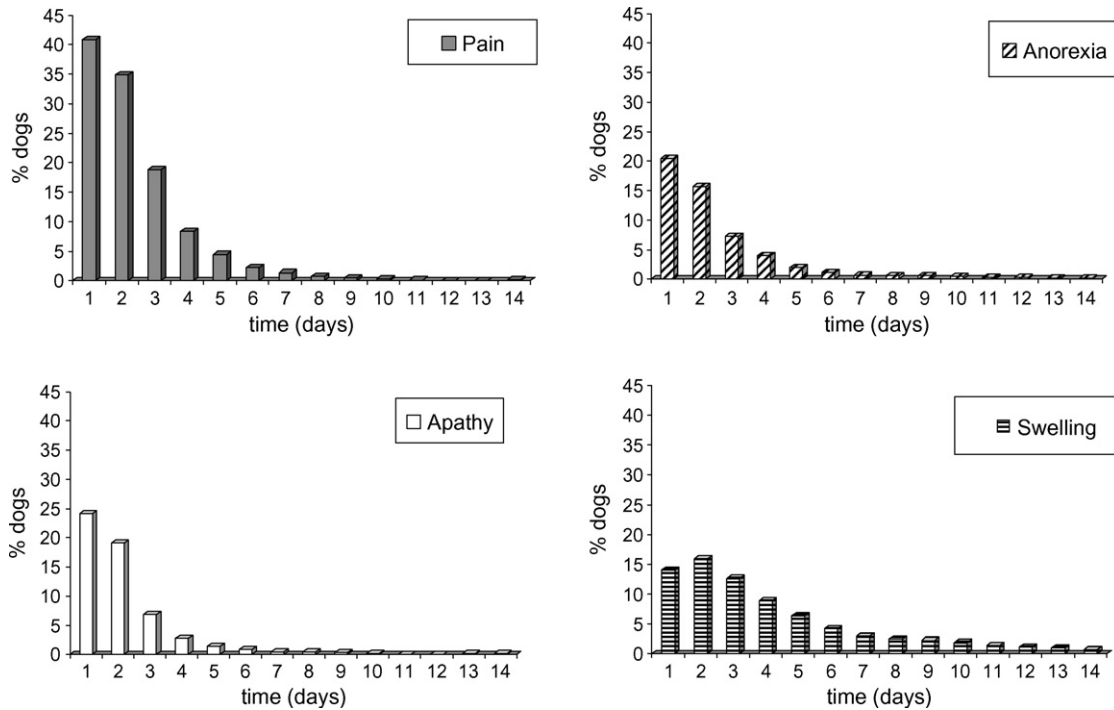


Fig. 1. Decrease in local pain, anorexia, apathy and local swelling along the time, after dog vaccination with Leishmune[®]. The number of reactive dogs among the 600 tested is represented as percent values and after each one of the three vaccine doses. Bars represent the average of these three values for each adverse effect: local pain, anorexia, apathy and local swelling. Dogs were monitored during the first 14 days after each vaccination dose.

diameters of the injection site swelling reactions (ANOVA, $p < 0.0001$), which showed significant declines from the first to the third dose ($p < 0.05$; Tukey honestly significant difference-SPSS) (Table 1).

Noteworthy, most of the noticed reactions of pain (73%), anorexia (79%) and local swelling (84.7%) were mild indicating that the vaccine was very tolerable. No significant differences between puppies and adults dogs were found in the number of adverse reactions of pain, anorexia, apathy and local swelling ($p > 0.05$). Adult dogs developed however 94.5% (1069/1134) of the small swelling reactions (<3 cm) while the puppies developed only 5.5% (65/1134) of them, indicating that adult dogs are more resistant to the inflammatory response promoted by the saponins. This was confirmed by the development of a higher number of >3 cm swelling reactions in puppies (22/2380) than in adults (191/21361). Both differences were significant ($p < 0.001$). No dead by anaphylaxis occurred and only two dogs (0.1%) showed allergic reactions (facial oedema and itching) after the third dose. Transient alopecia on injection site occurred in only five poodles (0.28%), 21–29 days after the vaccine injection and fur began to grow 10–21 days after, with total recovery and no need of treatment.

Besides the good tolerability to Leishmune[®] vaccine, all the mild adverse events described above were transient. Indeed, while the interval between the vaccine doses was 21 days, all the undesirable adverse effects decreased and disappeared before the end of this period (Fig. 1). The percent of reactive dogs with each adverse effect was scored, after

each one of the three vaccine doses. Bars in Fig. 1 represent the average of these three values. Each animal was monitored during the first 14 days after each vaccination dose. As previously described, the percent of dogs showing pain was the highest followed by those showing apathy, anorexia and local swelling. Hence, by day 3 after injection, the incidence of pain, anorexia and apathy was reduced to 53.9, 64.8 and 71.8%, respectively. The local swelling incidence, on the other hand, increased towards the day 2 and was reduced to 58.0% on day 5. One week after the injection only 1% of the dogs still showed pain, anorexia and apathy reactions while this value was achieved for the local swelling incidence only at day 11. Nevertheless, all adverse effects disappeared before the injection of the following vaccine dose, confirming their transitory condition.

Since the vaccine adjuvant is a *Q. saponaria* saponin mixture which already showed previously to be haemolytic in *in vitro* experiments, the haemolytic potential of Leishmune[®] was determined using a 0.5% human red blood cell suspension. A $HD_{50} = 180 \mu\text{g/ml}$ was disclosed for the Leishmune[®] vaccine formulation corresponding to a $45 \mu\text{g/ml}$ of the Riedel de Haen saponin adjuvant contained in the vaccine.

4. Discussion

Each dose of the Leishmune[®] vaccine is composed by 1.5 mg of FML and 0.5 mg of the Riedel de Haen saponin mixture which is a *Q. saponaria* Molina saponin extract con-

taining the QS21 saponin, which is related to toxicity [34,35], and two deacylated saponin deprived of the normonoterpene hydrophobic chain [18]. The QS21 represents 18%, and the deacylated saponin 19.4% of the Riedel de Haen mixture [18]. It means that for each dose of Leishmune[®] vaccine, the dog receives 90 µg of QS21 and 97 µg of the deacylated saponins. While the QS21 shows mild toxicity in mice (transient local swelling, loss of hair and skin lesion) [17,18,34,35], the deacylated saponins, in spite of their strong and specific adjuvant potential, are non-toxic and less haemolytic at all [18].

Slight variations of the haemolytic potential (HD₅₀) values were already reported for QS21: 7 ± 2 µg/ml [34], 13.3 µg/ml [40], 18 µg/ml [35] and 5.3 µg/ml [18] and attributed to the presence of side chains bearing aglycone (sugar chains) [32], acyl residues or the epoxy framework system (reviewed in [40]) or to the normonoterpene hydrophobic C-28 triterpen acylated chain [18]. Also related with the haemolysis, the reactions of local loss of hair and swelling after vaccine injection, detected in animals vaccinated with FML and the Riedel de Haen's saponin were enhanced in animals that received the purified QS21 fraction (HD₅₀ = 5.3 µg/ml) but abolished in animals treated with the purified deacylated saponins (HD₅₀ = 96 µg/ml) [18], which lack the hydrophobic chain that favors interactions between the saponin and membrane cholesterol promoting the haemolysis and toxicity.

The Riedel de Haen's saponin mixture, composed of QS21 and deacylated saponins of *Q. saponaria*, saccharose, glucose, rutine and quercetin, showed a lower HD₅₀ = 23.3–25 µg/ml [35,18] while the Leishmune[®] vaccine (this investigation) was even less haemolytic (HD₅₀ = 180 µg/ml). The increase in the saponin concentration needed to cause 50% haemolysis, from 25 to 45 µg/ml, is probably due to the interactions of saponins with the FML antigen forming micelles that block the direct lytic effect of the saponin on the red cells membrane.

Alternatively, other *Q. saponaria* adjuvant systems reduced their toxicity [41]. ISCOMs are nanoparticles (40 nm) used as delivery systems for vaccine antigens, made up of *Quillaja saponins*, lipids and antigen, usually held together by the hydrophobic interactions of the three components [41]. If no antigen is added in the mixture, ISCOM-MATRIX is formed. The *Quillaja* glycosides exhibit a unique affinity to cholesterol, stabilizing the complex [41]. Cholesterol is the ligand that binds to saponin forming 12 nm rings that are glued together by lipids (phosphatidylcholine) to form the spherical ISCOM-MATRIX or ISCOM. Thereby, the interactions of saponins with the cell membrane are blocked at the cholesterol level, and the lytic effect is virtually lost [41]. As expected for other micelles, in ISCOMs, the hydrophobic normonoterpene branch of QuilA saponins therefore, is probably oriented to the inner side of the vesicle, interacting with the other lipids. Confirming this idea, the inclusion of QuilA derivatives, increased the ISCOMs immunogenicity [41] and reduced the lytic properties of the saponins, attributed to the normonoterpene branch, which is

no more exposed [19]. ISCOMs containing QuilA saponins stimulate both the presentation of antigens by MHC classe II and class I receptors, inducing the increase of the CD4 helper T cells and the CD8 cytotoxic lymphocytes proportions, respectively, due to their capacity of antigen deposit both, in endosomal vesicles and/or directly into the cytosol [41].

The previous screening for adjuvants to the FML-vaccine identified the QS21 saponin isolated from the QuilA Superfos mixture and the Riedel de Haen saponin as the best candidates [35]. In this investigation using the Riedel de Haen mixture containing 90 µg of QS21 and 97 µg deacylated saponins we described mild undesirable effects in dogs: transient reactions of local pain (40.87%), anorexia (20.48%), apathy (24.17%), local swelling at the injection site (15.90%), vomit (2.45%) and soft stools (1.50%). Most of the noticed reactions of pain, anorexia and local swelling were mild, indicating that the vaccine was very tolerable and all the effects were spontaneously reverted before the next vaccine injection and with no need of symptomatic treatment. Schettters et al. [42] developed a dog vaccine using also saponins from *Q. saponaria* Molina and soluble antigens of *Babesia canis*. In his formulation the QuilA saponin was added at 1 mg/dose concentration [42] and tested in 15 dogs. The amount of QS21 in the QuilA saponin is closed to 40% [35] meaning that each *Babesia* vaccine contains at least 400 µg of QS21, which is 4.4 times the concentration of saponin included in Leishmune[®], and consequently is expected to develop a 2.4–6.2 times higher incidence of adverse effects. Indeed, this *Babesia* vaccine lead to 100% of local pain, 20% of anorexia, 33% of listless and 93% of swelling [42]. Aiming to minimize the adverse reactions at the injection site, the same authors improved their vaccine by reducing to 25% the amount of adjuvant [43]. Using only 250 µg of QuilA and monitoring the reaction for a period of 3 days only after each injection, a little local reaction after each one of the two doses was reported. No biologically significant effect on the body temperature was detected, since it deviated less than 1 °C after the vaccine injection and was restored to normal the next day [43]. Ma et al. [44] on the other hand, using less QS21 (50 µg/dose) and recombinant proteins of *Borrelia Burgdorferi*, vaccinated dogs against Lyme disease and described only slight swelling (<1 cm) at injection sites, in 80% of the animals (8/10 dogs). A clear dose-effect is then disclosed between the results obtained with the *B. canis* (400 µg) [42], the Leishmune[®] (90 µg) (this investigation) and the Lyme disease vaccines (50 µg) [44], particularly related to the QS21 adjuvant concentration.

A second finding of the present work is that the diameters of the injection site swelling reactions and the number of dogs showing local swelling significantly decreased from the first to the third dose. This might be related to the use of different sites for vaccine injection that show decreasing sensitivity for pain: flank for the first, ribs for the second and neck for the third vaccine dose. The aim of using different sites was to enable veterinarians to easily measure possible node formation at the site of injection after each dose, not masking one reaction with the subsequent. If this hypothesis

is correct, the decrease of anorexia, apathy and local swelling is a consequence of the decrease in pain. However, Ma et al. [44] reported swelling in 8/10 dogs after the first dose and in only 3/10 animals after the second and third dose, with no mention to the use of different injection sites. Also supporting our results, Ma et al. [44] described that the swelling usually occurred within the first 48 h post-vaccination and resolved completely within a week.

Different from our work that disclosed soft stools in 1.50% in dogs, Ma et al. [44] using half of the QS21 saponin found 33% (2/6) of the dogs experiencing light transient diarrhoea.

In spite of the previously described mild toxicity, the use of QS21 as adjuvant for human clinical trials has increased recently [19–21] and due to its tolerance and outstanding protective effect the accepted dose for human's vaccination also increased from 50 µg/dose [36] to 100 µg/dose [21].

The main contribution of this investigation was to screen the possible adverse effects of the first licensed vaccine against canine visceral leishmaniasis which is, in Brazil and Europe, a very severe zoonoses. Our results disclosed that Leishmune[®] vaccine is very tolerable and well accepted by dog owners and veterinarians for its large use in control of canine visceral leishmaniasis. The adverse effects noticed in this investigation for Leishmune[®] are compatible with those provoked by other veterinary vaccines such as rabies-vaccine which shows local pain, non-reversible alopecia, soreness, lameness, regional lymphadenopathy, fever, systemic signs, focal cutaneous vasculitis and anaphylaxis [45–47]. Phase I safety trials are supposed to be done on groups of dozen individuals initially from a non-endemic area [48]. To our knowledge, this is the first study dealing with safety and toxicity analysis of a licensed veterinary-vaccine in a very large cohort (600 dogs) of target individuals, at endemic areas. The large use of the prophylactic Leishmune[®] vaccine is of particular value in Brazil, where culling and euthanasia of *Leishmania*-seropositive infected dogs is regularly performed but is not enough effective to guarantee the visceral leishmaniasis epidemiological control.

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