Original Article

Immunotherapy Using Autoclaved *L. major* Antigens and *M. vaccae* with Meglumine Antimoniate, for the Treatment of Experimental Canine Visceral Leishmaniasis

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ABSTRACT

Background: To evaluate immunotherapy against canine visceral leishmaniasis, *Leishmania major* antigen and heat-killed *Mycobacterium vaccae* (SRL172) were used as stimulators of immune defense mechanisms and the results were compared with standard chemotherapy meglumine antimoniate.

Methods: Nineteen mongrel dogs aging 1-3 years old were used in this experiment. Infection was carried out in 15 out of 19 dogs using *L. infantum*, isolated from a naturally infected poly-symptomatic dog.

Results: All the cases showed positive serologic results by direct agglutination test during 30-60 days following inoculation. In the first group, which was under chemotherapy (Glucantime[R]), one of the members showed recurrence of the disease despite rapid effect of the therapeutic protocol. Immunotherapy using SRL172 caused complete cleaning of the parasite in group 2, but the speed was less than Glucantime. Immunotherapy using *L. major* antigen combined with *M. vaccae* in group 3 and combine administration of immunotherapy and chemotherapy in group 4 both were with relapsing of one case in each group. Group 5 and 6 were consisted of positive and negative control dogs, respectively.

Conclusion: Immunotherapy seems to be an adjuvant in treatment of canine leishmaniasis but it needs more investigation for final confirmation.

Keywords: Canine visceral leishmaniasis, Immunotherapy, Glucantime, *Mycobacterium vaccae*

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Introduction

Visceral leishmaniasis (VL), one of the most important chronic and systemic zoonosis, caused by a biphasic protozoan belongs to genus *Leishmania* (1). Due to considerable difficulties in eradication of the disease, visceral leishmaniasis is one of the most important parasitic diseases, which are endemic in different parts of Iran. The canidae family especially domestic dogs are the most important sources of *L. infantum* infection for human. There is a large dog population in Iran (7 dogs/100 humans in Meshkinshahr) and rate of infection may reach to 20% in some villages (2). Canine leishmaniasis in dogs is more resistant to therapy than human leishmaniasis, and only rarely *Leishmania* organisms are eliminated with available drugs. Killing the symptomatic and seropositive dogs is obviously unacceptable to the owners, and it is ineffective because nonsymptomatic dogs and occasionally seronegative dogs are sources of parasite transmission (1). Side effects have been reported following treatment with conventional chemical compounds (pentavalent antimony) and new substitutions such as allopurinol, aminosidine and antifungal drugs (3, 4). Although recurrence of the disease has also been frequent resulting in seeking new treatment protocols, immunotherapy as an efficient and alternative way can help in solving the problem. Gamma interferon, interleukin 12 and liposomal forms of some drugs are new treatment protocols with significant effect on treatment of visceral leishmaniasis (5, 6).

The aim of this interventional study was to determine the effectiveness of immunotherapy in experimental canine visceral leishmaniasis (CVL) in comparison with meglumine antimoniate.

Materials and Methods

Animals
Nineteen mongrel dogs aged 1-3 years were used in this experiment. The dogs were housed in individual cages in small animal hospital, Veterinary Faculty of Tehran University. All dogs appeared healthy, as determined by results of physical examination and normal hemogram. All of them were treated against internal and external parasites. In addition, the dogs were confirmed to be free of *Leishmania* infection using parasitological and serological tests.

Parasite
A symptomatic infected dog, positive by both direct agglutination test (DAT) and ELISA against *Leishmania* infection, was used as the donor source of the inoculum. During the necropsy, visceral leishmaniasis confirmed by observation of hepatosplenomegaly as well as enlargement of the abdominal lymph nodes, and presence of the parasite confirmed by examination of impression smears prepared from lymphoproliferative organs. Using RAPD-PCR technique the isolated parasite was identified as *L. infantum* (7).

Experimental design
Spleen from donor dog was minced to small cubes, macerated in Hank's balanced salt solution in a grinder, and dilution were made to provide the desired number of organisms. Fifteen out of 19 dogs were injected intravenously with 5 ml of the spleen homogenate containing at least 3×10^5 amastigotes/ml. Infection of the dogs was assessed by collecting bone marrow aspirates, ELISA and DAT. These infected dogs were randomly allocated into five treatment groups

of three dogs each. Group 1 dogs (Chemotherapy) were received glucantime (Rhone-poulenc Rorer Ltd., France) 100 mg/kg/day, IM for 30 consecutive days. Group 2 dogs (Immunotherapy) were administered intradermaly 0.1 ml suspension of heat-killed *M. vaccae* (SRL172), 3 times in a month intervals containing 10 mg/ml bacterial protein extract. Group 3 dogs (Dual Immunotherapy) were injected intradermaly with 0.1 ml autoclaved *L. major* antigen (ALm was prepared from the promastigotes of *L. major* (MRHO/IR/76/ER, vaccine, Razi Vaccine & Serum Research Institute, Iran), 3 times monthly in combination with SRL172 as the same dose in group 2. Each ml of autoclaved *L. major* antigen contained 3.6 mg parasite protein. Group 4 dogs (Immunotherapy & Chemotherapy) were injected with glucantime plus *L. major* antigen and SRL172 as the same doses in groups 1 and 3. Group 5 dogs (Positive control) were received sterile normal saline in a same volume as glucantime, IM, for 30 consecutive days. One more group comprising four dogs were served as negative control for evaluation of environmental factors and their treatment were performed as group 5.

The animals were monitored for parasite establishment and subsequently for the development of leishmaniasis. This was achieved by routine screening of the dog for classical clinical signs, hematological and serological examinations for ten months after inoculation. Treatments were started two months after infection reconfirmed by serological and parasitological examinations. The animals were clinically examined regularly each day to follow up the potential side effects as well as signs of infection during the study. Blood samples were taken before inoculation and at monthly intervals up to four months after cessation of treatment. The presence of specific antibodies against *Leishmania spp.* was evaluated monthly by DAT, using the cut off value of 1:320 and above (8). Doubtful results were confirmed by ELISA or Dipstick rK39 rapid tests (9). Four months after the end of treatment protocols, all of the animals were euthanized and samples of bone marrow puncture, spleen, and liver were prepared for parasitological examinations. The mentioned samples underwent Giemsa staining followed by microscopic examination by light microscope with high magnification ($\times$ 1000) for the presence of parasite.

Statistical analysis

Repeat measure one-way ANOVA test was performed for assess differences between hematological measurements and resistance or susceptibility to anti *Leishmania* therapy using SPSS software program. The influence of different treatment protocols on serological and parasitological examinations was evaluated using Fisher exact test. Data were considered significant at a *P* value < 0.05.

Results

The most common clinical signs in infected animals were skin lesions (13 cases), conjunctivitis (9 cases), and enlargement of popliteal (15 cases) and submandibular lymph nodes (15 cases). Enlargement of the popliteal lymph nodes also happened earlier in the course of the study (after 48-65 days) in comparison with submandibular and prescapular lymph nodes (after 53-75 days). In animals that received chemotherapy, the signs resolved gradually after 45 days, Whereas, this resolution took longer in groups which received immunotherapy with adjuvant (group 2) (70 days), killed parasite antigen with adjuvant (group 3) (85 days) and immunotherapy plus chemotherapy (group 4) (115 days). There were no significant changes in spleen and liver size against lymph nodes following treatment in different groups.

The most important and noticeable hematological changes were reduction in white and red blood cells (Table 2 and 3), PCV and hemoglobin. These factors returned to their normal values following treatment in groups 1 to 4. At the end of the experiment, all of the factors were in normal ranges for every subject except one dog in group 1 that was probably affected by the recurrence of clinical signs, and another one in group 4 that remained positive until the end of the experiment. All of the positive control animals also were shown reduction in hematological parameters until the end of study.

Based on the serological results, 30-60 days following inoculation, all of the animals had positive titers ($\geq$ 1:320). Performance of treatment protocols changed the titers to negative but with varying time courses: group 1 (1 month), 2 (2 months), 3 and 4 (3 months). One of the animal in group 1 encountered increase of the serum titer in period of follow up (4 months after cessation of treatment). In addition, one of the dogs in group 4 remained seropositive in all of the experiment. All of the positive control animals preserved their titers until the end of study and no serum titer was detected in negative control animals.

Results of parasitological examination on liver, spleen, and bone marrow samples for observation of Leishman bodies are depicted in Table 4. Out of the animals in therapeutic groups of 1, 3 and 4, two cases responded to treatment according to parasitological results. Whereas in animals treated only with SRL172, no parasite was detected. Based on Fisher exact test in spite of decrease in parasite population, there was no significant differences between groups 1, 3 and 4 with the control group.

Table 1: White blood cell count changes before and following anti-*Leishmania* therapy in different groups of dogs (Mean $\pm$ SE)

Stages Groups	WBC / μl			
	Before inoculation	After infection	After cessation of treatment	End of the study
Group I	11300 $\pm$ 529 [s]	9100 $\pm$ 230 [s]	11300 $\pm$ 378	10633 $\pm$ 384 [s]
Group II	10900 $\pm$ 435 [s]	8700 $\pm$ 288 [s]	10900 $\pm$ 450	10966 $\pm$ 371
Group III	10666 $\pm$ 554 [s]	7900 $\pm$ 321 [s]	10600 $\pm$ 458	10666 $\pm$ 566
Group IV	11100 $\pm$ 929 [s]	8600 $\pm$ 665 [s]	10700 $\pm$ 550	10700 $\pm$ 642 [s]
Group V	11466 $\pm$ 788	8700 $\pm$ 435	8466 $\pm$ 523	8300 $\pm$ 642
Group VI	10925 $\pm$ 436	11075 $\pm$ 526	11125 $\pm$ 520	11225 $\pm$ 558

[s] Significantly different (P<0.05)

Table 2: Red blood cell count changes before and following anti-*Leishmania* therapy in different groups of dogs (Mean ± SE)

Stages	RBC × 10^6/µl			
	Before inoculation	After infection	After cessation of treatment	End of the study
Groups				
Group I	7.0 ± 0.15 [s]	5.9 ± 0.21 [s]	6.9 ± 0.12	6.6 ± 0.46 [s]
Group II	7.0 ± 0.17 [s]	5.9 ± 0.06 [s]	7.1 ± 0.10	7.1 ± 0.06
Group III	7.1 ± 0.17 [s]	5.7 ± 0.12 [s]	7.0 ± 0.12	7.0 ± 0.10
Group IV	7.3 ± 0.23 [s]	6.0 ± 0.12 [s]	7.1 ± 0.31	7.2 ± 0.25
Group V	7.3 ± 0.23	5.9 ± 0.21	5.9 ± 0.12	5.7 ± 0.12
Group VI	7.2 ± 0.14	7.2 ± 0.13	7.2 ± 0.09	7.3 ± 0.09

[s] Significantly different (*P*<0.05)

Table 3: Observation of Leishman bodies from tissues removed at necropsy at the end of study

Tissue Group	Spleen	Liver	Bone marrow
Group I	1/3[§]	1/3	1/3
Group II	0/3	0/3	0/3
Group III	1/3	0/3	1/3
Group IV	1/3	0/3	1/3
Group V	3/3	3/3	3/3
Group VI	0/4	0/4	0/4

§ Number of positive/number of tested.

Table 3: Serological results (DAT=>1; 320) of experimental leishmaniasis under treatment with chemical and immunological procedures

Stage	Before in-oculation	After inoculation (month)									
		Before Treatment				During treatment		After treatment			
Group		Before in-fection		After in-fection							
		1	2	3	4	1	2	1	2	3	4
Group I	0/3[§]	3/3	3/3	3/3	3/3	1/3	0/3	0/3	0/3	0/3	1/3
Group II	0/3	2/3	3/3	3/3	3/3	3/3	3/3	1/3	0/3	0/3	0/3
Group III	0/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3	0/3
Group IV	0/3	1/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	1/3	1/3
Group V	3/3	2/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
Group VI	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

§ Number of positive/number of tested

Discussion

In this study, experimental leishmaniasis by intravenous injection of spleen homogenous extract including amastigote form of the parasite was performed for the first time in Iran. Abranches et al. (10) used both forms of the parasite in experimental infection and indicated that amastigote form could induce a stronger humoral immunity and more obvious clinical signs in dogs. Up to now the promastigote form of the parasite, which was acquired from culture, has been used in experimental infection. Induction of experimental infection using this form of parasite is time consuming and rather costly because it must be cultured on enriched medium.

Results of chemotherapy showed that in spite of relatively earlier effect it was not complete. Although the animals became serologically negative (< 320) one month after treatment, infection recurred in one dog four months later, which was confirmed by parasitology. Bergeaud (11) and Melo et al. (12) believed that glucantime have a complete effect on recovery of dogs either in natural or experimental cases. They did not

mention their follow up duration. Possibly, in shorter period, the treatment was considered complete. However, in respect to the persistence of parasite in various tissues of affected animals, parasitological examination is highly crucial. Likewise Neogy et al. (13) reported that 37.5% of dogs with visceral leishmaniasis responded parasitologically to chemotherapy.

M. vaccae had an efficient effect in treatment of canine visceral leishmaniasis. Efficacy of the SRL172 was confirmed previously in the treatment of tuberculosis, psoriasis, allergies, periodontal disorders, and some types of malignancies (14-16). It is said that this adjuvant could change immune pathways from antibody production (Th2) to cellular immunity (Th1) (17). In visceral leishmaniasis both types of immune responses develop and it is the balance of the Th1/Th2 responses that is considered important in controlling parasite replication, disease progression, or a cure. The immunoglobulin response is usually massive in clinical forms, however it not a protective response and can eventually be detrimental. Large amounts of

circulating immune complexes deposition in the walls of blood vessels may cause vasculitis, polyarthritis, uveitis, and glomerulonephritis.

There is still controversy about the effectiveness of immunotherapy against visceral leishmaniasis. Whereas some reports denote good effects (18-20), others point some unresponsiveness or even worsening of the signs following immunotherapy (21, 22). Incomplete and slower response in dogs treated with killed parasite antigen combined with adjuvant may be related to genetic resistance of the involved animals, state of the disease or type of used antigen. In this study, the effectiveness of *L. major* antigen was not evaluated, hence we lack a conclusive statement in this regards and it should be studied with more treatment groups.

The effects of adjuvant and killed parasite antigen combined with glucantime (group 4) were also slow and incomplete. Although there is some reports denote reduction in glucantime dose or its length of use following combination therapy (immunotherapy and chemotherapy) (23, 24) others report lack of complete response (25). In Neogy's study (13), which used double the dose of glucantime the efficacy was 100%. Failure of complete treatment in one of the animals in-group 4 may be due to the incomplete dose or duration of therapy, which certainly calls for more experiments.

In conclusion, immunotherapy in this study against visceral leishmaniasis was performed with the aim of controlling the disease in dogs as one of the most important reservoirs. Use of *M. vaccae* as an adjuvant in treatment of canine visceral leishmaniasis is the first pertinent experience and primary results are promising. Results of the groups 3 and 4 that were under treatment with killed parasite antigen combined with adjuvant showed that this type of therapy can reduce the number of parasites in experimental form of the disease, but its favorable effects should also be judged in the natural form of the disease.

Acknowledgments

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