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Original Article

Assessment of the Effects of a Novel Herbal Immunomodulator Drug (IMOD) on Cytokine Profiles in Experimental Canine Visceral Leishmaniasis: a Preliminary Survey

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Abstract

Background: Cytokines play a fundamental role in the regulation of immune responses in remission and/or relapsing of leishmaniasis. Therefore, immunotherapy for the treatment of canine visceral leishmaniasis (CVL) has represented a principle approach in control of the infection. The present research aimed to evaluating the immunotherapeutic potential of a novel herbal immunomodulator drug (IMOD) on CVL.

Methods: Twelve mongrel dogs were intravenously infected with Iranian strain of *L. infantum* and randomly divided into three groups; 1: negative control (non-infected), 2: immunotherapy with IMOD and 3: positive control (non-treated). Cell proliferation and Th1-/Th2-type cytokines were measured in peripheral blood mononuclear cell (PBMC) by cell proliferation kit I (MTT) and enzyme-linked immunospot (ELISpot) assays, respectively.

Results: At the 60 days follow-up assessment, no adverse effects were observed in treated interventional group. Cellular proliferation assay indicated that PBMCs of IMOD group had higher stimulation index (SI) than positive control group ($p < 0.05$). Enhancement of CD4+ T cells such as IL-2, IL-4 & IL-10 were detected in negative control group due to *in vitro* IMOD stimulation 30 days post-treatment. In accordance to decreasing trends of Th1 & Th2 cytokines in positive control group, the mean number of IFN- γ , IL-2, IL-4 and IL-10 spot forming cells (SFCs) down regulated for IMOD group during the study.

Conclusion: These data indicate that IMOD had immunomodulatory potential but is not sufficient for total parasitic cure due to balance of Th1/Th2 cytokines. This is a preliminary study and we propose to undertake a series of experiments to evaluate the CVL due to *in vitro* modulatory effects of IMOD.

Introduction

Visceral leishmaniasis (VL) is a fatal protozoan disease caused by *Leishmania donovani* complex in the Old and New Worlds. Domestic dogs (*Canis familiaris*) are the main reservoir hosts for zoonotic VL in Mediterranean region (1, 2). Hence, the most important approaches for interrupting domestic cycle of the disease are effective prevention and treatment protocols in endemic areas (3, 4).

Over 50 years, pentavalent antimonials were remained the mainstay compounds used to treat both human and canine VL (5). Poor results, relapse following treatment, toxicity and resistance against some of the most conventional treatments, such as the pentavalent antimonials, aminosidine and allopurinol provide great effort for identifying new agents for treatment of human and canine leishmaniasis (6-8).

The host immune response plays a very decisive role in the efficacy of drugs used against leishmaniasis (9, 10). Therefore, immunotherapy for treatment of CVL has represented a new approach to control this infection (10, 11).

Data on the mechanisms of immune response to CVL is lesser than experimental data on murine models and/or on human leishmaniasis (12). Recent studies of cell mediated immunity in CVL, suggest that resistance/susceptibility to the infection depends on the presence or absence of a specific proliferative response to *Leishmania* antigen (10).

Despite the scientific basis for biological activity of most herbal medicinal products is obscure, traditional medicine has been used for thousands of years with great contributions made by practitioners to human health in many countries (13, 14). Extracts of several plants have shown impressive spectrum of biological activities such as immunomodulatory and antiprotozoal effects (13, 15,

16). These potential activities have been attributed to compounds belonging to diverse chemical groups including alkaloids, flavonoids, steroids and terpenoids. "Herbal extracts have long been considered as suitable candidates for novel interventions due to their safety, low cost, availability and their potential functional activity as immunomodulators" (13, 15, 16, 17).

In this regard, IMOD is herbal mixture of *Rosa canina* (Rosaceae), *Urtica dioica* (Urticaceae), and *Tanacetum vulgare* (Asteraceae) comprising selenium. The mechanism of action of IMOD and its immune cell target are being investigated (17, 18). IMOD has been patented in USA and Europe for its anti-oxidative stress potential, improving T helper lymphocytes and reduction of tumor necrosis factor alpha. The herbs used in this complex have strong anti-oxidative potential that is useful in many oxidant-related diseases (19).

The safety of IMOD has already been approved by *in vitro* and *in vivo* studies (20, 21).

Due to increasing understanding of immunological mechanisms involved in *Leishmania* infection control, studies on anti-*Leishmania* agents have advanced in recent years (22). On the other hand, more awareness in CVL will help open new concepts for treating infected dogs and develop new medical protocol for human leishmaniasis (22, 23). Therefore in this preliminary investigation, we evaluated the potential activity of IMOD against experimental model of CVL.

Materials and Methods

Animals and Study design

In 2011, twelve mongrel dogs (males and females) aging from 1 to 3 yr old were used in this study. Dogs were kept in individual cages in small animal hospital, Faculty of Veterinary Medicine, University of Tehran. After one month adaption period, all dogs were exam-

ined, vaccinated against common diseases (DH2PPil and Rabvac-1, Fort Dodge Animal Health, USA) and treated with anti helminthic drug. All dogs were analyzed to be free of anti-*Leishmania* antibody by Direct Agglutination test (DAT) (24).

For inducing experimental infection, we injected 3×10^7 amastigotes of *L. infantum* (29) obtained from a naturally infected dog's spleen to 8/12 dogs by i.v. route as described previous studies (25).

Three months after establishment of the infection, these dogs were randomly divided into three groups with four animals each. Group 1: negative control which did not receive any injection and/or medicine; Group 2: treatment group received both amastigotes and IMOD infusion 2 mg/kg with 100 ml DW 5% over 1 h, every other day for one month (according to manufacturer recommendation) and Group 3: positive control received both amastigotes and phosphate-buffered saline (PBS) intramuscularly with no drugs.

Animals were monitored regularly after inoculation. For confirmation of parasite establishment and subsequent *Leishmania* infection, bone marrow aspiration followed by light microscopic observation was performed.

Treatment protocol was started after confirmation of *Leishmania* infection. Clinical signs associated with leishmaniasis, complete blood cell count and biochemistry profile were evaluated and monitored for all dogs at monthly interval up to 60 days after the end of treatment. Plasma and PBMC were also isolated from each dog to determine antibody, cell proliferation and cytokine responses.

At the end of experiment, all animals were anesthetized and spleen biopsy was carried out for parasitological evaluation (6, 23, 26).

Cell proliferation assay

Briefly, PBMCs were obtained from heparinized blood samples collected from jugular vein of all dogs and then separated using ficoll-hypaque gradient (Histopaque, Sigma, USA). The cells were washed three times with

PBS and after overnight storage at $-80\text{ }^{\circ}\text{C}$, frozen cell samples were transferred to liquid nitrogen for long-term storage. At the end of study, thawed cells were washed twice and resuspended in RPMI 1640 (Sigma, USA) containing fetal calf serum (FCS) 20%. Cells viability was determined by trypan blue dye exclusion that was more than 95%. In this stage, we used cell proliferation kit I (MTT), a colorimetric method for non-radioactive quantification of cell proliferation (Roche Diagnostic GmbH, Roche applied Science, Germany). PBMCs cultured at a density 5×10^5 cells in 200 μl R-10 medium (RPMI-1640 containing 10% heat-inactivated FCS, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin), in the presence or absence of IMOD (based on manufacturer recommendation) and phytohemagglutinin A (PHA; 20 $\mu\text{g}/\text{ml}$) as stimulator, in 96 wells flat-bottom cell culture specialized microplates (Nunc, Denmark) for 4 days at 37°C and 5% CO_2 .

Based on manufacturer's instructions, after the incubation period, 20 μl MTT labeling reagent (with a final concentration of 0.5 mg/ml) was added to each well and incubated for 4 h at $37\text{ }^{\circ}\text{C}$ and 5% CO_2 . Then 100 μl of the solubilization solution was added to each well and incubated overnight at 37°C and 5% CO_2 . Finally, the absorbance of the formazan product was measured at 550 nm by ELISA reader (Awareness Tech. Inc., USA) while the reference wavelength was 690 nm. Proliferative responses were expressed as stimulation index (SI), which represents the ratio between mean of the count per minute (cpm) obtained for stimulated cultures and cpm of unstimulated cultures.

T Cells response by ELISpot assay

ELISpot kits (R&D Systems, Minneapolis, USA) were used in this study for the detection of canine IFN- γ (EL781), IL-2 (EL1815), IL-4 (EL754) and IL-10 (EL735) from PBMCs of all dogs. The assays were optimized according to R&D System's guidelines and performed as

recommended by the manufacturer. In brief, plates were saturated with 200 μ l of RPMI-1640 and incubated for 20 min at room temperature. Culture media was aspirated and 5×10^4 PBMCs per 100 μ l R-10 medium were added to triplicate wells. Cells were stimulated as described before for 48 h at 37 °C in the presence of 5% CO₂. The ideal time and concentration for IMOD were established based a dose response curve in preliminary experiments (data not shown). Recombinant canine IFN- γ , IL-2, IL-4 and IL-10 were used in triplicate wells as positive control whereas unstimulated cells and R-10 medium without cell were used as negative and background controls, respectively. Following incubation, the cells were removed and the wells were washed and incubated with 100 μ l diluted detection antibody overnight followed by the addition of 100 μ l diluted streptavidin-AP into each well and incubated in room temperature for 2 h. Unbound enzyme was removed with 3 successive washes and 100 μ l of BCIP/NBT chromogen solution was added to each well. The color reaction was then stopped by washing with distilled water. Subsequently, the plates were air dried and spots were manually counted using a dissection microscope. Responses were considered to be positive if number of SFC were greater than double the number in negative control wells. The results were reported as the number of spots per 10⁶ cells per well. Background spots were subtracted from each well.

Ethical approval

This randomized, open labeled trial was reviewed and approved by the Ethical Committee of University of Tehran and conducted according to the Principles of Laboratory Animal Care.

Statistical analysis

All data were analyzed with *t-test* and Repeated measure one-way ANOVA. The influence of treatment protocol on clinical signs was evaluated using McNemar and Fisher's

exact tests. Differences between groups were considered significant for $P < 0.05$. All the analyses were performed by SPSS ver.17 for windows (Chicago, IL, USA).

Results

Physical and Clinicopathological examinations

Based on staging of CVL by Leish-Vet group (4), all infected dogs in this experiment were categorized in stages II (moderate disease) and III (severe disease). The most detected clinical signs were weight loss (7/8 or 87.5%) and lymphadenopathy (6/8 or 75%). At the end of study, clinical signs remission was not detected in IMOD group.

In post-inoculation period, normocytic/normochromic anemia was the significant hematological changes in all infected animals. Thirty days after treatment, hematologic changes such as neutropenia showed significant difference ($P=0.019$) between group II and group III. These differences were not significant in mentioned groups (II&III) 60 days after treatment.

Based on biochemical analyses, significant elevation of triglyceride were detected after inoculation. In IMOD group, significant differences were noticeable for cholesterol & LDL ($P=0.01, 0.02$ respectively) 30 days after treatment but these findings did not remain until the end of study.

Serology

Serological results by direct agglutination test (DAT) showed that all infected dogs had positive titers ($\geq 1:320$) 60 days following inoculation. In this experiment 60 days after treatment, 2/4 (50%) dogs in IMOD group showed reduction of anti-*Leishmania* antibody titers but seronegative results ($< 1:320$) were not obtained.

Parasitology

The spleen biopsies remained positive in all treated dogs at the end of this experiment.

Cell proliferation

Proliferation of PBMC stimulated with IMOD and PHA was used to investigate T cells proliferation of all dogs enrolled in the study. At the end of study (day 60), PBMC from dogs treated with IMOD had better re-

sponse to stimulation with this herbal agent than other groups (Fig. 1). In this assay, animals that received IMOD presented higher SI than that observed in the positive control group ($P=0.022$).

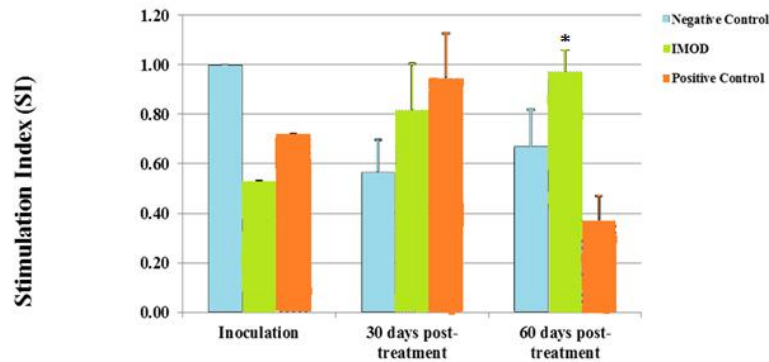


Fig. 1: Levels of lymphoproliferative response: 5×10^5 PBMCs from groups 1 to 3. The data are presented as mean \pm S.E. of four dogs per group. * P value: IMOD therapy *vs.* positive control group. * ($P = 0.022$)

ELISpot

Regarding ELISpot results for Th1- and Th2-type cytokines; unlike decreasing frequencies of IFN- γ SFC in control groups (groups I & III), the mean number of IFN- γ SFC was fairly constant (about $6000/10^6$ PBMC) for IMOD group until 30 days post-treatment and reduced at the end of study (day 60) (Fig. 2).

For IL-2, the mean number of SFC decreased during the experiment, having $24199/10^6$ PBMC at inoculation, about $10000/10^6$ PBMC 30 days post-treatment and about $7500/10^6$ PBMC at the end of study for positive control group and for IMOD group having $13148/10^6$ PBMC at inoculation, about $8318/10^6$ PBMC 30 days post-treatment and about $3022/10^6$ PBMC at 60 days post-treatment. In negative control group, the mean number of IL-2 SFC increased 30 days post-treatment due to *in vitro* IMOD stimulation and then decreased 60 days post-treatment (Fig. 2).

In positive control group, production of IL-4 SFC decreased over time. Like negative control group, the peak of IL-4 SFC was detected

30 days post-treatment in IMOD group and reduced at the end of study (Fig. 3).

Frequencies of IL-10 SFC changed over time in positive control group. Thus the mean number of IL-10 SFC decreased from $29898/10^6$ PBMC at inoculation to $11000/10^6$ PBMC 30 days post-treatment and then increased to $17200/10^6$ PBMC at the end of study. Down regulation of IL-10 was detected in animals treated with IMOD. Like mean number of IL-2 and IL-4 SFCs, IL-10 spot density enhanced 30 days post-treatment in negative control group due to *in vitro* IMOD stimulation (Fig. 3).

Combining the results of IFN- γ & IL-4 SFCs for evaluating an approximation of Th1/Th2 ratio showed significant differences between IMOD group and control groups at inoculation period by *in vitro* stimulation ($P=0.003, 0.009$). In post-treatment period, Th1/Th2 ratio changed during the evaluation and showed increasing trend for IMOD and negative control groups but decreasing trend for positive control group (Table 1).

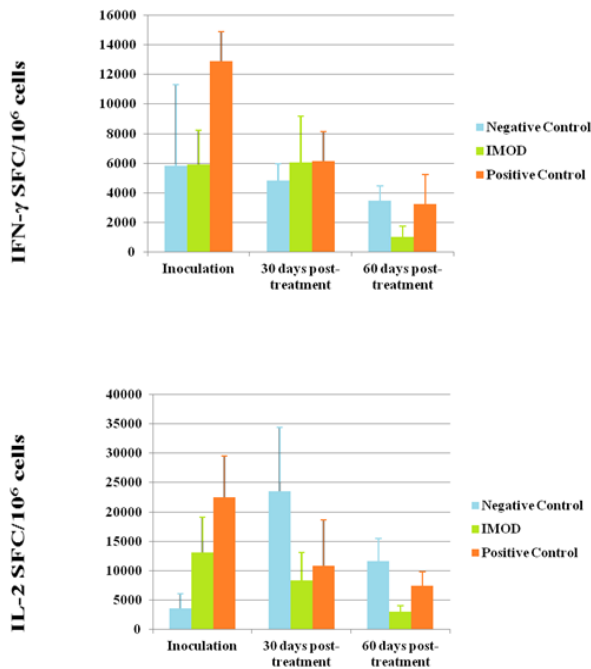


Fig. 2: Levels of Th1-type cytokines in PBMCs. Responses on y-axes are given as spot forming cells (SFC) per 10^6 cells. Numbers of SFCs are shown after subtracting the background. The data are presented as mean \pm S.E. of four dogs per group.

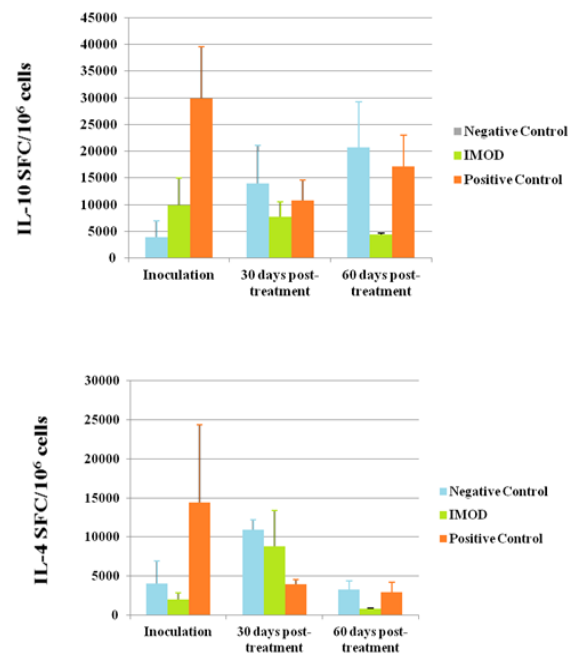


Fig. 3: Levels of Th2-type cytokines in PBMCs. Responses on y-axes are given as spot forming cells (SFC) per 10^6 cells. Numbers of SFCs are shown after subtracting the background. The data are presented as mean \pm S.E. of four dogs per group

Table 1: Levels of IFN- γ /IL-4 ratio. The data are presented as mean \pm S.E. of four dogs per group. * $P=0.003$ and † $p = 0.009$ vs. IMOD group

	Inoculation	30 days post-treatment	60 days post-treatment
Negative Control	1.00 \pm 0.73 †	0.43 \pm 0.10	2.37 \pm 1.07
IMOD	3.24 \pm 0.55	0.71 \pm 0.11	1.16 \pm 0.74
Positive Control	0.91 \pm 0.07 *	1.71 \pm 0.64	0.98 \pm 0.17

Discussion

Conventional treatments such as Glucan-time[®] and amphotericin are not effective in CVL, as reported. In fact, high rate of failures have reported using these agents as a result of disease progression, relapse or parasites drug resistance (27). Furthermore, experimental and clinical data from the reviewed studies indicate that full protection from CVL or complete parasite eradication is difficult. Therefore, infected dogs remain the main reservoir for hu-

man infection. This is why an effective treatment such as immunotherapy would represent a vital alternative (9, 12).

Protective and therapeutic responses in dogs have been related with the remission of clinical symptoms, low levels of anti-*Leishmania* antibodies and reduce the parasite load (4, 28). In this study, although IMOD administration was not associated with clinical adverse effects and the evaluation of renal and hepatic functions revealed no remarkable changes, signs of clinical remission were not detected in treat-

ment group. On the other hand, serological improvement (titers lower than 1:320) for anti-*Leishmania* antibody titers (24) were not obtained and parasitological evaluation showed no total parasite elimination.

Cytokines play a fundamental role in the regulation of immune response for leishmaniasis remission and/or relapsing (10). In comparison with experimental data on murine models of *Leishmania* and human leishmaniasis, scientific advances on the mechanisms of the immune response to CVL are limited. This is mainly due to the slower progression in development of laboratory products for the evaluation of canine immune system and few published works (12, 28, 29).

However, using of ELISpot assay has been scarce in veterinary immunology because of the inadequate monoclonal antibodies; it is a powerful and sensitive method for the quantitation of cytokine responses (30). The ELISpot assay preferred for basic research projects, clinical studies and vaccine trials and offers several advantages over other immunoassays (31, 32). It is approximately 10–200 times more sensitive than ELISA and displays similar sensitivity to RT-PCR analysis (32, 33), less expensive to perform and better suited to the analysis of frozen samples (31). Therefore, we analyzed Th1- & Th2-type cytokines by this method for evaluating the potential immunogenicity of IMOD against experimental model of CVL.

Early studies, particularly on the murine model of cutaneous leishmaniasis, largely defined the balance of Th1/Th2 = resistance/susceptibility to infection (28, 34). IFN- γ is considered to be one of the key cytokines in Th1 profiles, whereas IL-4 participates in the Th2 polarization and its secretion suggests a predominance of humoral responses. Regarding IL-10, it is thought that can be a contributor in both types of immune responses having a regulatory effect (30). There are several studies indicating that a Th2-type response predominates during acute leishmaniasis, such as increase the production

of IL-4 to IFN- γ ratio and polyclonal B-cell activation (7, 12).

Because of the few published data, understanding the profile of cytokine expression in CVL is a difficult task. It was described that cellular immune response in CVL is associated with producing IFN- γ & IL-2 and active disease is characterized by marked humoral response (35, 36). Based on other literatures, the role of IL-4 as a cytokine related to susceptibility remained controversial and IL-10 does not seem to have a predominant immunoregulatory pattern (28).

Recent advances in experimental model of CVL have allowed defining the mechanisms of cellular responses against *Leishmania*, similar to that investigated in the murine model of leishmaniasis (12, 28, 29). As it is believed control of VL in mice requires enhancement of IFN- γ /IL-4 ratio (37), we also considered this ratio in the present study.

Before initiating therapeutic protocol, the results of ELISpot assay showed all the symptomatic dogs had higher frequencies of IL-2 & IL-10 than un-infected dogs. On the other hand, higher levels of IFN- γ and IL-4 are expressed in 50% infected dogs than negative control group.

The IFN- γ /IL-4 ratio at the end of study, while reduced by disease progression in positive control dogs, was increased in IMOD group.

In general, lymphocytes from healthy dogs responded more vigorously to *in vitro* stimulation with IMOD and furthermore, the treated dogs with IMOD showed higher proliferation than did cells for control groups.

The hypothesis of present study was that IMOD as an immunomodulator agent could probably improve CVL. IMOD has been patented with the code of WO/2007/087825 for its immunomodulatory potential in Europe (19) and has USA-patent application with the code of P30280US. It is a combination of three herbal extracts treated with chemical trace elements and a special electromagnetic field which dispensed into sterile ampoules for

research use under the trademark IMOD (Rose Pharmed Biotechnology Co., Tehran, Iran). It was invented briefly by preparing ethanolic herbal extract from *Rosa canina*, *Urtica dioica* and *Tanacetum vulgare* with adding selenium and urea and having been exposed to a pulsed electromagnetic field (19). *Urtica dioica* leaf extract contains active blends that reduce TNF- α , IL-1 and other inflammatory cytokines (38). *Rosa canina* extract possesses abundant antioxidant agents containing flavonoids and some bioactive compounds which cause this plant to have anti-inflammatory and radical scavenging properties. *Tanacetum vulgare* is also another herb that has antioxidant and anti-inflammatory characteristics. These herbs are used in traditional therapy in different conditions (18).

The results of this experiment, however support the hypothesis indicating modulatory effects of IMOD most probably due to various effects of this herbal mixture, but did not show strong immunologic response for CVL as a model for an immune-mediated infectious disease; final outcome of IMOD as expected and not supposed to was not accompanied by total parasitological cure due to Th1/Th2 harmony. The poor response to therapy observed in dogs may be either due to serious immunosuppression induced by experimental CVL or due to short time for evaluating the medication which extending its period was beyond our planned program.

Conclusion

Understanding the cytokines expression in CVL is a difficult task and in comparison to the murine model, studies in CVL show more heterogeneity. Dogs are a major key to reduce the incidence of human leishmaniasis. Hence, it is essential matter to improve the knowledge of the protective cellular immune response in CVL for an immunomodulatory therapy and vaccine development.

Interestingly, CD4⁺ T cells enhancement in dogs, by *in vitro* IMOD stimulation, was the major result obtained in this preliminary study. Therefore, it is important to confirm this assumption by more studies with more animals in each studied groups and longer follow-up period whether IMOD monotherapy or combination therapy represent reasonable effects in leishmaniasis prevention and/or treatment as a model for improving cell-mediated immunity in a immunocompromised animal or human.

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