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Original Article

Evaluation of Apoptotic and Antileishmanial Activities of Artemisinin on Promastigotes and BALB/C Mice Infected with *Leishmania major*

***Fatemeh GHAFFARIFAR [1], Farzad ESAVAND HEYDARI [1], Abdolhosein DALIMI [1], Zuhair M. HASSAN [2], Mahdi DELAVARI [3], Hajar MIKAEILOO [1]**

1. *Dept. of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
2. *Dept. of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
3. *Dept. of Medical Parasitology and Mycology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran*

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***Correspondence**
Email:
ghafarif@modares.ac.ir

Abstract

Background: In leishmaniasis, some drugs prescribed for treatment have toxic effects and there are reports about drug resistance in some countries. Due to this fact, using herbal drugs such as artemisinin with good efficacy and low toxic effect might be suitable.

Methods: We evaluated the apoptotic effect of artemisinin on *Leishmania major* in vitro and the antileishmanial activities of artemisinin on leishmaniasis in BALB/c mice and at the end INF-γ and IL-4 cytokines levels were detected by ELISA in spleen cell culture supernatants. During treatment the lesion size and survival rate were measured each four and ten days, respectively.

Results: Percentage of early and late apoptosis in promastigotes of control group and promastigotes treated with 10, 25, 50 and 100 µg/ml of artemisinin after 48 h were 0.13, 16.04, 41.23, 49.03 and 81.83, respectively. The IFN-γ in ointment treated group were higher than those of other groups (P<0.05). The in vivo results showed that ointment compounds healed the lesions more effectively rather than intraperitoneal injection method (P<0.05). The survival rate of mice 150 days after challenge in treated group with ointment of artemisinin was 66% while all mice in control groups were died.

Conclusion: All of in vitro results represented that this drug had antileishmanial effects and these results were confirmed by evaluation effects in vivo condition of leishmaniasis. Interestingly, according to these results it can be concluded that this drug has antileishmanial effects in vitro and in vivo conditions. Artemisinin induces cytotoxic effect on *L. major* via apoptosis-related mechanism.

Introduction

Leishmaniasis is a major public health problem in many countries. Some drugs that are taken for treatment of leishmaniasis, have toxic effects and the emerg threat of drug resistance in some countries (1). Artemisinin is an herbal drug which its derivatives have been used for treatment and growth inhibition of Malaria (2). In BALB/c mice that infected with *Leishmania donovani*, artemisinin had anti-leishmanial activity (3). Th1 cytokines play a critical role in healing of leishmaniasis and the outcome is related to cytokine patterns (4, 5).

In one of the previous study, we found that artemether as a derivative of artemisinin has a good efficacy on treatment cutaneous and visceral leishmaniasis (6, 7). In a previous study for comparison between artemisinin and *Artemisia seiberi* extract in-vitro we found that both had anti-leishmanial activity however the effect of *A. seiberi* was higher (8).

Treatment of cutaneous leishmaniasis with 1%, 3% and 5% of *A. sieberi* in-vivo the ulcers got bigger with the more concentration (9). Other plants such as *Alkanna tincturia* and *Peganum harmala* extracts have in vitro effects on *L. major* (10).

The aim of this study was to study antiparasitic activity of artemisinin on *L. major* in both in-vitro and in-vivo and finding the cytokine pattern as well as percentage of apoptosis induced by artemisinin.

Materials and Methods

Ethics Statement

This project was approved by Ethical Committee of School of Medical Sciences of the Tarbiat Modares University (adopted from the Declaration of Helsinki (1975) and the Society for Neuroscience Animal Care and Use Guidelines (1998)), approved implementation by the Medical Ethics Committee on 17th April, 2006.

Parasites and artemisinin preparation

Artemisinin ($C_{15}H_{22}O_5$) was purchased from Enzo Life Sciences Company (Germany) with batch No (L16705). For preparation stock dissolution, 1mg of artemisinin was dissolved in 50% of ethanol and 50% distilled water. Promastigotes of *L. major* (MRHO/IR/75/ER) were cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) , 100 IU/ml of penicillin and 100 µg/ml of streptomycin.

In vitro assay

All *in vitro* experiments were repeated three times in triplicate wells.

Promastigote assay

The interactions of artemisinin directly on promastigotes of *L. major* were studied. In promastigote assay the interactions of artemisinin in five concentrations (5, 10, 25, 50, 100 µg/ml) were evaluated with promastigotes for 24, 48, 72 hours. After incubation, antileishmanial activity of this drug was evaluated by direct counting and was compared with control cultures (6).

Promastigote apoptosis assay by flow cytometry

We used Annexin V-FITC Apoptosis Detection Kit(Roche, Germany). Briefly,

2×10^6 promastigotes /ml were treated with different concentrations of artemisinin (0, 10, 25, 50 and 100 µg/ml) and incubated at 24°C for 48 h. Then the promastigotes were washed twice with cold PBS solution and centrifuged in 1400g and 4 °C for 10 min. Five µl annexin-V FITC, 5µl PI (propidium iodide) and 500µl binding buffer were added and incubated for 15 minutes at room temperature. The procedure was performed according to manufacturing protocol and using FACSCalibor system. Afterwards the flow cytometry results were then analyzed using FlowJo software on the

basis of 4 areas: the cells staining with annexin-V only as apoptotic cells (LR), the cells staining with PI as necrotic cells (UL), the cells staining with both of Annexin-V and PI as late apoptotic (UR) and those cells that did not stain as healthy cells (LL).

Macrophage cytotoxicity assay

For evaluation effects of artemisinin on un-infected mouse macrophages, we used female BALB/c mice that were purchased from the Razi Vaccine and Serum Research Institute, Hessarak, Karaj, Iran. Peritoneal macrophages of mice were collected and were cultured in RPMI medium (GIBCO) supplemented with 10% FCS. For evaluating the percentage of live macrophages the samples were stained with trypan blue and were estimated by direct counting method with a light microscope. The macrophages were seeded in exposure to five dilutions of artemisinin (5, 10, 25, 50, 100 µg/ml). These cultures were incubated at 37°C in 5% CO_2 for 24, 48, 72 hours. Direct counting of macrophages was applied for evaluation of cytotoxic effects of artemisinin and was compared with control groups (6).

Macrophage apoptosis assay by flow cytometry

For apoptosis detection of an un-infected BALB/c mouse macrophages treated with different doses of artemisinin, we used AnnexinV-FITC Apoptosis Detection Kit (Roche, Germany). The macrophages (1×10^5 cells/ml) were cultured in 24 well platesin RPMI 1640 medium (Gibco BRL) supplemented with 10% FBS (Gibco BRL) in presence of four concentrations of artemisinin (0, 10, 25, 50, 100 µg/ml). The cell cultures were incubated at 37 °C and 5% CO2. The samples were collected after 48h, centrifuged in 1400g and 4°C for 10 min. The macrophage apoptosis assay was done by flow cytometry like the procedure of promastigote apoptosis assay that was explained above.

Amastigote assay

After extraction of peritoneal cavity macrophages of BALB/c were cultured in 24-wells plates and infected with *L. major* promastigotes at a parasite/macrophage ratio of 10:1. Drug susceptibilities of intracellular amastigotes were determined with a brief modification that previously described (11). This plate culture was incubated at 37°C with 5% CO_2 for 24 h then each well of the plate was washed with PBS to remove the extracellular promastigotes. Number of amastigotes/macrophages was evaluated after staining with Gimsa and using light microscope. Then the infected macrophages were incubated with 5 concentrations of artemisinin (5, 10, 25, 50, 100 µg/ml) for 24, 48, 72 h. Finally, direct counting was applied to determine the mean of infected cells and also the number of amastigotes per macrophage cell after staining with Gimsa. Amastigote burden was compared for both test and control groups.

In vivo assay

Ointment preparation

Artemisinin ointment was prepared with a vaseline base in 25 µg/ml concentration. We prepared the ointment with mixing 250 µg of artemisinin in 10 ml of vaseline and the lesions were treated with 0.1 ml of ointment twice each day.

We have used the BALB/c female mice with the same age (6-8 weeks) and the average weight of 18-20 g. Sixty mice were randomly sorted into four groups: two test groups contain 15 infected mice as a test group with peritoneal injection of artemisinin and 15 infected mice as a test group with local ointment, 15 infected mice as a control group that treated with artemisinin solvent (control 1) and 15 infected mice that treated with vaseline as another control group (control 2). The mice were infected with 100 µl of promastigotes (2×10^7 cells/ml) in stationary phase subcutaneously in the base of the tail. The infected mice were kept about 6 weeks for the establishment of cutaneous leishmaniasis. Before

treatment initiation the presence of infection was assessed in three mice by culture and smears from lesions for amastigote detection.

Treatment with ointment and intraperitoneal injection (IP) of artemisinin

Drug treatments were started immediately after appear the lesions. Test groups were treated daily for 6 weeks for intraperitoneal injection of artemisinin and treated two times in each day for ointment of artemisinin.

Extraction of spleen lymphocytes for cytokine assay

All procedures for human care and animal were approved by the Ethical Committee on Research of Medical sciences faculty of Tarbiat Modares University.

In each group after treatment, nine mice were sacrificed and spleen of mice were removed and isolated under sterile conditions. Lymphocytes were isolated from spleen in test and control groups. Direct counting after staining by trypan blue was employed for evaluating the percentage of live lymphocytes. The suspension of lymphocytes in RPMI that supplemented with 10% FCS (2.5×10^6cells/ml) was cultured in 24 well plates. The lymphocyte culture was stimulated by SLA (soluble *Leishmania* antigens) with concentration of 40 µg/ml. The plates were incubated at 37°C with 5% CO_2 for 72 hours. After incubation time the supernatants were collected and then divided into 500µl tubes. The tubes were stored in -70°C until use. Finally for the evaluation of INF-γ and IL-4 cytokines in test and control groups DuoSet ELISA Development INF- γ and IL-4 Kits (R&D) was used. This procedure was performed according to manufacturing protocols and analyzed by ELISA system.

Measurement of lesion size, weight and survival rate in BALB/c mice

About 2×10^6 of stationary phase promastigotes of *L. major* were inoculated at the tail base of mice then after 6 weeks, the size of lesions was measured by a digital vernier caliper in 2 diameters each 4 days. The weight of mice was weekly measured with a digital balance. Six weeks after the parasite challenge, the survival rate of the treated and control mice was measured each 10 days.

Statistical analysis

The in vitro tests, lesion size, weight of the mice and cytokine assay were analyzed by non-parametric independent groups with Mann-Whitney test. The data were analyzed with SPSS version 16 and IC_{50} calculated by SPSS (Chicago, IL, USA) and Graph Pad Prism. The graphs were drawn by excel software. $P < 0.05$ was considered as the statistical differences.

Results

Results showed the effectiveness of artemisinin on *L. major* in both condition, in vitro and in vivo.

Promastigote, macrophage and amastigote assay

The cytotoxic effect of artemisinin on promastigotes was observed. These result show artemisinin has antileishmanial effects on promastigotes. There were significant differences with control group and treated groups in 24, 48 and 72 h ($P<0.05$) (Fig. 1).

The cytotoxic effect of artemisinin on uninfected macrophages of BALB/c mice was evaluated at 24, 48 and 72 hours. These results showed that artemisinin–had low cytotoxicity on uninfected macrophages of BALB/c mice. Percentage of BALB/c mice macrophages viability that cultured with 100 µg/ml concentrations of artemisinin at 24, 48 and 72h was 92.5, 80.0 and 76.3 respectively in comparison with control group that was 97.1, 94.6 and 93.1, respectively.

Amastigote assay of artemisinin effect on infected macrophages according to mean of amastigote per macrophage were done and

compared with control groups. The cytotoxic effect was evaluated by calculating 50% inhibitory concentration of artemisinin on amastigotes after 48 h (IC_{50}) according to usage of five dilutions of artemisinin in comparison with control (IC_{50}=25µg/ml). There were significant differences between the control group and 50 and 100 µg/ml after 24 h and between control group and 5, 10, 25, 50 and 100 µg/ml after 48 and 72h (P<0.05). These results are shown in Fig. 2.

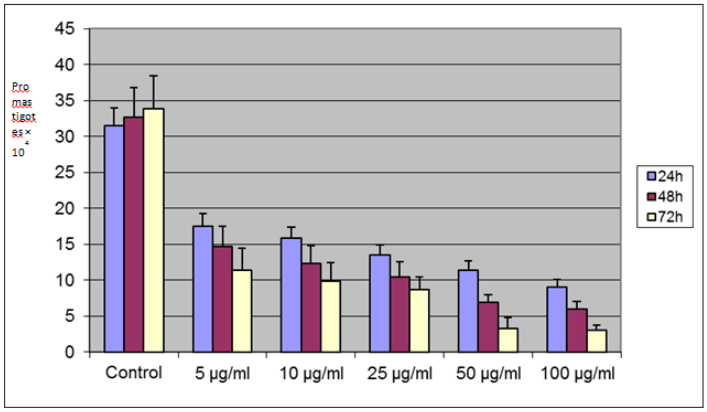

Fig. 1: Mean and SD of promastigotes of *Leishmania major* cultured with 5, 10, 25, 50, and 100 µg/ml concentrations of artemisininat 24, 48 and 72h and comparing them with control group. There are significant differences between the control group and treated groups in 24, 48 and 72 h (P<0.05)

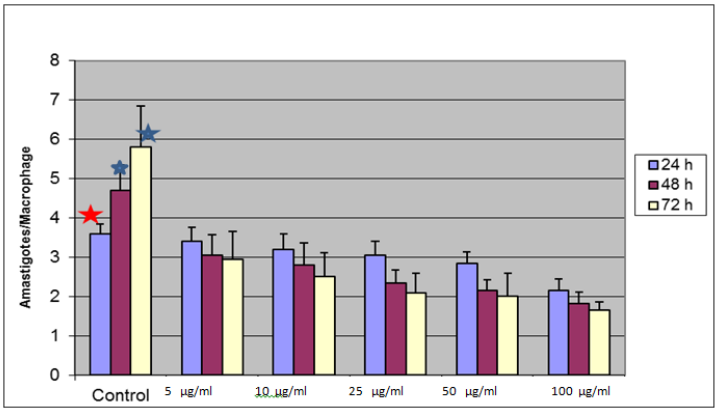

Fig. 2: Mean and SD of amastigotes per macrophage of BALB/c mice cultured with 5, 10, 25, 50, and 100 µg/ml concentrations of artemisininat 24, 48 and 72h and comparing them with control group/ ✿ There is statistical differences between control group and treated groups (P<0.05). / ✿ There is statistical differences only between control group and 50 and 100 µg/ml treated groups (P<0.05).

Promastigote and macrophage apoptosis assay by flow cytometry

Induction of apoptosis in promastigotes of *L. major* was analyzed by flow cytometry FACS Calibor system after staining with Annexin-V and PI. The percentages of apoptotic, necrotic and viable cells were determined: UL as necrotic cells in the upper left region (positive for PI), UR as the cells were banded with Annexin-V and PI in upper right region, LL as viable promastigotes in lower left region and LR as a marker of apoptosis in the lower right region of figures. The results were shown that artemisinin were induced apoptosis in promastigotes of *L.*

major at all concentrations of artemisinin. The most effects of artemisinin on promastigotes of *L. major* took place in apoptotic areas. The percentages of apoptosis in promastigotes stimulated with 10, 25, 50 and 100 µg/ml of artemisinin were calculated 12, 25.90, 40.52 and 68.16 respectively in comparison with control group that was 0.02. The results of necrosis, late apoptosis, apoptosis and live promastigotes are shown in Fig. 3.

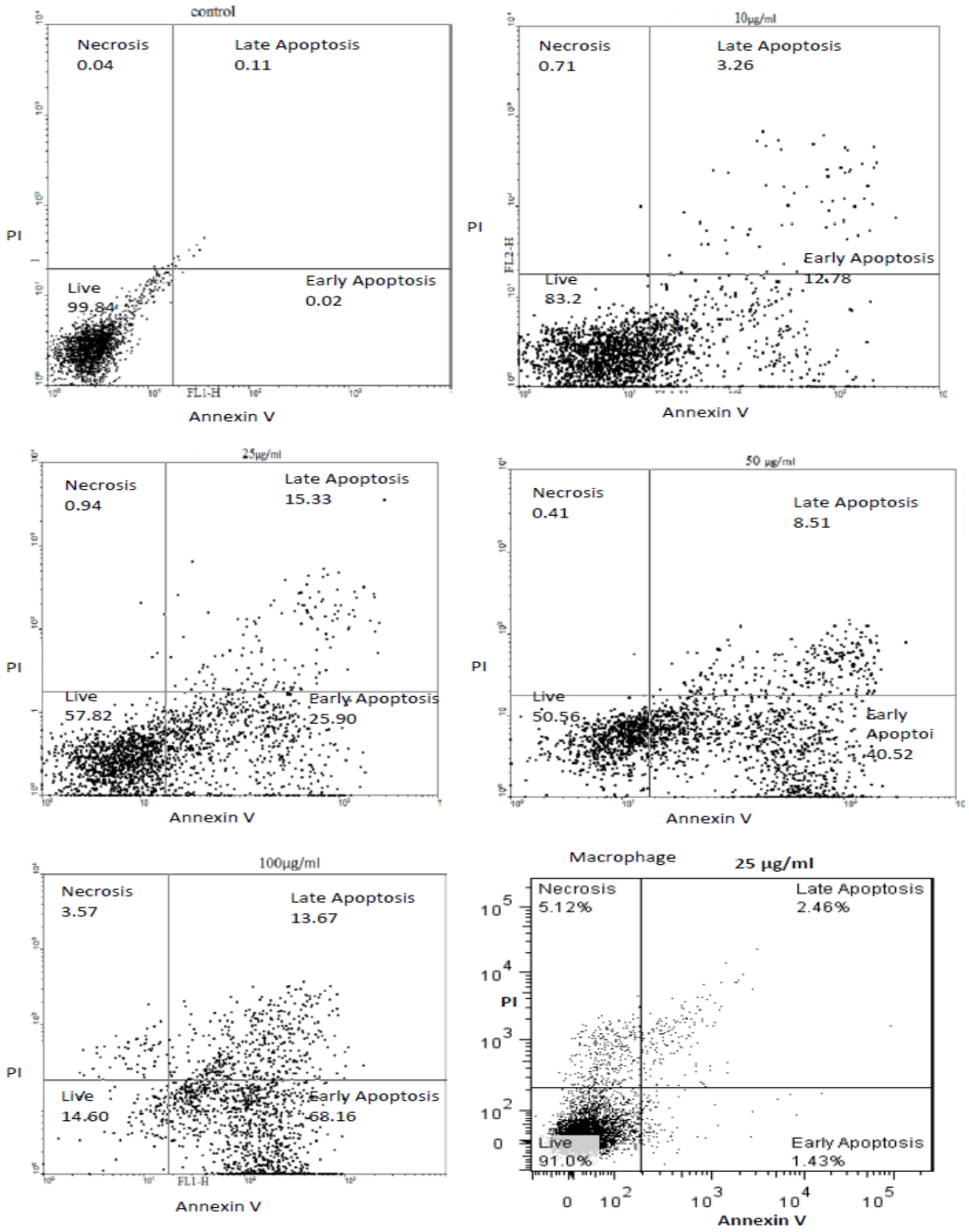

Fig. 3: Flow cytometry results of the effect of artemisinin with 10, 25, 50, and 100 µg/ml concentrations on the promastigotes (2×10^6 promastigotes /ml) of *Leishmania major* (MRHO/IR/75/ER) viability and comparing them with control group (promastigotes of *Leishmania major* without any treatment) and macrophages treated with 25 µg/ml of artemisinin (IC_{50} dose on promastigotes) after 48 h. Regions of quadrate show percentage necrosis, late apoptosis, apoptosis and live cells

The data of apoptosis in promastigotes of *L. major* obtained by flow cytometry had statistical differences in all concentrations of artemisinin in comparison with control group ($P<0.05$). The percentage of apoptosis in macrophages stimulated with 10, 25, 50 and 100 µg/ml of artemisinin were calculated 0.64, 1.43, 1.96 and 9.39 respectively in comparison with control group that was 0.6. The data of apoptosis in mice macrophages obtained by flow cytometry had statistical the differences only in 100 µg/ml concentration of artemisinin ($P<0.05$).

Survival rate and lesion size in treated mice and control groups

For determination effects of artemisinin on lesion size of leishmaniasis in vivo conditions, we used two forms of treatment (intraperitoneal injection and ointment) and lesion sizes were measured and compared with control groups. The results were shown Vaseline ointment containing artemisinin drug had antileishmanial effect and prevented of progression this disease. Results were analyzed by nonparametric Mann Whitney statistical test ($P<0.05$) (Table 1).

Table 1: Mean and standard deviation of lesion size in treated and control groups during the treatment of BALB/c mice with artemisinin

Days during the treatment	Injection	Ointment	Control 1	Control 2
4	7.1 ± 1.99	8.4 ± 1/04	7.1 ± 3.28	8.2 ± 1.04
8	7.36 ± 1.95	8.63 ± 0.96	7.51 ± 3.22	8.56 ± 1.02
12	7.70± 1.95	8.41 ± 1.09	8 ± 3.44	8.96 ± 0.55
16	8.03 ± 2.02	8.45 ± 1.62	8.26 ± 3.5	9.46 ± 0.5
20	8.38 ± 2.23	8.35 ± 2.12	8.88 ± 4.03	10.16 ± 0.65
24	8.63 ± 2.21	8.01 ± 2.20	9.56 ± 4.02	10.35 ± 0.49
28	8.88 ± 2.30*	7.41 ± 0.75*	10.71 ± 1.24	11.01 ± 0.21
32	9.08 ± 2.31*	7.28 ± 1.19*	11.60 ± 1.39	11.85 ± 0.21
36	9.71 ±1.47*	6.28 ± 1.44**	11.80 ± 1.94	12.65 ± 0.49
40	10.43 ±1.05*	5.46 ± 0.76**	12.50 ± 1.66	13.50 ± 0.7

* There-were significant differences with control groups ($P<0.05$)
** There were significant differences with other groups ($P<0.05$)
Control 1=mice treated with artemisinin solvent /control 2=mice treated with Vaseline

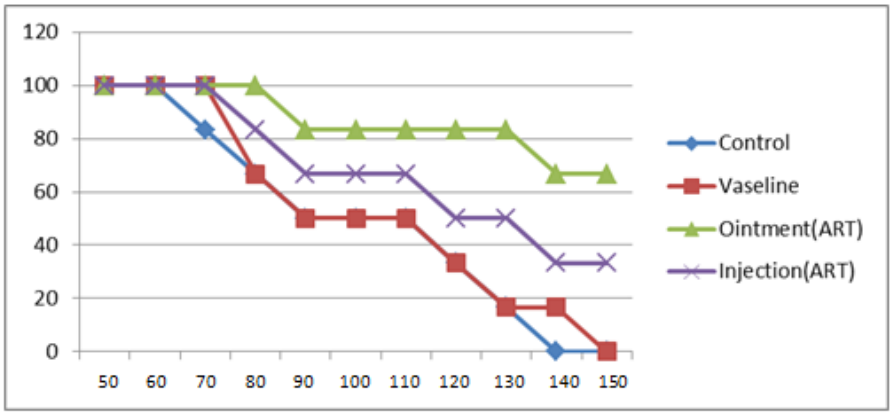

Fig. 4: Percentage of survival rate of infected BALB/c mice with 2×10^6 in treated groups with ointment and intraperitoneal injection form of artemisinin and control goups during 150 days after challenge. The number of mice in each group is six

For evaluation of survival rate, six mice in each group were treated then followed for 150 days. The results are shown in Fig. 4. The higher rate is related to a group that treated with ointment of artemisinin.

Cytokine assay

Evaluation of INF-γ and IL-4 cytokine levels in the test and control groups were analyzed by ELISA reader system at 72 hours after culture. Results were compared with INF-γ and IL-4 standard curves and control groups. These results are presented in Table II. The statistical tests were employed for multiple comparisons and analysis of IL-4 and INF-γ cytokines results (Table 2). These results showed that artemisinin makes increasing in the INF-γ and IL-4 cytokine levels in treated mice rather than control groups. But increasing the level of INF-γ was more than IL-4 and the differences were significant with control groups (P<0.05).

Table 2: Mean and standard deviation of cytokine assay (INF-γ and IL-4pg/ml) of lymphocytes culture in treated and control groups 72h after cultivation

Cytokines	Injection	Ointment	Control 1	Control 2
IL-4	40.17 ± 5.60*	52.00± 7.47*	26.83± 3.65	20.83 ± 4.70
INF-γ	13.33 ± 3.44*	79.50 ± 4.97**	7.67 ± 1.86	6.33 ± 1.50

* There were significant differences with control groups (P<0.05)
** There were significant differences with other groups (P<0.05)
control 1=mice treated with artemisinin solvent /control 2=mice treated with Vaseline

Discussion

Artemisinin is identified as a potent antileishmanial agent against promastigotes and amastigotes but in contrast it showed lower toxicity toward uninfected macrophages. Programmed cell death (apoptosis) was analyzed by flow cytometer and showed that artemisinin was induced apoptotic effects on promastigotes of *L. major*. Cell apoptosis can be very effective in elimination of parasite. In the present study, artemisinin showed apoptotic effect against promastigotes of *L. major*. The apoptosis and late apoptosis of promastigotes of *L. major* cultured with IC_{50} dose of artemisinin (25µg/ml) were calculated 25.90 and 15.33 respectively, while in previous study, IC_{50} dose of artemether on *L. major* were 42.28 and 3.83 respectively (6). *A. sieberi* essences were not effective on *L. major* ulcers of BALB/c mice in a study (9) whereas in this study the treatment by using artemisinin especially in ointment form could heal the lesions. The difference of findings may relate to the origin of artemisinin or the kind of application (we used ointment and intraperitoneal injection; however, Doroodgar et al. used dropped essence of *A. sieberi* on the lesions).

During the leishmaniasis, the cells produce various cytokines, such as tumor necrosis factor (TNF) and interferon (IFN) that facilitate activation of macrophages and the development of Th1 response in the mouse model (11-12). Interferon is a glycoprotein released by many cell types in responses against infection (13). The specific mechanisms are including the cellular and humeral immune responses (14). Macrophages are the main cells against to *Leishmania* infection and they have various roles in the immune response such as: antigen recognition, antigen presenting to T lymphocyte, phagocytosis, etc. (15-18). In the present study, artemisinin showed low toxicity effect on BALB/c macrophages.

The cells such as monocytes (macrophages), neutrophils and eosinophil are useful to control diseases for example against enzymes and oxidative radicals, but the parasite manages to evade most of these host defense mechanisms and these defenders molecules may also be involved in the development of clinical manifestations of leishmaniasis (19). Unfortunately,

Leishmania can interfere with these requirements and prevent the subsequent development of a protective response (20). In the presence of a pathogen, the chemokine can activate T lymphocytes and macrophages, and recruit appropriate effector cells to the infected area in the spleen of experimentally infected dogs by *L. donovani* (18-25). We demonstrated that the ability of lymphocytes of infected mice for produce INF-γ during treatment with artemisinin. This study revealed a significant IFN-γ response in the spleen cell cultures of test groups compared with the control group**.** IFN-γ is an important cytokine that is critical for immune cellular response against Leishmania. The importance of IFN-γ is immune stimulatory, immune regulatory and modulatory effects. IFN-γ is produced by (NK) as a part of the innate immune response and by T helper cells and cytotoxic T cells (16, 26, 27). IFN-γ is a promotive factor for macrophage activity, antigen presentation and lysosome inducible nitric oxide synthase activity of macrophages. In leishmaniasis, IFN-γ promotes Th1 differentiation and macrophage activity (28). In vivo experiments we have found that amastigotes of *L. major* are very susceptible to artemisinin and have increased the survival rate of treated mice especially in the group treated with ointment. The group that treated with ointment of artemisinin had better results in comparison with the group that treated with peritoneal injection form. The reason may be related to the short half-life of artemisinin (2-5 hours) and it needs to be used several times daily. The result of our previous study indicated that when we use the artemether 4 times per day for treating visceral leishmaniasis, better therapeutic effects could be obtained (7).

This study has shown that artemisinin can be used for treatment strategies against leishmaniasis. To sum up, these results demonstrated that artemisinin can be a potential candidate for further evaluation as a chemotherapeutic agent for the treatment of leishmaniasis. Finally we conclude that artemisinin had effect on leishmaniasis caused by *L. major* and could decrease progression of this disease in BALB/c mice.

Conclusion

All of in vitro results were shown that this artemisinin had antileishmanial effects. Artemisinin showed high toxicity and apoptotic effect on promasigotes and low toxicity and apoptotic effect on BALB/c macrophages. The results were confirmed by evaluation effects in vivo condition in BALB/c mice. IFN-γ increased significantly in the spleen cell cultures of treated group. Artemisinin has the ability to heal leishmaniasis caused by *L. major* in BALB/c mice.

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