International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

International Journal of Pharmacology 7 (8): 843-849, 2011
ISSN 1811-7775 / DOI: 10.3923/ijp.2011.843.849

INOS and IFNγ Gene Expression in *Leishmania major*-Infected J774 Cells Treated With Miltefosine

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Abstract: Miltefosine is the new drug of choice for the treatment of leishmaniasis. The aim of this work was to study the molecular mechanisms and immunomudolatory properties of miltefosine in J774 cell line infected with the *Leishmania major* (MRHO/IR/75/ER) parasite. In this experimental study infected J774 cell line by *L. major*, treated by miltefosine and incubated for 72 h. Total RNA was extracted and cDNA was synthesis. RT- PCR was used for study of IFNγ and iNOS gene expression. IFNγ and iNOS proteins were analyzed by Western blotting. Cell culture supernatant was examined by ELISA for of IL12 and IL10 concentration. After 4 h incubation, miltefosine increased iNOS and IFNγ gene expression in *L. major* infected J774 cell line. Western blot analysis of extracted cell proteins showed 130 and 17 kDa of bonds related to iNOS and IFNγ, respectively. After 48 h treatment with miltefosine, analysis of cell supernatant with ELISA showed a significant increase of IL12 but no change in IL10 Cytokine. Study showed that miltefosine in addition to its direct effect can improve cellular immunity with rising of IFNγ and of iNOS genes expression that are able to activate macrophages.

Key words: Miltefosine, *leishmania major,* IFNγ, iNOS, IL12, active macrophage

INTRODUCTION

Protozoan parasites genus *Leishmania* are obligatory intracellular organisms that manifested in several forms including visceral, mucocutaneous and cutaneous leishmaniasis. Approximately, 12 million people are involved in disease and threaten in additional 350 million people (Talari *et al*., 2006; WHO, 2010). In the present, Glucantime® and amphotericin B as current medications, have several disadvantages including side effects, resistance and parenteral rout of administration (Ndjonka *et al*., 2010; Goto and Lindoso, 2010; Khademvatan *et al*., 2011a; Saki and Khademvatan, 2011).

Miltefosine (HePC), an alkylphosphocholine which was originally developed as an anticancer drug has been proved an effective oral treatment for various types of leishmaniasis with a less side effects and a cure rate of about 98% (Terwogt *et al*., 1999; Soto *et al*., 2001, 2006; Soto and Berman, 2006; Sundar *et al*., 2002). Anti-leishmanial mechanism of miltefosine is not clearly understood (Berman, 2008). Previously direct effects of the HePC and induction of apoptotic cell death have been proved (Shaha, 2006; Khademvatan *et al*., 2009, 2011a). Some of studies indicate miltefosine may have indirect effect on different cell line with immunomodulatory functions (Hilgard *et al*., 1991; Hochhuth *et al*., 1992; Eue, 2002).

Macrophage cells are specific host of leishmania and activated macrophage by IFNγ cytokine, have fundamental role in elimination of the intracellular parasites. Two major mechanism of intracellular parasite eradication are producing activated oxygen radicals and Nitric Oxide (NO) (Sharma and Singh, 2009). A group of enzymes called Nitric Oxide Synthetase (NOS) makes Nitric Oxide (NO). There are three NOS isoforms that are named based on their activity include neural NOS (nNOS or NOS-I), endothelial NOS (eNOS or NOS-III) and under pathological conditions (e.g., infection), the inducible type of NO-synthase (NOS-II or iNOS) (Wanasen and Soong (2008). Both nNOS and eNOS enzymes mainly expressed in mammalian cells and NO in response to increased intracellular calcium levels are synthesized

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(Umar and van der Laarse, 2010). Production of NO by iNOS isoform is taking more time and higher concentrations of NO produces in the cell. NO production by iNOS can be controlled in transcription. iNOS protein levels in most cells is very low and unknown, but, stimulating these cells with cytokines or growth factors leads to increased iNOS gene transcription and subsequent production of high No concentration (Qadoumi *et al*., 2002).

In the present study, the leishmanicidal activity of miltefosine with its immunomudolatory function and Host-cell dependent effect in the J774 cell line infected with leishmania major (MRHO/IR/75/ER) parasite was evaluated. The mechanisms and pathways that activate leishmania-infected macrophages are particular interests as they will be potential targets for development of anti-leishmania medications.

MATERIALS AND METHODS

Materials: In this experimental study conducted in May to October, 2011 miltefosine (1-O-hexadecylphosphocholine) with molecular weight 407.57 were prepared from Zentaris GmbH (Zentaris, GmbH and Frankfurt, Germany). Mouse monoclonal anti-iNOS, IFNγ and goat anti-mouse antibody purchased from Sigma, Chemical Co., St. Louis, MO, USA. Taq DNA polymerase and random hexamer primers were purchased from Roche-applied-science, Germany. RNX™ isolation reagent purchased from Cinnagen Co., Tehran, IRAN and cDNA synthesis kit obtained from Fermentas, Vilnius, Lithuania. IL-10 and IL-12 ELISA kit purchased from (R and D Systems, Abingdon, U.K.).

Cell culture, parasite infection and drug exposure: Leishmania major (MRHO/IR/75/ER) was kindly provided by Dr. Mohebali (Tehran University of Medical Sciences). Briefly, 5×10^5 cells mL^{-1} were cultured in the $RPMI_{1640}$ media (pH 7.2, containing 25 mM HEPES with 10% fetal bovine serum) at 24°C for 48 h. J774 A. One mouse macrophage cell line purchased from Pasteur Institute (Tehran Iran) and cultured in RPMI medium, (containing 10% FCS and 100 μg mL^{-1} antibiotic) at 37 °C with 5% CO_2. Monolayer J774 cell line inoculated with *L. major* in a ratio of 5 parasites per macrophage and 4 h incubated at 32°C and flasks were washed two times to remove all free parasites. Microscopic slides were prepared from each cell suspension and stained by Giemsa (100 macrophages per treatment) to find percentage of infected cells and the number of parasites per infected macrophage. Miltefosine was added in ED50 (half-maximal effective concentration) 5.7 μM that was founded previously (Khademvatan *et al*., 2011a). Flasks were incubated in different time point (4, 18, 24, 48, 72 h) in 32°C with 5% CO_2. Each test was done triplicate. In parallel cultures, infected and non-infected cells were treated with or without miltefosine as well as the control.

Total RNA extraction, cDNA synthesis and RT-PCR: After 4 h of exposure, total RNA was extracted from monolayer cell cultures according to the manufacturer instructions. Briefly, 10^6 cells were collected and 1 mL of the RNA extraction solution of RNA™, was added and shacked gently for 1 min, then 200 μL chloroform was added and mixed to the supernatant and then Isopropanol was added. The vials were centrifuged in 12000 rpm and to precipitated RNA, 20 μL of distilled water contain DEPC (Diethylpyrocarbonate) was added. To produce cDNA, briefly 1 μg of RNA was mixed with 30 pmol of reverse primer. Obtained mixture was kept in 70°C for 5 minutes and put rapidly for 10 min on the ice and DEPC was added to reached to volume 20 μL and finally incubated for 60 min and 5 min in 42°C and 94°C, respectively.

RT-PCR was carried out to determine the mRNA expression levels of IFNγ and iNOS genes. Primers were design and built using Gen runner (Table 1). To RT-PCR test, 2.5 μL tag polymerase, 5 μL cDNA, 30 pmol of each sense and antisense primer, 200 μm dNTP, 2 μm $MgCl_2$, 2.5 μL Tag buffer were used. The mixture poured in 0.2 mL PCR vial and with distilled water reach to 25 μL. Cycling parameters for γ-ACTIN mRNA amplification was 94°C/30 sec, 63°C/45 sec and 72°C/30 sec for 30 cycles and for the amplification of IFNγ were 32 cycles and for the amplification of iNOS were 30 cycles in a DNA Eppendorf Mastercycler gradient thermal cycler (Eppendorf-Netherland, Hinz, Hamburg, Germany).

The PCR productions were stained by ethidium bromide and the obtained bands were graphed by Gel Documentation System (Uvidoc, Gel Documentation System, Cambridge, UK).

Table 1: Primers used for RT-PCR of iNOS, IFNγ and β-ACTIN.

Genes	Primers	Product size (bp)
iNOS	F: 5'-CAT GGC TTG CCCCTG GAA GTT TCT CTT CAA AG-3' R: 5'-GCA GCA TCC CCT CTG ATG GTG CCA TCG-3"	798
IFNγ	F: 5'-TAC TGC CAC GGC ACA GTC ATT GAA- 3' 5'-GCA GCG ACT CCT TTT CCG CTTCCT-3'	405
β-ACTIN	R: 5' GTGGGCCGCTCTAGGCACCAA3', R: 5'CTCTTTGATGTCACGCACGATTTC 3'	538

The amount of PCR products present in each lane was determined using the Molecular Analyst software (Bio-Rad, Philadelphia, PA, USA) version 1.4. The intensity of bands was measured by densitometry and normalized based on the β-ACTIN expression.

Cell lysate preparation and Western blotting analysis: Twenty-four hours after treatment, cells were washed two times with 1 mL of PBS, 100 μL of washing buffer was added (3 mM calcium chloride, 5 mM sodium fluoride, 0.32 M sucrose, 0.1 mm EDTA, 1 mm dithiothreitol (DTT), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 10 mm tris(hydroxyethyl) aminomethane (Tris, pH 8.0), 0.1 mm sodium orthovanadate). To each mL 20 μL L- protease inhibitor cocktail was added. Then 100 μL Lysis buffer was added (wash buffer plus 1% Nonidet P-40).

After centrifugation of suspension at 2500 *g*, the supernatant was stored at-80°C. For electrophoresis 8.5% Tris–glycine gels were used. Samples with maximum volume 30 μL including 1:1 SDS sample buffer of pH 8.8 (0.2 M Tris–HCl, 20% glycerol, 5 mM EDTA, 5% SDS and 5% mercaptoethanol) were used for the electrophoresis. Proteins were blotted on nitrocellulose filters. The membrane was blocked with 5% nonfat dry milk in PBS 0.1% Tween. Mouse monoclonal anti-iNOS and IFNγ were diluted to 2000 times and alkalinephosphatase conjugated secondary antibody (goat anti-mouse antibody) was used. Bands visualize by NBT and BCIP color reaction.

Determination of IL12 and IL10 cytokines concentration: IL12 and IL10 level were determined using cytokine detection ELISA kit according to the manufacturer's protocol. Culture supernatants were collected in different time point (18, 24, 48 and 72 h) after exposure with 5.7 M miltefosine, added to the plates, and incubated for 1 h at 37°C. After washing, 100 μL of HRP-conjugated streptavidin was then added to each plate and absorbance was read at 450 nm on Elisa reader. In addition, cytokines concentrations were measured by various miltefosine doses in 72 h later. All tests were performed in triplicates.

Statistics: Significance was determined by Student's paired t test, with a p. value of 0.05 considered significant. Data analyzed by SPSS ver. 16 software.

RESULTS

Miltefosine increase Endogenous IFN γ and iNOS genes expression in macrophage infected with *L. major*: The results of RT-PCR assay show that iNOS gene expression was significantly higher in the J774 cells infected with the *L. major* compared to both non treated-infected cells

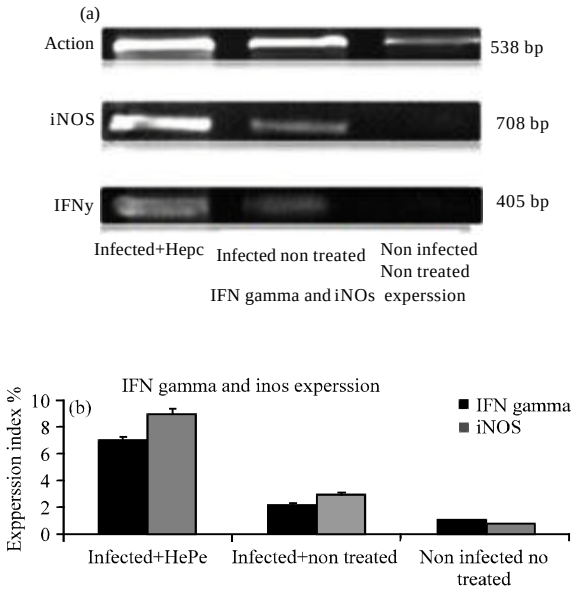

Fig. 1(a-b): RT-PCR analysis of iNOS and IFNγ gene expression in J774 cell line after 4 h incubation: A, semiquantitative RT-PCR analysis. Left to right, infected cell exposed with 5.7 μm miltefosine, infected cells without exposure to drug and control group (non infected non treated cells). γ-ACTIN gene was used as internal control. Density of amplified bands iNOS (708 bp) and IFNγ (405 bp) and γ-ACTIN (538 bp) shown in each group. B: Expression ratio of iNOS and IFN-γ in infected cell exposed with 5.7 μM miltefosine, infected cells without exposure to drug and control group after 4 h incubation. Results are shown relative to β-actin. The values (Mean±SEM) are derived from three independent experiments

and cell control (p-value= 0.05) (Fig. 1a, b). As shown in Fig. 1, expression of the iNOS mRNA was significantly increased (p-value = 0.0655) in *L. major* infected macrophages treated with miltefosine. In non-infected J774 cells, the noticeable lack of major up-regulations of iNOS characterised, 4 h after incubation in experiments

In addition results showed the miltefosine (5.7 μM). not only increased iNOS gene expression in *L. major* infected J774 cell line but also the amount of IFNγ mRNA also increased dramatically after 4 h incubation. This elevation was large in the early hours but decreased after 12 h (data not show). In contrast levels of mRNA was lower in non infected -J774 cells treated with miltefosine Densitometric analysis of iNOS and IFNγ genes showed that expression index in the infected group without

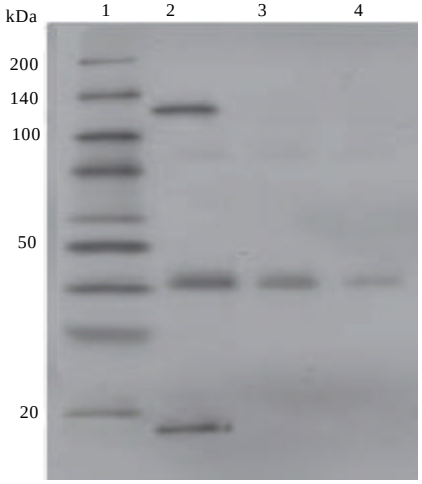

Fig. 2: The cell lysates of J774 cell line were subjected to SDS-PAGE and Western blot analysis. lane 1: Protein size marker, lane 2 *L. major* infected cell line treated with 5.7 μM miltefosine, lane 3 *L. major* infected cells without treatment. And lane 4 control cells. data are representative results of independent experiments. For positive control β-actin gene was used.

miltefosine exposure and also non- infected J774 cell line were lower than test group. To normalize gene expression for each gene of IFNγ and iNOS, γ-ACTIN gene was used (Fig. 1b).

Western blotting analysis of iNOS and IFN-γ: At this stage, Cell extracts were prepared after 24 h incubation and levels of iNOS and IFN-γ were determined in each sample by western blot analysis, as described in materials and methods. The results show that IFNγ protein was significantly higher in the *L. major* -infected J774 cells compared to both non treated/infected cells and cell control. In addition iNOS level increased in *L. major*-infected macrophages treated with miltefosine but in contrast levels of iNOS was lower in non infected cells treated with miltefosine. As the Fig. 2 shows bands of protein related to iNOS, IFNγ, respectively in positions 130 and 17 kDa after reaction with monoclonal antibody on the blot. Bands related to γ-actin with molecular weight 42 kDa were used to control.

Cytokine secretion patterns of J774 cells infected with *L. major* treated with miltefosine: The ability of mouse model macrophage to produce IL-12, or IL-10 in vitro in response to miltefosine was investigated by comparison to non infected cells and cells with medium that were used

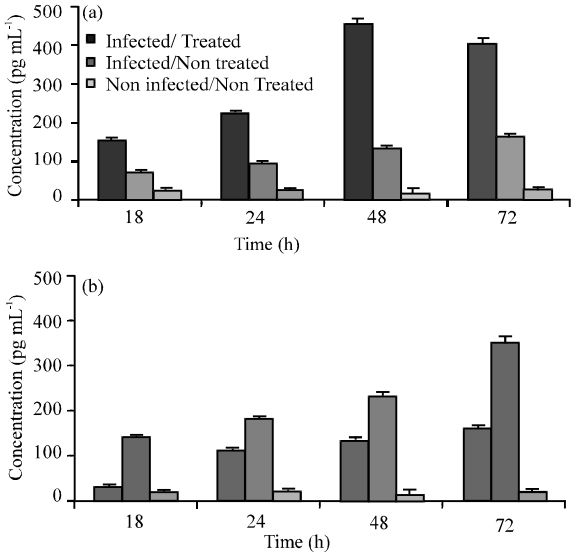

Fig. 3: Cytokines IL12 (a) and IL-10 (b) production by J774 cell line stimulated with miltefosine in different time pointe. Each point represent the mean of 3 independent tests.

as negative controls. IL-12 is important cytokine for stimulation of IFNγ secretion, changing macrophages to effector cells and control of the intracellular leishmania. Cell culture supernatant for endogenous production of IL12 was tested. Results showed that IL12 increased in *L. major*- infected cells, continuously in different time point as well as 48 and 72 h after exposure significantly. As far as IL12 production was concerned, compared to infected/ treated J774 cells, in the infected/ non treated cells cytokine was low in each phase of incubation (Fig. 3). In a typical macrophage response, besides IL-12, other cytokines such as IL10 have been shown to be important factors in the regulation of immune responses. In leishmaniasis the role of IL10 is suppressive cytokine. The pattern of production of this cytokine in this study is presented in Fig. 3 was lower compared with infected/ non treated cells ($p<0.05$). Results showed that ED50 dose of miltefosine The concentration of IL10 production in the culture supernatants from infected/treated J774 cells induce a significant increase level of IL12 and decrease IL10 after 72 h exposure. ($p<0.005$) (Fig. 4).

DISCUSSION

The purpose of this study was to determine whether the miltefosine, could induce leishmanicidal activity and, explore potential immunomodulatory mechanisms of action.

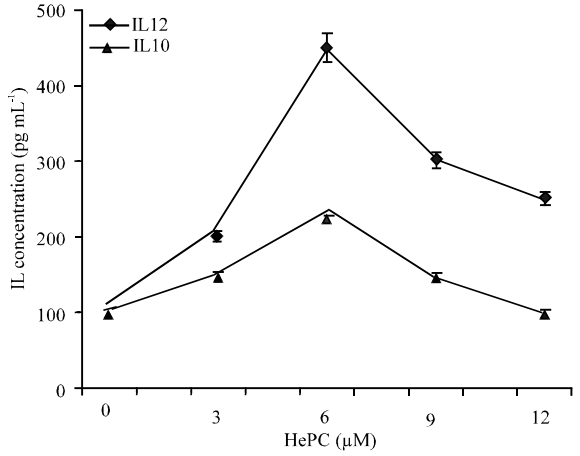

Fig. 4: Concentration of IL-10 and from treated J774-infected cell supernantant after 72 h. Each point represent the mean of 3 independent tests

The principal observation in this study was that miltefosine beside direct cytotoxic effect have macrophage-mediating leishmanicidal activity in vitro. Following increased cases of drug resistance to conventional treatment of Cutaneous Leishmaniasis (CL) in the Old World (Nilforoushzadeh *et al*., 2006), alkylphosphocholines such as miltefosine, edelphosine, perifosine been proposed as new drugs, but so far not enough studies on their effectiveness on the causative agents of old world cutaneous leishmaniasis has been conducted (Saki *et al*., 2009; Khademvatan *et al*., 2011c; Hadighi *et al*., 2007). Initial studies on the tumor cell lines, indicating a direct effect of the long-chain fatty acid of drug on membrane lipids synthesis resulting in inhibition of cell death signals (Berkovic *et al*., 1995). Previous studies indicated, miltefosine has direct effect and capacity to induce apoptosis in leishmania promastigotes (Khademvatan *et al*., 2009, 2011c, 2011b; Qadoumi *et al*., 2002; Croft *et al*., 1987).

Also some of studies indicated indirect effects and Immunomodulator ability of miltefosine (Buates and Matlashewski, 1999; Gupta *et al*., 2002; Zhang and Matlashewski, 2008). These findings showed host cell-dependent mechanism of anti-leishmanial action of miltefosine. Since functional tests such as No measurement not able to detect molecular mechanism of miltefosine, to highlight this rout regulate immune response by miltefosine we consider iNOS and IFNγ genes expression.

In this study RT-PCR method was detected IFNγ gene expression, in the *L. major* infected J774 cell line, 4 h after treatment with miltefosine. In a similar study *leishmania* infected macrophages exposed with miltefosine showing that the mRNA Cytokine have been increased dramatically (Wadhone *et al*., 2009).

Wadhone *et al*. (2009) showed that in addition to the direct effect miltefosine, activation of macrophages and enhance via IFNγ induced STAT-1 activation and also IFNγ receptors increasing could be the possible reason of the drug in the treatment of leishmaniasis. Macrophages activation by IFNγ what autocrine by macrophages or paracrine by T cells to control of parasite intracellular infection is essential.

Several *in vivo* and *in vitro* studies showed that IFNγ is very important factor in the activation of macrophages responsible against *Leishmania*.

In resistant mice to *L. major*, If IFNγ genes and their receptors be evacuated and inhibited, the infection becomes fatal (Sharma and Singh, 2009).

In addition our results, was found miltefosine are capable inducing iNOS gene expression in *L. major* infected macrophage 4 hours after treatment that eventually led to the production of nitric oxide radicals and elimination of the parasites. Also miltefosine was able to increase iNOS gene expression in non-infected cells in lower rate. NO is an important functional arms or destruction for parasites elimination. Inhibition of NO production causes macrophages fail to inhibit parasite proliferation. NO inhibitors in Leishmania major resistant mice make them unable to control of the infection. According to these findings, it is probable that increased iNOS expression is one of miltefosine effective mechanisms to control of the *Leishmania* infection and RT-PCR method in this study confirmed this true.

In support of our results, Wadhone *et al*. (2009) showed that IFNγ and iNOS genes in *L. infantum* infected-macrophages has important role in the response to treatment with miltefosine (Wadhone *et al*., 2009). These researchers showed that miltefosine able to activate signaling mediators (signaling intermediates in macrophages) such p38MAPK that plays an important role in iNOS gene expression and death of amastigotes within the macrophages.

In addition in vitro studies have shown that the addition of IFNγ to *Leishmania* infected macrophages from resistant or susceptible mice cause activation of these cells and the parasite was removed (Sharma and Singh, 2009).

In contrary of these results, Murray shown that miltefosine did not involved immune system to apply its anti-leishmanial ability (Murray and Delph-Etienne, 2000). Also Griewank *et al*. (2010), unlike our findings and

Wadhone *et al*. (2009) showed that miltefosine in dendrite cells infected with *Leishmania* will not be created iNOS gene expression therefore do not produce NO. These paradoxical findings, no activation by meltifosin in DC need to more study (Griewank *et al*., 2010).

IL10 cytokine inhibitory was assayed by ELISA in J774 cell line infected with and non-infected with *L. major* treated with miltefosine EC50 dose. Results shoed that showed that miltefosine is able to suppress IL-10 production. Although this issue requires further investigation. In contrast, IL-12 was observed increasing level at different time point. Previously Murray (1997) confirm that endogenous IL-12 regulates acquired resistance in experimental visceral leishmaniasis that this Pointe is another advantage of miltefosine To confirm the production of proteins iNOS and IFNγ involved in the cellular immunity, western blotting analysis showed significant increases in both proteins in the miltefosine exposed *L. major* infected cells. While the rate of increase in the control group had no significant rate this condition showed the miltefosine importance in activating cellular immunity against the parasite. In recent years, efforts to use immunomadulator compounds such as imiqumod and reziqumod have been used to treat CL that these activate the immune system (Buates and Matlashewski, 1999; Gupta *et al*., 2002; Zhang and Matlashewski, 2008).

Buates and Matlashewski (1999) indicate that tested imidazoquinolines did not demonstrate direct toxic effects against leishmania but could release of NO from both infected and noninfected macrophages (Gupta *et al*., 2002) but our results showed that the miltefosine not only has direct toxic effects against axenic promastigotes or amastigotes but also could induce the expression of the iNOS and IFNγ genes.

CONCLUSION

In conclusion these findings argue that the macrophage leishmanicidal activity induced by miltefosine is due to their ability to stimulate iNOS and IFNγ gene expression. This ability can candidate the miltefosine for the effective treatment of CL especially in case of inadequate immunity.

ACKNOWLEDGMENTS

This study is conducted and financially supported by grants No: U-90026 from Ahvaz Jundishapur Medical sciences. We should appreciate all staff of Protozoology laboratoryof Ahvaz Jundishapur Medical sciences. The authors declare no conflict of interests.

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