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Original Article

Induction of Apoptosis by Miltefosine in Iranian Strain of *Leishmania infantum* Promastigotes

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Abstract

Background: Miltefosine is a new drug of choice for the treatment of visceral leishmaniasis. Numerous experimental studies have shown miltefosine is effective on *Leishmania donovani*, however, effectiveness of miltefosine in treatment of *L. infantum* is not fully understood. The aims of the present study were to evaluate cytotoxic effects of miltefosine on Iranian strain of *L. infantum*, and to determine its 50% inhibitory concentration (IC50) as well as lethal dose.

Methods: Anti-*L. infantum* activity of miltefosine was studied by treatment of cultured promastigotes with various concentration of miltefosine. MTT assay was used to determine *L. infantum* viability and the results were expressed as IC50. Annexin-V FLUOS staining was performed to study apoptotic properties of this drug by using FACS flow cytometry.

Results: Miltefosine led to dose-dependent death of *L. infantum* with features compatible with apoptosis including cell shrinkage, DNA laddering, and externalization of phosphatidylserine with preservation of integrity of plasma membrane. The 100% effect was achieved at 22 µM and IC50 after 48 hours of incubation was 7 µM.

Conclusion: Miltefosine exerts cytotoxic effect on Iranian strain of *L. infantum* via an apoptosis-related mechanism.

Key words*:* Leishmania infantum*, Miltefosine, IC50, Apoptosis, Iran*

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Introduction

Leishmaniasis is one of the significant causes of morbidity and mortality in several countries. This disease affects 12 million people and threatens an additional 350 million people worldwide (1). It is manifested in several forms including visceral, mucocutaneous, or cutaneous. The most severe form is visceral leishmaniasis (VL) or kala azar, which is fatal in 90% of untreated patients. VL is more frequently observed in developing countries with an estimated incidence of 500,000 per year (1-3). *Leishmania infantum* is widespread in the Mediterranean areas and Iran. Infection by this species can lead to severe, often lethal, disease in dogs that are the main animal reservoir and children under 9 years old (4, 5).

Although pentavalent antimonials and amphotericin B have been used in the treatment of VL, these medications have several limitations including resistance to pentavalent antimonial drugs, parenteral rout of administration, long duration of treatment, and unwanted side effects (6). Miltefosine (HePC), an alkylphosphocholine, which was originally developed as an anticancer drug has been proved an effective oral treatment for VL with a less side effects and a cure rate of about 98% (7). It was also effective for treatment of patients with antimony-resistant visceral (8), and cutaneous leishmaniasis (9). Therefore, HePC is a suitable candidate to be based for designing new antileishmanial drugs. In Iran, limited studies have been done for use of miltefosine against cutaneous leishmaniasis (10, 11), but we have no any documented report about *L. infantum*.

A number of studies have been performed to elucidate the mechanism of action of HePC. The antineoplastic activity of HePC has been attributed to its apoptosis-inducing potential (12). Apoptosis has also been proposed as the mechanism of antiprotozoal activity of this medication (13). It was initially believed that apoptosis does not occur in unicellular organisms but, to date, there is enough evidence to confirm that this phenomenon also occurs in single-cell organisms (14, 15). The mechanisms and pathways that lead to induction or inhibition of apoptosis in *Leishmania spp.* are of particular interests as they will be potential targets for development of anti-*Leishmania* medications.

In most studies on the effects of HePC on *Leishmania*, *L. donovani* has been used as the model organism. These works have shown that HePC induces apoptosis or apoptosis-like cell death in *L. donovani* (13, 16). One of the identified mechanisms of action of HePC-induced apoptosis is targeting of DNA topoisomerases by this drug (17). However, it is not yet established whether miltefosine can induce apoptosis or apoptosis-like death in all forms of *Leishmania* parasites (13, 15, 16, 18). Meanwhile, despite many advances in the field biomedical research in Iran (19), data on effective dose of miltefosine and its exact mechanism on the Iranian strain of *L. infantum* (MCAN/IR/96/LON49), which leads to Mediterranean type of visceral leishmaniasis, is insufficient.

The aim of present work was to find IC50 of HePC and study the cell death process induced by HePC in Iranian standard strain of *L. infantum* promastigotes.

Material and Methods

Materials

Miltefosine (1-*O*-hexadecylphosphocholine) with structural formula C21H46NO4P and molecular weight 407.57 was prepared from Zentaris GmbH (Zentaris, GmbH, and Frankfurt, Germany).

Parasite culture

L. infantum (MCAN/IR/96/LON49) and *L. major* (MRHO/IR/75/ER) were kindly provided by Dr. Mohebali (Tehran University of Medical Sciences). Briefly, 5×10^5 cells/ml were cultured in RPMI 1640 medium (pH 7.2, containing 25 mM HEPES) (Sigma, Chemical Co., St.

Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics at 24 °C for 96 h and subcultured at cell densities of 2×10^7 to 2.5×10^7 cells/ml. After subculture, promastigotes were seeded in 96-well culture plates at a density of 2×10^6 cells/ml and treated with HePC in final concentrations ranging from 1-100 µM. HePC was added in triplicate at final dilutions ranging from 1 to 100 µM. The plates were incubated at 25 °C for 48 h before MTT assay. All tests were performed in triplicates.

Cell proliferation measurements by colorimetric MTT assay

MTT [3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] colorimetric assay measures reduction of MTT dye (tetrazolium) into formazan by mitochondrial enzymes in viable cells. Relative numbers of live cells were determined based on the optical absorbance of the treated and untreated samples and blank wells using the following formula (Verma and Dey 2004): Viable cells (%) = $(A_T - A_B) / (A_C - A_B) \times 100$.

Where, A_C is the absorbance of the untreated samples, A_T is the absorbance of the treated samples, and A_B is the absorbance of the blank. All values are means of triplicate wells. Results were expressed as the concentration that inhibited parasite growth by 50% (IC50).

Flow cytometry analysis of cell death

The Annexin-V FLUOS Staining Kit (Roche, Germany) was used for the detection of apoptotic and necrotic cells according to the manufacturer's protocol. Briefly, promastigotes were washed in cold phosphate-buffered saline (PBS) (×2) and centrifuged at 1400 g for 10 min. Then, they were incubated for 15 minutes in dark and at room temperature in 100 µl of Annexin-V FLUOS in the presence of PI. Afterwards, the samples were analyzed with FACSCalibur flow cytometer (Becton Dickinson and CellQuest software), and the percentage of positive cells was determined for each sample.

DNA ladder assay in the presence and absence of miltefosine

Qualitative analysis of total gDNA fragmentation was performed by agarose gel electrophoresis. Briefly, promastigotes (5×10^6 cells) were incubated and harvested in different time points. An apoptotic DNA ladder kit was used to extract DNA from apoptosis-induced and un-induced cells according to the manufacturer's instructions. DNA (10 µg DNA samples) was electrophoresed in 1.5% agarose gels at 100 V for 2 h, visualized by using an UV transilluminator and photographed.

Determination of promastigotes' morphology after treatment with HePC:

To observe changes in cell morphology, promastigotes treated with or without miltefosine (IC50), were examined. Briefly, cells were centrifuged at low speed (1000 g) and the pellets suspended in PBS. Changes in morphology were observed under ×100 objectives on a light microscope. Alteration of cellular morphology was studied in different time points, and for each sample, at least 10 microscopic fields were observed under ×100 objectives.

Statistical analysis

In vitro anti-leishmanial activity, expressed as IC50 (50% inhibitory concentration), was determined by linear regression analysis.

Results

Anti-leishmanial activity of miltefosine

Cytotoxic potential of miltefosine on *L. infantum (*MCAN/IR/96/LON49*)* promastigotes was tested using the MTT assay in order to determine 50% inhibitory concentration (IC50) of this drug. Miltefosine showed a dose-dependent cytotoxic effect with almost 100% death at a concentration of about 22 µM (Fig. 1). However, for Iranian strain of *L. major* (MRHO/IR/75/ER), 100% death occurred at

concentration of 32 µM. Treatment with HePC resulted in a concentration-dependent inhibition of Iranian strain of promastigotes' viability with an IC50 of 7 µM.

Direct microscopic examination

Cells were treated with 7 µM miltefosine and both treated and untreated (control) cells were observed with a light microscope at 4 h intervals up to 48 h. The number of promastigotes at the time of drug treatment was 2×10^6/ml. The untreated control cells continuously grew up to 10-12 $\times$ 10^6/ml in 48 h. The treated cells showed proliferative activity up to 8 h post-treatment during which they have grown up to 4-5 $\times 10^6$/ml in number. However, they were reduced in number from 8 h and were decreased to 50% at 16 h post–treatment. The number of live promastigotes remained unchanged from 16-48 h post-treatment (Fig. 2).

Microscopic examination of the treated cells showed that cell shrinkage starts around 4 h after drug treatment. Almost all the treated cells showed cytoplasmic condensation, shrinkage, and reduction in size compared to the control samples at the end of 48 h treatment (Fig. 3-A).

Flow cytometric analysis

Following treatment of promastigotes with 22 µM HePC for various time points [4, 12, 18, 24, 36, and 48 hours], flow cytometric analysis was performed after labeling with Annexin-V FLUOS and the percentages of viable, necrotic and apoptotic cells were determined for each time point. During the early stages of metazoan programmed cell death, several alterations occur in plasma membrane. One of these changes is translocation of phosphatidylserine from the inner side of cell membrane to its outer side. Fluorescein-conjugated Annexin-V is used to detect the externalized phosphatidylserine as it has a high binding affinity to this phospholipid component. Additionally, annexin-V FLUOS allows distinguishing between apoptotic cells (annexin V positive, PI negative), necrotic cells (annexin positive, PI positive), and surviving cells (annexin V negative, PI negative).

After 18 h of incubation of *L. infantum* promastigotes, 11.7% of the treated cells and 3% of the control cells were Annexin-VFLUOS positive. After 24 h of incubation, positivity was 22.6% for treated cells and 3.52% for the control cells. After 36 and 48 h of treatment, the percent of annexin-positive cells were 58% and 80%, respectively, whereas the corresponding figures in the control group were just 4% for both time points (Fig. 3-B).

In several time point miltefosine did not induce necrosis even after a prolonged incubation, as all the cells remained negative for PI.

DNA ladder assay

DNA fragmentation is one of the classic features of apoptosis in eukaryotic cells. HePC induced DNA fragmentation in promastigotes of *L. infantum (*MCAN/IR/96/LON49*)* confirmed by presence of DNA fragments in agarose gel electrophoresis. The fragments were in oligonucleosome size (in approximate multiples of 180-200 bp) in promastigotes treated with 22µM HePC for 24 h, whereas untreated cells did not show DNA fragmentation (Fig. 4A). No DNA fragmentation was observed in promastigotes treated with lower concentrations of HePC for 24 h.

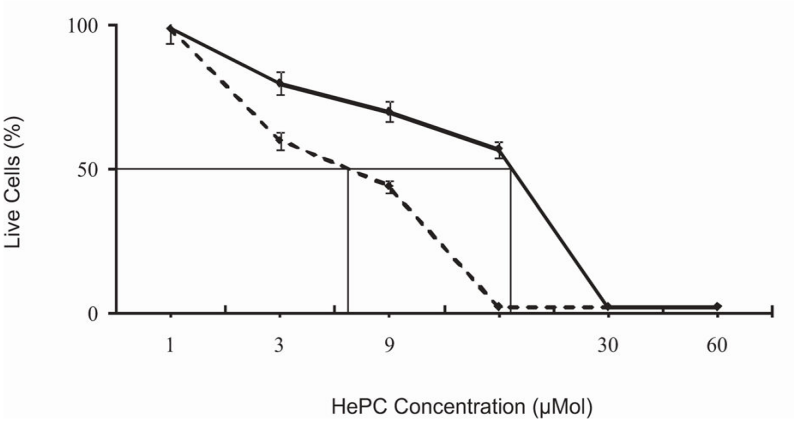

Fig. 1: The viability of *Leishmania infantum* promastigotes (MCAN/IR/96/LON49) and *Leishmania major* (MRHO/IR/75/ER) in the presence of various concentrations of HePC was assessed by MTT. Each point represents the means of 3 independent tests.
Dotted line: *Leishmania infantum* promastigotes (MCAN/IR/96/LON49), solid line: Leishmania major (MRHO/IR/75/ER)

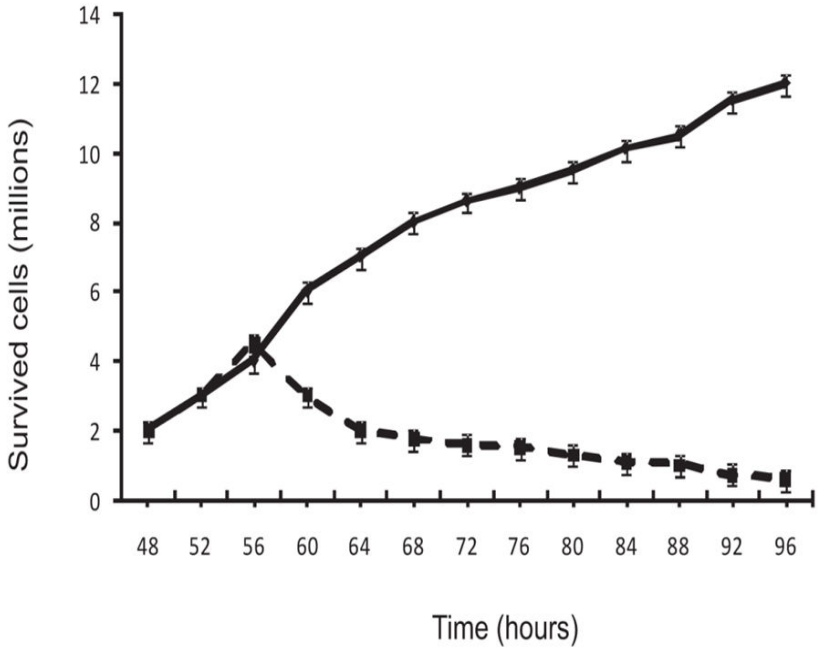

Fig. 2: Number of promastigotes treated with or without miltefosine (7 µM) at different time points after logarithmic phase (48 h).Solid line: control group and dotted line: treated group

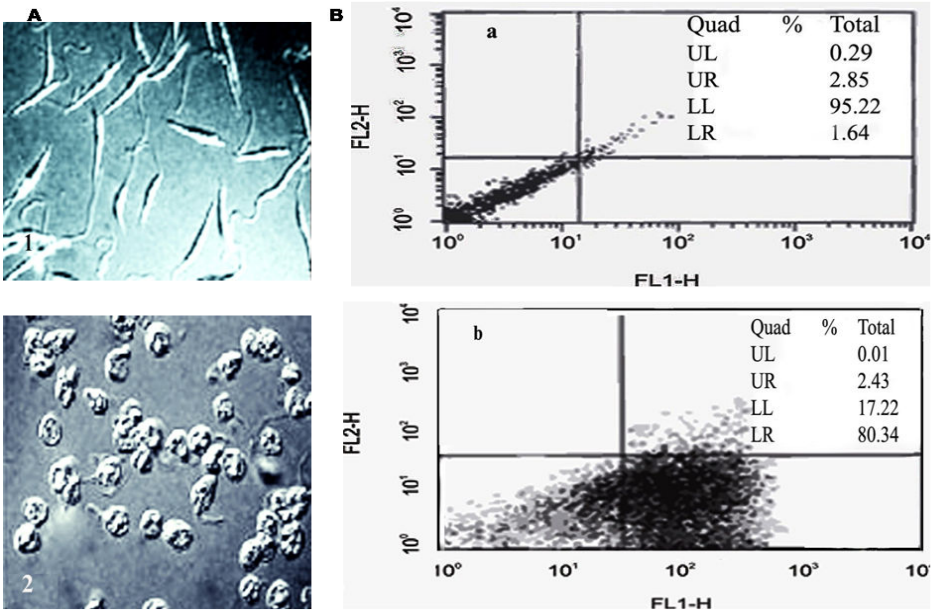

Fig. 3: A) Morphology of *Leishmania infantum* (MCAN/IR/96/*LON49*) in light microscopy (magnification, x 100) at different time points after Miltefosine (7µM) treatment. (1) 0 hours after treatment and (2) 48 hours after treatment. B) Flow cytometry analysis of promastigotes following treatment with 22 µM HePC and after labeling with annexin-V and PI. Miltefosine did not lead to necrosis even after a prolonged incubation, as all the cells remained negative for PI. Lower right region (LR) belongs to apoptotic cells (annexin positive) and upper left region (UL) belongs to necrotic cells (PI positive). (a) is control, and (b) is result after 48 h

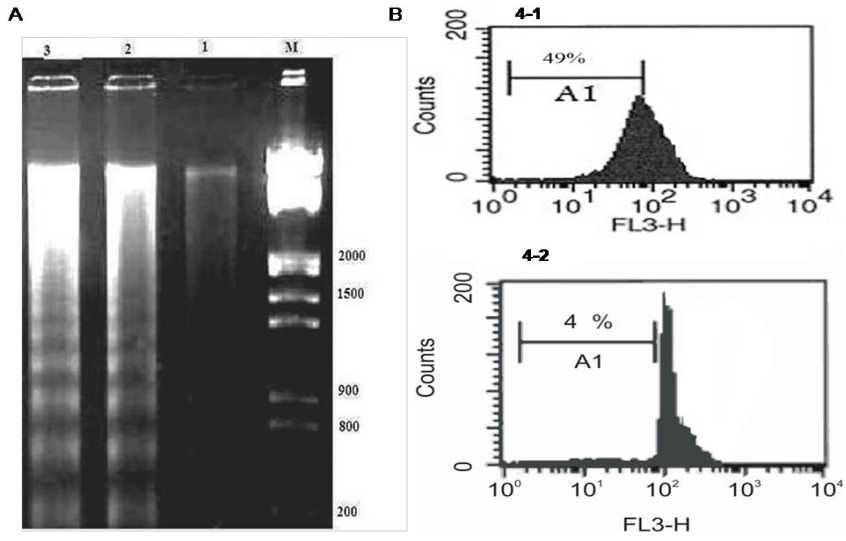

Fig. 4: A) DNA fragmentation analysis by agarose gel electrophoresis. The DNA of untreated (Lane 1), 22 µM HePC-treated *leishmania infantum* promastigotes (Lane 2) and 32 µM HePC-treated *leishmania major* promastigotes (Lane 3) after 24 h of incubation. B) The Percentages DNA content of *Leishmania infantum* promastigotes by flow cytometry, found in the sub-G1 peak: untreated (4-1) or treated with 22 µM miltefosine (4-2). FL3-H, fluorescence intensity

Discussion

In the present study, we evaluated the leishmanicidal activity of miltefosine on the Iranian strain of *L. infantum*. It showed cytotoxicity for this strain and exerts its effect by induction of apoptosis. The current medications for treatment of VL are pentavalent antimony, pentamidine and amphotericin B. However, their use is limited due to their high toxicity (20). Antimonials are the first line of treatment of VL, but their mechanism of actions is not fully understood. They inhibit some metabolic pathways in *L. spp*. The value of antimony therapy in treatment of VL is limited due to side effects of its high doses, development of drug resistance (21, 22), and marginal efficiency in treatment of patients co-infected with human immunodeficiency virus (21).

Miltefosine is the only oral drug that has recently developed for treatment of leishmaniasis. It is undergoing clinical trial in several countries. It has been shown that it induces apoptotic death in *L. donovani* marked by nuclear condensation, and DNA fragmentation and ladder formation (13, 16). Other compounds under study are dihydrobetulinic acid (DHBA), luteolin and quercetin. DHBA, a pentacyclic triterpenoid, is a novel lead compound that induces apoptosis by targeting DNA topoisomerases (15, 23). Luteolin and quercetin are effective compounds that exert their antileishmanial properties by cell cycle progression arrest and induction of apoptosis (24).

In our study, IC50 of HePC on the *L. infantum* promastigotes was determined by MTT assay. We have determined an IC50 of 7 µM for *L. infantum* (strain MCAN/IR/96/LON49), which is lower than what reported for other *Leishmania* species. The reported IC50 for two different strain of *L. donovani* are varying from our study. *Navin et al*. found IC50 of Indian strain of *L. donovani* (MHOM/80/IN/Dd8) 13 µM(13).

In another study, Paris *et al.* determined Promastigote forms of wild-type (WT) *L. donovani* (strain MHOM/ET/67/HU3/L82) 25 µM IC50 (16). These results show wide range of drug dosage for *L. donovani*. In contrast, Iranian strain of *L. infantum* promastigote is very sensitive to miltefosine.

Induction of apoptosis or apoptosis-like cell death is one of advantages of miltefosine against other currently used drugs, including antimony, as it does not lead to provocation of an acute immune response. So far, apoptosis has been described in at least nine species of unicellular organisms including *Leishmania* (25). Promastigotes of *L. donovani* were particularly studied for induction of apoptosis and it has been shown that they undergo apoptosis after exposure to some agents and nutrient deficiencies that cause apoptosis in higher eukaryotes (26). The process of apoptosis shares many similar features in both metazoan and protozoan organisms (14, 15). The two prominent features observed in both these eukaryotes are DNA condensation and DNA laddering. In the present work like Verma *et al.* study, *L. donovani* was treated with HePC (18). We have shown that treatment of *L. infantum* promastigotes with HePC induces a cell death that shares most features associated with metazoan apoptosis. Those include cell shrinkage, DNA laddering, and externalization of phosphatidylserine with preservation of integrity of the cell membrane (13, 16).

Cells after PI labeling were analyzed with Flow cytometry to quantify the percentage of pseudohypodiploid cells. The DNA content in cells had direct relation with the amount of fluorescence intensity and in apoptotic cells DNA degradation translates into PI intensity lower than that of G1 cells (sub-G1 peak). Treatment of cells with 22µM miltefosine after 24 h showed that 49% of the promastigotes were found in the sub-G1 peak region but in the control group only 4% of promastigotes were found in the sub-G1 peak region (Fig. 4B), thus this finding indi-

cates that miltefosine had induced DNA fragmentation in treated promastigotes.

From these data, we conclude that HePC kills *L .infantum* by apoptotic and not necrotic cell death. In addition, our finding of concentration- and time-dependence of DNA fragmentation induced by miltefosine is in agreement with other reports (13, 26). As miltefosine has been in clinical use for treatment of leishmaniasis in recent years, a better understanding of mechanisms of its action may help in finding of new targets for treatment of *Leishmania* parasites.

In conclusion, our findings indicate that HePC induces apoptosis in Iranian strain of *L. infantum* promastigotes with a dose lower than what reported for other species of *Leishmania* in their endemic areas.

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