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Original Article

Evaluation of In Vitro Cytotoxic and Apoptotic Effects of Miltefosine on the *Toxoplasma gondii* RH Strain

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Abstract

Background: We aimed to investigate the cytotoxic and apoptotic effects of miltefosine on *Toxoplasma gondii* RH strain by various techniques.

Methods: The study was conducted at the Department of Parasitology and Mycology, Urmia University of Medical Sciences, Iran in 2020. Four groups of five BALB/c mice were selected. The cytotoxicity test was conducted by adding miltefosine to *T. gondii* tachyzoites; control tachyzoites received PBS and MTT assay was done on each suspension. For evaluating the Th1-type immune responses, the serum levels of IFN-γ and nitric oxide (NO) were assessed in mice after injecting tachyzoites and miltefosine, respectively. The flow cytometry technique was performed on *T. gondii* tachyzoites challenged with IC_{50} and IC_{90} doses of miltefosine and unchallenged cells. DNA fragments in *T. gondii* tachyzoites were detected by Terminal dUTPnick-end labeling (TUNEL) method.

Results: Overall, 256, 64, 32, and 16 µg concentrations of miltefosine, respectively could kill more than 50% of viable *T. gondii* tachyzoites. The infected mice group, treated with miltefosine, significantly produced more IFN-γ relative to other groups ($P< 0.001$). Moreover, a significant difference was found in inducible NO synthase between the experimental and control groups ($P<0.05$). The flow cytometry results demonstrated a concentration-dependent apoptosis rate in tachyzoites incubated with miltefosine, though the necrosis rate was non-significant. DNA fragmentation analysis indicated oligonucleotides (18-200 bp) in tachyzoites treated with 11µg of miltefosine for 24, 48 and 72 h. However, this pattern was not observed in untreated control microorganisms.

Conclusion: Miltefosine could be a favorable candidate for use as a new treatment for toxoplasmosis.

Introduction

The intracellular parasite, *Toxoplasma gondii*, is a cosmopolitan apicomplexan protosa important in human and veterinary medicines(1-2). It is one of the most significant zoonotic feline gastrointestinal parasites transmitted to humans without the help of intermediate hosts or vectors (3). The protist could virtually propagate in all warm-blooded mammalian species on all continents (4). One-third of the global human population is seropositive for *T. gondii* infection (5).

Sexual replication occurs in the small intestine cells of the definitive hosts (Felidae family), while asexual replication happens in the nucleated cells of almost all warm-blooded animals and humans (1). Humans get the infection through various routes via oocyst-contaminated water, consumption of raw/undercooked meat products containing tissue cysts, and vertical transmission and tissue graft recipients and infected blood supplies (5). In immunocompetent individuals, toxoplasmosis is often a self-limiting mild disease with no pathognomonic symptoms, including fever, malaise, and lymphadenopathy. However, in pregnant women and immunocompromised patients, the infection is clinically significant because it may develop serious consequences such as splenomegaly, pneumonitis, retinochoroiditis, encephalitis, and fetal abortion or fetus birth with physical or mental abnormalities (4). The disease outcome is remarkably reliant on immune functions, the genetic background of the host, and the parasite genotype (6).

The treatment options for toxoplasmosis vary based on age, symptoms, the host's immune status, and the period of pregnancy in which the infection occurs (4). The standard therapy regimens for toxoplasmosis are pyrimethamine combined with sulfadiazine or clindamycin (7). The foremost limitations of these therapeutics reside in their side effects. Pyrimethamine shows antifolate activity and gives rise to dose-dependent bone marrow suppression that leads to neutropenia and thrombocytopenia. Regarding sulfonamides, hypersensitivity reactions are somewhat commonplace, particularly in AIDS patients. Moreover, anti-*Toxoplasma* medicines are chiefly active against acute infection; thus, it is possible that treated patients still possess the infection in a latent phase (7-8). By virtue of such issues, there is an urgent need to design, formulate and widely evaluate novel therapeutic agents for toxoplasmosis.

Recently, repurposing existing drugs has broadly been emphasized, which facilitates the intricate process of drug discovery process and avoids extra costs (9). Miltefosine, a hexadecyl phosphocholine (HePC), was first introduced as an anticancer agent in the early 1980s (10). Later, it was approved as a therapeutic drug for treating visceral leishmaniasis (VL) due to *Leishmania donovani* in India, antimony-resistant VL infections, cutaneous leishmaniasis in South America, *Trypanosoma* spp. and free-living amoeba infections such as *Acanthamoeba* spp., and the brain-eating amoeba infections such as *Naegleria fowleri*(11-15).

We aimed to address the cytotoxic and apoptotic effects of miltefosine on *T. gondii* RH strain using various techniques.

Materials and Methods

Ethical Issues

The study was approved by the Ethics Committee of Urmia University of Medical Sciences, West Azerbaijan Province, Iran (ethical code: IR.UMSU.REC.1397.395).

The study was conducted in 2020 at the Department of Parasitology and Mycology, Urmia University of Medical Sciences, Urmia, Iran.

Mice and parasites

Laboratory animals were 20 inbred BALB/c mice (five per each group) of 20-25 g weight aged 7-8 weeks. The mice were kept at the

optimum ambient temperature and had accessibility to water and food supplies according to the guidelines of the Ethics Committee of Urmia University of Medical Sciences, West Azerbaijan Province, Iran. Experimental groups were as follows: unchallenged mice treated with miltefosine (Healthy + HePC), challenged mice treated with miltefosine(Toxo + HePC), challenged mice without any treatment (Toxo + No treatment), and negative control receiving only PBS (control). *T. gondii* type I (RH strain) virulent tachyzoites harvested 3-4 days postinoculation(injection of 1×10^5 parasites into mice peritoneal cavity) were kept in sterile phosphate-buffered saline (PBS; pH 7.4) containing 100 µg/ml of streptomycin and 100 U/ml of penicillin and were used for intraperitoneal (IP) challenge (16).

Cytotoxicity test by methylthiazol tetrazolium (MTT) assay

For this aim, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/ml concentrations of miltefosine were added to1×10^5 fresh viable *T. gondii* tachyzoites in sterile tubes. The contents were mixed gently and incubated at 37 °C for6, 24, 48, and 72 h. Subsequently, alive tachyzoites were enumerated by trypan blue staining and light microscopy. The MTT assay was conducted on 200 µl of each suspension. This assay relies on the formation of formazan crystals within the mitochondria of alive cells. The total activity of this organelle is associated with the number of viable cells; therefore, the in vitro cytotoxic effects of drugs can be measured on various cell lines. Briefly, 10 µl of MTT substrate was added to the test and control samples, followed by incubation with 5-8% CO_2 at 37 °C for 24, 48, and 72 h. In the final step, formazan crystals were dissolved by adding 50 µl of dimethyl sulfoxide (DMSO) solution. The absorbance rate was determined at 570 nm (reference filter: 630 nm) by an automated ELISA reader (17-19). Non-viable *Toxoplasma* tachyzoites were calculated by the following formula, where AT refers to the optical density (OD) of the treated well, AB is the OD of blank well (containing culture medium), and AC implies the OD of negative control (without treatment):

$$\text{Non-viable (\%)} = 100 - \frac{AT-AB}{AC-AB} \times 100$$

The results were expressed as the concentration that inhibited Toxoplasma parasite growth by 50% (IC50: half maximal inhibitory concentration).

Measurement of IFN-γ

After four days of the treatment, blood samples were collected from infected mice of all groups, and the serum samples were tested for serum levels of IFN-γ. Sera were isolated by centrifugation (3000 rpm, 5 min), and the serum level of IFN-γ was measured (1) according to the manufacturer's kit protocol (Mouse Interferon gamma ELISA Kit [IFNG; ab100689 USA]).

Flow cytometry analysis

The flow cytometry technique was performed using Annexin-V to identify the type (apoptosis and necrosis) of the cell death. In brief, 100 µl of binding buffer (0.01 M of HEPES, pH 7.4, 0.14 M of NaCl, and 2.5 mM of $CaCl_2$) combined with 5 µl of each FITC-Annexin V conjugate and propidium iodide (PI) were added to 1×10^5*T. gondii* tachyzoites challenged with IC_{50} of miltefosine and unchallenged cells. Incubation was performed in the dark at room temperature for 15 min. In the next step, 400 µl of binding buffer was added, and the final analysis was accomplished using a flow cytometry device. The results obtained for 24-, 48-, and 72-h incubation were analyzed by CellQuest™software (20-21).

Nitric Oxide (NO) assessment

After four days of the treatment, blood samples were collected from infected mice of all groups, and the serum samples were tested for serum levels of iNOs. Sera were isolated by centrifugation (3000 rpm, 5 min)(22) and the serum level of iNOs was measured according to the manufacturer's kit protocol MouseiNOS ELISA Kit (ab253219, USA)

DNA fragmentation

DNA fragments in *T. gondii* tachyzoites can be detected by Terminal dUTP Nick-End Labeling (TUNEL) method (23). Briefly, 2% paraformaldehyde was added to all experimental organisms, being incubated at 20° C for an hour. Following centrifugation, the cells were impregnated in cold permeation solution (0.1% Triton-X100 in 0.1% sodium citrate) for two minutes, and then washed with PBS and finally incubated in TUNEL reaction agent in a dark room at 37 °C for an hour. A cytometer device (Beckman Coulter, California, USA) was used for the detection of dUTP labeling in the damaged sites of DNA. Qualitative and quantitative analyses of the fragmented genomic DNA (gDNA) were performed using agarose gel electrophoresis. In summary, tachyzoites ($^1\times10^5$) were incubated in a different time (24, 48, and 72 h), and their gDNA was extracted by the DNA ladder kit (DNA Fragmentation Kit. Cat. #6137.Takara, Japan), based on manufacturer's protocol. A volume of 10 µg of DNA was loaded on 1.5% agarose gel, and following the electrophoresis (100 V, 2 h), the results were shown in a UV illuminator.

Data processing and statistical analysis

Results were analyzed by descriptive statistics and non-parametric tests such as Kruskal-Wallis and Mann-Whitney U. A $P < 0.05$ was consideredstatistically significant. All statistics were conducted using SPSS 19 software (IBM Corp., Armonk, NY, USA).

Results

MTT test

Miltefosine at concentrations of 256, 64, 32, and 16 µg could kill more than 50% of viable *T. gondii* tachyzoites (Fig. 1A, B, and Fig. 2A, B).

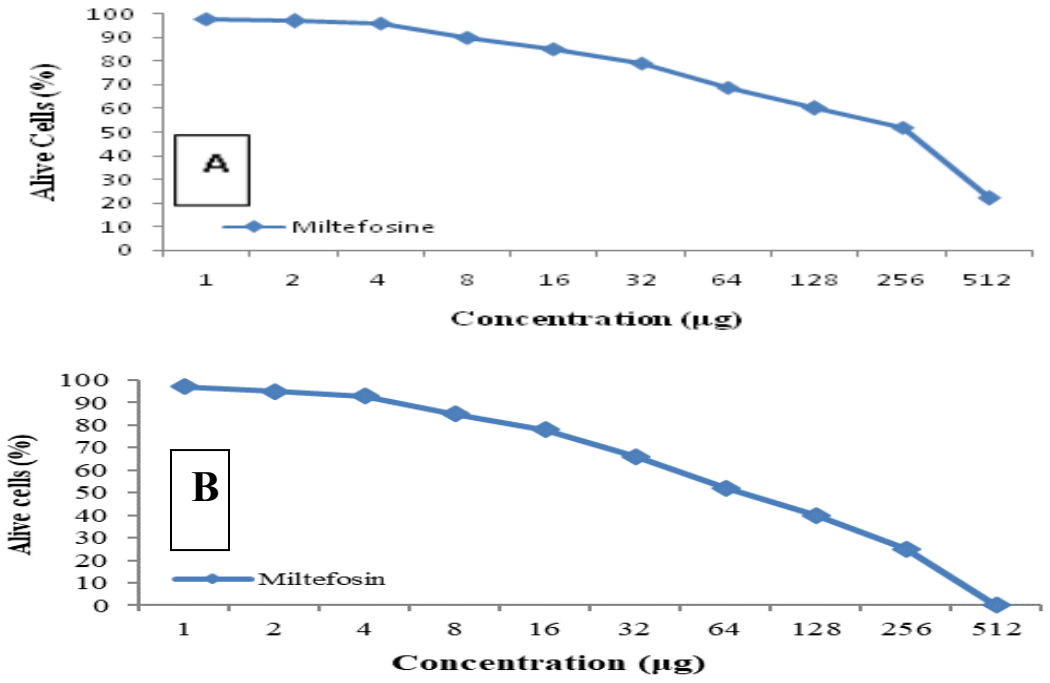

Fig. 1: In vitro infection and the effect of miltefosine on T. gondii tachyzoites after (A)6 and (B)24h of incubation

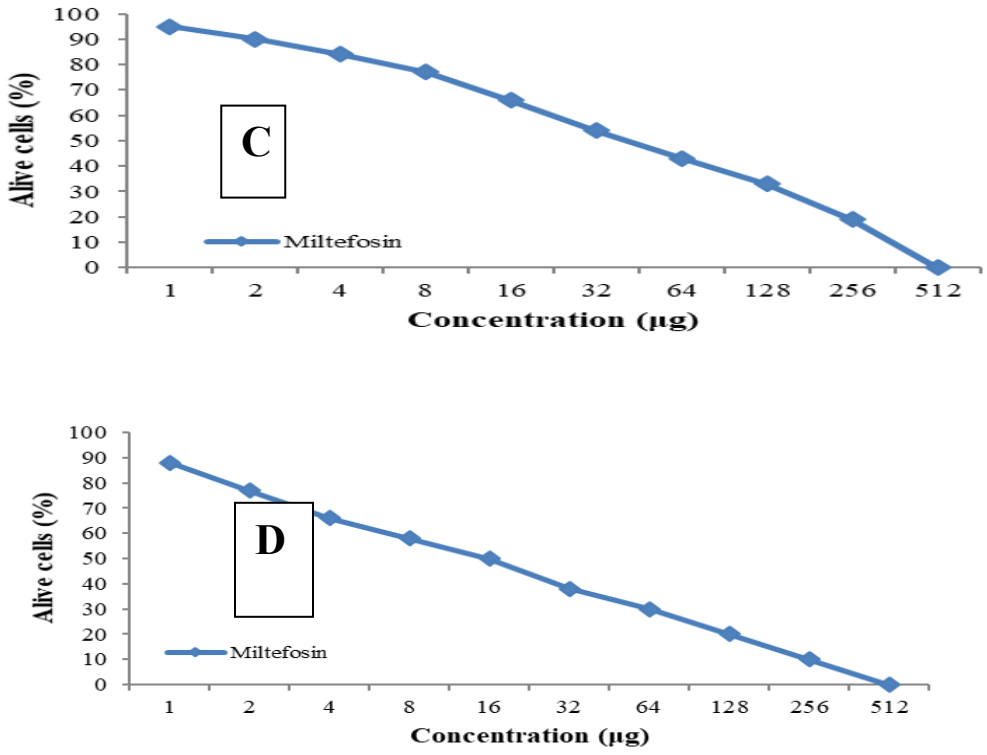

Fig.2: In-vitro infection and the effect of miltefosine on T.gondii tachyzoites after (C)48,and (D)72 h of incubation

Evaluation of IFN-γ production

The infected mouse group treated with miltefosine significantly produced more IFN-γ compared to other mouse groups ($P< 0.001$).

Flow cytometry

A concentration-dependent apoptosis rate was observed in tachyzoites after 24-, 48-, and 72-h incubation with miltefosine; however, the necrosis rate was insignificant. The apoptosis IC_{50} for different incubation periods were as follows: 15.53% (24 h), 47.99% (48 h), and 81.25% (72 h). The detailed results of the flow cytometry analysis are provided in Table 1. Also, the maximum apoptosis rates of the treatment with miltefosine after 48 h (B) and 72 h (C) post-treatment of *T. gondii* RH strain were 56.57% and 86.47%, respectively (Fig. 3A,B,C).

Table 1: Apoptosis and necrosis rates observed in tachyzoites after 24-, 48-, and 72-h incubation with miltefosine

Concentration (mg/ml) Time (h)	*IC_{50}*				*IC_{90}*			
	Apoptosis (%)		Necrosis (%)		Apoptosis (%)		Necrosis (%)	
	Control	Miltefosine	Control	Miltefosine	Control	Miltefosine	Control	Miltefosine
24	9.47	15.53	0.48	0.03	13.58	27.27	0.14	0.20
48	43.43	47.99	7.08	10.90	57.00	65.23	2.97	1.74
72	66.03	81.25	9.76	8.35	70.34	91.58	4.40	4.64

IC_{50}, half maximal inhibitory concentration; **IC_{90},** the concentration inhibiting 90% of the organism

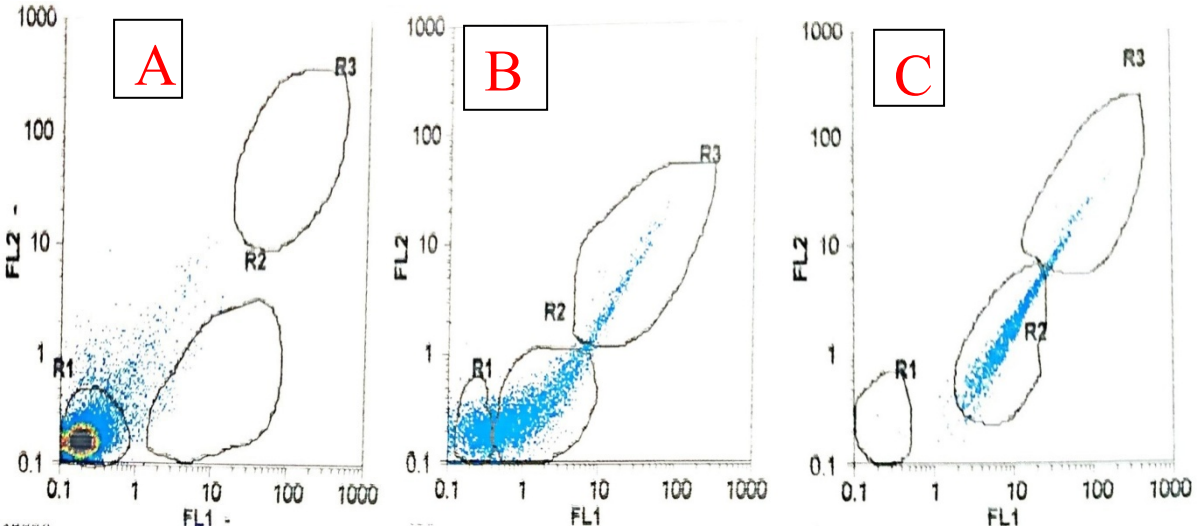

Fig.3: (A,B,C). Flow cytometry results. (A): Control. Maximum apoptosis rates of the treatment with miltefosine after 48 h (B) and72 h (C) post-treatment of T. gondii RH strain

Nitric oxide test

There was a significant difference in the level of inducible NO synthase (iNOS) between the experimental and control groups ($P< 0.05$); the highest iNOS level was found in the tachyzoite-infected group receiving miltefosine(Toxo + HePC).Also, the difference among the groups, Toxo + HePC, Toxo + No treatment, healthy + HePC, and control, was statistically significant ($P< 0.001$)

DNA fragmentation

The analysis of DNA fragmentation pattern using 1.5% agarose gel electrophoresis indicated that oligonucleotides (18-200 bp) in tachyzoites were treated with 11 µg of miltefosine for 24 h, though this pattern was not observed in untreated control organisms. In addition, the DNA fragmentation pattern was found in tachyzoites treated after 48 and 72 h.

Discussion

Notwithstanding the discovery of the cosmopolitan apicomplexan protist, *T. gondii*, 112 years, several biological aspects of this parasite are still open to question. The parasite readily infects human populations at risk, and there, regrettably, is no effective therapy without potential side effects to combat clinical infection (24). Thus, an urgent need seems to be essential to recognize novel drug targets in the protozoan organism, design unprecedented drug formulations, and/or repurpose drugs for use against toxoplasmosis. The last one is of the utmost interest such that it remarkably decreases costs and time for drug development, particularly when there is little inducement to invest in *de novo* drug discovery. Considering the side effects of sulfadiazine or clindamycin in the treatment of toxoplasmosis (21) and owing to the promising impacts of miltefosine on cancers and leishmaniasis etc., we used miltefosine instead of the standard therapy regimens, including pyrimethamine combined with sulfadiazine or clindamycin, for toxoplasmosis. The current original study was conducted to appraise the apoptotic and cytotoxic effects of miltefosine on *T. gondii* RH strains in vitro.

Over the past few years, researchers have conducted a multitude of research on miltefosine. In a study, the possible mechanism of miltefosine-mediated death of *L. donovani* promastigotes (in vitro) and of extra- and intra-cellular amastigotes were investigated. The

results indicated that miltefosine could induce apoptosis-like death in *L. donovani* based on the observed phenomena such as nuclear DNA condensation, DNA fragmentation with accompanying ladder formation, and in situ labeling of DNA fragments by the terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end labeling method. They believed that understanding miltefosine-mediated death will facilitate the design of new therapeutic strategies against Leishmania parasites (25). Our results of the inhibitory efficacy of miltefosine on *T. gondii* agrees with the findings achieved by another study where results showed the anti-parasitic activity of miltefosine against *T. gondii* cyst stage in the chronic experimental toxoplasmosis by a significant reduction in the brain cyst burden. Moreover, they detected considerable morphological changes in the cysts by light and electron microscopy and by the amelioration of pathological changes in the brain (26).

In a study on the anti-parasitic effects of miltefosine, Marinho et al. evaluated the action mechanism of miltefosine (as the first effective and safe oral treatment for visceral leishmaniasis) on *L. amazonensis* promastigotes. Miltefosine could induce a process of programmed cell death, which was determined by the externalization of phosphatidylserine, the incorporation of propidium iodide, cell cycle arrest at the sub-G0/G1 phase, and DNA fragmentation into oligonucleosome-sized fragments. They also elucidated that miltefosine gives rise to apoptosis-like death in *L. amazonensis* promastigote cells, as well as in *L. donovani* (27).

Miltefosine caused a dose-dependent apoptotic activity on the Iranian strain of *L. infantum* parasites, with IC_{50} of 7 μM after 48-h incubation (28). In the same incubation time and IC_{50} of 22 μM and 11 μM, this drug showed cytotoxic properties on *L. tropica* and *L. major* organisms (29). The toxic effect of miltefosine on *L. infantum* has been demonstrated through an apoptosis-associated mechanism in our former investigation in 2011. The results displayed the dose-dependent death of *L. infantum* by miltefosine and severely increased apoptosis and overexpression of metacaspase and PARP genes six hours after treatment (11).

Similar results to our present study were achieved from Khademvatan et al.'s research work, which studied molecular mechanisms and immunomudolatory properties of miltefosine in the J774 cell line infected with the *L. major* (MRHO/IR/75/ER) parasite. They also utilized RT-PCR and western blotting to assess the gene expression of IFN-γ and to analyze their proteins, respectively. Moreover, cell culture supernatant was examined by ELISA for IL-10and IL-12 concentrations. Based on their results, in addition to the direct effect, miltefosine could improve cellular immunity with rising IFN-γ and iNOS gene expression, thus activating macrophages (30).

Despite the positive results obtained from the current research, proving the effectiveness of a drug, especially for human use, has its own complications, which should be investigated by conducting clinical trial studies

Conclusion

More in vivo studies are required to uncover clear evidence of the anti-parasitic activity of miltefosine against *T. gondii* tachyzoites in chronic toxoplasmosis. Meanwhile, drug efficacy against toxoplasmosis depends on the severity of the pathogenicity of different strains of the parasite that needs to be considered .Moreover, using various infection routes can be another area of interest in future studies. We recommend that studies such as those performed on miltefosine can also be conducted on other medications to reduce costs and unwanted side effects of drugs.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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