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Original Article

Biological risk assessment of miltefosine in concomitant infection with opportunistic toxoplasmosis

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Abstract

Introduction: Although miltefosine is the first line for treatment of leishmaniasis, it could have multiple un-recognized effects if any infection accidentally takes place during therapy. The aim is to precisely evaluate the molecular and biochemical remarks of miltefosine on *Toxoplasma gondii* accidental infection during miltefosine therapeutic course.

Methodology: changes implied by miltefosine daily parenteral administration to *Toxoplasma*-infected mice, subcutaneously or intraperitoneal, have been investigated. Tumor necrosis factor-Alfa, immunoglobulin G and M, IL-12 and interferon-gamma release assay (IGRA) were measured in the animals' sera post-miltefosine administration in addition to monitoring Tissue parasite load by measuring the daily changes of copy number of B1 gene using quantitative PCR technique (qPCR).

Results: Miltefosine significantly increased inflammatory and immunological markers (TNF-α, IgG and IgM) measured on reference to control untreated group, with a significant increase in the parasite burden and distribution in all tested organs ($F = 390.9$, $df = 9$, $P < 0.0001$), ($F = 4478.98$, $df = 4.75$, $P< 0.0001$) and ($F = 247.3$, $df = 4$, $P < 0.0001$); heart, liver and lung, respectively, using MANOVA. Releasing capability of macrophages significantly increased during the first day of infection, however, it finally declined after seven consecutive doses of miltefosine ($t = 7.96$, $P < 0.001$).

Conclusion: Miltefosine could not control the pathogenesis and multiplication of accidental *Toxoplasma* infection. Cumulative low parenteral daily doses of miltefosine (1.5 µM) could inversely affected the normal humoral immunity against toxoplasmosis. Therefore, a periodical screening for accidental *Toxoplasma* infection during the course of therapy is strongly recommended.

Key words: miltefosine; toxoplasmosis; parasitic load; IGRA; TNF-alpha; B1 gene.

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Introduction

Miltefosine, (1-O-hexadecylphosphocholine) is a membrane-active synthetic ether-lipid analogue. In spite of the independent and simultaneous discovery of the antiprotozoal and antineoplastic activity of miltefosine by two different scientist groups in UK and Germany, the priority was given to the development of miltefosine as a local treatment for cutaneous metastases of breast cancer, which led to the approval of a topical formulation of miltefosine (Miltex, Baxter, UK). Later, then miltefosine was used orally for treatment of solid tumor after phases of study that were associated with profound gastrointestinal side effects including nausea, vomiting and loss of appetite [1]. However, the superior activity of oral miltefosine was as an anti-leishmanial drug over the standard anti-leishmanial therapy, which resulted in its approval as the only drug available for visceral and cutaneous leishmaniasis in 2002 by WHO [2].

Many of infection-related cancer, fungal, parasitic or viral, must be cured immediately and prevented along the therapeutic courses. About one fifth of cancer patients worldwide are incurred by infectious agents that increased the mortality rate of cancer cases [3,4]. One of the most frequent opportunistic infection that causes severe illness in cancer and immunocompromised patient is *Toxoplasma gondii*, an obligate intracellular protozoan, infecting up to one-third of human population [4]. Treatment of toxoplasma is not an easy procedure, so far, there is no available

drug capable of eliminating the parasite. Some drugs can limit the parasite multiplication during the active replication stage. However, once the parasite resides and encysted inside the tissue, the drug loses its activity [5].

Several studies suggest that cancer and immunocompromised patients are more susceptible to toxoplasmosis. In addition, toxoplasma patient could acquire some kinds of cancer as the abnormalities in the humoral and cellular immune system could induce a greater incidence of *Toxoplasma* infection and its recurrence [6–8].

According to clinical and epidemiological evidences, many reports highlighted a potential association between *T. gondii* infection and cancer [9,10].

Our study aimed at investigating the effect of miltefosine on *Toxoplasma in-vivo* by monitoring the tissue parasite load using q.PCR in addition to the biochemical and the immunological effects induced after exposure to miltefosine during *T. gondii* infection.

Methodology

Parasite

The virulent RH strain tachyzoites of *T. gondii* was maintained by intra-peritoneal (IP) inoculation of female albino mice in the zoonotic diseases department of the National Research Center (NRC), Egypt, according to the protocol suggested by McLeod *et al*., [11]. Following several passages, tachyzoites were aseptically collected from the ascetic fluid of infected mice after 4-5 days of inoculation, washed twice in a phosphate buffer saline (PBS, pH = 7.4), counted using hemocytometer counting chamber and used directly for *in-vivo* study.

Drug dosage and preparation

In order to establish a dose-response curve and optimal dosing regimen of miltefosine, the method described by Chamberland and Current [12] was applied. Briefly, 3×10^3 tachyzoites were exposed *in-vitro* to serial dilutions of previously used dose [13] (0.04, 0.25, 0.5, 0.98, 1.2 and 1.5 µM) of miltefosine (Sigma-Aldrich, Darmstadt, Germany) in 1 mL reaction volumes at 4ºC. The percentages of viable tachyzoites were determined microscopically by trypan blue exclusion assay using a hemocytometer at different time intervals of incubation with miltefosine (up to 120 hours).

Animals and experimental design

A total of 120 female Swiss albino mice, 8-10 weeks of age and weighing 20-25 g, maintained under ordinary diet at the NRC, were used for the experiment, following the approval of the ethical committee of the NRC (approval number 12-021). While forty mice were allocated into uninfected negative control group and miltefosine treated un-infected group (n = *20*), the remaining mice were classified according to the route of infection into two major groups; I: subcutaneously infected group (SC, n = 40) and II: intraperitoneally infected group (IP, n = *40*). Within each group I and II, animals infected with 3×10^3 RH strain tachyzoites, were randomly allocated into two subgroups: infected untreated control (n = *20)* and miltefosine-treated (n = *20*). Treatment started from the third day of infection; blood and tissue samples were collected daily from the first day of treatment up to the 5th day in the IP group, and 7th day in the SC group.

DNA extraction and qPCR analysis

Genomic DNA was extracted from approximately 20-25 mg of homogenized heart, liver, or lung tissues, from animals sacrificed daily at different time intervals of treatment, using Quick-gDNA Miniprep (Zymo research corporation, Bentley Cir. Tustin, USA), according to the manufacturer's instructions. The concentration and purity of DNA were determined spectrophotometrically (Nanodrop ND1000, Nanodrop, Wilmington, USA). The extracted DNA was used as a template in qPCR reactions with primer listed in Table 1, to amplify a 115-bp fragment of the B1 gene of *T. gondii* [14]. The real-time quantitative PCR run was carried out according to the method of El-Askalany [15], and the count of tachyzoites in each sample was estimated from the experimental threshold cycle (Ct) value with a standard curve generated using known count of *T. gondii* tachyzoites. The results were finally expressed as *T. gondii* tachyzoite-equivalents per mg of tissue [14].

Table 1. Sequence primer pairs used in quantitative real time PCR.

primer		Sequence 5-3	(bp)	Position gene	Reference
B1-B22	Forward	5'-AACGGGCGAGTAGCACCTGAGGAGA-3'	115	1793-1817	[24]
B1-B23	Reverse	5'-TGGGTCTACGTCGATGGCATGACAAC-3'		1907-1881	

Monitoring anti-toxoplasma IgG/IgM antibodies and TNF- alpha levels in drug-treated mice

Blood samples collected daily as described, IgG and IgM were measured using ELISA kit according to the manufacturer's instructions (Chemux Bioscience, INC, south San Fransisco USA). Briefly, *T. gondii* antibodies, if present, binds to purified *T. gondii* Ag coating the surface of 96 well plate, Enzyme conjugate was added to bind to all Ag-Ab complex which is colored later with a chromogenic substrate to be measured by a microwell reader at 450nm. Similarly, the serum level of TNF-alpha and IL-12 were measured using commercially available ELISA kits (Ani Biotech Orgenium Laboratories, Vanta, Finland), (Invitrogen, Thermofischer, NY, USA), respectively, according to the manufacturer's instructions. To exclude the possibility of changes in TNF-alpha and IL-12 due to miltefosine, immunomodulator, a group of uninfected miltefosine-treated mice was included in the assay.

Preparation of T. gondii Antigen

T. gondii lysate antigen (TLA) was prepared by collecting tachyzoites (RH strain) from Infected-VERO cell culture in a serum-free media and purified by filtration. Tachyzoites were washed and suspended in (PBS), disrupted by three cycles of freezing and thawing followed by sonication for one minute. The suspension was filtered and stored at -20 °C for future experiments.

Interferon-gamma release assay (IGRA)

Release assay was performed as described by Mahmoudi and his co-workers [16]. Briefly, Peripheral blood from each animal in the first and last day of the experiment were drawn on lithium heparin anticoagulant tubes. Diluted blood samples were cultured in sterile tubes containing *T. gondii* antigens previously prepared as described. Both positive and negative control consisted of Phytohemoaglutinin (PHA) (20 µg/mL) and PBS, respectively. All tubes were incubated at 37°C for 24 hours in 5% CO2. Centrifugation of the cultured tubes was performed to collect the supernatant. IFN-γ was assayed using a commercial ELISA kit (Invitrogen, Thermofischer, NY, USA). The mean of three replicates for each sample were subtracted from that of Antigen-stimulated samples. The released IFN-γ, expressed in pg/mL, was obtained from the kit standard curve.

Statistical analysis

Statistical significant differences among the subgroups were compared by multivariate analysis of variance (MANOVA), followed by Bonferroni's post hoc test (at $P \leq 0.01$) for multiple comparisons, using the Statistical Package for Social Science (SPSS) program, version 18.0 (SPSS Inc, Chicago, IL, USA).

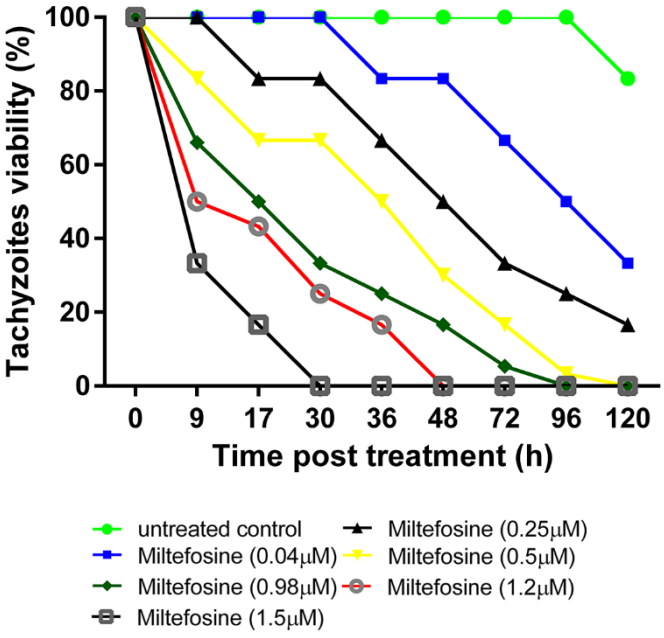
Results

Effect of miltefosine on viable T. gondii tachyzoites in-vitro

A preliminary experiment was performed to determine the dose-response curve. The percentages of viable tachyzoites were measured microscopically and plotted to determine the effective dose of miltefosine causing 50% reduction of the initial tachyzoites count (ED_{50}). As shown in Figure 1, miltefosine dose-dependently reduced the initial free tachyzoites count significantly ($P \leq 0.01$) better than the untreated control group starting from 0.25 µM or higher. The ED_{50} of miltefosine in treated-tachyzoites was 1.5, 1.2, 0.98, 0.5, and 0.25 µM at 7, 9, 17, 36, and 48 hours, respectively. On the other hand, in the untreated control, 120 hours (the maximum exposure time tested) was not enough to induce 50% reduction of the initial tachyzoites count.

After fitting this data into a dose-response curve, taking into consideration the maximum tolerable dose

Figure 1. Tachyzoite viability % as a function of incubation time with different concentrations of miltefosine.



In-vitro tachyzoites viability (%) after incubation of RH strain of *T. gondii* with different concentration of miltefosine (0.04, 0.25, 0.5, 0.98, 1.2 and 1.5 µM) as a function of time of exposure measured using Trypan blue exclusion assay by hemocytometer. Data represent mean ± SEM of 6-8 replicates.

of miltefosine with no observable side effects in mice (including locomotor or behavioral habits; data not shown), a dose of 1.5 µM of the drug was selected for conducting the remaining experiments described in this study.

Quantitative Follow-up of parasite load, in-vivo results

Quantitative follow up of the parasite load in various organs, at different time intervals following exposure to miltefosine, are plotted as in Figures 2 and 3 to validate the qPCR assay before being used in our study, amplification of known count of tachyzoites at various concentrations was conducted. The standard curve showed linearity within the concentration range studied with a correlation coefficient (R^2) of 1.0, a slope of -3.36, and a reaction efficiency of 92.34% (not shown).

Additionally, melting curve analysis was performed to confirm the qPCR specificity in detecting the B1 gene amplification product. A single product with a melting temperature of 83.2°C was detected only in *T. gondii* test samples but not in the negative controls (Figure 4A). Similarly, when the qPCR amplicons were evaluated by 1.5% agarose gel electrophoresis, a single band with an estimated size of ~115 bp was observed, which is in agreement with the expected size of the amplified fragment of the B1 gene (Figure 4B).

While all the samples tested were positive for *T. gondii*, the route of infection appeared to significantly affect the initial parasite load in some of the organs/tissues tested. As represented in Figure 3D. The parasite load in the liver on the first day of treatment was significantly higher in the IP-infected subgroups as compared to the SC-infected subgroups (620.04 *vs.* 11.4) (Table 2).

On the other hand, three doses of 1.5 µM miltefosine caused a significant elevation in liver parasite load of IP-infected subgroups ($F = 4478.98$, $df = 4.75$, $P \leq 0.0001$) compared to the control group (Figure 1A), heart ($F = 390.9$, $df = 9$, $P < 0.0001$) and lung ($F = 247.3$, $df = 4$, $P < 0.0001$) (Table 2, Figure 2C and 2D).

In Sc-infected group, the first three doses of miltefosine induced a significant increase in the parasite load of all tested organs ($P \leq 0.05$) as shown in Figure 3.

In SC group, miltefosine induced the tissue parasite load by 13.5 folds and 2.04 folds increase after three days of treatment for heart and lung respectively, as well as 22.6 folds and 7.6 folds of increase for heart and lung respectively when compared with first day of treatment, as expressed in Table 2.

Evaluation of immunological response

Serum TNF- alpha levels

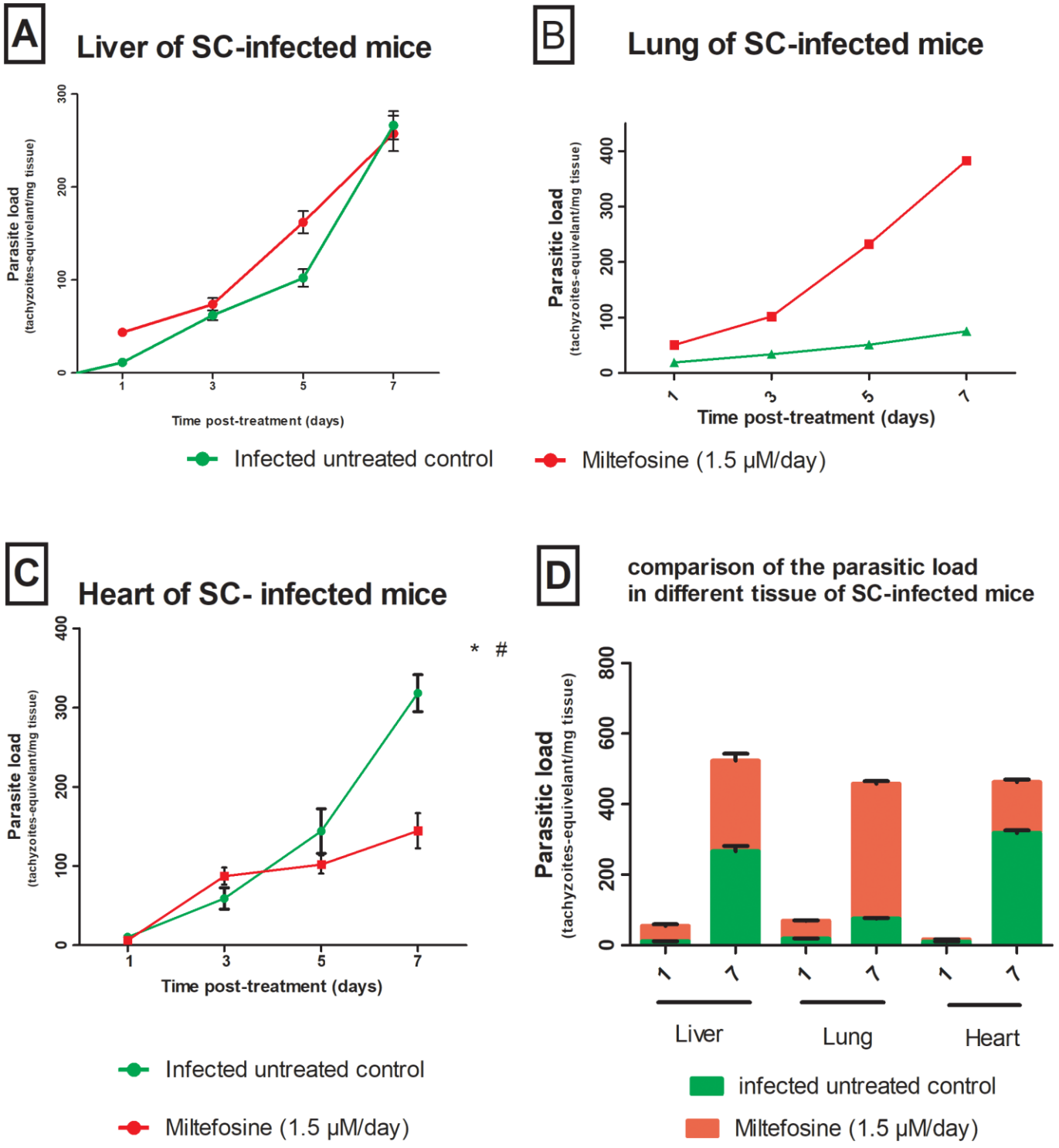
As seen in Figure 5A and 5B, miltefosine administration caused a significant increase in TNF-alpha levels ($F = 7.71$, $df = 6$, $P < 0.0001$) and ($F = 9.42$, $df = 6$, $P < 0.0001$) for SC and IP groups, respectively.

Table 2. Comparison and statistical evaluation of the parasitic load during post-treatment follow-up in different tissues of IP- and SC-infected mice. Following IP or SC infection of mice with 3×10^3 tachyzoites of *T. gondii*, the animals received (at 3 days post-infection) one of three experimental conditions: miltefosine, or no drug (in the infected untreated control group) ($n = 20$ in each group). Tissue samples from liver, heart, and lung were collected daily from the day of the first dose up to 5 days (in the IP-infected) or 7 days (in the SC-infected subgroup), and the parasitic load in these tissues was determined using qPCR assay, as detailed in methodology section. To allow for simpler comparisons, this table shows only the parasitic load on the first, third, and the last (the 5th day in the IP-infected, and the 7th day in the SC-infected subgroups) days of treatment. Results are expressed as tachyzoite-equivalents/mg of tissue (mean ± SD of at least three independent experiments). The fold change in the parasitic load relative to the load of the 1st day is shown.

Route of Infection	Tissues	Groups	Post-treatment parasitic load				
			1st day	3rd day		Last day (before animal sacrifice)	
			Mean ± SD	Mean ± SD	Fold change #	Mean ± SD	Fold change #
IP	Liver	Infected untreated (control)	620.04 ± 20.5	2368.9 ± 24	3.8	7575 ± 91.9 [a]	12.21
		Miltefosine-treated	1383.8 ± 93	7919.372	5.7	10534.6 ± 109 [a,b]	7.6
	Heart	Infected untreated (control)	176.1 ± 9.5	220.6 ± 11.1	0.014	280 ± 7.9 a	1.6
		Miltefosine-treated	109.1 ± 5.1	220.5 ± 10.6	2	620.2 ± 23 [a, b]	5.7
	Lung	Infected untreated (control)	220.6 ± 10.1	514 ± 16.8	2.3	947.6 ± 22.3 [a]	4.3
		Miltefosine-treated	379.2 ± 21.1	514.5 ± 16.1	1.35	1975.7 ± 77.7 [a ,b]	5.2
SC	Liver	Infected untreated (control)	11.4 ± 0.5	62 ± 5.1	5.4	266.4 ± 15.1[a]	23.3
		Miltefosine-treated	43.7 ± 4.31	73.9 ± 6.7	1.7	257.5 ± 19.1[a]	5.9
	Heart	Infected untreated (control)	10.3 ± 0.8	59.2 ± 4.2	5.74	318.1 ± 7.4[a]	30.9
		Miltefosine-treated	6.4 ± 0.4	87.3 ± 3.4	13.5	144.6 ± 13.8[a, b]	22.6
	Lung	Infected untreated (control)	19 ± 0.37	34.02 ± 2.4	1.8	75.4 ± 2.1[a]	4
		Miltefosine-treated	50.7 ± 1.4	102.1 ± 3.1	2.04	383.2 ± 6.7[a, b]	7.6

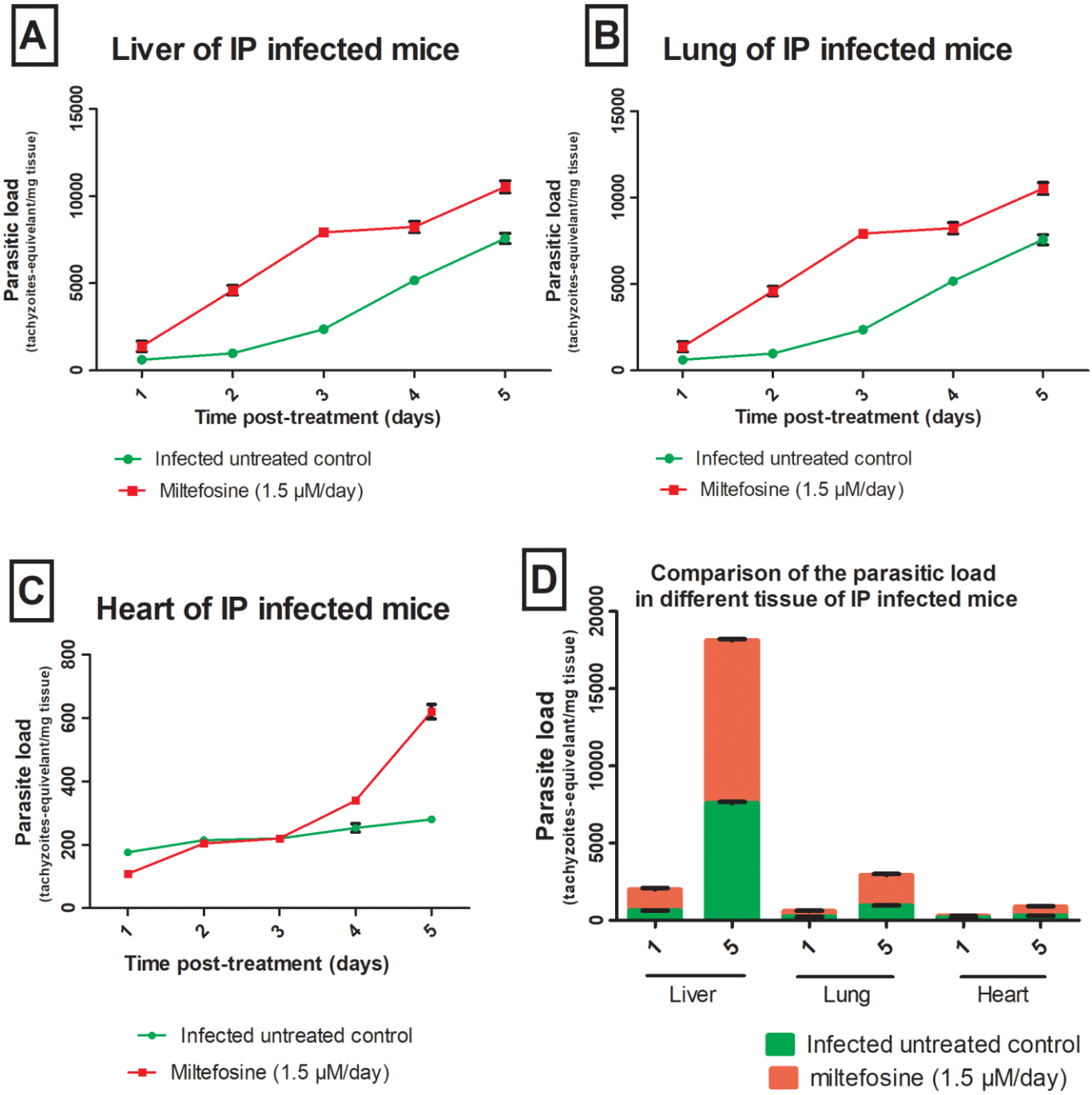
#: relative to the load of the 1st day; [a] Significant difference between the first and the last days of treatment, $P < 0.01$; [b] Significant difference between the untreated (control) and the miltefosine-treated groups at the last day of treatment, $P < 0.05$.

Figure 2. Follow-up evaluation of the parasite load in different tissues, with and without Miltefosine therapy, of IP-infected mice.



Parasite load in liver (A), Lung (B), and heart (C) of IP-infected mice with 3×10^3 of RH strain tachyzoites of *T. gondii* for both infected non-treated and infected-treated group, receiving daily parenteral dose of miltefosine for 5 days, samples are collected for daily follow up schedule, (D) Differences in the parasite load among these tissues, expressed as tachyzoite-equivalents/mg of tissue (mean ± SE).

Figure 3. Follow-up evaluation of the parasite load in different tissues, with and without Miltefosine therapy of SC- infected mice.



Parasite load in liver (A), Lung (B), and heart (C) of SC-infected mice with 3×10^3 of RH strain tachyzoites of *T. gondii* for both infected non-treated and infected-treated group with daily parenteral dose of miltefosine, receiving daily parenteral dose of miltefosine for 7 days, samples are collected for daily follow up schedule, (D) Differences in the parasite load among these tissues, expressed as tachyzoite-equivalents/mg of tissue (mean ± SE).

Serum IL-12 levels

Miltefosine administration induced a non-significant difference in the serum level of IL-12 ($p > 0.05$). However, as seen in Figure 6B, Infection with *T. gondii* tachyzoites induced a 24 times increase in the level of IL-12 at the third day of infection up to 40-55 times increase at the end of the experiment for both subgroups ($t = 25.5$, $P < 0.001$ and $t = 30.3$, $P < 0.0001$), respectively. Miltefosine treatment also had a non-significant difference when compared with infected non-treated groups in all days of the experiment ($p > 0.05$).

Serum immunoglobulins levels

A significant increase in anti-toxoplasma antibodies from the 3rd day of treatment, starting with IgM ($t = 29.24$, $df = 2$, $P = 0.0012$) followed by IgG at the 4th day of treatment ($t = 12.13$, $df = 2$, $P = 0.0067$) till they reached to a significant peak level at the 5th day for both IgG ($t = 21.91$, $df = 4$, $P < 0.0001$) and IgM ($t = 21.98$, $df = 4$, $P < 0.0001$) for infected un-treated group as shown in Figure 5C, 5D, 5E, 5F.

Miltefosine significantly increased the level of IgG ($t = 2.967$ $df = 4$, $P = 0.0413$) after the 4 th dose of treatment and IgM ($t = 2.962$ $df = 4$, $P = 0.0415$) after the 3rd dose of treatment compared to the control group, for both IP group (Figure 5C and 5D) and SC group (Figure 5E and 5F).

Interferon-gamma release assay (IGRA)

In our study we have used whole blood for measurement of interferon release assay, as shown in Figure 6A. No significant difference was detected after induction of blood of negative control group and group receiving miltefosine only as a control group in the first day of treatment. However, five doses of miltefosine induced a significant release of gamma INF ($t = 5.5$, $P < 0.001$) and ($t = 7.4$, $P < 0.001$) for IP and SC groups, respectively. About 5 –15-fold increase in the release ability of lymphocytes was measured after infection with toxoplasma in first and last day samples, respectively. Treatment of infected animals with miltefosine induced a non-significant difference in lymphocyte release after the first dose of treatment. However, the macrophage releasing ability has decreased significantly by 14 and 27% at the last day of treatment for IP and SC groups, respectively.

Discussion

The approval of intensive course of miltefosine by WHO to be used as an oral treatment of visceral leishmaniasis as well as its chemotherapeutic activity, necessitate the study of its chemicobiological effect on different immunological markers as well as its impact on accidental toxoplasmic infection during the course of therapy. Therefore, our study aimed at exploring the effect of miltefosine on the release of inflammatory markers as Interferon gamma, tumor necrosis factor and specific anti-toxoplasma antibodies, in addition to quantitative follow up of toxoplasma count in different organs encountered by successive doses of miltefosine in mice infected with acute toxoplasmosis using qPCR technique.

Although, all tissue organs collected from infected mice were positive during the experimental study, the liver tissue exhibited a relatively higher initial parasite load. This increase in the parasite load can be attributed to the high parasitic infiltration in blood, and thus, the abundance of quick trapping of *T. gondii* in liver as described by Daryani *et al*. [17].

Figure 4. A) Melting curve of q. PCR of *T. gondii* assay (RH strain) of different tissue samples from our experiment and standard samples after 40 cycles of amplification for each Real time PCR run: x axis of the melting temperature, y axis indicates fluorescent intensity; B) qPCR amplification product were analyzed by agarose gel electrophoresis (1.5%). Lane 1 to 5 parasite B1 gene from ascetic fluid of *T. gondii*-infected mice, from 6-8 DNA from *T. gondii*-infected clinical tissue samples, N negative control group and M, 100-bp DNA ladder (Biotools, Madrid, Spain).

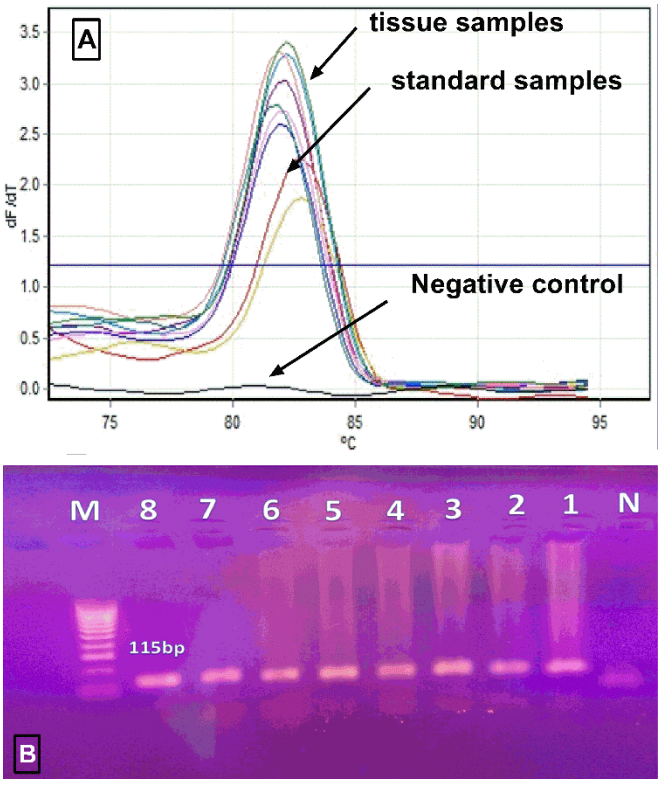
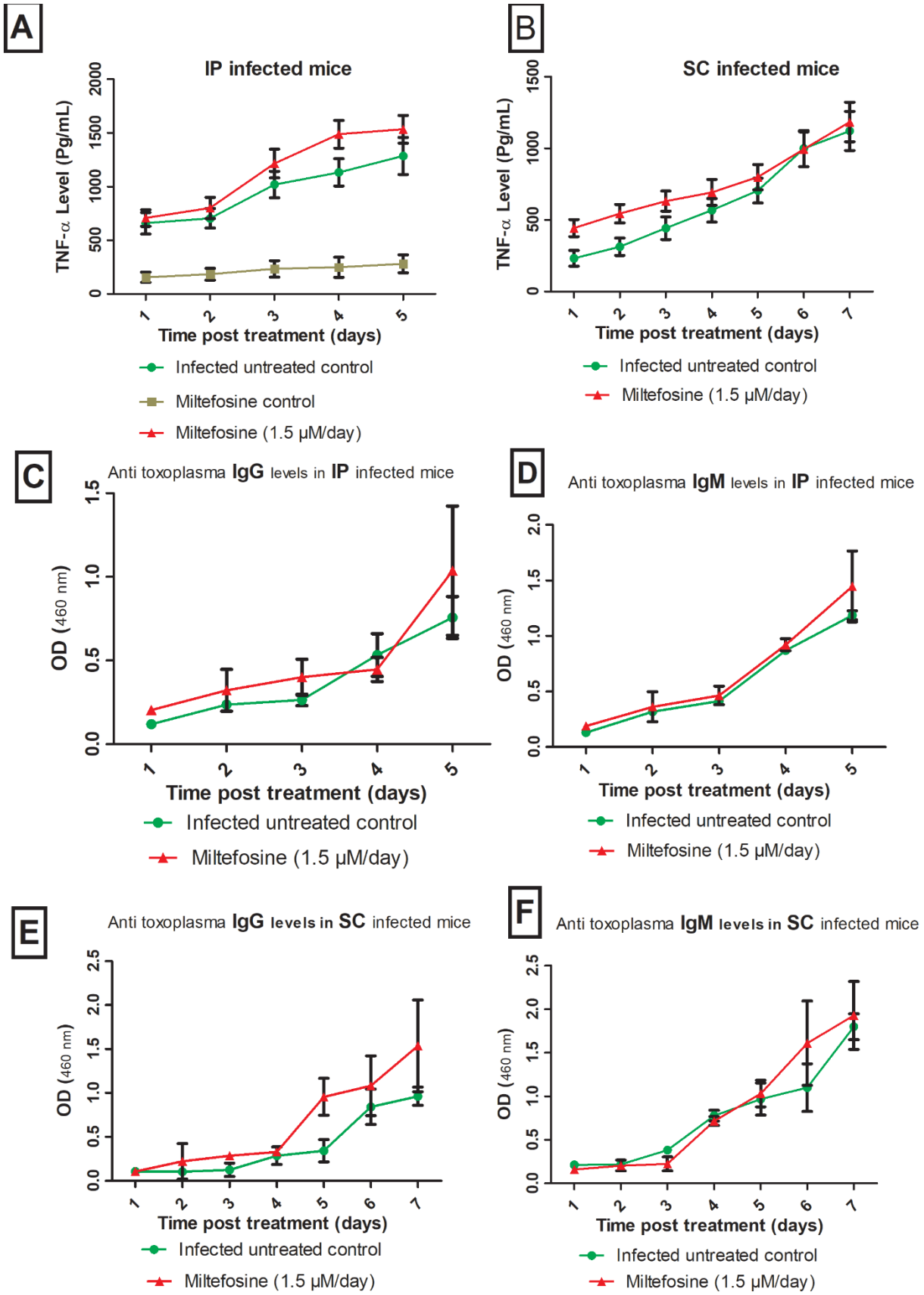


Figure 5. A, B) Daily levels of circulating TNF-α in control mice groups, drug-exposed and *T. gondii*-infected group with 3×10^3 tachyzoites RH strain, for both subgroups, the IP (A) or the SC (B) routes treated with daily parenteral dose of miltefosine for five days and seven days for IP and SC, respectively. Results are expressed as pg/mL (mean ± SD of three independent experiments); **C, D**) The daily anti-Toxoplasma IgG and IgM levels in control and *T. gondii*-infected group with 3×10^3 tachyzoites RH strain, for both subgroups, either the IP (C, D) or the SC (E, F) routes and as well as for treated group with daily parenteral dose of miltefosine for 5 and 7 days for both subgroups IP and SC, respectively. Values represent mean optical density ($OD_{460\ nm}$) (mean ± SE) of three independent experiments.



The more strikingly results detected by qPCR were a significant increase in the parasite load and distribution in different organs following miltefosine parenteral administration with 1.5µM for 5-7 days, which is 80 times less than the lowest dose used for VL, compared to the untreated control group for both subgroups. Also, induction of TNF- α, anti-toxoplasma Ig_s was observed. However, reduction of serum IL-12 and 27% decline in the releasing ability of INF- γ was observed, despite a high serum level, in addition to a marked increase in TNF- α, gave remarks for increasing the infectivity of toxoplasma and impairing the function of murine humoral immune system.

This decline in cytokine levels , in addition to marked increase in the parasite load in all organs by miltefosine could give a close overview of the vigorous invasion of the parasite, several explanations could be given for these findings.

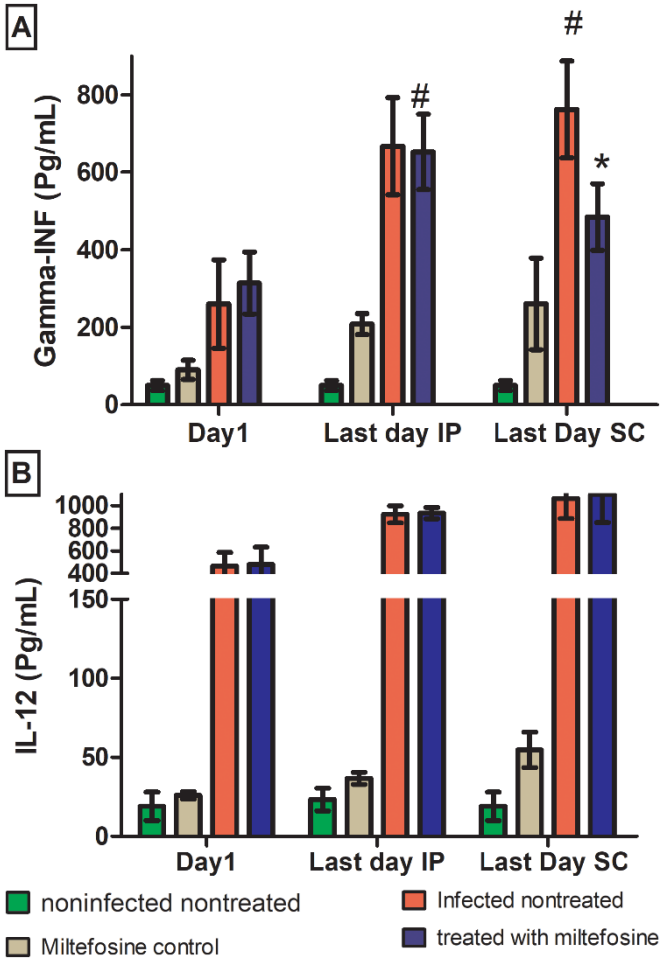
First, the blockage and interference of the host apoptotic machinery by miltefosine ,as described by [18–21], in leishmania and yeast, or by *Toxoplasma* invasion as the parasite itself is capable of interfering with host apoptotic signaling pathways to favor its survival [22,23].

The second explanation for the increase in tissue parasite load was as discussed by Rakotomanga [24] for leishmania is the development of a resistant strain due to rapid ultrastructural changes in tachyzoites membrane which alter absorption and accumulation of the drug, a key step for activity of miltefosine leading to loss of its activity and favoring the multiplication of the parasite [25,26].

The third explanation to be discussed is the powerful ability of *T. gondii* to use macrophages as host cells, inducing a different cellular strategy to ensure its survival and multiplication [27], resulting in the presence of highly infected macrophages that could escape the response towards INF- γ [28]. A successive decline in IFN- γ release measured by IGRA from macrophages could be due to inhibition of IFN-γ-induced gene expression resulted from *T. gondii* infection as explained by Emily E. Rosowski and co-workers [29] in various cell line types of multiple species, including human foreskin fibroblasts (HFFs) [30], macrophages derived from murine bone-marrow [31], and RAW264.7 murine macrophages [32]. In addition, the secreted INF- γ was only responsible for controlling the number of invaded cells without affecting the intracellular multiplication of the parasite to limit the disease within infected area that favor the increase in tissue parasite load which is in agreement with Gomez marine finding [33].

Induction of an aggressive Th1 cell-mediated response as an immunological response to *T. gondii*, resulted in early production of IL-12 which is necessary for stimulating the production of IFN-γ and vice versa [34]. IL-12-dependent- production of IFN- γ considered one of the most important mechanism required for elimination of parasite and its invasion as it activates macrophages for inducible nitric oxide synthetase 2 (iNOS2)-dependent elimination of Leishmania [35]. However, in our research, administration of miltefosine

Figure 6. A) Interferon gamma release assay performed on whole blood samples of different animal groups; non-infected non-treated, miltefosine only treated, infected group with 3×10^3 RH strain tachyzoites of *T. gondii*, infected treated group with daily parenteral dose of miltefosine for % days, IP group, seven days for SC groups at first and last day of treatment. Data represented by pg/mL (mean ± SE) three replicates for each (* a statistical difference between different groups-# a statistical difference between the same group); **B)** Daily levels of circulating IL-12 of different animal groups; non-infected non-treated, miltefosine only treated, infected group with 3×10^3 RH strain tachyzoites of *T. gondii*, infected treated group with daily parenteral dose of miltefosine for % days, IP group, seven days for SC groups at first and last day of treatment. Data represented by pg/mL (mean ± SE) three replicates for each.



for five days in IP subgroups, and seven days in SC subgroups, resulted in reduction of serum IL-12 and 27% decline in the releasing ability of INF- γ, in addition to a significant increase in TNF- α, that gave a remarks for increasing the infectivity of toxoplasma and impairing the function of murine humoral immune system.

The observation of Eissa and her collegues regarding the direct effect of miltefosine on free tachyzoites [13], in addition to our study performed *in-vitro*, could describe that invaded macrophage act as a shield to preserve the integrity of tachyzoites and favor its escape from the effect of cytokines that guarantee its survival as shown previously in our experiments. Further cytology level studies including electron microscopy imaging for intracellular tachyzoites are recommended to explain this point of view.

Thus, Due to the close association between *T. gondii* prevelance and cancer [36,37], and *T. gondii* prevelance and leishmaniasis [38] which are both fatal, clinicians should be more careful with toxoplasma-infected cancer or leishmanial patients receiving miltefosine. Moreover, parasitological surveys of miltefosine- receiving patients should be regularly carried out, aiming at preventing the possibility of severe accidental toxoplasmosis during therapy. Above all, these high-risk groups should be kept away from toxoplasmosis infection sources [6]. Strategy for treatment of cancer patients with miltefosine should take in consideration the risk of co-infection with other parasites as toxoplasmosis.

In addition, oral treatment of Leishmaniasis with miltefosine should be monitored frequently for the risk of concomitant infection with toxoplasma pathogen or any other parasites that are not investigated yet.

Conclusion

In conclusion, miltefosine, being a chemotherapeutic drug and one of the most effective drugs, if not the only, for treatment of all types of leishmaniasis, might not be an appropriate drug for treatment of cancer patients with opportunistic acute toxoplasmosis or even for treatment of leishmaniasis concomitant with *Toxoplasma* infection as it could aggravate the pathogenesis of toxoplasmosis. A periodical screening for accidental infection with toxoplasmosis during the course of miltefosine therapy is strongly recommended.

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