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EFFECT OF MILTEFOSINE ON THE NUMBER OF LEISHMANIA DONOVANI AMASTIGOTE IN VL INFECTED MACROPHAGE IN VITRO

Biology Science

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ABSTRACT

Introduction: Visceral leishmaniasis (VL), also known as kala-azar, is a diffuse protozoan infection caused by Leishmania donovani complex. VL is principally caused by L. donovani and L. infantum (synonym L. chagasi in South America). The parasite targets the reticulo-endothelial system, with penetration of the spleen, liver, bone marrow and lymph nodes lead to organomegaly and pancytopenia. Organic pentavalent antimonials have been the first-line drugs for the therapy of leishmaniasis for the latest six decades, and clinical resistance to these drugs has emerged as a primary obstacle to successful treatment and control. Miltefosine has been shown to be higher or equivalent to presently approved essential medicines for at least one of visceral, cutaneous or mucosal leishmaniasis.

Aim: The aim of this study is to clarify the effect of miltefosine on the number of amastigotes in the VL infected macrophage in vitro, in comparison to the effect of pentostam on the number of amastigote in the VL infected macrophage.

Materials & Method: Cells were plated in 96-well tissue culture plate, after incubation, adherent macrophages were infected with Leishmania donovani promastigotes. Infected macrophages were treated with the same concentration of pentostam and miltefosine (1, 2, 3, 4, 6, 7 µM). Treated macrophages incubated for 24, 48, 72 hours, and then stained with Gimsa stain. The results of L. donovani infected macrophages show that there were a significant differences between the percentage of infection macrophages in all used concentrations of both drugs.

Results: The results show that after 24, 48, 72 hour of treating L. donovani infected macrophages with Sb or HePC, the number of infected macrophages and number of amastigote per macrophage started to decline clearly in the case of HePC, especially at high concentrations of it, in comparison to the number of infected macrophages in the case of Sb. Conclusion: This suggested that miltefosine could be a good therapeutic option for treating all forms of leishmaniasis, including visceral leishmaniasis.

KEYWORDS

Leishmaniasis- Visceral leishmaniasis- VL- VL infected macrophage- Miltefosine.

INTRODUCTION

Leishmania, a protozoan parasite related to the family Trypanoso matidae, causes leishmaniasis, a group of diseases with clinical symptoms that range from self-healing cutaneous and mucocutaneous skin ulcers to a lethal visceral form (visceral leishmaniasis (VL) or kala-azar)(1). *Leishmania* parasite transmitted to humans through the bite of infected female sandflies from the genus *Phlebotomus* in the old world and *Lutzomiya* in the new world (2). The parasite leads a digenetic life cycle (3). Infected sandflies introduce the metacyclic forms of the promastigotes stage into the bloodstream of the vertebrate host when they bite to take a meal. The promastigotes transform into an amastigote stage in the phagolysosome of reticuloendothelial cells, where they multiply in the hostile environment of the macrophage and kill the cell (4). Most VL cases worldwide affect children and young adults. In some endemic areas, more than half of new infections are among children younger than 10 years (5). Leishmaniasis occurs worldwide; approximately 0.2–0.4 million visceral leishmaniasis (VL) cases and 0.7–1.2 million cutaneous leishmaniasis (CL) cases are estimated to occur each year (2, 6), as much as 20 percent of patients with VL infection may perish before their disease is recognized (7). The disease is a significant cause of morbidity and fatality in several countries of the world (1). The amastigote form of the parasite mainly infects the reticuloendothelial system and may be found in profusion in the bone marrow, spleen and liver. It lessens immunity, causes enduring fever, anaemia, liver and spleen enlargement, and if left untreated, death may happen due to opportunistic infection (8, 9). The classic treatment for all forms of leishmaniasis utilises pentavalent antimonials as sodium stibogluconate (SSG) and meglumine antimoniate (MA) administered intravenously or intramuscularly (10,11). However, in recent years, a large-scale increase in clinical resistance to pentavalent antimonials has been reported (12). For example, in India, as many as 65% of formerly untreated patients fail to respond directly or relapse after therapy with antimony drugs, due to the expansion of drug resistance (13). Miltefosine is the solely recognized oral agent with potential to treat leishmaniasis. Miltefosine had exhibited very good cure rates for visceral leishmaniasis (VL). It's long half-life and its oral administration could make it a good option for maintenance prophylaxis (14).

MATERIALS AND METHODS

Preparation of drugs :

Pentostam (Sb)

Pentostam present as ampouls for injection (100 mg/ml of pentostam) (Glaxo Operations UK Limited Castle) was obtained from Al-Yarmook hospital/ Baghdad.

Miltefosine (HePC)

Miltefosine present as powder form (10 g) with molecular weight 407.57 g/mol and purity 99%. It was manufactured by (Xian Wango Biopharm Co.,Ltd. China). 80 mg of HePC was dissolved in 20 ml of distilled water in order to prepare the stock solution of HePC (4mg/ml) or (104 µg/ml), then the used concentrations prepared from it.

Preparation of Fluid Thioglycollate Medium

1. The FTM medium (29.8 g) was dissolved in one liter of purified water.
2. Medium solution was heated with recurrent agitation to completely dissolve the medium, then autoclaved at 121°C for 15 minutes.

Protocol to obtain thioglycollate elicited macrophages: This method is used to get a higher yield of macrophages .

1- Five ml of FTM was injected into the peritoneal cavity of each mouse.
2- After 5 days, FTM injected mice were euthanized, and installed them on the styrofoam block on their back.
3- By using a scissors and forceps, the outer skin of the peritoneum was cut and gently pulled back to expose the inner skin lining the peritoneal cavity.
4- Five ml of ice cold RPMI 1640 (with 10% FCS) was injected into the peritoneal cavity.
5- After injection, the peritoneum was delicately massaged to dislodge any attached cells into the RPMI 1640 solution.
6- To collect the fluid, needle was inserted, bevel up, and moving the tip of the needle gently to avoid clogging by the fat tissue or other organs. Fluid was collected as much as possible and the collected cell suspension was deposited in tubes kept on ice after taking out the needle from the syringe.
7- The collected cell suspension was span at 1500 rpm for 8 minutes, the supernatant was discarded and the cells were re-suspended in RPMI 1640 media for counting .
8- Mouse peritoneal macrophages were washed with RPMI1640 medium supplemented with 10% FCS, 100U of gentamicin/ml and cultured overnight in flat bottom 96-well tissue culture plate (100 µl/ well) at a concentration of 2 × 104 cell/well. Non adherent cells were removed by two washes with RPMI medium.

***In vitro* macrophage infection**

1- Cells were plated in 96-well tissue culture plate, after incubation at 37°C.
2- Adherent macrophages were infected with logarithmic phase of Leishmania donovani promastigote at a parasites-to-macrophage ratio of 8:1.
3- After 2 hour of incubation at 37°C, extracellular parasites were removed via gentle washing.
4- Infected macrophages (except control groups) were then treated in duplicate with six concentrations (1, 2, 3, 4, 6, 7 µM) of Sb and HePC.
5- Treated macrophages then incubated for 24, 48, 72 hours.
6- After 24, 48 and 72 hours, the medium was removed and alcohol was added for 2 minutes and then Geimsa stain was added for 15-20 minutes in order to stain the infected and treated macrophages (15).

RESULTS

Infection rate

The infection rate was ranged between 75% - 80%, while the number of parasites (intracellular amastigotes) inside each macrophage cell was ranged between 1-10 parasites (Figure 1).

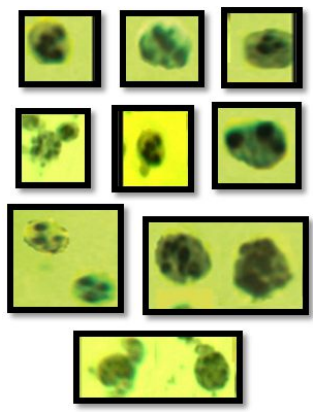

Figure (1) : Infected macrophages with several numbers of *L. donovani* amastigotes

Treatment of infected macrophages with Sb and HePC

After 24 hour

After 24 hour of treated L. donovani infected macrophages with (1, 2, 3, 4, 6 and 7 µM) of Sb or HePC, the number of infected macrophages started to decline clearly in the high concentrations of the HePC. There was a significant (P< 0.05) differences between them (Table 1), Figures (2), (3), (4), (5) and (6).

Table (1) : The percentage of infected macrophages after 24 hour of exposure to HePC and Sb

Drug Concentration (µM)	The mean of infected macrophages		LSD value
	HePC	Sb	
1	82.0	94.0	7.257 *
2	71.5	88.0	7.922 *
3	63.5	82.0	7.632 *
4	49.0	76.0	7.916 *
6	26.0	70.5	9.633 *
7	10.5	68.5	11.562 *
control	97		
LSD value	9.226	7.126	----
* (P<0.05).			

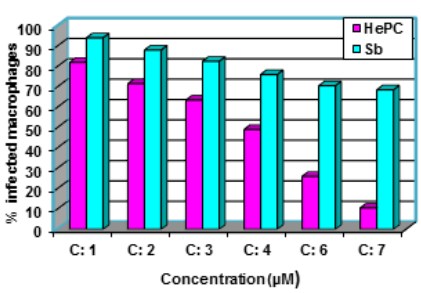

Figure (2) : The percentage of infected macrophages after 24 hour of exposure to different concentrations of HePC and Sb drugs

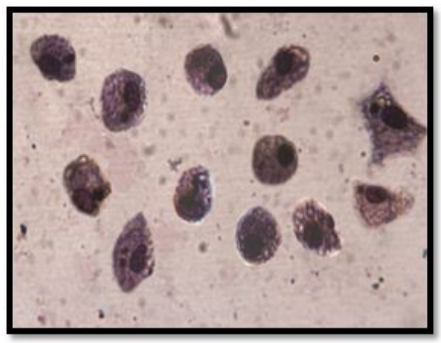

Figure (3): The infected macrophages after 24 hour of exposure to 1 µM of Sb

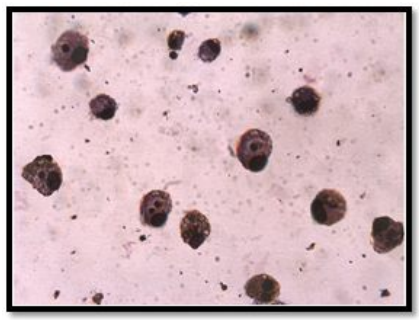

Figure (4) : The infected macrophages after24 hour of exposure to 1 µM of HePC

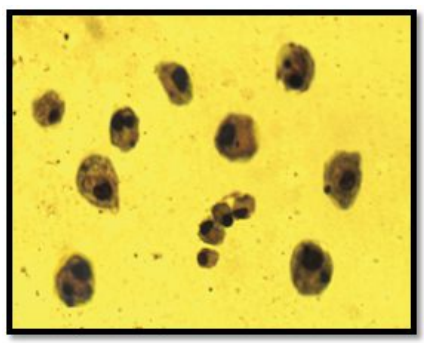

Figure (5) : The infected macrophages after 24 hour . of exposure to 7 µM of Sb

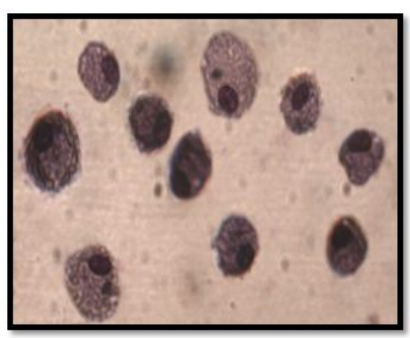

Figure (6) : The infected macrophages after 24 hour . . of exposure to 7 µM of HePC

After 48 hour

After 48 hour of exposure, the number of infected macrophages decreased clearly when treated with HePC in comparison to Sb especially in the high concentrations (Table 2), Figures (7), (8), (9), (10) and (11).

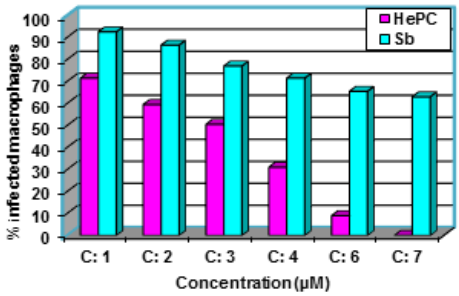

Figure (7) : The percentage of infected macrophages after 48 hour of exposure to different concentrations HePC and Sb drugs

Table (2) : The percentage of infected macrophages after 48 hour of exposure to HePC and Sb

Drug Concentration (µM)	The mean of infected macrophages		LSD value
	HePC	Sb	
1	71.5	92.5	8.411 *
2	59.5	86.5	7.038 *
3	50.5	77.0	7.462 *
4	31.0	71.5	9.667 *
6	9.0	65.5	9.035 *
7	0.0	63.0	9.558 *
control	98		
LSD value	10.966 *	9.352 *	----
* (P<0.05).			

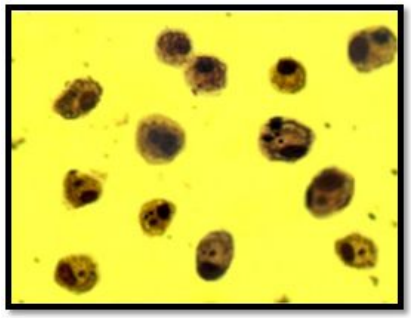

Figure (8) : The infected macrophages after 48 . hour of exposure to 1 µM of Sb

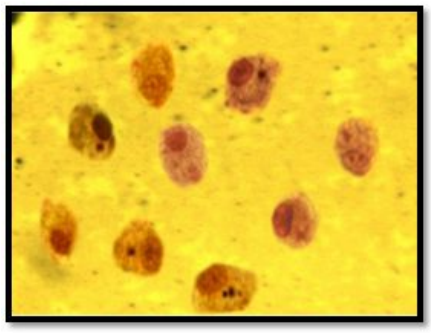

Figure (9) : The infected macrophages after 48 hour. of exposure to 1 µM of HePC

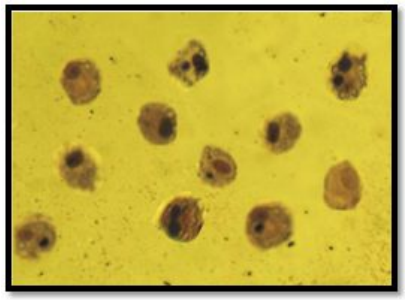

Figure (10) : The infected macrophages after 48 . . hour of exposure to 7 µM of Sb

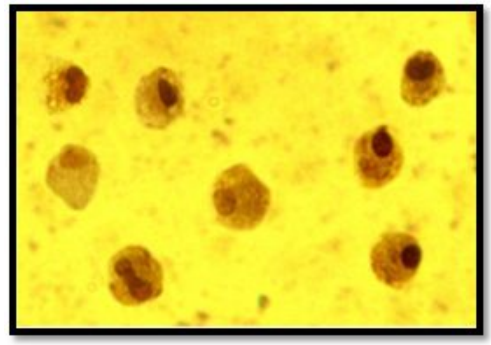

Figure (11) : The infected macrophage after 48 . . . hour of exposure to 7 µM of HePC

After 72 hour

The results after 72 hour were corresponding to these after 24 and 48 hour. It was shown obvious decline in the number of infected macrophages which treated with HePC in comparison to those with Sb (Table 3), Figures (12), (13), (14), (15) and (16).

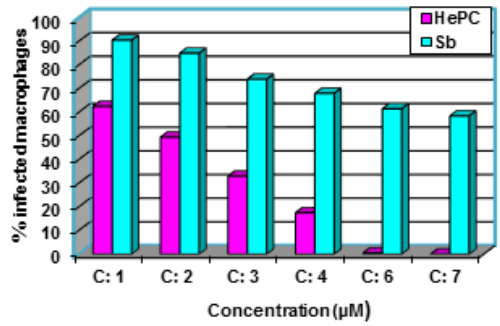

Figure (12) : The percentage of infected macrophages after 72 hour of exposure to different concentrations HePC and Sb drug

Table (3) : The percentage of infected macrophages after 72 hour of exposure to HePC and Sb

Drug Concentration (µM)	The mean of infected macrophages		LSD value
	HePC	Sb	
1	62.5	90.5	7.209 *
2	49.5	85.0	8.946 *
3	33.0	74.0	8.512 *
4	17.5	68.0	8.975 *
6	0.5	61.5	10.269 *
7	0.0	58.5	10.502 *
control	99		
LSD value	10.327 *	8.243 *	----
* (P<0.05).			

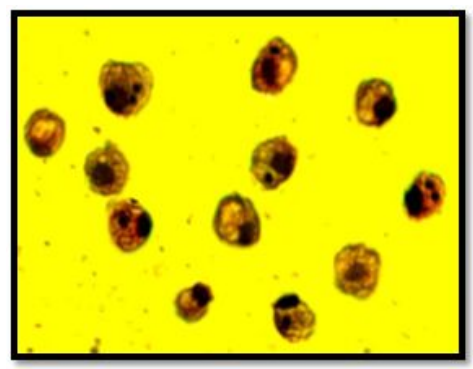

Figure (13) : The infected macrophages after 72 hour of exposure to 1 µM of Sb

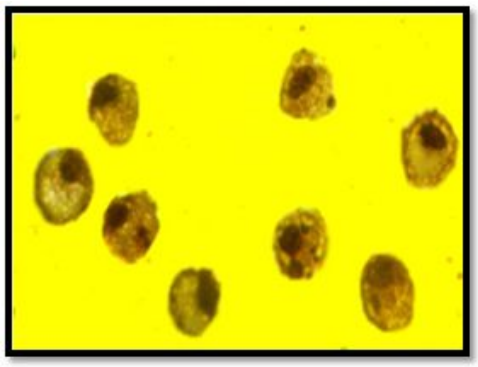

Figure (14) : The infected macrophages after 72 hour of exposure to 1 µM of HePC

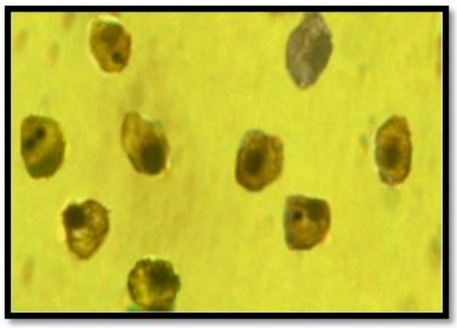

Figure (15) : The infected macrophages after 72 . . . . hour of exposure to 7 µM of Sb

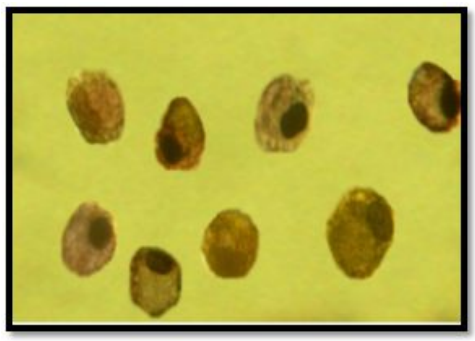

Figure (16) : The infected macrophages after 72 . hour of exposure to 7 µM of HePC

The 50% inhibitory concentration (IC50) of the drugs

The IC50 of the drugs were calculated to determine the most effective concentration on the intracellular parasites. The concentrations of HePC which could decrease half number of infected cells (IC50) were 15.01µM , 11µM and 3.44µM after 24, 48 and 72 hour, respectively, in comparison to Sb which doesn't revealed the IC50 in all used concentrations and periods.

Discussion

As for the results of *L. donovani* infected macrophages, the present study revealed that the rate of infected macrophages was between 75% -80%, and the number of amastigotes within the cell ranged between 1-10 parasites, as shown in figure (1). These results corresponds with Eliane de Morais-Teixeira *et al*. (16) results were they showed that at least 80% of the macrophages in the control wells were infected. In contrast, Prajapati *et al*. (15) were obtained 60–80% infected macrophages after 24 hours post-infection, while the infection level was approximately one to two amastigotes per macrophage.

Infected macrophages were treated with HePC and Sb, then they were determined after (24, 48 and 72) hours, the statistics analysis showed that there were a significant differences between the percentages of infected macrophages in all used concentrations of both drugs. These results were lower than those obtained by Verma and Dey (17), they recorded a very rapid and dose-dependent death occurred with HePC concentrations between 20 and 50µM, reaching approximately 100% at around 40µM.

Also, the HePC (IC50) for the current study was lower (3.44) after 72 hours of exposure than the IC50 obtained by the same researchers which was (25µM) when they used peritoneal macrophage isolated from Chinese hamsters. On the other hand, the current study recorded higher IC50 than those recorded previously by Eliane de Morais-Teixeira *et al.* (15) which was 0.2µM against *L.(L.) donovani*, while it was within the range when obtained by Inocêncio da Luz *et al*. (18) which was between (1.5-7.1 µM). In another study for Vermeersch *et al*. (19) showed that HePC exhibited IC50 of 2.8 to 5.8 µM on intracellular amastigotes.

It is expected that these differences in the proportions of IC50 between these different studies are due to the differences in parasite strains between different regions of the world, this reason confirmed by Griewank *et al*. (20) who revealed that the sensitivity to HePC appears to be strain dependent, also, may be due to different types of macrophages and different laboratory conditions.

Some studies show that a direct toxic effect of HePC on promastigote and amastigote forms of *Leishmania* in peritoneal macrophages has been good documented (21, 22). Previously, Wadhone *et al* (23) showed that HePC limited the growth of L. donovani in BALB/c peritoneal macrophages in a dose-dependent manner. They showed that the killing of Leishmania amastigotes requires iNOS2, the enzyme that catalyzes the production of NO, so the free radical responsible for killing of *Leishmania*. They concluded that HePC induced iNOS2 expression that peaked around 3–6 hour after HePC treatment, besides the reported direct effect on the amastigotes, HePC does have an immune interface by which it enhances IFN-γ responsiveness and facilitates IFN-γ-mediated amastigote killing, suggested that the drug may be primed the macrophages to respond to IFN-γ (24). Because the drug has a long fatty acid chain, it was also proposed that the drug might directly go into the lipid layers of the membrane and interfere with cellular signaling (25). When the drug was first observed to be active against Leishmania in vitro, it was proposed that the drug might act directly on the parasite (26) causing parasite apoptosis (27). In fact, studies on cellular uptake of the drug proposed that it went to the parasitophorous vesicle where the amastigotes reside. Although all these observations supported the direct action of the drug on the parasite, there were other observations that indicated to an indirect action of the drug.

Lux *et al.* (28) exhipted that the primary therapeutic mechanism of HePC is the direct toxicity to the parasite, it also affected the intracellular amastigotes in phagolysosomes. HePC is able to induce an increase in the [Ca2+] in some cancer cell lines and considering the well-known relevance of calcium homeostasis in *Leishmania* spp. (29), this researcher investigated the possible effect of HePC on Ca2+ regulation in these parasites and found that the drug generates a large and rapid increase in [Ca2+] in *L. mexicana* promastigotes and amastigotes. The specific increment in the [Ca2+] induced by HePC is probably due to the activation of a not-yet-characterized plasma membrane Ca2+ channel. There are some reports related to the existence of Ca2+ channels in Leishmania spp. Thus, an increase in the cytosolic Ca2+ levels through the activation of nonselective cation channel induced by oxidative stress in *L. donovani* has been demonstrated (30). HePC induces apoptosis-like death in *L. donovani*, a process that is associated with altered Ca2+homeostasis (31). Thus, it is conceivable that the disruption of Ca2+ homeostasis generated by HePC in *L. mexicana* could induce apoptotic-like phenomena seriously affecting the parasite's viability (32).

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