

# Introducing Miltefosine as an Anti-cryptosporidial Agent in Immunocompromised Mice

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## Abstract

Cryptosporidiosis is a clinically significant opportunistic infection causing diarrhea especially among immunocompromised patients includes those with immunodeficiency syndrome and certain immunosuppressed patients undergoing chemotherapy, organ transplant recipients treated with drugs that suppress the immune system, and patients with autoimmune disorders. The aim of this study was to evaluate the potential antiparasitic effect of miltefosine, (a phospholipid drug used for the treatment of visceral and cutaneous leishmaniasis) in immunocompromised (Dexamethasone treated) mice infected with *Cryptosporidium*. Parasitological examination of feces for oocysts count was performed ten and twenty days after start of treatment. Histopathological examination of intestinal, liver and spleen sections was held. Results revealed no significant reduction in mean *Cryptosporidium* oocyst number detected in immunocompromised infected group ten days post treatment (1.44%). Twenty days post treatment showed statistical significant reduction ( $p < 0.001$ ) reaching (38.63 %) when compared to the mean oocysts counts of infected untreated immunosuppressed group of mice. Histopathological sections of small intestine, liver and spleen showed several degrees of inflammatory changes before treatment. In treated group with miltefosine no improvements of small intestinal photomicrographs were seen; in contrast, significant improvement was observed in liver and spleen histopathological sections. Ziehl-Neelsen acid-fast stain was originally undertaken for the detection of *Cryptosporidium* oocysts within intestinal mice tissue. In conclusion, oral administration of miltefosine *in vivo* showed moderate efficiency against cryptosporidiosis in immunocompromised infected mice.

**Keywords:** Miltefosine; *Cryptosporidium*; Immunocompromised; Small intestine; Liver

## Introduction

*Cryptosporidium* species are obligate, intracellular, protozoan parasites that infect the epithelial cells of the distal jejunum and ileum of vertebrates. In immunocompetent individuals the disease is usually a self-limiting infection, but severe infection and diffusion to extra-intestinal sites or life-threatening can occur in high-risk individuals, such as the infant, children, and the old aged and in immunocompromised patients [1]. *Cryptosporidium* are spread by the ingestion of oocysts excreted by infected people or animals. Infection can be transmitted through consumption of contaminated water or food or via contact with contaminated environmental sources. Parasite development is relatively confined to the terminal jejunum and ileum yet, in immunosuppressed hosts the entire gastrointestinal tract as well as the biliary and pancreatic ducts may be infected and less frequently the respiratory tract [2]. The estimation of numerous chemotherapeutic agents as anti-cryptosporidial therapies to clear the host of these parasites is still limited [3].

Miltefosine (hexadecylphosphocholine, HePC), an alkylphosphocholine ester of hexadecanol, a membrane-active, alkylphospholipid, drug with demonstrated activity against various parasite species and cancer cells [4]. It is the first and still the only oral drug that can be used to treat visceral and cutaneous leishmaniasis and also other kinetoplastidae, *Trypanosoma cruzi* and *T. brucei* [4,5]. *Trichomonas vaginalis* [6] and other protozoan parasites, such as *Giardia lamblia* and *Balamuthia mandrillaris* [7,8].

Miltefosine was reported to have antihelminthic properties; it proved to have *in vitro* affected, against *Schistosoma* species (*Schistosoma mansoni*, *Schistosoma haematobium*). The authors added that miltefosine proved to have *in vitro* ovicidal, schistolarvicidal

and lethal activity on adult worms of both *Schistosoma* spp. and has considerable molluscicidal, activity on their snail hosts [9]. Recently, the Centers for Disease Control and Prevention (CDC) revealed miltefosine as the only and first line treatment for two fatal free living protozoal diseases, granulomatous amoebic encephalitis and primary amoebic meningoencephalitis [10]. Conclusions. Supportive therapy is very important in controlling cryptosporidiosis in immunocompromised patients but the search for effective antiparasitic agent is still recommended.

## Materials and Methods

### Mice

Twenty three, CD-1 male mice aged 7 week weight 20 gm were obtained from Schistosoma Biological. Supply. Program (SBSP) at Theodor. Bilharz Research Institute (TBRI) Cairo, Egypt. All mice were housed in separate sterilized plastic cages with filter top, and fed with sterilized food and water *ad libitum* under specific pathogen-free (SPF) conditions. To avoid physical, contact and minimize risk of infection by cross contamination, immunocompromised infected, mice were housed individually [11].

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## Drug

\*Miltefosine was manufactured and provided by Sigma-Aldrich Chemie GmbH, Germany. The administration doses of drug started after ten day post infection (PI).

\*Dexamethasone sodium phosphate (Dex) was manufactured and provided by (SIGMA-TEC, Egypt). Administration doses of drugs were calculated by extrapolation of human therapeutic dose to animal doses.

## Parasite

*Cryptosporidium* oocysts used for infection of CD1 mice included in the present study were obtained from [The Animal Reproduction Research Institute (ARRI)] Giza, Egypt. Samples were microscopically screened by floatation technique [12] and staining by modified. Ziehl-Neelsen. Acid fast stains [13]. Oocysts were suspended in phosphate buffered saline (PBS) with 0.01% Tween20, and stored at 4°C in the presence of antimicrobial (penicillin, streptomycin, and amphotericin B) [14].

## Experimental design

Animals were divided into two main groups: Control group (I) which represented normal non-infected non-treated group (five mice) and Group (II) including eighteen immunocompromised mice. Animals of this group (II) were administered with 20 mg/kg/day dexamethasone sodium phosphate (Dex) intramuscularly 3 times per week for 20 days prior to oocysts inoculation [11,15]. Then each mouse was inoculated with 10<sup>2</sup> *Cryptosporidium* oocysts in 200 µl of phosphate buffered saline (PBS) by oral gavage. This group was subdivided into two subgroups: Group (IIa) consisted of ten immunocompromised infected mice receiving no treatment, Group (IIb) comprised eight immunosuppressed infected animals treated with miltefosine 10 days post infection at a dose of 20 mg/kg /day [8] for 20 consecutive days. One mouse of group (IIb) died second day after treatment.

## Stool examination

Each infected mouse was transferred into a separate clean cage for sixty minute [14]. Fecal samples were collected, weighed then transferred into clean containers with tight-fitting covers and homogenized in PBS. Fecal samples, were, subjected to the modified Ziehl-Neelsen staining technique then examined for oocysts which were counted using light microscope under (1000X) objective. The stools were collected twice from mice of groups (IIa and IIb); the first samples being prepared and examined 10 days after start of treatment. Stools were collected and preceded for staining for the second time 20 days following start of miltefosine administration and in the same time at the end of treatment which was being given for 20 consecutive days.

## Histopathological study

Following termination of miltefosine administration and collection of stools, mice were sacrificed. Small part of the ileum and also small part of liver and spleen were fixed in 10% neutral buffered formalin. After fixation the tissues were processed for paraffin embedding. Histopathological sections of 4 µm thickness were stained with Hematoxylin and Eosin stain [16], Masson Trichrome for liver sections [17] and Periodic Acid stain (PAS) for spleen [18]. Ziehl-Neelsen acid fast stain [19] was also used to stain small intestinal tissue sections for the detection of *Cryptosporidium*.

## Statistical analysis

Statistics were expressed as mean values ± SD by the statistical

software package SPSS (version 16.0). Comparisons between, groups were done using the Student's *t*-test. Percent inhibition compared to infect untreated. Oocysts were determined using the following equation: [(Infected immunocompromised untreated - Treated)/Infected immunocompromised untreated] × 100. A *p*-value equal, to or less than 0.05 was appreciate, significant.

## Results

### Mean number of *Cryptosporidium* oocysts in faeces

The mean number of *Cryptosporidium* oocysts ten days and three weeks after administration of miltefosine are described in Table 1.

The mean number of *Cryptosporidium* oocysts ten days after start of treatment showed statistical non-significant (*p*>0.05) reduction (1.44%) in immunocompromised infected mice treated with miltefosine, group (IIb), when compared to the number of *Cryptosporidium* oocysts in feces of immunocompromised infected untreated mice of group (IIa). The mean number of oocysts counted in stools collected twenty days post treatment from animals of group (IIa) decreased when compared to that of group (II a) and revealed statistical significant (*p*<0.001) reduction ( 38.63% ) (Table 1).

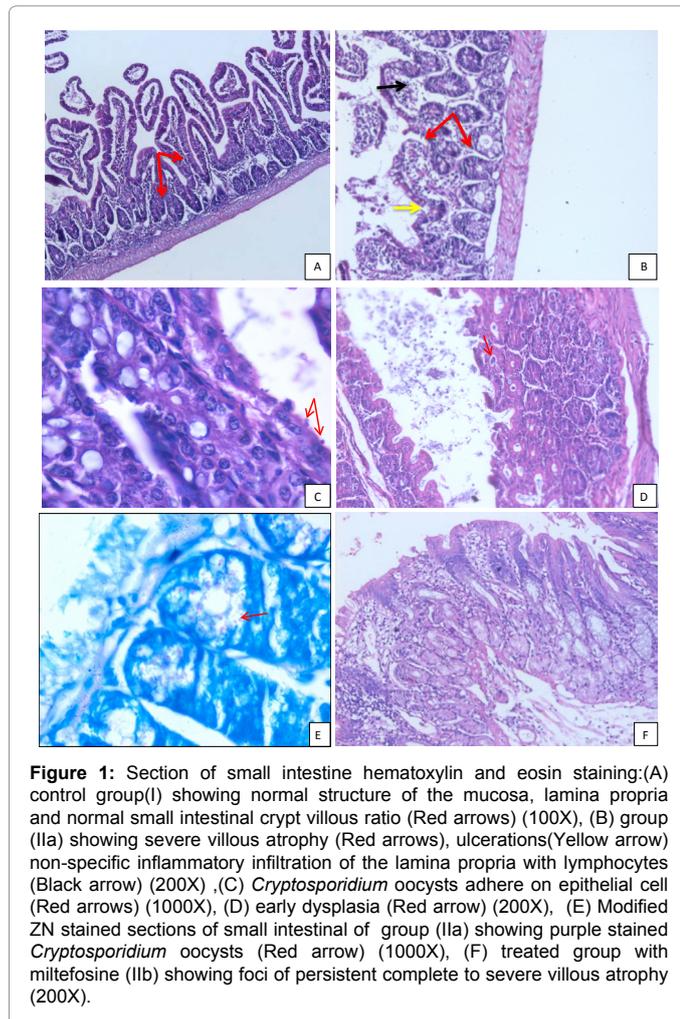
### Histopathological examination

Several changes in intestine tissue due to inflammation were seen in the group immunocompromised infected with the parasites. Histological examination of sections of small intestine of mice in control group (I) showed normal structure of the mucosa, lamina propria and normal small intestinal crypt villous ratio. Goblet cells were moderate in number with a healthy well defined brush border as demonstrated in Figure 1A. The immunocompromised untreated mice (IIa) showed noticeable histopathological changes in the morphology of the intestinal mucosa, intestinal ulcerations, and complete severe villous atrophy with severe depletion of goblet cell, non-specific inflammatory infiltration of the lamina propria with lymphocytes (Figure 1B). Also *Cryptosporidium* oocysts were appeared adhere on epithelial cell with destruction of brush border (Figure 1C). Early dysplasia manifested by hyperchromatic nuclei with irregular size (Figure 1D). Modified ZN stained sections of small intestinal of the group (IIa), showed purple stained *Cryptosporidium* in small sized (4-6 µm) that occur singly or in rows along the mucosal brush border from villi to crypts (Figure 1E). In miltefosine treated group (IIb) showed foci of persistent complete to severe villous atrophy. Epithelial cells presented a loss of their normal surface but no evidence of dysplasia was seeing (Figure 1F).

Liver sections of control group (I) showed the hepatocytes are organized into plates separated by vascular sinusoids in hexagonal arrangement centered by central veins and shows portal tracts on the periphery (Figure 2A). Histopathological liver sections among five mice of immunosuppressed infected untreated group (IIa) revealed few

Groups	No. of oocysts (Mean ± SD)	%Reduction in number of <i>Cryptosporidium</i> , oocysts	
GI	----	----	10 days
GIIa	15746.11 ± 1464.91	----	
GIIb	15520.11 ± 1457.61	1.44	
GIIa	15973.33 ± 2851.95	----	20 days
GIIb	9802.22 ± 1243.84**	38.63	

**Table 1:** The number and the percentage of reduction of *Cryptosporidium*. oocysts/g faeces ten and twenty days post-treatment with miltefosine. GI: Control uninfected non-treated (n=5), GIIa (n=10): Infected immunocompromised, GIIb (n=8): Infected immunocompromised miltefosine treated. Note: \*\* statistically significant (*p* < 0.001).



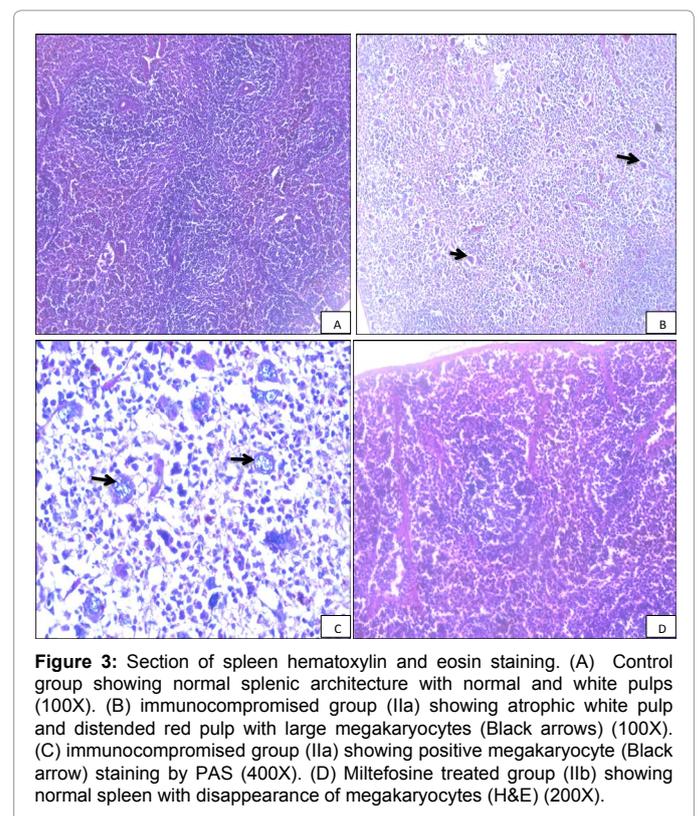
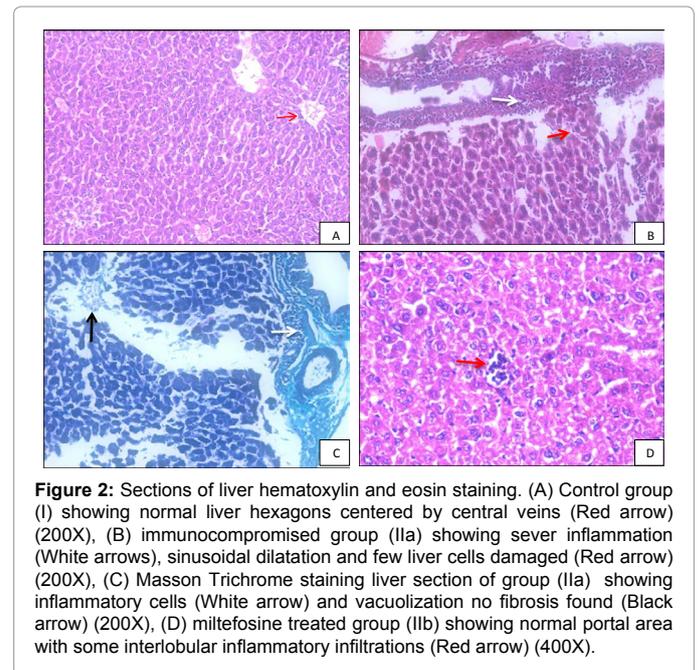
damaged liver cells at certain places and showing vacuole degeneration, with loss of radial arrangement of hepatocytes, also suffered from moderate to severe inflammation (Figure 2B). No evidence of dysplasia and no fibrosis were seen by Masson Trichrome (Figure 2C). Liver section of group (IIb) showed improvement in hepatocyte and nucleus seen in normal size. Inflammation reduced from moderate to mild lobular and/or portal inflammation (Figure 2D).

Histological examination of sections of spleen from (II) group showed normal red pulp and white pulp, central arterioles surrounded by lymphoid tissue (Figure 3A). Five mice of group (IIa) showed disorganization of the splenic tissue includes atrophic white pulp and distended red pulp with sheets of megakaryocytes representing the extra-medullary hematopoiesis (Figure 3B). Megakaryocytes (atypical large cells) showing high nucleic to cytoplasm (NC) ratio stained purple in PAS stain (Figure 3C). Twenty day post treatment all mice showed normal splenic lymphoid tissues. No evidence of megakaryocyte (Figure 3D).

## Discussion

In this study, the first main experiment was conducted to evaluate the potential effect of the anti-leishmanial agent miltefosine against experimental cryptosporidiosis in immunocompromised mice. Miltefosine showed broad anti-protozoal activity [4] it is currently the only effective oral treatment for leishmaniasis [20].

In infected immunocompromised mice (IIa), maximum shedding of oocysts in feces were observed on day 20 post-treatment. In a retrospective study it was shown that the level of oocysts excretion was higher in Dex- immunosuppressed animals [21,22]. This result is in agreement with Benamrouz et al. [14] who showed that in DexSCID mice inoculated with low doses of oocysts, the excretion of oocysts increased, reaching a mean of oocysts shedding of more than 10,000 oocysts per gram of faeces at forty five days post infection. Miltefosine when given



for 10 days to treat *Cryptosporidium* in immunocompromised infected group (IIb) yielded no significant reduction ( $p>0.05$ ) in the number of oocysts when compared with infected untreated groups being (1.44%). On the other hand, administration of miltefosine for Twenty days revealed significant reduction ( $p<0.001$ ) in the number of oocysts in infected treated groups the reduction being (38.63%) respectively. These results are in accordance with the study of Sinkala et al. [23] reported that miltefosine administration to Zambian adults with HIV-related cryptosporidiosis was terminated prematurely because of lack of efficacy and the development of severe adverse events. In another study the activity of miltefosine against *C. parvum* was demonstrated *in vitro* showing 78-98% inhibition of parasite at 45h post infection [24]. It seem that an intact immune system established a fair chemotherapeutic response, since treatment with miltefosine showed moderate effect in HIV -Ethiopian men with visceral leishmaniasis, initial treatment failure with miltefosine occurred in 18% of HIV-infected patients, compared with treatment failure in 5% of non-HIV-infected patients [25]. In this work, histopathological changes in-group (IIa) showed change in intestinal morphology and depletion of goblet cells in the infected villi. Goblet cells are the important cells which play good role as a defensive barrier and regulated physiological functions of the intestine [26]. The change of the intestinal surface was manifested by villous dystrophy and increased crypt length with cellular infiltration. Several reports similarly described crypt elongation and villous atrophy, and may be accompanied by mixed inflammatory-cell infiltrate in the lamina propria; in areas of severe injury, the epithelial cells showed blunted microvilli [11,27,28]. Twenty days after treatment with miltefosine did not ameliorate undesired result appeared in intestinal morphology with group (IIb), although a decrease in oocysts output percentage has been noticed. This finding is in agreement with [23] who reported that 6 of (41) HIV patient with cryptosporidiosis developed colonic infarction with intestinal obstruction on 6 day post treatment with miltefosine. Similarly pharmacokinetics studies have shown that oral administration of miltefosine has many adverse effects such as gastrointestinal symptoms and the severity may due to the immune state of mice [4]. On the other hand, no dysplasia was seen after treatment in group (IIb), while in group (IIa) early dysplasia manifested by hyperchromatic nuclei with irregular size. These results agree with Eissa and Amer [8] who studied the effect of miltefosine on murine giardiasis, miltefosine achieved a pronounced improvement in the pathological mucosal changes and a lesser degree of inflammatory infiltrate. In this study, liver section from five mice of group (IIa) showed inflammatory changes with no fibrosis, 30 days post-infection. Stephens et al. [29] reported that chronic infections of the biliary tract with *C. parvum* in SCID mice developed triaditis, cholangitis and lobular hepatitis. Moreover dysplastic changes in the liver after *C. parvum* infection have been reported in SCID mice [30]. According to Abdou et al. [31], only one experimental immunosuppressed mouse was demonstrating large cell dysplasia in the liver. Treated groups with miltefosine showed the improvement of liver cell architecture. Eissa et al. [9] showed that the efficacy of miltefosine in treatment of mice infected with either invasive, juvenile or adult stages of *Schistosoma mansoni* resulted in significant reduction of hepatic granulomata size and improvement of hepatic pathology. The improvement in liver pathology could be explained by the uptake of miltefosine by liver tissue and it may possibly play a role in healing liver tissue. The spleen contains vascular and lymphoid elements and it is a site of hematopoiesis, and in some species removal of aged red blood cells as well as particulate materials and circulating bacteria from the blood supply. The spleen is the site of direct and indirect toxicity and a target for some carcinogens and also a site for metastasis of

malignant neoplasms arising in other sites [32]. In these study mice from group (IIa) showed large megakaryocyte in spleen section. This means that extra-medullary hematopoiesis was the development and growth of blood cells outside the medullary spaces of the bone marrow. Spleen is a common site of extra-medullary hematopoiesis. It was refers to hematopoiesis occurring outside of the medulla of the bone. During foetal development, hematopoiesis occurs at various places, such as the spleen and liver [33]. However, it is more commonly associated with pathologic developments in cryptosporidiosis [34]. Miltefosine treated group showed improvement of spleen histopathology. These results are in agreement with [20] who found that administration of miltefosine cause decrease of spleen size leishmaniasis patient. Miltefosine had noticeable anti-cryptosporidial activity *in vitro* [24] but in the present study oral administration of miltefosine *in vivo* showed moderate efficiency against *Cryptosporidium* in immunocompromised infected mice. Changes in dose or treatment schedule to avoid its adverse side effects would be recommended or a possible trial of Nano drug delivery with minimal doses.

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