Global Veterinaria 15 (2): 137-143, 2015
ISSN 1992-6197
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DOI: 10.5829/idosi.gv.2015.15.02.9639

Efficacy of Low and High Dose of Paromomycin Sulfate for Treatment of Cryptosporidiosis in Immunosuppressed Infected-Mice

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Abstract: *Cryptosporidium parvum* is a protozoan parasite that infects the gastrointestinal epithelial cells causing several parasitological and pathological changes. It is incriminated in the development of colorectal cancer in immunosuppressed individuals. This study aimed to evaluate the effectiveness of low and high doses of paromomycin sulfate in the treatment of cryptosporidiosis in mice. Five groups of mice were included: group I, infected control; group II, infected and immunosuppressed; group III, infected immunosuppressed and treated with low dose of paromomycin sulfate; group IV, infected, immunosuppressed and treated with high dose of paromomycin sulfate; and groups V, non-infected control. Mice were subjected to stool examination for oocyst count prior to inoculation and every 5 days after infection until the end of the experiment (Day 35) and were later sacrificed for intestinal dissection and routine histopathological examination. Group II showed the highest numbers of oocysts shed and endogenous developmental stages compared to the other groups. Intestinal dysplastic changes were seen only in groups I and II, where these changes were in favor of group II compared to group I. This study was concluded that paromomycin sulfate was effective in the treatment of *Cryptosporidium* infection.

Key words: *Cryptosporidium parvum* · Paromomycin Sulfate · Immune suppression Oocysts Shed

INTRODUCTION

Cryptosporidium species are protozoan parasites that cause infection and diarrheal illness in a wide range of mammalian species [1]. *Cryptosporidium* belongs to the Apicomplexa, which are unicellular organisms possessing at some stage an apical complex; a specialized assembly of organelles believed to be involved in host invasion [2]. The genus *Cryptosporidium* contains many species, genotypes and subtypes that infect a wide range of vertebrates including humans. Each may have different sources of infection, transmission routes and pathogencity [3]. *Cryptosporidium hominis* and *C. parvum* are the most frequently detected. *C. hominis* infections are more common in developing countries [4]. *C. parvum* is an obligate intracellular parasite that infects the epithelial lining of luminal surfaces of gastrointestinal and respiratory tracts in a wide variety of hosts. In immunocompetent individuals, the organism is primarily localized in the distal small intestine and proximal colon, whereas in immune compromised hosts, the parasite had been identified throughout the gut, biliary and respiratory tracts [5]. There are many diagnostic tests for *Cryptosporidium*. They include microscopy, staining and detection of antibodies. Microscopy [6] can help identify oocysts in fecal matter [7]. To increase the chance of finding the oocysts, the diagnostician should inspect at least 3 stool samples [8]. There are several techniques to concentrate either the stool sample or the oocysts. The modified formalin-ethyl acetate (FEA) concentration method concentrates the stool [9]. Both the modified zinc sulfate centrifugal flotation technique and the Sheather's sugar flotation procedure can concentrate the oocysts by causing them to float [8]. Another form of microscopy is fluorescent microscopy done by staining with auramine [7]. PCR technology is a powerful alternative for the detection of *C. parvum* in both environmental and clinical samples [10]. To date, there is no totally effective and approved therapy for cryptosporidiosis and a healthy intact immune system seems to be the only solution [11]. Many vaccines and chemotherapeutic agents had been

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tested for prophylaxis against cryptosporidiosis [12]. Azithromycin showed partial results against the disease [13]. In vitro and in vivo effects of nitazoxanide had been demonstrated using different animal models and finally in clinical trials. Repeated doses of nitazoxanide and albendazole were also effective against cryptosporidiosis [14]. Paromomycin is most commonly used drug against cryptosporidiosis [15, 16] animal models [17-21] and uncontrolled clinical evaluations [22-25]. Paromomycin is probably the most promising compound for human treatment of cryptosporidiosis. It had been shown to be effective at a dosage of 50mg/kg/day or more for ileal infection and 200mg/kg/day or more for caecal infection. The effect was thus shown to differ according to the anatomical site of the infection [21]. These results confirmed the anti-cryptosporidial activity of paromomycin and underscored the limitations of this compound because of its potential toxicity at such high dosages and its inability to eradicate the infection. They suggested that only a beneficial effect on symptoms rather than a clearing of the infection might be expected from increasing the dose in humans [26]. Because of the great need to develop new anti-cryptosporidial agents, trials were designed to test the potency of different agents for treating cryptosporidiosis. Therefore, the present study aimed at investigating the antiparasitic effectiveness of paromomycin sulfate with low and high dose as treatment of *Cryptosporidium* infections in experimentally infected mice.

MATERIALS AND METHODS

Experimental Animals: Animals used in this work were male Swiss albino mice, aged five to six weeks, weighing 20-25 g, clean from any parasitic infection were obtained from Schistosome Biological Supply Centre (SBSC), Theodor Bilharz Research Institute (TBRI). They were housed in well ventilated cages with perforated covers, supplied with standard pellet food and water. Bedding was changed every day. The mice were allowed to adapt to the laboratory environment for one week before the experiment. This study was approved by the Ethics Committee of TBRI.

Parasites: Stool samples were collected from 30 immunosuppressed patients with chronic diarrhea, in the Kasr ELini Hospital and Fever Hospital from Haematology Department and Renal Dialysis Unit, from March 2013 to March 2014. Informed consents were obtained from the patients. The stool samples were transferred to the parasitology department to be screened by different techniques for the presence of intestinal protozoa. All samples were microscopically screened by modified Ziehl–Neelsen acid fast stain (MZN), aiming to identify the positive cases of *Cryptosporidium*.

Drugs:

- Paromomycin sulfate (Pfizer, Parkes-Davis, Courbevoie, France) was first dissolved in 5% dimethyl sulfoxide (DMSO) and then diluted in water prior to use.
- Dexamethasone (Qualimed, Puteaux, France) intraperitoneally injected with 0.80 mg/mice.

Studied Groups: Animals were divided into five groups:

Group I: (Infected control group), mice orally infected with 10^6 of *Cryptosporidium* oocysts /ml/mice.
Group II: (Immunosuppressed infected group), mice orally infected with 10^6 of *Cryptosporidium* oocysts/ml/mice and orally intraperitoneally injected with 0.8 mg/mice/ three times /week for 5 consecutive weeks.
Group III: Immunosuppressed infected and treated group, mice orally infected with 10^6 of *Cryptosporidium* oocysts/ml/mice and intraperitoneally injected with 0.8 mg/mice/ three times /week for 5 consecutive weeks and treated with low dose paromomycin orally (50mg/kg/ mice) daily for five consecutive days two weeks post infection.
Group IV: Immunosuppressed infected and treated group, mice orally infected with 10^6 of *Cryptosporidium* oocysts/ml/mice and intraperitoneally injected with 0.8 mg/mice/ three times /week for 5 consecutive weeks and treated with high dose Paromomycin orally (200mg/kg/ mice) daily for five consecutive days two weeks post infection.
Group V: Normal mice

Animals were sacrificed four weeks post infection.

Parasitological Examination: Mouse fecal samples were collected prior to inoculation and every 5 days after infection until the end of the experiment (Day 35). Briefly, Fresh fecal pellets from each mouse in the study groups were collected separately every 2 days over the 35 days of the experiment, according to the group to which they were assigned. Each sample was suspended in 10% formalin and homogenized. Then, 1 mg was prepared as a fecal smear and stained by the modified Ziehl-Neelsen staining method.

Modified Ziehl–Neelsen Staining Method: Stool samples were scooped and smeared on a clean glass slide, followed by fixing with 95% absolute methanol and stained using the modified Ziehl-Neelsen stain and air dried. Afterwards the smear was further stained with cold carbol fuchsin and allowed to stand for 10 minutes after which it was washed off with clean tap water. The smear was decolorized with 3% hydrochloric acid (HCl) in 95% ethanol, rinsed off and counterstained with 0.25% weight per volume malachite green for 30 s. The smear was, again, washed off with clean tap water and air dry. The slide was then observed microscopically for oocysts [27].

Sacrification of Mice: Sacrification of mice was done two weeks after administration of drugs by intraperitoneal anesthesia. The upper part of small intestine was removed; the duodenal contents were subjected to the previous parasitological examination and subjected to histopathological examination.

Efficacy of Selected Drugs: Efficacy of selected drugs against *Cryptosporidium* oocysts was calculated as per formula:

$$\text{Efficacy (\%)} = \frac{\text{Total oocysts before treatment} - \text{Total oocysts after treatment}}{\text{Total oocysts before treatment}} \times 100$$

Histopathological Examination: The small intestine of mice were fixed in 10% neutral buffered formalin. Sections, stained by hematoxylin and eosin (H&E) and (ZN stain) then examined by light microscopy according to standard operation procedures [28].

RESULTS

Efficacy of Paromomycin Sulfate on Mean Number of *C. parvum* Oocysts: Efficacy of low and high dose of paromomycin sulfate against cryptosporidiosis was evaluated on the basis of reduction in the oocyst per gram of feces and intestine post treatment in relation to time. The means of reduction in *Cryptosporidium* oocysts of treated and control groups were compared. Results for efficacy of paromomycin sulfate at different doses against *Cryptosporidium* oocysts in experimentally infected mice determine on the basis of reduction in oocyst per gram of feces (OPG) are presented at Table 1. OPG count showed an increasing trend in control (Untreated) animals. Low dose of 50mg/kg body weight of paromomycin sulfate caused a decrease in OPG count from 7th day post treatment and onward, the lowest count where 840 at the end of experiment. At 200 mg/kg body weight dose of paromomycin sulfate high significant reduction in OPG count of *Cryptosporidium* was recorded from 7th post treatment day and onward the lowest count where 100 at the end of experiment. (Table 1, 2).

Effect of Paromomycin Sulfate on Mean Count of *C. parvum* in Intestinal Contents: The ileum was the site with the heaviest burden of intestinal cryptosporidiosis. In immune suppressed infected group, the mean number of endogenous developmental stages of the parasite at the end of day 35 PI was (100.4±10.5) $x10^4$, while it was (80 ± 12.3) $x10^4$ in infected control group.; this difference was statistically significant increase ($p = 0.05$). However, on day 35 PI it was observed that there was a significant decrease in the mean number of endogenous developmental stages of *Cryptosporidium* in immunosuppressed infected treated with low dose (60.3 ±7.5) $x10^4$. In immunosuppressed infected treated with high dose, it continued to decrease to reach 20.1± 3.9 $x10^4$., with a significant difference compared to infected control group ($p < 0.0001$) (Table 3).

Effect of Paromomycin Sulfate on Histopathology of *C. parvum*-Infected Mice: Sections of the small intestine from *C. parvum*-infected mice without treatment displayed marked acute inflammation, mild villous blunting and abundance of *C. parvum* intracellular life cycle stages (Fig. 2B&C). Sections of small intestines from mice infected with *C. parvum* and treated dexamethasone showed Several degrees of inflammatory changes, focal inflammation as regards dysplasia and an abundance of *C. parvum* life cycle stages (Fig. 2D). In contrast, sections from the intestines of mice infected with *C. parvum* and treated with low dose of paromomycin sulfate (50 mg/kg)

Table 1: Effect of paromomycin sulfate on mean number of *C.* parvum oocysts

Groups	After 0days	After 7days	After 14days	After 21days	After 28days	After 35 days
Infected control	7450	8620	10480	14800	20500	26500
Immunosuppressed infected group	11200	15200	18700	23450	29700	33600
Immunosuppressed infected and treated group (Low dose)	5600	4200	4000	3200	2800	840
Immunosuppressed infected and treated group (High dose)	3200	2100	1600	880	420	100

Table 2: Efficacy of paromomycin sulfate at different doses against *Cryptosporidium* oocysts.

Groups	After 0days	After 7days	After 14days	After 21days	After 28days	After 35 days
Infected control	-	13.6 %↑	26.3 %↑	25 %↑	34.4 %↑	13.6 %↑
Immunosuppressed infected group	-	28.5%↑	40.1%↑	28.6%↑	50%↑	28.5%↑
Immunosuppressed infected and treated group (Low dose)	-	49.7%↓	52.2%↓	42.9%↓	72.5%↓	49.7%↓
Immunosuppressed infected and treated group (High dose)	-	63.7%↓	62.3%↓	50%↓	87%↓	63.7%↓

Table 3: Effect of treatment with paromomycin sulfate on mean count of *C. parvum* in intestinal contents

Groups	Mean count of *C.* parvum x10^4	%reduction
Infected control	80.3	-
Immunosuppressed infected group	100.4	20.1%↑
Immunosuppressed infected and treated group (Low dose)	60.3	24. 9%↓
Immunosuppressed infected and treated group (High dose)	20. 1	75 %↓

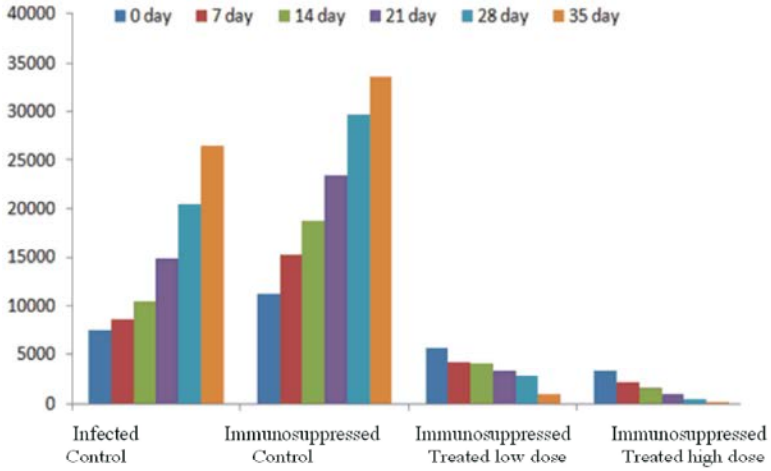

Fig. 1: Mean number of *C.* parvum oocysts in different studied groups

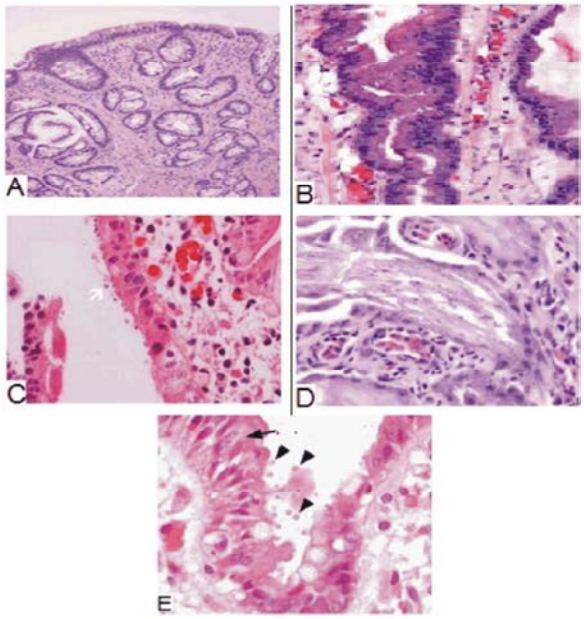

Fig. 2: Effect of low and high dose of paromomycin sulfate on histopathological changes of the small intestine from *C. parvum*-infected mice. (A) Normal mice. (B) Infected control mice. (C) Immunosuppressed infected mice. (D) Immunosuppressed infected mice and treated with low dose. (E) Immunosuppressed infected mice and treated with high dose

showed minimal focal inflammation in only 20% of the mice (Fig. 2D). Sections from the intestines of mice infected with *C. parvum* and treated with high dose of paromomycin sulfate (200 mg/kg) showed minimal focal inflammation in only 10% of the mice (Fig. 2E). The small intestines from uninfected mice were normal (Fig. 2A).

DISCUSSION

Cryptosporidiosis, also known as crypto, is a parasitic disease caused by *Cryptosporidium*, a protozoan parasite in the phylum Apicomplexa. It affects the intestines and is typically an acute short-term infection. It is spread through the fecal-oral route, often through contaminated water; Priest *et al*. [1] *Cryptosporidium* is a protozoan parasite that infects the gastrointestinal tract of vertebrate animals, including mammals, birds, reptiles, amphibians and fish [29, 30]. Cryptosporidiosis is found worldwide. It causes 50.8% of water-borne diseases that are attributed to parasites. In developing countries, 8-19% of diarrheal diseases can be attributed to *Cryptosporidium* [31]. Among the most commonly used treatments against cryptosporidiosis are paromomycin and azithromycin, which are partially effective [13]. In the current work, dexamethasone, a synthetic glucocorticoid, was used to induce chemical immunosuppression in the mice. Glucocorticoids are known to have an effect on the priming of the innate immune response and could suppress IFN-γ-regulated expression [32]. This study was carried out over a period of 35 days to evaluate the course of infection. Matsui *et al*. [33] reported that the interval which covered the natural shedding period of *Cryptosporidium* infection in mice was about 24 days. Lacroix-Lamande *et al*. [34] found that the duration of oocyst shedding was about 3–4 weeks. The intensity of oocyst shedding was higher in dexamethasone immune suppressed mice than in immunocompetent ones throughout the duration of the experiment. Similar results had been reported by Kapel *et al.* [35], Chai *et al.* [36] and Certad *et al*. [37]. In this work, the maximum shedding of oocysts in immunocomopetent infected group (Groups I) was observed on days 28 and 35 PI, in agreement with Miller *et al*. [38] and Certad *et al*. [37]. On the other hand, dexamethasone immunosuppressed mice (Group II) showed high levels of oocyst shedding throughout and at the end of the experiment. These results were in agreement with those of several studies [37-40]. paromomycin sulfate have broad antiparasitic activities. In the present study, paromomycin sulfate was tested as a treatment for cryptosporidiosis in at low and high dose against the experimentally infected dexamethasone-immunosuppressed groups (Group III and group IV). Our study demonstrated the effectiveness of paromomycin sulfate in infected groups with significant differences regarding levels of oocyst excretion in the stool and the number of endogenous developmental stages of the parasite in both groups, being lower in group IV (High dose) than in group III ($p < 0.05$) (Table 1 and Table 2). These results agreed with those of Bailey and Erramouspe [41] and Fox and Saravolatz [42] who found that the responses to the drug were lower in immunocompromised individuals. Although, the drug caused a marked decrease in the mean number of oocysts per milligram after the initiation of therapy in group III, the results showed high significant difference ($p < 0.01$) when compared to group I &II and few number of oocysts were detected in the stools of either treated group with low and high dose paromomycin sulfate by the end of the experiment. It is clear that the competent immune system rejected the parasite and caused a self-limited disease in the untreated group, while infected untreated immunosuppressed mice (Group II) still showed a high level of oocyst excretion until the last day of the experiment (Table 1). This might be due to a failure of the immune system to eradicate the parasite. Similar findings had been recorded in several studies including in vitro and in vivo studies using several animal models and in clinical trials, which had demonstrated the effectiveness of paromomycin sulfate in treating diarrhea and enteritis caused by *Cryptosporidium* species in immunocompetent patients [39]. In the present study, during and after treatment with low and high dose of paromomycin sulfate, oocyst shedding gradually decreased to very low levels or disappeared by day 35 postinfection. The overall reductions in oocyst numbers during treatment were more pronounced in the animals treated with paromomycin. Given that the location of *C. parvum* replication is in parasitophorous vacuoles at the apices of intestinal enterocytes, it is perhaps not surprising that the paromomycin route was more efficient than the i.p. route. This may also explain why relatively low doses of paromomycin sulfate (35 mg/kg or 70 mg/kg BID), had a significant impact on *C. parvum* infection. In conclusion, *Cryptosporidium parvum* is one of the infectious agents that may induce intestinal dysplasia, even of high-grade category, which is highly affected by immune suppression states and elevated endogenous parasite loads. paromomycin sulfate is a good and useful treatment for *Cryptosporidium parvum*.

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