Original article

Effects of two anticoccidial drugs, Monensin, Toltrazuril and the mixture of them on *Cryptosporidium parvum in vitro*

Maryam Fathi, MSc, Javid Sadraei, PhD*, Fatemeh Ghaffarifar, PhD

Parasitology Department of Medical School, Tarbiat Modares University, P. O. Box 14115-331, Tehran, Iran

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Abstract

Introduction and objective: *Cryptosporidium parvum* is a protozoan parasite that is a common cause of diarrhea in both animals and humans worldwide. There is no effective specific chemotherapeutic treatment. The aim of this study was to survey and compare the anticryptosporidial effect of two anticoccidial drugs, Monensin, Toltrazuril and synergic effect on oocysts of *C. parvum in vitro*.

Materials and methods: *Cryptosporidium parvum* oocysts were isolated from the fecal samples of calves after purification, stored in Hanks Balance Salt Solution at 4°C. They were exposed to different concentrations of the drugs, Monensin, Toltrazuril and the mixture of them (0, 0.1, 0.5, 1, 10, 20, 60 and 100µg/ml). The effects of the drugs were evaluated by counting the complete oocysts after 24h and 48h incubation at 37°C.

Results: The results showed a significant decrease in the oocysts number related to the increase in the concentration and exposure time of the drugs. The mixture of two drugs had the highest efficacy on the *C. parvum* oocysts than each of drugs alone ($P < 0.001$) and Monensin in contrast to Toltrazuril at the same concentration showed to be more effective ($P<0.001$). These drugs in all concentrations were effective on *C. parvum* oocysts, and at100µg/ml had the highest efficacy and at1µg/ml had the least.

Conclusion: This study showed that two drugs were effective on *C. parvum* oocysts and Monensin was more effective than Toltrazuril and the mixture of them was more effective than each of them alone because of their synergism.

Keywords: *Cryptosporidium parvum*; Anticoccidial drugs; Monensin; Toltrazuril; Hanks Balance Salt Solution (HBSS)

****Address for correspondence:***
Dr. Javid Sadraei, Parasitology Department of Medical School, Tarbiat Modares University, P. O. Box 14115-331, Tehran, Iran; Telefax: +9821 82883841; Email: Sadraeij@modares.ac.ir

Introduction

Cryptosporidium parvum is a protozoan parasite that infects the epithelial cells of small intestine and is highly infectious for human and animals. Cryptosporidiosis is usually a self-limiting disease in the immunocompetent host. In humans, commonly reported symptoms are diarrhea, vomiting, abdominal pain and headache [1]. Among immunocompromised individuals including those with acquired immune deficiency syndrome (AIDS), there can be extra intestinal spread of infection, particularly to the respiratory tract.

Additionally, diarrhea often persists and becomes life-threatening [1]. Although many antimicrobial compounds have been tested for the efficacy against cryptosporidiosis in animals and humans, choice drug is not available for prophylaxis or treatment [2]. The aim of this study was to compare the effects of two drugs; and the mixture of them with different concentrations on *C. parvum* oocysts.

Materials and methods

Source of Cryptosporidium parvum and sample preparation

Cryptosporidium parvum oocysts were collected from the stools of naturally infected calves of the stockyard of Shahriar (suburb of Tehran, Iran) in three months. The samples were identified as *C. parvum* by the oocyst morphology and modified Ziehl-Neelsen technique (Fig. 1). All samples were purified and centrifuged. Then the sediments were suspended with 1ml PBS, carefully layered on the top of a cold (4°C) Sheather's solution diluted 1:2(v/v) and centrifuged for 20min at 1500g. The white layer at the top of the sucrose solution was removed and layered again on the Sheather's solution. The oocysts were mixed with Hank's Balance Salt Solution (HBSS) and stored at 4°C [3].

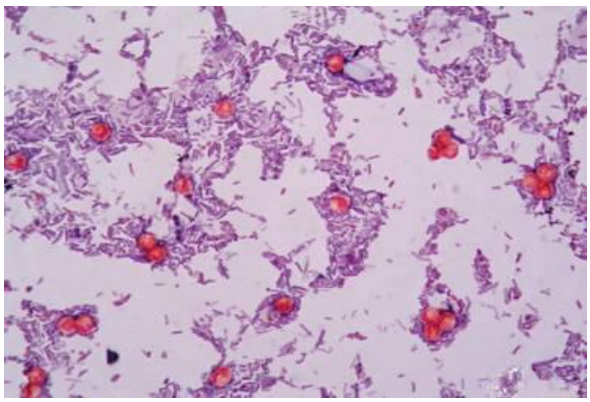

Fig. 1: *Cryptosporidium parvum* oocysts in stool (Ziehl-Neelsen technique, ×100)

Assessment of drugs activity on C. parvum oocysts

Serial concentrations (0, 0.1, 0.5, 1, 10, 20, 60 and 100µgml^{-1}) of Monensin (Monensin sodium 10%, Daroosazane Iran, Iran), Toltrazuril (Bycox 2.5%, Sepide Dahdasht, Iran) and the mixture of them, adjusted to pH 7 and filtered through 0.2µ filters [4]. The experiments were performed separately at 24h and 48h. We added 100µl of the stock suspension of *C. parvum* oocysts to 900µl of each concentration of drug in 1.5ml microcentrifuge tube. The samples were then vortexed and incubated for 24h and 48h at 37°C [4,5]. Following the incubation at first 24h, each sample was centrifuged (12.500g for 5mins), then 5µl of the pellet was sampled and counted under the bright field microscopy (1250×total magnification) for complete oocysts with four sporozoites. After 48h, the samples were taken out from the incubator and the same assay was repeated [6]. The controls in all steps contained no drugs.

Statistical analysis

The results are the mean of duplicate counts. Repeated Measure Analysis of Variance (ANOVA) (SPSS 17.0) was used for data analysis. The data were assumed to be normally distributed. The results were

expressed as mean ± standard deviation (SD). P values less than or equal to 0.05 were regarded as significant.

Results

Two anticoccidial drugs, Monensin sodium and Toltrazuril alone and a combination of them, were evaluated for anticryptosporidial activity in HBSS. The viability of purified *C. parvum* oocycts was evaluated after 24 and 48h incubated at 37°C with drugs. In this study, results showed that mixture of two drugs has the most efficacy on *C. parvum* oocycts than each of the alone ($p<0.001$). In addition Monensin sodium in contrast to Toltrazuril at the same concentration showed more efficacy ($p<0.001$).

In general all drugs in the highest concentration (100µg/ml) have the most efficacy because the number of viable and active oocycts remained was the least. The lower concentration of the drug (0.1µg/ml) has the least efficacy, because the number of viable and active remained oocycts was the most. As our data showed the exposure time had significant efficacy on decreasing the number of oocysts (both drugs were more effective at 48h than at 24h) ($P<0.001$) (Figs. 2,3)

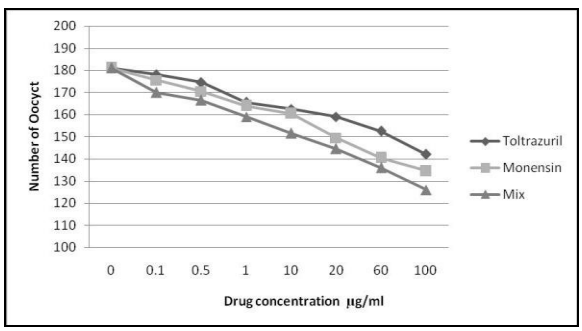

Fig. 2: Efficacy of Toltrazuril, Monensin and the mixture of them on *C. parvum* oocysts at 24h

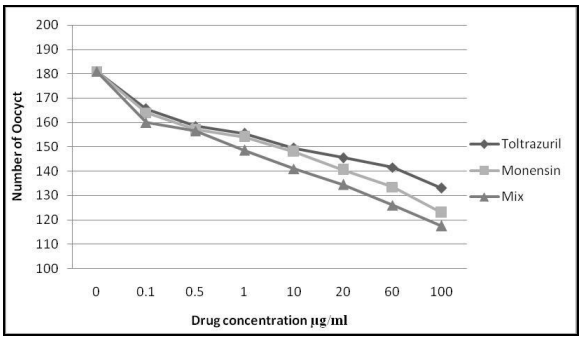

Fig. 3: Efficacy of Toltrazuril, Monensin and the mixture of them on *C. parvum* oocysts at 48h

Discussion

Due to the devastating effect of *C. parvum* in immunocompromised individuals especially AIDS patients and neonates, many antimicrobial drugs have been tested in animals or humans infected with a *Cryptosporidium* sp., but none has been consistently effective against this parasite [7]. The search for curative treatment for cryptosporidiosis must be considered [5]. A rapid method for isolating the potential chemotherapeutic agents is the first step in finding effective treatments.

In this study, using a simple screening assay, we showed that the mixture of two drugs (Monensin and Toltrazuril) was more effective on decreasing the number of *C. parvum* oocysts in all concentrations than two drugs alone at two measurements of 24h and 48h ($P<0.001$). In addition, Monensin was more effective than Toltrazuril with the same concentrations ($P<0.001$). Our findings also showed that the exposure time had significant efficacy on decreasing the number of oocysts (both drugs were more effective at 48h than at 24h) ($P<0.001$).

Armson *et al*. [6] have shown that Monensin had high efficacy (at 1µM) and Toltrazuril exhibited limited efficacy (at 20µM) against *C. parvum in vitro*. A study by Castro-Hermida *et al*. [7] has shown that

Toltrazuril had limited efficacy than α-β-Cyclodextrins on the excystation of *C. parvum* oocysts in sterile distilled water. Najdrowski *et al*. [8] showed that Monensin efficacy was related to its concentration and that it had more effect than halofuginone bromide on the *C. parvum* oocysts.

Conclusion

The present study showed that Monensin in all concentrations had more efficacy than Toltrazuril and the mixture of them had the highest effect on the *C. parvum* oocysts. The assay used in this study was to assess the drugs in HBSS had some advantages including speed, low cost and needless of unavailable measurement techniques. The chemotherapy of cryptosporidiosis yet remains a major challenge. The main goal of modern antiparasitic chemotherapies must be to bring the drugs as diversity to the target pathogens as possible and to minimize the potential side effects [9].

Acknowledgments

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