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PARASITOLOGICAL ASSESSMENT OF DIFFERENT CONCENTRATIONS OF PIPERAZINE CITRATE AGAINST CRYPTOSPORIDIOSIS INFECTION IN BOTH IMMUNOCOMPETENT AND IMMUNOCOMPROMISED MICE

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Abstract

Cryptosporidiosis is one of the risky zoonotic protozoan diseases of worldwide distribution. This present explored the efficacy of different concentrations of piperazine citrate with or without nitazoxanide against cryptosporidiosis infection in immuno-compromised and immuno-competent male mice. One hundred and thirty clean bred male Swiss Albino mice weighed about 20 gm were used. Of them 65 were given immunosuppressive drug (Dexamethasone®) for 15 day before infection. All mice put in separate labeled cages in the experimental laboratory under controlled condition and allowed suitable food. After confirming experimental infection, both types of mice were treated with Piperazine citrate as 100mg/kg in different doses (20, 30, & 40mg/kg). Besides, other groups of both types of mice were treated with Nitazoxanide® (NTZ) syrup 100mg/5ml, or Piperazine 30mg/kg and (NTZ). Assessment of drugs was done by stool examination of modified Zeil-Nelseen stained smears for oocysts.

The results showed that both Piperazine citrate and Nitazoxanide caused significant reduction in *C. parvum* oocysts as compared to control infected non-treated. Immuno-compromised mice treated by piperazine citrate (40mg/kg) for 7 days showed a higher significant reduction in number of oocysts as compared to immunocompromised mice treated by of Piperazine citrate (20&30) mg/kg for the same period. Combination of Piperazine 30 & NTZ for 7days in immuno-compromised mice gave much more reduction in number of oocysts than NTZ alone ($P<0.05$). Piperazine 30 & NTZ for 7 days in immuno-compromised mice gave much more reduction in oocysts than NTZ alone ($P<0.05$).

Keywords: *Cryptosporidium*, Oocysts, Piperazine citrate, Nitazoxanide, Stained smears.

Introduction

Cryptosporidiosis is an apicomplexan zoonotic worldwide gastrointestinal illness caused by *Cryptosporidium* spp. that infects many hosts including domestic animals, birds and human (Walter *et al,* 2021). There are 20 species of *Cryptosporidium*, of which *C. hominis* and *C. pa-rvum* are the most zoonotic ones (El-Helaly *et al,* 2012). In Egypt, in 19 studies examined the immunocompetent individuals with diarrhea presented to inpatient or outpatient clinics with cryptosporidiosis prevalence ranged from 0%-47% with median 9%, IQR 3-15% (Youssef *et al,* 2008). But, in immunocompromised individuals, *Cryptosporidium* infected gut, biliary and respiratory tracts (Mumtaz *et al,* 2010).

Cryptosporidium is an important food- and/or water-borne pathogenic disease of socioecon- omic significance (Putignani and Menichella, 2010). Also, infection occurred by man to man contact, or contact with pet or farm animals (Chalmers *et al,* 2011). Moreover, cryptosporidio-sis is still a major zoonotic health problem for two reasons, water purification are ineffective for its removal it from human water supply, and lack of effective drug and some outbreaks occurred in day care related to diaper changes (Abubakar *et al*, 2007).

In the last decade, so many active chemical and plant extracts were used as anti-cryptospor-idial therapies (Stockdale *et al,* 2008). Besides, some drugs reduced oocysts shedding, which presumably reduced environmental pathogen load and subsequent exposure and infection to susceptible hosts (Shahiduzzaman and Daugschies, 2012). Besides, several chemical drugs sh-owed an anti-cryptosporidial activity significantly potential in animal experiments and many

compounds with initially positive results ultimately were ineffective or only partially effective (Stockdale *et al,* 2008). Effective medical treatment of a patent cat-tle cryptosporidiosis is not available, despite many studies and testing of diverse active components (Shahiduzzaman and Daugschies, 2012). Massoud *et al.* (2008) in Egypt used *Commi-phora molmol* combined with paromomycin in treating cryptosporidiosis in immuno-compet-ent hospitalized patients. Abouel-Nour (2016) in Egypt found that garlic successfully eradica- ted oocysts of infected mice from stool and intestine, and ginger supplementation to infected mice markedly corrected elevation in t inflammatory risk factors and implied its potential antioxidant, anti-inflammatory and immunomodulatory capabilities.

Nitazoxanide (NTZ) has showed the most promise against cryptosporidiosis (Mor and Tzip-ori, 2008). It was well tolerated with relatively low incidence of adverse effects, and without significant known drug-to-drug interactions (Bobak, 2006). But, it was not effective against cryptosporidiosis in immunocompromised patients (Rossignol *et al*, 2006). A meta-analysis of randomized, placebo-controlled trial of NTZ (of which there were only 2) among immuno-compromised patients reported that NTZ was no more effective than placebo in resolving diarrhea and causing parasitological clearance in the HIV-patients (Abubakar, 2007). It was speculated that HIV-positive persons might benefit from longer-duration regimens or higher NTZ doses. However, a sustained clinical response was observed in only 59% of patients with HIV /AIDS who received off-label NTZ in a compassionate-use program (Rossignol, 2006). The efficacy of nitazoxanide without an efficient immune system (number of CD4 cells) was limited. Several authors therefore only attest a partial efficiency (Cabada and White, 2010).

Piperazine citrate acts as a γ-aminobutyric acid (GABA) agonist, causing chloride channel opening, neural hyperpolarization and flaccid paralysis of the susceptible parasites (Molina *et al,* 2014). Worms were expelled from their predilection sites by normal enteric movements and passed in the patients' stool. Piperazine citrate proved effective in treating *Ascaris lumbricoides* and *Enterobius vermicularis* (Carl *et al,* 2009).

The present work aimed to explore the parasitological efficacy of different concentrations of piperazine citrate with or without nitazoxanidagainst cryptosporidiosis in immunocomptent and immunocompromised Swiss Albino mice.

Materials and Methods

Drugs: 1- Piperazine citrate powder oral suspension given as (20, 30, & 40mg/kg), 2- Nitazoxanide® (Nitazode) syrup 100mg/5ml, & 3- Piperazine 30mg/kg, and nitazoxanide (NTZ).

Animals: Swiss Albino mice were divided into two groups: GA (immunocomptent) and GB (immunocompromised), then each group were subdivided in 7 subgroups:

GA1: Control infected not treated, GA2: Infected treated with nitazoxanide orally 100mg/kg daily for 5 consequent days, one week post infection (PI), GA3: Infected piperazine treated orally 40mg/kg divided in 2 doses daily for 7 days, a week PI, GA4: Infected treated with piperazine orally 30mg/kg divided in 2 doses daily for 7 days, a week PI, GA5: Infected treated with piperazine orally 20mg/kg divided in 2 doses daily for 7 days, a week PI, GA6: Infected treated with piperazine orally 30mg/kg divided in 2 doses & nitazoxanide orally 100 mg/kg daily for 7 days, a week PI, & GA7:Normal control neither infected nor treated.

GB1: Control infected not treated. GB2: Infected treated with nitazoxanide orally 100 mg/kg daily for 5 consequent days, a week P I. GB3: Infected treated with piperazine orally 40mg/kg divided in 2 doses daily for 7 days, a week PI, GB4: Infected treated with piperazine orally 30mg/kg divided in 2 doses daily for 7 days, a week PI, GB5: Infected treated with piperazine orally 20mg/kg were divided in 2 doses daily for 7 days, a week P

I. GB6: Infected mice treated with piperazine orally 30mg/kg divided in 2 doses & nitazoxanide orally 100mg/kg daily for 7 days, a week PI, & GB7: Normal control neither infected nor treated. Morning stools were examined for oocysts in modified Zeil-Nelseen stained smears (Henriksen and Pohlenz, 1981).

Results

Both piperazine citrate and nitazoxanide caused significant reduction in number of *C. parvum oocysts* compared with control infected non-treated mice. Immunocompromised mice piperazine citrate (40mg/kg) treated for 7 days gave higher significant reduction in number of oocysts as compared with immunocompromised mice of piperazine citrate 20 & 30mg/kg treated for the same period. Besides, immunocompromised mice treated with piperazine citrate 20 & 30mg/kg for 7 days gave significant reduction in number of oocysts than immunocompromised ones treated with nitazoxanide for 5 days with more reduction ($P<0.05$). The piperazine 30 & NTZ combined for 7 days in immunocompromised mice gave much more reduction in number of oocysts than NTZ alone ($P<0.05$). Treatment of immunocompromised mice with nitazoxanide for 5 days gave more reduction than immunocompromised mice piperazine citrate 40mg/kg treated for 7 days ($P<0.05$), with significant difference in outcome of immunocompromised mice treated for 7 days compared to immunocompromised ones treated by nitazoxanide for 5 days ($P<0.05$).

Also, both piperazine citrate and nitazoxanide gave significant reduction in number of *C. parvum* oocysts compared with control infected non-treated mice. Immunocompetent mice treated with piperazine citrate 40mg/kg for 7 days gave higher significant reduction in number of oocysts compared with immunocompromised mice piperazine citrate 20 & 30mg/kg treated for same period. The immunocompetent mice treated with piperazine citrate 20 & 30mg/kg for 7 days gave significant reduction in number of oocysts, but immunocompetent mice treated with nitazoxanide for 5 days gave more oocysts reduction ($P<0.05$). Piperazine 30 & NTZ for 7 days in immunocompetent mice gave more reduction in number of oocysts than NTZ given alone ($P<0.05$). Treatment of immunocompetent mice with nitazoxanide for 5days also gave more reduction in oocysts than immunocompetent treated piperazine citrate mice 40mg/kg for 7 days ($P<0.05$), with significant difference in outcome of immunocompetent mice treated with combined drugs for 7 days as compared with immunocompetent ones treated by nitazoxanide for 5days ($P<0.05$).

Details were given in tables (1, 2, & 3) and figures (1, 2, 3, 4, & 5).

Table 1: Effect of piperazine citrate and nitazoxanide (mg/kg) on *C. parvum* oocysts in immunocompetent mice

GA	Drug	Dose/day	TTTT duration	Total dose	Oocyst in stool	Post hoc test
GA2	NTZ	100	5days	500	12.8±1.69	
GA3	Piperazine40	40	7days	280	50.0±1.49	A
GA4	Piperazine30	30	7days	210	68.0±1.49	Ab
GA5	Piperazine20	20	7days	140	89.0±2.16	Abc
GA6	Piperazine & NTZ	30 &100	7days	700 & 210	10.0±2.16	Abcd
GA1	Control infected not treated				93.4±3.21	Abcde

A: significant with GA2, **b:** significant with GA3, **c:** significant with GA4, **d:** significant with GA5, **e:** significant with GA6
ANOVA=2963.74, P value<0.001**

Table 2: Effect of piperazine citrate and nitazoxanide (mg/kg) on *C. parvum* oocysts in immunocompromised mice

GB	Tested drug	Dose/day	TTT duration	Total dose	Oocyst in stool	Post hoc test
GB2	Nitazoxanide	100	5days	500	27.9±1.66	
GB3	Piperazine citrate40	40	7days	280	70.0 ±1.76	A
GB4	Piperazine citrate30	30	7days	210	90.1±1.91	Ab
GB5	Piperazine citrate20	20	7days	140	110.0±2.24	Abc
GB6	Piperazine & NTZ	100 & 30	7days	700 & 210	20.0±2.16	Abcd
GB1	Control infected not treated				407.4±3.21	Abcde

A: significant with GB2, **b:** significant with GB3, **c:** significant withGB4, **d:** significant with GB5, **e:** significant with GB6
ANOVA=27456.38, P value<0.001**

Table 3: Piperazine citrate and nitazoxanide on *C. parvum* oocysts in immunocompetent and immuneocompromised mice

Groups (A & B)	Immunocomptent	Immunocompromised	Paired t test	P value
G2 (NTZ)	12.8±1.69	27.9±1.66	21.38	<0.001**
G3(Piperazine40)	50.0±1.49	70 .0 ± 1.76	32.54	<0.001**
G4 (Piperazine30)	68.0±1.49	90.1±1.91	35.49	<0.001**
G5(Piperazine20)	89.0±2.16	110.0±2.24	21.33	<0.001**
G6 (NTZ+piperazine)	10.0±2.16	20.0 ±2.16	14.64	<0.001**
G1 Control	93.4±3.21	407.4 ±3.21	127.14	<0.001**

**Highly significant difference in immunocompetent and immunocompromised mice treated with nitazoxanide (P<0.01).

Discussion

Cryptosporidium is an obligate intracellular zoonotic parasite that infects epithelium of gastrointestinal and respiratory tracts (Putignani and Menichella, 2010). In immunocompetent individuals it is localized in small intestine, and in immunocompromised ones, it infects the gut, biliary and respiratory tracts (Leitch and He, 2011).

In the current study, that there was a highly significant reduction in mean number of *C. parvum* oocysts in infected mice treated with piperazine citrate as compared with controls. This agreed with Amadi *et al.* (2002) in Nigeria who reported three placebo controlled trials of cryptosporidiosis treatment with nitazoxanide in non-AIDS patients and response rate in malnourished children was only 56%. Also, Rossignol (2006) in Canada reported that up to 93% of treated patients experienced parasite clearance as opposed to 37% of placebo treated patients. Sparks *et al.* (2015) in USA found that nitazoxanide was only an antiparasitic treatment with proven efficacy for cryptosporidiosis in immunocompetent individuals and not effective in severely immunocompromised patients. Lee *et al.* (2019) in USA reported that MMV665917 significantly reduced fecal oocyst excretion, parasite colonization that damaged the intestinal mucosa, and peak diarrhea compared with infected untreated controls. Checkley *et al.* (2018) in USA reported that nitazoxanide had some effect in healthy hosts but, no proven efficacy in patients with AIDS. They added that use of cryptosporidium genomes might help to identify promising therapeutic targets. However, Tam *et al.* (2021) did not support CFZ for cryptosporidiosis treatment in severely immunocompromised HIV patients. They added that their trial gave a pathway to assess therapeutic potential of drugs for cryptosporidiosis treatment, and that screening HIV persons for diarrhea, and especially cryptosporidiosis, may identify those failing ARV therapy.

In the present study, the immunocompromised mice were treated with combination of NTZ & piperazine citrate 30mg/kg for 7 days started a week post infection, with high significant reduction in *C. parvum* oocysts that was more or less similar to nitazoxanide treated mice. This agreed with Mostafa *et al.* (2018) in Egypt who found that combination of artesunate and nanazoxide showed a synergistic effect by reducing number of *C. parvum* oocysts shed and improving dysplastic changes induced by cryptosporidiosis infection in the colon of immunosuppressed mice as compared to that induced by either artesunate or nanazoxide alone. Besides, Checkley *et al*. (2018) in USA reported that diagnostic tests for *Cryptosporidium* infection were the suboptimum necessitating specialized tests must be often insensitive. Antigen-detection and PCR improve sensitivity, and multiplexed antigen detection and molecular assays were underused. They added that therapy posseesed some effect in healthy hosts, but without proven efficacy in AIDS patients.

In the present study, the immunocompetent mice was treated with combination of the NTZ & piperazine citrate 30mg/kg for 7days a week PI showed the best therapeutic efficacy with reduction rate in *C. parvum* oocysts number was >89%, i.e. more or less similar effect with the nitazoxanide treated ones.

In the present study, piperazine citrate dose of 20mg/kg given daily for 7 days was significant as compared with infected controls, with best effect when used at doses of

30, &40mg/kg for 7 days in immunocompetent or immunocompromised mice. This agreed with Hussien *et al.* (2013) who stated that 86.6% of children treated with 100 or 200mg of nitazoxanide every 12 hours for three days were completely curried without clearance of oocysts passage and cessation of clinical symptoms. Also, Lee *et al.* (2019) reported that a dose of piperazine MMV 66591720mg/kg twice daily for 7 days was more effective than 10 mg/kg.

In the present study, the immunocompetent or immunocompromised given piperazine citrate 30mg/kg combined with nitazoxanide 100mg/kg for 7 days showed obvious effect in treating cryptosporidiosis, and the oocysts number was reduced to 89-95%. Meanwhile, nitazoxanide alone treated mice showed 86-93% reduction rate. However, mice treated with piperazine citrate 20mg/kg for 7days showed the lowest reduction rate of oocysts number among all groups, which was 4% in immunocompetent mice and 72% in immunocompromised ones. This more or less agreed with Jumani *et al.* (2018) who reported complete cryptosporidiosis clearance among hospitalized children treated with paromomycin compared to untreated ones. However, the response was significantly less than with nitazoxanide.

In the present study, highest reduction in *C. parvum* oocysts number was reached in mice treated with the combined therapies for a week in immunocompromised mice (95%) and immunocompetent group (89%), followed by mice treated with nitazoxanide 5 days in immunocompetent and immunocompromised mice (86-93%), followed by mice treated with piperazine 40mg/kg 7days in immunocompetent or immunocompromised ones (82-46%), and then mice treated by piperazine 30mg/kg7 days in immunocompetent or immunocompromised ones (26-77%). This agreed with Jumani *et al.* (2018), who found that piperazine MMV665917 given at 30mg/kg twice daily reduced oocyst shedding by > 90%. The lowest reduction was in mice treated with piperazine 20mg/kg 7days in immunocompetent or immunocompromised mice (4-72%). This agreed with *Desautels et al.* (2016) who found a significant difference ($P < 0.0001$) between Oleylphosphocholine treated and controls with 100nM due to the efficiency lack of such a dose to inhibit *C. parvum* infection.

Conclusion

Piperazine citrate and nitazoxanide gave significant reduction in number of *Cryptosporidium parvum oocysts* compared with control infected non-treated. Immunocompromised mice treated by piperazine citrate (40mg/kg) for 7 days gave higher significant reduction in number of oocy-sts compared with those mice treated by of piperazine citrate (20&30) mg/kg for same period. Piperazine 30 & NTZ combined for 7days to immunocompromised mice gave much more reduction in number of oocysts than NTZ alone ($P<0.05$). Piperazine 30 & NTZ combined for 7 days in immunocompromised mice gave much more reduction in number of oocysts than NTZ alone ($P<0.05$).

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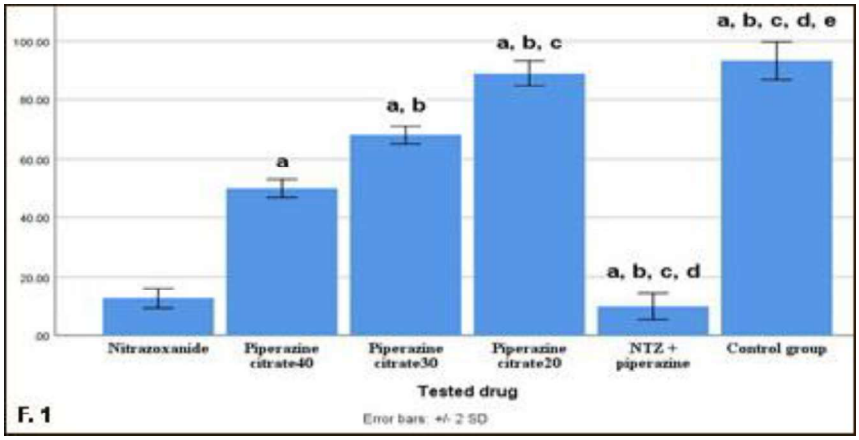
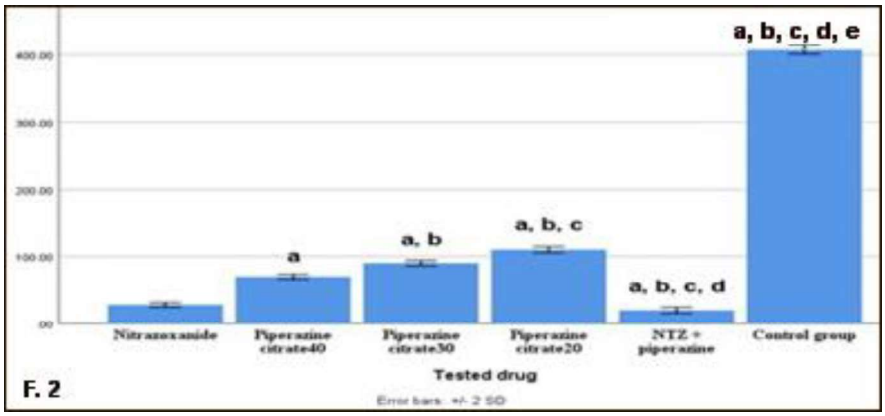
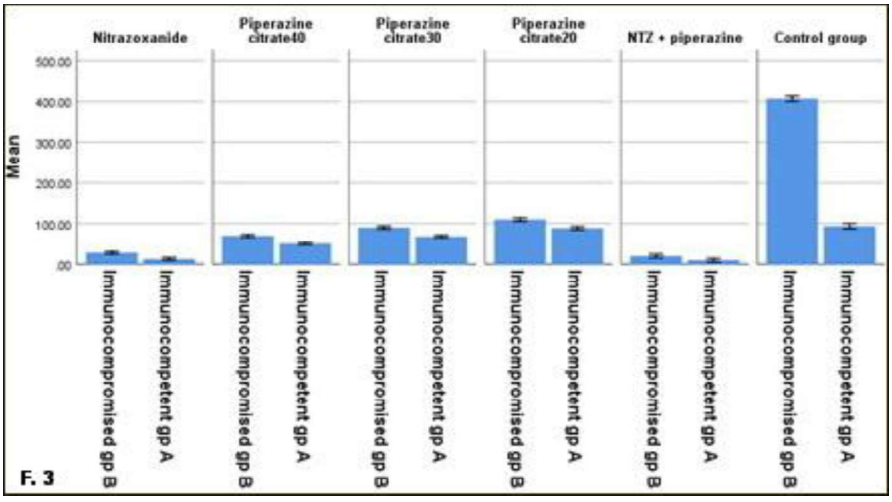
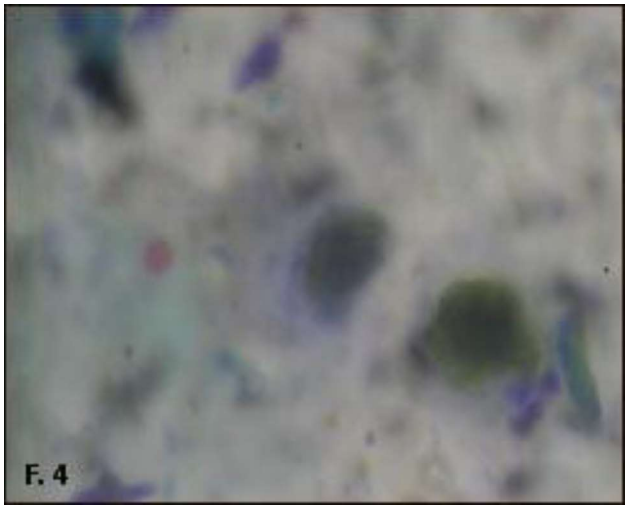
Explanation of figures

Fig. 1: Mean value among immunocompetent Albino mice treated versus control.
Fig. 2: Mean value among immmunocompromised Albino mice treated versus control.
Fig. 3: Treatment of immunocompetent and immunocompromised mice with different drugs.
Fig. 4: *Cryptosporidium parvum* oocysts in infected mice stool smear with Modified Zeil Nelseen stain x40.
Fig. 5: *C. parvum* oocysts in infected mice stool smear with Modified Zeil Nelseen stain x100









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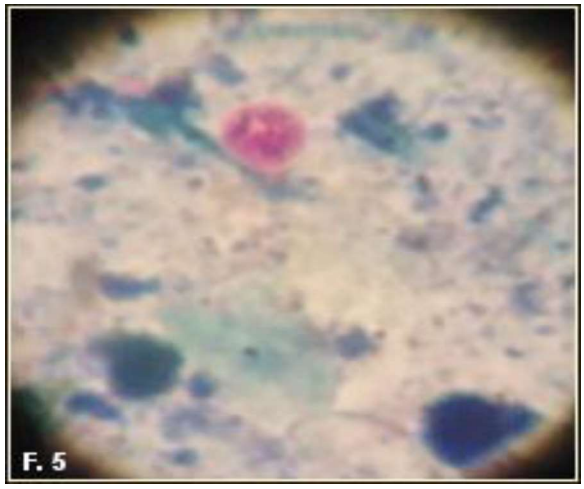

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