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

By
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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 immuno-compotent 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, cryptosporidiosis 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- cryptosporidial 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 Commiphora molmol combined with paromomycin in treating cryptosporidiosis in immuno-compertent 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 in-flammatory 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 immuno-nocompromised 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 nitazoxanid against cryptosporidiosis in immuno-nocomptent 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-Nelsen 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 immunocompetent mice treated with piperazine citrate 20 & 30mg/kg for 7 days. The 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 5 days 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 immunocompromised treated piperazine and nitazoxanide for 5 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 5 days 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 5 days (P<0.05).

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

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose/day</th>
<th>Oocysts in stool</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTZ</td>
<td>100</td>
<td>5days</td>
</tr>
<tr>
<td>Piperazine 40</td>
<td>40</td>
<td>7days</td>
</tr>
<tr>
<td>Piperazine 30</td>
<td>30</td>
<td>7days</td>
</tr>
<tr>
<td>Piperazine 20</td>
<td>20</td>
<td>7days</td>
</tr>
<tr>
<td>Piperazine &amp; NTZ</td>
<td>30 &amp; 100</td>
<td>7days</td>
</tr>
<tr>
<td>Control infected not treated</td>
<td>93.4±3.21</td>
<td></td>
</tr>
</tbody>
</table>

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 3: Piperazine citrate and nitazoxanide on C. parvum oocysts in immunocompetent and immunocompromised mice

<table>
<thead>
<tr>
<th>Groups (A &amp; B)</th>
<th>Immunocompetent</th>
<th>Immunocompromised</th>
<th>Paired t test</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2 (NTZ)</td>
<td>12.8±1.69</td>
<td>27.9±1.66</td>
<td>21.38</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>G3 (Piperazine 40)</td>
<td>50.0±1.49</td>
<td>70.0±1.76</td>
<td>32.54</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>G4 (Piperazine 30)</td>
<td>68.0±1.49</td>
<td>90.1±1.91</td>
<td>35.49</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>G5 (Piperazine 20)</td>
<td>89.0±2.16</td>
<td>110.0±2.24</td>
<td>21.33</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>G6 (NTZ + piperazine)</td>
<td>10.0±2.16</td>
<td>20.0±2.16</td>
<td>14.64</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>G1 Control</td>
<td>93.4±3.21</td>
<td>407.4±3.21</td>
<td>127.14</td>
<td>&lt;0.001 **</td>
</tr>
</tbody>
</table>

**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 (Putztognani 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 possessed 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 7 days 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 immunocompe-
tent 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
two days were completely cured 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 immunocompe-
tent 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 7 days
showed the lowest reduction rate of oocysts
number among all groups, which was 4% in
immunocompetent mice and 72% in immu-
nonocompromised ones. This more or less
agreed with Jumani et al. (2018) who reported
complete cryptosporidiosis clearance among
hospitalized children treated with paromom-
ycin 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
cells treated with the combined therapies for
a week in immunocompromised mice (95%)
and immunocompetent group (89%), follow-
ed by mice treated with nitazoxanide 5 days
in immunocompetent and immunocompro-
mised mice (86-93%), followed by mice tre-
ated with piperazine 40mg/kg 7days in immu-
nonocompetent or immunocompromised ones
(82-46%), and then mice treated by piperazine
30mg/kg 7 days in immunocompetent or immu
nonocompromised 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 tre-
ated with piperazine 20mg/kg 7days in imm-
nonocompetent 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 Cryptos-
poridium parvum oocysts compared with
control infected non-treated. Immunocom-
promised mice treated by piperazine citrate
(40mg/kg) for 7 days gave higher significant
reduction in number of oocysts compared
with those mice treated by of piperazine cit-
rate (20&30) mg/kg for same period. Pipera-
zine 30 & NTZ combined for 7days to immu
nonocompromised mice gave much more re-
duction in number of oocysts than NTZ alone
(P<0.05). Piperazine 30 & NTZ combined
for 7 days in immunocompromised mice ga-
ve much more reduction in number of oocy-
sts 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 immunocompromised 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 Zell Nelsen stain x40.

Fig. 5: C. parvum oocysts in infected mice stool smear with Modified Zell Nelsen stain x100