IVERMECTIN WITH NITAZOXANIDE ELIMINATE CRYPTOSPORIDIUM INTESTINAL INFECTION IN IMMUNOCOMPROMIZED MICE AND UPREGULATE CYTOKERATIN20 EXPRESSION, ENHANCING INTESTINAL CELLULAR HEALING

By
HALA M. EL-ASKARY¹, REHAM K. NAHNOUSH², AND MARWA A. ELMALLAWANY²

Department of Medical Parasitology¹, Faculties of Medicine, Beni-Suef University¹, and Cairo University², Egypt (*Correspondence: halaelaskary@hotmail.com)

Abstract
Cryptosporidiosis is an extensively spread protozoan parasite, but its immense cellular threat is limited to those suffering from immune suppression which may encounter fatal complications.

This study assessed the nitazoxanide effect alone or combined with ivermectin on eradicating cryptosporidiosis and enhancing cellular healing in immunocompromized infected mice using cytokeratin20 as an intestinal intermediate filament protein marker to reflect intestinal cellular integrity. Oocyst count in mice treated by nitazoxanide monotherapy was significantly higher (37×10³±7.3) than those treated with combined therapy (5×10³±4.1). Reduction rate was higher in mice treated with both therapy (95.7%) than mice treated with monotherapy (68.9%).

Considerably high cytokeratin expression was recorded in mice treated with combined therapy (34.64±6.65), possibly indicated the intestinal mucosa recovery, while a relatively lower expression was in mice treated with nitazoxanide monotherapy (21.28±9.41) (P<0.05).

Keywords: Cryptosporidium- cytokeratin20- intestine- Ivermectin- Nitazoxanide

Introduction
Cryptosporidiosis is an extensively world wide spread protozoan caused by several species, but C. parvum and C. hominis are the commonest zoontic ones (Ježková et al., 2021). The intestinal infection may extend to other organs with fatal complications, mainly in those suffering from immunological suppression (Dong et al., 2020). The problem faced the therapists in cryptosporidiosis was lack of effective treatment, not only capable of eliminating infection but, also restoring cellular function that damaged by infection (Wang et al., 2020).

The reported information concerning the outcomes of the current treatment by nitazoxanide® (NTZ) is not encouraging, either for its limited ability to eradicate the infection or its poor effect to restore cellular activity, specifically in immunocompromized hosts (Aboelsoued et al., 2020). There was a special interest in a broad antiparasitic Ivermectin®, which became globally well-known during the covid era for its ability to cure many cases as reported by several investigators and this was documented in meta-analysis (Bryant et al., 2021).

Adopting therapeutic strategies for parasitic infection relied on monotherapy may be problematic due to risk of resistance among other factors, thus finding another options depends on combined therapeutic agents may achieve better success regarding both infection and cryptosporidiosis mucosal healing (Love and Choy, 2021). Cytokeratins (CK) are a group of proteins found in the cytoplasm of all cells as markers to investigate cellular integrity considered among the vital intracytoplasmic cytoskeleton components, which resist variable stress and reflect cell mucosa healthiness (Herrmann et al., 2007).

This study aimed to assess the effect of nitazoxanide alone or combined with ivermectin on eradicating cryptosporidiosis and enhancing cellular healing in immunocompromized infected mice, using CK20 as a cytoplasmic cellular marker.

Materials and methods
Animals: Forty male Swiss Albino mice of CD1 strain, about 30g and aged 6 to 8 weeks were purchased from Theodor Bilharz Research Institute, Animal House. Mice were kept in separate wire cages under standard laboratory conditions and given food
pellets and water. Before Cryptosporidium oocysts infection mice were immune suppressed by oral dexamethasone (Dexazone, Al Kahira Pharmaceutical) at a dose of 0.25µg/g/day for 14 consecutive days till the end of the experiment after treatment (Rehg et al, 1988). Mice were divided into 4 immunosuppressed groups (10mice/each); GI: infected, G2: infected and treated with NTZ and G3: infected and treated with nitazoxanide + Ivermectin; and G4: negative control.

Fecal samples were collected from naturally infected diarrheic calves at Veterinary Clinic, Cairo Faculty of Veterinary Medicine were stained by Kinyoun's Acid Fast and examined for Cryptosporidium oocysts. The recovered oocysts were purified by sucrose density gradient flotation method (Arrowood and Sterling, 1989), suspended in PBS, and were kept in 0.01% Tween20, with 200 IU/ml penicillin, 0.2mg/ml streptomycin, 2.5µg/ml amphotericin B, and 10⁴oocysts were given to each mouse by an oral-gastric gavage (Love et al, 2017).

Nitazoxanide suspension 100mg/5ml (Uttopia Pharmaceuticals), 250mg/kg/day was governed from day 4 post-infection for 10 succeeding days in G2 & G3 Ivermectin (Un-ipharma, Egypt) was orally given in a single dose of 2mg/kg in G3 (Fahmy et al, 2020).

Parasitological analysis: Fecal pellets were collected at the experimental end and examined using formol/ether centrifugal sedimentation technique followed by Kinyoun’s Acid-Fast stain (cold method) to count oocysts in 50µl, and expressed per gram of feces (Benamrouz et al, 2012).

Histopathological and immunohistochemical (IHC) studies: Paraffin sections were prepared, stained by haematoxylin and eosin and examined microscopically (Drury and Wallington, 1980). For IHC, paraffin-embedded specimens were deparaffinized in xylene, rehydrated, incubated in 3% H₂O₂ for 5 min. to prevent endogenous peroxidation, and then specimens were washed twice in PBS. For antigen retrieval, sections were put in 0.01mol/L citrate buffer (pH 6) in a water bath, and then incubation was done with primary antibody murine anti-human cytokeratin monoclonal antibodies (Dako, USA) for an hour, to be washed 3 times later by PBS. Then, Bio-tinylated secondary antibody and streptavidin peroxidase enzyme were added successively for 10min. followed by washed in PBS. Visualized was done by adding diaminobenzidine chromogen for 5min. (Ramos-Vara and Miller, 2014). Counterstain with haematoxylin was done; dehydration and clearance in xylene were applied and then mounted by DPX. Positive control was provided with the kits and used according to the manufacturer’s recommendation. Negative controls were prepared by same protocol, except for the primary antibody use. Quantitative estimation of cytokeratin local expression was done using LIKA digital cytomorphic analysis.

Statistical analysis: Data were presented as mean and SD, and analyzed by STATA/IC Software version 16.1 (Stata Corp., Lake- way, TX, USA). ANOVA and post hoc test were used for multiple comparisons analysis between groups. P < 0.05 was considered significant.

Results

There was a significant difference among all oocysts counts (p<0.05) in groups. Oocyst count in mice treated by nitazoxanide monotherapy was significantly higher (37×10³±7.3) than mice treated with combined therapy (5×10³±4.1). Reduction rate (95.7%) was higher in combined therapy treated mice than in monotherapy treated ones (68.9%). Histopathological showed extensive damage of epithelial lining of intestinal villi in mice infected and non-treated, but mice treated with nitazoxanide monotherapy intestinal villi showed some improvement. Villi of infected mice treated with combined therapy showed complete recovery with restored intestinal mucosal lining. Cytokeratin20 local intestinal expression, infected non-treated mice exposed low expression as compared to normal (7.04 ±3.54 & 38.9±12.7
respectively), reflected intestinal mucosa pathogenecity. High cytokeratin20 expression was in mice treated with combined therapy (34.64±6.65), with the recovery of intestinal mucosa, while a relatively lower expression was in mice treated with nitazoxanide (21.28±9.41). Differences between CK20 values were significant (P<0.05). Details were given in table (1) and figures (1 & 2).

Table 1: Cryptosporidium oocyst count and reduction rate in groups.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>oocysts count</th>
<th>%Reduction</th>
<th>CK20 count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control normal</td>
<td>--</td>
<td>--</td>
<td>38.9±12.7</td>
</tr>
<tr>
<td>G1</td>
<td>119±10^3±23.5^</td>
<td></td>
<td>7.04±3.54^</td>
</tr>
<tr>
<td>G2</td>
<td>37±10^3±7.3</td>
<td>68.9%</td>
<td>21.28±9.41^</td>
</tr>
<tr>
<td>G3</td>
<td>5×10^3±4.1^</td>
<td>95.7%</td>
<td>34.64±6.65</td>
</tr>
</tbody>
</table>

Different symbols means significant difference between groups (P <0.05)

Discussion

In the present study, intestinal epithelial lining of Cryptosporidium-infected non-treated mice showed more cellular degeneration and pathological damage, probably the parasite protease activity. In a cell line study, Cryptosporidium infection reduced the cellular growth, led to its bad effect on the cellular maturation and function (Liu et al, 2008). Also, intestinal epithelium has a natural ability to regenerate through different manners, including cell division and maturation beside migration (Zhang et al, 2016).

In the present study, nitazoxanide/ivermectin combined therapy was successful not only in eliminating Cryptosporidium infection, but also regaining normal cellular structure and integrity. The nitazoxanide monotherapy gave a significantly less reduction rate (68.9%) and cellular healthiness, but when combined with ivermectin the reduction rate was significantly higher (95.7%). This agreed with Sparks et al. (2015) who reported limitation of nitazoxanide particularly with immunosupressed cases.

In general, Cryptosporidiosis is one of the significant enteropathogens worldwide, but its prevention was difficult, given the parasite’s high infectivity, robustness, and resistance to disinfection, highlighting the need to improve therapeutics particularly for immunocompromised individuals, and a safe and effective vaccine (Shirley et al, 2012). Many therapeutic agents were formerly reported to be efficient, but these agents were failed when applied in clinical trials, thus alternative therapeutic agents were recommended (Sparks et al, 2015; El-Bahnasawy et al, 2018).

Ivermectin is a broad spectrum anti-infectious agent against many parasites and viruses and studies are ongoing to evaluate its anti-cancer potential due to the best impact on cellular immunity (Momekov, 2020). Also, ivermectin killed Leishmania major promastigotes (Rasheid and Morsy, 1998), Rhipicephalus sanguineus vector of zoonotic babesiosis (Morsy and Hardiy, 2000) and scabmites and biting lice (Morsy et al, 2001). Ivermectin was used to treat chronic giardiasis, cryptosporidiosis and malaria (Smit et al, 2019), as well as used as a host-directed broad-spectrum antiviral agent, including the SARS-CoV-2 (Jans and Wagstaff, 2021).

However, cytokeratins (CKs) are proteins present in cytoskeleton of cellular cytoplasm as the vital components of intermediate filaments contribute in cell-matrix and cell-cell interactions (Herrmann et al, 2007). CKs provided the necessary cellular mechanical stability as well as many cellular activities as cell signaling, transport, cell cycle and cell death (Meyer and Gruss, 1993). Changes in CKs expression was associated in many abnormal cellular conditions, including inflammation, mutagenesis and epithelial barrier dysfunctions (Schreurs et al, 2020). Cytokeratin members keep the structural support needed for cellular components and defend cells from stress. The cytokeratin 20 is principally expressed in the cytoplasmic portion of small and large intestines (Moll et al, 1990). CK20 was used as a marker to differentiate between the mutagenic changes
and normal epithelium by using variable cellular technologies (Chen and Wang, 2004). The CK20 phosphorylation is linked to mucin secretion as well as cytoplasmic filaments regulation, loosening such activity in transgenic mice led to cellular collapse (Zhou et al, 2006), but regulation of this protein was yet not well known (Chan et al, 2009). Low expressions of CKs were recorded in many cellular abnormalities as alteration of cellular shape and structure beside loss of cellular functions (Karantza, 2011). However, certain CKs were found in certain types of tumors but lost in other types, and used as a diagnostic markers for carcinomas due to organ types and CKs types (Schreurs et al, 2020).

In the present study, CK20 in Cryptosporidium infected immunocompromised mice, proved valuable in reflecting cellular healing in mice treated with combined therapy. In infected non-treated ones, it significantly lower expression was recorded and abnormal distribution, possibly due to disturbed cellular structure and activity caused by infection.

**Conclusion**

The effect of nitazoxanide alone was less significant than combined therapy which successfully eliminate intestinal Cryptosporidium infection, and regain cellular integrity and healthiness.

**Acknowledgment**

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**References**


Jans, DA, Wagstaff, KM, 2021: The broad spe-


Explanation of figures

Fig. 1: Histopathological photographs represent longitudinal sections of intestinal villi stained by specific IHC stain for cytokeratin 20 marker with different local intestinal cellular expression within different groups in relation to normal (A). B: Low and abnormal expression in infected non-treated group, notice disturbed features of intestinal mucosa in section. C: A relatively lower expression in mice treated with monotherapy compared to normal ones. D: Intestinal villi regain a relatively normal CK20 distribution within cells with normal histological features in mice treated with combined therapy (IHC B×200; A, C&D×400).
Fig. 2: Histopathological pictures represent cross sections of histopathological changes in groups stained by H&E ×200 in A, B&C and by immunohistochemical stain (IHC×200) in D, E & F, in blue color= reflected local expression during image analysis. A: Infected non-treated group showed completely disturbed architecture. B: Villi of mice infected and treated with Nitazoxanide showed some improvement. C: Villi of infected mice treated with combined therapy showed complete recovery. Cytokeratin local expression was with low expression in infected non-treated mice (D), high expression in mice treated with combined therapy (F) and a relatively low expression in mice treated with monotherapy (E).