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

By

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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 protien marker to reflect intestinal cellular integrity. Oocyst count in mice treated by nitazoxanide monotherapy was significantly higher ($37\times10^3\pm7.3$) than those treated with combined therapy ($5\times10^3\pm4.1$). 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 theraputic 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 theraputic agents may achieve better success regarding both infection and cryptosporidiosis mucosal healing (Love and Choy, 2021). Cytokeratins (CK) are a group of protiens found in the cytoplasm of all cells as markers to investigate cellular integrity considered among the vital intracytoplamic cytoskeleton components, which resist varaible 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: Fourty 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 10^4oocysts were given to each mouse by an oral-gastric gavage (Love *et al,* 2017).

Nitazoxanide suspension 100mg/5ml (Utopia 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% H_2O_2 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 Soft- ware version 16.1 (Stata Corp., Lakeway, 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 nitazoxani-de monotherapy was significantly higher ($37\times 10^3\pm7.3$) than mice treated with combined therapy ($5\times10^3\pm4.1$). 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.

Study Group	oocysts count	%Reduction	CK20 count
Control normal	--	--	$38.9\pm12.7^{\neq}$
G1	$119\times10^{3}\pm23.5^{\neq}$		$7.04\pm3.54^{*}$
G2	$37\times10^{3}\pm7.3^{*}$	68.9%	$21.28\pm9.41^{\#}$
G3	$5\times10^{3}\pm4.1^{\#}$	95.7%	$34.64\pm6.65^{**}$

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 monotheapy 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 immunosuppressed 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 Haridy, 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 t 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, loosing such activity in transgenic mice led to cellular collapse (Zhou *et al,* 2006), but regulation of this protien 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 immunocompromized 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, posssibly 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.

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Explanation of figures

Fig. 1: Histopathological photographs represent longitudinal sections of intestinal villi stained by specific IHC stain for cytokeratin20 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: Vill 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).