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POTENTIAL IMMUNOMODULATORY EFFECT OF SINGLE AND COMBINED THERAPIES AGAINST *CRYPTOSPORIDIUM* INFECTION IN IMMUNOSUPPRESSED MOUSE MODEL

By

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Abstract

Cryptosporidium species are related to protozoan parasites that infect the gastrointestinal tract of a wide variety of animals, including humans. The host immune status is crucial for determining the susceptibility to infection, the outcome and the severity of the disease. Nitazoxanide® is the only FDA approved drug to treat such opportunistic infection, yet demonstrates limited and immune-dependent efficacy. The present work studied the effect of combination therapy in treating cryptosporidiosis, plus exploring the possible modulating effect on the local adaptive immune response in experimental dexamethasone immunocompromized mice model. The results showed that combination of selenium supplementation with ivermectin® or nitazoxanide improved the oocysts reduction percentage compared to groups receiving single anti-parasitic therapeutic drugs alone. Also, combination of ivermectin with nitazoxanide gave the best oocysts reduction rates with the lowest score of ileitis severity. But, local expression of CD4 T cells was down-regulated and unlikely could not be elevated enough even after all treatment types. CD8 was up regulated in all treated groups as compared with non-treated control group, indicating its possible role in reducing the serious threats of such opportunistic infection.

Keywords: *Cryptosporidium*- single therapy- combined therapies- CD4- CD8

Introduction

Cryptosporidium is an apicomplexan protozoon, which was reported as a human pathogen in 1976 by Nime. It is a leading cause of diarrheal death in children younger than five years, only second to rotaviral enteritis causing water- and food-borne diarrhea outbreaks in humans worldwide (Efstratiou *et al*, 2017; Ryan *et al*, 2018; Khalil *et al*, 2018). Considerable morbidity and mortality are frequently reported, specifically in immunodeficient individuals as a result of disease complications (Laurent and Lamande, 2017). Best possible therapy for cryptosporidiosis includes attention to fluids and electrolytes, antimotility agents, anti-parasitic drugs, nutritional support, and/or reversal of immunosuppression if achievable (Checkley *et al*, 2015; Wang *et al*, 2020). Effective drug treatment, particularly for infections in immunodeficient patients, has not been uniformly successful. The current standard of care to treat *Cryptosporidium* infections, nitazoxanide, demonstrates limited and immune-dependent efficacy (Sparks *et al*, 2015; Widmer *et al*, 2020). Thus, drug combinations were tried to give a better response against cryptosporidiosis than using nitazoxanide alone (Theodos *et al*, 1998; Krause *et al*, 2012; Bhadauria *et al*, 2015).

Ivermectin is a semi-synthetic derivative of a family of macrocyclic lactones, with a broad-spectrum anti-parasitic, antiviral, and even as a cancer chemotherapeutic (Laing *et al*, 2017; Momekov and Momekova, 2020). In contrast, Selenium (Se) is an essential micronutrient known to influence the functioning of all components of the immune system, and its deficiency correlated with susceptibility or resistance to *Cryptosporidium* infection (Wang *et al,* 2009). So, with the increasing numbers of immune-altered patie-

nts from various origins, cryptosporidiosis becomes an increasingly common problem which needs a treatment, not only as an anti-parasitic agent but also, to improve the immune status of the patients.

The present work aimed to assess the effect of nitazoxanide and ivermectin drugs alone or combined together or combined with selenium on eliminating *Cryptosporidium* infection in immunocompromized experimentally infected mice. Also, to investigate the immunomodulatory impact of these therapeutic agents on the local expression of ileal CD4 & CD8 T cell response.

Materials and Methods

Experimental animals: The study was carried out on 8 groups of laboratory-bred male Swiss albino mice of CD1 strain (6 mice/ each group); specific free pathogen (SFP), aged 7 weeks, and weighing 20-25gm each. Experimental mice were provided by *Schistosoma* Biological Supply Program (SBSP) at Theodor Bilharz Research Institute (TBRI). All experimental procedures were conducted in the Biological Unit of TBR, considering the internationally valid guidelines for animal experimentation. Each mouse was housed individually in a well-ventilated cage with clean wood-chip bedding, and provided with an ad libitum pelleted food and water under 18-23°C room temperature. Animals' stools were examined using direct wet saline smear and iodine, and then stained with acid fast stain to exclude any parasitic infection.

Experimental design: Mice were divided into 8 groups, 6 mice each as follows: I-Infected & treated with single therapeutic agent: G1: treated with nitazoxanide (NTZ), & G2: treated with ivermectin (IVC). II-Groups infected & treated with combined therapies: G3: treated with nitazoxanide combined with ivermectin, G4: treated with nitazoxanide combined with selenium (Se), & G5: treated with ivermectin combined with selenium. III- Control groups: Control 1 (C1): Normal non-infected non-treated (normal), Control 2 (C2): Non-infected (dexamethasone), & (C3): Infected non- treated mice.

Induction of immune suppression by dexamethasone: Dexamethasone induced immune suppression was performed by giving all mice groups (except control normal group, C1) synthetic corticosteroids, dexamethasone (Dexazone: Al Kahira Pharmaceutical and Chemical Industries Company, Cairo), orally at a dose of 0.25µg/g/day for 14 successive days before inoculation with *Cryptosporidium* oocysts (Rehg *et al*, 1988). Mice were continued to receive dexamethasone at the same dose throughout the experiment.

Amplification of cryptosporidium oocysts and preparation of inoculum: Propagation of *Cryptosporidium* oocysts (obtained from faeces of infected calves) was achieved by *in vivo* amplification in 20 Swiss albino CD1 strain mice. Animals were inoculated repeatedly every 2 weeks by gavage as 2000 oocysts/animal (Oettingen *et al*, 2008). The oocysts were isolated and purified using centrifugal flotation (Zeibig, 1997). Purified oocysts were then suspended in PBS and kept with 0.01%Tween-20, containing 200 IU/ mL penicillin, 0.2 mg/mL streptomycin and 2.5µg/mL amphotericin B eliminating any remaining bacterial or fungal contamination, and stored at 4°C before use. In order to adjust the infecting dose of the oocyst in the inoculum, the number of *Cryptosporidium* oocysts in the suspension was determined by counting oocysts in smears prepared from 50µl aliquots and stained with Kinyoun's Acid Fast stain (Operario *et al*, 2015).

Infection: All mice groups (except normal non-infected control, **C1**) were orally infected with *Cryptosporidium* oocysts using oral-gastric gavage. Each mouse was infected with *Cryptosporidium* oocysts in a dose of about 10^4 oocysts/ mouse (Love *et al*, 2017).

Drug administration: 1- Nitazoxanide (Nanazoxid; Medizen Pharmaceutical Industries for Utopia Pharmaceuticals) was given orally in a suspension form in a dose of 250mg/kg/body weight/day for 10 consecutive days (Theodos *et al*, 1998), and in a dose of 125mg/ kg/day for 10 consecutive days as a part of combined therapy. 2- Ivermectin (Iverzine; Unipharma Alobour Ci-

ty, Cairo) was given orally in a tablet form dissolved in distilled water in a dose of 2mg/kg as a single oral dose (Zhang *et al*, 2008), and in a dose of 1mg/kg single dose in combined therapy. 3- Selenium (Selenium-ACE; Sigma Pharmaceutical Industries for Interpharma, UK) was given orally a in a tablet form dissolved in distilled water in a dose of 2.5 µg/ml to for 14 consecutive days (Desowitz *et al*, 1980).

Mice scarification: Mice were sacrificed at the experimental end, 33 days post infection (PI) by receiving intraperitoneal anesthetic–anticoagulant solution (500mg/kg thiopental & 100 units/ml heparin) (Liang *et al,* 1987). Terminal ileum was removed from each mouse, stored in 10% formalin for histopathological and immunohistochemical studies. After fixation, the tissues were dehydrated, cleared in ascending grades of ethanol, followed by immersion in xylene and then impregnated in paraffin blocks. Three sections of 5µm thickness were prepared from each paraffin block of each mouse; one was stained with H&E stains, and other 2 sections were subjected to immunohistochemistry. Infection & drug action were evaluated parasitological, histological and immunochemicaly.

Parasitological examination: For monitoring oocyst shedding, fresh fecal pellets from each infected mouse were collected from the 3rd day PI & every 3 days until the experimental end. One gram of each fresh fecal sample was concentrated using formol/ether centrifugal sedimentation method (Arrowood, 1989), 50µl fecal smears was stained by Kinyoun's Acid-Fast stain (Garcia, 2001) and examined microscopically to count oocysts. For each animal, oocysts/gm feces were calculated (Benamrouz *et al*, 2012).

Histopathological examination: Intestinal (ileal) sections of mice were examined microsco-pically and the histopathological changes were recorded as mild, moderate, or marked, according to degree of inflammatory infiltrate in lamina propria, and villous mucosal architecture changes (villous height, brush border, and goblet cell content).

Immunohistochemical studies: For counting CD4+ & CD8+T lymphocytes, immunostaining of 2 intestinal sections (ileum) from each mouse was performed. Immunohistochemical staining was performed in an autostainer using a polymer-based detection system (DakoEnVision™ FLEX, K8000).

Tissue sections (5µm) of formalin-fixed & paraffin-embedded specimens, were deparaffinized in xylene, rehydrated in descending grades of alcohol, and then incubated in hydrogen peroxide 3% for 5min. to block endogenous peroxidase activity. This was followed by washing twice in PBS (5min. each time). For antigen retrieval, tissue sections were placed in 0.01mol/l citrate buffer (pH 6) in an automated water bath (Dako PT link). This was followed by sections incubation with the primary antibody murine anti-human CD4 & CD8 monoclonal antibodies (Dako, USA) at room temperature for 1hr, and washed 3 times in PBS for 15min. each. Biotinylated goat anti-polyvalent secondary antibody and streptavidin Peroxidase enzyme were added consecutively for 10min and washed in PBS. Peroxidase activity was visualized with diaminobenzidine (DAB) chromogen applied for 5 min (Ramos-Vara and Miller, 2014). Tissue sections were rinsed, counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted in DPX. Sections from tonsils were used as positive control according to the manufacturer's reco mmendation. Negative control was processed, except for the primary antibody usage. T lymphocytes expressing membranous or cytoplasmic brownish immunostaining for CD4 or CD8 were considered positive. Counting of CD4+ and CD8+ T lymphocytes in intestinal villi lamina propria were performed in 5 representative high-power fields (×400), and the average of cells/ HPF for each sacrificed mouse of groups (Casol *et al*, 2013).

Statistical analysis: Data was coded and entered using the statistical package SPSS version 25. Numerical data was summarized using mean, standard deviation and range. Comparisons between groups were done using analysis of variance (ANOVA) with mul-

tiple comparisons post hoc test. Chi-square test ($\chi 2$) was used to study comparison and association between two qualitative variables. Independent t-test used between every two groups having quantitative variables. A P-value of < 0.05 was considered significant and <0.001 for a high significant result.

Results

Comparison to control group (C3, infected, non-treated), gave the best reduction rate (81%) among mice infected & treated with combined nitzoxanide and ivermectin that gave the least oocysts count compared to group C3 (Fig. 1). Least reduction (39%) was in mice treated with nitazoxanide, followed by (59%) mean reduction in treated with combined nitazoxanide and selenium. Reduction rate in mice only treated with ivermectin (63%) and (70%) in mice treated with ivermectin and selenium. There was significant difference (p<0.001) between C3 and all dexamethasone infected and treated groups, as regard *Cryptosporidium* oocysts concentration in one gram feces (Tab. 1).

Among dexamethasone treated, infected and treated groups, there was a significant difference in mean number of excreted *Cryptosporidium* oocysts between G1 and other dexamethasone treated, infected and treated (p<0.001). G2, G4 & G5 or between G3 & G5 oocysts shedding were without significant (P> 0.05). The ileitis severity was maximized by the parasite in C3, infected non-treated, in which 83.3% of infected animals showed severe inflammatory response and marked villous changes including remarkable shortening and blunting of some villi, atrophy of other villi, sloughing of the brush border and depletion of goblet cell content. Only 16.7% showed moderate inflammatory and villous architectural changes. Significantly slighter inflammatory response and mild villous changes were noticed in all dexamethasone treated, infected and treated groups compared to control (C3) (P<0.001) (Tab. 2, Fig. 2). The best severity improvement after treatment was in mice received combined therapy (G3, NTZ + IVC), where only 16.7% showed moderate inflammatory and mild villous changes, but the other mice group (83.3%) showed mild inflammation and mild villous changes (Fig. 3), with significant difference in severity of ileitis between this group and all other treated group (P<0.001), except G5 (IVC & Se). There was no significant difference between G2 & G4 or G5) as to ileitis severity (P > 0.05).

Regarding the number of local ileal CD4 T-lymphocytes, there was a significant reduction in all groups compared to C1 and other groups (P<0.001), with the least count in dexa C2. There was significant elevation of T cell population in all treated groups (P<0.001) compared to control infected non-treated mice. Treated groups showed significant elevation compared to C2 (P<0.001) except mice treated with NTZ (P> 0.05). As regards the number of local ileal CD8 T-lymphocytes, there was a significant reduction in infected non-treated group, compared to control normal group (P<0.001). There was a significant elevation of CD8 T cell population in all treated groups (P<0.001) compared to control infected non-treated. Besides, treated groups showed significant elevation compared to C2, dexa only, non-infected (P<0.001) (Tab. 3 Fig.2).

Table 1: Mean number of *Cryptosporidium* oocysts in one gram of feces at experimental end.

Groups	Range		M±SD	Reduction %
	Minimum	Maximum		
Infected, non-treated (C3)	99000	170000	130166±28371.93 [a]	
Infected + NTZ (G1)	59500	105000	80000±19750.95 [b]	39%
Infected + IVC (G2)	33000	71000	48500±15056.56 [c]	63%
Infected + NTZ +IVC (G3)	16000	35000	25000±7429.67 [d]	81%
Infected + NTZ+Se (G4)	40000	69000	53000±11081.52 [c]	59%
Infected + IVC +Se (G5)	29000	55000	39000±9444.58 [c, d]	70%

a, b, c & d: No significant difference (P>0.05) between any two groups, within same column with same superscript letter.

Table 2: A comparison of histopathological changes included degree of inflammatory infiltrate and villous architectural changes between different groups.

Groups		Histopathologic Changes (Inflammatory & Villous Architectural Changes)				M ± SD
		No.	Mild	Moderate	Severe	
Infected, non-treated (C3)	No.	0	0	1	5	2.83 ± 0.408[a]
	%	0.0%	0.0%	16.7%	83.3%	
Infected + NTZ (G1)	No.	0	0	4	2	2.33 ± 0.516[b]
	%	0.0%	0.0%	66.7%	33.3%	
Infected + IVC (G2)	No.	0	0	6	0	2.00 ± 0.000[b,c]
	%	0.0%	0.0%	100.0%	0.0%	
Infected + NTZ + IVC (G3)	No.	0	5	1	0	1.17 ± 0.408[d]
	%	0.0%	83.3%	16.7%	0.0%	
Infected + NTZ+ Se (G4)	No.	0	0	5	1	2.17 ± 0.408[b]
	%	0.0%	0.0%	83.3%	16.7%	
Infected + IVC + Se (G5)	No.	0	2	4	0	1.67 ± 0.516[c]
	%	0.0%	33.3%	66.7%	0.0%	

a, b, c & d: No significant difference (P>0.05) between any two groups, within same column with same superscript letter.

Table 3: A comparison between groups, regarding CD4 &CD8 count in lamina propria of intestinal villi.

Groups	(CD4)	(CD8)
Normal (C1)	16.8 ± 1.49[a]	10.2 ± 0.87[e]
Dexa only (C2)	5.00 ± 0.75[d]	6.3 ± 0.8832[f]
Infected, non-treated (C3)	4.00 ± 0.58[e]	6.0 ± 0.8319[f]
Infected + NTZ (G1)	5.2 ± 0.62[d]	12.0 ± 1.15[d]
Infected + IVC (G2)	6.8 ± 0.64[c]	14.4 ± 1.02[c]
Infected + NTZ + IVC (G3)	6.6 ± 0.50[c]	15.0 ± 1.65[c]
Infected + NTZ+ Se (G4)	7.6 ± 0.70[b, c]	17.4 ± 1.63[b]
Infected + IVC + Se (G5)	8.0 ± 0.79[b]	20.0 ± 2.07[a]

a, b, c & d: No significant difference (P>0.05) between any two groups, within same column with same superscript letter.

Discussion

The immune status of the host generally plays an important role in determining susceptibility to cryptosporidiosis outcome and seriousness of the disease (Borad and Ward, 2010). Nitazoxanide was approved by the FDA to treat cryptosporidiosis. However, the efficacy of nitazoxanide depends upon a capable immune system. Thus, it demonstrates very poor efficacy in AIDS and other immunocompromized categories of patients which highlight an urgent medical need for such type of patients (Sparks *et al*, 2015; Love *et al*, 2017). Improvement in cellular immune function among these categories was a key priority for management of cryptosporidiosis (Checkley *et al*, 2015; Ahmadpour *et al*, 2020). Thus, resolution of this opportunistic infection was seen following CD4+ T cell reconstitution in immunocompromized patients given antiretroviral therapy, indicating importance of such set of cell population in serious infection (O'Connor *et al*, 2011; Ludington and ward, 2015). Combination drug therapy is recommended as alternative promising strategy against cryptosporidiosis. It is defined as the use of two or more pharmacologic agents administered separately or in a fixed-dose amalgamation with two or more active ingredients in a single-dosage formulation (Terrie, 2010). Thus, the latter was assessed in this study, in comparison to the commonly used single FDA approved therapeutic agent.

Ivermectin was chosen in the present study to combine nitazoxanide in one group and selenium was the other choice to be given in combination with each anti-parasitic agent, exclusively in separate groups. Ivermectin is a semi-synthetic derivative of macrocyclic lactones family with broad-spectrum anti-parasitic, antiviral, and anti-cancer chemotherapeutic (Chhaiya *et al*, 2012; Laing *et al*, 2017; Momekova, 2020). It is active against acute and chronic giardiasis, cryposporidiosis. and for mass treatment for malaria due to its ability to kill mosquitoes feeding on recently treated patients (Shalaby and Hagg ag, 2006; Smit *et al*, 2018). Selenium (Se) is an essential micronutrient with pivotal role in maintaining optimal health through its incorporation into selenoproteins (Gill

and Walker, 2008). Selenium is known to influence the functioning of all components of the immune system, and its deficiency has been reported to reduce the production of free radicals and killing capacity of neutrophils, T cell counts, proliferation and differentiation of T cells, lymphocyte toxicity and NK cell activity (Arthur *et al*, 2003; Ferencik *et al*, 2003; Gill and Walker, 2008). Wang *et al.* (2009) found that susceptibility or resistance to infection with *Cryptosporidium parvum* correlates with Selenium (Se) deficiency in response to infection.

In the current work, the highest percentage of oocysts reduction was recorded among the group that received combination therapy (IVC & NTZ, 81%), and the lowest oocysts reduction was among nitazoxanide only (39%), indicating its inefficiency to eradicate such intestinal opportunistic protozoal infection. While, therapy combined with Se supplementation improved the percentage of oocysts reduction with both IVC & NTZ to reach 70% & 59% with the single agent respectively, indicating the superiority of both IVC and combined therapy over NTZ alone. Blakley and Rousseaux (1991) found immunostimulatory properties for ivermectin associated with altered function of T lymphocytes, particularly T-helper lymphocytes. Ivermectin has influence on cellular and humoral immune responses, and immunopotentiating effect in rabbits (Sajid *et al*, 2007; Zhang *et al*, 2008; Omer *et al*, 2012). So, its immunomodulating effects gave an alternative treatment for diseases involving immunosuppression.

The histopathological changes of ileocecal sections observed in the current study within the infected non treated groups showed remarkable effect on the structure of the intestinal mucosa (mild, moderate & severe ileitis) compared with that of the non-infected control group. This effect was in the form of shortening or atrophy of the villi, goblet cell depletion, edema and infiltration of lamina propria with mononuclear inflammatory cells that agreed with Waters and Harp (1996) who found variable histopathologic changes in cryptosporidiosis ranged from partial to complete villous atrophy and inflammatory infiltrate. Similar histopathological findings were also reported (Al-Mathal and Alsalem, 2012; Al-Warid *et al*, 2013). There was a significant difference in severity of ileitis between infected treated and non-treated groups with the lowest score of ileitis severity in the treated group with combined treatment (83.3% mild & 16.7% moderate ileal inflammatory and villous changes), comp-0ared to infected non treated group (16.7% moderate and 83.3% severe ileal inflammatory changes). Therapy combined with supplementation in dexamethasone treated, infected and treated groups improved the severity of ileitis non-significantly ($P > 0.05$) in both groups treated with (NTZ & Se) and treated with (IVC & Se).

In the present study, T cells included the $CD4^+$ T cells and $CD8^+$ T cells and the ratio between these 2 immune elements were recognized as an important indicator for evaluating the immunomodulation state and response to homeostasis of intrinsic immune system (Wang *et al*, 2016). In all dexamethasone treated groups, CD4 were more affected but could not be elevated enough even after treatment. Thus, reflected the importance of early diagnosis and treatment to prevent unnecessary complications. CD8 significantly increased in all treated groups with a reversed and unbalanced ratio of CD4/CD8 within the intestinal lesions, compared to normal ones. This agreed with Rocamora-Reverte *et al*. (2019) who found that CD4+ Tcells were essential target cells affected by glucocorticoids (GC), and most affected by immune suppression. This explained the lower level within the local CD4, but did not explain the high level in CD8 in all infected and treated groups. Korbel *et al.* (2011) reported that the proportion of intestinal $CD4^+$ or $CD8^+$ T cells did not increase infection in infected mice during recovery. This suggested that even if $CD4^+$ T cells were important to eliminate *C. parvum*, but were possibly unnecessary for

controlling infection in mice. On the contrary, many authors suggested a greater role for CD8 in elimination of such infection and this may explain such elevation in the treated group to cope with the hard immunological task needed by the host to eradicate the infection helped by effective forms of the treatment (Rowe *et al*, 2007).

The present study, higher elevation in CD8 count over CD4 in all treated groups, compared to both normal and infected non treated groups were more or less agreed with Miller and Schaefer (2007) who studied the impact of dexamethasone induced immunosuppression on mice infected with *Cryptosporidium* oocysts. The authors reported that, the CD8 counts in such group were only 1.6% below controls, while CD4 counts was still depressed by 66%, reflected by GC much higher power to depress CD4 than CD8. They added that these corresponded to cryptosporidiosis elimination and that CD8 positive lymphocytes played a significant role parasite clearance in vivo. Pantenburg *et al*. (2010) found that CD8 (+) T cells eradicated intestinal epithelial zoonotic cryptosporidiosis. So, the CD8 high level was ineffective in dexamethasone treated groups to eradicate total infection.

Tessema *et al*. (2007) found that transfer of CD4+ and CD8+ T-cells (pan T-cells) did not provide a better protection of naive recipients than CD4+ T-cells alone, with irrelevance of CD8+ T-cells in protection against cryptosporidiosis. Possible scenarios from other intracellular pathogens rose existence of *Cryptosporidium* immune evasion mechanism by CD8+ T-cell-mediated cytotoxicity. Lysosomal degradation of *C. parvum* intracellular developmental stages was required to recognize *Cryptosporidium* infected host cells by CD8+ T-cells. Autophagy of intracellular pathogens contributed to effective elimination of viruses, bacteria & parasites, but, many pathogens escaped autophagy machinery as herpes simplex virus type 1 and *Shigella* use proteins to antagonize autophagy (Orvedahl *et al*, 2007). Ability of pathogens to escape autophagy allowed survival within host cells as a new immune mechanism evasion (Vyas *et al*, 2008), and *Cryptosporidium* location within cells was worth mentioned (Crotzer and Blum, 2008).

All stages of *Cryptosporidium* were located in a parasitophorous vacuole, formed extra-cytoplasmic, but intracellular within infected cells. The structure could hinder lysosomal degradation of *Cryptosporidium* proteins to be presented through MHC-I pathway. Lack of MHC-I *C. parvum* proteins was the cause why CD8+ T-cells do not kill infected cells. After degradation of antigens from intracellular pathogens, peptides from degraded antigen bind to MHC-I molecule on the infected cell surface for recognition by CD8+ T cells. Aguirre *et al.* (1994) found that MHC-II (important for CD4+ T-cells) deficient mice were more susceptible to *C. parvum* infection than MHC-I deficient mice (important for function of CD8+ T-cells). *C. parvum* modulated the MHC-I Ag presentation pathway without pathogenic antigen on epithelial infected cells surface to be recognized by CD8+T-cells (Hewitt, 2003).

Conclusion

The outcome results proved the superior effectiveness of the combined treatment over the other forms of treatment and the inferiority of NTZ alone. Studies to assess other drug combination with variable doses for more effective *Cryptosporidium* eradication specifically in immunocompromized models are ongoing and will be published later.

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Explanation of figures

Fig. 1: Column chart showed *Cryptosporidium* oocysts expelled/ gram feces in different treated groups.

Fig. 2: CD4 (left) & CD8 (right) within control normal and control non-infected groups. Notice apparently higher expression of CD4 in normal group (A) and relatively lower expression of CD4 in dexa group (C) & the higher expression of CD8 (D) than in control normal (B). CD4 & CD8 appear still down regulated within dexa infected non-treated group (E&F). On the contrary, expression of CD8 intensifies over CD4 within dexa infected treated group (G & H).

Fig. 3: Sections of ileum from different degrees of ileitis. A: infected, non-treated group showed severe villous change. B: Treated, infected group treated with NTZ only showing moderate to severe ileitis. C: Treated, infected group treated with IVC or NTZ & Se showed moderate ileitis. D: Mild villous changes in infected group treated with combined therapeutic agent with IVC (NTZ & IVC or IVC & Se) (Hx & E stain x 200).