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Original Article

Antiparasitic and immunomodulating effects of nitazoxanide, ivermectin and selenium on *Cryptosporidium* infection in diabetic mice

Efeitos antiparasitários e imunomodulantes da nitazoxanida, ivermectina e selênio sobre a infecção por *Cryptosporidium* em camundongos diabéticos

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Abstract

The present work aims to investigate the antiparasitic and the immunomodulating effects of nitazoxanide (NTZ) and ivermectin (IVC) alone or combined together or combined with selenium (Se), on *Cryptosporidium* infection in diabetic mice. The results revealed that the combined NTZ and IVC therapy achieved the highest reduction of fecal oocysts (92%), whereas single NTZ showed the lowest reduction (63%). Also, adding Se to either NTZ or IVC resulted in elevation of oocyst reduction from 63% to 71% and from 82% to 84% respectively. All treatment regimens, with the exception of NTZ monotherapy, showed a significant improvement in the intestinal histopathology, the highest score was in combined NTZ and IVC therapy. The unique results of immunohistochemistry in this study showed reversal of the normal CD4/CD8 T cell ratio in the infected untreated mice, however, following therapy it reverts back to a normal balanced ratio. The combined (NTZ+ IVC) treatment demonstrated the highest level of CD4 T cell expression. Taken together, NTZ and IVC combined therapy showed remarkable anti-parasitic and immunostimulatory effects, specifically towards the CD4 population that seem to be promising in controlling cryptosporidiosis in diabetic individuals. Further research is required to explore other effective treatment strategies for those comorbid patients.

Keywords: *Cryptosporidium*, ivermectin, selenium, CD4, CD8, diabetes.

Resumo

O presente trabalho tem como objetivo investigar os efeitos anti-parasitários e imunomodulantes da nitazoxanida (NTZ) e ivermectina (IVC), isoladas ou em associação, e do selênio (SE), associado à NTZ ou à IVC, sobre a infecção por *Cryptosporidium* em camundongos diabéticos. Os resultados revelaram que a terapia combinada com NTZ e IVC resultou em maior redução de oocistos fecais, enquanto a NTZ isolada mostrou a menor redução de oocistos fecais (63%). Além disso, a associação do SE com a NTZ ou IVC resultou em redução do número de oocistos fecais de 63% para 71% e de 82% para 84%, respectivamente. Todos os tratamentos, com exceção da monoterapia com NTZ, mostraram uma melhora significativa nos índices relacionados à histopatologia intestinal. Os resultados da imuno-histoquímica mostraram reversão da razão celular CD4/CD8 T normal nos camundongos infectados não

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tratados, no entanto, após a terapia, houve retorno à razão celular CD4/CD8 T normal. O tratamento combinado (NTZ+ IVC) demonstrou o mais alto nível de expressão celular CD4 T. Em conclusão, a terapia combinada com NTZ e IVC mostrou efeitos anti-parasitários e imunoestimuladores notáveis, especificamente para a população CD4, que parecem ser promissores para o controle da criptosporidiose em indivíduos diabéticos.

Palavras-chave: *Cryptosporidium*, ivermectina, selênio, CD4, CD8, diabetes.

Introduction

Cryptosporidium is an apicomplexan protozoan parasite that has been recognized, second to *Rotavirus*, as a significant cause of water and food-borne diarrhea outbreaks in humans, especially children and immunocompromised patients (Efstratiou et al., 2017; Ryan et al., 2018; Khalil et al., 2018).

The establishment of cryptosporidiosis is strongly related to the immune status of the host. Infected immunosuppressed individuals may develop severe persistent diarrhea that can lead to significant morbidity and mortality (Laurent & Lacroix-Lamandé, 2017). Several experimental and human studies on *Cryptosporidium* have linked cell-mediated immune responses, especially CD4+ count, to susceptibility to infection as well as outcome and severity of the disease. In patients with HIV, the intensity of the disease depends on the degree of immunosuppression, as indicated by the $CD4^+$ counts (Borad & Ward, 2010; Tinarwo et al., 2020).

Diabetes mellitus has been reported to increase the frequency and severity of common infections and raise the susceptibility to opportunistic infections (Chinen & Shearer, 2010; Knapp, 2013). Intestinal parasitic diseases have been reported among significant proportions of diabetic patients (Mohtashamipour et al., 2015; Tangi et al., 2016; Alemu et al., 2018). The hyperglycemic environment in DM has been suggested to induce weakening in both innate and acquired immune systems (Tanaka, 2008; Casqueiro et al., 2012). Furthermore, disturbed glucose metabolism, inadequate blood supply, and denervation are other reported factors that may contribute to the increased frequency of infections in DM (Chinen & Shearer, 2010). With the increasing numbers of potential immune-altered diabetic patients, cryptosporidiosis may represent a major public health concern. However, there is a general lack of scientific research regarding the management of such protozoa infection in diabetic patients.

Several previous studies have attempted to develop a satisfactory therapy for cryptosporidiosis, especially in AIDS patients (Hunter & Nichols, 2002). Effective drug treatment, especially for immunocompromised infected patients, has not been consistently successful (Checkley et al., 2015). The current therapeutic drug approved by the Food and Drug Administration (FDA) for *Cryptosporidium* infections is nitazoxanide (NTZ). This drug, however, exhibits limited and immune-dependent efficacy (Sparks et al., 2015; Widmer et al., 2020). This can be seen in HIV patients and malnourished children who demonstrate limited and weak response rates. Besides, the drug is not recommended for use in children younger than 12 months (Abubakar et al., 2007; Amadi et al., 2002). Some *in-vitro* and animal studies on cryptosporidiosis have reported a better response to NTZ in combination regimens than to NTZ alone (Theodos et al., 1998; Giacometti et al., 2000; Krause et al., 2012; Bhadauria et al., 2015).

Ivermectin (IVC) is a semi-synthetic derivative belonging to macrocyclic lactones and is approved by FDA. It possesses broad-spectrum anti-parasitic and antiviral activity as well as cancer chemotherapeutic effects (Laing et al., 2017; Momekov & Momekova, 2020). Ivermectin is used worldwide against a broad range of endoparasites (nematodes) and ectoparasites (acarine, insects) of humans and animals. Moreover, a number of studies have shown activity of the drug against protozoan parasites such as *Giardia lamblia*, *Cryptosporidium* spp. (Youssef et al., 1996; Zinada, 2000; Hassan et al., 2001), and recently, has been considered for mass drug administration for malaria (Smit et al., 2018). Furthermore, IVC has been reported to potentiate the immune system in rabbits and affect the cellular and humoral immune responses (Sajid et al., 2007; Zhang et al., 2008; Omer et al., 2012).

Selenium (Se) is an essential micronutrient which is of major importance to human health (Roman et al., 2014). It is a member of the selenoprotein family and has structural and enzymatic functions, moreover it is important for the proper functioning of the immune system (Gill & Walker, 2008; Majeed et al., 2018).

Numerous studies have reported dramatic effects of antiretroviral therapy on cryptosporidiosis in AIDS patients as a consequence of immune reconstitution and recovery of the CD4 count (Foudraine et al., 1998; Miao et al., 2000; Dillingham et al., 2011; Cabada & White, 2010). Therefore, recent evidence suggests that improvement in cell-mediated immune responses is fundamental for management of the infection in immunocompromised patients (Checkley et al., 2015; Ahmadpour et al., 2020). Hence, this highlights the need for a proper therapeutic approach that provides both anti-parasitic and immunostimulatory effects. The aim of the present work is to

assess the therapeutic effects of NTZ, IVC and Se drugs, using different regimens, on experimental *Cryptosporidium* infection in mice with chemically induced diabetes. Furthermore, the study attempts to investigate the possible immunomodulatory effects of these therapeutic agents on the local intestinal CD4 and CD8 T cell responses.

Materials and Methods

Experimental animals

The present study was carried out on 48 laboratory-bred male Swiss albino mice of CD1 strain; aged 7 weeks, specific free pathogen (SFP) and weighing 20-25gm each. The animals were provided by *Schistosoma* Biological Supply Program (SBSP) at Theodor Bilharz Research Institute (Giza, Egypt). All animal handling and experimental procedures were performed in accordance with the national and institutional guidelines for the care and use of laboratory animals. Each mouse was housed in a well-ventilated cage with clean wood-chip bedding, and kept between 18-23 °C room temperature with free access to a standard pelleted diet and water ad libitum. The animals' stool samples were examined using direct wet saline and iodine smears, then stained with acid fast stain to exclude the presence of parasites.

Ethical consideration

The experiment was approved by the ethical committee at Theodor Bilharz Research Institute (TBRI) (Approval code: 11/2018/00010609).

Experimental design and study groups

The animals were classified into 8 groups as follows, each containing 6 mice:

- Groups infected & treated with a single therapeutic drug:

Group 1: treated with nitazoxanide (NTZ);

Group 2: treated with ivermectin (IVC).

- Groups infected and treated with combined drug regimens:

Group 3: treated with NTZ combined with IVC;

Group 4: treated with NTZ combined with Se;

Group 5: treated with IVC combined with Se.

- Control groups:

Group 6: Normal non-infected non-treated (healthy- control);

Group 7: Non-infected (diabetic - control);

Group 8: Infected non-treated (infected - control).

Induction of type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) was induced by administration of multiple intraperitoneal, low doses of Streptozotocin (STZ, Sigma-Aldrich, USA) to all study groups (except normal control, group 6), in concordance with the previously described method (Wu & Huan, 2008). Briefly, freshly prepared STZ was dissolved (immediately prior to injection) in 50mM sodium citrate buffer (50 mM, pH 4.5) to a final concentration of 4 mg/ml. Streptozotocin solution then was injected intraperitoneally in a dose of 40 mg/kg (1.0 ml/100 g) for 5 consecutive days (starting from experimental day 1 to 5). Streptozotocin can cause pancreatic β-cell destruction leading to hyperglycemia (Wu & Huan, 2008). To check for induced hyperglycemia in the STZ- treated mice, the blood glucose level was measured on experimental day 14 (9 days after the last STZ injection), from a tail vein blood sample using a glucometer. Measurement of the blood glucose level was continued 3 times per week throughout the experiment.

Preparation of *Cryptosporidium* oocysts inoculum

Augmentation of *Cryptosporidium* oocysts (taken from the faeces of infected calves) was performed by passage in 20 Swiss albino CD1 strain mice. The animals were inoculated by gavage (2000 oocysts/animal) repeatedly every 2 weeks

(von Oettingen et al., 2008). Isolation and purification of oocysts was done by centrifugal flotation (Zeibig, 1997) and they were then suspended in PBS. The suspension was kept with 0.01%Tween-20, containing 200 IU/mL penicillin, 0.2 mg/mL streptomycin and 2.5 µg/mL amphotericin B to eliminate any remaining bacterial or fungal contamination, and stored at 4 °C before use. Finally, the number of *Cryptosporidium* oocysts in the prepared suspension was determined so as to adjust the inoculum infecting dose. This was performed by counting the number of *Cryptosporidium* oocysts in smears prepared from 50µl aliquots and stained with Kinyoun's Acid Fast stain (Operario et al., 2015).

Inoculation of mice

All experimental animal groups (except group 6 and 7) were orally infected with *Cryptosporidium* oocysts on experimental day 14 (9 days after the last STZ injection), using oral-gastric gavage. Each mouse received a dose of about 10^4oocysts/ mouse (Love et al., 2017).

Administration of the drugs

Drug administration to the treated mice groups was started from the fifth day post-infection. Nitazoxanide suspension (Nanazoxid; Medizen Pharmaceutical Industries for Utopia Pharmaceuticals) was administrated orally for 10 consecutive days as follows; in a dose of 250 mg/kg /day as a single therapy (Theodos et al., 1998), and in a dose of 125 mg/kg/day as a part of combined therapy. Ivermectin tablet (Iverzine; Unipharma Alobour City, Cairo, Egypt) dissolved in distilled water was given orally as a single dose as follows; 2 mg/kg as a single therapy, and 1 mg/kg in combined therapy. Selenium tablets (Selenium-ACE; Sigma Pharmaceutical Industries for Interpharma, UK) were dissolved in drinking water administrated orally in a dose of 2.5 µg/ml to for 14 consecutive days (Desowitz & Barnwell, 1980).

Euthanasia of mice

At the end of the experiment, 33 days post infection (PI), all mice were euthanized by intraperitoneal anesthetic–anticoagulant solution. The terminal ileum sections of the euthanized mice were removed and fixed in 10% formalin for histopathological and immunohistochemical analysis. The tissues were dehydrated, cleared in ascending grades of ethanol, then immersed in xylene and finally embedded in paraffin blocks (Drury & Wallington, 1980). For each mouse, 3 paraffin sections of 5 µm thickness were prepared; one was stained with haematoxylin and eosin (H&E) stains and the other 2 sections were processed for immunohistochemical studies.

Assessment of the infection & the drug effect

Parasitological examination

For estimation of *Cryptosporidium* oocyst shedding, fresh fecal samples were collected from each infected mouse at the end of the experiment. The samples were weighed, dissolved in known amounts of 10% formalin and passed through sterile gauze. Then, 50 µl from each prepared sample were taken, subjected to Kinyoun's Acid-Fast stain (Garcia, 2001) and examined microscopically for counting of the oocysts, using the oil immersion lens (1000×). For each mouse, the number of oocysts was expressed per gram of feces (Benamrouz et al., 2012). The mean number of oocysts was calculated for each group of mice.

Histopathological examination

Intestinal (ileal) sections of the euthanized mice were examined microscopically to evaluate the degree of inflammation in the lamina propria, and villous mucosal architecture changes (brush border, villous height and goblet cells). The histopathological changes were graded as mild, moderate, or marked according to (Sadek & El-Aswad, 2014), scored by 0 (no), 1 (mild), 2 (moderate) and 3 (severe) to produce, in each group, an average score for histopathological changes.

Immunohistochemical studies

Immunohistochemical staining of 2 intestinal sections (ileum) from each mouse was performed in order to count the number of CD4 and CD8 T lymphocytes. The immunostaining process was carried out in an auto-stainer using a

polymer-based detection system (DakoEnVision™ FLEX, K8000). Tissue sections (5µm) were deparaffinized in xylene, rehydrated in declining grades of alcohol, followed by incubation in hydrogen peroxide 3% for 5 minutes to block endogenous peroxidase activity and then washed twice in PBS (each 5 minutes). The slides were then immersed in citrate buffer (pH 6) in an automated water bath (Dako PT link) for antigen retrieval. Next, the sections were incubated with the primary antibody murine anti-human CD4 and CD8 monoclonal antibodies (Dako, USA) for 1 hour at room temperature and washed in PBS. This was followed by the addition of biotinylated goat anti-Polyvalent secondary antibody and streptavidin peroxidase enzyme consecutively for 10 min and washed in PBS. Visualization of peroxidase activity was carried out using diaminobenzidine (DAB) chromogen applied for 5 min (Ramos-Vara & Miller, 2014). The slides were then counterstained with haematoxylin and mounted by DPX. Sections from tonsils were stained as positive control according to the manufacturer's recommendation. Negative controls were prepared according to the same protocol, except for the use of the primary antibody. T lymphocytes were considered positive for CD4 or CD8, if there was expression of membranous or cytoplasmic brownish immunostaining. The number of CD4 and CD8 T lymphocytes in the lamina propria was counted in 5 representative high-power fields HPFs (×400). The results were recorded as the average number of cells/HPF for each mouse and for each designated group of mice (Cassol et al., 2013).

Statistical methods

The collected data was analyzed using the statistical package SPSS version 25. Quantitative data was expressed as mean, standard deviation and range. The data was tested for normality using Kolmogorov–Smirnov test, Shapiro–Wilk tests and it showed normal distribution. Analysis of variance (ANOVA) with multiple comparisons post hoc test was used for comparisons between groups. Differences were considered statistically significant if *P*-values were less than 0.05.

Results

Effect of different drug regimens on *Cryptosporidium* oocysts shedding

A significant decrease in the mean number of *Cryptosporidium* oocysts in feces, at the end of the experiment, was obtained in all treated groups ($p<0.001$). In comparison to the control group 8 (infected, non-treated), group 3 (received combined NTZ and IVC) showed the lowest oocyst count with a 92% reduction. This was followed by lower reduction rates 84%, 82% and 71% in group 5 (combined IVC and Se), group 2 (single IVC) and group 4 (combined NTZ and Se), respectively. The lowest percentage of reduction (63%) was reported in group 1(NTZ monotherapy). Also, it was observed a significant difference in the mean number of excreted oocysts between all treated groups ($p<0.001$) except between group 2 (IVC) and group 5 (IVC + Se) (Table 1 and Figure 1).

Histopathological examination

The infected, non-treated control **(**group 8) showed moderate to severe histopathological changes in the ileal mucosa in the form of moderate to severe inflammatory cellular infiltrate (plasma cells, lymphocytes and histiocytes), massive edema in the lamina propria, remarkable shortening of villi, crypt elongation and depletion of goblet cell content (Figure 2B). As Table 2 shows, there was a significant difference in the severity of intestinal inflammation between control group 8 and all other treated groups, except group1 (NTZ) which showed no significant difference ($P>0.05$). Among all treated groups, group 3 (NTZ+IVC) showed the weakest inflammatory reaction, where 16.7% of the animals did not reveal any inflammatory response, while 66.7% and 16.7% revealed mild and moderate inflammation respectively. The results also showed a significant difference in the severity of ileitis between group 3 (NTZ+ IVC) and all other treated groups ($P<0.001$), except group 5 (IVC + Se) ($P> 0.05$) which showed mild and moderate inflammation in 66.7% and 33.3% respectively. There was no significant difference between group 2 (IVC) and both; group 4 (NTZ+ Se) and group 5 (IVC+ Se) regarding the degree of pathological changes ($P>0.05$) (Table 2 and Figure 2).

Table 1. *Cryptosporidium* oocysts counts per gram feces in the infected control and treated groups. The least reduction is seen with group (1) treated with NTZ monotherapy, followed by group (4) NTZ & Se, while the highest reduction is achieved in group (3) treated with combined treatment NTZ +IVC, followed by group (5) treated with IVC +Se.

Group	Range		Mean ± SD	% of reduction
	Minimum	Maximum		
Group1 Infected + NTZ	27000	105000	35500±8117.88 [b]	63%
Group2 Infected + IVC	12000	25000	17500±4847.68 [d]	82%
Group 3 Infected + NTZ +IVC	5000	11000	7833.33 ±2041.24 [e]	92%
Group 4 Infected + NTZ+Se	19500	34000	27500 ±4795.83 [c]	71%
Group 5 Infected + IVC +Se	8500	22000	15000 ±4764.45 [d]	84%
Group 8 Infected non treated (control)	86000	105000	95666.67±6889.61 [a]	

[a,b,c,d,e]There is no significant difference (P>0.05) between any two groups, within the same column have the same superscript letter.

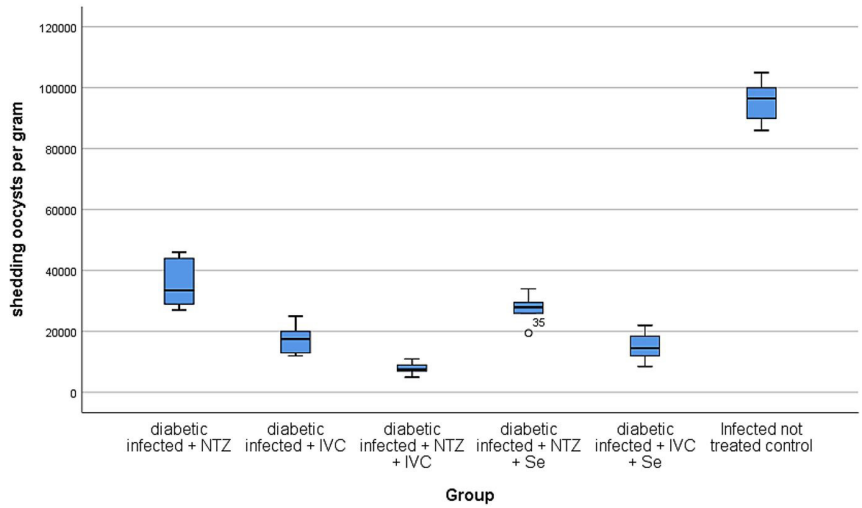

Figure 1. Box-plot diagram shows *Cryptosporidium* oocyst counts per gram feces in the infected control and treated groups. The least oocyst reduction is seen with the group treated with NTZ monotherapy, while the highest reduction is achieved in the group treated with combined treatment (NTZ +IVC).

Immunohistochemical results

In comparison to the control group (6, normoglycemic non-infected), there was no significant difference in the expression of local intestinal CD4 in either group 7 (diabetic control) or group 8 (infected control) (P> 0.05). However, group 8 presented significantly lower CD4 expression than all other groups that received treatment (Table 3 and Figure 3, 4 and 5). In addition, it was observed that CD4 expression in all treated groups was significantly higher than control group 6 (P<0.001), except group 1 (NTZ) which showed non-significant elevation (P>0.05). The highest level of CD4 expression among the treated groups was found in group 3 (NTZ+ IVC), this was statistically significant (P<0.001). This was followed by group 5 (IVC + Se), group 2 (IVC) and group 4 (NTZ+ Se), however, no significant difference was detected between these 3 groups (Table 3 and Figure 3).

In comparison to group 6 (healthy control), a significant increase in the expression of local intestinal cytotoxic CD8 T lymphocytes was observed in all diabetic groups (P<0.001) (Table 3 and Figure 4, 5 and 6). The highest level

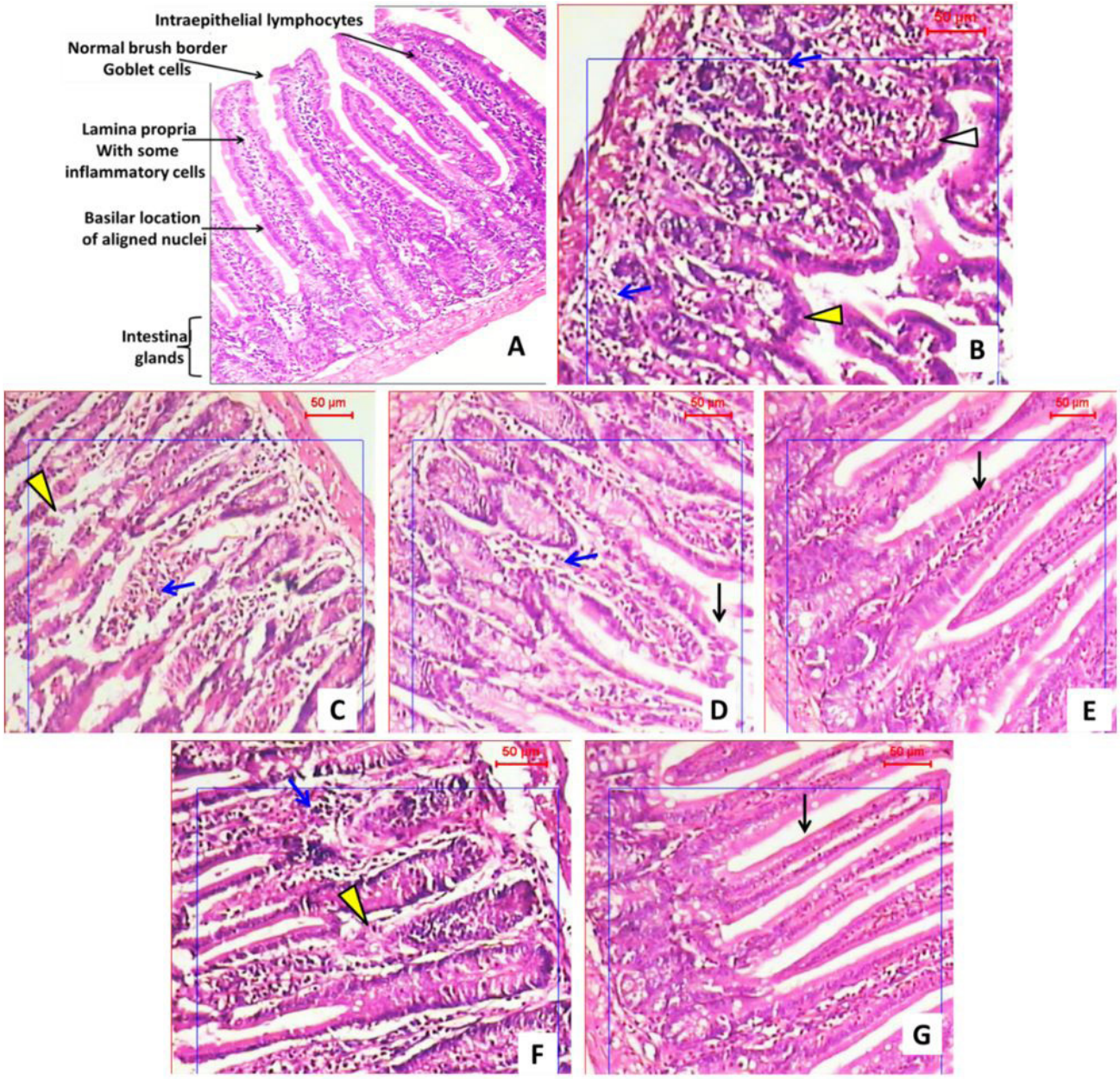

Figure 2. Photographs show sections of ileum from different study groups; (A) non-infected non diabetic control group showed features of normal villous architecture and components; (B) Diabetic infected non treated group showed severe ileitis with severely disrupted villus/crypt ratio "broadened villi" (white arrowhead), disturbed mucosa and basal nuclei (yellow arrowhead) with dense inflammatory infiltrate (blue arrows); (C) Diabetic infected group treated with NTZ showed moderate to severe ileitis; disrupted mucosal lining and basal nuclei (yellow arrowhead) with moderate inflammatory infiltrate (blue arrow); (D) Diabetic infected group treated with IVC showed moderate villous changes with slightly disrupted mucosal lining and basal nuclei (black arrow) and moderate collection of inflammatory cells; (E) Diabetic infected group treated with NTZ & IVC showed mild villous changes, in which most of the mucosa plus nuclei regain its normal distribution (black arrow) with improvement of villus/crypt ratio; (F) Diabetic infected group treated with NTZ & Se showed moderate villous changes; areas of disrupted mucosa are seen (yellow arrowhead) with dense collection of inflammatory cells (blue arrow); (G) Diabetic infected group treated with IVC & Se showed mild villous changes with areas with almost normal mucosa and normal villus/crypt ratio (black arrow) and very mild inflammatory infiltrate (Hx & E stain ×200).

was detected in group 8 (infected control) followed by group 7 (diabetic control). Regarding CD8 expression in differently treated groups, no statistically significant differences were found between the groups, except between group 3 (NTZ+ IVC) and both group 1 (NTZ) and group 2 (IVC). (Table 3 and Figure 6).

Table 2. Degree of intestinal histopathological changes in the infected control and treated groups. Minimum pathological changes are seen in group (3) treated with combined therapy NTZ + IVC, followed by group (5) treated with IVC +Se.

Group No (0)		Degree of pathological changes				Mean Score ± SD
		Mild (1)	Moderate (2)	Severe (3)		
Group 1 (Infected + NTZ)	N	0	0	5	1	2.17 ± 0.408 [a,b]
	%	0.0%	0.0%	83.3%	16.7%	
Group 2 (Infected + IVC)	N	0	2	4	0	1.67 ± 0.516 [b,c]
	%	0.0%	33.3%	66.7%	0.0%	
Group 3 (Infected + NTZ + IVC)	N	1	4	1	0	1.00 ± 0.632[d]
	%	16.7%	66.7%	16.7%	0.0%	
Group 4 (Infected + NTZ + Se)	N	0	2	4	0	1.67 ± 0.516 [b,c]
	%	0.0%	33.3%	66.7%	0.0%	
Group 5 (Infected + IVC + Se)	N	0	4	2	0	1.33 ± 0.516 [c,d]
	%	0.0%	66.7%	33.3%	0.0%	
Group 8 (Infected non treated) control	N	0	0	3	3	2.5 ± 0.548 [a]
	%	0.0%	0.0%	50.0%	50.0%	

[a,b,c,d]There is no significant difference (P>0.05) between any two groups, within the same column have the same superscript letter.

Table 3. Number of local CD4 and CD8 T lymphocytes in the lamina propria of immunostained intestinal sections (ileum). Reversal CD4/CD8 ratio is seen in group (7&8) with the highest CD8 expression, while treatment succeeded to raise CD4 with the best result seen again in group (3 & 5).

Group	(CD4) Mean ± SD	(CD8) Mean ± SD
Group 1 (Infected + NTZ)	17.8± 0.97 [c]	13.5 ± 1.20 [d]
Group 2 (Infected + IVC)	21.2 ± 1.94[b]	12.0± 1.62 [d]
Group 3 (Infected + NTZ + IVC)	24.4 ± 1.81 [a]	16.2 ± 2.15 [c]
Group 4 (Infected + NTZ+ Se)	20.8 ± 2.69 [b]	14.6 ± 1.59 [c, d]
Group 5 (Infected + IVC + Se)	22.8 ± 1.69 [a, b]	15.2 ± 1.29 [c, d]
Group 6 (Healthy control)	16.8 ± 1.49[c, d]	10.2 ± 0.87 [e]
Group 7 (Diabetic control)	18.4 ± 1.85 [c]	19.0± 1.40 [b]
Group 8 (Infected control)	14.3 ± 1.43 [d]	21.6 ± 1.69 [a]

[a,b,c,d,e]There is no significant difference (P>0.05) between any two groups, within the same column have the same superscript letter.

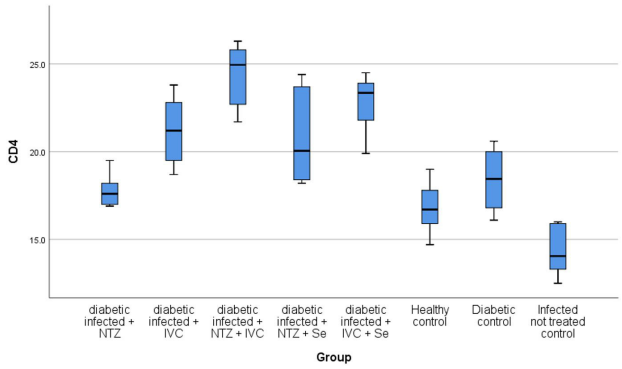

Figure 3. Box-plot diagram shows the number of local CD4 T lymphocytes in the lamina propria of immunostained intestinal sections (ileum). The least CD4 expression is seen in the infected non treated control group. The highest expression among the treated groups is seen in mice received combined therapy (NTZ+ IVC).

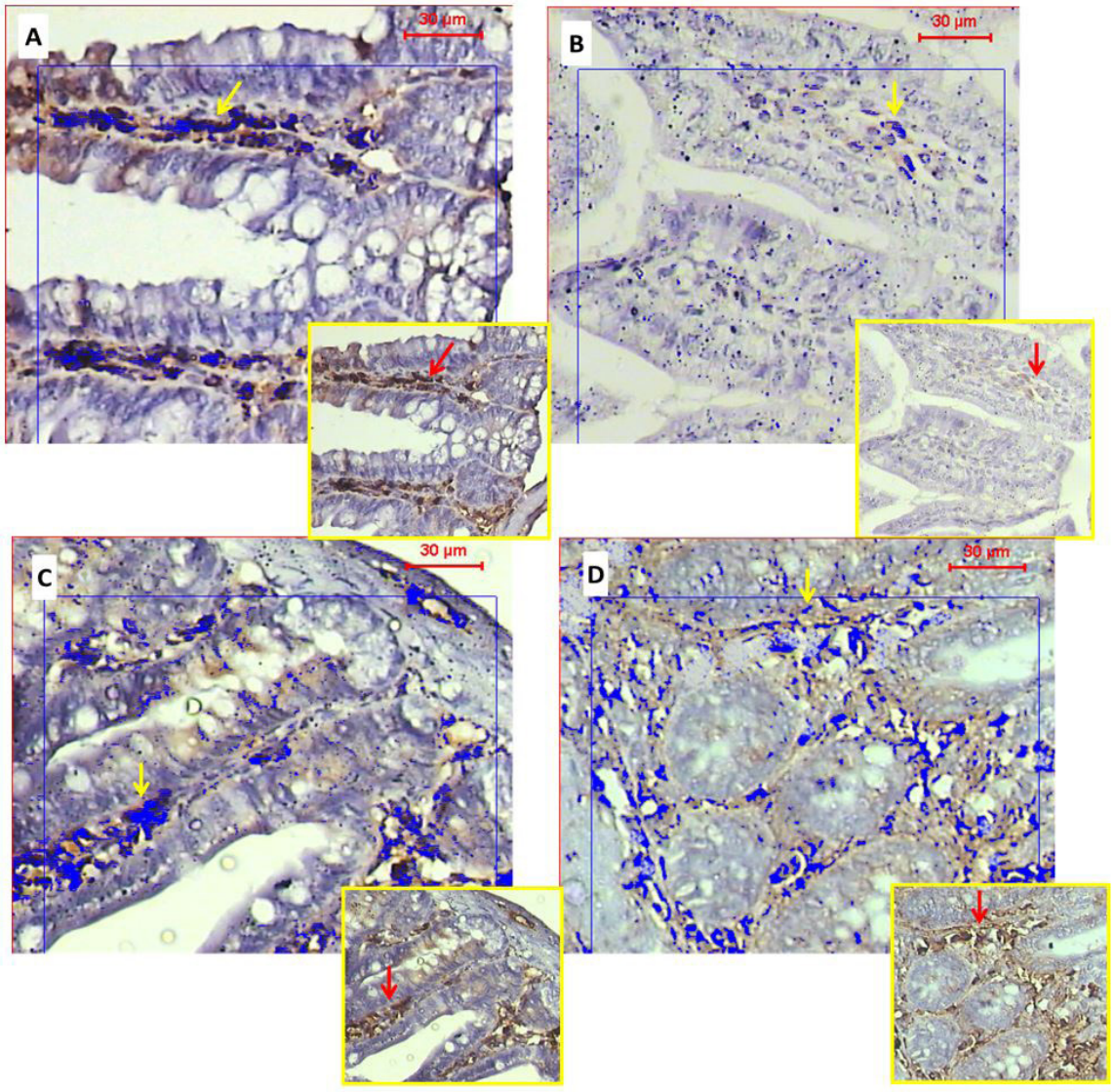

Figure 4. Photographs present immunohistochemical stained sections before image analysis (small one), in which the stain is identified by its brown discoloration (red arrow). Bluish colour (yellow arrow) is seen within the corresponding larger photo during image analysis. Figures show CD4 (left) & CD8 (right) within the control non-infected non diabetic group (A&B) and diabetic group (C&D). Notice the apparently higher expression of CD4 in normal group (A) than CD8 (B) and the almost equal expression in the diabetic group (C &D) (IHC ×200).

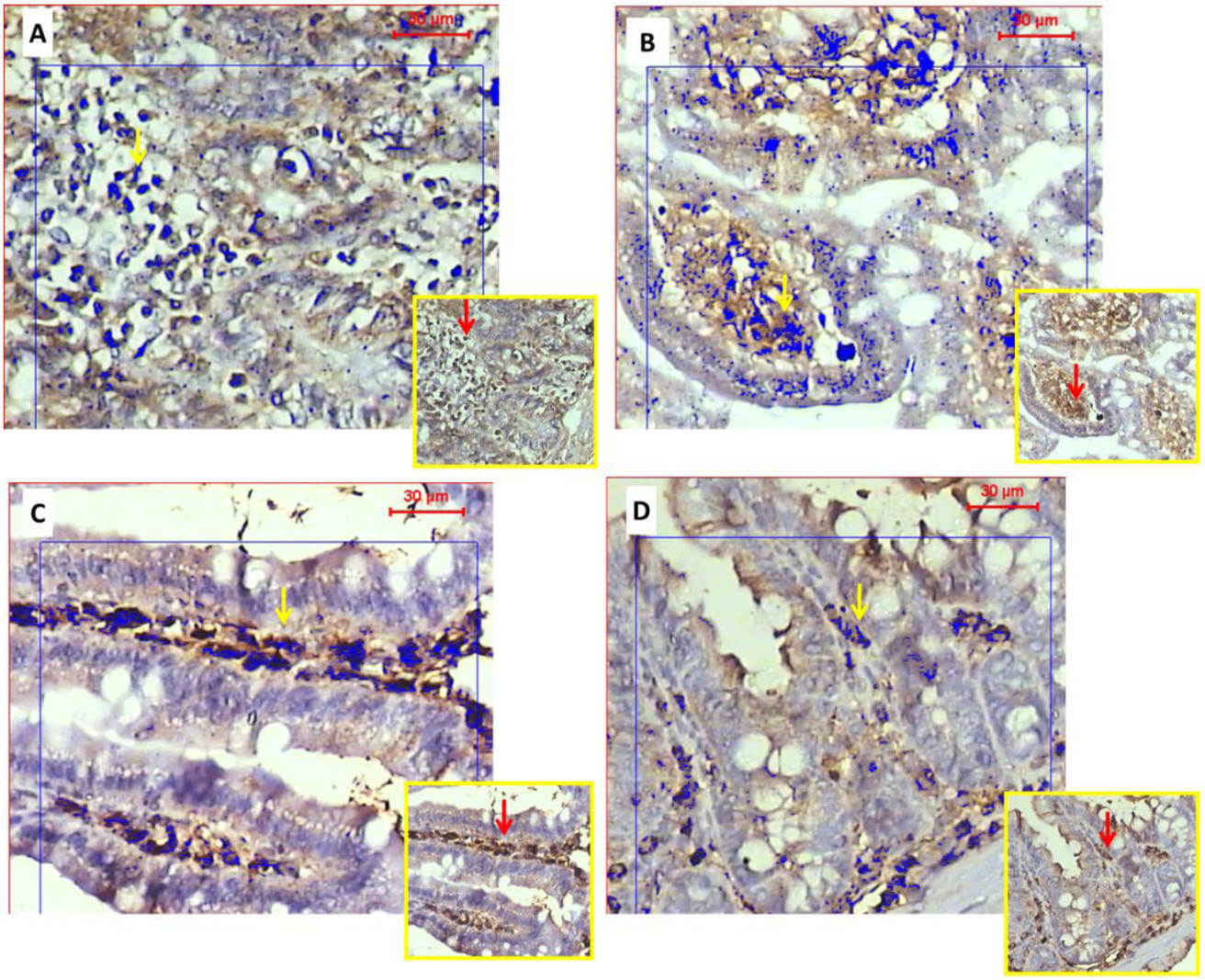

Figure 5. Photographs show CD4 (left) & CD8 (right) within diabetic infected non-treated group (A&B) and diabetic infected treated group (representative) (C&D). Expression of CD4 (A) apparently decreases in the non-treated group. Expression of CD4 (C) obviously increases over CD8 expression (D) in the treated group. Red arrows represent the expression before analysis and yellow arrows denote the expression during software analysis (IHC ×200).

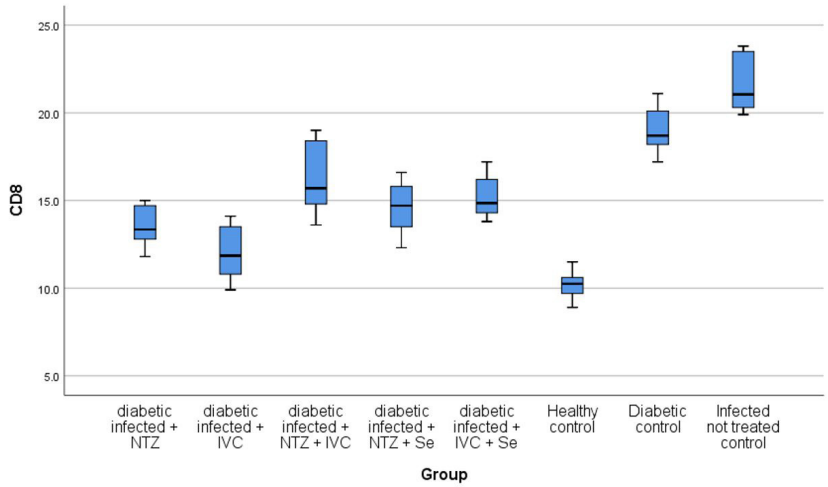

Figure 6. Box-plot diagram shows the number of local CD8 T lymphocytes in the lamina propria of immunostained intestinal sections (ileum). The highest level of CD8 expression is seen in the infected non treated control group.

Discussion

Cryptosporidiosis in immunocompromised patients is a major health problem that can result in chronic, debilitating, potentially serious diarrhea and severe consequences (Chinen & Shearer, 2010; Florescu & Sandkovsky, 2016; Wang et al., 2018; Gerace et al., 2019).

Current treatment for *Cryptosporidium* infections is not satisfactory (Sparks et al., 2015). Therefore, there is an intense need for safe and effective anti-cryptosporidiosis treatment, particularly for immunocompromised people (Love et al., 2017).

The present work assessed the effectiveness of different combination regimens comprising NTZ, IVC and Se, compared to NTZ and IVC mono-therapies in treating cryptosporidiosis in diabetic mice models.

In the present study, the group that received combined NTZ and IVC therapy showed the highest percentage of oocyst reduction (92%) in feces compared to the control group. On the other hand, the lowest percentage of reduction (63%) was found in the group that received single NTZ. This finding supports the theory of the insufficiency of NTZ treatment in infected immunocompromised hosts (Love et al., 2017). At the same time, IVC has been reported to potentiate the immune system in rabbits and affect both cellular and humoral immune responses (Sajid et al., 2007; Zhang et al., 2008; Omer et al., 2012).

The present results also proved that Se supplementation might have a role in improving the efficacy of NTZ and IVC. It was observed that adding Se supplements to either NTZ or IVC resulted in an increase of the percentages of oocyst reduction from 63% to 71% and from 82% to 84% respectively. The role of Se in *Cryptosporidium* infections has been demonstrated by Wang et al. (2009) who suggested that Se nutritional deficiency can decrease the immune response, thereby increasing susceptibility to the infection. In the same context, Se supplementation was proved in other studies to possess anti-leishmanial properties and to reduce parasitemia in the case of *Trypanosoma* spp. infections **(**Silva et al., 2014; Soflaei et al., 2014).

In the current study, histopathological examinations of the intestinal sections of the infected control group showed moderate to severe inflammatory cellular infiltrate, remarkable shortening of villi, edema and depletion of goblet cell content. These findings are consistent with those of Waters & Harp (1996) who reported intestinal inflammatory changes in the form of inflammatory infiltrate and villous atrophy in response to C*ryptosporidium* infection. Moreover, numerous studies have detected similar histopathological changes in infected animals (Abu El Ezz et al., 2011; Al-Mathal & Alsalem 2012; Al-Warid et al., 2013). On the other hand, in comparison to the control group, a significant improvement of the intestinal pathological changes was noticed in all treated mice, except in the NTZ-treated group, which showed no significant difference. Furthermore, previous studies performed on immunosuppressed mice, have reported a limited efficacy of single NTZ treatment in infected intestinal sections (Sadek & El-Aswad, 2014; Taha et al., 2017; Moawad et al., 2021).

The present results revealed that among all the treated groups, the group that received combined therapy (NTZ+IVC) showed the greatest improvement in the severity of ileitis, as only 16.7% of the animals presented moderate inflammation, while the remainder showed an absent or mild response. This finding could be explained, in part, by (Zhang et al., 2008) who proved experimentally that IVC has suppressing effects, in vivo and in vitro, on the production of pro-inflammatory cytokines. Another possible explanation is the effect of this combined (NTZ+IVC) regimen on oocyst clearance, mentioned above, leading to such improvement in the intestinal inflammatory changes. However, reports of Warren & Guerrant (2007) and Leitch & He (2012), have suggested that in *Cryptosporidium* infection, the histopathology of the small intestine does not fully correlate with the parasite load.

In fact, cell-mediated immune responses play a critical role in controlling cryptosporidiosis in both human and animal models. In addition, the balanced ratio between CD4 and CD8 immune cells (CD4/CD8) has been considered as an important marker for evaluating the immunomodulation status and response to the homeostasis of the intrinsic immune system (Dhur et al., 1991; Wang et al., 2017). Hence, this study intended to investigate the local (intestinal) expression of these 2 main subsets of the T lymphocyte populations in the infected mice.

The present immunohistochemical results demonstrated that the normal control group exhibited a higher expression of local CD4 T cells relative to CD8 cells. Similarly, Tauschmann et al. (2013) reported high CD4 and low CD8 T cell frequencies throughout the small and large intestine, which is important for immunomodulation in these regions.

In the present study, up-regulation of local ileal CD4 and CD8 was noticed in all diabetic groups compared to the normal group. These findings were in concordance with variable reports worked on T cells in type 1 diabetes (T1D), either chemically induced (Zhen et al., 2012) or due to autoimmune disorder (Rodriguez-Calvo et al., 2015).

Zhen et al. (2012) have investigated $CD4^+CD25^+$ T regulatory cells in mice with long-term STZ-induced diabetes and the results showed significant elevation of these cells in the peripheral lymphoid compartments, including peripheral and mesenteric lymph nodes, peripheral blood lymphocytes and the spleen. In the same context, a study done by Luo et al. (2007) indicated that the induced diabetic state following STZ administration causes rapid lymphopenia followed by homeostatic T cell proliferation. This may explain the relatively higher levels of both CD4 and CD8 in all diabetic groups compared to the healthy control. However, the infected non-treated control mice, in the present study, have demonstrated an overexpression of the local CD8 over CD4 cells leading to reversal of the balanced CD4/CD8 ratio, which again reverted to a normal ratio after therapy. Therefore, this overexpression of CD8 cells in the infected control group without improvement in either the parasite load or the pathological consequences supports the idea of inadequacy of CD8 cells in elimination of *Cryptosporidium* infection in murine models (Perryman et al., 1994; Abrahamsen et al., 1997; Tessema et al., 2009). For example, Tessema et al. (2009) reported that transferring both CD4 and CD8 T cells (pan T-cells) to naïve recipient mice doesn't confer better protection, against *C. parvum* than that of CD4 cells alone. In another study conducted on STZ-diabetic mice to investigate the role of CD8 cells in the removal of tumor cells, the authors concluded that CD8 (cytotoxic T lymphocytes) retained their proliferative capacity, yet exhibited diminished effector functions (Chen et al., 2014). On the contrary, these observations are in conflict with other studies which have demonstrated the importance of CD8 T cells in eradication of intestinal epithelial *Cryptosporidium* infection (Schaefer et al., 2000; Pantenburg et al., 2010).

As aforementioned, the diabetic treated groups in the present work have displayed a significant increase in the local expression of CD4 over CD8 cells thus inversing the ratio towards CD4, which has been considered essential for cure. The key role of CD4 cells in resistance to, and resolution of cryptosporidiosis has been investigated in previous animal and human studies (Borad & Ward, 2010; Checkley et al., 2015). For example, mice depleted with anti-CD4 antibody, have a reduced ability to resolve *C. parvum* infection (Chen et al., 1993). Furthermore, previous research showed that MHC-II (important for CD4 T cells) deficient mice are more susceptible to *C. parvum* infection than MHC-I (important for the function of CD8 T `cells) deficient mice (Aguirre et al., 1994). Moreover, numerous studies have reported resolution of *Cryptosporidium* infection after CD4 T cell reconstitution in AIDS patients given antiretroviral therapy, denoting the importance of such a cell population in the clearance of this opportunistic infection (Schmidt et al., 2004; O'Connor et al., 2011; Dillingham et al., 2011; Ahmadpour et al., 2020). In our earlier work on the dexamethasone immunocompromised mice model, CD4 expression in the treated animals was not elevated enough to the effective level that restores the balanced CD4/CD8 ratio (Fahmy et al., 2020)

The present work revealed that the highest level of CD4 expression among the treated mice was in the group which received combined regimen (NTZ+ IVC), whereas, the lowest level was in the single NTZ-treated group, thus suggesting the beneficial effect of the former therapy on the local cellular immune response. This data together with the results of oocyst excretion may signify the superiority of combined (NTZ+ IVC) regimen over the other forms of therapy. Aside from its anti-parasitic activity, IVC was reported in previous research to modulate the immune system, especially in conditions involving immunosuppression (Blakley & Rousseaux, 1991; López-Olvera et al., 2006). Blakley & Rousseaux (1991) have shown that the properties of IVC, as an immune stimulator in male CD-1 mice, are associated with altered function of T lymphocytes, particularly T-helper lymphocytes.

Conclusion

From the current work, it is concluded that NTZ and IVC combination regimen achieved the maximum reduction rate in the C*ryptosporidium* oocyst count, when compared to the other used regimens. In addition, it remarkably improved the intestinal histopathological features as well as the local cellular immune response. Therefore, the combination regimen appears to be promising in controlling cryptosporidiosis in the diabetic patient population. Further work is needed to better understand the immunomodulatory effect of IVC to determine the optimal strategy for its application in immunosuppressed conditions.

References

Abrahamsen MS, Lancto CA, Walcheck B, Layton W, Jutila MA. Localization of alpha/beta and gamma/delta T lymphocytes in *Cryptosporidium parvum*-infected tissues in naive and immune calves. *Infect Immun* 1997; 65(6): 2428-2433. http://dx.doi.org/10.1128/iai.65.6.2428-2433.1997. PMid:9169784.

Abu El Ezz NMT, Khalil FAM, Shaapan RM. Therapeutic effect of onion (*Allium cepa*) and cinnamon (*Cinnamomum zeylanicum*) oils on cryptosporidiosis in experimentally infected mice. *Glob Vet* 2011; 7(2): 179-183.

Abubakar I, Aliyu SH, Arumugam C, Usman NK, Hunter PR. Treatment of cryptosporidiosis in immunocompromised individuals: systematic review and meta-analysis. *Br J Clin Pharmacol* 2007; 63(4): 387-393. http://dx.doi.org/10.1111/j.1365-2125.2007.02873.x. PMid:17335543.

Aguirre SA, Mason PH, Perryman LE. Susceptibility of major histocompatibility (MHC) class I- and MHC class II-deficient mice to *Cryptosporidium parvum* infection. *Infect Immun* 1994; 62(2): 697-699. http://dx.doi.org/10.1128/iai.62.2.697-699.1994. PMid:7905464.

Ahmadpour E, Safarpour H, Xiao L, Zarean M, Hatam-Nahavandi K, Barac A, et al. Cryptosporidiosis in HIV-positive patients and related risk factors: a systematic review and meta-analysis. *Parasite* 2020; 27: 27. http://dx.doi.org/10.1051/parasite/2020025. PMid:32351207.

Alemu G, Jemal A, Zerdo Z. Intestinal parasitosis and associated factors among diabetic patients attending Arba Minch Hospital, Southern Ethiopia. *BMC Res Notes* 2018; 11(1): 689. http://dx.doi.org/10.1186/s13104-018-3791-x. PMid:30285833.

Al-Mathal EM, Alsalem MA. Pomegranate (*Punica granatum*) peel is effective in a murine model of experimental *Cryptosporidium parvum. Exp Parasitol* 2012; 131(3): 350-357. http://dx.doi.org/10.1016/j.exppara.2012.04.021. PMid:22580265.

Al-Warid HS, Al-Saqur IM, Mahmood SH. Histopathological changes in mice infected with *Cryptosporidium* spp. *Int J Pharma Bio Sci* 2013; 3(3): 220-227.

Amadi B, Mwiya M, Musuku J, Watuka A, Sianongo S, Ayoub A, et al. Effect of nitazoxanide on morbidity and mortality in Zambian children with cryptosporidiosis: a randomised controlled trial. *Lancet* 2002; 360(9343): 1375-1380. http://dx.doi.org/10.1016/S0140-6736(02)11401-2. PMid:12423984.

Benamrouz S, Guyot K, Gazzola S, Mouray A, Chassat T, Delaire B, et al. *Cryptosporidium parvum* infection in SCID Mice Infected with only one oocyst: qPCR Assessment of Parasite Replication in Tissues and Development of Digestive Cancer. *PLoS One* 2012; 7(12): e51232. http://dx.doi.org/10.1371/journal.pone.0051232. PMid:23272093.

Bhadauria D, Goel A, Kaul A, Sharma RK, Gupta A, Ruhela V, et al. *Cryptosporidium* infection after renal transplantation in an endemic area. *Transpl Infect Dis* 2015; 17(1): 48-55. http://dx.doi.org/10.1111/tid.12336. PMid:25620388.

Blakley BR, Rousseaux CG. Effect of ivermectin on the immune response in mice. *Am J Vet Res* 1991; 52(4): 593-595. PMid:1828942.

Borad A, Ward H. Human immune responses in cryptosporidiosis. *Future Microbiol* 2010; 5(3): 507-519. http://dx.doi.org/10.2217/fmb.09.128. PMid:20210556.

Cabada MM, White AC Jr. Treatment of cryptosporidiosis: do we know what we think we know? *Curr Opin Infect Dis* 2010; 23(5): 494-499. http://dx.doi.org/10.1097/QCO.0b013e32833de052. PMid:20689422.

Casqueiro J, Casqueiro J, Alves C. Infections in patients with diabetes mellitus: a review of pathogenesis. *Indian J Endocrinol Metab* 2012;16(7 Suppl. 1): S27-S36. http://dx.doi.org/10.4103/2230-8210.94253. PMid:22701840.

Cassol E, Malfeld S, Mahasha P, Bond R, Slavik T, Seebregts C, et al. Impaired CD4+ T-Cell restoration in the small versus large intestine of HIV-1–positive South Africans receiving combination antiretroviral therapy. *J Infect Dis* 2013; 208(7): 1113-1122. http://dx.doi.org/10.1093/infdis/jit249. PMid:23749968.

Checkley W, White AC Jr, Jaganath D, Arrowood MJ, Chalmers RM, Chen XM, et al. A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium. Lancet Infect Dis* 2015; 15(1): 85-94. http://dx.doi.org/10.1016/S1473-3099(14)70772-8. PMid:25278220.

Chen SC, Su YC, Lu YT, Ko PC, Chang PY, Lin HJ, et al. Defects in the acquisition of tumor-killing capability of $CD8^+$ cytotoxic T cells in streptozotocin-induced diabetic mice. *PLoS One* 2014; 9(11): e109961. http://dx.doi.org/10.1371/journal.pone.0109961. PMid:25390652.

Chen W, Harp JA, Harmsen AG. Requirements for CD4+ cells and gamma interferon in resolution of established *Cryptosporidium parvum* infection in mice. *Infect Immun* 1993; 61(9): 3928-3932. http://dx.doi.org/10.1128/iai.61.9.3928-3932.1993. PMid:8103040.

Chinen J, Shearer WT. Secondary immunodeficiencies, including HIV infection. *J Allergy Clin Immunol* 2010;125(2 Suppl. 2): S195-S203. http://dx.doi.org/10.1016/j.jaci.2009.08.040. PMid:20042227.

Desowitz RS, Barnwell JW. Effect of selenium and dimethyl dioctadecyl ammonium bromide on the vaccine-induced immunity of Swiss-Webster mice against malaria (*Plasmodium berghei*). *Infect Immun* 1980; 27(1): 87-89. http://dx.doi.org/10.1128/iai.27.1.87-89.1980. PMid:6987181.

Dhur A, Galan P, Preziosi P, Hercberg S. Lymphocyte subpopulations in the thymus, lymph nodes and spleen of iron-deficient and rehabilitated mice. *J Nutr* 1991; 121(9): 1418-1424. http://dx.doi.org/10.1093/jn/121.9.1418. PMid:1880620.

Dillingham R, Fitzgerald D, Eyma E, Miller E, Kashuba A, Dupnik K, et al. AIDS diarrhea and antiretroviral drug concentrations: A matched-pair cohort study in Port au Prince, Haiti. *Am J Trop Med Hyg* 2011; 84(6): 878-882. http://dx.doi.org/10.4269/ajtmh.2011.10-0541. PMid:21633022.

Drury RA, Wallington EA. *Carleton's histological technique*. 5th ed. Oxford: Oxford University Press; 1980.

Efstratiou A, Ongerth JE, Karanis P. Waterborne transmission of protozoan parasites: review of worldwide outbreaks—an update 2011-2016. *Water Res* 2017; 114: 14-22. http://dx.doi.org/10.1016/j.watres.2017.01.036. PMid:28214721.

Fahmy MEA, Abdelaal AA, Hassan SI, Shalaby MA, Ismail MAM, Khairy RA, et al. Potential immunomodulatory effect of single and combined therapies against *Cryptosporidium* infection in immunosuppressed mouse model. *J Egypt Soc Parasitol* 2020; 50(3): 673-682. http://dx.doi.org/10.21608/jesp.2020.131111.

Florescu DF, Sandkovsky U. *Cryptosporidium* infection in solid organ transplantation. *World J Transplant* 2016; 6(3): 460-471. http://dx.doi.org/10.5500/wjt.v6.i3.460. PMid:27683627.

Foudraine NA, Weverling GJ, van Gool T, Roos MT, de Wolf F, Koopmans PP, et al. Improvement of chronic diarrhoea in patients with advanced HIV-1 infection during potent antiretroviral therapy. *AIDS* 1998; 12(1): 35-41. http://dx.doi.org/10.1097/00002030-199801000-00005. PMid:9456253.

Garcia LS. *Diagnostic medical parasitology*. 4th ed. Washington, DC: ASM Press; 2001.

Gerace E, Lo Presti VD, Biondo C. *Cryptosporidium* Infection: epidemiology, pathogenesis, and differential diagnosis. *Eur J Microbiol Immunol* 2019; 9(4): 119-123. http://dx.doi.org/10.1556/1886.2019.00019. PMid:31934363.

Giacometti A, Cirioni O, Barchiesi F, Ancarani F, Scalise G. Activity of nitazoxanide alone and in combination with azithromycin and rifabutin against *Cryptosporidium parvum* in cell culture. *J Antimicrob Chemother* 2000; 45(4): 453-456. http://dx.doi.org/10.1093/jac/45.4.453. PMid:10747821.

Gill H, Walker G. Selenium, immune function and resistance to viral infections. *Nutr Diet* 2008; 65(3): S41-S47. http://dx.doi.org/10.1111/j.1747-0080.2008.00260.x.

Hassan SI, Nessim NG, Mahmoud SS, Nosseir MM. Effect of abroad spectrum antiparasitic drug "ivermectin" in acute and chronic experimental giardiasis using different dose regimens. *J Egypt Soc Parasitol* 2001; 31(2): 419-428. PMid:11478442.

Hunter PR, Nichols G. Epidemiology and clinical features of *Cryptosporidium* infection in immunocompromised patients. *Clin Microbiol Rev* 2002; 15(1): 145-154. http://dx.doi.org/10.1128/CMR.15.1.145-154.2002. PMid:11781272.

Khalil IA, Troeger C, Rao PC, Blacker BF, Brown A, Brewer TG, et al. Morbidity, mortality, and long-term consequences associated with diarrhoea from *Cryptosporidium* infection in children younger than 5 years: a meta-analyses study. *Lancet Glob Health* 2018; 6(7): e758-e768. http://dx.doi.org/10.1016/S2214-109X(18)30283-3. PMid:29903377.

Knapp S. Diabetes and infection: is there a link? A mini-review. *Gerontology* 2013; 59(2): 99-104. http://dx.doi.org/10.1159/000345107. PMid:23182884.

Krause I, Amir J, Cleper R, Dagan A, Behor J, Samra Z, et al. Cryptosporidiosis in children following solid organ transplantation. *Pediatr Infect Dis J* 2012; 31(11): 1135-1138. http://dx.doi.org/10.1097/INF.0b013e31826780f7. PMid:22810017.

Laing R, Gillan V, Devaney E. Ivermectin: old drug, new tricks? *Trends Parasitol* 2017; 33(6): 463-472. http://dx.doi.org/10.1016/j.pt.2017.02.004. PMid:28285851.

Laurent F, Lacroix-Lamandé S. Innate immune responses play a key role in controlling infection of the intestinal epithelium by *Cryptosporidium*. *Int J Parasitol* 2017; 47(12): 711-721. http://dx.doi.org/10.1016/j.ijpara.2017.08.001. PMid:28893638.

Leitch GJ, He Q. Cryptosporidiosis-an overview. *J Biomed Res* 2012; 25(1): 1-16. http://dx.doi.org/10.1016/S1674-8301(11)60001-8. PMid:22685452.

López-Olvera JR, Höfle U, Vicente J, Fernández-de-Mera IG, Gortázar C. Effects of parasitic helminths and ivermectin treatment on clinical parameters in the European wild boar (*Sus scrofa*). *Parasitol Res* 2006; 98(6): 582-587. http://dx.doi.org/10.1007/s00436-005-0099-2. PMid:16437240.

Love MS, Beasley FC, Jumani RS, Wright TM, Chatterjee AK, Huston CD, et al. A high- throughput phenotypic screen identifies clofazimine as a potential treatment for cryptosporidiosis. *PLoS Negl Trop Dis* 2017; 11(2): e0005373. http://dx.doi.org/10.1371/journal.pntd.0005373. PMid:28158186.

Luo B, Chan WFN, Lord SJ, Nanji SA, Rajotte RV, Shapiro AMJ, et al. Diabetes induces rapid suppression of adaptive immunity followed by homeostatic T-cell proliferation. *Scand J Immunol* 2007; 65(1): 22-31. http://dx.doi.org/10.1111/j.1365-3083.2006.01863.x. PMid:17212763.

Majeed W, Zafar M, Bhatti A, John P. Therapeutic potential of selenium nanoparticles. *J Nanomed Nanotechnol* 2018; 9(1): 1. http://dx.doi.org/10.4172/2157-7439.1000487.

Miao YM, Awad-EL-Kariem FM, Franzen C, Ellis DS, Müller A, Counihan HM, et al. Eradication of Cryptosporidia and Microsporidia following successful antiretroviral therapy. *J Acquir Immune Defic Syndr* 2000; 25(2): 124-129. http://dx.doi.org/10.1097/00126334-200010010-00006. PMid:11103042.

Moawad HSF, Hegab MH, Badawey MS, Ashoush SE, Ibrahim SM, Ali AA. Assessment of chitosan nanoparticles in improving the efficacy of nitazoxanide on cryptosporidiosis in immunosuppressed and immunocompetent murine models. *J Parasit Dis* 2021; 45(3): 606-619. http://dx.doi.org/10.1007/s12639-020-01337-y. PMid:34475640.

Mohtashamipour M, Ghaffari Hoseini SG, Pestehchian N, Yousefi H, Fallah E, Hazratian T. Intestinal parasitic infections in patients with Diabetes Mellitus: A case-control study. *J Anal Res Clin Med* 2015; 3(3): 157-163. http://dx.doi.org/10.15171/jarcm.2015.025.

Momekov G, Momekova D. Ivermectin as a potential COVID-19 treatment from the pharmacokinetic point of view: antiviral levels are not likely attainable with known dosing regimens. *Biotechnol Biotechnol Equip* 2020; 34(1): 469-474. http://dx.doi.org/10.1080/13102818.2020.1775118.

O'Connor RM, Shaffie R, Kang G, Ward HD. Cryptosporidiosis in patients with HIV/AIDS. *AIDS* 2011; 25(5): 549-560. http://dx.doi.org/10.1097/QAD.0b013e3283437e88. PMid:21160413.

Omer MO, Ashraf M, Javeed A, Maqbool A. Immunostimulatory effect of ivermectin on macrophage engulfment and delayed type hypersensitivity in broilers. *J Anim Plant Sci* [online]. 2012 [cited 2021 June 31]; 22(2): 250-255. Available from: http://www.thejaps.org.pk/docs/v-22-2/01.pdf

Operario DJ, Liotta J, Nydam DV, Bristol LS, Houpt ER. Correlation between diarrhea severity and oocyst count via quantitative PCR or fluorescence microscopy in experimental cryptosporidiosis in calves. *Am J Trop Med Hyg* 2015; 92(1): 45-49. http://dx.doi.org/10.4269/ajtmh.14-0488. PMid:25371182.

Pantenburg B, Ward HD, Dann SM, Connelly RL, Castellanos-Gonzalez A, Clinton White A, et al. Human $CD8^+$ T cells clear *Cryptosporidium parvum* from infected intestinal epithelial cells. *Am J Trop Med Hyg* 2010; 82(4): 600-607. http://dx.doi.org/10.4269/ajtmh.2010.09-0590. PMid:20348507.

Perryman LE, Mason PH, Chrisp CE. Effect of spleen cell populations on resolution of *Cryptosporidium parvum* infection in SCID mice. *Infect Immun* 1994; 62(4): 1474-1477. http://dx.doi.org/10.1128/iai.62.4.1474-1477.1994. PMid:7907581.

Ramos-Vara JA, Miller MA. When Tissue Antigens and Antibodies Get Along: Revisiting the Technical Aspects of Immunohistochemistry: the Red, Brown, and Blue Technique. *Vet Pathol* 2014; 51(1): 42-87. http://dx.doi.org/10.1177/0300985813505879. PMid:24129895.

Rodriguez-Calvo T, Suwandi JS, Amirian N, Zapardiel-Gonzalo J, Anquetil F, Sabouri S, et al. Heterogeneity and lobularity of pancreatic pathology in Type 1 diabetes during the prediabetic phase. *J Histochem Cytochem* 2015; 63(8): 626-636. http://dx.doi.org/10.1369/0022155415576543. PMid:26216138.

Roman M, Jitaru P, Barbante C. Selenium biochemistry and its role for human health. *Metallomics* 2014; 6(1): 25-54. http://dx.doi.org/10.1039/C3MT00185G. PMid:24185753.

Ryan U, Hijjawi N, Xiao L. Foodborne cryptosporidiosis. *Int J Parasitol* 2018; 48(1): 1-12. http://dx.doi.org/10.1016/j.ijpara.2017.09.004. PMid:29122606.

Sadek G, El-Aswad B. Role of COX-2 in pathogenesis of intestinal cryptosporidiosis and effect of some drugs on treatment of infection. *Res J Parasitol* 2014; 9(2): 21-40. http://dx.doi.org/10.3923/jp.2014.21.40.

Sajid MS, Iqbal Z, Muhammad G, Sandhu MA, Khan MN, Saqib M, et al. Effect of ivermectin on the cellular and humoral immune responses of rabbits. *Life Sci* 2007; 80(21): 1966-1970. http://dx.doi.org/10.1016/j.lfs.2007.02.025. PMid:17379254.

Schaefer DA, Auerbach-Dixon BA, Riggs MW. Characterization and formulation of multiple epitope-specific neutralizing monoclonal antibodies for passive immunization against cryptosporidiosis. *Infect Immun* 2000; 68(5): 2608-2616. http://dx.doi.org/10.1128/IAI.68.5.2608-2616.2000. PMid:10768951.

Schmidt B, Scott I, Whitmore RG, Foster H, Fujimura S, Schmitz J, et al. Low-level HIV infection of plasmacytoid dendritic cells: onset of cytopathic effects and cell death after PDC maturation. *Virology* 2004; 329(2): 280-288. http://dx.doi.org/10.1016/j.virol.2004.08.016. PMid:15518808.

Silva MTA, Silva-Jardim I, Thiemann OH. Biological implications of selenium and its role in trypanosomiasis treatment. *Curr Med Chem* 2014; 21(15): 1772-1780. http://dx.doi.org/10.2174/09298673206613119121108. PMid:24251578.

Smit MR, Ochomo EO, Aljayyoussi G, Kwambai TK, Abong'o BO, Chen T, et al. Safety and mosquitocidal efficacy of high-dose ivermectin when co-administered with dihydroartemisinin-piperaquine in Kenyan adults with uncomplicated malaria (IVERMAL): a randomised, double-blind, placebo-controlled trial. *Lancet Infect Dis* 2018; 18(6): 615-626. http://dx.doi.org/10.1016/S1473-3099(18)30163-4. PMid:29602751.

Soflaei S, Dalimi A, Abdoli A, Kamali M, Nasiri V, Shakibaie M, et al. Anti-leishmanial activities of selenium nanoparticles and selenium dioxide on *Leishmania infantum. Comp Clin Pathol* 2014; 23(1): 15-20. http://dx.doi.org/10.1007/s00580-012-1561-z.

Sparks H, Nair G, Castellanos-Gonzalez A, White JAC Jr. Treatment of *Cryptosporidium:* what we know, gaps, and the way forward. *Curr Trop Med Rep* 2015; 2(3): 181-187. http://dx.doi.org/10.1007/s40475-015-0056-9. PMid:26568906.

Taha NM, Yousof HA, El-Sayed SH, Younis AI, Negm MS. Atorvastatin repurposing for the treatment of cryptosporidiosis in experimentally immunosuppressed mice. *Exp Parasitol* 2017; 181: 57-69. http://dx.doi.org/10.1016/j.exppara.2017.07.010. PMid:28764965.

Tanaka Y. Immunosuppressive mechanisms in diabetes mellitus. *Nihon Rinsho* 2008; 66(12): 2233-2237. PMid:19069085.

Tangi FB, Fokam EB, Longdoh NA, Eteneneng EJ. Intestinal parasites in diabetes mellitus patients in the Limbe and Buea municipalities, Cameroon. *Diabetes Res Open J* 2016; 2(1): 1-7. http://dx.doi.org/10.17140/DROJ-2-123.

Tauschmann M, Prietl B, Treiber G, Gorkiewicz G, Kump P, Högenauer C, et al. Distribution of $CD4^{pos}$ -, $CD8^{pos}$ – and regulatory T Cells in the upper and lower gastrointestinal tract in healthy young subjects. *PLoS One* 2013; 8(11): e80362. http://dx.doi.org/10.1371/journal.pone.0080362. PMid:24265815.

Tessema TS, Dauber E, Petry F. Adoptive transfer of protective immunity from *Cryptosporidium parvum*-infected interferon- γand interleukin-12-deficient mice to naive recipients. *Vaccine* 2009; 27(47): 6575-6581. http://dx.doi.org/10.1016/j.vaccine.2009.08.036. PMid:19717136.

Theodos CM, Griffiths JK, D'Onfro J, Fairfield A, Tzipori S. Efficacy of nitazoxanide against *Cryptosporidium parvum* in cell culture and in animal models. *Antimicrob Agents Chemother* 1998; 42(8): 1959-1965. http://dx.doi.org/10.1128/AAC.42.8.1959. PMid:9687390.

Tinarwo P, Zewotir T, North D. Trends and Adaptive Optimal Set Points of CD4+ Count Clinical Covariates at Each Phase of the HIV Disease Progression. *Aids Res Treat* 2020; 2020: 1379676. http://dx.doi.org/10.1155/2020/1379676. PMid:32190387.

von Oettingen J, Nath-Chowdhury M, Ward BJ, Rodloff AC, Arrowood MJ, Ndao M. High-yield amplification of *Cryptosporidium parvum* in interferon gamma receptor knockout mice.. *Parasitology* 2008; 135(10): 1151-1156. http://dx.doi.org/10.1017/S0031182008004757. PMid:18667105.

Wang C, Wu Y, Qin J, Sun H, He H. Induced susceptibility of host is associated with an impaired antioxidant system following infection with *Cryptosporidium parvum* in Se-deficient mice. *PLoS One* 2009; 4(2): e4628. http://dx.doi.org/10.1371/journal.pone.0004628. PMid:19247447.

Wang Z, Wang Y, Xu B, Liu J, Ren Y, Dai Z, et al. Vitamin D improves immune function in immunosuppressant mice induced by glucocorticoid. *Biomed Rep* 2017; 6(1): 120-124. http://dx.doi.org/10.3892/br.2016.817. PMid:28123720.

Wang ZD, Liu Q, Liu HH, Li S, Zhang L, Zhao YK, et al. Prevalence of *Cryptosporidium*, microsporidia and *Isospora* infection in HIV-infected people: a global systematic review and meta-analysis. *Parasit Vectors* 2018; 11(1): 28. http://dx.doi.org/10.1186/s13071-017-2558-x. PMid:29316950.

Warren CA, Guerrant RL. Clinical disease and pathology. In: Fayer R, Xiao L, editors. *Cryptosporidium and cryptosporidiosis*. 2nd ed. Boca Raton: CRC Press; 2007. p. 235-254. http://dx.doi.org/10.1201/9781420052275-8.

Waters WR, Harp JA. *Cryptosporidium parvum* infection in T-cell receptor (TCR)-alpha- and TCR-delta-deficient mice. *Infect Immun* 1996; 64(5): 1854-1857. http://dx.doi.org/10.1128/iai.64.5.1854-1857.1996. PMid:8613403.

Widmer G, Carmena D, Kváč M, Chalmers RM, Kissinger JC, Xiao L, et al. Update on *Cryptosporidium* spp.: highlights from the Seventh International *Giardia* and *Cryptosporidium* Conference. *Parasite* 2020; 27: 14. http://dx.doi.org/10.1051/parasite/2020011. PMid:32167464.

Wu KK, Huan Y. Streptozotocin-induced diabetic models in mice and rats. *Curr Protocols Pharmacol* 2008; 40: 5.47.1-5.47.14. http://dx.doi.org/10.1002/0471141755.ph0547s40. PMid:22294227.

Youssef MY, Essa MM, Sadaka HA, Eissa MM, Rizk AM. Effect of ivermectin on combined intestinal protozoal infection (Giardiasis and Cryptosporidiosis*). J Egypt Soc Parasitol* 1996; 26(3): 543-553. PMid:8918027.

Zeibig EA. *Clinical parasitology.* Philadelphia: W.B. Saunders Company; 1997.

Zhang X, Song Y, Ci X, An N, Ju Y, Li H, et al. Ivermectin inhibits LPS-induced production of inflammatory cytokines and improves LPS-induced survival in mice. *Inflamm Res* 2008; 57(11): 524-529. http://dx.doi.org/10.1007/s00011-008-8007-8. PMid:19109745.

Zhen Y, Sun L, Liu H, Duan K, Zeng C, Zhang L, et al. Alterations of peripheral CD4+CD25+Foxp3+ T regulatory cells in mice with STZ-induced diabetes. *Cell Mol Immunol* 2012; 9(1): 75-85. http://dx.doi.org/10.1038/cmi.2011.37. PMid:21983870.

Zinada NY. The effect of ivermectin on *Cryptosporidium parvum* in experimentally infected rat. *J Egypt Soc Parasitol* 2000; 30(3): 747-752. PMid:11198372.