Original Article

Evaluation of potential prophylactic and therapeutic effect of azoximer bromide (polyoxidonium) on experimental cryptosporidiosis in immunocompromised mice

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ABSTRACT

Background: Considering the broad burden of cryptosporidiosis, there is still a limited choice of curative treatments. Nitazoxanide (NTZ) is the only anti-cryptosporidial agent currently available. Unfortunately, it showed low efficacy in children and AIDS patients. Accordingly, supplementation with immune-stimulation drugs is feasible.

Objective: To demonstrate the prophylactic immunomodulating effect of the immunostimulant Azoximer Bromide (AZB) and evaluate its potential therapeutic efficacy when combined with NTZ, for treatment of cryptosporidiosis in experimentally immunosuppressed mice.

Material and Methods: Ninety laboratory bred Swiss albino male mice were immunosuppressed and divided into three groups (30 mice each): control group (GI); prophylactic group, AZB treated then infected (GII); therapeutic group, oocysts infected then treated (GIII). Each group was divided equally into 3 sub-groups (10 mice each). Controls included: GIa, non-infected control negative; GIb, oocysts infected control positive; GIc, non-infected AZB treated drug control. Prophylactic subgroups included: GIIa, received AZB booster injection; GIIb, NTZ treated; GIIc, AZB+ NTZ treated. Therapeutic subgroups included: GIIIa, AZB treated; GIIIb, NTZ treated; GIIIc, AZB+NTZ treated. Oocysts shedding and the efficacy percentage of each drug were calculated. Other parameters used included histopathological examination and immunohistochemical assessment of small intestine and lung tissues, and serum analyses for biochemical, immunological and antioxidants evaluations.

Results: The prophylactic effect of AZB alone and its therapeutic effect when combined with NTZ gave the best reduction rate of oocyst shedding with marked improvement in histopathological features, and significantly reduced hepatic enzymes. Additionally, AZB enhanced the mice immunogenicity with significant upregulation of interleukin (IL)-1β, IL-6, tumor necrotic factor (TNF)-α and interferon (INF)-γ; overexpression of CD3 protein in pulmonary tissue, and significant elevation of antioxidant activity.

Conclusion: A powerful effect was achieved by AZB when administered with NTZ for treatment of experimental cryptosporidiosis with elicited high immune response of immunosuppressed mice.

Keywords: azoximer bromide; *Cryptosporidium*; immunostimulant; immunosuppressed; nitazoxanide; oxidative stress; prophylaxis.

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INTRODUCTION

Cryptosporidium spp. are common widely distributed, zoonotic protozoons that affect various livestock, and human[1]. Being an opportunistic parasite, it affects a wide range of immunosuppressed patients[2]. *Cryptosporidium* spp. oocysts passed in stools of infected humans or animals may subsequently contaminate the soil and water sources[3]. A low dose of 10–100 oocysts can transmit the infection[4]. Cryptosporidiosis causes prolonged episodes of severe diarrhea and is the second major cause of infant's death in Africa and South Asia[5]. The extent of pathology associated with cryptosporidiosis largely depends on the individual's immune status. In immunocompetent persons, the infection is usually limited to the small intestine, while in immunocompromised persons, it develops into severe, life-threatening gastrointestinal and disseminated cryptosporidiosis in other organ systems such as pancreatic, biliary, and respiratory tracts[6].

It was shown that the immunological control of cryptosporidiosis in mice depends on $CD3^+$/$CD4^+$ lymphocytes for recovery from the infection[7]. Upon antigen stimulation, $CD4^+$ T cells play an important role via secretion of abundant cytokines such as IL-2, IFN-α, IFN-γ secreted by Th1 cells, and IL-4, IL-6 secreted by Th2 cells. Notably, IFN-γ resists the invasion of *Cryptosporidium* pathogens

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and plays an important role in both innate and adaptive immune responses[8]. In the acute phase of infection, *Cryptosporidium* spp. sporozoites induce the production of IL-12 by macrophages and dendritic cells[9] that acts synergistically with IL-18 and TNF-α to activate natural killer (NK) cells[10]. In addition, TNF-α prevents the establishment of *Cryptosporidium* spp. infection in enterocytes[11]. Besides, other pro-inflammatory cytokines (IL-1, IL-6) released by multiple immunocompetent cells exert protective effect[12]. Adequate T helper cell responses are critical for hosts to orchestrate enough defensive mechanisms for infection control. This suggests a major role for host immune factors in controlling cryptosporidiosis[13].

Oxidative stress induced by *Cryptosporidium* spp. was reported to cause tissue damage in mice[14] and pigs[15]. So, decreasing oxidative stress allows the host to sustain a viable immune assemblage able to eradicate the pathogen and reduce host tissue damage[16]. Alterations in total antioxidant capacity (TAC) and malondialdehyde (MDA) concentration are valuable biomarkers to evaluate oxidative stress[17].

Currently approved therapeutics, NTZ and paromomycin, have limited activity in immunocompromised individuals[18]. Several drugs and drug combinations such as rifaximin and azithromycin were also investigated against cryptosporidiosis, with unsatisfactory results[19]. Limited treatment options create an urgent need for the development of new anti-parasitic drugs. For this purpose the additional use of non-specific immunostimulator drugs is a reasonable option to strengthen the body's resistance to parasitic infection[20].

Azoximer bromide (Polyoxidonium®) is a physiologically active compound from a new class of heterochain aliphatic polyamines. It is a high-molecular-weight synthetic immune modulator drug that increases the resistance to local and general infection and is indicated for the treatment of viral infections[21]. It is worth mentioning that AZB is approved in Russia as a vaccine adjuvant drug that stimulates antibody production. According to an analysis of about 50 million recipients, AZB complexed with antigen in a commercial influenza vaccine demonstrated high safety[22]. Furthermore, it is used in various conditions that include bronchial asthma[23], chronic recurrent herpes simplex infections[24], pneumonia[25], pyelonephritis[26], recurrent urogenital chlamydial infections[27] and atopic dermatitis[28]. The professed immunomodulatory action of AZB elicits or amplifies an immune response in immunosuppressed patients[29], and is acknowledged as an immune modulator for the treatment of parasitic diseases[21]. Difficulties in controlling unfavorable consequences of cryptosporidial diarrhea in immunodeficient individuals prompted us to consider the probable protective and/or curative value of AZB versus NTZ. Using AZB may prohibit the fulminant outcomes and/or ameliorate the immune response in *Cryptosporidium* infected immunocompromised hosts.

Our present work aimed to evaluate the prophylactic and therapeutic efficacy of immunostimulant AZB combined with NTZ, and their dual role in experimentally immunosuppressed mice exposed to cryptosporidiosis.

MATERIAL AND METHODS

This experimental case-control study began in April 2019 and was completed in May 2020. It was conducted at the Laboratories of the Medical Parasitology and Pathology Departments, Faculty of Medicine, Menoufia University.

Experimental animals: This study was performed on 90 Swiss albino male laboratory-bred mice weighing 20±3 gm. Mice were obtained from *Schistosoma* Biological Supply Program (SBSP), Theodor Bilharze Research Institute (TBRI), Giza, Egypt and kept under standard housing conditions in the animal house of TBRI. The mice were allowed to adapt to the experimental conditions for 10 days before *Cryptosporidium* infection. Mice were kept in separate cages under optimum conditions[30]. Private laboratory mouse pellets as food and water were also readily accessible. During this period, stool examination of all mice was conducted to ensure that they were parasites free.

Study design: Ninety mice were immunosuppressed for 14 days and then classified into three main groups (I, II, III). Each group was divided equally into three subgroups (a, b, c), consisting of 10 mice each (Table 1). All the survived mice were sacrificed on the 30th day post infection (dpi) by cervical dislocation[32]. The effect of the drugs on murine cryptosporidiosis was evaluated by parasitological, histopathological, immunohistochemical, and immunological examinations.

Mice immunosuppression: All mice were immunosuppressed by oral administration of synthetic corticosteroid (Dexazone tablets 0.5 mg, Al Kahira Pharmaceutical, and Chemical Industries Company, Egypt) at a dose of 25 µg/gm body weight/d for 14 successive days before oral inoculation with *Cryptosporidium* spp. oocysts[33,34].

Mice infection: *Cryptosporidium* oocysts collected from the feces of naturally infected calves[35] were identified by Modified Zeihl Neelsen (MZN) staining[36]. Oocysts were concentrated by floatation in Sheather's sugar solution and the sediment was collected and stored in a 2.5% potassium dichromate solution at 4°C[37]. Before infection, oocysts were concentrated and counted in

Table 1. Study groups and subgroups.

Groups and subgroups	Characteristics
Group I (Control)	30 immunosuppressed mice.
Subgroup Ia (Negative control)	Non infected, injected by phosphate buffer saline (PBS).
Subgroup Ib (Positive control)	Infected by *Cryptosporidium* oocysts and not treated.
Subgroup Ic (Drug control)	Non infected, injected twice intramuscularly with AZB with an interval of 48 hrs[31].
Group II (Prophylactic)	30 immunosuppressed mice, injected twice with AZB (interval of 48 hrs), then infected with *Cryptosporidium* oocysts.
Subgroup IIa	Injected IM with a booster dose of AZB on the 15th dpi.
Subgroup IIb	Received NTZ orally on the 15th dpi.
Subgroup IIc	Received both AZB and NTZ on the 15th dpi.
Group III (Therapeutic)	30 immunosuppressed mice, infected with *Cryptosporidium* oocysts.
Subgroup IIIa	Injected IM with AZB on the 15th dpi.
Subgroup IIIb	Received NTZ orally on the 15th dpi.
Subgroup IIIc	Received both AZB and NTZ on the 15th dpi.

PBS solution using a hemocytometer. The mice were infected intra-esophageally with 3000- 3500 oocysts using a tuberculin syringe[38]. All groups were observed daily for recording the mortality rate throughout the experimental period.

Drug regimen: NTZ was supplied as "Cryptonaz®" 60 ml suspension of 100 mg/5 ml by Copad Pharma (Egypt for Trade and Pharmaceutical industries, Obour City, Cairo, Egypt). It was given orally to mice of subgroups II b, II c, III b, and III c at a dose of 500 mg/kg twice daily starting on the 15th dpi for five consecutive days[32]. The dose was calculated according to the Paget and Barnes table[33]. AZB was supplied as "Polyoxidonium®" 6 mg lyophilisate for preparation of solution for injection by Cosmic Nootropic Russien company. It was given to the prophylactic groups by IM injection twice in a dose of 0.004 mg/mouse in 0.2 ml 0.9% NaCl with an interval of 48 h, and given to therapeutic groups (subgroup IIIa and IIIc) in the same dose starting on the 15th dpi[31].

Parasitological evaluation of the infection: On the last day of the experiment (30th dpi) fresh fecal pellets were collected from each mouse separately and labeled individually for oocyst count and examined by the MZN staining method to calculate *Cryptosporidium* spp. oocysts shedding[32]. The smears were examined using a laboratory microscope and examined by x40 and x100 objectives. The number of *Cryptosporidium* spp. oocysts was counted and estimated as the mean in 10 high power fields (HPFs)[33]. The reduction percent of each drug was calculated using the equation: Efficacy (%) = [(mean value of infected untreated group (G b)- mean value of all infected treated groups (either prophylactic or therapeutic)/mean value of infected untreated group] x100[39].

Histopathological examination: The terminal one cm of the ileum and the whole lung tissue were taken from each mouse and fixed in 10% neutral formalin. Ileum and lung tissues were embedded in paraffin, sectioned, mounted on glass slides, immersed in xylene, then dehydrated in graded alcohol solutions and stained by Hematoxylin and Eosin (HE)[40]. Stained sections were assessed regarding any pathological changes in the tissues, degree of inflammation, and severity of infection.

Immunohistochemical staining of CD3 in lung tissues and inducible nitric oxide synthetase (iNOS) in the ileal tissues: Immunoassay of the formalin fixed ileal and lung tissue was performed using the streptavidin-biotin-amplified system. The primary antibodies used were mouse polyclonal anti-CD3 (diluted as 1:100) and anti-iNOS antibody (dilution 1: 50; Santa Cruz Biotechnology, USA). After deparaffinization and rehydration of tissue, the slides were incubated overnight at 4°C with primary antibodies. Secondary antibody using Ultravision detection system anti-polyvalent HRP/DAB, was utilized. Neomarker was applied, and staining was visualized using DAB chromogen substrate and Mayer's hematoxylin as a counterstain. Positive and negative controls were used in each run[41].

Positive cells for iNOS and CD3 showed brownish cytoplasmic staining that was semi-quantitatively scored using binocular Olympus light microscope at high magnification (×40). Based on assessment of the proportion of expressed positive cells as (0-100%), they were scaled as follows: 0 (< 10% positive cells), +1 (10-40% positive cells), +2 (40-70% positive cells), +3 (>70% positive cells)[42]. Staining intensity was graded as follows: 0 (no staining), +1: weak, +2: moderate and +3: strong[43]. Each antibody was assessed using histoscore (H score) as strong intensity: 3x percentage, moderate intensity: 2x percentage, weak intensity: 1x percentage, negative staining: 0x percentage. The final score ranged from 0-300[44].

Measurement of serum biochemical parameters: The serum was separated and stored at -20ºC. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using commercially available spectrophotometric diagnostic kits (Sigma-Aldrich) according to the manufacturer's instructions[45].

Quantitative estimation of pro-inflammatory cytokines by sandwich ELISA: This was performed

by validated manual microplate ELISA kits following the manufacturer instructions. The kits were supplied from Affymetrix e Bioscience co., Catalog N. BMS6002/ BMS6002TWO/BMS6002TEN for IL-1β; Affymetrix e Bioscience co., Catalog N. BMS603/2/BMS603/2TWO/ BMS603/2TEN for IL-6; RnDsystems co., Catalog N. MTA00B/SMTA00B/PMTA00B for TNF-α; Invitrogen, Thermofisher, USA co., Catalog N KMC4021 for IFN-γ. According to standards' concentration and the corresponding optical density (OD=450nm) values, the standard curve linear regression equation was calculated and the OD values of the sample were applied to the regression equation to calculate the corresponding sample's concentration.

Determination of oxidative stress markers: Superoxide dismutase (SOD) activity was estimated according to the method described by Misra and Fridovich[46]. This method is based on the ability of SOD to inhibit the autoxidation of epinephrine to adrenochrome in an alkaline medium (pH 10.2). Biochemical parameters were performed using a spectrophotometer (Jasco-V530). Serum level of MDA was determined according to the modified method of Ratty and Das[47], based on thiobarbituric acid reaction with MDA. The standard absorption curve for MDA quantification was prepared using 1,1,3,3-tetraethoxypropane and the values were expressed as mmol/l.

Ethical consideration: All procedures related to animal experimentation met the International Guiding Principles for Biomedical Research Involving Animals as issued by the International Organization of Medicine.

Statistical method: Results were tabulated, and statistically analyzed by IBM personal computer and Statistical Package for Social Sciences (SPSS) software, version 22 (Armonk, NY: IBM Corp, 2013). They were expressed in number (No.), percentage (%), mean (x) and standard deviation (SD). After testing homogeneity of data, ANOVA (one-way analysis of variance) test was used for normally quantitative variables to compare between more than two studied groups; followed by Tamhane's test as a post hoc test. Chi-square test (χ^2): was used to study the association between qualitative variables. *P*-value of < 0.05 was considered statistically significant.

RESULTS

Mortality rate: At the end of the study, the mortality rate in mice was 30% in GIb (infected untreated control positive), 20% in GIIIa (treated by AZB), 10% in GIIa (prophylaxis by AZB), and 10% in GIIIb (treated by NTZ). The mortality rate was 0% in the other prophylactic subgroups (GIIb, and GIIc) and therapeutic GIIIc (received combined treatment) (Figure 1A).

Oocyst shedding: In comparison with G1b (infected untreated control positive; 8008.571±7.48), the lowest mean number of oocyst shedding in mice stool (310±7.45) was observed in the prophylactic subgroup GIIc (received AZB+NTZ). In descending order, reduction was recorded in the therapeutic group GIIIc (received AZB+NTZ) with a mean of 1508±7.15, then therapeutic group GIIIb (NTZ treated) with a mean of 2007.78±5.65. In the therapeutic GIIIa (AZB treated) mean number of oocyst shedding (4508.13±5.94) was not as low as in the other groups. The difference was statistically significant (*P*>0.001) in the four groups (Table 2).

The reduction percent of oocysts was 96.13%, 89.89%, 81.17%, 62.45% in GIIc, GII, GIIIc, GIIa, respectively, while the lowest reduction percent of oocyst was 43.71% in the therapeutic subgroup GIIIa (Figure 1B). The onset of oocyst shedding in stool, started on the 2nd day PI in immunosuppressed mice. Oocysts were evident in large numbers in the stools of the infected untreated control positive (GIb) (Figure 1C).

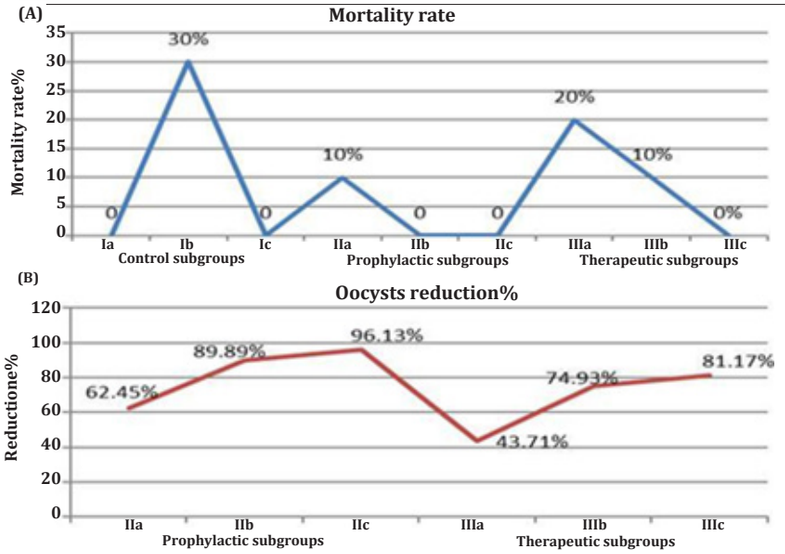
Histopathological examination

Intestine: Histopathological examination of ileal sections from control negative GIa (immunosuppressed non-infected mice) showed normal structure with average length and width of villi without any pathological changes in the mucosa or the lamina propria (Figure 2A) while, control positive GIb (immunosuppressed infected) showed loss of villous architecture, shortening, broadening of the villi and even villous atrophy. Additionally, there were mucosal ulcerations, edema, low-grade dysplasia, and goblet cells depletion in the covering epithelium and inflammatory cellular infiltrate observed mainly in the core of the villi and extending into the submucosa. *Cryptosporidium*

Table 2. Comparison between mean oocyst shedding in the studied groups on last day of experiment (30th day).

Study subgroups	Mean± SD	ANOVA test	*P* value	Post Hoc test
Ib (No. = 7)	8008.57±7.48			
IIa (No. = 10)	3007.00±5.38			***P*1:** <0.001, ***P*2:** <0.001
IIb (No. = 10)	810.00±7.45			***P*3:** <0.001, ***P*4:** <0.001
IIc (No. = 10)	310.00±7.45	1157381.32	<0.001	***P*5:** <0.001, ***P*6:** <0.001
IIIa (No. = 8)	4508.13±5.94			***P*7:** <0.001, ***P*8:** <0.001
IIIb (No. = 9)	2007.78±5.65			***P*9:** <0.001
IIIc (No. = 10)	1508.00±7.15			

***P*1:** IIa vs IIIa; ***P*2:** IIa vs IIIb; ***P*3:** IIa vs IIIc; ***P*4:** IIb vs IIIa; ***P*5:** IIb vs IIIb; ***P*6:** IIb vs IIIc; ***P*7:** IIc vs IIIa; ***P*8:** IIc vs IIIb; ***P*9:** IIc vs IIIc.



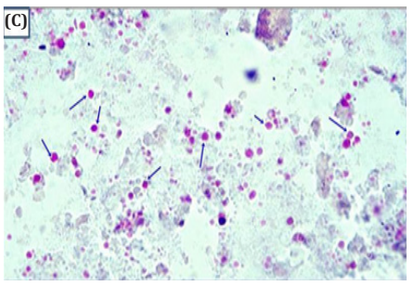


Fig. 1. Comparison between mortality rates (A) and mean oocysts reduction percent (B) in all studied groups on the last day of the experiment (30th dpi). (C) *Cryptosporidium* oocysts in stool sample of an immunocompromised mouse (MZN, X1000).

oocysts appeared along the brush border of the villi and in the intestinal lumen as rounded to oval, purple-stained bodies (Figure 2 B-C).

Improvement of ileal tissue sections appeared in groups GIIc and GIIIc (received combined treatment with AZB and NTZ in both prophylactic and therapeutic groups), in the form of mild inflammation with intact mucosa, intestinal villi, and crypts. Additionally in these two groups *Cryptosporidium* endogenous developmental stages were hardly detected, and the epithelial cells showed preserved polarity, and were devoid of any cytological atypia (Figure 2F). While GIIa, GIIb, and GIIIb showed partial improvement in the histopathological changes with mild to moderate inflammatory infiltrates in the core of villi with intact mucosa and mild blunting, shortening and ulceration (Figure 2D-E).

Lung: Histopathological examination of lung sections from control negative GIa mice (immunosuppressed non-infected) showed normal alveoli (Figure 3A). Sections from control positive GIb (immunosuppressed infected mice) revealed distorted and dilated airspaces with hyper-distension of alveolar spaces associated with rupture of alveolar septa and severe inflammation (HE stain ×200) (Figure 3B). These changes improved with treatment in both prophylactic and therapeutic combined AZB+NTZ treatments, (GIIc and GIIIc), that showed the best results with mild inflammation and normal alveolar spaces (Figure 3 C-F).

Immunohistochemical staining of CD3 in lung tissue: The highest expression of the CD3 protein in lymphocytes infiltrates was detected in the pulmonary tissue of the groups GIIc and GIIIc that received combined AZB and NTZ treatment in both prophylactic

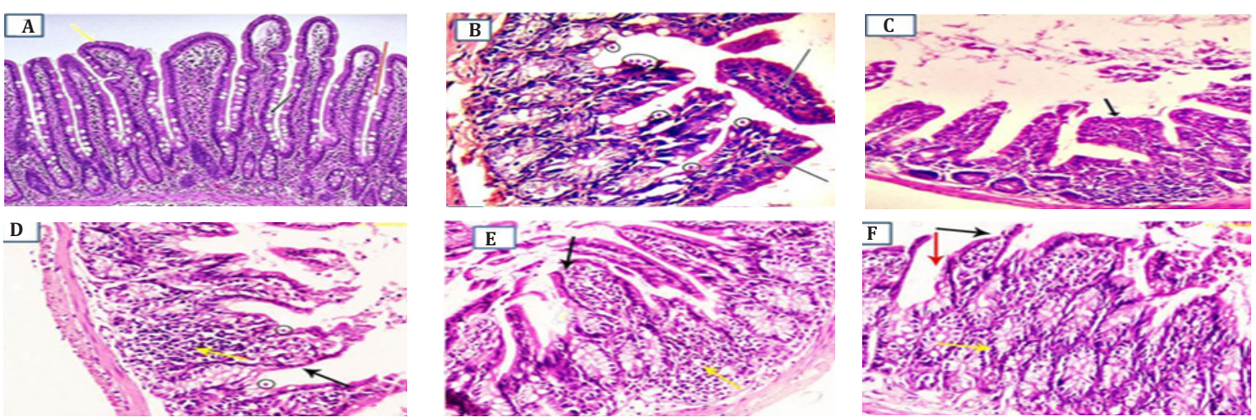
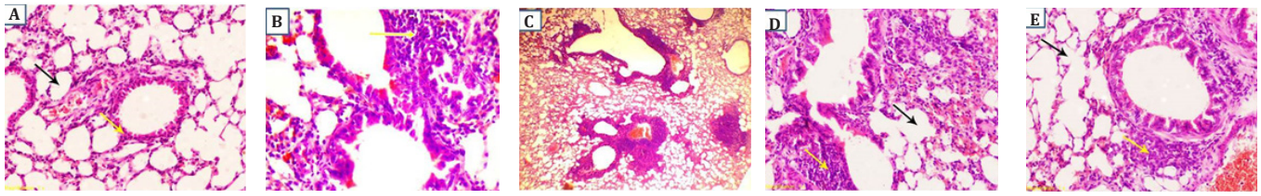


Fig. 2. Cross sections of ileum from studied groups: **(A)** GIa (control negative) showing normal villous architecture (yellow arrow) with preserved goblet cells and normal crypts. **(B)** GIb (control positive) showing inflammatory cellular infiltrate observed mainly in the core of the villi and extending into the submucosa (black arrow), edema, and destruction of intestinal villi with depletion of goblet cells. The endogenous developmental stages of *Cryptosporidium* are highlighted with circles on or in the lining epithelium; and **(C)** showing high-grade dysplasia manifested by great pleomorphism, absence of mucin, and frequent mitosis (arrow). **(D)** GIIa (prophylactic AZB treated) showing moderate inflammation with inflammatory infiltrate in the core of villi (yellow arrow), moderate edema, and moderate blunting and shortening of villi (black arrow). Few endogenous developmental stages of *Cryptosporidium* are highlighted with circles. **(E)** GIIb (prophylactic NTZ treated) and GIIIb (therapeutic NTZ treated) showing, mild to moderate inflammation with inflammatory infiltrates in the core of villi (yellow arrow) with intact mucosa, mild blunting and shortening of villi (black arrow) **(F)** GIIc (prophylactic AZB + NTZ treated) and GIIIc (therapeutic AZB +NTZ treated) showing, mild inflammation in the core of villi (yellow arrow) with intact mucosa, intestinal villi, and crypts (black and red arrow). HE x200.



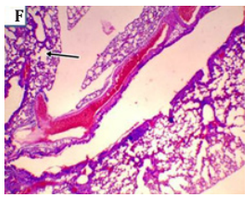


Fig. 3. Cross sections of lung tissue from studied groups: **(A)** GIa (control negative) showing thin and delicate alveolar walls. The alveoli are well-aerated (black and yellow arrows). **(B)** GIb (control positive) showing lung destruction (distorted and dilated airspaces) with hyper-distension of alveolar spaces associated with rupture of many alveolar septa and severe inflammation (yellow arrow); and **(C)** with bronchial epithelial hyperplasia, ulceration, and congestion (HE ×100). **(D)** GIIa (prophylactic AZB treated) and GIIIa (therapeutic AZB treated) showing, moderate inflammation of alveolar spaces (yellow arrow) associated with rupture of many alveolar septa (black arrow) and congestion of blood vessels. **(E)** GIIb (prophylactic NTZ treated) and GIIIb (therapeutic NTZ treated) showing, mild to moderate inflammation near alveolar spaces (yellow arrow) associated with rupture of some alveolar septa (black arrow) and congestion of blood vessels. **(F)** GIIc (prophylactic AZB + NTZ treated) and GIIIc (therapeutic AZB + NTZ treated) showing, very mild inflammation near-normal alveolar spaces associated with few ruptures of alveolar septa (black arrow). HE ×200.

and therapeutic groups respectively (79.50±8.32 and 71.50±4.77 percentages and mean H score=173.00± 7.03 and 155.50±7.39, respectively). The infected control positive group GIb showed mild expression with lowest mean percent (25.57±3.78) and mean H score (40.00±7.07). Results were statistically significant (*P*<0.001) (Table 3; and Figure 4 A-D).

Immunohistochemical staining of iNOS in ileal tissue: Strong cytoplasmic expression of iNOS occurred in the dysplastic intestinal epithelium infected by *Cryptosporidium* spp. (GIb control positive) with the highest mean percent (87.14±7.56) and mean H score=202.86±3.18). While GIIc and GIIIc (received combined treatment in both prophylactic and therapeutic groups), showed weak cytoplasmic expression of iNOS with the lowest mean percent (12.80±7.63 and 23.00±7.57, and mean H score=35.80±5.04 and 50.00±1.29, respectively) (Table 3; and Figure 4 E- H).

Serum biochemical parameters: *Cryptosporidium* GIb (infected control positive) exhibited marked liver damage indicated by significantly increased serum ALT and AST levels (*P*<0.001) compared to GIa (non-infected control negative). Meanwhile, the concomitant treatment in both prophylactic and therapeutic groups, significantly reduced (*P*<0.001) hepatic damage as demonstrated in the groups GIIc and GIIIc in comparison to GIb (infected control positive) (Table 4).

Table 3. Comparison between CD3 expression in the lung and iNOS in ileum sections of different studied groups.

Study subgroups	CD3 expression in the lung		iNOS expression in the ileim	
	% of positive cells Mean ± SD	H score Mean ± SD	% of positive cells Mean ± SD	H score Mean ± SD
Ib (No. = 7)	25.57±3.78	40.00±7.07	87.14±7.56	202.86±3.18
IIa (No. = 10)	55.00±3.33	123.00±9.93	40.10±9.57	73.10±6.1
IIb (No. = 10)	43.00±8.76	67.10±6.61	55.00±7.87	111.65±3.56
IIc (No. = 10)	79.50±8.32	173.00±7.03	12.80±7.63	35.80±5.04
IIIa (No. = 8)	39.50±9.58	62.63±6.26	47.50±6.16	97.00±2.95
IIIb (No. = 9)	34.67±9.15	58.22±6.89	61.67±7.91	123.94±5.62
IIIc (No. = 10)	71.50±4.77	155.50±7.39	23.00±7.57	50.00±1.29
ANOVA test	**23.73**	**70.03**	**82.03**	**74.79**
***P* value**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

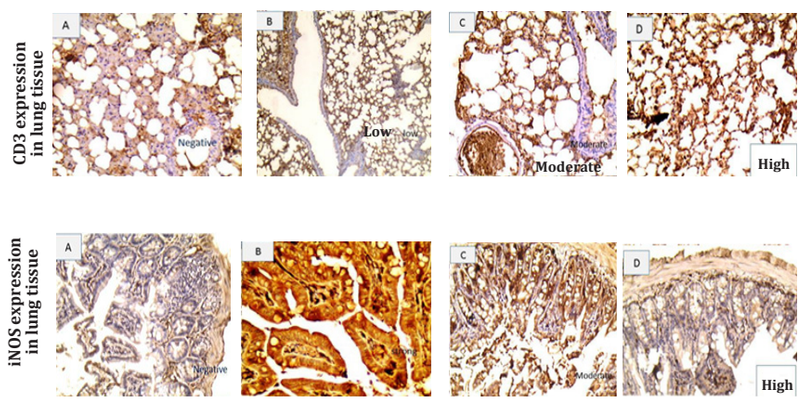


Fig. 4. Cross sections of lung tissue showing CD3 expression in epithelium and inflammatory cells. **(A)** Negative expression in GIa (control negative) **(B)** Mild expression in GIb (control positive) **(C)** Moderate expression in GIIa and GIIb. **(D)** Strong cytoplasmic expression in GIIc and GIIIc. CD-3 immunostain, X200. Cross sections of ileum showing INOS expression in epithelium and inflammatory cells: **(A)** Negative expression in GIa (control negative) **(B)** Mild expression in GIIc and GIIIc (prophylactic and therapeutic AZB+NTZ treated, respectively). **(C)** Moderate expression in GIIb and GIIIb (prophylactic and therapeutic NTZ treated, respectively) with diffuse expression in inflammatory cells within the core of intestinal villi **(D)** Strong diffuse expression in GIb (infected control positive) in inflammatory cells within the core of intestinal villi with high expression in mucosal epithelial cells. iNOS immunostain, X200

Immunological findings: Mean levels of IL-1β, IL-6, INF-γ and TNF-α in GIb (infected control positive) were 25.40±0.14, 30.40±0.13, 32.23±011, 90.39±0.13 pg/ml respectively. Comparatively the mean levels were significantly increased (*P*<0.001) in the other groups recording 65.43±18, 77.4±0.15, 80.75±0.16 and 155.40±0.15, respectively in prophylactic AZB+NTZ treated GIIc. The prophylactic AZB treated GIIa recorded 55.23±0.13, 67.31±0.12, 71.48±0.14 and 140.51±0.20, respectively; followed by the prophylactic NTZ treated GIIb recording 49.73±0.17, 61.56±0.19, 66.66±0.17 and 128.61±0.18, respectively (Figure 5A- D).

Oxidative parameters: There was a significant decrease (*P*<0.001) in the mean serum level of MDA in AZB treated GIIc, GIIa, and NTZ treated GIIIc and GIIb (1.85±0.08, 2.22±0.13, 2.72±0.13, 2.93±0.14, respectively) compared to control positive GI b (4.96±0.11) (Table 4). The mean of serum SOD levels showed significant increase (*P*<0.001) in AZB treated GIIc, GIIa, GIIIc and NTZ treated GIIb (53.08±0.28, 48.75±0.45, 48.80±0.35, and 37.79±0.38, respectively) compared to control positive GI b (30.83±0.26) (Table 4).

Table 4. Comparison between mean serum biochemical parameters (AST and ALT) and oxidative parameters (MDA and SOD) in all studied groups.

Study subgroups	AST (U/L) Mean ± SD	ALT (U/L) Mean ± SD	MDA (nmol/ml) Mean ± SD	SOD (nmol/ml) Mean ± SD
Ia	31.00±1.49	41.00±0.82	1.20 ±0.15	55.77±0.36
Ib	70.14±1.35	74.00±2.00	4.96 ±0.11	30.83±0.26
Ic	29.00±1.29	39.00±0.62	1.38 ±0.12	57.45±0.24
IIa	34.20±1.14	44.00±1.33	2.22 ±0.13	48.75±0.45
IIb	35.00±0.82	43.10±1.45	2.93 ±0.14	37.79±0.38
IIc	34.20±1.14	44.00±1.33	1.85 ±0.08	53.08±0.28
IIIa	40.00±1.51	46.00±1.41	4.51 ±0.18	40.56±0.37
IIIb	37.00±0.87	46.00±1.50	3.54 ±0.27	35.88±0.52
IIIc	35.00±1.05	43.00±1.63	2.72 ±0.13	48.80±0.35
ANOVA test	**683.42**	**384.67**	**466.18**	**5531.35**
***P* value**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

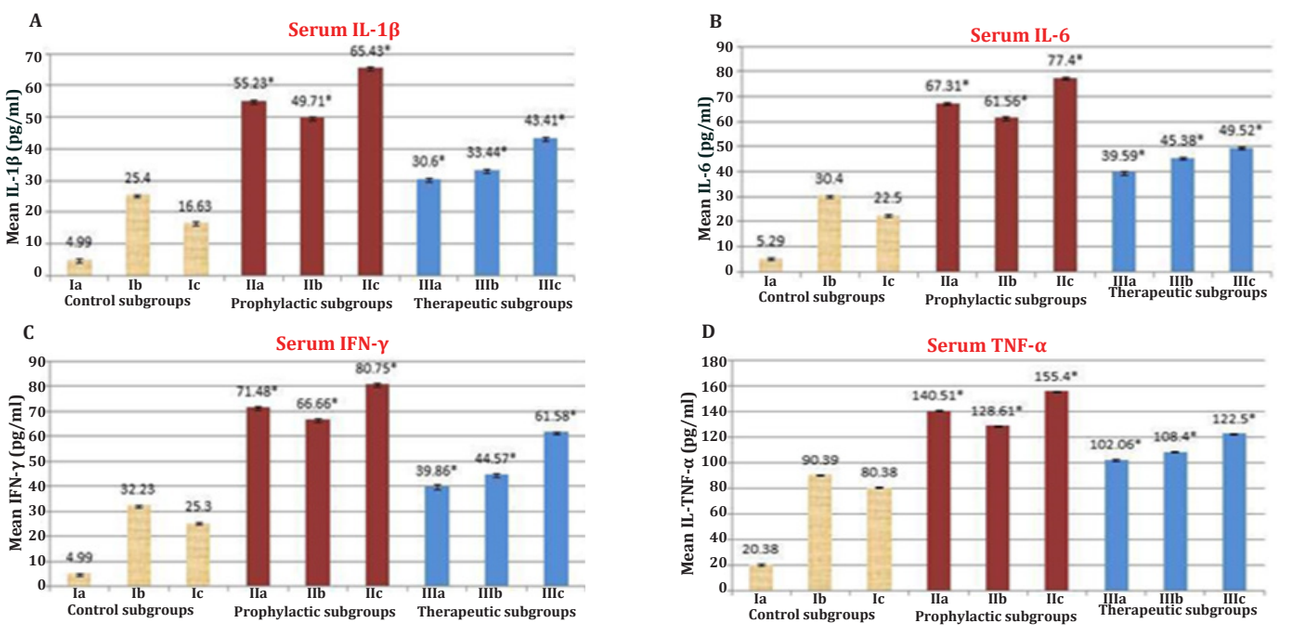


Fig. 5. Comparison in the studied groups between mean pro-inflammatory cytokines levels of IL-1β **(A)**; IL-6 **(B)**; IFN-γ **(C)**; and TNF-α **(D)**. All the values were expressed as mean±S.D with statistically significant differences (*P*<0.001) in comparison to GIb (infected control positive).

DISCUSSION

Considering the broad burden of cryptosporidiosis there is still a shortage of curative treatments despite the imperative need for it[48]. Nitazoxanide is the only accepted anti-cryptosporidial agent. However, it proved inefficient with the absence of an effective immune response specially in immunodeficient individuals. The use of immune-stimulating supplements is therefore recommended[49,50].

Immunosuppressed mice as models for cryptosporidiosis presented substitutes for the study of acquiring rapid uptake and prolonged duration of infection, allowing evaluation of the efficacy of anti-*Cryptosporidium* therapy[8]. By the last day of our

experiment (30th dpi), the mortality rate in mice was 30% in the infected non treated control positive GIb, which generally coincides with another report by El Shafei *et al*.[51]. The mortality of mice may be attributed to the parasite or to the immunosuppression state that promotes a more severe course of infection[52]. Meanwhile, the mortality rate was 0% in the non infected control AZB treated (GIc), prophylactic NTZ treated (GIIb), and AZB+NTZ treated (GIIc), and the therapeutic AZB+NTZ (GIIIc) subgroups, as reported with other treatments as *Echinacea Purpurea*[32] and *Allium Sativum*[53] previously used in experimental cryptosporidiosis[32,53].

In the current study, shedding of the oocysts started from the 2nd dpi. in the immunosuppressed mice as formerly reported by El Shafei *et al*.[51]. The *Cryptosporidium* oocysts continued to be shed with varying degrees throughout the experiment in all infected groups. This result agrees with the reports of Abdou *et al*.[33] and Atia *et al*.[32] who stated that shedding of oocysts continued until day 30th dpi in the infected control group. Clearance of *Cryptosporidium* oocytes from stools is very difficult as rejection of the parasite requires a competent host immune system[49]. The lowest shedding of *Cryptosporidium* oocyst was observed in the prophylactic subgroups GIIc (received combined treatment) and GIIb (received NTZ) with high reduction percentages, 96.13% and 89.89%, respectively; followed by the therapeutic subgroup GIIIc (received combined treatment) with a reduction percent of 81.17%. In immunosuppressed mice prophylaxis with AZB followed by treatment with AZB+NTZ (GIIc) produced a good synergistic prophylactic effect on eradication of oocytes with the highest reduction percentage than either one alone. These results conform with other experimental studies explaining that the combined use of antiparasitic drugs and immunomodulators lead to successful immunization against cryptosporidiosis[32,54-56]. In the current study, the use of AZB in the prophylactic group followed by a booster dose to GIIa stimulated the immune response leading to reduction of oocyst shedding to 62.45%. The high-power effect of AZB as immunostimulant was also reported in experimental trichinosis by Rudneva *et al*.[33].

For further evaluation, we studied the histopathological changes in the intestine and the lung. The terminal part of the ileum was chosen to assess the course of infection because it was reported to be the site with the heaviest burden of intestinal cryptosporidiosis in both immunocompetent and immunosuppressed mice. This was attributed to the favorable biochemical conditions in this part of the ileum for the parasite, in addition to the presence of specific receptors[33]. The histopathological examination of the ileal sections of GIb (infected control positive) revealed marked pathological changes in the intestinal mucosa in comparison with the non-infected control group. Similarly, Abdelhamed *et al*.[57] and Moawad *et al*.[58] reported that *Cryptosporidium* infection induces dysplastic changes of the intestinal tract in mice. The histopathological effect was also described by Soufy *et al*.[54] as displacement of the brush borders resulting in shortening and broadening of the villi and villous atrophy, probably due to secretion of toxins by the pathogen that directly damage the epithelial cells.

In the present study, the prophylactic GIIc and therapeutic GIIIc subgroups that received combined AZB and NTZ treatments showed mild inflammation with improvement of mucosa, intestinal villi and crypts. Our results confirm previous studies that showed promising results with combined treatment than using NTZ alone[57-59].

The pathogenesis of pulmonary cryptosporidiosis has not been fully clarified. According to previous studies, respiratory cryptosporidiosis may occur in immunocompetent individuals and complicate immune-deficient ones especially those co-infected with HIV[60]. Similar reports on the establishment of various developmental stages of *Cryptosporidium* spp. oocysts within the lung were reported by Heo *et al*.[61]. In our current work, lung tissue examination of immunosuppressed infected control positive mice (GIb) revealed marked inflammation with hyper-distension of alveolar spaces associated with rupture of alveolar septa. While the mice in GIIc and GIIIc (who received combined treatment either prophylactically or therapeutically respectively) showed improvement in the form of mild inflammation. Similar findings were detected by Moawad *et al*.[58]. Moreover, Madbouly *et al*.[62] reported that there are pulmonary hemorrhage and interstitial inflammations in the lung tissue of the infected immunosuppressed mice, and these changes improved following Atorvastatin (a statin drug used to treat abnormal lipid levels) and NTZ combination treatment.

Furthermore, the immunohistochemical examination of iNOS antibody revealed strong cytoplasmic expression in the dysplastic intestinal epithelium of GIb (immunosuppressed infected control positive). On the other hand, cytoplasmic expression of iNOs was weak in mice that received prophylactic and therapeutic combined treatments (GIIc and GIIIc respectively). This confirmed the strong oxidative stress exerted by the parasite, and asserted the effect of the drugs in lowering oxidative stress on the tissue. This result coincides with the report of Abdelhamed *et al*.[57], and that of Gookin *et al*.[63] who observed that piglets treated with either an iNOS inhibitor or a peroxynitrite scavenger are capable of normal recovery from cryptosporidiosis. The latter researchers suggested that reactive nitrogen intermediates may serve as an early and innate defense against intestinal epithelial infection. Synthesis of NO is significantly increased in cryptosporidiosis and the absence or inhibition of iNOS

significantly exacerbates epithelial infection and oocyst shedding[64].

CD3 has an important role in T cell function either by stimulation of division or inhibition of the cytotoxic effector functions[65]. In our present study, there was mild expression of CD3 proteins in lymphocytes infiltrating the lung tissue of the infected control positive subgroup (GIb). This result is incompatible with the result reported by Gaber *et al*.[66] who revealed overexpression of CD3 protein in lymphocytes infiltrating intestinal, pulmonary and brain tissues of mice infected with *Cryptosporidium* spp.; possibly because they used immunocompetent mice in their experiment. The role of $CD3^+/CD4^+$ lymphocytes proved to be vital for cryptosporidiosis recovery[7]. Meanwhile, overexpression of CD3 proteins detected in the prophylactic combined therapy subgroup GIIc, corresponded with the result of Alexia *et al*.[67]. They reported that AZB increased T-cell proliferation and may be used in the treatment and prophylaxis of diseases associated with damage to the immune system. This was based on its promotion of the normalization of T-cell indices ($CD3^+$, $CD4^+$, $CD8^+$) and phagocytic activity[68].

Serum elevation of ALT is caused by injury of hepatic cells membranes while AST refers to mitochondrial damage of hepatic tissue[69]. Any applied damage to the liver releases more ALT into the blood raising its levels. In the present study the hepatocytes damage mediated by cryptosporidiosis in mice were assessed by measuring serum levels of ALT and AST

The recorded increased levels of these enzymes confirmed that hepatocytes damages was caused by cryptosporidiosis in the immunosuppressed mice. Our findings corroborate with results reported by Aboelsoued *et al*.[70] and Elmahallawy *et al*.[71] who also recorded elevation in ALT and AST in experimentally infected mice with cryptosporidiosis. Notably the serum levels of liver ALT and AST enzymes in prophylactic and therapeutic combined therapy of GIIc and GIIIc mice decreased near to the control level, confirming the therapeutic effects of AZB and NTZ in cryptosporidiosis. The combination therapy either in prophylactic or therapeutic subgroups, strongly ameliorated the harmful effects of cryptosporidiosis by minimizing hepatocyte damage and decreasing serum concentrations of the enzymes. This amelioration was also attributed to antioxidant properties of Mefloquine and NTZ *via* inhibiting lipid peroxidation[56].

Additionally, we found that the cytokines IL-1β, IL-6, IFN-γ and TNF-α levels were upregulated in prophylactic and therapeutic subgroups. Prophylaxis of immunosuppressed mice by AZB then followed by combined treatment with AZB and NTZ (GIIc) produced a significant increase of cytokines in comparison with infected control positive subgroup GIb ($P<0.001$). These results coincided with former report by El-Wakil *et al*.[56], and confirmed by Chang *et al*.[72] demonstration of the important role of cytokines in controlling cryptosporidiosis. It has been shown that cytokines including IFN-γ, TNF-α, IL-2, IL-4, and IL-6 play a vital role in immune regulation and are capable of hindering the development of disease due to their rapid recruitment after cryptosporidiosis[73]. An immunomodulator as AZB, when combined with a probiotic can elicit or amplify an immune response in immune suppressed patients[29]. The main immunomodulatory mechanism of AZB is by direct action on phagocytic cells and NK lymphocytes, and the promotion of antibody formation, IFN-α, and IFN-γ synthesis and stimulation of the production of IL-1β and IL-6[74]. The anti-inflammatory action of AZB succeeds in normalization of pro- and anti-inflammatory cytokines synthesis and suppression of neutrophil extracellular traps formation[75].

Antioxidants also inhibit the scavenging of free radicals and give significant protection against infectious diseases in humans and animals. Moreover, SOD catalyzes the toxic superoxide radical into less toxic hydrogen peroxide[76]. On the other hand, MDA, as a product of lipid peroxidation is an important indicator of oxidative damage of cell membrane as it is the most abundant aldehyde formed[77]. In the current study, cryptosporidiosis generated oxidative stress by reducing SOD activity and elevating MDA content in the infected control positive mice (GIb) verifying results published by El-Sayed and Fathy[8], and Bhagat *et al*.[14]. The oxidative injury following cryptosporidiosis was also previously reported by other studies that demonstrated a decrease in SOD activity[14,71].

In our study, subgroup GIIc that received prophylactic combined treatment, showed increase in SOD and decrease MDA due to early antioxidant properties of AZB, and was followed by the subgroup GIIIc that received combined therapeutic treatment. Zarubina and Shabanov[78] reported that a more pronounced antioxidant effect was observed during combined use of AZB with another antioxidant, Metaprot, resulting in decreased pulmonary MDA by 73%, and restoration of SOD activity up to the normal level of intact animals. Moreover, another report revealed that the antioxidant action of AZB is manifested when used in high doses, while low doses exerted a moderate pro-oxidant effect *in vitro*[79].

The antitoxic and antioxidant properties of AZB is achieved by penetration of the cell endosomes to link with increased macromolecular concentrations of hydrogen peroxide. This in turn activates signaling molecules and transcription factors, particularly nuclear factor-kappa B which has detoxifying and antioxidant properties[80]. This can explain the ability of AZB to enhance resistance to infections and stimulation of the immune response, by promoting antigen

presentation and cytotoxic activity[67]. Moreover, it is used as an immune adjuvant particularly for vaccines, and as an immune modulator for the treatment of acute and chronic bacterial, viral, parasitic, or fungal infectious diseases[21].

It was concluded that AZB exerted a promising effect in achieving the best protection when combined with NTZ in experimental cryptosporidiosis. Also, it can elicit or amplify an immune response in immune-suppressed mice and stimulate the immune systems. Moreover, it stimulated scavenger systems, which control oxidative stress. In this way, immune-stimulating drugs significantly increase the protective reaction against the organism and can prevent subsequent infection by different helminths.

Author contribution: Atia AF, EL Sobky MM, Harba BN, Abou Hussien NM and Kamel RA conceived and designed the research topic. Atia AF, Abou Hussien NM and Kamel RA were responsible for acquisition, analysis and interpretation of the research data. Allam DM analyzed the pathological and immunohistochemical data. Atia AF wrote the draft of the manuscript. Harba NM, EL Sobky MM, and Abou Hussien NM critically revised the article for publication. All the author approved the final revision.

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REFERENCES

1. Xiao L. Molecular epidemiology of cryptosporidiosis: an update. Exp Parasitol 2010; 124(1):80-89.
2. Shirley DT, Moonah SN, Kotloff KL. Burden of disease from cryptosporidiosis. Curr Opin Infect Dis 2012; 25(5):555-563.
3. Zaki NE, Hamed NA, Sadek NA, Mishriky RI, Abd El-Latif NF, Mikhael IL. Intestinal protozoan infections among Egyptian neutropenic patients with acute leukemia. Trop Biomed 2021; 38(1):50-56.
4. Vanathy K, Parija SC, Mandal J, Hamide A, Krishnamurthy S. Cryptosporidiosis: A mini review. Trop. Parasitol 2017; 7(2): 72-80.
5. Checkley W, Jr ACW, 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.
6. Lunde CS, Stebbins EE, Jumani RS, Hasan MM, Miller P, Barlow J, *et al*. Identification of a potent benzoxaborole drug candidate for treating cryptosporidiosis. Nat Commun 2019; 10(1):1-11.
7. White J. Cryptosporidiosis (*Cryptosporidium hominis*, *Cryptosporidium parvum*, and other species). In: Bennett JE, Dolin R, M.J. Blaser MJ (eds). Mandell, Douglas and Bennett's infectious disease essentials (principles and practice of infectious diseases). Elsevier. Churchill Livingstone, Philadelphia, Pennsylvania 2005; 3547-3560.
8. El-Sayed NM, Fathy GM. Prophylactic and therapeutic treatments' effect of *Moringa oleifera* methanol extract on *Cryptosporidium* infection in immunosuppressed mice. Anti-Infective Agents 2019; 17(2):130-137.
9. Bedi B, Mead JR. *Cryptosporidium parvum* antigens induce mouse and human dendritic cells to generate Th1-enhancing cytokines. Parasite Immunol 2012; 34(10):473-485.
10. Barakat FM, McDonald V, Foster GR, Tovey MG, Korbel DS. *Cryptosporidium parvum* infection rapidly induces a protective innate immune response involving type I interferon. J Infect Dis 2009; 200(10):1548-1555.
11. Lean IS, Lacroix-Lamandé S, Laurent F, McDonald V. Role of tumor necrosis factor alpha in development of immunity against *Cryptosporidium parvum* infection. Infect Immun 2006; 74 (7): 4379-4382.
12. McDonald V. (Eds.). *Cryptosporidium* and cryptosporidiosis IN: Fayer R, Xiao L (eds). CRC press 2007.
13. Drinkall E, Wass MJ, Coffey TJ, Flynn RJ. A rapid IL-17 response to *Cryptosporidium parvum* in the bovine intestine. Vet Immunol Immunopathol 2017; 191:1-4.
14. Bhagat M, Sood S, Yadav A, Verma P, Manzoor N, Chakraborty D, *et al*. Alterations in oxidative stress parameters and its associated correlation with clinical disease on experimental *Cryptosporidium parvum* infection in Swiss albino mice. J Parasit Dis 2017; 41:707-712.
15. Ujjwal k, Mukherjee R, Nandi S, Bhushan B, Dimri U, Verma AK. Disturbance of oxidant/antioxidant balance, acute phase response and high mobility group box-1 protein in acute undifferentiated diarrhea in cross bred piglets. Indian J Anim Sci 2014; 84:860-863.
16. Tsikas D. Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. Anal Biochem 2017; 524:13-30.
17. Asadpour M, Namazi F, Razavi SM, Nazifi S. Comparative efficacy of curcumin and paromomycin against *Cryptosporidium parvum* infection in a BALB/c model. Vet Parasitol 2018; 250:7-14.
18. Kumar VP, Frey KM, Wang Y, Jain HK, Gangjee A, Anderson KS. Substituted pyrrolo [2,3-d] pyrimidines as *Cryptosporidium hominis* thymidylate synthase inhibitors. Bioorg Med Chem Lett 2013; 23(19):5426-5428.
19. Bamaiyi PH, Redhuan NEM. Prevalence and risk factors for cryptosporidiosis: a global, emerging, neglected zoonosis. Asian Biomedicine 2016; 10:309-325.
20. Sparks H, Nair G, Castellanos-Gonzalez A, White AC. Treatment of *Cryptosporidium*: what we know, gaps, and the way forward. Current trop med reports 2015: 2(3): 181-187.
21. Powell BS, Andrianov AK, Fusco PC. Polyionic vaccine adjuvants: another look at aluminum salts and polyelectrolytes. Clin Exp Vaccine Res 2015; 4:23-45.
22. Kabanov VA. From synthetic polyelectrolytes to polymer-subunit vaccines. Pure Appl Chem J 2004; 76:1659-1677.
23. Jurkiewicz D, Zielnik-Jurkiewicz B. Bacterial lysates in the prevention of respiratory tract infections. Otolaryngol Pol 2018; 72(5):1-8.

24. Shulzhenko AE. The immunomodulator polyoxidonium: A new approach to treatment of HSV-2 infection in its resistance to acyclic nucleosides. Immunology 2000; 5:29-32.
25. Ilek JJ, Galanina AV, Zajceva GA. Efficacy of Polyoxidonium in severe pneumonia in young children. Teppa Медика 2003; 3:12-14.
26. Kazeko NI, Zhmurov VA, Borovskij AA, Lerner Gia, Katrenko IV, Nechaĭ EN, *et al*. The use of polyoxidonium in the treatment of calculous pyelonephritis. Urologiia 2006; 2:59-62.
27. Kulagin VI, Batkaev EA, Ponomarev BA, Šaparenko MV, Rjumin DV. Treatment of urogenital chlamydiosis with combination of antibiotics and immunomodulator polyoxidonium (a multicenter study). J Postgraduate Med Education 2001; 3:21-23.
28. Pružinec P, Chirun N, Sveikata A. The safety profile of polyoxidonium in daily practice: results from post authorization safety study in Slovakia. Immunotherapy 2018; 10 (2):131-137.
29. Sajjadieh SM, Edalati M, Ajami A, Sajjadieh SE. The effect of azoximer bromide in treatment irritable bowel syndrome. Allergy 2017; 72:758-823.
30. Fahmy A, Fahmy Z, Aly E, Elshenawy A, ELWakil E. Therapeutic potential of *Commiphora molmol* extract loaded on chitosan nanofibers against experimental cryptosporidiosis. PUJ. 2021; 14(1):39-45.
31. Rudneva OV, Napisanova LA, Berezhko VK: Immunostimulating effects of azoximer bromide and cyclopheron in experimental *T. spiralis* infection. Agrofor Int J 2018;3(1):99-105.
32. Atia AF, Dawoud MA, EL-Refai SA. Effects of *Echinacea Purpurea* on cryptosporidiosis in immunosuppressed experimentally infected mice. Med J Cairo Univ 2018; 86: 3209-3222.
33. Abdou AG, Harba NM, Afifi AF, Elnaidany NF. Assessment of *Cryptosporidium parvum* infection in immunocompetent and immunocompromised mice and its role in triggering intestinal dysplasia. Int J Infect Dis 2013; 17:593-600.
34. Mohamed AW, Koura1AE, Rabee I, Hammam AO, Ismail MH. The efficacy of chitosan nanoparticule alone versus conjucated with *Nigella sativa* (El-Baraka seed oil) against *Cryptosporidium parvum* in infected immunocompetent and immunosuppressed mice. WJPPS 2019; 8(10):139-161.
35. Anderson BC. Moist heat inactivation of *Cryptosporidium* spp. Am J Public Health 1985; 75:1433-1434.
36. Henriksen SA, Pohlenze JFL. Staining of *Cryptosporidium* by a modified Ziehl-Neelsen technique. Acta Vet Scand 1981; 22: 594-596.
37. Current WL, Reese NC, Ernst JV, Bailey WS, Heyman MB, Weinstein WM. Human cryptosporidiosis in immunocompetent and immunodeficient persons: Studies of an outbreak and experimental transmission. N Engl J Med 1983; 308 (21):1252-1257.
38. Operario DJ, Bristol LS, Liotta J, Nydam DV, 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.
39. Hosking BC, Watson TG, Leathwick DM. Multigeneric resistance to oxfendazole bynematodes in cattle. Vet Rec 1996; 138(3): 67-68.
40. Gamble M (Eds). (The hematoxylin and eosin) In: Bancroft JD, Gamble M (eds.). Theory and Practice of Histological Techniques, Elsevier health sciences; 2008; pp. 121.
41. Kim SW, Roh J, Park CS. Immunohistochemistry for pathologists: protocols, pitfalls, and tips. J Pathol Transl Med 2016; 50(6):411-418.
42. Al-Rajhi N, Soudy H, Ahmed SA, Elhassan T, Mohammed SF, Khoja H, *et al*. $CD3^+$ T-lymphocyte infiltration is an independent prognostic factor for advanced nasopharyngeal carcinoma. BMC cancer 2020; 20(1):1-13.
43. Brennan PA, Conroy B, Spedding AV. Expression of inducible nitric oxide synthase and p53 in oral epithelial dysplasia. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2000; 90(5):624-629.
44. Nie H, Zheng Y, Li R, Guo TB, He D, Fang L, *et al*. Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF-α in rheumatoid arthritis. Nature Med 2013; 19: 322–328.
45. Lee LY, Chi-Ming W, Wang CC, Song JY, Liang Y, Huang K , *et al*. Expression of matrix metalloproteinases MMP-2 and MMP-9 in gastric cancer and their relation to claudin-4 expression. Histol Histopathol 2008; 23:515-521.
46. Misra HP, Fridovich I. The role of superoxide anion in the auto oxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972; 247:3170-3174
47. Ratty A, Das NP. Lipid peroxidation in the rat brain mitochondria in the presence of ascorbic acid. Medical Science 1986; 14:815-816.
48. Striepen B. Parasitic infections: Time to tackle cryptosporidiosis. Nature 2013; 503:189-191.
49. Gargala G. Drug treatment and novel drug target against *Cryptosporidium*. Parasite 2008; 15(3):275-281.
50. Atia MM, Abdul Fattah MM, Abdel Rahman HA, Mohammed FA, Al-Ghandour AMF. Assessing the efficacy of nitazoxanide in treatment of cryptosporidiosis using PCR examination. J Egy Soc Parasitol 2016; 46(3):683-692.
51. El Shafei OK, Saad AGE, Harba NM, Sharaf OF, Samaka RM, Farag SA. Therapeutic effect of phenyl vinyl sulfone and nitazoxanide on experimentally infected mice with cryptosporidiosis. Menoufia Med J 2018; 31(3):786-794.
52. Sadek GS, El-Aswad BE. Role of COX-2 in Pathogenesis of intestinal cryptosporidiosis and effect of some drugs on treatment of infection. Res J Parasitol 2014; 9:21-40.
53. Kiros H, Bitsue FZ, Gebreyesus N, Hadush B, Afera B, Tekele Y, *et al*. *In vivo* evaluation of the therapeutic efficacy of *Allium Sativum* against cryptosporidiosis. Ethio J Vet Science and Animal Production (EJVSAP) 2017; 1(1):46-56.
54. Soufy H, Nadia M, Nasr SM, Abd El-Aziz TH, Khalil FA, Ahmed YF, *et al*. Effect of Egyptian propolis on cryptosporidiosis in immunosuppressed rats with special emphasis on oocysts shedding, leukogram, protein profile and ileum histopathology. Asian Pac J Trop Med 2017;10(3):253-262.
55. Al-Ghandour AMF, Yousef AM, Mohamed RMSM, Tealeb AM, Ahmed HK, Atwa HA *et al*. Prophylactic

anticryptosporidial activity of atorvastatin versus nitazoxanide on experimentally infected immunosuppressed murine models. J Egypt Soc Parasitol 2020; 50(3):535-546.
56. El-Wakil ES, Salem AE, Al-Ghandour AMF. Evaluation of possible prophylactic and therapeutic effect of mefloquine on experimental cryptosporidiosis in immunocompromised mice. J Parasit Dis 2021; 45(2):380-393.
57. Abdelhamed EF, Fawzy EM, Ahmed SM, Zalat RS, Rashed HE. Effect of nitazoxanide, artesunate loaded polymeric nano fiber and their combination on experimental cryptosporidiosis. Iran J Parasitol 2019; 14(2):240-249.
58. Moawad HSF, Hegab MHA, Badawey MSR, Ashoush SE, Ibrahim SM, Ali AAELS. Assessment of chitosan nanoparticles in improving the efficacy of nitazoxanide on cryptosporidiosis in immunosuppressed and immunocompetent murine models. J Parasitic Dis 2021; 45:1-14.
59. Metawae AG, Bayoumy AM, Ali IR, Hammam OA and Temsah KAT. Efficacy of nitazoxanide alone or loaded with silica nanoparticle for treatment of cryptosporidiosis in immunocompetent hosts. IJMA 2020; 3(2):1229- 1239.
60. Sponseller JK, Griffiths JK, Tzipori S. The evolution of respiratory cryptosporidiosis: evidence for transmission by inhalation. Clin Microbiol Rev 2014; 27(3):575-586.
61. Heo I, Dutta D, Schaefer DA, Iakobachvili N, Artegiani B, Sachs N, *et al*. Modelling *Cryptosporidium* infection in human small intestinal and lung organoids. Nat Microbiol 2018; 3(7): 814-823.
62. Madbouly NT, Hebat SA, Yousof HA, El-Sayed SH, Younis AI, Mohamed SJ. Atorvastatin repurposing for the treatment, of cryptosporidiosis in, experimentally immunosuppressed mice. Exp Parasitol 2017; 181:57-69.
63. Gookin JL, Allen J, Chiang S, Duckett L, Armstrong MU. Local peroxynitrite formation contributes to early control of *Cryptosporidium parvum* infection. Infect Immun 2005; 73(7):3929-3936.
64. Gookin JL, Duckett LL, Armstrong MU, Stauffer SH, Finnegan CP, Murtaugh MP, *et al*. Nitric oxide synthase stimulates prostaglandin synthesis and barrier function in *C. parvum*-infected porcine ileum. Am J Physiol Gastrointest Liver Physiol 2004; 287:571-581.
65. Mizoguchi A, Andoh A. Animal models of inflammatory bowel disease for drug discovery. In: Animal models for the study of human disease. (Ed.P.M. Conn). Academic Press, London 2013; 499-527.
66. Gaber M, Galal LAA, Hassan D, Badary DM, Israa M.A. Mohamed IMA, *et al*. Evidences of brain and lung invasion of a local water *Cryptosporidium parvum* isolate in comparison to Iowa strain serological and immunohistochemical cytokine evaluation. Ann Parasitol 2020; 66(3):311-318.
67. Alexia C, Cren M, Louis-Plence P, Vo DN, El Ahmadi Y, Dufourcq-Lopez E, *et al*. Polyoxidonium® activates cytotoxic lymphocyte responses through dendritic cell maturation: clinical effects in breast cancer. Front Immunol 2019; 10: 2693.
68. Kharit SM, Galustyan AN. Azoximer bromide is a safe and effective preparation for the treatment of acute respiratory infections of the upper- respiratory tract in children: an overview of the results of double-blind, placebo-controlled, randomized clinical trials of phase II and III. Pediatrics (Suppl. Consilium Medicum); 2017; 2:55-61.
69. Abou Gabal A, Aboul-Ela HM, Ali E, Khaled AEM, Shalaby OK. Hepatoprotective, DNA damage prevention and antioxidant potential of *Spirulina platensis* on CCl4-induced hepatotoxicity in mice. Am J Biomed Res 2015; 3: 29-34.
70. Aboelsoued D, Abo-Aziza F, Mahmoud M, Megeed KA, El Ezz NA, Abu-Salem F. Anticryptosporidial effect of pomegranate peels water extract in experimentally infected mice with special reference to some biochemical parameters and antioxidant activity. J Parasit Dis; 2019; 43: 215-228.
71. Elmahallawy EK, Elshopakey GE, Saleh AA, Agil A, El-Morsey A, El-shewehy DMM, *et al*. S-Methylcysteine (SMC) ameliorates intestinal, hepatic, and splenic damage induced by *Cryptosporidium parvum* infection *via* targeting inflammatory modulators and oxidative stress in Swiss albino mice. Biomedicines 2020; 8(10):423-440.
72. Chang L, Chen Y, Qian Kang J, Liu Z. Detection of expression alteration of cytokines in the intestine of Balb/c mice infected with *Cryptosporidium parvum* using relative fluorescence quantitative PCR method. Pakistan J Zool 2021; 1-11.
73. Han M, Cai Y, Huang X, He Y, Bo X, Zhong F. Establishment and application of a real time fluorescent quantitative RT-PCR assay for detection of bovine Th1/Th2 cytokines. Chin J Vet Sci 2011; 31:513-520.
74. Dyakonova VA, Dambaeva SV, Pinegin BV, Khaitov RM. Study of interaction between the polyoxidonium immunomodulator and the human immune system cells. Intern Immunopharmacol 2004; 4:1615-1623.
75. Karaulov AV, Gorelov AV. The use of azoximer bromide in therapy of infectious and inflammatory diseases of respiratory system in children: meta-analysis of controlled clinical trials. J Infectology 2019; 11:31-41.
76. Quílez J, Vergara-Castiblanco C, Monteagudo L, del Cacho E, Sánchez Acedo C. Host association of *Cryptosporidium parvum* populations infecting domestic ruminants in Spain. Appl Environ Microbiol 2013; 79: 5363-5371.
77. Petry F, Jakobi V, Tessema TS. Host immune response to *Cryptosporidium parvum* infection. Exp Parasitol 2010; 126: 304-309
78. Zarubina IV, Shabanov PD. Antioxidant effect of polyoxidonium and metaprot during bronchopulmonary inflammation in rats. Bull Exp Biol Med. 2015; 160(2):234-237.
79. Huang K, Yang S. Inhibitory effect of selenium on *Cryptosporidium parvum* infection *in vitro* and *in vivo*. Biol Trace Elem Res 2002; 90:261-272.
80. Menzella F, Fontana M, Salvarani C, Massari M, Ruggiero P, Scelfo C, *et al*. Efficacy of tocilizumab in patients with COVID-19 ARDS undergoing noninvasive ventilation. Crit Care 2020; 24(1):1-9.