INTRODUCTION

Cryptosporidium spp. are common widely distributed, zoonotic protozoans 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.
and plays an important role in both innate and adaptive immune responses\[^9\]. 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 immune-competent 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 antiparasitic 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 professcd 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 Bilharz 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 30\(^{th}\) day post infection (dpi) by cervical dislocation\[^31\]. 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\[^32\].

**Mice infection:** *Cryptosporidium* oocysts collected from the feces of naturally infected calves\[^33\] were identified by Modified Zehl Neelsen (MZN) staining\[^34\]. 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\[^35\]. Before infection, oocysts were concentrated and counted in...
Azoximer bromide in experimental cryptosporidiosis

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All groups were observed. The smears were examined using th

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dpi.

Each antibody was assessed using

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dpi.

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were assessed regarding any pathological changes in

dehydrated in graded alcohol solutions and stained

and lung tissues were embedded in paraffin, sectioned,

Histopathological examination: The terminal one cm

do 0.9% NaCl with an interval

PBS solution using a hemocytometer. The mice were infected intra-esophageally with 3000- 3500 oocysts using a tuberculin syringe[31]. 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 Russian 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[34].

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[35]. 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)[36]. 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][37].

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)[38]. 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 Ultrasound 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 (x40). 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 -20°C. 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

<table>
<thead>
<tr>
<th>Study groups and subgroups</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group I (Control)</strong></td>
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</tr>
<tr>
<td>Subgroup Ia (Negative control)</td>
<td>30 immunosuppressed mice. Non infected, injected by phosphate buffer saline (PBS).</td>
</tr>
<tr>
<td>Subgroup Ib (Positive control)</td>
<td>Infected by Cryptosporidium oocysts and not treated.</td>
</tr>
<tr>
<td>Subgroup Ic (Drug control)</td>
<td>Non infected, injected twice intramuscularly with AZB with an interval of 48 hrs[31].</td>
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<tr>
<td><strong>Group II (Prophylactic)</strong></td>
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<tr>
<td>Subgroup IIa</td>
<td>Injected IM with a booster dose of AZB on the 15th dpi.</td>
</tr>
<tr>
<td>Subgroup IIb</td>
<td>Received NTZ orally on the 15th dpi.</td>
</tr>
<tr>
<td>Subgroup IIc</td>
<td>Received both AZB and NTZ on the 15th dpi.</td>
</tr>
<tr>
<td><strong>Group III (Therapeutic)</strong></td>
<td></td>
</tr>
<tr>
<td>Subgroup IIIa</td>
<td>Injected IM with AZB on the 15th dpi.</td>
</tr>
<tr>
<td>Subgroup IIIb</td>
<td>Received NTZ orally on the 15th dpi.</td>
</tr>
<tr>
<td>Subgroup IIIc</td>
<td>Received both AZB and NTZ on the 15th dpi.</td>
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</tbody>
</table>
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/MTA00B/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 autooxidation 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 statistically analyzed by IBM personal computer and Statistical Package for Social Sciences (SPSS) software, version 22 (Armonk, NY: IBM Corp, 2013). They were statistically analyzed by IBM personal computer and Statistical Package for Social Sciences (SPSS) software, version 22 (Armonk, NY: IBM Corp, 2013).

**RESULTS**

**Mortality rate:** At the end of the study, the mortality rate in mice was 30% in G1b (infected untreated control positive), 20% in GIIa (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 GIIc (received combined treatment) (Figure 1A).

**Oocyst shedding:** In comparison with G1b (infected untreated control positive; 8008.57±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 (Glb) (Figure 1C).

**Histopathological examination**

**Intestine:** Histopathological examination of ileal sections from control negative Gla (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 Glb (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 cell 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).

<table>
<thead>
<tr>
<th>Study subgroups</th>
<th>Mean ± SD</th>
<th>ANOVA test</th>
<th>P value</th>
<th>Post Hoc test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iib (No. = 7)</td>
<td>8008.57±7.48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iia (No. = 10)</td>
<td>3007.00±5.38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iib (No. = 10)</td>
<td>810.00±7.45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iic (No. = 10)</td>
<td>310.00±7.45</td>
<td>1157381.32</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>IIia (No. = 8)</td>
<td>4508.13±5.94</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IIib (No. = 9)</td>
<td>2007.78±5.65</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IIic (No. = 10)</td>
<td>1508.00±7.15</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

P1: Iia vs Iib; P2: Iia vs Iic; P3: Iia vs IIic; P4: Iib vs Iib; P5: Iib vs IIia; P6: Iib vs IIIc; P7: Iic vs IIia; P8: Iic vs IIib; P9: Iic vs IIIc.
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 Gla mice (immunosuppressed non-infected) showed normal alveoli (Figure 3A). Sections from control positive GIIb (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

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**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).

**Fig. 2.** Cross sections of ileum from studied groups: (A) Gla (control negative) showing normal villous architecture (yellow arrow) with preserved goblet cells and normal crypts. (B) GIIb (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.
High iNOS expression in the ileal tissue:

Cross sections of lung tissue showing CD3 expression in inflammatory cells in Cryptosporidium-infected ileal tissue: Strong cytoplasmic expression in GIIc and GIIIc (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 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 Gib and GIIc (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 A-D).

<table>
<thead>
<tr>
<th>Study subgroups</th>
<th>% of positive cells</th>
<th>H score</th>
<th>% of positive cells</th>
<th>H score</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td></td>
<td>Mean ± SD</td>
<td></td>
</tr>
<tr>
<td>Ib (No. = 7)</td>
<td>25.57±3.78</td>
<td>4.00±0.70</td>
<td>87.14±5.66</td>
<td>202.86±3.18</td>
</tr>
<tr>
<td>Ila (No. = 10)</td>
<td>55.00±3.33</td>
<td>123.00±9.93</td>
<td>40.10±9.57</td>
<td>73.10±6.1</td>
</tr>
<tr>
<td>Iib (No. = 10)</td>
<td>43.00±8.76</td>
<td>67.10±6.61</td>
<td>55.00±7.87</td>
<td>111.65±5.56</td>
</tr>
<tr>
<td>IIC (No. = 10)</td>
<td>79.50±8.32</td>
<td>173.00±7.03</td>
<td>12.80±7.63</td>
<td>35.80±5.04</td>
</tr>
<tr>
<td>IIIa (No. = 8)</td>
<td>39.50±9.58</td>
<td>62.62±6.26</td>
<td>47.50±6.16</td>
<td>97.00±2.95</td>
</tr>
<tr>
<td>IIIb (No. = 9)</td>
<td>34.67±9.15</td>
<td>58.22±6.89</td>
<td>61.67±7.91</td>
<td>123.94±5.62</td>
</tr>
<tr>
<td>IIIc (No. = 10)</td>
<td>71.50±4.77</td>
<td>155.50±7.39</td>
<td>23.00±7.57</td>
<td>50.00±1.29</td>
</tr>
</tbody>
</table>

ANova test: 23.73 <0.001  70.03 <0.001  82.03 <0.001  74.79 <0.001

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 Gla (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.
Immunological findings: Mean levels of IL-1β, IL-6, INF-γ and TNF-α in GIb (infected control positive) were 25.4±0.14, 30.4±0.13, 32.2±0.11, 90.3±0.13 pg/ml respectively. Comparatively, the mean levels were significantly increased (P<0.001) in the other groups recording 65.4±18, 77.4±0.15, 80.7±0.16 and 155.4±0.15 respectively in prophylactic AZB+NTZ treated GIIc. The prophylactic AZB treated GIIa recorded 55.2±0.13, 67.3±0.12, 71.4±0.14 and 140.5±0.20 respectively; followed by the prophylactic NTZ treated GIIb recording 49.7±0.17, 61.6±0.19, 66.6±0.17 and 128.6±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 GIIc and GIIb (1.8±0.08, 2.2±0.13, 2.7±0.13, 2.9±0.14 respectively) compared to control positive GIb (4.9±0.11) (Table 4). The mean of serum SOD levels showed significant increase (P<0.001) in AZB treated GIIc, GIIa, GIIc and NTZ treated GIIb (53.1±0.28, 48.7±0.45, 37.7±0.38 respectively) compared to control positive GIb (30.8±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.

<table>
<thead>
<tr>
<th>Study subgroups</th>
<th>AST (U/L) Mean ± SD</th>
<th>ALT (U/L) Mean ± SD</th>
<th>MDA (nmol/ml) Mean ± SD</th>
<th>SOD (nmol/ml) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>31.00±1.49</td>
<td>41.00±0.82</td>
<td>1.20±0.15</td>
<td>55.77±0.36</td>
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<tr>
<td>Ib</td>
<td>70.1±1.35</td>
<td>74.00±2.00</td>
<td>4.9±0.11</td>
<td>30.83±0.26</td>
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<tr>
<td>Ic</td>
<td>29.00±1.29</td>
<td>39.00±0.62</td>
<td>1.38±0.12</td>
<td>57.45±0.24</td>
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<tr>
<td>IIa</td>
<td>34.2±1.14</td>
<td>44.00±1.33</td>
<td>2.2±0.13</td>
<td>48.75±0.45</td>
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<tr>
<td>IIb</td>
<td>35.00±0.82</td>
<td>43.1±1.45</td>
<td>2.9±0.14</td>
<td>37.79±0.38</td>
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<td>IIc</td>
<td>34.2±1.14</td>
<td>44.00±1.33</td>
<td>1.8±0.08</td>
<td>53.00±0.28</td>
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<td>IIIa</td>
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<td>IIIb</td>
<td>37.00±0.87</td>
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<td>IIIc</td>
<td>35.00±0.10</td>
<td>43.00±1.63</td>
<td>2.7±0.13</td>
<td>48.80±0.35</td>
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ANOVA test

<table>
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<tr>
<td>&lt;0.001</td>
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DISCUSSION

Considering the broad burden of cryptosporidiosis there is still a shortage of curative treatments despite the imperative need for it⁴⁸. 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 recommended⁴⁹,⁵⁰.

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 therapy⁵¹. 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 (Glc), prophylactic NTZ treated (GIb), and AZB+NTZ treated (Glc), and the therapeutic AZB+NTZ (GIIc) 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 oocysts from stools is very difficult as rejection of the parasite requires a competent host immune system[69]. 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 GIic (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 oocysts 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[32,54-66]. In the current study, the use of AZB in the prophylactic group followed by a booster dose to GIa 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.[31].

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[32]. 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 GIic 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[57-69].

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.[51]. In our current work, lung tissue examination of immunosuppressed infected control positive mice (GIb) revealed marked inflammation with hyperdistension of alveolar spaces associated with rupture of alveolar septa. While the mice in GIic and GIic (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.[59]. Moreover, Madbouly et al.[60] 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 GIic 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.[61] 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

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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 (Glb). 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 Elmahlawy 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 GIIc 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.[64].

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 Glb (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 (Glb) verifying results published by El-Sayed and Fathy[9], and Bhagat et al.[14]. The oxidative injury following cryptosporidiosis was also previously reported by other studies that demonstrated a decrease in SOD activity.[78,79].

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 GIIc that received combined therapeutic treatment. Zarubina and Shabanov[79] 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.[78].

The anti-toxic 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. 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 authors approved the final revision.

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