

Control of the Waterborne Cryptosporidiosis: Evaluation of the Protective Role of *Cryptosporidium parvum* Oocysts Antigen in Infected Immunocompetent and Immunosuppressed Mice

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ABSTRACT

Cryptosporidium parvum causes acute, persistent, and chronic diarrhea with fatal consequences in children and immunocompromised individuals. Due to the absence of effective drug treatments, vaccine development is a relevant option. The present work was carried out to evaluate the protective role of *Cryptosporidium parvum* oocysts antigen against Cryptosporidiosis in infected immunocompetent and immunosuppressed mice. *C. parvum* oocysts were collected from infected calves and mice were infected with 10000 oocysts/mouse. Sonicated *C. parvum* oocysts (vaccine) were used for the immunization of mice in three booster doses. Seven days post-infection, Nitazoxanide (NTZ) was used for the treatment of mice for seven successive days. Mice immunosuppression was performed orally by (Dexamethasone) for 14 successive days prior to infection, immunosuppressed mice continued to receive Dexamethasone at the same dose throughout the experiment. Twenty-one days post-infection mice were sacrificed. The highest percentage of reduction in the mean number of oocysts was observed in vaccinated, infected mice treated with NTZ in both immunocompetent and immunosuppressed infected mice (vaccinated before or after immunosuppression). The highest percentage of reduction in sera mean levels of IgG, IgM and IgA were detected in the immunocompetent group (vaccinated, infected, then treated with NTZ). Vaccination before immunosuppression, the C10 group (vaccinated, immunosuppressed, infected, then treated with NTZ) showed the highest improvement in the mean IgG, IgM, and IgA sera levels. The findings indicated improvement of the immune status in vaccinated, infected, NTZ-treatment groups in both immunocompetent and immunosuppressed (vaccinated before or after immunosuppression), and the vaccination before immunosuppression was better than vaccination after immunosuppression.

INTRODUCTION

Cryptosporidium sp. is a protozoan parasite that causes diarrheal disease (cryptosporidiosis) in humans by infecting the epithelial cells of the small intestine (Huston & Petri 2001). One of 2 species of *Cryptosporidium* infect human: *C. hominis* which is transmitted primarily person to person or *C. parvum*, a species that can be transmitted person to person or zoonotically (Priest et al., 2001; Frost et al., 2004).

Cryptosporidium sp. causes moderate to severe diarrhea, which is more common in young and immunocompromised people (Urrea-Quezada *et al.*, 2022). Infections can develop from low doses of *Cryptosporidium* oocysts; so, it has major public health implications and can persist in the environment for long periods of time. Humans can be infected with *Cryptosporidium* through direct contact with infected people or animals, ingestion of contaminated food (food borne transmission), and drinking contaminated water (waterborne transmission) (Xiao and Cama 2006).

Profuse watery diarrhea, fever, anorexia, weight loss, weakness, abdominal pains, vomiting, and swollen joints are the most common symptoms of cryptosporidiosis. Recurrence of symptoms after apparent resolution has been frequently reported; nevertheless, in immunocompetent people, the illness is self-limiting, and symptoms usually resolved entirely within 2–3 weeks (Hunter *et al.*, 2004). The highest prevalence has been reported in young children and immunocompromised patients (Hunter and Nichols 2002; Hunter *et al.*, 2004). Cryptosporidiosis infection can be recurrent in certain immunocompetent hosts, it can be fatal in immunocompromised people such AIDS patients and those who take immunosuppressive drugs (Shane *et al.*, 2017). Drug resistance may lead to a resurgence in viral loads and, as a result, an increase in opportunistic infections. Malnutrition can contribute to greater rates of infection among children in underdeveloped countries (Amadi *et al.*, 2001; Saleem and Haque 2009). Furthermore, cryptosporidiosis has the potential to cause severe illness and mortality in humans, particularly in children living in resource-poor environments in developing nations (Bouزيد *et al.*, 2018).

The prevalence of cryptosporidiosis in 19 investigations conducted in immunocompetent persons with diarrheal illnesses in Egypt revealed significant heterogeneity, with prevalence ranging from 0% to 47% (Youssef *et al.*, 2008). Cryptosporidiosis was found to be present in 5.9% of diarrheic patients in Cairo (Abd El Kader *et al.*, 2012). Moreover, In Egypt, the rate of *Cryptosporidium* infection prevalence among children was 13.51% with a peak among the age period (5-10) years old with significant relation between males and females, between infection and low socioeconomic level in rural areas and between the infection and the presence of animal contact (Shalaby 2015). Helmy *et al.*, (2015) reported that in Ismailia, the prevalence of *Cryptosporidium* was 49.1% among diarrheal children. He discovered that *Cryptosporidium hominis* dominated (60%) and *Cryptosporidium parvum* (38%) respectively.

Several *Cryptosporidium* antibiotics have been described, spiramycin producing partial responses against the parasite (partial decrease in diarrhea and decreases in stool oocyst number). Paromomycin has been shown to decrease the intensity of infection and improve intestinal function and morphology, paromomycin is a poorly absorbed broad-spectrum antibiotic like neomycin (Gargala 2008). Cryptosporidiosis can be treated by nitazoxanide, which was approved by the Food and Drug Administration (FDA) for all immunocompetent patients aged ≥ 1 years (CDC 2012). However, nitazoxanide is useless in the absence of adequate immune responses, it is ineffective in immunocompromised people (Gargala 2008).

As an effective drug treatment is almost absent, vaccine development that prevents disease or reduces the severity of infection is a relevant option, especially for immunocompromised individuals and children (Dillingham *et al.*, 2002). We evaluated the protective role of *Cryptosporidium parvum* oocysts antigen or/and nitazoxanide against cryptosporidiosis infected immunocompetent and immunosuppressed mice by parasitological, immunological and histopathological parameters.

MATERIALS AND METHODS

1. Animals

160 male CD-1 Swiss mice, aged 6–7 weeks with a weight range of 20–25 gm, were provided by the Schistosome biological supply program (SBSP) at Theodor Bilharz Research Institute (TBRI), Giza, Egypt.

2. Parasite Isolation

Cryptosporidium parvum oocysts used for infection of mice involved in the present study were obtained from the Animal Reproduction Research Institute, Giza, Egypt. The faecal samples of infected diarrhetic calves were collected in sterile clean faecal cups to be isolated by sedimentation and flotation method (Waldman *et al.*, 1986; Zeibig 1997). The genotype of the present *Cryptosporidium* species was molecularly determined in a previous study (Mahmood *et al.*, 2016) by some present authors.

3. *Cryptosporidium parvum* Oocysts Antigen Preparation

Cleaned oocysts suspended in phosphate buffered saline (PBS) (pH 7.2), and homogenized, then the oocyst suspension was sonicated on ice, and left overnight at 4°C under magnetic stirring. The oocyst suspension was centrifuged 500x g for 10 min, the supernatant was divided into aliquots and stored at -70°C to be used (Gomez Morales *et al.*, 1995). The protein content of the centrifuged and autoclaved supernatant was measured by the Bradford method (Bradford 1976).

4. Immunization

CD1 immunized mice groups (10 mice/group) were intramuscularly injected with *C. parvum* oocysts crude antigen through three consecutive doses within three weeks. The priming dose in the form of intramuscular injection (i.m) with 100 µg antigen/200 µl PBS mixed in complete Freund's Adjuvant, (Sigma). Followed by two booster doses, each was 50 µg antigen emulsified in incomplete Freund's Adjuvant (Sigma). The first boosting dose was two weeks after the priming dose. After a week, the second boosting dose was given according to (Fagbemi *et al.*, 1995; Guobadia and Fagbemi 1997).

5. Infection

The mice were divided into an immunocompetent group, composed of 50 mice, and an immunosuppressed group composed of 100 mice. Each mouse was infected by oral inoculation using oral-gastric gavage with the isolated *C. parvum* oocysts (200 µl *C. parvum* oocyst/PBS) in a dose of about 10000 oocysts/ mouse (Gaafar 2007). 21 days post-infection, mice were sacrificed for parasitological, immunological, and histopathological studies.

6. Drugs

6.1. Dexamethasone

Immunosuppression of the mice was performed by giving synthetic corticosteroids (Dexamethasone) (Dexazone) orally at a dose of 0.25 µg/g/day for 14 successive days prior to inoculation with *C. parvum* oocysts (Rehg *et al.*, 1988; Abdou *et al.*, 2013). The immunosuppressed mice continued to receive Dexamethasone at the same dose throughout the experiment. Dexazone (0.5mg) was manufactured and provided by Kahira Pharmaceuticals and

Chemical Industries Company [Shoubra, Cairo, Egypt].

6.2. Nitazoxanide (NTZ)

Nanazoxid tablets (Batch No. 26428/2009) are labelled to contain 500 mg of nitazoxanide (NTZ) per tablet, produced by Pharmed Healthcare Pharmaceuticals, Menofeya, Egypt for Utopia Pharmaceuticals. Seven days post-infection nitazoxanide was orally given to mice in a dose of 200 mg/kg/body weight for seven successive days (**Abd El-Aziz *et al.*, 2014**).

7. Experimental Design

Two experiments were carried out independently. Animals were divided as follows: Control group (A): comprising of 10 mice were left unvaccinated, uninfected, and untreated (negative control group).

Experiment 1 was done on immunocompetent mice; Group (B): 50 mice were subdivided into 5 subgroups (10 for each); Subgroup B₁: infected untreated mice. Subgroup B₂: infected & treated mice with (NTZ). Subgroup B₃: vaccinated mice. Subgroup B₄: vaccinated then infected mice. Subgroup B₅: vaccinated, infected, then treated with (NTZ).

Experiment 2 was done on immunosuppressed mice; Group (C): 100 mice were subdivided into 10 subgroups (10 for each); Subgroup C₁: immunosuppressed, unvaccinated, uninfected, and untreated mice. Subgroup C₂: immunosuppressed, infected, and untreated mice. Subgroup C₃: immunosuppressed, infected, then treated with (NTZ). Subgroup C₄: immunosuppressed, vaccinated mice. Subgroup C₅: immunosuppressed, vaccinated, then infected mice. Subgroup C₆: immunosuppressed, vaccinated, infected then treated mice. Subgroup C₇: vaccinated mice (equivalent to B₃ group). Subgroup C₈: vaccinated then immunosuppressed mice group. Subgroup C₉: vaccinated, immunosuppressed, then infected mice. Subgroup C₁₀: vaccinated, immunosuppressed, infected, then treated mice.

8. Oocysts' count

Twenty-one days post-infection; individual faecal samples were collected after dissection from the last part of intestine. The stool samples were leaved to dry, each sample was weighted and dissolved in saline solution. The oocysts were stained by modified Ziehl-Neelsen (MZN) staining technique according to (**John and Petri 2006**), counted and calculated as "oocyst/gm" of faeces.

9. Sera Preparation

Sacrification of animals was performed by rapid decapitation in 21 days post-infection. Blood was individually collected into tubes and centrifuged at 3000 rpm for 5 minutes. The clear, non-haemolyzed supernatant serum was removed in clean tubes and stored at -20°C until use.

10. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was used to assess the antibodies in response to *C. parvum* infection in both immunocompetent and immunosuppressed groups in comparison to the control group. This method was performed, with some modifications from the original method of **Engvall and Perlmann (1971) & Voller *et al.*, (1976)**. Mice sera were assessed for cryptosporidiosis antibodies (IgG, IgM, and IgA) against *C. parvum* oocysts' antigen (1 µg/well) in duplicate wells. Horseradish peroxidase (HRP)-labelled goat anti-mouse conjugate (IgG, IgM, and IgA) 100 µl/well, were used at dilution 1:1000 to detect bound antibodies.

Reactivity was estimated spectrophotometrically at 450 nm after adding SureBlue™ TMB (3,3',5,5'-Tetramethylbenzidine) Microwell Peroxidase Substrate.

11. Histopathological Studies

Histopathological examination was accomplished at the Pathology Department, TBRI to clarify the histological changes in duodenum. About 1cm long of duodenal segments were cut off and immediately fixed in 10% buffered formalin. After fixation the segments were processed for routine histopathological examination by Hematoxylin and Eosin stain (H&E) (Drury and Wallington 1980).

12. Statistical Analysis

Independent-samples t-test of significance was used to compare between two means. A one-way analysis of variance (ANOVA) was used when comparing between more than two means.

RESULTS

1. Effect of Nitazoxanide treatment or / and Cryptosporidium parvum oocysts crude antigen on oocysts' count in immunocompetent and immunosuppressed groups

The mean number and percentage of reduction in *C. parvum* oocysts/g faeces in infected immunocompetent and immunosuppressed groups, 21 days post-infection were shown in (Table 1 & Fig. 1). In group B1 (infected-untreated), the mean number of oocysts was ($172.04 \times 10^3 \pm 19.21 \times 10^3$) while it was ($122.83 \times 10^3 \pm 13.06 \times 10^3$) in group B2 (infected then treated with NTZ), and the percentage of reduction in number of *C. parvum* oocysts after 21 days post-infection was (28.60%). A high statistical significance ($P < 0.01$) was observed in group B4 (vaccinated, then infected) the mean number of oocysts was ($51.001 \times 10^3 \pm 5.56 \times 10^3$) and the percentage of reduction was (70.35%). In group B5 (vaccinated, infected, then treated with Nitazoxanide) the mean number of oocysts was ($30.88 \times 10^3 \pm 5.38 \times 10^3$) and the percentage of reduction reached (82.05%) which was very highly significant ($P < 0.001$).

In immunosuppressed groups, mice were given orally Dexamethasone ($0.25 \mu\text{g/g/day}$) for Immunosuppression. Mice were subdivided into C2 (immunosuppressed infected- untreated group), the mean number of oocysts was ($478.64 \times 10^3 \pm 23.89 \times 10^3$) while it was ($361.95 \times 10^3 \pm 30.28 \times 10^3$) in group C3 (immunosuppressed, infected, then treated with NTZ) and the percentage of reduction was (24.40%). A highly statistically significant ($P < 0.01$) was observed in group C5 (immunosuppressed, vaccinated, then infected group) the mean number of oocysts was ($150.06 \times 10^3 \pm 8.38 \times 10^3$) and the percentage of reduction in number of *C. parvum* oocysts was (68.60%). In group C6 (immunosuppressed, vaccinated, infected, then treated with NTZ) the mean number of oocysts was ($120.95 \times 10^3 \pm 5.14 \times 10^3$) and the percentage of reduction reached (74.70%), which is very highly statistically significant ($P < 0.001$) (Fig.1).

Table (1): The mean number and percentage of reduction in *C. Parvum* Oocysts/g faeces in infected immunocompetent & immunosuppressed groups.

		Animals' groups	Mean No. of oocysts (\pm SD $\times 10^3$)	% Reduction in number of <i>C. Parvum</i> oocysts
Immunocompetent groups		B1 (infected –untreated)	172.04 \pm 19.21 ^a	-----
		B2 (infected then treated with Nitazoxanide)	122.83 \pm 13.06 ^b	28.60% *
		B4 (vaccinated, infected)	51.001 \pm 5.56 ^c	70.35% **
		B5 (vaccinated, infected, then treated with Nitazoxanide)	30.88 \pm 5.38 ^d	82.05% ***
Immunosuppressed groups	Immunosuppression before vaccination	C2 (immunosuppressed, infected-untreated)	478.64 \pm 23.89 ^a	-----
		C3 (immunosuppressed, infected, then treated with Nitazoxanide).	361.95 \pm 30.28 ^b	24.40% *
		C5 (immunosuppressed, vaccinated, then infected).	150.06 \pm 8.38 ^c	68.60% **
		C6 (immunosuppressed, vaccinated, infected, then treated with Nitazoxanide).	120.95 \pm 5.14 ^d	74.70% ***
	Immunosuppression	C9 (vaccinated, immunosuppressed, then infected)	123.96 \pm 15.11 ^{de}	74.10% ***
		C10 (vaccinated, immunosuppressed, Infected, then treated with Nitazoxanide)	103.20 \pm 7.49 ^{df}	78.40% ***

Independent Sample t-test: * P -value <0.05 significant; ** (P-value <0.01) highly significant, *** (P-value <0.001) very highly significant.

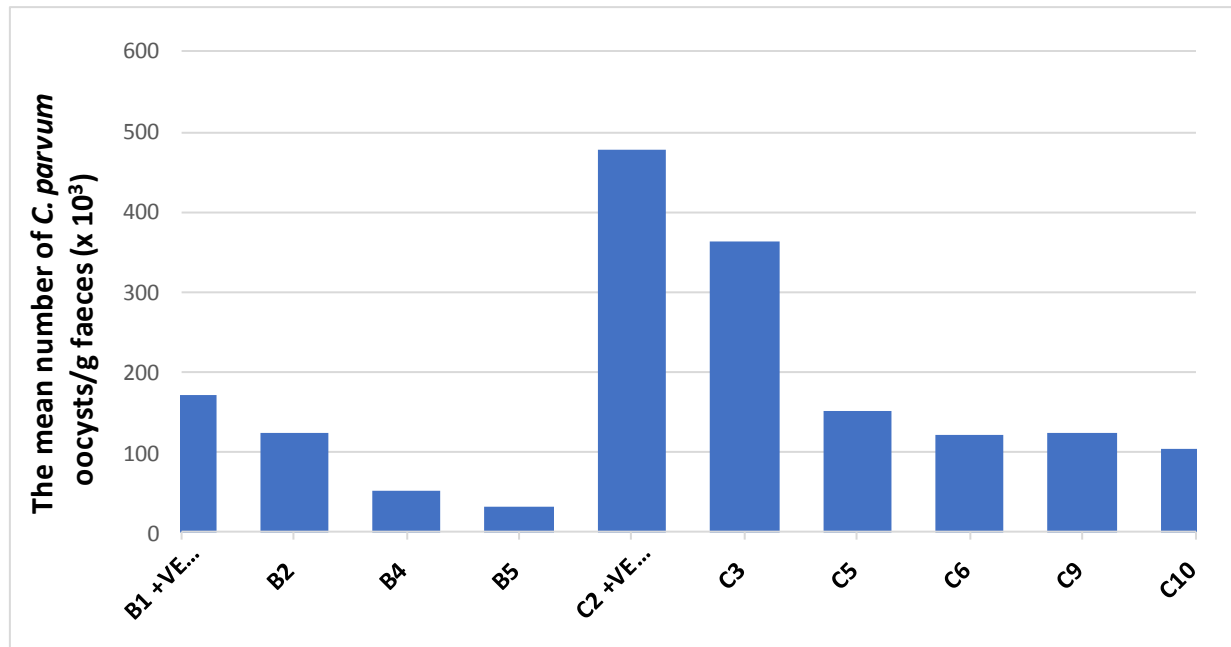


Fig 1 The mean number of *Cryptosporidium parvum* oocysts/g faeces in immunocompetent (B) and immunosuppressed (C) infected groups; **B1**: Infected untreated, **B2**: Infected treated with NTZ, **B4**: Vaccinated infected, **B5**: Vaccinated infected, treated with NTZ, **C2**: Immunosuppressed infected untreated, **C3**: Immunosuppressed infected, treated with NTZ, **C5**: Immunosuppressed vaccinated infected, **C6**: Immunosuppressed vaccinated infected, treated with NTZ, **C9**: Vaccinated immunosuppressed infected, **C10**: Vaccinated immunosuppressed Infected, treated with NTZ.

A very highly statistically significant ($P < 0.001$) was observed in group C9 (vaccinated, immunosuppressed, then infected) the mean number of oocysts was ($123.96 \times 10^3 \pm 15.11 \times 10^3$) and the percentage of reduction in number of *C. parvum* oocysts was (74.10%). In group C10 (vaccinated, immunosuppressed, Infected, then treated with NTZ) the mean number of oocysts was ($103.20 \times 10^3 \pm 7.49 \times 10^3$) and the percentage of reduction reached (78.40%), which is very highly statistically significant ($P < 0.001$) (**Fig. 1**).

2. Immune responses against *C. parvum* oocysts' antigen in Immunocompetent groups

Mice sera were investigated for serum antibodies levels (IgG, IgM & IgA) against *C. parvum* oocysts' antigen in immunocompetent groups, 21 days post-infection (**Table 2**). Compared to B1 group (infected –untreated), all treated immunocompetent mice groups showed a significant decrease in mean sera IgG levels.

A highly statistically significant decrease (P -value < 0.01) was found in mean sera levels of IgG in groups B5 (vaccine, infection, then treated with NTZ) and B2 (infected then treated with NTZ) (0.585 ± 0.040 & 0.654 ± 0.065 , respectively), with percentages of reduction reached 56.54% & 51.41%, respectively. Statistically, a significant decrease (P -value < 0.05) was found in mean sera IgG levels in both groups B3 (vaccinated) and B4 (vaccinated then infected) (0.839 ± 0.083 & 0.860 ± 0.083), with percentages of reduction were (37.67% & 36.11%, respectively) (**Fig. 2**).

Concerning IgM sera levels, a significant decrease (P -value < 0.05) was observed in both groups B5 (vaccine, infection, then treated with NTZ) and B2 (infected then treated with NTZ) (0.175 ± 0.031 & 0.216 ± 0.057 , respectively), with percentages of reduction were (42.24% & 28.71%, respectively). On the other hand, an insignificant increase (P -value > 0.05), in mean IgM sera levels, was found in both groups B3 (vaccinated) and B4 (vaccinated then infected) groups.

Belonging to IgA serum levels, a highly statistically significant decrease (P -value < 0.01) was found in group B2 (infected then treated with NTZ) (0.391 ± 0.056) with a percentage of reduction reached (43.98%). Furthermore, a significant decrease (P -value < 0.05) was found in both groups B5 (vaccine, infection, then treated with NTZ) and B3 (vaccinated) (0.491 ± 0.043 & 0.596 ± 0.037), with percentages of reduction were (29.66% & 14.61%, respectively). Group B4 (vaccinated then infected), statistically, showed an insignificant decrease (P -value > 0.05) (0.652 ± 0.034) (**Fig.2**).

Table (2): Mean sera antibodies' levels of IgG, IgM & IgA in immunocompetent & immnosuppressed groups

		Animals' groups	IgG		IgM		IgA	
			Mean absorbance (450 nm) ±SD	% Change (to infec./untre.)	Mean absorbance (450 nm) ±SD	% Change (to infec./untre.)	Mean absorbance (450 nm) ±SD	% Change (to infec./untre.)
Immu-competent groups		A (uninfected)	0.201±0.005a		0.101±0.015a		0.038±0.014a	
		B1 (infected, untreated)	1.346±0.072 ^b	---	0.303±0.017 ^b	---	0.698±0.051 ^b	---
		B2 (infected, treated with NTZ)	0.654±0.065 ^c	51.41% **↓	0.216±0.057 ^c	28.71% *↓	0.391±0.056 ^c	43.98% **↓
		B3(vaccinated)	0.839±0.083 ^d	37.67% *↓	0.305±0.039 ^{bd}	0.66%	0.596±0.037 ^d	14.61% *↓
		B4 (vaccinated, infected)	0.860±0.083 ^{de}	36.11% *↓	0.309±0.069 ^{bde}	1.98%	0.652±0.034 ^{be}	6.59%
		B5 (vaccinated, infected, treated with NTZ)	0.585±0.040 ^f	56.54% **↓	0.175±0.031 ^f	42.24% *↓	0.491±0.043 ^f	29.66% *↓
Immunosuppressed groups	Immunosuppression before vaccination	C2 (immunosuppressed, infected, untreated)	0.766±0.040 ^c	---	0.191±0.046 ^c	---	0.620±0.087 ^c	---
		C3 (immunosuppressed, infected, treated with NTZ).	0.667±0.032 ^d	12.92% * ↓	0.188±0.007 ^{cd}	1.57%	0.539±0.026 ^d	13.06% *↓
		C4 (immunosuppressed, vaccinated)	0.600±0.048 ^{de}	21.67% **↓	0.229±0.040 ^e	19.90% *↑	0.607±0.050 ^{ce}	2.10
		C5 (immunosuppressed, vaccinated, infected).	0.963±0.088 ^f	25.72% ** ↑	0.316±0.016 ^f	65.45% **↑	0.791±0.031 ^f	27.58% **↑
		C6 (immunosuppressed, vaccinated, infected, treated with NTZ).	0.752±0.080 ^{cg}	1.83%	0.229±0.020 ^{eg}	19.90% *↑	0.697±0.015 ^g	12.42% *↑
	C7 (vaccinated)	0.822±0.077 ^h	6.8% *↑	0.307±0.035 ^{fh}	59.7% **↑	0.591±0.033 ^{cehk}	4.9%	
	Immunosuppression after vaccination	C8(vaccinated, immunosuppressed)	0.707±0.045 ^{dgl}	7.70% *↓	0.282±0.042 ^{hi}	47.64% **↑	0.653±0.081 ^{cehgl}	5.32%
		C9 (vaccinated, immunosuppressed, infected)	0.893±0.010 ^{hjl}	16.58% ** ↑	0.311±0.015 ^{fhj}	62.83% **↑	0.729±0.046 ^{gj}	17.58% **↑
		C10 (vaccinated, immunosuppressed, Infected, treated with NZ)	0.658±0.082 ^{dK}	14.10% *↓	0.199±0.008 ^{cdk}	4.19%	0.635±0.052 ^{cehK}	2.42%

Using One Way Analysis of Variance: ***P-value <0.001 very highly significant. Using: Independent Sample t-test: * (P-value <0.05) significant; ** (P-value <0.01) highly significant, (P-value >0.05) insignificant. Categories labelled with different letters are statistically significant.

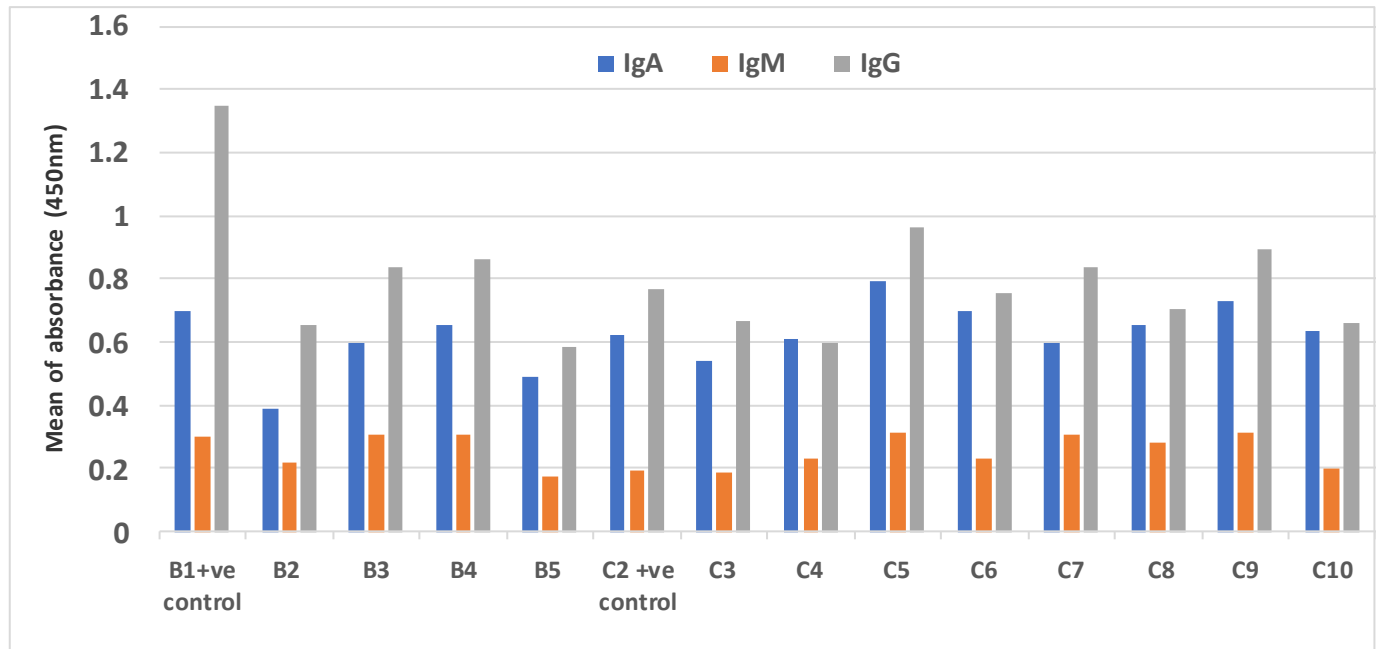


Fig 2 The mean sera antibodies' levels (IgG, IgM & IgA) in immunocompetent groups; **B1**: Infected untreated, **B2**: Infected treated with NTZ, **B3**: Vaccinated, **B4**: Vaccinated infected, **B5**: Vaccinated infected, treated with NTZ.

3. Immune responses against *C. parvum* oocysts' antigen in immunosuppressed groups

In immunosuppressed groups, 21 days post infection, mice sera were investigated for serum antibodies levels against *C. parvum* oocysts' antigen. A highly statistically significant decrease ($P < 0.01$) in the mean IgG sera levels was only observed in group; C4 (immunosuppressed then vaccinated) (0.600 ± 0.048), with a percentage (21.67%) in comparison to infected untreated group. A statistically significant decrease (P -value < 0.05) in the mean IgG sera level (0.667 ± 0.032 , 0.707 ± 0.045 , & 0.658 ± 0.082) was observed in group C3 (immunosuppressed, infected, then treated with NTZ), C8 (vaccinated then immunosuppressed) and C10 (vaccinated, immunosuppressed, infected, then treated with NTZ), respectively. The percentage of reduction was (12.92%, 7.70%, & 14.10%), respectively in comparison to infected untreated group. Statistically insignificant decrease (P -value > 0.05) was observed in the mean IgG sera level in group C6 (immunosuppressed, vaccinated, infected, then treated with NTZ). On the other hand, the mean sera IgG levels in group C5 (immunosuppressed, vaccinated, then infected), C7 (vaccinated), and C9 (vaccinated, immunosuppressed, then infected) (0.963 ± 0.088 , 0.839 ± 0.083 , and 0.893 ± 0.01), respectively, showed highly significant increase (P -value < 0.01) as compared to infected untreated group (**Table 2**)

In case of IgM sera levels, statistically insignificant decrease (P -value > 0.05) in the mean IgM sera levels (0.188 ± 0.007) was observed in group C3 (immunosuppressed, infected, then treated with NTZ) as compared to infected untreated group. Although, a significant increase (P -value < 0.05) in the mean IgM sera level (0.229 ± 0.040 , 0.316 ± 0.016 , 0.229 ± 0.020 , 0.305 ± 0.039 , 0.282 ± 0.04 , & 0.311 ± 0.015) was observed in groups C4 (immunosuppressed then vaccinated), C5 (immunosuppressed, vaccinated, then infected), C6 (immunosuppressed, vaccinated, infected, then treated with NTZ), C7 (vaccinated), C8 (vaccinated then immunosuppressed), and C9 (vaccinated, immunosuppressed, then infected). And the percentage of increase was

(19.90%, 65.45%, 19.90%, 59.69%, 47.64%, & 62.83%), respectively in comparison to infected untreated group.

As regards IgA sera levels, a statistically significant decrease (P-value <0.05) in the mean IgA sera level (0.539 ± 0.026) was observed in group C3 (immunosuppressed, infected, then treated with NTZ) and the percentage of reduction was (13.06%) compared to infected untreated group. A statistically insignificant decrease (P-value >0.05) in the mean IgA sera level was observed in group C4 (immunosuppressed then vaccinated) and C7 (vaccinated) compared to infected untreated group. However, the mean IgA sera levels (0.791 ± 0.031 , 0.697 ± 0.015 , & 0.729 ± 0.046) showed a significant increase (P-value <0.05), in groups C5 (immunosuppressed, vaccinated, then infected) C6 (immunosuppressed, vaccinated, infected, then treated with NTZ), and C9 (vaccinated, immunosuppressed, then infected). And the percentage of increase was (27.58%, 12.42%, and 17.58%) compared to infected untreated group (**Fig.3**).

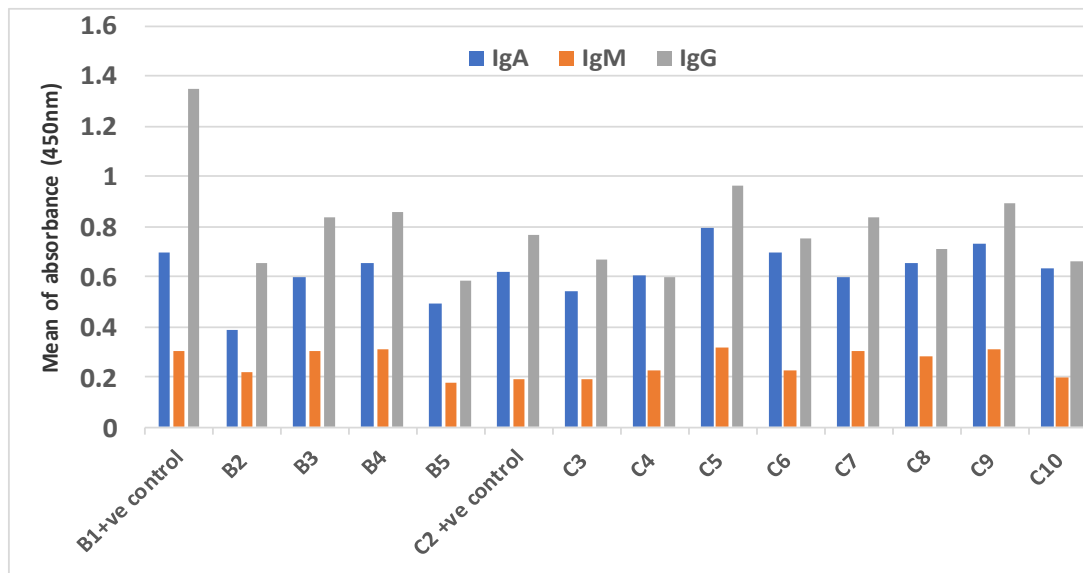


Fig 3 The mean sera antibodies' levels (IgG, IgM & IgA) in immunosuppressed groups; **C2**: Immunosuppressed infected untreated, **C3**: Immunosuppressed infected treated with NTZ, **C4**: Vaccinated, **C5**: Immunosuppressed vaccinated infected, **C6**: Immunosuppressed vaccinated infected, treated with NTZ, **C7**: Vaccinated, **C8**: Vaccinated immunosuppressed, **C9**: Vaccinated immunosuppressed infected, **C10**: Vaccinated immunosuppressed infected, treated with NTZ.

4. Histopathological study

Histopathological examination of negative control groups (uninfected/untreated) showed normal villous architecture with average length and width of villi (crypt-villous ratio is 1:3 to 1:5). Besides, no inflammatory exudation in the lamina propria (**Fig. 4A**). Immunocompetent group B1 (infected, untreated) revealed the many histopathological changes in the intestinal mucosa which characterizes cryptosporidiosis, including villous shortening (villous atrophy), accompanied with broaden and epithelial proliferations (**Fig.4B**), besides, fusion of some villi, goblet cell hyperplasia and vacuolations in many enterocytes. (**Fig.4C**). Comparing to Immunocompetent group B1, immunosuppressed, infected & untreated group C2 showed more

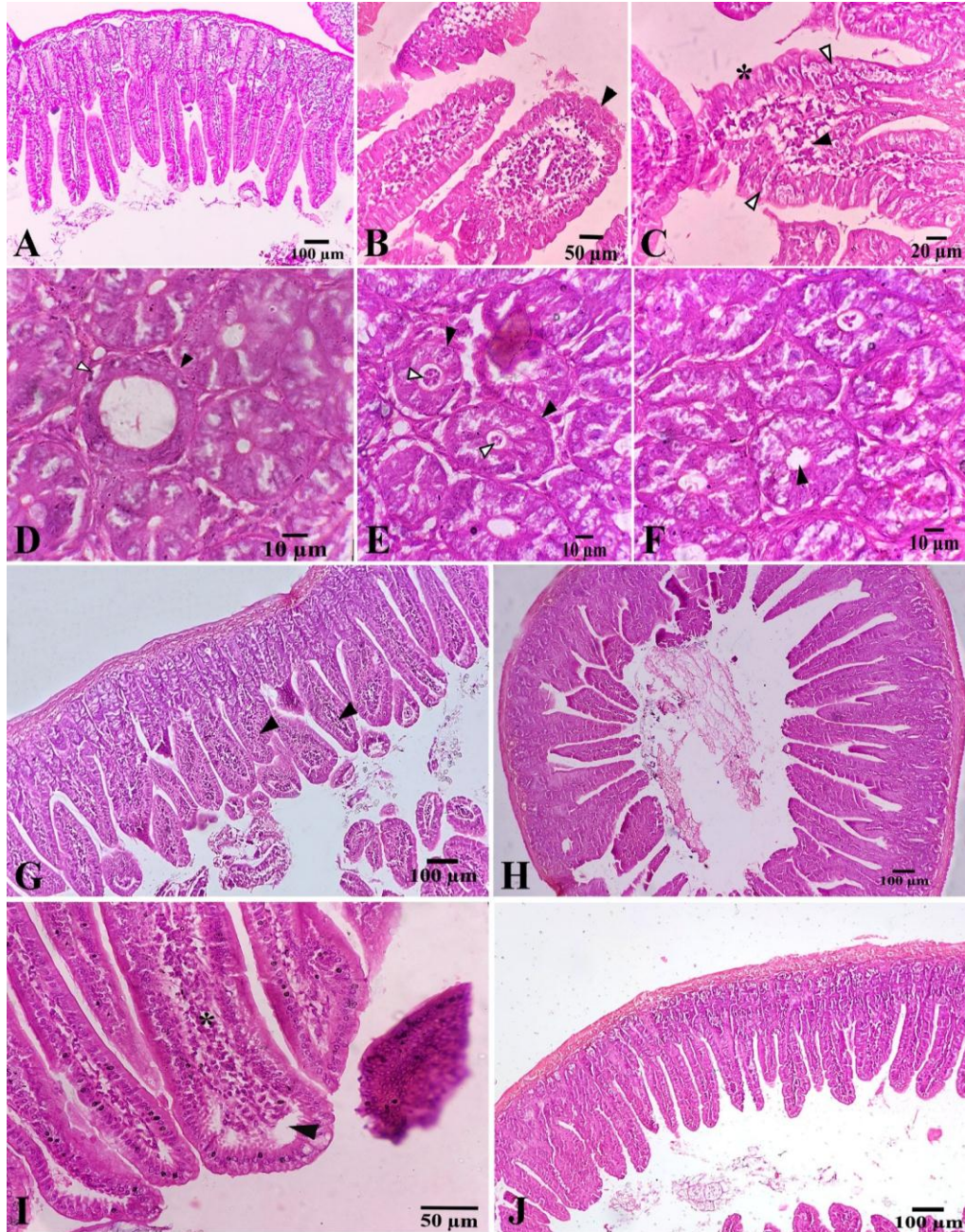


Fig.4 Sections of small intestine of (A) Uninfected untreated group, showing normal crypt-villous ratio of 1:3 to 1:5 with normal mucosa (B & C) Infected untreated (B1) B: showing short blunt villous with subendothelial edema (Gruenhang space) and epithelial proliferation (black arrowhead). H&E; C: showing fusion in some villi (black arrowhead), goblet cell hyperplasia (white arrow heads) and vacuolations in many enterocytes (asterisks) (D, E & F) Immunosuppressed infected untreated group (C2) D: showing apoptotic bodies (white arrowhead) and cryptic degeneration (black arrowhead); E: showing cryptic abscesses (black arrowheads) with neutrophils aggregations (white arrowheads); F: showing developmental stages of *C. parvum* into cryptic cavity (black arrowhead) (G) Vaccinated infected treated with NTZ (B5) showing normal healthy villi with normal villous architecture and inflammatory infiltrates in lamina propria (black arrowheads) (H & I) Vaccinated, immunosuppressed, infected NTZ-treated group (C10) H: showing normal healthy villi with crypt-villous ratio 1-3 and 1-5; I: showing rarely seen hypertrophied villous ends with sub epithelial edema (black arrowheads) and mild inflammatory infiltrates in lamina propria (asterisks) (J) Immunosuppressed vaccinated infected and NTZ-treated group (C6) showing normal villous architecture and mostly healthy epithelia. "Hematoxylin and eosin staining"

additional tremendous histopathological aspects, which include: cryptic degeneration with apoptotic bodies, (**Fig.4D**) & cryptic abscesses with neutrophils aggregation (**Fig.4E**). besides, developmental stages of *C. parvum* were observed into the cryptic cavity (**Fig.4F**). From the comparative histopathological examinations for experimental groups in this study (excluding the vaccinated uninfected ones) it can be observed that the groups B5 (vaccinated and infected), C10 (vaccinated, immunosuppressed, infected, treated with NTZ) and C6 (immunosuppressed, vaccinated, infected, treated with NTZ) were among the groups that showed the highest degree of improvements in profiles of intestinal tissues. In group B5, few histopathological changes in the intestinal mucosa were observed including inflammatory infiltrates in lamina propria but normal healthy villi with normal villous architecture (**Fig.4G**). Group C10 showed major histological improvements in the intestinal mucosa (**Fig.4H**) but still shows some histopathological aspects represented in hypertrophied villous ends with slight subepithelial edema and mild inflammatory infiltrates in lamina propria (**Fig.4I**). Concerning to group C6, it was the least in histopathological changes, which include few areas of mild villous atrophy (**Fig.4J**). Away from NTZ-treated groups, the vaccinated infected groups, B4, C5 & C9 revealed fewer improvements (than B5, C6 & C10) with more histopathological effects. Comparatively, C9 still show more histopathological impacts [paracellular spaces among enterocytes, degeneration in many epithelial apical regions (**Fig.5A**) & some developmental stages of parasite (**Fig.5B**)] than B4 & C5 (**Fig.5C & 5D**), respectively. In vaccinated groups B3, C4 & C8 showed the normal histological features of healthy intestinal mucosa.

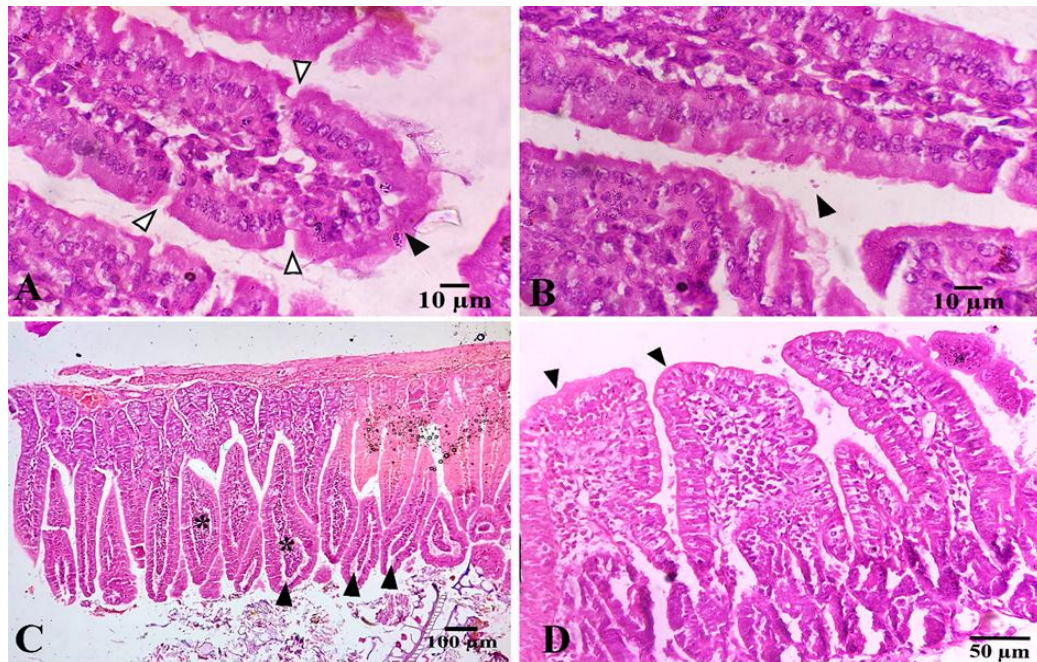


Fig.5 Sections of small intestine of (A) Immunosuppressed infected untreated group (C2), showing degeneration in enterocytes (black arrowheads) and pyknotic nuclei (white arrowheads) (B) Vaccinated immunosuppressed infected untreated group (C9) showing some developmental stages of parasites seen among intervillus spaces (black arrowhead) (C) Vaccinated infected group (B4) showing few villi with villous hypertrophy with subepithelial edema (black arrowheads) and mononuclear infiltrates in lamina propria (asterisk) (D) Immunosuppressed vaccinated infected group (C5) showing few blunt short broad villi (black arrowheads). “Hematoxylin and eosin staining”

DISCUSSION

Cryptosporidium spp. are apicomplexan protozoans that form oocysts and complete their life cycles in humans and animals via zoonotic and anthroponotic transmission, resulting in cryptosporidiosis. They cause moderate to severe diarrhea which is more common in young and immunocompromised people (**Putignani and Menichella 2010; Robertson et al., 2020 & Urrea-Quezada et al., 2022**).

In the current study, Dexamethasone was used to induce immunosuppression in the mice as it was considered as a good immunosuppressive agent in numerous studies mainly in mice (**Miller and Schaefer 2007**). It has a high glucocorticoid activity, with inhibitory effects on the immune response (**Stojadinovic et al., 2007**).

The intensity of oocyst shedding continued until day 21 (PI) and oocyst count was significantly higher in dexamethasone immunosuppressed mice than in immunocompetent ones. These results are in agreements with **Abdou et al., (2013) & Atia et al., (2018)**, who also stated that Swiss albino immunosuppressed mice continued to shed oocysts of *C. parvum* until day 30 (PI). Also, **Matsui et al., (2001)** revealed that the interval which covers the natural shedding period of *Cryptosporidium* infection in mice is about 24 days PI. As well as patients with acute infection of cryptosporidiosis, acute watery diarrhea can be persistent and last for up to 5 weeks (**Borad and Ward 2010**). Clearance of *Cryptosporidium* oocysts from stools is very difficult as elimination of the parasite needs a competent host immune system with defense mechanisms which are able to fight the infection and reject the parasite rapidly (**Gargala 2008; Takeuchi et al., 2008**).

Cryptosporidiosis is resistant to the majority of chemotherapeutic agents used to treat parasitic diseases. The current therapeutic drug (NTZ) has been approved by the Food and Drug Administration (FDA) for *Cryptosporidium* infection, on the other hand, it only shows moderate effectiveness in immunocompetent children and produces no benefit in immunosuppressed patients (**Atia et al., 2016**). Finding a reliable drug remains an important goal (**Mead and Arrowood 2014; Widmer et al., 2020**). Some in vitro and in vivo studies on cryptosporidiosis have reported a better response to NTZ in combination treatments than to NTZ alone (**Krause et al., 2012; Bhadauria et al., 2015**). This study was conducted to evaluate the anti-cryptosporidiosis effectiveness of (NTZ) or / and the protective role of *C. parvum* oocysts crude antigen (vaccine), besides, the role of NTZ in improving the efficacy of the vaccine in *C. parvum* infected immunocompetent and immunosuppressed mice groups.

In comparison with NTZ-treated infected groups B2, C3, the vaccinated infected groups, B4 & C5, C9, showed more significant (P-value <0.01, 70.35% & 68.60% in B4 & C5, respectively) and high significant reductions (P-value <0.001, 74.10% in C9) in oocyst count. These results reflect the remarkable effectiveness of the vaccine, in both immunocompetent & immunosuppressed mice, when compared to chemotherapy treatment only (NTZ). This strong efficacy of the oocyst antigenic vaccine may be attributed by the fact that the cyst wall contains 17 different proteins (**de Graaf et al., 1999**), mainly, CP15 has been identified as an immunodominant antigen (**Reperant et al., 1994; Sagodira et al., 1999**). Also, CP15 was

identified and shed from the surface of sporozoites during gliding motility on the host enterocyte cells before the invasion (**Arrowood *et al.*, 1991**). The present oocyst crude vaccine (containing CP15 and other proteins) may act as an immunogenic substrate (stimulating the adaptive immunity) which indirectly hamper the attachment or/and the invasion process by sporozoites, consequently, less oocyst shedding.

Harp & Goff (1995) used lyophilized *C. parvum* oocysts as an oral vaccine in newborn calves, 3 out of 9 vaccinated ones (33.3%) did not shed any detectable oocysts. The limited effectivity of their vaccine can be attributed to using the contact oocysts (not homogenized & sonicated as in the present study) which in turn leads to the detection of a larger number of internal surface proteins in the oocyst wall, besides, the oral route of immunization which may be inefficient like as intramuscular one, applied in this study, which could evoke more powerful immunological action. In another study, **Jenkins *et al.*, (1999)** stated that immunosuppressed mice receiving immune colostrum (obtained by direct injection of recombinant pCP15/60 plasmid DNA in preparturient cows) showed partial protection (50% reduction) against intestinal *C. parvum* development compared to mice receiving control colostrum. The authors added that this partial protection was evident at a challenge dose of 10^3 *C. parvum* oocysts per mouse while at a dose of 10^4 *C. parvum* oocysts, no protection was established. In contrast in the present study, the reduction in oocyst shedding reach 68.60% (in the immunosuppressed group C5) when challenged with a dose of *C. parvum* oocysts (10^4). **Yu *et al.*, (2003)** reported that attenuated *Cryptosporidium* oocysts (using γ -irradiation) was revealed to decrease parasite reproduction (57%) in C57BL/6 mice after oral administration. Besides, they showed that the elimination of infectivity of oocysts in environmental media required gamma radiation dose reach 50.000 Gy, 25-83 times more than the amount needed to control *Toxoplasma gondi* & *Eimeria necatrix*.

In light of the multiple treatments, in both immunocompetent & immunosuppressed groups, separately, the highest percentage of reduction in oocysts shedding was found in group B5 (vaccinated, infected, and treated with NTZ) and C10 (vaccinated, immunosuppressed, infected, treated with NTZ), respectively. The percentage of reductions reached (82.05% & 78.40%) which were very highly significant. Several studies assessed using multiple treatments against Cryptosporidiosis, especially with NTZ. **Mostafa *et al.*, (2018)**, found that the combination of Artesunate and NTZ showed a synergistic effect by reducing the number of *C. parvum* oocysts in immunosuppressed mice with percentage of reductions (68.5, 75.9, 99%) after 7, 14 and 21 days when compared with that treated by either Artesunate or Nitazoxanide alone. In addition, **Atia *et al.*, (2021)** revealed that in immunosuppressed infected mice prophylaxis with immunostimulant Azoximer Bromide (AZB) followed by treatment with AZB+NTZ produced a good synergistic prophylactic effect on reduction of oocysts with the highest percentage of reduction. **Moawad *et al.*, (2021)** reported that treatment of *Cryptosporidium* infected mice with NTZ loaded on chitosan nanoparticles (CS NPs) resulted in the highest significant reduction in oocysts shedding in both immunocompetent and immunosuppressed groups followed by treatment with NTZ than by treatment with CS NPs

alone at 19th day PI, with percentage of reductions (57.1%, 75.7, & 14.3%) respectively. Above results confirmed with other experimental studies that the combined use of antiparasitic drugs and immunomodulators leads to successful immunization against cryptosporidiosis.

Regarding the immune response, the specific antibodies of *Cryptosporidium* sp. (serum IgM, IgA and IgG) are generated following infection. These antibodies are insufficient to prevent and control *Cryptosporidium* infection (**Kassa et al., 1991**) and are not essential for the recovery and clearance of the parasites (**Mead, 2014**). In contrast, gut-associated lymphoid tissue (GALT) is a key component of the immune mucosal response against *Cryptosporidium* (**Patricio et al., 2011**). Few studies reported that neonatal and weaned mice (with low immune responses) have a high susceptibility to *Cryptosporidium* infection accomplished with decreased mucosal IFN- γ secretion (**Costa et al., 2011**). So, some studies elucidated that IFN- γ levels can be enhanced by the systemic exposure to immunogenic antigens of the parasite, intranasally (**Manque et al., 2012; Roche et al., 2013**), prior to infection, which can partially attenuate *Cryptosporidium* susceptibility. Accordingly, in the present study, vaccination (*C. parvum* oocysts crude) was introduced through the intramuscular route to enhance the systemic immune response (IgM, IgA and IgG), especially, the IFN- γ -producing peripheral blood mononuclear cells (**Preidis et al., 2007**). Besides, **Aguirre et al., (1998)** stated that IL-4-producing CD4+ T cells (Humoral immune response) were important to accelerate the resolution of infection. These findings explain the present result that immunosuppressed/vaccinated (C5) & vaccinated/immunosuppressed infected groups (C9) showed highly significant increases in serum concentrations of all three types of immunoglobulins (IgG, IgM and IgA) (25.72%, 65.45%, 27.58% & 16.58%, 62.83%, 17.58%, respectively) where the vaccination process (intramuscularly injected oocyst crude antigen) caused a prolonged enhancing effect on the adaptive immune response.

NTZ has a significant feature, its ability to promote balance between proinflammatory and anti-inflammatory responses (**Shakya et al., 2018**). **Castillo-Salazar et al., (2021)** reported that NTZ exerts an immunomodulatory effect on peripheral blood mononuclear cells and activates Th₁ immune response, lowering their secreted proinflammatory cytokines, besides (IL-2, IL-6, IL-10, and IL-12), it decreases M1 macrophages subpopulation (produce proinflammatory cytokines) and increases M2 macrophage anti-inflammatory subpopulation by its action on miR-155-5p & miR-146a-5p, respectively. These findings can likely be the main explanations for significant decreases in most of the present serum immunoglobulin titers in infected NTZ-treated groups (B2 & C3) after 21 post-infection. However, when NTZ was used as a co-therapeutic drug in vaccinated immunosuppressed infected groups (C6 & C10), also remarkable decreases in immunoglobulin levels were observed if compared with corresponding ones in C5 & C9, respectively. These results indicate the immunoregulatory action of NTZ (**Lokhande & Devarajan 2021**). In terms of influence on the parasite, NTZ is metabolized to tizoxanide and tizoxanide-glucuronide which inhibit the growth of *C. parvum* sporozoites & oocysts (**Theodos et al., 1998**). This inhibitory effect was caused by the fact that NTZ interferes with pyruvate-ferrodoxin oxidoreductase enzyme which is important for anaerobic glucose energy metabolism

(Hoffman *et al.*, 2007). On the other hand, most of the metabolized effective form of NTZ (tizoxanide) is excreted with feces by bile section, explaining the effectiveness of the drug in directly affecting the parasite over a period of 240 h (Broekhuysen *et al.*, 2000).

In conclusion group B5 (vaccinated, infected, treatment with NTZ) showed the highest improvement in the immune status of the mice where they may be Th₁/Th₂ response and they were the nearest group to recovery. These results are in agreement with Mohamed *et al.*, (2019), who reported that immunological studies against *C. parvum* as IgG and IgM showed improvement of immune status after treatment by Baraka and Chitosan nanoparticles. IgM, IgG, and IgA titers normally increase during infection and decrease after recovery, while IgG in serum might last for months longer than IgM (Ungar *et al.*, 1986). In addition, Wolska-Kusnierz *et al.*, (2007) revealed that IgG, IgM, and IgA were found in the serum and mucosa of people and animals with diarrhea and oocyst shedding.

Generally, the immunocompetent groups showed better immune response than immunosuppressed groups. The highest mean levels were detected in group C5 (immunosuppressed, vaccinated, then infected) and group C9 (vaccinated, immunosuppressed, then infected) compared to normal control group A (uninfected - untreated). These different results in immune response between the immunocompetent and the immunosuppressed groups due to the decrease in the efficiency of the immune system of mice, so took longer time for recovery. These results are in accordance with Cozon *et al.*, (1994) who found that AIDS patients with chronic cryptosporidiosis, a high sera IgA titer has been seen. In another study AIDS patients with cryptosporidiosis have larger amounts of IgA and IgM plasma cells in the duodenal lamina propria, as well as higher total and specific faecal IgA levels than AIDS patients with other enteric illnesses or healthy controls (Benhamou *et al.*, 1995).

In immunosuppressed patients, extraintestinal cryptosporidiosis may occur in the biliary tract, pancreas, and respiratory tract (Dirim *et al.*, 2003; Reina *et al.*, 2016; Dupuy *et al.*, 2021). Recently, it seems to be approved presence of *Cryptosporidium* DNA in blood and cerebrospinal fluid of HIV patients (Velásquez 2018), that open a probability to multi-organ dissemination of *Cryptosporidium*. Administration of the present vaccine intramuscularly can be interestingly valuable in immunodeficiency patients to increase the protection level against the development of extraintestinal cryptosporidiosis. The abovementioned concept is supported by the present fact that our vaccination evoked the serum levels of immunoglobulins in uninfected immunosuppressed groups.

Concerning histopathological results, it was similar to other cryptosporidium-included experimental works; hyperplasia and atrophy in villi (Casemore *et al.*, 1985), shortening, broadening, and ulceration of villi with mild inflammation (Abdel-maksoud *et al.*, 2022), epithelial paracellular gaps (France & Turner 2017), mucosal damage and neutrophil infiltration in immunocompromised mice (Laurent & Lacroix-Lamande 2017). In this study, the histopathological changes were focused on the duodenal region, but the infection was observed in other regions of the ileum. The intestinal epithelial cells are considered an initial mechanical and functional barrier against cryptosporidiosis invasion (Peterson & Artis 2014),

besides they are the essential sites for the life cycle of the parasite. Accordingly, these cells are among the first to be severely affected. As in this study, numerous studies have reported that *Cryptosporidium*-infected immunosuppressed mice elucidated more tremendous histopathological changes, especially in intestinal epithelial cells than in immunocompetent ones (Abdou *et al.*, 2013; Yang *et al.*, 2000). That is explained by that in immunocompetent host, T cells play an important role in immunity to *C. parvum*, on contrast, immunosuppressed mice which have depleted levels of CD4+T cells. (Ungar *et al.*, 1991; Gardner *et al.*, 1991). Few studies revealed that *C. parvum* infection increased epithelial paracellular permeability via disruption of epithelial junctional complexes as part of their pathogenesis (Guttman & Finlay 2009) this action is mediated by alterations in the expression of junctional complexes proteins (claudin 4 & occludin and ZO1) (France & Turner 2017). The above histopathological changes were observed in immunosuppressed vaccinated group C5 & vaccinated immunosuppressed C9 group. Enhancing of the histopathological recovery in *C. parvum*-infected and NTZ-treated mice was done by the accompanied therapy with other treatments, this approach was reported in many studies; Secnidazole with NTZ (Madbouly *et al.*, 2021), Silica nanoparticle with NTZ (Metawae *et al.*, 2021) & chitosan nanoparticles with NTZ (Moawad *et al.*, 2021) & phenyl vinyl sulfone with NTZ (El Shafei *et al.*, 2018). The present histopathological results elucidated that using the oocyst antigens, as a vaccine, combined with NTZ showed a high efficacy in immune response, especially in immunosuppressed mice group (C10).

CONCLUSION

In this research, it is concluded that the vaccination by oocyst crude antigen with the NTZ treatment if used together showed a highly significant decrease in the number of oocyst counts, especially in immunosuppressed mice when compared to the mice groups treated with NTZ alone. Besides, applying the vaccination before immunosuppression was preferred. The above finding was supported by a noticeable improvement in the histological characteristics of the intestine. Besides, the highest improvement of the immune status. NTZ shows a coordinated anti-cryptosporidium immune response, so, additional research should be done in the future to understand the immunological effect of the NTZ and its interfering action with different types of vaccines.

DECLARATIONS

Conflict of interest

The authors declare no competing interests.

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Compliance with ethical standards

All animal procedures and experimental protocols concerning this work were approved by the TBRI Research Ethics Committee (TBRI-REC) at the Schistosome Biological Supply Program, Animal House of Theodor Bilharz Research Institute (TBRI), Egypt (No. 00010609).

Authors' contributions

Shadia H. Mohamed & Ibrahim R. Shalash, conceived and developed the study. Amira M. Lotfy & Marwa M. Abou El Dahab participated in the design and implementation of parasitological studies. Amira M. Lotfy & Ahmed Nigm carried out the histopathological studies with writing the related observations. All authors did data analysis and interpretation. Marwa M. Abou El Dahab & Ahmed Nigm wrote the first draft of the manuscript. The final manuscript was edited by Ahmed Nigm and Marwa M. Abou El Dahab. All authors read and approved the final manuscript.

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