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Cellulose Nanocrystals as a Drug Delivery System to Improve the Efficiency of Nitazoxanide against *Cryptosporidium parvum* Infected Mice

Nabila M. Mira[1], Shrouk R. Khaleil[1], Nora F. Ghanem[1], Rewaida Abdel-Gaber[2], Mohamed A. Dkhil[3], Felwa A. Thagfan[4], Ibrahim B. Helal[5], Shaimaa M. Kasem[1]

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ABSTRACT

Background: Cryptosporidiosis poses several health risks, particularly due to its potential severity and the populations it can affect. Comprehending these hazards is crucial for efficient prevention and treatment. This study was to investigate the efficiency of cellulose nanocrystals (CNC), nitazoxanide (NTZ) and nitazoxanide-cellulose nanocrystals (NTZ-CNC) in treating experimental infected mice of cryptosporidiosis.

Methods: A total of 75 male swiss albino mice were allocated into eight groups (5 mice/ group) with triplicates of each group. Infection with cryptosporidiosis was conducted with 3000 *Cryptosporidium parvum* oocysts. All treatments commenced on the initial day of oocyst emergence and persisted for five succeeding days across all groups, except for the negative control group. Oocysts shedding was estimated daily from 1st till 20 days post treatment. Hematological and blood biochemical analysis, immunoglobulins levels as well as histopathological studies were performed.

Result: It was detected that NTZ-CNC group had the lowest *Cryptosporidium* oocysts shedding. Also, these treatments induced significant improvements in hematological, blood biochemical, immunoglobulins levels including immunoglobulin G and immunoglobulin M with best improvements recorded in NTZ-CNC treated groups. A remarkable amelioration of the intestinal histopathological lesions was observed, especially in NTZ-CNC treated group. In conclusion, this study reported that CNC could significantly enhance the therapeutic effects of NTZ, making it a promising treatment for cryptosporidiosis due to its potent anti-parasitic and anti-inflammatory capabilities.

Key words: Anti-inflammatory, Cellulose nanocrystals, *Cryptosporidium*, Immunomodulatory, Nitazoxanide.

INTRODUCTION

Cryptosporidium spp. is an intestinal Apicomplexan protozoan parasite responsible for inducing diarrhea in people and animals all over the world (Ryan, 2010; Zahedi *et al*., 2016). It poses a concern to public health and generates significant financial losses (Adam *et al*., 2019). The fecal–oral pathway can spread cryptosporidiosis by coming into contact with infected individuals and animals or by consuming tainted water or food that has a minimal number of 10 oocysts dose (Zahedi *et al*., 2016). Nitazoxanide is the sole medication sanctioned by the Food and Drug Administration (FDA) for cryptosporidiosis remedy (Vandenberg *et al*., 2012; Hussien *et al*., 2013) though it is largely in effective, particularly in immuno compromised patients (Amadi *et al*., 2009). Due to the absence of licensed vaccinations for cryptosporidiosis and the development of resistance against the available drug treatments, it became essential to explore effective anti-parasitic natural medications (Ryan *et al*., 2016).

Nanoparticles represent a promising innovation for the treatment of parasitic infections, functioning as an advanced medication carrier to augment their efficacy. The integration of these nanoparticles into intelligent systems for the coating of therapeutic agents facilitates targeted drug delivery with precise release mechanisms. Furthermore, this focused delivery approach reduces the side effects of used drugs and enhances patient adherence by reducing the frequency of dosing (Rizvi and Saleh, 2018). Numerous studies have focused on identifying the optimal

[1]Department of Zoology, Faculty of Science, Kafrelsheikh University, Kafr El-Sheikh-33516, Egypt.
[2]Department of Zoology, College of Science, King Saud University, Riyadh-11451, Saudi Arabia.
[3]Department of Zoology and Entomology, Faculty of Science, Helwan University, Cairo-11759, Egypt.
[4]Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, Riyadh-11546, Saudi Arabia.
[5]Department of Zoology, Faculty of Science, Tanta University, Tanta-31527, Egypt.

Corresponding Author: Shaimaa M. Kasem, Department of Zoology, Faculty of Science, Kafrelsheikh University, Kafr El-Sheikh-33516, Egypt. Email: shaimaakasem48@yahoo.com

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combination of nanoparticles and targeted anti-protozoal medications for cryptosporidiosis therapy (Sedighi *et al*., 2016; Fahmy *et al*., 2017). This is due to the inherent benefits of nanoparticles, including their reduced particle size, greater surface areaand improvement in their dissolution rate (Priotti *et al*., 2016). Cellulose nanocrystals are biopolymers that demonstrate safety for applications in medical fields, such as drug delivery systems (El-Shafai *et al*., 2022; Mehany *et al*., 2022). The recent study conducted by Khaleil *et al*. (2023) concluded that the self-assembly of cellulose nanocrystals with nitazoxanide exhibited potential in vitro anti-cryptosporidial effects.

Therefore, the current work aimed to investigate the potential in vivo anti-cryptosporidial and immunomodulatory effects of cellulose nanocrystals-nitazoxanide against *C. parvum.*

MATERIALS AND METHODS

Synthesis of nanomaterials

Cellulose nanocrystals (CNC) and nitazoxanide-cellulose nanocrystals (NTZ-CNC) were formulated and obtained as previously detailed by Khaleil *et al*. (2023). Cotton fiber (5 g) was soaked in NaOH (2M), subsequently washed with double distilled H_2O and then transferred into an acidic medium of H_2SO_4 at 60°C with continuous stirring for a duration of 24 hours. The CNC mixture was subsequently washed, filteredand dried at 40°C to obtain a powdered product. For the preparation of NTZ-CNC, approximately 0.04 grams of CNC was dissolved in double distilled H_2O (100 ml), thoroughly mixedand then 0.1 grams of NTZ was incorporated. The mixture underwent ultrasonication for a duration of 30 minutes, followed by washing and drying at a temperature of 40°C. The characterization of CNC and NTZ-CNC was previously published in Khaleil *et al*. (2023) showing that CNC resembles tiny crystal rods with an average size of 43.37±5.72 µm and NTZ appeared in an amorphous shape on CNC surface in NTZ-CNC complex. Our study also proved that CNC and NTZ-CNC had an *in vitro* safety state against normal mouse liver cells (BNL) at low and high concentrations. The cell viability was 93.88±1.75% and 91.29±1.14% with 10 µg/ml, while was 84.039±3.24% and 86.41±1.42% for CNC and NTZ- CNC, respectively.

Collection of *Cryptosporidium parvum* oocysts

C. parvum oocysts were collected from feces of naturally infected calves, aged three months, that were experiencing watery diarrhea on a private farm in Kafr ElSheikh city, Egypt. Using a modified Ziehl-Neelsen staining method, *C. parvum* oocysts existence was verified (Henriksen and Pohlenz, 1981).

In vivo infectivity of mice with *C. parvum*

This study was carried out between February 2024 and July 2024 at the Zoology Department, Faculty of Science, Kafrelsheikh University, Kafr Elsheikh, Egypt, while formal analysis was done in Saudi Arabia. Five male Swiss albino mice, (28-35 days old) with a body weight of approximately 20-25 g, were acquired from a mice farm located in Kafrelsheikh city, Egypt. Mice received 3000 oocysts in 100 µl normal saline solution (Abdelmaksoud *et al*., 2023) utilizing a 19-gauge, ball-tipped stainless-steel oral gavage. Five days post-infection (DPI), fecal samples from mice were collectedand oocysts were concentrated utilizing the flotation technique through the zinc sulfate ($ZnSO_4$) flotation centrifugal method as described by Current and Reese (1986). For later usage, precipitated oocysts were gathered in a potassium dichromate solution (2.5%) and preserved at a temperature of 4°C. Nitazoxanide (NTZ) was acquired from Medizen Pharmaceutical Industries for Utopia Pharmaceuticals.

Experimental animals and laboratory conditions

A total of 75 male mice were housed in suitable good ventilated cages featuring perforated coverings and an appropriate layer of wood shavings as bedding material to facilitate the collection of fecal pellets. The food and drink were supplied *ad libitum* at a temperature of 23-24°C, with a 12/12-light/dark cycle. The mice were housed in the laboratory for a duration of one week to facilitate adaptation.

Experimental design

Following a one-week adaptation period, the mice were categorized into eight experimental groups, with five mice per group and triplicates for each group. Group 1 served as a negative control (non-infected, non-treated) group, while Group 2 acted as a positive control (infected, non-treated) group, which was infected with 3000 *C. parvum* oocysts. The remaining three groups were administered 3000 *C. parvum* oocysts and subsequently treated with cellulose nanocrystals (CNC) at a dosage of 200 mg/kg mice weight (Group 3) (Yanamala *et al*., 2014), nitazoxanide (NTZ) at 100 mg/kg mice weight (Group 4) (El-Wakil *et al*., 2021) and a combination of cellulose nanocrystals and nitazoxanide (NTZ-CNC) at 200 mg/kg mice weight (Group 5). All treatments commenced on the initial day of oocyst shedding and persisted for five succeeding days following infection for all groups, with the exception of the negative control group.

Oocyst shedding in feces

Samples of fecal pellets were collected daily from the first day post treatment (DPT) until the termination of the experimental period (20 DPT) for the determination of *C. parvum* oocysts per gram (OPG) count, calculated as outlined by Benamrouz *et al.* (2012).

Hematology and blood biochemistry

Blood samples were collected during cervical dislocation from mice jugular vein in vacutainer EDTA tubes for the analysis of red blood cells (RBCs) and white blood cells (WBCs) counts, as well as lymphocyte percentages. An additional blood quantity was obtained using non-

anticoagulated weatherman tubes. The tubes were incubated at 4°C for 30 minutes to allow clotting, followed by centrifugation at 2500 rpm for 20 min to facilitate serum separation. The serum samples were meticulously collected and preserved at -20°C until required for analysis. Hepatic function tests were conducted to measure levels of liver enzymes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), utilizing commercial kits; specifically, comprehensive liver function kits (MxP® Quant 500) were employed for kinetic measurement.

Humoral immune response (Serum immunoglobulins Level)

Serum levels of immunoglobulin G and M (IgG and IgM) were measured according to the manufacture instructions provided by BD Pharmingen™ mouse immunoglobulin isotyping ELIZA kit (B 1500).

Histopathological investigations

At the end of the experiment, ilea sections were immersed and fixed directly in 10% neutral buffered formalin, let to dry, then cleaned in xylene. They were impregnated and embedded with paraffin, sectioned at a thickness of 4 mmand marked with H and E (Bancroft and Stevens, 1990). Stained sections were inspected with a light microscope (LEICA DM650, Germany) for histopathological alterations and explanations.

Statistical analysis

Means and standard deviation were calculated. One-way analysis of variance (ANOVA) in SigmaPlot® version 15.0 (Systat Software, Inc., Chicago, IL, USA) was used with Tukey Test for comparison of means. As P value was less than 0.05, the results were statistically judged significant.

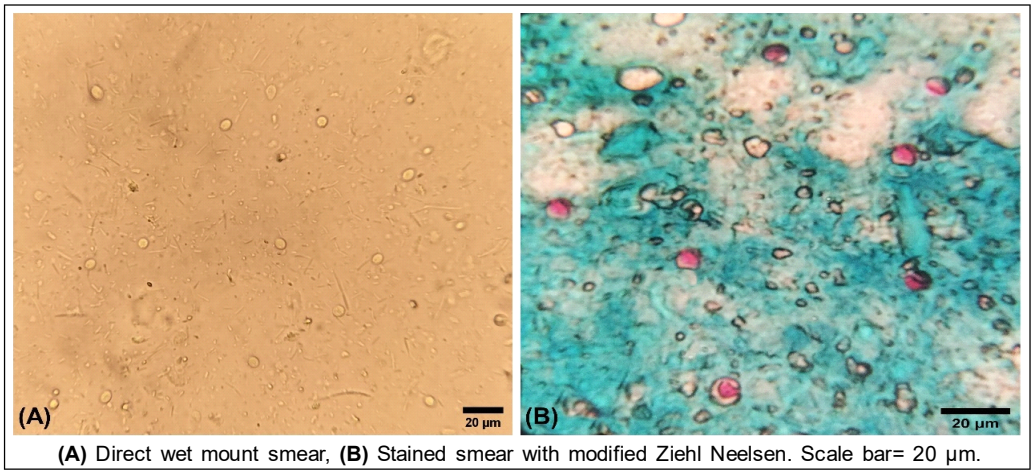
RESULTS AND DISCUSSION

This study demonstrated that the nitazoxanide-cellulose nanocrystals (NTZ-CNC) complex significantly reduced oocyst shedding and improved hematological and biochemical parameters in infected mice with *Cryptosporidium parvum*, compared to nitazoxanide (NTZ) alone. Additionally, NTZ-CNC treatment showed a beneficial effect on serum immunoglobulin levels and alleviated histopathological damage. These findings suggest that NTZ-CNC may enhance the therapeutic efficacy of NTZ, offering a more potent intervention for cryptosporidiosis, particularly in immunocompromised populations.

The oocysts of *C. parvum* observed in this study through direct wet mount presented a rounded to oval shape, with dark sporozoites visible in certain oocysts and had an average diameter of 4.6±0.75 µm (Fig 1A). In addition, the stained *C. parvum* oocysts using the modified Ziehl Neelsen acid-fast method exhibit a distinct pinkish-red coloration, contrasted against a blue-green background (Fig 1B).

In the current study, the analysis of fecal smears indicated a progressive decrease in oocyst shedding across all experimental groups from 1 to 20 days post-treatment. All treatment groups showed a reduction in oocyst shedding, against the positive group, with the most significant reduction observed in the NTZ-CNC treated group, surpassing the effects of CNC and NTZ used individually (Fig 2). Similarly, Fahmy *et al*. (2017) found a substantial decrease in *Cryptosporidium* oocysts per gram count in nitazoxanide-loaded nano-gold-treated immunocompetent mice. A separate investigation revealed that *Commiphora molmol* extract incorporated into chitosan nanofibers demonstrates greater efficacy against the oocyst of *Cryptosporidium* compared to NTZ, the primary standard treatment for infections in both adults and children (Jin *et al*., 2019). Cellulose has been conjugated with several inorganic-based antimicrobial agents and exerts pharmacological efficacy (Hamedi and Shojaosadati, 2021). The decrease and cessation of fecal oocyst shedding could be linked to a direct impact on the growth of parasites within the intestines, the generation of sexual stages and/or the development of oocysts (Abdelgelil *et al*., 2023).

The hematological analysis revealed a notable significant ($P<0.05$) reduction in RBCs counts in positive



(A) Direct wet mount smear, **(B)** Stained smear with modified Ziehl Neelsen. Scale bar= 20 µm.

Fig 1: Oocysts of *C. parvum*.

mice, when compared to the negative ones. Nonetheless, all groups receiving treatment with CNC, NTZ and NTZ-CNC showed notable increases in their counts when contrasted with the positive group (Table 1). Kabu (2023) showed that the concentrations of RBCs in calves with Cryptosporidiosis experiencing diarrhea exhibited statistically significant differences before and after treatment with halofuginon, with levels being higher post-treatment. Alterations in RBCs counts were observed in calves suffering from diarrhea caused by Cryptosporidiosis (Aiello, 2016). Additionally, in this study, significant ($P<0.05$) increase in WBCs and lymphocytes percent were observed in positive group, when compared to the negative group (Table 1). These results are the same of Bhagat *et al*. (2018). Also, Soufy *et al*. (2017) indicated lymphocytes counts were markedly increased in infected mice with cryptosporidiosis. The current study showed a significant ($P<0.05$) decrease in WBCs count and lymphocytes percentages of all treated groups with CNC, NTZ and NTZ-CNC, compared to the positive group (Table 1). This is also line with Soufy *et al*. (2017), who reported that rats treated with NTZ showed a significant decrease in absolute lymphocytes. When nanoparticles first come into contact with blood and its constituents, they can result in a variety of immunogenic

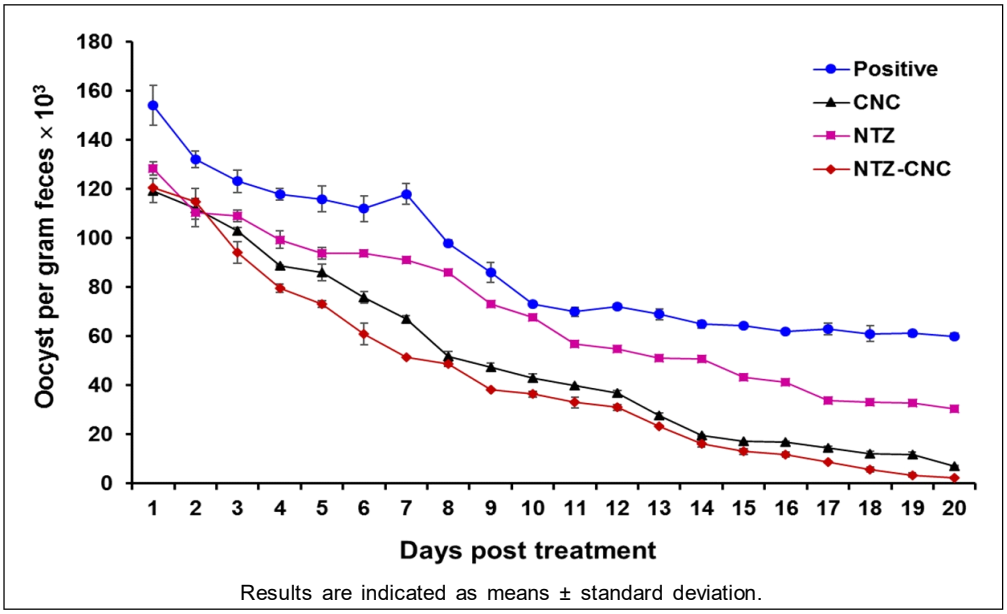


Fig 2: Oocysts shedding per gram feces from *C. parvum*-infected mice and treated with CNC, NTZ and a formulated NTZ-CNC from 1st to 20th days post treatment.

Table 1: RBCs, WBCs counts and lymphocytes (%) of *C. parvum*-infected mice and treated with CNC, NTZ and a formulated NTZ-CNC.

Parameter	Groups	Days post treatment		
		5	10	20
RBCs ($\times 10^6$/UL)	Negative	10.30±0.25	9.40±0.22	9.20±0.35
	Positive	10.10±0.38	8.70±0.25*	8.50±0.22*
	CNC	8.80±0.20*#	8.10±0.35*#	9.10±0.21#
	NTZ	8.20±0.18*#	8.90±0.13*	9.50±0.28*#
	NTZ-CNC	9.00±0.25*#	9.50±0.31#	8.00±0.20*#
WBCs ($\times 10^3$/UL)	Negative	7.50±2.25	8.10±0.19	7.20±2.13
	Positive	11.00±0.49*	12.40±1.68*	13.10±3.23*
	CNC	10.60±0.49*#	9.20±0.75*#	8.40±0.19*#
	NTZ	10.10±1.82*#	9.30±2.38*#	8.20±0.18*#
	NTZ-CNC	9.60±2.41*#	8.20±0.19#	7.50±2.25#
Lymphocytes (%)	Negative	59.00±5.95	58.70±15.15	59.30±6.45
	Positive	67.20±5.90*	68.60±4.00*	69.3⁰±5.10*
	CNC	63.20±5.60*#	62.00±1.65*#	61.40±3.05*#
	NTZ	61.50±6.45*#	61.20±0.50*#	60.00±1.56*#
	NTZ-CNC	61.30±10.35*#	60.60±9.90*#	59.80±8.05*#

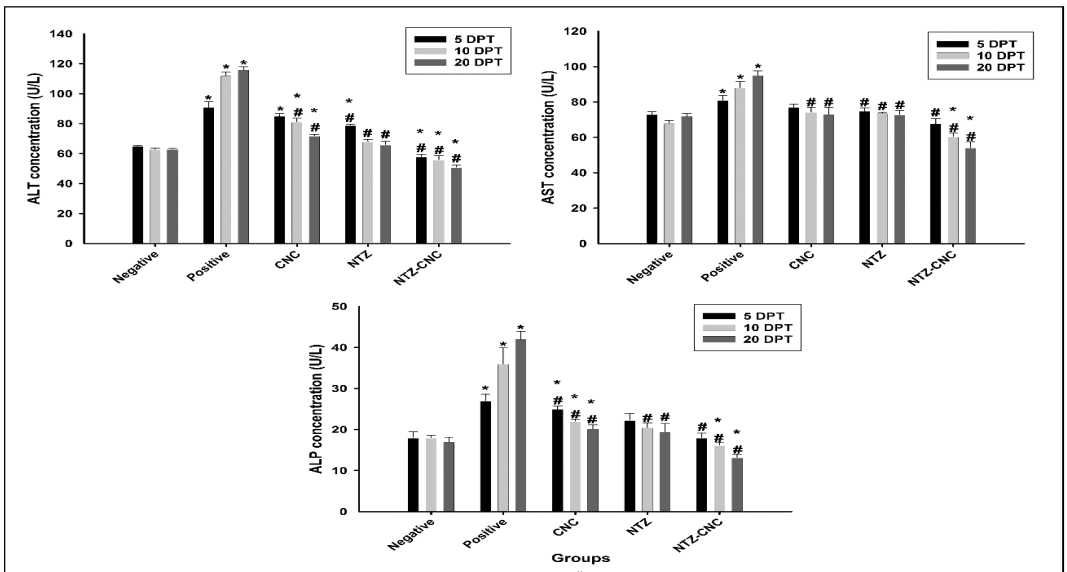
Results are indicated as means ± standard deviation. *, # Significant when $P<0.05$ against negative and positive groups, respectively.

reactions, inflammationand alterations to hematological parameters, such as white blood cells (Kim *et al*., 2009). The observed alterations in red blood cells and white blood cells may be the result of elevated immunogenic response (Duffin *et al*., 2007; Shin *et al*., 2007; Kawata *et al*., 2009) or disruptions in signaling pathways and cell maturation (Gopinath *et al*., 2010), which can impact not only red blood cells but also the division and development of other cells.

The liver function tests determine the presence of infections and offer precise information for illness diagnosis (Bertolini *et al*., 2020). Because the two ALT and AST enzymes are produced in the liver and can be discharged into the blood when liver damage occurs, their activities in serum are frequently employed as markers of liver health (Goodla *et al*., 2010; McGill, 2016). Mice with cryptosporidiosis may develop a persistent illness that spreads throughout their gastrointestinal tract or extraintestinal areas, resulting in liver damage (Mead *et al*., 1994; Sood *et al*., 2019). In the present study, levels of liver enzymes including ALT, AST and ALP displayed significant ($P<0.05$) increases in the positive group, when compared to the negative group (Fig 3). These findings supported the possibility of extraintestinal consequences of cryptosporidiosis (Chalmers and Davies, 2010). This study also showed that all therapeutic treatment groups had noted decreased liver enzyme levels of ALT, AST and ALP with the best reduction was observed in NTZ-CNC group in comparison to the positive group (Fig 3). These results are in similar with that in previous studies on mice and piglets (Wang *et al*., 2016; Wang *et al*., 2017; Wang *et al*., 2018).

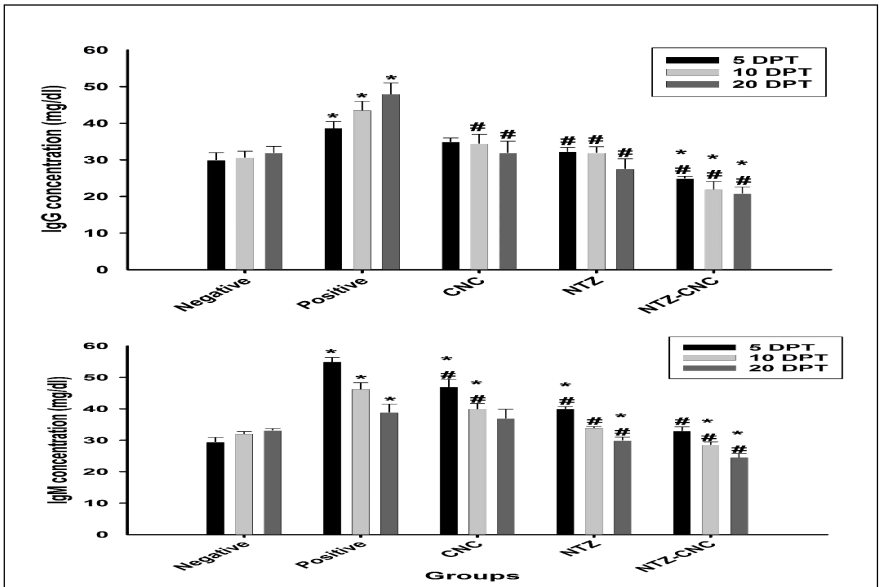
In the present study, in the positive group, serum levels of IgG were increased, while IgM levels were decreased significantly ($P<0.05$) during the infection with increase in time, linked to the negative group (Fig 4). Similarly, Metawae *et al*. (2021) reported that serum levels of IgG and IgM were increased during the infection with *Cryptosporidium*. The current study also showed that the serum levels of IgG and IgM differs between the treated and positive groups indicating that NTZ-CNC group exhibited the lowest serum titres of both IgG and IgM in relation to the positive group (Fig 4). Compared to untreated infected groups, NTZ and NTZ-loaded silica demonstrated decreased positive antibody titers, suggesting that pharmaceutical therapy is helpful in reducing and mitigating antigen presentation and parasite development inside the cells of intestine (Metawae *et al*., 2021). According to Abdelgelil *et al*. (2023), NTZ therapies have the potential to alter immunological responses. Moreover, Li *et al*. (2007) showed that serum IgG and IgM can defend the extravascular compartment from harmful viruses and microbes.

Histopathological analysis is still a popular way to assess tissue degeneration and cytoarchitectural changes (Mikail *et al*., 2019). The histological findings of the negative control group revealed a normal ileal tissue structure, characterized by intact mucosa, villi, lamina propria, and goblet cells (Fig 5A). In the present study, infected mice with *C. parvum* exhibited marked villous atrophy and damaged ileal lining with sloughing epithelium and intraluminal heavy infection with *Cryptosporidium* oocysts as well as massive inflammatory infiltrate in the lamina propria (Fig 5B). A several Previous studies were also in agreement as Metawae *et al*. (2021), Abd El Wahab *et al*. (2022), Hamdy *et al*. (2023) and Ganai *et al*. (2023). The pathological changes brought on by a cryptosporidiosis infection are reflected in the dislodging of the brush boundary, asymmetrical loss of epithelial cellsand villi that are shortened and fused (Al-Mathal and Alsalem, 2012; Hemphill *et al*., 2019). The significant lymphocyte infiltration and *Cryptosporidium* are the causes of these developments



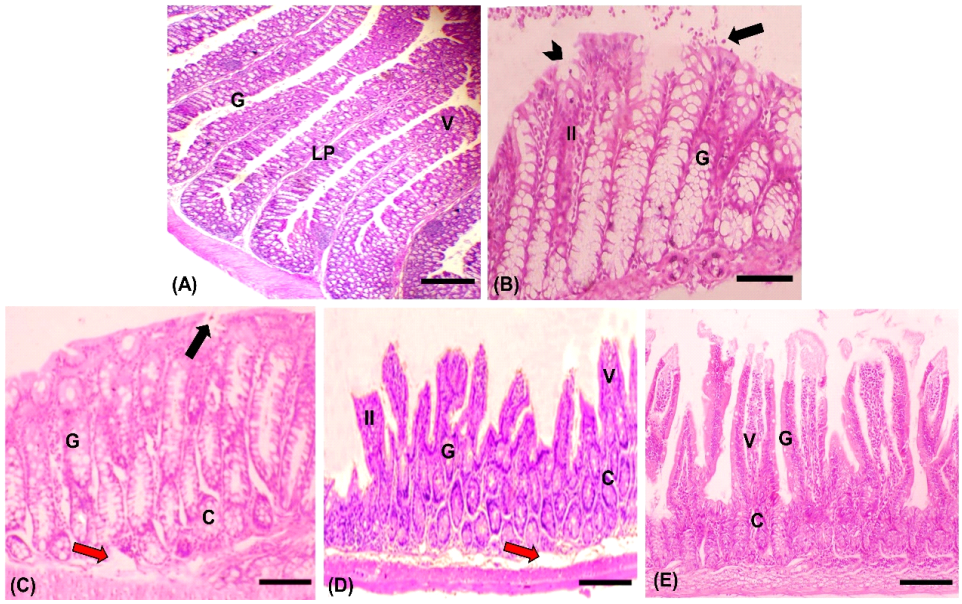
Results are indicated as means ± standard deviation. *, # Significant when $P<0.05$ against negative and positive groups, respectively.

Fig 3: Liver enzyme levels of *C. parvum*-infected mice and treated with CNC, NTZ and a formulated NTZ-CNC.



Results indicated as means ± standard deviation. *, # Significant when P < 0.05 against negative and positive groups, respectively.

Fig 4: Serum immunoglobulins levels of *C. parvum*-infected mice and treated with CNC, NTZ and a formulated NTZ-CNC.



(A) negative control group showing normal ilea tisuue structure with normal mucosa have intact villi (V), lamina propria (LP) and goblet cells (G). **(B)** positive control group showing marked villous atrophy (black arrowhead) and broadening of the brush borders of villi with intraluminal heavy infection with Cryptosporidium oocysts (black arrow)and massive inflammatory infiltrate (II) in lamina propria of intestinal villi and huge numbers of goblet cells. **(C)** CNC treated group showing moderate disorganized crypts (C) with broadening of villi with a number of goblet cells (G) and moderate edema (red arrow). **(D)** NTZ treated group showing disorganized crypts, inflammatory infiltrate (II) in lamina propria of intestinal villi with a number of goblet cells (G) and mild edema (red arrow). **(E)** NTZ-CNC treated group showing almost normality intestinal villi (V) and crypts (C) with no Cryptosporidium oocysts. Scale bar = 50 µm.

Fig 5: Photomicrograph of H and E stained paraffin sections in ilea intestinal tissues.

(Soufy *et al*., 2017; El-Shafei *et al*., 2018). The current study also documented that the use of CNC and NTZ could partially improve the ileal tissue structure with appearance of a number of goblet cells (Fig 5C, D). This is consistent with the findings of Hamdy *et al*. (2023), who found that somewhat enhanced intestinal pathological lesions were observed in infected mice treated with NTZ alone following infection with *Cryptosporidium*. Additionally, Moawad *et al*. (2021) and Abd El Wahab *et al*. (2022) found that rats treated with NTZ showed moderate improvements over a positive control group that had serious pathological abnormalities. In the present study, appearance of a number of goblet cells in treated groups by CNC and NTZ play an important defensive role in the intestine by their mucin secretion. The intestinal mucus helps heal the damaged epithelium and has a significant preventive function against chemically produced ulcers. (Zhang *et al*., 2018; Zhang and Merlin, 2018; Kim and Kim, 2018). In addition, this study revealed that the use of NTZ-CNC showed some intestinal architechture improvemrnts with absence of intraluminal *Cryptosporidium* oocysts (Fig 5E). The intestinal histological sections exhibited a documented improvement, evidenced by the presence of reduced *Cryptosporidium* oocysts inside the intestinal epithelium, accompanied by modest inflammatory cellular infiltration in mice treated with a combination of NTZ-artesunate delivered by polymeric nano-fibre and NTZ-loaded Chitosan nanoparticles (Abdelhamed *et al*., 2019; Moawad *et al*., 2021). Cheng *et al*. (2011) and Hemphill *et al*. (2019) stated that the enhancement in the clinical presentation, seen by the restoration of the brush border to its normal architecture, can be ascribed to the diminished quantity of oocysts and/ or a decrease in cytokine and inflammatory cell production.

CONCLUSION

In conclusion, our study reveals that CNC can considerably improve the therapeutic effects of NTZ, making it a promising treatment for cryptosporidiosis due to its powerful anti-parasitic and anti-inflammatory properties. Future study should focus on analyzing the long-term safety, pharmacokineticsand potential of CNC for delivering various antiparasitic drugs, as well as investigating the processes underlying the observed synergistic effects to optimize CNC-based drug delivery systems.

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Disclaimers

The views and conclusions expressed in this article are for the authors solely and do not represent the views of the affiliated institutions.

Informed consent

All animal procedures for experiments were in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of Kafrelsheikh University, under approval number KSA-IACUC/201/2024.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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