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Comparative Evaluation of Diosmectite versus Nitazoxanide and their Combination in the Treatment of Cryptosporidiosis in Immunosuppressed Mice

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Cover Page Footnote

Acknowledgment: We would like to express our gratitude to the animal house of Theodor Bilharz Research Institute which provided us with mice and oocysts of *Cryptosporidium* spp., and offered a well-acclimatized place for animals till the end of the experiment.

Comparative Evaluation of Diosmectite Versus Nitazoxanide and Their Combination in the Treatment of Cryptosporidiosis in Immunosuppressed Mice

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Abstract

Background: Cryptosporidiosis is a parasitic infection that affects immunocompromised patients and can result in systemic involvement of different organs and death.

Aim: To compare nitazoxanide (an antiprotozoal agent) and diosmectite (an antidiarrheal agent) and their combination in the treatment of cryptosporidiosis infection.

Materials and methods: Mice were divided into five groups: negative control, the infected untreated (positive control), diosmectite-treated, nitazoxanide-treated, and the group treated with nitazoxanide and diosmectite simultaneously. The evaluation was through measuring body weight, oocyst shedding, intestinal, and hepatic histopathological examination, as well as estimation of some inflammatory and oxidative markers.

Results: Body weight was improved significantly in all treatment groups. The highest oocyst reduction rate was observed in nitazoxanide-treated mice. Inflammatory scores of both intestine and liver were markedly improved in mice treated with diosmectite and nitazoxanide compared with each of them separately. Combined treatment with diosmectite and nitazoxanide significantly decreased interleukin-6, tumor necrosis factor alpha, and transforming growth factor- β levels while increasing interleukin-10 and catalase activity.

Conclusion: Combined treatment with diosmectite and nitazoxanide demonstrated potent synergism by improving body weight, lessening oocyst shedding, and ameliorating the immunopathological changes in the intestine and liver.

Keywords: *Cryptosporidium parvum*, Diarrhea, Diosmectite, Immunocompromised, Nitazoxanide

1. Introduction

Cryptosporidiosis is an opportunistic parasitic disease characterized by its fecal transmission of the oocyst stage. It affects the intestinal tract, resulting in villous atrophy, mucosal erosion, and diarrhea that sometimes necessitate hospitalization.¹

Cryptosporidium poses significant resistance to various environmental stresses and harsh chemical

management. Therefore, it is recognized as one of the principal agents of mortalities and long-term sequelae concerning growth in children under 5 years in developing countries.²

Immunocompromised individuals are more susceptible to infection; the parasite often triggers a chronic and protracted form of the disease, which is challenging to cure and can even lead to death. Another concern is the systemic dissemination of

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cryptosporidiosis infections as it can invade the biliary tree and the right upper quadrant of the liver, which results in an elevation in liver functions, biliary tract obstruction, sclerosing cholangitis, and pancreatitis.³ Moreover, Velásquez *et al.*⁴ detected cryptosporidium DNA in the brain specimens of AIDS patients. Hence, cryptosporidiosis is recognized as one of the hazardous infections.

Searching for the optimal treatment protocol to inhibit the parasite's growth while simultaneously alleviating the immunopathological changes is recommended. A notable debate was sparked among clinicians regarding the efficacy of antiprotozoal and anti-diarrheal therapeutic approaches to achieve the best outcome.

Nitazoxanide exhibits its action by disrupting the reaction that is required for the anaerobic metabolism of various pathogens; thus, it has a wide spectrum of antiparasitic activity.⁵ However, diosmectite is an aluminosilicate of phyletic structure that effectively treats acute diarrhea through the adsorption of toxins, bacteria, and mucosal barrier reinforcement by changes in the mucopolysaccharide's chemical nature.⁶

This study aimed to evaluate the effect of nitazoxanide and diosmectite on cryptosporidiosis treatment individually and in combination.

ribosomal gene amplification and development of 422 bp fragments specific for *Cryptosporidium parvum*. Oocysts were preserved for mice infection in 2.5% aqueous potassium dichromate.⁷ Immunosuppressed mice were infected orally with 1×10^3 *C. parvum* oocysts through an esophageal tube to ensure successful infection, fecal pellets were obtained, and oocyst shedding was assessed using the MZN stain.

Nitazoxanide preparation was received as Nita-zode (Alandalous Pharma, Cairo, Egypt), commercially sold as a 60-ml syrup bottle of concentration 100 mg/5 ml. Diosmectite preparation was received as Smecta (Amriya Pharma, Cairo, Egypt), commercially available in a sachet containing 3 g to be dissolved in distilled water.

Fifty immunosuppressed mice were allocated into five groups (10 mice each) as follows (Table 1).

During the 7-day treatment, dynamic changes, including general health status, mortality, and body weight, were monitored, and recorded.

Fecal pellets were obtained from each group. Physical features of fecal pellets and the count of *C. parvum* oocysts/g stool were assessed by MZN stain. The reduction rate in oocyst shedding was estimated according to the following equation to assess the efficacy of drugs:

$$\frac{\text{MN of } C. \textit{parvum} \text{ oocysts of CG} - \text{MN of } C. \textit{parvum} \text{ oocysts of TG}}{\text{MN of } C. \textit{parvum} \text{ oocysts of CG}} \times 100.$$

2. Materials and methods

The Ethics Committee at Theodor Bilharz Research Institute reviewed and approved this study protocol under serial number PT (711). Immunosuppression was induced by oral dexamethasone (Dexazone, Al Kahira Pharmaceutical Company, Cairo, Egypt) in male Swiss albino mice, aged 6–8 weeks, C57BL/6 strain, weighing 20–25 g body weight, using a maintenance dose of 0.25 mg/g/day for 14 days by oral-gastric gavage.

The Parasitology Department in the above-mentioned institute supplied the oocyst of *Cryptosporidium spp.* It was characterized by a modified Ziehl–Neelsen (MZN) stain. The specimen was stored in -20°C to explore the copro-DNA of the parasite by PCR. DNA extraction was performed using the QIAamp DNA Stool Mini Kit from QIAGEN, Germany, followed by *Cryptosporidium* 18 S

where MN is the mean number; CG is the control group; and TG is the treated group. Any detected microscopic alteration or deformation in the morphology, loss of integrity of the wall of oocysts, and the presence of granular content were indicative of loss of viability.

Serum samples were obtained on day 7 after the start of treatment, for measuring the levels of

Table 1. Animal groups.

Group 1	Negative control group
Group 2	Infected with <i>Cryptosporidium parvum</i> oocysts, untreated (positive control)
Group 3	Infected with <i>C. parvum</i> oocysts, then treated with diosmectite (500 mg/kg/day orally for 7 days)
Group 4	Infected with <i>C. parvum</i> oocysts, then treated with nitazoxanide (500 mg/kg/day twice orally)
Group 5	Infected with <i>C. parvum</i> oocysts, then treated with diosmectite and nitazoxanide

interleukin-10 (IL-10), transforming growth factor- β (TGF- β), IL-6, and tumor necrosis factor alpha (TNF- α) levels by enzyme-linked immunosorbent assay (IL-10, TGF- β , and TNF- α ; SUN LONG, Hangzhou, China; IL-6, Cloud Clone Crop, Wuhan, China). In addition, catalase activity was measured by enzyme-linked immunosorbent assay (SUNLONG, Hangzhou, China) to evaluate oxidative stress.

Segments of the liver and ileum were stained with hematoxylin and eosin. Histopathological findings were scored on a scale from 0 to 5. The lesions in the intestine were evaluated based on features of enteritis such as crypt degeneration, desquamation, necrosis, hyperplasia, or inflammation, while hepatic lesions were evaluated depending on the degree of congestion, degeneration, necrosis, apoptosis, and granuloma formation in the hepatic tissue.

Data were analyzed using the statistical program (SPSS), version 26 (IBM Corp., Armonk, New York, USA). Data were presented using mean \pm SD. Quantitative variables were compared using the nonparametric Kruskal–Wallis and Mann–Whitney tests. *P* values less than 0.05 were considered statistically significant.⁸

3. Results

Successful infection was characterized by persistent diarrhea and weight loss. Infected mice showed a significant reduction in body weight (25.54 ± 0.60) compared with the noninfected mice (30.60 ± 0.33). The reduced body weight was alleviated in all treatment groups, especially the group treated by the combination of diosmectite + nitazoxanide (29.19 ± 0.38) ($P < 0.05$) (Table 2).

Concerning the shedding of the *C. parvum* oocyst, infected untreated mice showed a significant increase in oocyst counts; in contrast, the nitazoxanide-treated group showed the highest reduction rate (67.59%), followed by the combination treatment group (57%), and then the diosmectite-treated group (45.6%).

Histopathological examination of intestinal tissues revealed normal intestinal mucosa in the negative control, in the form of a single layer of tall columnar cells with oval basal nuclei lining the villi. Columnar

cells were interspersed with many goblet cells. Columnar epithelial cells lined the regularly spaced intestinal crypts. Digestive glands seemed healthy and had high cuboidal epithelial cells lining them (score 0, Fig. 1a).

However, the infected untreated group exhibited catarrhal enteritis associated with parasitic invasion in the villus and glandular epithelium lining, marked desquamative changes, and hyperplastic changes in the crypts' basal aspect. Subepithelial edema, partial loss of goblet cells, intestinal crypt dilatation, and shedding of the epithelial lining were seen. The intestinal villi appeared short and blunted compared with the negative control. Both mucosa and submucosa were infiltrated with mononuclear cells and eosinophils. In addition, intestinal glands showed hyperplasia of their epithelial lining (score 4, Fig. 1b).

Treatment by diosmectite showed mild improvement in the intestinal structure compared with the untreated group. There was epithelial shedding at the apices of the intestinal villi, and subepithelial edema was noticed. Parasitic invasion in intestinal crypts and glandular epithelium lining was seen. Moderate inflammatory cell infiltrations were observed in the mucosa and submucosa. Intestinal glands showed an intact epithelial lining with basal basophilic nuclei (score 3, Fig. 1c).

However, the nitazoxanide-treated group showed better improvement characterized by mild shedding in the epithelial cells at the apices of some intestinal villi with moderate loss of goblet cells and subepithelial edema. Few parasites invading the glandular epithelium were seen. Moderate inflammatory cell infiltrations were detected in mucosa and submucosa (score 2, Fig. 1d). However, a more obvious improvement was observed in combination therapy (diosmectite + nitazoxanide), where mild epithelial shedding at the apices of some intestinal villi was observed with subepithelial edema. A small number of goblet cells were located along the intestinal villi. Mild inflammatory cell infiltrations were seen in the mucosa and submucosa. Intestinal glands showed an intact epithelial lining without detection of any parasites (score 1, Fig. 1e).

Concerning liver affection, histopathological examination showed a normal structure of hepatic lobules with normal organization of hepatic cords and prominent central hepatic veins in the negative control group (score 0, Fig. 2a).

The infected, untreated mice demonstrated numerous necrotic foci in the hepatic tissues, with inflammatory cells, lymphocytes, eosinophils, and macrophages having gathered there in a noticeable way forming a granulomatous reaction.

Table 2. Comparison of animals' weight at the end of the treatment.

Studied groups	Body weight (g) (mean \pm SD)
Negative control	30.60 ± 0.33
Infected untreated	25.54 ± 0.60
Diosmectite treated	27.42 ± 0.61
NTZ treated	27.19 ± 0.56
Diosmectite + NTZ treated	29.19 ± 0.38

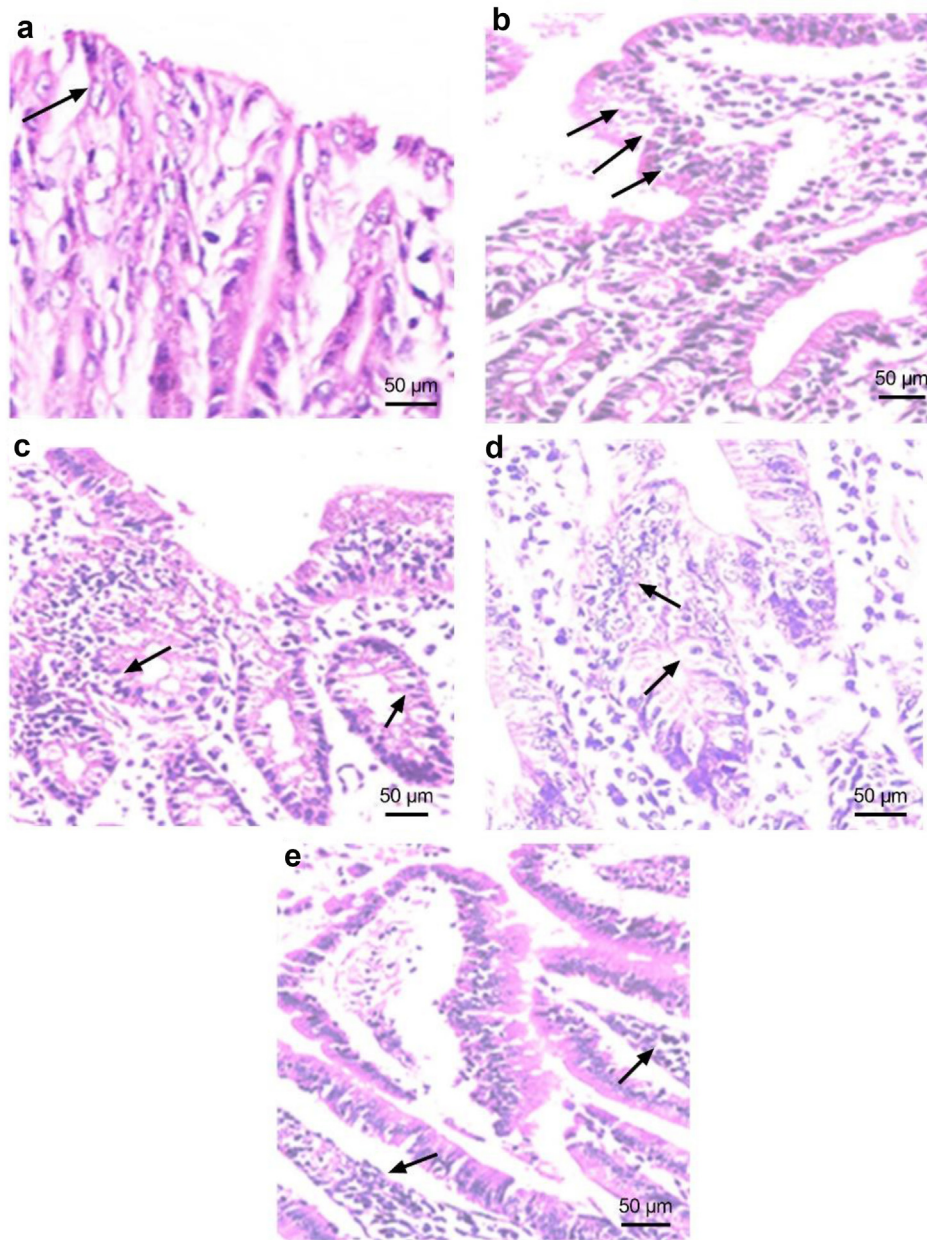


Fig. 1. Photomicrographs of intestinal mucosa after treatment with diosmectite and nitazoxanide. (a) The negative control group shows a normal histological structure of intestinal mucosa. (b) The positive control group shows the parasitic invasion of the villus and glandular epithelial lining. (c) The diosmectite-treated group shows that numerous parasites invade the glandular epithelial lining. (d) The nitazoxanide-treated group shows that parasites invade the crypt epithelial lining with moderate inflammatory cell infiltration. (e) The group treated with diosmectite and nitazoxanide shows mild inflammatory cell infiltration in the mucosa (hematoxylin and eosin, $\times 200$).

Disorganization in the hepatic cords and necrobiotic changes was seen in hepatocytes. Eosinophilic bodies were scattered all over the hepatic lobules, showing apoptosis of hepatocytes. Hyperplasia of Kupffer cells with narrowing of hepatic sinusoids was also noticed (score 4, Fig. 2b).

Diosmectite-treated group showed ballooning degeneration. Narrowing of sinusoids, hyperplasia of Kupffer cells, and numerous binucleated cells, especially in the centrilobular zone, were observed.

Hepatocytes in the peripheral zone appeared swollen with narrowed sinusoids (score 2, Fig. 2c). In the nitazoxanide-treated group, moderate necrotic changes were observed with few numbers of apoptotic hepatocytes and hyperplasia of Kupffer cells (score 3, Fig. 2d).

The combination therapy (diosmectite + nitazoxanide) showed better improvement than treatment with each drug alone. Hepatocytes showed little swelling, granular cytoplasm, and

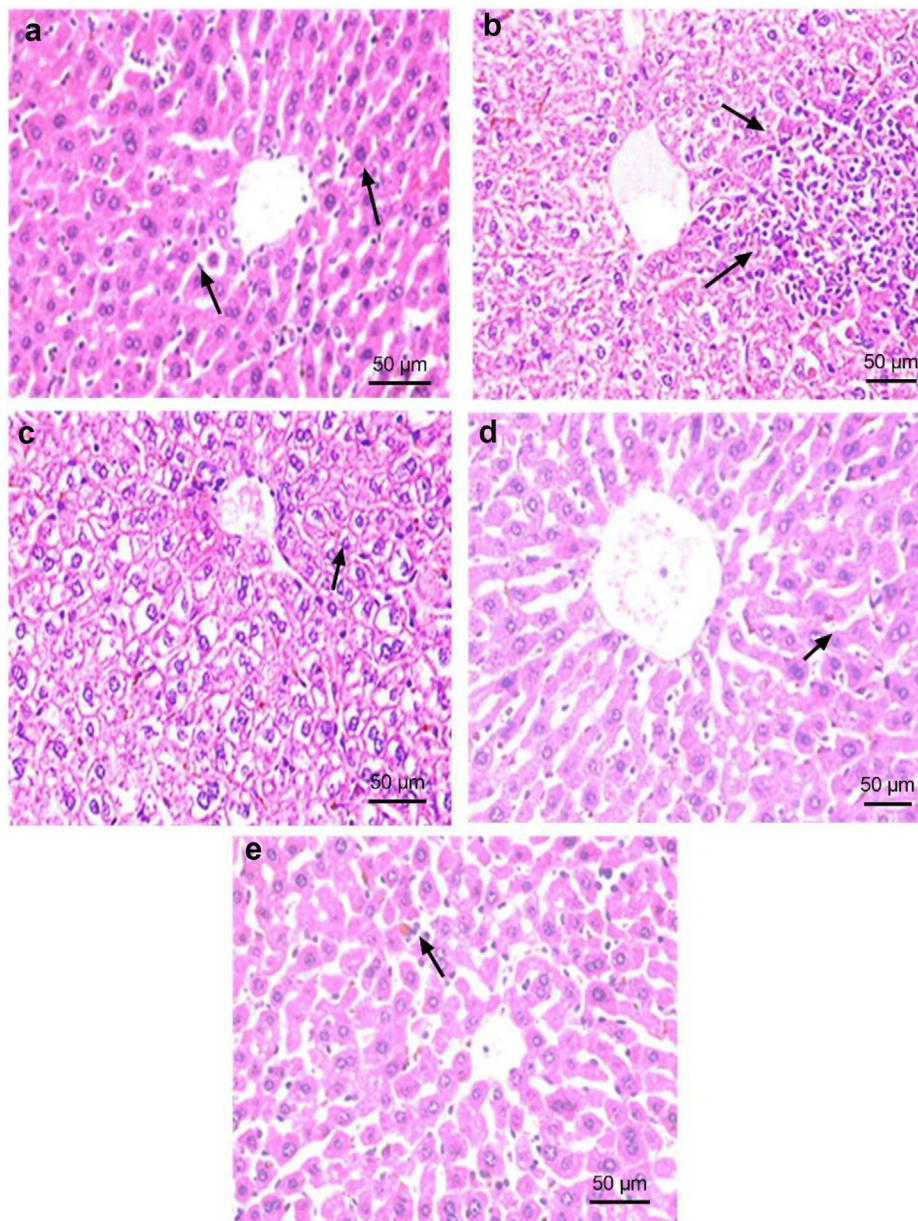


Fig. 2. Photomicrographs of hepatic tissues after treatment with diosmectite and nitazoxanide. (a) The negative control group shows a normal hepatic structure. (b) The positive control group shows necrotic foci within the hepatic tissues associated with marked aggregation of inflammatory cells. (c) The diosmectite-treated mice show ballooning degeneration of hepatocytes. (d) The nitazoxanide-treated mice show few numbers of apoptotic hepatocytes with hyperplasia of Kupffer cells. (e) The group treated with diosmectite and nitazoxanide shows mild swelling of hepatocytes and granularity of the cytoplasm (hematoxylin and eosin, $\times 200$).

central vesiculated nuclei with peripheral nuclear chromatin condensation. Mild dilatation of hepatic sinusoids and hyperplasia of Kupffer cells were also noticed (score 1, Fig. 2e).

As shown in Fig. 3, the positive control group showed a significant increase in IL-6, TNF- α , and TGF- β levels but decreased IL-10 levels compared with the negative control group ($P < 0.05$). Treatment with diosmectite and nitazoxanide alone or in

combination remarkably diminished IL-6 and TNF- α levels and enhanced IL-10 levels ($P < 0.05$). However, the TGF- β level was reduced by nitazoxanide and the combination therapy ($P < 0.05$).

In addition, catalase activity was measured to evaluate the antioxidant capacity of tissues, where its level decreased in the positive control group compared with the negative control. The combination therapy (diosmectite + nitazoxanide) and

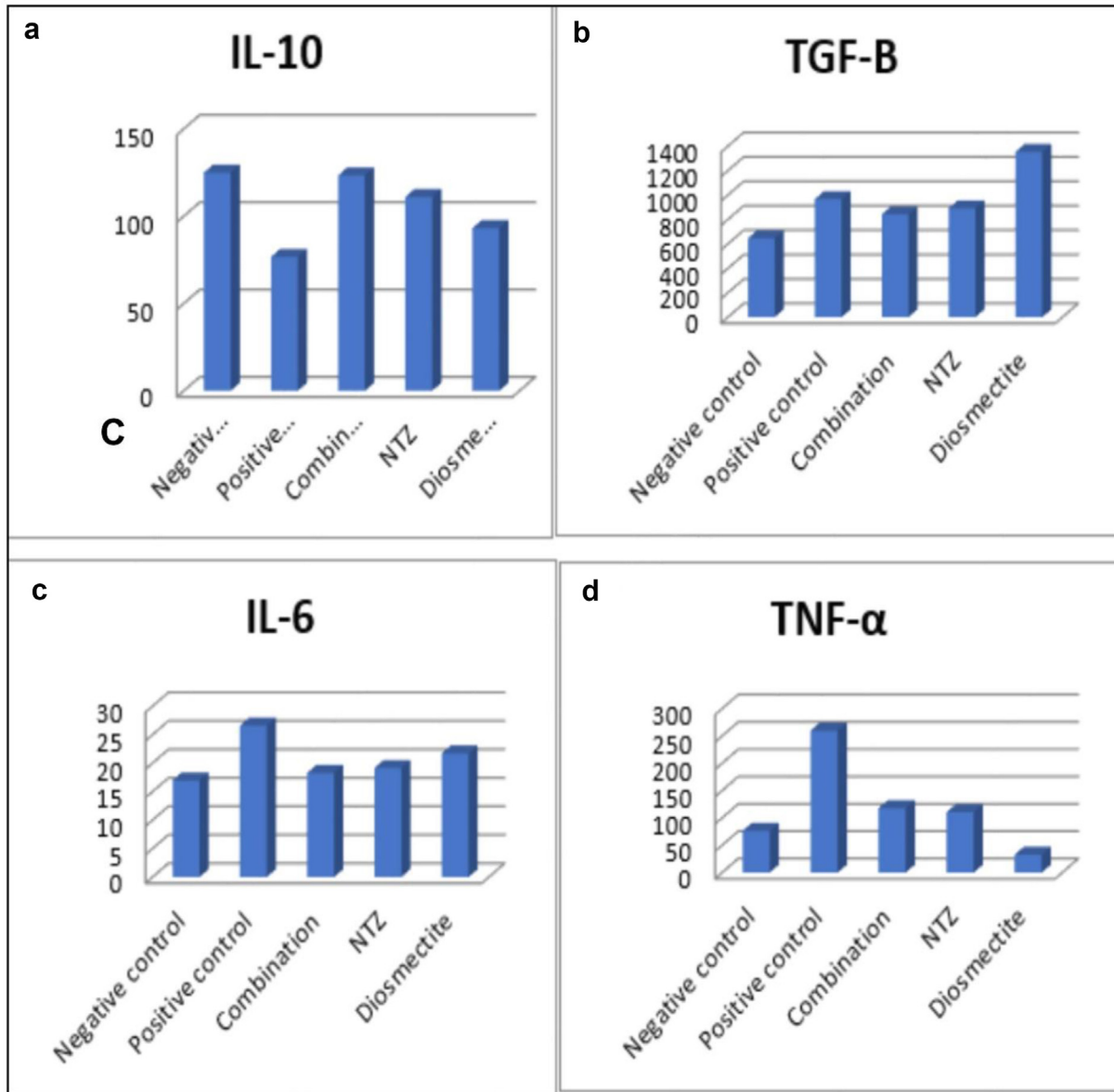


Fig. 3. Effect of diosmectite and nitazoxanide and their combination on (a) IL-10, (b) TGF- β , (c) IL-6, and (d) TNF- α levels. The data are expressed as mean \pm SD. IL, interleukin; TGF- β , transforming growth factor- β ; TNF- α , tumor necrosis factor alpha.

diosmectite showed a significant increase in catalase activity, while nitazoxanide showed lowered catalase activity ($P < 0.05$) (Fig. 4).

4. Discussion

In the current study, the combination therapy (diosmectite + nitazoxanide) significantly improved body weight, indicating healing of intestinal absorption. As diosmectite forms polyvalent junctions with the mucus glycoproteins protecting against the harmful outcomes of enteric germs and their toxins. These changes in mucopolysaccharides reduce toxin penetration through the mucosa. Consequently, this

opposes the pathogenicity of cryptosporidium parasites that rely on the reorganization of the cytoskeleton of enterocytes.⁶ In addition, nitazoxanide has been reported to heal various forms of gastroenteritis by reducing stool frequency and duration.⁹

A significant reduction in the shedding of *C. parvum* oocysts was observed in groups treated with nitazoxanide. Our result was consistent with previous studies that showed that nitazoxanide can inhibit the development of the parasite by more than 50%. El-Ashkar *et al.*¹⁰ reported that nitazoxanide terminates the shedding of oocysts earlier than infected untreated controls. Al-Ghandour *et al.*¹¹ suggested that nitazoxanide activity sustains

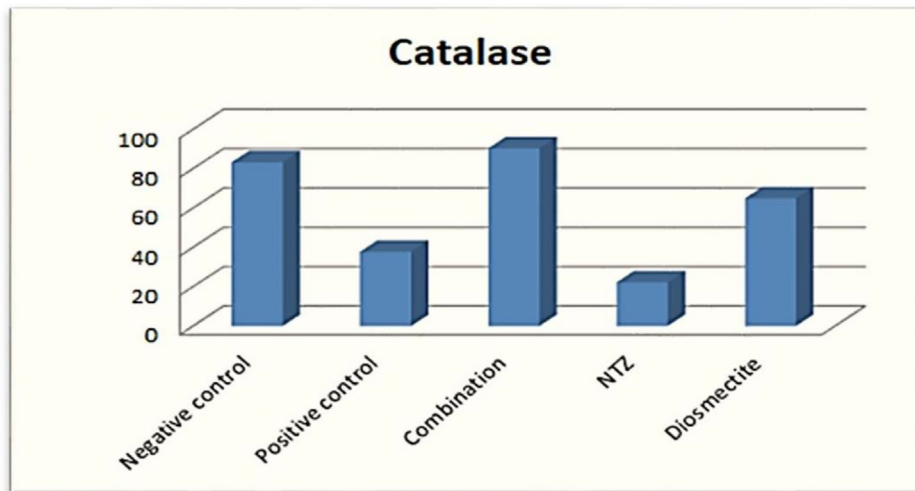


Fig. 4. Effect of diosmectite and nitazoxanide and their combination on catalase level. The data are expressed as mean \pm SD.

after terminating treatment, thus assuming a possible action of nitazoxanide on the sequestered parasites.

Although nitazoxanide is the only FDA-approved treatment for cryptosporidiosis, its efficacy is incompetent in AIDS patients. Prior studies recommended the potentiation of nitazoxanide by an adjuvant agent.¹² El Shafei *et al.*¹³ suggested the potentiation of nitazoxanide with phenyl vinyl sulfone (a cysteine protease inhibitor). Metawae *et al.*¹⁴ reported the significant efficacy of silica nanoparticles as a delivery system with nitazoxanide to reduce oocyst shedding and trigger histopathological cure.

In the current study, the histopathological examination of the infected untreated mice showed parasitic invasion of the villus, while the glandular epithelium lining showed marked desquamation and crypt hyperplasia. There were eosinophil and mononuclear infiltrations in the mucosa and sub-mucosa. Similar findings were found in an earlier study where the villous surface area was reduced, affecting the absorptive capacity. Histological alterations seemed to be partially related to the cell-mediated immunity against the invading parasite.¹⁵

Diosmectite showed mild improvement in intestinal pathology induced by cryptosporidiosis, which explained the insufficient antidiarrhetic and spasmolytic effect of diosmectite as sole therapy. However, in rotavirus infection, diosmectite is efficient in adsorbing the virus to the intestinal mucosa,¹⁶ and its long-term administration preserves gut microbiota, hence improving host metabolism.^{17,18}

Although nitazoxanide showed moderate improvement in the intestinal mucosa, the combination treatment (diosmectite + nitazoxanide)

showed marked improvement in the integrity of the mucosal barrier and inflammatory infiltrations. This result was in accordance with other studies that showed the potentiation of nitazoxanide with other compounds, such as chitosan nanoparticles and azithromycin.^{19,20}

Regarding the histopathological examination of the liver, the combination treatment (diosmectite + nitazoxanide) showed much more improvement in liver tissues than other treated groups. This result might be attributed to the adjuvant effects of the diosmectite as an adsorbent and anti-inflammatory agent.⁶ Nitazoxanide-treated group showed less improvement in the hepatic pathology, this result was supported by Shams *et al.*²¹ who reported fatty changes and congestion of central veins and fatty changes in the liver treated with nitazoxanide.

The results of the present study showed an increase in IL-6, TGF- β , and TNF- α in the infected untreated model. This was in accordance with previous studies that showed cryptosporidiosis infection results in changes in Th1 and Th2 cytokine profiles.^{22,23}

Groups treated with nitazoxanide alone or in combination with diosmectite showed comparable results in levels of IL-6, TGF- β , and TNF- α , while the group treated with diosmectite alone showed a significant decrease in TNF- α level, which could explain the increase in oocyst shedding count in this group. Lacroix *et al.*²⁴ suggested that TNF- α may have a role in the regulation of parasite development and that the resolution of infection depends on Th1-type cytokine expression in the mucosa of C57BL/6 mice. However, Lean *et al.*²⁵ found that when TNF- α was applied to a mouse enterocyte cell line, it drastically limits *C. parvum* growth.

Diosmectite has an anti-inflammatory effect that has been proved in previous studies,⁶ which explains the lessening in the histopathological score of groups treated with it; however, the significant elevation in TGF- β in the diosmectite-treated mice that even exceeds the infected untreated group could induce a tumorigenic effect in intestinal tissue and abolish interferon- γ activation in the intestine during *C. parvum* infection.^{26,27}

Despite the potent activity of nitazoxanide in reducing oocyst shedding and increasing body weight in cryptosporidiosis infection nitazoxanide individually has shown less improvement in hepatic histopathological score, most probably due to the reduction in the antioxidant activity in the form of inhibition of catalase activity. This agreed with the results of Shams *et al.*,²¹ who found that nitazoxanide causes an obvious decline in catalase and superoxide dismutase with a significant increase in MDA, while Gong *et al.*²⁸ documented that NTZ increased reactive oxygen species contents in the hearts of zebrafish.

Adding diosmectite to nitazoxanide in treating cryptosporidiosis in immunocompromised mice has been found to improve the overall outcome parameters, mostly due to the adsorption of luminal antigens and toxins, an increase in colonic mucin, that interfere with pathogen invasion, as well as direct modulation in cytokine production by mucosal cells.⁶

4.1. Conclusion

This study significantly highlighted the synergistic effect of diosmectite in the potentiation of the therapeutic properties of nitazoxanide. The combination therapy remarkably improved hepatic and intestinal structure and regulated inflammatory parameters, which can be further explored as a better regimen for treating cryptosporidiosis than using only nitazoxanide.

Authors' contributions

E.A.E.S. proposed the idea, shared in writing the manuscript, and arranged to establish the practical aspect. R.M.S. revised the manuscript and participated in the practical interpretation of the results. R.S.Z. supervised the induction of infection in the experiment, performed the parasitological examination of fecal pellets, and recorded all physical changes of murine models throughout the experiment. W.A.O. shared in proposing the idea, revised the pharmacology of the paper and calculating the doses, measuring body weight, shared in writing and revising the manuscript, and was responsible

for interpreting the pathology of specimens. S.A.A. performed the immunological investigations in the study.

Conflicts of interest

There are no conflicts of interest.

Acknowledgment

We would like to express our gratitude to the animal house of Theodor Bilharz Research Institute which provided us with mice and oocysts of *Cryptosporidium spp.*, and offered a well-acclimatized place for animals till the end of the experiment.

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