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Original article

Efficacy of Nitazoxanide Alone or Loaded with Silica Nanoparticle for Treatment of Cryptosporidiosis in Immunocompetent Hosts

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ABSTRACT

Background: Cryptosporidiosis is a major health problem for humans and animals with severe consequences in immune deficient hosts. There is no effective approved drug therapy against Cryptosporidium till now and it is increasingly necessary for evaluating new potential drugs. Nanoparticles are promising for effective treatment of parasitic diseases, as an emerging drug carriers.

The aim of the work: Studying nitazoxanide efficacy alone and compared to nitazoxanide loaded with silica nanoparticles in the treatment of cryptosporidiosis in immunocompetent mice infected with Cryptosporidium.

Materials and methods: The study included 50 Swiss albino mice subdivided into five subgroups, including treatment either with silica nanoparticles alone, with Nitazoxanide alone or by Nitazoxanide loaded with silica nanoparticles. We included infected non-treated and non- infected non treated mice in the study as the positive and the negative controls, respectively. The post-treatment evaluation at two and three weeks was done using parasitological stool examination, histological examination of the intestine and liver, and serological screening for anti-cryptosporidium IgG and IgM using ELISA at the 3rd week only.

Results: Decreased percentages of cryptosporidium oocyst passage in all treated immunocompetent mice groups and an improvement on the intestinal and liver histopathology observed after two weeks and a significant oocyst count reduction and near total histopathological cure observed after the third week with superior results observed in groups treated with Nitazoxanide loaded silica.

Conclusion: Cryptosporidium infection is a potentially harmful condition. Major histopathological changes and clinical deterioration occur in immunocompromized hosts requiring more available therapeutic options. The nitazoxanide loaded silica showed promising results in the treated groups superior to nitazoxanide alone. This gives promising results encouraging further evaluation studies.

Keywords: Cryptosporidium; Nitazoxanide; Silica; Nanoparticles; Parasitic diarrhea.

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* Main subject and any subcategories have been classified according to the research topic.

INTRODUCTION

Cryptosporidium is a protozoan parasite of the phylum Apicomplexa, with other intestinal coccidea [1]. Humans and most vertebrate animals are prone to *Cryptosporidium* infection. *Cryptosporidium* infection led to 25 foodborne and 905 waterborne outbreaks worldwide [2]. There are more than 60 genotypes for *Cryptosporidium* with 41 species identified till now [3]. *Cryptosporidium hominis* [*C. hominis*] *and Cryptosporidium Parvum* [*C. Parvum*] constitute the most prevalent pathogenic species in humans. The host preference in *C. hominis* is mainly anthroponotic, whereas *C. Parvum* is characterized by a zoonotic transmission [4]. Global burden of disease studies revealed that, diarrheal diseases are very important causes of mortality and morbidity in children worldwide. Studies attributed diarrheal diseases to few infectious agents. Providing effective treatment for these agents could have a global impact [2].

In developing countries, *Cryptosporidium* is a major cause of diarrhea in children and comes directly after rotavirus as causes of diarrhea in infancy [children <5 years]. The introduction of a highly effective rotavirus vaccine decreased overall incidence of rotavirus diarrhea. Thus, *Crypto-sporidium* is expected to become the leading cause of childhood diarrhea worldwide [5-6]. Few pharmaco-logical interventions are available for *Cryptosporidium* infection. Nitazoxanide is the only approved drug by the Food and Drug Administration [FDA] [7,8].

Results in HIV-infected patients are not promising with large controversy regarding efficacy of all available drugs [9-11]. The search for new drugs to treat cryptosporidiosis is a high priority to alleviate the high burden of disease. Furthermore, new technologies to recognize the potential drug targets are needed for advancing anticryptosporidial drug design [12].

Recent studies showed that the delivery of new classes of antiprotozoal drugs to treat crypto-sporidiosis are pharmacologically possible [13].

Nano-particles are promising for effective treatment of parasitic diseases, as an emerging drug carrier. Multiple studies addressed the need to reach the perfect formula for combination of nanoparticles and specific anti-protozoal drugs to treat cryptosporidiosis [14-5].

AIM OF WORK

The aim of the present study was to evaluate nitazoxide efficacy alone compared to nitazoxide loaded with silica nanoparticles in the treatment of cryptosporidiosis in immunocompetent mice infected with *Cryptosporidium.*

SUBJECTS AND METHODS

Experimental Animals:

Our study included Fifty laboratory bred immunocompetent mice. The weight of them ranged from 20 to 25 gm. The mice were provided from the Schistosome biological supply program [SBSP] at Theodor Bilharz Research Institute [TBRI], Egypt. Also, silica nanoparticles and nitazoxide silica conjugate preparations were prepared and supplied by the evaluation of drug unit, at TBRI. Fifty mice were divided into Five groups. The first group included 10 animals [10 uninfected and untreated mice; the negative control].

The remaining four groups are infected by *Cryptosporidium* oocysts [infected by oral-gastric gavage. Each mouse was inoculated with 200µl of Phosphate-buffered saline [PBS] containing 10^3 sporulated *C. Parvum* oocysts]. The second group included 10 infected non treated [the positive control]. The third group included 10 infected mice treated with nitazoxanide alone [by oral nitazoxanide, 100mg/kg/ day]. The fourth group included 10 infected mice treated by silica alone and Fifth group included 10 mice treated by Nitazoxanide loaded silica.

Fecal analysis was performed for fecal samples of all included mice to calculate the number of *Cryptosporidium* oocysts/gm for parasitological studies two and three weeks after treatment. Histopathological examination for sections of intestinal and liver biopsies was performed for all groups three weeks after treatment. Serological screening for anti-cryptosporidium IgG and IgM were done using ELISA at the third week only.

Data analysis:

Quantitative data were presented as mean ± standard deviation [SD] and groups included compared by student [*t*] test. On the other side, categorical variables presented as frequency and percentages with groups compared with the Chi-

square test. P-value < 0.05 was considered significant. All data processing and analysis were performed by statistical package for social sciences [SPSS] version 16 [SPSS Inc., Chicago, Illinois, USA].

RESULTS

This study was carried out at the Immuno-parasitology Unit, Immunology and Evaluation of Drug Department, Theodor Bilharz Research Institute [TBRI] located at Cairo, Egypt. The post mice and drug preparation' laboratory work extended for 30 days to evaluate the infection course and to cover the period of natural shedding of Cryptosporidium infection in mice, that is stated to be about 24 days.

There was oocyst reduction at two and three weeks after therapy for all treated mice groups compared with the infected non-treated mice group [Table 1] with a significant deference between all groups and the infected non treated group and highest efficacy for nitazoxanide loaded silica compared with the silica or nitazoxanide alone at both tow and three weeks after therapy. Histopathological examination of sections of small intestine [SI] of the negative control mice group [non- infected non- treated] showed normal structure of the mucosa, normal lamina propria and Bsmall intestinal crypt villous ratio. Goblet cells were normal in number and the mucosal brush border was healthy and well defined as demonstrated in [Figures 1 and 2]. Liver sections of this negative control group also showed intact [preserved] lobular hepatic architecture with hepatocytes showing normal morphological appearance, organized into plates in hexagonal arrangement centered by central veins and separated by vascular sinusoids with portal tracts located on the periphery [Figure 3].

Compared with findings in the negative control group, there were different degrees of inflammatory changes seen in the positive control group. These histopathological changes include changes in intestinal morphology as blunting and shortening of villi in mucosa and villous atrophy, ulcerations and non-specific lymphocytic infiltration of the lamina propria, with numerous numbers of *Cryptosporidium* oocysts present intra and extracellularly with depletion of goblet cells in the infected villi [Figure 4].

Cryptosporidium oocysts were observed adhering to the surface of epithelial cells and intraepithelial with brush border destruction, the terminal part of the ileum was found to be the heaviest infected site with *Cryptosporidium* in infected mice groups [Figure 4].

Sections of Liver of infected immunocompetent positive control group showed intact [preserved] lobular hepatic architecture. The plates of hepato-cytes were thin with moderate hydropic degeneration, binucleated hepatocytes and inter-lobular collection of lymphocytes [Figure 5].

Treated mice with Nitazoxanide alone, silica *and Nitazoxanide loaded with silica*, showed an improvement of the lining epithelium of the intestine, while complete healing of intestinal mucosa with superior results obtained with *Nitazoxanide loaded with silica* treatment [Figures 6,8,10, respectively].

Also, Liver sections of Treated mice with Nitazoxanide alone, silica *and Nitazoxanide loaded with silica* showed an improvement of pathological changes. In the silica treated group still present congested central vein, congested dilated sinusoids with a reduction of an improvement in the previously shown collection of interlobular lymphocytes with intact [preserved] lobular hepatic architecture with apparently thin plates of hepatocytes. Infected nitazoxanide treated mice group showed intact [preserved] lobular hepatic architecture with thin plates of almost normal hepatocytes, small collection of interlobular lymphocytes. Maximal improvement was observed in mice group treated by Nitazoxanide loaded with silica revealing thin plates of almost normal hepatocytes and resolving central vein congestion, and the previous congested dilated sinusoids to normal [Figure 7, 9, 11 respectively].

Serum IgG and IgM were increased during the infection and treatment period in mice groups compared with the negative control group with a highly significant difference between them [Table 2]. But the extent of IgG and IgM differs between the treated and untreated infected groups where the silica treated group had lower serum titres of both IgG and IgM, Whereas Nitazoxanide and the Nitazoxanide loaded silica showed a significant lower positive antibody titers denoting effective role of the drug therapy in abating and decreasing antigen presentation and parasite production in the intestinal cells.

Table [1]: Comparison between means of oocyst numbers in infected non-treated control, NTZ treated, silica treated and NTZ loaded silica treated immunocompetent mice groups.

Variable	Noninfected non treated	Infected Non-treated	Infected NTZ treated	Infected Silica treated	Infected NTZ Silica treated	p
Oocyst 2 weeks	-	34437.10±1341.58	18209.80±1358.59	28897.20±2149.99	13161.90±1342.05	<0.001*
Oocyst 3 weeks	-	13304.90±974.66	7966.80±1453.21	11680.30±1066.28	2181.80±655.90	<0.001*

P-value < 0.05 was significant*

Table [2]: Comparison between groups regarding IgG and IgM levels

Variable	Non infected non treated	Infected Non-Treated	Infected NTZ treated	Infected Silica treated	Infected NTZ Silica treated	p
IgG	0.245±0.011	0.949±0.049	0.557±0.025	0.854±0.033	0.397±0.006	**<0.001***
IgM	0.126±0.008	0.458±0.034	0.255±0.026	0.362±0.021	0.188±0.010	**<0.001***

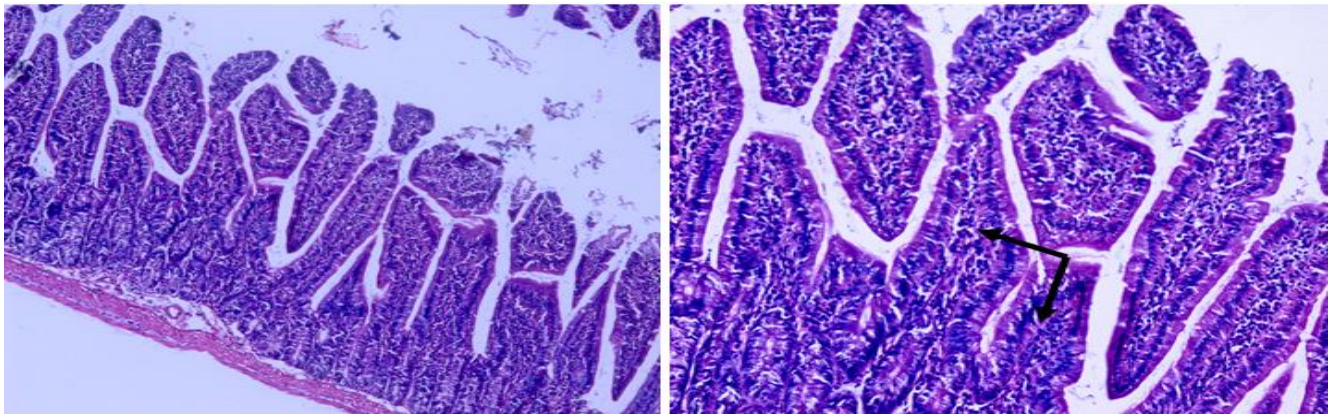

Figure [1,2]: Section of small intestine from the negative control group showing normal mucosal structure and lamina propria with normal crypt villous ratio [black arrows] [H&E,x100,x200].

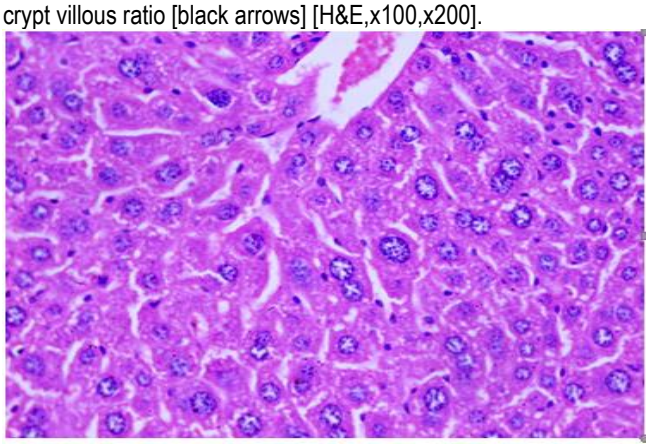

Figure [3]: liver section from the negative control group [-ve control] showed intact [preserved] lobular hepatic architecture, thin plates of normal hepatocytes [black arrow] and normal morphology of hepatocytes [H&Ex400].

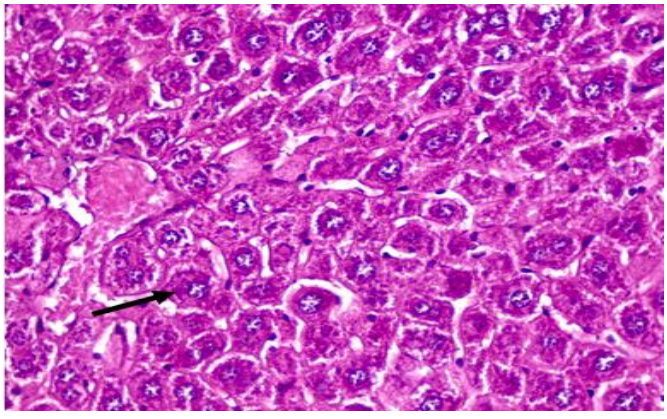

Figure [3]: liver section from normal control group [-ve control] showed intact [preserved] lobular hepatic architecture, thin plates of normal hepatocytes [black arrow] and normal morphology of hepatocytes [H&E x 400].

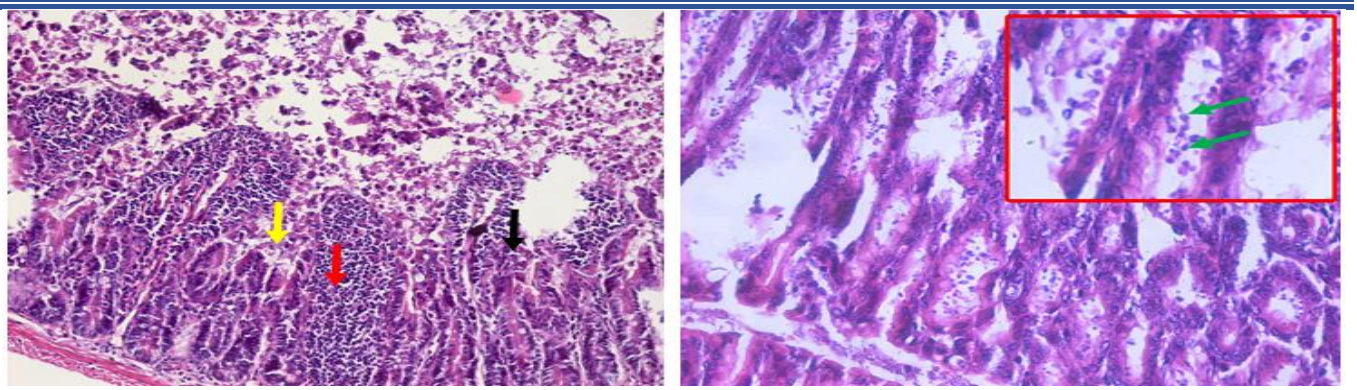

Figure [4]: Sections of small intestine of infected animals [+ve control] showing, blunting and shortening of villous in mucosa and villous atrophy as [red arrows], ulcerations as [yellow arrow] and non-specific inflammatory infiltration of the lamina propria with lymphocytes as [black arrow] [H&E x200] numerous numbers of *Cryptosporidium* oocysts adhere on epithelial cell [green arrow] [H&E, x400].

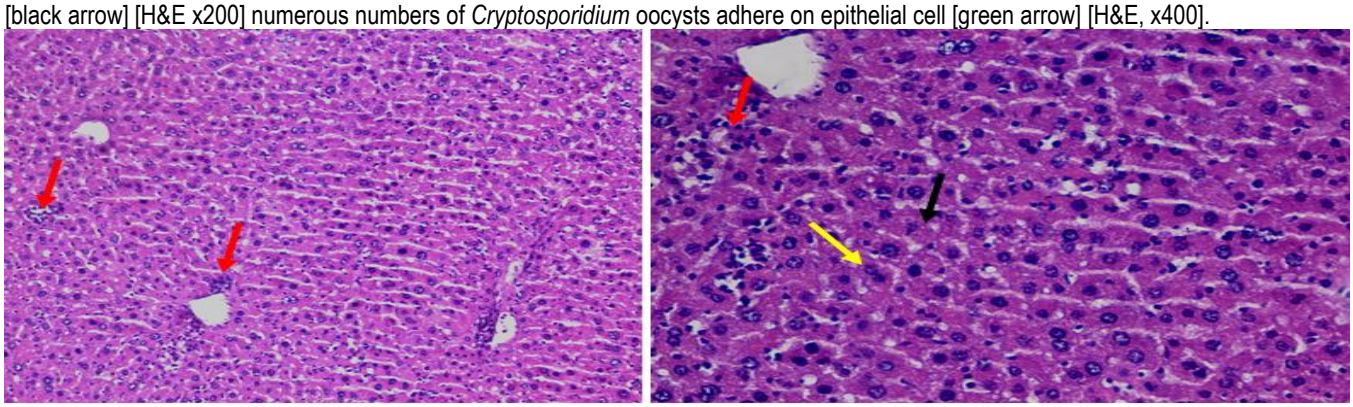

Figure [5]: Sections of Liver of infected animals [+ve control] showing intact [preserved] lobular hepatic architecture, thin plates of hepatocytes with moderate hydropic degeneration [arrows black], binucleated hepatocytes [arrows yellow], interlobular collection of lymphocytes [red arrow] [H&E,x200,x400].

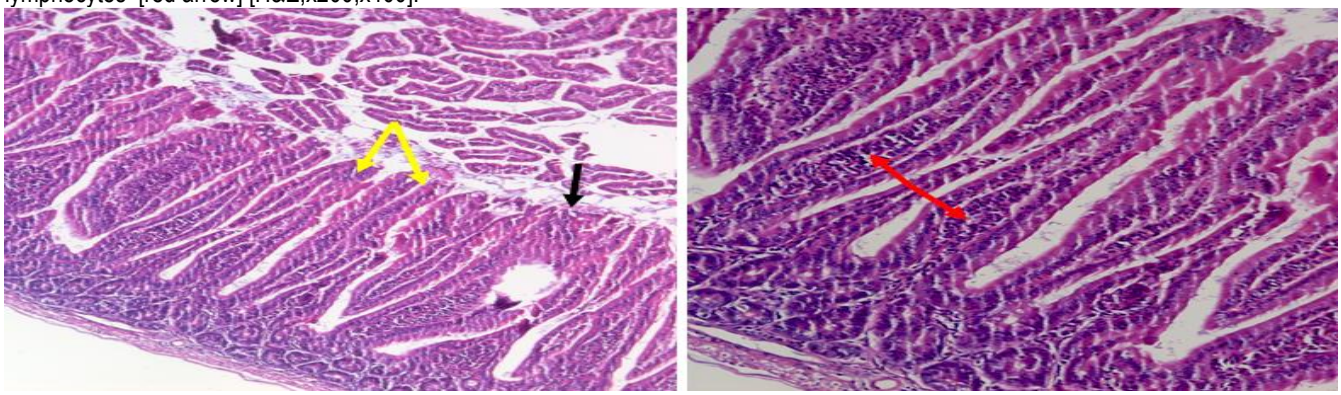

Figure [6]: Sections of small intestine of infected animals and treated with nitazoxanide showed almost normal villous pattern, [yellow arrow], with healed superficial erosion [black arrow], mild lymphocytic inflammatory response noticed in villi [red arrow] [H&Ex200,x400]

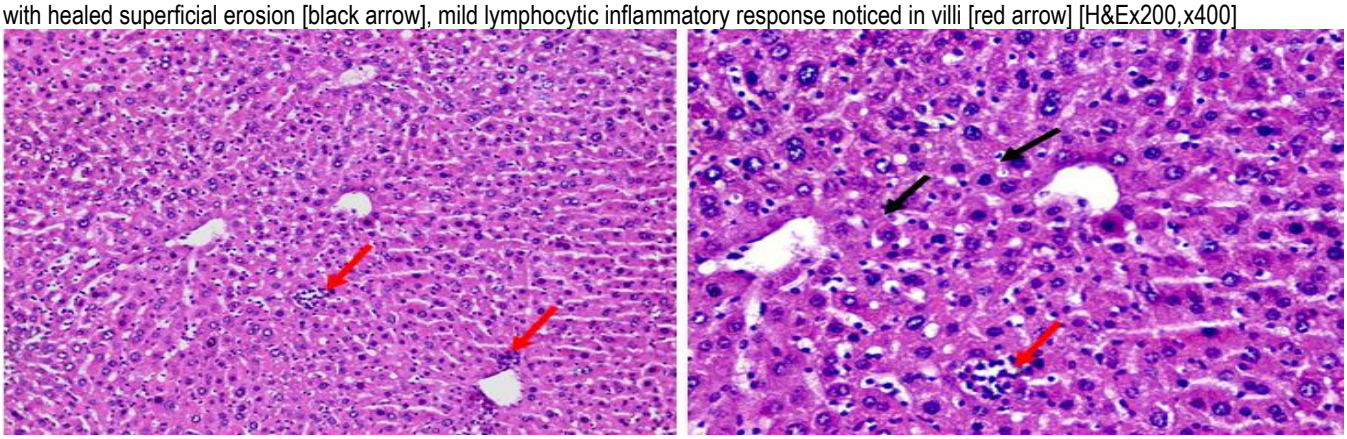

Figure [7]: Sections of liver of infected animals and treated with nitazoxanide showing intact [preserved] lobular hepatic architecture with thin plates of almost normal hepatocytes [black arrows], small collection of interlobular lymphocytes [red arrow], congested dilated sinusoids [arrow green][H&E,x200,x400].

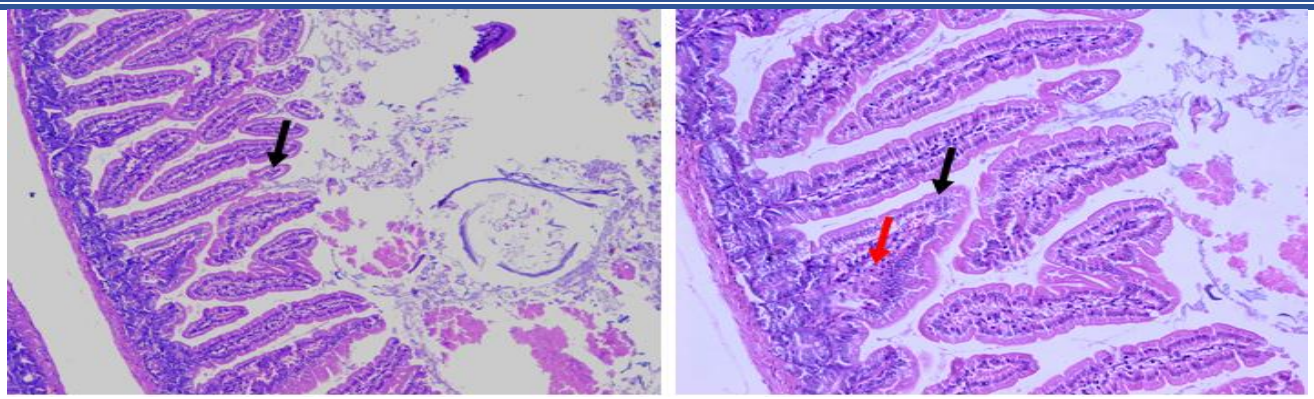

Figure [8]: Sections of small intestine of infected animals and treated with silica nano particles showed almost normal villous pattern, [black arrow], mild lymphocytic inflammatory response noticed in villi [red arrow] [H&Ex100,x200].

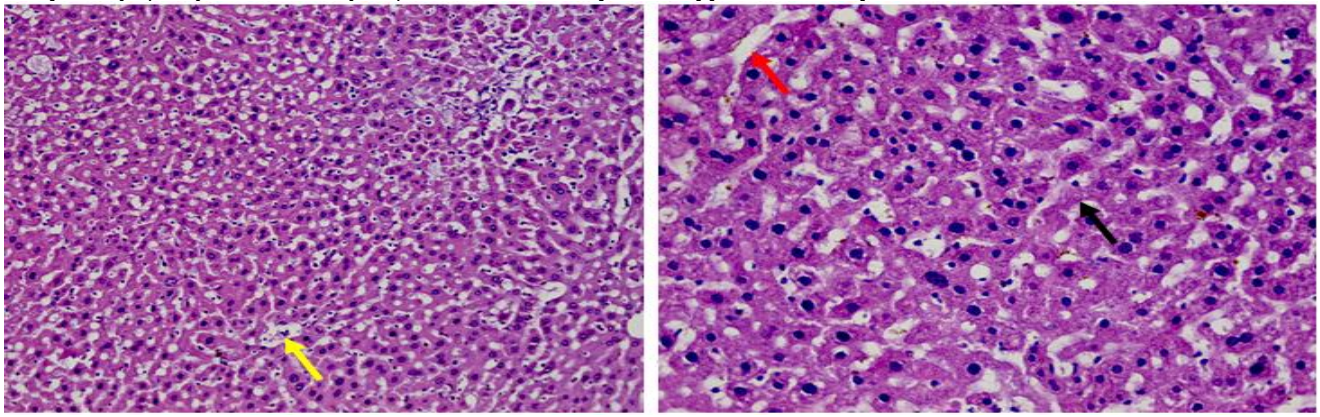

Figure [9]: Sections of Liver of infected animals and treated with silica nano particles showing preserved [intact] lobular hepatic architecture, thin plates of hepatocytes [black arrows], central vein congestion [yellow arrow], dilated congested sinusoids [red arrow][H&E,x200,x400].

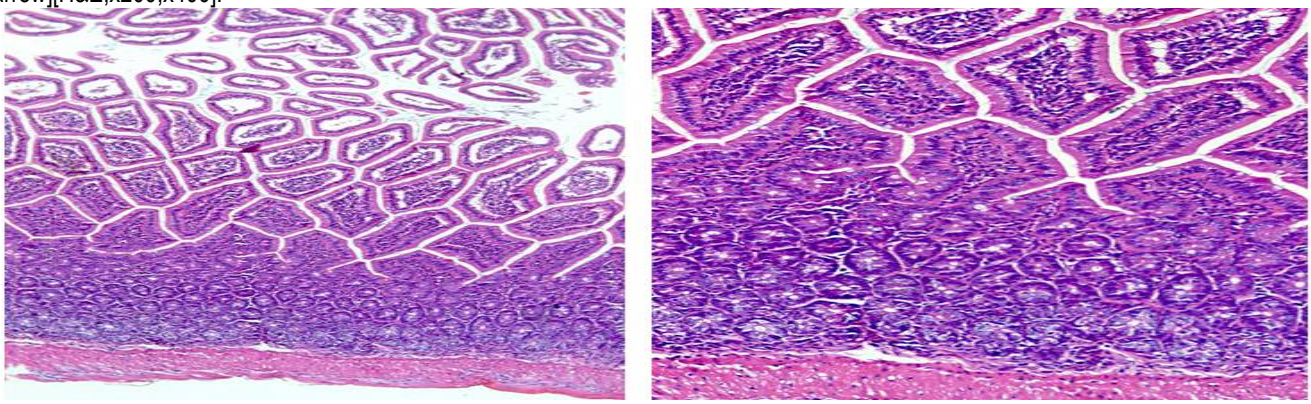

Figure [10]: Sections of small intestine of infected animals and treated with nitazoxanide +silica nano particles showed almost normal villous pattern [H&Ex100,x200].

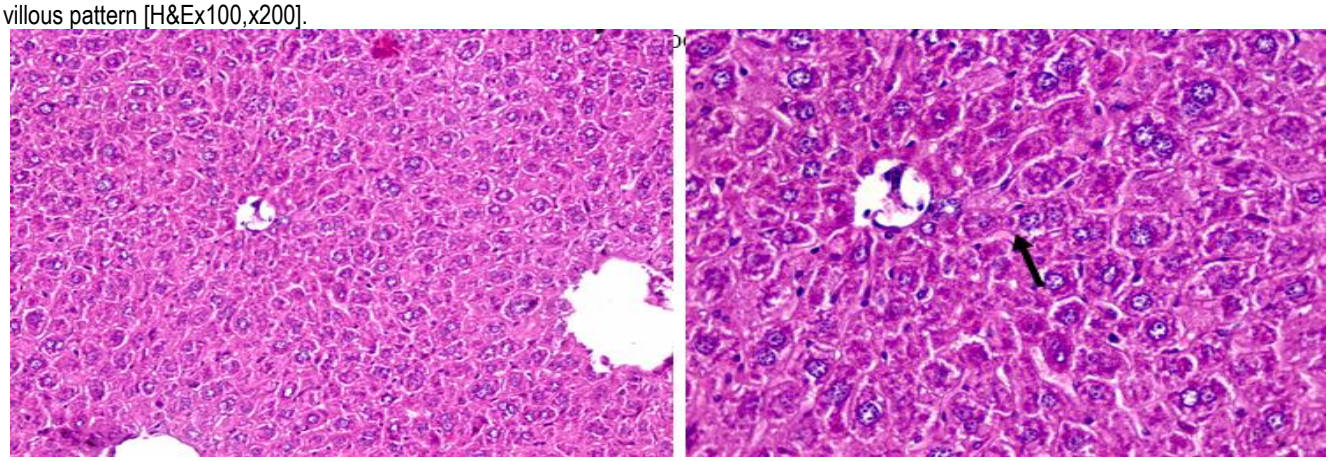

Figure [11]: Sections of Liver of infected animals and treated with nitazoxanide +silica nano particles showing preserved [intact] lobular hepatic architecture with thin plates of almost normal hepatocytes [black arrows], [H&E, x200, x400].

DISCUSSION

Cryptosporidium is considered a serious problem for infected individuals especially the immunosuppressed patients in developed and developing countries [16].

Cryptosporidium is one of the most prevalent waterborne transmitted protozoa in developing countries [17].

It is considered as the fourth major cause of diarrhea in many regions of Sub-Saharan Africa and Asia [2].

Egypt took the lead to study and estimate the presence of pathogenic and nonpathogenic waterborne protozoa with about 1/3 [36/120] of study reports in Africa [18], these observations pavement the road to the largest and most successful Egyptian projects that gained recognition worldwide, the Egyptian presidential campaign to purify water supplies for agricultural use has gained the best ENR [Engineering News Record] construction project prize in 2020 for Al-Mahsamah Water Recycling Mega Station in Ismailia Governorate and also the best water desalination project to Al-Alamin desalination Mega station in the northern coast [19].

Silica nano particles [SiNPs], also named nanoform [with size <100 nm] of silicon dioxide possesses appealing physicochemical properties, they are now used in agriculture extensively, also in food and consumer products including cosmetics. Nanoparticles provide an effective alternate therapy for treatment of parasitic diseases, as an emerging drug carriers, they possess ability to overcome barriers of low bioavailability, low cellular permeability, non- targeted distribution and rapid excretion and inactivation of antiparasitic drugs in the body [20].

The post mice and drug preparation' laboratory work extended for 30 days to evaluate the infection course and to cover the period of natural shedding of Cryptosporidium infection in mice, that is stated to be about 24-30 days [21-22].

The duration of therapy was a positive determinant of parasitological and histopatho-logical cure with an improvement observed on the 15th day of therapy with a significant oocyst count reduction and total histopathological cure observed after the 3rd week of therapy.

Our result comes in agreement with Fahmy *et al*. [15], who reported that, oocyst per gram [OPG] count was higher in the positive control [untreated] mice as compared with treated ones.

In line with our results, Sedighi *et al*. [14], observed that neither nitazoxanide alone nor nano-nitazoxanide were able to eliminate completely Cryptosporidium shedding in treated rats.

Also, Fahmy *et al*. [15], found that, the outputs of oocysts were still high at the end of the experimental work in [all group] more in infected immune-suppressed control group.

This result is in agreement with Abdou *et al*.[23], where Swiss albino mice continued to shed Cryptosporidium oocysts until day 30 post infection.

In spite of the oocyst reduction denoting a self-limited course of disease in the untreated immunocompetent mice group [23].

In our present study nitazoxanide reduced number of Cryptosporidium oocysts compared to the positive control group both after two and three weeks of treatment.

Sedighi *et al*. [14] experiment showed that, nitazoxanide treatment decreased the number of parasites in the treated groups significantly.

Another study by Fahmy *et al*. [15] showed a significant effect of multiple nitazoxanide doses of 10mg/kg body weight, decreasing oocyst per gram [OPG] count in immunocompetent mice from 6th day post treatment and onward [66.1%].

Abdou *et al*. [23], demonstrated the effectiveness of NTZ both in decreasing levels of oocyst excretion in stool and number of intestinal developmental stages of parasite.

Besides, Gargala [24] demonstrated marked reduction in the duration of diarrhea and mortality in infected adults and debilitated children treated with NTZ. However, latter study also addressed the need for hosts' intact immune response for NTZ optimal effect to help in the host rejection for the parasite.

Rossignol [25] reported the NTZ effect variability, depending on the degree of host's immune-suppression and CD4 counts.

In addition, Hussien *et al*. [8] in Egypt reported that among 90 children with C. Parvum infection at Al-Azhar University Hospitals in Assiut, nitazoxanide treatment caused a significant improvement and shortening of the course of diarrhea occurring in 39 of 45 patients [86.6%] with optimal clinical and laboratory cure, while 5 patients showed clinical improvement and a reduction in oocysts' number of while one studied case showed no cure.

In addition, Hussien *et al*. [8] reported that children received Paromomycin 31 out of 45 cases [68.8%] showed complete clinical and laboratory cure, 8 cases showed clinical improvement with a reduction of oocysts number while six cases not cured. Nitazoxanide was highly effective than Paromomycin in treatment of cryptosporidiosis.

On the other hand, Schnyder *et al*. [26], reported that both prophylactic and therapeutic doses of NTZ failed to improve the clinical appearance, intensity and the duration of oocyst excretion in experimentally infected neonatal calves. The studied therapeutic group even had longer and more intense diarrheic episodes compared to the control [untreated] group.

NTZ appeared to be ineffective in both prophylactic and therapeutic doses [26].

The results of the present study demonstrated that a combination of both nitazoxanide and silica NP had the best therapeutic effect on experimentally infected mice. This combination showed the highest reduction of oocyst shedding in mice groups when compared with other infected groups.

Similar results by Sedighi *et al*. [14] experiment showed that encapsulating nitazoxanide in solid lipid nano-particles [SLN] has notable and a significant therapeutic activity in animal model [immune-competent neonatal rat]. The results showed that nano-nitazoxanide is more effective than free drug for control of infection. As oocyst count day three post treatment with nitazoxanide [free drug] and nanonitazoxanide was 10.96 and 9.63, respectively with no significant difference between the two groups. While day 6 post treatment showed significant differences in oocysts count between two groups that were 0.38 and 0.046, respectively. The mean of oocysts' number in both groups decreased significantly on days 3, and 6 after treatment intervention, in comparison to untreated groups, they attribute this difference to the possible increase the bio-availability and slow release of nano drug in the intestine [14].

Other studies also revealed the same results, where Nano nitazoxanide proved to be more effective than free drug on parasites [15], Abdelhamed *et al*. [27], reported that the combination of both nitazoxide and ALPN had the best therapeutic effect on experimentally infected mice with Cryptosporidium.

Fahmy *et al*. [15], detected a highly significant decrease in oocyst per gram [OPG] count more in nitazoxanide loaded with the nano-gold treated immunocompetent mice group [81.5%]. Also, highest reduction of oocysts shed was observed in this group [93.7%].

Joob and Wiwanitkit, study stated that, gold nanoparticle solution has the effect against cryptosporidium oocyst. As average change [reduction] of the mean Cryptosporidium oocyst number after mixing [28].

Compared to normal histopathology in the negative control group in the present study, the small intestine histopathological biopsies of the positive control groups showed significant changes with several degrees of inflammatory changes including changes in intestinal morphology as blunting and shortening of villous mucosa and villous atrophy, ulcerations, non-specific infiltration of the lamina propria with inflammatory lymphocytes and numerous number of Cryptosporidium oocysts and goblet cells depletion of the infected villi.

Several reports similarly show similar histopatho-logical findings in areas of severe injury with vesiculated enterocytes and inconspicuous brush borders in some cases blunted of microvilli may show [28,29-31].

These findings are also in agreement with results of Certad *et al*. [32], Abdou *et al*. [23], and later studies who all addressed similar histopatho-logical changes in either the negative controls or the infected positive control mice [33-34].

Histological examination of small intestinal [SI] sections of the negative control mice showed normal structure of the intestinal mucosa, lamina propria with normal crypt villous ratio [23,32-34].

Healthy well defined brush border of the small intestine and moderate number of Goblet cells were also reported [35].

Studies by Abdou *et al*. [23], Mahmood *et al*. [36], and Abdelhamed *et al*. [27], reported the presence of villus blunting associated with greater inflammation in the lamina propria and hyperplastic crypts. Few cells showed had high nuclear cytoplasmic ratio and hyper chromatic nuclei [low grade dysplasia].

Compared to normal histopathology of Liver sections in the negative control group, liver sections of infected untreated mice group revealed intact [preserved] hepatic lobular architecture, thin plates of hepatocytes with moderate hydropic degeneration with binucleated hepatocytes and interlobular collection of lymphocytes.

This is in accordance with Mahmood *et al*. [33] results in which, histopathological liver sections among the same group revealed some areas of damaged liver cells with vacuole degeneration and loss of radial arrangement of hepatocytes. All small intestinal and liver biopsies of treated groups revealed remarkable improvement with almost normal villous pattern, mild lymphocytic inflammatory response and few number of Cryptosporidium oocysts, with preserved lobular hepatic architecture with thin plates of almost normal hepatocytes, with silica alone having moderate improvement on the histopathology, nitazoxanide [free drug] and nano-nitazoxanide appeared to have a better ameliorating effect on the histopathology of the intestinal infected cell and liver affected tissues with superior results for nano-nitazoxanide treated groups.

After therapy Abdelhamed *et al*. [27], stated that nanazoxide loaded artesunate, showed few numbers of Cryptosporidium parasite surrounded by inflammatory cells at the brush border of the intestinal villi. The infected non treated samples showed sever villous atrophy and inflammatory cells infiltrations.

Aly *et al*. [34] observed mild to moderate inflammatory reaction in the mefloquine treated mice samples while the intensity of inflammation ranged from moderate to marked in the corresponding NTZ samples as compared with the control samples.

In a study by Mahmood *et al*.[36], histopathological finding of infected mice group treated with aqueous extract of P. dactylifera revealed an improvement of the intestinal affection with healing of mucosa, normal goblet cells and increase in the crypt/villous ratio and absent ulceration with straight brush border.

In our study, liver sections of treated mice showed an improvement of pathological changes either with Nitazoxanide alone, silica and Nitazoxanide loaded with silica. Moderate improvement in liver sections of silica treated group is observed with near normal hepatocytes, congested central vein, congested dilated sinusoids with a reduction of an improvement in the previously shown collection of interlobular lympho-cytes. Infected mice treated with nitazoxanide showed moderate improvement with preserved [intact] lobular hepatic architecture with thin plates of almost normal hepatocytes, small collection of interlobular lymphocytes. Maximal improvement was observed in mice group treated by Nitazoxanide loaded with silica revealing thin plates of almost normal hepatocytes and resolving central vein congestion, and the previous congested dilated sinusoids to normal.

Mahmood *et al*. [33] reported a significant improvement in histopathological sections of, liver and spleen from mice treated with miltefosine with the best results obtained in the liver and spleen sections treated with aqueous extract of P. dactylifera [36].

Serum IgG and IgM were increased during the infection and treatment period in mice groups when compared to the negative control group. Nitazoxanide and the Nitazoxanide loaded silica showed lower positive antibody titers than untreated infected groups denoting effective role of drug therapy in abating and decreasing antigen presentation and parasite production in the intestinal cells.

Mahmood *et al*. [33], observed that all cytokines [IL-10 and IL-17, INF-γ, IL-4] level raised after C. Parvum infection compared to the corresponding normal levels in the control group. The markers decreased after treatment with miltefosine except INF-γ and IL-17. While treatment with P. dactylifera showed a decrease in the mean level of all inflammatory cytokines including IL-17 denoting superior immunoregulatory effect [36].

Conclusion:

Cryptosporidium infection can cause disease in both immunocompetent and immune-compromised hosts with several degrees of histopathological abnormalities and clinical deterioration. the nitazoxanide loaded silica showed promising results with near complete resolution of the histopathological changes in the treated groups superior to nitazoxanide alone. While both nitazoxanide loaded silica and nitazoxanide alone decreased significantly the Cryptosporidium oocyst count in treated mice' fecal specimens neither of them

induced complete eradication of oocysts. The clinical cure noticed in the treated groups especially those with nitazoxanide loaded silica gives promising results in treated mice. The positive and likely therapeutic effect of silica nano particles observed in silica treated groups and the absence of apparent toxic effect on the studied liver and intestinal specimens encourages further evaluation studies.

Conflict of Interest Statement

The authors hereby, declare that this research was conducted in the absence of any financial or commercial relations that might have any potential conflict of interest.

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