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Effect of Silver Nanoparticles loaded Nitazoxanide in The Treatment of Murine Cryptosporidiosis in Immunocompetent and Immunosuppressed Mice

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Abstract

Cryptosporidium is a protozoan that can result in severe diarrhea which is usually self-limiting in immunocompetent individuals but may be chronic and life threatening to those who are immunocompromised. Aim of the work to study the effect of silver nanoparticle (AgNps) -loaded nitazoxanide (NTZ) in treatment of cryptosporidiosis in the experimentally infected immunocompetent and immunosuppressed mice. Methods is the study included two major groups of immunocompetent and immunosuppressed mice. Each group had the following subgroups; normal control, infected control, infected treated with nitazoxanide, infected treated with silver nanoparticle, infected treated silver nanoparticle- loaded nitazoxanide 100ug/g and infected treated silver nanoparticles loaded nitazoxanide 200ug/g. Mice were subjected to stool examination for oocysts count and were later sacrificed for organs dissection, histopathological examination of intestinal tissue and assessment of the toxicity of silver nanoparticles in mice tissues. Also, immunological assessment of circulating cytokines (IFN-γ, IL-4, IL-10 and IL-17) in the serum of the mice was done. The results revealed that the silver nanoparticle loaded nitazoxanide (200ug/g) showed the lowest number of oocysts shedding and the highest improvement of histopathological changes with a remarkable decrease in all of the immune mediators in both immunocompetent and immunosuppressed mice. No tissue toxicity in all silver nanoparticles treated mice. Conclusion AgNps loaded NTZ proved its effectiveness against the experimental cryptosporidiosis than nitazoxanide alone.

Keywords: *Cryptosporidium*, Nitazoxanide, Silver nanoparticles.

1. Introduction

Cryptosporidiosis is a worldwide zoonotic parasitic disease caused by various *Cryptosporidium* species [1]. *Cryptosporidium* is a leading cause of diarrheal disease in young children and untreated AIDS patients in resource-limited countries worldwide. Transmission occurs via the fecal–oral route, where

infection occurs through contaminated water or food, or contact with infected people or animals [2]. Globally, cryptosporidiosis is estimated to be responsible for 30-50% of the deaths in children under 5 years of age and is considered the second greatest cause of diarrhea and death in children after rotavirus [3],[4]. Immunocompetent individuals can develop a transient self-limiting illness. However, for immunocompromised patients such as HIV infected individuals, symptoms may include chronic diarrhea and prior to the use of antiretroviral therapy cryptosporidiosis was associated with significant mortality, also extra-intestinal, spreading to other sites including the gall bladder, biliary tract, pancreas and pulmonary system may occur [5],[6]. Nitazoxanide was an important advance in cryptosporidiosis treatment in children, but it has limited efficacy in immunocompromised and malnourished individuals, which raised the importance for development of better drugs for cryptosporidiosis therapy [7]. Currently, there is no vaccine, and only one drug (nitazoxanide), which has limited efficacy in those most susceptible [2]. Silver nanoparticles (AgNps) have attracted attention for medical and chemical applications due to their exceptional properties, including antibacterial activity, high resistance to oxidation, and high thermal conductivity [8]. Cameron [9] reported a duplicate mode of interaction between AgNps and *Cryptosporidium* oocyst, where released Ag ions can enter the oocyst and demolish the sporozoites while nanosized particles of silver can react with the cell wall, resulting in leakage. It significantly decreased the *Cryptosporidium parvum* oocysts count and viability in a safe, effective and cheap manner Hassan [10]. In the present study, AgNps loaded with NTZ was evaluated for the augmented therapeutic and immune modulating effects on Albino mice infected with cryptospordiosis.

2. Materials and Methods

2.1 The Animal:

The present study was carried out on 120 laboratory bred Swiss albino male mice weighing about 20-25g. The animals were provided by Theodor Bilharz Research Institute (TBRI). They were all free from any parasitic infection, as determined by examining their stools using the formol-ether concentration method and modified Ziehl-Neelsen (MZN) technique Henriksen and Pohlenz [11]. Animals were divided into two main groups with 10 mice for each subgroup; Group I (40 mice) (Control group) were subdivided into 2 groups; Group A (normal control group) which included 2 subgroups (A1) non infected immunocompetent and non-infected immunosuppressed mice (A2); Group B included 2 subgroups; (B1) infected non treated immunocompetent mice & Subgroup (B2) infected non treated immunosuppressed mice. Group II (80 mice) infected treated groups; were divided into: Group C nitazoxanide (200 ug/g) treated mice, which was divided into subgroup (C1) treated immunocompetent mice & subgroup (C2) treated immunosuppressed mice; Group D silver nanoparticles (5ug/g) - loaded nitazoxanide treated mice, which divided into I-subgroup (D1) silver nanoparticle (5 ug/g) - loaded nitazoxanide (200 ug/g) treated mice which included (D1a) 10 immunocompetent mice & subgroup (D1b) 10 immunosuppressed mice; II-subgroup (D2) silver nanoparticle (5ug/g)-loaded nitazoxanide (100 ug/g) treated mice included subgroup (D2a) immunocompetent mice & subgroup (D2b) immunosuppressed mice; Group E silver nanoparticle (5ug/g) treated mice, Which included subgroup (E1) immunocompetent mice & subgroup (E2) immunosuppressed mice.

2.2 Oocysts Preparation and Isolation:

The stool samples containing the parasite strain were obtained from TBRI of

experimentally infected mice. The samples were examined then stained by modified Ziehl-Neelsen acid fast stain (MZN) (Kinyoun's Acid-Fast stain, cold method) according to Garcia [12]. The provided sample after sieving was centrifuged at 500xg for 5 min and the supernatant fluid was discarded & the sediment was washed twice in 1 ml of phosphate buffer saline (PBS) with centrifugation at 13,000xg for 2 min. After repeated washing followed by centrifugation, fecal debris was totally eliminated [13]. The infecting dose was calculated by taking the mean of 3 counts of *Cryptosporidium* oocysts per High Power Field (HPF) stained by the Kinyoun's Acid-Fast stain according to Garcia and Bruckner [14]. After that the samples containing *Cryptosporidium* oocysts were ready for infection.

2.3 The Infection:

Mice were infected with *Cryptosporidium* oocysts using oral-gastric gavage in a dose of about 10,000 oocysts/ mouse except normal control [15].

2.4 Immunosuppression:

Immunosuppression was performed by giving synthetic corticosteroids (dexamethasone) (Dexazone) orally at a dose of 0.25 mg/g/day before and after infection till the end of the experiment Abdou [16].

2.5 Drugs Preparation:

2.5.1 Nitazoxanide Tablets:

(500 mg) was dissolved in distilled water and administrated orally at a dose of 200 ug/g body weight/day for four consecutive days [16].

2.5.2 Silver Nanoparticles:

(AgNps) were purchased from Nano tech Egypt Company (6 October City, Cairo, Egypt), and were administrated in a dose of 5ug /g/mouse daily for four days according to Said [17].

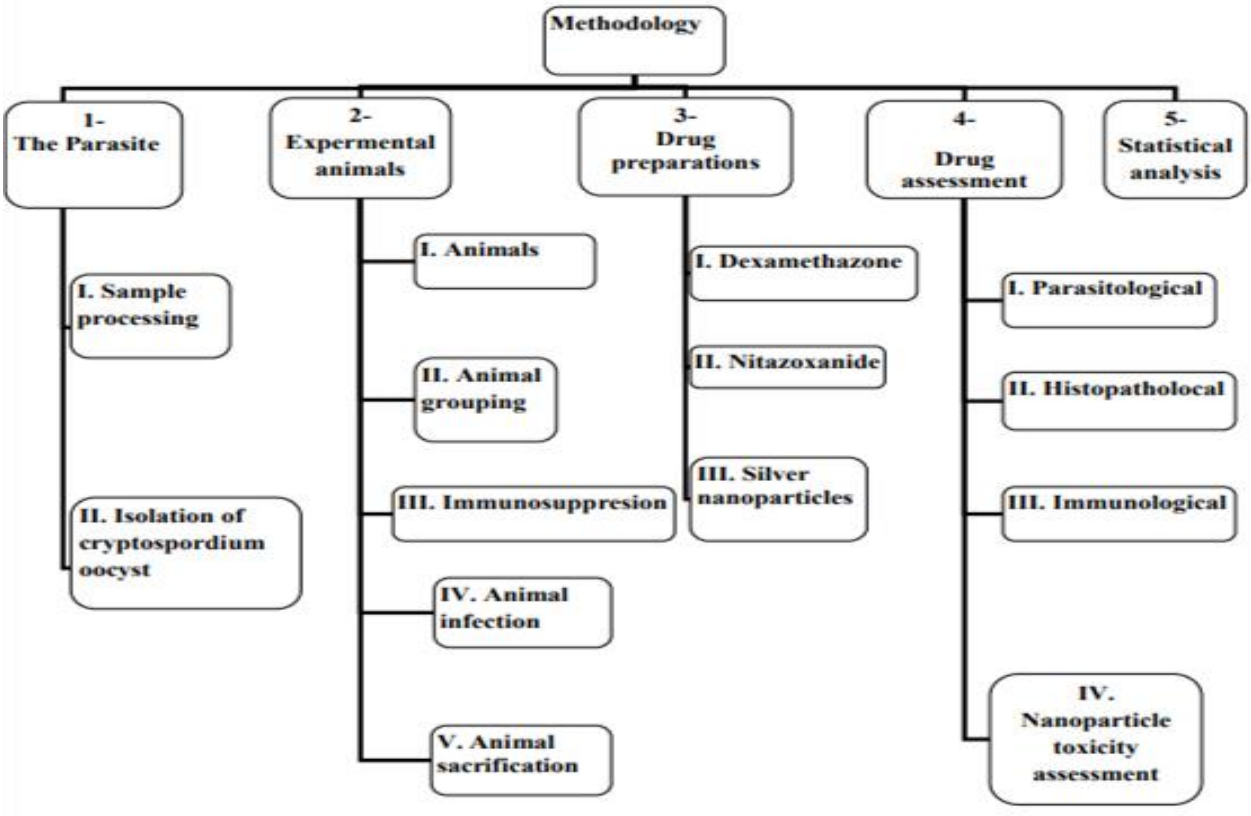

Figure (1): Flow chart of methodology design.

2.5.3 Silver Nanoparticles Loaded Nitazoxanide:

(AgNps (5ug /g) + NTZ 200 ug/g), and (AgNps (5 ug/g) + NTZ 100 ug/g) were given for both groups for four days.

2.6 Drug Assessment:

2.6.1. Stool Examination:

After administration of drugs fresh fecal pellets were collected from infected mice at 7th, 10th ,15th and 30th days post infection and subjected to parasitological examination using the Kinyoun's Acid-Fast stain (cold method) to count the number of *Cryptosporidium* oocysts Garcia [12]. The number of parasites was expressed per gram of faeces Benamrouz [15].

2.6.2. Histopathological Examination:

Segments of about 1cm long from the ileum were immediately fixed in 10% formalin and processed for paraffin embedding. All histopathological sections of 4 µm thickness were stained with Haematoxylin & Eosin stain (Hx&E) and examined to clarify any pathological changes and *Cryptosporidium* developmental forms Drury and Wallington [18].

2.6.3. Immunological Assessment:

Sera were taken out from -20Cº freezer and kept at room temperature to avoid freezing thawing. The Boster Immuonoleader Mouse enzyme-linked immunosorbent assay (ELISA) kits (BOSTER BIOLOGICAL TECHNOLOGY Co., Ltd.) were used to quantify mouse serum (IFN-γ, IL-4, IL-10, and IL-17) Engvall and Perlmann, [19].

2.6.4. Assessment The Toxicity of Silver Nanoparticle in Tissues:

Assessment the toxicity of silver nanoparticle in liver, kidney and spleen tissues of mice was done by using catalase enzyme activity kit (from Bio diagnostic Co.) an antioxidant enzyme in tissues Aebi [20].

2.7. Statistical Analysis:

The collected data was revised, coded, tabulated and introduced to a personal computer. Data was presented and suitable analysis was done according to the type of data obtained. A p-value ≤ 0.05 was considered significant.

3. Results

3.1. Parasitological Results:

The shedding oocyst was observed with Modified Ziehl–Neelsen stain (MZN) as spherical pink organisms (Figure 2) in the stool started from the 4^{th} day in the immunosuppressed mice and at the 5^{th} day in the immunocompetent mice. The peak of oocysts shedding was on the 10^{th} day post infection for both. There was significant difference ($P<0.01$) in *Cryptosporidium* oocysts shedding between immunosuppressed and immunocompetent mice in different days post infection.

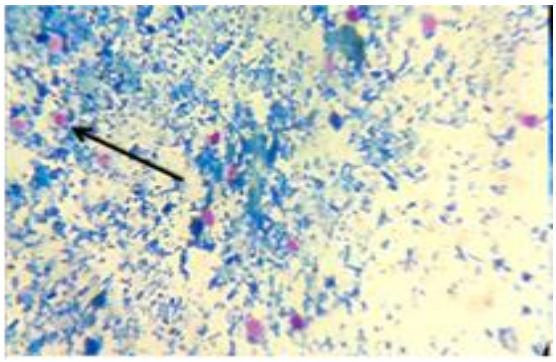

Figure (2): *Cryptosporidium* oocysts stained by modified Ziehl Neelsen (MZN) in stool sample of normal control group. The black arrow referred to *Cryptosporidium* oocysts (x1000).

3.2. Drug Assessment

Among all immunocompetent and immunosuppressed mice there was highly significant difference ($p<0.001$) between *Cryptosporidium* oocysts sheded before and after treatment (Tables 1& 2).

Table (1): *Cryptosporidium* oocysts shedding in one gram stool before and after treatment among all immunocompetent mice.

Immunocompetent subgroups	Day Post Infection		Mean±SD	P value
NTZ (C1)	8 th Before Treatment		51600 ± 8173.1	
	10th	Days After Treatment	16000 ± 3391.2	P<0.001***
	15th		4400 ± 547.7	P<0.001***
	30th		114 ± 49.8	P<0.001***
AgNps (E1)	8 th Before treatment		51600 ± 8173.1	
	10th	Days after treatment	22000 ± 5700.9	P<0.001***
	15th		2900 ± 1181.1	P<0.001***
	30th		240 ± 114.02	P<0.001***
AgNps 100 ug/g (D2a)	8 th Before treatment		51600 ± 8173.1	
	10th	Days after treatment	3400 ± 2607.7	P<0.001***
	15th		1080 ± 334.7	P<0.001***
	30th		0	P<0.001***
AgNps+NTZ200ug/g (D1a)	8 th Before treatment		51600 ± 8173.1	
	10th	Days After Treatment	1040 ± 384.7	P<0.001***
	15th		240 ± 114.01	P<0.001***
	30th		0	P<0.001***

Table (2): *Cryptosporidium* oocysts shedding in one gram stool before and after treatment among all immunosuppressed mice.

Immunosuppressed Subgroups	Day Post Infection		(Mean±SD)	P value
NTZ (C2)	8 th Before Treatment		68000± 8366.7	
	10 th	Days after treatment	37600± 4335.9	P<0.001***
	15 th		14600± 8820.4	P<0.001***
	30 th		720± 130.4	P<0.001***
AgNps (E2)	8 th before treatment		68000± 8366.7	
	10 th	Days after treatment	29400± 12915.1	P<0.001***
	15 th		6600± 1341.6	P<0.001***
	30 th		540± 250.9	P<0.001***
AgNps +NTZ 100ug/g (D2b)	8 th before treatment		68000± 8366.7	
	10 th	Days after treatment	7200± 1643.2	P<0.001***
	15 th		3600± 1140.2	P<0.001***
	30 th		82± 24.9	P<0.001***
AgNps +NTZ 200ug/g (D1b)	8 th before treatment		68000± 8366.7	
	10 th	Days after treatment	2620± 1105.4	P<0.001***
	15th		420± 192.4	P<0.001***
	30th		0	P<0.001***

3.3 Histopathological Results:

Histopathological sections of small intestine of the immunocompetent normal control group showed an average normal villous architecture with average villi/crypt ratio (crypt villous ratio is 1:3 to 1:5). Goblet cells are moderate in number with a well-defined brush border. Immunosuppressed mice showed normal villous architecture and mild inflammation. Different histopathological changes in different treated groups of sections of ileum can be detected in **Figures (3-6).**

3.3.1 Infected Non-Treated Control Group:

Histopathological examination of sections of small intestine of infected non treated immunocompetent and immunosuppressed mice revealed profound changes included shortening and broadening of villi, loss of villous architecture with decreased ratio of villous height to crypt length. Additionally,

there were goblet cell depletion, mucosal ulceration and infiltration of lamina propria with inflammatory cells mainly lymphocytes, eosinophils with diffuse loss of brush border microvillous surface area. These changes were more sever in immunosuppressed than immunocompetent mice**.** Also, *Cryptosporidium* oocysts were detected as tiny purple stained structures about 4-6 µm in Hx & E stain. *Cryptosporidium* oocyst-stained purple in MZN stain.

3.3.2 Infected Nitazoxanide Treated group:

No improvement in the histopathological changes following *Cryptosporidium* infection in the form of persistent moderate to severe villous atrophy, broad villi and infiltration of the lamina propria with inflammatory cells in both immunocompetent and immunosuppressed mice**.**

3.3.3 Infected Silver Nanoparticles Treated group:

Evidence of partial improvement included partial healing of the intestinal mucosa with mild to moderate inflammation and decrease in the ratio between villous heights to crypt length in both immunocompetent and immunosuppressed mice.

3.3.4 Silver Nanoparticles Loaded Nitazoxanide (100 ug/g) group:

This group revealed partial improvement in the histopathological changes following *Cryptosporidium* infection. Evidence of improvement included partial healing of the intestinal mucosa with mild to moderate inflammation and moderate decrease in the ratio between villous heights to crypt length in both immunocompetent and immunosuppressed mice.

3.3.5 Silver nanoparticles loaded nitazoxanide (200 ug/g) group:

Histopathological examination of sections of small intestine in the group treated with silver nanoparticles loaded nitazoxanide (200 ug/g) revealed improvement in the histopathological changes following cryptosporidiosis.

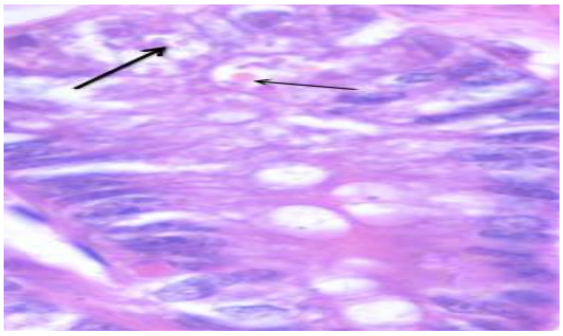

Figure (3): Section of small intestine in infected immunocompetent mice group is showing *Cryptosporidium* oocytes (black arrows) (Hx& E x1000).

Figure (4): Section of small intestine in infected immunosuppressed mice group is showing *Cryptosporidium* oocytes (black arrow) stained tiny purple in MZN (x1000).

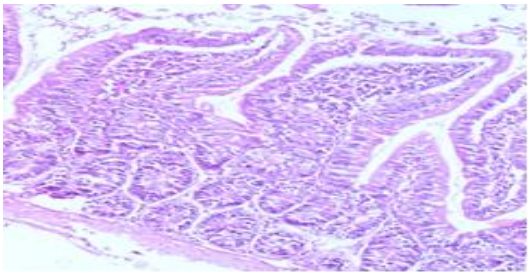

Figure (5): Section of small intestine in infected treated AgNps+ NTZ (200 ug/g) immunocompetent mice group is showing persistent broad villi (Hx &E x200).

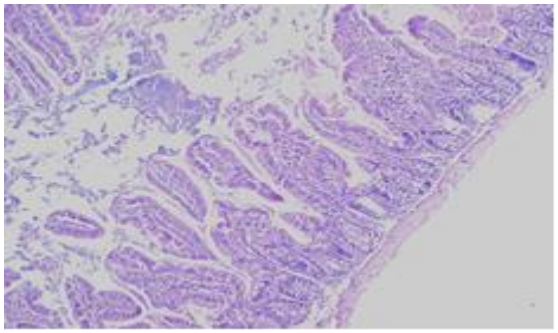

Figure (6): Section of small intestine in infected treated AgNps+ NTZ (200 ug/g) immunocompetent mice group with returning of the villous like pattern with focal broad villi (Hx &E x100).

3.4. Immunological Results (Determination of Cytokines Serum Levels):

Immunological assessment was done by measuring the level of cytokines (IFN-γ, IL-4, IL-10 and IL-17) in mice sera of both immunocompetent and immunosuppressed subgroups using quantitative indirect sandwich Enzyme-Linked Immune-Sorbent Assay (ELISA).
Immunological assessment of both immunocompetent and immunosuppressed mice groups during the infection recorded that all the circulating cytokines (IFN-γ, IL-4, IL-10 and IL-17) increased when compared to the control negative group, but all of the immune mediators decreased after treatment with all used drugs, but the best results were in mice treated with AgNps loaded NTZ (200 ug/g) **(Charts 1&2).**

3.5. Assessment The Toxicity of Silver Nanoparticles:

Assessment was done through estimation of the level of antioxidant catalase enzyme in the tissue homogenates of liver, spleen and kidney organs of both immunocompetent and immunosuppressed mice. Results showed non-significant (P> 0.05) changes in the level of catalase enzyme in all groups of both immunocompetent and immunosuppressed mice treated with silver nanoparticles (5ug/g) either alone or loaded with nitazoxanide in comparison to non-infected non treated control groups **(Tables 3&4).**

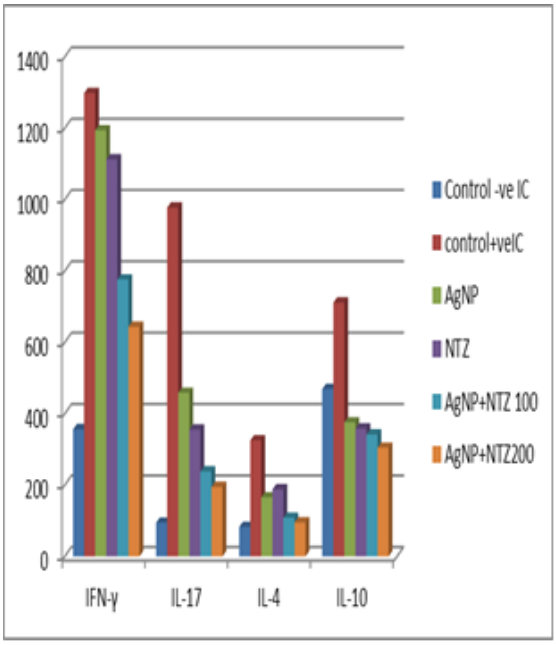

Chart (1): Mean level of all cytokines in immunocompetent mice before and after treatment.

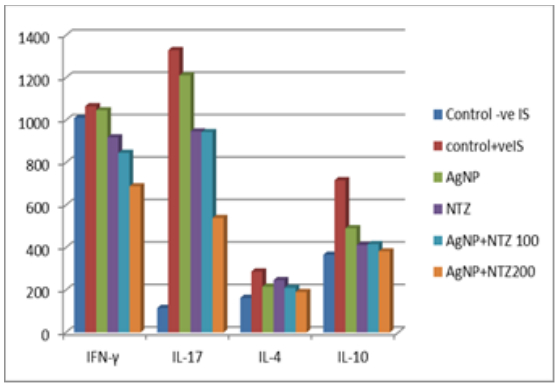

Chart (2): Mean level of all cytokines in immunosuppressed mice before and after treatment.

4. Discussion

Cryptosporidium is one of the most important causes of gastroenteritis in the world, especially in low- and middle-income countries. It mainly affects children and immunocompromised people, in whom it can pose a serious threat to their health,

or even be life threatening Sergio Betancourth [21].

Table (3): Changes in catalase enzyme in all immunocompetent mice treated with silver nanoparticles.

Organ / IC mice	Liver		Spleen		Kidney	
	Mean ± SD	**P value**	**Mean ± SD**	**P value**	**Mean ± SD**	**P value**
Control negative	1.2 ± 0.17		0.34 ± 0.016		0.27±0.02	
AgNps	0.98 ± 0.11	P > 0.05	0.29 ± 0.00	P > 0.05	0.24 ± 0.009	P > 0.05
AgNps +NTZ 100	1 ± 0.11	P > 0.05	0.225 ± 0.04	P > 0.05	0.26 ± 0.009	P > 0.05
AgNps +NTZ 200	1.02 ± 0.15	P > 0.05	0.24 ± 0.02	P > 0.05	0.25 ± 0.03	P > 0.05

Table (4): Changes in catalase enzyme in immunosuppressed mice treated with silver nanoparticles.

Organ / IS mice	Liver		Spleen		Kidney	
	Mean ± SD	**P value**	**Mean ± SD**	**P value**	**Mean ± SD**	**P value**
Control negative	1.02 ± 0.17		0.35 ± 0.014		0.24±0.017	
AgNps	0.8 ± 0.04	P>0.05	0.22 ± 0.008	P>0.05	0.23 ± 0.009	P>0.05
AgNps +NTZ 100 ug/g	1.13 ± 0.4	P>0.05	0.34 ± 0.08	P>0.05	0.3 ± 0.02	P>0.05
AgNps +NTZ 200 ug/g	0.786 ± 0.02	P>0.05	0.29 ± 0.02	P>0.05	0.29 ± 0.02	P>0.05

In the current work, the intensity of oocysts shedding in dexamethasone immunosuppressed mice was significantly higher (P<0.01) than in immunocompetent ones throughout the duration of the experiment. This result agrees with that which has been reported by Certad [22].

The shedding of oocysts continued until 30 days post infection and this result is in agreements with that reported by Abdou [16], where Swiss albino mice continued to shed oocysts until day 30 post infection.

Regarding the effect of nitazoxanide (200 ug/g) on *Cryptosporidium* infection in mice it was found that there was high significant reduction in oocysts shedding in both immunocompetent and immunosuppressed mice with mean percentage reduction 65% and 49% (P<0.001) respectively on 15th and 30th day post infection 88% and 74% (P<0.001) respectively.

These results agreed with that of Amadi [23], who reported that nitazoxanide reduced the duration of diarrhea and oocyst shedding in both, immunocompetent and immunosuppressed patients.

Rossignol [24] recorded higher percentage of reduction (80%) in non-immunodeficient adults and children with cryptosporidial diarrhea in the Nile Delta of Egypt four days post full course administration of nitazoxanide treatment.

Abdou [16] evaluated the effectiveness of nitazoxanide in both immunocompetent and immunosuppressed mice and reported significant differences ($p < 0.05$) regarding levels of oocysts excretion in the stool and the number of endogenous developmental stages of the *Cryptosporidium* in both groups, being lower in immunocompetent mice than immunosuppressed mice.

Regarding the immunosuppressed group our results on 15th day post infection agreed with that recorded by Gargala [25] who tested nitazoxanide and three halogeno-thiazolides (RM-850, RM- 4865 and RM-5038) against *Cryptosporidium* in experimentally infected immunosuppressed Mongolian gerbils. RM-5038 reduced oocysts shedding by 95% while nitazoxanide reduced oocysts shedding by 47% only suggesting that RM-5038 is more effective than nitazoxanide under the experimental conditions used.

These results disagree with the study done by Amadi et al [26], who founded that in Zambian children with HIV-related immunosuppression nitazoxanide does not eradicate this infection nor provide clinical symptom reduction.

On the other hands, the results of reduction in oocysts shedding on 30th day post infection are in relative agreement with the results reported by Rossignol [27], who done a double-blind, placebo-controlled crossover study of NTZ in 66 AIDS patients in Mexico reported parasitological cure (no oocysts detected in fecal samples) rates that were significantly superior to the placebo response in 65% of the patients. Of these, 86% of the patients also reported resolution of diarrhea.

The results of the current study revealed that the percentage of reduction in oocysts shedding in immunosuppressed mice treated with NTZ increased from 49% (on the 15th) to 74% (on the 30th day) and this may be due to improving the immune response in these mice after longer duration of infection.

The effect of 5ug /g of silver nanoparticles when used alone on *Cryptosporidium* infection in mice represent high significant reduction in oocysts shedding in both immunocompetent and immunosuppressed mice with percentage reduction 77% (P<0.001) and 77% (P<0.001) respectively on 15th day post infection while on 30th day post infection it was 85% (P<0.001) and 81% (P<0.001) respectively. These results demonstrated that relatively no difference between the reductions in oocysts shedding of immunocompetent and immunosuppressed mice after treatment with AgNps and this mean that AgNps exert its effect independent on the immune response. Our results agree with the results which reported by Saad [28], who found that AgNps had higher activity in vitro

against *C. parvum* oocysts than copper oxide nanoparticle (CuO Nps).

Regarding the effect of silver nanoparticles loaded nitazoxanide (100 ug/g) on *Cryptosporidium* infection in mice it was found that there was high significant reduction in oocysts shedding in both immunocompetent and immunosuppressed mice with mean percentage reduction 91% (P<0.001) and 87% (P<0.001) respectively on 15th day post infection, while on 30th day post infection it was 100% (P<0.001) and 99% (P<0.001) respectively.

In the same way regarding the effect of silver nanoparticles loaded nitazoxanide (200 ug/g) on cryptosporidiosis in mice, it was found that there was high significant reduction in oocysts shedding in both immunocompetent and immunosuppressed mice with mean percentage reduction 99% (P<0.001) and 98% (P<0.001) respectively on 15th day, while on 30th day post infection it was 100% (P<0.001) and 100% (P<0.001) respectively.

The results revealed that the reduction in oocysts shedding in immunosuppressed mice after AgNps treatment alone was higher than nitazoxanide alone. On the other hand, although silver nanoparticles loaded nitazoxanide (100 ug/g) had good effect on reduction in oocysts shedding in both immunocompetent and immunosuppressed mice, but still silver nanoparticles loaded nitazoxanide (200 ug/g) had the best effect on reduction in oocysts shedding in both immunocompetent and immunosuppressed mice.

Gaafar [29] reported that silver nanoparticles proved their effectiveness against the experimental *Toxoplasma* infection when used singly or combined with chitosan.

Many studies reported significant acaricidal, larvicidal and lousicidal properties in biologically produced AgNPs, proving that they represent an innovative approach to control arthropods and prevent spread of vector-borne diseases Salunkhe [30].

In the present study, histopathological examination of sections of small intestine of the non-infected immunosuppressed control group revealed normal villous architecture and only mild inflammation and this may be due to the effect of dexamethasone administration, while the infected control groups showed profound effects on the structure of the intestinal mucosa in comparison with the non-infected control group and they were severe in immunosuppressed mice than immunocompetent ones. These effects were in the form of villous shortening and atrophy, broadening, decrease in the ratio of villous height to crypt length, goblet cell depletion, mucosal ulceration and infiltration of lamina propria with inflammatory cells mainly lymphocytes and eosinophils.

Several reports similarly show histopathological findings as variable changes ranging from partial to complete villous atrophy and inflammatory infiltrate attributed to *Cryptosporydium* infection Waters and Harp [31], Enemark [32] & Gaafar [33].

Histopathological examination of sections of small intestine in nitazoxanide treated subgroups showed no improvement in the histopathological changes following *Cryptosporidium* infection in both immunocompetent and immunosuppressed mice in the form of persistent moderate to severe villous atrophy, broad villi and infiltration of the lamina propria with inflammatory cells. Similar histopathological finding was reported by Elwakil [34].

On the other hand, histopathological examination of sections of small intestine in silver nanoparticle treated group revealed partial improvement in the histopathological changes following *Cryptosporidium* infection. The group treated with silver nanoparticles loaded nitazoxanide (100 ug/g) revealed partial improvement in the histopathological changes following *Cryptosporidium infection*. Evidence of improvement

included partial healing of the intestinal mucosa with mild to moderate inflammation and moderate decrease in the ratio between villous heights to crypt length.

Histopathological examination of sections of small intestine in the group treated with silver nanoparticles loaded nitazoxanide (200 ug/g) revealed improvement in the histopathological changes following *Cryptosporidium* infection. Evidence of improvement included focal villous atrophy with focal flattening of surface enterocytes. Mild depletion of goblet cells, mild decrease in the ratio between villous heights to crypt length.

The immunocompetent subgroups showed more improvement in the histopathological changes than the immunosuppressed subgroups and the best results were with silver nanoparticles loaded nitazoxanide (200 ug/g) treated subgroups, while the worst results were with nitazoxanide treated subgroups.

This study estimated the level of antioxidant catalase enzyme in the tissue homogenates of liver, spleen and kidney organs of both immunocompetent and immunosuppressed mice as an assessment of the toxicity of silver nanoparticle. The results showed non-significant ($P>0.05$) changes in the level of catalase enzyme in all subgroups of both immunocompetent and immunosuppressed mice treated with silver nanoparticle (5ug/g) either alone or loaded with nitazoxanide in comparison to non-infected non treated control group.

These results agree with many studies have shown that small dose AgNps are more effective antioxidant than large dose AgNps. Also, other studies reported that micro-sized particles are less toxic than their smaller counterparts Rai [35] & Schluesener and Schluesener [36].

Also, Akram [37] investigated antioxidative and oxidative properties of AgNps in different doses and they found that AgNps had beneficial effects for the protection oxidative liver injury in low doses (5& 50 mg/kg/day) and considered toxic in high doses (500 mg/kg/day) after two weeks of treatment.

On the other hand, AgNps being a metal, Ag can be deposited in vital organs causing harmful effects. The dose of Ag NPs (5ug/g) which used in this study was in accordance with Kim [38], who stated that no observable adverse effect was detected by using AgNps of 30 mg/kg, and the dose used in this study was less than this value.

On the other side, Adeyemi and Faniyan [39] reported that silver nanoparticles in doses (100, 1000 and 5000 mg/kg /day for 7-10 days) may cause lipid peroxidation and alter antioxidant status in a manner that may cause oxidative stress. Their findings were not completely in accordance with those reported by Banlunara [40], in which the LD50 of silver nanoparticles in mice was >5000 mg/kg.

5. Conclusion

The effect of nitazoxanide in treatment for cryptosporidiosis in both immunocompetent and immunosuppressed mice improved after loading with silver nanoparticles (AgNps) and it was higher in silver nanoparticles loaded nitazoxanide (200ug/g) than silver nanoparticles loaded nitazoxanide (100 ug/g) groups.

Additionally, the treatment with silver nanoparticles loaded nitazoxanide (200 ug/g) displayed a remarkable improvement of the histopathological changes of the small intestine tissues caused by *Cryptosporidium* infection in immunocompetent and immunosuppressed mice. The study showed that the used dose of silver nanoparticle had no toxological effects in mice after treatment.

So, the results of this study proved that silver nanoparticle loaded nitazoxanide had a remarkable anti *Cryptosporidium* effect than nitazoxanide alone.

References

1. Moawad, H. S. F.; Hegab, M. H. A.; Badawey, M.S. R. et al., (2021): Assessment of chitosan nanoparticles in improving the efficacy of nitazoxanide on cryptosporidiosis in immunosuppressed and immunocompetent murine models. Journal of Parasitic Diseases. 45: 606–619.

2. Dumaine, J.E.; Tandel, J.; Striepen, B. (2020): Cryptosporidium parvum. Trends Parasitol, 36: 485–486.

3. Striepen, B. (2013): Parasitic infections: time to tackle cryptosporidiosis. Nature, (503): 189-191.

4. Khalil, I.A.; Troeger, C.; Rao, P.C. et al. (2018): Morbidity, mortality, and long-term consequences associated with diarrhoea from *Cryptosporidium* infection in children younger than 5 years: a meta-analyses study. Lancet Glob Health.;6: e758–68.

5. Hunter, P.R. and Nichols G. (2002): Epidemiology and clinical features of *Cryptosporidium* infection in immunocompromised patients. Clin. Microbiol. Rev., 15 (1): 145-154.

6. Khan A. A. and Somasundaram, K. (2021): Cryptosporidiosis -A Plausible Cause for Relapse of Guillain-Barré Syndrome. Cureus, 13(4): e14652.

7. Checkley, W.; White, J. A.; Jaganath, D. et al. (2015): A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium.* Lancet Infect. Dis., 15(1):85–94.

8. Soni, N. and Prakash, S. (2011): Efficacy of fungus mediated silver and gold nanoparticles against *Aedes aegypti* larvae. Parasitol. Res., 110 (1):175–84.

9. Cameron, P.; Gaiser, B.K.; Bhandari, B. et al., (2016): Silver Nanoparticles Decrease the Viability of *Cryptosporidium parvum* Oocysts. Appl Environ Microbiol., Vol. 82(2): 431–437.

10. Hassan, D.; Farghali, M.; Eldeek, H. et al. (2019): Antiprotozoal activity of silver nanoparticles against *Cryptosporidium parvum* oocysts: New insights on their feasibility as a water disinfectant. J. Microbiol. Meth. 165:105698.

11. Henriksen, S.A.; Pohlenz, J.F. (1981): Staining of cryptosporidia by a modified Ziehl–Neelsen technique. Acta. Vet. Scand. 22:594–596.

12. Garcia, L.S. (2007): Clinically important human parasites: Intestinal protozoa: *Cryptosporidium spp*. In: Diagnostic Medical Parasitology. L.S. Garcia 5th ed, ASM press, Washington DC., 2: 771-812.

13. Lumb, R.; Swift, J.; James, C. et al. (1993): Identification of the microsporidian parasite, *Enterocytozoon bieneusi* in faecal samples and intestinal biopsies from an AIDS patient. Int. J. Parasitol., 23:793–801.

14. Garcia, L.S. and Bruckner, D.A. (1997): Macroscopic and microscopic examination of fecal specimens. Diagnostic Medical Parasitology. 3rd ed. Washington D.C., AMS press, 608–49.

15. Benamrouz, S.; Guyot, K.; Gazzola, S. et al. (2012): *Cryptosporidium parvum* infection in SCID Mice infected with only one oocyst: qPCR assessment of

parasite replication in tissues and development of digestive cancer. PLOS ONE, 7(12): 512-532.

16. Abdou, A.G.; Harba, N.M.; Afifi, A.F. et al. (2013): Assessment of Cryptosporidium parvum infection in immunocompetent and immunocompromised mice and its role in triggering intestinal dysplasia. Int. J. Infect. Dis., 17: 593-600.

17. Said, D.E.; El Samad, L.M.; Gohar, Y.M. (2012): Validity of silver, chitosan and curcumin nanoparticles as anti-Giardia agents. Parasitol. Res., 111:545–554

18. Drury, R.A.B. and Wallington, E.A. (1980): Carleton's Histological Technique. 5th ed. Oxford, New York, Toronto: Oxford University Press.

19. Engvall, E. and Perlmann, P. (1971): Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunohistochemistry, 8: 871-874.

20. Aebi, H. (1984): Catalase in vitro. Methods Enzymol., 105: 121-126.

21. Betancourth, S.; Archaga, O.; Moncada, W. et al., (2021): First Molecular Characterization of *Cryptosporidium spp*. in Patients Living with HIV in Honduras. Pathogens 2021, 10(3), 336.

22. Certad, G.; Benamrouz, S.; Guyot, K. et al. (2012): Fulminant cryptosporidiosis after near drowning: a human *Cryptosporidium parvum* strain implicated in invasive gastrointestinal adenocarcinoma and cholangiocarcinoma in an experimental model. Appl. Environ. Microbiol., 78: 1746-1751.

23. Amadi, B.; Mwiya, M.; Musuku, J. et al. (2002): Effect of nitazoxanide on morbidity and mortality in Zambian children with cryptosporidiosis: A randomized controlled trial. Lancet, 360: 1375–1380.

24. Rossignol, J.F.; Ayoub, A. and Ayers, M.S. (2001): Treatment of diarrhea caused by *Cryptosporidium parvum*: a prospective randomized, double-blind, placebo-controlled study of nitazoxanide. J. Infect. Dis., 184: 103-106.

25. Gargala, G.; Francois, A.; Favennec, L. et al. (2013): Activity of halogeno-thiazolides against *Cryptosporidium parvum* in experimentally infected immunosuppressed gerbils (*Meriones unguiculatus*). Antimicrob. Agents Chemother., 10:1128.

26. Amadi, B.; Mwiya, M.; Sianongo, S. et al. (2009): High dose prolonged treatment with nitazoxanide is not effective for cryptosporidiosis in HIV positive Zambian children: a randomized controlled trial. BMC Infect. Dis., 9: 195.

27. Rossignol, J.F.; Hidalgo, H.; Feregrino, M. et al. (1998): A double- 'blind' placebo-controlled study of nitazoxanide in the treatment of cryptosporidial diarrhoea in AIDS patients in Mexico. Trans. R. Soc. Trop. Med. Hyg., 92 (1998): 663–666.

28. Saad, A.A.; Soliman, H.I.; Azzam, A.M. et al. (2015): Antiparasitic activity of silver and copper oxide nanoparticle against *Entamoeba histolytica* and *Cryptosporidium parvum* cysts. J. Egypt. Soc. Parasitol., 45(3):593-602.

29. Gaafar, M.R.; Mady, R.F.; Diab, R.G. et al. (2014): Chitosan and silver nanoparticle: promising anti-toxoplasma agents. Exp. Parasitol., 143:30-8.

30. Salunkhe, R.B.; Patil, S.V.; Patil, C.D. et al. (2011): Larvicidal potential of silver nanoparticles synthesized using fungus *Cochliobolus lunatus* against *Aedes aegypti* and *Anopheles stephensi Liston* (*Diptera; Culicidae*). Parasitol. Res.,109: 823–831.

31. Waters, W.R. and Harp, J.A. (1996): *Cryptosporidium parvum* infection in T-cell receptor (TCR)-alpha- and TCR-delta-deficient mice. Infect. Immun., 64:1854-1857.

32. Enemark, H.L.; Bille-Hansen, V.; Lind, P. et al. (2003): Pathogenicity of *Cryptosporidium parvum* evaluation of an animal infection model. Vet. Parasitol., 113: 35-57.

33. Gaafar, M.R. (2012): Efficacy of *Allium sativum* (garlic) against experimental cryptosporidiosis. Alex. J. Med., 48:59–66.

34. Elwakil, E.S. (2015): Effect of natural therapeutic agent on experimental cryptosporidiosis in immunocompetent and immunocompromised mice. Thesis submitted for M.Sc. in Parasitology, Al-Azhar University.

35. Rai, M., Yadav, A. and Gade, A. (2009): Silver nanoparticles as a new generation of antimicrobials. Biotechnol. Adv., 27(1): 76-83.

36. Schluesener, J.K. and Schluesener, H.J. (2013): Nanosilver: application and novel aspects of toxicology. Arch. Toxicol., 87(4):569-76.

37. Akram, R.; Tavakol, H. S.; Farzad, K. et al. (2014): Effects of silver nanoparticle (AgNps) on oxidative stress, liver function in rat: hepatotoxic or hepatoprotective? Biological Sciences and Pharmaceutical Research, 2(5): 040-044.

38. Kim, Y.S.; Song, M.Y.; Park, J.D. et al. (2010): Subchronic oral toxicity of silver nanoparticles. Part. Fibre Toxicol., 7: 20–25.

39. Adeyemi, O.S. and Faniyan, T.O. (2014): Antioxidant status of rats administered silver nanoparticles orally. Journal of Taibah University Medical Sciences, 9(3): 182-186.

40. Banlunara, W.; Maneewattanapinyo, P.; Thammacharoen, C. et al. (2011): An evaluation of acute toxicity of colloidal silver nanoparticles. J. Vet. Med. Sci., 73: 1417e 1423.