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Original article

Efficacy of Atorvastatin Loaded on Nano Particles on Cryptosporidium Parvum in Experimentally Infected Mice

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Abstract:

Background: Cryptosporidium parvum is an intracellular coccidial protozoa that can lead to life-threatening impacts in immunocompromised hosts. Atorvastatin may be used to treat human cryptosporidiosis. Nanomedicine developments appear promising in treatment of cryptosporidiosis. Purpose: The aim of this work is to evaluate the efficacy of atorvastatin loaded on nano-particles on treatment of cryptosporidiosis. **Methods:** Cryptosporidium oocysts were obtained by collection of stools from naturally infected calves. Both Nitazoxanide (500 mg/kg/day) and Atorvastatin (40 mg/kg/day) either alone or loaded on silver nano particles (SNP)- were given to the selected immunosuppressed 110 mice. Assessment of drugs effect was done by parasitological, histopathological and serological assessment. **Results:** The highest group in reduction of number of oocysts recorded in combination group of Atorvastatin and Nitazoxanide loaded on SNP (95.1%). The highest level of serum GSH was recorded in negative control group (26±.5) and the least value recorded in positive control group (7.1±0.9). Combination of Nitazoxanide and Atorvastatin loaded on SNP achieved the highest increase in serum GSH level among treated groups (24.8±1.4). Examination of sections from different treated groups showed remarkable decrease in intestinal inflammation with decreased number of Cryptosporidium oocysts with best results in groups BVII (combination of Atorvastatin and Nitazoxanide) and BVIII (combination group of Atorvastatin and Nitazoxanide loaded on SNP)- showing significant statistical difference compared to positive control group BII. **Conclusion:** Atorvastatin (either alone or loaded on silver nanoparticles) in combination with Nitazoxanide seems to be more efficient and could be a good candidate in the treatment of cryptosporidiosis.

Keywords: Cryptosporidium parvum; Nitazoxanide; Atorvastatin; Silver nanoparticles.

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Introduction

Cryptosporidium parvum is an intracellular coccidial protozoa that can infect the microvillous region of epithelial cells in human and other mammals' digestive tract [(1)]. Infection among HIV-infected individuals may also become extra-intestinal, spreading to other sites including the gall bladder, pancreas, and pulmonary system[(2)].In low-income nations, cryptosporidiosis is a highly prevalent enteric infection of children having prolonged episodes of severe diarrhea and is the second major cause of diarrheal disease and infants' death in Africa and South Asia [(3)].

Despite the fact that cryptosporidiosis has been recognized as an important cause of diarrheal disease for over 3 decades, anti-parasitic treatment has been limited [(4)]. Nitazoxanide has been licensed by the U.S. Food and Drug Administration (FDA) for the treatment of cryptosporidiosis in patients with strong TH-1 response [(5)].

Atorvastatin (ATV) was first approved in the UK as a synthetic statin [(6)]. HMG-CoA reductase inhibition held by ATV and its family in the host liver is well tolerated in man; this made the statins an excellent applicant for repurposing as an anti-cryptosporidial agent [(7)]. Nanoparticles are promising for effective treatment of parasitic diseases, as an emerging drug carrier and multiple studies addressed the need to reach the perfect formula for combination of nanoparticles and specific anti-protozoal drugs to treat cryptosporidiosis [(8)].

The aim of this work is to evaluate the efficacy of Atorvastatin loaded on nanoparticle on treatment of cryptosporidiosis.

Materials and methods

I-Place of the work:

This experimental study was conducted at parasitology department, Faculty of Medicine, Benha University and Zoonotic Diseases Department, Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. The study was started from September 2021 to November 2022. The Ethics Committee of Faculty of Medicine, Benha University, Egypt approved this study code {M.D.15.10.2021}.

II-Drugs:

1) **Nitazoxanide:** 500 mg tablets were used. The used dose was 500 mg/kg /day [(9)].

2) **Atorvastatin:** Drug provided in tablets form (20 mg tabs). The used dose was (40 mg/kg/day) according to AL-Ghandour et al. [(10)].

3) **Dexamethasone:** Drug provided in tablets form (Kahira Pharmaceuticals and Chemical Industries Company, Egypt). The used dose was 0.25 µg/g/day for 14 days before infection according to Rehg et al. [(11)]. All the tablets used were crushed and dissolved in distilled water to make oral suspension and given orally to mice via esophageal tube [(12)].

The preparation of silver nano particles: Silver nanoparticles were prepared by chemical reduction. Commonly used reducing agents are borohydride, citrate, and elemental hydrogen [(13)].

Procedure

Thirty mL of 0.002M sodium borohydride ($NaBH_4$) was added to an Erlenmeyer flask. Dripping 2 mL of 0.001M silver nitrate ($AgNO_3$) into the stirring $NaBH_4$ solution at approximately 1 drop per second. A few drops of 1.5 M sodium chloride solution were added to the suspension [(13)].

Conjugation

- The previously prepared, 1% AgNO3 (10 mL) was added under constant stirring on a magnetic stirrer assembly for 5 min, followed by incorporation of Nitazoxanide (500 mg) and Atorvastatin (40 mg) to obtain [Ag (Drug/MS)] + dispersion.
- Twenty-five mL of a freshly prepared aqueous solution of Nitazoxanide and Atorvastatin- was added under

constant stirring on a magnetic stirrer assembly for 5 min. to the resultant nano silver and maintained at 40°C temperature for 24 h.

Characterization of Silver nanoparticles

The morphology and size of the silver nanoparticles were analyzed by Scanning Electron Microscopy. The Scanning Electron Microscopic analysis was done using LEO 1430 VP, SEM machine **(14).** Scanning electron microscopy images showing that most particles are almost spherical with smooth surface**.**

III- Parasite: *Cryptosporidium* oocysts were obtained by collection of stools from naturally infected calves. The specimens were identified by modified Ziehl-Nelsen technique **(15).**

IV-Experimental animal:

Inclusion criteria: One hundred and ten laboratory-bred male Swiss albino mice, selected from the animal house of TBRI**,** 10 weeks old and approximately 20-25 gram. Mice were housed in plastic cages, fed by commercial complete food mixture and tape water for drinking and maintained under controlled conditions of lighting (12 h light/dark cycle) and temperature (25+/-2^{O}C).

Exclusion criteria: Diseased mice or drug injected mice should be excluded.

V- Mice infection and experimental design: After immunosuppression, all mice in the studied groups except drug control groups and normal control were deprived of water overnight and then inoculated intra-esophageal with the prepared inoculums. The amount given to each mouse was adjusted to contain approximately 10^5 oocysts/ml and each mice received 1ml. The number of *Cryptosporidium* oocysts in the inoculums was determined using a hemocytometer **(12).**

Division of immunosuppressed animals into 2 groups (A & B):

Groups A: Non- infected (drug control) 5 mice in each group, divided into:

- **GA I:** mice received Nitazoxanide orally 500 mg/kg daily for 5 consequent days (5 mice). **GA II:** mice received Nitazoxanide loaded on nano particles orally 500 mg/kg/day for 5 days. **GA III:** mice received Atorvastatin orally 40 mg/kg/day for 5 days. **GA IV:** mice received Atorvastatin loaded on nano particles orally 40 mg/kg/day for 5 days. **GA V:** mice received Atorvastatin orally 40 mg/kg/day in combination with Nitazoxanide orally 500 mg/kg daily for 5 days. **GA VI:** mice received Atorvastatin loaded on nano particles orally 40 mg/kg/day in combination with Nitazoxanide loaded on nano particles orally 500 mg/kg daily for 5 days.

Groups B, which are (normal control +infected groups) 10 mice each, all treated groups received drugs for 5 days, one week post infection:

- **GB I:** Non- infected, non -treated mice. **GB II:** Infected, non- treated mice. **GB III:** Infected mice treated with Nitazoxanide orally 500 mg/kg daily. **GB Iv:** Infected mice treated with Nitazoxanide loaded on nano particles orally 500 mg/kg/day. **GB V:** Infected mice treated with Atorvastatin orally 40 mg/kg/day. **GB VI:** Infected mice treated with Atorvastatin loaded on nano particles orally 40 mg/kg/day. **GB VII:** Infected mice treated with Atorvastatin orally 40 mg/kg/day in combination with Nitazoxanide orally 500 mg/kg daily. **GB VIII:** Infected mice treated with Atorvastatin loaded on nano particles orally 40 mg/kg/day in combination with Nitazoxanide loaded on nano particles orally 500 mg/kg daily.

Assessment of drugs effect:

- **I- Parasitological assessment (Counting the mean No. of oocyst)** according to **Henriksen et al (15):**

  The mice were sacrificed after 4 weeks of housing. The faeces were collected immediately before scarification; faeces were collected from 7 groups (the infected groups B). The faeces of each group were weighted, mixed with saline and filtered. Twenty microliters of the suspension were taken for examination

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under oil immersion lens at x100 magnification of light microscope for oocyst counting. The efficacy percentage of each drug was calculated using the equation: Efficacy (%) =mean value of infected untreated group - mean value of infected treated group x100 / mean value of infected untreated group [16].

II- Histopathological assessment: Tissue samples from ilium of studied groups were fixed in 10% neutral buffered formalin for 24h, dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin blocks. Sections 4micron thick were stained with H&E and Geimsa stain for light microscopic examination [17].

III-Serology

Sample preparation

1. Collect whole blood into tubes without additives.
2. Keep at room temperature for 20 minutes.
3. Centrifuge 10 minutes at 3,000 rpm.
4. Aliquot into small tubes and store at -80°C until use.

Glutathione Colorimetric Technique:

Glutathione reduced (GSH) procedure:

Principle: The method based on the reduction of 5, 5` dithiobis (2 - nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm[18].

IV- Assessment of mortality rate.

Statistical Methods of Analysis

The collected data were coded then entered and analyzed using the SPSS version 26-2020 (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) for Microsoft Windows 10.

The following tests were used:

- **Descriptive analysis** of the results in the form of (Mean value ± SD) calculation for quantitative data.
- **ANOVA test:** For comparison between related groups with normal distribution.
- Differences were considered significant at P-values ≤0.01. P-values of post hoc analysis were written as *** superscript symbol and expressed by ABC letters.
- **LSD** (Post hoc test) was determined between the examined groups [19].
- **The percentage of reduction (efficacy)** was calculated between the treated groups and the control groups in the same column by:

Reduction rate (%) = $\frac{\mathbf{A-B}}{\mathbf{A}}\mathbf{x100}$

A = Number of *Cryptosporidium* oocyst of control group (positively infected without drug treatment).

B = Number of *Cryptosporidium* oocyst of treated groups (positively infected with different treatments).

Ethical consideration: All the animal experiments were performed according to the rules of the Scientific Research Ethical Committee, Faculty of Medicine Benha University, Egypt **{M.D.15.10.2021}** Also, animal handling and all procedures were done in agreement with the TBRI ethical guidelines.

Results:

All treated groups made a statistically significant reduction in the number of *Cryptosporidium parvum* oocysts when compared with control infected non-treated group (GBII). The highest group in reduction of number of oocysts recorded in combination group of Atorvastatin and Nitazoxanide loaded on SNP for 5days (GBVIII) (95.1%).

Table (1): Effect of studied tested drugs on *Cryptosporidium* oocytes shedding in stool of male Swiss albino mice after 5 days treatment.

Groups	Tested drug	Dose/day mg/kg	Treatment duration	Number of oocytes/HPF (mean ± SD)	Percentage of reduction in the number of oocytes	Duncan (Post hoc)
GB I	ND	_-_	-	-	-	
GB II	ND	-	-	**20.5±1.04**	**0**	**A**
GB III	NTZ	500	5 days	**10.7±1.03*** **	**47.8**	**B**
GB IV	NTZ (nano)	500	5 days	**4.0±0.2*** **	**80.5**	**C**
GB V	Ator	40	5 days	**11.7±0.4*** **	**42.9**	**D**
GB VI	Ator (nano)	40	5 days	**5.0±0.4*** **	**75.6**	**E**
GB VII	Ator+NTZ	40+500	5 days	**3.0±0.4*** **	**85.4**	**F**
GB VIII	Ator+NTZ (nano)	40+500	5 days	**1.0±0.2*** **	**95.1**	**G**

Abbreviations: NTZ Nitazoxanide /Ator Atorvastatin/ HPF high power field /SD standered deviation / ND no drug.

Table (2): Mean GSH values in sera of male Swiss albino mice in drug control groups after 5 days from drug receiving.

Groups	Tested drug	Dose/day mg/kg	GSH (mean ± SD)	P value	Duncan (post hoc)
GB I	Control –(ND)	-	26.0±0.5***	<0.01	A
GA I	NTZ	500	19.7±.5***	<0.01	C
GA II	NTZ (nano)	500	22.7±0.2***	<0.01	B
GA III	Ator	40	22.6±1.9***	<0.01	B
GA IV	Ator (nano)	40	22.5±1.1***	<0.01	B
GA V	Ator+NTZ	40+500	22.9±0.4***	<0.01	B
GA VI	Ator+NTZ (nano)	40+500	23.2±0.6***	<0.01	B

Table (2) showing the effect of tested drugs on glutathione (GSH) level in sera of drug control non infected mice compared to normal non infected non treated (GBI) (26.0±0.5). The group received Nitazoxanide alone (GAI) recorded the least value (19.7±0.5) and the group received combination of Atorvastatin + Nitazoxanide loaded on SNP (GAVI) achieved the highest level of GSH (23.2±0.6) among groups received the drug.

All groups made a statistically significant difference in the level of serum GSH when compared with normal control (non-infected non-treated) group (GBI). The highest level of serum GSH was recorded in negative control group (GBI) (26±.5) and the least value recorded in positive control group (GBII) (7.1±0.9). Combination of Nitazoxanide and Atorvastatin loaded on SNP for 5 days (GBVIII) achieved the highest increase in serum GSH level among treated groups (24.8±1.4).

Table (3): Mean GSH values in sera of male Swiss albino mice of the control and infected treated groups after 5 days treatment.

Groups	Tested drug	Dose/day mg/kg	GSH (mean ± SD)	P value	Duncan (Post hoc)
GB I	Control -	-	26.0±0.5***	<0.01	A
GB II	Control +	-	7.1±0.9***	<0.01	H
GB III	NTZ	500	13.7±0.8***	0.01	G
GB IV	NTZ (nano)	500	20.8±1.3***	<0.01	F
GB V	Ator	40	11.9±0.9***	<0.01	E
GB VI	Ator (nano)	40	18.9±1.1***	<0.01	D
GB VII	Ator + NTZ	40+500	21.4±1.3***	<0.01	C
GB VIII	Ator+NTZ (nano)	40+500	24.8±1.4***	<0.01	B

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Table (4): Histopathological lesion score in control and treated groups.

Group		Geimsa			Total	Inflammatory infiltrate				Total	Villous broadening and fusion				Total
		No	Few	Many		Minimal	Mild	moderate	marked		normal	Mild	Moderate	marked	
BI	No. (%)	6(100%)	0 (0.00%)	0 (0.00%)	6 (100%)	6 (100%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	6 (100%)	6 (100%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	6 (100%)
BII	No. (%)	0 (0.00%)	2 (33.3%)	4 (66.7%)	6 (100%)	0 (0.00%)	0 (0.00%)	3 (50%)	3 (50%)	6 (100%)	0 (0.00%)	1 (16.7%)	2 (33.3%)	3 (50%)	6 (100%)
BIII	No. (%)	2 (33.30%)	4(66.70%)	0[a] (0.00%)	6 (100%)	0 (0.00%)	4 (66.7%)	2 (33.3%)	0[b] (0.00%)	6(100%)	0 (0.00%)	4 (66.7%)	2 (33.3%)	0 (0%)	6 (100%)
BIV	No. (%)	3 (50%)	3 (50%)	0[c] (0.00%)	6 (100%)	0 (0.00%)	3 (50%)	3 (50%)	0[b] (0.00%)	6 (100%)	0 (0.00%)	3 (50%)	3 (50%)	0 (0%)	6 (100%)
BV	No. (%)	3 (50%)	2 (33.3%)	1 (16.7%)	6 (100%)	0 (0.00%)	2 (33.3%)	4 (66.7%)	0 (0.00%)	6 (100%)	0 (0.00%)	3 (50%)	3 (50%)	0 (0%)	6 (100%)
BVI	No. (%)	3 (50%)	3 (50%)	0 (0.00%)	6 (100%)	0 (0.00%)	2 (33.3%)	4 (66.7%)	0 (0.00%)	6 (100%)	0 (0.00%)	3 (50%)	3 (50%)	0 (0%)	6 (100%)
BVII	No. (%)	4 (66.7%)	2 (33.3%)	0[a] (0.00%)	6 (100%)	2 (33.3%)	3 (50%)	1 (16.7%)	0[a] (0.00%)	6 (100%)	2 (33.3%)	2 (33.3%)	2 (33.3%)	0 (0%)	6 (100%)
BVIII	No. (%)	5 (83.3%)	1 (16.7%)	0[a] (0.00%)	6 (100%)	3 (50%)	3 (50%)	0 (0.00%)	0[a] (0.00%)	6 (100%)	5 (83.3%)	1 (16.7%)	0 (0%)	0 (0%)	6 (100%)
Total	No. (%)	25 (52.1%)	18 (37.5%)	5 (10.4%)	48 (100.0%)	11 (22.9%)	17 (35.4%)	17 (35.4%)	3 (6.3%)	48 100.0%	13 27.1%	17 35.4%	15 31.3%	3 6.3%	48 100.0%

[a] Significant statistical difference compared to positive control group p value 0.036
[b] Significant statistical difference compared to positive control group p value 0.027
[c] Significant statistical difference compared to positive control group p value 0.018
No.= number, %= percentage.

Results of histopathological examination:

Histopathological examination of sections stained for hematoxylin & eosin from positive control group (BII) showed moderate to marked inflammatory infiltration by lymphocytes, plasma cells and macrophages, Fig. (1). There was moderate to marked villous broadening, fusion and blunting. There were many *Cryptosporidium* oocysts related to brush border highlighted by Geimsa stain, Fig. (1). Examination of sections from different treated groups showed remarkable decrease in intestinal inflammation and less affection of intestinal villi with decreased number of *Cryptosporidium* oocysts, Fig. (2) with best results in groups BVII (combination of Atorvastatin and Nitazoxanide) and BVIII (combination group of Atorvastatin and Nitazoxanide loaded on SNP), Fig (3) showing significant statistical difference compared to positive control group BII. No significant difference between groups treated with oral drug doses when compared to drugs loaded on nanoparticles. All negative control groups (BI, AI to AVI) showed unremarkable histopathological changes.

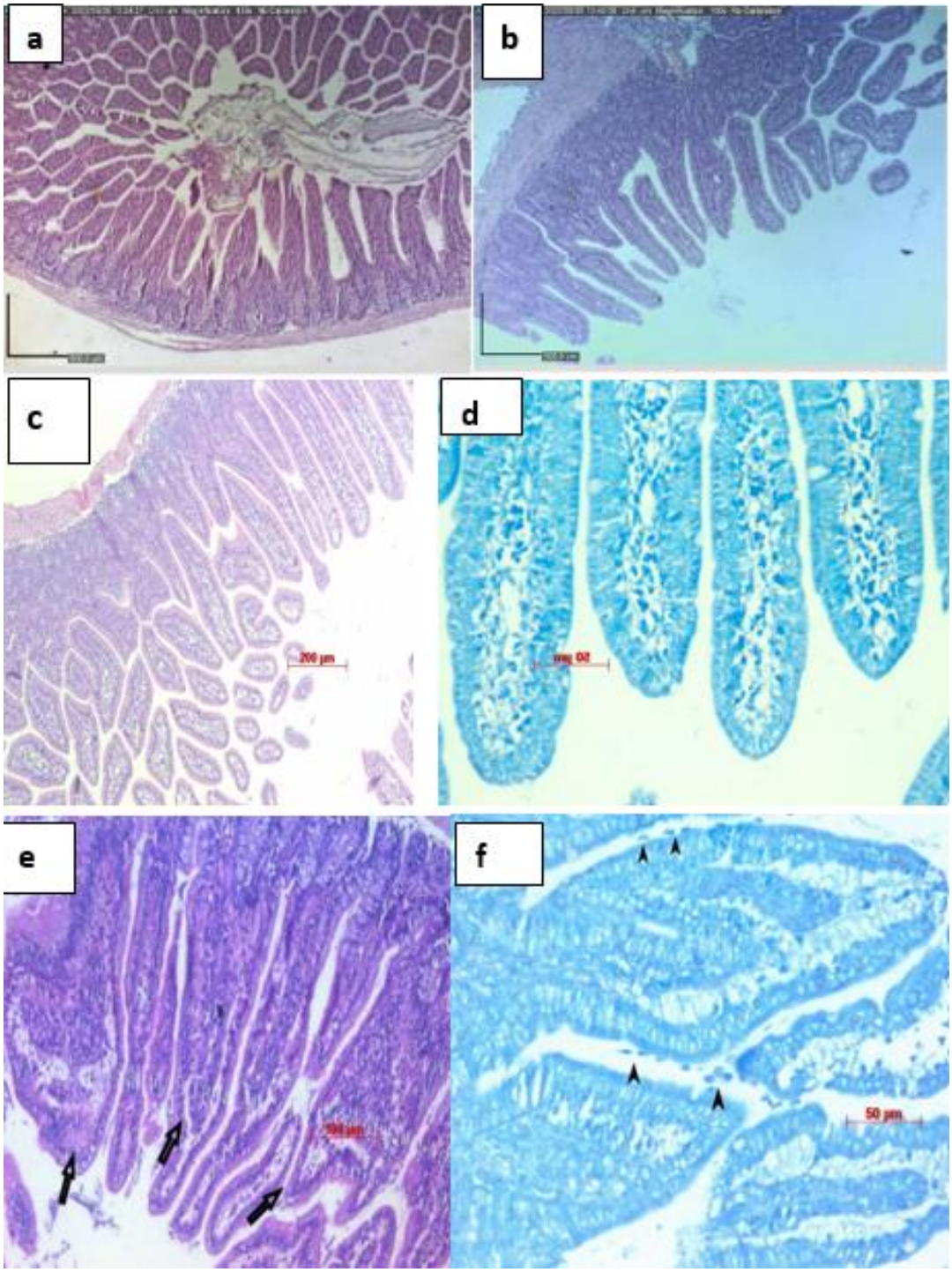

Fig. (1): Histopathological sections of intestinal tissue showing **(a)** and **(b)** Sections in intestinal tissue of non-infected drug control groups showing normal villous architecture with minimal inflammatory infiltrate (Hx&E stain, x100). **(c)** Section in intestinal tissues of non-infected non treated group (GBI) showing normal intestinal villous architecture with minimal chronic inflammatory cell infiltrate (Hx&E stain, x100). **(d)** Section in intestinal tissues of non-infected non treated group (GBI) showing no *Cryptosporidium* oocysts (Geimsa stain, x400). **(e)** Hx & E Stained section in positive control group (BII) (x100). [arrows on fused & braodened villi with marked chronic inflammatory infiltrate]. **(f)** Geimsa stained section showing many *Cryptosporidium* oocysts in positive control group (BII) (x400).

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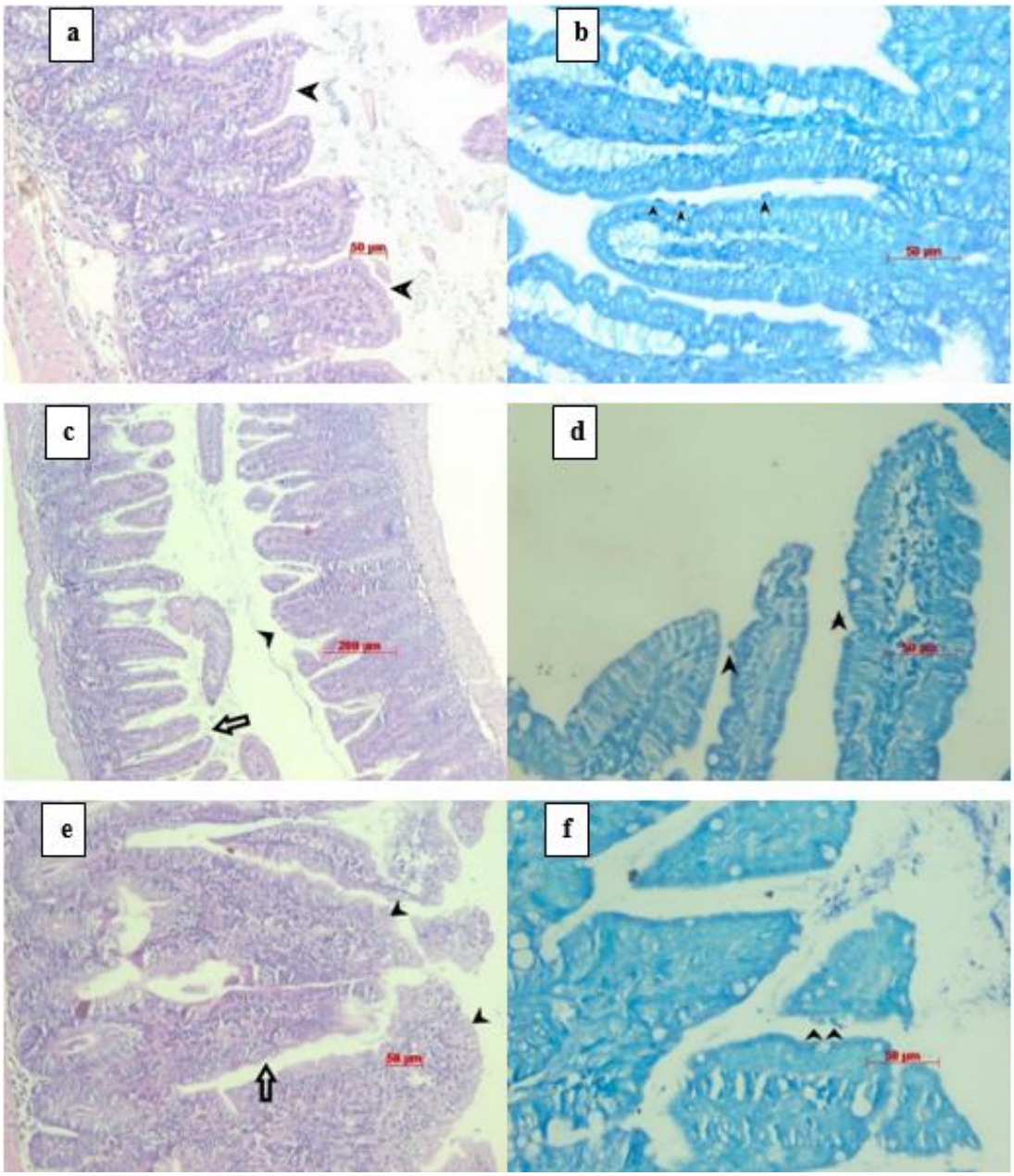

Fig. (2): Histopathological sections of intestinal tissue showing **(a)** Section from group BIII showing moderately fused & braodened villi with mild chronic inflammatory infiltrate (short arrow) (Hx & E Stained, x200). **(b)** Section from group BIII showing few *Cryptosporidium* oocysts (short arrow) (Geimsa stained, x400**).(c)** Section from group BIV showing focally fused & braodened villi with mild chronic inflammatory infiltrate (short arrow) & other normal villi (long arrow) (Hx & E Stained, x100). **(d)** Geimsa stained section from group BIV showing few *Cryptosporidium* oocysts (short arrow) (x400**).(e)** Section from group BV showing moderately fused & braodened villi (short arrow) with moderate chronic inflammatory infiltrate (long arrow) (Hx & E Stained, x200). **(f)** Geimsa stained section from group BV showing few *Cryptosporidium* oocysts (short arrow) (x400).

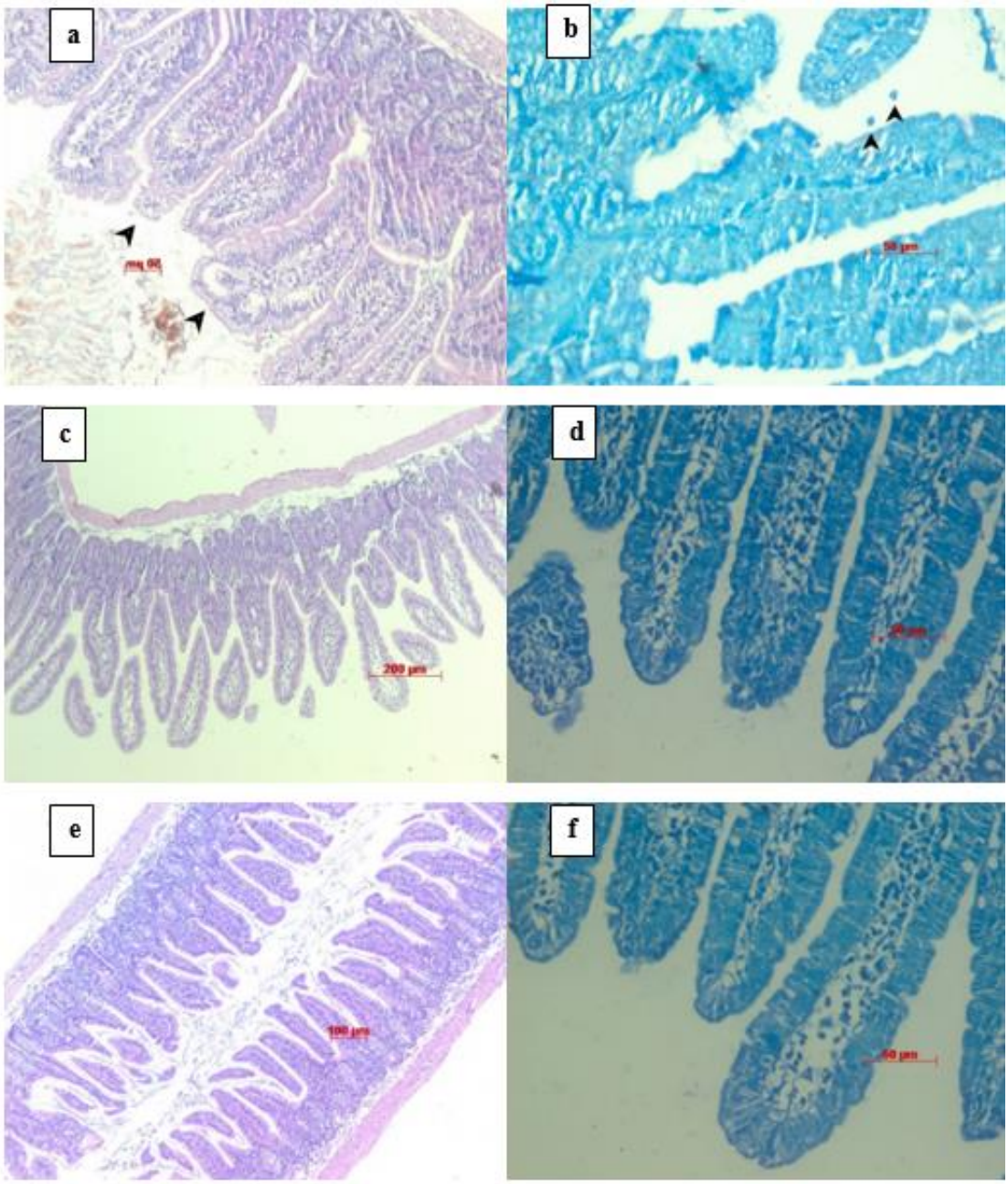

Fig. (3): Histopathological sections of intestinal tissue showing **(a)** Section from group BVI showing mildly fused & braodened villi with mild chronic inflammatory infiltrate (arrow) (Hx & E Stained, x200). **(b)** Geimsa stained section from group BVI showing few *Cryptosporidium* oocysts (short arrow) (x400). **(c)** Section from group BVII showing normal intestinal villous architecture with minimal chronic inflammatory cell infiltrate (Hx&E stain, x100**). (d)** Section from group BVII showing no *Cryptosporidium* oocysts (Geimsa stain, x400). **(e)** Section from group BVIII showing normal intestinal villous architecture with minimal chronic inflammatory cell infiltrate (Hx&E stain, x100). **(f)** Section from group BVIII showing no *Cryptosporidium* oocysts (Geimsa stain, x400).

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Discussion

Cryptosporidium is an important food-borne pathogen causing a disease of socioeconomic significance worldwide [21]. The infection is self-limited in immune-competents. However, in immune-compromised persons, it can cause life threating diarrhea [22]. The lack of successful cryptosporidiosis therapies and vaccinations conserves a cycle of infection, malnutrition and immunosuppression- which can lead to a spectrum of conditions termed as "environmental enteropathy[3].

Recently, Statins have gained a lot of attention due to many factors that includes inhibition the growth of both procyclic and epimastigote forms of *Trypanosomes* [23], an activity against the proliferation of Toxoplasma gondii inside the macrophages [24].

According to this study results, Nitazoxanide (NTZ) was used in a dose of 500 mg/kg/day and gave (47.8%) reduction in oocyst shedding (Table 1). This agreed with Li et al [25] who studied the long-lasting anti-cryptosporidial activity of NTZ in an immunosuppressed rat and showed that NTZ at either 50mg/ kg/day, 100mg/kg/day or 200mg/kg/day, in seven days duration gave a dose-dependent reduction (45.1%) in oocyst shedding. In accordance with this study results, the therapeutic dose of Atorvastatin (40mg/kg) enhanced a high significant oocyst reduction (42.9%) when used alone or in combined regimen with Nitazoxanide (85.4%). The drug control group by Nitazoxanide (500mg/kg) alone achieved oocyst reduction by (47.8%) as compared with the infected control group (Table 1). This agreed with Madbouly Taha et al. [26] who proved the therapeutic role of Atorvastatin in cryptosporidiosis challenged infection in immunocompromised rats. They tested the therapeutic efficacy of 2 different doses of ATV (20 & 40mg/kg) alone and when combined with NTZ (1000 mg/kg) in treatment of cryptosporidiosis experimental infection. The percentage of oocyst reduction on 21st day post infection was 53.7%, 67.2%, 70.1% and 77.5% respectively compared to the infected untreated group. The Nitazoxanide treated group showed 52.7% reduction.

The current results showed that combined regimen of Atorvastatin and NTZ- gave (85.4 %) oocyst reduction, and this is in parallel to AL-Ghandour et al. [10] we used Atorvastatin and high dose NTZ as prophylactic regimens to ameliorate the severity of cryptosporidiosis on immunosuppressed mice. The best efficacy was by dual therapy after prophylaxis (ATV 40+ NTZ 500) the reduction was (87.43%) at 14th day post infection and (94.71%) at 21st day post infection. According to our results, combinations of NTZ and Atorvastatin have given better results in oocyst reduction either alone (85.4 %) or loaded on silver nano particles (SNP) (95.1 %) and this agreed with AL-Attar et al. [27]who reported that the immunocompetent or immunosuppressed mice given daflon coupled with NTZ cured cryptosporidiosis, with oocysts reduction number of 79.2-85.5%. Meanwhile, NTZ alone gave a decrease rate of 61.3-70%. But mice given daflon alone gave the lowest oocyst reduction rate among all groups 32.6% in immunocompetent mice and 27.4% in immunosuppressed ones.

In the last decade, the use of nano particles received considerable interest because of their defined properties, and they were used in the development of diagnostic methods, therapeutic targets, and in protection and vaccination of tropical parasitic diseases [28].The present experiment revealed that administration of Nitazoxanide loaded on SNP (500 mg/kg/day) for 5 days gave more reduction (80.5%) in the number of oocysts when compared with group treated with Nitazoxanide alone (500 mg/kg/day) for 5 days(47.8%) (Table 1) and this agreed with Sedighi et al [8] who stated that treatment of *C. parvum* infection in

neonatal rats with NTZ loaded on solid lipid nanoparticles was more effective than free drug in reducing the parasite number.

The previous results were explained by Said et al. **(29)** who stated that the mucoadhesive character of SNPs increases resident times with a prolonged action and reduces elimination in the gut. SNPs tend to stick to the intestinal wall, thus they can directly interact with the pathogen in infected gastrointestinal tract.

The present study was the first trial of testing Atorvastatin loaded on SNP in vivo against *Cryptosporidium* spp. infection, Atorvastatin was tested at a dose of 40 mg/kg for 5 consecutive days after *Cryptosporidium* spp. shed had been started; as the standard concentration of Atorvastatin that is commonly used and well tolerated in mouse experiments as reported by Li et al. (24).

Also, Bhagat et al. **(30)** proved the valuable role of statins as antioxidants and anti-inflammatory agents beside their cholesterol-lowering activity. Statins induce inhibition of cysteine protease and protect endothelial barrier integrity **(31).** In accordance with this study results, the group received combination of Atorvastatin and Nitazoxanide loaded on SNP (GAVI) achieved the highest level of GSH (23.2±0.6) among non-infected drug control groups (Table 2) and this agreed with Esmail et al. **(32)** who stated tha tatorvastatin ameliorated the increased level of serum reduced glutathione levels and increased the depleted level and activity of glutathione reductase in rats fed on high fatty diet. In the current study, cryptosporidiosis generated oxidative stress by reducing GSH activity in the infected control positive mice (7.1±0.9) (Table 3) verifying results published by Bhagat et al **(30)** who stated that experimental infection with *C. parvum* in immunocompromised mice causes decrease in level of GSH, CAT and SOD at the peak of infection and these findings implicate production of ROS in pathogenesis of experimental *C. parvum* infection in mice. The oxidative injury following cryptosporidiosis was also previously reported by other studies that demonstrated a decrease in SOD activity **(33).** In the current study, serum level of GSH in group treated with combination of Nitazoxanide and Atorvastatin loaded on SNP reported more amelioration than other treated groups and observed a significant increase in levels of GSH (24.8±1.4) toward the normal group (26.0±0.5), (Table 3). This can be explained by the fact that Atorvastatin exerts vasculo-protective effects independent of its lipid-lowering properties, also known as the pleiotropic effects of statins. These effects include improvement in endothelial function, reduced oxidative stress and inflammation **(34).**

According to the current results, histopathological examination of intestinal sections stained with (H&E) from positive control group (BII) showed moderate to marked inflammatory infiltration by lymphocytes, plasma cells and macrophages with scattered eosinophils and mast cells. There was moderate to marked villous broadening, fusion and blunting (Table 5). There were many *Cryptosporidium* oocysts related to brush border highlighted by Geimsa stain and this agreed with Madbouly et al. **(26),** who stated that histopathological examination of sections from ileum in infected untreated mice showed severe active ileitis in the form of shortening and broadening of villi. Villous architecture was lost with decreased ratio of villous height to crypt length, goblet cell depletion, lamina propria showed edema and infiltration with inflammatory cells.

Examination of intestinal sections from different treated groups showed remarkable decrease in intestinal inflammation and less affection of intestinal villi with decreased number of *Cryptosporidium* oocysts and this can be explained by Karvaly et al. (35) who stated that statins drive cyclooxygenase 2

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induction and the generation of lipoxins that downregulate inflammation.

According to this study, group BV treated with Atorvastatin (40 mg/kg/day) for 5 days showed moderately fused and histopathological moderate chronic inflammatory infiltrate and this result in parallel with Madbouly et al. [26] who found that oral administration of Atorvastatin (40 mg) resulted in partial improvement in the histopathological changes. These changes were in the form of partial healing of the intestinal mucosa with moderate restoring of the villous architecture and this may be due to treatment with Atorvastatin produces lower production of tissue-damaging cytokines and chemokines [36].

The previous result of Ator treated group also agreed with AL-Ghandour et al. [10] who found that ATV-treated group showed mild inflammatory cellular infiltrate in comparison to infected untreated group or drug control therapy. According to this study results, the best results in groups BVII and BVIII (combination groups treated with Ator + NTZ) in the form of normal intestinal villous architecture with minimal chronic inflammatory cell infiltrate and no *Cryptosporidium* oocysts also those groups showed significant statistical difference compared to positive control group BII(Table 4) and this agreed with Madbouly et al. **[26]**who stated that the groups treated with the combinations of (Atorvastatin and Nitazoxanide) showed significant effect in the form of remarkable improvement of the histopathological changes. These changes were in the form of mild inflammation without activity indicating synergistic effect of drug combinations, while administration of Nitazoxanide alone didn't show a significant improvement in the histopathological changes.

Conclusion

In conclusion, Atorvastatin had a good effect on *Cryptosporidium parvum* infection in mice. Loading of Atorvastatin on silver nano particles improved its efficacy as it increased the drug delivery to tissues. Therefore, Atorvastatin (either alone or loaded on nanoparticles) in combination with Nitazoxanide seems to be a good candidate in treatment of *Cryptosporidium parvum* infection.

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