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AMELIORATING EFFECT OF NITAZOXANIDE LOADED CHITOSAN NANOPARTICLES ON INTESTINAL DYSPLASTIC CHANGES IN IMMUNOSUPPRESSED CRYPTOSPORIDIOSIS MURINE MODEL

By
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Abstract

Cryptosporidiosis is a major cause of human diarrhea worldwide in immunocompromised individuals causing severe, chronic and possibly life threatening diarrhea. Nitazoxanide (NTZ) has been approved for treatment of diarrhea in immunocompetent children and adults, but not effective in immunosuppressed individuals**.** The present study evaluated the effect of NTZ-loaded chitosan nanoparticles on intestinal dysplastic changes of experimental *Cryptosporidium* infection in immunosuppressed murine model using parasitological, histopathological and immunohistochemical studies. Fifty immunosuppressed male mice, divided into 5 groups (10 each) as following: G1: non infected control, G2: non-treated infected control, G3: infected then treated by nitazoxanide, G4: infected then treated by nitazoxanide loaded on chitosan nanoparticles, G5: infected then treated by chitosan nanoparticles (CS NPs). NTZ loaded on chitosan NPs treated mice showed the highest significant reduction in oocysts shedding, a remarkable improvement in histopathological changes and the least expression of cyclin D1 marker denoting the best protection presented to intestinal dysplastic changes caused by *Cryptosporidium* infection of the intestine followed by treatment with NTZ then by treatment with CS NPs alone that showed some improvement in histopathological and immunohistochemical changes.

Key words: Chitosan nanoparticles, Nitazoxanide, *Cryptosporidium,* immunosuppression and Dysplasia

Introduction

Cryptosporidium is a coccidian parasite that infects the epithelial intestinal cells with more than 27 species (Moore *et al*, 2016), the common zoonotic ones are *Cryptosporidium parvum* and *C hominis* worldwide (Lendner and Daugschies, 2014). It is transmitted fecal-oral, particularly contaminated water supply and rarely by fomites. Oocysts may be found in all types of water including chlorinated water (Meinhardt *et al.,* 1996). In Egypt, cryptosporidiosis were reported in man especially immunocompetent (El Bahnasawy *et al,* 2018), animals (Shoukry *et al,* 2009), and birds (El-Shahawy and Elenien, 2015). But, in immunocompromised patients especially HIV/AIDS, parasite may cause severe, chronic and possibly life threatening diarrhea and wasting (Chen *et al,* 2002).

In man *C. parvum* can induce gastrointestinal and biliary adenocarcinomas in murine models (Certad *et al.* 2012). Also, the risk of developing cancer colon is significantly high among AIDS patients with cryptosporidiosis (Shebl *et al,* 2012).

Nitazoxanide (NTZ) proved to treat diarrhea caused by *Cryptosporidium* in immunocompetent children and adults (Mainali *et al*, 2013), but not effective in immunosuppre-ssed ones (Yacoub *et al*, 2014). It inhibits the pyruvate ferredoxin/flavodoxin oxidoreductases (PFORs) enzyme-dependent electron transfer reaction essential for its anaerobic metabolism (Hoffman *et al,* 2007). Also, NTZ can inhibit protein disulphide isomerases (PDI2 & PDI4) in protozoa (Muller *et al,* 2007), altering expression of genes involved in stress response such as heat-shock proteins (Muller et *al.,* 2008).

Chitosan is a polysaccharide derived by partial deacetylation of chitin, with important nano-medical agent biodegradable, biocompatible and nontoxic (Ing *et al,* 2012). Thus, it is used as an antibacterial and antifungal product (Kean and Thanou, 2010). Chitosan also can increase the intestinal ab-

sorption and drug bioavailability ultimately by increasing the mucosal epithelium permeability (Thanou *et al.,* 2001).

Polymeric nanoparticles made from synthetic and natural polymers have received the majority of interest due to their stability and facility of surface modification (Herrero-Vanrell *et al*., 2005). Chitosan nanoparticles (CS NPs) produced mild protection in treatment of Giardiasis (Said *et al.,* 2012), and also were effective against *cryptosporidium* in a neonatal CD-1 mice (Mammeri *et al.,* 2018).

Cell proliferation can be assessed by immunohistochemistry, it can identify cellular changes that are not visible with H&E and can detect the earliest changes in transformed tissues, it can be used to distinguish hyperplasia from neoplasia (Okoye and Nnatuanya, 2015). Cyclin D1 is a good and beneficial marker for detection of intestinal dysplasia (Bartkova *et al,* 1995). Expression of this marker has been found to be essential for cell cycle progression in both transformed and non-transformed cells (Lukas *et al,* 1997). Overexpression of cyclin Dl protein has been detected in colon carcinoma (Bartkova *et al,* 1994) and colorectal polyps in humans (Arber *et al,* 1996).

Moawad et al. (2021) previously approved that loading NTZ on CS NPs improved NTZ efficacy on cryptosporidiosis in both immunocompetent and immunosuppressed mice. Also, NTZ loaded CS NPs showed better results than free NTZ in reduction of Cryptosporidium oocysts shedding and improving histopathological changes caused by Cryptosporidium infection in the liver, intestine and lung of mice.

This study aimed to evaluate Nitazoxanide® (NTZ) loaded chitosan nanoparticles effect on intestinal dysplastic changes of experimental *Cryptosporidium* infected immunosuppressed mice parasitologically, histopathologically and immunohistochemically.

Material and Methods

Animals: Fifty apparently normal laboratory bred male Swiss albino mice, 20-25gm and aged 4-6 weeks, purchased from Biological Supply Center at Theodor Bilharz Research Institute, Cairo were put in insect proof cages on a standard diet and water. All mice stools were examined to exclude any parasitic infection (Garcia, 2007).

Ethical aspects: The study was approved by the Research Ethics Committee, Faculty of Medicine, Zagazig University. All procedures related to animal studies met the International Guiding Principles for Biomedical Research Involving Animals as issued by the International Organizations of Medical Sciences approval no 4243/1-1-2018.

Experimental design: Mice were divided into 5 groups of 10 mice each. G1: non infected control, G2: infected non-treated control, G3: infected & treated by NTZ, G4: infected & treated by NTZ loaded on chitosan nanoparticles, and G5: infected & treated by chitosan nanoparticles (CS NPs).

Immunosuppression: Dexamethasone (Dexazone) tablets 0.5mg were purchased (Kahira Pharmaceuticals and Chemical Industries Co.), and given orally for mice immunosuppression at a dose of 0.25µg/gm/day for 14 successive days prior to infection, and continued on dexamethasone for 21 successive days post infection (PI) experimental end (Rehg *et al*,1988).

Parasitic infection: *Cryptosporidium* oocysts were obtained from Department of Parasitology Theodor Bilharz Research Institute, and confirmed by modified Ziehl-Neelsen staining. Positive stools were preserved in an equal mixture of 2.5% potassium dichromate, stored at (4^{o}C) until needed and oocysts were isolated by Lumb's technique (Lumb *et al,* 1993).

Oocysts infection: Inoculum was prepared as 50µl from the positive stools, stained by modified ZN stain, and a mean of three oocysts counts by high power field was multiplied by 20 to have average number in 1ml (Garcia, 2007). Mice except the normal control ones were orally infected with oocysts on day 15th of dexamethasone (Moon *et al,* 1982). Mice were water deprived overnight,

and inoculum was given by an esophageal tube, as 10^4oocysts/mouse. Stools were collected from infected mice on the 3rd to 7th day PI to ensure infection (Gaafar, 2007).

Treatment: Nitazoxanide (Nanazoxid) 500 mg tablets purchased from Utopia Pharmaceuticals were given to G3 in a dose of 200 mg/kg/day and NZ loaded on CS NPS were given to G4 in same dose (Li *et al,* 2003), and G5 received 20µg of CS NPs in 200µl PBS/day/mouse.

Preparation of CS NPs: Ionotropic gelation method of CS with tripolyphosphate (TPP) anions was used for synthesis of CS NPs. Chitosan was dissolved in acetic acid as 3-mg/ml. Acetic acid concentration was 1.5 times higher than CS. TPP as 1mg/ml was prepared in double-distilled water. CS NPs were prepared by drop-wise adding of CS solution 5ml to 2ml TPP solution with magnetic stirring (1000 rpm) at room temperature for an hour. The opalescent suspension was formed under the same conditions. NPs were separated by centrifugation at 20.000g and 14°C for 30 min, freeze-dried and stored at 4°C, after evaluated weights of NPs. The CS NPs were characterized by using SEM (SU1510 model; Hitachi Ltd., Tokyo, Japan) and Zetasizer (Malvern Instruments, UK) (Somnuk *et al,* 2011). The CSNPs was nearly spherical in shape with smooth surface and size range was about 20-30nm.

Nitazoxanide loaded chitosan nanoparticles: NZ-loaded chitosan nanoparticles were done by drop-wise addition of chitosan solution to aqueous sodium TPP of nitazoxanide at concentration of 100mg/ml with constant stirring, followed by sonication. NZ -loaded nanoparticles were separated by centrifugation at 20,000xg for 30 minutes at 14°C.

Histopathology: Three weeks PI, all mice were scarified by rapid decapitation, and intestinal parts from each one were removed and processed for microscopy examinations. Small intestinal and caeca were scrapped, weighted and parasitological examined.

Drugs' efficacy was done by 1-Parasitological examination: Fecal samples were collected from infected mice at 19th & 21st days PI, and dissolved in 1ml formalin 7%. From each one 50µl smear was taken and stained with modified Ziehl-Neelsen stain to count oocysts number (Garcia, 2007). Number was expressed per gram of feces and percentage reduction (%R) was calculated according to the following equation: %R= 100 (C-E)/C where C: control group and E: experimental groups of mice (Penido *et al.* 1994).

2- Histopathological examination: Intestinal pieces from each mouse were fixed in 10% neutral formalin, dehydrated in ascending grades of ethanol, cleared in xylol, processed for paraffin 5µ sections and stained with H&E) for microscopic examination (Abdel-Bary *et al,* 2012).

3- Immuno-histochemical cyclin D1 examination: Immuno-histochemical staining stained 3-5µm thickness thin sections and xylol deparaffinized them. They were rehydrated in descending ethanol series and incubated for 10 minutes in 3% hydrogen peroxide to block endogenous peroxidase activity. Dako target retrieval solution (pH 6.0) was added for 20 minutes and samples were incubated with primary antibody anti-cyclin D1 (SP4, ab16663, Abcam, UK, diluted in 1/100 PBS) for an hour. Antibody binding was detected by Dako's HRP Envision Kit (Dako Cytomation, Denmark). Human colon cancer tissue samples were used as positive control. Positive reaction was detected by cyclin D1 dark brownish nuclear staining. Stained areas were graded based on its intensity as grade-0: none or <5% of cells stained, grade-1: mild to moderate nuclear staining or 5 to 50% cells stained, and grade-2: strong nuclear staining or >50% cells stained (Ramasubramanian *et al,* 2013)

Statistical analysis: Data were collected, tabulated and statistically analyzed using SPSS program version 25. ANOVA F-test was used for comparison between several quantitative variables among groups while the Student's t-test was used to compare between two quantitative variables. Post hoc using LSD was used to compare each two

groups in ANOVA test. Fisher's exact test was used to detect difference between qualitative variables (Kirkwood, 2003).P-value (≤ 0.05) was significant (Leslie *et al,* 1991).

Results

Cryptosporidium oocysts started to shed from the 3rd day post infection (PI) with a peak on the 7th day PI. Quantitative assessment of oocysts intensity per gram stool in treated mice at 15th & 21st days PI showed a significant difference (P < 0.001), in both follow up days except G2 versus G5. Also, there was high significant difference in oocysts' counts in both days in G3 & G4 There were increase in reduction percentage of oocyst counts among different groups with increased on follow up with the highest rate in G4 at 21st day. The significant oocysts reduction in nitazoxanide and NTZ loaded on CS NPs groups were 37.6%, 44.85% and 53.21%, 70% respectively. The NTZ loaded on CS NPs gave highest percentage oocysts reduction when compared to other groups.

Histopathology of intestinal tissue in infected non treated mice showed intense pathological changes in mucosa included shortening and boarding of villi with loss nor-mal villous architecture. There were mucosal ulceration and infiltration of lamina propria with inflammatory cells with loss of brush border microvillus surface area, with dysplasia of various grades. Pathological changes were moderate in NTZ treated mice and marked in chitosan treated ones. NTZ-loaded on chitosan nanoparticles treated mice showed improved pathological changes in the form of intestinal mucosa healing, preserved brush border and normal villous architecture. Mild inflammatory cellular infiltration of lamina propria and superficial mucosal ulceration were detected in few mice.

Immunohistochemical studies of intestinal tissues by cyclin D1 marker showed immuno-reactivity represented as intestinal dysplasia, with high significant difference among all as to grading of cyclin D1 expression.

Details were given in table (1, 2 & 3) and figures (1, 2 & 3)

Table 1: Cryptosporidium oocysts mean counts in 1gm stool and reductions percentage among groups:

Groups	Day 19 PI		Day 21 PI		Paired t	P
	Mean ± SD×10^3	(R %)	Mean ± SD×10^3	(R %)		
G 2	1379.16 ± 434.98		1250.90± 382.32		0.37	0.68
G 3	860.12 ± 98.73	(37.6%)	689.85± 93.06	(44.85%)	5.03	<0.001**
G 4	645.30 ± 75.34	(53.21%)	375.25 ± 63.08	(70%)	8.63	<0.001**
G 5	1300.57 ± 164.85	(5.7%)	1125 ± 152.63	(10.06%)	0.61	0.45
F^	21.33		26.79			
P	<0.001**		<0.001**			
LSD	<0.001**[1], <0.001**[2], 0.68 [3]		<0.001**[1], <0.001**[2], 0.30			

*Significant (P<0.05), **Highly significant (P<0.01), P1:G2 vs. G3, P2:G2 vs. G4, & P3:G2 vs. G5

Table 2: Dysplastic changes observed by H&E among immunosuppressed groups:

	G1(N=10)	G2(N=6)	G3(N=6)	G4(N=8)	G5(N=6)	P
No dysplasia	10 (100%)	0 (0%)	2 (33.3%)	4 (50%)	1 (16.7%)	<0.001**
Low grade dysplasia	0 (0%)	1 (16.7%)	3 (50%)	4 (50%)	1 (16.7%)	
High grade dysplasia	0 (0%)	5 (83.3%)	1 (16.7%)	0 (0%)	4 (66.6%)	

**Highly significant (P<0.01)

Table 3: Degrees of immunohistochemical staining of Cyclin D1 among immunosuppressed groups:

Cyclin D1 expression	G1(N=10)	G2(N=6)	G3(N=6)	G4(N=8)	G5(N=6)	P
Grade 0 N (%)	10 (100%)	0 (0%)	2 (33.3%)	5 (62.5%)	0 (0%)	<0.001**
Grade 1 N (%)	0 (0%)	0 (0%)	2 (33.3%)	3 (37.5%)	2 (33.3%)	
Grade 2 N (%)	0 (0%)	6 (100%)	2 (33.3%)	0 (0%)	4 (66.7%)	

**Highly significant (P<0.01)

Discussion

Cryptosporidium is one of the most important waterborne pathogen especially in developing countries; it resists all practical levels of chlorination (Ng *et al,* 2010). *Cryptosporidium* is also resistant to the majority of the anti-parasitic chemotherapeutic agents (Mead and Arrowood, 2014). So, it was urgent to have new medications for cryptosporidiosis. There has been a great interest in

developing drug delivery system using nanoparticles. NPs can provide significant advantages in terms of high stability, high specificity, high drug carrying capacity and ability for controlled drug release (Pal *et al,* 2011). Chitosan nanoparticles have been used in several studies as carriers of drugs and vaccines. It is biocompatible, biodegradable and nontoxic. CS NPs are able to protect unstable drug molecules from strong gastric acids. In addition, they are able to adhere to mucosal tissues to improve the drug absorption; all these characteristics make chitosan an ideal pharmaceutical excipient and can be used extensively in drug delivery (Li *et al.,* 2018).

In the present study, immunosuppressed mice induced by dexamethasone proved to be a good immunosuppressive agent especially in murine model (Rasmussen and Healey, 1992; Madbouly *et al.* 2017). Mice received dexamethasone before were high susceptibility to cryptosporidiosis (Benamrouz *et al*, 2012).

In the present study, *Cryptosporidium* oocysts appeared in mice feces on 3rd day post infection. This agreed with Abo Sheishaa *et al.* (2020) and El Shafei *et al.* (2018) who found that the oocysts shedding in stool was on 2nd day PI in immunosuppressed mice.

The *Cryptosporidium* oocyst shedding was continued throughout the study in infected mice. This agreed with Lacroix *et al.* (2001) who found that the duration of oocysts shedding was about 3 to 4 weeks. Also, this agreed with El Shafei *et al.* (2018) who found that infected mice continued to shed oocysts until 30th PI with *Cryptosporidium*. But, Chai *et al*. (1999) reported a short patent period about 9 days, which may be due to the different dose and duration of immunosuppression.

In the present work, treatment with NTZ loaded on chitosan NPs resulted in the highest percentages reduction in oocysts counts in stool (53.21%,70%) on days 15th & 21st PI respectively. This agreed with Abo Sheishaa *et al*. (2020) who found that using NTZ loaded CS NPs in treating *C. parvum* infection in immunosuppressed mice resulted in reduction of 78.17%, and that NPs augment the therapeutic effect of NTZ with safety profile. Sedighi *et al.* (2016) found that NTZ loaded on solid lipid nanoparticles was more effective than free NTZ in treating *C. parvum* infection in neonatal rats.

Mohamed *et al.* (2019) also stated that, using *Nigella sativa* in combination with CS NPs for treatment of cryptosporidiosis gave strong reactivity in reducing the oocysts shedding in mice. Loading several drugs on CS NPs resulted in enhancement its efficacy as good targeted drug delivery system for parasites as leishmaniasis (Esfandiari *et al*, 2019), trichinellosis (Nassef *et al*, 2019) and toxoplasmosis (Hagras *et al,* 2019).

As regards the NTZ effect on *Cryptosporidium* infection in mice, it was found that there was significant reduction in oocyst shedding in immunosuppressed mice with percentages reduction of (37.6%,44.85%) on 19th & 21st days PI respectively. This agreed with Baishanbo *et al.* (2005) who found that NTZ treatment significantly reduced *Cryptosporidium* oocysts excretion in immunosuppressed infected gerbils. Also it was in relative agreement with the results reported by Rossignol, (2010) who found that in NTZ treated group there was resolution of diarrhea in (32%) of adult AIDS patients with cryptosporidial infection. Also, Abdelhamed *et al.* (2019) recorded 42.5% reduction in oocysts counts at 21st day PI in NTZ treating *Cryptosporidium* infection in immunosuppressed mice. But, this disagreed with Amadi *et al.* (2009) who found that NTZ wasn't effective in treating *Cryptosporidium* in HIV infected children even with high dose for prolonged treatment. Also, Manjunatha *et al.* (2016) reported that NTZ has limited efficacy in presence of immunosuppression status.

In the present study, CS NPs didn't give significant reduction in oocysts/gm stool as reduction was 5.7%, & 10.06% on 19th & 21st days PI respectively compared to corresponding infected untreated ones. This more

or less agreed with Mohamed *et al.* (2019) who found that treating cryptosporidiosis in immunosuppressed mice with CSNPs reduced oocyst counts by 11.7% at 18 day PI. But, Ahmed *et al.* (2019) reported that CS NPs killed most *Cryptosporidium* oocysts in culture and that CS NPs treated oocysts didn''t cause any infection in mice compared with mice fed with untreated oocysts. This may be explained by different chitosan forms, methods of CS preparation, dose, models, *Cryptosporidium* species and evaluation test.

In the present study, infected control mice showed marked changes in intestinal mucosal structure compared to normal control ones. These were villous atrophy, mucosal ulceration, inflammatory infiltration, loss of brush border and intestinal dysplasia in most of the mice. This agreed with Gaafar (2012) and Madbouly *et al.* (2017).

In the present NTZ treated mice, pathological changes were partially improved, with moderate villous atrophy, partial mucosal ulceration and moderate inflammatory infilt-ration of lamina propria. This agreed with Sadek and El‑Aswad (2014) reported that changes were moderate in mice received NTZ, which were severe in untreated ones.

In the present study, the intestine in NTZ -loaded on chitosan NPs treated mice showed marked histopathological improvement in the form of mild inflammation and with more or less normal villous pattern. This agreed with Abdelhamed *et al.* (2019) who reported pathological changes improvement with few *Cryptosporidium* parasites at the brush border of intestinal cells with mild inflammation as compared to combined NTZ and artesunate loaded polymeric nano-fiber. Also, Etewa *et al.* (2018) reported that spiramycin-loaded chitosan nanoparticles exhibited an anti-inflammatory effect with decreasing perivascular inflammatory infiltration leading to marked pathological improvement of various organs in *Toxoplasma* infected mice.

In the present study, histopathological examination of intestinal sections showed that 83.3% of infected control mice developed high grade intestinal dysplasia and 16.7% of them developed low grade dysplasia. In NTZ- loaded on CS NPs treated mice, 50% of them didn't show dysplasia, but 50% of them showed low grade dysplasia. While in NTZ treated mice, 33.3% were without dysplasia, 50% of them showed with low grade dysplasia and 16.7% were with high grade intestinal dysplasia. In CS NPS treated ones, 16.7% showed low grade dysplasia and 66.6% showed high grade intestinal dysplasia. Both Abdou *et al.* (2013) and Abdelhamed *et al.* (2019) reported that cryptosporidiosis might trigger intestinal dysplasia of high grade in murine models, which were improved by treatment.

Certad *et al.* (2007) proved the association between cryptosporidiosis and induction of intestinal neoplasia in mice even in low infectious doses. Shebl *et al.* (2012) stated also that, *Cryptosporidium* infection was associated with cancer colon in HIV infected individuals. This was clarified by Lantier *et al.* (2013) that immune cell recruitment and retention in the infected intestine are essential in the protection mechanism against *C. parvum*.

In the present study, immunohistochemical expression of cyclin D1 immunoreactivities showed significant increase in immunohistochemical changes and intestinal dysplasia after *Cryptosporidium* infection. Also, NTZ and NTZ-loaded on CSNPs treated mice showed an improvement in the alterations. Cyclin D1 expression in infected control mice intestine showed 100% (grade 2), in NTZ-loaded on CS NPs treated mice 62.5% of mice showed negative cyclin D1 expression (grade 0) and 37.5 % of them showed mild to moderate cyclin D1 expression (grade 1). While in NTZ treated group, 33.3% were in grade (1) and 33.3%in grade (2). In CS NPS treated group, 33.3% were in grade (1) and 66.7% were in grade (2). This agreed with Abdou *et al.* (2013) who reported that *Cryptosporidium* infection mice induced intestinal dysplasia was 60% in immunosuppress-

ed ones sacrificed on 18th day PI and 100% in mice sacrificed at 30th day PI, with high-grade dysplasia (40%) in immunosuppressed mice. They added that cyclin D1 was a useful marker for detecting intestinal dysplasia and in NTZ improved these changes. This agreed with Mostafa *et al.* (2018) who found histochemical stained immunocompromised improvement *Cryptosporidium* infected mice showed high grade dysplasia of colonic epithelium with marked nuclear expression of cyclin D1 compared to mild dysplastic ones without nuclear expression of cyclin D1 in infected mice then treated with combined artesunate and NTZ. This atypical changes secondary to inflammation and infection affected the GIT epithelium causing true dysplastic changes (Abdou *et al,* 2013).

Conclusion

NTZ loaded on CS NPs was an effective alternative treatment of murine cryptosporidiosis on immunosuppressed mice inducing marked reduction in ocysts output, improving histopathological changes and protecting intestinal dysplasia. Consequently, the NTZ loaded on CS NPs proved to be an effective cryptosporidiosis drug.

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Explanation of figures

Fig. 1: a- Characterization of chitosan nanoparticles, SEM micrograph, and b- size of chitosan nanoparticles by Zeta sizer.

Fig. 2: a- H&E stained intestinal cut sections of immunosuppressed mice of different groups showed marked villous atrophy with broad villi (100x) (Fig. 2a), b- dysplastic changes to nuclear stratification (arrows) and loss of mucin in infected untreated G2 (100x), c- sloughed and distorted villi (100x) and dysplastic changes in the form of enlarged hyperchromatic nuclei, d- nuclear stratification and loss of mucin in NTZ treated G3 (400x), e- mild inflammatory infiltration and mild foci of broad villi with returning of normal villous pattern (100x), f- mild dysplastic changes in form of nuclear hyperchromatism in NTZ loaded on chitosan treated G4 (400x). g- distorted and atrophied villi with marked inflammatory infiltration (100x) and h- marked dysplastic changes of nuclear hyperchromatism in chitosan treated G5 (400x).

Fig. 3: a-Immunohistochemical stained section in intestine groups (IHC x400) showed negative cyclin D1 expression in intestinal epithelium of normal G1, b- strong cyclin D1 expression in dysplastic intestinal epithelium black arrows G2, c- Moderate cyclin D1 expression in NTZ treaded immunosuppressed G3, d- mild cyclin D1 expression in intestinal epithelium in NTZ loaded on chitosan treaded immunosuppressed G4, & e- marked cyclin D1 expression in intestinal epithelium in chitosan treaded immunosuppressed G5.