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ASSESSMENT OF IMMUNOLOGICAL, PARASITOLOGICAL AND HISTOPATHOLOGICAL PARAMETERS AFTER TREATMENT WITH COMBINED THERAPY AGAINST *CRYPTOSPORIDIUM PARVUM*

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ABSTRACT

Background: *Cryptosporidium parvum* is a protozoan parasite that infects the gastrointestinal epithelial cells causing several parasitological and pathological changes. This work aimed to evaluate the effectiveness of paromomycin and nitroalanin alone or in combination to each other in the treatment of cryptosporidiosis. **Methodology:** This study included five groups of mice: group I, infected control; group II, infected and treated with paromomycin; group III, infected and treated with nitoanalin; group IV, infected treated with both paromomycin and nitroanalin; and groups V non-infected control. Mice were subjected to stool examination for oocyst counts prior to and after 2 weeks post infection and were later sacrificed for intestinal dissection and routine histo-pathological examination, beside measurement of fecal IgA in stool samples and different cytokines in serum samples. **Results**: Infected control group showed the highest numbers of oocysts shed compared to the other groups. The highest reduction of oocysts shed was observed in group of mice with combined treatment 93.7 %. Low-grade dysplastic changes were seen in group of mice with combined treatment. The highest significant reduction of fecal IgA was observed in combined therapy. Mice with combined treatment showed a high significant ($P<0.001$) increase in serum levels of both IFN-γ and IL-10 and moderate significant

(P<0.01) reduction in serum level of IL-5 when compared to group infected non-treated mice, while no significant difference between all treated groups and group infected non-treated mice in serum level of IL-2. **Conclusion:** This study was concluded that the combination of paromomycin and nitroanalin was effective in the treatment of *Cryptosporidium* infection.

Keywords: *Cryptosporidium parvum*- paromomycin- nitroanalin- oocysts shed

INTRODUCTION

Cryptosporidium is a ubiquitous protozoan parasite thatinfects humans and a wide range of domestic and wild animals [1]. Transmission of Cryptosporidium is mainly through fecal-oral route, as well as through contaminated water and food, person-to-person spread and contact with infected animals [2-4]. In developing countries where there is low hygiene level, poor sanitation, no good water management, and frequent contact with animals, the burden of cryptosporidiosis remains to be a major health problem [5]. The genus *cryptosporidium* contains many species, genotypes and subtypes that infect a wide range of vertebrates including humans. Each may have different sources of infection, transmission routes and pathogenicity **[6]**.*Cryptosporidium parvum* is an obligate intracellular parasite that infects the epithelial lining ofluminal surfaces of gastrointestinal and respiratory tracts in a wide array of hosts. The parasite is ingested as oocysts which undergoes excystation to sporozoite that parasitise the host. Infection can occur in esophagus and any portion of gastrointestinal tract can be involved; it usually starts in the lower small intestine. Other areas include the gall bladder, bile ducts, pancreas and respiratory tract. The infection provokes symptoms such as abdominal cramps, diarrhea, vomiting, loss of appetite, low grade fever, generalized malaise and nausea **[7]**. While the infection can resolve without intervention in immune-competent individuals, cryptosporidiosis is increasingly becoming a major public health threat as an opportunistic infection in immunosuppressed and immunocompromised individuals, especially in HIV/AIDS [8, 9]. In Egypt, limited studies were devoted to ascertain cryptosporidiosis in human and animals. Cryptosporidiosis is increasing in 1986 and 1987, its prevalence was 16.6% [10] and 11.6% [11] in Cairo and Alexandria respectively. Another study gave a prevalence of 27.9% in diarrheic children in Alexandria and Behira **[12]**. However, *Cryptosporidium* was responsible for considerable part of diarrheal illness among American military personnel participated in a military exercise in the northwestern Egyptian desert [13]. Human cryptosporidiosis was also reported in

different ages [14]. *Cryptosporidium* infection frequently affects domestic animals with strong relation between *C.parvum* infection and diarrhea among Egyptian buffalo calves **[15, 16]**. Recently, epidemiological and molecular findings on *Cryptosporidium* spp., from Egyptian dairy calves and stray dogs were performed by **Amer et al. [17]**, **El-Madawy et al. [18]** and **Hassanain et al. [19]**. There exists a broad spectrum of illnesses caused by this parasite, and their occurrence is closely related to the host's immune response [20]. Cryptosporidiosis continues to be a cause of morbidity in human immunodeficiency virus (HIV)-infected persons. A relationship among the clinical picture of cryptosporidiosis, the extra-intestinal localization of the parasite, and immunosuppression has been demonstrated in HIV-positive persons [20 - 22]. **Colford et al. [23]**, suggested that *C. parvum* infection may occur early in the course of HIV progression and that it may resemble other AIDS-related conditions, such as tuberculosis, in that it can occur before a severe status of immunosuppression is reached.There is a history of inadequate and unreliable treatments for *Cryptosporidium* enteritis. Certain antiparasitic agents such as paromomycin, nitazoxanide and azithromycin are sometimes used, but they usually have only temporary effects and sometimes relapses happened. Currently, the best approach is to improve the immune status in immune-deficient individuals, for example, by using antiviral therapy in patients with AIDS and supportive treatment for symptoms [23-26]. Because of the great need to develop new anti-cryptosporidial agents, trials were designed to test the potency of different agents for treating cryptosporidiosis. Therefore, the present study aimed at investigating the antiparasitic effectiveness of Paromomycin and Nitroanalin treatment of *Cryptosporidium* infections in experimentally infected mice.

MATERIALS AND METHODS

Experimental animals

Animals used in this work were male Swiss albino mice, aged five to six weeks, weighing 20–25 g, clean from any parasitic infection were obtained from Schistosome Biological Supply Centre (SBSC), Theodor Bilharz Research Institute (TBRI). They were housed in well ventilated cages with perforated covers, supplied with standard pellet food and water. Bedding was changed every day. The mice were allowed to adapt to the laboratory environment for one week before the experiment. This study was approved by the Ethics Committee of TBRI**.**

2.1. Parasites

Stool samples were collected from 30 immunosuppressed patients with chronic

diarrhea, in the Kasr ELini Hospital and Fever Hospital from Hematology Department and Renal Dialysis Unit, from March 2013 to March 2014. Informed consents were obtained from the patients. The stool samples were transferred to the Parasitology Department to be screened by different techniques for the presence of intestinal protozoa. All samples were microscopically screened by direct smear, iodine smear and modified Ziehl–Neelsen acid fast stain (MZN), aiming to identify the positive cases of *Cryptosporidium*.

Oocyst purification on discontinuous sucrose gradient

The sucrose gradient was prepared as described previously by **Stone, [27]**. Briefly, sucrose solution (500 mg of sucrose, 9 ml of phenol, 0.5 ml of Tween 80, in 320 ml of deionizedwater) was diluted 1:2 and 1:4 with sterile phosphate-buffered saline (PBS) (0.1M, pH 7.2). The sucrose gradient was prepared by placing 10 ml of the 1:2solution into 50 ml of polypropylene centrifuge tubes (Falcon; Becton Dickinson**,** Franklin Lakes, N.J.) and then slowly adding 10 ml of the 1:4 solution. Thirtymilliliters of the fecal oocyst suspension (1.5 x 10^6/ml) was layered onto the 1 /4 gradient. The sucrose gradient was centrifuged at 1,700 xg for 30 min at 4^{o}C. Following centrifugation, the potassium dichromate layer and half of the 1/4 gradient were collected with a pipette and then discarded. The remainder ofthe 1:4 gradient and 0.5 ml of the 1:2 gradient above the interface were recovered**.** This aspirate was dispersed into 80 ml of PBS, and the oocysts werecounted**.**

Drugs:-

Paromomycin was purchased from MP Biomedicals (Solon, OH) and Nitroanalin from Sigma (St. Louis, MO). Paromomycin was diluted in water just prior to use. Nitroanalin was first dissolved in dimethyl sulfoxide (DMSO) or ethanol and then diluted in waterprior to use.

Studied Groups:-

Animals were divided into four groups. All infected animals received 10^4 oocysts/ml Cryptosporidium oocysts orally.

Group I: Infected control mice.

Group II: Infected animals given Paromomycin orally (100mg/kg/ mice) daily for five consequence days two weeks post infection.

Group III: Infected mice given Nitroanalin orally (200 mg/kg/mice) daily for five consequence days two weeks post infection.

Group VI: infected mice combined Paromomycin orally (100mg/kg/ mice) and Nitroanalin orally (200 mg/kg/mice) daily for five consequence days two weeks post infection.

Group V: Normal mice

Animals were sacrificed four weeks post infection.

Parasitological examination:-

Mouse fecal samples were collected prior to inoculation and every 2 days after infection until the end of the experiment (day 35). Briefly, fresh fecal pellets from each mouse in the study groups werecollected separately every 2 days over the 35 days of theexperiment, according to the group to which they were assigned.Each sample was suspended in 10% formalin and homogenized.Then, 1 mg was prepared as a fecal smear and stained by the modified Ziehl–Neelsen staining method. The stained fecal smear was examined microscopically and the number of cryptosporidium oocysts was counted in 10 high-power fields (HPF); the number of oocysts per mg for each animal and then for each group of animals was calculated.

Fecal smear examinations.

Bright-field and fluorescence observations of fecal smears (1 slide per smear for each detection method) were performed at x200 and x400 magnifications. The entire smear was examined to verify the presence of oocysts. When smears contained many oocysts, only a portion of the smear was examined. Oocyst numbers demonstrated per x400 field were classified as follows: 1+ for <2 oocysts; 2+ for 3 to 5 oocysts; 3+ for 5 to 7 oocysts; and 4+ for >8 oocysts. Epifluorescence microscopy employed an Optiphot microscope (Nikon Inc., Garden City, N.Y.) equipped with a halogen UV light source, a 520- nm-wavelength barrier filter, a 510-nm-wavelength dichroic mirror, and a 450- to 490-nm-wavelength excitation filter.

Acid-fast staining of fecal oocysts

A commercially availableacid-fast staining kit (MedicalIndustries Inc., Las Vegas, Nev.) was applied as recommendedto fecal smears. Briefly, the primary stain wasapplied at room temperature to the fecal smear for 2 min,rinsed off with tap H2O, decolorized for 5 to 10 s, rinsed withtap HO, counterstained for 2 min, and rinsed with tap HO.After the smears were dried, they were coated with a thinlayer of immersion oil and observed by bright-field microscopy.

Scarification of mice

Scarification of mice was done two weeks after administration of drugs by intraperitoneal anesthesia. The upper part of small intestine was removed, the duodenal contents were subjected to the previous parasitological examination and subjected to histopathological examination.

Efficacy of selected drugs

Efficacy of selected drugs against *Cryptosporidium* oocysts was calculated as per formula:

$$\text{Efficacy (\%)} = \frac{\text{Total oocysts before treatment} - \text{Total oocysts after treatment}}{\text{Total oocysts before treatment}} \times 100$$

Histopathological examination:-

The small intestine of mice were fixed in 10% neutral buffered formalin. Sections, stained by hematoxylin and eosin (H&E) and (ZN stain) then examined by light microscopy according to standard operation procedures [28].

Measurement of IgA by ELISA.

ELISAs were performed to detect and quantify IgA in the fecal extracts. Unless otherwise stated, all incubation steps were performed for 1 h at 37°C and followed by three wash steps using 0.15 M PBS (pH 7.2) containing 0.1% polysorbate 20 (Tween 20) (ICI Americas, Inc., Wilmington, Del.). Total fecal IgA was quantified using a sandwich ELISA. Microtitration plates (Nunc, Roskilde, Denmark) were coated for 16 h at 4°C with anti-human IgA secretory component (Sigma Immuno Chemicals, St. Louis, Mo.). The wells were blocked with a 5% dry milk solution for 1 h. Fecal extracts (50 μl) were then added to duplicate wells and incubated. Each plate also contained duplicate wells of six known concentrations (15.6 to 0.49 ng) of purified human secretory IgA (Cappel, Durham, N.C.). Addition of peroxidase-conjugated anti-human secretory IgA (Cappel) diluted 1:1,000 in PBS was followed by the incubation of H2O2-activated 1 M 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS; Boehringer Mannheim GmbH, Mannheim, Germany) at room temperature. Optical densities were read at 15 and 30 min with a Titer tek Multiskan MCC/340 ELISA reader (Flow Laboratories, McLean, Va.) using a 414-nm-pore-size filter. For each plate, a standard curve was constructed from the known IgA protein and used to determine the IgA concentration of each fecal extract. The concentrations determined were expressed as nano grams of IgA per 50 μl of extract [29].

Cytokine production measurements.

IL-2, IFN-γ, IL-5, and IL-10 were measured in serum samples of different studied groups by commercial ELISA kits (R&D Systems, Minneapolis).

Ethical considerations:-

The experimental animal studies were conducted in accordance with international valid guidelines and they were maintained under convenient conditions at the SBSP animal house of TBRI.

RESULTS

Oocyst shedding by parasitological examination:-

The minimum shedding of *C. parvum* oocysts (Figure 1A) was observed in mice treated with combined therapy of paromomycin and nitroanalin (group VI), with a mean number of oocysts1.3± 0.23, when compared to the infected control (group I) this difference was statistically significant ($p < 0.0001$), while the mean

number of oocysts shed in thestools was 9.2 ±0.43 in group treated with paromomycin only (group II). After nitroanalin treatment, infected mice in (group III) showed a marked decrease in oocyst shedding, the mean number of oocysts shed in the stools was 7.6± 0.8. (Table 1).

Detection of endogenous developmental stages of cryptosporidiosis

The ileum was the site with the heaviest burden of intestinal cryptosporidiosis (Figure 1, B and C). In group I, the mean numberof endogenous developmental stages of the parasite was 75.63 ± 2.5, while it was 42.91 ± 2.3in mice treated with paromomycin group II; this difference was statistically significant ($p = 0.0003$). However, the highest reduction in the mean numberof endogenous developmental stages was observed in group of mice treated with combined therapy (group VI) 23.61± 0.28 with a significant difference ($p < 0.0001$). In group III, after receiving Nitroanalin, it was found that the mean number of theendogenous developmental stages of the parasite dropped markedly to.39.56 ± 0.49 this difference was highly significant ($p < 0.0001$). (Table 2).

Histopathological examination:-

Several degrees of inflammatory changes were seen in thegroups infected with the *Cryptosporidium*. As regards dysplasia, most dysplastic changes were seen in group I (a and b). Low-grade dysplastic changes were also seen in mice belonging to group VI, with a significant difference compared to group I ($p < 0.0001$). No evidence of dysplasia was seen in the other groups and no frank carcinoma developed throughout the experiment. In group II, the development of high-grade dysplastic changes was significantly($p < 0.05$) associated with the number of endogenous developmental stages of the parasite, since high-grade dysplasia was associated with a higher mean number compared to low-grade dysplasia. (Fig. 2 and Table 4).

Measurement of IgA by ELISA

Fecal IgA was measure in mice referred to relevant group. Known concentrations of secretory IgA (0.49 to 150.60 ng) constituted the standard curve and had typical absorbance values in the range of 0.150 to 2.610 for the respective concentrations. Total IgA value in normal specimens was 0.241 ± 0.04 which is equivalent to 0.5 µg of IgA per g of stool. Total IgA value in infected non-treated mice was 2.147 ± 0.37 which is equivalent to 140 µg of IgA per g of stool. After treatment with paromomycin or nitroanalin alone the concentration of IgA was 67 and 59 µg of IgA per g of stool respectively while after combined treatment the concentration of IgA was decreased to 25 µg of IgA per g of stool. Concentration of

IgA was significantly associated with infection and oocyst shedding (Table3).

Effect of treatment on serum levels of cytokines

To assess the induction of Cryptosporidium-specific T-cell responses after treatment with the selected drugs, we measured production of IL-2, IL-5, IL-10, and IFN-γ by spleen cells from mice treated using our protocol. Mice in group treated with combined treatment showed a high significant (P<0.001) increase in serum levels of both IFN-γ and IL-10 when compared to group infected non-treated mice (Table 4). On the other, a moderate significant (P<0.01) reduction in serum level of IL-5 when compared to group infected non-treated mice, while no significant difference between all treated groups and group infected non-treated mice in serum level of IL-2 (Table 5).

Table 1:- Measurement of Oocyst shedding in stool samples of different studied groups

Mouse groups	Oocyst shedding (No./g stool) ± SD	% Reduction
Normal Mice	-	-
Infected Mice	20.6± 6.4	-
Infected treated with Paromomycine	9.2 ± 0.43	53.4 %
Infected treated withNitroanalin	7.6± 0.8	63.1 %
Infected treated with combined	1.3 ± 0.23	93.7 %

Table 2:- Measurement of Oocyst shedding in intestine of different studied groups

Mouse groups	Oocyst shedding (No./ml) ± SD	% Reduction
Normal Mice	-	-
Infected Mice	75.63 ± 2.5	-
Infected treated with Paromomycine	42.91 ± 2.3	43.3 %
Infected treated with Nitroanalin	39.56 ± 0.49	47.7 %
Infected treated with combined	23.61± 0.28	68.8 %

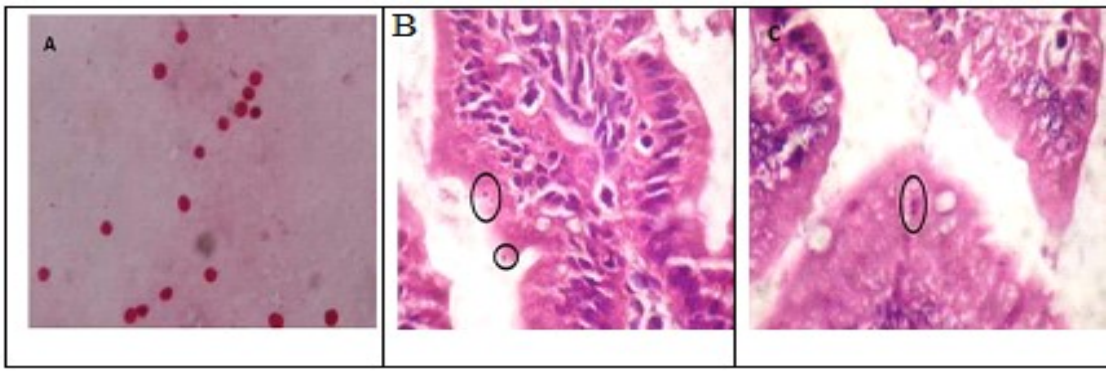

Figure 1. (A) shedding of *C. parvum* oocysts in fecal smears B) shedding of *C. parvum* oocysts in illeum c) shedding of *C. parvum* oocysts in intestine.

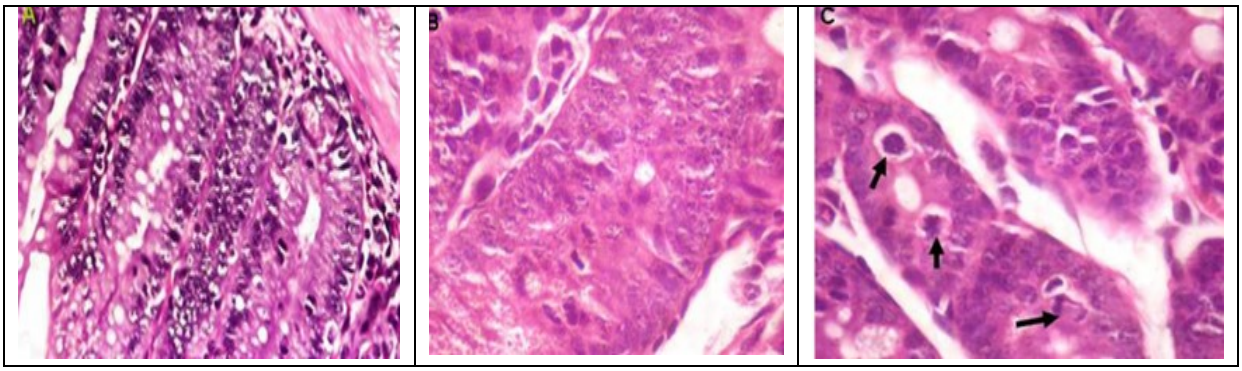

Figure 2. Several degrees of inflammatory changes were seen in the groups infected with the *Cryptosporidium* a) high-grade dysplasia was associated with a higher number off oocysts. b) No evidence of dysplasia was seen in the other groups and no frank carcinoma developed throughout the experiment c) Low-grade dysplastic changes.

Table 3:- Measurement of IgA in serum samples of different studied groups

Mouse groups	IgA level (µg/ml) ± SD
Normal Mice	0.241± 0.04
Infected Mice	2.147± 0.37
Infected treated with Paromomycine	0.86± 0.24**
Infected treated withNitroanalin	0.74 ± 0.19**
Infected treated with combined	0.543± 0.14**

** Statistically significant difference at p< 0.05 compared to infected control group

Table 4:- Measurement of IFN-γ in serum samples of different studied groups

Mouse groups	IFN- γ level (pg/ml) ± SD
Normal Mice	118± 3.9
Infected Mice	209± 5.1
Infected treated with Paromomycine	338± 7.4**
Infected treated withNitroanalin	299± 6.1*
Infected treated with combined	404 ± 7.8***

* Statistically significant difference at p< 0.01 compared to infected control group
** Statistically significant difference at p< 0.05 compared to infected control group
*** Statistically significant difference at p< 0.001 compared to infected control group

Table 5:- Measurement of IL-2, IL-5, and IL-10 in serum samples of different studied groups

Mouse groups	IL-2	IL-5	IL-10
Normal Mice	26.39± 3.28	22.12 ± 1.8	20.00 ± 2.71
Infected Mice	35.83± 4.52	252.02 ± 11.3	160.00 ± 12.34
Infected treated with Paromomycine	24.15 ± 1.2	123.53 ± 1.5**	238.11 ± 2.1*
Infected treated with Nitroanalin	29.45 ± 2.30	217.25 ± 4.7	271.2 ± 3.3**
Infected treated with combined	28.59 ± 1.90	162.91 ± 4.2*	398.72 ± 27.4***

* Statistically significant difference at p< 0.01 compared to infected control group
** Statistically significant difference at p< 0.05 compared to infected control group
*** Statistically significant difference at p< 0.001 compared to infected control group

DISCUSSION

Cryptosporidiosis is an important zoonotic disease of domestic and wild animals. Infection with *Cryptosporidium parvum* is common in cattle, buffaloes, goats, sheep, horses, cats, human beings and other vertebrates [30]. The infection is endemic in ruminants as well as human beings in Pakistan. In present study occurrence of disease in cattle reared at Government dairy farm, Military dairy farm, Household dairy and Gawala colonies was determined. Efficacy of selected drugs against *Cryptosporidium* oocysts was evaluated under experimental conditions in cattle.Oocyst per gram (OPG) count showed an increasing trend in control (untreated) animals. Asingle dose of 10mg/kg body

weight of albendazole caused a significant decrease in OPG count from 6th day post treatment and onward (P<0.05). Similarfindings in relation to the efficacy of albendazole at 20mg/kg body weight in calves against Cryptosporidiosis were reported by **Xia *et al.* [31] and Johny *et al.* [32]**. Metronidazole treatment caused a significant decrease in OPG count from 6th day post treatment and onward (P<0.05). Paromomycin used against Cryptosporidiosis under experimental conditions showed better results than albendazole and metronidazole. Although a number of compounds have beentested against Cryptosporidiosis and only limited showed effective results. **Kelly *et al.* [33]** observed improvement in symptoms of Cryptosporidiosis and eradication in four Zambian AIDS patients by albendazole used at dose 80mg twice. However control large scale study was recommended. Only paromomycin has been shown to have an anti-cryptosporidial activity [34]. Comparable results were documented in relation to efficacy of paromomycin by **Leitch and He [30]**. Similar findings were reported by **Sharling *et al.* [35]**, **Tzipori *et al.* [36] and Verdon *et al.* [37]** while working on animal models regarding efficacy of paromomycin against Cryptosporidiosis. Paromomycin in a dose of 25 to 35 mg/kg/day has a beneficial but limitedeffect upon oocyst shedding and stool frequency in AIDS patients **[38]**. Paromomycin was suggested to bethe most valuable drug for the treatment of *Cryptosporidium* infection based on the clinical trials **[39]**. Post treatment side effects observed were sweating and diarrhea. In the present study, a mixture of paromomycin and Nitroanalin was assessed and used to prevent and treat intestinal disorders.

The significant reduction in the number of oocysts and in percent reduction in oocysts shedding detected in fecal samples collected from the combined treated group in comparison to the treated mice, together with the significant decrease in the number of endogenous parasite stages in ileum sections along the intestinal mucosal surface of the early treated mice in comparison with the non-treated, all these results confirm the effective therapeutic role of paromomycin and Nitroanalin used in the present study against *Cryptosporidium* and are in agreement with **McDonald, *et al* [40]** and **Alak *et al* [41]**. Histologically, morphological and cellular alteration of microvillus membrane integrity revealed that Paromomycin and Nitroanalin administration ameliorated the mucosal damage in treated mice, compared with the severe microvillus damage, edematous and vacuolated epithelial cells in non-treated mice. These results clearly show the anti-cryptosporidial effect of the Paromomycin *in vivo* by modulating

the gut cells to inhibit the colonization and multiplication of *Cryptosporidium* , thus reducing the severity of cryptosporidiosis.Close results were reported by **Alak *et al* [42]** since daily ingestion of *L. reuteri* was efficient to prevent *C. parvum* intestine colonization and tissue lesions in immune-deficient mice. **Waters *et al.* [43]** suggested that protection was due to secretion of as yet unidentified antimicrobial products. **Arvola *et al* [44]** added that probiotics have been shown to modulate release of cytokines (TNF-α IFN-γ, IL-10, IL-12) which play a central role in maintaining the delicate balance between necessary and excessive defense mechanisms. **Gill [45]** reported that the underlying mechanisms are however not clear, involving stimulation of different subsets of immune system cells to produce cytokines, which in turn play a role in the induction and regulation of the immune response, and to enhance intestinal IgA immune responses and increase intestinal mucin production. Similarly, **Borchers *et al* [46]** found that IL-10 and secretory IgA response, important actors in an efficient anti-*Cryptosporidium* immune response, have been shown to be induced by some probiotic strains. In the present study, the increased serum level of IFN-y ininfected treated groups is in agreement with **Mead and You [47]**and **Riggs [48]** who found that protection against thisparasite has been largely associated with production of IFN- γ; a major player not only in cell-mediated immunity, but in early innate immune responses as well. In vitro studies have demonstrated that IFN- γ directly prevents the parasite from invading host cells [**49**]. Additionally, a case of persistent cryptosporidiosis has been described in a child with primary IFN- γ deficiency [50], suggesting that this cytokine is also important in human infections. The important protective roles for IFN- γ may also include apoptosis induction in infected intestinal epithelial cells, and modulation of mucosa epithelial integrity **[51]**. In conclusion combined therapy of paromomycin and nitoanalin is probably the most promising compound for treatment of Cryptosporidiosis in experimental mice.

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