Changes in parasitic load, phagocytosis and ultra-structural pattern in experimental cryptosporidiosis following combined (Antox and Nitazode) treatment

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Infection with cryptosporidium produces mild and self-limited diarrhea in immunocompetent persons and a prolonged, life-threatening cholera-like illness in immunocompromised patients and underweight children. On the other hand, cells are protected from the damaging effects of reactive oxygen intermediates by specific endogenous anti-oxidant enzymes and the level of these anti-oxidants in blood is closely dependent on the nutritional status of the host. This study was done to assess the effect of administration of the antioxidant Antox alone and/or in combination with nitazoxanide (nitazode) in experimental cryptosporidiosis. Lab bred albino mice were divided into 4 groups; infected control (gr I), infected treated with Antox (gr II), infected treated with Nitazode (gr III) and group IV, infected and treated with combination of Antox and Nitazode. All drugs were given orally for 7 consecutive days. The number of cryptosporidium oocysts was counted in small intestinal contents after scraping & staining with modified ZN stain. Phagocytosis index was examined in duodenal section. After animal sacrifice, ileal intestinal region was subjected to transmission electron microscopic examination. Treatment with Nitazode (gr III) resulted in significant reduction in the number of oocysts one week (52.2%) &two weeks (84.1%) post treatment. The combination regimen (gr IV) resulted in the highest significant reduction one week (68.1%) and two weeks (84.1%) post-treatment. The group receiving Antox (gr II), showed slight reduction one week post treatment, but was not significantly different from infected control (gr I) after two weeks. The phagocytic index increased significantly in all treated groups one week post treatment. It started to decrease in all groups at the 2nd week post treatment except group II. The ultrathin sections prepared from ileum of infected treated mice (group IV) revealed degenerated epithelial microvilli with exposure of cryptosporidia. In conclusion, combination of NTZ and Antox revealed the best antiprotozoal effect according to oocysts count stained with modified ZN. Antox alone, ameliorated the ultra-structural findings, but did not decrease oocyst burden in intestinal contents. The data of this study are reported in immunocompetent mice, the outcome would differ in immunosuppressed model.

Keywords: Immunocompetent, Immunosuppressed, Phagocytosis, Oocysts, Antox, Cryptosporidia, Nitazide

INTRODUCTION

Cryptosporidium parvum is an important cause of diarrheal disease in children and adults worldwide [Ungar 2000, Guerrant et al 2001]. Although many antimicrobial drugs are used to treat C. parvum infections, none has proved effective in treating the disease [Current & Garcia 1991]. Nitazoxanide, a nitrothiazolyl-salicylamide derivative [Rossignol, Stachulski 1999], has shown activity against C. parvum in cell culture and in animal models [Theodos et al 1998, Blagburn et al 1998; Gargala et al 2000]. In humans, a 3-day course of nitazoxanide is effective in treating enteric protozoan infections caused by Giardia intestinalis, Entamoeba histolytica, E. dispar, Blastocystis hominis, Balantidium coli, Isospora belli, and Cyclosporacayetanensis [Romero et al 1997, Abaza et al

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1998; Va´quez et al 1998]. It is also effective in treating diarrhea caused by C. parvum in patients with AIDS. Its activity varies depending on the degree of immunosuppression and the duration of treatment [Davis et al 1996, Rossignol et al 1998]. In this study, the treatment of diarrhea caused by C. parvum in immunocompetent adults and children is displayed.

Although Cryptosporidium is originally classified as a coccidian, yet it demonstrates several peculiarities that differentiate it from other coccidian. These include the location of Cryptosporidium within the host cell where the endogenous developmental stages are confined to the apical surfaces of epithelial cells. Also the attachment of the parasite to the host cell is characteristic, where a multi-membranous attachment or feeder organelle is formed at the base of the parasitophorous vacuole to facilitate the uptake of nutrients from the host cell (Levine, 1980).

In immune competent hosts, parasite development is relatively confined to the terminal jejunum and ileum. In immunosuppressed hosts, the entire gastrointestinal tract, as well as the biliary and pancreatic ducts may be infected. Less frequently, there could also be involvement of the respiratory tract (Mc Cole et al., 2000).

Cryptosporidia are spread by the ingestion of oocysts excreted by infected people or animals. Infection can be transmitted through the consumption of faecally contaminated water or food, via direct person-to-person or animal- to- person contact, and contact with contaminated environmental sources (Kosek et al., 2001).

In humans, C. parvum is now one of the most prevalent enteropathogens worldwide, and the etiological agent of a diarrheal disease which course is largely dependent on the immunological status of the affected individual (Flannigan et al., 1992). Consequently, it affects malnourished children in developing countries (Mc Cole et al., 2000). It also affects up to 50% of AIDS patients, (Agnew et al., 1998).

MATERIAL AND METHODS

Ethics

Anesthetic procedures complied with the ethical guidelines approved by the Ethical Committee of the Federal Legislation and National Institutes of Health Guidelines in USA were approved by the Medical Ethical Committee of Theodor Bilharz Research Institute (TBRI) in Egypt.

Experimental Animals

Laboratory-bred male albino mice (Mus musculus) of CDI strain, weighing 18–20 g, were used under a protocol in accordance with guidelines approved by the institutional committee for care of laboratory animals of the Theodor Bilharz Research Institute, Guiza, Egypt. Animals were maintained in air-conditioned rooms at 21 C, receiving food containing 24% protein fed ad libitum. The animals were supplied and housed throughout the study in animal house at the Theodor Bilharz Research Institute. The experimental design included 4 main groups:

Group 1- Experimental animals infected with microscopically proven Cryptosporidium parvum.
Group 2- Experimental animals infected with Cryptosporidium parvum and given Antox drug.
Group 3- Experimental animals infected with microscopically proven Cryptosporidium parvum and treated with (Nitazode).
Group 4- Experimental animals infected with microscopically proven Cryptosporidium parvum and administered a combination regimen (Antox and Nitazode).

Animal Infection: experimental animals were infected each by 1000 oocysts/mouse orally using tuberculin tube (Rehg et al., 1988). The oocysts were concentrated from stool samples collected from infected diarrheic calves by repeated sedimentation, washing and centrifugation to obtain the infecting oocysts.

Drugs

1. Antox was given orally in a suspension form in a dose of (0.86 mg /mouse) for seven consecutive days
2. Nitazode(nitazoxanide) was given orally in a suspension form in a dose of (500 mg /kg ) for seven consecutive days.
3. Antox was given orally in a suspension form in a dose of (0.86 mg/mouse) in combination with Nitazode in a suspension form in a dose of (500 mg /kg) for seven consecutive days.

Direct parasitological methods

The fresh samples of diarrheic stools collected from calves were stained with Modified Zeihl –Nelsen stain. Microscopic identification of oocysts in 10 oil-immersion (100xobjective) fields was done before concentration and count as previously described (Casemore et al., 1985 and Theodos et al., 1997).

Animal sacrifice

Sacrifice of half the animals in each group was done one week post-treatment; the rest of mice in these groups were sacrificed 2 weeks after administration of drugs. The scraped small intestinal contents (duodenal and proximal ileum) were stained with modified Zeil-Neelsen
then subjected to parasitological examination in order to count the number of Cryptosporidium parvum in 10 successive fields/animal (Tzipori et al., 1981).

Phagocytic Index

The method is based on assessing the percentage of macrophage cells that phagocytize heat-killed yeast (Metcalf et al.1986).

Statistical analysis

-Data were coded and entered using the statistical packages SPSS version 7.5.
-Data were summarized using mean and standard deviation for quantitative and qualitative variables.
-Comparison between two groups was done using T test.
-P values lower than 0.05 were considered as statistically significant.

Transmission Electron Microscopy of small intestine

Small part from the ileal intestinal region was excised and divided into tiny pieces of 1mm3 They were fixed in buffered 4% glutaraldehyde with 0.2M sodium cacodylate for 2hours. The fixed pieces were washed twice in equal volume of 0.3M cacodylate and 0.4 M sucrose. After washing the samples were postfixed in 2% osmium tetroxide, washed in bidistilled water, and dehydrated in ascending concentration of alcohol till reaching the concentration of absolute alcohol. The tiny pieces of intestine were infiltrated with epoxy resin, embedded in mould by using freshly prepared resin then polymerized at 60 0C for 48 hours. Semithin and ultrathin sections were performed using Ultramicrotome (Leica Ultracut R). Ultrathin sections were stained with uranyl acetate and lead citrate. Examination of the stained grids was done using a Philips EM 208 S electron microscope.

RESULTS

Electron microscopic examination of the ultrathin sections prepared from the ileal intestinal region of infected treated mice revealed absence of the glycocalyx in most of the examined sections. The ultrathin sections of treated mice with Antox revealed mainly the envelope of some oocysts at the surface of the enterocytes. The intestinal microvilli at the site of these oocytes were short, atrophic or degenerated (Figure1). Also areas of intact enterocytes displaying microvilli in the form of striated border, junctional complex, appearance of glycocalex on the tips of some microvilli were evident (Figure 2-3).

The ultrathin sections prepared from the ileum of infected treated mice with the combination of the two drugs Antox and Nitazode revealed degenerated epithelial microvilli with the exposure of the parasite in different stages of development in some sections (Figure 4). Most of these developing stages of the cryptosporidia revealed some disintegration changes. The Exfoliating surface cells allowed the exposure of vessels in the lamina propria (Figure 5-6).
Figure 1: Electron micrograph of intestinal mucosa of treated mouse with Antox shows the apical surface of enterocytes with evident microvilli, remnant of a cryptosporidium oocyst (arrow). At the site of contact with the envelope of the oocyst the microvilli are short or atrophic. No glycocalyx could be depicted along the microvilli.

Figure 2: Electron micrograph of two adjacent intestinal epithelial cells of treated mouse with Antox showing the junctional complex and well-formed apical brush border.

Figure 3: Enterocytes of treated mouse with Antox show striated border. Some tips of the microvilli are covered with glycocalyx.
Figure 4: Electron micrograph of enterocyte of infected treated mice with Antox and Nitozole reveals complete damage of the epithelial microvilli. The cellular rough endoplasmic reticulum studded with ribosomes are still evident also remnant of a mitochondrion is seen in the photo (white arrow). Note the two developing stages of the cryptosporidiawith undergoing collapse and degenerative changes (arrow).

Figure 5: Electron micrograph showing the exfoliation of the apical portion of an enterocyte with degenerative changes of the rest of the cell in the examined section from the ileum of infected treated mice with Antox and Nitozole.

Figure 6: Electron micrograph of ileal section from infected treated mice with Antox and Nitozole shows exposed blood capillary (arrow), the enterocyte reveals intracellular lysosomes.
DISCUSSION

In this study, the combination of Nitazode and Antox revealed the best antiprotozoal effect according to oocysts count. Marked improvement of ultra-structural findings, following antioxidant administration was reported, yet the decrease in oocyst burden in intestinal contents was less significant.

Phagocytic index increased in both groups receiving Antox. Eid et al. (2014) postulated that anti parasitic chemotherapy significantly improved oxidative/nitrosative stress and DNA damage in infected mice.

Abdel Maksoud et al. (2014) evaluated the effect of lauric acid and ginger against Cryptosporidium parvum in infected mice they found significant reduction in the Cryptosporidium parvum oocysts count following treatment with the highest percentages of reduction in the group receiving nitazoxanide (96.7%) followed by lauric acid (92.5%) treated group followed by the combined treated group that received half the doses of lauric acid and ginger (72.3%). Lastly, came the ginger treated group (84.4%). The authors added that Lauric acid significantly improved the histopathological pattern as compared to infected control.

Administration of oxi–guard (antioxidant agent) had led to eradication of free radicals which in turn diminished their lethal effects on cryptosporidium leading to increased parasitic colonization in the small intestine (Yassein et al. 2001). The activity of endogenous anti-oxidant enzymes in cryptosporidium oocysts didn't increase in the group given anti oxidant; the infected group exhibited a significant increase in super oxide dismutase (Yassein et al. 2001).

The immunosuppressed individuals are usually subjected to infection with opportunistic protozoa, most commonly cryptosporidium. The use of antioxidant drugs in these patients should better be monitored with periodic stool examination specially if they suffer diarrhea since a potential increase in oocysts burden might be encountered in such condition.
REFERENCES


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