The efficacy of platelet rich plasma as adjuvant therapy in the treatment of cryptosporidiosis in experimentally infected immunosuppressed rats

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ABSTRACT

Background: Cryptosporidium species are zoonotic opportunistic coccidian parasites that could cause disseminated life-threatening infection in the immunocompromised host. Unfortunately, few available drugs effectively eradicate the infectious oocyst with limited availability in developing countries. Although Nitazoxanide (NTZ) is the drug of choice for the treatment of cryptosporidiosis, it has limited efficacy in malnourished and immunocompromised patients. Moreover, platelet-rich plasma (PRP) successfully ameliorated the hepatic granuloma size in patients with parasitic schistosomiasis mansoni.

Objective: This study aims to test the potential therapeutic effect of PRP versus the currently used NTZ, and/or using PRP as adjuvant therapy.

Material and Methods: Sixty-five immunosuppressed rats were divided into 5 groups: non-infected as negative control (GI), infected non-treated as the positive control (GII), infected with Cryptosporidium spp. and treated with either intraperitoneal PRP (GIII), or NTZ (GIV), or a combination of intraperitoneal PRP and NTZ (GV). Parameters used for evaluation of the therapeutic efficacy included parasitological examination, histopathological examination of ileocaecal and liver specimens, and quantitative analysis of reduced glutathione (GSH) and malondialdehyde (MDA) for evaluation of oxidative stress markers, glutamic-oxaloacetic transaminase (SGOT), and glutamic pyruvic transaminase (SGPT) for evaluation of liver functions.

Results: Parasitological and histopathological examinations revealed minimal improvement in GIII, marked improvement in GIV, and the best results were recorded in GV. The administration of PRP in GIII produced no significant changes in GSH, MDA, SGOT compared to positive control GII. Treatment with NTZ in GIV, and in addition to PRP in GV showed significant difference (P<0.05) compared to GII regarding serum results of GSH, MDA, and SGOT with the best results recorded in GV. GIV and GV showed reduction of serum levels of SGPT although there was statistically insignificant difference between the study groups.

Conclusion: PRP could be used as a potential adjuvant therapy with NTZ to ameliorate the pathologic and inflammatory effects of cryptosporidiosis on the ileocaecal region. It also improves liver function in the immunocompromised hosts.

Keywords: cryptosporidiosis; glutathione; liver function tests; malondialdehyde; nitazoxanide; platelet-rich plasma.

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INTRODUCTION

The genus Cryptosporidium and its species belong to the phylum Apicomplexa. They are intestinal coccidian protozoa that mainly inhabit the brush border of the small intestine acquiring the unusual position of being intracellular, but extra-cytoplasmic[1]. Cryptosporidiosis is a globally distributed zoonotic disease-causing enteritis and aqueous diarrhea[2]. Implicated species are transmitted via consumption of contaminated food or drink with sporulated oocysts, inhalation of oocysts from the soil, and self-infection[3-4].

In 2015, 27 species of Cryptosporidium were characterized[5]. Despite reports on infection in man by several species, the majority of human cryptosporidiosis proved to be due to C. hominis and C. parvum[6-8]. Cryptosporidiosis may cause serious complications in immunocompromised patients including severe diarrhea with subsequent dehydration and electrolyte imbalance, hepatic and respiratory problems[9]. There are several major mechanisms responsible for the associated diarrhea, the most important of which is morphologic atrophy of small intestinal villi, blunting and fusion of the villi, in addition to inflammatory infiltrates in the lamina

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Platelet-rich plasma in cryptosporidiosis. Besides, PRP products are [21][18][14-17]® 5 [33][18,19]. Although, there is no accessible effective anti-fibrotic therapy for preventing damage by oxidative stress. There are very few reports in the literature documenting the role of ROS in the pathogenesis of experimental C. parvum infection in mice[31].

Keeping the previous layout in mind, our study aimed to evaluate the synergistic effect of PRP as an adjuvant therapy with NTZ, to revert the pathologic changes in the ileocecal region, livers, and portal tracts of white albino rats that were subjected to immunosuppression then infection with Cryptosporidium oocysts. In addition to evaluation of liver functions and oxidative stress markers.

MATERIAL AND METHODS

This experimental study was performed in the biological unit of Theodor Bilharz Research Institute (TBRI) during the period from September 2020 to March 2021. Pathology sections were prepared and examined in the Pathology Department Laboratory at Ain-Shams University Hospital.

Animals: Sixty-five laboratory rats of Sprague Dawley strain, ~10 weeks old, weighing 130-150 gm, were purchased from the Animal House of TBRI. The animals were kept in plastic cages with clean woodchip bedding, in well-ventilated, sanitary, conditioned rooms with the mean temperature (27±2°C), away from direct sunlight. Animals were provided by chow and water ad lib.

Study groups: Fifty immunosuppressed rats were divided into 5 groups; 10 in each. Groups I (non-infected) and II (infected with Cryptosporidium oocysts, non-treated) were used as negative and positive controls, respectively. Groups III-V included infected rats treated with intraperitoneal PRP, NTZ, and combined therapy, respectively. Fifteen non-immunosuppressed rats served for PRP preparation.

Immunosuppression: Rats were immunosuppressed by oral administration of dexamethasone sodium phosphate (Dexazone) with a dose of 0.25 ug/g/day for two weeks by gavage technique using an esophageal tube; and was maintained by a weekly dose during the whole experiment for all the study groups[32].

Infection: After two weeks, immunosuppressed rats were orally infected by a suspension of 10^6 Cryptosporidium oocysts in 200 μL PBS, by gavage using an esophageal tube[19]. Cryptosporidium oocysts were kindly obtained from the Animal Research Institute in Giza governorate, Egypt.

Drug and PRP preparation: NTZ was obtained from a local pharmacy in the form of powder to prepare 100 mg suspension/5 ml (Nanazoxide®). To prepare PRP, 15 non-immunosuppressed rats were anesthetized using ether. After laparotomy, four ml venous blood...
was collected from the inferior vena cava in tubes containing 3.2% sodium citrate (Merck, Darmstadt, Germany). Centrifugation of blood samples was performed at 400 g for 10 min. The supernatant was centrifuged again at 800 g for 10 min. From each tube, two-thirds of the serum were discarded from the top, and the remaining pooled portions were accepted as PRP. After the aforementioned procedure, ~15 ml of PRP was obtained.

**Therapeutic doses:** Rats of GII received PRP in a dose of 0.5 ml/kg by intraperitoneal injection twice weekly for 4 weeks starting one week post-infection (pi). Rats of GIV received 65 mg of NTZ oral suspension. The drug was administered daily for 4 weeks starting one-week pi. The doses were adjusted by an extrapolation table for the therapeutic doses of man and animals. Whereas GV received a similar regimen and doses of PRP and NTZ.

**Parasitological examination:** To ensure rats’ infection, stool samples were obtained a week after inoculation with oocyst for microscopic examination using the oil immersion lens (x100) after staining the stool smears by modified Ziehl Neelsen (MZN) stain. Parasite load was calculated by counting the number of Cryptosporidium spp. oocysts in the fecal pellets collected from infected rats at 7th, 14th, 21st, 28th, and 35th day pi. One mg stool pellet was weighed, then preserved in 1 ml 10% formalin; the stool suspension was concentrated by centrifugation at 500 g for 10 min. The oocysts were counted in 1 ml of stool sample by staining 100 ul of stool sediment by MZN and examining microscopically (x100). The average of 3 counts multiplied by 10 equaled the number of oocysts in 1 ml of stool. The number of oocysts was expressed per gram of feces. Oocyst shedding one week after inoculation was considered a sure sign of viability and establishment of infection.

Measurement of serum liver enzymes and oxidative stress markers: One ml venous blood was collected from each rat from the retro-orbital vein 4 weeks after the establishment of infection at the end of the treatment regimen. Levels of GSH(Merck, Darmstadt, Germany). Measurement of serum liver enzymes and oxidative stress markers: One ml venous blood was collected from each rat from the retro-orbital vein 4 weeks after the establishment of infection at the end of the treatment regimen. Levels of GSH, MDA, SGOT and SGPT were measured using Sigma Diagnostic kits (USA).

**Histopathological examination:** All rats were euthanized 4 weeks pi to remove the ileocecal region of the small intestine and liver with the portal tracts. The specimens were fixed in 10% buffered formalin, embedded in paraffin wax blocks, sectioned, and stained by Hematoxylin and Eosin (H&E). The internationally valid guidelines were applied on all animal experiments after acceptance by the institutional ethical committee.

**RESULTS**

**Parasitological studies:** After inoculation of rats with Cryptosporidium spp. oocysts (Fig. 1), fecal pellets were collected from GII (infected non-treated) at different days pi to determine the number of Cryptosporidium spp. oocysts/gm of feces on each day (Fig. 2). It was observed that oocysts shedding started from the 7th day pi with 715 ±48.65 x10³ oocysts/gm of feces.

Table (1) shows the therapeutic effects of drugs used on oocysts shedding in collected stool pellets. The number of Cryptosporidium spp. oocysts/gm of feces that were shed by GII rats on the 35th day of infection was 2292.8 ±227.32 x10³ oocysts. There was 59.17%, 65.14% and 71.32% reduction of oocyst shedding in GIII, GIV and GV compared to GII, respectively. It is worth mentioning that the mortality rate was around 10% in all the study groups.

**Histopathological examination of ileocecal and liver specimens:** In GI, sections of the small intestine at ileocecal junction showed normal small intestinal mucosa with normal villous architecture length and width. A well-formed intact brush border was detected.
with an average number of goblet cells. Hepatic sections showed normal hepatic architecture with the hepatocytes arranged in thin regular plates around the central vein and unremarkable portal tracts (Fig. 3).

In GII, the ileocecal junction of the small intestine revealed marked histopathological changes as a result of cryptosporidiosis. Variable grades of villous atrophy in addition to decreased villous height to crypt ratio and shortened broad villi were detected. Foci of mucosal ulceration were observed together with the depletion of goblet cells. Significant inflammatory infiltrate was also seen in the lamina propria composed mainly of plasma cells, lymphocytes, and macrophages with lymphoid aggregates. Examination of liver sections showed expanded portal tracts with moderate lymphoid infiltrate. Dispersed spotty necrosis foci were detected together with mild interface activity and mild fibrosing reaction forming porto-portal bridges (Fig. 4).

In GIII, there was minimal amelioration of the histopathological changes at the ileocecal junction. A mild reduction in villous height to crypt ratio with moderate villous blunting was detected. Foci of mucosal ulceration were seen with mild exhaustion of goblet cells. Lamina propria showed moderate inflammatory infiltration. Liver sections showed foci of lobular activity with mildly dilated portal tracts and moderate lymphocytic infiltrate (Fig. 5).

In GIV, marked amelioration of the histopathological changes occurred. Restoration of normal villous architecture was detected and the ratio between villous height and the crypt became close to normal. Regular brush border and normal goblet cells were seen. There were minimal surface mucosal erosions, and the mucosal lining showed a nearly normal pattern. The lamina propria showed a mild inflammatory reaction. The liver sections showed mild portal inflammatory infiltrate (Fig. 6).

In GV, the ileocecal junction showed remarkable amelioration of the histopathological changes, with preservation of the villous to crypt height ratio.
The normal villous architecture was restored. The mucosal lining, brush border, and goblet cells were nearly normal. The lamina propria showed minimal inflammatory infiltration. Liver sections showed maintenance of hepatic architecture with minimal inflammatory infiltrate (Fig. 7).

Biochemical analysis of serum GSH, MDA, SGOT, and SGPT: There was a significant difference between all the study groups regarding serum GSH, and SGOT (P<0.01), and serum MDA (P<0.05), with a non-significant difference regarding serum SGPT (P=0.1257). Compared to GI, there was a significant difference regarding serum GSH, MDA, SGOT, and SGPT (P<0.001) in GIII. However, when compared to GI, there was a non-significant change regarding the same parameters. The difference between GIV and GI regarding serum GSH, MDA, SGOT, and SGPT was significant (P<0.001), and there was also a significant difference when compared to GI regarding the same parameters (P<0.001). Similarly, GV versus GI showed a significant difference for the results of serum GSH and SGPT (P<0.001) and a non-significant difference regarding serum MDA and SGOT, while GV versus GII showed a significant difference for the serum levels of GSH, MDA, SGOT, and SGPT (P<0.001) and MDA (P<0.05) (Table 2). The administration of PRP in GII produced no significant changes in GSH, MDA, SGOT compared to positive control GII. Treatment with NTZ in GIV, and in addition to PRP in GV showed significant difference (P<0.05).

Table 2. Biochemical changes of the different study groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>GSH</th>
<th>MDA</th>
<th>SGPT</th>
<th>SGOT</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>136.3±5.467</td>
<td>2.060±0.2875</td>
<td>47.07±0.8512</td>
<td>51.09±0.9893</td>
</tr>
<tr>
<td>II</td>
<td>91.7±2.080</td>
<td>3.920±0.5865</td>
<td>59.08±1.405</td>
<td>52.03±1.239</td>
</tr>
<tr>
<td>III</td>
<td>93.9±1.527</td>
<td>3.780±0.5473</td>
<td>61.17±3.029</td>
<td>68.19±1.686</td>
</tr>
<tr>
<td>IV</td>
<td>110.2±4.626</td>
<td>2.950±0.3923</td>
<td>41.09±1.166</td>
<td>44.11±1.453</td>
</tr>
<tr>
<td>V</td>
<td>123.1±1.567</td>
<td>2.390±0.2331</td>
<td>49.03±1.467</td>
<td>40.06±0.701</td>
</tr>
<tr>
<td>P value*</td>
<td>0.0027*</td>
<td>0.0400*</td>
<td>0.0019*</td>
<td>0.1257</td>
</tr>
</tbody>
</table>

* Non-infected, non-treated (negative control); II: Infected, non-treated (positive control); III: Infected and received PRP; IV: Infected and received NTZ; V: Infected received PRP and NTZ. Values are presented as mean ± SD. @ One-way ANOVA and post hoc Tukey’s multiple comparison test. a: Significant difference between all groups and the negative control. b: Significant difference between GII, GIV, and GV versus the positive control. c: Significant difference between GIV in comparison to GV. **: Significant P < 0.05.

DISCUSSION

Cryptosporidium spp. are well-known parasites causing waterborne epidemics all over the world[43]. The outcome of cryptosporidiosis is greatly dependent on the immune status of the patient, giving clinical manifestations that vary greatly from mild self-limited diarrhea within two to three weeks in the immunocompetent patients, to severe life-threatening illness with dehydration and electrolyte imbalance in the immunocompromised patients[44-46]. Cryptosporidiosis is associated with high mortality among immunodeficient patients[47,48]. Also, cryptosporidiosis may lead to atypical clinical manifestations in immunodeficient patients such as pancreatitis, respiratory and biliary tract infections[49].
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Tissue sections of the small intestinal ileocaecal junction from immunocompromised mice infected with cryptosporidiosis revealed short, broad, and atrophied intestinal villi with the reduction of the villous to crypt height ratio. Mucosal ulceration, goblet cell depletion, and inflammatory infiltration of the lamina propria were noted. Liver sections showed congested sinusoids with dilated fibrotic portal tracts.

According to results of the present study, following cryptosporidiosis under immunocompromised conditions, treatment with the PRP showed significant improvement in the histopathological changes of small intestinal villi at the ileocaecal region and restored the liver architecture with reduction of portal tracts pathology. These results were marked when PRP was used in combination with NTZ giving results better than PRP injection alone, or NTZ administration alone.

Marx et al. were the pioneers in studying PRP prepared by centrifugation of blood then separation of the supernatant that is very rich in platelets. Platelet-rich plasma proved to contain many important GFs. It is a breakthrough in the field of medicine to discover the ability to use PRP in tissue regeneration as platelets contain proteins required for hemostasis as well as numerous GFs such as basic fibroblast growth factor (BFGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), endothelial growth factor (EGF), and insulin-like growth factor (IGF). Platelet-rich plasma also releases special chemokines and cytokines (e.g., IL-1 and platelet factor 4) in addition to important proteins and peptides (e.g., fibrinogen, fibronectin, osteocalcin, osteonectin, vitronectin, and thrombospondin). Therefore, many PRP types have been used by clinicians for years due to their enhancing effects on wound healing, cellular mitogenesis, osteogenesis, and angiogenesis. Moreover, advocates of PRP treatment prefer PRP because of its beneficial effects in increasing tissue regeneration. Accordingly, it was used in reconstructive surgery to decrease the rate of postoperative necrosis and enhance healing, as well as lowering infection due to its antibiotic-like-action of contained leukocytes; in addition to decreasing pain and blood loss. Platelet-rich plasma is a cheap easily obtained product without any risk for rejection or harmful immune responses against it. The use of PRP has been reported in many fields of medicine, including maxillofacial surgery and the treatment of problematic soft tissue ulcers. Also, PRP became one of the best treatment options in the field of sports medicine for the treatment of tears in the rotator cuff, and the ulnar collateral ligament, hamstring injuries, in addition to knee osteoarthritis. Also, transfusion of platelets can improve liver function in patients with chronic liver disease and cirrhosis due to their ability to produce many types of GFs in addition to the antifibrotic properties. Platelet-rich plasma proved to reduce chemically induced liver fibrosis in experimental animals as it significantly diminished fibrotic areas and hepatic hydroxyproline in carbon tetrachloride (CCL4) induced hepatic fibrosis. Also, Takahashi et al. found that human PRP lessened significantly the fibrotic index and hepatic hydroxyproline content in CCL4-initiated liver fibrosis in severe combined immunodeficient mice. Moreover, a recent study revealed that rat PRP markedly improved dimethylnitrosamine-induced liver fibrosis as demonstrated by significant lowering of liver hydroxyproline content.

Glutathione has a significant role in the detoxification of oxygen free radicals produced at the inflammation sites. A low level of GSH is of pathogenic importance in hepatic parasitic infections as in schistosomiasis. Accordingly, elevated GSH levels in target cells and the plasma might have a beneficial effect. Inhibition of oxidative stress and decreasing the intracellular peroxides level depend mainly on elevating the level of GSH and fostering its metabolizing enzymes to face the liberated free radicals. In our study involving immunocompromised rats infected with Cryptosporidium spp., the MDA lipid peroxidation product was elevated, whereas the GSH level was reduced. This concurs with many studies in which there was liver affection in experimentally infected mice with S. mansoni. In our study, biochemical measurements of GSH, MDA, SGOT and SGPT confirmed the beneficial therapeutic effect of NTZ against cryptosporidiosis. This concurs with the study stating that NTZ is the only Food and Drug Administration-approved therapy, although it is not consistently effective for therapy of cryptosporidiosis in the majority of vulnerable patients. In the present study, GV versus GI showed a non-significant difference regarding...
serum MDA and SGOT indicating the strong effect of the combination therapy in lowering their levels near to the normal values; and a significant difference regarding decreased serum GSH and SGPT, \((P<0.001)\). However, when compared to GII, there was a significant change in decreasing serum GSH, and SGPT with increasing serum levels of MDA and SGOT \((P<0.001)\).

Results of GV were better than GIV, being very similar to the negative control with a non-significant statistical difference regarding results of serum MDA and SGOT and had the best results between the study groups regarding serum GSH and SGPT compared to the negative control. This denotes the strong therapeutic effect for the PRP/NTZ combination that is superior to the effect of NTZ alone where there was a significant difference between GV and GIV regarding the results of serum GSH, SGOT, SGPT \((P<0.001)\), and MDA \((P<0.05)\). Accordingly, we could conclude that GV showed the best results due to statistically significant elevation of GSH with reduction of MDA, and SGOT indicating improvement compared to the GII. Also, GV showed a reduction of serum SGPT although there was statistically insignificant difference between the study groups. This is in agreement with the study that proved the strong synergistic effect of PRP with the adipose-derived stem cells in inducing healing of tissues in mice exposed to pressure injury\(^{[50]}\). This also concurs with the usage of PRP-gel as an adjuvant treatment in human fibrosarcoma, to stimulate tissue repair and speed up recovery, thus impairing tumor growth and slowing tumor progress\(^{[72]}\). Similar conclusions were reported regarding the results of serum GSH, MDA, and SGOT when ginger \((Zingiber Officinale)\) loaded nanoparticles were used in the treatment of murine schistosomiasis \textit{mansoni}\(^{[70]}\). Hence, it seems that combination therapies will play an important role to ameliorate the pathologic effects of parasitic infections.

In conclusion, results of former studies\(^{[6,11,12,25,31,35,42,46,50,52,64,67,72]}\) in addition to the findings of the current research elucidate the important effects of PRP on tissue healing and fibrosis reduction that could be considered a breakthrough in the treatment of the pathologic changes following parasitic infections. Although the PRP therapy had no direct effect on the parasite, our results indicated that PRP could be considered as a novel potential adjuvant therapy with NTZ, to ameliorate the pathologic and inflammatory effects of cryptosporidiosis on the small intestinal villi and on the liver and portal tracts in the immunocompromised hosts.

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Author contribution: El-Kholy WAMS designed the plan of work, performed the practical part, and shared in writing and revising the manuscript. Elghohary SA performed the histopathological study. El-Ashkar AM shared in designing the plan of work, analyzing the data, writing, and revising the manuscript. The manuscript has been read and approved by all named authors. We further confirm that the order of authors listed has been approved by all of us.

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