Alterations in oxidative stress parameters and its associated correlation with clinical disease on experimental *Cryptosporidium parvum* infection in Swiss albino mice

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Abstract The present study was conducted to evaluate the changes in oxidative stress parameters on experimental infection with *Cryptosporidium parvum* in Swiss albino mice. The mice were divided into four groups viz., group I–IV, each group comprising of 15 mice. Group I mice served as healthy control. In Group II mice, *C. parvum* oocysts @ 10^4 /os were administered, mice of group III were given dexamethasone @ 30 µg/ml in drinking water whereas group IV mice were given dexamethasone @ 30 µg/ml along with *C. parvum* oocysts @ 10^4 /os. Significant changes were seen in oxidative stress parameters which included significant increase in LPO and decrease in levels of SOD, CAT and GSH in liver and intestine in group IV mice at 10th DPI when compared to others indicating an important role played by free radical induced oxidative stress in the development of *C. parvum* infection in mice which was clinically characterized by loss of body condition, profuse bloody diarrhoea and peak oocyst shedding intensity occurring at 10th DPI.

Keywords *Cryptosporidium parvum* · LPO · SOD · CAT · GSH · Oxidative stress

Introduction

Cryptosporidium parvum belongs to a ubiquitous and diverse group of intracellular apicomplexan parasites of both veterinary and public health importance. *C. parvum* is associated with gastrointestinal tract illness in a variety of host species viz., cattle, sheep, goat, reptiles and man (O'Hara et al. 2004). The parasite causes a self-limiting watery diarrhoea in immunocompetent hosts but has a devastating effect in immunocompromised hosts like neonates, people with AIDS, hematological malignancies, organ transplantation, or people undergoing chemotherapy (Hunter and Nichols 2002). The prevalence of *Cryptosporidium parvum* infection in livestock and humans has been reported from all across the world (Becher et al. 2004; Brook et al. 2009; Cocklin et al. 2007; Uga et al. 2000) including India (Khan et al. 2010; Venu et al. 2012; Maurya et al. 2013; Singla et al. 2013).

Oxidative stress is defined as an alteration in the steady-state balance between oxidant and antioxidant agents in the cells. It is caused by an over production of reactive oxygen species (ROS). A balance between ROS and primary antioxidant defenses which mainly include antioxidant scavenging enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH) is needed in preventing damage by oxidative stress. There are very few reports in literature documenting the role of ROS in the pathogenesis of *C. parvum* infection. Immunocompromised mouse model has been used to study pathogenic potential of *C. parvum* by numerous workers (Harp et al. 1988; Fayer et al. 1990; Bjorneby et al. 1991). Here, our

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endeavour was to measure any alterations in oxidative stress parameters occurring in response to experimental *C. parvum* Jammu isolate infection in mice in tissues particularly liver and intestine thus ascertaining the role of ROS in development of experimental cryptosporidiosis in mice.

Materials and methods

Faecal samples were collected from a naturally infected cattle calf (20 days old) having severe mucoid diarrhoea from a private farm in Jammu. Examination of collected faecal samples for cryptosporidiosis was done by modified Ziehl-Neelsen (mZN) staining technique (Henricksen and Pohlenz 1981) and quantification of *Cryptosporidium* oocysts was done by as per the method of OIE (2008). Molecular confirmation of *C. parvum* was done by nested PCR & RFLP following 18S SSU rRNA (Xiao et al. 1999; Feng et al. 2007). The purified oocysts of *C. parvum* were counted by Neubauer Chamber. Final concentration of purified oocysts was adjusted to 10^7 /ml and these were fortified with streptomycin @100 µg/ml and penicillin @ 100 IU/ml and stored at 4 °C for further use.

The study was conducted on 60 female Swiss albino mice, 3 weeks age, procured from Indian Institute of Integrative Medicine (IIIM), Jammu. Mice were found to be free from parasitic infections for three consecutive days prior to the start of experiment as determined by diethyl ether sedimentation and mZN technique. Strict hygienic conditions were maintained and animals were given feed and water ad libitum. All the mice were acclimatized to the experimental conditions for one week. The mice were randomly divided into four groups viz., group I to IV, each group comprising of 15 mice and housed in separate cages during the experiment. Group I mice served as healthy control. Group II mice were administered *C. parvum* oocysts @ 10^4 per os (Tarazona et al. 1998). Group III mice served as control for immunosuppression and individual mice in this group were administered dexamethasone @ 30 µg/ml in drinking water. Group IV mice were given dexamethasone @ 30 µg/ml in drinking water and also were infected with *C. parvum* oocysts @ 10^4 per os. Dexamethasone was administered in group III and IV mice 7 days prior to the start of experiment and was continued till the end of experiment. Faecal samples were collected at regular intervals. Mice were sacrificed at 2nd, 4th, 6th, 10th and 15th day intervals and liver and intestines were collected. Estimations of different oxidative stress-related biochemical parameters (SOD, CAT, LPO & GSH) in the infected tissues of intestines and liver were done using a UV spectrophotometer.

200 mg of intestine and liver were weighed and taken in 2 ml of ice- cold PBS. Another set of samples of intestine,

liver (200 mg) were weighed separately and taken in 2 ml of 0.02 EDTA in distilled water for GSH estimation. The samples were homogenized with tissue homogenizer under ice- cold condition and were centrifuged for 10 min at 3000 rpm. The supernatants were stored at -20 °C until the assay was done. The activity of lipid peroxidation in tissue homogenate of liver and intestine was determined according to the methodology of Shafiq-ur-Rahman (1984). Membrane peroxidative damage in tissues was determined in terms of MDA (malondialdehyde) production, using TBA (thiobarbituric acid). The amount of LPO was expressed as nM of MDA formed per gram of wet tissue. Superoxide dismutase (SOD) was estimated as per the method described by Madesh and Balasubramanian (1997). A unit of enzyme activity was measured as the amount of enzyme causing 50 per cent inhibition of the auto-oxidation of pyrogallol observed in blank. The activity of superoxide dismutase was expressed as SOD units/gm of tissue.

Catalase enzyme activity in intestine and liver homogenate was estimated by the method of Aebi (1983) and was expressed as k/g of wet tissue (K is mM H₂O₂ utilised/min/g of wet tissue). GSH was determined by estimating free –SH groups, USING 5–5' dithiobios 2-nitrobenzoic acid (DTNB) (Sedlak and Lindsay 1968).

Data analysis

Data generated from various parameters were presented as Mean $\pm$ SE. The growth performance parameters and other parameters were subjected to a two way ANOVA employing Tukey's descriptive statistical analysis as per method described by Snedecor and Cochran (1994).

Results and discussion

Mice in group IV started shedding oocysts at 4th DPI. The intensity of shedding went on increasing and a peak intensity of +3 was seen at 10th DPI which subsequently decreased to +1 as the mice recovered at the end of trial at 15th DPI. Clinically mice were found to be dull, depressed, lethargic and had poor fur condition, decreased body weight and food intake at peak infection period. In contrast, Group II mice showed only a mild infection with the intensity being +1 at the time of peak infection and the infection completely resolved in these mice at 15th DPI.

The average LPO, SOD, GSH and CAT levels in intestine of mice of different groups measured at different intervals is summarised in Table 1. No significant ($p < 0.05$) difference in LPO levels of intestinal homogenates was observed in any of the four groups at any interval except at 10th DPI, when significant ($p < 0.05$) increase in LPO levels of intestinal homogenates was

Table 1 Oxidative stress parameters in intestine of Swiss albino mice infected with *C. parvum*

Parameters	Days PI	G-I	G-II	G-III	G-IV
LPO intestine (nM MDA/g)	2nd	38.24 ^{aA} ± 0.56	38.23 ^{aA} ± 0.53	38.87 ^{aA} ± 0.87	38.53 ^{aA} ± 1.44
	4th	38.18 ^{aA} ± 0.58	38.65 ^{aA} ± 0.40	39.33 ^{aA} ± 0.56	38.98 ^{aA} ± 0.94
	6th	38.50 ^{aA} ± 1.17	39.73 ^{aA} ± 0.95	39.65 ^{aA} ± 0.97	39.35 ^{aA} ± 1.21
	10th	39.00 ^{aA} ± 1.43	39.05 ^{aA} ± 0.52	39.07 ^{aA} ± 0.84	44.02 ^{bB} ± 1.85
	15th	39.72 ^{aA} ± 0.92	39.67 ^{aA} ± 0.82	39.75 ^{aA} ± 0.95	39.83 ^{aA} ± 0.65
GSH Intestine (mM/g)	2nd	0.302 ^{aA} ± 0.010	0.303 ^{aA} ± 0.020	0.299 ^{aA} ± 0.009	0.300 ^{aB} ± 0.008
	4th	0.301 ^{aA} ± 0.006	0.302 ^{aA} ± 0.001	0.306 ^{aA} ± 0.008	0.305 ^{aB} ± 0.009
	6th	0.300 ^{aA} ± 0.007	0.302 ^{aA} ± 0.020	0.302 ^{aA} ± 0.009	0.303 ^{aB} ± 0.008
	10th	0.302 ^{bA} ± 0.008	0.301 ^{bA} ± 0.010	0.303 ^{bA} ± 0.001	0.285 ^{aA} ± 0.000
	15th	0.302 ^{aA} ± 0.009	0.300 ^{aA} ± 0.009	0.300 ^{aA} ± 0.020	0.300 ^{aB} ± 0.009
Catalase intestine (μMH ₂ O ₂ decom pose/min/g of tissue)	2nd	70.45 ^{aA} ± 0.64	69.00 ^{aA} ± 0.70	68.25 ^{aA} ± 1.080	69.08 ^{aB} ± 0.96
	4th	69.53 ^{aA} ± 1.20	68.87 ^{aA} ± 1.03	68.92 ^{aA} ± 0.92	68.52 ^{aB} ± 1.28
	6th	69.43 ^{bA} ± 0.59	68.32 ^{aA} ± 1.07	67.77 ^{aA} ± 1.04	68.90 ^{aB} ± 1.05
	10th	69.08 ^{bA} ± 1.77	67.10 ^{bA} ± 1.70	67.50 ^{bA} ± 0.59	63.62 ^{aA} ± 1.23
	15th	70.00 ^{aA} ± 0.57	69.05 ^{aA} ± 0.80	67.68 ^{aA} ± 1.87	66.02 ^{aB} ± 0.97
SOD intestine (U/mg)	2nd	11.15 ^{aA} ± 0.24	10.98 ^{aA} ± 0.29	10.85 ^{aC} ± 0.26	11.15 ^{aC} ± 0.23
	4th	11.04 ^{aA} ± 0.30	10.55 ^{aA} ± 0.24	10.75 ^{aA} ± 0.24	11.03 ^{aB} ± 0.31
	6th	11.06 ^{aA} ± 0.29	11.25 ^{aA} ± 0.26	11.00 ^{aA} ± 0.31	10.89 ^{aB} ± 0.27
	10th	11.00 ^{bA} ± 0.54	10.45 ^{bA} ± 0.30	11.10 ^{bA} ± 0.53	8.89 ^{aA} ± 0.22
	15th	11.05 ^{bA} ± 0.27	11.15 ^{bA} ± 0.89	10.15 ^{aA} ± 0.90	10.08 ^{aB} ± 0.91

Mean bearing at least one common superscript (a, b, c and A, B, C, D) do not differ significantly between groups and weeks ($p < 0.05$), respectively

observed in group IV when compared with others. LPO levels measured in the intestinal tissues at 10th DPI in group IV were also significantly ($p < 0.05$) decreased when compared to than the LPO levels at other intervals in this group.

SOD levels in intestinal tissues did not vary significantly on 2nd, 4th, 6th DPI among different groups. But on 10th DPI, SOD levels of intestine in the group IV mice, were significantly ($p < 0.05$) lower in comparison to SOD levels in homogenates of intestine in other groups. SOD intestinal levels at 10th DPI in group IV were also significantly lower than the corresponding values at other intervals for this group. At the end of experiment on 15th DPI, SOD levels in various groups again did not show any significant variation among different groups. Similar to the trend of variation noticed in SOD values, CAT and GSH levels in intestines of different groups did not differ significantly upto 6th DPI but at 10th DPI, group IV mice had significantly ($p < 0.05$) lower CAT and GSH concentration in intestine when compared with the corresponding values of mice of other groups. At 15th DPI the comparison of SOD, CAT and GSH did not reveal any significant variation.

The average LPO, SOD, CAT and GSH levels in liver homogenates of mice of different groups observed at different intervals is summarised in Table 2. No significant ($p < 0.05$) difference in the LPO levels of liver samples is concerned was observed in any of the four groups at any

interval at 2nd, 4th, 6th or 15th DPI. But at 10th DPI, a significant increase in LPO levels in livers of group III and IV was observed when compared group I and group II. More significantly, the LPO values in liver samples of group IV were found to be higher than the LPO levels measured in liver tissues of group III.

The SOD levels in liver homogenates did not differ among different groups until 6th DPI. SOD levels in liver of group III and IV mice were significantly lower as compared to these levels in group I and II on 10th and 15th DPI. Remarkably, SOD levels in liver of group IV animals were significantly lower as compared to the corresponding values in group III animals on 10th DPI. The average CAT and GSH levels in liver samples were found to follow a similar trend as was observed for liver SOD levels wherein significantly lower CAT and GSH concentrations were seen in group IV at the 10th DPI of experiment when compared to others. At 15th DPI the difference in values of SOD, CAT and GSH in liver tissues became non-significant among different groups.

Estimation of oxidative stress biomarkers gives an assessment of injury caused by free radicals. Oxidative stress plays an important role in pathogenesis of enteric diseases. Alterations in oxidant and antioxidant steady state were found to exacerbate the infectivity and pathogenicity of *Blastocystis hominis*, a common intestinal protozoan parasite of humans, in a mouse model (Chandramathi et al.

Table 2 Oxidative stress parameters in liver of Swiss albino mice infected with *C. parvum*

Parameters	Days PI	G-I	G-II	G-III	G-IV
LPO liver (nM MDA/g)	2nd	38.20 ^{AA} ± 0.59	37.95 ^{AA} ± 0.93	38.23 ^{AA} ± 0.58	38.75 ^{AA} ± 0.61
	4th	37.65 ^{AA} ± 1.22	38.35 ^{AA} ± 0.47	38.40 ^{AA} ± 0.57	38.00 ^{AA} ± 0.55
	6th	37.86 ^{AA} ± 0.95	38.37 ^{AA} ± 0.48	37.28 ^{AA} ± 1.47	39.23 ^{AA} ± 0.52
	10th	38.25 ^{AA} ± 0.28	38.08 ^{AA} ± 0.87	38.10 ^{AA} ± 0.80	41.83 ^{BB} ± 1.76
	15th	38.05 ^{AA} ± 0.81	38.42 ^{AA} ± 0.65	38.83 ^{AA} ± 0.51	38.96 ^{AA} ± 0.55
GSH liver (mM/g)	2nd	0.359 ^{AA} ± 0.010	0.358 ^{AA} ± 0.010	0.357 ^{AB} ± 0.008	0.356 ^{AB} ± 0.009
	4th	0.360 ^{AA} ± 0.007	0.355 ^{AA} ± 0.006	0.352 ^{AB} ± 0.009	0.350 ^{AB} ± 0.008
	6th	0.358 ^{AA} ± 0.008	0.355 ^{AA} ± 0.010	0.350 ^{AB} ± 0.010	0.355 ^{AB} ± 0.006
	10th	0.359 ^{CA} ± 0.009	0.356 ^{CA} ± 0.008	0.310 ^{BA} ± 0.007	0.300 ^{AA} ± 0.000
	15th	0.356 ^{BA} ± 0.009	0.354 ^{BA} ± 0.008	0.315 ^{AB} ± 0.009	0.320 ^{AB} ± 0.027
Catalase liver (μMH ₂ O ₂ decompose/min/g of tissue)	2nd	70.47 ^{AA} ± 1.17	69.70 ^{AA} ± 1.68	69.67 ^{AB} ± 0.87	68.43 ^{AB} ± 0.99
	4th	70.40 ^{AA} ± 1.57	69.40 ^{AA} ± 0.64	69.40 ^{AB} ± 0.51	68.00 ^{AB} ± 0.65
	6th	70.07 ^{BA} ± 0.70	69.00 ^{ABA} ± 1.23	68.40 ^{AB} ± 1.08	67.45 ^{AB} ± 0.58
	10th	70.09 ^{CA} ± 0.96	69.00 ^{CA} ± 0.38	66.38 ^{BA} ± 0.99	62.50 ^{AA} ± 1.40
	15th	69.07 ^{BA} ± 0.91	68.40 ^{BA} ± 0.85	65.98 ^{AB} ± 1.20	64.93 ^{AB} ± 0.94
SOD liver (U/mg)	2nd	10.90 ^{AA} ± 0.30	10.78 ^{AA} ± 0.24	10.65 ^{AB} ± 0.31	10.69 ^{AB} ± 0.31
	4th	11.00 ^{AA} ± 0.26	10.68 ^{AA} ± 0.42	10.40 ^{AB} ± 0.31	10.60 ^{AB} ± 0.31
	6th	11.01 ^{AA} ± 0.22	10.96 ^{AA} ± 0.51	10.20 ^{AB} ± 0.41	10.01 ^{AB} ± 0.28
	10th	10.98 ^{CA} ± 0.23	10.35 ^{CA} ± 0.61	9.09 ^{BA} ± 0.34	8.60 ^{AA} ± 0.22
	15th	10.55 ^{BA} ± 0.27	10.22 ^{BA} ± 0.33	9.11 ^{AA} ± 0.34	9.10 ^{AB} ± 0.11

Mean bearing at least one common superscript (a, b, c and A, B, C, D) do not differ significantly between groups and weeks ($p < 0.05$), respectively

2014). Oxidative damage induced by ROS has been incriminated in many pathological conditions of gastrointestinal tract including inflammatory bowel disease (IBD) (Grisham 1994; Keshavarzian et al. 2003). The role of SOD is to catalyzes the dismutation of the superoxide radical produced as a by-product of oxygen metabolism which, if not regulated, causes cell damage. Studies conducted by Quílez et al. (2013) have revealed a positive role of SOD in amelioration of TNBS (Trinitrobenzene sulfonic acid)-induced colitis by reducing oxidative stress in the intestine. CAT on the other hand, catalyzes the decomposition of hydrogen peroxide to water and oxygen and therefore plays an important role in protecting the cell from oxidative damage by reactive oxygen species. GSH also prevents damage to important cellular components caused by ROS. Also, peroxidation of cell membrane lipid layer due to free radicals is an important feature of cellular injury of intestinal tract infections (Metwaly et al. 2015).

In the present study, SOD, CAT and GSH values in intestine were significantly lower in group IV as compared to others at 10th DPI underscoring their utilization in usurping the ROS generated in response to *C. parvum* infection. These biomarkers in liver of mice in group III and IV were decreased significantly as compared to group I and II mice during the later part of the experimental trial i.e. day 10 and 15 PI. The decrease seen in liver

homogenates was probably due to the stress of immunosuppression. However, the significant decrease in the level of these markers in group IV as compared to group III on 10th DPI shows that *C. parvum* infection exacerbated the stress of immunosuppression. LPO levels of liver and intestinal homogenates on the other hand, were increased significantly in group IV at 10th DPI pointing towards the damage to lipid component of cell membrane. This period also correlated with peak intensity of oocyst shedding and severe clinical signs exhibited by the mice of this group. Aziz et al. (2014) found significant decrease in GSH level in *C. parvum* infected rats. Selenium deficient C57BL/6 mice were shown to possess decreased levels of GSH, CAT and SOD and these impaired antioxidant systems correlated with increased susceptibility of such mice for *C. parvum* infection (Wang et al. 2009). Morada et al. (2013) noted that *C. parvum* induces endoplasmic reticulum stress responses. Scanning of literature revealed that few similar studies have been conducted in apicomplexan protozoan parasites. Decreased GSH levels and elevated levels of ROS were seen in mice infected with *Toxoplasma gondii* (Xu et al. 2015). *Eimeria papillata* was found to induced a state of oxidative damage and disturbance in antioxidant system within jejunum tissue as exhibited by enhanced lipid peroxidation and decrease in CAT and GSH levels in infected jejunum tissue (Metwaly et al. 2015). Moreover,

decrease in catalase and glutathione activity was seen in *E. papillata* infection in mice and the oxidative stress responses were attenuated by treatment with berberin (Dkhil et al. 2015).

To summarize, the present study, show that experimental infection with *C. parvum* in immunocompromised Swiss albino mice causes an increase in LPO and decrease in GSH, CAT and SOD at the peak of infection. These findings clearly implicate production of ROS in pathogenesis of experimental *C. parvum* infection in mice.

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Compliance with ethical statements

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement The institutional ethics committee for animal experiments from the Sher-e- Kashmir University of Agricultural Sciences and Technology of Jammu granted an approval for the conduct of the present work (Order No. 862/ac/04/CPCSEA).

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