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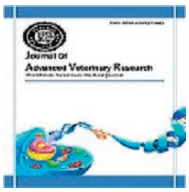

Oxidative Stress Biomarkers and Pathological Alterations Induced by *Cryptosporidium* Infection in Buffalo Calves at Assiut Governorate, Egypt

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ABSTRACT

This study aimed to examine the histopathological changes and some biochemical parameters including oxidative stress indices during the course of a natural *Cryptosporidium parvum* infection in newborn buffalo calves. A total of 102 buffalo calves of 1-3 weeks of age, suffering from diarrhea were examined for the presence of *C. parvum* oocysts. Out of them, 16 buffalo calves were positive for *C. parvum* and 15 calves were free from *Cryptosporidium* infection and represented the control group. The histopathological study was also included two newly born buffalo calves that were died and proved to be positive for *C. parvum* oocysts. Intestinal and abomasal mucosa of infected calves showed villous atrophy and architectural abnormalities characterized by rounded edges, with markedly dilated glands filled with necrotic material, and numerous cryptosporidia at different stages of life cycle. Serum biochemical constituents revealed decreases ($P < 0.05$) in concentrations of total proteins (-14.94%), albumin (-17.22%), sodium (-7.532%), potassium (-16.02%) and chloride (-9.628%) when compared with healthy calves. There were increases ($P < 0.05$) in serum concentrations of malondialdehyde (62.524%) and total peroxides (30.31%). In contrast, there were an inhibition ($P < 0.05$) in serum concentrations of total antioxidant capacity (-35.49%) and the activity of superoxide dismutase (-30.43%) in comparison with the control group. Pearson's correlation coefficients (r) and linear regression (R2) analysis (n. = 16) showed that TPX was inversely correlates with albumin (r=0.61, R2=0.43, P<0.001) and sodium (r=0.67, R2=0.48, P<0.001) concentration in serum of *C. parvum* infected calves. It can be concluded that Cryptosporidiosis had an adverse effect on biochemical parameters with increased reactive oxygen metabolites and lipid peroxide production in infected buffalo calves, which may be responsible for tissue damage and villus atrophy in infected calves.

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Introduction

Cryptosporidium spp. (phylum Apicomplexa, family Cryptosporidiidae) are coccidian protozoa affecting the gastrointestinal tract in several mammalian and amphibian species. Some of the members of this family possess also a well expressed zoo-anthropogenic potential (Brar *et al.*, 2017). Between the most common food and waterborne diseases with worldwide spread, Cryptosporidiosis comes the first that causing diarrhea in animals and man (Abdul Rahman *et al.*, 2017). Oocyst can survive through regular wastewater treatment and it is resistant to inactivation by commonly used drinking water sterilizers (Vanathy *et al.*, 2017).

Infection starts with the ingestion of the oocysts by the host. The exogenous stage, is oocyst containing four sporozoites. (Fayer and Xiao, 2007). The oocyst undergoes excystation, releasing four sporozoites in the small intestine (ileum), which invades epithelial cells (O'Donoghue, 1995). There are two types of oocysts, oocysts with thick walls, which are expelled in the faeces and thin-walled oocysts, which re-circulate in the intestinal tract causing autoinfection (Hijjawi *et al.*, 2004).

Abou El-Ella *et al.* (2013) found that *C. parvum* was the most frequently encountered causative agent among diarrheic cattle and buffalo calves in Assiut governorate, Egypt with a prevalence of 59.2% and 39.6%, respectively. Ibrahim *et al.* (2016) found that *C. parvum* was the only species detected in cattle and buffaloes in Upper Egypt; the authors highlighted the potential role of these animals as significant reservoirs of infection to humans. The neonatal diarrheic syndrome caused by *C. parvum* is usually observed in 5–35-day-old claves with maximum incidence in the second week of life (Fayer and Xiao, 2007), resulting in high mortality and morbidity rates, reduced weight gain after remission from the disease and disease treatment costs (Cho and Yoon, 2014; Abdul Rahman *et al.*, 2017).

Cryptosporidiosis duration and intensity correspond to the localization of the infection and the extent of morphological aberrations of the intestinal mucosa (Fayer and Xiao, 2007). *Cryptosporidium* spp. multiply at the microvillus borders of the

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intestinal epithelium, giving serious damage to the villi thereby reduce the absorptive surface, resulting in mal-digestion and mal-absorption followed by diarrhea (Vanathy *et al*., 2017). Diarrhoea in calves with cryptosporidiosis causes clinical findings and metabolic changes associated with dehydration, hemoconcentration and electrolyte imbalance (Yagci *et al*., 2017).

Oxidative stress is the consequence of an imbalance of pro-oxidants and antioxidants leading to cell damage and tissue injury (Celi, 2011). This disturbance produces reactive oxygen species (ROS). Oxidative stress is common in organs and tissues with high metabolic and energy demands (Kim *et al*., 2012). When ROS are not effectively and safely removed, oxidative stress may impair the health both directly and indirectly. Changes induced by ROS in cellular membranes are among the indirect effects (Gutteridge and Halliwell, 2018). Oxidative stress and ROS due to tissue damage has a crucial role in the pathogenesis of the enteric damage in farm animals (Kim *et al*., 2012). Oxidative stress induced by *C. parvum* infection was reported to induce tissue damage in a rat model (Abd El-Aziz *et al*., 2014; Bhagat *et al*., 2017; Sood *et al*., 2018). Decreased the antioxidant, superoxide dismutase (SOD) and increased the lipid peroxide marker, malondialdehyde (MDA) was reported in cattle infected by *C. andersoni* (Zhou *et al*., 2012). This study aimed to evaluate the histopathological changes during the course of a natural *Cryptosporidium parvum* outbreak and to estimate the oxidative stress status in buffalo calves naturally infected with *C. parvum*.

Materials and methods

Animals and sampling

This study was carried out in rural areas in the east of Assiut province (Upper Egypt). Buffalos in this area are reared in small sized flocks under unorganized farming with unsatisfactory standards of animal management and feeding. A total of 102 buffalo calves of 1-3 weeks of age, suffered from diarrhea and rapid dehydration were examined for the presence of *C. parvum* oocysts. Accordingly, 16 buffalo calves harbored *C. parvum* oocysts were selected as an infected group. Another clinically healthy buffalo (n.=15) calves with the same ages, and free from *Cryptosporidium* infection were selected from the same area and represented the control group. In addition, two newly born buffalo calves were died due to chronic diarrhea and proven to be positive for *C. parvum* oocysts were included in this study for histopathological studies. All the selected calves were free from other internal or blood parasites according to the routine laboratory diagnosis.

Blood was sampled from the selected positive (n.=16) and negative calves (n.=15) in 10-ml plain vacutainer tubes (Terumo Europe N.V, Interleuvenlaan 40, 3001Leuven, Belgium) and used for separation of serum, which stored at -20˚C until biochemical analysis.

Faecal samples were collected from all the investigated live and dead calves directly from the rectum into labelled screw-top specimen containers, which were placed in an insulated portable cooler, taken to the laboratory within few hours of collection and stored at 4 °C until examined within 24 h.

This study was done in accordance to the Institutional Animal Care and use ethical Committee (IACUC), and approved by Faculty of Veterinary Medicine, Assiut University, Egypt

Parasitological examinations

Faecal samples were processed immediately for detection of *Cryptosporidium* spp. oocysts by staining the faecal smears with modified Ziehl-Neelsen method as described by Henriksen and Pohlenz (1981). The presence of *C. parvum* coproantigens was confirmed through Rainbow calf scour 5 BIO K 306 (BIOX Diagnostics, Belgium), a coprological rapid antigenic strip test to detect *Cryptosporidium* in faecal samples according to Klein *et al*. (2009) and manufacturer's instruction.

Histopathological examination

Carcasses were submitted to routine necropsy using the standard protocol. Tissue samples (size 2 cm) were collected from the affected and intact gastrointestinal tract areas (abomasum, duodenum, jejunum and ileum) for histopathological examination. Specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. From paraffin blocks, one µm sections were cut and conventionally stained with haematoxylin-eosin and 1% toluidine blue (Drury and Wallington, 1980). Villus height and crypt depth from all affected and healthy samples were measured using eyepiece micrometer according to Argenzio *et al*. (1990).

Biochemical analyses

Blood serum was used for determination of total proteins, albumin, sodium, potassium and chloride levels by using specific commercially available test kits (Biodiagnostic, Dokki-Giza – Egypt) according to the manufacture instructions.

Determination of oxidative stress markers

Serum level of MDA was determined using commercial kit (Biodiagnostic, Dokki- Giza - Egypt) according to Placer *et al*. (1966). Serum total peroxide (TPX) concentration was measured as a H_2O_2 equivalent after the method described by (Erel, 2005). Serum total antioxidant capacity (TAC) was determined using standardized kit (Biodiagnostic, Dokki- Giza - Egypt) after the method described by Erel (2004). Epinephrine and H_2O_2 were obtained from local sources with highest analytical grade.

Serum superoxide dismutase (SOD) activity was estimated according to the method described by Misra and Fridovich (1972). This method is based on the ability of SOD to inhibit the autoxidation of epinephrine to adrenochrome in an alkaline medium (pH 10.2). Colorimetric and kinetic determinations of biochemical parameters were performed using spectrophotometer (Jasco-V530).

Statistical analysis

Data were analyzed using the packaged SPSS program for windows version 21.0.1 (SPSS Inc., Chicago, IL.) according to Borenstein *et al*. (1997). Data were expressed as Mean±Standard error (SE). Differences between groups were determined using an analysis of variance followed by the Student t-test. Pearson's correlation coefficients (r) and linear regression analysis (R2) were determined between paired variables of blood samples (n = 16). Significance level was set at $P < 0.05$.

Results

Repeated parasitological examinations of feces (acid fast modified Ziehl–Neelsen staining) revealed presence of numerous *Cryptosporidium* spp. sporulated oocysts, i.e., 20–50 oocysts/10 fields. The oocysts were ovoid or rounded with single layer wall of 4-6 um diameter, stained red or pink with a granular appearance against a whitish blue background. Black dot in oocyst could be seen. Developmental forms of daughter merozoites appear in the oocyst (Fig. 1a,b). Microscopic examination of the fecal smear showed that 15.69 % of the ex-

amined buffalo calves were positive for *C. parvum*.

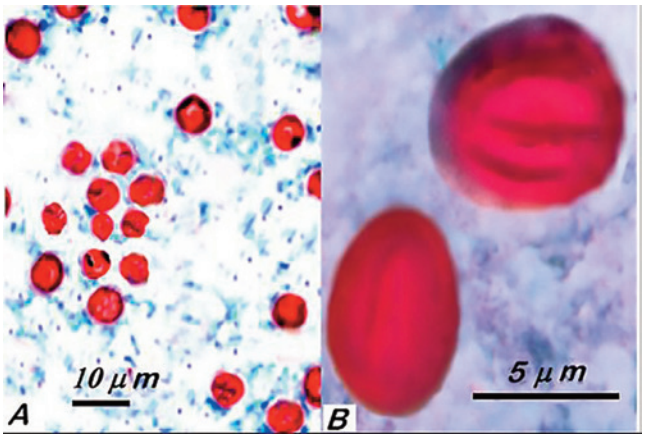

Fig. 1. Sporulated oocysts of *Cryptosporidium parvum* by acid fast modified Ziehl–Neelsen staining.

The performed histopathological investigations on Cryptosporidial enteritis in calves demonstrated changes in the small intestine include villous architectural abnormalities characterized by presence of desquamative catarrh of the intestinal mucosa, intestinal villous atrophy, metaplasia and desquamation of the surface epithelium.

Analysis of villous height and crypt depth indicated significant differences in the ileum (Fig. 2). A comparison of control and infected ileum, villous height was reduced to as much as half of the control. Reduction of villous height and expansion of the crypts is seen to give an approximate 2:l villus-crypt ratio in the infected tissue as compared with the 4:1 ratio in the controls.

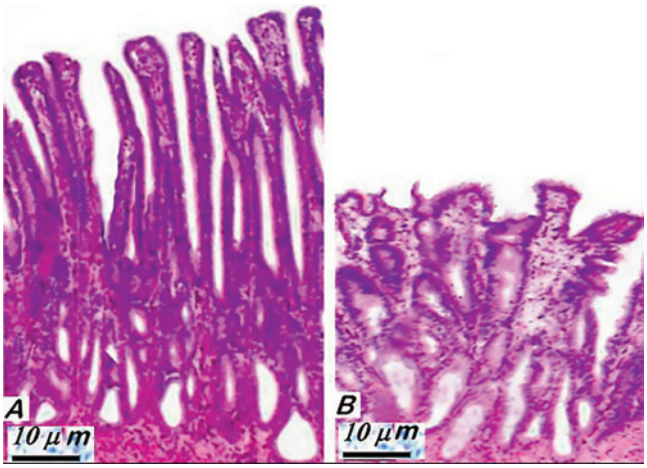

Fig. 2. Transverse section in ileum of control (A) and infected (B). Villous height is reduced to as much as half of the control. Reduction of villous height and expansion of the crypts is seen to give an approximate 2:l villus-crypt ratio in the infected tissue as compared with the 4:1 ratio in the controls. Infected calve showing villous architectural abnormalities characterized by rounded edges, intestinal villous atrophy and desquamation of the surface epithelium. *C. parvum* organisms were embedded in the microvillous border (H&E).

The villi were with rounded edges (Fig. 2b). There was local or diffuse infiltration of the mucosa and submucosa of some areas of small intestine with neutrophil leukocytes. The affected intestinal epithelium contained large numbers of developmental forms of *C. parvum* organisms were embedded in the microvillus border of the jejunal and ileal absorptive enterocytes. The normal vacuolation in absorptive cells was absent in the infected animals. Infection is confined to the apical surfaces of enterocytes, from the base of the crypts to the tips of the villi. Because of their small size and indistinct structure, they may be confused with cellular debris or mucus (Fig. 3).

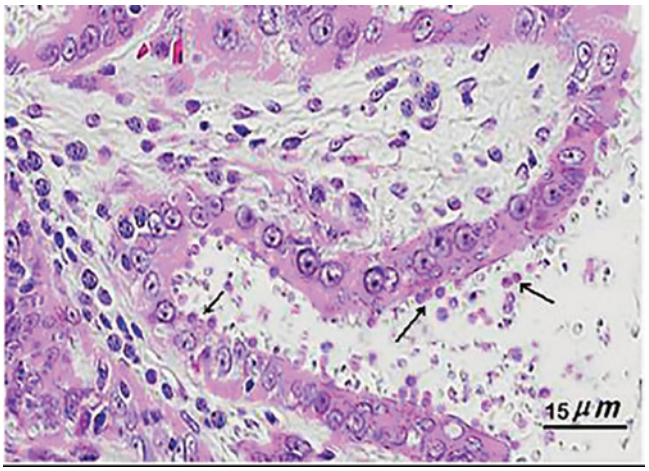

Fig. 3. Transverse section in jejunum of infected calf. There is local or diffuse infiltration of the mucosa and submucosa of some areas of small intestine with neutrophil leukocytes. The affected epithelium contained large numbers of developmental forms of *C. parvum* organisms were embedded in the microvillous border of the absorptive enterocytes (arrows). The normal vacuolation in absorptive cells was absent in the infected animals. Infection is confined to the apical surfaces of enterocytes, from the base of the crypts to the tips of the villi, (H&E).

Microscopic histopathological lesions in the abomasum consisted mainly in dilated glands, filled with necrotic material, mucus and numerous cryptosporidia at different stages of life cycle. Epithelial cells of infected glands were atrophic and exhibited cuboidal or squamous metaplasia. The lamina propria was slightly edematous. Markedly few eosinophils were present in the lamina propria of infected areas of the abomasum. In the infected parts of glands, mucous epithelial cells were activated and in the lumen of glands mucus was abundant (Fig. 4a, b).

Blood serum biochemical parameters in control and infected calves are presented in Table 1. Serum biochemical constituents revealed significant decreases ($P < 0.05$) in serum concentrations of total proteins (-14.94%), albumin (-17.22%), sodium (-7.532%), potassium (-16.02%) and chloride (-9.628%) levels.

The oxidative stress parameters, MDA, TPX, TAC, and SOD in blood serum of control and infected calves are shown in Table 2. There were significant increases ($P < 0.05$) in serum

Table 1. Blood serum biochemical parameters in control and infected calves

	Control calves (n.=15)	Infected calves (n.=16)	Change (%)
Total proteins (g/l)	63.91 ± 0.87	54.36 ± 0.77	- 14.94*
Albumin (g/l)	32.75 ± 0.99	27.11 ± 0.79	- 17.22*
Sodium (mmol/l)	139.4 ± 1.32	128.9 ± 1.09	- 7.532*
Potassium (mmol/l)	4.781 ± 0.15	4.015 ± 0.12	- 16.02*
Chloride (mmol/l)	107.6 ± 0.83	97.24 ± 0.91	- 9.628*

Data were expressed as Mean±SE, *: Significant different than control at $P < 0.05$

concentrations of MDA (62.524%) and TPX (30.31%). In contrast, there were decreases (P < 0.05) in serum concentrations of TAC (-35.49%) and the activity of SOD (-30.43%).

As shown in Fig. (5), Pearson's correlation coefficients (r) and linear regression (R2) analysis (n = 16) showed that TPX was inversely correlates with albumin (r=0.61, R2=0.43, P<0.001) and sodium (r=0.67, R2=0.48, P<0.001) concentration in serum of *C. parvum* infected calves.

Discussion

Cryptosporidiosis in neonatal calves has been reported from different geographical regions of the globe and it is an

Table 2. Values of malondialdehyde, total Peroxide, total antioxidant capacity and superoxide dismutase in blood serum of control and infected calves.

	Control calves (n.=15)	Infected calves (n.=16)	Change (%)
MDA (nmol/ml)	0.531 ± 0.014	0.863 ± 0.024	62.524*
TPX (µmol H_2O_2 Equiv/L)	17.55 ± 0.151	22.87 ± 0.201	30.313*
TAC (mmol trolox Equiv/L)	0.541 ±0.084	0.349 ± 0.076	- 35.490*
SOD (U/ml)	11.27 ±1.401	7.841 ± 1318	- 30.426*

Data were expressed as Mean±SE. Malondialdehyde: MDA; Total peroxide: TPX; Total antioxidant capacity: TAC; Superoxide dismutase: SOD. Significant different than control at P < 0.05

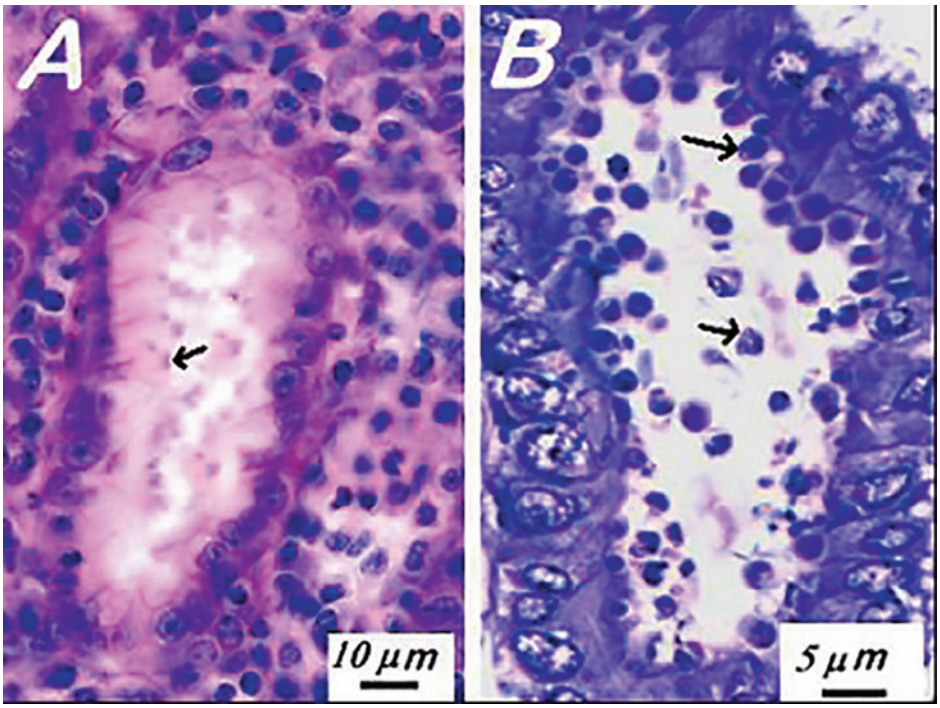

Fig. 4. Transverse section in abomasum of infected calf, A (H&E) and B (toluidine blue): abomasum consisted mainly of markedly dilated glands, numerous cryptosporidia (arrows) are shown on the brush border and in the lumen which is filled with necrotic material and mucus. Epithelial cells of infected glands were cuboidally or squamously metaplasied and atrophic. Mucous epithelial cells were activated in the infected parts of glands and the lumen of glands mucus was abundant.

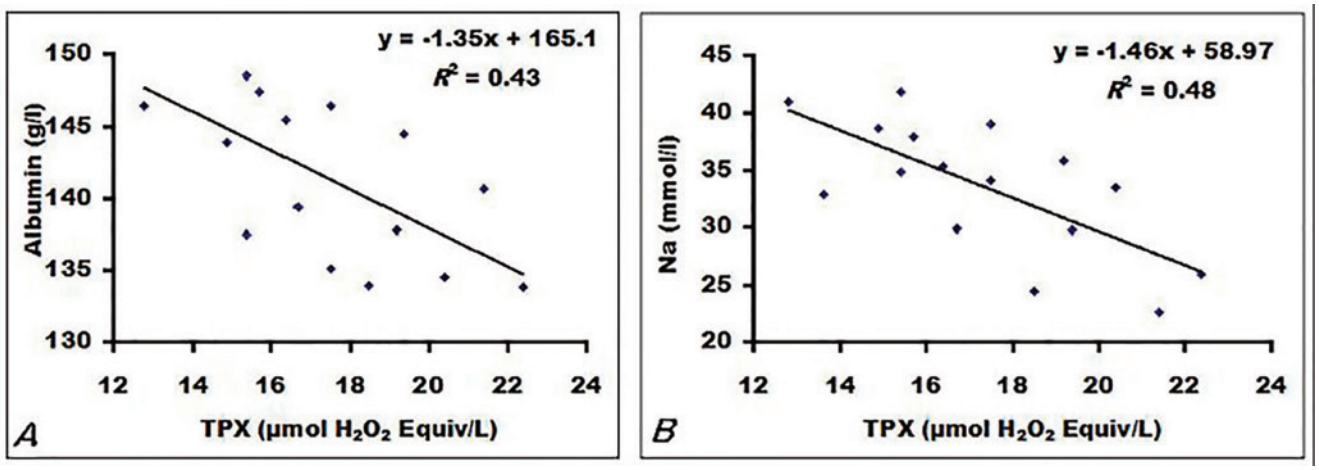

Fig. 5. Linear regression analysis (n. = 16) of total Peroxide (TPX) with albumin (A) and sodium (B) concentration in serum of *C. parvum* infected calves.

important cause of neonatal diarrhoea causing real economic damage to animal production farms.

Microscopic examination of the fecal smear showed that 15.69 % of the examined buffalo calves were positive for *C. parvum*. These results are lower than those reported by Abou El-Ella *et al*. (2013), who found that the prevalence of *C. parvum* among diarrheic buffalo calves in Assiut governorate was 39.6%. These differences probably related to managemental, nutritional or age factors (Abdul Rahman *et al*., 2017). Poor managemental practices, including the method of cleaning, the type of flooring and the frequency of cleaning, deprivation of colostrum in suckling calves and sudden weather changes were found to be the major epidemiological factors associated with *Cryptosporidium* infections (Brar *et al*., 2017).

In the present study, the histopathologic analysis of the abomasal and intestinal mucosa demonstrated epithelial cell damage and significant acute inflammation. Gastric and intestinal mucosa associated with the presence of *C. parvum* have shown nonspecific inflammation together with epithelial reactive changes and demonstrated a close relationship between the intensity of *C. parvum* infection and the degree of histological alterations (Rivasi *et al*., 1999).

The whole life cycle occurs in one host on the luminal surface of the intestinal epithelium (Garcia, 2007). The intensive loss of enterocytes is attributed to the pathogenic mechanism of *Cryptosporidium* infection, namely formation of parasitic vacuole after penetration of the agent into the cytoplasm (O'Handley and Olson, 2006). When the *Cryptosporidium* infection becomes well established, it will be associated with lack of intestinal villi, principally in the distal small intestinal portion (Rosales *et al*., 1998). The histological results confirmed some of morphogenetic features of *C. parvum* attachment to the surface of enterocytes in the distal small intestine and proximal colon (Fayer and Xiao, 2007).

Compared to the control group, the infected ileum possessed reduced villous height to as much as half of the controls. Reduction of villous height and expansion of the crypts is seen to give an approximate 2:l villus-crypt ratio in the infected tissue as compared with the 5:1 ratio in control. the present results are similar to those reported for *C. suis* in pigs. It was postulated that such an event aided the "restitution" process by reducing the size of the injured surface area to be re-epithelialized. The histological findings of acute villous atrophy, crypt hyperplasia, and inflammatory cell infiltration of the connective layer beneath the epithelium were associated with altered Na-glucose and fluid absorption by the small intestine. reduction of villus height was accompanied by an increase in width (Argenzio *et al*., 1990).

This pathology can lead to the establishment of clinical signs such as mal-digestion, diarrhea, and osmotic effects. When diarrhea lasting for 5-7 days, it may lead to abdominal tension, inappetence, dehydration and death (Vanathy *et al*., 2017). Therefore, results of the current study The relative importance of absorptive surface area, direct enterocyte damage, or an immature population of cells on the villus are suggested causes of the malabsorption (Hunt *et al*., 2002). These factors may lead to reduced intestinal permeability in infected calves compared with controls due to a decrease in surface area caused by infection, which would reduce the number of paracellular pathways in the epithelium and decline permeability across infected tissue (Hunt *et al*., 2002). The intestinal barrier function could be disrupted by *Cryptosporidium* parasite with increasing its permeability leading to weaken absorption and increased secretion of fluid, which leads to malnutrition and watery diarrhea. overtime, the parasitophorous vacuole was formed as a result of attachment of motile sporozoites to the gastric epithelium, this structure can protect *Cryptosporidium* within the host gastrointestinal tract (Vanathy *et al*., 2017).

Serum biochemical constituents revealed decreases in serum total proteins, albumin, sodium, potassium and chloride levels. The present findings were in agreement with the previous observations of Shobhamani *et al*. (2007). The hypoalbuminemia and hypoproteinemia in the infected group could be due to malabsorption through the damaged intestinal mucosa caused by *Cryptosporidium* infection. In neonatal diarrhoeic calves, chloride, sodium and potassium losses occur along with fluid loss. consequently, the electrolyte balance of the body is impaired (Yagci *et al*., 2017).

The *Cryptosporidium* parasite causes the release of inflammatory mediators such as interleukin-8, and tumor necrosis factor, which in turn liberates the soluble factors that increase the secretion of chloride and water and decrease the sodium absorption resulting in osmotic diarrhea (Vanathy, *et al*., 2017). Interestingly, these cytokines activate polymorphonuclear cells and macrophages in the ileum of infected animals at the peak of infection. These phagocytes are a major source of oxidants, TPX concentration and reactive oxygen metabolites (ROM) in the intestine (Argenzio and Rhoads, 1997). Phagocyte-derived ROM production contributed to increased concentrations of MDA during the infection. Increased levels of MDA in ileal mucosa in response to exogenous ROM in C. suis infection in pigs and rats was reported in previous studies (Argenzio and Rhoads, 1997; Sood *et al*., 2018). It has been reported that concentration of MDA increased by 59.9% during cryptosporidial infection in cattle (Zhou *et al*., 2012). 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).

Reactive oxygen metabolites have been implicated in the mucosal damage induced by various inflammatory conditions and could theoretically be responsible for both the villous damage and diarrhea induced by *Cryptosporidium* (Tamai, *et al*., 1991). The obtained results suggested that there is an evidence of increased ROM and lipid peroxide production in cryptosporidial infection in buffalo calves, which may be responsible for tissue damage and villus atrophy in infected calves. The negative correlation between TPX and sodium concentrations in the current work indicates a link between tissue oxidation and sodium concentrations and may suggest that the oxidative damage of intestinal mucosa caused by *C. parvum* infection might impaired the absorption of sodium ions. Previous reports showed that disequilibrium in the redox state of gut is important for its absorption function (de Barboza, *et al*., 2017).

A balance between ROS and primary antioxidant defenses is needed in preventing damage by oxidative stress. Oxidative damage induced by ROS may cause many pathological conditions of gastrointestinal tract including inflammatory bowel disease (Metwaly *et al*., 2015). There are few reports documenting the role of ROS in the pathogenesis of *C. parvum* infection in ruminants (Zhou *et al*., 2012). Results from this study revealed significant decreases in SOD and TAC. However, Bhagat, *et al*. (2017) and Sood, *et al*. (2018) observed that the infection of Swiss albino mice with *C. parvum* caused an increase in LPO and decrease in SOD activity at the peak of infection. The negative correlation between TPX and albumin levels in the current work suggests a connection between oxidative products and albumin status in the blood. Albumin by its thiol molecule is considered a major extracellular antioxidant fraction (Zheng, *et al*., 2019). So that it may be exhausted as an antioxidant compound to cope and scavenge the extra-peroxidation in the blood. These findings clearly implicate the production of oxidative stress in pathogenesis of *C. parvum* infection in neonatal calves. Bhagat *et al*. (2017) indicated that free radical induced-oxidative stress played an important role

in the development of *C. parvum* infection in mice, which the profuse bloody diarrhoea was one of its most important features.

Conclusion

Cryptosporidium infected-calves has an epithelial cell damage and significant acute inflammation in the abomasal and intestinal mucosa with intensive loss of enterocytes. The infection is greatly affected by the managemental system. The present study provides possible consequences of diarrhea in neonatal calves, like malabsorption, disrupted intestinal barrier function and malnutrition. *C. parvum* infection in neonatal calves is associated with oxidative stress.

Conflict of interest

Authors declare that there is no conflict of interest exist.

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