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

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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 (-7.53%), 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 was 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.

Keywords:
Buffalo calves, Cryptosporidium parvum, Oxidative stress, pathological alterations, Assiut

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 calves 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...
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 Pohlzen (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 H202 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 H202 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 μm diameter, stained red or pink with a granular appearance against a whistish 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-
aminated buffalo calves were positive for C. parvum.

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 2:1 villus-crypt ratio in the infected tissue as compared with the 4:1 ratio in the controls.

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).

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 (-7.53%), potassium (-16.02%) and chloride (-9.62%) levels.

<table>
<thead>
<tr>
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<th>Control calves (n=15)</th>
<th>Infected calves (n=16)</th>
<th>Change (%)</th>
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<tbody>
<tr>
<td>Total proteins (g/l)</td>
<td>63.91 ± 0.87</td>
<td>54.36 ± 0.77</td>
<td>-14.94*</td>
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<tr>
<td>Albumin (g/l)</td>
<td>32.75 ± 0.99</td>
<td>27.11 ± 0.79</td>
<td>-17.22*</td>
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<tr>
<td>Sodium (mmol/l)</td>
<td>139.4 ± 1.32</td>
<td>128.9 ± 1.09</td>
<td>-7.532*</td>
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<tr>
<td>Potassium (mmol/l)</td>
<td>4.781 ± 0.15</td>
<td>4.015 ± 0.12</td>
<td>-16.02*</td>
</tr>
<tr>
<td>Chloride (mmol/l)</td>
<td>107.6 ± 0.83</td>
<td>97.24 ± 0.91</td>
<td>-9.628*</td>
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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 correlated 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>
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<th>Table 2. Values of malondialdehyde, total Peroxide, total antioxidant capacity and superoxide dismutase in blood serum of control and infected calves.</th>
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<tr>
<td>Control calves (n.=15)</td>
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<tr>
<td>MDA (nmol/ml)</td>
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<tr>
<td>TPX (µmol H₂O₂ Equiv/L)</td>
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<tr>
<td>TAC (mmol trolox Equiv/L)</td>
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<td>SOD (U/ml)</td>
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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 manage-
ment, nutritional or age factors (Abdul Rahman et al., 2017).

In the present study, the histopathological analysis of the
intestinal mucosa associated with the presence of C. parvum formed some of morphogenetic features of
enterocytes in the distal small intestine (Brar et al., 2017).

In the present study, the histopathological analysis of the
abomasal and intestinal mucosa demonstrated epithelial cell
damage and significant acute inflammation. Gastric and intesti-
nal mucosa associated with the presence of C. parvum have shown nonspecific inflammation together with epithelial re-
active changes and demonstrated a close relationship be-
tween 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 sur-
face 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 in-
fected becomes well established, it will be associated with lack of intestinal villi, principally in the distal small intestinal
portion (Rosalès et al., 1998). The histological results con-
firm some of morphogenetic features of C. parvum attach-
ment 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 pos-
Posessed reduced villous height to as much as half of the con-
trols. Reduction of villous height and expansion of the crypts
is seen to give an approximate 2:1 villus-crypt ratio in the in-
fected 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 at-
rophy, 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 intes-
inite. Reduction of villus height was accompanied by an in-
crease 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 im-
portance of absorptive surface area, direct enterocyte dam-
age, 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 in-
testinal barrier function could be disrupted by Cryptospori-
dium parasite with increasing its permeability leading to
weaken absorption and increased secretion of fluid, which
leads to malnutrition and watery diarrhea. overtime, the para-
sitophorous 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 previ-
ous observations of Shobhamani et al. (2007). The hypoalu-
minemia and hypoproteinemia in the infected group could be
due to malabsorption through the damaged intestinal mucosa
caused by Cryptosporidium infection. In neonatal diarrheic
calves, chloride, sodium and potassium losses occur along
with fluid loss. consequently, the electrolyte balance of the
body is impaired (Yagi et al., 2017).

The Cryptosporidium parasite causes the release of infam-
matory 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 impor-
tant feature of cellular injury of intestinal tract infections (Met-
walay 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 (Tamaí et
al., 1991). The obtained results suggested that there is an evi-
dence of increased ROM and lipid peroxide production in
 Cryptosporidium infection in cattle (Zhou et al., 2012). Peroxidation
of cell membrane lipid layer due to free radicals is an impor-
tant feature of cellular injury of intestinal tract infections
(Metwalay et al., 2015).

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 condi-
tions of gastrointestinal tract including inflammatory bowel
disease (Metwalay et al., 2015). There are few reports docu-
menting the role of ROS in the pathogenesis of C. parvum in-
fec tion in ruminants (Zhou et al., 2012). Results from this study
revealed significant decreases in SOD and TAC. However, Bha-
gat et al. (2017) and Sood et al. (2018) observed that the in-
fec tion in 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 frac-
tion (Zheng et al., 2019). So that it may be exhausted as an
antioxidant compound to cope and scavenge the extra-perox-
interface oxidation 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 management 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.

**References**


