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Original Research

Effect of Neem (*Azadirachta indica*) Leaf Powder on Clostridium perfringens Induced Infection in Broiler Chicks

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Abstract

The experimental chicks (n=150) were equally divided into five groups (n =30, CON: healthy control, NLP: Neem leaf powder control, CLP: Clostridium perfringens infected, CLB: CLP + Bacitracin and CLN: CLP + Neem leaf powder). On necropsy, the CLP group birds duodenum, jejunum/ileum frequently found distended containing large amounts blood mixed mucoid contents. Histopathological examination of intestines from CLP group birds showed extensive coagulative necrosis and sloughing of villi. Overall, the severity of gross and histopathological lesions in various organs was comparatively less in CLN group as compared to CLP group. In conclusion, the oral supplementation of neem leaf powder although could not completely prevent the chicks from Clostridium perfringens infection, but it showed moderate ameliorating effect in affected chicks in terms of severity of clinical symptoms and pathological lesions.

Keywords: Clostridium perfringens, Gross and Histopathological Lesions, Neem Leaf Powder

Introduction

Clostridium perfringens type A is the most important bacterium, which is a Gram-positive, rod-shaped, extremely prolific facultative anaerobic endospore forming encapsulated bacterium (Hafez, 2011; Pawade *et al.,* 2019). *Clostridium perfringens* is widespread and commonly found in environmental materials such as soil, polluted feed, declining vegetation, aquatic debris, poultry litter and is a component of ordinary flora in the animal and human gastrointestinal tract (Miller *et al.,* 2010; Vierheiling *et al.,* 2013). Infections with this bacterium in poultry can cause some clinical manifestations and lesions such as necrotic enteritis, necrotic dermatitis, cholangiohepatitis and gizzard erosions. The economic effect of necrotic enteritis on the worldwide poultry industry was estimated over five to six billion dollars per year mostly due to the losses through growth depression, high morbidity, mortality and medicine costs (Wade and Keyburn, 2015).

Synthetic drugs and chemicals have their inherent drawbacks such as high cost of production, toxicity due to continuous usage, contraindications, development of resistance, environmental and health hazards (Devegowda, 1996). This scenario has challenged the poultry industry to search for safer alternative products to prevent necrotic enteritis. In this context, the utilization of natural plant extracts with antimicrobial properties appears as a provision and a feasible tool to control necrotic enteritis (Carrasco *et al*., 2016).

The herbal products make a harmless substitute due to its suitability and preference, lower price of production and being environment-friendly by nature, reduced risk of toxicity and health hazards (Devegowda, 1996). Neem is botanically recognized as *Azadirachta indica*. Its active components have shown several pharmacological actions such as anti-inflammatory, antiarthritic, antipyretic, hypoglycaemic, diuretic, spermicidal, antifungal, antibacterial, anti-gastric ulcer, antiviraland anti-psoriasis actions (Barman *et al*., 2019). Hence, the present work was undertaken with an aim to study the protective effects of *Azadirachta indica* leaf powder on *Clostridium perfringens* infection in broilers.

Materials and Methods

Experimental Design

One hundred and fifty (150) day old broiler chicks vaccinated against Marek's disease were used for this study. The total duration of study was 32 days. On the 7th day, the broiler chicks were randomly divided into five experimental groups each comprised of 30 birds each (CON: healthy control, NLP: Neem leaf powder control, CLP: *Clostridium perfringens* infected, CLB: *Clostridium perfringens* infected + Bacitracin and CLN: *Clostridium perfringens* infected + Neem leaf powder. After day 7, birds from groups CON and CLP were provided with non-medicated basal diet. Fresh leaves of the neem tree were collected from the college campus and shade dried leaves were powdered, sieved and stored in bags until they were used. Group NLP and CLN birds were fed on non-medicated basal diet mixed with neem (*Azadirachta indica*) leaf powder @ 1% of basal diet. Group CLB birds were fed on non-medicated basal diet mixed with Bacitracin Methylene Disalicyclate (BMD) powder @ 50 mg/kg of basal diet.

Experimental Induction of *Clostridium perfringens* Infection

For experimental induction of necrotic enteritis, the intestine should be previously damaged. This damage was induced through experimental administration of coccidial oocysts. Coccidial oocysts were collected from the field sample. The *Eimeria* spp. (*Eimeria maxima* and *Eimeria acervulina*) were isolated from the collected samples. These were sporulated and stored in 2% (w/v) potassium dichromate solution until use. Before administration, the potassium dichromate solution was removed by washing in water by repeated centrifugations. The total number of oocysts in the suspension was calculated by using a McMaster's chamber and the final concentration of oocysts was obtained by adjusting the volume of the suspension by the adding water. Birds from all groups except control groups (CON, NLP) were orally administered with 3000 sporulated coccidial oocysts in normal saline on 13th day of age.

The pure culture of pathogenic *Clostridium perfringens* type A (ATCC 13124) strain was obtained from Hi-Media Laboratories Pvt. Limited, Mumbai, India. The culture was maintained on egg yolk agar in anaerobic jar till the completion of experiment. It was sub-cultured for cultivations in 100 ml soybean casein broth medium and incubated in the anaerobic jar at 37ºC for 24 hours. The culture was centrifuged and bacterial pellet was washed with sterile PBS (pH 7.2). The washed bacterial pellet was resuspended in PBS to obtain final concentration of 10^7CFU/ml,

using Mac Farland Tube No.2. The freshly prepared suspension was used to challenge the birds. Birds of CLP, CLB and CLN groups were orally challenged at the dose rate of 10^7CFU/ml per bird with the help of gastric gavage on 20th and 21st day of the experiment.

Clinical Signs and Gross Pathology

All birds were examined twice daily for any clinical sign throughout the experiment and observations were recorded group wise. The number of dead birds were recorded as and when seen in any group and mortality percentage was computed. The carcasses were necropsied to ascertain the cause of death. At the age of 25th, 28th and 32nd day, six birds were randomly selected from each group. The selected birds were sacrificed by cervical dislocation, then detailed necropsy examination was conducted and gross observations were recoded.

Histopathology

The 3 cm segments of the lower duodenal, jejunum, ileum and other visceral organ were collected and fixed in 10% neutral buffered formalin. Then, these tissue samples were processed for histopathology as per standard protocol described by Culling (1974).

Results and Discussion

The birds from CON and NLP groups did not showed any abnormal clinical symptoms and remained healthy throughout the experimental trial. The birds from CLP, CLB and CLN showed clinical signs of successful induction of coccidiosis on 20th day of age (on day 8 after administration of sporulated oocysts). The birds from this coccidiosis induced groups showed clinical signs mainly reduced feed intake, huddling in corner, dullness, depression and blood mixed watery droppings. Similar findings were also observed by previous researchers (Sharma *et al*., 2013; Abdisa *et al*., 2019; Gogoi *et al*., 2019).

From 24th day of age and onwards (i.e. on 4th day and onwards after administration of *Clostridium perfringens*), the birds from CLP, CLB and CLN groups started to show the clinical signs like severe depression, reluctance to move, ruffled feather and foamy diarrhoea frequently admixed with bloody mucous content. Das *et al.* (2008) and Alnassan *et al.* (2014) reported similar clinical signs of *Clostridium perfringens* infection in broiler birds. However, the severity of clinical signs in case of birds from CLB and CLN groups found milder in nature than birds from CLP group. No mortality was observed in birds from healthy control (CON) and neem leaf powder fed control (NLP) groups throughout study period. Two birds from CLP group (6.67 % mortality) and one bird each from CLB and CLN groups (3.33 % mortality) were died during the present study. The findings regarding mortality in *Clostridium perfringens* infection in present study are in consonance with mortality recorded by Shane *et al.* (1985) and Dinev (2010). Birds from CON and NLP groups did not showed any appreciable gross pathological changes and all organs appeared normal at all intervals of study during necropsy.

On necropsy (on 20th day of age or on day 8 after administration of sporulated oocysts), the intestinal tracts of CLP, CLB and CLN group birds showed marked distension and haemorrhagic patches visible externally from duodenal, jejunal and ileum serosa. The intestinal lumen of affected birds showed presence of blood mixed watery mucoid content and ecchymotic haemorrhages over the mucosa of duodenum, jejunum and ileum indicating induction of coccidiosis. The other gross pathological changes observed in coccidiosis affected birds included marked paleness of breast muscles and liver. The droppings of affected birds were appeared mucoid and blood mixed indicating bloody diarrhoea. The faecal samples from affected birds were subjected for microscopic examination by floatation technique showed presence of numerous unsporulated coccidial oocysts (*Eimeria spp.*) indicating successful induction of coccidiosis in experimental birds from CLP, CLB and CLN groups (Figure 1). The histopathological examination of duodenum, jejunum and ileum from affected birds of CLP, CLB and CLN groups revealed presence of various developmental stages (trophozoites and macrogametes/zygotes) of coccidia (*Eimeria spp.*) within cytoplasm of crypt epithelium and villi enterocytes (Figure 2). Sharma *et al.* (2013) and Abdisa *et al.* (2019) recorded similar gross and histopathological lesions in coccidiosis affected broiler birds. These findings are in agreement with lesions observed in present study.

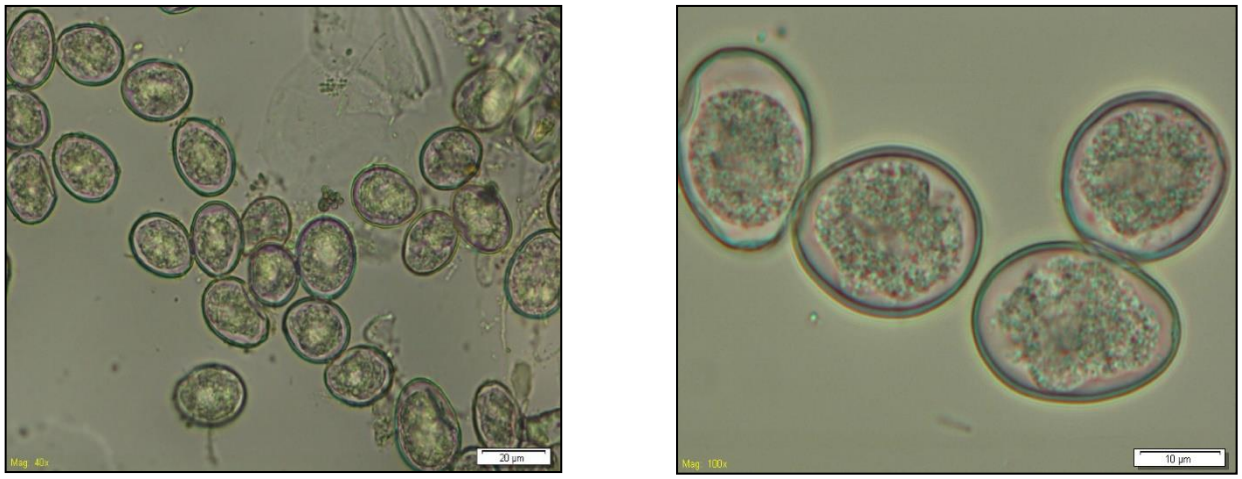

Figure 1: CLP & CLN Group: Coccidiosis: Unsporulated coccidial oocysts in faecal sample collected from affected birds. Floatation Technique. Bar= 20µm (L) & 10µm (R)

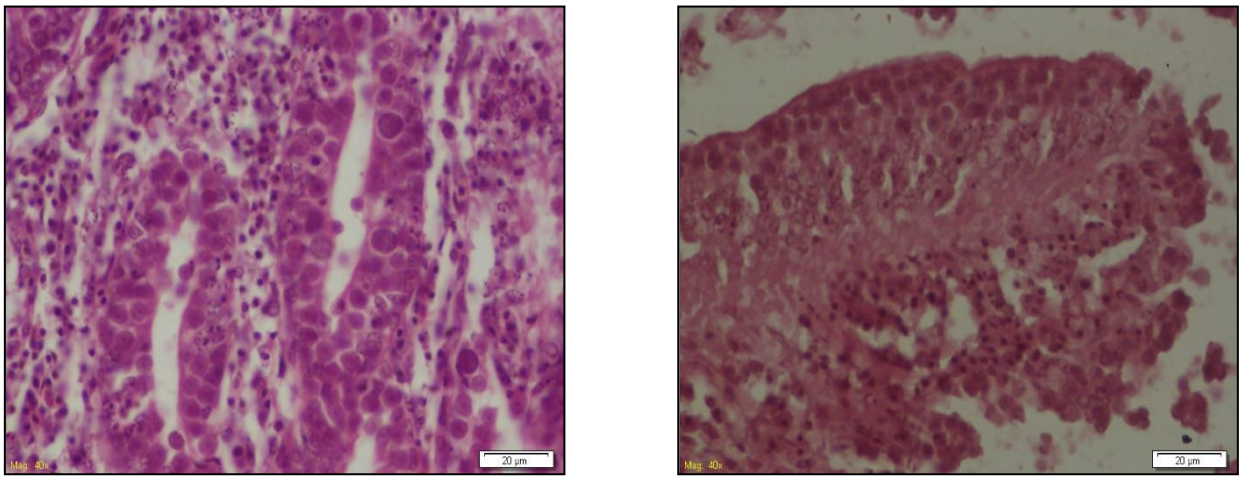

Figure 2: CLP & CLN Group: Coccidiosis: Note the presence of numerous trophozoites (small basophilic intracytoplasmic bodies surrounded by halo) of *Eimeria spp.* in duodenal crypt epithelium (left) and jejunal mucosal epithelium (right). H & E Stain. Bar=20µm.

Birds from groups CON and NLP did not showed any appreciable gross pathological changes and all organs appeared normal at all intervals of study during necropsy. The unopened intestine from CLP group birds frequently found distended containing large amount blood mixed mucoid contents and appeared friable. The mucosal surface of affected intestine showed excessive accumulation of yellow-brown coloured blood mixed necrotic debris adhered over the entire affected surface (diphtheritic membrane) along with ecchymotic haemorrhages. These changes were most frequently observed in jejunum than duodenum, ileum and less frequently in caeca (Figure 3). The intestine of chicks from CLB and CLN groups showed less damage and accumulation of necrotic debris than chicks from CLP group. The heart of chicks from CLP group frequently showed presence of petechial haemorrhages in epicardium and endocardium indicating septicaemic changes while no such gross lesions were observed in heart of chicks from CLB and CLN groups (Figure 4). The liver of chicks from CLP group showed marked enlargement, increased fragility most probably due to bacterial toxin induced hepatic damage and paleness. These changes in liver of chicks from CLB and CLN groups were milder or moderate in nature (Figure 5). The breast muscles from CLP, CLB and CLN groups showed marked paleness during necropsy. The gross pathological lesions recorded in *Clostridium perfringens* infected birds of present study were in close proximity with findings of Long *et al.* (1974), Timbermont *et al.* (2011), Smyth (2016) and Umar *et al.* (2016).

Figure 3: CLP Group: Presence of mucosal haemorrhages and blood mixed debris adhered to the mucosa of duodenum, jejunum and ileum

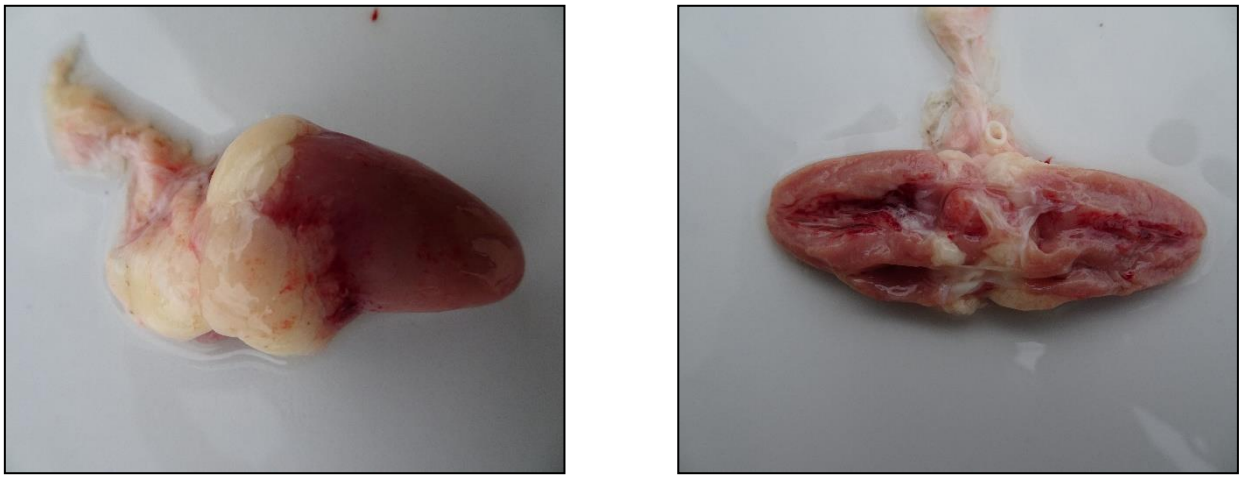

Figure 4: CLP Group: Note the presence of epicardial (left) and endocardial haemorrhages (right) indicating septicaemia

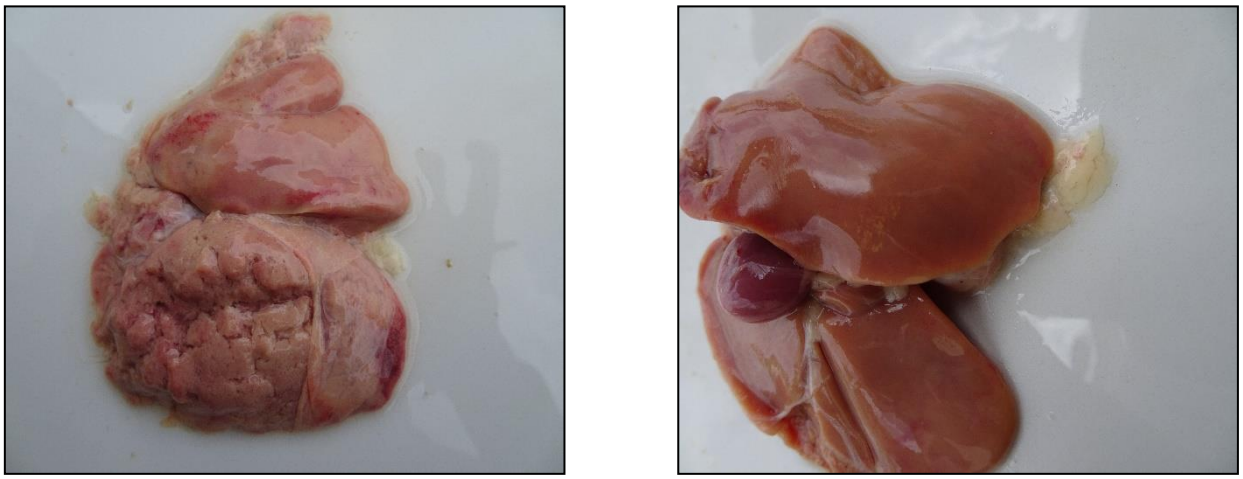

Figure 5: Liver from CLP group showing marked paleness and increased fragility (left) as compared to liver from CLN group (right)

The following histopathological lesions in various organs were observed in the experimental birds from different groups-

Intestine

The intestine (jejunum/ileum) from CLP group birds showed extensive coagulative necrosis in the epithelium of villi/enterocytes and lamina propria leading sloughing of villi or mucosal epithelium into lumen. The necrosed debris found admixed with abundant eosinophilic fibrin-like material which was frequently intermixed with bacterial colonies (rods). In some cases, there was extensive necrosis of villi causing sloughing and fusion of affected villi and the fibrino-necrotic debris adhered to the damaged villi (formation of diphtheritic membrane consisting degenerated epithelial cells, erythrocytes, heterophils, mononuclear cells, fibrin and bacilli). The inflammatory exudate primarily consists of heterophils and mononuclear cells mainly lymphocytes. The affected villi were also surrounded by abundant eosinophilic material and showed goblet cell hyperplasia (Figs. 6 and 7).

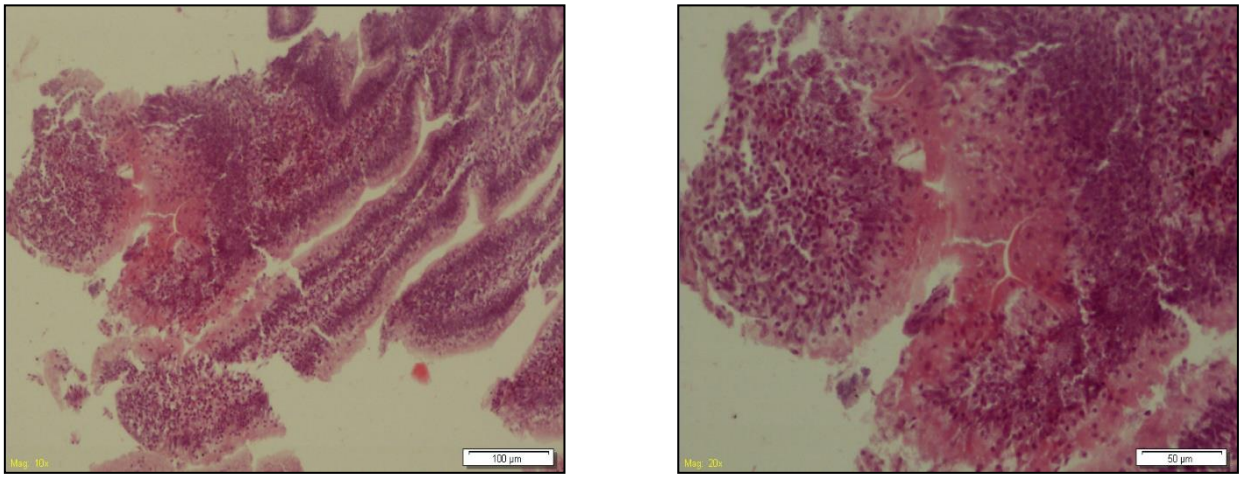

Figure 6: CLP Group: Jejunum/Ileum: Note marked sloughing, coagulative necrosis of villi epithelium along with heterophilic infiltration (left). Also note the presence of zone of demarcation between necrosed and viable tissue separated by inflammatory cells primarily consisting heterophils (right). H& E Stain, Bar=100 µm (L) & 50 µm (R)

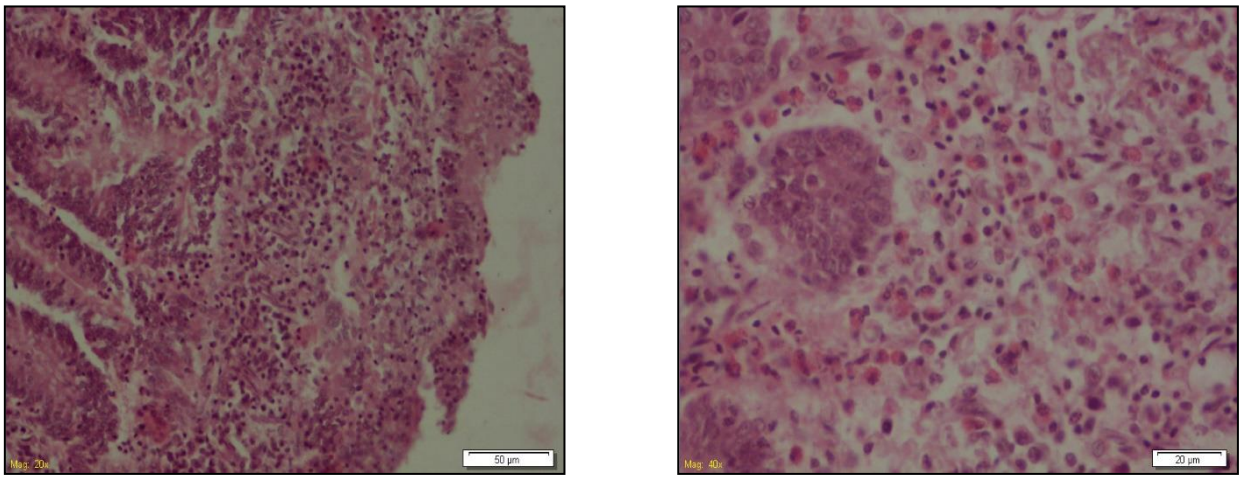

Figure 7: CLP Group: Jejunum/Ileum: Note the presence of thick diffused layer of fibrino-necrotic cellular debris attached to damaged villi. Bar=50 µm (L). Also, note presence of predominantly heterophils. Bar=20 µm (R)

The intestine (jejunum/ileum) from CLB group showed minimal damage, which was frequently limited to tips of villi as compared to extensive damage observed in CLP group birds, while the jejunum/ileum from CLN group birds showed moderate damage to epithelium of villi in terms of coagulative necrosis and sloughing of enterocytes, which was frequently limited to apical portions of villi as compared to damage observed in CLP group birds. The normal histological architecture of jejunum/ileum was observed in both control groups (Figs. 8 and 9).

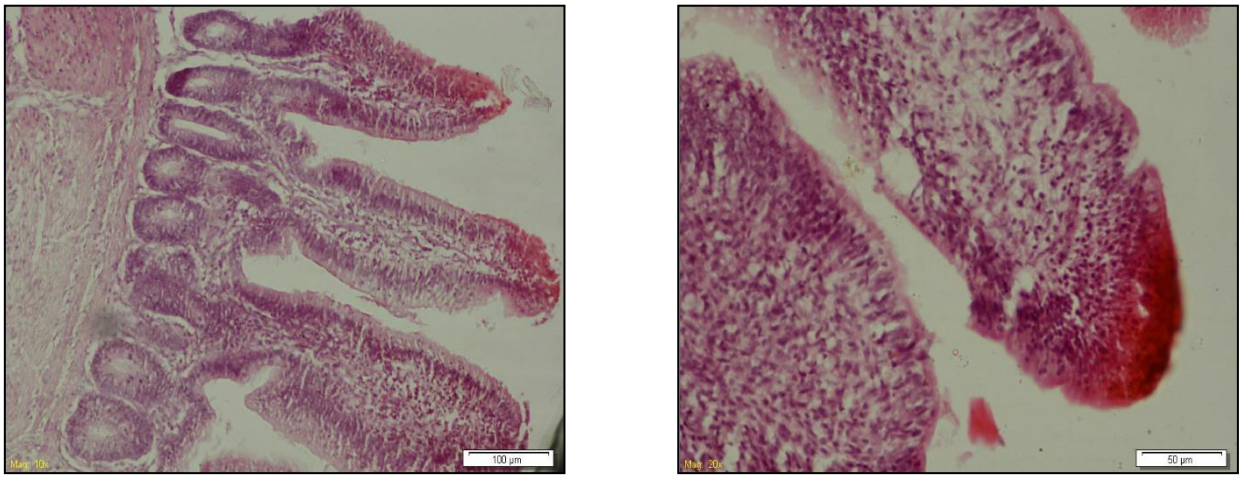

Figure 8: CLB Group: Jejunum/ Ileum: Note minimal damage to villi epithelium/enterocytes, which was frequently limited to tips of villi as compared to damage observed in CLP group birds. H& E Stain, Bar=100 µm (L) & Bar=50 µm (R)

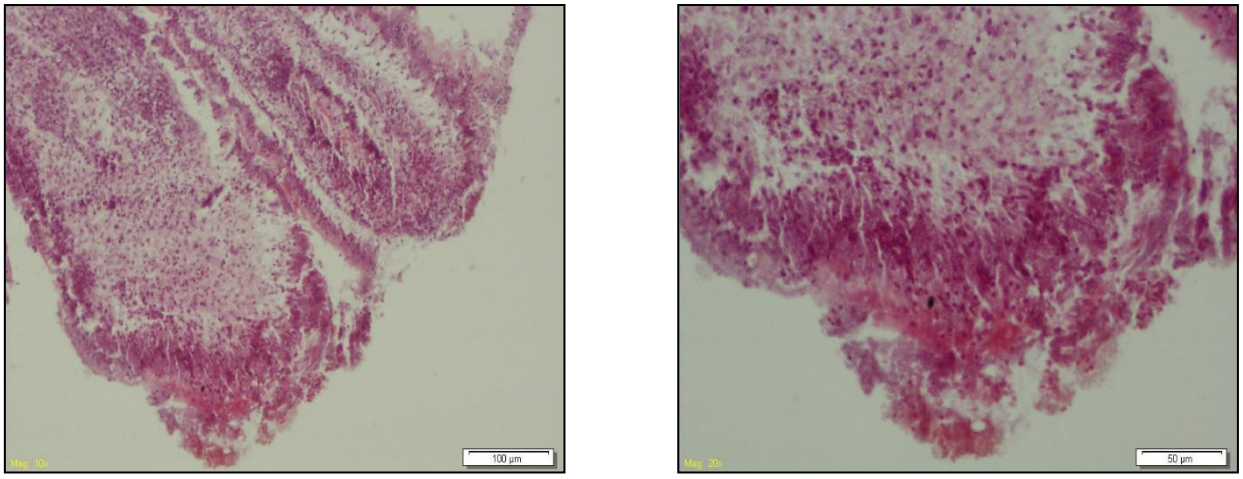

Figure 9: CLN Group: Jejunum/Ileum: Note moderate damage to villi epithelium in terms of coagulative necrosis and sloughing of enterocytes, which was frequently limited to apical portions of villi as compared to damage observed in CLP group birds. H& E Stain, Bar=100 µm (L) & Bar=50 µm (R)

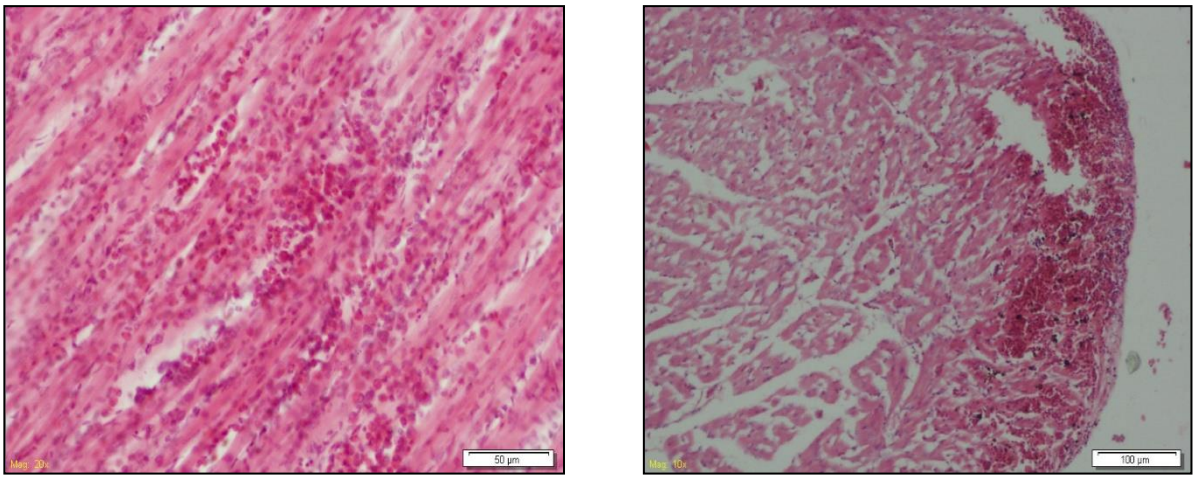

Figure 10: CLP Group: Heart: Myocarditis characterized by marked infiltration of heterophils. Bar=50 µm (L). Also note marked endocardial and sub-endocardial inflammation (predominantly heterophils) and haemorrhages. Bar=100 µm (R)

Heart: The marked heterophilic infiltration along with myocardial degeneration was evident in CLP group birds

while the endocardium and sub-endocardium showed marked mononuclear cells mainly lymphocytes infiltration and haemorrhages indicating haemorrhagic endocarditis (Fig. 10). No such lesions were observed in birds from CLB and CLN groups.

Kidneys: The histopathological changes like cortical haemorrhages along with heavy infiltration of mononuclear cells mainly lymphocytes and mild tubular degeneration were evident in kidneys from CLP group birds. The kidneys from CLB and CLN group showed mild tubular degeneration.

Liver: The liver from CLP group birds showed focal periductular and periportal lympho-mononuclear aggregates along with moderate vacuolar hepatocellular degeneration. The mild hepatocellular degeneration was also evident in CLB and CLN group birds.

Lungs and Spleen: Marked hyperaemia was evident in lungs and spleen from birds from CLP, CLB & CLN groups.

Overall, the severity of histopathological lesions in various organs were comparatively more in the chicks from CLP group followed by CLN group and then CLB group (CLP>CLN>CLB). More or less similar histopathological lesions in *Clostridium perfringens* infected birds in various organs as mentioned above were reported earlier by previous researchers (Long *et al.,* 1974; Kaldhusdal *et al.,* 1995; Keyburn *et al.,* 2006; Olkowski *et al.,* 2008; Timbermont *et al.,* 2011; Allaart *et al.,* 2012; Alnassan *et al.,* 2014; Prescott *et al.,* 2016; Smyth, 2016; Umar *et al.,* 2016).

Furthermore, Olkowski *et al.* (2008) postulated that the initial pathological changes causing damage to intestine were due to the activity of collagenolytic enzymes secreted by *C. perfringens*. Initially, the damage to villi occurred at the level of the basement membrane and lateral domain of the enterocytes, spreading throughout the lamina propria, while epithelial damage occurred later in the process. The initiation of the pathological process leading to necrotic enteritis involved proteolytic factors affecting the extracellular matrix and cellular junctions. Thus, it was assumed that the pathogenesis of necrotic enteritis might be the result of bacterial collagenases, whose action get enhanced when mucosal damage (e.g. induced by coccidia) is present or host matrix metalloproteinases that were activated by the host-pathogen interaction. Timbermont *et al.* (2011) stated that the intestinal damage allowed the bacteria to reach the bile duct and portal blood stream. Colonization of the liver by high numbers of *C. perfringens* resulted in cholangiohepatitis.

Conclusion

In conclusion, it may be stated that the oral supplementation of neem leaf powder although could not completely prevent the birds from *Clostridium perfringens* infection, but it showed moderate ameliorating effect in affected birds in terms of comparatively less severity of clinical symptoms and pathological lesions.

Conflict of Interests

There is no conflict of interest.

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