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Ameliorative effect of neem (*Azadirachta indica*) leaf extract on the pathology of experimental *Escherichia coli* infection in broiler chicken

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ABSTRACT

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The present study was conducted to evaluate the effects of supplementation of neem leaf extracton pathology of different organs in experimentally *Escherichia coli* (*E. coli*) challenged broiler chickens. One hundred and ninety two, day old broiler chicks were divided into two groups (A and B) with ninety six birds each. Diet of group Achicks was supplemented with 10% neem leaf extract in water, whereas group B chicks were given feed and water without supplementation. After one week, chicks of both the groups were again divided into two subgroups (A1 & A2 and B1 & B2), respectively. At the age of 7 days, A1 and B1 groups were injected with *Escherichia coli* O78 @ 10^7 CFU/0.5 ml intraperitoneally. Necropsyof chicks sacrificed at 0^{th}, 2^{nd}, 4^{th}, 7^{th}, 14^{th}, 21^{st} and 28^{th} day post infection revealed gross lesions of fibrinous pericarditis and perihepatitis, congestion in visceral organs, pneumonia, peritonitis and enteritisin *E. coli* infected chicks. These gross lesions were of lesser intensity in NLE supplemented infected group. The histopathological lesions observed in *E. coli* infected groups were fibrinous pericarditis, myocarditis, fibrinous perihepatitis, hepatitis, enteritis, pneumonia and nephritis. The severity of these lesions in group A1 was of mild magnitude as compared to group B1. No gross and histopathological changes could be observed in chicks from non- infected groups. It may be inferred that supplementation of 10% NLE has protective effect on limiting the pathology of *E. coli* infection in broiler chicken.

Keywords: Broiler chicken, *Escherichia coli*, neem leaf extract, pathological lesions

INTRODUCTION

In recent years, commercial farms have grown in size and the poultry has undergone dramatic improvements in growth, feed efficiency and production. But a major problem affecting the growth of the poultry industry in India is the occurrence of disease outbreaks. Some regions have reported a dramatic increase in the incidence of infectious disease outbreaks during this time of rapid expansion. Amongst these infections, *Escherichia coli* infection is quite common and causes a large number of disease conditions such as pericarditis, perihepatitis, airsacculitis, peritonitis, salpingitis, panophthalmitis, omphalitis, cellulitis, colisepticemia, coligranuloma and swollen-head syndrome[1]. *E. coli* is a gram-negative, rod-shaped bacterium normally found in the intestine of poultry and most other animals. Although most serotypes are non-pathogenic, a limited number produce extra intestinal infections[2]. Avian pathogenic *E. coli* (APEC) strains are commonly of the O1, O2, and O78 serogroups, but many others have also been associated with cellulitis and colibacillosis[2].

Various plant extracts have been used worldwide for a wide range of medicinal properties like antibacterial, antiviral, antifungal, antiprotozoal and hepatoprotective without adverse effects[3]. One of the alternatives could be the leaves extract of *Azadirachta indica*. Aqueous extract of neem leaves extract has a good therapeutic potential as anti-hyperglycemic agent, antibacterial agent and could be used for controlling airborne bacterial contamination in the residential premise[4]. The present study was planned to investigate the effects of neem leaf extract on the pathology of *E. coli* infectionin chickens.

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MATERIALS AND METHODS

Experimental design

One hundred and ninety two, day old broiler chicks were procured from a local hatchery. All the birds were given *ad libitum* aflatoxin free standard chick feed and provided clean drinking water throughout the experiment. The approval for conducting the experiment was taken from the Institutional Animal Ethics Committee (IAEC), Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar. These chicks were divided randomly into two groups (A and B) containing ninety six birds each on the first day. Diet of all the chicks of group A was supplemented with 10% neem leaf extract (NLE) in water whereas, chicks of group B were given feed and water devoid of NLE supplementation throughout the experiment. After rearing for one week chicks of both the groups (A and B) were again divided

into two subgroups (group A into A1 & A2 and group B into B1 & B2) of 54 and 42 birds each, respectively. At the age of one week all the chicks of groups A1 and B1 were injected with *E. coli* O78 @ 10^7 CFU/0.5 ml intraperitoneally (I/P). Thorough post-mortem examination of the sacrificed chicks was conducted and for histopathological examination of tissue pieces of various organs showing lesions were collected in 10% buffered formalin on 0, 2, 4, 7, 14, 21 and 28 day post infection.

Experimental chicks and feed

One hundred and ninety two, day old healthy broiler chicks were procured from a local hatchery. The chicks were kept under strict hygienic conditions in the departmental animal house under cage system. All the birds were provided with fresh, clean drinking water and fed *ad libitum* throughout the experiment. The experiment was undertaken after taking prior permission from the Institutional Animal Ethics Committee (IAEC) of the university.

Preparation of neem leaf extract

Neem leaves collected from campus of Chaudhary Charan Singh Haryana Agricultural University, Hisar were shade dried and powdered. The aqueous extract was then prepared from powdered neem leaves. Hundred grams neem leaves powder was boiled in 1 litre of water for 15 mins and the extract obtained after straining was added to drinking water to make volume of 1 litre[5].

Preparation of *E. coli* inoculums

O78 serotype of *E. coli* isolated from natural cases was inoculated into Brain Heart Infusion Broth (BHIB) and incubated at 37°C for 24h. Viable count of *E. coli* organism per ml of BHIB was determined by surface spread method[6]. Serial 10 fold dilutions of the above culture were prepared in the sterile phosphate buffer saline (PBS) and 0.1ml of each dilution was pipetted onto three MacConkey's Lactose Agar (MLA) plates. The inoculum on the plates was spread with the help of a sterile spreader and then these plates were incubated at 37°C for 24h. The average count of three plates of particular dilution having colonies in the range of 30-300 was calculated. This bacterial count for particular dilution was made in 0.1 ml, the inoculum used for each dilution. Then the viable count per ml was determined which was considered as Colony Forming Units (CFU) of the *E. coli*. The infective dose @ 10^7 CFU of *E. coli*/0.5 ml was prepared for the experiment as *E. coli* inoculum[7].

Clinical signs and mortality

Birds were closely observed daily for clinical signs and mortality, if any.

Gross pathology

The chicks sacrificed or died naturally during the experiment were thoroughly examined for gross lesions, if any.

Histopathology

The formalin fixed tissues were processed for paraffin embedding technique. The tissues were properly trimmed, washed in running tap water, dehydrated in graded ethyl alcohol, cleared in cedar wood oil and embedded in paraffin wax (melting point 60-62°C). Sections of 4-5 μ thickness were cut using semiautomatic microtome and stained with haematoxylin and eosin[8].

Lesion score

The colibacillosis specific gross lesion score (GLS) and histopathological lesion score (HLS) in different experimental groups were calculated for different organs/ tissues at scale of 0 to 4 as detailed below:

0 = No lesion; 1 = Mild lesions; 2 = Moderate lesions; 3 = Moderately severe lesions; 4 = Severe lesions.

% mean gross and histopathological lesions were calculated as per method described by Witter[9] with slight modifications with following formula:

% mean gross/histopathological lesion score (GLS/HLS) of organs

$$= \frac{\text{Mean GLS/HLS of an organ}}{\text{4 (Maximum GLS/HLS of an organ)}} \times 100$$

Overall % mean GLS/HLS of organs irrespective of post infection period

$$= \frac{\text{Sum of \% mean GLS/HLS of an organ at different post infection period}}{\text{7 (Total number of post infection periods)}}$$

Overall %mean GLS/HLS irrespective of post infection period and organs

$$= \frac{\text{Sum of overall \% mean GLS/HLS of organs Irrespective of post infection period}}{\text{Total number of organs}}$$

Protective effect

% protective effect due to neem leaf extract (NLE) supplementation in *E.coli* infected chickens was calculated on the basis of GLS and HLS as per method of Witter[9] using following formula:

% protective effect due to neem leaf extract (NLE) supplementation in *E. coli* infected chickens

$$= \frac{\text{Overall \%mean GLS/HLS in group B1–overall \% mean GLS/HLS in groupA1}}{\text{Overall \%mean GLS/HLS in group B1}} \times 100$$

RESULTS

Clinical Signs

No clinical signs were observed in both the control groups A2 and B2 throughout the experiment. Clinical signs of *E. coli* infection in the non-supplemented infected group (B1) started to appear at 12 hours post infection. These clinical signs were dullness, depression, drooping of the head and neck, closing of eyes and ruffled feathers. They were huddling together near the heat source. Thereafter, the chicks showed anorexia, listlessness, inappetance, ruffled feathers and closing of eyes. The birds showed outstretching and drooping of wings and they were not able to bear weight on their legs (Fig. 1) and some of the infected birds exhibited respiratory distress and diarrhoea which was watery or pasty white in few birds soiling the vent and resulting in dehydration. These clinical signs were more severe at 5 DPI. Thereafter the severity of clinical signs in survived birds was started to decline and they were significantly reduced from 18 DPI. There was almost complete recovery in clinical signs at 21 DPI. On the other hand clinical signs of *E. coli* infection in group A1 in which drinking water was supplemented with 10% neem leaf extract started appearing at 24 hours post infection. Clinical signs were almost identical to those observed in group B1 but the severity of the signs was of less intensity as compared to group B1. The survived birds in group A1 appeared almost normal after 15 DPI.

Mortality

Mortality in non-supplemented infected group B1 started from 24 hours post infection and there was death of two birds. Thereafter, two birds died on 2 DPI, one each on 3, 4 and 5 DPI, two on 6 DPI and one each on 7, 8 and 10 DPI. Total numbers of birds died in group B1 throughout the experiment were 12 and percentage of overall mortality was 22.22%. On the other hand, mortality in NLE supplemented infected group A1 started from 2 DPI onwards and number of birds died at different intervals was considerably less as compared to group B1. Total death in group A1 was 7 and percentage of overall mortality was 12.96%. No mortality was noticed in group A2 and B2.

Gross pathological changes

No gross pathological changes could be observed in chicks from non-infected groups (A2 and B2) at different intervals throughout the experiment. On 2 DPI, in group B1 congestion in various organs and thin fibrinous layer on the surface of heart and liver was observed. On 4 DPI, the fibrinous layer became relatively thick in both the organs indicating fibrinous perihepatitis and pericarditis, respectively. There was congestion in intestine, kidneys and lungs. On 7 DPI, liver and heart were almost covered with thick fibrinous mass resulting in adhesions with abdominal wall as well as with other visceral organs. Kidneys, lungs and intestine showed severe congestion. On 14 DPI, heart was covered with thick fibrin layer though not as thick as it was on 4 and 7 DPI and liver was severely congested. Lungs and kidneys were still congested. On 21 DPI, heart and liver showed severe congestion. Kidneys, lungs and intestine showed only mild congestion. On 28 DPI, there were no significant lesions except heart and liver showed mild congestion.

NLE supplemented *E. coli* infected group (A1) showed no significant lesions on 2 DPI except mild congestion in heart and liver. On 4 DPI, heart revealed a thin layer of fibrin on the pericardium (Fig. 2). Liver was also covered with thin fibrinous layer. Intestines, lungs and kidneys revealed mild congestion. On 7 DPI, heart was covered with thick fibrinous layer on its surface. Liver was also congested and covered with fibrinous layer. Overall severity of lesions in different organs in supplemented infected group was comparatively less in comparison to non-supplemented infected group. On 14 DPI, heart was covered with thin fibrinous layer in a few cases. Liver revealed severe congestion. Lungs, intestines and kidneys were also congested. However, these lesions were of lesser intensity as compared to those observed on 7 DPI. Furthermore, the severity of lesions was also of mild degree as compared to non-supplemented infected group. On 21 DPI, there was only mild congestion in heart, liver, lungs, intestines and kidneys. On 28 DPI, no noticeable lesions could be seen in various organs.

Histopathological changes

In non-supplemented infected group (B1), heart revealed fibrinous pericarditis characterized by congestion, presence of fibrin and leucocytic infiltration in pericardium extending into myocardium leading to myocarditis on 2 DPI. Liver revealed congestion in portal vein, dilatation and congestion in sinusoid, atrophy of hepatocyte (Fig. 3). Fibrinous perihepatitis was also noticed in some cases characterized by accumulation of fibrin and heterophils. Intestine revealed enteritis characterized by congestion, desquamation of villi, serous exudation in submucosa and leucocytic infiltration in mucosa and submucosa. Lungs revealed pneumonia characterized by haemorrhages and leucocytic infiltration leading to obliteration of the alveoli. Kidneys revealed noticeable fatty changes and focal areas of necrosis with mononuclear cells infiltration and fibroblast proliferation. On 4 DPI, heartrevealed severe fibrinous pericarditis characterized by presence of fibrin andleucocytic infiltration in pericardium extending into myocardium (Fig. 4). Liver exhibited fibrinous perihepatitis characterized by large amount of fibrin and infiltration of leucocytes extending in parenchyma (Fig. 5). In some cases there were lymphoid follicle formation in perivascular area of liver along with hydropic changes. Lungs revealed severe pneumonia characterized by

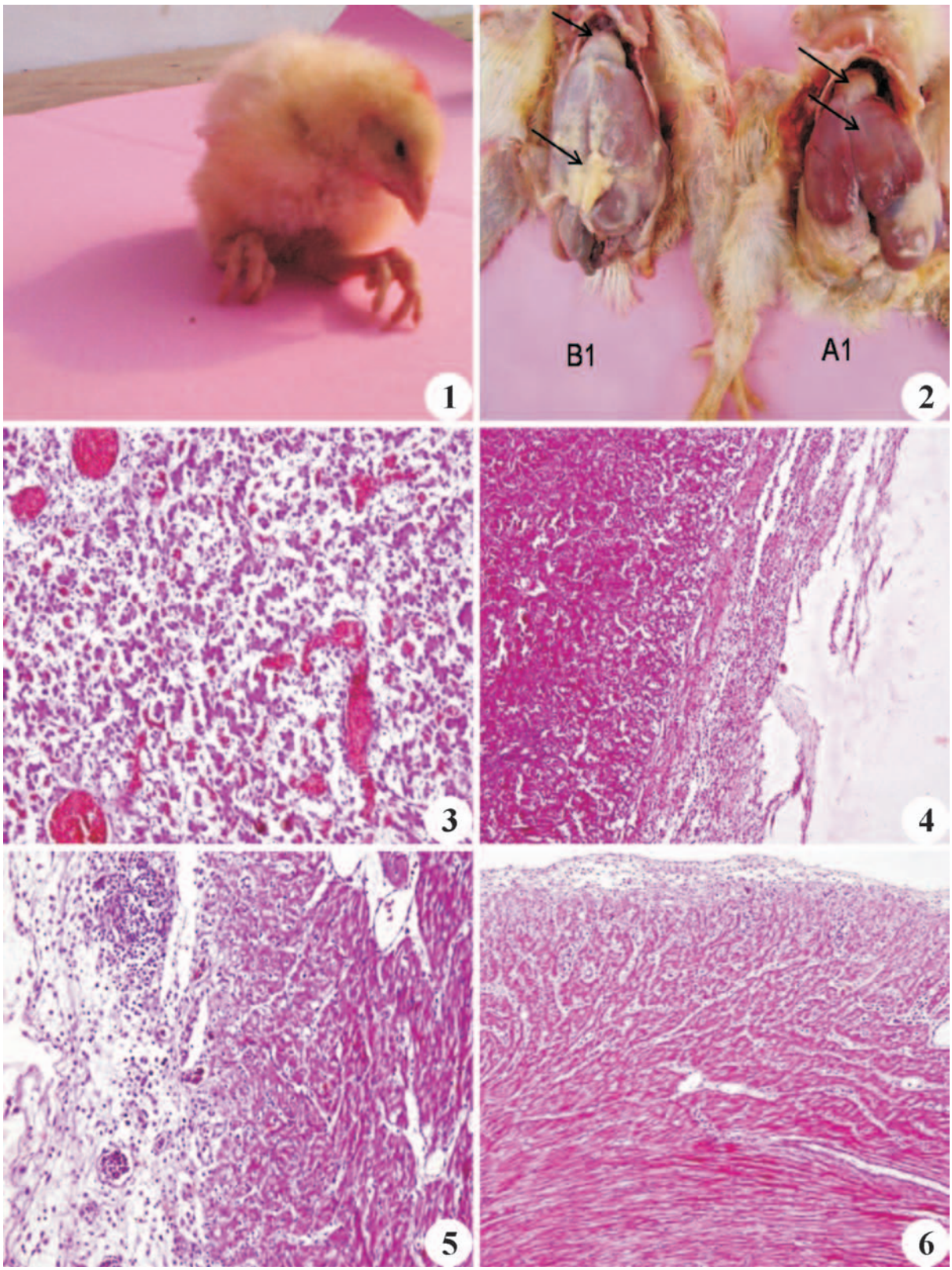

Fig.1. Bird from group B1 at 4 DPI showing sitting on hock and unable to bear weight; **Fig.2.** Bird from group B1 at 4 DPI showing comparatively thick layer of fibrin on heart and liver than group A1; **Fig.3.** Liver (Group B1-2DPI): Congestion and dilatation in sinusoid and atrophy of hepatocyte. H&E ×200; **Fig.4.** Heart (Group B1-4DPI): Severe fibrinous pericarditis characterised by presence of fibrin and leucocytic infiltration in pericardium extending intomyocardium. H&E ×200**; Fig.5.** Liver (Group B1-4DPI): Fibrinous perihepatitis characterized by deposition of fibrin and leucocytic infiltration.H&E ×100; **Fig.6.** Heart (Group A1-4DPI):Pericarditis characterized by heterophil and mononuclear cells infiltration in pericardium extending into myocardium.H&E ×100.

Table 1. Overall % mean gross and histopathological lesion scores (GLS/HLS) in *E. coli* infected groups and % protective effect due to neem leaf extract supplementation in chickens.

Lesion Score	Groups	Overall % mean scores in different organs irrespective of post infection period				Overall % mean lesionscores irrespective of post infection period and organs	% protective effectdue to 10 % NLE supple-mentation
		Liver	Heart	Lung	Intestine		
Gross Lesion	Group A1	32.18	31.5	20.78	15.5	24.99	31.17
Score	Group B1	39.85	45.28	32.14	27.96	36.31	
Histopathological	Group A1	34.53	36.32	29.75	19.61	30.05	28.52
Lesion Score	Group B1	44.64	48.57	39.86	35.1	42.04	

haemorrhages, serofibrinous exudation with heterophil and mononuclear cells infiltration, exudate containing fibrin, erythrocyte and leucocytes in bronchiole. Fatty changes and focal area of necrosiswere seen in kidneys with mild leucocytic infiltration. Enteritis was noticed in the intestine, characterized by severe congestion, hemorrhages and infiltration of heterophils and lymphocytes in mucosa along with desquamation of epithelium of mucosa. Pancreas revealed focal area of necrosis replaced by mononuclear cells. On 7 DPI, there was mild pericarditis as well as myocarditis characterized by leucocytic infiltration in the pericardium as well as myocardium and fibrinous perihepatitis was characterized by deposition of fibrin on liver and leucocytic infiltration. In lungs there were haemorrhages, emphysema, serous exudate in perivascular area containing leucocytic infiltration and few RBC. The intestines revealed enteritis characterized by serofibrinous exudation and leucocytic infiltration in serosa, leucocytic infiltration was also seen in mucosa. Kidneys revealed congestion, degenerative changes in the renal tubules and lymphocytic infiltration in the interstitial tissue. Pancreas revealed congestion and serofibrinous exudation in peripancreatic tissue. On 14 DPI, heart revealed severe fibrinous pericarditis characterized by deposition of fibrin alongwith leucocytic infiltration mainly mononuclear cells in pericardium. Liver revealed fibrinous perihepatitisas well as hepatitis. In lungs, there was pneumonia characterized by congestion and infiltration of lymphocytes and few heterophils. Intestine exhibited enteritis characterized by severe necrosis of villi in mucosa and glands in submucosa with massive leucocytic infiltration. Kidneys revealed congestion. On 21 DPI, pericarditis was evident in heart though of mild degree. Similarly liver revealed mild perihepatitis and hepatitis at focal areas associated with infiltration of few lymphocytes and macrophages. Mild congestion was noticed in various organs such as lungs, kidneys, intestines and pancreas. On 28 DPI, mild congestion was noticed in heart and liver. Other organs did not reveal any significant lesions.

In NLE supplemented infected group (A1), on 2 DPI there was congestion in a few blood vessels in the myocardium of heart along with deposition of thin layer of fibrin in pericardium. Liver revealed congestion in central veins and in sinusoid. Lungs revealed congestion. Mild congestion was also noticed in kidneys. Intestine revealed goblet cell hyperplasia and infiltration of leucocyte in mucosa and submucosa. On 4 DPI, heart exhibitedpericarditis characterized by heterophil and mononuclear cells infiltration in pericardium extending into myocardium (Fig. 6). In liver, there was fibrinous perihepatitis characterized by deposition of fibrin on liver and leucocytic infiltration. Mild congestion was present in lungs. In intestines congestion and mild desquamation of villi was noticed. Kidney, revealed haemorrhages in intertubular tissue, necrosis of tubular epithelium and mild leucocytic infiltration. On 7 DPI, heart revealed mild pericarditis as well as myocarditis. In liver, fibrinous perihepatitis was characterized by deposition of fibrin on liver and leucocytic infiltration. Enteritis was noticed in intestines characterized by infiltration of heterophils and lymphocytes in the mucosa along with desquamation of the mucosal epithelium. Lungs revealed pneumonia characterized by severe leucocytic infiltration in interalveolar tissue leading to obliteration of alveoli and serofibrinous exudation in interlobular septa. Kidneys revealed congestion and haemorrhages. On 14 DPI, heart revealed pericarditis consisting infiltration of heterophils and macrophages along with myocarditis. Liver revealed telangiectasis and perihepatitis characterized by infiltration of heterophils and lymphocytes. Lungs revealed congestion and mononuclear cell infiltration in alveoli. Intestines revealed enteritis characterized by desquamation of villi and lymphoid aggregates replacing glands in submucosa. Kidneys showed congestion. On 21 DPI, mild pericarditis was observed in the heart of few birds characterized by mononuclear cells infiltration. In liver, there was only dilatation of sinusoids. Mild congestion and edema was noticed in lungs. On 28 DPI, different organs did not reveal any significant lesions.

Lesion scores and percent protective effect due to NLE supplementation

Colibacillosis specific mean gross lesion scores (GLS) and histopathological lesion scores (HLS), overall % mean lesion scores irrespective of post infection period and

organs in different experimental groups and % protective effect on gross and histopathological lesions of *E. coli* infection due to supplementation of neem leaf extract are illustrated in Table 1.

DISCUSSION

The purpose of present study was to evaluate the effects of supplementation of neem leaf extracton pathology of different organs in experimentally *E. coli* infected broiler chickens. During this study no clinical signs were observed in the pure control groups. The clinical signs observed in experimental colibacillosis in the present study were dullness, depression, weakness, inappetance, ruffled feathers, closing of eyes, drooping of head and neck, respiratory distress, watery and white pasty diarrhea, dehydration and huddling near the heat source. Similar clinical signs have also been reported by other workers 10-14 in natural and experimental cases of colibacillosis. The clinical signs of colibacillosis in NLE supplemented group were of mild degree at different intervals, appeared later and persisted only for shorter period as compared to non-supplemented group. These results indicate that NLE has protective effect on disease manifestation which might be due to its immunomodulatory effect[15]. Rudraswamy and Chaithanya[16] also reported protective effect of Actovet CRD (A.CRD) a blend of ayurvedic herbs (*Glycorrhyzaglabra*, *Adhatodavasika*, *Pipperlongum*, *Abiswebbiana*, *Azadirachtaindica*, *Curcuma longa*etc) on clinical signs of chronic respiratory disease in poultry.

Overall mortality observed in the group with infection alone was 22.22%. and overall mortality was 12.96% in group A1. These results are in accordance with the findings of Raheja[5] and Saini[11].

The characteristic gross lesions of colibacillosis observed in the present study were fibrinous pericarditis, fibrinous perihepatitis, cloudy air sac, congestion in visceral organs, peritonitis and enteritis. Other workers 17-20 have also reported similar lesions in natural and experimental colibacillosis. The gross lesions in NLE supplemented infected group were of lesser intensity at different intervals as compared to those observed in non-supplemented infected group. These findings support hepatoprotective and cardioprotective properties of NLE.

Histopathological changes observed in heart due to colibacillosis in the present study were fibrinous pericarditis and myocarditis characterized by accumulation of fibrin, infiltration of heterophils and lymphocytes in early stage and macrophages in later stage. Similarly, liver revealed fibrinous perihepatitis and hepatitis along with degenerative changes hydropic and fatty changes in hepatocytes and dilatation of sinusoids at different intervals of post infection. Perihepatitis was also characterized by presence of fibrin and infiltration of heterophil and mononuclear cells. Almost similar findings have also been reported by other workers[7,10,11,20-23] in chickens infected with *E. coli*. These histopathological features of fibrinous pericarditis and fibrinous perihepatitis were of lesser severity in NLE supplemented infected group at different intervals and appeared at later stage. This difference in severity of lesions revealed the hepatoprotective and cardioprotective effect of neem leaf extract supplementation. Similar effect of NLE supplementation has also been reported by Saini[12] against *E. coli* infection. Histopathological lesions observed in lungs due to colibacillosis in the present study were congestion, hemorrhages in early stages and emphysema, serofibrinous exudation along with severe leucocytic infiltration in later stages. Similar respiratory lesions have also been reported by other workers[11,18,20] in *E. coli* infected chickens. However, severity of these lesions in NLE supplemented infected group was less in nature as compared to non-NLE supplemented group. Intestines revealed enteritis characterized by congestion, leucocytic infiltration, goblet cell hyperplasia and desquamation of villi in broiler chicks infected with *E. coli* though the lesions at different intervals in NLE supplemented infected group were not as severe as in non-NLE supplemented infected group. Similar lesions in *E. coli* infection were also reported by many workers[11,18,20,23].

On the basis of gross and histopathological lesions in different organs, the lesion scores in both the infected groups were calculated to quantify protective effect of NLE supplementation. It was observed that protective effect on gross and histopathological lesions of *E. coli* infection due to supplementation of neem leaf extract was 31.17% and 28.52%, respectively.

It is concluded that supplementation of 10% NLE causes reduction in severity as well as recovery period of *E. coli* infection, suggesting its protective effect on limiting the pathology and pathogenesis of *E. coli* infection in broiler chickens.

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