Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.292

RESEARCH ARTICLE

In Vivo Assessment of the Antibacterial Action of Zinc Oxide Nanoparticles against Colibacillosis-Induced Infection in Broilers: Comparison with Colistin

Sami Ullah Khan Bahadur[1*], Muhammad Tariq Javed[1], Muhammad Hunain Ahmed[1], Muhammad Ehtisham Tariq[1], Muhammad Hassan Tahir[1], Hafeez Anwar[2], Razia Kausar[3], Shaza Zarnab[1], Rana Hamas Ahmed[4] and Mo Salman[5]

[1]Department of Pathology, University of Agriculture Faisalabad Pakistan-38040.[2]Department of Physics, University of Agriculture Faisalabad Pakistan-38040.[3]Department of Anatomy, University of Agriculture Faisalabad Pakistan-38040.[4]Institute of Animal Breeding and Husbandry, Christian-Albrechts-University Kiel, Hermann-Rodewald-Straße 6, Kiel, Germany 24118. [5]College of Veterinary Medicine and Biomedical Sciences, Colorado State University Fort Collins, CO USA 80523.
*Corresponding author: drsamiuaf@gmail.com

ARTICLE HISTORY (24-340)

Received: June 23, 2024
Revised: October 02, 2024
Accepted: October 07, 20-24
Published online: December 9, 2024

Key words:
Colistin, Broilers
E. coli
Immune organs
Zinc oxide nanoparticle

ABSTRACT

This study was conducted to assess in-vivo antibacterial action of different levels of zinc oxide nanoparticles (ZnO NPs) against *E. coli*-induced infection in broilers. At 12 days old, 90 birds were randomly divided into six groups: two controls and four treatment groups. On day 19, all groups except the negative control were infected with *E. coli*. Once signs of colibacillosis appeared, birds received oral ZnO-NPs at varying doses and colistin. After six days of treatment, the birds were slaughtered for sample collection on the 8th and 11th days post induced infection. Mortality rate, hematological, biochemical, and immunological parameters, as well as gross and histopathological changes in immune organs, were assessed. Statistical analysis was conducted to compare differences among the groups. There was non-significant ($P>0.05$) difference of NDV titers among all the groups. Hematology, biochemical parameters, lymphoproliferative response and phagocytic index were significantly ($P<0.05$) higher in groups treated with zinc oxide NPs 100 mg/kg than control positive group. The mortality rate, gross pathology and quantitative histopathology indicated the better response in the 100 mg/kg ZnO-NPs treated group. *E. coli* induced infection in broilers can be treated with zinc oxide nanoparticles @ 100 mg/kg.

To Cite This Article: Bahadur SUK, Javed MT, Ahmed MH, Tariq ME, Tahir MH, Anwar H, Kausar R, Zarnab S, Ahmed RH and Salman M, 2024. In vivo assessment of the antibacterial action of zinc oxide nanoparticles against colibacillosis-induced infection in broilers: comparison with colistin. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2024.292

INTRODUCTION

Bacterial infections pose a significant challenge to poultry farming, leading to considerable economic losses attributed to increased mortality rates, decreased productivity, and the condemnation of carcasses (Niemi, 2021). Colibacillosis, caused by Avian Pathogenic *E. coli* (APEC), is one of the most prevalent bacterial diseases among poultry (Joseph *et al.,* 2023). This disease has significant impact on poultry industry contributing to high mortality rates, carcass condemnations, and decreased productivity (Souza *et al.,* 2019). Similarly, *E. coli* infection causes decreased egg production due to salpingitis and is one of the most common causes of mortality in commercial layer and breeder chickens (Nolan *et al.,* 2013). To mitigate the impact of bacterial infections, various strategies can be employed. Most important is maintenance of cleanliness through regular cleaning practices, including feed and water hygiene, replacement of bedding, and thorough disinfection between bird batches. Additionally, the administration of probiotics and prebiotics serves, implementation of "all in, all out" flock management practice with down time of at least two weeks help in minimizing outbreaks (Laurent, 2018).

Although bacterial diseases including colibacillosis can be treated with antibiotics, emergence of antibiotic resistance is of foremost concern. Antimicrobial resistance (AMR) has emerged in poultry, poultry products, carcasses, litter, and fecal matter of birds which can pose a risk to consumers, handlers, and public health (Thongratsakul *et al.,* 2023). Therefore, there is a need to either make careful choices of antibiotics based on an

effective monitoring programs (Kazemnia et al., 2014) or shift treatment and prevention to antibiotic alternatives. To achieve the later goal, there are research studies to use different antibiotic substitutes against different infections e.g., bacteriophages had been used against *E. coli* (Eid *et al.*, 2022). Similarly, egg yolk antibodies have been assessed to replace antibiotics against bacteria including *E. coli* in the poultry industry (Yegani and Korver, 2010).

Nanoparticles are materials whose particle size is less than 100 nm in at least one dimension. Zinc oxide NPs inhibit the activity of bacteria by penetrating and rupturing the bacterial cell wall (Wang, 2012). The other proposed mechanism of action is production of reactive oxygen species (ROS), which destroys the bacteria due to their oxidizing ability. Zinc oxide nanoparticles have been used in vitro against *E. coli* and remarkable minimum inhibitory concentration (MIC) has been observed (Tayel, 2011). Based on in-vitro antibacterial action of ZnO NPs, it was hypothesized that these nanoparticles can treat the colibacillosis (*E. coli* infection) in broiler birds. Copper oxide nanoparticles have also shown antibacterial properties against colibacillosis in broiler (Ahmed *et al.*, 2022). The objective of the present study was to investigate the activity of zinc oxide nanoparticles against *E. coli* induced infection in broilers through pathological parameters.

MATERIALS AND METHODS

Ethics approval: The study was conducted according to the Responsible Conduct in Research (RCR) Training Policy (Policy#10.07.001) of the University of Agriculture Faisalabad. The RCR policy is followed by the instructions of National Institute of Health (NIH publication No. 8023, revised 1978) regarding animal welfare and housing in research.

Nanoparticle preparation and characterization: Zinc oxide nanoparticles were synthesized using zinc sulphate ($ZnSO_4$) and NaOH precursors by the co-precipitation method following the procedure described by Manyasree et al. (2018). The synthesized nanoparticles were commercially characterized using X-ray diffraction (XRD) with the D8 Advance (Bruker™), scanning electron microscopy (SEM) at the University of Agriculture Faisalabad, and Zeta sizing with the Zetasizer Nano ZS™ at the National Textile University Faisalabad.

Isolation and pathogenicity testing of *Escherichia coli* for inoculum: Pathogens were isolated from spleen and liver tissues collected from broiler birds, suspected of colibacillosis, presented at Diagnostic Laboratory of University of Agriculture Faisalabad on MacConkey agar (Dufour, 2008) and identified through Gram's staining and culturing on TSI agar. For confirmation, biochemical analysis using analytical profile index (API-20E kit) was used. For pathogenicity testing, PCR for the identification of pathogenic gene stx1A was performed using primers F=5'-CAGTTAATGTGGTGGCGAAG-3' and R=5'CTGCTAATAGTTCTGCGCATC-3'. For this purpose, DNA was extracted from bacterial colonies isolated on MacConkey agar using Thermo Scientific® DNA extraction kit (LOT#00777285). Thermal cycling was programmed following the method used by Jeong *et al.* (2012). PCR products were run on 2% agarose gel for 45min at 90V and read Gel Doc. For in vitro pathogenicity testing of *E. coli* culture, growth on MacConkey agar was shifted on tryptic soy agar supplemented with 0.03% Congo red dye. For in vivo pathogenicity testing, 0.2ml of suspension dilution 10^7CFU/ml was injected into each bird via intramuscular route.

Duration of death since inoculation and necropsy of birds was performed on their death. After the 10 days of inoculation, the rest of the birds were euthanized humanely, and necropsy lesions were recorded.

Antibiotic sensitivity testing: Following CLSI (Clinical and Laboratory Standards Institute) guidelines, disc diffusion antibiotic sensitivity testing was performed against identified pathogenic *E. coli* using colistin and doxycycline at different concentrations. For this purpose, antibiotic discs of colistin with 10ug and 20ug and doxycycline with 10ug and 30ug were prepared in the lab using antibiotic powder as per protocol described by Epoke *et al.* (2003).

Housing and management of the experimental birds: The poultry shed, at Department of Clinical Medicine and Surgery, University of Agriculture Faisalabad, was thoroughly cleaned and disinfected. Ninety (90) broiler day-old birds were purchased from a local hatchery in Faisalabad. Thirty-six hours before the chicks' arrival, the poultry shed was fumigated and was open for six hours. Temperature and light maintenance at the shed were started before the birds' arrival and was continued throughout the whole trial. Commercially available broiler feed was provided to all the birds *ad libitum* with clean drinking water.

Inoculation of birds: To prepare the inoculum, *E. coli* colonies, prepared as above, were suspended in sterile Phosphate buffer saline at pH 7.0. The turbidity of inoculum was compared with McFarland standard 1. Ten-fold serial dilution of suspension was made. Colonies were counted using colony counter. Suspension dilution with 10^7CFU/ml was used as inoculum. The experimental design is provided in Table 1, which outlines the schedule for inoculation, infection, treatment, sampling, and testing.

Outcomes and other related factors: Mortality of each group was recorded after the induction of infection. Gross pathology of the spleen, thymus and bursa of Fabricius were examined, and changes present were recorded on both sampling days. Birds were humanely euthanized, and blood from each bird was collected in test tubes with and without added anti-coagulant for hematological analysis and serum separation, respectively. Total erythrocytic count and total leukocytic count were determined by using a hemocytometer while hematocrit level was determined by using the micro-hematocrit method (Coles, 1986). Hemoglobin was measured as described by Kuttner (1916). Leukocytes were deferentially counted (DLC) under 100X after staining the smear with Giemsa stain.

Table 1: Experimental design: timeline for vaccination, induction of infection, treatment, and sampling.

Group/Days	Negative control	Positive control	Colistin	A	B	C
3rd	ND+IB vaccination (eye drop)					
12th	Birds divided in groups					
14th	ND+IB vaccination (drinking water) + collection of serum for ND titres					
19th	------	Challenge of *E. coli* with 10^7 CFU/ml				
21st-26th (Treatment days)	------	------	Colistin continuous supply via drinking water	Divided doses, twice a day via oral route with syringe 50 mg/kg ZnO-NPs	75 mg/kg ZnO-NPs	100 mg/kg ZnO-NPs
27th	Sampling I (day 8 post-infection) Immune organs collection in 10% formalin, blood, and serum collection					
28th	Avian tuberculin injection					
29th	Carbon clearance assay + 24-hour tuberculin response					
30th	48-hour tuberculin response + Sampling II (day 11 post-infection) Immune organs collection in 10% formalin, blood, and serum collection					

Serum total proteins and albumin were determined using Bioclin® kit; by applying formulas total proteins (g/dL) = (Serum sample absorbance × 4) / Standard absorbance; and albumin (g/dL) = (Sample absorbance×3.8) / Standard absorbance, respectively. Serum globulins were calculated by differentiation method by applying formula: serum globulins = total serum protein−serum albumin.

For lipid analysis, serum high-density lipids (HDL) were determined for all groups using the Human™ kit (Lot # 20005). The HDL was calculated by using formula: HDL = 150* (Samples absorbance / Standards absorbance). Similarly, the assessment of low-density lipids (LDL) and triglycerides employed the Human™ kit (Lot # CF1000050) and LABKIT® kit (Lot # TI253), respectively, with corresponding calculation formulas: LDL = 200* (Samples absorbance / Standards absorbance) and Triglyceride = 200* (Samples absorbance / Standards absorbance). Total cholesterol was computed as the sum of LDL, HDL, and one-fifth of the triglyceride level: Total cholesterol = LDL + HDL + (triglyceride/5). Additionally, very low-density lipids (VLDL) were determined by the formula: VLDL = triglycerides/5.

For Histopathology, immune organs including the spleen, thymus and bursa of Fabricius were collected in 10% formalin containers and fixed for 10 days at room temperature, followed by processing, sectioning and staining as described by Bancroft and Stevens (1996). Histopathological examination was quantified for immune cells and other pathological parameters using Qupath™ software.

Mortality pattern, hematology, biochemical parameters, gross and histopathological changes in immune organs were primary outcomes to be considered as these organs exhibit significant changes during colibacillosis infection.

Apart from these, antibody titer against ND was determined by Hemagglutination (HA) and Hemagglutination Inhibition (HI) assay (OIE, 2012) on serum collected from all groups on day 14, 21, 27 and 30. A carbon clearance assay was performed as described by Hudson and Hay (1989) on day 29 (day 10th post-infection) on randomly selected birds from each group. Avian tuberculin response/lymphoproliferative response was seen in the form of thickness by injecting tuberculin (Corrier, 1990) 48 hours before the second sampling.

Statistical analysis: Complete Randomized Design was applied for Analysis of Variance (ANOVA), and Tukey's test to check the significance and to compare group mean values of the hematological, biochemistry, lipid profile, ND titers, carbon clearance assay, tuberculin response, and histopathological parameters, using SAS® University Edition online software SAS 15.1 (2018). In this study, a 5% significance level (α) was used, indicating a 95% confidence level for assessing statistical significance.

RESULTS

Nanoparticles' characterization: Size distribution intensity determined through zeta sizer had depicted that the average size of synthesized zinc oxide nanoparticles was 97.5nm (Fig. 1a). Scanning Electron Microscopy revealed that zinc oxide nanoparticles had oval to spherical shape with no agglomeration with other particles (Fig. 1b) at 500X. The broad peaks in XRD (Fig. 1c) indicated that the synthesized material has particles in the nanoscale range with no impurity. These peaks are indexed as the hexagonal wurtzite phase of ZnO NPs with lattice constants a = b = 0.32495 nm and c = 0.52069 nm (COD 03-065-3411).

Isolation and pathogenicity testing of *Escherichia coli* for inoculum: Pink colonies on MacConkey agar were indication of isolation of *Escherichia coli.* Gram's staining revealed the pink rods with no purple bacteria (Suppl. file). Bacteria inoculated in TSI appeared as yellowish growth on the slant and there was gas production in the butt. Results indicated the acidic slant and butt, without the production of H_2S. Analytical Profile Index® 20E system confirmed that isolated bacteria were *E. coli* (Suppl. file). For the pathogenicity confirmation, stx1A gene was targeted, whose bands were seen at 895 bp. Out of five (05) samples subjected to PCR, four (04) showed positive results for pathogenic *E. coli* (Suppl. file). For *in vitro* pathogenicity testing, inoculated plates showed red color growth. This was due to the binding of Congo red with *E. coli* and was found on Congo red agar. Birds inoculated to test the *in-vivo* pathogenicity showed clinical signs including respiratory distress, and greenish diarrhea after 48 hours of injection. One bird died after 56 hours of infection, while others on the fifth day of infection. The dead birds had perihepatitis and pericarditis, and it was very severe (adhered fibrinous pericarditis) in the birds (Suppl. file).

Antibiotic sensitivity testing: Zone of inhibition (Suppl. file) by doxycycline having concentration 10ug and 30ug

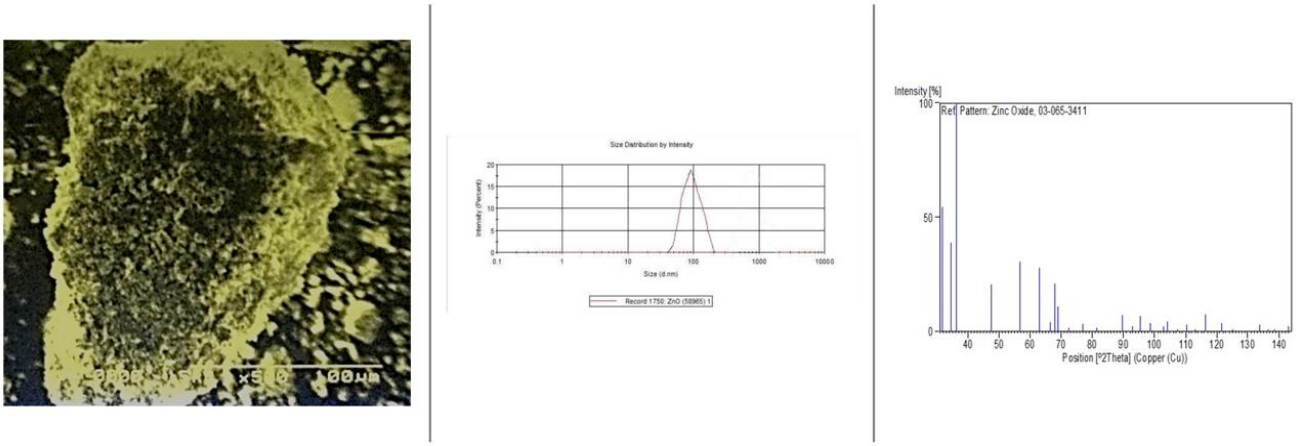

Fig. 1: a) Scanning Electron micrograph revealing oval to spherical shape of ZnO nanoparticles at 500X; b) Size distribution intensity determined through zeta sizing indicating average size of nanoparticles as 97.5 nm; c) XRD spectrum showing no peaks other than characteristic ZnO peaks, indicating the purity of nanoparticles.

was 8.83±1.04 (I) and 11.0±1.0 mm (S), respectively, while the disc prepared from colistin having concentration of 10ug and 20ug showed 14.16±0.76 (S) and 17.33±1.15 mm (S), respectively.

Mortality rate: The mortality rate for each group during the period of post-infection from day 19 to 30 is given in Supplementary File (Fig. 6).

Gross Pathology

Spleen: There was severe congestion at the 1st and moderate at 2nd sampling in the positive control group, but other groups treated with colistin, ZnO-NPs at 50, 75 and 100mg/kg had mild to moderate congestion at the 1st sampling, while no congestion in groups treated with colistin and 100 mg/kg ZnO-NPs. The enlargement was moderate and severe in control positive at the 1st and 2nd sampling, respectively. The treated groups had mild to moderate enlargement at the 1st sampling and mild in colistin and 100 mg/kg ZnO-NPs treated groups at 2nd sampling. Mild mottled grayish areas were seen in positive control as well as ZnO-NPs 50 and 75mg/kg only at the 1st sampling (Suppl. File).

Thymus: There was mild congestion at the 1st sampling and moderate in the 2nd sampling in the positive control group, but other groups treated with colistin, ZnO NPs at 50, 75 and 100mg/kg had mild to moderate congestion at the 1st sampling which was not seen on the 2nd sampling. Severe atrophy in colistin treated and mild in ZnO NPs 50 and 75mg/kg at the 1st sampling was seen. At the 2nd sampling, positive control and colistin treated groups had moderate atrophy. Control positive had moderate hemorrhages while mild changes in all treated groups at the 1st sampling. Mild hemorrhages were still present in the positive control group and groups treated with colistin, ZnO NPs 75 and 100mg/kg at the 2nd sampling (Suppl. File).

Bursa of Fabricius: There was only mild bursal atrophy in groups treated with ZnO NPs 50 and 75mg/kg at the 1st sampling, while moderate bursal atrophy was seen in groups treated with colistin and ZnO NPs 100mg/kg but not observed in groups treated with 50 and 75mg/kg ZnO NPs (Suppl. File).

Hematology: At the 1st sampling (day 8 post-infection), the control negative, ZnO NPs (100mg/kg) and colistin groups had significantly higher RBCs levels than control positive, with varying effects for 50 and 75 mg/kg ZnO NPs (Table 2). At the 2nd sampling (day 11 post-infection), the positive control group had significantly lower RBCs than the control negative, colistin and ZnO NPs (75 and 100 mg/kg). At the 1st sampling, control positive had significantly higher WBCs than control negative, ZnO NPs (100mg/kg) and colistin, with non-significant difference from 50 and 75mg/kg ZnO NPs; while at the 2nd sampling, the positive control had significantly higher WBCs than control negative, colistin and ZnO NPs (75, 100mg/kg).

Regarding hematocrit, at the 1st sampling, there was non-significant difference among all groups. At the 2nd sampling, control negative, ZnO NPs (75, 100mg/kg) groups had significantly higher hematocrit than positive control. At the 1st sampling, the control positive group had significantly lower hemoglobin than control negative, colistin and ZnO NPs 75, 100mg/kg, while 50mg/kg ZnO NPs showed a non-significant increase. At the 2nd sampling, colistin, ZnO NPs 50, 75, and 100mg/kg groups had significantly increased hemoglobin compared to control positive group.

Differential leucocytic count (DLC): At the 1st sampling, the colistin treated group had significantly higher lymphocytes than control positive, while ZnO NPs (50, 75, 100mg/kg) groups had non-significantly higher lymphocytes (Table 2); at the 2nd sampling, colistin, ZnO NPs 75mg/kg and ZnO NPs 100mg/kg treated groups showed significantly higher level of lymphocytes. At the 1st slaughtering, all treated groups showed non-significant lower heterophils than the control positive group while control positive had significantly higher lymphocytes than the control negative group. At the 2nd sampling, there was a non-significant difference of lymphocytes among all groups. At the 1st sampling, colistin and ZnO NPs (75, 100mg/kg) treated groups had significantly lower monocytes than control positive, while 50mg/kg was non-significantly lower. At the 2nd sampling, colistin and all ZnO NPs (50, 75, 100mg/kg) treated groups showed significantly lower monocytes than the control positive group.

Table 2: Hematological and biochemical parameters comparison across treatment groups.

Group	Total Erythrocytic Count (x10^6/µL)		Total Leucocytic Count (x10^3/µL)		Haematocrit Level (%)		Haemoglobin Conc. (µg/dL)	
	Sampling I	Sampling II	Sampling I	Sampling II	Sampling I	Sampling II	Sampling I	Sampling II
NC	5.47±0.60 (a)	5.78±0.29 (a)	3.81±0.86 (d)	3.56±0.33 (c)	33.33±4.04	32.66±3.05 (a)	10.50±0.79 (abc)	10.53±0.41 (a)
PC	3.63±0.61* (bcd)	3.67±0.54* (b)	15.24±0.52* (b)	8.44±1.18* (a)	26.80±2.32	26.20±1.18* (b)	8.20±1.18 (c)	7.94±0.12* (b)
Colistin	5.40±0.84 (ab)	5.49±0.34 (a)	9.82±1.28* (c)	4.58±0.42 (bc)	31.50±4.63	30.60±0.77 (ab)	11.17±1.04 (ab)	10.96±1.41 (a)
A	3.01±0.89* (d)	4.98±0.63 (ab)	11.57±1.17* (bc)	6.22±2.12* (ab)	26.00±2.00	29.00±1.00 (ab)	9.37±1.00 (bc)	10.95±0.30 (a)
B	3.58±0.89* (cd)	5.95±0.84 (a)	12.52±0.47* (b)	5.76±0.58 (bc)	26.50±2.12	31.66±2.08 (a)	11.05±0.63 (ab)	11.56±0.40 (a)
C	5.32±0.39 (abc)	6.11±0.68 (a)	10.06±0.52* (c)	4.63±0.50 (bc)	32.33±2.08	31.25±2.31 (a)	12.20±1.21 (a)	11.70±0.46 (a)

	Lymphocytes		Heterophils		Monocytes	
	Sampling I	Sampling II	Sampling I	Sampling II	Sampling I	Sampling II
NC	61.00±2.00 (a)	60.33±2.64 (a)	30.10±3.11 (b)	29.66±2.52	6.67±0.57 (c)	7.09±0.79 (b)
PC	34.20±11.33* (b)	46.00±1.73* (c)	46.40±8.69* (a)	35.4±5.79	18.20±1.54* (a)	16.6±4.07* (a)
Colistin	53.25±8.96 (a)	59.80±4.04 (ab)	35.00±6.00 (ab)	29.40±3.54	10.75±1.83 (bc)	7.20±1.54 (b)
A	48.00±3.46 (ab)	51.50±6.67* (bc)	35.25±2.52 (ab)	33.75±3.92	14.50±2.34* (ab)	12.25±1.54 (b)
B	49.25±2.31 (ab)	56.33±1.52 (ab)	37.00±6.92 (ab)	31.33±2.51	11.75±2.89* (b)	8.33±2.08 (b)
C	50.00±6.24 (ab)	56.25±2.52 (ab)	35.33±5.85 (ab)	31.25±2.52	13.33±1.52* (b)	7.50±4.63 (b)

	Serum Total Proteins		Serum Albumin		Serum Globulins	
	Sampling I	Sampling II	Sampling I	Sampling II	Sampling I	Sampling II
NC	3.61±0.21 (c)	3.40±0.25 (c)	1.84±0.16 (b)	1.65±0.27 (c)	1.77±0.05 (ab)	1.75±0.20
PC	4.56±0.25* (ab)	5.08±0.23* (a)	3.25±0.41* (a)	3.62±0.42* (a)	1.31±0.15 (b)	1.45±0.20
Colistin	5.02±0.32* (a)	4.60±0.37* (ab)	3.07±0.21* (a)	3.16±0.95* (ab)	1.95±0.12 (ab)	1.43±1.33
A	5.15±0.46* (a)	4.55±0.40* (ab)	2.67±0.57 (ab)	2.88±0.31* (ab)	2.48±1.03 (a)	1.66±0.08
B	4.45±0.36* (ab)	4.40±0.08* (ab)	3.17±0.27* (a)	3.04±0.38* (ab)	1.28±0.11 (b)	1.36±0.46
C	3.87±0.26 (bc)	4.15±0.44 (bc)	2.92±0.33* (a)	1.93±0.68 (bc)	0.95±0.16 (b)	2.21±1.12

Note: Group values with * are significantly different from control negative while **bold** values are significantly different from control positive. NC = negative control group; PC = positive control group; A = ZnO NPs 50 mg/kg group; B = ZnO NPs 75 mg/kg group; C= ZnO NPs 100 mg/kg group.

Serum Biochemistry: At the 1st and 2nd samplings (day 8 & 11 post-infection), control positive and ZnO NPs 100mg/kg had significantly higher serum total proteins than control negative, while colistin and ZnO NPs (50, 75mg/kg) showed non-significantly lower total proteins than control positive (Table 2). At the 1st sampling, all groups except ZnO NPs 50mg/kg had significantly higher serum albumin than control negative group. At the 2nd sampling, only the group treated with ZnO NPs 100mg/kg had significantly lower albumin than control positive group. At the 1st sampling, only the group treated with ZnO NPs 50mg/kg had significantly higher serum globulins than control positive group, while at the 2nd sampling there was non-significant difference among all groups.

Lipid Profile: There was non-significant (P>0.05) difference of high-density lipids (HDLs) and triglycerides and very low-density lipids (VLDLs) among all groups at the 1st and 2nd sampling.

At the 1st sampling, colistin and ZnO NPs 100mg/kg treated groups had non-significantly higher low-density lipids (LDL) than control positive group while ZnO NPs 50 and amp; 75mg/kg treated group had non-significantly lower LDLs than control positive group. At the 2nd sampling, all groups showed non-significant differences in LDL. The colistin treated group showed significantly higher total cholesterol than positive control, while 75 and 100mg/kg ZnO NPs groups had a non-significantly higher level. However, at the 2nd sampling, all groups showed non-significant difference.

Immunological parameters: All birds were vaccinated on day 3 (ocular) and day 14 (drinking water) using ND vaccine. Titers against NDV were tested from serum collected on day 14, 21, 27 and 30. Results indicated that there was non-significant (P>0.05) difference of antibody titers among all groups on all days. However, groups treated with colistin and ZnO NPs 100mg/kg had a better response than control positive group.

Tuberculin was injected 48 hours before sampling II (day 9 PI). After 24 hours of injection, only control positive group had significantly (P<0.05) lower response than control negative group. After 48 hours, all treated groups had non-significant (P>0.05) difference from control positive; also, from control negative except ZnO NPs 75mg/kg.

Carbon clearance assay has indicated that absorbance at 3 min was significantly (P<0.05) higher in control positive than control negative by 64.57%. Similarly, colistin and ZnO NPs 50, 75 and 100mg/kg treated groups had significantly lower absorbance than control positive by 81.48, 77.35, 73.53 and 70.79%, respectively. At 15 min, absorbance was significantly (P<0.05) higher in control positive than control negative by 91.46%, while all treated groups had non-significant difference from control negative group.

Histopathology of Immune organs: Histopathological examination of spleen, thymus and bursa of Fabricius have been shown in Fig. 2, 3 and 4, respectively. It was revealed (Suppl. File: Table 1) that number of lymphocytes in specific randomly selected areas of each 138400 µm 2 in spleen, thymus and bursa of Fabricius were significantly (P<0.05) decreased/lowered in positive group as compared to control negative. Group treated with colistin had significantly (P<0.05) higher/increased lymphocytes than control positive in spleen, thymus and bursa. There was non-significant (P>0.05) difference of groups treated with 50, 75 and 100mg/kg ZnO NPs than positive control group in thymus and spleen, while in bursa groups treated with 75 and 100mg/kg ZnO NPs had significantly (P<0.05) higher/increased lymphocytes than control positive group.

Congested area percentage in positive control group at the 1st and 2nd sampling was significantly (P<0.05) higher than control negative in spleen and thymus. All groups treated with colistin, 50, 75 and 100mg/kg ZnO NPs had significantly lower than control positive group.

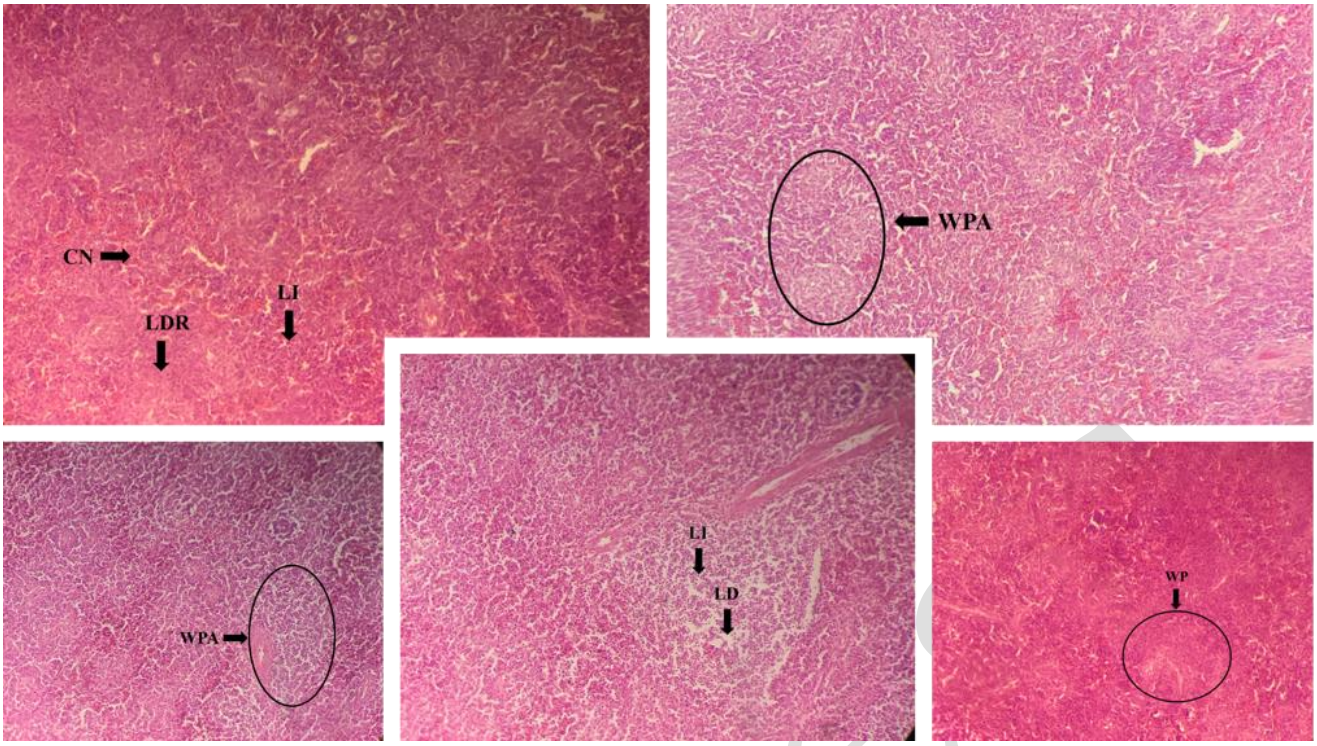

Fig. 2: Histopathology of spleen from group a) treated with 75 mg/kg ZnO NPs with lymphocytic depleted region (LDR), leukocytic infiltration (LI) and congestion (CN); b) treated with colistin with mild lymphocytic depletion; c) treated with 50 mg/kg ZnO NPs having severely depleted white pulp area; d) control positive with severe lymphocytic depletion (LD); e) treated with 100 mg/kg ZnO NPs with very mild lymphocytic depletion.

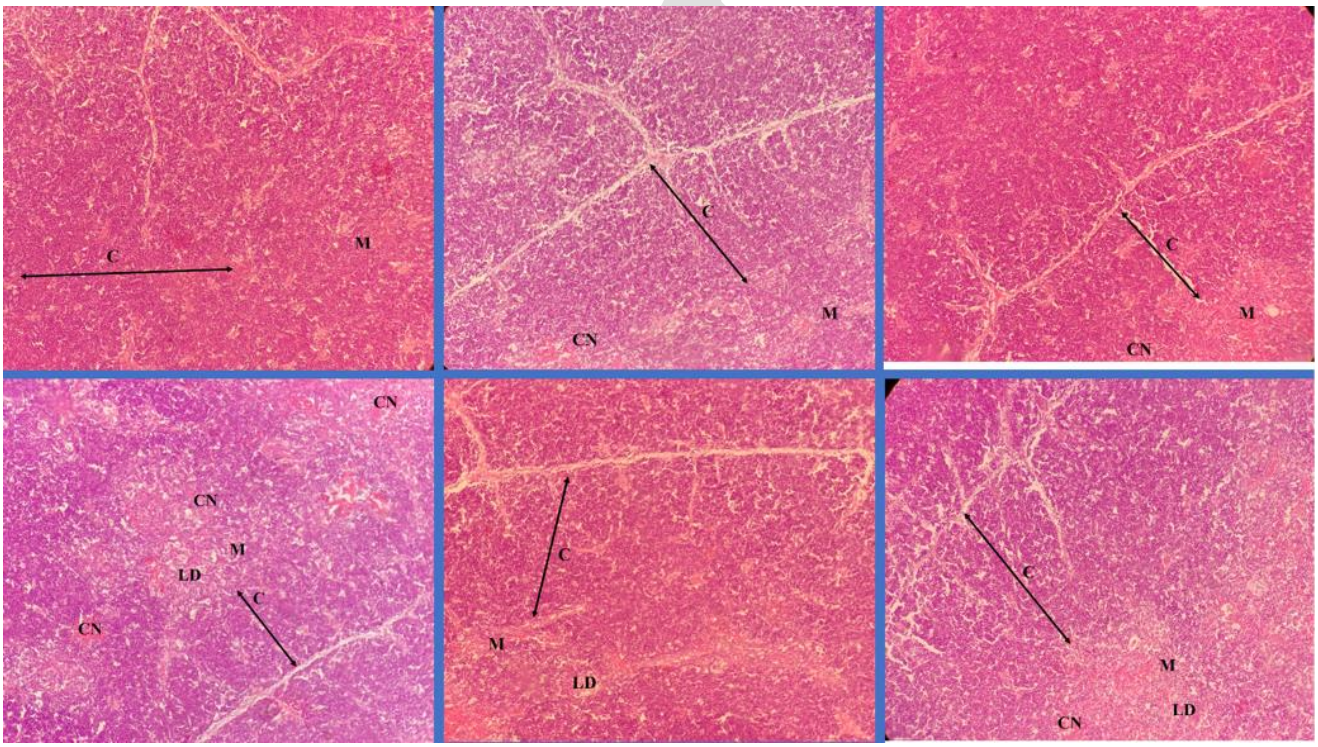

Fig. 3: Histopathology of Thymus from group a) control negative; b) treated with 100 mg/kg ZnO NPs having mild congestion; c) treated with colistin; d) control positive with with severe lymphocytic depletion (LD) and congestion (CN); e) treated with 75 mg/kg lymphocytic depletion (LD); f) treated with 50 mg/kg ZnO NPs having severe congestion (CN) and lymphocytic depletion (LD).

At the 1st and 2nd sampling, interfollicular space in bursa of Fabricius in positive control group was significantly ($P<0.05$) higher than control negative. All groups treated with colistin, 50, 75 and 100mg/kg ZnO NPs had significantly lower than control positive group. Group received 50mg/kg ZnO NPs was having significantly ($p<0.05$) higher interfollicular space in bursa of Fabricius than colistin treated group.

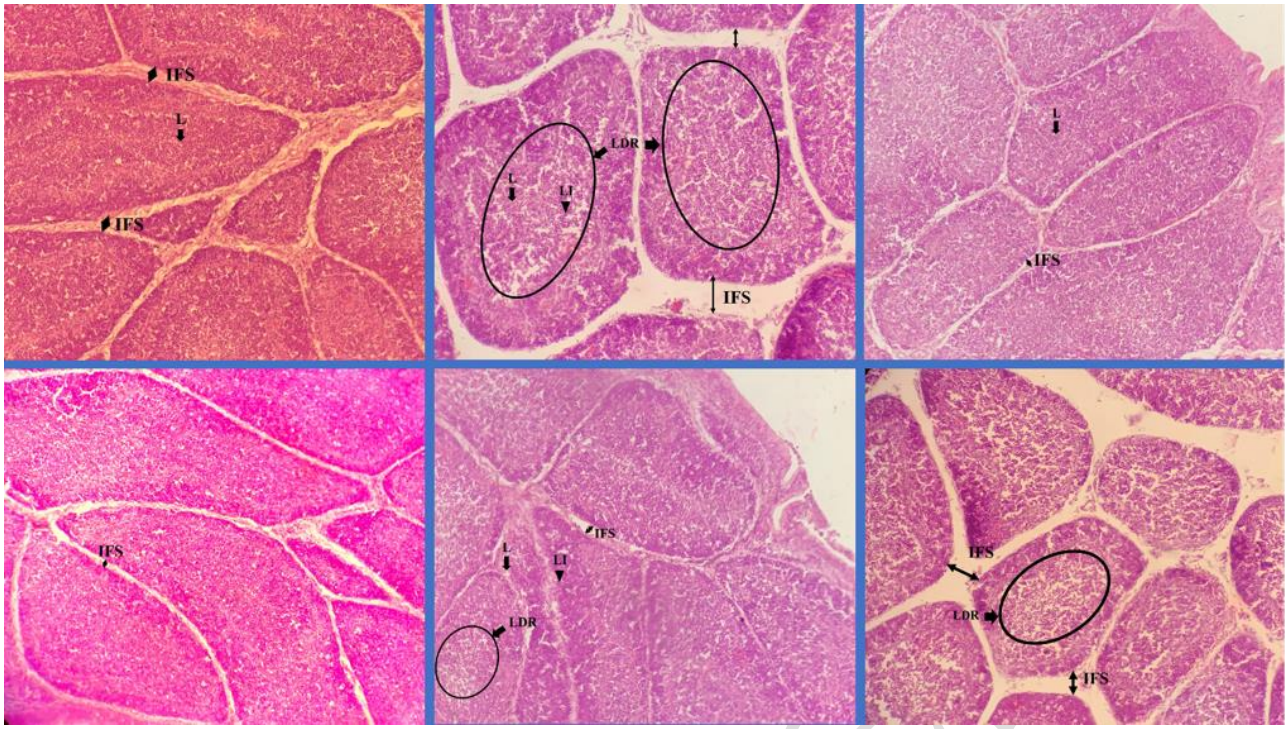

Fig. 4: Histopathology of bursa of Fabricius from group a) treated with 100 mg/kg with mild increased interfollicular space (IFS); b) treated with 50 mg/kg ZnO NPs having increased IFS and lymphocytic depletion; c) treated with colistin; d) control negative group; e) treated with ZnO NPs 75 mg/kg ZnO NPs having lymphocytic depleted region (LDR) and narrow IFS; f) control positive: severe pathological changes.

DISCUSSION

Nanoparticles are materials whose particle size is less than 100nm in at least one dimension. Zinc oxide nanoparticles, inorganic antibacterial material, are regarded as safe for humans and animals, having antimicrobial activity. There was a need to know the antibacterial activity of zinc oxide against *E. coli* in in-vivo in broilers, which had been assessed in this study. To compare the results of zinc oxide nanoparticles, colistin antibiotic has been used due to its higher susceptibility towards our isolated bacterial pathogen. In this study, the isolated *E. coli* had shown more zone of inhibition towards colistin than Doxycycline (Osman and Elhariri, 2014). There is also a significantly ($P<0.05$) higher susceptibility of colistin toward avian *E. coli* than doxycycline, but Eltai *et al.* (2018) had seen comparatively higher colistin resistance toward the *E. coli* isolated from poultry in Qatar. Thus, one of the goals of this research was to contribute to the intention which discourages colistin use in the chicken industry (Eltai *et al.* 2018).

Although zinc oxide nanoparticles have been used as antibacterial agent against *E. coli* in only in-vitro studies, but to our best knowledge, ZnO NPs have not been used as antibacterial in poultry (in-vivo). As far as in-vivo studies are concerned, ZnO NPs have been supplemented as growth promotors, for better performance and to improve blood profile in poultry birds (Ahmadi *et al.*, 2013; Ali *et al.*, 2017; Abedini *et al.*, 2018; Hafez *et al.*, 2019). The average size of nanoparticles was 97.5nm (Fig. 4.2), falling under the category of nanoparticles. Salem *et al.* (2015) had used Zn NPs against *E. coli* in their in-vitro study with the average particle size of 120–169nm. Most often ZnO NPs size ranges between 90-100nm (Souza *et al.*, 2019), and they had found 0.05mg/mL and 0.5 mg/mL MBC against *S. aureus* and *S. Typhimurium*, respectively with this nanorod size.

For this study, nanoparticles were synthesized in-house but were characterized by Zeta sizer, Scanning Electron Microscopy and X-ray Diffraction for quality assessment; similarly, in the absence of available *E. coli* isolates to induce infection, we isolated the *E. coli* from broiler birds suspected of colibacillosis. Thus, pathogenicity of isolated *E. coli* was confirmed before preparing inoculum. Pink colonies of isolated *E. coli* used for inoculum, appeared on MacConkey agar, confirming the lactose fermentation as justified by Jacob *et al.* (2020). Apart from pathogenicity confirmation through PCR, in-vivo pathogenicity testing revealed the fibrinous pericarditis and perihepatitis, which are considered to be gross lesions of colibacillosis (Daud *et al.*, 2014; Zahid *et al.*, 2016). In our study, the concentration of *E. coli* at 10^7CFU/mL was used to prepare inoculum to test in-vivo pathogenicity. Earlier, Hassanin *et al.* (2014) had used $10^{7.5}$ CFU/mL of inoculum, while Rao *et al.* (1999) injected 0.1ml of 10^{-4} dilution of *E. coli* (serotype O2) after incubation of 18 hours in nutrient broth, to induce colibacillosis in broiler birds. Clinical signs of colibacillosis as dyspnea and greenish diarrhea appeared after 42 hours of injection, earlier than the reported incubation period (3-5 days) of *E. coli* in poultry (Daud *et al.*, 2014).

A possible reason for the earlier appearance can be attributed to route of infection, i.e., intramuscular in our study. For in-vitro pathogenicity confirmation, red color colonies of avian pathogenic *E. coli* on Congo red agar, has been considered as epidemiological marker to distinguish between pathogenic and non-pathogenic *E. coli* (Zahid *et al.*, 2016).

RBC levels in groups treated with 50 and 75mg/kg were significantly lower at the 1st sampling but became

non-significantly lower at the 2nd sampling compared to control negative. At the 2nd sampling, RBC levels in colistin, ZnO NPs 75, and 100mg/kg groups were significantly higher than control positive. A similar increase in RBCs was observed in colibacillosis in turkeys (Ahmed and El Nabarawy, 2013) and Fish *Labeo rohita* treated with zinc oxide nanoparticles (Thangapandiyan and Monika, 2019).

At the 1st sampling, all groups had non-significant differences in hematocrit levels, but at the 2nd sampling, it was significantly higher in ZnO NPs 75 and 100mg/kg treated groups than control positive, showing the improvement in treated groups. The significant increase in hemoglobin in colistin, ZnO NPs 75 and 100mg/kg treated groups than control positive also aligns with previous findings of increased Hb due to ZnO NPs in fish (Thangapandiyan and Monika, 2019).

Regarding bacterial infection, total leucocytic count is a useful parameter as this is always increased in colibacillosis (Hassan *et al.*, 2013). At the 1st and 2nd sampling, control positive had significantly higher WBCs than control negative while colistin and ZnO NPs 100mg/kg treated groups showed significantly lower levels than control positive group. There were significantly higher lymphocytes and monocytes while significantly lower heterophils in colistin, ZnO NPs 75 and 100mg/kg treated groups. Previous studies also reported the same results in poultry with ZnO NPs supplementation (Abedini *et al.*, 2018; Hafez *et al.*, 2019). Apart from cellular component of blood, there are always changes in serum constituents. So, serum total proteins, serum albumin and serum globulins were determined to see their level in colibacillosis as well as the effect of colistin and zinc oxide nanoparticles in broilers. Groups treated with ZnO NPs at 100mg/kg had significantly ($P<0.05$) lower total proteins than untreated groups while there was a non-significant ($P>0.05$) difference from control negative. Serum total proteins are also significantly higher in case of calf colibacillosis (Singh *et al.*, 2014). So, this could be an indication that ZnO NPs affected avian colibacillosis. The serum albumin levels in the positive control and ZnO NPs treated groups also support this indication i.e., the group treated with ZnO NPs at 100mg/kg showed a non-significant difference initially but later significantly lower albumin than the untreated group. Lipid profile was not significantly affected in this study. Nanoparticles treatment might primarily influence the immune response to infection rather than directly modulating lipid metabolism.

Immunological parameters studied include humoral response through NDV titers, cell mediated response through carbon clearance assay/phagocytic activity analysis and avian tuberculin response/lymphoproliferative response. Although, there was non-significant ($P>0.05$) difference of NDV titers among all groups at day 14 (pre-infection), 21 (3rd day post-infection), day 27 (8th day post-infection) and day 20 (11th day post-infection) but the group treated with colistin and ZnO NPs at 100mg/kg/day had a protective level even on day 8 and 11 post-infection. According to Bami *et al.* (2018) ZnO NPs at a dose of 50mg/kg had shown the enhanced humoral response; however, in our investigation, infection was also induced, so the protective titers were only obtained at a dose of 100mg/kg/day. Additionally, Abedini *et al.* (2018) reported a significant ($P<0.05$) increase in NDV titers in the ZnO NP-treated group compared to the control group. Again, the same explanation may account for our study's non-significant difference from the negative control. The groups treated with colistin and ZnO NPs at 50, 75, and 100 mg/kg/day showed significantly higher phagocytic activity ($P<0.05$) compared to the untreated group (control positive). The phagocytic response at ZnO NPs 80 mg/kg was also significantly higher than that at 40 mg/kg, as reported by Hafez *et al.* (2019). This cellular response due to use of zinc oxide nanoparticles may be attributed to role of zinc (Zn) in enhancing nutrient absorption and assimilation, antioxidant defense, and endocrine status in poultry nutrition (Naz *et al.*, 2016). In contrast to phagocytic activity, the lymphoproliferative response was significantly ($P<0.05$) higher in the group treated with the highest level of ZnO NPs (100mg/kg/day) than in the control group. However, the increase was non-significant ($P>0.05$) in the groups treated with 50 and 75 mg/kg ZnO NPs. Similar to phagocytic activity, our findings about the lymphoproliferative response following a 24-hour injection were also supported by Abedini *et al.* (2018) and Hafez *et al.* (2019).

Anatomical pathology (histopathology of immune organs) has been quantified in this study using Qupath™ software. In this way, histopathology results have been described statistically. In the spleen, lymphocytes decreased significantly in the positive group, while the colistin-treated group had higher counts than the positive control; no significant difference in lymphocyte counts was observed among groups treated with 50, 75, and 100mg/kg ZnO NPs. Severe lymphocytic depletion had been seen in colibacillosis affected birds by Abalaka *et al.* (2017). Similarly, Nakamura *et al. (*1986) also clearly reported the transient lymphocytic depletion in lymphoid tissues of *E. coli* induced infected chicken. Therefore, the non-significant ($P<0.05$) difference between control negative group and colistin, 75 and 100mg/kg ZnO NPs treated groups can be considered as indication of treatment response against induced colibacillosis in broilers.

Further investigation of the histopathology of other immune organ provides insights on increased number of lymphocytes due to ZnO NPs. In the thymus, lymphocytes significantly decreased in the positive group compared to the negative control and colistin-treated group. Although no significant difference was observed in groups treated with 50, 75, and 100mg/kg ZnO NPs when compared to the positive control, all groups treated with colistin, 50, 75 and 100mg/kg ZnO NPs, besides control negative group, had significantly lower congested area percentage in thymus than control positive group. When compared to the colistin-treated group, the group treated with 50mg/kg ZnO NPs demonstrated a significantly ($p<0.05$) higher congested area, which indicates that a higher level of ZnO NPs were more protective. Bursa of Fabricius from positive control group had significantly ($P<0.05$) decreased/lowered lymphocytes as compared to control negative groups treated with colistin, 75 and 100mg/kg ZnO NPs, while there was non-significant ($P>0.05$) difference between the positive control and the 50mg/kg ZnO NPs treated group. Like other immune organs, severe

lymphocytic depletion with increased interfollicular space had been seen in untreated group. Wani *et al.* (2017) also revealed the depletion as well as congestion in thymus with increased interfollicular space in bursa of Fabricius of birds affected with colibacillosis.

However, in our study even in the presence of induced infection apart from adequate immune response there were minor lymphocytic depletion in the immune organs from ZnO NPs treated birds. It has already been reported that ZnO NPs support the body of the chicken for significant increased immune response (Naz *et al.*, 2016). Therefore, in the current study, survival of birds in ZnO nanoparticle treated groups having comparable hematological, biochemical, immunological and pathological parameters with non-infected birds can be attributed to increased immune response due to given ZnO NPs. The zinc oxide nanoparticles were not soluble in water and therefore were given orally, which was a source of stress for the birds. There is possibility to synthesize the water-soluble nanoparticles to address the limitation of this study.

Conclusion: From this study, it can be concluded that *E. coli* induced infection in broilers can be treated with zinc oxide nanoparticles @ 100mg/kg with particle size ranging 90-100nm. Results of zinc oxide nanoparticles were comparable with Colistin when used against *E. coli* induced infection in broilers. Zinc oxide nanoparticles could be a possible substitute for antibiotics to treat *E. coli* induced infection in broilers. Now, levels more than 100mg/kg should be assessed, testing the toxicity level of ZnO-NPs. There is also the need to prepare a type of nanoparticle which can be dissolved in water.

Authors' contributions: Conceptualization: MTJ; Methodology: SUKB, MHA, MET, MHT and SZ; Resources (Nanoparticles synthesis support): HA; Supervision: MTJ; Writing – original draft: SUKB, MHA, MET and MHT; Writing – review & editing: RHA, RA and MS.

Acknowledgement: We are highly thankful to Fiaz Ahmad Chatha (GM Sales and Marketing at Mukhtar Feeds Pvt. Ltd. Pakistan) and Manshad Bashir (GM Marketing Unique Feeds Pvt. Ltd. Pakistan), for their support in providing the poultry feed.

REFERENCES

Abalaka S, Sani N, Idoko I, *et al.*, 2017. Pathological changes associated with an outbreak of colibacillosis in a commercial broiler flock. Sokoto J Vet Sci 15:95-102. DOI: 10.4314/sokjvs.v15i3.14.

Abedini M, Shariatmadari F, Karimi Torshizi MA, *et al.*, 2018. Effects of zinc oxide nanoparticles on egg quality, immune response, zinc retention, and blood parameters of laying hens in the late phase of production. J Anim Physiol Anim Nutr 102:736-745. https://doi.org/10.1111/jpn.12871.

Ahmadi F, Ebrahimnezhad Y, Sis NM, *et al.*, 2013. The effects of zinc oxide nanoparticles on performance, digestive organs, and serum lipid concentrations in broiler chickens during the starter period. Int J Biosci 3:23-29. http://dx.doi.org/10.12692/ijb/3.7.23-29.

Ahmed MH, Javed MT, Bahadur SUK, *et al.*, 2022. Antibacterial effects of copper oxide nanoparticles against *E. coli* induced infection in broilers. Appl Nanosci 12:2031-2044.

Ahmed TI, El Nabarawy E, Aly Salah B, *et al.*, 2013. Effect of apramycin on pathological, hematological, and biochemical changes in turkeys infected with colibacillosis. Zagazig Vet J 41:124-136.

Ali S, Masood S, Zaneb H, *et al.*, 2017. Supplementation of zinc oxide nanoparticles has beneficial effects on intestinal morphology in broiler chicken. Pakistan Vet J 37:335-339.

Bami MK, Afsharmanesh M, Salarmoini M, *et al.*, 2018. Effect of zinc oxide nanoparticles and Bacillus coagulans as probiotic on growth, histomorphology of intestine, and immune parameters in broiler chickens. Comp Clin Pathol 27:399-406. https://doi.org/10.1007/s00580-017-2605-1.

Bancroft J and Stevens A, 1996. Theory and practice of histological techniques. Churchill Livingstone, New York.

Coles EH, 1986. Veterinary Clinical Pathology. 4th ed. W.B. Saunders, Philadelphia.

Corrier DE, 1990. Comparison of phytohemagglutinin-induced cutaneous hypersensitivity reactions in the interdigital skin of broiler and layer chicks. Avian Dis 34:369-373.

Daud NHA, Htin N, Abba Y, *et al.,* 2014. An outbreak of colibacillosis in a broiler farm. J Vet Adv 4:646. DOI: 10.36478/javaa.2014.545.548.

Dufour ZL, 2008. A laboratory manual for the isolation, identification, and characterization of avian pathogens. American Association of Avian Pathologists.

Eid S, Tolba HMN, Hamed RI, *et al.,* 2022. Bacteriophage therapy as an alternative biocontrol against emerging multidrug resistant *E. coli* in broilers. Saudi Journal of Biological Sciences 29(5):3380-3389. https://doi.org/10.1016/j.sjbs.2022.02.015.

Eltai NO, Abdfarag EA, Al-Romaihi H, *et al.,* 2018. Antibiotic resistance profile of commensal *Escherichia coli* isolated from broiler chickens in Qatar. J Food Prot 81:302-307. https://doi.org/10.4315/0362-028X.JFP-17-191.

Epoke J, Igumbor E, Asuquo A, *et al.,* 2003. Locally prepared antibiotic sensitivity discs: a substitute for imported commercial discs. Global J Pure Appl Sci 9:453-456. DOI: 10.4314/gjpas.v9i4.16051.

Hafez A, Nassef E, Fahmy M, *et al.,* 2019. Impact of dietary nano-zinc oxide on immune response and antioxidant defense of broiler chickens. Environ Sci Pollut Res 27(16):19108-19114.

Hassan N, Sheikh G, Malik H, *et al.,* 2013. Hemato-biochemical and therapeutic studies on s*Escherichia coli* associated with concurrent enteric infection in lambs. Vet World 6:870.

Hassanin O, Abdallah F, Awad A, 2014. Effects of florfenicol on the immune responses and the interferon-inducible genes in broiler chickens under the impact of *Escherichia coli* infection. Vet Res Commun 38:51-58. DOI: 10.1007/s11259-013-9585-7.

Hudson L, Hay FC, 1989. Practical Immunology, 3rd edition. Blackwell Scientific Publications, Oxford, pp. 71-73.

Jacob ME, Keelara S, Aidara-Kane A, *et al.,* 2020. Optimizing a screening protocol for potential extended-spectrum β-lactamase *Escherichia coli* on MacConkey agar for use in a global surveillance program. J Clin Microbiol 58:01039-01019. https://doi.org/10.1128/JCM.01039-19.

Jeong YW, Kim TE, Kim JH, *et al.,* 2012. Pathotyping avian pathogenic *Escherichia coli* strains in Korea. J Vet Sci 13:145-152. https://doi.org/10.4142/jvs.2012.13.2.145.

Joseph J, Zhang L, Adhikari P, *et al.,* 2023. Avian Pathogenic *Escherichia coli* (APEC) in broiler breeders: An overview. Pathogens 12(11):1280. https://doi.org/10.3390/pathogens12111280.

Kazemnia A, Ahmadi M, Dilmaghani M, 2014. Antibiotic resistance pattern of different *Escherichia coli* phylogenetic groups isolated from human urinary tract infection and avian colibacillosis. Iran Biomed J 18:219-224. doi: 10.6091/ibj.1394.2014.

Kuttner T, 1916. Hemoglobin estimation. J Am Med Assoc 66(18):1370-3.

Laurent JW, 2018. Alternatives to common preventive uses of antibiotics for cattle, swine, and chickens. NRDC Report.

Manyasree D, Kiranmayi P, Venkata RK, 2018. Characterization and antibacterial activity of ZnO nanoparticles synthesized by co-precipitation method. Int J Appl Pharmacol 10:224-228. https://doi.org/10.22159/ijap.2018v10i6.29376.

Nakamura K, Imada Y, Maeda M, 1986. Lymphocytic depletion of bursa of Fabricius and thymus in chickens inoculated with *Escherichia coli*. Vet Pathol 23(6):712-717.

Naz S, Idris M, Khalique M, *et al.,* 2016. The activity and use of zinc in poultry diets - ERRATUM. World's Poult Sci J 72:432-432. https://doi.org/10.1017/S0043933916000155.

Niemi JK, 2021. The economic cost of bacterial infections. In: Advancements and Technologies in Pig and Poultry Bacterial Disease Control, PP 1-23. https://doi.org/10.1016/B978-0-12-818030-3.00010-6

Nolan LK, Barnes HJ, Vaillancourt JP, *et al.,* 2013. Colibacillosis. In: Diseases of Poultry. 751-805.

OIE, 2012. Newcastle disease, Chapter 2.3.14. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 7th edition. Office International des Epizooties (World Organization for Animal Health), Paris, France, pp. 555-574.

Osman K, Elhariri M, 2014. Mass gatherings pose a threat to the spread of antimicrobial resistant *Escherichia coli* strains. Int J Adv Res 2:592-601.

Rao SR, Praharaj N, Reddy M, *et al.,* 1999. Immune competence, resistance to *Escherichia coli*, and growth in male broiler parent chicks fed different levels of crude protein. Vet Res Commun 23:323-336. https://doi.org/10.1023/A:1006318307103.

Salem W, Leitner DR, Zingl FG, *et al.,* 2015. Antibacterial activity of silver and zinc nanoparticles against Vibrio cholerae and enterotoxic *Escherichia coli*. Int J Med Microbiol 305:85-95. https://doi.org/10.1016/j.ijmm.2014.11.005.

SAS Institute Inc, 2018. SAS Software, Version 15.1. Cary, NC: SAS Institute Inc.

Singh M, Gupta V, Mondal D, *et al.,* 2014. A study on alteration in haemato-biochemical parameters in colibacillosis affected calves. Int J Adv Res 2:746-750.

Souza RC, Haberbeck LU, Riella HG, *et al.,* 2019. Antibacterial activity of zinc oxide nanoparticles synthesized by solvothermal process. Braz J Chem Eng 36:885-893. https://doi.org/10.1590/0104-6632.20190362s20180027.

Souza WF, Granjeiro MDB, Procópio DP, 2019. Analysis of the economic loss and the main causes of total condemnation of poultry carcasses under Brazilian federal inspection between 2013 and 2017. Arch Vet Sci 24(4):36-49.

Tayel AA, El-Tras WF, Moussa S, *et al.,* 2011. Antibacterial action of zinc oxide nanoparticles against foodborne pathogens. J Food Safety 31:211-218. https://doi.org/10.1111/j.1745-4565.2010.00287.x.

Thangapandiyan S, Monika S, 2019. Green synthesized zinc oxide nanoparticles as feed additives to improve growth, biochemical, and hematological parameters in freshwater fish Labeo rohita. Biol Trace Elem Res 195:1-12.

Thongratsakul S, Amavisit P, Poolkhet C, 2023. Antimicrobial resistance in poultry farming: A look back at environmental *Escherichia coli* isolated from poultry farms during the growing and resting periods. Vet Med Int 8354235:1-8.

Wang C, 2012. Antibacterial effects of zinc oxide nanoparticles on *Escherichia coli* K88. J Appl Microbiol 11:1193-1201.

Wani BM, Darzi MM, Mir MS, et al., 2017. Pathological and pharmacochemical evaluation of broiler chicken affected naturally with colibacillosis in Kashmir valley. Int J Pharmacol 13:388-395. DOI: 10.3923/ijp.2017.388.395.

Yegani M, Korver DR, 2010. Application of egg yolk antibodies as replacement for antibiotics in poultry. World's Poult Sci J 66:27-38. https://doi.org/10.1017/S0043933910000048.

Zahid A, AL-Mossawei M, Mahmood AB, 2016. In vitro and in vivo pathogenicity tests of local isolates APEC from naturally infected broiler in Baghdad. Int J Adv Res Biol Sci 3:89-100. http://s-o-i.org/1.15/ijarbs-2016-3-3-12.