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Evaluation of zinc oxide and copper oxide nanoparticles as potential alternatives to antibiotics for managing fowl typhoid in broilers

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Abstract

Antimicrobial resistance poses challenges to humans and animals, especially to the poultry sector in control of fowl typhoid with antibiotics, leading to increased mortality and food insecurity. Therefore, it is essential to develop more effective medications as alternatives to antibiotics. Currently, zinc oxide and copper oxide nanoparticles are of such significant interest due to their antibacterial properties. This study aimed to evaluate antimicrobial activity of zinc oxide and copper oxide nanoparticles against fowl typhoid in broilers. Ninety broiler chicks were raised under suitable management conditions. On day 10 of age, chicks were divided into six groups: control negative, control positive, T_1, T_2, T_3, and T_4. On day 19 of age, chicks in all groups except control negative were infected with *Salmonella gallinarum* (0.2 mL, 10^8 CFU/mL). After appearance of clinical signs, the treatments (Florfenicol; 50 mg/L drinking water [T_1], and zinc oxide + copper oxide nanoparticles; 25 + 10 mg/kg/d [T_2], 37.5 + 15 mg/kg/d [T_3], and 50 + 20 mg/kg/d [T_4]) were administered to chicks. Chicks were sacrificed on 26th and 30th day of age, and samples of blood and tissue were obtained. Hematological analysis with gross and histopathological examination of spleen, thymus and bursa of Fabricius was performed. Results revealed that there was no visible congestion in spleen and thymus of T_3 and T_4 at 11th day post infection. Antibody level against new castle's disease and lymphoproliferative response showed no significant difference in all groups. However, phagocytic response in nanoparticles treated groups exhibited a notable ($p < 0.01$) distinction compared to control positive. Notably, T_3 demonstrated the highest level of phagocytic activity. Hematological parameters, including lymphocytes, heterophils, eosinophils, and heterophils/lymphocytes ratio in groups T_2, T_3, and T_4, indicated significant ($p < 0.01$) difference compared to control positive. However, lymphocytes, heterophils, and heterophils/lymphocytes ratio in groups T_2, T_3, and T_4 showed no significant difference when compared to T_1. Nanoparticle treated groups

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Availability of data and material
Upon reasonable request, the datasets of this study can be available from the corresponding author.

Authors' contributions
Conceptualization: Raza MA, Javed MT, Kim MO.
Data curation: Kim E, Fiaz M.
Formal analysis: Shakeel M, Park K.
Methodology: Javed MT.
Software: Ma L, Kim H, Kim CY, Liu Z, Huang K, Park K.
Validation: Raza MA.
Writing - original draft: Raza MA.
Writing - review & editing: Raza MA, Kim E, Shakeel M, Fiaz M, Ma L, Kim H, Kim CY, Liu Z, Huang K, Park K, Javed MT, Kim MO.

Ethics approval and consent to participate
The animal care and experimental protocols used in the present study were approved by the Graduate Study Research Board, in accordance with the guidelines of Institution of Animal Care and Use Committee, University of Agriculture Faisalabad, Pakistan (approval number: DGS/.7049-52).

showed decreased ($p < 0.01$) congestion of spleen and thymus as compared to control positive. Overall, zinc oxide and copper oxide nanoparticles have potential to serve as an alternative to florfenicol in treatment of fowl typhoid.

Keywords: Antimicrobial resistance, *Salmonella gallinarum*, Infection, Poultry health, Immunology

INTRODUCTION

The poultry sector has emerged as a vital industry in growing economies [1]. It not only meets the daily protein requirements of the growing population but also provides opportunities for employment [2] through the supply of high-quality food items such as chicken meat and eggs, ensuring global food security and nutrition [3,4]. Poultry meat and eggs are important for human health because the protein and vitamins they contain play a crucial role in the development of immunity [5].

However, the poultry industry faces several challenges that jeopardize economic output and the health of animals and humans in many countries [6]. Poultry mortality is one of the main issues that hinder continuous food supply to the population and high mortality may be largely attributed to the spread of infectious diseases [7]. Fowl typhoid is a septicemic disease of poultry caused by gram negative bacterium *Salmonella gallinarum* which produces the endotoxins in the blood circulation of host. The disease is associated with substantial losses to the country's economy through high mortality and decreased egg production [8]. Infectious diseases like salmonellosis, new castle's disease (NDV), infectious bursal disease etc. are highly occurring diseases in poultry around the world causing high mortality in the birds [9]. These diseases are associated with the immunosuppression of the affected birds by damaging the immune organs leading to mortality [10]. The birds affected by fowl typhoid manifest clinical signs like depression, pale and shrunken comb, ruffled feathers, anorexia, dyspnea, huddling, diarrhea, and adherence of the excreta to the vent [8], and inflammation in the liver, spleen, cecum, and yolk sac [11]. The morbidity rate is very high, resulting in 93%–100% mortality occurring when the birds are infected with a bacterial load of 10^8 colony forming unit per milli liter (CFU/mL) [12]. Therefore, it is necessary to reduce the mortality in poultry from infectious diseases.

Antibiotics are widely used for the prevention and treatment of various infectious diseases as well as growth promoters in poultry [13]. Although antimicrobial agents play a vital role in the control of morbidity and mortality in animals as well as humans, the extensive use of these agents has led to the generation of antimicrobial resistance (AMR) in pathogenic bacteria [14]. The irrational or irresponsible use of different antibiotics, especially florfenicol, promotes the prevalence of AMR in poultry farms [15]. The emergence and transmission of AMR strains of different bacteria not only affects poultry production but also threatens human health [16]. The AMR salmonella species can be transferred to humans while handling or slaughtering the infected birds which leads to human illness [17]. Therefore, the presence of AMR in animals raised for food is a significant concern [18] as it poses a substantial zoonotic risk to human health. This is especially true considering the abundance of AMR bacteria such as Salmonella, Campylobacter, and Listeria [19]. Hence, there is an urgent requirement to develop alternative therapeutic treatments that can replace antibiotics.

Nanotechnology is a new field of science with extensive applications in the development of nanomedicine [20]. Several metals including zinc oxide (ZnO) and copper oxide (CuO) nanoparticles have excellent antibacterial activity against gram-positive and gram-negative bacteria [21]. Hameed et al. [22] reported that ZnO nanoparticles can inhibit the growth of *Escherichia coli* and *Klebsiella pneumoniae* on the culture plates and increased the zone of inhibition. Similarly, the

in vitro antibacterial activity of ZnO nanoparticles against *E. coli, Enterobacter aerogenes, Micrococcus luteus,* and *Bacillus subtilis* were also documented [23]. Recently, the in vitro antibacterial activity of ZnO and CuO nanoparticles has been studied against *B. subtilis, E. coli, Staphylococcus aureus*, *Salmonella typhimurium,* and *Pseudomonas aeruginosa* [24]. Kim et al. [25] reported that pigs treated with nano ZnO showed increased average daily weight gain and decreased incidence of fecal score and diarrhoea. Nano ZnO also inhibited the colonization of *E. coli, S. typhimurium,* and *Listeria monocytogenes*.

The gram-positive and gram-negative bacteria have different structures of cell wall. The gram-positive bacteria have a thick layer of peptidoglycan in the cell wall while the gram-negative bacteria have thin layer of peptidoglycan and an additional layer of lipopolysaccharide molecules in the cell wall which carry a negative charge. The negative charge have more affinity for positive ions released from nanoparticles, causing an increased uptake of ions leading to intracellular damage in bacterial cell [26]. The antibacterial activity of ZnO and CuO nanoparticles is due to the generation of free radicals and reactive oxygen species that bind to the bacterial cell wall and cause bacterial cell destruction [27]. The CuO nanoparticles are extremely reactive because of their high surface area to volume ratio which improves their antimicrobial efficiency [6]. The metal oxides show antibacterial properties by generating reactive oxygen species and free radicals. The oxygen reacts with the CuO and forms cupric ion (Cu^{2+}); the cation reacts with superoxide ion (O_2^-), leading to oxidative stress. The O_2^- ion reduces the Cu^{2+} ion to cuprous ion and produces hydrogen peroxide (H_2O_2) which reacts with copper and again produces hydroxyl ion. Similarly, ZnO nanoparticles also produce H_2O_2 and O_2^- ion. The H_2O_2 penetrates the bacterial cells and causes cellular membrane damage, lipid peroxidation, and ultimately bacterial cell growth inhibition and bacterial cell destruction by damaging the cellular components such as deoxyribose nucleic acid and proteins [26,28,29]. Supplementation of nano copper to the poultry diet can improve the daily weight gain, erythrocyte count, and haematocrit level in chicken [30]. Recently, Kim et al. [31] reported that copper is frequently used as growth promoter in monogastric animals. Copper can shift intestinal microbiota in pigs which may be attributed with its antimicrobial activities [32]. It is anticipated that nanoparticles will become the most appropriate antibacterial drugs in the future. Therefore, this study was designed to determine the antibacterial effects of ZnO and CuO nanoparticles in *S. gallinarum* induced infection in broilers in terms of their hematological, pathological, and immunological parameters.

MATERIALS AND METHODS

Ethics approval and consent to participate

The animal care and experimental protocols used in the present study were approved by the Graduate Study Research Board, in accordance with the guidelines of Institution of Animal Care and Use Committee, University of Agriculture Faisalabad, Pakistan (approval number: DGS/.7049-52).

Experimental birds and study plan

A total of 90, one-day-old broiler chicks (Hubbard) were selected for this study. The birds were kept under the same environment and management conditions for the first 18 days. The ZnO and CuO nanoparticles were prepared at the Department of Physics, University of Agriculture Faisalabad (Pakistan). The chemicals cupric chloride ($CuCl_2$), zinc sulphate ($ZnSO_4$) and sodium hydroxide (NaOH) were kindly provided by Dr. Muhammad Yasir Javed (Department of Physics, University of Agriculture Faisalabad, Pakistan) for the preparation of ZnO and CuO nanoparticles. The CuO

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nanoparticles were prepared by co-precipitation method as previously documented by Phiwdang et al. [33] using $CuCl_2$ and NaOH as precursor. Briefly, $CuCl_2$ (1 M) was dissolved in distilled water (1 L) and constantly stirred at magnetic stirrer until completion dissolution of $CuCl_2$. After, NaOH (1 M) was added gently drop by drop under vigorous stirring on magnetic stirrer. The black precipitates of CuO were obtained and washed with distilled water several times. Later, the washed precipitates were dried in oven at 80℃ overnight and dried product was kept in muffle furnace (500℃) for 4 hours. Finally, CuO was crushed into fine powder. The size and purity of CuO nanoparticles used in the present study were 33.20 nm and 99.9% respectively, as the nanoparticles were from same batch already reported by our research group [6]. The ZnO nanoparticles were prepared by co-precipitation method as previously documented by D et al. [34] using $ZnSO_4$ and NaOH as precursor. Briefly, $ZnSO_4$ (1 M) was dissolved in distilled water (1 L) and constantly stirred at magnetic stirrer for 1 hour. After complete dissolution of $ZnSO_4$, NaOH (2 M) solution was added drop by drop under continuous stirring conditions for two hours. Subsequently, a white creamy suspension was formed and was allowed to settle overnight. The precipitate was several times with distilled water and dried in the oven at 80℃. During drying, zinc hydroxide is completely converted into ZnO. The ZnO was kept in the muffle furnace (500℃) for 4 hours. Finally, ZnO was crushed into fine powder. The size and purity of ZnO nanoparticles used in the present study were 97.5 nm and 99.9% respectively, as the nanoparticles were from same batch already documented by Bahadur [35].

The birds were divided (15 birds/group) into six groups: control negative, control positive, T_1 (Florfenicol; 50 mg/L drinking water), T_2 (ZnO nanoparticles; 25 mg/kg + CuO nanoparticles; 10 mg/kg), T_3 (ZnO nanoparticles; 37.5 mg/kg + CuO nanoparticles; 15 mg/kg), and T_4 (ZnO nanoparticles; 50 mg/kg + CuO nanoparticles; 20 mg/kg). The birds were maintained in six individual compartments with wood shavings as litter material. On day 19, the birds of all the groups except the control negative were orally infected with *S. gallinarum* at a dose of 10^8 CFU/mL as shown in the experimental design (Fig. 1). All birds were provided with clean water and commercially available feed *ad libitum* throughout the study. The treatments were given to the birds three days post-infection (after the appearance of clinical signs).

Parameters and data collection

The birds were sacrificed on day 26 and 30 of the study. The blood samples were collected in ethylene diamine tetra acetic acid vacutainers (LOT: 07072014, Lab Vac, Bayswater North VIC, Australia).

Gross pathology and histopathology of spleen, thymus, bursa of Fabricius

Spleen, thymus, and bursa of Fabricius were isolated after sacrifice and inspected for abnormal morphology changes. The scoring of the congestion was performed using an arbitrary scoring system. The congestion was described as none (–), mild (+), moderate (++), or severe (+++).

The tissue samples were taken spleen, thymus, and bursa of Fabricius, cut into small pieces of 2–3 cm with a thickness of 1–2 mm, and placed in containers with 10% neutral buffered formalin solution for fixation, followed by histopathological examination. The tissue samples were dipped in a series of ethanol solutions with different concentrations. After the tissue samples were cleaned with xylene-I and xylene-II to remove the dehydrating agent. Finally, the tissue section slides were prepared, and staining was done. The previously described protocols [36] were used for the processing of the tissue sections and staining with hematoxylin and eosin stains. The quantitative analysis of the histopathological slides was analyzed by using QuPath™ 0.2.2. Software. The lymphocytes were counted in spleen, thymus, and bursa of Fabricius. The congestion percentage was

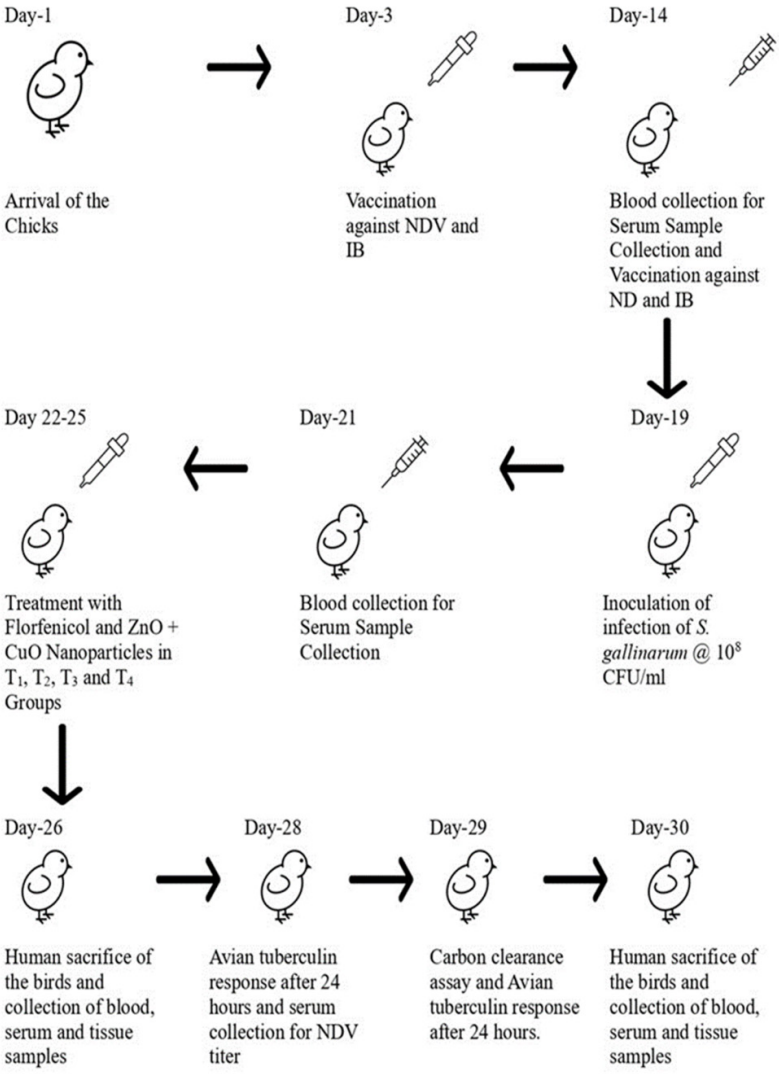

Fig. 1. Experimental design operation layout.

determined in the spleen and thymus. The interfollicular space in the bursa of Fabricius was also determined.

Immunological and hematological parameters

The antibody titer against NDV was determined by performing the hemagglutination and hemagglutination inhibition tests as previously described [37]. The phagocytic activity of the macrophages present in the blood of the infected birds was determined by a carbon clearance assay, as previously described [38]. 1 mL of Pelican® Black Indian No. 4001 was injected into the wing vein of the birds. 0.2 mL of blood was collected at 0, 3, and 15 minutes intervals and added to 4 mL of 0.1% sodium citrate solution in a 15 mL falcon tube. Centrifuged at 6,000×g for 4 minutes. 50 μL of supernatant was transferred to a 96 well plate, and the optical density value was determined at 650 nm. The lymphoproliferative response against avian tuberculin was determined as previously described [39] by injecting 0.1 mL avian tuberculin into the interdigital space of the right claw of the bird and 0.1 mL normal saline into the interdigital space of the left claw and comparing their

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immune responses. The hematological parameters (complete blood count) were determined as previously described [40].

Statistical analysis

The statistical analysis of the collected data was performed using the complete randomized design through the analysis of variance technique and Tukey's test was performed for the comparison of the group mean values using SAS® University edition online software SAS 15.1. p values < 0.01 and < 0.05 were considered significantly different.

RESULTS

Hematological parameters at day 7 and 11 post-infection

A complete blood count analysis of the blood samples infected from *S. gallinarum* was performed to find the effects of ZnO and CuO nanoparticles. The antibacterial effect of different levels of the ZnO and CuO nanoparticles and florfenicol on the *S. gallinarum* induced infection in the broilers in terms of the hematological parameters is presented in Table 1.

On day 7 post-infection, the influence of the various levels of ZnO and CuO nanoparticles (T_2, T_3, T_4) on the total erythrocyte count, basophils, mean corpuscular volume, and mean corpuscular hemoglobin concentration showed no significant differences with that of the control negative and group T_1. The total leukocyte count of T_3 and T_4 was not significantly different from group T_1, however, significantly different ($p < 0.01$) from control positive, while that of T_2 was significantly different ($p < 0.01$) as compared to T_1. The hematocrit level of group T_1 and groups T_2, T_3, and T_4 was not significantly different. The lymphocyte, heterophil, monocyte, and eosinophil percentages, and heterophils to lymphocyte (H/L) ratio of groups T_2, T_3, and T_4 were not significantly different from that of group T_1, however, lymphocyte percentage of T_2, T_3, and T_4 was found significantly different ($p < 0.01$) as compared to that of control positive. At day 11 post-infection, the influence of the various levels of ZnO and CuO nanoparticles (T_2, T_3, and T_4) on the total erythrocyte count, total leukocyte count, hematocrit level, hemoglobin concentration, basophils, mean corpuscular hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin concentration was not significantly different from that of groups; control negative, control positive, and T_1. The heterophil and eosinophil percentage of groups; T_2 and T_3 were significantly different ($p < 0.01$) as compared to that of control positive. The heterophil, monocyte, and eosinophil percentages of groups T_2, T_3, and T_4 were not significantly different from that of group T_1. The lymphocyte percentage and H/L ratio of groups; T_2, T_3 and T_4 was found significantly different ($p < 0.01$) to that of control positive and not significantly different to that of groups; control negative and T_1.

Immunological parameters

The influence of ZnO and CuO nanoparticles on the immune parameters of the *S. gallinarum* infected birds was evaluated in terms of antibody titer against NDV, lymphoproliferative response of lymphocytes, and phagocytic power of the macrophages. The log antibody titer against NDV (on days 14, 21, and 28) and lymphoproliferative response of lymphocytes against avian tuberculin (at 24, 48 and 72 hours post injection of avian tuberculin) was not significantly different in the treatment groups (T_1, T_2, T_3, and T_4) including the control negative and control positive as described in Figs. 2 and 3 respectively. After 3 minutes, the phagocytic index was significantly ($p < 0.01$) decreased in the lower and medium treatment groups T_2 and T_3 as compared to that of control positive group and not significantly different to that of T_1 as depicted in Fig. 4A. However, after 15 minutes, the phagocytic index in all groups treated with ZnO and CuO nanoparticles was

Table 1. Antibacterial effect of various levels of mixed zinc oxide (ZnO) and copper oxide (CuO) nanoparticles, and florfenicol against *Salmonella gallinarum* induced infection in broiler in terms of hematological parameters at day 7 and 11 post-infection

Hematological Parameters	Treatments						*p*-value
	Control negative	Control positive	Florfenicol (mg/L)	Nanoparticle levels of ZnO and CuO (mg/kg/d)			
			T_1 (50)	T_2 (25 + 10)	T_3 (37.5 + 15)	T_4 (50 + 20)	
S1							
TEC (×10^6 /μL)	5.57 ± 0.70^{a}	4.04 ± 0.30^{a}	3.81 ± 0.47^{a}	4.5 ± 0.47^{a}	4.98 ± 1.60^{a}	4.6 ± 0.33^{a}	0.098
TLC (×10^3 /μL)	4.47 ± 0.51^{a}	11.9 ± 0.98^{b}	7.03 ± 0.55^{c}	10.03 ± 0.81^{bd}	9.16 ± 0.72^{cd}	8.15 ± 1.55^{cd}	0.000
Hematocrit level (%)	32.25 ± 1.84^{a}	22.6 ± 3.54^{b}	27.5 ± 3.67^{ab}	24.0 ± 2.44^{b}	26.0 ± 4.35^{ab}	22.7 ± 2.52^{b}	0.003
Hemoglobin concentration (μg/dL)	10.4 ± 0.60^{a}	8.76 ± 0.46^{b}	10.3 ± 3.67^{a}	8.4 ± 0.84^{b}	8.1 ± 0.58^{b}	7.8 ± 0.20^{b}	0.000
Lymphocytes (%)	55.02 ± 5.80^{a}	31.34 ± 3.18^{b}	49.67 ± 2.33^{a}	48.7 ± 3.25^{a}	51.74 ± 5.31^{a}	50.9 ± 1.55^{a}	0.000
Heterophils (%)	29.13 ± 1.60^{a}	42.22 ± 4.86^{b}	34.19 ± 2.29^{a}	34.21 ± 1.54^{a}	33.04 ± 4.57^{a}	34.98 ± 2.76^{ab}	0.006
Monocytes (%)	5.55 ± 0.58^{a}	15.29 ± 1.55^{b}	8.97 ± 1.07^{ac}	13.68 ± 4.86^{bc}	11.73 ± 3.39^{bc}	11.29 ± 0.52^{ac}	0.001
Eosinophils (%)	0.53 ± 0.04^{a}	1.29 ± 0.25^{a}	1.65 ± 0.18^{ab}	2.58 ± 0.80^{b}	2.74 ± 0.96^{b}	1.79 ± 0.29^{ab}	0.000
Basophils (%)	1.16 ± 0.25^{a}	0.51 ± 0.13^{a}	0.68 ± 0.02^{a}	0.81 ± 0.50^{a}	0.74 ± 0.78^{a}	1.01 ± 0.62^{a}	0.636
Heterophil lymphocyte ratio	0.53 ± 0.09^{a}	1.35 ± 0.27^{b}	0.68 ± 0.02^{a}	0.7 ± 0.04^{a}	0.64 ± 0.14^{a}	0.68 ± 0.07^{a}	0.000
MCH (pg)	18.72 ± 1.71^{a}	21.7± 1.42^{ab}	27.22 ± 2.91^{b}	18.78 ± 3.47^{a}	17.01 ± 4.91^{a}	16.9 ± 1.48^{a}	0.004
MCV (fL)	58.33 ± 9.78^{a}	55.79 ± 5.13^{a}	73.04 ± 16.65^{a}	53.58 ± 8.76^{a}	53.61 ± 9.74^{a}	49.27 ± 8.09^{a}	0.540
MCHC (g/dL)	32.34 ± 3.53^{a}	39.13 ± 6.90^{a}	37.89 ± 6.61^{a}	35.01 ± 1.91^{a}	31.43 ± 3.50^{a}	34.63 ± 3.06^{a}	0.255
S2							
TEC (×10^6 /μL)	5.6 ± 0.70^{a}	4.2 ± 0.25^{a}	4.44 ± 0.73^{a}	4.38 ± 1.69^{a}	3.97 ± 1.98^{a}	3.99 ± 0.63^{a}	0.162
TLC (×10^3 /μL)	6.09 ± 0.69^{a}	6.1 ± 0.43^{a}	5.24 ± 0.21^{a}	4.94 ± 0.74^{a}	4.87 ± 1.43^{a}	6.0 ± 0.46^{a}	0.068
Hematocrit level (%)	30.75 ± 4.04^{a}	29.0 ± 3.60^{a}	25.0 ± 6.63^{a}	32.8 ± 2.32^{a}	29.0 ± 2.44^{a}	28.0 ± 1.73^{a}	0.379
Hemoglobin concentration (μg/dL)	10.2 ± 0.34^{a}	8.93 ± 0.46^{a}	10.25 ± 0.46^{a}	10.24 ± 0.77^{a}	9.2 ± 1.23^{a}	10.35 ± 0.46^{a}	0.065
Lymphocytes (%)	56.04 ± 5.33^{a}	31.54 ± 3.04^{b}	49.09 ± 5.49^{a}	49.0 ± 4.38^{a}	49.98 ± 0.74^{a}	48.96 ± 4.69^{a}	0.000
Heterophils (%)	25.37 ± 2.67^{a}	49.09 ± 5.49^{b}	33.11 ± 1.73^{c}	34.48 ± 2.00^{c}	34.96 ± 2.01^{c}	37.06 ± 3.55^{bc}	0.000
Monocytes (%)	8.1 ± 1.65^{a}	13.51 ± 2.59^{b}	8.78 ± 2.33^{ab}	12.06 ± 2.70^{ab}	11.19 ± 2.11^{ab}	10.74 ± 1.32^{ab}	0.030
Eosinophils (%)	0.79 ± 0.13^{ab}	1.89 ± 0.38^{b}	2.03 ± 0.09^{ac}	3.21 ± 0.68^{c}	2.42 ± 0.74^{ac}	1.87 ± 0.69^{ab}	0.000
Basophils (%)	1.26 ± 0.40^{a}	0.92 ± 0.12^{a}	0.71 ± 0.06^{a}	1.23 ± 0.31^{a}	1.44 ± 0.94^{a}	1.35 ± 0.49^{a}	0.224
Heterophil lymphocyte ratio	0.68 ± 0.07^{a}	0.45 ± 0.09^{b}	0.67 ± 0.05^{ac}	0.71 ± 0.09^{ac}	0.69 ± 0.03^{ac}	0.76 ± 0.13^{c}	0.000
MCH (pg)	18.37 ± 2.83^{a}	21.29 ± 1.83^{a}	23.42 ± 4.59^{a}	24.56 ± 7.82^{a}	23.93 ± 6.30^{a}	26.19 ± 3.77^{a}	0.198
MCV (fL)	55.69 ± 13.46^{a}	69.05 ± 8.87^{a}	58.36 ± 27.39^{a}	78.66 ± 24.27^{a}	75.97 ± 23.06^{a}	71.16 ± 13.67^{a}	0.406
MCHC (g/dL)	33.4 ± 3.58^{a}	31.0 ± 2.44^{a}	42.32 ± 10.38^{a}	31.26 ± 2.73^{a}	31.75 ± 3.90^{a}	37.02 ± 2.58^{a}	0.114

$^{a-d}$Mean Values in rows with various superscripts are significantly different ($p < 0.01$) and ($p < 0.05$), S1 (day 7), S2 (day 11) post-infection sampling.

TEC, total erythrocyte count; TLC, total leukocyte count; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration.

significantly ($p < 0.01$) decreased as compared to that of control positive group and not different to that of T_1 as depicted in Fig. 4B.

Pathological parameters

Gross pathology of spleen, thymus, bursa of Fabricius

The scoring of congestion of spleen, thymus, and bursa of Fabricius are shown in Table 2, Figs. 5 and 6. At day 7 and 11 post-infection, the congestion in the spleen and thymus of the control positive was high in comparison to the control negative and treatment groups (T_1, T_2, T_3, and T_4) as shown in Figs. 5 and 6.

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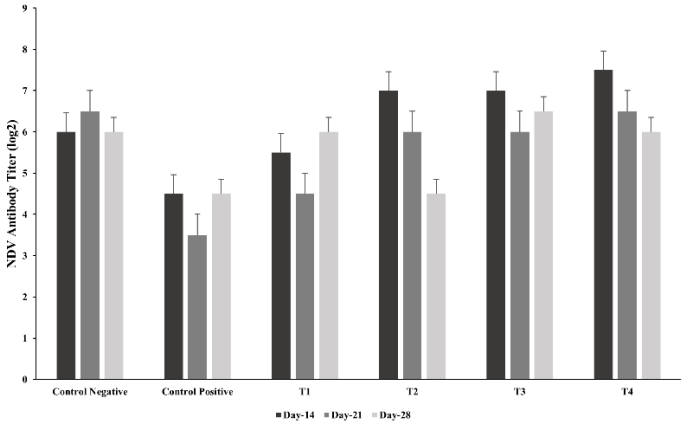

Fig. 2. New castle's disease (NDV) titer of *Salmonella gallinarum* infected birds treated with florfenicol and zinc oxide (ZnO), and copper oxide (CuO) nanoparticles. Groups: Control negative (no infection, no treatment); control positive (*S. gallinarum* infection, no treatment); T_1 (*S. gallinarum* infection and florfenicol treatment at dose rate 50 mg/L in drinking water); T_2 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 25 + 10 mg/kg/d); T_3 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d); T_4 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d). Mean ± SD, n = 3 each group.

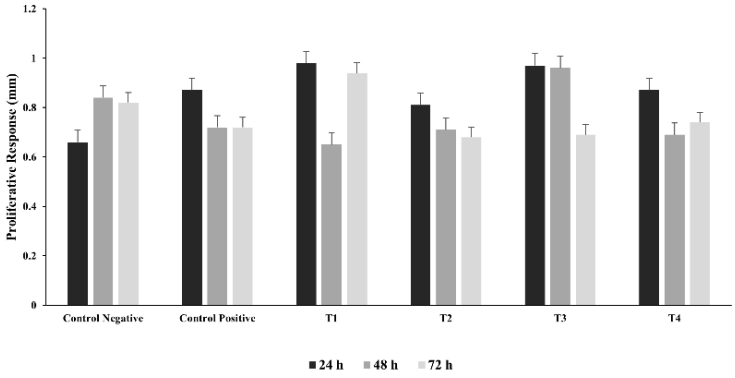

Fig. 3. Lymphoproliferative response (skin thickness in mm) to injection avian tuberculin in *Salmonella gallinarum* infected birds treated with florfenicol, zinc oxide (ZnO), and copper oxide (CuO) nanoparticles. Groups: Control negative (no infection, no treatment); control positive (*S. gallinarum* infection, no treatment); T_1 (*S. gallinarum* infection and florfenicol treatment at dose rate 50 mg/L in drinking water); T_2 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 25 + 10 mg/kg/d); T_3 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d); T_4 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d). Mean ± SD, n = 3 each group.

Histopathology of spleen, thymus, bursa of Fabricius

The congestion, lymphocyte count, and interfollicular bursal space in spleen, thymus, and bursa of Fabricius at day 7 and 11 post-infection is described in Table 3 and Figs. 7, 8, and 9, respectively. At day 7 post-infection, the congestion and lymphocytic count in the spleen and thymus of the ZnO and CuO nanoparticles treated groups; T_2, T_3, and T_4 was not significantly different from that of the control negative and T_1, however, the congestion and lymphocytic count of spleen in ZnO and CuO nanoparticles treated groups; T_2, T_3, and T_4 were significantly ($p < 0.01$) different from that of the control positive. ZnO and CuO nanoparticles treated groups; T_2, T_3, and T_4 were significantly different from that of control positive group in terms of congestion ($p < 0.01$) and lymphocytic

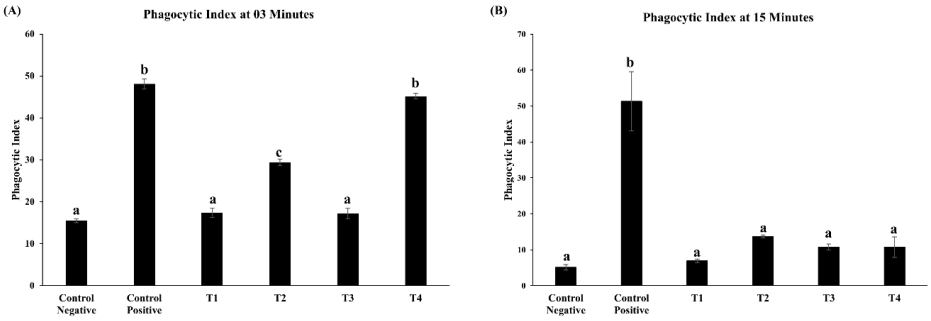

Fig. 4. Phagocytic activity of lymphocytes via carbon clearance assay of *Salmonella gallinarum* infected birds treated with florfenicol, zinc oxide (ZnO), and copper oxide (CuO) nanoparticles. (A) Phagocytic index at 3 minutes. (B) Phagocytic index at 15 minutes. Groups: Control negative (no infection, no treatment); COntrol positive (*S. gallinarum* infection, No Treatment); T_1 (*S. gallinarum* infection and florfenicol treatment at dose rate 50 mg/L in drinking water); T_2 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 25 + 10 mg/kg/d); T_3 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d); T_4 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d). Mean ± SD, n = 3 each group. Values with different letters ($^{a-c}$) indicate a significantly difference ($p < 0.01$) and ($p < 0.05$) phagocytic index.

Table 2. Antibacterial effect of various levels of mixed zinc oxide (ZnO) and copper oxide (CuO) nanoparticles, and florfenicol on *Salmonella gallinarum* induced infection in broiler in terms of scoring of gross pathological lesions (congestion) of thymus, spleen, and bursa of Fabricius at day 7 and 11 post-infection

Organ	Treatments					
	Control negative	Control positive	Florfenicol (mg/L)	Nanoparticle levels of ZnO and CuO (mg/kg/d)		
			T_1 (50)	T_2 (25 + 10)	T_3 (37.5 + 15)	T_4 (50 + 20)
S1						
Spleen	−	+++	++	+	++	++
Thymus	−	+++	++	++	++	+
Bursa of Fabricius	−	++	−	+	++	+
S2						
Spleen	−	++	−	+	−	−
Thymus	−	++	−	+	−	−
Bursa of Fabricius	−	++	−	−	−	−

No congestion (−), mild congestion (+), moderate congestion (++), severe congestion (+++). S1 (day 7), S2 (day 11) post-infection sampling.

count ($p < 0.05$). The interfollicular space of bursa of Fabricius in ZnO and CuO nanoparticles treated groups was significantly ($p < 0.01$) different from that of control positive group, however, it was found not different to that T_1. At day 11 post-infection, the congestion in the spleen and thymus of ZnO and CuO nanoparticles treated groups (T_2, T_3, and T_4) was not significantly different from that of the control negative and T_1, however, was significantly ($p < 0.01$) different from that of the control positive (Table 3). Congestion and lymphocytic depletion in the spleen and thymus of the control positive was observed while the ZnO and CuO nanoparticles treated groups showed decreased ($p < 0.01$) congestion of spleen and lymphocytic depletion (Figs. 7 and 8).

DISCUSSION

Fowl typhoid, caused by gram-negative bacterium *S. gallinarum,* poses a significant economic burden on the global poultry industry [28]. Multiple antibiotics such as florfenicol, ciprofloxacin,

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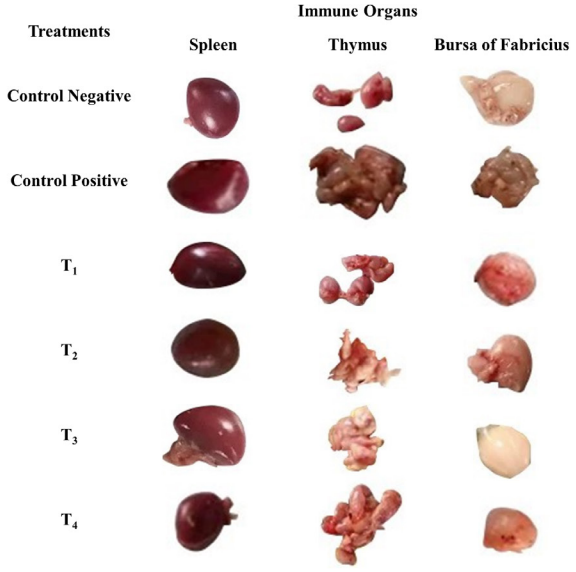

Fig. 5. Antibacterial effect of various levels of mixed zinc oxide (ZnO) and copper oxide (CuO) nanoparticles, and florfenicol on *Salmonella gallinarum* induced infection in broiler in terms of gross pathology of spleen, thymus, and bursa of Fabricius at day 7 post-infection. Groups: Control negative (no infection, no treatment); control positive (*S. gallinarum* infection, no treatment); T_1 (*S. gallinarum* infection and florfenicol treatment at dose rate 50 mg/L in drinking water); T_2 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 25 + 10 mg/kg/d); T_3 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d); T_4 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d).

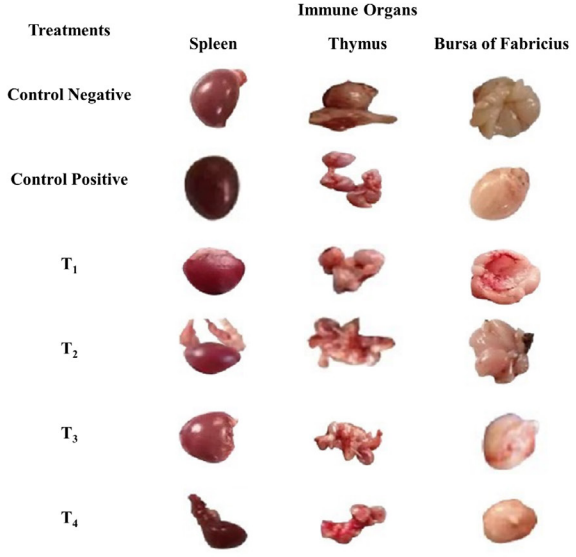

Fig. 6. Antibacterial effect of various levels of mixed zinc oxide (ZnO) and copper oxide (CuO) nanoparticles, and florfenicol on *Salmonella gallinarum* induced infection in broiler in terms of gross pathology of spleen, thymus, and bursa of Fabricius at day 11 post-infection. Groups: Control negative (no infection, no treatment); control positive (S. gallinarum infection, no treatment); T_1 (*S. gallinarum* infection and florfenicol treatment at dose rate 50 mg/L in drinking water); T_2 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 25 + 10 mg/kg/d); T_3 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d); T_4 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d).

Table 3. Antibacterial effect of various levels of mixed zinc oxide (ZnO) and copper oxide (CuO) nanoparticles, and florfenicol on *Salmonella gallinarum* induced infection in broiler in terms of quantitative histopathology of thymus, spleen, and bursa of Fabricius at day 7 and day 11 post-infection

Organ	Parameters	Treatment						*p*-value
		Control negative	Control positive	Florfenicol (mg/L)	Nanoparticle levels of ZnO and CuO (mg/kg/d)			
				T_1 (50)	T_2 (25 + 10)	T_3 (37.5 + 15)	T_4 (50 + 20)	
S1								
Spleen	Congestion (%)	4.33 ± 7.50^{a}	38.02 ± 7.52^{b}	20.62 ± 1.14^{c}	13.38 ± 0.73^{ac}	10.24 ± 0.48^{ac}	13.5 ± 2.74^{ac}	0.000
	Lymphocytes	1,179.67 ± 165.32^{a}	631.33 ± 178.55^{b}	983.33 ± 40.69^{a}	957.67 ± 40.00^{a}	1,121.67 ± 137.90^{a}	1,085 ± 58.59^{a}	0.001
Thymus	Congestion (%)	6.00 ± 2.00^{a}	41.42 ± 6.30^{b}	19.12 ± 5.80^{c}	20.65 ± 5.07^{c}	14.6 ± 1.80^{ac}	22.34 ± 1.51^{c}	0.000
	Lymphocytes	1,221.67 ± 241.61^{a}	703.33 ± 85.13^{b}	1,055 ± 279.77^{ab}	915 ± 112.80^{ab}	1,011.33 ± 26.00^{ab}	1,020 ± 118.29^{ab}	0.051
Bursa of Fabricius	Interfollicular space	1.06 ± 0.12^{a}	3.98 ± 0.30^{b}	2.66 ± 1.07^{ab}	3.98 ± 0.75^{b}	2.7 ± 0.81^{ab}	3.1 ± 0.58^{b}	0.002
	Lymphocytes	1,103 ± 197.40^{a}	662.66 ± 86.10^{b}	858 ± 115.90^{ab}	968 ± 53.20^{ab}	994 ± 55.60^{ab}	996.67 ± 148.90^{a}	0.012
S2								
Spleen	Congestion (%)	3.66 ± 2.08^{a}	41.99 ± 9.37^{b}	15.38 ± 5.56^{a}	15.51 ± 3.19^{a}	13.07 ± 1.23^{a}	12.5 ± 1.23^{a}	0.000
	Lymphocytes	1,331.67 ± 334.33^{a}	862.66 ± 92.52^{a}	1,130.33 ± 390.37^{a}	959.33 ± 50.00^{a}	1,039.67 ± 187.70^{a}	962 ± 43.55^{a}	0.276
Thymus	Congestion (%)	3.66 ± 2.08^{a}	41.99 ± 9.37^{b}	15.38 ± 5.56^{a}	15.51 ± 3.19^{a}	13.07 ± 1.23^{a}	12.5 ± 1.23^{a}	0.000
	Lymphocytes	1,192.33 ± 335.90^{a}	700 ± 92.70^{a}	1,109.67 ± 401.50^{a}	973 ± 106.60^{a}	1,057 ± 139.20^{a}	1,036.33 ± 192.30^{a}	0.268
Bursa of Fabricius	Interfollicular space	0.94 ± 0.10^{a}	4.09 ± 0.30^{b}	2.38 ± 1.20^{ab}	2.68 ± 1.10^{ab}	2.17 ± 0.10^{ab}	2.34 ± 0.10^{ab}	0.003
	Lymphocytes	1,133 ± 352.20^{a}	747 ± 78.00^{a}	927.66 ± 64.40^{a}	630.67 ± 393.90^{a}	960 ± 175.00^{a}	1,102.33 ± 283.80^{a}	0.200

$^{a-c}$Mean Values in rows with various superscripts are significantly different ($p < 0.01$) and ($p < 0.05$), S1 (day 7), S2 (day 11) post-infection sampling.

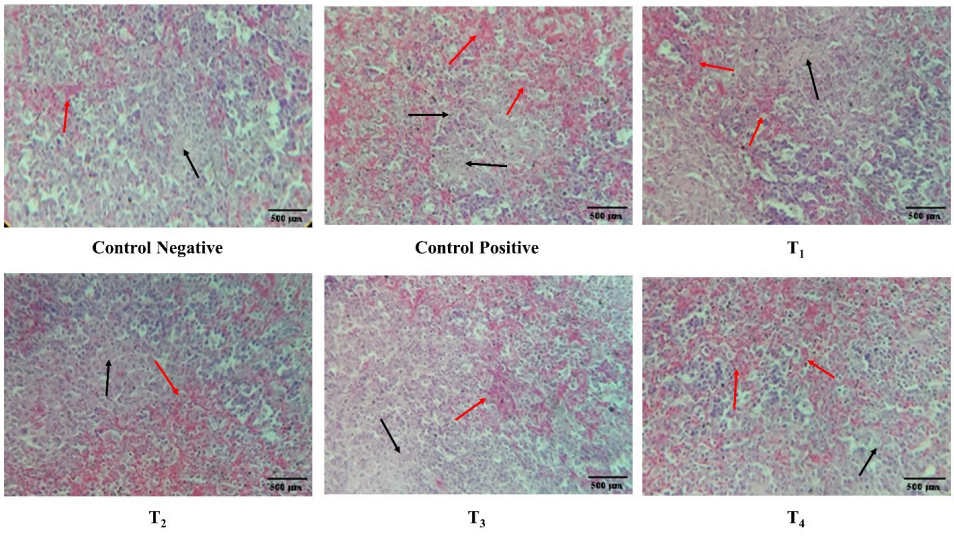

Fig. 7. Antibacterial effect of various levels of mixed zinc oxide (ZnO) and copper oxide (ZnO) nanoparticles, and florfenicol on *Salmonella gallinarum* induced infection in broiler in terms of histopathology of spleen. Red arrow indicates congestion black arrow indicates lymphocytic depletion. Groups: Control negative (no infection, no treatment); control positive (*S. gallinarum* infection, no treatment); T1 (*S. gallinarum* infection and florfenicol treatment at dose rate 50 mg/L in drinking water); T2 (S. gallinarum infection and ZnO + CuO nanoparticles treatment at dose rate 25 + 10 mg/kg/d); T3 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d); T4 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d); H & E stain, Magnification 10x.

and enrofloxacin are being used against *S. gallinarum* at poultry farms however, the irrational use of these antibiotics created AMR in *S. gallinarum*. Morsy et al. [41] reported that ZnO and CuO nanoparticles have no cytotoxic effects in the broiler chickens at low doses, however, at high dose

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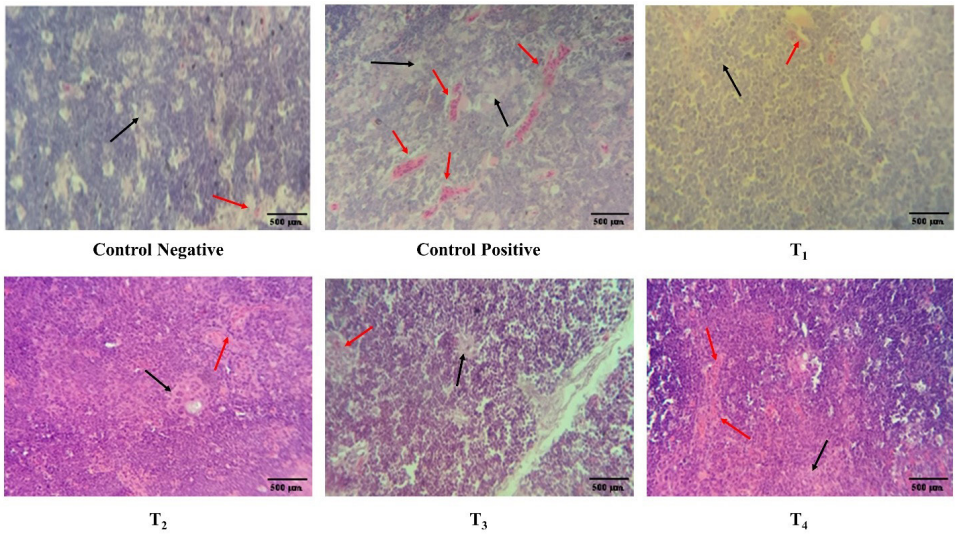

Fig. 8. Antibacterial effect of various levels of mixed zinc oxide (ZnO) and copper oxide (CuO) nanoparticles, and florfenicol on *Salmonella gallinarum* induced infection in broiler in terms of histopathology of thymus. Red arrow indicates congestion, black arrow indicates lymphocytic depletion. Groups: Control negative (no infection, no treatment); control positive (*S. gallinarum* infection, no treatment); T1 (*S. gallinarum* infection and florfenicol treatment at dose rate 50 mg/L in drinking water); T2 (S. gallinarum infection and ZnO + CuO nanoparticles treatment at dose rate 25 + 10 mg/kg/d); T3 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d); T4 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d); H & E stain, Magnification 10x.

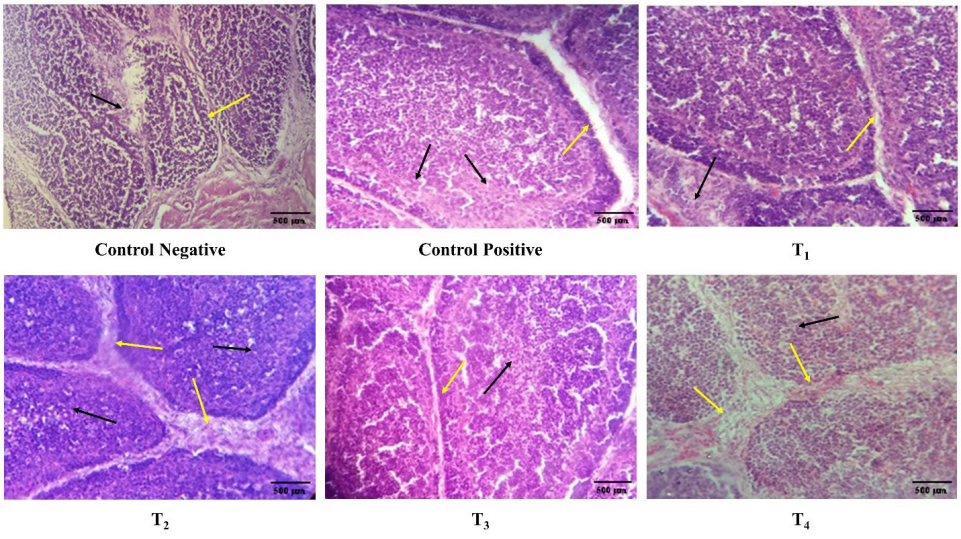

Fig. 9. Antibacterial effect of various levels of mixed zinc oxide (ZnO) and copper oxide (CuO) nanoparticles, and florfenicol on *Salmonella gallinarum* induced infection in broiler in terms of histopathology of bursa of Fabricius. Yellow arrow indicates interfollicular space, black arrow indicates lymphocytic depletion. Groups: Control negative (no infection, no treatment); control positive (*S. gallinarum* infection, no treatment); T1 (*S. gallinarum* infection and florfenicol treatment at dose rate 50 mg/L in drinking water); T2 (S. gallinarum infection and ZnO + CuO nanoparticles treatment at dose rate 25 + 10 mg/kg/d); T3 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d); T4 (*S. gallinarum* infection and ZnO + CuO nanoparticles treatment at dose rate 37.5 + 15 mg/kg/d); H & E stain, Magnification 10x.

it can cause cytotoxicity. However, in another study reported that ZnO, CuO, and Ferric oxide nanocomposite can ameliorate the toxic effects of ochratoxins in broilers and can improve the body weight, liver, and kidney functions [42]. To address the issue of AMR, this study aimed to assess the antibacterial activity of ZnO and CuO nanoparticles against *S. gallinarum* infection in broiler chicken.

The findings of this study highlight the antibacterial potential of nanoparticles as a significant alternative treatment approach for combating *S. gallinarum* infection in broiler chickens. In response to *S. gallinarum* infection at day 19 with 10^8 CFU/mL, the clinical signs like huddling, anorexia, depression, pasty yellow diarrhea, and postmortem lesions including bronzed colored liver, splenomegaly, necrotic foci on the visceral organs (liver and heart), and mortality greater than 60% in the birds were observed. These findings are consistent with previous studies [43–45] where they reported 50% mortality, rough feathers, yellow-green diarrhea, and sunken eyes. The bursa of Fabricius is very important and unique immune organ responsible for B lymphocytes production and humoral immunity in birds [46]. Therefore, the bursa of Fabricius was observed and based on evaluation it was noted that after nanoparticle treatment no congestion in the bursa of Fabricius was observed. The treatment of nanoparticles can increase the relative weight of bursa of Fabricius [47]. The recovery of the bursa of Fabricius from congestion was attributed to the antibacterial activity of the combination of nanoparticles [44].

The total erythrocytic count, total leukocyte count, hemoglobin concentration, and hematocrit level were found similar in all groups which was also reported in previous studies [48,49]. The intraperitoneal administered infection of *S. gallinarum* causes a significant decrease in hematocrit and hemoglobin concentration as compared to per oral infection [46]. The hematocrit and hemoglobin was decreased in the *S. gallinarum* infected birds as compared to the control negative which was in agreement with another study [50]. An arithmetic decrease in erythrocyte count and substantial decrease in hemoglobin concentration and hematocrit of the *S. gallinarum* infected birds was observed. The decreased hemoglobin and hematocrit level caused anemia in the birds [51]. The anemia in the control positive might be attributed to the increased ability of the reticuloendothelial system to take up modified erythrocytes [52]. The increase in the erythrocyte count in the nanoparticles treated groups may be attributed to the efficacy of CuO nanoparticles, because copper plays a vital role in iron metabolism for hemoglobin synthesis [53] and erythrocyte production [54]. After inducing *S. gallinarum* infection, an increased total leukocyte count was observed in the infected groups which is in line with a previous study [50] because they play a key role in the defense mechanism of the host and active removal of the bacteria from circulation [55]. The increase in leukocyte count indicates the severity of infection while CuO nanoparticles reduces the leukocyte count in the blood [56]. After the treatment with ZnO and CuO nanoparticles, total leukocyte counts decreased. However, the total leukocyte count in the group T_3 (day 7 post-infection) was found comparable with that of T_1 group treated with florfenicol antibiotic, whereas the leukocyte number was not different with that of the control negative at the second sampling at day 11 post-infection, which indicates that nanoparticles have similar efficacy as the antibiotics.

In contrast, Fathi et al. [48] obtained contrasting findings where they observed that nanoparticles had no impact on the leukocytic count. This discrepancy might be attributed to the induced *S. gallinarum* infection in our study as bacterial infections cause an increase in leukocytic count [57]. An increase in the leukocyte count of infected birds and the substantial decrease due to nanoparticle treatment is due to the adequate efficacy of the nanoparticles as they contribute to reducing the leukocytic count [58]. Ahmed et al. [6] also reported that the CuO nanoparticles decreased the leukocyte count in birds infected with *E. coli* and elaborates on the efficacy of ZnO and CuO nanoparticles against *S. gallinarum* infection in broilers. A significant increase in

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heterophil percentage was observed in the control positive as compared to the control negative. The nanoparticles treatment can decrease the heterophils percentage. Heterophilia in the control positive could be an indication of acute inflammatory degenerative changes in the internal organs [43]. The stress can also be linked with the impaired immunity of the birds. The infection of *S. gallinarum* can cause the increased level of corticosterone [59]. The heterophils percentage can be increased with increased level of corticosterone in the blood [60]. However, the treatment groups T_1, T_2, and T_3 showed a significantly decreased heterophil percentage as compared to the control positive, while the heterophil percentage in the groups (T_2, T_3, and T_4) treated with nanoparticles was found comparable to group T_1. However, group T_3 showed a lower heterophil percentage as compared to T_2 and T_4 indicating that the group T_3 may have the minimum inflammatory degenerative changes which endorse the efficacy of treatment with ZnO and CuO nanoparticles at the levels of 37.5 + 15 mg/kg/d. At day 7 post-infection, the decreased H/L ratio in the nanoparticle-treated groups might be attributed to the efficacy of nanoparticles in the alleviation of stress due to the *S. gallinarum* infection. The leukocytosis and heterophilia in the control positive could be due to the inflammatory response to the *S. gallinarum* induced tissue damage. The decreased leukocyte count and heterophil percentage in the nanoparticle-treated groups indicates the improved health status of the birds and antibacterial activity of ZnO and CuO nanoparticles which cause a decrease in bacterial load and inflammatory degeneration in the infected birds.

The CuO nanoparticles can inhibit the growth of NDV [61]. The nonsignificant difference between all the treatments in NDV antibody titer at day 14, 21, and 27 in the current study was endorsed by previous in vivo studies [62]. On the other hand, a previous study reported that the humoral immune response was increased when using ZnO nanoparticles [63]. The nonsignificant titers against NDV in the present study could be due to the induced *S. gallinarum* infection. The NDV antibody titer was highest in group T_2 receiving a low dose of nanoparticles and lowest in group T_4 receiving a high dose of nanoparticles which is in line with the previous study reported by Morsy et al. [41]. The low NDV antibody titer in group T_4 may be attributed to the oxidative stress induced by the high level of nanoparticles [64].

The macrophage phagocytic activity of the nanoparticle-treated groups T_2, T_3, and T_4 were enhanced. Macrophages are involved in the initiation of cellular and humoral immune responses by activating the B and T lymphocytes [38]. Copper plays an important role in the production of arachidonic acid and prostaglandin which enhances the production of macrophages [65]. The minimum light absorption percentage in T_3 indicates the increased phagocytic activity of macrophages which may be attributed to the efficacy of the nanoparticles dose level in T_3. The decreased light absorption in the nanoparticle-treated groups demonstrates the increased phagocytic activity of the macrophages indicating improved immune status of the nanoparticle-treated groups.

The histopathological examination of spleen, thymus, and bursa of Fabricius in our study indicated lymphocytic depletion and congestion in the *S. gallinarum* infected birds which also endorsed by a previous in vivo study [66]. The histological sections of spleen, thymus and bursa of Fabricius showed lymphocytic depletion and congestion which is in line with the previous studies [67–69]. The lymphocytic depletion in the *S. gallinarum* infected groups may be attributed to immunosuppression in the presence of the *S. gallinarum* induced infection [50]. In our study, the lymphocyte count in spleen, thymus and bursa of Fabricius was increased while the congestion was decreased after treatment with ZnO and CuO nanoparticles in the *S. gallinarum* induced infected birds which indicates the efficacy of the nanoparticle treatment.

In conclusion, ZnO and CuO nanoparticles at the dose level of 37.5 + 15 mg/kg/d and 50 + 20 mg/kg/d, respectively, showed optimum therapeutic activity against *S. gallinarum* infection in

broilers. As the two dose levels show equal therapeutic results against *S. gallinarum*, the lower dose 37.5 + 15 mg/kg/d recommended.

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