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PAPER

The impact of dietary inclusion of silver nanoparticles on growth performance, intestinal morphology, caecal microflora, carcass traits and blood parameters of broiler chickens

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ABSTRACT

We evaluated the fects of different levels of dietary silver nanoparticle (AgNP) powder on performance, intestinal microflora, carcass traits and blood parameters of broiler chickens. Three hundred seven-day-old Ross broiler chicks were randomly divided into five groups, each group replicated three times with 20 birds per replication. Chickens were fed the basal diet with 2.5, 5, 10 and 20 mg AgNPs per kg feed. Dietary inclusion of AgNPs improved the final body weight, cumulative weight gain and feed conversion ratio. The best broiler performance, carcass traits, and relative organ weights were observed in the group supplemented with 2.5 ppm AgNPs. Increasing the AgNP dose resulted in a significant decrease in the caecal lactose positive and enterococci bacteria populations, while lactobacilli counts were numerically increased. The silver residues in the breast and thigh muscle significantly increased ($p < .05$) in a dose-dependent manner. Dietary inclusion of AgNPs induced dose-dependent lesions in liver, kidney, spleen and duodenum tissues involving degeneration, necrosis, mononuclear infiltration and focal aggregation of inflammatory cells. In conclusion, despite its potential positive impacts on growth performance, carcass traits and caecal microbial population diversity at a dose of 2.5 ppm, dietary inclusion of AgNPs had the following negative effects on broilers: 1) silver residues in breast and thigh muscle, which may result in AgNPs transmission to consumers, and 2) cytotoxicity in intestinal, liver, spleen and kidney cells in a dose-dependent manner. Therefore, we suggest the use of lower doses of AgNPs (< 2.5 ppm diet) in poultry production in the future studies.

HIGHLIGHTS

- Dietary inclusion of silver nanoparticles (AgNPs) in broiler diets more than 2.5 mg/kg diets had many negative effects represented by accumulation of silver residue in broiler meat and the possibility of transmission of nanosilver to consumers.
- AgNPs had a cytotoxic effect on intestine, liver, spleen and kidney cells in a dose-dependent manner in broilers and might be harmful to chicken and human health.
- Therefore, we do not recommend using AgNPs as a dietary growth promotor or antibacterial agent in broiler diets and their use and marketing should be controlled and restricted.

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Introduction

Antibiotic growth promoters have long been used in chicken feed to enhance feed efficiency and minimise the occurrence of certain diseases (Chattopadhyay 2014). However, to avoid the development of microbial resistance, their usage in animal production has been restricted to varying degrees. As a result, alternatives to antibiotics are urgently needed. Numerous nutritional supplements are currently available in the market, including organic acids, probiotics,

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prebiotics, essential oils (Awad et al. 2009; Al-Sultan et al. 2016) and nanoparticles.

Nanoparticles are considered promising in livestock and poultry production due to their chemical and physical properties. They are used in various applications in nutrition, therapy, and medicine. Although it has not yet been made mandatory to use AgNPs and neither it has been recommended by NRC. It has been suggested by researchers to be used in animal production and chicken feed additives for improving various aspects as a result of their antibacterial, antifungal and immune stimulatory capabilities (Małaczewska 2014; Hassanpour et al. 2015; Patra and Lalhriatpuii 2020). Silver nanoparticles (AgNPs) have a diameter of less than 100 nanometres, making them ideal for penetration and accumulation in bacteria (improving antibacterial action) and enhancing absorption into the intestinal lining epithelia (Atiyeh et al. 2007). Due to their toxicity to microorganisms, AgNPs are used as growth promoters in animal nutrition and to modify the gut flora (Percival et al. 2005; Bolandi et al. 2021). According to previous studies, the use of nanoparticles improved the growth performance, immune response, digestive efficiency and reduced the mortality rate of the poultry (Kumar and Bhattacharya 2019, Kumar et al. 2020, Dosoky et al. 2021).

On the other hand, supplementation of the larger dose of AgNPs (8 mg/kg feed) resulted in deterioration in growth performance and significant negative impacts on all the measured parameters in broilers (Awaad et al. 2021). Similarly, the use of AgNPs in drinking water at a dosage of 50 ppm decreased broiler growth, diminished immune function, and had no antibacterial effect on various intestinal bacterial groups (Vadalasetty et al. 2018).

Many researchers used varied concentrations of AgNPs in the drinking water of broiler chickens and detected residues in the edible parts of broiler muscle at all concentrations (Ahmadi and Rahimi 2011, Kulak et al. 2018, Salem et al. 2021). There was a linear increase in AgNPs retention in meat with increasing dose in growing rabbits (Abdelsalam et al. 2019) and in poultry (Ahmadi and Rahimi 2011, Saleh and El-Magd 2018). Ag retention was higher in liver followed by spleen than that in muscular tissue in broiler chickens (Fondevila 2010, Wang et al. 2013). In addition, Nabinejad et al. (2016) stated that the muscles and organs of the poultry may transfer AgNPs to consumers which may cause negative effects.

Several studies on the effects of silver nanoparticles on chicken growth performance, intestinal histomorphology and microflora, tissue residue, and carcass characteristics have recently been published, in spite of that there is insufficient data as the results are inconsistent. Therefore, our primary goal for this study was to elucidate the effect of dietary AgNP powder on growth performance, carcass traits, intestinal histopathology and microflora, silver residues in meat and blood metabolites.

Materials and methods

Statement of animal rights

The ethics committee for Laboratory Animal Research at King Faisal University approved all study procedures (KFU-REC-2022-JAN-EA000337). The institutional and national guidelines that ensure animal rights were strictly followed during the study.

Nanoparticles

Chemical reduction was used to synthesise AgNPs, as described by Pal et al. (2009). AgNPs were created by exposing a silver nitrate solution to microwave radiation in an ethanolic medium, including polyvinylpyrrolidone as a stabilising agent. In the presence of microwave, ethanol was applied as a reducing agent. To obtain AgNPs in powdered form, the solvent was evaporated at a mild temperature (mild degree of microwave oven at 2450 MHz for 5 seconds). Particle sizes were measured using a JEOL JEM-2100 high-resolution transmission electron microscope with an accelerating voltage of 80 kV (Figure 1). The AgNPs were spherical, with particle size ranging from 11.9 to 45 nm, according to morphological examination.

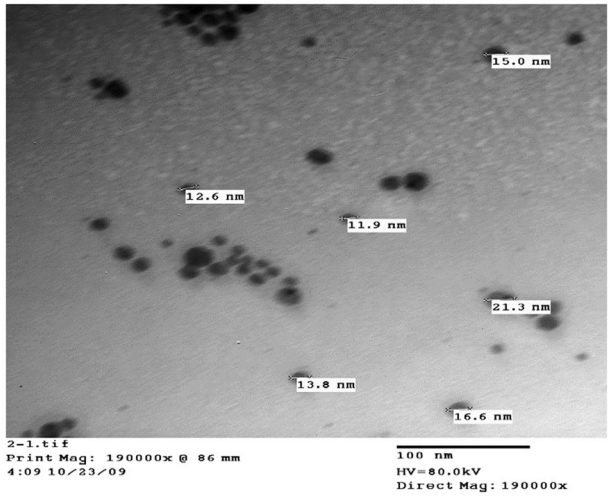

Figure 1. Size and Shape of silver nano particles.

Animal and dietary management

Three hundred (50% male) seven-day-old Ross 308 broiler chickens were raised until the age of 42 days. Broiler chicks were randomly allocated into five treatment groups, each replicated three times with 20 birds per replication. Five AgNP concentrations (0, 2.5, 5, 10, and 20 ppm, respectively) were added to the basal diet from day 7 to day 42 post-hatching. Five broiler starter (8–28 d) and finisher (28–42 d) diets were formulated incorporating five concentrations of AgNPs (0, 2.5, 5, 10 and 20 ppm diet). The following were the five dietary treatments: 1) basal diet without any addition (C), 2) basal diet containing 2.5 ppm AgNPs (NS 2.5), 3) basal diet containing 5 ppm AgNPs (NS5), 4) basal diet containing 10 ppm AgNPs (NS10), and 5) basal diet containing 20 ppm AgNPs (NS20). The birds were housed in standard conditions in a building with controlled temperature and humidity in pens with sawdust litter. Water and experimental diets were offered *ad libitum*. The experimental diet formulations satisfied the nutritional requirements for broiler chickens according to the NRC (1994) guidelines (Table 1). During the first week, the temperature was maintained at 32 ± 1 °C, then reduced to 27 °C in the second week and to 24 °C in the third week of age.

Table 1. Composition and chemical analysis of basal diets (% as fed basis).

Ingredient, %	Starter	Grower and Finisher
Corn	54.85	63
Corn gluten meal (60%)	4.5	4
Soybean meal, (48.5 %)	32	25
Sunflower oil	4.3	3.65
Limestone	1.5	1.5
Dicalcium phosphate	1.8	1.8
Salt	0.4	0.4
Vitamin - mineral Premix[a]	0.3	0.3
DL-methionine	0.2	0.2
L- lysine	0.1	0.1
Antioxidants	0.05	0.05
Total	100	100
Chemical analysis		
Dry matter (%)	90.10	89.93
Crude protein (%)	23	20
ME (Kcal/kg diet) [b]	3200	3200
Calcium (%)	1.08	1.06
Total phosphorus (%)	0.77	0.69
Non-phytae phosphorus (%)	0.44	0.43
Na (%)	0.17	0.17
Lysine (%)	1.24	1.05
DL-Methionine (%)	0.58	0.54
Methionine + cysteine (%)	1.06	0.88
Crude fibre (%)	2.95	2.92
Crude fat (%)	6.39	6.02

[a]The vitamin and mineral premix provided per kg of diet: vitamin A, 4000000 IU; vitamin D3, 667000 IU; vitamin E 3334 mg; vitamin K3, 1167 mg; vitamin B1, 334 mg; vitamin B2, 1667 mg; vitamin B3, 3334 mg; B6, 500 mg; vitamin B12 33.4 mg, Folic acid, 334 mg; Biotin, 17 mg; Iron, 10; Copper, 2.167; Zinc, 18.334; Manganese 20.0; Iodine, 0.167; Cobalt, 0.034 and Selenium, 0.034.
[b]Calculated based on NRC (1994) feed composition tables.

From the start of the 4th week the temperature was set to 23 ± 1 °C until the end of the experiment. From 7 to 42 days of age, the broilers' body weight (BW) and feed intake were measured and recorded weekly.

Growth performance

To calculate feed consumption each week, we measured daily feed intake per replicate. A digital balance was used to record chick body weight at the time of delivery and every week thereafter. FCR was calculated using the formula FCR = g feed/g weight gain.

Carcass yield

At the end of the experiment, broiler carcass characteristics and organ weights were assessed by randomly selecting and euthanizing two chickens from each replicate. A digital balance was used to weigh the warm carcass, heart, liver, breast, thigh, and abdominal fat to the nearest 0.01 g. Dressing percentage is the ratio of dressed carcass weight to the weight of the live bird, expressed as a percentage.

Water-holding capacity

To estimate the water-holding capacities (WHCs) of post-rigor chilled chicken leg and breast muscle the method Honikel and Hamm (1994) was applied. By squeezing the sample (≈300 mg) between 2 filter papers (70 mmφ) and ceramic plates for 4 min. The compressed sample was carefully separated from the moist filter paper after pressing and weighed immediately. RW % was estimated by the formula: RW%= (W1-W2)/W1X 100; where W1= Weight of meat sample, W2= Weight of meat sample after pressing. WHC was expressed as percent water retained by the meat sample; calculated as following: WHC (% water retained) = 100 − RW%.

Evaluation of silver residue from nanoparticles

Meat sample from breast and thigh muscle (1 gram) was placed in a 30-ml galzzed porcelain crucible then the crucible placed in muffle furnace at 500 °C for 2 hours. After cooling the crucible was removed from the muffle furnace and 3 mL of HNO3 (1 + 1 (deionised water (DW)) was added and heating on a hot plate at 100–120 °C until dryness. Then the crucible was set back into the muffle furnace at 500 °C for additional 1 hour. After cooling crucible was removed and 10 mL Hcl (1 + 1 DW) was added. Then the sample transferred into a 50 ml volumetric flask and dilute to the volume with deionised water and well mixed. Then the sample was placed in the inductively coupled

Table 2. Effect of different dietary treatments on body weight, feed intake and feed conversion ratio in broiler chickens.

	AgNPs levels (mg/kg diet)						
Item	C	NS2.5	NS5	NS10	NS20	SEM[A]	Sig.
Body weight (g)							
Initial (day 8)	179.83	182.00	183.27	178.87	181.40	1.28	0.87
Day 28	1343.33	1398.33	1401.11	1368.89	1423.33	14.78	0.53
Final BW (day 42)	2500[c]	2600[a]	2580[ab]	2560[ab]	2535[bc]	11.2	0.01
Feed consumption (g)							
From day 8–28	1970	1945	1964.07	1958.33	1911.66	10.62	0.56
From day 28–42	2595.74	2624.07	2638.27	2622.41	2612.37	8.05	0.61
From day 8–42	4565.74	4569.07	4602.34	4580.74	4524.03	13.64	0.13
Weight gain (g)							
From day 8–28	1163.5	1216.33	1217.84	1190.02	1241.93	14.62	0.55
From day 28–42	1156.67	1201.67	1179.56	1191.11	1111.67	18.57	0.63
From day 8–42	2320.167[c]	2418[a]	2397.4[ab]	2381.13[ab]	2353.6[bc]	10.67	0.006
Feed conversion ratio							
From day 8–28	1.69	1.60	1.61	1.65	1.54	0.02	0.17
From day 28–42	2.24	2.20	2.23	2.20	2.36	0.03	0.64
From day 8–42	1.97[a]	1.89[b]	1.91[b]	1.92[b]	1.91[b]	0.02	0.01

[A]SEM: pooled standard errors of means; Sig.: significant.
C (Control, basal diet); NS2.5, NS5, NS10, NS20 (Basal diet with 2.5, 5, 10, 20 ppm AgNPs), respectively.
[a,b]Means with different superscript letters in the same row are significantly different ($p \leq 0.05$).

plasma optical emission spectrometry to evaluate total silver concentration in breast and thigh muscle samples, as described in a previous study (Isaac and Johnson 2019).

Histopathological studies

Duodenum, liver, kidney, and spleen specimens were fixed in a 10% neutral buffered formalin solution. Increasing concentrations of ethyl alcohol were used to dehydrate the specimens. Samples were then clarified with xylene before being embedded in paraffin, sectioned 4–5 μm thick and stained with haematoxylin and eosin, then Masson trichrome for fibrosis detection (Banchroft et al. 1996).

Transmission electron microscopes (TEM)

Tissue samples (1 mm) were obtained from the duodenum. They were fixed in 3% buffered glutaraldehyde for 2 hours, then washed three times (10 minutes each) with cacodylate buffer. The post was then fixed for 1.5 hours in 1% buffered osmic acid, then washed three times with distilled water (10 minutes each time). The samples were dehydrated using increasing alcohol concentrations (30–50–70–90–100%), changed every 15 minutes. The samples were embedded in a mixture of epon and araldite. From the embedded blocks, ultrathin (70 nm) sections were cut and stained with uranyl acetate and lead citrate. Finally, the TEM 100 eXII electron microscope in the Electron Microscope Unit, Assiut University, Assiut, Egypt, was used to examine the samples at 80 kV according to the protocol of Bozzola and Russell (1999).

Enumeration of bacteria

Intestinal content from the caecum was collected shortly after slaughter in weighted screw-capped sterile Falcon tubes (50 ml capacity). The collected digesta were stored on ice until they were brought to the lab for enumeration. microbial population enumeration. The sealed containers were kept at 4 °C in the laboratory, and the fresh mass was diluted 1:10 with a sterile 0.1% peptone solution. ten-fold serial dilutions up to 10^7 of each sample were made. The spread-plate technique was used to count coliform bacteria, lactose-negative enterobacteria, enterococci, and lactobacilli. Coliform and lactose-negative enterobacteria were enumerated as red and colourless colonies on MacConkey agar (BBL), respectively. Enterococci were enumerated as red colonies mostly surrounded by a yellow zone on KF Streptococcus agar basis (Merck, 110707). By the use of DeMan, Rogosa, and Sharpe (MRS) agar (Biolife) lactobacilli was enumerated and the plates were incubated with 5% CO2 for 48 hours. Lactobacilli appear as little opaque and white colonies that are compact or feathery. Colonies were counted after incubation based on colony morphology. The average of the counts from two plates was calculated. The number of colony-forming units per gram of digesta was given as log colony-forming units.

Blood biochemical parameters

At day 42, 2 birds from each replicate were randomly chosen for collection of blood samples from wing vien. The serum was obtained by centrifugation of the collected blood samples at 4000 x g for 15 min and saved at −20 °C until further analysis. Blood metabolites (total Protein, albumin, cholesterol, triglycerides,

Table 3. Effect of different dietary treatments on carcass traits and relative organ weight and WHC in broiler chickens.

	AgNPs levels (mg/kg diet)						
Item	C	NS2.5	NS5	NS10	NS20	SEM[A]	Sig.
Presloughter weight (g)	2515^{ab}	2594.00^{a}	2590.00^{a}	2573.33^{ab}	2500.00^{b}	13.90	0.05
Worm carcass weight (g)	1610^{c}	1788.33^{a}	1723.19^{ab}	1686.68^{bc}	1634.79^{c}	19.63	0.005
Dressing %	64.00^{b}	68.95^{a}	66.53^{ab}	65.54^{b}	65.39^{b}	0.53	0.01
Liver %	1.73^{b}	2.03^{a}	1.97^{a}	1.87^{ab}	1.72^{b}	0.04	0.006
Gizzard %	1.72^{c}	1.95^{a}	1.92^{ab}	1.83^{b}	1.69^{c}	0.03	0.001
Kidney %	0.61^{a}	0.67^{a}	0.63^{a}	0.64^{a}	0.51^{b}	0.02	0.014
Spleen %	0.13^{b}	0.18^{a}	0.17^{a}	0.15^{ab}	0.14^{b}	0.005	0.017
Proventriculus %	0.36	0.38	0.42	0.42	0.42	0.008	0.06
Heart %	0.46^{b}	0.53^{a}	0.49^{ab}	0.47^{ab}	0.42^{b}	0.13	0.05
Bursa %	0.13	0.15	0.14	0.14	0.15	0.003	0.06
Water-holding capacity (WHC)							
Breast muscle, (WHC %)	70.55^{a}	73.42^{a}	71.41^{a}	65.58^{b}	63.87^{b}	0.82	0.003
Thigh muscle (WHC,%)	70.27^{ab}	73.71^{a}	70.95^{a}	70.10^{ab}	67.10^{b}	0.69	0.02

WHC: water-holding capacity.
[A]SEM: pooled standard errors of means; Sig.: significant.
C (Control, basal diet); NS2.5, NS5, NS10, NS20 (Basal diet with 2.5, 5, 10, 20 ppm AgNPs), respectively.
[a,b]Means with different superscript letters in the same row are significantly different ($p \leq 0.05$).

uric acid (Spectrum Egypt), urea, creatine (Diamond, Egypt), calcium, phosphorus (Spinreact, Spain), Igg, IgA (QCA, Spain) were investigated by spectrophotometer (Unico, USA) using commercial test kits in accordance with the manufacturer company procedures.

Statistical analysis

Kolmogorov–Smirnov tests were used to examine all data for normality and homogeneity of variance. All bacterial enumeration information was converted to $\log_{10}$ CFU/mL. Data were analysed by ANOVA using SPSS software (version 19) (SPSS 2010). We used the Duncan multiple range test post-hoc when significant differences ($p < .05$) were found (Steel and Torrie, 1980). The data are presented for each variable as means with pooled standard errors.

Results

Effect of AgNPs on the growth performance parameters

The effects of AgNP concentration on the growth performance of broiler chickens are shown in Table 2. No significant differences were observed between AgNP concentration groups in live body weight (g) in the starter phase (initially and at day 28) and body weight gain (g) for the periods of 8–28 days and the finisher phase of 28–42 days. However, there were significant increases in the final body weight and weight gain with increasing AgNP dose from 2.5 to 10 mg/kg, followed by a decrease at 20 mg/kg. The highest body weight (2600 g) and weight gain (2480 g) were observed after 42 days in the group consuming 2.5 mg/kg AgNPs. Feed consumption (g/bird) was not affected ($p > .05$) by AgNP concentration during all phases of the experiment. However, the FCR was significantly ($p < .05$) improved by dietary treatments compared to the control group from 8–42 days. Moreover, birds receiving 2.5 mg AgNPs/kg diet demonstrated the lowest ($p < .05$) FCR value (1.89) from 8–42 days (Table 2). In addition, the FCR was not significantly ($p > .05$) affected by the dietary treatments during the periods from 8–28 and 28–42 days.

Effect of AgNPs on carcass traits and water holding capacity

The data in Table 3 indicated that the AgNP concentration significantly ($p < .05$) affected worm carcass weight, dressing percentage and relative organ weights (Table 3). The dressing percentages were significantly ($p < .05$) increased by the addition of 2.5 mg AgNPs/kg diet compared to the control and other treatment groups. The relative weights of the liver, gizzard, kidney, spleen and heart were significantly ($p < .05$) decreased by the dietary inclusion of 20 mg AgNPs/kg diet. The relative weight for the proventriculus and bursa were not significantly ($p > .05$) affected by the concentration of AgNPs. The percentages of WHC of both thigh and breast muscle were significantly ($p < .05$) decreased at 10 and 20 mg AgNPs/kg diet.

Effect of AgNPs on silver residues in broiler meat

Silver residues were detectable (in ppm) in all samples, even the control group. The average silver residue in the breast and thigh muscle significantly increased ($p < .05$) with increasing AgNP dosage (Table 4).

Table 4. The effect of dietary AgNPs level on residues of silver (mg/kg) in meat of broilers.

	AgNPs levels (mg/kg diet)						
Item	C	NS2.5	NS5	NS10	NS20	SEM[A]	Sig.
Breast muscle	0.01[b]	0.04[b]	0.06[b]	0.07[ab]	0.11[a]	0.01	0.02
Thigh muscle	0.02[c]	0.10[c]	0.15[b]	0.17[b]	0.29[a]	0.02	0.001

[a,b]Means with different superscript letters in the same row are significantly different ($p \leq 0.05$).
[A]SEM: pooled standard errors of means; Sig.: significant.
C (Control, basal diet); NS2.5, NS5, NS10, NS20 (Basal diet with 2.5, 5, 10, 20 ppm AgNPs), respectively.

Table 5. Numbers of dominant bacterial groups in the contents of caeca [log cfu/g] of broilers after treatment with increasing concentration of AgNano.

	AgNPs levels (mg/kg diet)						
Item	C	NS2.5	NS5	NS10	NS20	SEM[A]	Sig.
Day 28							
Lactose negative bacteria	4.78	3.99	3.98	3.06	2.52	0.33	0.22
Lactose positive bacteria	6.41[a]	6.31[a]	5.35[ab]	4.78[b]	4.47[b]	0.25	0.02
Enterococci	7.19[a]	7.16[a]	7.08[a]	7.09[a]	6.37[b]	0.09	0.002
Lactobacilli	8.77	8.84	9.05	8.86	8.63	0.14	0.95
Day 42							
Lactose negative bacteria	3.38	2.13	1.97	1.57	1.43	0.26	0.11
Lactose positive bacteria	4.98	4.62	4.61	4.46	4.21	0.14	0.61
Enterococci	6.19	5.16	5.91	5.65	6.09	0.18	0.45
Lactobacilli	9.29	9.31	9.61	9.83	9.37	0.13	0.72

[a,b]Means with different superscript letters in the same row are significantly different ($p \leq 0.05$).
[A]SEM: pooled standard errors of means; Sig.: significant
C (Control, basal diet); NS2.5, NS5, NS10, NS20 (Basal diet with 2.5, 5, 10, 20 ppm AgNPs), respectively.

Effect of AgNPs on caecal microbial population

The populations of caecal lactose-negative, lactose-positive, and enterococcal bacteria on day 28 significantly decreased with dietary inclusion of AgNPs, but not ($p > .05$) on day 42 (Table 5). A numerical increase in the lactobacilli population was observed in groups fed 2.5 to 10 mg/kg dietary AgNPs; however, this increase was not statically significant ($p > .05$).

Effect of AgNPs on blood serum constituents

The results of blood serum analyses of 6-week-old broiler chickens are shown in Table 6. Total protein (mg/dl), total cholesterol, urea, creatinine, and phosphorus exhibited significant ($p < .05$) differences between treated and control broiler groups. However, differences in other blood serum constituents were insignificant. Groups supplemented with 2.5 and 20 mg/kg dietary AgNPs had the highest total serum protein (4 mg/dl) compared to the control group (2.73 g/dl). Serum total cholesterol significantly decreased between the groups fed 2.5 to 20 mg/kg dietary AgNPs. Groups supplemented with AgNPs had lower ($p < .05$) serum phosphorus than the control group.

Histopathological evaluation

Duodenum

Figure 2 shows that the duodenum from the control group had normal epithelium with no lesions (A). The duodenum from birds supplemented with a dose of 2.5 mg/kg AgNPs were observed with normal intestinal villi structure (B). The duodenum from the 5 mg/kg supplemented group showed necrosis and sloughing of the epithelium (arrow) (C). The duodenum from the 10 mg/kg AgNPs group exhibited epithelial metaplasia (arrow) (D). The duodenum from 20 mg/kg AgNPs also showed epithelial metaplasia (arrow) (E).

Spleen

As seen in Figure 2, the spleen from the control group demonstrated normal red and white pulp with normal lymphoid follicles (F). The spleen from the 2.5 mg/kg supplemented group had normal red and white pulp with normal lymphoid follicles (G). The spleen from the 5 mg supplemented group showed congestion of the blood vessels, desquamation of endothelium (arrow) and perivascular infiltration (H). The spleen from the 10 mg/kg supplemented group demonstrated necrosis of the lymphoid follicle (arrow) (I), as did the spleen from the 20 mg/kg supplemented group (arrow) (G).

Representative micrographs for liver (A–E) and kidney (F–G) of broiler chickens treated with different dietary AgNP doses for 42 days, H&E stain (Figure 3).

Liver: The liver from the control group showed normal hepatic architecture (A). The liver from the 2.5 mg/kg group demonstrated focal mononuclear infiltration (arrow) (B). The liver from the 5 mg/kg supplemented group exhibited congestion and vacuolar degeneration (C). The liver from the 10 mg/kg supplemented group showed hepatocellular vacuolation (arrow) (D), as did the liver from the 20 mg/kg group (arrow) (E).

Kidney: In the control group, the kidney exhibited normal glomerulus and renal tubules (F). Kidneys from the 2.5 mg/kg group showed normal glomerulus and renal tubules (G). Kidneys from the 5 mg/kg group presented congestion of the glomerulus (arrow) (H). Kidneys from the 10 mg/kg group showed severe congestion of the blood vessels (arrow) (I). Kidneys from the 20 mg/kg group showed congestion of the glomerulus (arrow) (G).

Representative transmission electron microscope micrographs of duodenums from broiler chickens treated with 20 mg/kg AgNPs for 42 days showing AgNPs of different sizes inside the nucleus (N) of the enterocyte (E) (Figure 4).

Table 6. Effect of different dietary treatments on blood metabolites (mg/dL) in broiler chickens.

	AgNPs levels (mg/kg diet)						
Item	C	NS2.5	NS5	NS10	NS20	SEM[A]	Sig.
Total protein (mg/dlìL)	2.73[b]	4[a]	4[a]	3.7[a]	3.17[ab]	0.18	0.03
Albumen (mg/dL)	1.43	1.77	1.93	1.86	1.73	0.07	0.14
Globulin (mg/dL)	1.3	2.32	2.07	1.84	1.43	0.14	0.09
Total Cholesterol (mg/dL)	52.00[b]	75.00[a]	74.67[a]	69.00[ab]	51.67[b]	3.77	0.03
Triglycerides (mg/dL)	49	84.67	82.5	69.00	61.33	5.53	0.17
Urea (mg/dL)	11[c]	13[bc]	15[ab]	16.5[a]	14.5[ab]	0.62	0.01
Creatinine (mg/dL)	0.30	0.27	0.30	0.55	0.35	0.03	0.05
IgG (mg/dL)	371.66	507	415.67	367.33	266.5	25	0.09
IgA (mg/dL)	60.33	69.66	65.33	52.5	45.5	3.54	0.25
Uric acid (mg/dL)	6.66	5.27	5.3	5.8	5.45	0.34	0.7
Calcium (mg/dL)	6.73	7.83	7.73	7.45	6.85	0.19	0.24
Phosphorus (mg/dL)	6.47[a]	5.66[ab]	4.7[b]	4.60[b]	4.60[b]	0.27	0.05

[a,b]Means with different superscript letters in the same row are significantly different ($p \leq 0.05$), [A]SEM: pooled standard errors of means; Sig.: significant.
C (Control, basal diet); NS2.5, NS5, NS10, NS20 (Basal diet with 2.5, 5, 10, 20 ppm AgNPs), respectively.

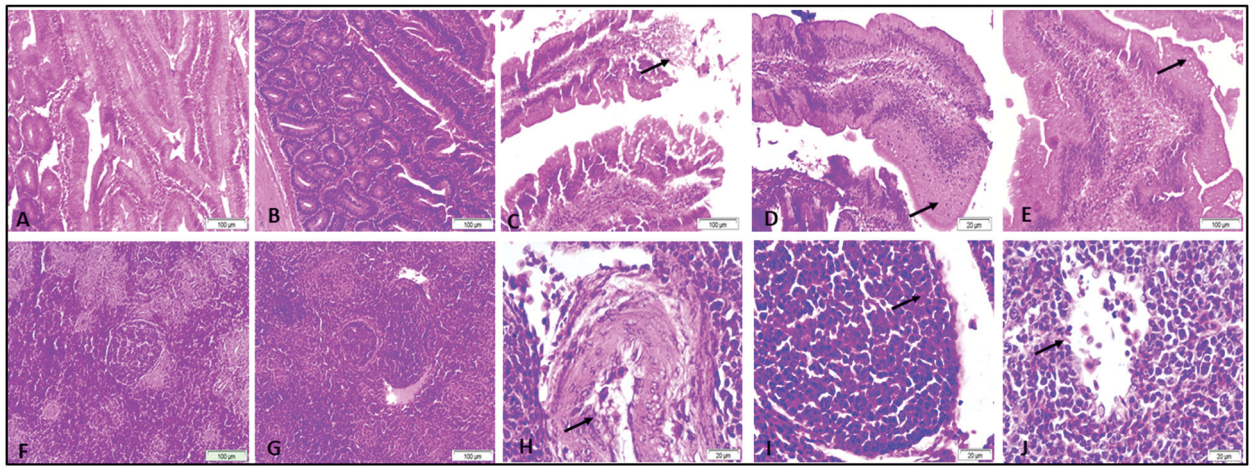

Figure 2. Representative micrographs for duodenum (A–E) and spleen (F-G) of broiler chickens treated with different levels AgNPs for 42 days, H&E stain. A) Duodenum from control group showing normal epithelium with no lesions. (B) Duodenum from 2.5 mg supplemented group showing normal structure of intestinal villi. (C) Duodenum from 5 mg group showing necrosis and sloughing of epithelium (arrow). (D) Duodenum from 10 mg showing epithelial metaplasia (arrow). (E) Duodenum from 20 mg group showing epithelial metaplasia (arrow). F) Spleen from control group showing normal red and white pulp with normal lymphoid follicle. (G) Spleen from 2.5 mg group showing normal red and white pulp with normal lymphoid follicle. (H) Spleen from 5 mg group showing congestion of the blood vessels, desquamation of endothelium (arrow) and perivascular infiltration. (I) Spleen from 10 mg group showing necrosis of the lymphoid follicle (arrow). (G) Spleen from 20 mg group showing necrosis of the lymphoid follicle (arrow).

A summary of the lesions at 42 days in all examined organs is presented in Table 7. The severity of lesions increased in all examined tissues and organs (duodenum, liver and kidney) in a dose-dependent manner. The smallest lesions were observed at 2.5 mg/kg dietary AgNPs.

Discussion

Several authors have suggested that the addition of a solution of AgNPs to broilers under ideal conditions either inhibits or stimulates growth performance (Saleh and El-Magd 2018; Kumar and Bhattacharya 2019; Kumar et al. 2020; Bolandi et al. 2021; Dosoky et al. 2021). In the current study, growth performance improvement observed after incorporating different levels of AgNPs (Saleh and El-Magd 2018 [50 ppm]; Kumar and Bhattacharya 2019 [50 ppm], Kumar et al. 2020 [50 ppm], Bolandi et al. 2021 [25, 50, 75 ppm]; Dosoky et al. 2021 [2, 4, 8 ppm]) was consistent with the results of these previous studies. The improvement could be attributed to the antimicrobial effect of AgNPs on harmful intestinal bacteria, improving gut health and the absorption of nutrients at low doses 2.5 ppm because the higher dose $>$ 2.5 ppm induce had a negative impact. The improvement in gut health

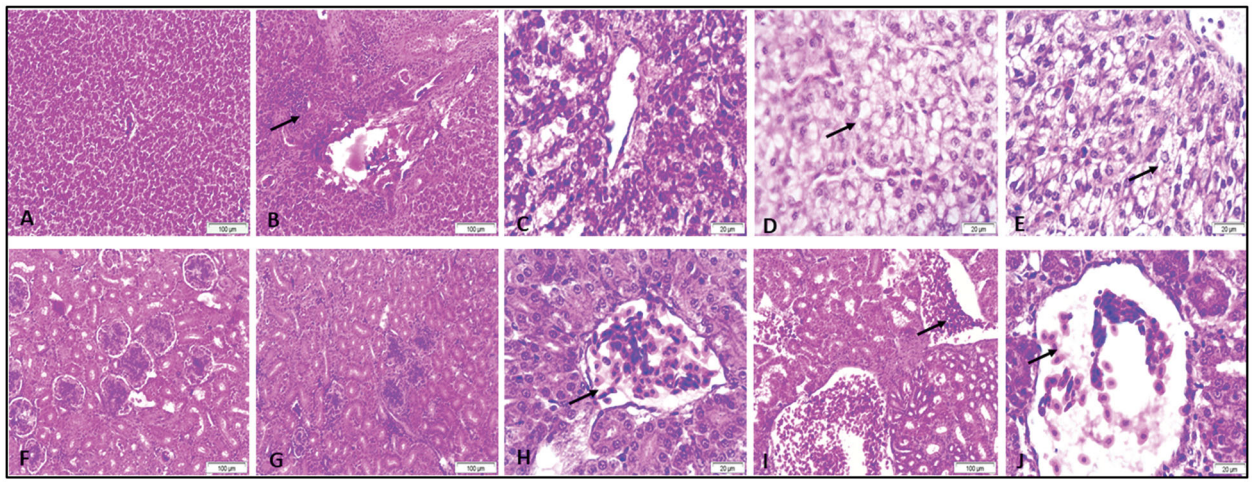

Figure 3. Representative micrographs for liver (A–E) and kidney (F-J) of broiler chickens treated with different levels AgNPs for 42 days, H&E stain. A) Liver from control group showing normal hepatic architecture. (B) Liver from 2.5 mg group showing focal mononuclear infiltration (arrow). (C) Liver from 5 mg group showing congestion and vacuolar degeneration. (D) Liver from 10 mg group showing hepatocellular vacuolation (arrow). (E) Liver from 20 mg group showing hepatocellular vacuolation (arrow). F) Kidney from control group showing normal glomerulus and renal tubules. (G) Kidney from 2.5 mg group showing normal glomerulus and renal tubules. (H) Kidney from 5 mg group showing congestion of the glomerulus (arrow). (I) Kidney from 10 mg group showing severe congestion of the blood vessels (arrow). (J) Kidney from 20 mg group showing congestion of the glomerulus (arrow).

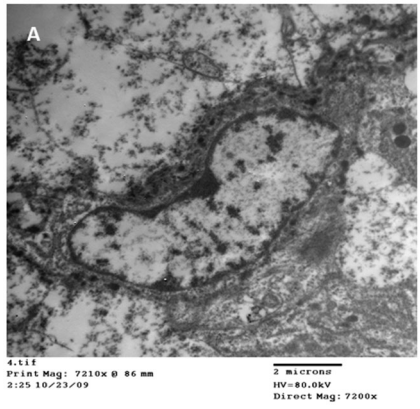

Figure 4. Representative micrographs of Transmission electron microscope of duodenum from broiler chickens treated with 20 mg AgNPs for 42 days showing nano particles of different size inside the nucleus (A) of the enterocyte.

leads to enhanced nutrient absorption, as seen by increased weight gain, feed conversion ratio and feed intake in broilers fed AgNP diets (Andi et al. 2011).

AgNPs also have anti-inflammatory properties because they regulate the expression of matrix metalloproteinases, proteolytic enzymes involved in various inflammatory and healing processes (Nadworny et al. 2010). Stimulating digestive enzyme activity is another proposed reason for the AgNPs growth stimulatory effect. AgNPs improve the animal's health and immunological state by allowing them to expend fewer nutrients for the metabolic effort required for immunological control, allowing them to utilise those extra nutrients for other physiological and productive functions (Fondevila 2010). In contrast, other studies found that a diet supplemented with AgNPs negatively impacted chicken performance (Ahmadi and Rahimi 2011 [0, 4, 8, 12 ppm]; Pineda et al. 2012 [0, 10, 20 ppm]; Vadalasetty et al. 2018 [50 ppm]). These inconsistent results are possibly attributable to differences in nanoparticle size, the method of synthesis, the dosage, or the means of administration.

The decrease in cumulative body weight and relative organ weights of organs in broilers supplemented with a high dose (20 mg/kg dietary AgNP) might be due to AgNPs reducing protein enzymatic digestibility and blocking the absorption of sugars and amino acids. Another possible reason might be that the birds

Table 7. Summary of lesion score at 42 days in all studied groups.

	AgNPs levels (mg/kg diet)				
Item	C	NS2.5	NS5	NS10	NS20
Duodenum lesions:					
Necrosis & sloughing of epithelium	–	+	+	++	+++
Mononuclear infiltration	–	+	++	+++	+++
Spleen lesions:					
Necrosis of Lymphoid follicle	–	–	+	++	++
Changes in bl.vs.	–	–	+	++	++
Liver Lesions:					
Congestion	–	+	+	+++	+++
Focal accumulation of mononuclear cells	–	+	++	+++	+++
Hepatocellular vacuolation	–	+	+	+++	+++
Kidney Lesions:					
Congestion of bl.vs	–	–	++	++	+++
Hemorrhages	–	–	–	+++	+++
Glomerular congestion	–	–	+	++	+++
Glomerular swelling	–	–	++	+++	+++
Mononuclear infiltration	–	+	++	+++	+++
Renal tubular degeneration	–	+	++	++	++

(- No lesions, + lesions present in 3–5 sections, ++ lesions present in 6–12 sections, +++ lesions present in 13–20 sections).
C (Control, basal diet); NS2.5, NS5, NS10, NS20 (Basal diet with 2.5, 5, 10, 20 ppm AgNPs), respectively.

undergo more cellular stress and excessive cellular interactions when consuming AgNPs (Vadalasetty et al. 2018).

The improvement in dressing percentages and relative organ weight in chickens administered low AgNP doses is partially consistent with Elkloub et al. (2015). In contrast, Ahmadi and Rahimi (2011) reported that the levels of AgNPs 4, 8 and 12 ppm silver nanoparticles increased the weight of small intestine and abdominal fat and had non-significant effects on liver and gizzard % weight. Moreover, Felehgari et al. (2013) found that the relative weight of the small intestine and liver increased, but the gizzard, proventriculus and pancreas were not affected by the addition of different levels of AgNPs and inorganic selenium.

The improvement in the water holding capacity of breast and thigh muscle from chickens administered the lowest dose of AgNPs (2.5 mg/kg) is consistent with results obtained previously (Hashemi 2014; Hashemi et al. 2017). These results indicate that the lower dose induces low protein oxidation levels (Hashemi 2014).

The histopathology of the duodenum, liver, kidneys, and spleen tissues from control and NS2.5 chickens were within normal histologic limits without particular abnormalities, indicating that AgNPs have no deleterious influence on the histologic structure at such dose levels. However, birds given higher doses of AgNPs (5, 10 and 20 mg/kg) had mild to moderate pathologic lesions ranging from the congestion of blood vessels, necrosis and sloughing of epithelium, necrosis in the lymphoid tissue, and focal accumulation of mononuclear cells. Similar findings were reported previously (Loghman et al. 2012; Samani et al. 2018; Dosoky et al. 2021). Those researchers found that the administration of high doses (Loghman et al. 2012 [8 and 12 ppm]; Samani et al. 2018 [10 and 100 μg/ml]; Dosoky et al. 2021 [8 and 12 ppm]) of AgNPs induced mild necrotic changes and inflammatory cell infiltration in the liver, spleen and kidney tissues of broiler chickens. Several authors proposed that the toxic action of AgNPs was due to its ability to generate reactive oxygen species and consequently cause DNA damage by oxidative stress in mammalian cells (Hussain et al. 2005; Choi et al. 2010; Chen et al. 2014; El Mahdy et al. 2015). These findings could also be explained by AgNPs reaching the cells of numerous organs, such as the kidneys, liver and lymphoid organs, by binding to plasma proteins (Wijnhoven et al. 2009). In contrast, other authors found that a variable dose of oral AgNPs (2.87–63.74 mg/bird) or 50 mg/kg in drinking water did not result in silver accumulation in broiler breast muscles and did not affect tissue histology or morphology (Kulak et al. 2018; Kumar et al. 2020).

Previous research has shown that adding dietary AgNP powder reduces the population of lactose-positive and enterococcal bacteria and increases the *Lactobacillus* population after three weeks from treatments (day 28) (Ahmadi and Kurdestany 2010; Elkloub et al. 2015; Bolandi et al. 2021). Results of several studies (Fondevila 2010, Vadalasetty et al. 2018) indicated that the application of AgNPs in drinking water at 5, 15 and 25 mg/L had no impact on growth or intestinal microbial count, but increased the number of lactobacilli. Differences in AgNP concentrations, species, dietary ingredients, and even bacterial counting methods could explain some discrepancies in the results in different studies.

The addition of AgNPs significantly impacted the mean values of various blood parameters (Table 7**)**. This finding is consistent with Ahmadi (2012), who fed broiler chicks with feed supplemented with 20, 40, 60 ppm AgNPs and observed significant changes in total protein, albumin, and globulin. Serum total cholesterol significantly ($p < .05$) and triglycerides non-significantly ($p > .05$) decreased in all treatments relative to the control. Those results indicate that AgNPs had no negative effect on lipid profile or blood indices as reported previously (Sawosz et al. 2009; Andi et al. 2011; Ognik et al. 2016; Kumar et al. 2020). Ognik et al. (2016) reported that the decrese in the total cholesterol could be due to degradation of polyunsaturated fatty acid and as a result of lipid peroxidation induced by AgNPs.

Previous research demonstrated that AgNP dietary supplementation resulted in silver residue accumulation in broiler tissues and organs in a dose-dependent manner and that this accumulation is beneficial, which supports the current findings (Kulak et al. 2018; Kumar et al. 2020). Our data support evidence from previous studies that excess silver is not efficiently removed (Hadrup and Lam 2014). Furthermore, because silver has a long elimination half-life, it accumulates in the human body. The European Food Safety Authority established a maximum silver concentration of 0.05 mg/L in water and 0.05 mg/kg in food (EFSA. 2016). For chronic oral silver exposure, the United States Environmental Protection Agency established a reference dose of 5 g/kg body weight/d (SCENIHR 2014). Although the level of silver in broiler breast and thigh muscle may be below the safety limits set for silver in food, it may still be toxic because of its small size and $Ag+$ release capabilities. Incorporating AgNPs into a poultry diet can induce a wide range of adverse effects and can be harmful to both birds and humans (Leino et al. 2021). The results of the present study indicate that the accumulation of AgNPs increases in a dose dependent manner in different broiler meat parts and the hazards from the transmission of nanosilver to humans requires that their use and marketing as feed additives or growth promotors be controlled and restricted.

Conclusions

We found that despite its potential effects on growth performance, carcass traits and caecal microbial population diversity at a dose of 2.5 ppm diet, dietary inclusion of AgNPs in broiler diets had many negative effects in terms of 1) silver residue in breast and thigh muscle in a dose dependent manner and the possibility of transmission of nanosilver to consumers and 2) the cytotoxic effects of AgNPs on intestinal, liver, spleen and kidney cells. Thus, the use of dietary AgNP powder might be harmful to chicken and human health. Therefore, we suggest the use of lower dose of AgNPs (< 2.5 ppm) to be tried in further studies.

Ethical approval

All experimental protocols were approved by the ethics committee for Laboratory Animal use and care at King Faisal University in accordance with the institutional and national guidelines of King Faisal University for laboratory animal research.

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Author contributions

All of the authors contributed equally to the experimental design, sample collection and analysis, and manuscript writing and revision. All authors approved the final draft of the work.

Disclosure statement

The authors report no potential conflicts of interest regarding the publication of this paper.

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Data availability statement

The corresponding author is committed to providing data that support the findings of the study upon request.

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