Supplemental chromium-loaded chitosan nanoparticles affect growth, serum metabolites and intestinal histology in broilers

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Abstract

The goal of the present research was to evaluate the effects of chromium-loaded chitosan nanoparticles (Cr-CNPs) on production performance, viscera development, serum metabolites and intestinal histology in broilers. Two hundred (200) day-old broilers were randomly divided into five groups with five replicates (n = 8). Birds in the first group served as control and were fed a corn soybean-based diet, while the remaining four supplemented groups were offered 200, 400, 800, and 1200 µg Cr-CNPs/kg of feed, respectively, for 35 days. Weight gain, feed intake and feed conversion ratio (FCR) remained unaffected with Cr-CNPs supplementation. No changes were observed in the relative weights of viscera. The relative length of the small intestine was decreased in birds supplemented with 200 and 800 µg Cr-CNPs/kg compared with the 1200 µg Cr-CNPs-supplemented group and control. Serum metabolites remained unaffected with Cr-CNPs supplementation except for serum HDL, which was increased. Cr-CNPs decreased the retention of chromium in the bone at higher concentrations. Jejunal villus height, villus surface area, and villus height to crypt depth ratio were increased in the 800 µg Cr-CNP-supplemented group. In conclusion, Cr-CNPs did not affect growth performance, viscera development, and most of the serum metabolites, but enhanced jejunal morphological attributes at 800 µg Cr-CNPs/kg of feed.

Keywords: blood biochemistry, health, nano-biotechnology, prebiotics, poultry, trace mineral

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Introduction

Chromium (Cr), though regarded as an important trace element and a constituent of the glucose tolerance factor is necessary for carbohydrate, fat, and protein metabolism (Mertz, 1993). Chromium supplementation is used to enhance growth performance, reproduction, carcass characteristics and tissue deposition in pigs and broilers (Page et al., 1993; Lindemann et al., 1995; Mooney & Cromwell, 1995; Ghanbari et al., 2012). Most of the Cr contents of a corn-soybean diet are unavailable to animals owing to feed processing. The size of the particle, the nature of its polymers and zeta potential are some of the factors that determine the absorption rate through the intestine (Wang et al., 2007).

Chitosan is a non-toxic and biodegradable carbohydrate polymer and is well digested by birds (Hirano et al., 1990). Functional attributes of intestinal mucosa, including absorptive surface area, expression of brush border enzymes, and nutrient transport systems, are dependent on the shape and size of villi. Chitosan supplementation in birds showed improved intestinal morphology and increased villus size (Khambualai et al., 2009). Its beneficial effects on weight gain, FCR, and nitrogen retention were also investigated in broiler chickens and ducks (Shi et al., 2005; Shi-bin & Hong, 2012).

The application of nanoparticles gained more attention because of their novel properties. Nanoparticles are different in properties from bulk materials because of small size, greater surface area, and shape of particles (Awad et al., 2012). Nano-composites have higher absorption rates in the gastrointestinal tract and are absorbed by gastrointestinal lymphatics (Desai et al., 1996; Hussain et al., 2001). Chromium (III) loaded chitosan nanoparticles have been reported to increase carcass lean percentage, decrease the fat
percentage, and reduce backfat thickness in pigs (Wang & Xu, 2004). They reduce the cholesterol levels in serum as they are involved in fatty acid metabolism and potentiate cellular, humoral, and mucosal immune responses (He et al., 2000). Positive effects of Cr nanoparticles on serum biochemistry, hormones, and immune status are also reported in pigs (Wang et al., 2007).

Organic salts of chromium have greater bioavailability than inorganic salts, but they are not cost effective. Also, prebiotics such as chitosan polysaccharides have been used extensively to improve the gut microbiota and morphology, under both normal and heat stress conditions. To minimize their dosage and excretion and enhance their absorption and solubility, the present research is planned to evaluate the effects of the supplementation of Cr-CNPs on growth performance, viscera development, serum metabolites and intestinal histology in broilers under standard conditions. To the best of the authors' knowledge, no such work is being done on the use of Cr-CNPs in broilers, and only limited studies are available in pigs.

Materials and Methods

All the procedures adopted to perform this experiment were approved by the Ethical Review Committee of the University of Veterinary and Animal Sciences, Lahore, Pakistan, vide letter no. DR/498, dated 09-05-2018.

The Cr-CNPs were prepared and characterized at the Interdisciplinary Research Centre in Biomedical Research at COMSATS University, Islamabad (Lahore Campus), Pakistan, according to the method described by Wang et al. (2012). Briefly, 1% chitosan solution was prepared by dissolving chitosan in 0.5% acetic acid with pH adjusted at 3.5. Afterwards, the chitosan solution was stirred continuously for one hour and 200 mg/l chromium chloride solution was added to the chitosan solution during stirring to obtain a suspension of chitosan and chromium chloride. The pH of the suspension was adjusted to 6.5 and stirring continued for five hours. Then the precipitate was centrifuged at 12,000 g for 15 minutes at room temperature and washed with water and dried to obtain Cr-CNPs.

Day-old broiler chicks (n = 200), purchased from a commercial hatchery, were randomly divided into five groups (n = 40/group), each group having five replicates (n=8/replicate). Birds in the first group were labelled the control group and offered corn a soybean-based diet. Birds in the remaining four groups were labelled 200 Cr-CNPs, 400 Cr-CNPs, 800 Cr-CNPs, and 1200 Cr-CNPs, and were offered the same diet supplemented with graded levels of Cr-CNPs at the dose level of 200, 400, 800 and 1200 µg/kg of feed, respectively, for 35 days. The feed and water were provided ad libitum. Temperature and relative humidity on day 1 were kept at 35 ± 1.1 °C and 65 ± 5%, respectively. The temperature was decreased by 3 °C each week till it reached 26 °C. Birds were vaccinated against Newcastle disease and Infectious Bursal disease, as mentioned by Giambron and Clay (1986). The composition of the diet is presented in Table 1 (Khan et al., 2016).

Feed intake was measured daily, while bodyweight gain and FCR were determined weekly. On day 35 two birds from each replicate were randomly selected and slaughtered. The blood sample was collected and allowed to clot to collect serum that was stored at -40 °C until the analyses of serum metabolites. Viscera were collected to calculate the weights and lengths of the relative organs. The liver and whole tibial bone were stored at -40 °C to estimate their chromium contents. Intestinal segments (duodenum, jejunum, and ileum) were removed, washed with saline and stored in neutral buffered formalin for histology.

Prior to analysis, serum samples were thawed, vortexed and then analysed for lipid profile (total cholesterol, triglycerides and HDL-cholesterol), serum proteins (total proteins, albumin and globulins), urea and uric acid using commercial kits (HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany) according to the manufacturer's recommendations with the help of Epoch™ micro-plate spectrophotometer (Biotek Instruments Inc., Winooski, USA). The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatinine levels were determined by commercial kinetic kits (HUMAN Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany, and Diasys, Holzheim, Germany) using UV/VIS spectrophotometer (UV-2800, Thermo Fischer Scientific, Waltham, Massachusetts , USA). Chromium concentration in samples was estimated using flame atomic absorption spectrophotometer (iCE 3300 double beam AA spectrometer, Thermo Fischer Scientific, Waltham, Massachusetts , USA) after wet digestion (Yousaf et al., 2009).

The intestinal mucosal morphometry was determined by analysing the duodenum, jejunum and ileum villus height, crypt depth, villus surface area, and villus height to crypt depth ratio. Intestines, collected from birds (two birds from each group), were processed according to a conventional method of haematoxylin and eosin (Ashraf et al., 2013) and examined with a light microscope (Olympus CX31, Olympus, Center Valley, Pennsylvania, USA) fitted with a digital imaging system (Olympus DP20, Olympus USA). Five villi with intact lamina propria and well orientation were used for observations. Villus height was measured from the tip of the villus to the villus crypt junction, and the crypt depth was measured from its base up to the region of
transition between the crypt and villus. The villus surface area was measured by using the formula \((2\pi)\left(\frac{\text{villus width}}{2}\right)\left(\text{villus length}\right)\).

Table 1 Composition of the diet to be supplemented with chromium-loaded chitosan nanoparticles and fed to broilers

| Ingredients (g/kg)        | Percentage |
|--------------------------|------------|
| Corn                     | 58.50      |
| Soybean meal 44%         | 25.00      |
| Sunflower meal           | 3.50       |
| Canola meal              | 8.00       |
| Vegetable oil            | 1.50       |
| Dicalcium phosphate      | 0.90       |
| Limestone                | 1.51       |
| Common salt              | 0.50       |
| DL-Methionine            | 0.21       |
| L-Lysine HCl             | 0.12       |
| Vitamin premix\(^1\)     | 0.13       |
| Micro mineral premix\(^2\)| 0.13      |

Nutrient contents

|                |          |
|----------------|----------|
| Crude protein  | 20.72    |
| Metabolizable energy (MJ) | 12.20 |
| Calcium        | 0.91     |
| Phosphorus     | 0.61     |

\(^1\) Vitamins A (retinol): 11000 IU; B12 (cyanocobalamin): 0.0132 mg; D\(_3\) (cholecalciferol): 2200 IU; E (alpha-tocopherol): 22 IU; choline chloride: 440 mg; riboflavin: 8.8 mg; pantothenic acid: 22 mg; ethoxyquin: 250 mg; menadione: 2.2 mg; pyridoxine: 4.4 mg; folic acid: 1.1 mg; biotin: 0.22; thiamine: 4.4 mg

\(^2\) Copper (CuSO\(_4\)): 20 mg; zinc (ZnO): 20 0mg; manganese (MnSO\(_4\)): 240 mg; iron (FeSO\(_4\)): 120 mg; iodine (KI): 0.92 mg; calcium: 150–180mg

Data were analysed statistically using SPSS for Windows version 20.0 (IBM Inc., Armonk, New York, USA). Data were presented as mean ± SEM and analysed using one-way analysis of variance (ANOVA). For group differences, Duncan's multiple range test was used. Orthogonal contrasts were used to determine the linear, quadratic and cubic effects of Cr-CNPs at \(P<0.05\).

Results

The weight gain of birds supplemented with 200 µg Cr-CNPs/kg was found to be higher \((P<0.05)\) compared with the 800, and 1200 Cr-CNPs and control groups during week 2. But in weeks 1, 3, 4, and 5, no effect was found on weight gain in all the groups (Table 2). The mean feed intake remained unaffected by Cr-CNP-supplementation during all the weeks (Table 3). The FCR was significantly lower in birds supplemented with 400 and 200 µg Cr-CNPs/kg compared with the control and 1200 µg Cr-CNPs/kg supplemented groups during weeks 1 and 2, respectively. However, the FCR remained unchanged with Cr-CNP supplementation in weeks 3, 4, and 5 as shown in Table 4.
Table 2 Effects of supplementation with chromium-loaded chitosan nanoparticle on weekly weight gain (g) in broilers

| Weeks | Treatments | Control | 200 Cr-CNPs | 400 Cr-CNPs | 800 Cr-CNPs | 1200 Cr-CNPs | SEM | P-Value | Linear | Quadratic | Cubic |
|-------|------------|---------|-------------|-------------|-------------|-------------|-----|---------|--------|-----------|-------|
| Week 1 |             | 78      | 91          | 93          | 87          | 85          | 1.8 | 0.05    | 0.33   | 0.01      | 0.24  |
| Week 2 |             | 184<sup>a</sup> | 220<sup>b</sup> | 203<sup>ab</sup> | 184<sup>bc</sup> | 166<sup>c</sup> | 5.4 | 0.004   | 0.02   | 0.003     | 0.06  |
| Week 3 |             | 345     | 353         | 359         | 350         | 317         | 8.2 | 0.57    | 0.33   | 0.19      | 0.73  |
| Week 4 |             | 528     | 576         | 544         | 531         | 534         | 12.8 | 0.80     | 0.75   | 0.55      | 0.34  |
| Week 5 |             | 625     | 594         | 481         | 571         | 482         | 23.8 | 0.17     | 0.07   | 0.63      | 0.56  |

<sup>1</sup> Data presented as mean ± SEM of five replicated (n = 8 birds/replicate)

<sup>2</sup> Within the row, different superscript indicates significantly different means at P <0.05

<sup>3</sup> Control: without chromium-loaded chitosan nanoparticles (Cr-CNPs); 200 Cr-CNPs: fed with 200 µg Cr-CNPs per kg of feed; 400 Cr-CNPs: fed with 400 µg Cr-CNPs per kg of feed; 800 Cr-CNPs: fed with 800 µg Cr-CNPs per kg of feed; 1200 Cr-CNPs: fed with 1200 µg Cr-CNPs per kg of feed

Table 3 Effects of supplementation with chromium-loaded chitosan nanoparticles on weekly feed intake (g) in broilers

| Weeks | Treatments | Control | 200 Cr-CNPs | 400 Cr-CNPs | 800 Cr-CNPs | 1200 Cr-CNPs | SEM | P-Value | Linear | Quadratic | Cubic |
|-------|------------|---------|-------------|-------------|-------------|-------------|-----|---------|--------|-----------|-------|
| Week 1 |             | 126     | 138         | 137         | 135         | 137         | 1.7 | 0.13    | 0.10   | 0.12      | 0.13  |
| Week 2 |             | 311     | 323         | 320         | 284         | 291         | 5.6 | 0.07    | 0.03   | 0.30      | 0.11  |
| Week 3 |             | 631     | 613         | 629         | 599         | 542         | 14.4 | 0.29    | 0.07   | 0.31      | 0.54  |
| Week 4 |             | 870     | 912         | 862         | 917         | 819         | 15.1 | 0.29    | 0.36   | 0.16      | 0.56  |
| Week 5 |             | 1244    | 1196        | 1075        | 1182        | 1007        | 35.3 | 0.17    | 0.05   | 0.92      | 0.39  |

<sup>1</sup> Data presented as mean ± SEM of five replicated (n = 8 birds/replicate)

<sup>2</sup> Control: without chromium-loaded chitosan nanoparticles (Cr-CNPs); 200 Cr-CNPs: fed with 200 µg Cr-CNPs per kg of feed; 400 Cr-CNPs: fed with 400 µg Cr-CNPs per kg of feed; 800 Cr-CNPs: fed with 800 µg Cr-CNPs per kg of feed; 1200 Cr-CNPs: fed with 1200 µg Cr-CNPs per kg of feed

Table 4 Effects of supplementation with chromium-loaded chitosan nanoparticles on weekly feed conversion ratio in broilers

| Weeks | Treatments | Control | 200 Cr-CNPs | 400 Cr-CNPs | 800 Cr-CNPs | 1200 Cr-CNPs | SEM | P-Value | Linear | Quadratic | Cubic |
|-------|------------|---------|-------------|-------------|-------------|-------------|-----|---------|--------|-----------|-------|
| Week 1 |             | 1.63<sup>a</sup> | 1.53<sup>ab</sup> | 1.48<sup>b</sup> | 1.55<sup>ab</sup> | 1.62<sup>a</sup> | 0.02 | 0.03    | 0.92   | 0.002     | 0.67  |
| Week 2 |             | 1.69<sup>ab</sup> | 1.47<sup>b</sup> | 1.58<sup>bc</sup> | 1.55<sup>bc</sup> | 1.76<sup>a</sup> | 0.03 | 0.01    | 0.22   | 0.002     | 0.55  |
| Week 3 |             | 1.84     | 1.73         | 1.78         | 1.72         | 1.71         | 0.03 | 0.70    | 0.26   | 0.69      | 0.64  |
| Week 4 |             | 1.67     | 1.60         | 1.60         | 1.73         | 1.54         | 0.04 | 0.57    | 0.59   | 0.73      | 0.17  |
| Week 5 |             | 2.00     | 2.01         | 2.26         | 2.08         | 2.12         | 0.04 | 0.34    | 0.33   | 0.33      | 0.94  |

<sup>1</sup> Data presented as mean ± SEM of five replicated (n = 8 birds/replicate)

<sup>2</sup> Within the row, different superscript indicates significantly different means at P <0.05

<sup>3</sup> Control: without chromium-loaded chitosan nanoparticles (Cr-CNPs); 200 Cr-CNPs: fed with 200 µg Cr-CNPs per kg of feed; 400 Cr-CNPs: fed with 400 µg Cr-CNPs per kg of feed; 800 Cr-CNPs: fed with 800 µg Cr-CNPs per kg of feed; 1200 Cr-CNPs: fed with 1200 µg Cr-CNPs per kg of feed
The relative viscera weights and caecal length remained unaffected with Cr-CNP supplementation. But, the relative length of the small intestine was significantly reduced (P < 0.05) with Cr-CNP supplementation compared with the control group (Table 5). The serum metabolites remained unaffected with Cr-CNP supplementation except for the serum HDL-cholesterol which was increased (P < 0.05) with Cr-CNP supplementation compared with the control group (Table 6). The chromium concentration in serum and liver remained unchanged with Cr-CNP supplementation while in bone, the Cr concentration was decreased (P < 0.001) in 1200 µg Cr-CNPs/kg compared with the 200 µg Cr-CNPs/kg supplemented and control groups (Table 7).

Table 5 Effects of supplementation with chromium-loaded chitosan nanoparticles on relative viscera weights and lengths in broilers

| Treatments 2 | Control | 200 Cr-CNPs | 400 Cr-CNPs | 800 Cr-CNPs | 1200 Cr-CNPs | SEM | P-Value | Linear | Quadratic | Cubic |
|--------------|---------|-------------|-------------|-------------|-------------|-----|---------|--------|-----------|-------|
| Liver        | 3.21    | 2.66        | 2.54        | 2.59        | 2.48        | 0.10| 0.12   | 0.03   | 0.11      | 0.38  |
| Pancreas     | 0.27    | 0.26        | 0.24        | 0.25        | 0.26        | 0.01| 0.31   | 0.60   | 0.12      | 0.56  |
| Gizzard      | 1.88    | 1.85        | 1.71        | 1.90        | 2.01        | 0.04| 0.13   | 0.21   | 0.05      | 0.92  |
| Proventriculus| 0.44   | 0.45        | 0.37        | 0.43        | 0.44        | 0.01| 0.19   | 0.78   | 0.18      | 0.69  |
| Heart        | 0.48    | 0.98        | 0.43        | 0.47        | 0.44        | 0.10| 0.36   | 0.42   | 0.57      | 0.17  |
| Spleen       | 0.20    | 0.13        | 0.10        | 0.13        | 0.15        | 0.02| 0.49   | 0.48   | 0.11      | 0.62  |
| Bursa        | 0.05    | 0.05        | 0.05        | 0.05        | 0.06        | 0.01| 0.68   | 0.29   | 0.61      | 0.38  |
| Caecal Tonsils| 0.02   | 0.02        | 0.02        | 0.01        | 0.02        | 0.01| 0.73   | 0.57   | 0.58      | 0.41  |
| Small Intestine | 2.26  | 2.21        | 2.19        | 2.48        | 2.37        | 0.08| 0.78   | 0.40   | 0.81      | 0.46  |
| Caecum       | 0.13    | 0.13        | 0.14        | 0.12        | 0.13        | 0.01| 0.95   | 0.98   | 0.96      | 0.72  |
| Small Intestine | 9.95a | 8.20b       | 9.16ab      | 8.36b       | 9.95a       | 0.23| 0.02   | 0.92   | 0.01      | 0.83  |
| Caecum       | 0.99    | 0.82        | 0.87        | 0.87        | 0.93        | 0.02| 0.23   | 0.72   | 0.06      | 0.30  |

1 Data were presented as mean ± SEM of five replicated (n = 8 birds/replicate)
2 Within the row different superscript indicates significantly different means at P < 0.05
3 Control: without chromium-loaded chitosan nanoparticles (Cr-CNPs); 200 Cr-CNPs: fed with 200 µg Cr-CNPs per kg of feed; 400 Cr-CNPs: fed with 400 µg Cr-CNPs per kg of feed; 800 Cr-CNPs: fed with 800 µg Cr-CNPs per kg of feed; 1200 Cr-CNPs: fed with 1200 µg Cr-CNPs per kg of feed
4 Intestinal Length = (organ length/body weight)*100

Table 6 Effects of supplementation with chromium-loaded chitosan nanoparticles on serum biochemical metabolites in broilers

| Treatments 2 | Control | 200 Cr-CNPs | 400 Cr-CNPs | 800 Cr-CNPs | 1200 Cr-CNPs | SEM | P-Value | Linear | Quadratic | Cubic |
|--------------|---------|-------------|-------------|-------------|-------------|-----|---------|--------|-----------|-------|
| Liver        | 3.21    | 2.66        | 2.54        | 2.59        | 2.48        | 0.10| 0.12   | 0.03   | 0.11      | 0.38  |
| Pancreas     | 0.27    | 0.26        | 0.24        | 0.25        | 0.26        | 0.01| 0.31   | 0.60   | 0.12      | 0.56  |
| Gizzard      | 1.88    | 1.85        | 1.71        | 1.90        | 2.01        | 0.04| 0.13   | 0.21   | 0.05      | 0.92  |
| Proventriculus| 0.44   | 0.45        | 0.37        | 0.43        | 0.44        | 0.01| 0.19   | 0.78   | 0.18      | 0.69  |
| Heart        | 0.48    | 0.98        | 0.43        | 0.47        | 0.44        | 0.10| 0.36   | 0.42   | 0.57      | 0.17  |
| Spleen       | 0.20    | 0.13        | 0.10        | 0.13        | 0.15        | 0.02| 0.49   | 0.48   | 0.11      | 0.62  |
| Bursa        | 0.05    | 0.05        | 0.05        | 0.05        | 0.06        | 0.01| 0.68   | 0.29   | 0.61      | 0.38  |
| Caecal Tonsils| 0.02   | 0.02        | 0.02        | 0.01        | 0.02        | 0.01| 0.73   | 0.57   | 0.58      | 0.41  |
| Small Intestine | 2.26  | 2.21        | 2.19        | 2.48        | 2.37        | 0.08| 0.78   | 0.40   | 0.81      | 0.46  |
| Caecum       | 0.13    | 0.13        | 0.14        | 0.12        | 0.13        | 0.01| 0.95   | 0.98   | 0.96      | 0.72  |
| Small Intestine | 9.95a | 8.20b       | 9.16ab      | 8.36b       | 9.95a       | 0.23| 0.02   | 0.92   | 0.01      | 0.83  |
| Caecum       | 0.99    | 0.82        | 0.87        | 0.87        | 0.93        | 0.02| 0.23   | 0.72   | 0.06      | 0.30  |
Table 7: Effects of supplementation with chromium-loaded chitosan nanoparticles on chromium concentration (ppm) in various analytes in broilers

| Parameters       | Control | 200 Cr-CNPs | 400 Cr-CNPs | 800 Cr-CNPs | 1200 Cr-CNPs | SEM | P-Value | Linear | Quadratic | Cubic |
|------------------|---------|-------------|-------------|-------------|--------------|-----|---------|--------|-----------|-------|
| Cholesterol (mg/dL) | 126.52  | 141.48      | 131.56      | 143.11      | 147.70       | 2.89| 0.09    | 0.03   | 0.97      | 0.38  |
| Triglycerides (mg/dL) | 195.49  | 201.74      | 202.92      | 203.65      | 211.11       | 2.31| 0.28    | 0.04   | 0.92      | 0.48  |
| HDL (mg/dL)       | 36.07a  | 56.68a      | 46.69a      | 49.22a      | 53.63a       | 2.22| 0.02    | 0.04   | 0.21      | 0.03  |
| ALT (U/L)         | 15.44   | 13.41       | 15.44       | 15.89       | 11.30        | 0.74| 0.22    | 0.25   | 0.27      | 0.09  |
| AST (U/L)         | 50.11   | 61.26       | 61.95       | 56.52       | 62.00        | 1.81| 0.15    | 0.11   | 0.23      | 0.08  |
| Total Proteins (g/dL) | 4.72    | 4.90        | 5.06        | 4.79        | 4.96         | 0.06| 0.44    | 0.41   | 0.37      | 0.38  |
| Albumin (g/dL)    | 3.08    | 2.97        | 3.02        | 3.24        | 3.07         | 0.03| 0.32    | 0.33   | 0.82      | 0.06  |
| Globulins (g/dL)  | 1.64    | 1.92        | 2.05        | 1.56        | 1.89         | 0.07| 0.25    | 0.80   | 0.38      | 0.10  |
| A/G Ratio        | 2.03    | 1.56        | 1.52        | 2.31        | 1.65         | 0.10| 0.09    | 0.97   | 0.54      | 0.02  |
| Creatinine (mg/dL) | 1.70    | 1.76        | 1.74        | 2.03        | 2.05         | 0.22| 0.98    | 0.55   | 0.91      | 0.90  |
| Urea (mg/dL)      | 29.39   | 27.50       | 29.44       | 29.67       | 28.33        | 0.76| 0.90    | 0.99   | 0.93      | 0.41  |
| Uric Acid (mg/dL) | 6.23    | 6.37        | 6.25        | 6.65        | 5.61         | 0.20| 0.25    | 0.33   | 0.10      | 0.31  |

Data presented as mean ± SEM of five replicated (n = 8 birds/replicate)
a,b Within the row different superscript indicates significantly different means at P <0.05

The results of the study show that supplementation with chromium-loaded chitosan nanoparticles (Cr-CNPs) at different concentrations (200, 400, 800, and 1200 µg/kg feed) had significant effects on various parameters of broiler chickens. The Villus height to crypt depth ratio in the duodenum and jejunum of birds supplemented with 800 µg Cr-CNPs/kg supplementation compared with 1200 µg Cr-CNPs/kg supplemented group, while ileal villus height remained unchanged. Crypt depth of duodenum was increased (P <0.001) with 200 and 400 µg Cr-CNPs supplementation. But no effects were observed in jejunal and ileal crypt depth with Cr-CNPs supplementation. The villus width, villus surface area, and villus height to crypt depth ratio remained unaffected in duodenum and ileum with Cr-CNPs supplementation. However, villus width and villus surface area in the jejunum of birds supplemented with 400 and 800 µg Cr-CNPs/kg was found higher (P <0.001) compared with the control group. Villus height to crypt depth ratio of jejunum in birds supplemented with 800 µg Cr-CNPs/kg was found higher (P <0.05) compared with the 1200 Cr-CNPs group.
Table 8 Effects of supplementation with chromium-loaded chitosan nanoparticles on intestinal microarchitecture in broilers

| Parameters | Control | 200 Cr-CNPs | 400 Cr-CNPs | 800 Cr-CNPs | 1200 Cr-CNPs | SEM | P-Value | Linear | Quadratic | Cubic |
|------------|---------|-------------|-------------|-------------|-------------|-----|--------|--------|----------|-------|
| **Duodenum** |         |             |             |             |             |     |        |        |          |       |
| VH (µm)    | 1230ab  | 899bc       | 955ab       | 1226a       | 624c        | 66.01| 0.001  | 0.01  | 0.39     | 0.002 |
| VW (µm)    | 77      | 68          | 64          | 49          | 69          | 3.30 | 0.06   | 0.11  | 0.09     | 0.17  |
| CD (µm)    | 118b    | 227a        | 248a        | 150b        | 144b        | 10.79| <0.001 | 0.57  | <0.001   | 0.001 |
| VSA (mm²)  | 0.24    | 0.17        | 0.20        | 0.16        | 0.17        | 0.01 | 0.18   | 0.06  | 0.38     | 0.56  |
| VH:CD      | 8.69    | 5.12        | 5.11        | 5.83        | 5.38        | 0.44 | 0.13   | 0.07  | 0.06     | 0.12  |
| **Jejunum** |         |             |             |             |             |     |        |        |          |       |
| VH (µm)    | 464b    | 513b        | 635b        | 645b        | 473b        | 23.52| 0.02   | 0.47  | 0.02     | 0.13  |
| VW (µm)    | 49c     | 48b         | 83a         | 65b         | 61bc        | 2.42 | <0.001 | 0.02  | 0.003    | 0.12  |
| CD (µm)    | 100     | 130         | 119         | 109         | 124         | 4.99 | 0.35   | 0.57  | 0.54     | 0.08  |
| VSA (mm²)  | 0.07b   | 0.08b       | 0.16a       | 0.13a       | 0.09b       | 0.01 | <0.001 | 0.11  | 0.001    | 0.04  |
| VH:CD      | 4.81ab  | 4.65b       | 5.67bc      | 6.49a       | 3.80b       | 0.26 | 0.03   | 0.94  | 0.04     | 0.02  |
| **Ileum**  |         |             |             |             |             |     |        |        |          |       |
| VH (µm)    | 535     | 447         | 536         | 502         | 451         | 12.88| 0.39   | 0.23  | 0.65     | 0.49  |
| VW (µm)    | 78      | 72          | 79          | 75          | 82          | 2.14 | 0.66   | 0.49  | 0.49     | 0.94  |
| CD (µm)    | 147     | 132         | 156         | 130         | 146         | 3.90 | 0.19   | 0.90  | 0.70     | 0.93  |
| VSA (mm²)  | 1.30    | 1.02        | 1.35        | 1.19        | 1.23        | 0.05 | 0.17   | 0.97  | 0.73     | 0.28  |
| VH:CD      | 3.91    | 3.64        | 3.62        | 3.92        | 3.18        | 0.11 | 0.32   | 0.16  | 0.52     | 0.15  |

+ Data presented as mean ± SEM of five replicated (n = 8 birds/replicate)
+ a-c within the row different superscript indicates significantly different means at P <0.05
+ Control: without chromium-loaded chitosan nanoparticles (Cr-CNPs); 200 Cr-CNPs: fed with 200 µg Cr-CNPs per kg of feed; 400 Cr-CNPs: fed with 400 µg Cr-CNPs per kg of feed; 800 Cr-CNPs: fed with 800 µg Cr-CNPs per kg of feed; 1200 Cr-CNPs: fed with 1200 µg Cr-CNPs per kg of feed
+ VH: villus height; VW: villus width; CD: crypt depth; VSA: villus surface area; VH:CD: villus height to crypt depth ratio

Discussion

Improved production performance with Cr supplementation is reported in some experiments in broilers, steers and pigs (Rosebrough & Steele, 1981; Chang et al., 1992; Page et al., 1993; Lien et al.,1999), while others reported no effect (Kegly et al., 1996). In the current study, no change was observed in weight gain, feed intake and FCR with Cr-CNPs supplementation in weeks 1, 3, 4, and 5. Unayik et al. (2002) reported no significant effect of Cr supplementation on the bodyweight and weight gain of broilers supplemented with inorganic chromium chloride, but they reported reduced feed intake and improved feed efficiency at the dose of 20 mg/kg Chromium (III) Chloride (CrCl₃). Sirirat et al. (2012) also found no effect of nano-chromium picolinate supplementation on the bodyweight and weight gain but observed decreased feed intake at 500 and 3000 ppb nano-chromium picolinate and improved FCR at 3000 ppb nano-chromium picolinate in broilers. Lin et al. (2015) also found no effect on bodyweight and FCR of broilers with Cr supplementation from either organic or inorganic sources. Zheng et al. (2016) also reported no effect of Cr supplementation from Cr-picolinate, Cr-propionate and CrCl₃ at the concentration of 0.4 and 2 mg/kg feed on the weight gain, feed intake and FCR of broilers. Rajalekshmi et al. (2014) also reported no change in weight gain, feed intake, or FCR with various dietary concentrations of Cr-picolinate ranging from 0 to 3.2 mg Cr/kg diet for 42 days kept under normal conditions. Similarly, Wang et al. (2012) reported that Cr-CNPs (100, 200 and 400 µg/kg feed) supplementation did not affect the performance of the finishing pigs. Under heat stress conditions, Cr supplementation from organic and inorganic sources did not affect bodyweight, feed intake and FCR (Amatya et al., 2004; Meine et al., 2011; Toghyani et al., 2012). The effect on the growth performance of broilers with Cr supplementation remained inconsistent (Rajalekshmi et al. 2014). However,
the differences in studies in terms of growth performance could be because of the differences in the breeds or species, and source and size of chromium, which that greatly affect the bioavailability, as well as environmental variations.

The relative viscera weights and caecal length were not affected by Cr-CNPs supplementation in this study. However, the relative length of the small intestine was decreased significantly in birds supplemented with 200 and 800 μg Cr-CNPs/kg of feed. The relative weight of liver and bursa was increased with CrCl₃ supplementation (Unayik et al., 2002), but no effect was observed on the relative weight of liver and spleen in broilers with nano Cr-picolinate supplementation (Srirat et al., 2012). Similarly, no effect was found in the liver and pancreas weights in chicks fed with Cr-picolinate (Ghanbari et al., 2012). However, the relative weight of liver, heart, spleen, and gizzards was increased linearly with supplementation of Cr-picolinate in broilers reared under heat stress (Sahin et al., 2002), but no effect was seen on the relative mass of liver (Mohini et al., 2011; Toghyani et al., 2012; Akbari & Torki, 2014), bursa (Mohini et al., 2011), spleen (Mohini et al., 2011; Hamidi et al., 2016), pancreas (Toghyani et al., 2012), and heart (Mohini et al., 2011; Habibian et al., 2013; Hamidi et al., 2016) in birds reared during heat stress conditions.

Serum cholesterol and triglycerides were not affected by Cr-CNPs supplementation, but serum HDL-cholesterol was increased linearly and cubically in the present research. Similar observations were reported by Uyanik et al. (2002). They found no effect on serum cholesterol concentration with CrCl₃ supplementation at the doses of 20, 40 and 80 mg/kg of feed in broilers. Motozono et al. (1998) also found no effect on serum cholesterol and triglycerides levels with the addition of Cr-picolinate to the diet. Lin et al. (2015) reported no effect of trivalent chromium supplementation on serum cholesterol and HDL but decreased triglycerides concentration with 1200 μg/kg CrCl₃. In another study by Wang et al. (2012) on Cr-CNPs supplementation at the levels of 100, 200, and 400 μg Cr-CNPs/kg in pigs reported no effect on serum cholesterol, triglycerides, and HDL levels, but Mohini et al. (2011) reported a slight improvement in the serum HDL with Cr supplementation in broilers. However, under heat stress, Cr supplementation reduces the serum cholesterol levels in broilers (Sahin et al., 2002; Habibian et al., 2013) and in quails (Sahin et al., 2005). These variations in results can be owing to the different sources of Cr being supplemented and therefore need further insights. The serum total proteins, albumin, globulin concentrations and the albumin to globulin ratio remained unaffected with Cr-CNPs supplementation. Some studies also indicated no effect on serum proteins with Cr as Cr-yeast (Krolczewska et al., 2004), and CrCl₃ (Bakhiet et al., 2007; Samanta et al., 2008) in boilers and as Cr-CNPs (Wang et al., 2012) in pigs, but other studies reported an increase in serum proteins with supplementation of Cr as CrCl₃ (Unayik et al., 2002; Ahmed et al., 2005) in broilers. During heat stress, serum proteins were increased with Cr supplementation as Cr-picolinate (Sahin et al., 2002) but there was no effect on serum albumin (Akbari & Torki, 2014). Serum urea, creatinine, and uric acid were assessed to investigate Cr toxicity, but Cr as Cr-CNPs did not affect the urea, creatinine and uric acid, which indicates that supplemented doses of Cr were not nephrotoxic. Serum ALT and AST were not changed with Cr-CNPs supplementation, which indicates the supplemented doses of Cr-CNPs were not hepatotoxic. Wang et al. (2012) also reported that serum ALT and AST remained unchanged with the supplementation of Cr as Cr-CNPs in pigs. The current results are in line with those of Bakhtee et al. (2007), who found no effect of Cr supplementation as CrCl₃ on serum AST and uric acid in broilers.

In the present study, Cr concentration in serum and liver remained unchanged with Cr supplementation, but Cr retention was lower in the bone of birds supplemented with 1200 μg Cr-CNPs. The current results are in line with the study conducted by Krolczewska et al. (2004), who reported no effect on serum Cr in chicks supplemented with Cr-yeast at the level of 300 and 500 μg/kg of diet. Similarly, Lin et al. (2015) observed no change in serum Cr of broilers supplemented with CrCl₃ at the dose of 1200 μg/kg, but broilers supplemented with Cr as Cr-picolinate and nano-Cr-picolinate had increased serum chromium at the dose of 1200 μg/kg of diet. Toghyani et al. (2012) found that liver Cr remains unaffected in birds supplemented with CrCl₃ at the levels of 500, 1000, or 1500 μg/kg diet or Cr-nickel at the levels of 500 or 1000μg/kg diet, but increased with Cr-nickel at the level of 1500 μg/kg diet. Similarly, Zha et al. (2009) observed an increase in liver retention of Cr in birds supplemented with CrCl₃, Cr-picolinate or Cr-nanoparticles at the level of 500 μg/kg diet. However, Amatya et al. (2004) found a decrease in liver Cr concentration with Cr supplementation as CrCl₃ or Cr-yeast at the dose of 200 μg/kg diet. In another study by Wang et al. (2012), they reported that Cr retention in blood, muscle, liver, kidney, heart, and pancreas increased linearly with Cr supplementation as Cr-CNPs in pigs.

Improvement in the mucosal morphology of the gut is regarded as a health indicator and growth in birds (Awad et al., 2009). The intestine mucosal barrier, which consists of epithelial cells, provides selective permeability, which allows only essential nutrients and prevents the entry of harmful components, such as bacteria and its toxins, from the intestinal lumen (Lee et al., 2015). Any damage to intestinal cells breaches the barrier, and results in the entry of harmful substances that may lead to the shortening of villi and epithelial sloughing (Sikandar et al., 2017). The absorption of nutrients across the intestine is increased
greatly by the intestinal villi and microvilli (Awad et al., 2009). Intestinal health and integrity are associated with villus height, width, surface area, and villus height to crypt depth ratio, which are the important indicators of intestinal digestion and absorption (Li et al., 2018). The literature about the effects of chromium on intestinal histology in broilers is scarce. In the current research, the villus height of duodenum and jejunum was found to be significantly higher in birds supplemented with 800 µg Cr-CNPs but remained unaffected in the ileum. Crypt depth of duodenum in birds supplemented with 200 and 400 µg Cr-CNPs/kg was found to be higher (P < 0.001) compared with other supplemented and control groups. The jejunal and ileal crypt depth remained unaffected with supplementation compared with the control group. The duodenal and ileal villus surface area remained unaffected with Cr-CNPs supplementation. The jejunal villus surface area of birds supplemented with 400 and 800 µg Cr-CNPs/kg was found higher (P < 0.001) compared with the control group. The villus height to crypt depth ratio of birds supplemented with 800 µg Cr-CNPs/kg was found to be higher (P < 0.001) compared with the 1200 µg Cr-CNPs/kg supplemented group. Li et al. (2018) conducted a study to observe the effects of Cr-picolinate in duck reared under heat stress and found that Cr-picolinate did not affect the villus height and crypt depth in duodenum, jejunum, and ileum at days 14, 21 and 35. However, the villus height to crypt depth ratio was increased significantly with Cr-picolinate supplementation in the jejunum and ileum.

Conclusion
Supplemental Cr-CNPs did not affect growth and development of the viscera or cause hepatic damage or nephrotoxicity. Supplementation with Cr-CNPs did affect retention of chromium in the bone. Intestinal histology was also improved with Cr-CNPs supplementation. However, future investigations are needed to gain insight into the exact mechanism that underlies the effects of supplemental Cr-CNPs in broilers.

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Authors’ Contributions
SKT, MSY, MAR and HR participated in the research design and conduction of an experimental trial. AFK helped in the preparation and characterization of nanoparticles. SKT, SA, HZ and IK participated in the data collection and laboratory analyses. SKT, MSY and HR analysed the data and prepared the manuscript.

Conflict of Interest Declaration
The authors declare that they have no conflict of interest.

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