

RESEARCH ARTICLE

Effects of Copper nanoparticle supplementation on growth, intestinal morphology and antioxidant status in broilers exposed to cyclic cold stress

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ABSTRACT

The purpose of this research was to determine the effects of copper nanoparticles (CuNP) supplementation on growth performance, intestinal morphology, intraepithelial lymphocytes (IELs), goblet cell (GC) count, stress hormones and oxidant/antioxidant balance of broilers exposed to cold stress. One day old broiler chickens (n = 400) were allocated into five different groups and were raised under standard conditions until the 21st day. The five groups were designated as thermo-neutral (TN reared in 26°C), cold stress (CS raised under 13°C ± 2°C for 8 hours/day from d 22- d 35), CS5 (CS + 5mg/kg diet CuNP), CS10 (CS + 10mg/kg diet CuNP) and CS15 (CS + 15mg/kg diet CuNP). On 35th day, 16 birds per group were selected randomly for blood collection and were then humanely sacrificed for tissue sample collection. CS group had a reduced (P<0.05) body weight (BW), villus surface area (VSA), villus height to crypt depth ratio (VH: CD) but had a higher (P<0.05) feed conversion ratio (FCR), goblet cell count, serum corticosterone and malondialdehyde (MDA) levels than the TN group. Inclusion of CuNP in diet linearly increased (P<0.05) BW, VH, VW, CD, VSA and GC count in jejunum and ileum. Conversely, MDA and corticosterone hormone levels and FCR, decreased linearly (P<0.05) with CuNP supplementation. In conclusion, during cold stress conditions, CuNP is a valuable feed additive in broilers with positive effects on growth performance and intestinal morphology.

Keywords: Corticosterone; Epithelium; Histomorphometry; Malondialdehyde; Poultry

INTRODUCTION

Continued research and development have improved broiler genetics and as a result, there is an improvement in early weight gain with better feed conversion as compared to the past (Havenstein et al., 2003). This improvement has resulted an increased sensitivity of broilers to a variety of stressors including temperature stress (Burkholder et al., 2008) which can lead to production losses (Shini et al., 2010). Broilers are sensitive to changes in environmental temperature and 18°C to 21°C is the optimum range for production. Temperatures below 18°C are considered as cold stress, which reduces weight gain, feed efficiency,

damages intestinal mucosa and leads to early mortality in broilers (Zhang et al., 2017). Cold stress also results in an increased corticosterone levels by activating the (HPI) hypothalamic-pituitary-thyroid axis (Slota et al., 2015). It has been reported that high levels of corticosterone are related to stress, and immunosuppression. Cold stress disturbs gastrointestinal micro flora resulting in the growth of pathogens such as *Escherichia coli* and *Salmonella heidelberg* (Borsoi et al., 2015).

Cold stress is a major barrier in the development of the poultry industry in cold regions (Tsiouris et al., 2015). Controlled temperature housing systems have substantially

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overcome the effects of thermal stress in broilers and have aided in maintaining a comfortable temperature for proper growth, however; they are expensive to operate (Yang, et al., 2014).

Antibiotics growth promoters have been used in the poultry diet to compensate for the effects of cold stress (Huff et al., 2015) but these growth promoters cause microbial resistance and results in the disturbance of normal gut flora (Gunal et al., 2006). Due to these challenges, the use of antibiotic growth promoters was banned in 2006 (EC Regulation No. 1831/2003). In broiler nutrition, today nanoparticles are used as a substitute to antibiotic growth promoters (Ahmadi, and Rahimi, 2011). Encouraging results have been obtained with the use of nanoparticles (silver, zinc oxide and selenium) when used as an alternative to growth promoters in livestock and poultry (Fondevila and Herrer, 2009).

Copper (Cu) is a vital microelement necessary for the proper growth and development of various organs such as bones, heart and connective tissues. It is also important in immune system development and acts as an antioxidant agent in the body. Cu cannot be stored in the body and, therefore, it is added to the poultry diet in higher doses (Świątkiewicz et al., 2014). Due to their physiochemical properties, copper nanoparticles can easily penetrate the cell membrane of intestinal endothelial cells and can be rapidly distributed (Anjum et al., 2016). Due to the high absorbance capability of nanoparticles, lower doses of nanoparticles have been used in the poultry diet recently (Pineda et al., 2013; Mroczek-Sosnowska et al., 2015; Mroczek-Sosnowska et al. 2016; Scott et al., 2017). Copper nanoparticles help in blood vessels development in embryos (Mroczek-Sosnowska et al. 2015). *In ovo* use of copper nanoparticles results in a decrease of metabolic rate in embryos without affecting the immunity of chicks (Pineda et al., 2013) as well as improved body weight gain and FCR later on (Mroczek-Sosnowska et al., 2016). Improved FCR and body weight (Scott et al., 2018), accumulation of Cu in the intestinal walls and increased Cu levels in blood have also been observed when nanoparticles were used in drinking water (Ognik et al., 2016). Cu is not stored in the body and its excess is excreted from the body, thus, a regular supply of copper is required in the diet of the animals (Scott et al., 2018). A twenty times increase in the dose of Cu than normal requirements have beneficial health effects. Dietary Cu is toxic when it exceeds a hundred folds compared to the normal requirements. Cu is mainly absorbed in the duodenum by endocytosis/pinocytosis. Cu is also transported by proteins such as divalent metal transporter 1 (DMT1) and Copper transport 1 (CTR1) from intestinal cells into the blood (Ognik et al., 2016).

Cold stress results in immunosuppression and causes a decrease of lymphocytes in broilers (Yang et al., 2014). Cold stress also results in oxidative stress in the broiler and ultimately affects the digestive system (Fu et al., 2012). Cu is a major part of several antioxidant enzymes and the addition of copper nanoparticles in the broiler diet can improve the antioxidant status of the poultry. It has been proved that copper nanoparticles in the broiler feed improved SOD and malondialdehyde activity (Ognik et al., 2017). Copper nanoparticles (CuNP) have been used in numerous ways in broiler diets and have shown positive results on growth performance (Mroczek-Sosnowska et al., 2016), hematological parameters (Scott, et al., 2018) and immune status (Ognik et al., 2017) and acts as an antibacterial agent (DeAlba-Montero et al., 2017), but their efficacy has not yet been tested under cold stress conditions in broilers yet and there is a scarcity of scientific reports in this regard. We formed the hypothesis that copper nanoparticles, owing to their antioxidant and antibacterial properties, would lead to a reduction in the overall cold induced oxidative stress.

The objective of this research was an evaluation of the effects of CuNP on broiler growth performance, intestinal morphometry, intraepithelial lymphocyte count, number of goblet cells and stress hormones under cold stress conditions.

MATERIALS AND METHODS

The research was carried out following the procedures of the Ethical Review Committee, University of Veterinary and Animal Sciences, Lahore, Pakistan (no. DR/391-1).

Copper nanoparticles

Copper nanoparticles with 99.8% purity and 25nm in size were obtained from sky-Spring (Nanomaterials Inc., USA). Copper nanoparticles were mixed in broiler according to the method described by Ewa Sawosz et al. (2018).

Experimental Design

Four hundred one-day Hubbard broiler chicks were randomly separated into five treatments (eight replicates/treatment and ten birds/pen). A corn-based diet (Table 1) was used during the experiment and was formulated according to the NRC recommendations (NRC, 1994) for the starter and grower phase. Birds in control (thermo-neutral) and cold stress (CS) groups were fed with the basal diet only while, birds in other three groups received CuNP mixed in the diet at the dose rate of 5mg/kg (CS5), 10mg/kg (CS10) and 15mg/kg (CS15) respectively. Birds were fed *ad libitum* throughout the experimental period.

During first week, temperature was maintained at $35 \pm 2^\circ\text{C}$ and a relative humidity $65 \pm 5\%$. After first week, temperature was decreased $3^\circ\text{C}/\text{week}$ until it reached 26°C by 21st day. From 22nd to 35th day, birds in TNG continued to receive temperature of 26°C for 24 hours and were separated from other groups. All other groups i.e.; CS, C5, CS10 and CS15 were exposed to lower temperature treatment of $13^\circ\text{C} \pm 2^\circ\text{C}$ for eight hours/day (12 am - 8 am) and 26°C for the rest of the day until the end of the experiment i.e. 35th day. Light was supplied for 24 hours throughout the experimental period.

Growth performance

Feed intake (FI) was monitored on a day-to-day basis while body weight (BW) and feed conversion ratio (FCR) were documented every week.

Table 1: Composition of basal diet used during the experiment

| Ingredients (%) | Starter Phase | Grower Phase |
|--|---------------|--------------|
| Corn | 40 | 57 |
| Rice Broken | 15 | 0 |
| Rice Polish | 0 | 4.5 |
| Wheat Bran | 1.2 | 0 |
| Soybean meal | 11 | 10 |
| Sunflower meal | 12 | 13 |
| Canola meal | 9.3 | 5 |
| Rapeseed meal | 5 | 6 |
| Guar meal | 1 | 0 |
| Molasses | 2 | 1 |
| DCP ¹ | 1.76 | 2 |
| Vitamins and mineralsPremix ² | 1 | 1 |
| Sodium chloride | 0.21 | 0.41 |
| Sodium bicarbonate | 0.03 | 0.09 |
| Chemical composition (% DM) | | |
| Crude protein | 19.5 | 18.5 |
| Crude fat | 2.1 | 2.30 |
| Crude fiber | 1.25 | 1.75 |
| Total Ash | 5.7 | 5.5 |
| Calculated ME (Kcal/kg) | 2750 | 2850 |

¹Dicalcium Phosphate

²Vitamin mineral premix (in 1 kg) : Ca, 190 g; K, 80 g; Na, 20 g; Mg, 8 g; Zn, 3500 mg; Fe, 7500 mg; Mn, 6,000 mg; Se, 12 mg; Co, 40 mg; I, 35 mg; Cu 8.5g; vitamin A, 200,000 IU; vitamin D3, 75,000 IU; vitamin E, 1100 IU; vitamin K3, 30 mg; ascorbic acid, 1,400 mg; thiamine, 135 mg; riboflavin, 350 mg; niacin, 3,340 mg; vitamin B6, 350 mg; folic acid, 45 mg; vitamin B12, 0.67 mg; and biotin, 13.5mg. ME, Metabolizable energy.

Tissue sampling

On 35th day, 16 birds from each group (2 from each replicate) were randomly selected. Before killing them through cervical dislocation, 3ml blood was sampled from the brachial vein in a non-heparinized tube for hormonal studies.

For histomorphometric analysis, 3cm samples from all the intestinal segments (duodenum, jejunum and ileum) were collected from all the sampled birds. For the sake of sampling, small intestine was distributed into duodenum (pylorus to the last part of duodenal loop), jejunum (duodenal loop to Meckel's diverticulum) and ileum (Meckel's diverticulum to anterior portion of ileocecal connection). Samples were washed with saline solution before fixation in 10 % formaldehyde solution and stored prior to histological processing (Aiyen et al., 2018).

Tissue processing, staining and morphometric analysis

After fixation, samples were trimmed and placed in a tissue cassette. The specimens were processed by paraffin embedding technique and stained using hematoxylin and eosin (H&E) and Alcian blue-PAS (Periodic acid Schiff) staining technique (Bancroft et al., 2018).

H&E stained slides were used for the morphometric evaluation of small intestine. Villus height, width, crypt depth and villus surface area were measured using villus crypts units with intact lamina propria. The surface area was calculated using a formula $(2\pi)(VW/2)(VL)$ (Ashraf et al., 2013).

Round shaped nuclei help to distinguish IELs from oval nucleated enterocytes in H&E stained slides. (Ashraf et al., 2013).

Alcian blue-PAS (Periodic acid Schiff) stained tissue specimens were used for counting and differentiation of goblet cells in different sections of the intestine as described previously (Bancroft et al., 2018).

Serum processing and analysis

Blood samples were centrifuged at 2500 rpm for 10 minutes and serum was stored at -20°C . Levels of Corticosterone, Triiodothyronine (T3), Thyroxine (T4) levels were analyzed

Table 2: Body weight and feed intake at different levels of copper nanoparticles (CuNP) during the thermo-neutral period.

| Parameters | Week | Treatments | | | | P-Value | | |
|------------|------|------------|-----|------|------|---------|--------|-----------|
| | | TNG | CS5 | CS10 | CS15 | SEM | Linear | Quadratic |
| BW (g) | 1 | 143 | 148 | 149 | 147 | 1.38 | 0.001 | 0.02 |
| | 2 | 420 | 415 | 416 | 425 | 2.18 | 0.14 | 0.001 |
| | 3 | 720 | 731 | 731 | 756 | 2.47 | <0.001 | 0.009 |
| FI (g) | 1 | 111 | 110 | 112 | 112 | 0.82 | 0.25 | 0.34 |
| | 2 | 341 | 340 | 347 | 341 | 1.89 | 0.18 | 0.19 |
| | 3 | 516 | 521 | 521 | 522 | 2.55 | 0.04 | 0.29 |

Control, Thermo- neutral group; CS5: CS+ 5mg CuNP per kg of feed; CS10: CS+10mg CuNP per kg of feed and CS15: CS+ 15mg CuNP per kg of feed; SEM=standard error of the mean; BW=Body weight; FI=Feed intake.

with the help of ELISA kits (IBL International GmbH, Hamburg, Germany). SOD (superoxide dismutase) concentrations were measured as per instructions (Randox, UK). MDA (Malondialdehyde) levels were accessed using previously described method. (Ali et al., 2017).

Statistical analysis

The Statistical analyses were executed in R version R-4.1.2 (R Core Team, 2021). T- test was used to compare the means of different parameters between cold stress (CS) and thermo neutral (TN) groups. The linear and quadratic polynomial contrast were used to examine the response to increasing dose to CuNP using package dplyr (Hadley et al., 2021). Results were assumed significant at P-value ≤ 0.05 .

RESULTS

Growth Performance

The average BW and FI of the thermo-neutral period are shown in Table 2. The results show that addition CuNP had a linear increase ($P \leq 0.05$) in BW during 1st and 3rd weeks. CuNP supplementation also showed a quadratic effect ($P \leq 0.05$) in BW during the first three weeks. Inclusion of different levels of CuNP into broilers diet showed no linear and quadratic effect on FI during the thermo-neutral period ($P > 0.05$).

The BW, FI and FCR of TNG and cold stress groups during the cold stress period are presented in Table 3. BW of TNG significantly improved ($P \leq 0.05$) during 5th week. There was no significant difference ($P > 0.05$) on FI amongst CS and TNG during the cold stress period. At the end of

the experiment, a significant difference ($P \leq 0.05$) in FCR was observed between TNG and CS groups.

Inclusion of CuNP in broiler diets had a linear increase in BW during the cold exposure period. CuNP supplementation also had a quadratic effect ($P \leq 0.05$) on BW during the 4th and 5th weeks. At the end of the experiment, inclusion of CuNP in broiler diets linearly decreased ($P \leq 0.05$) FCR and maximum FCR was recorded in the CS group (1.72). No significant linear effect ($P > 0.05$) was noticed on FI during 4th and 5th week but FI differ quadratically ($P \leq 0.05$) during 4th week FI in broilers (Table 4)

Histomorphometry of small intestine

Results from histomorphometric measurements of the small intestine between TNG and CS are shown in Table 5. Cold stress (CS) had a reduced ($P \leq 0.05$) VSA in duodenum, jejunum and ileum compared to TNG. CS also resulted in a decreased ($P \leq 0.05$) VH, VW and CD in all the intestinal segments and in jejunum and ileum, reduced ($P \leq 0.05$) VH: CD ratio was observed as compared to TNG. However, VH: CD ratio was non-significant ($P > 0.05$) between CS and TNG in duodenum.

Effects of different dietary levels of CuNP during cold stress on histomorphometric parameters are displayed in Table 6. CuNP supplementation in broiler diets linearly increased ($P \leq 0.05$) VH, VW, CD and VSA in small intestine. A significant linear effect ($P \leq 0.05$) was also observed in VH: CD ratio in duodenum and ileum. CuNP also had a quadratic effect ($P \leq 0.05$) on VW, CD and VSA in duodenum and ileum. Quadratic effect ($P \leq 0.05$) was also observed for VH: CD ratio in whole small intestine and for villus height in duodenum only

Goblet cells (GC) and intraepithelial lymphocytes count (IELs)

A significant increase ($P < 0.05$) in goblet cells was observed (acidic and total goblet cells) in jejunum and ileum (acidic, mixed and total) in CS group as compared to the TNG. IELs did not differ significantly ($P > 0.05$) between CS and TNG. (Table 7)

Table 3: Body weight, feed intake and FCR of cold stress (CS) and thermoneutral (TN) group.

| Parameters | Week | CS | TN | SEM | P-value |
|------------|------|------|------|------|---------|
| BW (g) | 4 | 1109 | 1106 | 3.42 | 0.54 |
| | 5 | 1633 | 1680 | 8.02 | <0.001 |
| FI (g) | 4 | 727 | 734 | 4.54 | 0.30 |
| | 5 | 1054 | 1037 | 14.2 | 0.39 |
| FCR | | 1.72 | 1.67 | 0.01 | 0.009 |

CS, cold stress group; TN, thermo-neutral group; SEM=standard error of the mean; BW=Body weight; FI=Feed intake; FCR=Feed conversion ratio.

Table 4: Body weight, feed intake and feed FCR during cold stress period at different dietary levels of copper nanoparticles (CuNP)

| Parameters | Week | Treatments | | | | | SEM | P-Value | |
|------------|------|------------|------|------|------|--------|---------|-----------|--|
| | | CS | CS5 | CS10 | CS15 | Linear | | Quadratic | |
| BW (g) | 4 | 1109 | 1124 | 1123 | 1207 | 3.8 | < 0.001 | < 0.001 | |
| | 5 | 1633 | 1658 | 1681 | 1725 | 6.1 | < 0.001 | < 0.001 | |
| FI (g) | 4 | 727 | 736 | 742 | 728 | 3.9 | 0.6 | 0.006 | |
| | 5 | 1054 | 1060 | 1055 | 1031 | 11.1 | 0.15 | 0.19 | |
| FCR | | 1.72 | 1.71 | 1.70 | 1.64 | 0.009 | < 0.001 | 0.06 | |

CS, cold stress group; CS5: CS+5mg CuNP per kg of feed; CS10: CS+10mg CuNP per kg of feed and CS15: CS+15mg CuNP per kg of feed; SEM=standard error of the mean; BW=Body weight; FI=Feed intake; FCR=Feed conversion ratio.

CuNP supplementation had a linear effect ($P < 0.05$) on acidic and total goblet cells in jejunum and ileum and mixed goblet cells in ileum alone. Acid goblet cells in jejunum and ileum, total goblet cells in ileum and mixed goblet cells in duodenum also displayed a quadratic response ($P \leq 0.05$) with increased dose of copper nanoparticles (Table 8).

CuNP supplementation did not show any linear or quadratic effect on IELs in all the segments of the small intestine.

Table 5: Histomorphometric parameters of small intestine of cold stress (CS) and thermoneutral (TN) group

| Parameters | CS | TN | SEM | P-Value |
|------------------------|------|------|--------|---------|
| Duodenum | | | | |
| VH (mm) | 1.11 | 1.28 | 0.002 | <0.001 |
| VW (mm) | 0.12 | 0.15 | 0.0008 | <0.001 |
| CD | 0.11 | 0.13 | 0.001 | 0.003 |
| VSA (mm ²) | 0.43 | 0.61 | 0.003 | <0.001 |
| VH: CD | 8.40 | 8.39 | 0.045 | 0.29 |
| Jejunum | | | | |
| VH (mm) | 0.72 | 0.74 | 0.002 | <0.001 |
| VW (mm) | 0.11 | 0.10 | 0.0001 | <0.001 |
| CD | 0.12 | 0.11 | 0.006 | <0.001 |
| VSA (mm ²) | 0.25 | 0.23 | 0.003 | 0.006 |
| VH: CD | 5.72 | 6.35 | 0.036 | <0.001 |
| Ileum | | | | |
| VH (mm) | 0.50 | 0.54 | 0.003 | <0.001 |
| VW (mm) | 0.11 | 0.13 | 0.0009 | <0.001 |
| CD | 0.10 | 0.11 | 0.001 | <0.001 |
| VSA (mm ²) | 0.18 | 0.22 | 0.001 | <0.001 |
| VH: CD | 4.82 | 4.93 | 0.06 | <0.001 |

CS, cold stress group; TN, thermo-neutral group; SEM=standard error of the mean; VH=Villus height; VW=Villus width; CD=Crypt Depth; VSA=Villus surface Area; VH: CD=Villus height to crypt depth ratio.

Serum Corticosterone, Triiodothyronine (T3), Thyroxine (T4) and oxidant/antioxidant concentrations

The serum level of T4 and SOD were significantly higher ($P \leq 0.05$) in TNG compared to CS (Table 9). Whereas serum levels of T3, MDA and corticosterone were found to be lowered ($P \leq 0.05$) in TNG.

CuNP linearly increased ($P \leq 0.05$) SOD and T4 levels in the blood during cold stress in broilers (Table 10). CuNP supplementation linearly decreased ($P \leq 0.05$) the levels of corticosterone, T3 and MDA. CuNP supplementation also showed a quadratic decrease and increase in T3 and T4 respectively ($P \leq 0.05$).

DISCUSSION

The study found a broiler response to an altered level of CuNP under cold stress conditions. Results suggest that birds in cold stress (CS) had a reduced body weight and a higher FCR as compared to all other experimental groups. The findings of the current study regarding cold stress are in accordance with previous studies (Ipek and Shahan, 2006; Blahova et al., 2007; Yang et al., 2014; Ashraf et al., 2017; Marzieh, et al., 2018). Cold exposed broilers might have an increased metabolic rate due to the activation of the hypothalamic-pituitary-thyroid axis (HPT) resulting in additional feed consumption. This additional feed might have helped in maintaining body temperature rather than making any influence on the body weight (Ipek and Shahan, 2006). Therefore, birds had a poor body weight and higher FCR. All supplemented groups with CuNP had an improved

Table 6: Histomorphometric parameters of small intestine at different dietary levels of copper nanoparticles (CuNP)

| Parameters | Treatments | | | | | P-Value | |
|------------------------|------------|------|------|------|-------|---------|-----------|
| | CS | CS5 | CS10 | CS15 | SEM | Linear | Quadratic |
| Duodenum | | | | | | | |
| VH (mm) | 1.11 | 1.17 | 1.13 | 1.39 | 0.007 | <0.001 | < 0.001 |
| VW (mm) | 0.12 | 0.14 | 0.13 | 0.16 | 0.001 | <0.001 | < 0.001 |
| CD | 0.11 | 0.13 | 0.12 | 0.13 | 0.009 | <0.001 | 0.03 |
| VSA (mm ²) | 0.43 | 0.53 | 0.47 | 0.72 | 0.006 | <0.001 | 0.01 |
| VH: CD | 8.42 | 8.07 | 8.39 | 8.94 | 0.077 | <0.001 | <0.001 |
| Jejunum | | | | | | | |
| VH (mm) | 0.72 | 0.78 | 0.79 | 0.83 | 0.003 | <0.001 | 0.09 |
| VW (mm) | 0.11 | 0.11 | 0.15 | 0.16 | 0.002 | <0.001 | 0.07 |
| CD | 0.12 | 0.14 | 0.13 | 0.14 | 0.001 | <0.001 | 0.48 |
| VSA (mm ²) | 0.25 | 0.28 | 0.38 | 0.42 | 0.005 | <0.001 | 0.52 |
| VH: CD | 5.72 | 5.53 | 5.99 | 5.63 | 0.052 | 0.41 | < 0.11 |
| Ileum | | | | | | | |
| VH (mm) | 0.50 | 0.56 | 0.58 | 0.6 | 0.002 | <0.001 | 0.082 |
| VW (mm) | 0.11 | 0.13 | 0.15 | 0.16 | 0.001 | <0.001 | 0.001 |
| CD | 0.1 | 0.11 | 0.12 | 0.14 | 0.001 | <0.001 | 0.004 |
| VSA (mm ²) | 0.18 | 0.24 | 0.28 | 0.31 | 0.002 | <0.001 | <0.001 |
| VH: CD | 4.83 | 4.91 | 4.68 | 4.27 | 0.044 | <0.001 | <0.001 |

CS, cold stress group; CS5: CS+5mg CuNP per kg of feed; CS10: CS+10mg CuNP per kg of feed and CS15: CS+15mg CuNP per kg of feed; SEM=standard error of the mean; VH=Villus height; VW=Villus width; CD=Crypt Depth; VSA=Villus surface Area; VH: CD=Villus height to crypt depth ratio.

body weight and FCR as compared to the cold stress (CS) group. These results on broiler performance are consistent with the studies (El-kazaz and Hafez, 2020). Scott et al. (2018) reported similar results on body weight and FCR, that when broilers were fed with CuNP. In addition, it has been reported that copper loaded chitosan nanoparticles at the dose rate of 50 and 100mg/kg had positive effects on the growth parameters of broiler (Wang et al., 2011). Miroshnikov et al. (2015) observed an improvement in broiler's performance while using CuNP injections. Similar effects have been reported in other animal species, for example, body weight in pigs was improved by copper nanoparticles in diet at the dose rate 50 and 100 mg/kg (Wang et al., 2012). Likewise, similar effects on weight gain were also observed by dietary copper nanoparticles in fish (El-Basuini et al., 2016) and rabbits (Refaie et al., 2015).

These findings may be explained by improved uptake and absorption of CuNP. Copper nanoparticles have a

smaller size and this might have helped to diffuse and absorb them from the gastrointestinal tract to the blood stream (Singh, 2016). Cu has a higher bioavailability that might have helped in improved growth (Gonzales-Eguia et al., 2009). Fatty acids and vitamins absorption might have been boosted by CuNP which helped to increase uptake of nutrients and, enhance growth performance by prompting the enzymes responsible for metabolism (Das et al., 2010). Furthermore, the antibacterial effects of CuNP might have helped improving performance of broilers (Usman et al., 2013). Additionally, copper bioavailability in CuNP supplemented chickens minimized the effects of cold stress probably due to the role of copper in thyroid hormone regulation and heat production (Lukaski et al., 1995).

Histomorphometric results exposed that low temperature was harmful to intestinal microarchitecture. CS group had a reduced villus height and VSA in small intestine. Slota et al. (2015) observed similar results in the small intestine morphometry when broilers were exposed to cold. Stress decreases the proliferation of intestinal cells and causes regression in VH (Huo and Guo, 2008). Studies have shown that cold stress resulted in the damage of DNA (Jia et al., 2009) and intestinal tissues (Zhang et al., 2011). In current study, poor VH and VSA in the CS group might be due to the negative effects of the cold on intestine.

VH and VSA are linked with better absorption of available nutrients (Awad et al., 2008). Longer villi are indicators of a healthy gut (Baurhoo et al., 2007). In the current study, CuNP administered group (CS+15mg/kg) had the highest VH and VS. In addition to this, VH: CD ratio was also observed greatest in duodenum and ileum in the CS+15mg/kg group. The stem cells in crypt have a key role in the renewal of villous epithelial cells (Baurhoo

Table 7: Goblet cells and intraepithelial lymphocytes (IELs) in small intestine of cold stress (CS) and thermo-neutral (TN) group

| Intestinal Segments | Cells | CS | TN | SEM | P-value |
|---------------------|-------|-----|-----|-----|---------|
| Duodenum | AGC | 50 | 50 | 3.1 | 0.28 |
| | MGC | 29 | 32 | 1.8 | 0.15 |
| | TGC | 78 | 83 | 4.2 | 0.41 |
| | IELs | 86 | 86 | 0.7 | 0.28 |
| Jejunum | AGC | 60 | 56 | 1.2 | 0.01 |
| | MGC | 61 | 59 | 1.8 | 0.33 |
| | TGC | 122 | 114 | 2.4 | 0.04 |
| | IELs | 74 | 73 | 0.6 | 0.72 |
| Ileum | AGC | 60 | 49 | 1.7 | <0.001 |
| | MGC | 51 | 45 | 1.2 | 0.01 |
| | TGC | 111 | 94 | 2.0 | <0.001 |
| | IELs | 80 | 81 | 0.4 | 0.95 |

CS, cold stress group; TN, thermo-neutral group; SEM=standard error of the mean; AGC=Acidic goblet cells, MGC=Mixed goblet cells, TGC=Total goblet cells, IELs=Intraepithelial lymphocytes.

Table 8: Goblet cells and IELs count in small intestine at different dietary levels of copper nanoparticles (CuNP)

| Intestinal Segments | Cells | Treatments | | | | SEM | P-Value | |
|---------------------|-------|------------|-----|------|------|------|---------|-----------|
| | | CS | CS5 | CS10 | CS15 | | Linear | Quadratic |
| Duodenum | AGC | 50 | 47 | 50 | 54 | 3.68 | 0.34 | 0.302 |
| | MGC | 28 | 37 | 32 | 29 | 1.85 | 0.70 | 0.001 |
| | TGC | 78 | 84 | 82 | 83 | 4.71 | 0.54 | 0.60 |
| | IELs | 86 | 85 | 86 | 83 | 0.96 | 0.08 | 0.70 |
| Jejunum | AGC | 60 | 67 | 75 | 75 | 1.24 | <0.001 | 0.02 |
| | MGC | 61 | 59 | 60 | 60 | 1.41 | 0.52 | 0.69 |
| | TGC | 122 | 126 | 134 | 134 | 1.85 | <0.001 | 0.20 |
| | IELs | 74 | 74 | 75 | 75 | 0.63 | 0.45 | 0.76 |
| Ileum | AGC | 60 | 64 | 65 | 76 | 1.58 | <0.001 | 0.02 |
| | MGC | 51 | 58 | 63 | 75 | 1.37 | <0.001 | 0.08 |
| | TGC | 111 | 121 | 128 | 151 | 1.86 | <0.001 | 0.002 |
| | IELs | 80 | 81 | 81 | 81 | 0.57 | 0.44 | 1 |

CS, cold stress group; CS5: CS+5mg CuNP per kg of feed; CS10: CS+10mg CuNP per kg of feed and CS15: CS+15mg CuNP per kg of feed; SEM=standard error of the mean; AGC=Acidic goblet cells, MGC=Mixed goblet cells, TGC=Total goblet cells, IELs=Intraepithelial lymphocytes.

et al., 2007). VH: CD ratio is linked with regeneration and mitosis (Hu et al., 2012). These results may be attributed to the fact that copper has the ability to actively influence the enzyme function to enhance the metabolic pathways and better energy digestibility (Singh, 2016).

It is well known that cold stress has adverse effects on the immune system (Hangalapura et al., 2006). Humoral and cell mediated immunity was decreased by cold in rats and chicken respectively (Regnier and Kelley, 1981; Rybakina et al., 1997). In this study, IELs count did not show any significant difference but had a tendency of higher IELs in CS group. This tendency may be due to an increase in the expression level of immunoglobulins (IgG, IgM and IgA) due to cold stress (Zhao et al., 2013).

Goblet cells are present in all the segments of the intestine and produce mucus by the secretion of mucin glycoprotein (Majidi-Mosleh et al., 2017). Studies have shown that the population, activity and mucin diversity of goblet cells are affected by diet (Deplancke and Gaskins, 2001). In the present study, goblet cells in CS group increased as compared to the TNG, which can be a protective mechanism during cold due to disturbance in micro flora of the intestine against cold (Söderholm and Perdue, 2001). An increase in acidic and total goblet cell count was noted in jejunum and acidic, mixed and total goblet cells increased with 15mg/kg CuNP but no effect was observed in duodenum. Acidic mucin acts as a defense mechanism against invading microbes and mixed mucin helps in the lubrication of the ingested food (Duritis and

Mugurevics, 2015). The higher number of goblet cells of jejunum in CuNP supplemented groups may be linked to the antibacterial effects of CuNP and their role in the lubrication of feed for proper absorption. The increase in all types of goblet cells in ileum maybe because ileum has maximum number of bacterial burden and it describes bactericidal effects of copper nanoparticles (CuNP) on different bacterial strains (DeAlba-Montero et al., 2017).

Oxidative stress and antioxidant parameters are indicators of the metabolic status of any organism (Świątkiewicz et al., 2014). Cuplays an important role as an antioxidant agent in the diet (Pineda et al., 2013). A decrease in the oxidative process in chickens was observed because of in ovo injections of copper nanoparticles (Pineda et al., 2013). In current study, copper nanoparticles treated birds showed an increase in serum SOD activity and a reduction in MDA level which indicated that oxidative stress was reduced in nanoparticles administered groups. Oxidation processes leads results in an increased number of free radicals in body that cause an increase and a decrease later on in the activity of antioxidant enzymes (Ognik and Krauze, 2017). During any disturbances in the organism's redox balance, copper in the body may play a defensive role against oxidative stress (Pál et al., 2015). Ognik and Krauze (2017) also demonstrated that the nano form of copper helps to improve the antioxidant status of broiler chicken.

Serum corticosterone is used as a major indicator of stress in broilers (Pál et al., 2015). In current research, serum corticosterone level was highest in broilers, which were exposed to cold stress (CS) and were not supplemented with copper nanoparticles. An increase in corticosterone level in blood during cold stress was also observed by Ashraf et al. (2020), Borsoi et al. (2015) and Aarif et al. (2013), whereas a decrease in corticosterone level was observed by Hangalapura et al. (2006) and no effect of stress was observed on levels of corticosterone by Mills et al. (1997). Stress depends on different factors such as duration, intensity and genetics, therefore a different result of hormonal studies during cold stress is possible (Ashraf et al., 2020). In current research, a linear decrease in the

Table 9: Serum Corticosterone, Triiodothyronine (T3), Thyroxin (T4) and oxidant/antioxidant concentrations of cold stress (CS) and thermo-neutral (TN) group

| Parameters | CS | TN | SEM | P-value |
|------------------------|------|------|------|---------|
| Corticosterone (ng/ml) | 1.87 | 1.43 | 0.07 | <0.001 |
| T3(ng/ml) | 3.15 | 1.41 | 0.04 | <0.001 |
| T4(µg/dl) | 2.56 | 3.04 | 0.06 | <0.001 |
| MDA(µmol/l) | 1.85 | 1.50 | 0.03 | <0.001 |
| SOD (U/ml) | 1.24 | 1.46 | 0.01 | <0.001 |

CS, cold stress group; TN, thermo-neutral group; SEM=standard error of the mean; T3=Triiodothyronine; T4=Thyroxin; MDA=Malondialdehyde; SOD=Superoxide dismutase.

Table: 10. Serum Corticosterone, Triiodothyronine (T3), Thyroxin (T4) and oxidant/antioxidant concentrations at different dietary levels of copper nanoparticles (CuNP)

| Parameters | Treatments | | | | | P-Value | |
|------------------------|------------|------|------|------|------|---------|-----------|
| | CS | CS5 | CS10 | CS15 | SEM | Linear | Quadratic |
| Corticosterone (ng/ml) | 1.87 | 1.50 | 1.4 | 1.06 | 0.02 | <0.001 | 0.32 |
| T3(ng/ml) | 3.15 | 1.92 | 1.87 | 1.86 | 0.10 | <0.001 | <0.001 |
| T4(µg/dl) | 2.58 | 2.91 | 3.29 | 3.15 | 0.09 | <0.001 | 0.01 |
| MDA(µmol/l) | 1.85 | 1.47 | 1.24 | 1.02 | 0.04 | <0.001 | 0.09 |
| SOD (U/ml) | 1.24 | 1.56 | 1.61 | 1.82 | 0.04 | <0.001 | 0.16 |

CS, cold stress group; CS5: CS+5mg CuNP per kg of feed; CS10: CS+10mg CuNP per kg of feed and CS15: CS+15mg CuNP per kg of feed; SEM=standard error of the mean; T3=Triiodothyronine; T4=Thyroxin; MDA=Malondialdehyde; SOD=Superoxide dismutase

serum corticosterone level was observed in CuNP treated groups which indicates overcoming the negative effects of stress in treated groups. An increase in lipid peroxidation levels is observed as a result of exogenous corticosterone supplementation in broilers (Lin et al., 2004). MDA and SOD levels indicate that the oxidant/antioxidant status of CuNP fed broilers were improved, as a result, serum corticosterone level was declined. Contrary to this, oxidant/antioxidant was poor in non-treated groups and therefore, a rise in serum corticosterone level was recorded in CS and TNG group. The improvement in all these parameters might be due to the antioxidant properties of copper and because copper nanoparticles are absorbed rapidly due to their smaller size and are more readily available instead of larger particles (Hafez and Gad, 2018).

A maximum increase in serum concentration of T3 level was observed because of cold stress in the CS group. However, T4 decreased in the same cold stress group. Previous studies involving the effects of cold stress on these hormones show inconsistent results as well (Ashraf et al., 2020; Blahova et al., 2007; Hangalapura et al., 2006; Mills et al., 1997). Various changes in hormonal levels occur during stress due to the activation of HPA (Hypothalamo adrenal axis) (Ashraf et al., 2020). However, many other factors like duration of stress, health status, and genetics of individual apart from these HPA and HPT axis also play a vital role during stress (Borsoi et al., 2015). Increased T3 level might be due to the effects of cold as this hormone has an inverse relationship with temperature (Stojević et al., 2000) or owing to the conversion of T4 to T3 as the phenomenon is dependent on temperature (Hangalapura et al., 2004).

CONCLUSIONS

In conclusion, dietary copper nanoparticles at 15mg/kg of diet enhanced gut health by improving the villus height, width and villus surface area in broilers which results in higher weight gains and improved FCR during cold stress in broilers. However, further studies are required to observe the response of immune cells to dietary copper nanoparticles.

CONFLICTS OF INTEREST

The authors have no conflict of interest to declare.

Author's contributions

Saqib Saleem Abdullah, Saima Masood, Hafsa Zaneb and Imtiaz Rabbani designed experiment and methodology. Saqib Saleem Abdullah, Jamil Akbar, Zulfiqar Hussain Kuthu performed experiment. Saqib Saleem Abdullah,

Rajan Dhakal and Einar Vargas Bello Perez performed data curation. Saqib Saleem Abdullah, Saima Masood, Aysha performed formal analysis. Saima Masood and Zulfiqar Hussain Kuthu were supervising the research project. Saqib Saleem Abdullah prepared the original draft. Saqib Saleem Abdullah, Saima Masood, Rajan Dhakal and Einar Vargas Bello Perez were involved in writing, review and editing.

REFERENCES

- Aarif, O., S. Mahapatra, M. A. Yattoo and S. A. Dar. 2013. Impact of cold stress on physiological, hormonal and immune status in male and female broad breasted white Turkeys. *J. Stress. Physio. Biochem.* 9: 54-60.
- Ahmadi, F and F. Rahimi. 2011. The effect of different levels of nano silver on the performance and retention of silver in edible tissues of broilers. *World Appl. Sci.* 12: 1-4.
- Aiyan, A. A., K. Richardson, T. Shawaf, S. Abdullah and R. Barigye. 2018. A histologic and histomorphometric study of the first compartment of stomach in the dromedary (*Camelus dromedarius*) *J. Camel Pract. Res.* 25: 221-230.
- Ali, S. F., W. Chao, W. Xiaoli, H. Jintian, W. Mingfa, M. E. Abd El-Hack, Z. Lili, Z. Xiang and W. Tian. 2017. Growth, serum biochemical indices, antioxidant status and meat quality of broiler chickens fed diet supplemented with sodium stearoyl-2 Lactylate. *Pak. Vet. J.* 37: 445-449.
- Anjum, N. A., M. A. M. Rodrigo, A. Moulik, Z. Heger, P. Kopel, O. Ziftka, A. S. Lukatin, A. C. Duarte, E. Pereira and R. Kizek. 2016. Transport phenomena of nanoparticles in plants and animals/humans. *Environ. Res.* 151: 233-243.
- Ashraf, S., H. Zaneb, S. Masood, M. S. Yousaf, M. M. Usman, H. F. Rehman, A. Sikandar, M. Shah and H. Rehman. 2020. Growth performance, hormonal dynamics and intestinal microarchitecture in broilers fed B-galacto-oligosaccharides during cyclic cold stress. *J. Anim. Plant. Sci.* 30: 288-297.
- Ashraf, S., H. Zaneb, M. S. Yousaf, A. Ijaz, M. U. Sohail, S. Muti, M. M. Usman, S. Ijaz and H. Rehman. 2013. Effect of dietary supplementation of prebiotics and probiotics on intestinal microarchitecture in broilers reared under cyclic heat stress. *J. Anim. Physiol Anim. Nutr.* 1: 68-73.
- Awad, W., K. Ghareeb and J. Böhm. 2008. Intestinal structure and function of broiler chickens on diets supplemented with a synbiotic containing *Enterococcus faecium* and oligosaccharides. *Int. J. Mol. Sci.* 9: 2205-2216.
- Baurhoo, B., L. Phillip and C. A. Ruiz-Feria. 2007. Effects of purified lignin and mannan oligosaccharides on intestinal integrity and microbial populations in the ceca and litter of broiler chickens. *Poult. Sci.* 86: 1070-1078.
- Blahova, J., R. Dobsikova, E. Strakova and P. Suchy. 2007. Effect of low environmental temperature on performance and blood system in broiler chickens (*Gallus domesticus*). *Acta. Vet. Brno.* 76: 17-23.
- Borsoi, A., W. M. Quinteiro-Filho, A. S. Calefi, A. J. P. Ferreira, C. S. Astoffi-Ferreira, J. C. Florio and J. Palermo-Neto. 2015. Effects of cold stress and *Salmonella* Heidelberg infection on bacterial load and immunity of chickens. *Avian. Pathol.* 44: 490-497.
- Burkholder, K. M., K. Thompson, M. E. Einstein, T. J. Applegate and J. A. Patterson. 2008. Influence of stressors on normal intestinal microbiota, intestinal morphology, and susceptibility to *Salmonella* Enteritidis colonization in broilers. *Poult. Sci.* 87: 1734-1741.

- Das, T. K., M. K. Mondal, P. Biswas, B. Bairagi and C. C. Samanta. 2010. Influence of level of dietary inorganic and organic copper and energy level on the performance and nutrient utilization of broiler chickens. *Asian Aust. J. Anim. Sci.* 23: 82-89.
- DeAlba-Montero, I., J. Guajardo-Pacheco, E. Morales-Sánchez, R. Araujo-Martínez, G. M. Loredó-Becerra, G. A. Mar-Tínez-Castañón, F. Ruiz and M. E. C. Jasso. 2017. Antimicrobial properties of copper nanoparticles and amino acid chelated copper nanoparticles produced by using a soya extract. *Bioinorg. Chem. Appl.* 2017: 1064918.
- Deplancke, B. and H. R. Gaskins. 2001. Microbial modulation of innate defense: Goblet cells and the intestinal mucus layer. *Am. J. Clin. Nutr.* 73: 1131-1141.
- Duritis, I and A. Mugurevics. 2015. Distribution and characterization of goblet cells in the large intestine of ostriches during the pre- and post-hatch period. *Anat. Histol. Embryol.* 45: 457-462.
- El-Basuini, M. F., A. M. El-Hais, M. A. O. Dawood, A. E. S. Abou-Zeid, S. Z. Eidamrawy, M. M. E. Khalafalla and S. Dossou. 2016. Effect of different levels of dietary copper nanoparticles and copper sulfate on growth performance, blood biochemical profiles, antioxidant status and immune response of red sea bream (*Pagrus major*). *Aquacult.* 455: 32-40.
- El-Kazaz, S. E. and M. H. Hafez. 2020. Evaluation of copper nanoparticles and copper sulfate effect on immune status, behavior, and productive performance of broilers. *J. Adv. Vet. Anim. Res.* 7: 16-25.
- Ewa, S., L. Monika, L. Andrzej, S. Malwina, J. Sławomir, N. Jan, S. Abdullah, J. Jan, J. Damian and C. André. 2018. Effect of copper nanoparticles on the mineral content of tissues and droppings, and growth of chickens. *Arch. Anim. Nutri.* 72: 396-406.
- Fondevila, M. and R. Herrero. 2009. Silver nanoparticles as a potential antimicrobial additive for weaned pigs. *Anim. Feed Sci. Technol.* 150: 259-269.
- Fu, J., C. P. Liu, Z. W. Zhang, W. Liao and S. W. Xu. 2012. Effects of acute and chronic cold stress on expression of cyclooxygenase-2 and prostaglandin synthase mRNA in quail intestine. *Pak. Vet. J.* 33: 358-363.
- Gonzales-Eguia, A., C. M. Fu, F. Y. Lu and T. F. Lien. 2009. Effects of nanocopper on copper availability and nutrients digestibility, growth performance and serum traits of piglets. *Livest. Sci.* 126: 122-129.
- Gunal, M., G. Yayli, O. Kaya, N. Karahan and O. Sulak. 2006. The effects of antibiotic growth promoter, probiotic or organic acid supplementation on performance, intestinal microflora and tissue of broilers. *Int. J. Poult. Sci.* 5: 149-155.
- Hadley, W., F. Romain, L. Henry and K. Müller. 2021. Dplyr: A Grammar of Data Manipulation. R Package Version 1.0.6. Available from: <https://CRAN.R-project.org/package=dplyr>
- Hafez, M. H. and S. B. Gad. 2018. Zinc oxide nanoparticles effect on oxidative status, brain activity, anxiety-like behavior and memory in adult and aged male rats. *Pak. Vet. J.* 38: 311-315.
- Hangalapura, B. N., M. G. Kaiser, J. J. Poel, H. K. Parmentier and S. J. Lamont. 2006. Cold stress equally enhances *in vivo* pro-inflammatory cytokine gene expression in chicken lines divergently selected for antibody responses. *Dev. Comp. Immunol.* 30: 503-511.
- Hangalapura, N., M. G. B. Nieuwland, J. Buyse, B. Kemp and H. K. Parmentier. 2004. Effect of duration of cold stress on plasma adrenal and thyroid hormone levels and immune responses in chicken lines divergently selected for antibody responses. *Poult. Sci.* 83: 1644-1649.
- Havenstein, G., P. R. Ferret and M. A. Qureshi. 2003. Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. *Poult. Sci.* 82: 1500-1508.
- Hu, C. H., L. Y. Gu and Z. S. Luan. 2012. Effects of montmorillonite-zinc oxide hybrid on performance, diarrhea, intestinal permeability and morphology of weanling pigs. *Anim. Feed Sci. Technol.* 177: 108-115.
- Hu, X. and Y. Guo. 2008. Corticosterone administration alters small intestinal morphology and function of broiler chickens. *Asian Aust. J. Anim. Sci.* 21: 1773-1778.
- Huff, G. R., W. E. Huff, N. C. Rath, F. A. El-Gohary, Z. Y. Zhou and S. Shini. 2015. Efficacy of a novel prebiotic and a commercial probiotic in reducing mortality and production losses due to cold stress and *Escherichia coli* challenge of broiler chicks. *Poult. Sci.* 94: 918-926.
- Ipek, A and U. Sahan. 2006. Effects of cold stress on broiler performance and ascites susceptibility. *Anim. Sci.* 19: 734-738.
- Jia, H. Y., J. M. Li, Q. Yu, J. J. Wang and S. Li. 2009. The effect of cold stress on DNA oxidative damage of lung in chicken. *Zhongguo. Ying. Yong. Sheng. Li. Xue.* 25: 373-376.
- Lin, H., E. Decuyper and J. Buyse. 2004. Oxidative stress induced by corticosterone administration in broiler chickens (*Gallus gallus domesticus*): 2. Short-term effect. *Comp. Biochem. Physiol. Biochem. Mol. Biol.* 139: 745-751.
- Lukaski, H. C., C. B. Hall and M. J. Marchello. 1995. Body temperature and thyroid hormone metabolism of copper-deficient rats. *J. Nutr. Biochem.* 6: 445-451.
- Majidi-Mosleh, A., A. A. Sadeghi, S. N. Mousavi, M. Chamani and A. Zarei. 2017. Influence of in ovo inoculation of probiotic strains on the jejunal goblet cell counts and morphometry in peri- and post-hatching chicks. *Kafkas Univ. Vet. Fak. Derg.* 23: 169-172.
- Marzieh, R., G. Abolghasem, K. Hassan and R. B. Mohammad. 2018. Effects of curcumin or nanocurcumin on blood biochemical parameters, intestinal morphology and microbial population of broiler chickens reared under normal and cold stress conditions. *J. Appl. Anim. Res.* 46: 200-209.
- Mills, P. J., M. G. Ziegler, T. Patterson, J. E. Dimsdale, R. Hauger, M. Irwin and I. Grant. 1997. Plasma catecholamine and lymphocyte beta 2- adrenergic receptor alterations in elderly Alzheimer caregivers under stress. *Psychosom. Med.* 59: 251-256.
- Miroshnikov, S., E. Yausheva, E. Sizova and E. Miroshnikova. 2015. Comparative assessment of effect of copper nano- and micro-particles in chicken. *Orient. J. Chem.* 31: 2327-2336.
- Mroczek-Sosnowska, N., E. Sawosz, K. P. Vadalasetty, M. Łukasiewicz, J. Niemiec, M. Wierzbicki, M. Kutwin, S. Jaworski and A. Chwalibog. 2015. Nanoparticles of copper stimulate angiogenesis at systemic and molecular level. *Int. J. Mol. Sci.* 16: 4838-4849.
- Mroczek-Sosnowska, N., M. Łukasiewicz, A. Wnuk, W. Sawosz, J. Niemiec, A. Scot, S. Jaworski and A. Chwalibog. 2016. In ovo administration of copper and copper sulphate positively influences chicken performance. *J. Sci. Food Agric.* 96: 3058-3062.
- Ognik, K and M. Krauze. 2016. The potential for using enzymatic assays to assess the health of Turkeys. *World Poult. Sci. J.* 72: 535-550.
- Ognik, K., A. Stępnowska, E. Cholewińska and K. Kozłowski. 2016. The effect of administration of copper nanoparticles to chickens in drinking water on estimated intestinal absorption of iron, zinc, and calcium. *Poult. Sci.* 95: 2045-2051.
- Ognik, K., I. Sembratowicz, E. Cholewińska, J. Jankowski, K. Kozłowski, J. Juśkiewicz and Z. Zduńczyk. 2017. The effect of administration of copper nanoparticles to chickens in their drinking water on the immune and antioxidant status of the blood. *Anim. Sci. J.* 89: 579-588.

- Pál, L., M. Kulcsár, J. Poór, L. Wágner, S. Nagy, K. Dublec and F. Husvéth. 2015. Effect of feeding different oils on plasma corticosterone in broiler chickens. *Acta Vet. Hung.* 63: 179-188.
- Pineda, L., E. Sawosz, K. Vadalasetty and A. Chwalibog. 2013. Effect of copper nanoparticles on metabolic rate and development of chicken embryos. *Anim. Feed Sci. Tech.* 186: 125-129.
- R Core Team. 2021. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. Available from: <https://www.R-project.org>
- Refaie, A. M., M. N. Ghazal, F. M. Easa, S. A. Barakat, G. E. Younan and W. H. Eisa. 2015. Nano-copper as a new growth promoter in the diet of growing New Zealand white rabbits. *Egypt. J. Rabbit. Sci.* 25: 39-57.
- Regnier, J. A and K. W. Kelley. 1981. Heat and cold-stress suppresses *in vivo* and *in vitro* cellular immune responses of chickens. *Am. J. Vet. Res.* 42: 294-299.
- Rybakina, E. G., S. N. Shanin, I. A. Kozinets, E. E. Fomicheva and E. A. Korneva. 1997. Cellular mechanisms of cold stress-related immunosuppression and the action of interleukin 1. *Int. J. Tissue React.* 19: 135-140.
- Scott, A., K.P. Vadalasetty and M. Łukasiewicz. 2018. Effect of different levels of copper nanoparticles and copper sulphate on performance, metabolism and blood biochemical profiles in broiler chicken. *J. Anim. Physiol. Anim. Nutr.* 102: e364-e373.
- Scott, A., K. P. Vadalasetty, M. Łukasiewicz, S. Jaworski, M. Wierzbicki and A. Chwalibog. 2017. Effect of different level of copper nanoparticles and copper sulphate on performance, metabolism and blood biochemical profile in broiler chicken. *J. Anim. Physiol. Anim. Nutr.* 102: 1-10.
- Shini, S., G. R. Huff, A. Shini and P. Kaiser. 2010. Understanding stress-induced immunosuppression: Exploration of cytokine and chemokine gene profiles in chicken peripheral leukocyte. *Poult. Sci.* 89: 841-851.
- Singh, P. 2016. Use of nano feed additives in livestock feeding. *Int. J. Livest. Res.* 6: 1-14.
- Slota, C., A. Chi, G. Chen, M. Bevans, N. P. Weng. 2015. Norepinephrine preferentially modulates memory CD8 T cell function inducing inflammatory cytokine production and reducing proliferation in response to activation. *Brain Behav. Immun.* 46: 168-179.
- Söderholm, J. D. and M. H. Perdue. 2001. Stress and gastrointestinal tract. II. Stress and intestinal barrier function. *Am. J. Physiol. Gastrointest. Liver Physiol.* 280: G7-G13.
- Stojević, Z., S. Milinković-Tur and K. Curčija. 2000. Changes in thyroid hormones concentrations in chicken blood plasma during fattening. *Vet. Arhiv.* 70: 31-37.
- Świątkiewicz, S., A. Arczewska-Włosek and D. Jozefiak. 2014. The efficacy of organic minerals in poultry nutrition: Review and implications of recent studies. *Worlds Poult. Sci. J.* 70: 475-486.
- Tsiouris, V., I. Georgopoulou, C. Batzios, N. Pappaioannou, R. Ducatelle and P. Fortomaris. 2015. The effect of cold stress on the pathogenesis of necrotic enteritis in broiler chicks. *Avian. Pathol.* 44: 430-435.
- Usman, M., M. El Zowalaty, K. Shamel, N. Zainuddin, M. Salam and M. A. Ibrahim. 2013. Synthesis, characterization and antimicrobial properties of copper nanoparticles. *Int. J. Nanomed.* 8: 4467-4479.
- Wang, C., M. Q. Wang, S. S. Ye, W. J. Tao and Y. J. Du. 2011. Effects of copper-loaded chitosan nanoparticles on growth and immunity in broilers. *Poult. Sci.* 90: 2223-2228.
- Wang, M. Q., Y. J. Du, C. Wang, W. J. Tao, Y. D. He, Y. D. and H. Li. 2012. Effects of copper-loaded chitosan nanoparticles on intestinal microflora and morphology in weaned piglets. *Biol. Trace Elem. Res.* 149: 184-189.
- Yang, X., Y. H. Luo, Q. F. Zeng, K. Y. Zhang, X. M. Ding, S. P. Bai, J. Wang. 2014. Effects of low ambient temperatures and dietary Vitamin C supplement on growth performance, blood parameters, and antioxidant capacity of 21-day-old broilers. *Poult. Sci.* 93: 898-905.
- Zhang, Z. W., Z. H. Lv, J. L. Li, S. Li, S. W. Xu and X. L. Wang. 2011. Effects of cold stress on nitric oxide in duodenum of chicks. *Poult. Sci.* 90: 1555-1561.
- Zhao, F. Q., Z. W. Zhang, Y. Hai-Dong, Y. W. Liang-liang, L. Tao, Y. Xian-Yi, L. Shu and X. Shi-Wen. 2013. Effects of cold stress on mRNA expression of immunoglobulin and cytokine in the small intestine of broilers. *Res. Vet. Sci.* 95: 146-155.