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Copper Nanoparticles Supplementation Improves Intestinal Morphology and Antioxidant Status in Broilers

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Abstract
The study was planned to determine the effects of copper nanoparticles on intestinal histomorphometric parameters, goblet cells, intra-epithelial lymphocytes and antioxidant status in broiler. One day old Hubbard broiler chicks (n=320) were divided in to four treatment groups with eight replicates in each group. The birds were raised under standard management conditions for 35 days. Control group was fed with basal diet whereas three other groups received basal diet supplemented with copper nanoparticles at 5mg/kg (A), 10mg/kg (B) and 15mg/kg (C) respectively. On 35th day, two birds from each replicate were selected randomly for blood and tissue sample collection. Supplementation of CuNP linearly increased ($P \leq 0.05$) villus height (VH), villus width (VW), crypt depth (CD) and villus surface area (VSA) in duodenum, jejunum and ileum. Villus height to crypt depth ratio (VH: CD) was also improved with copper nanoparticles supplementation in jejunum and ileum ($P \leq 0.05$). Copper nanoparticles supplementation also increased ($P \leq 0.05$) acidic and mixed goblet cells (GC) population in duodenum, acidic and total GC in jejunum and acidic, mixed and total GC in ileum. Increasing levels of copper nanoparticles linearly ($P \leq 0.05$), increased mixed and total GC number in ileum and mixed GC and intra-epithelial lymphocytes IELs in duodenum. Blood Malondialdehyde (MDA) and corticosterone levels decreased linearly with copper nanoparticles supplementation ($P \leq 0.05$) whereas superoxide dismutase (SOD) levels increased linearly and quadratically with increasing supplementation. In conclusion, copper nanoparticles is a useful additive in broilers feed with beneficial effects on and intestinal microarchitecture, goblet cells and antioxidant status.

Keywords: Corticosterone, goblet cells, Malondialdehyde, morphometry, villi

Introduction

Nanotechnology is the modern scientific invention and is broadly applied in agriculture, food and textile industry (Abdullah *et al.,* 2024). Nanoparticles are also been used as additives and as a substitute of antibiotic growth promoters in broilers (Ahmadi and Rahimi, 2011). Nanoparticles of silver, selenium and zinc oxide showed promising results as a substitute to antibiotic growth promoters (Fondevila and Herrer, 2009).
Copper is an important micro mineral required for proper growth and development of the body. Copper is also involved in the development of immune system and also play antioxidant role in the body (Scott *et al.,* 2018). A continuous supply of copper is required as it cannot be stored in the body. Currently copper sulphate is being used as the main source of copper in poultry diets and it is added in higher doses. Due to its reduced bioavailability, it is mostly excreted from the body which results in economic losses (Scott *et al.,* 2018).
Diet plays an important role in poultry gut reinforcement. Epithelial cells, goblet cells (GC) and intraepithelial lymphocytes (IELs) act as a protection barrier and prevent bacteria and other microbes from entering into the blood vessels (Deplancke and Gaskins, 2001). Surface area of the intestine is increased by the presence of small finger like projections called villi (Yazdani *et al.,* 2013). Elongated villi provide have a higher capability for absorption of nutrients. Villus height decreases, whereas mucus secretion and goblet cells number is increased as one move from duodenum to ileum (Choct, 2009).

Copper nanoparticles have enhanced bioavailability because of their smaller size and an increased surface area. Small sized copper nanoparticles easily cross the gastrointestinal mucosa and rapidly absorb in blood. Digestive system of the broiler is also affected by oxidative stress and antioxidant properties of copper help to minimize this stress (Scott *et al.,* 2018). SOD (superoxide dismutase) and MDA (Malondialdehyde) activity was enhanced by the use of copper nanoparticles in broiler feed (Ognik *et al.,* 2017)

We hypothesized that Copper nanoparticles could overcome the oxidative damage through the stimulation of the antioxidant system and due to its antibacterial activity, will help minimize the bacterial burden that will help to support villus growth and will help in better absorption of feed particles.

Data regarding the effects of Copper nanoparticles on intestinal micro structural alterations in poultry is insufficient. This research was designed to determine the effect of different doses of CuNP on intestinal morphology, intraepithelial lymphocytes, intestinal goblet cells and antioxidant status in broilers.

Materials and Methods

Copper nanoparticles: Copper nanoparticles with 99.8% purity and 25nm in size were obtained from skySpring (Nanomaterials Inc., USA). Copper nanoparticles were added in the diet by mixing nanoparticles with starch. *(*Ewa *et al., 2018).*

Experimental design and diets: One day old Hubbard broiler chicken (n=320) were divided into four groups. Each group was further divided into of eight replicates having ten birds per replicate. On day 1, the temperature of the shed was retained at 35°C with a relative humidity at 65 ± 5%. After each week, temperature was reduced to 3°C until it reached 26°C and was kept constant till the end of the experiment. A corn based basal diet was given to control group (Table 1) while the remaining groups were given basal diet with supplemented copper nanoparticles (5mg/kg) A, (10mg/kg) B and (15mg/kg) C respectively. Birds were fed *ad libitum* during the whole experimental period.

Blood and Tissue sampling: At the end of the trial, 16 birds from each group (2/replicate) were selected through random sampling for sample collection. 3ml blood was collected from brachial vein; serum was separated and was stored. Birds were slaughtered and samples of duodenum, jejunum and ileum were collected. Collected samples were fixed in 10% buffered formalin. Samples were processed with paraffin embedding technique and stained with Haematoxylin and Eosin (H&E) and Alcian blue-PAS techniques.

With the help of histomorphometry program (Progress capture Pro 2.7.7. Labomed USA), well-oriented villus crypts units with intact lamina propria were selected for the measurement of villus length (VL), width (VW), crypt depth (CD) and villus surface area (VSA). VL was measured from the top of villi to the crypt junction, VW was measured at three points (mid, base and top) and their average was considered as VW. CD was measured from the bottom of the crypt up to the transition region of villi and crypt. VSA was calculated using the formula $(2\pi)(VW/2)(VH)$, where VW is the villus width and VH is the villus height (Aliyan *et al.,* 2018). IELs were also observed in H&E stained slides whereas PAS stained slides were used for goblet cells identification according to previously described method (Masood *et al.,* 2020).

Serum Corticosterone levels were analyzed using ELISA kits (IBL International GmbH, Hamburg, Germany) whereas SOD (superoxide dismutase) concentrations were analyzed using diagnostic kits (Randox, UK) and Malondialdehyde (MDA) was analyzed as described earlier (Ali *et al.,* 2017)

Statistical analysis: The data was analyzed by Statistical Packages for Social Sciences software (SPSS Inc. Version 20, Chicago IL, USA) using analysis of variance (ANOVA) and mean differences were calculated using Duncan's multiple range tests. Linear and quadratic effects of different doses of copper nanoparticles was examined using orthogonal polynomial. The significance was declared at $P \leq 0.05$.

Table 1: Ingredients and chemical composition of basal diets

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
Di-calcium phosphate	1.76	2
Vitamins and mineralsPremix1	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
ME (Kcal/kg)	2750	2850

[1]Vitamin 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; 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.

Table 2: Effects of supplementation of copper nanoparticles on histomorphometric analysis of small intestine

Parameters	Treatments					P-Value	
	Control	A	B	C		Linear	Quadratic
			Duodenum				
VH(mm)	1.19±0.05^{c}	1.20±0.04^{c}	1.25±0.001^{b}	1.32±0.001^{a}	<0.001	<0.001	< 0.001
VW(mm)	0.12±0.02^{c}	0.14±0.06^{b}	0.15±0.04^{a}	0.16±0.04^{a}	<0.001	<0.001	< 0.001
CD	0.18±0.001^{c}	0.17±0.07^{c}	0.18±0.001^{b}	0.19±0.04^{a}	<0.001	<0.001	0.010
VSA(mm^2)	0.47±0.001^{d}	0.53±0.04^{c}	0.62±0.07^{b}	0.68±0.02^{a}	<0.001	<0.001	0.511
VH:CD	6.61±0.07	6.78±0.29	6.62±0.17	6.77±0.13	0.16	0.062	0.065
			Jejunum				
VH(mm)	0. 71±0.03^{d}	0.73±0.001^{c}	0. 77±0.07^{b}	0.80±0.001^{a}	<0.001	<0.001	< 0.001
VW(mm)	0.09±0.06^{d}	0.10±0.08^{c}	0.11±0.02^{b}	0.14±0.08^{a}	<0.001	<0.001	< 0.001
CD	0.11±0.02^{d}	0.12±0.02^{c}	0.13±0.03^{b}	0.14±0.02^{a}	<0.001	<0.001	< 0.001
VSA(mm^2)	0.20±0.001^{d}	0.24±0.02^{c}	0.29±0.08^{b}	0.35±0.02^{a}	<0.001	<0.001	< 0.001
VH:CD	6.34±0.17^{a}	6.00±0.20^{b}	5.78±0.14^{c}	5.73±0.09^{c}	<0.001	<0.001	< 0.001
			Ileum				
VH(mm)	0.50±0.08^{d}	0.57±0.08^{c}	0.58±0.09^{b}	0.63±0.001^{a}	<0.001	<0.001	0.082
VW(mm)	0.12±0.02^{d}	0.14±0.09^{c}	0.16±0.073^{b}	0.17±0.050^{a}	<0.001	<0.001	0.001
CD	0.19±0.06^{d}	0.26±0.001^{c}	0.29±0.001^{b}	0.34±0.001^{a}	<0.001	<0.001	0.122
VSA(mm^2)	0.11±0.00^{d}	0.12±0.02^{c}	0.13±0.02^{b}	0.14±0.004^{a}	<0.001	<0.001	0.006
VH:CD	4.58±0.16^{b}	4.74±0.05^{a}	4.32±0.05^{c}	4.23±0.12^{c}	<0.001	<0.001	0.025

$^{a-d}$ Means within a row with different superscripts differ significantly ($P \leq 0.05$). Values represent the Mean±SEM of eight replicates.
VH= Villus height; VW= Villus width; CD= Crypt Depth; VSA= Villus surface Area; VH: CD=Villus height to crypt depth ratio

Table 3: Effects of supplementation of Copper nanoparticles on Goblet cells and Intra-epithelial lymphocytes

Treatments							P-Value	
	Cells	Control	A	B	C		Linear	Quadratic
Duodenum	AGC	53.1±2.3^{ab}	53.6±2.9^{a}	50.5±2.0^{b}	51.3±5.8^{ab}	0.06	0.64	0.10
	MGC	29.6±2.5^{b}	30.8±3.2^{ab}	33±2.7^{a}	32.6±3.6^{a}	0.014	0.01	0.14
	TGC	82.8±3.1	84.5±4.0	83.5±3.2	84±7.7	0.80	0.26	0.93
	IELs	68±1.4^{a}	69.6±2.0^{ab}	68.7±3.2^{ab}	70.4±2.4^{a}	0.02	0.04	0.2
Jejunum	AGC	68.0±4.4	68±5.7	68.9±2.2	70.1±1.1	0.47	0.37	0.52
	MGC	33.8±1.7^{b}	33.9±3.4^{b}	35.7±1.9^{ab}	36.6±4.2^{a}	0.02	0.64	0.15
	TGC	101.8±4.2^{b}	102.3±6.6^{b}	104±3.0^{ab}	106.7±4.0^{a}	0.01	0.61	0.72
	IELs	66.2±3.9	67.3±4.1	67.5±3.0	68.4±3.2	0.41	0.56	0.71
Ileum	AGC	61.3±2.5^{b}	61.7±3.6^{ab}	63.7±3.0^{a}	63.7±2.2^{a}	0.03	0.25	0.38
	MGC	45.2±2.6^{d}	53.0±3.2^{c}	56.8±5.0^{b}	64.9±1.5^{a}	<0.001	<0.001	0.12
	TGC	106.5±3.2^{d}	114.7±4.7^{c}	120.6±5.5^{b}	128.6±2.6^{a}	<0.001	<0.001	0.54
	IELs	67.3±5.9	67.8±4.7	64±4.3	67.7±5.0	0.15	0.44	0.84

$^{a-d}$ Means within a row with different superscripts differ significantly ($P \leq 0.05$). Values represent the Mean±SEM of eight replicates.
AGC = Acidic goblet cells, MGC = Mixed goblet cells, TGC = Total goblet cells, IELs= Intraepithelial lymphocytes

Table. 4. Effects of supplementation of Copper nanoparticles Serum Corticosterone, MDA and SOD

Parameters	Treatments					P-Value	
	Control	A	B	C		Linear	Quadratic
Corticosterone(ng/ml)	2.0±0.23^{a}	1.8±0.20^{b}	1.7±0.15^{b}	1.3±0.32^{c}	<0.001	<0.001	0.11
MDA(μmol/l)	1.8±0.13^{a}	1.6±0.18^{b}	1.24±0.19^{c}	1.1±0.18^{d}	<0.001	<0.001	0.08
SOD(U/ml)	1.1±0.14^{d}	1.3±0.18^{c}	1.5±0.20^{b}	1.9±0.11^{a}	<0.001	<0.001	0.04

$^{a-d}$ Means within a row with different superscripts differ significantly ($P \leq 0.05$). Values represent the Mean±SEM of eight replicates.
MDA=Malondialdehyde; SOD=Superoxide dismutase

Results

Histomorphometry of small intestine: Results from histomorphometric measurements are shown in Table 2. VH, VW, VSA and CD of duodenum, jejunum and ileum linearly increased ($P < 0.05$) with copper nanoparticles supplementation. Villus height to crypt depth (VH: CD) ratio remained unaffected in duodenum ($P>0.05$) but a significant linear decrease ($P <0.05$) was observed in VH: CD in jejunum and ileum. Highest VH: CD ratio was observed in jejunum of control group and by Group A (5mg/kg CuNP) in ileum followed by control, B and C respectively. Copper nanoparticles supplementation also had a quadratic effect on VH, VW and CD in duodenum and jejunum and VSA in jejunum ($P <0.05$) respectively.

Histochemistry of Goblet cells (GC) and Intraepithelial lymphocytes count (IEL): Results of GC and IEls are depicted in Table. 4. There was a significant effect on mixed and total goblet cells and acidic and mixed goblet cells count and count in jejunum and duodenum respectively ($P<0.05$) but no liear or quadrtatic effect was observed ($P>0.05$). In ileum, acid, mixed and total goblet cells were signifcantly affected by the use of copper nanoparticles ($P<0.05$). Mixed and total goblet cell count also linearly as dietary copper concentration increased ($P<0.05$). Quadratic effect was not observed on goblet cells by the use of copper nanoparticles.

A significant linear increase ($P<0.05$) was observed on intraepithelial lymphocytes count in duodenum. However, IEL number did not differ significintally in jejunum and ileum ($P>0.05$).

Serum Corticosterone, MDA and SOD: Effetcs of Copper nanoparticles supplementation on Corticosterone, MDA and SOD are shown in 5. Copper nanoparticles supplementation linerily decreased ($P<0.05$) coeticosterone and MDA levles in blood. However, serum SOD levles increased both linearly and quadratically with an increased supplementation ($P<0.05$).

Discussion:

Poultry industry suffers a huge economic loss due to intestinal health issues as pathogens have developed resistance due to endless use of antibiotics as growth promoters. Consumption and absorption of nutrients is affectd by poor gut health and results in lowered growth and feed efficiency in broilers. Intestinal mucosa is important for nutrients absorption. Function of intestine is dependent on villus morphology. Longer villi provide greater surface area and help to absorb nutrients efficiently whereas shorter villi are an indicator of poor intestinal healt (Awad *et al.,* 2008).

The results of the histomorphometric analysis showed that an increase in VH, VW and VSA was observed in all the sections of the small intestine. VH and VSA absorb nutrients from the diet and villus height indicates gut health status (Awad *et al.*, 2008). Improvements in morphometric parameters may be attributed to the activation of mitosis as VH: CD ratio is associated with cell renewal and mitosis (Ali *et al.,* 2017). In current study, a linear increase in VH: CD ratio was observed by the use of copper nanoparticles in diet. Additionally, copper nanoparticles promotes energy digestibility and their smaller size and increased surface area helps penetration into the intestinal tissues through capillaries and causes an increased uptake of nutrients in the intestine (Kumar *et al.,* 2013). Copper helps in the villus growth and development by influencing the gut microbioata and ultimately intestinal morphology (Nguyen *et al.* 2022). Otto and Carlos (2014) also observed an increase in villus height by the use of copper in broiler diet. Production of short-chain fatty acid (SCFAs) in intestine might be another reason of improved villus parameters as SCFAs play a vital role in enterocyte production and copper is known to increase SCFA production (Nguyen *et al.,* 2022).

Additionally, increased acid goblet cell count is observed in duodenum and ileum which might have helped villi to gain an increased height as acidic mucin protect against bacterial degradation (Deplancke and Gaskins, 2001). An increased villus height was also observed by the use of Copper hydroxychloride at the 200mg/kg but higher levels of copper my result in copper resistant bacteria (Nguyen *et al.*, 2022).

Mucus which is an antibacterial agent is produced by goblet cells of the intestine. Goblet cells, their secretory activity and nature of mucus is affected by diet (Deplancke and Gaskins, 2001). Current findings show that there was an increase of acidic and mixed goblet cells in duodenum, mixed and total goblet cells in jejunum and acidic, mixed and total goblet cells count in ileum. Acidic mucin have defensive properties for different microbes whereas, mixed mucin help in the movement of feed due to their less viscous nature (Duritis *et al.,* 2015). Acidic and mixed goblet cells increase in duodenum as a result of nanoparticles supplementation may be a protective mechanism against pathogens and additionally helping the feed transfer for absorption. The increase of goblet cells in jejunum may be an indicator of increased maturation of goblet cells by copper nanoparticles. The increased number of all the observed goblet cell types may be due to the bactericidal or bacteriostatic properties of copper nanoparticles (DeAlba-Montero *et al.,* 2017) as ileum has maximum bacterial load as compared to duodenum and jejunum (Bogusławska-Tryk *et al.,* 2015).

Copper nanoparticles supplementation increased the number of IELs in duodenum but no significant effect was observed in jejunum and ileum. Previous studies also reported that a decreased leucocyte count by the use of copper nanoparticles (Mroczek-sosnowska *et al.,* 2013).

Oxidative stress and antioxidant parameters indicate the health status of animals (Abdullah *et al.,* 2022). Copper acts as an antioxidant agent due to its antioxidant and pro oxidant properties (Fry *et al.,* 2012). In current study, a linear increase in SOD level and a linear decrease MDA level indicates that oxidative stress decreased by the use of copper nanoparticles in diet as compared to control group. Similar findings were observed on MDA levels respectively by the use of copper nanoparticles (Nassiri and Ahmadi, 2015; Ognik and Krauze, 2016). Improvement in oxidative status was also observed in chickens by *in ovo* administration of copper nanoparticles (Pineda *et al.,* 2013). Different free radicals are produced in the body during oxidative processes which initially increase the activity of antioxidant enzymes (Ognik and Krauze, 2016). During oxidative stress, production of MDA increases which causes damage to cells and tissues (Afsar *et al.*, 2018). Copper plays a defensive against oxidative stress during any imbalance in the body's redox mechanism (Pal *et al.,* 2015).

Serum corticosterone is another stress marker in broilers. In current study, control group presented highest level of serum corticosterone in blood which indicates that stress level was high in non-supplemented group. Previous studies on broilers indicate that corticosterone level increased as a result of stress (King *et al.,* 2016; Borosi *et al.,* 2015). In current study, corticosterone level decreased linearly with copper nanoparticles supplementation. This might be due to the fact that lipid peroxidation is not initiated due to as lipid peroxidation increases with increased corticosterone level (Lin *et al.,* 2004). These results suggest that oxidant/antioxidant status of copper nanoparticles treated birds was better as compared to control. Antioxidant properties of copper might be a reason for this improvement and rapid absorptive properties of nanoparticles might be the other reason as nanoparticles are rapidly absorbed due to their smaller size (Scott *et al.,* 2018).

Conclusion

Copper nanoparticles supplementation improved gut health status by increasing villus height, villus width, villus surface area and goblet cells in intestine. Furthermore, antioxidant status of broilers also improved by the use of copper nanoparticles.

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