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Research Article

Effect of biosynthesised nano copper on blood biochemical profile, antioxidant status and immune response in Vanaraja chicken[#]

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Abstract

This study aimed to elucidate the response of Vanaraja chicken to the dietary biosynthesised nano copper supplementation in terms of blood biochemical profile, antioxidant status and immune response. A total of 324 one-day old chicks were randomly assigned to one of the six dietary treatments, each consisting of 54 birds with 9 replicate pens of 6 chicks. The groups were basal diet without any supplemental copper (control), basal diet supplemented with 12 mg Cu/kg diet from $CuSO_4$ and the remaining four groups were fed with nano copper at 3, 6, 9 and 12 mg Cu per kg diet, respectively. The trial lasted for 56 days. The results indicated that nano copper causes significant ($P<0.05$) improvement in humoral immunity as compared to the control group. Absence of any significant changes in the serum enzyme profile and biochemical parameters indicated that nano copper is safe to feed. From the current study, it was concluded that the conventional $CuSO_4$ can be replaced with nanoparticle forms at a 50% reduced level, i.e., 6 mg per kg feed without any adverse effect on health as assessed through serum biochemical profile. In order to recommend the level of dose reduction, more studies with different breeds and in different agro-climatic zones during different seasons are recommended.

Keywords: Antioxidant status, Immunity, Nano copper, Vanaraja

Highlights

- Copper was replaced by biosynthesized nano copper at four different levels in the diets of Vanaraja poultry.
- Effect was studied in terms of immunity status as expressed by serum antibody levels against Newcastle disease, serum parameters, and antioxidant status of birds that received different levels of nano copper.
- Nano copper caused improved immunity in terms of antibody concentration against Newcastle disease.
- Nano copper has no negative impact on health as expressed by serum biochemical parameters.
- Dietary copper can be reduced by 50% if traditional copper is replaced with bio-synthesized nano copper.

INTRODUCTION

Copper is an important trace element involved in various metabolic processes in animals and birds. In poultry, copper is required for growth, bone development, synthesis of collagen, stimulation of immune system, antioxidant defence system, keratinization and pigmentation of tissue, and in many enzyme systems like ceruloplasmin, cytochrome oxidase, dopamine monooxygenase, lysyl oxidase, copper-zinc superoxide dismutase, and tyrosinase (Underwood and Suttle, 1999; Richards *et al*., 2010).

Copper is supplemented in the diet as inorganic salts, mainly as copper sulphate. Inorganic salts are poorly absorbed and hence may have to be dietary supplemented in excess of actual requirements, as a result of which most of them are excreted, contaminating the soil and water in the region and slowly accumulating in the water bodies causing environmental pollution (Sharif *et al*., 2021). Though organic sources are better absorbed compared to inorganic sources of copper (Zafar and Fathima, 2018) however, due to high cost and inconsistent results, these are not routinely used in commercial poultry production. Therefore, efforts have been made to find alternative sources of Cu to increase absorption, reduce dose levels and improve performance and

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health of poultry. Supplementation of nano form of copper is one of the recently developed technologies. Several studies have shown that nano form of minerals is more bioavailable than its bulk form because of its small size and increased surface area (Hassan *et al*., 2020).

El-Wardany *et al.* (2020) reported that organic and nano copper sources showed similar performance, but both were significantly (P<0.05) better than the inorganic source of copper in broiler diets. El-Katcha *et al.* (2020) concluded that nano copper at 50% supplementation was recommended as sufficient, and no additional benefits were observed by supplementing 100% of copper requirements as nano copper. According to Sawosz *et al.* (2018), nano copper at 25% level is sufficient to support production similar to 100% inorganic copper. Sharif *et al*. (2021) reviewed the existing research related to potential nano copper as an antioxidant agent in poultry.

Most of the earlier investigations concluded that nano copper would help in reducing copper content of the feed without any negative impact on health and production of the birds. Earlier investigators used nano copper prepared from either chemical or physical methods. However, there are concerns about using physical or chemically synthesised nanominerals for the food and feed industry because of the possibility of toxic chemicals involved in the process. Hence, the use of green chemicals from plant sources would be safer for inclusion in the feed (Punjabi *et al.*, 2015). Recently, biosynthesised nanomineral particles have been developed for use in biomedical applications and the food industry. These biosynthesised nanoparticles are considered relatively safe and cheap to produce. This study, therefore, aimed to investigate the effect of biosynthesised nano copper in comparison to inorganic copper sulphate in terms of immune status, blood biochemical profile, and antioxidant status in Vanaraja chicken.

MATERIALS AND METHODS

The experimental procedures were approved and undertaken in accordance with the guidelines of the animal ethics committee of the ICAR-Directorate of Poultry Research, Hyderabad.

A total of 324 one-day old Vanaraja chicks (mean BW 37.86±0.02 g) were wing banded and randomly assigned to six dietary treatments of nine replicates with six chicks in each replicate. The groups were basal diet without any supplemental copper (control, Cu_0), basal diet supplemented with 12 mg Cu/kg diet from $CuSO_4$ (CuS_12) and the remaining four groups were fed with nano copper at 3, 6, 9 and 12 mg Cu per kg diet (CuNP_3, CuNP_6, CuNP_9 and CuNP_12), respectively. Diets were formulated to meet nutrient requirements of chicken to meet the standards of broiler diets as per BIS (1992) for all nutrients except copper. Copper was added separately as per the treatment levels. Nano copper was produced in the laboratory using leaf extract of *Azadirachta indica* (neem) as a reducing agent and copper nitrate as substrate. Once the method was standardized, the nanoparticles were subjected to characterization. Once the particle size below 100 nm was achieved, it was produced in sufficient quantities to conduct feeding trial. Nano copper mixing method was adapted from Sawosz *et al.* (2018). The nanoparticle suspension was added to 1 kg feed to achieve 240 ppm, and the feed was dried in a hot air oven to remove moisture. This feed was mixed in the experimental rations in calculated proportions to achieve the desired level of nano copper in the experimental feeds. The ingredient composition and nutrient composition of chick feed and grower feed are presented in Table 1. Copper content of different treatment feeds is presented in Table 2.

Table 1. Ingredient and chemical composition of basal diets

Ingredient (kg)	Chick diet (0-3 weeks)	Grower diet (4-8 weeks)
Maize	58.07	65.54
Soybean meal	37.32	29.85
Stone grit	1.60	1.6
Dicalcium phosphate	1.90	1.90
Common salt	0.35	0.35
Sodium bicarbonate	0.10	0.10
DL-methionine	0.19	0.19
Lysine	0.04	0.04
Trace mineral mix (without copper)*	0.10	0.10
Vitamin ADK pre mix**	0.015	0.015
Vitamin B-Complex mix***	0.015	0.015

Cont. Table 1.

Table 1., Cont. ...

Ingredient (kg)	Chick diet (0-3 weeks)	Grower diet (4-8 weeks)
Choline chloride	0.10	0.10
Toxin binder	0.10	0.10
Tylosine	0.05	0.05
Coccidiostat	0.05	0.05
Total	**100.00**	**100.00**
Chemical composition		
ME (kcal/kg)[1]	2803	2881
Crude protein (%)	22.45	20.12
Ether extract (%)	1.78	1.92
Crude fibre (%)	3.12	3.26
Calcium (%)	1.02	1.03
Available phosphorus (%)	0.45	0.42
Lysine[1]	1.36	1.28
Methionine[1]	0.60	0.52

*Contains Ca- 32%, P- 9%, Fe- 2000 ppm, I- 0.01%, Mn- 0.4% and Zinc- 0.4%; **Each gram contains Vitamin A- 82500 IU, Vitamin B2- 50 mg, Vitamin D3- 12000 IU and Vitamin K- 10 mg; ***Each gram contains Vitamin B_1- 4 mg, B_6- 8 mg, B_{12}- 40 mg, E- 40 mg, Calcium pantothenate- 40 mg, Niacin- 60 mg; [1]Calculated value

Table 2. Copper concentration in different dietary treatments

Treatment	Copper source	Copper added (mg/kg)	Analysed copper concentration[#] (mg/kg)
Cu_0	-	0	35.00
CuS_12	Copper sulphate	12	50.00
CuNP_3	Biosynthesised nano copper	3	39.00
CuNP_6	Biosynthesised nano copper	6	42.00
CuNP_9	Biosynthesised nano copper	9	47.00
CuNP_12	Biosynthesised nano copper	12	51.00

[#]Values based on triplicate analysis

The birds were housed in 3-tiered wire floored SS battery brooders. The chicks were brooded by providing supplementary heat till 4 weeks of age using incandescent bulbs. The chicks were vaccinated against Newcastle and Infectious bursal diseases as per the standard vaccination schedule.

On the 56th day of the experiment, about two to three mL of blood was collected through the brachial vein from one chick in each replicate (9 chicks per treatment) into non-heparinised tubes. Subsequently, serum was separated and analysed for the serum biochemical parameters (glucose, total protein, albumin, cholesterol, triglycerides and blood urea nitrogen) and enzymes by using reagent kits (Jeev Diagnostics Pvt. Ltd. Chennai, India & ERBA Diagnostics, Mumbai, India). The serum antioxidant enzymes, glutathione peroxidase and glutathione reductase were estimated according to the procedures of Paglia and Valentine (1967) and Cohen *et al.* (1970), respectively and superoxide dismutase by Madesh and Balasubramanian (1998). Serum enzymes were estimated as per Bergmeyer *et al.* (1978).

The humoral immunity was estimated by comparing the antibody titres against Newcastle disease. Additional vaccination of R2B strain was given to the experimental birds in different groups on 42nd day of age for the study. At 56th day of age, 2 mL blood was collected from one bird per replicate, and the antibody titres in sera against ND virus were measured by haemagglutination inhibition (HI) test.

In vivo, cell-mediated immune response to phyto-haemagglutinin type P (PHA-P) was evaluated at 30 days of age as per the method described by Cheng and Lamont (1988). Solution of phyto haemagglutinin P (PHA-P) antigen of 1 mg/mL in sterile phosphate buffer saline was injected intradermally in inter-digital space between 3rd and 4th toe of the right foot of the individual chick. One bird per replicate, i.e., 9 birds per treatment were subjected to this test. The swelling thickness was

measured to calculate Foot web index (FWI) as the cell-mediated immune response of the bird.

One bird per replicate near to the mean body weight of the respective group was selected on 56th day for sacrifice to assess the immunity status as per the size of bursa of Fabricius.

The replicate pens were the experimental units, and all data obtained were pooled replicate-wise. The data were subjected to one way analysis of variance (ANOVA) (Snedecor and Cochran, 1994) using the software of Statistical Package for Social Sciences IBM (2012). Whenever significant differences were found (at $P<0.05$), the treatment means were compared using Tukey's test. The results were expressed as mean and standard error of mean.

RESULTS

The effect of nano copper on immunity has been presented in Table 3. The antibody titres ranged between 6.78 and 9.77. The data indicate that, in the present study, supplementation of nano copper increased the antibody titres significantly ($P = 0.01$) compared to the negative control, while the positive control had shown similar antibody titres to the nano copper fed groups. The numerical trend suggests that 6 ppm of nano copper or 50% of the inorganic copper-fed group had shown better antibody titres, while 3 ppm copper could fall short of positive control and 6 ppm copper-fed groups. Other immunity parameters, viz., cell-mediated immunity and bursal weight, did not differ due to nano copper feeding or between positive and negative controls.

Table 3. Effect of feeding copper nanoparticles on immunity of Vanaraja chicken

Treatment	Antibody titres	CMI (Ratio)	Weight of Bursa (g)
Cu_0	6.78[a]	0.46	2.22
CuS_12	9.56[b]	0.57	2.40
CuNP_3	8.44[ab]	0.39	2.38
CuNP_6	9.77[b]	0.61	2.34
CuNP_9	9.33[b]	0.47	2.37
CuNP_12	8.78[b]	0.47	2.41
SEM	0.23	0.04	0.04
P Value	0.01	0.740	0.835

[a,b] figures within a column bearing different superscripts differ significantly; CMI- Cell-mediated immunity

The effect of nano copper on the antioxidant parameters of serum is presented in Table 4. The data indicate that all the tested antioxidant parameters, viz., lipid peroxidation per mg protein, glutathione peroxidase activity, glutathione reductase activity and superoxide dismutase activity, were all statistically similar among all the treatments.

Table 4. Effect of feeding copper nanoparticles on antioxidant status of Vanaraja chicken

Treatment	LP/mg protein	GPX (U/l)	GRX (U/l)	SOD (U/l)
Cu_0	0.76	1224	3294	4.68
CuS_12	0.87	1447	2978	3.79
CuNP_3	0.87	1306	2975	2.48
CuNP_6	0.75	1292	3220	4.33
CuNP_9	0.83	1297	2762	5.07
CuNP_12	0.81	1239	2736	3.49
SEM	0.03	75	73	0.45
P Value	0.803	0.973	0.142	0.311

LP/mg protein: Lipid peroxidation per mg of serum protein; GPX– Activity of glutathione peroxidase

The effect of feeding copper nanoparticles to Vanaraja chicken on serum biochemical parameters is presented in Table 5. The data suggested that there was no significant difference due to nano copper supplementation in any of the tested parameters, viz., glucose, total protein, albumin, globulin, cholesterol, triglycerides and blood urea nitrogen.

The effect of feeding copper nanoparticles to Vanaraja chicken on serum enzyme content, serum

Table 5. Effect of feeding copper nanoparticles on serum biochemical parameters of Vanaraja chicken

Treatment	Glucose (mg/dL)	Total Protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	BUN (mg/dL)
Cu_0	156.03	4.91	3.47	1.44	105.44	113.61	2.13
CuS_12	126.11	4.67	2.90	1.77	109.00	108.57	2.12
CuNP_3	146.11	4.03	2.74	1.30	108.58	105.25	2.10
CuNP_6	155.11	4.81	2.92	1.89	109.14	117.58	2.13
CuNP_9	136.28	4.10	2.49	1.61	116.04	107.61	2.14
CuNP_12	140.01	4.54	2.86	1.68	117.50	109.65	2.10
SEM	6.01	0.16	0.15	0.08	3.83	4.76	0.006
P Value	0.717	0.519	0.616	0.337	0.782	0.862	0.961

Table 6. Effect of feeding copper nanoparticles on serum enzymes and minerals of Vanaraja chicken

Treatment	Copper (ppm)	Calcium (mg/dL)	Phosphorus (mg/dL)	SGPT (U/l)	SGOT (U/l)	ALP (U/l)
Cu_0	116.33	10.35	5.10	5.62	142.06	351.59
CuS_12	118.01	9.81	5.10	6.98	139.08	346.99
CuNP_3	120.54	9.61	5.11	5.43	134.56	301.76
CuNP_6	127.61	9.62	5.03	5.04	115.69	351.75
CuNP_9	132.32	9.68	5.19	5.24	158.34	353.89
CuNP_12	125.25	9.69	5.17	5.43	152.14	325.53
SEM	2.92	0.26	0.11	0.53	6.28	22.41
P Value	0.618	0.712	0.611	0.337	0.186	0.587

copper, calcium and phosphorus content is presented in Table 6. The data indicate no effect of nano copper on the serum enzyme activities of SGOT, SGPT and ALP. These results indicate that nanoparticles of copper oxide are safe to feed to Vanaraja chicken.

DISCUSSION

Better immunity with feeding of nano copper, in terms of higher immunoglobulin levels, was reported by Wang *et al.* (2011), El-Kazaz and Hafez (2020) and El-Wardany *et al.* (2020), who too reported better immunity with lesser doses of nano copper compared to negative control and similar to positive control indicating the potential of nano copper as a tool to reduce dietary copper supplementation without compromising on immunity. The current results agree with the review by Scott *et al.* (2018), who concluded that copper nanoparticles have higher bioavailability compared to traditional sources.

The results of this study, in terms of antioxidant parameters, agree with Rao *et al.* (2013), who took Vanaraja birds to evaluate the effect of organic trace minerals and found no effect on antioxidant status. Contrasting results were observed in commercial broilers in the study reported by Ognik *et al.* (2019), who found the antioxidant potential of both liver and breast muscle to be increased significantly ($P<0.05$) due to nano copper supplementation. The results of the current study also differ from the findings of Ognik *et al.* (2017) that nano copper supplementation above 54% of NRC requirement had enhanced antioxidant activity and reduced lipid peroxidation in broilers. The nano copper doses under the current investigation were either equal or less than the requirements, which could be the factor for different results in the present study. Also, the period of the current research was during the winter months from December to February, and there was no heat stress. The lack of antioxidant response could be also attributed to lack of changes in antioxidant status of experimental birds.

The effect of nano copper on serum biochemical parameters of this study differed from the findings of El-Wardany *et al.* (2020), who reported significantly ($P<0.05$) higher serum protein, globulin, glucose and cholesterol in broiler chicks fed nano copper compared to inorganic copper fed group. The breed of experimental bird and environment could be the factors responsible for the different results.

The results of this study in terms of serum enzyme concentration indicate that nanoparticles of copper oxide are safe to feed to Vanaraja chicken. Similar results were reported by Wang *et al.* (2011), who investigated the effects of dietary copper-loaded chitosan nanoparticle in broilers. They too did not observe any significant changes in the three enzymes, GOT, GPT and ALP of the serum samples. The results indicate no significant effect of nano copper on serum copper, calcium and phosphorous content. These results differ from Lee *et al.* (2021) and Sui *et al.* (2011), who observed significantly ($P<0.01$) higher serum copper because of nano copper feeding.

From the current study, it may be concluded that the conventional copper sulphate of 12 mg/kg in broiler feeds can be replaced with nanoparticle forms at 50% level or 6 mg/kg feed without any adverse effect on health as assessed by serum biochemical profile. The humoral immunity of birds fed nano copper at 6 mg/kg was better than birds of negative control and similar to the birds in positive control. Hence, nano copper can be considered beneficial overfeeding of traditional copper sources in terms of reduced dosage. Absence of any difference in antioxidant parameters of negative control with all other five treatments in the current study could be attributed to the winter season during which the study was carried out. In order to establish this conclusion, it may be further investigated with similar experiments using commercial broilers in different agro-climatic zones and during different seasons.

Conflict of interest: The authors declare no conflict of interest with the research activity conducted during the current investigation.

Author's contribution: MR, BR, AK: Conceptualization and design; MR, AK, MS, MV, RK, RM: Data

compilation; MR, AK: Performed statistical analysis of date; MR, BR, AK: Performed interpretation of results.

Ethical Statement: The animal experiment was performed as per the guidelines issued by the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) regarding the use of animals in scientific experiments in India. The experiment protocol used in the study was approved by the Institute's Animal Ethics committee for experiment on live animals/birds, and the approval no. is IAEC/DPR/21/01.

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