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Effect of organic and nano copper at reduced dietary levels on biochemical profile and immune response of Giriraja and Swarnadhara birds

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ABSTRACT

Experiments were conducted to assess the effect of dietary organic and nano-particle forms of Cu at reduced levels on serum biochemical profile and immune response in improved chickens. Giriraja chicks (n=420) were assigned to starter (1-6 weeks) and grower diets (7-10 weeks) and Swarnadhara breeders (n=224) to breeder diets (28-48 weeks) during experimentation. Control diet prepared for each phase was supplemented with inorganic $CuSO_4$ as per ICAR recommendation and test diets with organic Cu at 100 (OC-100), 75 (OC-75) and 50% (OC-50) or with nanoparticle Cu at 75 (NC-75), 50 (NC-50) and 25% (NC-25) of control. During each phase, blood samples were collected from 8 birds in each treatment. The serum total protein and globulin was higher in OC-50 and lower in NC-50 at 3rd and 48th week. Glucose content was higher in control and OC-50 as compared to NC-50 group at 3rd week and lower in NC-75 as compared to all other groups at 10th week. Total cholesterol was lower in OC-100 and NC-75 at 10th week and in NC-75 at 48th week. Serum albumin, calcium, phosphorus, triglyceride, creatinine, SGOT and SGPT were similar among the groups during 3rd, 10th and 48th week. Antibody titre against Newcastle disease virus was higher in OC-75 and lower in NC-75 and titre against infectious Bursal disease virus was higher in OC-75 at 3rd week, while similar among the groups at 10th week. It was concluded that the dietary inclusion of organic and Nano Cu at reduced levels had no adverse effect on biochemical profile except nano Cu on immune response at 3rd week of chicken age.

Keywords: Blood biochemical profile, Immune response, Nano copper, Organic copper

Copper (Cu) plays a key metabolic role in the body as a component of more than 200 enzymes including cytochrome oxidase and superoxide dismutase (Wu *et al.* 2015). Poultry diets are supplemented with Cu salts such as Cu sulphate, Cu oxide or Cu carbonate to meet the birds' Cu requirement. However, bioavailability of Cu from such inorganic sources is poor (Scott *et al.* 2018a) and a large quantity of Cu is excreted. Accumulation of Cu in the environment and the potential toxic effect of applying manure of high Cu content to pasture grazed by ruminants has alarmed to limit the Cu added to poultry diets. Therefore, interventions are required to increase Cu bioavailability from its conventional sources and reduce its environmental pollution. Due to better absorption, utilization, and metabolism properties, organic Cu can be included at reduced level in poultry diets (Nollet *et al.* 2007). Considering the physicochemical characteristics, nano Cu is another form to replace conventional $CuSO_4$ in the poultry diets (Hefnawy and El-khaiat 2015). Improved growth performance, nutrients' metabolizability and reduced pathogenic microorganisms in the intestine of poultry due to organic or nanoparticles form of Cu in the diet were observed (Raje *et al.* 2018). The Cu inclusion levels can be reduced by 50 and 75% of conventional form $CuSO_4$ using organic and nanoparticle forms, respectively without any adverse effect on the growth performance and nutrients utilization in Giriraja birds (Aminullah *et al.* 2022) and without affecting egg production performance, quality and hatchability in Swarnadhara breeder hens (Aminullah *et al.* 2021). However, organic and nano forms of Cu can affect the blood serum parameters (Mroczek *et al.* 2013) and immune status in chicken (Mroczek *et al.* 2015). Excess of Cu in the diet can also have adverse effects on health including calcium deficiency due to antagonism and increased stress and toxicity in chicken (Cao *et al.* 2016). Therefore, the present study was conducted to evaluate the effect of reduced Cu inclusion levels sourced from organic and nano Cu forms on the serum profile and immune response in improved native breeds of chicken.

MATERIALS AND METHODS

Experimental design: In experiment 1, 420 day-old Giriraja chicks were randomly assigned to seven treatment groups of four replicates, with 15 chicks in each for a

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period of 10 weeks. In experiment 2, 224 Swarnadhara breeder hens were wing banded and randomly assigned to seven treatment groups of four replicates, with 8 hens in each for a period of 20 weeks (from 28 to 48 week). All the birds were maintained under deep litter system with all standard management practices. Light was scheduled 23 h in experiment 1 and 16 h in experiment 2. Birds were immunized using live vaccine against Newcastle Disease (ND) on 7th and 21st day and against Infectious Bursal Disease (IBD) on 14th and 28th day of age. Both the trials were conducted at the Department of Poultry Science, Veterinary College, Bengaluru located at latitude 13º04’ N, longitude 77º61’ E and altitude 916 m MSL. All the procedures with regard to the management and care of the birds followed during the trials were approved by the Institutional Animal Ethics Committee with approval number VCH/IAEC/2020/01, dated March 3, 2020.

Experimental diets: In experiment 1, the basal diet was supplemented with 20 ppm Cu in starter (1-6 weeks) and 30 ppm in grower phase (7-10 weeks) from the conventional form of inorganic $CuSO_4$ as control. In experiment II, the basal breeder diet was supplemented with 20 ppm Cu using conventional inorganic $CuSO_4$. The treatment diets in both experiments were supplemented with different reduced levels of Cu from organic and nano particle sources (Table 1). The ingredients and chemical composition of basal diets are presented in Table 2. All treatment groups received respective iso-nitrogenous, iso-caloric diets according to their growth stage, viz. starter, grower and production phases. The inorganic copper sulphate, organic copper (copper proteinate) and nano Cu were procured from local market. The particle size of Nano Cu was 50-80 nm with 98% purity as measured by Atomic Absorption Spectrophotometer (AAS). The basal diets were formulated as per ICAR (2013) nutrient specifications for poultry, except for Cu.

Sample collection: In experiment 1, blood samples were collected at 3rd and 10th week of chicken growth and in experiment 2, the samples were collected during 48th week from two birds from each replicate by puncturing the brachial vein of the bird into 5 ml test tubes without anti-coagulant. The serum was separated and collected as per the standard procedures and analyzed for biochemical parameters such as total protein, albumin, glucose, total cholesterol, triglycerides, calcium, phosphorus, creatinine, serum glutamic oxaloacetic transaminase (SGOT or aspartate aminotransferase) and serum glutamate pyruvate transaminase (SGPT or alanine aminotransferase) using biochemical semi-auto analyser (MICRO LAB, RX-50) and Erba analysis reagent kits as per the manufacturer’s specification. The antibody titre against ND virus was measured by Hemagglutination (HA) followed by Hemagglutination inhibition (HI). The antibodies titre against IBD virus was determined using indirect ELISA Kit.

Statistical analysis: The data was subjected to one way ANOVA using Statistical Package for the Social Sciences (SPSS-16). If, the $p<0.05$ value of traits was considered as significant difference among the treatment groups.

RESULTS AND DISCUSSION

Biochemical profile: Serum biochemical indices are vital indicators of general health and physiological stress reaction in chicken. The serum total protein and globulin at 3rd and 48th week of age (Table 3) were significantly ($p<0.05$) higher in OC-50 as compared to NC-50, while values were comparable with the remaining groups. The total protein and globulin contents of group fed organic Cu in both experiments was dose-dependent and improved with a lower (50%) inclusion level, while it was dose-independent in nano Cu supplemented groups. The increased total protein and globulin levels at 50% organic Cu may have accelerated tissue protein bio-synthesis, while decelerated at 50% nano Cu. However, improved body weight gain at 100% organic and better feed conversion ratio at 75% nano Cu supplementation in Giriraja birds (Aminullah *et al.* 2022) and similar egg production performance in Swarnadhara breeder birds (Aminullah *et al.* 2021) were observed. In this study, no adverse effect on protein metabolism in Giriraja and Swarnadhara birds at 3rd and 48th week, respectively was observed in spite of reduced supplemental level of organic or nano Cu in the diet. The better effect at 100% Cu supplementation from organic source on serum total protein and globulin contents was due to greater Cu bioavailability (Zafar and Fatima 2018, Elsherif *et al.* 2019). The improved protein bio-synthesis in the body of chicken was due to growth hormone stimulating properties of Cu (Yang *et al.* 2011).

Table 1. Description of experimental diets and copper content

Group	Copper source and level (% of standard recommendation)	Supplemental Cu (ppm)			Analyzed Cu (ppm)		
		Starter diet (1-6 weeks)	Grower diet (7-10 weeks)	Breeder diet (28-48 week)	Starter diet (1-6 weeks)	Grower diet (7-10 weeks)	Breeder diet (28-48 week)
Control	Inorganic Cu (100)	20	30	20	27.94	38.35	28.04
OC-100	Organic Cu (100)	20	30	20	27.62	37.56	28.07
OC-75	Organic Cu (75)	15	22.5	15	22.65	29.37	23.03
OC-50	Organic Cu (50)	10	15	10	17.70	23.52	18.05
NC-75	Nano Cu (75)	15	22.5	15	22.73	28.41	23.06
NC-50	Nano Cu (50)	10	15	10	18.06	24.41	18.05
NC-25	Nano Cu (25)	5	7.5	5	12.77	16.43	13.04

OC, Organic copper (Cu proteinate); NC, Nano copper.

Table 2. Ingredient and nutrient composition of basal diets

Ingredient (%)	Starter diet (1-6 week)	Grower diet (7-10 week)	Breeder diet (28-48 week)
Yellow maize	61.30	57.00	58.86
Soybean meal	34.50	24.36	22.5
De-oiled rice bran	-	15.00	9.00
Oyster shell grit (kg)	-	-	4.50
Dicalcium phosphate	1.55	-	2.50
Mineral mixture-without copper*	2.00	3.00	2.12
Bacitracin methylene disalicylate	0.03	0.03	0.07
Salinomycin	0.05	0.05	-
Vitamin premix**	0.025	0.03	0.015
Vitamin B complex***	0.035	0.04	0.025
DL-Methionine	0.10	0.08	0.10
Hepatocare	0.10	0.10	0.10
Common salt	0.31	0.31	0.21
Total	100.00	100.00	100.00
Nutrient composition			
ME[a] (kcal/kg)	2881	2795	2692
Crude protein (%)	21.20	18.34	16.65
Calcium (%)	1.09	1.10	3.03
Total phosphorus (%)	0.86	0.65	0.77
Ca:P ratio	0.79	0.59	0.25
Lysine[a] (%)	1.17	0.97	0.78
Methionine[a] (%)	0.46	0.41	0.41
Selenium[a] (ppm)	0.25	0.27	0.25
Zinc (ppm)	105	124	86.51
Iron (ppm)	104	118	103.6
Manganese (ppm)	94	127	59.55
Copper (ppm)	7.97	8.27	8.03

*Contains: Ca-32%, P-9%, Fe-2000 ppm, I-0.01%, Mn-0.4% and Zn-0.4%. ** Each gram contains: Vitamin A-82500 IU, Vitamin B_2-50 mg, Vitamin D_3-12000 IU and Vitamin K-10 mg. ***Each gram contains: Vitamin B_1-4 mg, Vitamin B_6-8 mg, Vitamin B_{12}-40 mg, Vitamin E-40 mg, Pantothenate-40 mg, Niacin- 60 mg. [a]Calculated value.

The serum glucose content (Table 3) of Giriraja chicken was significantly ($p<0.05$) higher in control and OC-75 in comparison to the NC-50 group at 3rd week and significantly ($p<0.05$) lowered in NC-75 as compared to all groups at 10th week. The effect of organic or NP forms of Cu on serum glucose contents was non-significant ($p>0.05$) among the groups at 48th week. This indicates that blood glucose metabolism can be regulated using a specific dose of Cu derived from either organic or nano form. The insulin-like properties of organic Cu are also reported by Kwiecien *et al*. (2015). The serum total protein content at 3rd week was also affected at 50% nano Cu supplementation, which indicates that the Nano Cu at particular dose (10 ppm) can reduce glucose and protein bio-synthesis up to 3rd week of chicken age. The glucose level at 48th week remained similar at dietary Cu inclusion levels, while significantly ($p<0.05$) reduced in NC-50 at 3rd week and in NC-75 group at 10th week, similar to findings of Mroczek *et al*. (2013). Cu plays important role in cytochrome C oxidase activities that contributes to the most efficient production of adenosine triphosphate within the cell resulting in better energy utilization (Hill *et al.* 2000).

Serum total cholesterol level (Table 3) remained similar among the groups at 3rd week and significantly ($p<0.05$) lowered in OC-100 and NC-75 at 10th week and in NC-75 at 48th week of age. The results are in agreement with Payvastegan *et al*. (2013) and Pekel and Alp (2011). The Cu regulate cholesterol metabolism in the body (Tapiero *et al.* 2003) by increasing the oxidised form of glutathione that stimulate 3-hydroxy 3-methylglutraryl-coenzyme A (HMG-CoA) reductase (Friesen and Rodwell 2004). The reduced serum total cholesterol level due to organic (Jegede *et al.* 2011) and nano sources of Cu (Payvastegan *et al.* 2013, Mroczek *et al.* 2015) was also reported. In contrast, Pekel and Alp (2011) reported no significant ($p>0.05$) effect of organic Cu on serum total cholesterol. Cu can reduce the serum triglyceride level in poultry (Mroczek *et al.* 2013), whereas in the current experiment no such effect was observed on serum triglycerides due to variation in Cu source and levels which influences lipid metabolism. Kumar *et al*. (2013) also reported no effect of different Cu level on blood triglyceride and creatinine level in poultry.

The serum Ca and P contents at 3rd and 10th week (Table 3) remained unaffected by different treatments and were within normal physiological range. However, Cu can bind with phytic acid forming insoluble Cu-phytate complex that suppress phytase activity in gut (Maenz *et al.* 1999) leading to reduction in P and other phytic acid

Table 3. Serum biochemical profile in Giriraja birds at 3rd and 10th week and Swarnadhara breeder at 48th week under different groups

Attribute	Control	OC-100	OC-75	OC-50	NC-75	NC-50	NC-25	SEM	P-value
Total protein (g/dl)									
3rd week	3.205^{ab}	3.203^{ab}	3.504^{ab}	3.735^{a}	3.537^{ab}	2.874^{b}	3.254^{ab}	0.110	0.039
10th week	3.762^{b}	4.275^{a}	3.775^{b}	4.150^{ab}	4.142^{ab}	3.913^{ab}	3.855^{ab}	0.200	0.047
48th week	5.487^{ab}	5.587^{ab}	5.487^{ab}	5.830^{a}	5.300^{ab}	4.700^{b}	4.950^{ab}	0.338	0.013
Albumin (g/dl)									
3rd week	1.003	0.972	1.053	1.102	1.007	0.972	0.979	0.065	0.721
10th week	1.475	1.500	1.522	1.450	1.471	1.447	1.447	0.084	0.945
48th week	2.175	2.375	2.225	2.147	1.887	1.900	2.212	0.230	0.250
Globulin (g/dl)									
3rd week	2.203^{ab}	2.330^{ab}	2.454^{ab}	2.633^{a}	2.534^{ab}	1.906^{b}	2.272^{ab}	0.015	0.025
10th week	2.287	2.775	2.262	2.650	2.671	2.475	2.437	0.211	0.085
48th week	3.354^{ab}	3.265^{ab}	3.263^{ab}	3.706^{a}	3.412^{ab}	2.806^{b}	2.737^{b}	0.294	0.014
Glucose (mg/dl)									
3rd week	181.78^{a}	151.99^{bc}	184.14^{a}	149.40^{bc}	168.10^{ab}	131.53^{c}	171.52^{ab}	6.390	0.000
10th week	186.5^{a}	160.9^{a}	174.2^{a}	172.0^{a}	127.8^{b}	173.0^{a}	171.8^{a}	16.927	0.021
48th week	242.8	255.7	237.0	256.2	241.8	246.6	246.8	9.023	0.224
Total cholesterol (mg/dl)									
3rd week	87.65	85.75	88.05	91.77	92.40	103.7	86.52	6.555	0.433
10th week	144.4^{a}	124.2^{b}	141.4^{a}	140.1^{a}	123.7^{b}	131.9^{ab}	139.4^{a}	5.044	0.000
48th week	213.6^{a}	186.4^{ab}	165.6^{bc}	180.2^{abc}	146.3^{c}	159.5^{bc}	189.8^{ab}	12.379	<0.001
Calcium (mg/dl)									
3rd week	9.973	10.45	10.37	11.02	10.55	10.51	10.15	0.823	0.979
10th week	9.850	10.82	10.76	10.50	10.57	10.62	9.787	0.762	0.648
Phosphorus (mg/dl)									
3rd week	6.374	7.573	6.955	6.632	6.570	6.741	7.033	0.528	0.701
10th week	6.056	4.831	5.350	5.475	6.028	5.135	5.400	0.491	0.104
Triglyceride (g/dl)									
10th week	81.93	74.67	78.60	82.51	82.22	80.00	84.05	4.006	0.230
48th week	142.3	133.4	137.1	134.3	130.4	139.8	143.1	5.349	0.115
Creatinine (mg/dl)									
10th week	0.818	0.867	0.786	0.887	0.791	0.727	0.890	0.067	0.106
48th week	0.898	0.787	0.876	0.896	0.866	0.857	0.773	0.056	0.982
SGOT (IU/L)									
10th week	149.2	165.9	154.8	146.9	155.10	144.6	164.1	11.697	0.357
SGPT (IU/L)									
10th week	16.08	17.16	16.57	18.52	16.37	15.62	16.54	0.401	0.149

$^{a-c}$Mean values bearing different superscripts within the column differ significantly ($p<0.05$).

bound nutrients utilization (Pang and Applegate 2006). The antibacterial properties of nano Cu can also affect gut normal flora population (Zhang *et al.* 2017, Di Giancamillo *et al.* 2018) and reduce phytase producing bacteria, thereby reducing minerals solubility. In such condition, P-phytate complex can be formed which is insoluble at intestinal pH leading to reduced P utilization. Increased serum Ca and P level when broilers were injected with nano-Cu at 50mg/kg BW was observed (Mroczek *et al.* 2013).

The serum creatinine level (Table 3) at 10th and 48th week remained similar among the groups and was within normal physiological range indicating tested organic or nano Cu levels safety for kidney. Similarly, the liver enzymes, viz. SGOT and SGPT in Giriraja chicken at 10th week was unaffected ($p>0.05$). The Cu toxicity occurs through damaging of hepatic cells and generation of reactive oxygen species, altering the liver enzymatic level (Dojlido 1995). The normal serum SGOT and SGPT levels indicate normal liver function confirming the organic or nano form of Cu at the tested levels has no toxic effect in the body

Table 4. Immune competence profile in Giriraja birds under different treatments

Attribute	Control	OC-100	OC-75	OC-50	NC-75	NC-50	NC-25	SEM	P-value
ND titre (log_2)									
3rd week	15.00[bc]	18.00[bc]	39.00[a]	15.00[bc]	10.50[c]	29.25[ab]	22.00[bc]	3.706	<0.001
10th week	2.00	3.50	4.00	4.00	2.00	4.00	2.50	1.030	0.474
IBD titre									
3rd week	1377[b]	1094[c]	1512[a]	1160[c]	1167[c]	1191[c]	1132[c]	4.123	<0.001
10th week	1625	1422	1584	1535	1380	1511	1234	27.907	0.399

[a-c]Mean values bearing different superscripts within the column differ significantly ($p<0.05$)

(Mroczek *et al.* 2013).

Immune competence: The antibodies titre against ND and IBD was highly significant ($p\leq0.05$) at 3rd week, while non-significant ($p>0.05$) at 10th week (Table 4). The antibodies titre against ND was higher ($p<0.05$) in OC-75 as compared to NC-75, and against IBD, the antibodies titre in general was higher in OC-75 as compared to all the remaining groups. The antibodies titre was low in OC-100 and NC-75 groups. The higher level (75% of control) nano Cu supplementation tends to suppress the immune response probably due to inappropriate stimulation of immune response with nano particles (Shannahan and Brown 2014). Nano Cu is involved in promoting inflammatory responses due to their physicochemical properties (Scott *et al.* 2018a). However, no effect on immune status was reported as a result of nano Cu injection into chicken embryos which was attributed to inappropriate recognition of Cu nanoparticles by antigen-presenting cells (APCs) (Scott *et al.* 2018b). Similar non-responsive result in broiler birds against *in-ovo* injected nano Cu was noticed (Scott *et al.* 2016) which might be due to inadequate recognition of Cu nanoparticles by macrophages (Kagan *et al.* 2006). The uptake of nano particles and phagocytic recognition might be important in determining of their effect on immune system (Konduru *et al.* 2009).

The immune response depends on nano particles uptake by APCs which may impair immune function and induce toxicity (Pineda *et al.* 2013). However, no negative effect of reduced dietary nano Cu levels on immune response was observed against ND virus. While reports indicate improved antibodies titre as result of organic (Saleh *et al.* 2019) or nanoparticle (El-kazaz *et al.* 2020) form of Cu incorporation in the poultry ration.

However, immune response was suppressed at 75% of nano Cu inclusion level in the birds' diet. It was concluded that the conventional $CuSO_4$ can be replaced with organic and nanoparticle forms at a reduced level without any adverse effect on serum biochemical profile.

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