An Acad Bras Cienc (2024) 96(3): e20231121 DOI 10.1590/0001-3765202420231121
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

ANIMAL SCIENCE

Nano copper in the diet of laying quails: productive performance, metabolism, and tissue concentration

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Abstract: The study evaluated the use of nano copper in semi-purified diets for laying quails and its effect on performance, metabolic state, and bioavailability. A total of 160 (180-days-old) quails were distributed in a completely randomized design, in a 3x3+1 factorial. The copper sources used were copper sulfate, copper oxide, and nano copper oxide, at levels of 200, 400, and 800 ppm each, totaling nine treatments plus a negative control (with no copper inclusion). The following variables were determined: weight gain, feed intake, egg production, egg weight, hemoglobin, hematocrit, Cu in the tissues and Cu bioavailability. Data were subjected to analysis of variance at 5% probability. The effect of sources and levels, as well as the interaction between the factors were evaluated. When interaction was observed, the effect of sources was evaluated separately by the Tukey's test and the effect of levels by regression, both at 5% probability. Copper nano oxide can be used at up to 800 ppm in the diet of laying quails without altering the productive performance, and with higher bioavailability than conventional copper oxide. Hemoglobin increases with the inclusion of 200 and 400 ppm of nano copper oxide and the hematocrit with 400 ppm.

Key words: Bioavailability, *Coturnix coturnix japonica*, gelatin, hematology, nano minerals, semi-purified diet.

INTRODUCTION

Brazilian poultry farming began with family farmers and for many years was considered a subsistence activity, in which only surplus production was sold (Zen et al. 2015). Over the years, production has intensified and reached the current level, with excellent production rates and worldwide recognition, since Brazil is one of the largest producers and the largest exporter of broiler meat in the world (ABPA 2023). Among the different sectors of poultry farming, quail farming consists of raising quails for meat or laying. Due to their unique taste and nutritional value, quail eggs and meat are important products in the diet of many countries, in addition to being considered a potential niche market (Knaga et al. 2018).

In poultry, among the nutritional limitations for laying chickens, mineral deficiency is highlighted, since the feeds that form the basis of their diet, such as corn and soybean meal, commonly do not meet the requirements of these animals (Araujo et al. 2008). One of the minerals that are supplemented in poultry diets is copper (Cu), which despite being present in the animal body in low concentrations is a fundamental element for metabolism (Richards et al. 2010). Cu is a chemical element belonging to group 11 of the periodic table and has atomic number 29. This orange-colored mineral was discovered around the year 9,000 BC, in the Middle East. In addition to its characteristic color, it also has high malleability and good electrical and thermal conduction. Around 1928, working with rats as an animal model, researchers observed that it was an essential mineral for the body, noting that it was extremely important for growth, hemoglobin formation and acting in the prevention of pathologies, such as anemia (Díaz et al. 2015). Additionally, Cu participates in several biochemical processes necessary for development, mainly in the skeletal system, central nervous system and cardiac muscle functioning (Richards et al. 2010, Díaz et al. 2015).

Cu is part of the composition of numerous enzymes that need it to carry out their metabolic activities, such as cytochrome oxidase, fundamental in the process of cellular respiration (Díaz et al. 2015). Other enzymes are also dependent on Cu, such as superoxide dismutase, which acts in the detoxification of free radicals; lysyl oxidase, which is responsible for the formation of connective tissue; and ceruloplasmin, responsible for the mobilization of iron (Suttle 2010). In addition, Cu also acts in hemoglobin synthesis and erythrocyte production (Ognik et al. 2018).

Chloride (CuCl), oxide (CuO) and sulfate ($CuSO_4$), are the main Cu sources for animal supplementation. $CuSO_4$ has high bioavailability of Cu (considered 100% bioavailable), but it has only 25% Cu in its molecule (Gattás & Barbosa 2004), which makes a greater inclusion of the product in the animals' diet necessary. Furthermore, when Cu is used at levels higher than the nutritional requirements, it acts as a performance-enhancing additive, (Broom et al. 2021). Additionally, the banning of antimicrobial growth promoters in several countries, due to the concern about risks to human health, whether due to bacterial resistance or even the toxicity and allergic reactions that these compounds can cause (Santos et al. 2005), makes the search for alternatives to these products necessary.

In this sense, nanoparticles have unique characteristics, such as small size and high surface area (Khurana et al. 2019). According to Raje et al. (2018) the small particle size facilitates absorption, as the smaller the particle the faster the diffusion through the intestinal mucosa, reaching the epithelial cells and overcoming the gastrointestinal tract barrier, causing the product to have better absorption, reaching deeper the tissue. Taking advantage of these characteristics, studies suggest that nanoparticles, such as nano CuO, have greater bioavailability and, therefore, better use by the animal, when compared to the conventional forms regularly used in the diets (Raje et al. 2018).

The search for answers about the bioavailability and action of different sources of Cu on animal performance is necessary for a better understanding about the effects on the animal organism. Thus, the aim of this study was to evaluate the use of Cu, in the form of nanoparticles (< 50 nm), in semi-purified diets for laying quails and its effect on performance, metabolic state and bioavailability, to define the best level of inclusion of this mineral in the diet as a performance promoter.

MATERIALS AND METHODS

The trial was conducted at the Poultry Farm of the Animal Sciences Teaching and Experimentation Station "Professor Renato Rodrigues Peixoto" (31°48'30.8"S 52°24'40.7"W) of the Department of Animal Sciences – Faculty of Agronomy Eliseu Maciel – Federal University of Pelotas (UFPel), Capão do Leão, Brazil.

The methods and protocols used in the experiment were approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Pelotas, under number 36970.

Animals

A total of 160 (180-days-old and 95% laying rate) Japanese quails (*Coturnix coturnix japonica*) were used. The birds were purchased from a commercial farm. At the beginning of the experiment, all birds were weighed, identified, and housed in pairs in metallic cages (50 x 50 cm), equipped with automatic nipple waterers and a manual trough-type feeder. The experimental room was maintained at a temperature controlled by air conditioning devices, with an average of 23ºC. And the lighting was provided artificially, following a program of 17 hours of light per day, controlled with an analog timer, in accordance with the recommendations of Della-Flora & Dionello (2012).

The temperature and humidity of the room were both recorded daily with thermo-hygrometers. Additionally, the maximum and the minimum temperatures were registered throughout the entire experimental period, and the values varied around 2ºC, in relation to the average. The humidity remained at values between 54.0% and 68.4%.

The birds had *ad libitum* access to water and feed throughout the whole experimental period (three weeks).

Experimental treatments and diets

The semi-purified diets were formulated to meet the nutritional requirements of laying Japanese quails, according to Silva & Costa (2009) and Rostagno et al. (2017). The diets were distributed in 10 treatments, in which three had $CuSO_4$, three had CuO and three other treatments had nano CuO as the mineral source, at the levels of 200, 400 and 800 ppm each one. And a negative control diet, without the addition of Cu. The centesimal composition of the diets and calculated nutritional levels are shown in Table I.

The protein source used in the study was unflavored gelatin powder, from C2 Alimentos, Brazil, with the following composition: 90% crude protein; 11.0% alanine; 9.0% arginine; 6.7% aspartic acid; 0.1% cystine; 11.4% glutamic acid; 27.0% glycine; 0.8% histidine; 13.8% hydroxyproline; 1.5% isoleucine; 3.3% leucine; 4.3% lysine; 0.8% methionine; 2.4% phenylalanine; 16.0% proline; 4.1% serine; 2.2% threonine; 0.3% tyrosine; and 2.7% valine. The nano CuO used was Sigma Aldrich, in powder form, smaller than 50 nm, molecular weight of 79.55 g/mol (product code: 544868).

Animal performance

The quails were weighed weekly to verify weight gain. The leftover feed was also weighed weekly to calculate feed intake and feed conversion rate.

Hematological analyzes

At the end of the experimental period, blood was collected from four quails per treatment, at random, to hematocrit and hemoglobin analyses. Blood was collected from the ulnar vein and placed in vials with anticoagulant (EDTA). The hematocrit was performed using the micro-hematocrit technique, while the hemoglobin concentration was measured using the hemoglobin cyanide colorimetry technique, with a commercial kit (Hemoglobin Kit, ref. as described by Van Kampen & Zijlstra 1961).

Table I. Proximate composition and calculated nutritional levels of semi-purified diets, based on sugar and gelatin, for laying Japanese quails.

	Treatments									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Ingredients (%)										
Sugar	62.50	62.42	62.34	62.18	62.48	62.45	62.40	62.48	62.45	62.40
Gelatin	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00	20.00
$CuSO_4.5H_2O$ (25% Cu)	-	0.0800	0.1600	0.3200	-	-	-	-	-	-
CuO conventional (75% Cu)	-	-	-	-	0.02666	0.05332	0.10667	-	-	-
CuO nano (75% Cu)	-	-	-	-	-	-	-	0.02666	0.05332	0.10667
Soy oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Monosodium phosphate	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Limestone	8.55	8.55	8.55	8.55	8.55	8.55	8.55	8.55	8.55	8.55
Salt common	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Inert (sand)	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Magnesium sulfate	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43	0.43
Potassium chloride	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Zinc oxide	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004
Manganous oxide	0.0012	0.0012	0.0012	0.0012	0.0012	0.0012	0.0012	0.0012	0.0012	0.0012
Potassium iodate	0.00016	0.00016	0.00016	0.00016	0.00016	0.00016	0.00016	0.00016	0.00016	0.00016
Sodium selenite	0.00011	0.00011	0.00011	0.00011	0.00011	0.00011	0.00011	0.00011	0.00011	0.00011
Ferrous sulfate	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032	0.032
L-lysine HCl	0.31	0.31	0.31	0.31	0.31	0.31	0.31	0.31	0.31	0.31
DL-methionine	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34
L-threonine	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23
L-tryptophan	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23
L-cystine	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39
Vitamin premix[1]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Choline chloride-60	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
	Calculated analysis (%)									
	1	2	3	4	5	6	7	8	9	10
Metabolizable energy, kcal/kg	2,935	2,932	2,929	2,923	2,934	2,933	2,931	2,934	2,933	2,931
Calcium	3.161	3.161	3.161	3.161	3.161	3.161	3.161	3.161	3.161	3.161
Total phosphorus	0.336	0.336	0.336	0.336	0.336	0.336	0.336	0.336	0.336	0.336
Available phosphorus	0.336	0.336	0.336	0.336	0.336	0.336	0.336	0.336	0.336	0.336
Copper	0.00001	0.020001	0.04001	0.08001	0.020001	0.04000	0.08001	0.020001	0.04000	0.08001
Magnesium	0.051	0.051	0.051	0.051	0.051	0.051	0.051	0.051	0.051	0.051
Potassium	0.462	0.462	0.462	0.462	0.462	0.462	0.462	0.462	0.462	0.462
Crude protein	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00	18.00
Zinc	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
Manganese	0.00072	0.00072	0.00072	0.00072	0.00072	0.00072	0.00072	0.00072	0.00072	0.00072
Iodine	0.00010	0.00010	0.00010	0.00010	0.00010	0.00010	0.00010	0.00010	0.00010	0.00010
Selenium	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005
Iron	0.0096	0.0096	0.0096	0.0096	0.0096	0.0096	0.0096	0.0096	0.0096	0.0096
Lysine	1.10	1.10	1.10	1.10	1.10	1.10	1.10	1.10	1.10	1.10
Methionine	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50

Table I. Continuation.

Cystine	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41	0.41
Methionine + Cystine	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Threonine	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67	0.67
Tryptophan	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23	0.23
Arginine	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80	1.80
Glycine	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Choline (mg/kg)	2098	2098	2098	2098	2098	2098	2098	2098	2098	2098

[1]Composition per kg of product: Vitamin A: 8,000,000 UI/kg; Vitamin D3: 2,500,000 UI/kg; Vitamin E: 6,000 UI/kg; Vitamin K3: 1,000 mg/kg; Vitamin B1: 1,000 mg/kg; Vitamin B2: 4,500 mg/kg; Vitamin B6: 2,000 mg/kg; Vitamin B12: 12,000 mcg/kg; Niancin: 15 g/kg; Calcium pantothenate: 6,000 mg/kg; Folic acid: 400 mg/kg; Biotin: 25 mg/kg. Product used: Poli-vita poedeiras®, provided by Polinutri Alimentos S.A. Diet 1: no added Cu; diet 2: 200 ppm $CuSO_4$; diet 3: 400 ppm $CuSO_4$; diet 4: 800 ppm $CuSO_4$; diet 5: 200 pm CuO; diet 6: 400 ppm CuO; diet 7: 800 ppm CuO; diet 8: 200 ppm nano CuO; diet 9: 400 ppm nano CuO; diet 10: 800 ppm nano CuO.

Copper determination

The analysis of Cu concentration in the tissues was performed in the liver, as this is the main deposit organ for this mineral in the body, after absorption. The determination of Cu was also performed in the tibias, since the deposition of minerals in this structure in response to dietary supplementation is a widely used parameter, as it presents good sensitivity when compared to other tissues since Cu plays an important role in the bone formation. The technique used was flame atomic absorption spectrophotometry. A 2.0 g tissue sample (liver and tibia) was added to 7.5 mL of nitric acid in test tubes. Subsequently, this material was digested in the digester block for 180 minutes at 130ºC, as described by Pinto et al. (2019). After digestion, the samples were filled with deionized water until reaching 20 mL, for subsequent reading in the spectrophotometer (PerkinElmer, model Analyst 200, Waltham, Massachusetts, USA).

Copper bioavailability

Cu bioavailability was determined by slope ratio, using the concentration of Cu in the liver, from which linear regression equations were obtained. Bioavailability was calculated as a function of the standard source, $CuSO_4$, in which Cu is considered 100% bioavailable. For each Cu source, a linear regression equation (Y = a + bX) was obtained and the b value (*slope*) of the test source was divided by the b value obtained for the standard source and expressed as a percentage.

Statistical analysis

A completely randomized design in a 3 x 3 + 1 factorial was used (3 sources of Cu, 3 levels of Cu and a negative control, with no added Cu). A total of eight replications per treatment were used.

The data were subjected to analysis of variance at 5% probability. The effects of the sources and levels of Cu and the presence or absence of interaction were evaluated. When no significant interaction was observed, the effect of sources and levels of Cu inclusion were evaluated separately by the Tukey's test at 5% probability. In split analysis, the effect of sources within each Cu level was compared by the Tukey's test at 5% probability, and the effect of levels within each Cu source was evaluated by polynomial regression analysis at 5% probability. The basal diet (treatment 1) was considered as zero level for comparison with the Cu sources tested.

RESULTS AND DISCUSSION

Performance

There was no significant interaction (P>0.05) between the sources of Cu and the levels of inclusion of the mineral on the performance variables (egg production, egg weight, weight gain and feed intake), similar results were found by Aminullah et al. (2021), not observing significant differences, on the productive performance of layers fed with different levels and sources of copper. Also, no significant effect was observed on the performance variables when the effect of the sources of Cu and the levels of its inclusion were evaluated separately (Table II).

There was no significant difference between mineral sources within each level tested, as well as there no significant effect of inclusion levels within each source on egg production and egg weight (Table III). However, egg production tended to decrease when the level of 800 ppm of Cu was used, regardless of the source

Table II. **Performance of quails fed semi-purified diets containing different sources and levels of copper (mean ± standard deviation).**

Copper sources	Egg production, %	Egg weight, g	Weight gain (1-21 days), g/bird	Feed intake (1-21 days), g
$CuSO_4$	21.71±6.19	9.66±0.64	-63.20±18.96	413.18±87.18
CuO	24.50±6.05	9.50±0.69	-52.65±13.80	427.89±101.16
Nano CuO	21.82±5.42	9.62±0.53	-62.01±23.84	375.47±109.56
Negative control	24.33±7.21	9.30±0.71	-55.25±20.81	410.16±148.32
Inclusion levels (ppm)				
0	24.33±7.21	9.30±0.71	-55.25±20.81	410.16±148.32
200	23.61±7.36	9.58±0.73	-58.61±19.77	414.83±94.62
400	23.71±4.73	9.48±0.56	-55.10±15.29	414.04±101.54
800	20.76±5.25	9.71±0.56	-65.14±22.80	388.70±107.05
		P - values		
Source	0.2117	0.6776	0.1868	0.2317
Level	0.1733	0.4919	0.2977	0.6171
Source x level	0.5336	0.8435	0.8632	0.9490

$CuSO_4$ – copper sulfate; CuO – copper oxide; and Nano CuO – nano copper oxide.

Table III. **Egg production and egg weight of quails fed semi-purified diets containing different sources and levels of copper.**

	Egg production, %				Egg weight, g			
Levels/ sources	$CuSO_4$	CuO	Nano CuO	**p	$CuSO_4$	CuO	Nano CuO	**p
0	24.33±7.21	24.33±7.21	24.33±7.21	1.000	9.30±0.71	9.30±0.71	9.30±0.71	1.000
200	23.51±7.68	26.78±9.06	20.53±3.80	0.244	9.70±0.58	9.34±0.95	9.71±0.64	0.541
400	23.21±4.17	23.80±3.81	24.10±6.41	0.934	9.47±0.62	9.42±0.60	9.55±0.51	0.900
800	18.78±5.76	22.91±3.80	20.83±5.65	0.280	9.79±0.74	9.72±0.46	9.60±0.48	0.803
*p linear	0.055	0.452	0.396		0.195	0.186	0.445	
P quadratic	0.140	0.676	0.701		0.430	0.404	0.517	

***p- significance level by regression equation. **p – significance level by analysis of variance. $CuSO_4$ – copper sulfate; CuO – copper oxide; and Nano CuO – nano copper oxide.**

(Table III). For birds fed diets containing $CuSO_4$, a tendency of linear reduction was observed as the Cu levels increased. As expected, the opposite was observed in relation to egg weight, in which numerically lighter eggs were produced when Cu was not included in the diets. It is well known that the lower the egg production, the greater the weight of the eggs, since they remain longer in the follicle.

Similarly, no significant difference was observed for weight gain and feed intake (Table IV). The performance data from the present study corroborate those found by Lee et al. (2021), who working with levels of up to 120 ppm of copper sulfate, in conventional form and nanoparticles, found no difference in weight gain and feed consumption of broiler fed with supplemental doses of copper. However, when feed intake tended to be higher, the weight loss tended to be lower since there was no weight gain in the present study. Nonetheless, in all sources and levels of Cu inclusion in the diets, it was possible to observe the weight loss of the quails. Previously, Olgun et al. (2020) tested the levels of 0, 5, 10, and 20 mg/kg of organic Cu in practical diets for laying quails and did not observe a significant effect of the levels used on egg production, egg weight, egg mass, and egg mass conversion.

Prior to the study, the quails were fed a practical standard diet based on corn and soybean meal, with a commercial vitamin and mineral mix for laying quails, formulated according to Silva & Costa (2009) and Rostagno et al. (2017). The birds were in the same facilities and subjected to the same management as previously described. However, a low performance of quails was observed when semi-purified diets based on sugar and gelatin were introduced, which caused an immediate reduction of feed intake. Consequently, the overall performance of the quails was negatively affected. A reduction of body weight and egg production was observed, demonstrating the sensitivity of the quails and their lack of adaptation to the new semi-purified diets provided. In addition to the low feed intake and drop in performance, as the experiment progressed (about 15 days) a considerable increase in the mortality rate of the birds was verified, along with a change in their behavior, which visibly appeared to be downcast and prostrated, with low feathering, possibly due to low feed intake and poor nutritional intake. The utilization of semi-purified diets was necessary to avoid the interference of Cu levels from

Table IV. Weight gain and total feed intake of laying quails fed semi-purified diets containing different sources and levels of copper (mean ± standard deviation).

	Weight gain (1-21 days), g/bird				Total feed intake (1-21 days), g			
Levels/ sources	**$CuSO_4$**	**CuO**	**Nano CuO**	****p**	**$CuSO_4$**	**CuO**	**Nano CuO**	****p**
0	-55.25±20.81	-55.25±20.81	-55.25±20.81	1.000	410.16±148.32	410.16±148.32	410.16±148.32	1.000
200	-64.65±16.98	-48.39±11.13	-61.92±26.93	0.255	405.62±68.86	453.00±105.21	385.87±104.33	0.361
400	-54.40±12.45	-51.93±13.23	-59.53±20.88	0.644	429.18±90.93	436.37±108.09	376.56±107.19	0.456
800	-70.56±24.18	-58.58±17.30	-64.57±26.81	0.644	405.66±105.07	394.31±93.70	364.00±129.59	0.731
*p linear	0.184	0.584	0.495		0.977	0.637	0.445	
P quadratic	0.365	0.544	0.788		0.944	0.583	0.726	

***p- significance level by regression equation. **p – significance level by analysis of variance. $CuSO_4$ – copper sulfate; CuO – copper oxide; and Nano CuO – nano copper oxide.**

other sources, such as corn and soybean meal. Therefore, the only source of Cu in the diets came from $CuSO_4$, CuO, and nano CuO.

Funk & Baker (1991) used a semi-purified diet (based on dextrose and casein) and conventional diets (based on corn and soybean meal) to compare Cu toxicity in different diets for broilers. The authors observed a drop in performance, in which chickens fed semi-purified diets had lower weight gain than birds fed conventional diets. Nonetheless, the use of semi-purified diets is recommended when working with minerals to minimize or even eliminate the negative effects caused by anti-nutritional factors that may be present in ingredients conventionally used in diets (Lovell 1998, Hardy & Barrows 2002, NRC 2011). In other study with nano Cu, instead of providing the mineral in the diet, Scott et al. (2018) evaluated the use of *in ovo* injection of nanoparticles of a Cu colloid. The authors found that it improved the performance of broilers more efficiently than the injection of $CuSO_4$ and concluded that supplementation with Cu nanoparticles improved not only the performance of broilers, but also the use of energy and nitrogen.

Blood analysis

No significant interactions (P>0.05) were found between the different Cu sources and their inclusion levels in the quails hematocrit. Likewise, when evaluating the factors separately, no significant effect (P>0.05) was observed for inclusion levels and Cu sources on this variable (Table V). On the other hand, the hemoglobin assessment showed a significant effect (P=0.0007) of Cu sources (Table V), in which quails fed diets containing nano CuO supplementation had higher concentrations of hemoglobin than those fed diets supplemented with conventional CuO and $CuSO_4$. A significant interaction (P=0.0046) between Cu sources and inclusion levels on hemoglobin was also observed (Table V). Table VI shows that the quails fed diets containing 200 ppm of nano CuO had a higher hemoglobin rate (36.70 g/dL), differing from those that were fed diets supplemented with conventional CuO (24.77 g/dL) and $CuSO_4$ (22.73 g/dL) (P = 0.0007), which did not differ from each other.

Table V. Hematocrit and hemoglobin in quails fed semi-purified diets containing different sources and levels of copper (mean ± standard deviation).

Copper sources	Hematocrit (%)	Hemoglobin (g/dL)
$CuSO_4$	32.64± 4.61	25.07±4.16 B
CuO	30.00± 6.15	25.71±7.59 B
Nano CuO	31.46±5.84	32.24±5.73 A
Negative control	28.40± 3.36	26.52±2.53 AB
Inclusion levels (ppm)		
0	28.40±3.36	26.52±2.53
200	31.86±4.77	28.07±7.62
400	31.60±4.48	26.40±6.35
800	30.50±7.43	28.80±6.41
P - values		
Source	0.3638	0.0007
Level	0.8694	0.4879
Source x level	0.1270	0.0046

Different capital letters in the column differ from each other by the Tukey test at 5%.
$CuSO_4$ – copper sulfate; CuO – copper oxide; and Nano CuO – nano copper oxide.

Similarly, quails fed diets containing 400 ppm of nano CuO had a higher hemoglobin rate (31.34 g/dL) than birds fed diets containing conventional CuO (20.86 g/dL), and not differing from those that received diets with $CuSO_4$ (27.01) (P = 0.017). When evaluating the effect of Cu levels within each of the sources used in the diets, only CuO showed a significant quadratic response (P=0.04), in which the increase of CuO levels in the diet led to a reduction in hemoglobin up to the level of 400 ppm, increasing later, when 800 ppm of the mineral were used.

Quails fed diets supplemented with growing levels of $CuSO_4$ showed a linear increase in hematocrit (P = 0.013), while the ones fed with increasing levels of other sources of Cu did not show any significant difference in hematocrit (P>0.05) (Table VII). Similarly, Olgun et al. 2020 did not observe any effect on the hematocrit and hemoglobin of quails fed diets containing organic sources and lower levels of Cu (0, 5, 10, 20 mg/kg).

Cu is known to play a significant role in iron metabolism, hemoglobin synthesis, and erythrocyte production (Sharma et al. 2009, Samanta et al. 2011). The results of the present study showed a higher concentration of hemoglobin in the blood of birds fed diets containing nano CuO. The increase in hemoglobin suggests an increase in the number of red blood cells, but as the hematocrit was not changed and the cell count was not performed, it cannot be affirmed that there was an increase in the number of red blood cells. The increase in hemoglobin can be explained by the fact that there are more molecules being inserted into the cells to supply an oxygen demand, not changing the number of red blood cells. Similarly, Miroshnikov et al. (2015), observed an increase in hemoglobin in the blood of birds that received nano Cu intramuscularly.

Table VI. **Hemoglobin (g/dL) in quails fed semi-purified diets containing different sources and levels of copper (mean ± standard deviation).**

Level/sources	$CuSO_4$	CuO	Nano CuO	**p
0	26.52±2.53	26.52±2.53	26.52 ±2.53	1.0000
200	22.73±3.91 B	24.77±6.64 B	36.70±1.25 A	0.0007
400	27.01±4.45 AB	20.86±5.96 B	31.34± 4.08 A	0.017
800	25.58±3.52	31.49±7.07	28.69± 7.42	0.421
*p linear *p quadratic	0.873 0.942	0.215 0.04	0.841 0.085	
$Y=27.20-0.03x+0.00004x^2$ $r^2=0.31$				

***p- significance level by regression equation. **p– significance level by analysis of variance. Different capital letters in the same row differ from each by the Tukey test at 5%. $CuSO_4$ – copper sulfate; CuO – copper oxide; and Nano CuO – nano copper oxide.**

Table VII. **Hematocrit (%) in quails fed semi-purified diets containing different sources and levels of copper (mean ± standard deviation).**

Levels/sources	$CuSO_4$	CuO	Nano CuO	**p
0	28.40±3.36	28.40± 3.36	28.40 ± 3.36	1.000
200	31.00±4.94	34.00± 3.80	30.60± 5.68	0.503
400	31.80± 3.27	29.60± 6.30	33.40± 3.28	0.435
800	35.75± 5.18	26.40 ± 6.46	30.40± 8.32	0.177
*p linear	0.013	0.263	0.581	
*p quadratic	0.053	0.211	0.390	
	$Y= 28.64 + 0.008x.$ $r^2=0.30$			

***p- significance level by regression equation. **p– significance level by the analysis of variance. $CuSO_4$ – copper sulfate; CuO – copper oxide; and Nano CuO – nano copper oxide.**

Copper determination

The concentration of Cu in the liver and tibiae is shown in Table VIII. A significant interaction ($P<0.0001$) between sources and levels of Cu was observed, in which as the level of Cu increased in the diets a corresponding increase was observed in the concentration of the mineral in both tissues. The breakdown of interaction for liver concentration between Cu sources and Cu levels is shown in Table IX. Quails fed diets containing 200 ppm of nano CuO showed a lower concentration of Cu in the liver (3.90 mg/kg), differing from those fed diets containing the same concentration (200 ppm) of CuO (8.14 mg/kg) or $CuSO_4$ (7.49 mg/kg) ($P<0.0001$). When the birds were fed diets with 400 ppm of nano CuO, the quails had a lower concentration of the mineral in the liver (6.43 mg/kg) than birds that received the diet with the same concentration (400 ppm) of CuO (10.67 mg /kg), not differing from the birds that were fed a diet containing 400 ppm of $CuSO_4$ (9.12 mg/kg).

Quails fed diets containing 800 ppm of $CuSO_4$ showed the highest concentration of Cu in the liver (45.48 mg/kg), a value practically three times greater than the concentration verified in the liver of the quails fed diets containing the same concentration of CuO (12.33 mg/kg) or nano CuO (16.06 mg/kg), evidencing the high bioavailability of the mineral in the $CuSO_4$ form. Lee et al. (2021), found no significant difference in relation to the concentration of copper in the liver of broiler chickens fed with levels of 16 to 120 ppm of nano copper, obtained by hot extrusion of copper sulfate.

When evaluating the effect of the levels in the different sources of Cu, through regression analysis, an increased linear response ($P<0.0001$) was observed for both sources of Cu. As the level of Cu increased in the diets, a corresponding increase was observed in the mineral concentration in the liver. This result was expected since the liver is one of the main Cu storage organs in the body.

The breakdown of the interaction for Cu concentration in the tibias is also shown in Table IX. Regardless of the Cu source, as its concentration in the diet increased there was a quadratic response in the Cu concentration in the quail tibiae ($P<0.001$). Quails fed diets containing the highest levels of Cu (400 and 800 ppm) in the form of nano CuO showed significantly higher concentrations of the mineral in the tibias ($P<0.0001$), practically four times higher when compared to other sources of copper used, showing that this is a tissue in which the accumulation of the mineral in this form is also considerable.

Copper bioavailability

Table X shows the bioavailability of Cu determined as a function of its concentration in the liver. The results indicated values of relative bioavailability of Cu in relation to the standard form, $CuSO_4$, considered as 100% bioavailable. As shown in figure 1, the relative bioavailability of Cu was 37.2% for nano CuO and 28.3% for conventional CuO, confirming the higher bioavailability of Cu in the nano size CuO in comparison to Cu in the conventional size CuO, regularly utilized in diets.

As previously described, the liver is one of the main Cu storage organs in the body. Table IX shows the highest concentration of Cu in the liver, in the form of $CuSO_4$, especially when high concentrations of the mineral were added to the diets. The bioavailability of trace minerals for animals is defined according to the degree to which ingested minerals are absorbed in a form that can be metabolized by the animal. However, the low bioavailability, mainly of nano CuO in the present study, is probably due to the low consumption of semi purified diets.

Table VIII. Copper concentration in the liver and tibias of quails fed semi-purified diets containing different sources and levels of copper.

Copper sources	Copper concentration in the liver (mg/kg)	Copper concentration in the tibia (mg/kg)
$CuSO_4$	18.44±17.59	1.73±0.53
CuO	10.20±2.38	1.81±0.20
Nano CuO	9.01±5.99	6.30±3.68
Negative control	2.31±0.66	1.40±0.12
Inclusion levels (ppm)		
0	2.31±0.66	1.40±0.12
200	6.51±2.054	1.58±0.28
400	8.95±2.28	3.69±3.52
800	23.77±15.46	4.35±3.29
P - values		
Source	<0.0001	<0.0001
Level	<0.0001	<0.0001
Source x level	<0.0001	<0.0001

$CuSO_4$ – copper sulfate; CuO – copper oxide; and Nano CuO – nano copper oxide.

Table IX. Breakdown of the interaction of copper concentration in the liver and tibias (mg/kg) of quails fed semi-purified diets containing different sources and levels of copper.

Levels / sources	Copper concentration in the liver (mg/kg)				Copper concentration in the tibia (mg/kg)			
	$CuSO_4$	CuO	Nano CuO	**p	$CuSO_4$	CuO	Nano CuO	**p
0	2.31±0.66	2.31±0.66	2.31±0.66	1.000	1.40±0.12	1.40±0.12	1.40±0.12	1.000
200	7.49±0.70 A	8.14±0.60 A	3.90±0.87 B	<0.0001	1.35±0.33	1.69±0.16	1.69±0.24	0.153
400	9.12±1.30 AB	10.67±2.24 A	6.43±0.75 B	0.027	1.45±0.22 B	1.84±0.28 B	9.13±0.71 A	<0.0001
800	45.48±5.77 A	12.33±2.14B	16.06±3.28 B	<0.0001	2.38±0.15 B	1.89±0.11 B	8.79±0.59 A	<0.0001
*p	<0.0001	<0.0001	<0.0001		0.0001	0.0041	<0.0001	
	$Y= -0.886+0.0414x$ $r^2=0.90$	$Y=4.530+0.0117x$ $r^2=0.76$	$Y=1.492+0.0154x$ $r^2=0.97$		$Y=1.4079-0.0009x +0.00003x^2$ $r^2= 0.99$	$Y=1.4046+0.0016x -0.00001x^2$ $r^2=0.99$	$Y= 0.3999+ 0.0227x-0.00001x^2$ $r^2= 0.78$	

Different capital letters in the same row differ from each other by the Tukey test at 5%. *p- significance level by regression equation. **p– significance level by the analysis of variance. $CuSO_4$ – copper sulfate; CuO – copper oxide; and Nano CuO – nano copper oxide.

Table X. Regression equation, coefficient of determination (r^2) and copper bioavailability based on the concentration of the mineral in the liver.

	Regression equation	r^2	Relative bioavailability (%)
Copper concentration in the liver (mg/kg)			
$CuSO_4$	$Y= -0.886 + 0.0414x$	0.90	100
CuO	$Y= 4.530 + 0.0117x$	0.76	28.3
Nano CuO	$Y= 1.492 + 0.0154x$	0.97	37.2

$CuSO_4$ – copper sulfate; CuO – copper oxide; and Nano CuO – nano copper oxide.

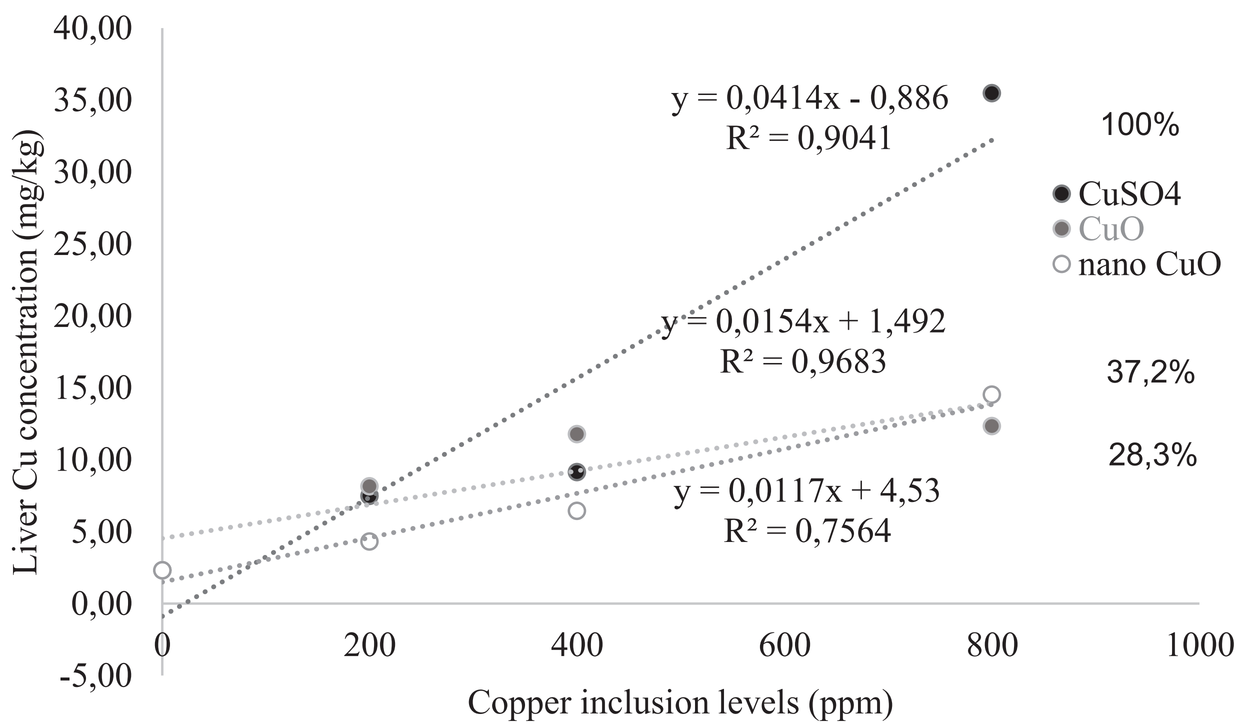

Figure 1. Relative bioavailability (%) of Cu of laying quails fed semi-purified diets containing different sources and levels of the mineral.

CONCLUSIONS

The performance of quails was not affected by different sources or levels of copper inclusion in the diet. The inclusion of nano CuO at the 200 or 400 ppm levels increased the hemoglobin levels and, at the 400 ppm level, the hematocrit of the birds. CuO in nano form (< 50 nm) has greater bioavailability than its conventional form.

Acknowledgments

To the Universidade Federal de Pelotas and the Programa de Pós-Graduação em Zootecnia for the opportunity. To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support. To the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the research scholarship of Professor Eduardo Gonçalves Xavier. To the Universidade Federal do Rio Grande (FURG), Instituto de Ciências Biológicas (ICB), Laboratórios de Citologia e Determinações, for carrying out the hematological analyses. To Polinutri Alimentos S.A. represented by Dr. Everton Elibert Rodrigues, for supplying the mineral and vitamin premix.

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How to cite
LOPES JP ET AL. 2024. Nano copper in the diet of laying quails: productive performance, metabolism, and tissue concentration. An Acad Cienc Bras 96: e20231121. DOI 10.1590/0001-3765202420231121.

Manuscript received on October 8, 2023;
accepted for publication on April 7, 2024

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JOYCE PEREIRA LOPES: developed the project, responsible for monitoring all stages of the experiment and for preparing the manuscript. EDUARDO GONÇALVES XAVIER: prepared the manuscript, designed the study, coordinated the steps of the experiment and reviewed the manuscript. SUELEN NUNES DA SILVA: participated in the experimental setup and laboratory analysis. DÉBORA CRISTINA NICHELLE LOPES: formulated the diets. ALINE ARASSIANA PICCINI ROLL, DÉBORA MINETTI SARTURI, CAROLINA OREQUES DE OLIVEIRA, RENATA CEDRES DIAS and BRENNA KELEN MELLO DE FREITAS: participated in data collection. ANDERSON SCHWINGEL RIBEIRO and DÁISA BÖNEMANN: responsible for the analysis of copper in the tissues. ISABEL SOARES CHAVES: guided and assisted in hematological analyses.