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Effect of different levels of copper nanoparticles and copper sulphate on performance, metabolism and blood biochemical profiles in broiler chicken

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Summary

A study was conducted to investigate the influence of copper administration in ovo to chicken embryos and/or supplied in drinking water to growing chickens in the form copper nanoparticles (Cu-NP) or copper sulphate ($CuSO_4$). The fertilised eggs were assigned to three groups (n = 50 per group): control (not injected), injected with 50 mg/kg Cu-NP or with 50 mg/kg $CuSO_4$ at day 1 of incubation. Thereafter, 126 one-day-old broiler chickens were randomly assigned to seven post-hatched groups: control not injected and not provided with Cu in the drinking water, injected with 50 mg/kg Cu-NP + 20 mg/kg in water, not injected + 20 mg/kg Cu-NP in water, injected with 50 mg/kg $CuSO_4$ + 20 mg/kg in water, not injected + 20 mg/kg $CuSO_4$ in water, injected with 50 mg/kg Cu-NP and injected with 50 mg/kg $CuSO_4$. The experiment was carried out from day 1 to 35 post-hatching. The in ovo injection of Cu improved the final body weight, average daily gain and feed conversion ratio in relation to the control group. Conversely, the provision of Cu in the drinking water had less of an effect on growth performance in comparison with the injected groups. A significant improvement was shown in energy and nitrogen utilisation, being better for Cu-NP than $CuSO_4$. The cholesterol, urea and glucose levels in the blood were reduced by Cu-NP treatment in relation to the other groups. The relative weight of the liver was decreased, while bursa of Fabricius was increased in Cu groups in relation to the control group. Cu excretion was only reduced in chickens injected with 50 mg/kg Cu-NP + 20 mg/kg in water. The immune-related genes were not affected by the treatments. The in ovo injection of Cu-NP might improve broiler performance more efficiently than the injection of $CuSO_4$ or the provision of Cu-NP and/or $CuSO_4$ in drinking water.

KEYWORDS
broiler, growth performance, metabolic rate, nanoparticles

1 | INTRODUCTION

Copper (Cu), a crucial microelement required for proper physiological functions in chickens, is frequently added to poultry diets at high concentrations, at levels that often exceed the birds' requirements (Świątkiewicz, Arczewska-Włosek, & Józefiak, 2014), as a possible alternative to an antibiotic growth promoter. It enhances animal performance, but an excess of Cu in the diet can also have adverse effects,

including iron and calcium deficiency due to antagonism between those elements; this may cause a reduction in viability (Miroshnikova, Arinzhanov, Kilyakova, Sizova, & Miroshnikov, 2015) and increased toxicity (Cao et al., 2016). The digestibility of Cu salts is very low, and approximately 80% of Cu is excreted in the faeces (McDowell, 1992), causing the environmental pollution (Leeson, 2009). Many studies have shown the beneficial effects of Cu supplementation in different forms and levels (inorganic and organic), from 4 to 400 mg/kg in poultry diets. However, the delivery technique and bioavailability of Cu might have different effects on Cu utilisation. In this experiment, we used copper nanoparticles (Cu-NP) as a feed additive delivered by in ovo injection to embryos and/or in drinking water given to post-hatched chickens. Considering their unique physicochemical characteristics and based on recent results, we hypothesised that Cu-NP would have greater bioavailability and thus be more efficient than Cu salts (Joshua, Valli, & Balakrishnan, 2016; Mroczek-Sosnowska, Sawosz et al., 2015). Additionally, it has been demonstrated that in ovo feeding may be a new and safe method to provide external nutrients to the embryo before it hatches. This could increase nutrient utilisation, to a greater extent than post-hatched supplementation, thereby reducing the excretion of these elements into the environment (Das et al., 2010; Mroczek-Sosnowska, Łukasiewicz et al., 2015). It has been documented that Cu-NP has beneficial effects on the animal performance and could be used to replace copper sulphate ($CuSO_4$) (Wang, Wang, Ye, Tao, & Du, 2011; Mroczek-Sosnowska, Sawosz et al., 2015; Mroczek-Sosnowska, Łukasiewicz et al., 2015; Miroshnikov, Yausheva, Sizova, & Miroshnikova, 2015; Muralisankar, Saravana Bhavan, Radhakrishnan, Seenivasan, & Srinivasan, 2016; El-Basuini et al., 2016). However, far less is known about the mechanism of action of Cu-NP in improving chicken performance, particularly regarding nutrient digestion and metabolism.

It has been well documented that increasing Cu levels in the diet could reduce cholesterol and triglyceride levels in the blood of chickens (Rahman et al., 2001; Skrivanova, Skrivan, Marounek, Tumova, & Sevcikova, 2004). Furthermore, it was shown that in ovo administration of Cu-NP and $CuSO_4$ affected the blood serum, which reduced concentrations of glucose and cholesterol, but increased levels of calcium, phosphorus and iron in broiler chickens (Mroczek-Sosnowska et al., 2013).

It has been demonstrated that Cu-NP and $CuSO_4$ can affect the immune status and muscle development of chickens and chicken embryos (Goel, Bhanja, Mehra, Majumdar, & Pande, 2013; Mroczek-Sosnowska, Sawosz et al., 2015; Mroczek-Sosnowska, Łukasiewicz et al., 2015). Furthermore, it was demonstrated that Cu-NP and $CuSO_4$ positively affected fish performance and immune response (El-Basuini et al., 2016). To evaluate whether the whole body responses to the treatments could be linked to molecular responses, the expression of selected genes related to immune status and muscle development was measured.

Recently, we reported that the metabolic rate of broiler embryos could be affected by the in ovo administration of Cu-NP (Scott et al., 2016). We demonstrated that the administration of 50 mg/kg of Cu-NP increased the metabolic rate of broiler embryos; however, this improvement did not alter the growth of the embryos. Therefore, we hypothesised that in ovo injection of Cu-NP could have prolonged effects on broiler performance during the rearing period. The objectives of the current study were thus to investigate the effects of in ovo administration of Cu-NP or $CuSO_4$ in the pre-hatch period and the subsequent delivery of Cu-NP and $CuSO_4$ in the drinking water during the post-hatch period on the growth performance, energy and nitrogen utilisation, Cu excretion with faeces, blood profile, organ weight and mRNA gene expression of broiler chickens.

2 | MATERIALS AND METHODS

2.1 | Nano-solution

A colloid of Cu-NP was obtained from Nano-Tech (Warsaw, Poland) and was produced by a patented non-explosive high-voltage method (Polish Patent 3883399) from high-purity metals (99.99%) and high-purity demineralised water. The concentration of nanoparticles in the colloids was 50 mg/kg and the particle size ranged from 2 to 15 nm, according to the transmission electron microscope evaluation, as described by Chwalibog et al. (2010). The desired concentrations of 20 mg/kg Cu-NP or $CuSO_4$ used in the study were prepared by diluting the original concentration of Cu-NP solution (50 mg/kg) and the powder form of $CuSO_4$ in distilled water.

2.2 | Study design

On day 1 of incubation, fertilised eggs from a 37-week-old Ross × Ross 308 breeder flock were assigned into three groups (n = 50 per group): control (not injected), injected with 50 mg/kg Cu-NP and injected with 50 mg/kg $CuSO_4$. After incubation, 126 one-day-old broiler chickens were randomly assigned to seven post-hatched subgroups (n = 12 + 6 reserve): control: not injected and not provided with Cu in the drinking water: (i) injected with 50 mg/kg Cu-NP and provided with 20 mg/kg Cu-NP in the drinking water, (ii) not injected and provided with 20 mg/kg Cu-NP in the drinking water, (iii) injected with 50 mg/kg $CuSO_4$ and provided with 20 mg/kg $CuSO_4$ in the drinking water, (iv) not injected and provided with 20 mg/kg $CuSO_4$ in the drinking water, (v) injected with 50 mg/kg Cu-NP, and (vi) injected with 50 mg/kg $CuSO_4$.

2.3 | Animals and management

The eggs were incubated for 21 days under standard conditions (37.8°C, 65% humidity, turned once per hour for the first 18 days, and at 37°C, 70% humidity from day 19 until hatching). After hatching, broilers were brooded in furnished pens with a heat lamp with an ambient temperature of approximately 25°C and a 23L:1D lighting programme for 6 days post-hatching.

On day 7, 12 chickens from each group were randomly selected, weighed, leg-banded and transferred to metabolic cages (0.5 × 0.5 × 0.5 m), provided with a feeder and nipple drinker. Four chickens per cage were kept until day 35. The birds were fed ad libitum with a commercial broiler (grower) diet (Table 1) with free access to

drinking water containing one of the treatments, i.e., Cu-NP or $CuSO_4$ (at 0 or 20 mg/kg), for 4 weeks.

Body weight (BW), feed consumption and water intake were recorded each week starting at 7–35 days of age. The average BW gain was calculated from the initial and final weights of the broilers. Feed and water intakes were calculated from the difference between the amount of feed or water offered and the amount refused. The feed conversion ratio (FCR) was calculated as the feed consumption-to-weight gain ratio.

The experiment was carried out in accordance with the Danish Ministry of Justice regarding the housing and treatment of experimental animals (Law no. 726, September 1993).

2.4 | Balance and respiration experiments

Balance and respiration experiments followed the methodology described by Pineda et al. (2012). Four sets of five-day-long balance experiments with 4 days of excreta collection were performed. Each group had twelve replicates, and the ages of the chickens were 7, 14, 21 and 28 days at the start of the four balance experiments respectively. All excreta was collected between 08:00 hr to 12:00 hr noon. After collection, the excreta was weighed and frozen at −18°C.

Gas exchange was measured for 22 hr in an open-air circuit indirect calorimeter according to the method described by Chwalibog, Jakobsen, Tauson, and Thorbek (2005). Subsequently, heat production (HP) was calculated according to the formula used by Brouwer (1965). The chickens were placed in metabolic cages in the respiration unit from 11:00 to next day 09:00 hr and returned to their respective metabolic cages.

2.5 | Chemical analyses

Dropping samples were analysed by each week for the dry matter, nitrogen, gross energy and crude fat as described by Pineda et al. (2012).

2.6 | Blood and organ sampling

At the end of the experiment (day 35), the chickens were weighed and euthanised, and blood samples (n = 10 per each group) were taken directly from the right wing of the birds and collected in heparinised tubes. After centrifugation at 2,000 g for 10 min at 4°C, blood plasma was obtained and kept at −20°C for biochemical analysis. The broilers were then dissected, and the heart, liver, bursa, breast and spleen were weighed.

2.7 | Copper content in faeces

Dry dropping samples were analysed each week for the content of Cu in the faeces. The sample was weighed on an analytical balance quartz dish and burned in a muffle furnace at a temperature of 480°C for 16 hr. After cooling, 1 ml of HNO_3 (65%) and 1 ml of H_2O_2 were added; then, the sample was burned in a muffle furnace at 480°C for 16 hr. After cooling, 5 ml HCl (37%) was added and transferred quantitatively using double-distilled water to 25 ml. The samples were filtered quantitatively.

Cu analyses were performed using an atomic absorption spectrometer (Z-5300 Polarized Flame Atomic Absorption Spectrophotometer, Hitachi-Science & Technology, Tokyo, Japan). The method is based on the phenomenon of the absorption of radiation at a specific wavelength (324.8 nm for Cu) by free metal atoms.

TABLE 1 Ingredients and nutrient composition of the diet, %

Wheat	61.8
Soya bean meal	16.3
Corn	7
Oat	5.4
Sunflower meal	5
Calcium carbonate	1.5
Monocalcium phosphate	1
L-lysine (VitaLys® Dry 53)[a]	0.36
Agro Denmark 40[b]	0.3
Rock salt	0.2
Sodium bicarbonate	0.17
DL-methionine	0.15
Threonine (98%)	0.05
Phytase pre-mix[c]	0.03
Enzyme[d]	0.02
Analysed values	
Crude protein	17.6
Crude fat	3.3
Crude fibre	3.8
Ash	5.2
Feed table values	
Metabolisable energy [MJ/kg]	12.2
Lysine [g/kg][e]	8.7
Methionine [g/kg]	4.1
Cysteine [g/kg]	3.2

[a]VitaLys1 Dry 53 (VitaLys I/S, Esbjerg, Denmark) provides L-lysine sulphate produced by fermentation (*Corynebacterium glutamicum*) with a lysine content of 530 g/kg.
[b]Supplied per kg diet: 500 units phytase; 10 mg copper sulphate; 0.30 mg selenium; 13.50 IU vitamin A; 75 mg α-tocopherol; 50 mg choline; 3.0 IU vitamin D3.
[c]Contains per g of pre-mix 1,666.67 FTU phytase (6-phytase; EC 3.13.26; EU number 4a1640).
[d]Specific active enzymes: 3,000 units endo-1,4-ß-glucanase; 7,200 units endo-1,4-ß-xylanase;
[e]1.0 g lysine sulphate and bioproducts.

2.8 | Gene expression

Gene expression on the mRNA level of nuclear factor kappa-light-chain-enhancer of activated B cells (*NF-kB*) and tumour necrosis factor alpha (*TNFα*) was measured in the liver, and fibroblast growth factor 2

TABLE 2 The growth performance of broiler chicks treated with Cu-NP or $CuSO_4$ either by injection or/and in drinking water

	Treatment								
Parameter	Control	A	B	C	D	E	F	SE	*p* Value
BW (g)	1,354^{e}	1,605^{b}	1,622^{b}	1,601^{b}	1,418^{d}	1,591^{c}	1,679^{a}	11.4	<.01
ADFI (g)	75.9^{ab}	76.5^{ab}	77.5^{ab}	74.5^{b}	70.6^{c}	78.5^{a}	78.2^{a}	1.20	<.05
FCR	1.73^{a}	1.52^{c}	1.65^{b}	1.48^{d}	1.54^{c}	1.62^{b}	1.54^{c}	0.02	<.001
ADG (g)	43.5^{c}	49.1^{a}	45.6^{bc}	47^{b}	44.6^{bc}	47.4^{b}	49.4^{a}	1.08	<.002

Control, not injected. A, injected 50 mg/kg Cu-NP + provided 20 mg/kg Cu-NP in drinking water. B, provided 20 mg/kg Cu-NP in drinking water. C, injected 50 mg/kg $CuSO_4$ + provided 20 mg/kg $CuSO_4$ in drinking water. D, provided 20 mg/kg $CuSO_4$ in drinking water. E, injected 50 mg/kg Cu-NP. F, injected 50 mg/kg $CuSO_4$. SE, pooled standard error. BW, Final body weight. ADFI, average daily feed intake (g/bird/day). ADG, average daily gain (g/bird/day). FCR, feed conversion ratio. Within rows not sharing the same superscript letters indicate a significant difference ($p < .05$).

(*FGF2*), and vascular endothelial growth factor A (*VEGFA*) in the breast muscle, using quantitative polymerase chain reaction (qPCR) method as described by Sawosz et al. (2012).

2.9 | Calculations

The N retained in the body (RN) was calculated as the difference between nitrogen intake (IN) and N excretion (EN). Nitrogen utilisation was calculated as an RN/IN. The intake of metabolisable energy (ME) was calculated as the difference between gross energy consumed and energy excreted in the droppings. Heat production (HE) was calculated without a correction for urinary nitrogen, according to Brouwer (1965):

$$\mathrm{HE\,(kJ)} = 16.18\,(\mathrm{kJ/L}) \times \mathrm{O_2\,(L)} + 5.02\,(\mathrm{kJ/L}) \times \mathrm{CO_2\,(L)}.$$

The retained energy (RE) was determined as the difference between ME intake and HE. Energy utilisation was calculated as RE/ME.

2.10 | Statistical analyses

All data were normally distributed and analysed using the general linear model procedure. Tukey's range test was used to test the separation of the means. All results were analysed using one-way analysis of variance followed by the specific contrasts test; however, data obtained for energy and nitrogen metabolism were analysed by two-way analysis of variance followed by a specific contrast test for unequal observations to examine the effects of treatment, age and the interaction between treatment × age. Statistical analyses were performed using the statistical software SPSS 22 (SPSS, Chicago, IL, USA). Mean values with $p < .05$ were considered significantly different.

3 | RESULTS

3.1 | Growth performance

The in ovo administration of 50 mg/kg Cu-NP or 50 mg/kg of $CuSO_4$ did not affect development during embryogenesis, and there were no significant differences observed in the hatching weight and hatchability as compared to the control group (46 ± 0.45 g and 78 ± 2.30% respectively; data not shown). However, Cu supplementation affected the final body weight in all treated groups as compared to the control group (Table 2; $p < .01$). The highest body weight (BW) was recorded in group F administered 50 mg/kg $CuSO_4$ in ovo followed by heavier chicks recorded in groups A, B and C; the lowest BW was in the control group. The average daily feed intake (ADFI) was significantly lower in group D than in all other groups. The feed conversion ratio (FCR) was improved in all treated groups in relation to the control group. The average daily weight gain (ADG) was significantly higher ($p < .002$) in groups A and F in relation to other treated and control groups. Furthermore, the age of broilers had a significant effect ($p < .0001$) on all growth performance variables (data not shown).

3.2 | Energy metabolism

The ME per kg metabolic body size ($kg^{0.75}$) was higher for the Cu-NP-injected groups (A and E) as compared to other treated and control groups, excluding group D which had lower values (Table 3). The measured HE/$kg^{0.75}$ was significantly lower ($p < .05$) in all treated groups (except for group E) in relation to the control group. There were significant effects on RE/$kg^{0.75}$ in all treated groups as compared to the control group ($p = .001$), and group A showed a higher average in relation to all other groups. RE/ME was higher in all treated groups in relation to the control group; group A had the highest value. The period had a significant effect on all parameters ($p < .0001$), which increased with the age of the broilers (data not shown).

3.3 | Nitrogen metabolism

The statistical analyses showed that there was no treatment effect on nitrogen intake (IN) between groups ($p = .14$; Table 3); however, there was a significant reduction in nitrogen excretion (EN) in all treated groups in relation to the control group ($p = .006$). Therefore, there was a significant effect on nitrogen retention (RN) per $kg^{0.75}$ in groups A, C and E, which retained more nitrogen as compared to the other groups. RN/IN was enhanced in treated groups; the highest levels were recorded in groups A, C, F and E as compared to other treated and control groups. Age had a significant effect ($p < .0001$) on all variables measured (data not shown). IN decreased from weeks 2 to 5,

TABLE 3 Energy and nitrogen metabolism of pooled means of four cages, each containing three birds per cage, measured in the period between 7 and 28 days of age of the chickens treated with Cu-NP or $CuSO_4$

	Treatment								
Parameter	Control	A	B	C	D	E	F	SE	*p* Value
ME, kJ/kg$^{0.75}$	1,684^{c}	1,721^{a}	1,652^{e}	1,715^{b}	1,587^{f}	1,719^{a}	1,673^{d}	1.13	<.001
HE, kJ/kg$^{0.75}$	1,113^{a}	960^{b}	967^{b}	972^{b}	918^{c}	1,073^{ab}	1,011^{b}	34.73	<.05
RE, kJ/kg$^{0.75}$	571^{g}	760^{a}	685^{c}	743^{b}	670^{d}	646^{f}	662^{e}	1.52	<.001
RE/ME%	34^{d}	44^{a}	41^{b}	43^{ab}	42^{ab}	37^{c}	39^{bc}	0.83	<.002
IN, g/kg$^{0.75}$	4.04	3.81	3.79	3.74	3.62	3.88	3.74	0.190	>.14
EN, g/kg$^{0.75}$	1.50^{a}	1.07^{d}	1.20^{b}	0.99^{e}	1.14^{c}	1.18^{bc}	1.14^{c}	0.021	<.006
RN, g/kg$^{0.75}$	2.54^{b}	2.74^{a}	2.59^{b}	2.75^{a}	2.47^{b}	2.69^{a}	2.61^{ab}	0.050	<.001
RN/IN%	61.6^{c}	70.5^{a}	66.4^{b}	69.1^{a}	67.2^{b}	68^{ab}	69.6^{a}	1.17	<.005

Control, not injected. A, injected 50 mg/kg Cu-NP + provided 20 mg/kg Cu-NP in drinking water. B, provided 20 mg/kg Cu-NP in drinking water. C, injected 50 mg/kg $CuSO_4$ + provided 20 mg/kg $CuSO_4$ in drinking water. D, provided 20 mg/kg $CuSO_4$ in drinking water. E, injected 50 mg/kg Cu-NP. F, injected 50 mg/kg $CuSO_4$. SE, pooled standard error. ME, metabolisable intake (kJ/kg$^{0.75}$). HE, heat production (kJ/kg$^{0.75}$). RE, retained energy (kJ/kg$^{0.75}$). RE/ME, retained energy: intake of metabolisable energy. IN, Intake of nitrogen (g/kg$^{0.75}$). EN, excreted nitrogen (g/kg$^{0.75}$). RN, retained nitrogen (g/kg$^{0.75}$). RN/IN, retained nitrogen: intake of nitrogen. Within rows not sharing the same superscript letters indicate a significant difference ($p < .05$).

TABLE 4 Biochemical blood indices of chickens

	Treatment								
Parameter	Control	A	B	C	D	E	F	SE	*p* Value
Albumin (g/L)	13.9	12.9	12.3	13.6	14.0	12.4	13.9	0.60	>.21
ALP-DEA (U/L)	5,848	4,745	4,278	4,875	5,028	4,934	5,061	187.5	>.31
ALT (U/L)	9.2^{b}	10.0^{a}	8.2^{c}	9.4^{b}	10.0^{a}	7.8^{c}	8.0^{c}	0.17	<.005
Cholesterol (mmol/L)	3.46^{b}	3.47^{b}	2.85^{e}	3.18^{c}	3.65^{a}	2.97^{d}	3.25^{c}	0.048	<.006
Creatinine (mmol/L)	5.1^{a}	4.3^{b}	4.2^{b}	4.2^{b}	4.3^{c}	3.2^{c}	4.0^{b}	0.13	<.002
Triglycerides(mmol/L)	0.48^{a}	0.37^{d}	0.37^{d}	0.41^{b}	0.37^{d}	0.36^{d}	0.39^{c}	0.008	<.003
Phosphate (mmol/L)	2.40	2.21	2.07	2.24	2.37	2.21	2.44	0.300	>.12
AST (U/L)	328^{a}	247^{c}	216^{d}	243^{c}	265^{b}	232^{c}	206^{c}	5.1	<.001
LDH (U/L)	1,262	1,009	1,287	1,054	962	1,315	1,203	125.2	>.27
Urea (mmol/L)	1.13^{a}	0.76^{c}	0.94^{b}	0.83^{b}	0.92^{b}	0.72^{d}	0.77^{c}	0.020	<.001
Calcium (mmol/L)	2.4	2.5	2.4	2.5	2.4	2.6	2.4	0.11	>.15
Magnesium (mmol/L)	0.96	0.92	0.87	0.90	0.96	0.87	0.93	0.041	>.12
Glucose (mmol/L)	14.8^{a}	13.8^{c}	13.5^{c}	13.6^{c}	14.1^{b}	13.6^{c}	13.7^{c}	0.10	<.01

SE, pooled standard error; ALP-DEA, Alkaline phosphatase; ALT, Alanine transferase; AST, Aspartate transferase; LDH, Lactate dehydrogenase.
Control, not injected. A, injected 50 mg/kg Cu-NP + provided 20 mg/kg Cu-NP in drinking water. B, provided 20 mg/kg Cu-NP in drinking water. C, injected 50 mg/kg $CuSO_4$ + provided 20 mg/kg $CuSO_4$ in drinking water. D, provided 20 mg/kg $CuSO_4$ in drinking water. E, injected 50 mg/kg Cu-NP. F, injected 50 mg/kg $CuSO_4$.
Within rows not sharing the same superscript letters indicate a significant difference ($p < .05$).

while retention increased. EN was higher in weeks 2 and 3 and then declined in weeks 4 and 5.

3.4 | Blood analyses

All treated groups resulted in significantly ($p < .05$) lower levels of the blood parameters as compared to the control group (Table 4). However, the decline in albumin, alkaline phosphatase, lactate dehydrogenase, calcium and magnesium was not significant ($p > .05$). The levels of alanine transferase, cholesterol, triglycerides, aspartate transferase and glucose were significantly lower in groups treated with Cu-NP in relation to $CuSO_4$ and the control groups. Groups B and E had the lowest values.

3.5 | Relative organ weight

There was a significant effect of the treatments on the relative organ weight in relation to the control group (Table 5). Group E had the

TABLE 5 Organs weight relative to the body weight of chickens

Treatments	Heart (g)	Liver (g)	Bursa (g)	Breast (g)	Spleen (g)
Control	0.41^{c}	2.3^{a}	0.12^{e}	16.47^{b}	0.09
A	0.43^{c}	1.9^{c}	0.16^{c}	17.47^{a}	0.08
B	0.47^{bc}	2.1^{b}	0.17^{c}	15.64^{b}	0.09
C	0.48^{bc}	1.9^{c}	0.25^{a}	17.73^{a}	0.11
D	0.48^{bc}	1.9^{c}	0.22^{b}	15.08^{c}	0.10
E	0.56^{a}	2.0^{bc}	0.21^{b}	15.29^{c}	0.14
F	0.45^{c}	2.1^{b}	0.15^{d}	15.82^{b}	0.09
SE	0.014	0.05	0.007	0.296	0.03
p Value	<.03	<.002	<.01	<.02	>.22

SE, pooled standard error.
Control, not injected. A, injected 50 mg/kg Cu-NP + provided 20 mg/kg Cu-NP in drinking water. B, provided 20 mg/kg Cu-NP in drinking water. C, injected 50 mg/kg $CuSO_4$ + provided 20 mg/kg $CuSO_4$ in drinking water. D, provided 20 mg/kg $CuSO_4$ in drinking water. E, injected 50 mg/kg Cu-NP. F, injected 50 mg/kg $CuSO_4$.
Within columns not sharing the same superscript letters indicate a significant difference ($p < .05$).

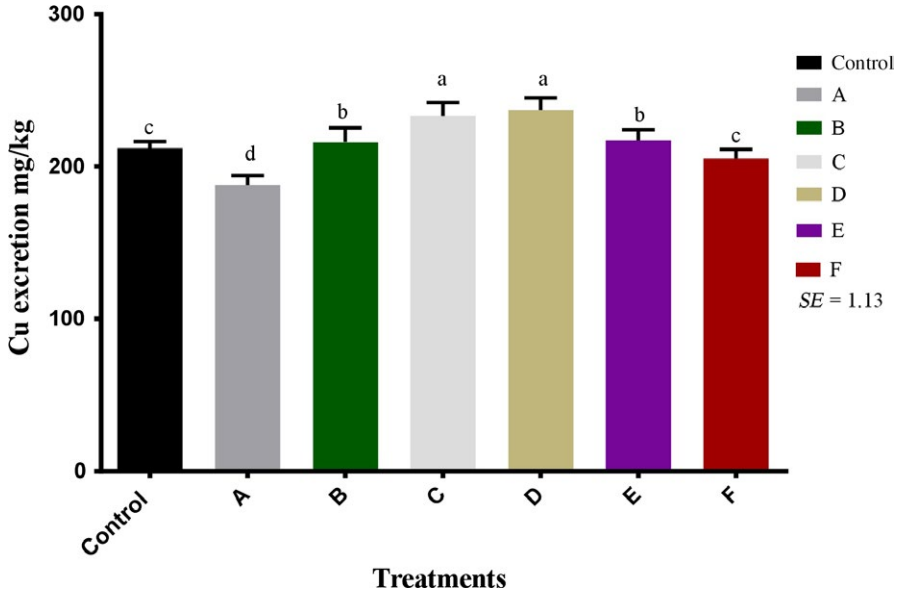

FIGURE 1 The analysis of copper contents in the faeces of chickens treated with different concentrations of Cu-NP or $CuSO_4$ via injection, drinking water or injection + drinking water from 7 to 35 days of age. Control, not injected. (A) injected 50 mg/kg Cu-NP + provided 20 mg/kg Cu-NP in drinking water. (B) provided 20 mg/kg Cu-NP in drinking water. (C) injected 50 mg/kg $CuSO_4$ + provided 20 mg/kg $CuSO_4$ in drinking water. (D) provided 20 mg/kg $CuSO_4$ in drinking water. (E) injected 50 mg/kg Cu-NP. (F) injected 50 mg/kg $CuSO_4$. SE, pooled standard error. Columns with different letters are statistically different at $p < .05$

highest heart weight of all the treated and control groups. The weight of the liver was significantly reduced ($p < .002$) in all treated groups as compared to the control group. The weight of the bursa of Fabricius was significantly higher ($p < .01$) in all treated groups in relation to the control group; group C had the highest value among all treated groups. The breast weight was higher in groups A and C in relation to other treated and control groups. The weight of the spleen was not different.

3.6 | Copper analyses in faeces

The Cu content in the faeces (Figure 1) was significantly higher ($p < .001$) in groups C and D as compared to other treated and control groups, while the content in group A was significantly lower than other treated and control groups. Generally, Cu excretion was lower in chickens treated with Cu-NP as compared to the $CuSO_4$-treated groups. The period had a significant effect ($p < .001$) on Cu excretion, which was higher in weeks 2 and 3 then declined remarkably at 4 and 5 weeks of age (data not shown).

3.7 | Gene expression

The expression of *NF-kB*, *TNF*α and *FGF2* at the mRNA level was not affected in the Cu-NP and $CuSO_4$ groups. However, *VEGFA* was up-regulated significantly ($p < .05$) in the $CuSO_4$ group in comparison with the other groups (data not shown).

4 | DISCUSSION

4.1 | Growth performance

The present experiment was conducted to examine broiler responses to different Cu sources and levels supplemented either as administration in ovo and/or in drinking water. In the previous investigation,

during the hatching period (Scott et al., 2016), the BW was not affected by either Cu-NP or $CuSO_4$ injection. Furthermore, it was demonstrated that in ovo injection of Cu-NP had no effect on BW of hatching broiler embryos (Joshua et al., 2016). However, the present results indicate that the in ovo injection and the subsequent provision of Cu-NP and $CuSO_4$ in drinking water affected the final BW of chickens (Table 2).This result is consistent with our previous study (Mroczek-Sosnowska, Łukasiewicz et al., 2015), demonstrating that the administration of Cu in ovo could have long-lasting effects on broiler performance. Similar to our results, it was shown that 50 and 100 mg/kg of Cu-loaded chitosan nanoparticles improved the growth performance of broiler chickens (Wang et al., 2011). Furthermore, it was revealed that intramuscular injection of Cu-NP stimulates the growth of broiler chickens (Miroshnikov et al., 2015). The ADFI was not enhanced by the treatments, except in groups E and F, while the FCR, ADG and the final BW were improved in all treated groups compared with the control group. Similar to our results, it was shown that supplementation with 50 and 100 mg/kg of Cu-NP significantly enhanced growth performance in piglets (Gonzales-Eguia, Fu, Lu, & Lien, 2009; Wang et al., 2012). Furthermore, similar effects of Cu-NP on the final BW and ADG in fish were reported (El-Basuini et al., 2016).

Different sources of Cu may have different effects on feed utilisation; Igbasan and Akinsanmi (2012) reported that chickens treated with copper oxide utilise feed better than those supplemented with $CuSO_4$ and Cu acetate. However, Mondal, Haldar, Saha, and Ghosh (2010) did not find any effect nutrient metabolism of broilers supplemented with inorganic or organic Cu, which could be due to lower Cu absorption as it was provided as a feed additive.

4.2 | Energy and nitrogen metabolism

The present study revealed that supplementation with Cu significantly increased the $ME/kg^{0.75}$ as compared to the control; furthermore, the Cu-NP had a greater effect than $CuSO_4$, which might be due to the better bioavailability and higher uptake of Cu-NP in the gastrointestinal tract (GIT). The small size of nanoparticles might result in faster diffusion through the GIT mucus to reach the cells of the intestinal lining, followed by uptake through the GIT barrier to reach the blood (Singh, 2016). Furthermore, Cu supplementation decreased $HE/kg^{0.75}$. Previously, it was demonstrated that in ovo administration of Cu-NP reduced the HE of chicken embryos (Pineda, Sawosz, Vadalasetty, & Chwalibog, 2013). It is possible that increased Cu levels might enhance the activities of the enzymes involved in nutrient utilisation (Das et al., 2010; Han, Du, Huang, Xu, & Wang, 2012), making metabolic pathways more efficient and hence reducing HE. Therefore, the $RE/kg^{0.75}$ increased in all treated groups in relation to the control group. Furthermore, all treated groups utilised energy better than the control group.

Our results are in agreement with a study on piglets (Gonzales-Eguia et al., 2009), demonstrating that the improvement in pig growth could be attributed to better energy digestibility in animals treated with Cu-NP than with $CuSO_4$. Furthermore, the same authors showed that the bioavailability of Cu-NP was higher in comparison with $CuSO_4$. However, it was reported that adding 100 mg/kg Cu-NP in poultry feed had no significant effect on growth performance and nutrient digestibility (Sarvestani et al., 2016). Generally, supplementation with Cu in broilers could increase the absorption of fatty acids and fat-soluble vitamins, thereby enhancing nutrient metabolism and consequently influencing the growth of broiler chickens. Additionally, Cu might increase lipase and phospholipase activity in the small intestine (Das et al., 2010).

The accumulation of the nitrogen in tissues depends on feed intake, digestibility and the metabolic rate; these factors can be affected by Cu (Berntssen, Lundebye, & Maage, 1999). Our results indicate that both forms of Cu (Cu-NP or $CuSO_4$) supplied by in ovo injection and/or provided in the drinking water did not affect IN but significantly reduced excretion, improved retention and utilisation of N, in relation to the control group. Similar to our result, it was shown that Cu-NP supplemented in fish diet significantly improved the protein efficiency ratio, protein gain and protein retention in relation to $CuSO_4$ (El-Basuini et al., 2016). It could be speculated that Cu-NP supplementation may boost hormone and growth factor production in chickens (Das et al., 2010), thereby enhancing protein synthesis. In contrast, it was shown that high levels of $CuSO_4$ decrease the protein level in the liver tissues of fish (Tomar, Vyas, Bainerjee, & Abhishek, 2015).

4.3 | Blood characteristics

Blood biochemical indices are vital indicators of general health and the physiological stress reaction in broiler chickens. The results of the blood analysis are considered to be within the normal range for a healthy chicken. Generally, our results reveal that providing Cu by in ovo injection and/or in drinking water decreased the concentration of the measured parameters in plasma in relation to the control group. However, groups treated with Cu-NP showed lower levels of some blood parameters to a greater extent than the other groups. However, the results did not indicate a significant effect on albumin, alkaline phosphatase, lactate dehydrogenase, phosphate, calcium or magnesium levels. Albumin works as a protein source under the circumstances of subnormal intake and is known to decrease in young broilers under feed-restricted conditions. In our results, the ADFI did not cause a reduction by Cu supplementation; hence, the albumin level did not alter significantly, which means that the examined levels of Cu did not cause harmful effects on chicken feed intake. Conversely to our results, it was shown that albumin, alanine transferase and uric acid levels were increased in broilers fed 100–400 mg/kg of $CuSO_4$ (Kumar, Biswas, Bharti, & Srivastava, 2013). This might be related to the concentration used. The results of the present study are similar to the observations of (Mroczek-Sosnowska et al., 2013) who reported that in ovo injection of 50 mg/kg of Cu-NP decreased the levels of alanine transferase, glucose and cholesterol. However, the same authors showed an increase in calcium, magnesium and phosphorus levels in the groups treated with Cu-NP and $CuSO_4$ in relation to the control group, while these levels did not alter significantly in the present experiment. Furthermore, it was reported that dietary supplemented with Cu-NP causes a significant reduction in glucose levels, more so than $CuSO_4$ in fish (El-Basuini et al., 2016).

Many studies (Jegede, Oduguwa, Bamgbose, Fanimo, & Nollet, 2011; Kumar et al., 2013; Payvastegan, Farhoomand, & Delfani, 2013; Samanta, Biswas, & Ghosh, 2011; Zahedi, Ghalehkandi, Ebrahimnezhad, & Emami, 2013) have shown a reduction in cholesterol levels after adding Cu to poultry diets, which is in agreement with our results. This can be explained, in that adding higher levels of Cu will regulate cholesterol biosynthesis indirectly by decreasing the reduced form of glutathione and increasing the oxidised form of glutathione (Bakalli, Pesti, Ragland, & Konjufca, 1995; Kim, Chao, & Allen, 1992).

Lactate dehydrogenase (LDH) is released during tissue damage and is an indicator of common injuries and diseases. Changes in LDH activity could be related to liver damage; however, the results of this study do not indicate a significant reduction in the LDH. In contrast, it was reported that the concentration of LDH was increased with increasing the supply of organic Cu; however, this increase did not lead to any changes to the organs (Kwiecień, Winiarska-Mieczan, Piedra, Bujanowicz-Haraś, & Chałabis-Mazurek, 2015).

The concentration of urea in plasma was reduced significantly in the groups treated with Cu-NP more than those treated with $CuSO_4$ and the control group, which could be an indicator that Cu-NP-treated birds utilised amino acids more efficiently for growth (Yang, Guo et al., 2009). Furthermore, the results demonstrate a reduction in the concentration of blood urea in relation to the control group. The same results were reported when the chickens were provided with 100 mg/kg Cu-loaded chitosan nanoparticles in the feed (Wang et al., 2011), suggesting that this reduction could be an indicator that protein synthesis is enhanced (Fukawa, Nishimura, Irino, & Nitta, 1982).

4.4 | Relative organ weight

Previously, we demonstrated that 50 mg/kg of Cu-NP had no significant effects on the broiler dressing percentage and carcass content of the leg muscle (Mroczek-Sosnowska, Łukasiewicz et al., 2015). The same authors reported that Cu supplementation reduced the heart weight. However, our results showed that the relative heart weight was not affected. Similar to our results, it was reported that the relative heart weight at 42 days age was not affected in birds treated with $CuSO_4$ (Shahzad, Javed, Shabir, Irfan, & Hussain, 2012).

The relative liver weight was affected by both forms of Cu and was significantly lower at higher concentrations of Cu in relation to the control group. The liver results are consistent with the results of (Shahzad et al., 2012; Skrivan, Skrivanova, Marounek, Tumova, & Wolf, 2000), while in other studies (Mroczek-Sosnowska, Łukasiewicz et al., 2015; Payvastegan et al., 2013; Upadhaya, Lee, & Kim, 2016; Wang et al., 2011), the liver weight was not affected. It was reported that a high concentration of Cu-NP negatively affected the histology of the liver, kidneys and spleen, but not the heart and lungs in rats, and these changes were well supported by organ weight changes (Lee et al., 2016). Furthermore, it was demonstrated that the greatest accumulation of Cu was observed in the liver and spleen (Mroczek-Sosnowska, Lukasiewicz, Wnuk, Sawosz, & Niemiec, 2014).

The bursa of Fabricius is a primary lymphoid organ and a major pathway through which environmental antigens stimulate the immune system in the chicken (Ifrah, Perelman, Finger, & Uni, 2016). In this study, the weight of the bursa of Fabricius increased by both forms of Cu. Moreover, the $CuSO_4$-treated groups had a higher bursa weight in relation to the other groups. However, previous studies (Shahzad et al., 2012; Upadhaya et al., 2016; Wang et al., 2011) did not find any changes in bursa weight with increasing Cu levels. Conversely, it has been reported that higher levels of Cu (up to 400 mg/kg) inhibit the development of the bursa, causing pathological changes and impaired humoral immunity in ducklings (Yang, Zhao, Peng, Deng, & Cui, 2009).

Previously, it has been observed that injected Cu-NP and/or $CuSO_4$ increases the content of breast muscle (Joshua et al., 2016; Mroczek-Sosnowska, Łukasiewicz et al., 2015). The same results were obtained in our study, with the highest breast muscle weight recorded at the higher concentration of Cu (groups A and C).

4.5 | Copper content in faeces

One of the objectives of using different sources of Cu is to decrease Cu excretion in the faeces, thus reducing environmental pollution. It was hypothesised that in ovo injection of Cu-NP could decrease Cu excretion in the faeces; however, the results do not fully support this hypothesis. Only the group injected 50 mg/kg Cu-NP + provided 20 mg/kg Cu-NP in drinking water (A) showed significant reduction, while the Cu excretion in the other groups were similar or even higher than in the control group. The results are inconsistent and not conclusive, probably due to limited number of animals, suggesting further investigations with a broader range of Cu concentrations.

4.6 | Gene expression

To evaluate the immune response of broiler chickens after treatment with Cu-NP and $CuSO_4$, the expression of *NF-KB* and *TNF*α of mRNA was measured in the liver samples. The immune-related genes were not affected by the treatments in relation to the control, probably due to the biocompatible properties of Cu-NP and $CuSO_4$ (Scott et al., 2016).

The expression of *VEGFA* was significantly higher for $CuSO_4$ than in the other groups, which might be related to the different transport mechanisms and signalling pathways of the metallic form. Furthermore, it may be due to the existence of local hypoxia in muscle (Hoppeler & Vogt, 2001; Pugh & Ratcliffe, 2003). The HIF-1 (hypoxia-inducible factors-1) plays a key role in the induction of VEGFA gene expression under hypoxic conditions (Hannon, Kudla, McAvoy, Clase, & Olwin, 1996). The expression of *FGF2* was not affected for all treatments, suggesting that this gene lacks a direct role (Cao & Pettersson, 1990) and a classical signal sequence for secretion (Cao et al., 2004).

5 | CONCLUSION

The supplementation with Cu as nanoparticles or as a salt improved broiler performance and utilisation of energy and nitrogen.

Furthermore, in ovo administration of 50 mg/kg Cu-NP led to better energy and nitrogen utilisation than provision in drinking water. These results suggest that in ovo injection of Cu-NP could be an alternative to $CuSO_4$ supplementation to growing chickens.

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