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Effect of copper nanoparticles and copper sulphate on metabolic rate and development of broiler embryos

A. Scott[a], K.P. Vadalasetty[a], E. Sawosz[b], M. Łukasiewicz[c], R.K.P. Vadalasetty[a], S. Jaworski[b], A. Chwalibog[a,*]

[a] Department of Large Animal Sciences, University of Copenhagen, Groennegaardsvej 3, DK-1870 Frederiksberg C, Denmark
[b] Department of Animal Nutrition and Biotechnology, Warsaw University of Life Sciences, Ciszewskiego 8, 02-786 Warsaw, Poland
[c] Department of Poultry Breeding, Warsaw University of Life Sciences, Ciszewskiego 8, 02-786 Warsaw, Poland

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ABSTRACT

Copper (Cu) is regularly used as a growth promoter in poultry production. However, it has been demonstrated that the content of Cu inside eggs might not be sufficient to support the embryonic development. It is possible to supply the embryo with extra nutrients by *in-ovo* administration. Recently, it has been shown that *in-ovo* administration of copper nanoparticles (Cu-NP) and copper sulphate ($CuSO_4$) remarkably improved the body weights of growing chickens. Thus, the objective of the present experiment was to elucidate the potential effects of Cu-NP and $CuSO_4$ on the metabolic rate (oxygen consumption — O_2 and energy expenditure — EE) and development during embryogenesis.

Fertilised broiler eggs were divided into six groups: a non-injected control, a placebo injected with demineralised water, two groups injected, at day one of incubation, with $CuSO_4$ (50 and 100 mg/kg) and two groups injected with Cu-NP (50 and 100 mg/kg). Gaseous exchange was measured in an open-air-circuit respiration unit, and EE was estimated from day 10 to day 19 of embryogenesis. Body weight at 24 h after hatching and the relative organ weights were used as a measure of hatching development. *In-ovo* injection of 50 mg/kg Cu-NP and $CuSO_4$ significantly increased O_2 consumption and EE on the 16th and 19th day of incubation compared with the control group; Cu-NP had the largest effect on the metabolic rate. However, organ weights (intestine, heart, liver, and breast) relative to the yolk-free body weight were not affected in the injected groups. In addition, blood parameters did not show any changes among the groups. This result demonstrates that *in-ovo* injection of Cu-NP affects the metabolic rate of embryos, which might explain their improved performance after hatching.

1. Introduction

Copper (Cu) is a fundamental trace element required for several biochemical processes such as enzyme-coenzyme catalytic reactions, oxygen transport and haemoglobin synthesis. (Kim et al., 2008). It is also used as an efficient growth and health promoter for poultry (Richards et al., 2010). In poultry, there is substantial interest in using Cu as an alternative to antibiotics that can produce equivalent effects on chicken performance. In fact, feed mixtures are enhanced with high levels

* Corresponding author.
E-mail address: ach@sund.ku.dk (A. Chwalibog).

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of Cu as growth promoters; however, increasing Cu in animal diet could result in low digestibility and absorption in pigs and poultry, causing more Cu to be excreted in faeces and leading to environmental pollution (Gonzales-Eguia et al., 2009; Zhao et al., 2010; Karimi et al., 2011).

Some authors have suggested that Cu salts are less bioavailable than organic Cu and nano-sized Cu (Creech et al., 2004; Gonzales-Eguia et al., 2009); however, the results regarding organic Cu are inconsistent and need to be supported by further studies. Chickens spend 40% of their entire lifespan inside the egg as embryo; thus embryogenesis is the most critical period of growth and development of chickens (Azaranova et al., 2012). During the last days of incubation, the amount of Cu in the yolk is low leading to mineral deficiency in the embryo; consequently, the embryo consumes less Cu during that period (Yair and Uni, 2011). Therefore, efforts had been made to supplement broiler eggs with Cu (*in-ovo* nutrition) to improve hatchability and chicken performance (Bakyaraj et al., 2012; Mroczek-Sosnowska et al., 2015a). Recently, it has been reported that *in-ovo* injection of copper nanoparticles (Cu-NP) might achieve more efficient penetration into embryonic tissue than copper sulphate ($CuSO_4$), consequently enhancing chicken performance (Mroczek-Sosnowska et al., 2015a). Moreover, the injection of Cu-NP does not harm the development of the embryos or affect chick mortality (Joshua et al., 2016).

Cu-NP can move across cellular and also nuclear membranes and can penetrate cells and intracellular structures, and move to defined target points within the body (Xia et al., 2010; Maojo et al., 2012). It has been demonstrated that 50 mg/kg of Cu-NP stimulates the development of embryonic blood vessels at the molecular and systemic level, more effectively than $CuSO_4$ (Mroczek-Sosnowska et al., 2015b). Considering that the mechanism of action of Cu-NP during embryogenesis is not clear, we hypothesised that *in-ovo* injection of Cu-NP may affect the metabolic rate of embryos. Therefore, determination of oxygen consumption (O_2) and energy expenditure (EE) might be a valuable parameter for predicting the metabolic rate during embryogenesis (Tona et al., 2004; Hamidu et al., 2010).

The objective of the present study was to evaluate whether *in-ovo* supplementation of Cu-NP or $CuSO_4$ would affect the metabolic rate and development of broiler embryos.

1.1. Material and methods

1.1.1. Experimental design

The experimental procedures followed the Danish National Legislation. Broiler eggs (n = 300) from commercial breeder Ross 308 chickens (37 weeks old) were obtained from a Danish hatchery, and were randomly distributed into six groups (45 eggs per group): a non-injected control, a placebo injected with demineralised water, two groups injected with Cu-NP (50 and 100 mg/kg) and two groups injected with $CuSO_4$ (50 and 100 mg/kg). On day 1 of incubation, the eggs were weighed then injected into the air sac with 0.3 mL (15 and 30 μg/egg) of the appropriate solution using a sterile 27 gauge, 20 mm needle. Before and directly after injection the hole was sanitised with an alcohol swab, and was sealed with hypoallergenic tape. The eggs were incubated for 21 days under standard conditions (37.8 °C, 60% humidity, turned once per hour during the first 18 days, and at 37 °C and 70% humidity from day 19 until hatching).

1.1.2. Colloids

Colloidal Cu solutions with concentrations of 50 and 100 mg/kg and a particle size 2–15 nm were purchased from Nano-Tech, Warsaw, Poland. The solutions were manufactured by a patented non-explosive high voltage method (Polish Patent 3883399) from high purity metals (99.9999%) and high purity demineralised water.

The $CuSO_4$ solution was dissolved in ultra-pure water purchased from Sigma-Aldrich, St Louis, MO, USA. The placebo group was injected with the high purity (99.9999%) demineralised water obtained from Nano-Tech, Warsaw, Poland.

1.1.3. Gaseous exchange measurement

The eggs were candled and weighed prior to measurements. Eggs without an embryo or with dead embryo were discarded and replaced with eggs of the same age from the same treatment kept in the incubator as reserves. The measurements were carried out on the 10th, 13th, 16th and 19th day of incubation. Oxygen consumption and carbon dioxide production (CO_2) were measured according to the procedure described by Chwalibog et al. (2007) in an open-air circuit respiration unit (Micro-Oxymax calorimeter from Columbus Instruments, Columbus, OH, USA), equipped with four respiration chambers, each with a capacity of 2000 cm^3. The temperature and relative humidity were maintained similar to the conditions in the incubator (37.8 °C,60% humidity). Six eggs from each treatment group were placed in each chamber and measured for 3 h in the morning from 8:00–11:00, followed by another six eggs from the same groups in the afternoon from 12:00–15:00. After each measurement, the eggs were put back into the incubator. All gas exchange results were standardised to a 50 g egg mass to account for weight differences. Energy expenditure was calculated from O_2 consumption and CO_2 production by the formula: $EE\ (J) = 16.18 \times O_2\ (ml) + 5.02 \times CO_2\ (ml)$ (Brouwer, 1965).

1.1.4. Blood parameters and organ weight

The hatched chickens were weighed and euthanized and blood samples (n = 10 per group) were taken directly from the neck of the one-day old broilers and collected in heparinized tubes. After centrifugation at 2000*g* for 10 min at 4 °C, blood plasma was obtained and kept at −20 °C for biochemical analysis. The chicks were then dissected and the yolk sac, heart, liver, breast and intestine were weighed in order to measure their development.

Table 1
Primer sequences used for the investigated genes.

Target gene	Forward primer	Reverse primer
ACTB	GTCCACCTTCCAGCAGATGT	ATAAAGCCATGCCAATCTCG
NF-kB	TTGCTGCTGGAGTTGATGTC	TGCTATGTGAAGAGGCGTTG
TNF-α	TTCAGATGAGTTGCCCTTCC	TCAGAGCATCAACGCAAAAG
FGF2	GGCACTGAAATGTGCAACAG	TCCAGGTCCAGTTTTTGGTC
VEGF-A	TGAGGGCCTAGAATGTGTCC	TCTTTTGACCCTTCCCCTTT

β-actin (ACTB), nuclear factor (NF-κB), tumour necrosis factor alpha (TNF-α), vascular endothelial growth factor A (VEGFA), fibroblast growth factor 2 (FGF2).

Table 2
Oxygen consumption (ml/h^{-1}) of chicken embryos injected with different doses of copper sulphate ($CuSO_4$) and copper nanoparticles (Cu-NP).

Treatment	Day 10	Day 13	Day 16	Day 19	Average
Control	10.75^{c}	11.63^{d}	25.24^{c}	27.04^{d}	26.14
Placebo	10.37^{c}	11.93^{c}	23.84^{e}	27.71^{b}	25.78
50 mg/kg $CuSO_4$	9.93^{d}	12.85^{a}	25.94^{a}	27.82^{b}	26.88
50 mg/kg Cu-NP	11.48^{b}	12.28^{b}	26.10^{a}	28.14^{a}	27.12
100 mg/kg $CuSO_4$	11.67^{a}	12.32^{b}	24.90^{d}	27.32^{c}	26.11
100 mg/kg Cu-NP	10.94^{c}	11.37^{d}	25.71^{b}	27.61^{b}	26.66
SE = 0.066					
P-value <0.0001					

Means in columns with different letters are significantly different at $P \leq 0.05$.
$CuSO_4$, groups treated with copper sulphate; Cu-NP, groups treated with copper nanoparticles.
SE, pooled standard error of means.

1.1.5. Gene expression at the mRNA level

To measure gene expression, quantitative polymerase chain reaction (qPCR) was conducted using a Light Cycler® 480 real-time PCR system (Roche Applied Science, Penzberg, Germany). The method was described previously in Sawosz et al. (2012). The tissues were dissected and homogenised in TRIzol® Reagent (Life Technologies, Naerun, Denmark). RNA was isolated and purified using the SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA) and quantified using a Nano-Drop ND 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Genomic DNA was removed by DNase and 2 μg of RNA was used to synthesize cDNA using a reverse transcriptase kit from Promega. Real-time PCR was performed with cDNA and gene-specific primer pairs (TAG, Copenhagen A/S, Copenhagen, Denmark) mixed in a 2 μL reaction with LightCycler® 480 SYBR Green I Master mix (Roche Applied Science, Penzberg, Germany) in a LightCycler® 480 real-time PCR system (Roche Applied Science, Penzberg, Germany). For each cDNA, the reaction was performed in triplicate and for analyses; relative quantification was conducted using *ACTB* as the housekeeping gene. The following primers were used in this study (Table 1):

1.1.6. Statistical analysis

All data were normally distributed and analysed using the General Linear Model procedure. Duncan's multiple range test was used to test the separation of the means. All results were analysed using one way analysis of variance followed by LSD (least significant differences); however, result obtained for O_2 consumption and EE were analysed using two-way analysis of variance followed by LSD for unequal observations and to examine the effects of treatments, age and the interaction between treatment × age by using a linear model as described below:

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ij}$$

where μ was the population mean, α_i was the fixed effect of treatments, β_j was the fixed effect of incubation days and $(\alpha\beta)_{ij}$ was the interaction between fixed effects. The e_{ij} was the residual error. Statistical analyses were performed using the SPSS 22 software (SPSS, Chicago, IL, USA), and P-value of <0.05 was considered as statistically significant.

2. Results

2.1. Metabolic rate

The egg weights were similar for all groups at setting (62.5 ± 0.5). O_2 consumption and CO_2 production were measured at days 10, 13, 16 and 19 of incubation. Oxygen-consumption showed increments during the incubation period from days 10–19 for all groups (Table 2).

The results showed increased O_2 consumption in the groups injected with Cu-NP and $CuSO_4$ compared with the control and placebo groups (Table 2). The injection of 50 mg/kg Cu-NP caused particularly and significantly higher ($P < 0.05$) O_2 consumption on days 16 and 19 compared with all other groups, while the injection of 100 and 50 mg/kg of $CuSO_4$ showed higher O_2 consumption at days 10 and 13 of incubation. However, there was no treatment effect on the respiratory coefficient (RQ) values ($P > 0.05$; 0.73 ± 0.025) compared to the control and placebo groups.

Table 3
Energy expenditure (J/h^{-1}) of chicken embryos injected with different doses of copper sulphate ($CuSO_4$) and copper nanoparticles (Cu-NP).

Treatment	Day 10	Day 13	Day 16	Day 19	Average
Control	175[f]	302[b]	452[f]	538[b]	367
Placebo	183[e]	300[b]	511[c]	532[c]	381
50 mg/kg $CuSO_4$	191[d]	304[b]	469[e]	536[b]	375
50 mg/kgCu-NP	202[b]	329[a]	517[b]	537[b]	396
100 mg/kg $CuSO_4$	225[a]	267[d]	494[d]	521[d]	377
100 mg/kgCu-NP	196[c]	277[c]	535[a]	549[a]	389
SE = 1.1					
P-value < 0.0001					

Means in columns with different letters are significantly different at $P \leq 0.05$.
$CuSO_4$, groups treated with copper sulphate; Cu-NP, groups treated with copper nanoparticles.
SE, pooled standard error of means.

Table 4
Biochemical indicators measured in the blood plasma of chickens.

Substance measured	Control	Placebo	50 mg/kg $CuSO_4$	50 mg/kg Cu-NP	100 mg/kg $CuSO_4$	100 mg/kg Cu-NP	SE
Albumin (g/L)	6.1	5.7	6.0	5.2	5.6	6.1	0.02
ALP-DEA (U/L)	14.2	7.2	1.0	0.0	0.0	0.0	10.59
ALT (U/L)	9.0	10.2	11.2	9.6	9.6	11.4	0.40
Cholesterol (mmol/l)	9.2	8.7	9.1	8.2	8.4	8.3	0.30
Creatinine (mmol/l)	7.0	7.6	10.0	9.0	12.2	8.2	0.60
Triglyceride (mmol/l)	1.3	1.2	1.3	0.9	1.5	0.9	0.02
Phosphate (mmol/l)	2.0	2.0	2.1	2.0	2.1	2.1	0.05
AST (U/L)	179.4	188.6	212.0	171.6	146.6	194.8	7.60
LDH (U/L)	1832	2278	2277	1820	1560	1994	92.9
Urea (mmol/l)	3.9	3.8	3.9	3.6	4.3	3.4	0.05
Glucose (mmol/l)	11.7	11.3	12.2	11.6	11.2	11.7	0.10

Means in rows with different letters are significantly different at $P \leq 0.05$.
SE, pooled standard error of means.
$CuSO_4$, groups treated with copper sulphate; Cu-NP, groups treated with copper nanoparticles.
ALP-DEA, Alkaline phosphatase; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; LDH, Lactate dehydrogenase.

Table 5
Yolk-free body weight (YFBW), residual yolk-sac weight (RSY), and organ weight relative to the YFBW of chickens injected *in-ovo* with different doses of copper sulphate ($CuSO_4$) and copper nanoparticles (Cu-NP).

Treatment	YFBW (g)	RSY(g)	Heart (g)	Liver (g)	Breast (g)	Intestine (g)
Control	41.0	6.42[d]	0.65	1.97	0.58	3.33
Placebo	41.1	8.04[b]	0.59	1.72	0.63	2.98
50 mg/kg $CuSO_4$	41.0	7.86[c]	0.66	1.77	0.70	3.08
50 mg/kg Cu-NP	41.2	8.43[a]	0.56	1.69	0.57	2.94
100 mg/kg $CuSO_4$	40.6	7.99[c]	0.68	1.72	0.70	2.73
100 mg/kg Cu-NP	40.9	8.07[b]	0.44	1.72	0.88	2.70
SE	0.783	0.443	0.063	0.110	0.103	0.183
P-value				<0.01		

Means in columns with different letters are significantly different at $P \leq 0.05$.
$CuSO_4$, groups treated with copper sulphate; Cu-NP, groups treated with copper nanoparticles.
SE, pooled standard error of means.

EE followed the pattern of O_2 consumption, being higher in the treated groups than in the control group, particularly for the Cu-NP groups (Table 3). However, the injection of 100 mg/kg of $CuSO_4$ caused a higher EE only at day 10 of incubation.

2.2. Biochemical indices

There were no significant differences ($P \geq 0.05$) among the groups (Table 4).

However, there was a tendency of decreasing cholesterol and triglyceride levels in the Cu-NP groups compared with the other treated groups and the control group.

2.3. Body and relative organ weights

Neither the yolk-free body weight (YFBW) nor the organ weight relative to YFBW (heart, liver, breast, intestine) was affected by *in-ovo* injection of the Cu-NP or $CuSO_4$ ($P > 0.05$) (Table 5). However, we observed an increase in the residual yolk sac ($P < 0.05$) for the treated groups and it was highest in the group injected with 50 mg/kg Cu-NP.

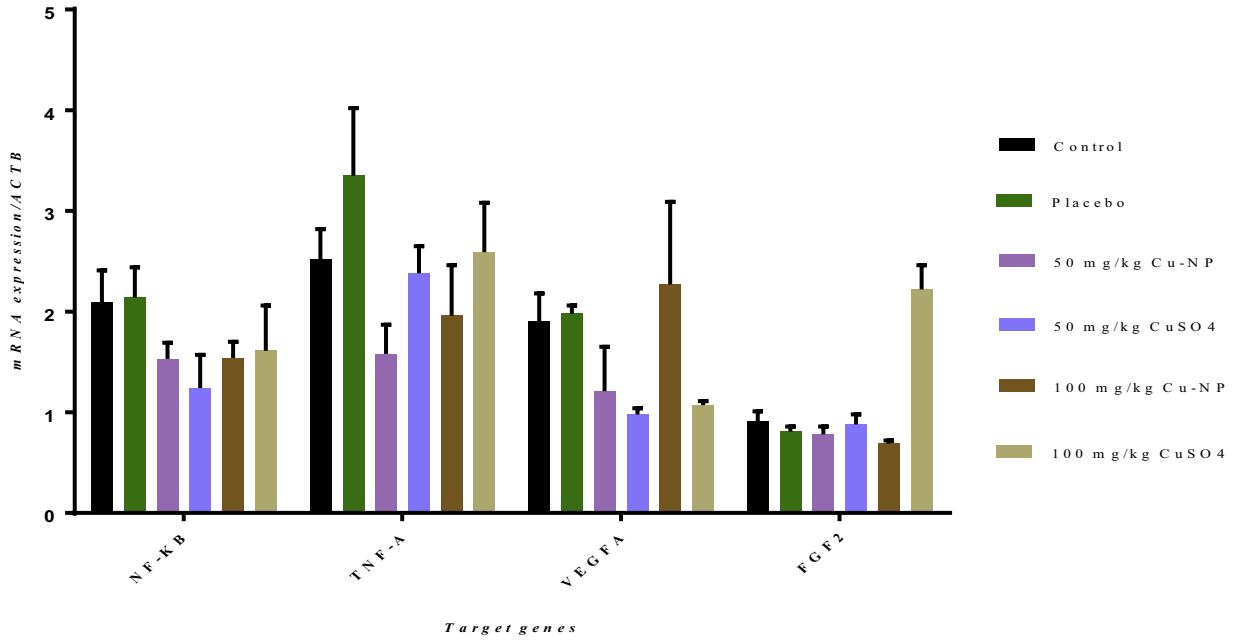

Fig. 1. The expression of mRNA *nuclear factor* (*NF-κB*) and *tumour necrosis factor alpha* (*TNF-α*) in the liver tissue, *vascular endothelial growth factor A* (*VEGFA*) and *fibroblast growth factor 2* (*FGF2*) in breast sample normalised to the housekeeping gene β-*actin* (*ACTB*). Samples were analysed at day one after hatching. Each data bar represents the mean values and standard errors of ten samples per treatment.

2.4. Gene expression

The mRNA expression of *NF-kB* and *TNF-α* in the liver tissue, and *VEGFA* in the breast tissue of new-hatched chickens was not significantly different ($P > 0.05$) among the groups, but there was a significant difference ($P = 0.003$) in the *FGF2* gene for the 100 mg/kg $CuSO_4$ group compared with the other groups (Fig. 1).

3. Discussion

The mechanisms behind the improvement in animal performance after treatment with copper nanoparticles are still unclear. Several researchers have attributed the improvement to the antimicrobial and antibacterial properties of Cu-NP (Usman et al., 2013), but others have suggested that improved digestibility of energy and fat is responsible (Gonzales-Eguia et al., 2009). It was demonstrated that nanoparticles of silver are capable of carrying oxygen (Pineda et al., 2012) while Cu-NP are major components of protein transporting O_2 into cells (Pineda et al., 2013). We speculated that because of their size and O_2 carrying capacity, injected Cu-NP could enhance the contribution of O_2 to cellular oxidation. Cu is an important component of proteins responsible for O_2 transport in cells (haemoglobin) and for the activities of essential enzymes (cuproenzymes such as cytochrome c oxidase) that are vital in the cellular energy generation (Pineda et al., 2013). Therefore, it was hypothesised that the *in-ovo* injection of Cu-NP would affect the metabolic rate, thereby increasing O_2 consumption and consequently improving growth and development during embryogenesis. In the present study, the values of gas exchange and EE of the embryos were typical of metabolic rates for chicken embryos as reported by Chwalibog et al. (2007). It was revealed that the Cu-NP, especially at the concentration of 50 mg/kg at 16 and 19 days of incubation, stimulated the metabolic rate of the broiler embryos to a greater level than did $CuSO_4$. This result could be associated to that Cu-NP has a stronger bioactivity affecting the blood vessel formation and growth to a greater degree than $CuSO_4$ (Mroczek-Sosnowska et al., 2015b). Furthermore, the biological effect of Cu-NP is linked directly to their size and high surface area, which result in specific physicochemical activities and a better ability to penetrate into the organism than bulk materials (Mamonova et al., 2013). Moreover, it was observed that intramuscular administration of Cu-NP stimulated the growth of chickens and the arginine level in their livers, which is a prime factor for stimulating growth in animals (Miroshnikov et al., 2015). Additionally, it was reported that the concentration of haemoglobin was higher in chickens treated with Cu-NP compared with chickens treated with $CuSO_4$ or control groups, which allowed more oxygen to be transported and contributed to cellular oxidation (Mroczek-Sosnowska et al., 2013).

In the present study, the YFBW and organ weights relative to YFBW of newly hatched chickens were used as an indicator of broiler development, but the differences in the metabolic rate did not affect the YFBW or relative organ weight compared with the control and placebo groups. This may suggest that the difference in the metabolic rate was not large enough to significantly affect the growth of the embryos. We could also suppose that modern broilers are selected for their high

feed utilisation, body gain and increased muscle mass (Vieira and Angel, 2012) and because of genetic selection the broiler embryos cannot increase further in body and organ weights, as previously demonstrated by Mroczek-Sosnowska et al., (2015b).

In the current study, we observed an increase in the metabolic rate in the group treated with 50 mg/kg Cu-NP, which is in contrast with the results of a study with layer embryos (Pineda et al., 2013). This conflict could be explained to the hatching weight and metabolic rate of broiler embryos are greater than those of layer embryos (Druyan, 2010). Broiler embryos have different embryonic development compared to layer embryos, which affects their energy utilisation (Nangsuay et al., 2015). Furthermore, broiler embryos are selected for faster growth and have a higher yolk mass, which contains a larger lipid fraction, therefore using more energy for growth (Buzala et al., 2015). It has been reported that broiler embryos have more muscle tissue to support metabolism and consequently require more O_2 and produce more heat compared with layer embryos (Janke et al., 2004; Hamidu et al., 2007; Tona et al., 2010). Moreover, it has been demonstrated that the larger muscle mass of broilers contains more water and is less mature than that of layers, which might allow increased O_2 diffusion (Meuer et al., 1992; Konarzewski et al., 2000).

Hamidu et al. (2010) reported that the embryo metabolism in different environmental conditions could be evaluated from heat production and respiratory coefficient (RQ) values. However, in the present study, the RQ values were approximately (0.73) for all groups, which is consistent with the values reported by Chwalibog et al. (2007), demonstrating that the main source of energy was from the oxidation of fat. The rate of O_2 consumption critically influenced the amount of fat used by the embryo (Speake et al., 1998). However, our results indicated a reduction in fat oxidation in the treated group, as demonstrated by the higher weight of the residual yolk sac (RSY) compared with the control group. Similar results were observed by (Pineda et al., 2013) on layer embryos. It could be speculated that Cu-NP may reduce the need to use yolk fat as an energy source during embryogenesis and consequently it might serve as an effective source of energy for a few days after hatching (Ali et al., 2007). Thus, it might be that the increased weight of the RSY in groups treated with Cu could provide more nutrients after hatching, contributing to better performance of the broilers as reported by Mroczek-Sosnowska et al. (2015a).

Other organs (heart, liver, breast and intestine) were not affected by the treatments; however, there was a tendency of decreased liver weight, compared with the control group.

Payvastegan et al., (2013) reported that supplementation of Cu in broilers diets leads to decreased levels of cholesterol and triglyceride synthesis in blood plasma. In the present study, the biochemical analyses of blood plasma (Table 4) did not indicate any significant differences among the treated groups. However, a tendency to reduce cholesterol and triglyceride levels in Cu-NP groups compared to the $CuSO_4$ groups and the control group was observed. The reduced cholesterol level confirms the findings of Mroczek-Sosnowska et al. (2013).

In the present experiment, the expression of immune-related genes (*NF-kB* and *TNF-α*) and the growth related (*VEGF-A*) was not affected by the treatments. There were no differences in *FGF2* between the treatments, except the higher value for the group treated with 100 mg/kg $CuSO_4$. Generally, the results indicate that the injection at the beginning of embryogenesis does not interact with cellular responses in hatchlings. In the present experiment, the expression of immune-related genes (NF-*k*B and TNF-α) and growth-related genes (VEGF-A and FGF2) was not affected, indicating that the injection at the beginning of embryogenesis does not interact with cellular responses in hatchlings. The reason for this could be that antigen-presenting cells did not uptake nanoparticles in the hatchlings, and immunostimulatory properties were absent (Pineda et al., 2013). Furthermore, nanoparticles might be poorly recognised by macrophages (Shvedova, 2005; Kagan et al., 2006). The phagocytic recognition and uptake of nanoparticles might be important in determining their impact on the immune system (Konduru et al., 2009).

Similar to our results, Cu-NP did not show inflammatory properties when injected into embryos at 50 mg/kg (Pineda et al., 2013; Mroczek-Sosnowska et al., 2015b). These responses might be species specific; hence, 2 mg of Cu-NP added to the diet improved the immune status and antioxidant defence systems in fish (El Basuini et al., 2016).

4. Conclusion

The obtained results indicated that the *in-ovo* administration of 50 mg/kg Cu-NP stimulated the metabolic rate in broilers during embryogenesis. Moreover, *in-ovo* application of Cu-NP was not harmful and showed good immunological biocompatibility with chicken embryos.

The authors declare no conflict of interest.

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