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Effect of zinc nanoparticles on embryo and chicken growth, and the content of zinc in tissues and faeces

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Abstract

The hypothesis was that owing to their high bioavailability, zinc oxide nanoparticles (NanoZnO) can effectively replace (Zn) salts and reduce Zn excretion with faeces. The objective of this study was to investigate the effects of NanoZnO on the development of chicken embryos, the growth of broiler chickens, and Zn excretion with faeces. At day 1 of incubation, 120 eggs were randomly divided between a control group (not injected) and groups injected with a hydrocolloid of NanoZnO in increasing concentrations (50, 100, 500 mg/L). At day 19 of incubation, no differences were observed in the bodyweight, but 100 and 500 mg/L affected liver and heart weights, indicating that high levels of NanoZnO may induce differential organ development. In the subsequent experiment, 308 chickens were randomly divided into six groups. The control diet was supplemented with 55 mg Zn/kg (standard level), the 0 group received no Zn supplement, and groups fed NanoZnO received 25%, 50%, 75%, and 100% of the standard level. The 100% replacement of ZnO with NanoZnO increased the chickens' bodyweight compared with the standard level of ZnO, but to the same level as the diet without ZnO supplementation. Furthermore, NanoZnO did not reduce the content of Zn in faeces, which was only significantly lower in the group without ZnO supplementation in comparison with other groups. The results indicate that the replacement of ZnO with NanoZnO had no negative effects on chicken growth. Compared with ZnO, NanoZnO did not reduce Zn excretion with faeces.

Keywords: broiler, development, excretion, mineral, nanonutrition
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Introduction

Nanoparticles are increasingly being considered for inclusion in formulated diets and supplementary nutrition for livestock production. The biological advantages of nano nutraceuticals include greater bioavailability owing to formation of micelles for absorption with lower residence in the intestinal tract, less excretion via the faeces, and easier movement across membranes (Gopi *et al*., 2017). Zinc is an essential supplement in poultry diets and plays crucial roles in the regulation of growth, the immune system and reproduction.

Nanoparticles of Zn, primarily zinc oxide nano particles (NanoZnO) are potential alternatives to inorganic and organic Zn sources, specifically as an enhancer of broiler growth performance, as reviewed by Swain *et al*. (2016) and indicated by Fathi (2016), Asheer (2017), Badawi and Behairy (2017), Ali *et al*. (2017) and Bami *et al*. (2018). However, although the chickens supplemented with NanoZnO had a higher body gain and better feed conversion rate (FCR) than birds without Zn supplement, the growth performance was not different between groups fed with different Zn sources (inorganic, organic and nano) (Badawi *et al*., 2017). The results of growth responses are not consistent, and render it difficult to draw general conclusions, largely owing to the lack of characterization (size, shape) of these nanoparticles and the varying content of Zn in dietary feed ingredients.

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Post-hatch survival, growth and development are continuations of embryonic survival, growth and development, which are dependent on the nutrients in the yolk and albumen that are available to the developing embryo. To this effect, nano-copper (NanoCu) can replace copper (Cu) salts effectively in embryonic and post-hatch health and growth (Mroczek-Sosnowska *et al*., 2015a, 2015b; Mroczek-Sosnowska *et al*., 2017; Scott *et al*., 2016; Scott *et al*., 2017; Sawosz *et al*., 2018; Scott *et al*., 2018).

This research pursued the hypotheses that NanoZnO does not affect embryo growth and that NanoZnO could replace Zn salts as a health and growth promoter and, given their high reactivity and bioavailability, may reduce the content of Zn in faeces. The objective of the study was to establish whether NanoZnO could replace ZnO supplement in the diets of broilers, reducing excretion of Zn into the environment.

Materials and Methods

Ethical approval for this research is documented in I.dz.Ike 46/2015, Warsaw University of Life Sciences.

NanoZnO powder (99.8% purity) was purchased from Sky Spring Nanomaterials Inc., Houston, USA, (Product no. 8410DL). For characterization, nanoparticles were suspended in Milli-Q water at a concentration of 50 mg/L water. The size and shape of the nanoparticles were determined with a JEM-2000EX Transmission Electron Microscope at 80 keV (JOEL, Tokyo, Japan). The zeta potential was measured in a colloidal solution of 50 mg/L NanoZnO using Zetasizer Nano ZS, model ZEN3500 (Malvern Instruments, Worcestershire, UK). Each sample was measured after 120 seconds of stabilization at 25 °C, in 20 replicates. Furthermore, colloidal impurities were determined using a Z-5300 polarised flame atomic absorption spectrometer (Hitachi-Science & Technology, Tokyo, Japan).

The study consisted of Phases 1 and 2, in which Phase 1 examined the effects of NanoZnO in developing embryos, and Phase 2 examined the effects on post-hatch growth and development.

In Phase 1, 120 eggs were randomly divided into four groups of 30 eggs per group. The hydrocolloids of NanoZnO were injected at the first day of incubation in concentrations 50, 100 and 500 mg/L. The control group (C) was not injected. At 19 days of incubation, the body and organ weights and biochemical indices of blood serum were measured. The administration of the NanoZnO incubation procedure and evaluation of embryo growth were performed as described by Pineda *et al*. (2013).

In Phase 2, given that Zn nanoparticles in powder form were dusty and were added to the basal diets (Table 1) feeds in small quantities, it would have been difficult to obtain a homogeneous feed mixture. This limitation was overcome by mixing the NanoZnO with starch as a carrier before being added to the diets. NanoZnO powder and starch were suspended in ultrapure water in the proportion 1: 100 (NanoZnO: starch, w/w) and were subsequently dried to powder with a dry matter content not less than 90%. The NanoZnO and starch complex was then added to a premix (Rovmix Broiler Grower, 0.5%, DSM) and the premix (Table 2) was added to the basal diets. Dry matter, nitrogen, crude fat, crude fibre and energy contents were determined in feed samples (Pineda *et al*., 2012).

Day-old Ross 308 chicks were obtained from a commercial hatchery and were randomly divided into six groups of six birds each, with a treatment allocated to a group, and six replicates in a treatment. The treatments of this post-hatch growth phase were designed to include a positive control (C) of 55 mg/kg ZnO in the diet as the standard that was in the mineral-vitamin premix, a group without ZnO, which received no Zn supplement (0) and groups that were fed with complexes of NanoZnO and starch received 25%, 50%, 75%, and 100% of the standard level of Zn used in the control group (Table 3). The calculated total levels of exposure to dietary Zn were 114 and 59 mg/kg provided as ZnO, and 72.8, 86.5, 100 and 114 mg/kg provided as NanoZnO. The treatment period was from day 1 for 42 days.

Table 1 Ingredients and chemical composition of the basal starter and grower diets

Ingredients (%)	Starter (1 - 14 days)	Grower (15 - 42 days)
Wheat	45.10	47.50
Soybean meal	25.65	22.00
Maize	10.00	10.00
Rape meal	10.00	10.00
Soybean oil	5.23	7.19
Dicalcium phosphate	2.25	1.54
Alimet 88%	0.33	0.27
L-lysine hydrochloride 98%	0.24	0.21
Sodium chloride	0.21	0.22
Chalk	0.20	0.32
Sodium carbonate	0.15	0.10
L-Threonine	0.14	0.15
Mineral-vitamin premix	0.50	0.50
Analysed chemical composition (%)		
Crude protein	21.70	19.42
Crude fat	4.95	6.73
Crude fibre	3.32	3.37
Metabolizable energy, calculated (MJ/kg DM)	12.56	13.31

The chickens were kept in groups during the first week and then individually in metabolic cages until they were 42 days old. The temperature was 32 °C in the first week, and lowered 2 °C weekly to 20 °C. The average humidity was 60% and 24-hour lighting was applied. The birds had free access to water and were fed ad libitum. The applied feed mixtures were starter for chickens 1 - 14 days and grower for 15 - 42 days (Table 2). Bodyweight (BW) was recorded at 1, 14, 35 and 42 days. The feed intake was registered daily as total values for each group. Individual excreta were collected daily over the period 28 to 35 days to determine the amount of Zn excreted.

The animals were euthanised at 42 days following a 12 hour fast. Carcass yield (%) and the weight yield of leg and breast muscles, gizzard, liver, heart and adipose tissue were measured following 24-hour air chilling at 4 °C. The tissue samples, organs and excreta (n = 6 per group) were kept at -80 °C for further analyses. The Zn content in the feed, breast muscle, liver and femoral bone at 42 days, and in excreta (28 to 35 days) was determined using a Z-5300 polarized flame atomic absorption spectrometer (Hitachi High-Tech Science Corporation, Tokyo, Japan).

The data distribution was evaluated using the Shapiro-Wilk test. Data were normally distributed and the results were analysed with one-way analysis of variance (ANOVA) using Statgraphics® Centurion XVI software (StatPoint Technologies, Inc., Warrenton, Virginia, USA) at a significance level of $P < 0.05$.

Table 2 Composition of the mineral-vitamin premix to which zinc was added to administer treatments

Ingredient	Unit	Quantity
A (retinol acetate)	IU/kg	2 200 000
D3 (E671)	IU/kg	500 000
E (di-alpha-tocopherol acetate)	mg/kg	10 000
D (D-pantothenate calcium)	mg/kg	2 722
K3 (MNB)	mg/kg	500
B1 (thiamine mononitrate)	mg/kg	400
B2 (riboflavin)	mg/kg	1400
B6 (pyridoxine hydrochloride)	mg/kg	800
B12 (cyanocobalamin)	µg/kg	400
Niacin (nicotinic acid)	mg/kg	8 000
Folic acid	mg/kg	200
Biotin	µg/kg	30 000
Choline chloride	mg/kg	60 000
Copper	mg/kg	
Zinc (zinc oxide)	Amount depending on the treatment applied	
Manganese (manganese oxide)	mg/kg	14 000
Iodine (calcium iodate)	mg/kg	120
Selenium (sodium selenate)	mg/kg	70.0
Iron (iron sulphate)	mg/kg	9 000
Citric acid	mg/kg	19.0
Etoxyquin	mg/kg	34.8
Propyl gallate	mg/kg	5.4
Calcium carbonate 25.07%	g/kg	251
Magnesium 0.22%	mg/kg	2200

Table 3 Zinc sources and content of zinc oxide and nano-zinc oxide to be used in the experimental diets

Zinc (Zn) source	Zinc oxide		Nano-zinc oxide			
Zn (%) of standard level[1]	100	0	25%	50%	75%	100%
Zn (mg/kg) in premix	11000	0	2750	5500	8250	11000
Zn supplement (mg/kg) diet[2]	55	0	13.8	27.5	41.3	55.0
Zn total (mg/kg) diet[3]	114	59	72.8	86.5	100.0	114.0

[1] Level of Zn reduction compared with the standard level of 55.0 mg/kg diet: 100%
[2] Level of Zn supplemented from premix in a diet
[3] Total level of Zn from premix + feed ingredients in the grower diet

Results

NanoZnO were slightly angular, rounded or elongated with an average size of 37 nm (10 - 70 nm) (Figure 1). The specific surface area was 30–50 m^2/g. The mean zeta potential of the colloid was -32 mV, which indicated an intermediate stability of the solution. The total impurities were below 0.01%.

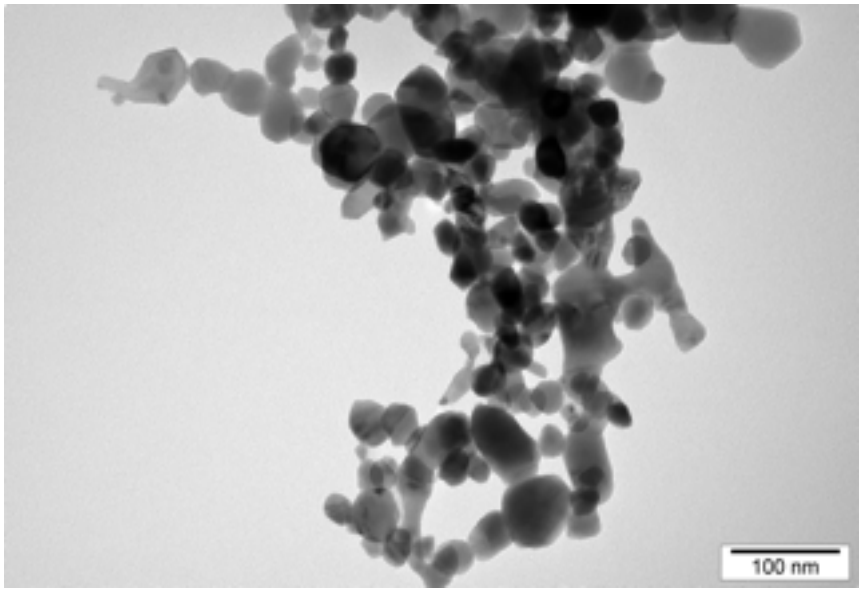

Figure 1 Image of zinc oxide nanoparticles by transmission electron microscopy

Live embryos at 19 days of incubation constituted 67% in the NanoZnO-treated groups compared with 73% in the control group. Bodyweights were not significantly different between groups (Table 4). However, groups treated with 100 and 500 mg/L had significantly higher heart weights than the other groups, while liver weights were lower in NanoZnO groups compared with the control.

Table 4 Pre-hatch embryo and organ weights of chicks at day 19 of incubation

Treatment[1] (mg/L)	Embryo, g	Heart, g	Liver, g
0 (C)	43.9	0.44^{B}	1.21^{Aa}
50	41.2	0.36^{B}	0.95^{B}
100	43.3	0.51^{A}	1.13^{A}
500	42.1	1.13^{A}	1.03^{b}
SE	1.0	0.03	0.04
P-value	0.20	0.005	0.000

[1] C: control; treated with 50, 100 and 500 mg/kg of ZnO nanoparticles
[a, b] Significant differences at $P \leq 0.05$; [A,B] Significant differences at $P \leq 0.01$

The only differences in blood biochemical parameters were higher concentrations of alanine aminotransferase (ALT) and lower triglycerides (TRG) in groups treated with 100 and 500 mg/L of NanoZnO (Table 5).

Bodyweight at 35 days did not differ significantly among the groups. At 42 days, the BWs of the ZnO-supplementation (0) and the 100% NanoZnO groups were significantly higher than the control. However, BW did not differ between 0 and NanoZnO groups (Table 6). Feed conversion ratio was measured on a group basis, which prevented statistical comparisons, but numerically FCR was higher (1.72) in the control relative to other groups (1.63). No mortality was recorded.

Table 5 Biochemical indices in the blood serum of chicken embryos as affected by level of supplementation with zinc oxide nanoparticles

	Treatment[1]				SE	*P*-value
	Control	50	100	500		
AST (U/l)	133^{A}	122^{A}	72.0^{B}	122^{A}	17.0	0.017
ALT (U/l)	11.6^{B}	9.90^{B}	20.6^{A}	17.0^{A}	1.71	0.000
ALP (U/l)	2222	2284	2540	2444	254.4	0.619
Glc (mmol/l)	277	263	260	249	10.4	0.154
Cr (mmol/l)	0.31^{ab}	0.37^{a}	0.27^{b}	0.32^{ab}	0.271	0.014
TP (mmol/l)	20.5	19.5	22.5	17.5	2.69	0.415
ALB (mmol/)	9.50	9.50	10.0	9.50	1.141	0.970
TC (mmol/l)	360	390	415	334	22.4	0.020
TRG (mmol/l)	84.4^{A}	86.7^{A}	71.9^{B}	71.4^{B}	3.70	0.001
LDH (U/l)	655	691	628	660	37.9	0.528
Ca (mmol/l)	9.00	10.2	9.70	9.60	0.492	0.246
P (mmol/l)	6.51	5.82	5.71	5.47	1.053	0.051

NanoZnO: zinc oxide nanoparticles , AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, Glc: d-glucose, Cr: Creatinine, TP: total protein, ALB: albumin, TC: number of white blood cells, TRG: triglycerides, LDH: lactate dehydrogenase, Ca: calcium, P: phosphorus
[1] C: control; and treated with 50, 100 and 500 mg/kg of ZnO nanoparticles
[a, b] Significant differences at $P \leq 0.05$; [A, B] Significant differences at $P \leq 0.01$

Table 6 Weights of chickens (g) over time as affected by level of supplementation with zinc oxide nanoparticles

Treatment[1]	Age (days)			
	0	14	35	42
C (55 mg/kg)	45	417	1936	2425^{b}
0%	43	376	2014	2612^{a}
25%	46	370	1927	2486^{ab}
50%	44	408	1975	2516^{ab}
75%	44	415	2094	2652^{ab}
100%	44	412	2063	2706^{a}
SEM	1	16	84	100
P-value	0.665	0.192	0.703	0.042

[1] C: control; 0: without zinc oxide (ZnO) nanoparticles; 25%, 50%, 75%, and 100% in groups provided with ZnO nanoparticles in proportion to the control
[a, b] Significant differences at $P \leq 0.05$

The analysis of carcasses showed that the Zn content of leg muscle was highest in the control and in the 0 group. The dressing percentage and the content of breast muscle and giblets were not affected (Table 7).

Table 7 Dressing percentage and carcass content of chickens (%) as affected by level of supplementation with zinc oxide nanoparticles

Treatment[1]	Dressing percentage	Muscle		Giblets		
		Breast	Leg	Gizzard	Liver	Heart
C (55 mg/kg)	79.1	27.5	20.0[a]	1.63	2.58	0.69
0%	80.6	27.1	20.0[a]	1.77	2.05	0.61
25%	77.4	26.5	18.6[ab]	1.82	2.05	0.61
50%	77.9	24.9	16.2[b]	1.68	2.19	0.93
75%	78.0	26.6	16.7[b]	1.84	2.33	0.72
100%	76.7	27.6	18.3[ab]	1.35	2.08	0.67
SE	1.2	1.2	0.8	0.19	0.20	0.06
P-value	0.293	0.626	0.022	0.512	0.414	0.054

[1] C: control; 0: without zinc oxide (ZnO) nanoparticles; 25%, 50%, 75% and 100% in groups provided with ZnO nanoparticles in proportion to the control
[a, b] Significant differences at $P \leq 0.05$

Zinc concentration in breast muscle at 42 days did not differ significantly among the groups (Table 8). However, the liver and femoral bone showed some differences, although these were inconsistent. The lowest concentration in faeces was observed in chickens that did not receive the ZnO supplement and highest in chickens that received 75 and 100% of Zn level and in the control.

Table 8 Zinc content (mg/kg DM) of tissues and faeces from chickens provided different levels of dietary supplementation with zinc oxide nanoparticles

Treatment[1]	Breast muscles	Liver	Femoral bone	Faeces
C (55 mg/kg)	34.0	130[b]	105[d]	281[AD]
0%	34.6	164[a]	98.1[Bb]	164[B]
25%	30.8	160[a]	113[a]	255[AE]
50%	33.0	133[ab]	108[abcd]	243[AC]
75%	25.0	121[b]	117[a]	293[ADF]
100%	34.2	105[b]	119[Ac]	303[ADF]
SE	4.3	12.0	4.3	8.6
P-value	0.627	0.031	0.040	0.000

[1] C: control; 0: without zinc oxide (ZnO) nanoparticles; 25%, 50%, 75%, and 100% in groups provided with ZnO nanoparticles in proportion to the control
[a-d] Significant differences at $P \leq 0.05$; [A-F] Significant differences at $P \leq 0.01$

The treatments had no effects on the biochemical indices of the blood serum (Table 9).

Table 9 Biochemical indices in the blood serum of chickens provided different levels of dietary supplementation with zinc oxide nanoparticles

Treatment[1]	AST, U/l	ALT, U/l	Glc, mmol/dl	Urea, mg/dl	TP, g/l	ALB. g/l	TRG, mg/dl
C (55 mg/kg)	487	19.7	226	8.73	24.0	16.7	22.2
0%	511	21.2	204	6.53	26.0	16.6	20.5
25%	508	20.7	199	9.20	23.3	15.3	19.5
50%	485	20.3	191	8.13	24.0	15.0	18.6
75%	429	22.9	194	7.61	24.2	16.0	18.5
100%	417	19.0	188	7.33	24.0	15.3	20.2
SE	50	3.2	10	0.97	0.8	0.6	4.2
P-value	0.682	0.416	0.175	0.094	0.298	0.333	0.987

[1] C: control; 0: without zinc oxide (ZnO) nanoparticles; 25%, 50%, 75%, and 100% in groups provided with ZnO nanoparticles in proportion to the control
AST: aspartate aminotransferase; ALT: alanine aminotransferase, Glc: glucose; TP: total protein; ALB: albumins; TRG: triglycerides

Discussion

In Phase 1, administration of NanoZnO at the beginning of embryogenesis had no effect on BW, but increased the heart weight and decreased the liver weight of embryos at 19 days. The lack of influence on BW at hatch was also demonstrated by Josuha *et al*. (2016), who injected NanoZnO at 18 days of incubation, and by Hassan (2018) when the injection took place at the beginning of incubation. The percentage of live embryos was lower in the NanoZnO treated groups than in the Control. This finding, however, is not congruent with those of Josuha *et al*. (2016) and Hassan (2018), who found no differences in hatchability, albeit using smaller doses of Zn nanoparticles. Whether the injection per se could affect the hatchability cannot be answered no group was injected with a placebo, but Hassan (2018) demonstrated no differences between embryos administered with nanoparticles and those that were not injected or were injected with phosphate buffered saline.

Comparable results for the effect of NanoZnO on organ weights in the developing embryo could not be traced in the literature. The biochemical indices of blood serum were different only for ALT with an elevated level, and decreased TRG level in the 100 and 500 mg/L groups. These changes were within the normal range for chickens (Chattopadhyay *et al*., 2006; Hassan, 2018). The changes are an interesting phenomenon since the ALT was increased significantly in the 100 and 500 mg/L treatments, and the ratios of AST to ALT, which indicates clinical health in liver function, were 11.5, 12.3, 3.5, and 7.2 for the control and groups that were injected with 50, 100, and 500 mg/L, respectively. This decreasing ratio, aligned with the lower liver mass and increased heart mass, indicated that the higher levels of NanoZnO may induce differential organ development in chicken embryos. Chicken embryos are highly sensitive models for testing potential toxic effects of injected substances by monitoring total and differential organ development rates and survivability of the embryos (Korhonen *et al.*, 1982; Sawosz *et al.*, 2014; Lucht *et al*., 2018).

In Phase 2, the chickens were kept in metabolic cages. Consequently, the number of animals was limited. However, the experiment was not a performance study, but it included individual measurements of the content of Zn in faeces, requiring individual cages for quantitative sample collection. The feed intake and FCR were recorded on a group basis. The control group was supplemented with a standard level of 55 mg Zn per kg of feed, as recommended by the NRC (1994). The group without ZnO did not receive Zn supplementation, while the other groups were administered with NanoZnO in incremental proportions of the control (55 mg/kg).

There was no clear indication of differences in BW at 42 days since treatments 0%, 25%, 50%, 75%, and 100% did not differ. However, the treatment with 100% of NanoZnO significantly increased BW compared with the standard level of ZnO. Several reports show enhanced growth performance (Lina *et al*., 2009; Ahmadi *et al*., 2013; Mishra *et al*., 2014; Zhao *et al*., 2014; Khah *et al*., 2015; Mohammadi *et al*., 2015; Fathi, 2016; Sahoo *et al*., 2016; Hassan, 2018), but others show no effects of Zn nanoparticles on BW (Lina *et al*., 2009; Asheer, 2017; Bami, 2018). The literature is conflicting, and it is impossible to explain incongruence, which might be attributed to the type, size and dose of nanoparticles, experimental design, and differences in feeding and management of chickens.

The numeric values of FCR indicate that this was improved in the group without ZnO supplement and in all NanoZnO groups compared with the control group. Improved FCR had been demonstrated by Ahmadi *et al*. (2013), Zhao *et al*. (2014), Fathi (2016) and Sahoo *et al*. (2016) when comparing increased doses of NanoZnO with the standard supplement of Zn. However, when inorganic Zn was replaced with NanoZnO (25 - 100% replacement), only the 100% replacement reduced FCR (Asheer, 2017).

It is striking that BW and FCR, although improved by NanoZnO supplementation, were not different from the birds fed without ZnO. The feed ingredient provided 59 mg Zn/kg diet, which was sufficient to meet the birds' requirements. In the control, the total amount of Zn was 114 mg/kg (feed ingredients 59 mg/kg + ZnO supplement 55 mg/kg), which was probably too high, but nevertheless low enough to avoid causing harmful health effects. The diets with NanoZnO provided increasing levels of Zn (from 73 to 114 mg/kg), causing higher body gain and superior FCR to the control group, but no different from the group without ZnO supplementation. This might indicate that the replacement of ZnO with NanoZnO can enhance chicken performance, but that it is not necessary when the supply of Zn from feed ingredients covers Zn requirements. The feed ingredients used in the present diet are standard compounds, suggesting that the results might be applicable to commercial poultry mixtures, which provide enough Zn from feed ingredients. Nevertheless, this postulate has to be justified in extended performance experiments.

The effect of NanoZnO on dressing percentage has been reported as being both positive (Lina, 2009; Khah *et al*., 2015) and negative (Sahoo *et al*., 2016). The present results indicated an absence of effects on dressing percentage and the carcass content of breast muscle and giblets at 42 days. However, the content of leg muscle decreased in NanoZnO-treated groups. Again, it is difficult to compare results from different methodologies, but the findings of Kahah *et al*. (2015) and Sahoo *et al*. (2016) showed no effects on the proportion of thigh.

After absorption in the intestine, nanoparticles can enter the bloodstream and be stored in various organs and be distributed independently of the blood circulation (Anjum *et al*., 2016). In the present experiment, the content of Zn in breast muscle was generally lower in NanoZnO groups than in the control and 0 groups. The differences between Zn retained in the liver and femoral bone were not conclusive. Sahoo *et al*. (2014) noted that NanoZnO could increase Zn content in the tibia bone, liver and blood serum, but that study is not directly comparable with the present study.

The content of Zn in faeces was significantly lower in the group without ZnO supplementation, relative to the other groups, which did not differ significantly. The administration of NanoZnO did not reduce Zn excretion in comparison with the control group. The increased excretion in the faeces with increasing supplementation of NanoZnO can be ascribed to residual non-absorbed dietary Zn in the digestive tract plus endogenous Zn that is excreted via the liver and biliary tract and kidneys. As noted, individual feed intake could not be recorded, nor could the total exposure to dietary Zn calculated. However, the non-significant differences in faecal Zn between groups C, 25%, 50%, 75%, and 100% could indicate similar homeostatic processes between the treatments. The deduction is that homeostasis of Zn was not dependent on the level of NanoZnO intake.

The biochemical parameters of the blood serum were not affected. The present results correspond with findings by Fathi (2016) and Hassan (2018), but contradict those of Ahmadi *et al*. (2013), Mishra *et al*. (2014), and Sahoo *et al*. (2014), who did identify some effects. As in performance, the literature is inconsistent. Furthermore, there was no mortality in any of the groups, indicating good health status of all birds.

It emerged from Phases 1 and 2 that differential development may have been induced in embryonic development that was carried through to the post-hatch growth and development. The embryos showed increased liver and heart weights and the chickens showed lighter livers with increasing NanoZnO, though non-significant, and significant differential development of the leg muscle in which the 50%, 75%, and 100% treatments were lower that the C and 0 groups. The biochemical indices also showed interesting tendencies. It would appear, though the results do not show significance, that the NanoZnO could have an effect at mitochondrial level. The embryos show decreasing TP and increasing Cr. Using these few values, the correlation is -0.6, and reciprocal. Because the leg muscle is lower percentagewise, this may indicate differential growth that is affecting red muscle fibre. In early development, all the muscle fibres have the characteristic of red aerobic muscles. As development advances in chickens, a differentiation occurs with the breast muscles, which become white muscle types, glycolytic, with the leg muscle, for example the gastrocnemius, retaining red muscle fibre characteristics.

Conclusion

Injection of NanoZnO into eggs at the start of incubation did not affect BW, survival rate and health status of embryos in the final stage of embryogenesis. There was an indication that concentrations greater than or equal to 100 mg/L may induce differential organ development. In post-hatch growth, the replacement

of the standard level of ZnO (55 mg/kg) with 100% NanoZnO in the diet increased the chickens' BW, but to the same level as in the group without ZnO supplementation. Furthermore, NanoZnO did not reduce the content of Zn in faeces, which was only significantly lower in the group without ZnO supplementation in comparison with other groups. The results indicate that the replacement of ZnO with NanoZnO has no negative effects on chicken growth.

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Authors' Contributions

ES conceived the study and helped draft the manuscript. MŁ carried out chicken experiments and drafted the manuscript. AŁ conducted statistical analyses and edited the draft version of the manuscript. AC participated in the statistical analyses and prepared the final manuscript. NHC participated in the evaluation of data and manuscript preparation. JN participated in the design and coordination of experiments. AM participated in chicken experiments and data collection. MS and MW carried out embryo experiments and participated in the evaluation of data. MZ performed and evaluated blood analyses and participated in embryo experiments..

Conflict of Interest Declaration

No potential conflicts of interest were reported by the authors.

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