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RESPONSE OF BROILER CHICKEN TO *IN OVO* ADMINISTRATION OF INORGANIC SALTS OF ZINC, SELENIUM AND COPPER OR THEIR COMBINATION

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ABSTRACT

This study determined the hatchability, post-hatch growth performance, immune response and bone morphometry of broiler chicken to *in ovo* administration of inorganic salts of zinc, selenium, copper or their combination. A total of 330 fertile eggs of Cobb broiler strain were procured from commercial breeder hatchery, fumigated, weighed and set in the incubator. The eggs were candled on the 14th day of incubation and distributed into five treatment groups: Control (without *in ovo* administration), Group II, *in ovo* administration of 80 µg.egg^{-1} inorganic zinc; Group III, *in ovo* administration of 0.3 µg.egg^{-1} inorganic selenium; Group IV, *in ovo* administration of 16 µg.egg^{-1} inorganic copper and Group V, *in ovo* administration with the combination of the inorganic salts of zinc, selenium and copper. The *in ovo* administration was carried out on the 18th day. The post-hatch chicks were distributed into 5 treatment groups of 6 replicates containing 7 chicks each. Data obtained were subjected to Analysis of Variance in a completely randomized design. The results showed highest percentage of hatchability in Zn-injected hatching eggs. The final weight of birds from Zn-injected eggs was significantly ($P < 0.05$) highest on day 7. On day 35, the final weight and weight gain were significantly ($P < 0.05$) affected by the *in ovo* administration of Zn, Se, Cu and their combination with the highest values obtained in birds on Cu-injected eggs. Birds from Zn-injected eggs, Cu-injected eggs and eggs on the combination of the inorganic salts had significantly ($P < 0.05$) highest proportion of heart than obtained in the control. The tibiae ash of birds from Zn and Se-injected eggs recorded the highest values of 40.96 and 40.37 %, respectively, while the lowest value (35.47 %) was recorded in the tibiae of birds from eggs injected with the combination of the inorganic salts. It was concluded that Zn impacts mostly on hatchability, but the combination of the mineral sources at the ratio injected impacted negatively the growth performance of the broiler chickens.

Key words: hatchability; *in ovo* injection; inorganic salts; broiler chicken; gut morphology; tibiae mineralization

INTRODUCTION

The period of embryonic development becomes a greater proportion of a bird's life to match up with the decreasing generation interval that takes meat birds to achieve market size. Most poultry research (Peebles *et al*., 2005; Collin *et al*., 2007; Elibol and Brake, 2008) is now designed towards realizing gains in genetic and production potential of poultry from advancements made during the incubation period and embryogenesis.

Under a practical condition, birds have access to feed after hatching only between 3 and 4 days and this brings about reduction in the body weight, while the intestine and muscle development are also retarded. Noy and Uni (2010) then suggested that a continuous feeding process could be established to ensure continuous supply of nutrients *in ovo* to the developing embryo, feed and water to the newly hatched chicks within the hatchery. The *in ovo*

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injection is, thereby, adopted and widely used for many purposes, such as fertilizing an avian egg in the shell (Cantrell and Wooten, 2003), injecting avian eggs with immunological material (Jochemsen and Jeurissen, 2002), a trial for sex reversal in birds (Kagmi and Hanada, 1997), increasing the post-hatching body weights of birds by *in ovo* injection of growth promoters (Ohta *et al*., 1999) and enhancing the growth of avian embryo by injecting eggs with a special liquid as nutritional supplements. Nutrients in *in ovo* injection have a lot of benefits: greater efficiency of feed utilization (Bhanja *et al*., 2004); reduced post-hatch mortality and morbidity, improved immune response (Gore and Qureshi, 1997), enhanced early growth by improving intestinal function and development through an enhanced absorption by the villi (Tako *et al*., 2004; Noy and Uni, 2010), and increased skeletal growth (Hargis *et al*., 1989), breast muscle yield (Hajihosaini and Mottaghitalab, 2004) and marketing body weight (Selim *et al*., 2012).

It is noteworthy that the rapid growth rate and increasing incidence of leg problems in broiler chickens are highly related to an acceleration of bone deposition at the periosteal surface, which increases the porosity of the cortical bone, subsequently causing poorer biomechanical properties of the bone (Williams *et al*., 2004). Micro-minerals that are of major importance to bone formation and strength include Cu, Zn, and Mn, but they are greatly reduced in concentration in the egg by the 17th day of incubation (Yair and Uni, 2011). These minerals also participate through their contribution to enzyme activity along metabolic pathways that are related to the formation of the skeletal system (Bao *et al*., 2007). The metalloenzymes of which zinc is grouped, play an important role in the bird's immune response and in hormone production (O'Dell, 1981). The involution of the thymus and small spleen weights are characterized by zinc deficiency and primarily the absence of white cells (Vruwink *et al*., 1993). The authors also reported that inadequate intracellular concentration of zinc also causes damage to the lymphocyte function that is responsible for the ability of T-and B- cell proliferation. Zinc is noted to be responsible for normal growth and maintenance and includes among other functions bone development, feathering, enzyme structure and function as well as appetite regulation for all poultry (Batal *et al*., 2001). Zinc sulphate is highly water soluble, allowing reactive metal ions to promote free-radical formation, which can facilitate reactions that lead to the breakdown of vitamins and ultimately to the degradation of fats and oils, decreasing the nutrient value of the diet (Batal *et al*., 2001). Although, zinc interferes with iron metabolism in chicks, iron-deficient chicks are more susceptible to the effects of zinc toxicity than are iron-adequate chicks (Blalock and Hill, 1988).

Rotruck *et al*. (1973) reported that selenium was first associated with toxicity in 1950's but its importance in the diet was elucidated when it was deemed essential in the prevention of liver necrosis in rats. This was further strengthened in the 1970's, when it was found to be an essential component of the enzyme glutathione peroxidase. Since selenium (Se) deficiency in the poultry diets has shown to cause several pathological conditions that can impact growth and development, research is thereby focused on how to ensure its adequacy. Se is often added to the animal's diet using inorganic Se (Na_2SeO_3). It has been reported that Se is readily transferred from breeder hens to the eggs and thus, to the embryo (Paton *et al*., 2002). The authors further reported that the amount of Se that can be derived from the hen's diet is limited, because the maximum level of dietary Se supplementation is limited to 0.3 ppm by the FDA (2002). Hence, the introduction of Se *in ovo* to the incubating embryo was found to be a suitable alternative.

Copper is essential for the normal maturation of collagen (Rucker and Murray, 1978), hence laying hens fed a copper-deficient diet produced eggs with abnormal shell membrane (Baumgartner *et al*., 1978). The activation of lysil oxidase also appears to involve a role of copper as cofactor, which may also act on the regulation of lysil oxidase synthesis (Harris, 1976). A well-structured outer shell membrane is required for mineralization and strong eggshells. In addition, adequate membrane structure allows the separation between the inner and the outer shell membranes, promoting the proper formation of the air chamber immediately after oviposition. This is an important oxygen reservoir used by the embryo during pipping. Copper in the shell seems to be essential for the chick embryo metabolism, as it was shown that shell-less cultures led to a failure in accumulation

of normal amounts of hepatic copper during the latter half of incubation (Richards *et al*., 1984). Copper was also found to play vital role in haemoglobin synthesis and it is associated with many enzymes (Gaetke and Chow, 2003). In the findings of Goel *et al*. (2013), Cu was found to enhance the immune response of broiler chickens on *in ovo* administration of 8 µg.egg^{-1} of inorganic Cu ($CuSO_4$).

Most studies on trace mineral requirements in poultry production (Nollet *et al*., 2007; Jegede *et al*., 2011) focused on supplementation of the diets and not the embryo with a single trace mineral. However, supplementation with a single trace mineral could be a disadvantage because of negative interactions, so that over-supplementation of one trace mineral will interfere with other trace minerals' availability (Watts, 1990; Scheideler, 1991). The most common antagonism occurs between Zn and Cu, and a ratio greater than 4:1 of Zn/Cu can be considered antagonistic (Scheideler, 1991). The author further stated that high levels of dietary Zn will inhibit Cu absorption, resulting in hepatic accumulation and deposition in the egg. High levels of Cu and Fe can interfere with Zn availability and potentially induce anaemia in poultry. Hence, this study assessed the effects of *in ovo* administration of inorganic Zn, Se, Cu and their combination on the growth performance, development of the gastro-intestinal tract, carcass yield, bone (tibia) morphology and mineralization and *in vivo* immune response of broiler chickens.

MATERIALS AND METHODS

The entire experiment including the feeding trials of broiler chicken were carried out at the Experimental Livestock Unit, Indian Council of Agricultural Research - National Institute of Animal Nutrition and Physiology (ICAR-NIANP), Bengaluru, Karnataka, India.

Hatching of eggs

Three hundred and thirty fertile eggs of Cobb strain of broiler chickens procured from the commercial breeder hatchery were fumigated, weighed and set into the incubator. On the 14th day of incubation, the eggs were candled and eggs showing viable embryo were distributed into five groups of control and *in ovo* supplemented groups (Table 1).

In ovo supplementation

On 18th day of embryonic age, the eggs showing viable embryo were injected with nutrients into amnion using a 24-gauge hypodermic needle (25 mm long) under laminar flow system, with handling temperature not lower than 35 °C (Bhanja *et al*., 2004). The *in ovo* injection of each treatment was completed within 30 minutes of taking out from the incubator. Before injection, the site was suitably sterilized and the injection was done at the broad end of the egg. Following *in ovo* feeding, the injection site was sealed with a sterile paraffin and the eggs were transferred to hatching compartment.

Table 1. Groups of eggs for *in ovo* injection

Treatment group	*In ovo* injection
Group I	Control
Group II	*In ovo* supplementation with 80 µg.egg^{-1} of inorganic Zinc (Zn sulphate 351.80 µg. 0.5 ml^{-1} deionised water)
Group III	*In ovo* supplementation with 0.3 µg.egg^{-1} of inorganic Selenium (Sodium Selenite 0.657 µg. 0.5 ml^{-1} deionised water)
Group IV	*In ovo* supplementation with 16 µg.egg^{-1} of inorganic Copper (Copper Sulphate 62.87 µg. 0.5 ml^{-1} deionised water)
Group V	*In ovo* supplementation with 80 µg.egg^{-1} of inorganic Zinc, 0.3 µg.egg^{-1} of inorganic Selenium and 16 µg.egg^{-1} of inorganic Copper

Post-hatch chick

A total of 265 eggs were fertile from the 298 egg set (88.93 % fertility) and a total of 234 chicks hatched from 260 fertile eggs (90.00 % hatchability) transited to the hatching compartment. Post-hatch chicks were distributed into 5 treatment groups (Table 1), into 6 replicates of 7 chicks per replicate, and reared in electrically heated battery cages with a provision of wire mesh floor, feeders and waterers under uniform and standard management condition. Standard broiler pre-starter (0-7 d), starter (7-21 d) and finisher (21-35 d) diets were prepared with maize and soybean meal, as the major ingredients (Table 2). Feed and drinking water were provided *ad libitum*. The experiment lasted for 35 days.

Assessment of the following parameters

i) Hatching: Egg weight, percentage of hatchability, chick weight and chick-to-egg ratio.

The percentage of hatchability was calculated as follows:

% Hatchability = (№ of hatched chicks/№ of fertile eggs) *100

ii) Growth: Weekly body weight and feed intake were recorded. The feed conversion ratio (FCR) was calculated using the formula:

FCR = (Feed intake/weight gain)

iii) Gut morphometry: On 7th day post-hatch, one chick from each replicate and on 35th day post-hatch, two birds from each replicate were slaughtered by cervical dislocation for gut development studies. Gut morphometry was done by recording the weights of gizzard, proventriculus, liver as well as the weight and length of the duodenum, jejunum, ileum and caecum.

Table 2. Ingredient and nutrient composition (%) of experimental diets

Ingredient	Pre-starter (0-7 days)	Starter (1-3 wk)	Finisher (3-5 wk)
Maize	57.00	58.60	62.50
Soybean meal	37.00	36.10	31.50
Fat / oil (soybean oil)	1.87	1.65	2.20
Limestone	1.00	1.00	1.10
Dicalcium Phosphate	1.75	1.75	1.75
Salt (NaCl)	0.35	0.35	0.35
Lysine	0.40	0.10	0.12
Methionine	0.20	0.20	0.20
Threonine	0.18	0.00	0.00
*Vit & Minerals premix	0.25	0.25	0.28
Total	100.00	100.00	100.00
ME (kCal.kg^{-1})	2995.50	2991.50	3047.75
CP (%)	22.51	21.89	20.07
Lysine (%)	1.52	1.26	1.15
Methionine (%)	0.55	0.51	0.44
Threonine	0.98	0.78	0.72
Tryptophan	0.23	0.23	0.21
Valine	0.89	0.88	0.80
Arginine	1.37	1.35	1.21
Ca (%)	1.00	1.00	1.00
P, avail. (%)	0.45	0.45	0.45

*Trace mineral premix 0.1 %, Vit. Premix 0.1 %, B - Complex 0.02 %, Choline 0.05 % and Salt 0.3 %
Trace mineral premix supplied mg.kg^{-1} diet: Mg, 300; Mn, 55; I, 0.4; Fe, 56; Zn, 30; Cu, 4
The vitamin premix supplied per kg diet: Vit. A, 8250 IU; Vit. D3,1200 ICU; Vit. K, 1 mg; Vit. E, 40 IU; Vit. B1, 2 mg; Vit. B2 4 mg; Vit. B12, 10 mcg; niacin, 60 mg; pantothenic acid, 10 mg; choline, 500 mg

iv) Carcass characteristic and weight of immune organs:

Two birds from each replicate were sacrificed through cervical dislocation to evaluate

a) Eviscerated yield, cut-up parts yield (breast, back, drumsticks, and thighs) and giblet yields (gizzard, heart and liver).
b) Weight of immune organs: Weight of lymphoid organs (spleen and bursa only) was expressed as mg.100 g^{-1} live weight at the 35th day.

v) Bone morphology and mineralization

Tibia-Osteo - morphometry

(i) Length.
(ii) Proximal width
(iii) Mid shaft width
(iv) Distal width

Tibia – Osteo-mineralization

(i) Bone weight
(ii) Calcium
(iii) Phosphorous

Procedures

Tibia bone morphometric measurements

The left tibial bones were collected and their adhering muscles together with connective tissues were thoroughly removed manually and dipped into a boiling water for 5 minutes to remove any remaining soft tissues. The length, proximal and distal width, as well as the mid shaft width of the tibia bone were measured with Vernier callipers. These were expressed in mm.kg^{-1} live weight.

The tibia weight, tibia length, diaphysis diameter, tibia weight/length index and tibia robusticity index were determined as described by Mutus *et al.* (2006). The bone weight/length index was obtained by dividing the tibia weight by its length (Seedor *et al*., 1991), while robusticity index was determined using the following formula as described by Reisenfeld (1972):

Robusticity index = Bone length/Cube root of bone weight

Tibia-Osteo-mineralization

Each tibia was defatted for 16 hours in petroleum ether (boiling point of 60-80 °C), dried and weighed before ashing. The samples were digested with diluted hydrochloric acid (1:2) and the mineral extract was prepared according to AOAC (1995). The extract was used in the estimation of the minerals. The mineral extract from each treatment replicate group was selected and the concentration of Zn, Mn, Cu, Ca and P were determined by Inductively Coupled Plasma Optical Emission Spectrometry (method 6010B).

vi) *In vivo* immune response of the bird

a) Cell-mediated immunity:

Reagents: Phosphate-buffered saline (PBS):
Sodium chloride, 8.0 g; Potassium chloride, 0.20 g; Potassium dihydrogen phosphate, 0.20 g; Disodium hydrogen phosphate, 1.44 g; distilled water, 1 litre; pH 7.2. (The producer of the reagents is Zunche Pharmaceutical Private Ltd., India)

Procedure

The cell-mediated immune response to phytohemagglutinin type P (PHA-P) was studied using the method of Corrier and Deloach (1990). At 21 days post-hatch, 0.1 ml (concentration 1 mg.ml^{-1}) of PHA-P was injected at 3rd and 4th inter--digital space of the right foot. The left foot served as control and injected with 0.1 ml phosphate--buffered saline (PBS). The foot web index was calculated as a difference between the swelling in the right and left feet before and after 24 hours of injection and expressed in millimetres.

The foot web/pad index was calculated as follows:

Cell-mediated immune response (CMIR): (R2-R1) – (L2-L1)

Where:

R2 = Thickness of right foot web after 24 hours of injection
R1 = Thickness of the right foot web before injection
L2 = Thickness of left foot web after 24 hours of injection
L1 = Thickness of the left foot web before injection

Humoral immunity

Reagents: Alsever's solution: Dextrose (Wuhan Yuancheng Gongchuang Tech. Co. Ltd, China), 2.05 g; Trisodium citrate dehydrate (Lianyungang Longyi Industry C. Ltd, China), 0.80 g; Sodium chloride (Zunche Pharmaceutical Private

Ltd., India), 0.42 g; Citric acid (A.+E, Fisher Chemie), 0.055 g; Distilled water, 100 ml; pH 6.5.

Procedure

The antibody response to the Sheep Red Blood Cell (SRBC) was studied at 29th day post-hatch, wherein 1 ml of 1 % SRBC was injected i/v to the birds. The SRBC was washed thrice and centrifuged at 704 g for 10 minutes after each washing. After 5 days of SRBC immunization, 2 ml of blood was collected from the wing vein and the antibody titre was recorded by hemagglutination (HA) titre (Siegel and Gross, 1980; Vander Zipp, 1983). The blood was kept in a slightly slanting position for 1 hour to clot. The clot was then allowed to retract after detaching it from the side of the tube. Centrifugation was carried out at 313 g for 5 to 10 minutes for rapid collection of serum.

For the HA test, 50 µl of PBS were poured into each well of the micro titre plate and 50 µl of serum from the chicken were added into the first well. Thereafter, two-fold serial dilution was made up to row 11, while the 12th row was left as a control and then 50 µl of 1 % SRBC were added into each well. The plates were covered and shaken on automatic shaking machine for proper mixing. The micro titre plates were then kept at 37 °C for 1 hour in an incubator. The plates were read under light and the titre expressed as log2 of the highest dilution in which there were complete haemagglutination.

Statistical analysis

The data collected during the experiment were subjected to one-way analysis of variance for completely randomized design. Significantly ($P < 0.05$) different means among variables were separated using Tukey test as contained in the Minitab® version 17.1.0 (Minitab, 2013).

RESULTS AND DISCUSSION

The percentage of hatchability was highest in the Zn-injected hatching eggs resulting from an increase in hatched chicks (Figure 1). This finding corroborates earlier reports (Luscombe *et al*., 2000; Batal *et al*., 2001; Bartsevich *et al.*, 2003) that zinc is essential for embryonic development of all species including poultry. Zn was reported to control the differentiation of many cell types including T-lymphocytes (Staal *et al*., 2001)

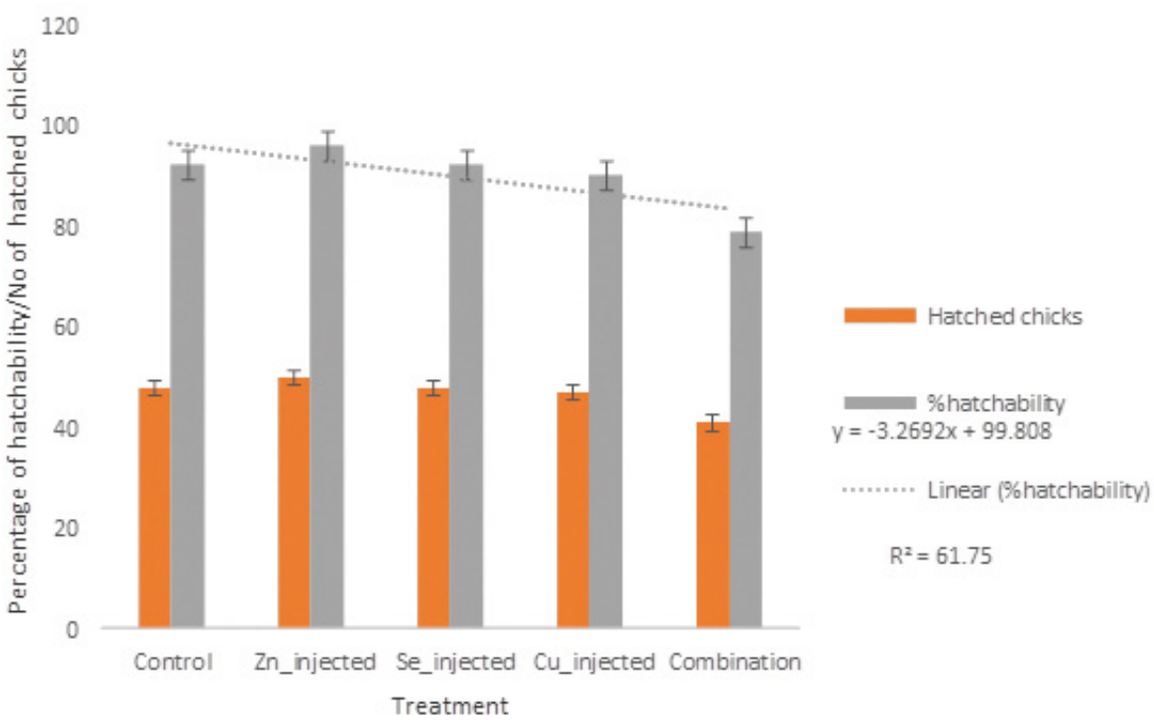

Figure 1. Effects of *in ovo* injection on percentage of hatchability

and myeloid precursor cells (Shivdasani, 2001). However, there had been varying hatchability results with *in ovo* administration in broiler chickens; decreased hatchability (McGruder *et al*., 2011); increased hatchability (Bottje *et al*., 2010) and no effect (Zhai *et al*., 2011).

The effect of *in ovo* injection of inorganic salts of Zn, Se, Cu and their combination on growth performance of broiler chickens at days 7 and 35 is shown in Table 3. The final weight of birds from Zn-injected eggs was significantly ($P < 0.05$) highest at day 7 than the values recorded in birds in other treatment groups except in birds from Se-injected and Cu-injected eggs. At day 35, the final weight and weight gain were significantly ($P < 0.05$) affected by the *in ovo* administration of Zn, Se, Cu and their combination. The highest final weight and weight gain were obtained in birds on *in ovo* administration of Cu, though not significantly different from the values recorded in birds from Se-injected eggs and those on the combination of inorganic salts. This is contrary to the report by Richards (1997), that during incubation the mineral use is not constant but shows peak for Zn at 1-2 days post-hatch, particularly in turkey poults. However, the observed development of the birds from Zn-injected eggs at day 7 attest to the role of Zn in post-hatch development by the regulation of the cell turnover (Cui *et al.*, 2003; Joshua *et al*., 2016). The combination of the mineral sources depressed growth possibly from the antagonism occurring between Zn and Cu (Scheideler, 1991). In addition, earlier works (Yair *et al*., 2013; Oliveira *et al*., 2015) indicated that *in ovo* injection of trace minerals singly or combined did not influence increased growth performance of post-hatch chicks. However, Bakyaraj *et al.* (2012) reported that the trace mineral supplemented group (Zn, 80 µg; Se, 0.3 µg; Fe, 160 µg and I, 0.7 µg) gave significantly higher body weight than the control.

The Table 4 shows the effects of *in ovo* injection of inorganic salts of Zn, Se, Cu and their combination on organ development and gut morphology of broiler chickens at days 7 and 35. Significant difference ($P < 0.05$) was obtained only in the proportion of heart at day 7. Birds from Zn-injected eggs, Cu-injected eggs and eggs on the combination of the inorganic salts had significantly ($P < 0.05$) highest values: 1.13, 1.13 and 1.11, respectively. The highest heart percentage obtained was relative to the weight of birds in the treatment group but it corroborates the relevance of Zn in embryonic and early post hatch development (Bartsevich *et al*., 2003). The findings at days 7 and 35 are however opposed to the reports by Uni *et al.* (2003), that *in ovo* feeding results in the improvement of the development of gastrointestinal tracts.

Table 3. Effect of *in ovo* injection of inorganic salts and their combination on growth performance of broiler chicks at days 7 and 35

Parameter	Treatment						
	Control	Zn	Se	Cu	Zn*Se*Cu	SEM	P-Value
At day 7							
Initial weight (g.bird^{-1})	50.08	50.78	51.12	50.12	49.20	0.25	0.13
Final weight (g.bird^{-1})	182.79^{b}	191.74^{a}	186.51^{ab}	185.95^{ab}	183.23^{b}	1.24	0.01
Weight gain (g.bird^{-1})	132.71	140.96	135.39	135.84	134.03	1.19	0.24
Feed intake (g.bird^{-1})	144.26	154.09	151.30	148.44	163.70	3.05	0.32
FCR	1.08	1.10	1.12	1.09	1.23	0.03	0.39
At day 35							
Final weight (g.bird^{-1})	1358.83^{b}	1367.17^{b}	1411.00^{ab}	1481.43^{a}	1445.40^{ab}	16.71	0.05
Weight gain (g.bird^{-1})	1308.76^{b}	1316.39^{b}	1359.89^{ab}	1431.38^{a}	1396.29^{ab}	16.73	0.05
Feed intake (g.bird^{-1})	2052.69	2170.38	2127.65	2074.70	2195.97	27.91	0.27
FCR	1.57	1.66	1.57	1.45	1.56	0.03	0.06

^{a,b} Means in the same row with different superscripts differ significantly ($P < 0.05$); Zn = Zinc-injected; Se = Selenium-injected; Cu = Copper-injected; Zn*Se*Cu = Combination of Zn, Se and Cu; SEM = Standard error of means

Table 4. Effect of *in ovo* injection of inorganic salts and their combination on organ development and gut morphology of broiler chicks at days 7 and 35

Parameter	Treatment						
	Control	Zn	Se	Cu	Zn*Se*Cu	SEM	P-Value
At day 7							
Live weight, g	188.97	184.85	183.47	177.66	194.01	2.65	0.386
Proventriculus, %	1.30	1.19	1.23	1.35	1.21	0.04	0.590
Gizzard, %	7.47	7.67	8.45	8.36	8.01	0.18	0.371
Liver, %	3.94	3.99	3.94	4.08	4.08	0.08	0.968
Heart, %	0.90[b]	1.13[a]	0.92[b]	1.13[a]	1.11[a]	0.03	0.032
Duodenum length, cm.100 g^{-1}	9.22	9.81	10.19	10.41	9.31	0.23	0.379
Duodenum, %	3.03	3.02	3.24	2.99	2.82	0.06	0.394
Jejunum length, cm.100 g^{-1}	24.53	26.56	25.94	26.99	25.08	0.46	0.430
Jejunum, %	5.07	5.19	5.25	5.56	5.55	0.13	0.730
Ileum length, cm.100 g^{-1}	20.10	21.49	22.86	22.41	20.64	0.51	0.395
Ileum, %	3.21	3.71	3.89	3.27	3.67	0.14	0.467
Caecum length, cm.100 g^{-1}	3.60	3.60	3.29	4.11	3.69	0.13	0.371
Caecum, %	1.31	1.01	1.21	1.76	1.92	0.12	0.093
At day 35							
Live weight, g	1346.67	1382.08	1367.00	1327.67	1406.83	24.67	0.880
Proventriculus, %	0.81	0.63	0.79	0.76	0.81	0.04	0.628
Gizzard, %	2.69	2.58	2.61	3.14	2.69	0.09	0.254
Liver, %	2.44	2.39	2.49	2.66	2.53	0.05	0.625
Heart, %	0.75	0.74	0.84	0.81	0.82	0.03	0.816
Duodenum length, cm.100 g^{-1}	2.76	2.62	2.45	2.72	2.65	0.06	0.474
Duodenum, %	1.31	1.29	1.33	1.28	1.33	0.04	0.993
Jejunum length, cm.100 g^{-1}	5.80	6.02	5.63	6.38	6.32	0.12	0.235
Jejunum, %	2.28	2.49	2.37	2.48	2.57	0.07	0.731
Ileum length, cm.100 g^{-1}	5.89	5.97	5.41	6.15	6.32	0.13	0.192
Ileum, %	2.32	2.19	2.24	2.17	2.55	0.06	0.316
Caecum length, cm.100 g^{-1}	1.19	1.15	1.16	1.21	1.20	0.02	0.907
Caecum, %	1.01	0.88	1.01	1.10	1.10	0.05	0.560
Spleen, mg.100 g^{-1}	0.12	0.13	0.14	0.16	0.14	0.005	0.099
Bursa, mg.100 g^{-1}	0.24	0.25	0.20	0.28	0.25	0.01	0.296

[a,b] Means in the same row with different superscripts differ significantly ($P < 0.05$); Zn = Zinc-injected; Se = Selenium-injected; Cu = Copper-injected; Zn*Se*Cu = Combination of Zn, Se and Cu; SEM = Standard error of means

Table 5. Effect of *in ovo* injection of inorganic salts and their combination on cell-mediated immunity and humoral immunity

Treatment	Parameter	
	CMI	Humoral
Control	0.53	4.11
Zn	0.53	4.50
Se	0.57	4.69
Cu	0.58	4.87
Zn*Se*Cu	0.54	4.18
SEM	0.04	0.15
P-value	0.99	0.46

The effect of *in ovo* injection of inorganic salts of Zn, Se, Cu and their combination on cell-mediated immunity and humoral immunity is shown in Table 5. The observed results of non-significance in the growth of immune organs and response to PHA-P or SRBC suggested that *in ovo* injection of Zn, Se, Cu and their combination might not be immunomodulatory in broiler chickens of improved genetic lines as in the study. This is at variance with the findings on *in ovo* injection of lysine (Lotan *et al.*, 1980), arginine (Kidd *et al.*, 2001) and 8 µg.egg^{-1} of inorganic Cu ($CuSO_4$), which were found to enhance the immune response of

Table 6. Effect of *in ovo* injection of inorganic salts and their combination on bone morphometry and mineralization of tibia bone

Parameter	Treatment						
	Control	Zn	Se	Cu	Zn*Se*Cu	SEM	P-Value
Bone morphometry							
Bone weight, g	4.36	4.38	4.63	4.16	4.38	0.33	0.76
Tibia Length, mm	85.39	86.40	86.24	85.35	86.43	0.17	0.05
Tibia bone weight/length index, mg.mm^{-1}	50.97	50.52	53.36	48.64	50.59	3.00	0.70
Tibia Length, mm.kg^{-1}	63.99	64.43	62.47	70.22	62.99	1.67	0.65
Proximal Length, mm	18.85	19.30	19.43	19.39	19.42	0.51	0.79
Proximal Length, mm.kg^{-1}	14.09	14.45	14.10	15.88	14.16	0.37	0.55
Distal width, mm	15.71	15.61	15.83	15.46	15.83	0.05	0.93
Distal width, mm.kg^{-1}	11.77	11.68	11.47	12.58	11.54	0.27	0.76
Mid-Shaft width, mm	7.86	7.57	8.10	7.39	7.82	0.04	0.37
Mid-shaft width, mm.kg^{-1}	5.90	5.65	5.86	6.02	5.69	0.14	0.93
Robusticity index	5.95	6.02	5.77	6.24	6.02	0.37	0.84
Relative Tibia Bone Density	0.33	0.32	0.33	0.34	0.32	0.03	0.96
Mineralization of Tibia bone							
Ash, %	36.79^{ab}	40.96^{a}	40.37^{a}	37.77^{ab}	35.47^{b}	29.84	0.01
Zinc, ppm	118.52	125.55	131.50	125.77	116.96	82.72	0.75
Manganese, ppm	3.49	3.76	4.10	3.78	3.55	2.27	0.73
Copper, ppm	5.06	4.21	3.92	4.12	3.95	2.79	0.15
Calcium, %	17.17	18.79	20.00	18.25	17.51	0.55	0.55
Phosphorus, %	9.97	10.55	11.31	10.21	9.80	6.96	0.57

^{a,b} Means in the same row with different superscripts differ significantly (P < 0.05); Zn = Zinc-injected; Se = Selenium-injected; Cu = Copper-injected; Zn*Se*Cu = Combination of Zn, Se and Cu; SEM = Standard error of means

broiler chickens (Goel *et al*., 2013).

The effects of *in ovo* injection of inorganic salts of Zn, Se, Cu and their combination on bone morphometry and mineralization of tibia bone are shown in Table 6. Significant (P < 0.05) difference was obtained only in the percentage of ash of the tibia bone. The tibiae ash of birds from Zn and Se-injected eggs recorded the highest values: 40.96 and 40.37 %, respectively, while the lowest value (35.47 %) was recorded in the tibiae of birds from eggs injected with the combination of the inorganic salts. This was similar to the ash obtained in the tibiae of birds from the control and Cu-injected eggs. Significant difference in the tibiae bone ash did not influence the robusticity and relative tibiae bone density. Yair *et al.* (2013) observed that bone ash of birds on *in ovo* injection of Zn, Cu and Mn was increased on 19th day of incubation. However, Bello *et al*. (2014) did not observe differences in the tibia ash concentrations of hatchlings +on *in ovo* injection of different levels of 25 (OH)D_3.

CONCLUSION

Research on *in ovo* feeding has established a new science of neonatal nutrition, and this has engendered greater understanding of the developmental transition from embryo to chick.

Zn impacts mostly hatchability, but the combination of the mineral sources at the ratio injected in this study affected negatively the growth performance of the broiler chickens.

Conflict of interest statement

There is absolutely no conflict of interest with any individual or organisation regarding the materials discussed in the manuscript.

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REFERENCES

AOAC. 1995. Official methods of analysis, 16th edition. Association of Official Analytical Chemists, Arlington, Virginia, 1995, 22201 USA.

BAKYARAJ, S. – BHANJA, S.K. – MAJUMDAR, S. – DASH, B. 2012. Modulation of post-hatch growth and immunity through *in ovo* supplemented nutrients in broiler chickens. J*ournal of Science Food and Agriculture*, vol. 92, 2012, p. 313–320.

BAO, Y.M. – CHOCT, M. – IJI, P.A. – BRUERTON, K. 2007. Effect of organically complexed copper, iron, manganese and zinc on broiler performance, mineral excretion and accumulation in tissues. *Journal of Applied Poultry Research*, vol. 16, 2007, p. 448–455.

BARTSEVICH, V.V. – JEFFREY, C. – MILLER, C.C.C. – CARL, O.P. 2003. Engineered Zinc finger proteins for controlling stem cell fat. *Stem Cells*, vol. 21 , 2003, p. 632–637.

BATAL, A.B. – PARR, T.M. – BAKER, D.H. 2001. Zinc bio-availability in tetrabasic zinc chloride and the dietary zinc requirement of young chicks fed a soy concentrate diet. *Poultry Science*, vol. 80, 2001, p. 87–90.

BAUMGARTNER, S. – BROWN, D.J. – SALVSKY, E. Jr. – LEACH, M. Jr. 1978. Copper deficiency in the laying hen. *Journal of Nutrition*, vol. 108, 1978, p. 804–811.

BELLO, A. – HESTER, P.Y. – GERARD, P.D. – ZHAI, W. – PEEBLES, E.D. 2014. Effects of commercial *in ovo* injection of 25-hydroxycholecalciferol on bone development and mineralization in male and female broilers. *Poultry Science*, vol. 93, 2014, p. 1–6.

BHANJA, S.K. – MANDAL, A.B. – JOHRI, T.S. 2004. Standardization of injection site, needle length, embryonic age and concentration of amino acids for *in ovo* injection in broiler breeder eggs. *Indian Journal of Poultry Science*, vol. 39, 2004, p. 105–111.

BLALOCK, T.L. – HILL, C.H. 1988. Studies on the role of iron and zinc toxicity in chicks. *Biological Trace Element Research*, vol. 17, 1988 p. 17–29.

BOTTJE, W. – WOLFENDEN, A. – DING, L. –WOLFENDEN, R. – MORGAN, M. – PUMFORD, N. – LASSITER, K. – DUNCAN, G. – SMITH, T. – SLAGLE, T. – HARGIS, B. 2010. Improved hatchability and post-hatch performance in turkey poults receiving a dextrin-iodinated casein solution *in ovo*. *Poultry Science*, vol. 89, 2010, p. 2646–2650.

CANTRELL, T. – WOOTEN, A. 2003. Methods of fertilizing an avian egg in the shell. United States ovo Biosciences, Inc. (Athens,GA). 6573097.http://www.freepatentsonline.com/65 73097.html.

COLLIN, A. – BERRY, C. – TESSERAUD, S. – REDON, F.E. – SKIBA-CASSY, S. – CROCHET, S. – DUCLOS, M.J. – RIDEAU, N. – TONA, K. – BUYSE, J. – BRUGGEMAN, V. – DECUYPER, E. – PICARD, M. – YAHAV, S. 2007. Effects of thermal manipulation during early and late embryogenesis on thermotolerance and breast muscle characteristics in broiler chickens. *Poultry Science*, vol. 86, 2007, p. 795–800.

CORRIER, D.E. – DELOACH, J.R. 1990. Evaluation of cell-mediated, cutaneous basophilic hypersensitivity in young chickens by an inter-digital skin test. *Poultry Science*, vol. 69. 1990, p. 403–408.

CUI, H. – JING, J. – XI, P. 2003. Pathology of the thymus, spleen and bursa of Fabricius in zinc-deficient ducklings. *Avian Pathology*, vol. 33, 2003, p. 519–524.

ELIBOL, O. – BRAKE, J. 2008. Effect of egg weight and position relative to incubator fan on broiler hatchability and chick quality. *Poultry Science*, vol. 87, 2008, p. 1913–1918.

FDA. 2002. Food additive permitted in feed and drinking water of animals: Selenium yeast. *Federal Register*, vol. 67 (137), 2002, p. 46850–46851.

GAETKE, I.M. – CHOW, C.K. 2003. Copper toxicity, oxidative stress and antioxidant nutrients. *Toxicology*, vol. 189, 2003, p. 147–163.

GOEL, A. – BHANJA, S.K. – MEHRA, M. – PANDE, V. – MAJUMDAR, S. 2013. Effect of *in ovo* copper and iron feeding on post-hatch growth and differential expression of growth immunity related genes in broiler chickens. *Indian Journal of Poultry Science*, vol. 48 (3), 2013 p. 279–285.

GORE, A.B. – QURESHI, M.A. 1997. Enhancement of humoral and cellular immunity by vitamin E after embryonic exposure. *Poultry Science*, vol. 76, 1997, p. 984–991.

GRAU, C.R. – ROUDYBUSH, T.E. – MCGIBBON, W.H. 1979. Mineral composition of yolk fractions and whole yolk from eggs of restricted ovulatory hens. *Poultry Science*, vol. 58, 1979, p. 1143–1148.

HAJIHOSAINI, M. – MOTTAGHITALAB, M. 2004. Effect of amino acid injection in broiler breeder eggs on hatchability and growth of hatched chicken. *Journal of Agricultural Science*, vol. 1, 2004, p. 23–32.

HARGIS, P. – PARDUE, S. – LEE, A. – SANDEL, G. 1989. *In ovo* growth hormone alters growth and adipose tissue development of chicken. *Growth Development and Aging*, vol. 53, 1989, p. 93–99.

HARRIS, E. 1976. Copper-induced activation of aortic lysyl oxidase *in vivo*. *Proceedings of the National Academy of Sciences USA*, 73, 1976 p. 371-374.

JEGEDE, A.V. – ODUGUWA, O.O. – BAMGBOSE, A. M. – FANIMO, A.O. – NOLLET, L. 2011. Growth response, blood characteristics and copper accumulation in organs of broilers fed on diets supplemented with organic and inorganic dietary copper sources. *British Poultry Science*, vol. 52 (1), 2011, p. 133–139.

JOCHEMSEN, P. – JEURISSEN, S.H.M. 2002. The localization and uptake of *in ovo* injected soluble and particulate substances in the chicken. *Poultry Science*, vol. 81, 2002, p. 1811-1817.

JOSHUA, P.P. – VALLI, C. – BALAKRISHNAN, V. 2016. Effects of *in ovo* supplementation of Nano form of Zinc, Copper and Selenium on post-hatch performance of broiler chicken. *Veterinary World*, vol. 9 (3), 2016, p. 287–294.

KAGMI, H. – HANADA, H. 1997. Current knowledge of sexual differentiation in domestic fowl. *World's Poultry Science Journal*, vol. 53, 1997, p. 111–123.

KIDD, M.T. – PEEBLES, E.D. – WHITMARSH, S.K. – YEATMAN, J.B. – WIDERMAN, R.F.J. 2001. Growth and immunity of broiler chicks as affected by dietary arginine. *Poultry Science*, vol. 80, 2001, p. 1535- 1542.

LOTAN, R. – MOKADY, S. – HORENSTEIN, L. 1980. The effect of lysine and threonine supplementation on the immune response of growing rat fed wheat gluten diets. *Nutrition Report International*, vol. 22, 1980, p. 313–318.

LUSCOMBE, N.M. – AUSTIN, S.E. – BERMAN, H.M. – THORNTON, J.M. 2000. Review: An overview of the structures of protein-DNA complexes. *Genome Biology*, 2000, 1:001. 1:001.10.

MCGRUDER, B.M. – ZHAI, W. – KERALAPURATH, M.M. – BENNETT, L.W. – GERARD, P.D. – PEEBLES, E.D. 2011. Effects of *in ovo* injection of electrolyte solutions on the pre- and post-hatch physiological characteristics of broilers. *Poultry Science*, vol. 90, 2011, p. 1058–1066.

MINITAB 17 Statistical Software. 2013. Stable release 17.1.0

MUTUS, M.N. – KOCABAGLI, M.A. – ACAR, N. – EREN, M. – GEZEN, S.S. 2006. The effect of dietary probiotic supplementation on tibial bone characteristics and strength in broilers. *Journal of Poultry Science*, vol. 85, 2006, p. 1621–1625.

NOLLET, L. – VANDERKLIS, J.D. – LENSING, M. – SPRING, P. 2007. The effect of replacing inorganic with organic trace minerals in broiler diets on productive performance and mineral excretion. *Journal of Applied Poultry Research*, vol. 16 (4), 2007, p. 592–597.

NOY, Y. – UNI, Z. 2010. Early nutritional strategies. *World's Poultry Science Journal*, vol. 66, 2010, p. 639–646.

O'DELL, B.L. 1981. Trace elements metabolism in man and animals (TEMA-4). In: Howell, J. M.C., Gawthome, J.M. and White, C.L. (eds.) *Proc. Australian Academy of Science*, Canberra, Australia, 1981, p. 319.

OHTA, Y. – TSUSHIMA, N. – KOIDE, K. – KIDD, M.T. – ISHIBASHI, T. 1999. Effect of amino acid injection in broiler breeder egg on embryonic growth and hatchability of chicks. *Poultry Science*, vol. 78, 1999, p. 1493–1498.

OLIVEIRA, T.F.B. – BERTECHINI, A.G. – BRICKA, R.M. – KIM, E.J. – GERARD, P.D. – PEEBLES, E.D. 2015. Effects of *in ovo* injection of organic zinc, manganese and copper on the hatchability and bone parameters of broiler hatchlings. *Poultry Science*, vol. 94, 2015, p. 2488–2494.

PATON, N.D. – CANTOR, A.H. – PESCATORE, A.J. – FORD, M.J. – SMITH, C.A. 2002. The effect of dietary selenium source and level on the uptake of selenium by developing chick embryos. *Poultry Science*, vol. 81, 2002, p. 1548–1554.

PEEBLES, E.D. – KEIRS, R.W. – BENNETT, L.W. – CUMMINGS, T.S. – WHITMARSH, S.K. – GERARD, P.D. 2005. Relationships among pre-hatch and post-hatch physiological parameters in early nutrient restricted broilers hatched from eggs laid by young breeder hens. *Poultry Science*, vol. 84, 2005, p. 454–461.

REISENFELD, A. 1972. "Metatarsal robusticity in bipedal rats." *American Journal of Physiology*, vol. 40, 1972, p. 229–234.

RICHARDS, M.P. 1997. Trace mineral metabolism in the avian embryo. *Poultry Science*, vol. 76, 1997, p. 152–164.

RICHARDS, M.P. – ROSEBROUGH, R.W. – STEELE, N.C. 1984. Hepatic zinc, copper and iron of turkey embryos (*Meleagris gallopavo*) maintained in long-term, shell-less culture. *Comparative Biochemistry and Physiology*, vol. 78A, 1984, p. 525–531.

ROTRUCK, J.T. – POPE, A.L. – GANTHER, H.E. – SWANSON, A.B. – HAFEMAN, D.G. – HOEKSTRA, W.G. 1973. Selenium biochemical role as a component of glutathione peroxidase. *Science*, vol. 179, 1973, p. 588–590.

RUCKER, R. – MURRAY, J. 1978. Cross-linking amino acids in collagen and elastin. *American Journal of Clinical Nutrition*, vol. 31, 1978, p. 1221–1236.

SCHEIDELER, S.E. 1991. Interaction of dietary calcium, manganese and manganese source (manganese oxide or manganese methionine chelate) on chick performance and manganese utilization. *Biological Trace Element Research*, vol. 29, 1991, p. 217–223.

SEEDOR, J.G. – QUARRUCCIO, H.A. – THOMPSON, D.D. 1991. "The biophosphonate alendronate (MK-217) inhibits bone loss due to ovariectomy in rats." *Journal Bone Mineral Research*, vol. 6 1991, p. 339–346.

SELIM, SH.A. – GAAFAR, K.M. – EL-BALLAL, S.S. 2012. Influence of in-ovo administration with vitamin E and ascorbic acid on the performance of Muscovy ducks. *Emirate Journal of Food Agriculture*, vol. 24, 2012, p. 264–271.

SHIVDASANI, R.A. 2001. Molecular and Transcriptional regulation of megakaryocyte differentiation. *Stem Cells*, vol. 19 (5), 2001, p. 397–407.

SIEGEL, P.B. – GROSS, W.B. 1980. Production and persistency of antibodies in chickens to sheep erythrocytes. 1. Directional selection. *Poultry Science*, vol. 59, 1980, p. 1–5.

STAAL, F.J.T. – WEERKAMP, F. – LANGERAK, A.W. – HENDRIKS, R.W. – CLEVERS, H.S. 2001. Transcriptional control of T-lymphocyte differentiation. *Stem Cells*, vol. 19, 2001, p. 165–179.

TAKO, E. – FERKET, P.R. – UNI, Z. 2004. Effects of *in ovo* feeding of carbohydrates and beta-hydroxy-beta--methylbutyrate on the development of chicken intestine. *Poultry Science*, vol. 83, 2004, p. 2023–2028.

UNI, Z. – SMIRNOV, A. – SKLAN, D. 2003. Pre- and post-hatch development of goblet cells in the broiler small intestine: effect of delayed access to feed. *Poultry Science*, vol. 82, 2003, p. 320–327.

VANDER ZIPP, A.J. 1983. The effect of genetic origin, source of antigen and dose of antigen on immune response of cockerels. *Poultry Science*, vol. 62, 1983, p. 205–211.

VRUWINK, K.G. – KEEN, C.L. – GERSHWIN, M.E. – MARESCHI, J.P. – HURLEY, L.S. 1993. The effect of experimental zinc deficiency on development of the immune system. In: Nutrient Modulation of Immune Response, Cunningham-Rundles, S. (ed.). Marcel Dekker, New York, 1993, p. 263–279.

WATTS, D.L. 1990. Nutrient interrelationships of minerals vitamins and endocrines. *Journal of Orthomology Medicine*, vol. 5 (1), 1990, p. 11–19.

WILLIAMS, B. – WADDINGTON, D. – MURRAY, D.H. – FARQUHARSON, C. 2004. Bone strength during growth: Influence of growth rate on cortical porosity and mineralization. *Calciferous Tissue International*, vol. 74, 2004, p. 236–245.

YAIR, R. – UNI, Z. 2011. Content and uptake of minerals in the yolk of broiler embryos during incubation and effect of nutrient enrichment. *Poultry Science*, vol. 90, 2011, p. 1523–1531.

YAIR, R. – SHAHAR, R. – UNI, Z. 2013. Prenatal nutritional manipulation by *in ovo* enrichment influences bone structure, composition, and mechanical properties. *Journal of Animal Science*, vol. 91, 2013, p. 2784–2793.

ZHAI, W. – BENNETT, L.W. – GERARD, P.D. –PULIKANTI, R. – PEEBLES, E.D. 2011. Effects of *in ovo* injection of carbohydrates on somatic characteristics and liver nutrient profiles of broiler embryos and hatchlings. *Poultry Science*, vol. 90, 2011, p. 2681–2688.