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Influence of *in ovo* Injection of Nano forms of Zinc, Copper and Chromium on Morphometry of Intestine and Immune Response in Broiler Chicken

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A B S T R A C T

The perinatal period is a most crucial time in the development of a young chick as this is a transitional period in which the chicks undergo metabolic and physiological shifts from the utilization of egg nutrients to exogenous feed. The *in ovo* feeding allows the delivery of various supplements directly to chicken embryos which enhances digestive capacity, growth rate, muscle development, feed conversion efficiency and immunity. Hence, the present study was aimed to examine the effect of *in ovo* injection of nano forms of zinc, copper and chromium on morphometry of digestive organs and immune response in broiler chicken. In this study, four hundred fertile broiler eggs from Cobb 430 flock were randomly divided into five treatment (80 eggs each) groups. First treatment was without injection (control), second was injectedwith0.5ml normal saline, third, fourth and fifth treatment eggs were injected with 40, 12 and 0.5 µg/egg of nano zinc, nano copper and nano chromium, respectively. After hatching, 240 day old straight run broiler chicks were allocated into five treatment groups each consisting of four replicates with twelve chicks and all birds were fed with broiler starter diet (1-21 days) and finisher diet (22-42 days) as per NRC (1994). *In ovo* feeding of nano forms of zinc, copper and chromium at18th day of incubation through amniotic route did not influence the growth performance of broiler chicken. However, better serum glucose, total protein and globulin levels were observed in nano trace mineral supplemented groups.

Introduction

The *in ovo* feeding allows the delivery of various supplements directly to chicken embryos. *In ovo* feeding of nutrients improves the early development of digestive function in newly hatched chicks and therefore it enables better utilize nutrients, growth function of gastrointestinal tract and also increases active immunity. Nano technology deals with the conversion of larger molecules to nanometer size. The process of conversion of these larger molecules into tiny one cause changes in the innate physical and chemical nature of the base material.

As the technology engineers to nano level, their properties differ fundamentally and unpredictably compared to a larger scale. The mineral nano particles not only increase the bioavailability of

minerals and also reduce their requirements and excretion (Gopi *et al.*, 2017).

Microminerals that are important to bone formation and strength include Cu, Zn, and Mn which are greatly reduced in concentration in the egg by 17th day of incubation (Yair and Uni, 2011). Cardoso *et al.*, (2007) reported that additional zinc in diet of broiler has improved antibody production. Zinc enhances cells mediating non-specific immunity such as neutrophils and natural killer cells (Shankar and Prasad, 1998). Copper is part of the linkage between elastin and collagen, which gives the bone its tensile strength (Carlton and Henderson, 1964). Supplemental nano chromium picolinate improved the retention of Zn, Fe, Ca and also increased the number of lymphocytes in broiler chicken (Nattapon *et al.*, 2012).

Hence, the objective of this study was to evaluate the effect of *in ovo* injection of nano forms of zinc, copper and chromium on morphometry of digestive organs and immune response in broiler chicken.

Materials and Methods

The present nutritional study was carried out at the Department of Poultry Science, Veterinary College, Hebbal, Bengaluru, Karnataka. The eggs were fumigated and cleaned with egg shell sanitizer and incubated with broad end up in forced draft automatic chicken incubator. Throughout incubation, a dry-bulb temperature ranging from 99-100°F and wet-bulb temperature of 85-87°F were maintained from day 1 to 18 day of incubation. The hatching eggs in the setter were turned by 45° angle on either side at hourly interval until they were transferred to the hatcher.

In ovo injection was carried out on 18th day of incubation with various trace mineral solutions. The trace mineral nano particles were procured from M/s. Matrix Nano Pvt. Ltd., Noida, India. Mineral nano particles were charecterized by Scanning Electron Microscopy (SEM) method. The average particle size were found to be 50-80 nm and the purity was 99 per cent. Required amount of nano trace minerals were weighed and dissolved in the normal saline in such that a concentration of 0.5 ml contained the required amount of trace mineral to be injected in one egg.

On 18th day of embryonic age, the eggs showing viable embryo were injected with nano particles of minerals at the broad end of the egg 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). A validation test using a water soluble dye was carried out to confirm the site (amnion) of deposition of mineral solution. Prior to each injection (between eggs) the needle was immersed in 99.90 % ethanol and replaced between treatments. The injection area was disinfected with 99.90 % ethyl alcohol and the hole was sealed with melted paraffin wax and transferred to hatching trays. After completion of *in ovo* injection, all the eggs were transferred and incubated in hatching trays at the dry bulb temperature of 97.34°F and the wet bulb temperature of 86.36°F without turning from 19- 21 days.

The design of biological experiment is summarized in table 1.A total of 400 fertile eggs with uniform weight were randomly divided into 5 treatment groups with four replicates of 20 eggs each.

After hatching, 240 day old straight run broiler chicks were allocated into five treatment groups each consisting of four replicates with twelve chicks and all birds were fed with broiler starter diet (1-21 days) and finisher diet (22-42 days) as per NRC (1994). The experimental chicks were vaccinated against Marek's disease on day one with HVT strain, against Newcastle Disease on day seven with LaSota strain and against Infectious Bursal Disease on day 14 with Intermediate strain. Booster doses against ND and IBD were given on 21st day and 28th day with F1 and intermediate strains, respectively.

At the end of the experiment, one male and one female from each replicate (six birds from each

treatment) were randomly selected and slaughtered as per the method of Arumugam and Panda (1970). The gut samples (2 cm) were taken from duodenum, jejunum and ileum for his to morphometric study at the end sixth weeks of age. The duodenum was collected from duodenal loop, jejunum (between the duodenal loop and Meckel's diverticulum) and ileum between Meckel's diverticulum and ileo-cecal junction as per the method described by Miller (2007). After fixing the tissues in10 per cent neutral buffered formalin, the tissues were embedded in paraffin. Serial tissue sections of 5 µm thick were cut by a microtome and were fixed on slides. The tissue sections were stained with haematoxylin and eosin stain. The sections were examined for histomorphological studies *viz.*, villus height, width and crypt depth. Blood samples were collected, serum was separated and antibody titer against New Castle Disease virus and Infectious Bursal Disease virus were estimated. The antibody titer against New castle Disease Virus was carried out by HA followed by HI test. The micro-test method as described by Allan and Gouch (1974) was used for the detection of HI titers. The antibodies against IBDV were measured by using Poultry Diagnostic and Research Center (PDRC) indirect ELISA Kit. Each of the steps was followed as per the manufacturer's instructions.

The data obtained in this study were statistically analyzed with general linear models procedure of SPSS statistical software (Version 20 for windows, SPSS). Significant differences among treatment means were tested by Duncan multiple range test. A level of $P \leq 0.05$ was used as the criterion for statistical significance. All the experimental procedures were assessed and approved by the Institutional Animal Ethics Committee from Karnataka veterinary, Animal and Fisheries Sciences University, Bidar and all the institutional guidelines were followed.

Results and Discussion

Effects of *in ovo* nutrition with nano forms of zinc, copper and chromium on duodenal, jejunal and ileal histomorphometry and antibody titers against NDV and IBDV in broiler chicken are summarized in table 2 and table 3, respectively.

The duodenal villi height was significantly ($P \leq 0.05$) higher in nano zinc injected group (1971.88 µm) followed by nano copper (1915.63 µm) and nano chromium (1898.12 µm) injected groups.

There was no significant difference in duodenal villi height between nano chromium and nano copper injected groups. The shortest duodenal villi was noticed in non-injected group (1722.59 µm). The same trend was followed in duodenal crypt depth and jejunal villi height.

Nano zinc injected group recorded significantly ($P \leq 0.05$) wider duodenal villi (206.48 µm) followed by nano copper (191.20 µm) injected group. The non-injected group birds recorded lowest duodenal villi width of 143.48 µm. No significant difference recorded in jejunal villi width among *in ovo* injected groups. Also there was no significant difference recorded in jejunal crypt depth among nano trace mineral groups and also between negative and positive control groups.

The ileal villi height, width and crypt depth at six weeks of age differed significantly ($P \leq 0.05$) between treatment groups. No significant difference noticed in ileal villi height between nano zinc and nano copper injected groups (1470.00 and 1409.55 µm, respectively) and between nano chromium and nano copper injected groups. The shortest ileal villi recorded in non-injected group (1288.18 µm). Significantly widest ($P \leq 0.05$) ileal villi recorded in nano zinc injected group (215.11 µm) and the narrowest ileal villi recorded in non-injected group (153.40 µm).

Significantly ($P \leq 0.05$) higher ileal crypt depth noticed in nano copper injected group (139.33 µm) followed by nano zinc injected group (136.04 µm) than other treatment groups. There was no significant difference noticed between positive and negative control groups.

The log_2 HI titer against NDV was significantly higher (P≤0.05) in *in ovo* injected groups than control groups. Among trace mineral injected groups, the log_2 HI titer against NDV was significantly similar between nano chromium (6.00) and nano zinc (6.02) injected groups and between nano copper (5.34) and normal saline (4.81) injected groups. The lowest log_2 HI titer against NDV was recorded in non-injected group (4.14).

The antibody titer values against IBDV were significantly higher (P≤0.05) in nano trace mineral injected groups than control groups. Nano chromium injected group recorded the highest antibody titer (2752.67) against IBDV than control groups. There was no significant difference noticed between nano trace mineral injected groups and the values ranged from 2632.00 to 2752.67. The least antibody titer value against IBDV was recorded in non-injected group (1816.67).

Increased duodenal and jejunal villi height were recorded by Ferket and Uni (2012); Chous *et al.,* (2009) as has been observed in this study. Smirnov *et al.,* (2006) observed that *in ovo* feeding of carbohydrates to the amniotic fluid of Cobb embryos at 17.5 days of incubation increased jejunal villus surface area at hatch and 3days post hatch about 27% and 21%, respectively than non-injected control. The present study results also supported by Chous *et al.,* (2009) who reported that *in ovo* supplementation of 25-hydroxycholecalciferol increased duodenum and jejunum villi length.

Sogunlei *et al.,* (2018) studied the effect of *in ovo* injection of inorganic salts of Zn (80 µg. egg-1), Se (0.3 µg. egg-1) and Cu (16 µg. egg-1) and found significantly (P<0.05) higher gut morphometry values than control.

In ovo feeding results in gastrointestinal tract (GIT) of hatchlings to be functionally similar to that of conventional 2 day old chicks offered feed immediately after hatch (Uni *et al.,* 2003). They also indicated that during the last 3 days of incubation, the weight of the intestine with a proportion of embryo weight increased from approximately 1% at 17 days of embryonic age to 3.5% at hatch. Rapid intestinal growth is due to increase in cell number and size, accelerated enterocyte proliferation and differentiation and intestinal crypt formation (Uni *et al.,* 2000; Geyra *et al.,* 2001).

Sahoo *et al.,* (2015) found that *in ovo* injection with 0.06 ppm nano zinc improved health status and immune response in broilers. Kadam *et al.,* (2008) concluded that *in ovo* feeding of threonine improved the humoral immune response of broiler chicks. Goel *et al.,* (2012) observed that *in ovo* feeding of Zn enhanced expression of cellular immunity in broilers. Pineda *et al.,* (2012) used silver nanoparticles for *in ovo* administration in broiler eggs and observed beneficial effects in immune status.

No changes were reported in the levels of IgG or IgM when the broiler eggs injected with a hydro colloid of nano copper into the air chamber (Pineda *et al.,* 2013). Jose *et al.,* (2017) concluded that *in ovo* injection in broiler eggs with nano zinc at 0.04 and 0.08 mg per egg had no significant difference (P>0.05) between treatments for cell-mediated immune response and humoral immune response.

Birla *et al.,* (2019) injected 50, 75 and 100 ppm nano ZnO on the first day of incubation into the air cell of broiler eggs and concluded that antibody titer against the influenza virus at 10 days of age did not differ between experimental treatments (P>0.05).

In ovo feeding of nano forms of zinc, copper and chromium at 18^{th} day of incubation through amniotic route improved intestinal histomorphometry and increased antibody titers against NDV and IBDV.

Table.1 Design of biological experiment

Treatments	Nano forms of minerals	*In ovo* feeding of nano trace minerals		No. of 18th day incubated eggs for *in ovo* injection	Growth performance study after *in ovo* injection (No. of birds)
		Basal solvent (ml/egg)	Levels (µg/egg)		
T_1	Control (Non injected)	0	0	80	48
T_2	Injected control	0.5	0	80	48
T_3	Nano zinc	0.5	40	80	48
T_4	Nano copper	0.5	12	80	48
T_5	Nano chromium	0.5	0.5	80	48

Table.2 Mean (±SE) duodenal, jejunal and ileal histomorphometry of broiler chicken at sixth week of age as influenced by *in ovo* feeding of nano trace minerals on18th day of incubation

Experimental group	Description of the treatment	Duodenal histomorphometry			Jejunal histomorphometry			Ileal histomorphometry		
		Villi height (µm)	Villi width (µm)	Crypt depth (µm)	Villi height (µm)	Villi width (µm)	Crypt depth (µm)	Villi height (µm)	Villi width (µm)	Crypt depth (µm)
T_1	Negative control	1722.59 ± 5.91^{c}	143.48 ±2.28^{d}	115.86 ± 2.50^{c}	1397.14 ± 24.48^{c}	145.03 ± 2.09^{b}	119.28 ± 1.16^{b}	1288.18 ± 31.24^{c}	153.40 ±1.93^{d}	124.12 ±1.42^{c}
T_2	Positive control	1785.63 ±26.18^{c}	167.68 ±1.28^{c}	113.42 ± 2.69^{c}	1420.20 ± 6.85^{c}	149.39 ± 3.48^{ab}	122.35 ± 1.93^{b}	1367.41 ±29.86^{b}	168.95 ±1.69^{c}	120.10 ±0.90^{c}
T_3	Nano Zn	1971.88 ±38.20^{a}	206.48 ±10.30^{a}	136.78 ± 2.04^{a}	1644.83 ±26.82^{a}	162.49 ± 2.02^{a}	134.91 ± 1.30^{a}	1470.00 ±19.83^{a}	215.11 ±3.25^{a}	136.04 ±2.67^{a}
T_4	Nano Cu	1915.63 ± 33.26^{b}	191.20 ± 1.6 3^{b}	122.58 ± 1.14^{b}	1559.48 ± 4.75^{b}	163.00 ± 8.96^{a}	135.10 ± 1.45^{a}	1409.55 ± 16.31^{ab}	171.29 ± 3.92^{c}	139.33 ± 1.74^{a}
T_5	Nano Cr	1898.12 ± 14.55^{b}	180.43 ± 3.37b^{c}	125.38 ± 1.68^{b}	1569.36 ± 27.66^{b}	155.89 ± 6.23^{ab}	136.48 ± 1.98^{a}	1372.36 ± 50.7^{b}	187.22 ± 4.06^{b}	127.89 ± 2.59^{b}

Means with in a column bearing different superscripts differ significantly (P≤0.05)

Table.3 Mean (±SE) antibody titers against NDV and IBDV in broiler chicken as influenced by *in ovo* feeding of nano trace minerals on18th day of incubation

Experimental group	Description of the treatment	NDV (log2) HI titer	IBDV titer (ELISA)
T_1	Negative control	4.14 ± 0.39c	1816.67 ± 60.1^{d}
T_2	Positive control	4.81 ± 0.34^{b}	2586.67 ± 25.23^{c}
T_3	Nano Zn	6.02 ± 0.67^{a}	2665.67 ± 44.10^{ab}
T_4	Nano Cu	5.34 ± 0.34^{b}	2632.00 ± 10.00c^{ab}
T_5	Nano Cr	6.00± 0.14^{a}	2752.67 ± 14.53^{a}

Means with in a column bearing different superscripts differ significantly (P≤0.05)

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