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Zinc methionine supplementation during the summer season enhanced the growth, immunity, antioxidants and zinc retention of broiler chickens

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ABSTRACT

The current study is aimed for determining the optimal dose of zinc methionine (Zn-met) in broiler birds during the summer season in order to improve growth, immunity, stress tolerance, and concentration of Zn in vital organs. Total of 240 number of one day-old Vencobb chicks were randomly allocated into six dietary groups, each group consisting of five replicates of 08 birds. The dietary interventions consisted of: T1: Basal diet; T2: Basal diet + inorganic Zn ($ZnSO_4$) 80 ppm; T3: Basal diet + Zn-met 20 ppm; T4: Basal diet + Zn-met 40 ppm; T5: Basal diet + Zn-met 60 ppm, and T6: Basal diet + Zn-met 80 ppm. The trial lasted for 5 weeks. The weight and FCR under T5 and T6 increased significantly. Antibody titres were significantly increased in treated chickens. The experimental chickens in all the Zn treated groups showed a significant increment in catalase and superoxide dismutase activities with a decreased lipid peroxidation rate. Zn concentrations in blood along with different organ samples *viz*. liver, kidney, and breast muscle were significantly higher in all the supplemented birds. There were no observable histological abnormalities evident in liver and kidney tissues in the T5 and T6 groups with the highest Zn-met dose. It was concluded that adding Zn at 60 ppm above basal level resulted in improved performance of the chickens along with protection from heat stress.

Keywords: Antioxidant, Chicken, Growth, Immunity, Zinc methionine

High environmental temperature in the summer season is a major problem for the poultry sector due to poor growth rates, reduced intake of feed, feed efficiency and an increased mortality rate (Liao *et al*. 2018). Along with poor productive performance, it has also been reported that summer stress in broilers affects the redox balance, and the exponential increase in reactive oxygen species production leading to oxidative stress (Tan *et al*. 2010, Xie *et al*. 2015). Chicks are at disadvantage with respect to thermoregulation owing to feather cover and absence of sweat glands, for which they have a quite narrow range of thermal comfort zones. This has been a growing concern in the summer season resulting in huge economic losses in this sector (Rao *et al*. 2016). Low visceral blood flow during heat stress leads to hypoxia, hampering digestion and absorption of nutrients in chickens (Ahmad and Sarwar 2006). Zinc (Zn) has been documented to play a vital role in mitigating such losses in birds. It is essential for various biological functions, including nutrient metabolism, and acts as a regulator, catalyst, and structural component of more than 300 enzymes. Zn supplementation in broiler diets has tremendous health benefits like improved growth, better FCR, as well as improved antioxidants and immunity in birds (Zhang *et al*. 2018).

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Since an animal's body cannot store Zn, there is regular requirement of dietary supplementations (Bao and Choct 2009). The NRC (1994) recommended 40 mg Zn/kg of feed for broiler birds, which may be increased during the summer season. However, in broiler diets, Zn may be used either in an inorganic (Zn oxide or Zn sulphate) or organic form (Zn-met and Zn-glycinate). Zn-met is a highly bioavailable and effective form of Zn supplementation for broilers (Salim *et al*. 2011). Zn-met is lacking in free divalent cations in order to chelate within the lumen of the intestine with phytate, leading to higher absorption and lower excretion to the environment. Since there is paucity of reports on Zn-met in broiler chickens, especially during the stress period of summer, the present research trial was designed in order to estimate an optimum dose of Zn-met in broiler birds during this stress period.

MATERALS AND METHODS

Place of study: The said research trial was carried out during the summer season at the Livestock Farm Complex in the College of Veterinary Science and Animal Husbandry, which is situated between the latitudes of 20.12 N and 20.25 N and the longitudes of 85.44 E and 85.55E. During the experimental period (April and May), the lowest and highest recorded temperatures were 24.8°C (76.64°F) and 41.6°C (106.88°F), respectively, accompanied by 59%. A calculated THI of 95.86 with the corresponding maximum temperature and relative humidity level, clearly indicated prevalence of heat stress conditions especially during peak hours.

Experimental birds and dietary treatments: The study was carried out as per the guidelines laid down by the Institutional Animal Ethics Committee (433/GO/Re/S/01CCSEA). Randomly 240 number of one-day-old Vencobb chicks were assigned to six treatments in 05 replicates having 08 chicks in each. The experimental diet was prepared as per Bureau of Indian Standards (BIS 2007). The ingredient composition and proximate compositions of basal feed (T1) are shown in Table 1. A deep-litter housing system was followed for rearing the experimental birds with regular management practices. In group T1, birds were fed with the basal diet (without Zn addition) considered it as a control. In T2, basal diet with the addition of 80 ppm of inorganic Zn ($ZnSO_4$), and in T3, T4, T5, and T6 birds fed basal diet in addition with 20, 40, 60 and 80 ppm of Zn-met, respectively. The highest dose of Zn supplementation (ppm) by feed was 80, as indicated in the guidelines of Indian standards of BIS 2007 for broiler feed. The chicks received electrolyte infused water, had their wings banded, and were weighed when they arrived. The brooding period was spent providing adequate light, warmth, and ventilation for the chicks. Routine healthcare practices were followed along with recommended vaccination schedule.

Preparation of zinc methionine: Zn-met was prepared by diluting 250 mg of methionine (equivalent to 1.75 mmol) with 8 mL of NaOH (1 mol/L), followed by stirring for 30 minutes. Subsequently, 250 mg of $ZnSO_4$ heptahydrate (equivalent to 0.875 mmol) was introduced into the stirring mixture until the formation of white sediment occurred. The resulting product underwent filtration and was subjected to vacuum drying at 100°C for 2 hours, ultimately producing the Zn-methionine complex in the form of white sediment (Nastiti and Jatmika 2018).

Growth and feed efficiency: Weekly computation of different production parameters was carried out to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) for different phases for growth in broiler chickens.

Blood collection and processing: Two birds from each replication (ten birds in each group) were slaughtered on 36th day i.e. after completion of the research trial in order to collect organs for Zn profiling. Before watering and feeding in the morning, 5 milliliters of blood was collected from heart in a centrifuge tube. The blood was then kept in an inclined posture for 45 minutes to aid in the separation of the serum. Blood was centrifuged at 800x g for 15 minutes to extract the serum, which were preserved (-40°C) for analysis. Further, 2 mL of blood were drawn in a collection vial with acid citrate dextrose as an anticoagulant to estimate the erythrocytic antioxidant status. The blood samples with anticoagulant were centrifuged for 10 minutes at 1000x g to separate the plasma and buffy coats and after that, three rounds of washings in sodium chloride solution were performed on the obtained red blood cells (RBC). 10% RBC hemolysates were prepared by diluting the RBC pellets in distilled water at a 1:10 ratio.

Erythrocytic antioxidant analysis: Nitro blue tetrazolium was used as a substrate to assess the amount of superoxide dismutase (SOD) present in 10% RBC hemolysates (Marklund and Marklund 1974). Lipid peroxidation (LPO) was estimated by Placer *et al*. (1966), to calculate the quantity of malonaldialdehyde (MDA) produced. Catalase was measured in erythrocytes (10% RBC hemolysates) using Bergmeyer's (1983) technique.

Cellular immune response: To test the cell mediated immune response, Phytohaemaglutinin-P (PHAP) (100 µg) in normal saline (0.1 mL) was injected intradermally in the foot-web of 03 birds per replicate on the 36th day of the experiment (Edelman *et al*. 1986). The foot web thickness was measured with a digital vernier scale before and 24 hours post inoculation. Cutaneous Basophilic Hypersensitivity (CBH) is calculated *as:*

$$\text{CBH response} = \frac{\text{Post injection skin thickness}}{\text{Pre-injection thickness}} \times 100$$

Humoral immune response: On day 35, chickens (three from each replicate) were vaccinated against NDV to determine humoral immunity. Seven days after injection, blood was collected and antibody production was assessed by hemagglutination techniques using 96 well U-shaped micro titer plates (Abdallah *et al*. 2009).

Table 1. Ingredients and chemical composition of the basal diet

Particular	Prestarter (%) (1-7th day)	Starter (%) (8-21st day)	Finisher (%) (22-35th day)
	Ingredients		
Maize	51.50	53.50	56.50
Soyabean meal	43.00	39.00	35.00
Vegetable oil	2.00	4.00	5.00
Dicalcium phosphate	1.83	1.83	1.83
Limestone	0.93	0.93	0.93
DL-Methionine	0.05	0.05	0.05
L-Lysine	0.03	0.03	0.03
Vitamin and mineral premix	0.15	0.15	0.15
Common salt	0.51	0.51	0.51
	Chemical composition (% DM)		
Crude protein	22.50	21.20	19.60
Ether Extract	2.90	5.20	5.80
Crude fibre	4.80	4.60	5.10
Total Ash	9.20	8.30	8.90
Nitrogen free extract*	60.60	60.70	60.60
Calcium	1.30	1.20	1.02
Phosphorus	0.55	0.62	0.50
Zinc (ppm)	6.20	5.95	6.18
Metabolisable energy (Kcal/kg)*	2990	3110	3222

*Calculated value

Zn concentration in serum and vital organs: After 5 weeks of the experiment (36th day), serum, vital organs (liver, kidney) and breast muscle were collected from 10 chickens in each treatment group. The samples were wet digested in a Triacid mixture (Nitric acid: Sulphuric acid: Perchloric acid in the ratio of 4: 2: 1) and Zn was estimated by using ICP-OES (Toplab India, Mumbai).

Histopathological examination of vital organ: In order to evaluate the adverse impact of Zn at its maximum concentration using histopathological analysis, samples of the liver and kidney from three broiler chicks in each group (T5 and T6) that were five weeks old were taken and preserved in a 10% formalin solution. Routine histological procedures were used to process the tissues that had been formalin fixed. Following fixation, the tissues underwent an overnight washing in tap water, alcohol dehydration, and clearing by xylene. Tissue samples were placed in paraffin blocks, and 5-micron slices were then created. Hematoxylin and eosin (H&E) staining of the slides was done, and then the slides were examined with a light microscope (Leica DM500).

Statistical analysis: Data collected from the above experiments were analysed using SPSS 20.0 version analysis of variance and Tukey least significant difference test for post-hoc comparisons (Snedecor and Cochran 1994).

RESULTS AND DISCUSSION

Experimental diets and synthesized Zn-met: The protein (CP) and energy (ME) content of the various rations were in the line of the needs of the chicken, as outlined in BIS (2007). Likewise, the Zn levels of the three basal diets were around 6.20, 5.95, and 6.18 ppm, respectively (Table 1). The synthesis of white sediment denotes the formation of a zinc methionine complex. The final product was a white powder with a measured Zn content of 12%. The Zn content in the basal diet was very low as compared to the requirements of birds as suggested by BIS (2007). For efficient chicken production in the summer season, supplementation of Zn within the basal diet is highly recommended.

Effect on production parameters: In 4th and 5th week the average weekly weight of the birds increased significantly ($P<0.05$) in T5 and T6 compared to other treatments (Table 2). In addition, dietary Zn significantly ($p< 0.05$) increased the mean weekly feed intake (Table 2). Similarly, the FCR of T5 and T6 chicks at 5th week was significantly ($p<0.05$) more than T1. This study also showed increased Zn met (T5 and T6) supplementation during summer increased weight gain and FCR compared to inorganic supplemented birds (T2). The birds under T5 and T6 groups showed enhanced ($p<0.05$) average BW gain, feed consumption, as well as FCR in the last two (4th & 5th) weeks of the trial, while no improvement was seen during the first three weeks. It was also observed that Zn-met supplementation at 60 ppm and above improved the BW gain and FCR compared to inorganic Zn (80 ppm) supplemented birds

Table 2. Effect of dietary Zn met supplementation on growth performance of broiler chickens

Attribute	Treatment T1	T2	T3	T4	T5	T6	SEM	p-Value
				Pre-starter (1-7 days)				
FBW Day 7 (g)	107.47	108.93	107.73	109.83	111.03	108.17	4.50	0.485
ADG (g)	9.29	9.50	9.20	9.54	9.85	9.32	0.66	0.180
ADFI (g)	10.24	10.67	10.33	10.48	10.65	10.38	0.52	0.620
FCR	1.10	1.12	1.12	1.10	1.08	1.11	0.07	0.104
				Starter (8-21 days)				
FBW Day 21 (g)	710.43	726.67	708.40	700.60	711.20	713.00	11.20	0.290
ADG (g)	43.07	44.12	42.90	42.19	42.87	43.20	1.75	0.162
ADFI (g)	61.81	62.52	62.76	62.43	63.05	62.19	2.05	0.220
FCR	1.44	1.42	1.46	1.48	1.47	1.44	0.06	0.088
				Finisher (22-35 days)				
FBW Day 35 (g)	1753.80^{c}	1913.90^{b}	1880.00^{bc}	1910.50^{b}	2020.20^{a}	2010.40^{a}	40.45	0.020
ADG (g)	74.53^{b}	84.80^{ab}	83.68^{ab}	86.42^{ab}	93.50^{a}	92.67^{a}	3.28	0.023
ADFI (g)	141.30^{b}	152.50^{a}	152.24^{a}	151.62^{a}	154.09^{a}	154.76^{a}	3.80	0.040
FCR	1.90^{a}	1.80^{b}	1.82^{b}	1.75^{bc}	1.65^{c}	1.67^{c}	0.02	0.028
				Whole phase (1-35 days)				
ADG (g)	48.89^{c}	53.47^{ab}	52.47^{ab}	53.34^{ab}	56.52^{a}	56.21^{a}	1.27	0.025
ADFI (g)	83.29^{b}	88.14^{a}	88.06^{a}	87.71^{a}	88.99^{a}	88.86^{a}	2.80	0.041
FCR	1.70^{a}	1.65^{b}	1.68^{ab}	1.64^{b}	1.57^{c}	1.58^{c}	0.02	0.033

^{abc}Values bearing different superscripts in a row differ significantly ($p<0.05$), FBW: final bodyweight, ADG: average daily weight gain, ADFI: average daily feed intake, FCR: feed conversion ratio during the summer days. This may be due to higher Zn bioavailability, better cell metabolism in Zn-met (60 ppm) supplemented birds compared to $ZnSO_4$ (80 ppm) treated birds. Results on similar lines were observed by Larasati *et al*. (2022), who noted significantly elevated BW gain and feed intake, while concurrently reducing the FCR with inclusion of 60 ppm Zn in the diet. Farhadi *et al*. (2021) also reported that Zn threonine chelate @ 80 and 120 ppm showed the lowest ($p<0.05$) FCR when compared with the inorganic (80 ppm), and unsupplemented control birds. Additionally, Rao *et al*. (2016) found that in broilers raised under heat-stressed conditions, supplementing with organic Zn elevated ($p<0.05$) weight gain and feed intake in comparison to the control. Furthermore, Sahin *et al*. (2005) stated that supplementing quails under heat stress with Zn picolinate (30 or 60 mg/kg) enhanced their growth performance. Zhao *et al*. (2016) reported that supplementing the organic form of Zn (Zn-methionine) at 30 ppm increased feed intake by 12% compared to inorganic Zn ($ZnSO_4$). Similarly, the study by Hosseini *et al*. (2018) demonstrated that the addition of zinc-methionine resulted in higher feed intake, suggesting an improvement in appetite ($p<0.05$). Saleh *et al*. (2018) also revealed that Zn-met supplementation at 60 ppm augmented body mass gain and FCR ($p<0.05$) in broiler chickens. The increased growth may be due to the fact that Zn is required for various metabolic processes like energy and protein metabolism and the synthesis of digestive enzymes, leading to higher feed intake, better nutrient utilisation and a higher growth rate in supplemented birds than control birds (Sunder *et al*. 2013). The role of organic Zn (Zn-met) in synthesis of variety of metalloproteins such as insulin, growth hormone, and IGF-I (insulin-like growth factor-I) for enhancing body metabolism might be another possible explanation for such improvement (Khan *et al*. 2024).

Antioxidant enzyme activity: Zn–met significantly ($p<0.05$) increased erythrocyte SOD and catalase levels in all the treatments and reduced lipid peroxidation by decreasing MDA levels in chickens during summer stress condition (Table 3). It has also been observed that Zn met at higher concentration (T5 and T6) have better stress prevention ability compared to inorganic supplemented birds (T2). Zn prevents the depletion of glutathione (GSH), hence inhibiting lipid peroxidation (Prasad 1998). Additionally, Zn stimulates the synthesis of Zn metallothionein, which effectively scavenges hydroxyl radicals protecting from oxidative stress (Laudadio *et al*. 2012). In the current study, Zn-met supplementation significantly ($p<0.05$) resulted in enhanced levels of erythrocytic SOD and catalase as well as a reduced ($p<0.05$) level of MDA owing to decreased lipid peroxidation, during the summer months. Supplementation of Zn-met @ 60 ppm and above showed better antioxidant status compared to inorganic Zn supplemented birds (T2). Enhanced SOD and catalase activity in the red blood cells of broiler chickens denotes better resiliency to stress, because these are important radical scavenging enzymes against oxygen metabolites during summer stress conditions (El-Moghazy *et al*. 2019). Similar results were also observed when Zn-met was added to the diet. This significantly boosted the activity of SOD and catalase in the tissues and erythrocytes of chickens by scavenging superoxide ions and inhibiting the generation of oxygen free radicals (Saleh *et al*. 2018, Li *et al*. 2019). Zn stimulates synthesis of variety of antioxidant molecules and proteins like glutathione and Cu/Zn superoxide dismutase as well as ameliorates the effects of oxidant promoting enzymes like inducible nitrate synthetase. These effects appear to enhance growth and immunity in broiler chickens (Kloubert and Rink 2015).

Effect on immunity: The antibody titres (log_2) against ND vaccine were significantly ($p<0.05$) improved in T5 and T6 and the CBH response was significantly ($p<0.05$) better in all the Zn supplemented birds in comparison to control in summer season (Table 4). Zn plays an important role in the development and functioning of cells of the immune (innate and adaptive) systems. Zn-met supplementation improved the immune cells from

Table 3. Antioxidant status (U/ mg haemoglobin) of broiler birds

Attribute	Treatment T1	T2	T3	T4	T5	T6	SEM	p-Value
SOD	26.25^{c}	33.39^{ab}	30.85^{b}	33.08^{ab}	40.60^{a}	42.87^{a}	1.30	<0.01
Catalase	14.82^{c}	19.82^{b}	17.34^{b}	20.98^{b}	21.61^{ab}	22.09^{ab}	2.55	0.033
LPO	3.10^{a}	2.03^{bc}	2.56^{b}	1.97^{bc}	1.70^{c}	1.55^{c}	0.05	0.018

^{abcd}Values bearing different superscripts in a row differ significantly ($p<0.05$)

Table 4. Immune response of broiler birds

Attribute	Treatment T1	T2	T3	T4	T5	T6	SEM	p-Value
CBH Response (%)	167.12^{b}	195.16^{a}	189.88^{ab}	194.82^{a}	198.85^{a}	208.88^{a}	8.73	<0.031
Log_2 value HA titre	4.60^{b}	5.65^{ab}	4.80^{b}	6.00^{ab}	6.60^{a}	7.00^{a}	0.03	<0.01

^{abc}Values bearing different superscripts in a row differ significantly ($p<0.05$)

Table 5. Zinc concentration (ppm) in different organs of broiler birds

	Treatment							
Concentration of Zn	T1	T2	T3	T4	T5	T6	SEM	*p*-Value
Serum	10.60^{d}	14.65^{b}	12.37^{c}	14.80^{b}	15.10^{a}	15.95^{a}	0.22	0.022
Liver	20.30^{d}	34.40^{b}	26.20^{cd}	32.00^{c}	37.50^{b}	41.02^{a}	0.05	0.017
Kidney	13.80^{c}	23.50^{b}	18.80^{bc}	19.50^{bc}	26.90^{a}	27.40^{a}	0.04	0.041
Muscle	4.50^{d}	7.30^{b}	5.60^{c}	6.08^{c}	7.50^{b}	8.92^{a}	0.14	0.036

^{abc}Values bearing different superscripts in a row differ significantly ($p<0.05$)

undergoing apoptosis and also aid in maintaining the normal operations of natural killer cells (Fraker 2005). Zn is an essential cofactor for thymulin, which is a thymic hormone needed for the differentiation of immature T-cells (Jena *et al*. 2022). In conjunction with IL-2, it also regulates the release of cytokines and the proliferation of CD8 T-cells (Chand *et al*. 2014). In alignment with our findings, Chitithoti *et al*. (2012) demonstrated that Zn-met supplementation exhibited a superior immune response compared to inorganic supplementation. Both SRBC and ND titers demonstrated enhanced humoral immune response at 60 ppm and 80 ppm dietary Zn-met supplementation. This response is consistent with earlier findings (Bartlett and Smith 2003, Chand *et al*. 2014, Sethy *et al*. 2018), which demonstrated the critical role of Zn in every facet of immunity under heat stress. In male broilers grown in high ambient temperatures, Bartlett and Smith (2003) reported that dietary supplementation of Zn resulted in superior immunity responses elicited through higher weights of lymphoid organs, antibody responses, macrophage phagocytic capacity, and overall IgM and IgG antibody titres. These findings are consistent with findings reported by Chand *et al*. (2014), who showed that dietary Zn supplementation under heat stress conditions significantly raised antibody titres against ND and IBD

Zinc concentration in vital organs: The Zn content in the serum, liver, kidney and breast muscle in the experimental birds under all the treatments was distinctly ($p<0.05$) higher when compared with the birds in control (Table 5), which exhibited better Zn bioavailability in Zn-met supplemented birds. As the Zn concentration increases, the concentration in vital organs also increases linearly. It was also observed that the availability of Zn from Zn-met (T5 as well as T6) was significantly ($p<0.05$) greater compared to T2 birds (inorganic Zn). In the current experiment, the addition of dietary Zn- met significantly raised the Zn concentration in the serum and vital organs such as the liver, kidney, and breast muscle of broiler chickens. It was also observed that the concentration of Zn in organic supplemented birds (60 and 80 ppm) was significantly ($p<0.05$) higher than inorganic supplemented birds. Organic forms of minerals have more bioavailability and utilisation efficiency than their inorganic counterparts. According to Sunder *et al*. (2008), the amount of Zn deposited in the bone, liver, and kidney rose correspondingly with the amount of dietary Zn supplementation. According to Ledoux *et al*. (1991), organic trace elements are stable in the digestive system and are prevented from forming compounds with other feed components that would prevent interactions and maximize absorption. This could be the cause of the increased Zn concentration in muscle, liver, kidney, and serum. Additionally, Kakhki *et al.* (2016) demonstrated a linear link between the broiler chickens' muscle Zn concentration and their dietary Zn level.

Histological examination of vital organ: The histological analysis of the kidney and liver in the highest Zn-met supplemented broilers (T5 and T6) were shown in Fig. 1 to Fig 4. After feeding Zn-met at 60 ppm and 80 ppm in chickens, histological analysis revealed no abnormalities or alterations in the liver and kidney tissues during summer season. Excessive Zn supplementation can produce adverse toxic effects and may damage the organs of animals (Wang *et al.* 2017). In the present experiment, histopathological examination confirmed that Zn-met up to a concentration of 80 ppm have no adverse effects on the vital organs such as the liver and kidney. This corroborated the reports of Chen *et al.,* (2018), who did not find any significant effect of Zn-met supplementation up to 700 ppm on the tissue histology. Similarly, a normal cellular architecture was obtained

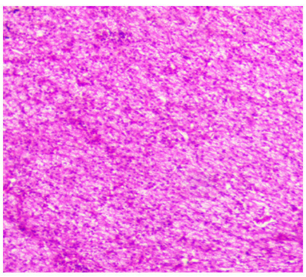

Fig. 1. Liver (T5) showing normal architecture, with healthy parenchyma (H&E ×100) at 5 weeks age

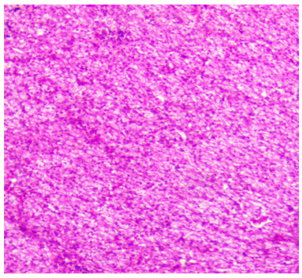

Fig. 2. Liver (T6) showing normal architecture, with healthy parenchyma (H&E ×100) at 5 weeks age

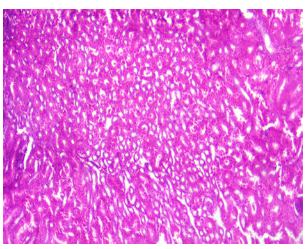

Fig. 3. Kidney (T5) showing normal architecture of tubular epithelium (H&E ×100) at 5 weeks age

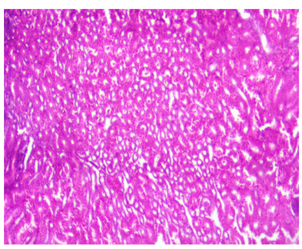

Fig. 4. Kidney (T6) showing normal architecture of tubular epithelium (H&E ×100) at 5 weeks age

in the hepatic and renal histology of Cobb-500 broiler chickens supplemented with 40 ppm Zn for 35 days (Hatab *et al.* 2022).

From this experiment, it may be concluded that dietary supplementation of Zn-met at 60 and 80 ppm in broiler chickens enhanced growth and feed conversion ratio by increasing immunity as well as stress response without any evident negative effect in the summer season. However, responses of 60 ppm and 80 ppm doses of Zn-met supplementation were statistically similar. Therefore, broiler birds can benefit from a 60 ppm Zn-met supplement, which will enhance their growth, immunity, antioxidants and Zn retention the summer season.

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