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Effect of zinc mono glycinate and zinc proteinate on production performance, antioxidant profile, metallothionein gene expression and tibia bone parameters in commercial broiler chicken

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ABSTRACT

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An experiment was conducted for 35 days on Ven Cobb 430Y strain of broilers (n=180, 3 replicates/group, each comprising 20 birds) to evaluate the effect of inorganic and organic forms of Zinc (Zn). The broilers were randomly divided into three groups *viz.*, T1 (basal diet with inorganic Zn as Zinc Sulfate (ZnS) @ 80g/ton of feed), T2 (basal diet with organic Zn as Zinc Mono Glycinate (ZnGly) @ 27.5 g/ton of feed) and T3 (basal diet with organic Zn as Zinc Proteinate (ZnPro) @ 30 g/ton of feed). The overall body weight gain (BWG), feed consumption (FC), feed conversion ratio (FCR), and European Production Efficiency Factor (EPEF) were recorded for five weeks. Dressing parameters, antioxidant profile, metallothionein (MT) mRNA gene expression, and tibia parameters were evaluated at the end of the experiment. Supplementation of organic Zn as Zinc Mono Glycinate (ZnGly) @ 27.5g/ton and Zn Proteinates @ 30.0 g/ton of feed was beneficial in improving ($p<0.05$) FCR without affecting BWG and dressing parameters in broilers. However, EPEF and FC were significantly lower in T3 than T1 and T2. The malondialdehyde (MDA) lipid peroxidation and glutathione peroxidase levels in blood were significantly ($p\leq0.05$) lower and higher, respectively, in T2 than T1, while T2 and T3, as well as T3 and T1, were comparable. Non-significant differences were observed for blood lipid peroxidation/MDA level and plasma total antioxidant capacity between T1, T2, and T3. Percent tibia ash and tibia bone weight were comparable between T1, T2, and T3. However, the tibia Zn content was significantly improved in T2 and T3 than T1. The significantly higher MT mRNA gene expression in liver and duodenal tissue was recorded in T2 and T3 than T1, indicating higher bioavailability of ZnGly and ZnPro than ZnS.

Keywords: Broiler Chicken, Zinc Glycinate, Metallothionein gene, Tibia, Antioxidant

INTRODUCTION

Diets for poultry are generally supplemented with a higher inorganic form of Zinc (Zn) to ensure dietary adequacy and to enhance immune response; however, excess Zn addition could interfere absorption and metabolism of other minerals and result in more fecal excretion of minerals (Ao *et al.*, 2009). Organic minerals are chemically inert, more stable, and less prone to interactions (Ao *et al.*, 2009) and have greater bioavailability (Zhao *et al.*, 2010). Several authors reported the positive effect of the organic form of Zn trace minerals @ less than 50% of the dose level recommended for an inorganic form of Zn. The Zn is a necessary trace mineral (TM) for the functional and structural integrity of more than 300 Zn-dependent enzymes and a large number of functional proteins (Jahanian *et al.*, 2008). It is involved in gene expression, modulation of appetite control, fat absorption, and antioxidant defense (Salim *et al.*, 2008). The weight gain may not be the ideal index to evaluate trace mineral requirements. Mineral-dependent enzyme activities are considered sensitive criteria for determining their requirements (Ao *et al.*, 2011). Hence, the present experiment was carried out to evaluate the efficacy of the organic Zn Glycinate and Zn Proteinate on growth performance, antioxidant profile, and Metallothionein (MT) mRNA gene expression of broiler chicken.

MATERIALS AND METHODS

Experimental design

The study was conducted at Krantisinh Nana Patil College of Veterinary Science, Shirwal, Dist. Satara, Maharashtra Animal & Fishery Sciences University, Nagpur, Maharashtra. Total 180 day-old straight-run broiler chicks of Ven Cobb 430Y strain were randomly distributed to three groups *viz.*, T1 (basal diet with inorganic Zn as Zinc Sulfate (ZnS) @ 80g/ton of feed), T2 (basal diet with organic Zn as Zinc Mono Glycinate (ZnGly) @ 27.5 g/ton of feed) and T3 (basal diet with organic Zn as Zinc Proteinate (ZnPro) @ 30 g/ton of feed). Each treatment had three replicates containing 20 chicks each. The iso-caloric and iso-nitrogenous pre-starter, starter, and finisher basal diets were fed to the birds from 0-10, 11-22, and 23-35 days of age, respectively as per BIS (2007). The ingredient composition of the basal pre-starter, starter, and finisher diet is depicted in Table 1. The mineral premix

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composition of different experimental diets is depicted in Table 2.

Table 1: Percent ingredient composition of broiler Pre-starter, Starter, and Finisher basal diet

Percent Ingredients (%)	Pre-starter 0-10 days	Starter 11-22 days	Finisher 23-35 days
Maize	60.020	64.950	68.932
Soy Bean Meal-De-oiled (46%)	32.430	27.700	23.048
De-Oiled Rice Bran (14%)	0.000	0.000	0.000
Meat cum Bone Meal (45%)	4.000	4.000	4.000
Oil	1.000	1.000	1.770
Lime Stone Powder (LSP)	0.657	0.682	0.557
Di-Calcium Phosphate (DCP)	0.000	0.000	0.000
Mineral Premix	0.010	0.010	0.010
Vitamin Premix	0.050	0.050	0.050
Antioxidant	0.0125	0.0125	0.0125
Salt	0.300	0.300	0.300
Soda bi carb	0.200	0.150	0.100
L - Lysine	0.490	0.405	0.500
DL- Methionine	0.290	0.230	0.200
L-Threonine	0.130	0.100	0.110
Livertonic	0.050	0.050	0.050
Choline Cl (60%)	0.100	0.100	0.100
Toxin Binder	0.050	0.050	0.050
Phytase 5000	0.010	0.010	0.010
NSP Zyme 2000 /Protease	0.010	0.010	0.010
Fixer	0.000	0.000	0.000
Betaine	0.050	0.050	0.050
Maduramycin	0.050	0.050	0.050
Total	100.000	100.000	100.000

Table 2: Mineral premix composition of different experimental diets

Mineral	Required Level in the Premix in mg/kg		
	T1	T2	T3
Copper (Cu)	15.00	15.00	15.00
Iodine (I)	2.00	2.00	2.00
Iron (Fe)	90.00	90.00	90.00
Manganese (Mn)	100.00	100.00	100.00
Selenium (Se)	0.30	0.30	0.30
Zinc (Zn)	80.00*	27.50**	30.00***

*Inorganic Zn as Zinc Sulphate (ZnS), **Organic Zn as Zinc Mono Glycinate (ZnGly), ***Organic Zn as Zinc Proteinate (ZnPro).

Parameters studied

Growth and carcass studies

The broiler birds were weighed individually replicate-wise on day-old and subsequently at weekly intervals to arrive at the overall (0-5 week) body weight gain (BWG) in each treatment. The average feed consumption per bird (FC) was recorded weekly to arrive at the overall FC (0-5 week). The feed conversion ratio (FCR) was calculated from the BWG and FC data weekly to arrive at an overall FCR (0-5 week). The European Production Efficiency Factor (EPEF) was calculated at the end of the fifth week as per Mavromati *et al.* (2018).

EPEF = (Livability (%) x live weight (kg)) / age (days) x FCR) x 100

Two birds per replicate were slaughtered (6 birds/ treatment) on 35th day to evaluate carcass parameters *viz.*, dressing, evisceration, giblet and abdominal fat pad percentage.

Antioxidant profile and MT mRNA gene expression studies

Sample collection

Blood and tissue samples *viz.*, liver and duodenum were collected from six broilers each group. A total of six ml of blood was collected in the morning from various groups on the 35th day. Out of this, 1 ml was transferred to a heparinized vial and used for hemolysate preparation. 2ml of blood was kept at -20^{o}C for extraction of RNA for real-time PCR experiment. 3ml of blood was used for plasma preparation. Approximately 2 gm of tissue samples were collected aseptically in the sterile phosphate-buffered saline, and total RNA was extracted from all tissue samples. A 1g liver tissue sample was homogenized using a tissue homogenizer to measure lipid peroxidation level by malondialdehyde (MDA) assay.

Preparation of Haemolysate

Approximately 2.5 ml of blood samples were centrifuged at 3000 rpm for 15 minutes. Plasma and buffy coats were discarded, and the supernatant was separated. The sedimented RBC cells were washed thrice with chilled 0.85% NaCl solution. The washed erythrocytes were then hemolysed with nine volumes of ice-cold distilled water (100 µl of RBC to 900 µl of water) to prepare 10% RBC hemolysate. Hemolysate was stored in aliquots at -20°C to determine hemoglobin concentration in hemolysate and oxidative stress markers. For Glutathione peroxidase (GSH-Px) measurement, water was replaced with stabilizing solution (2.7 mM ethylenediaminetetraacetic acid and 0.7 mM 2-mercaptoethanol). Hemoglobin concentration in hemolysate was estimated as per Drabkin and Austin (1935).

Antioxidant status parameters

The erythrocyte and liver tissue lipid peroxidation level by MDA assay was estimated as per Placer *et al.* (1966). The GSH-Px was analysed in RBC hemolysate as per Flohe and Gunzier (1984). Plasma total antioxidant activity was measured by using ferric reducing antioxidant power (FRAP) assay (Benzie and Strain, 1999) and final vlues of FRAP were expressed as µmol/ ml $FeSO_4.7H_2O$ equivalent.

Gene expression studies

The Gene expression studies for the following genes were carried out for various groups.

1. *Gallus gallus* metallothionein 4 gene expression
2. *Gallus gallus* Beta actin genes were tested for use as internal control.

Polymorphic blood mononuclear cells (PBMC) were isolated from the blood samples. The total RNA extraction from blood, liver and duodenum tissues was carried out by Trizol method. The cDNA synthesis kit of TAKARA was used for cDNA synthesis.

Primer design and synthesis

Gene-specific primers were designed using online gene script real-time PCR primer design software available online, and the specificity was checked using NCBI BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The details of the primers are as follows:

Gene	F/R	Sequence (3'-5')	Product size (bp)	Reference
Metallothionein	Forward	GAACCGACCCGAACCGAACC	100	NCBI Reference
	Reverse	CAGCGGCAGTTCTTGCACTTG		Sequence: NM_205275.2
Beta Actin	Forward	GAGAAATTGTGCGTGACATCA	152	NM_205518.2 *Gallus gallus*
	Reverse	CCTGAACCTCTCATTGCCA		actin, beta (ACTB), mRNA

The slected gene expression was studied using SYBR green chemistry. The real-time qPCR reaction conditions were

Segment	Remark	Thermal Profile	Time	No of cycles
1	Initial Denaturation	95°C	15 min	1
2	Denaturation	95°C	10 sec	35
	Annealing	58°C	30 Sec	
	Extension	72°C	30 sec	
3	Melt Curve/Dissociation curve analysis	95°C	1 min	1
		65°C	30 sec	
		65°C -95°C	2 degree per min	
		95°C	30 sec	

After the run has ended, cycle threshold (Ct) values and amplification plot for all determined. Mean ΔCt of each target gene with reference to internal control was calculated. The relative gene expression change was expressed as fold gene expression (2^- ΔΔCt).

Tibia bone parameters

The six right tibia bones per group were collected and soft tissue was removed before drying and weighing the bone. The bones were placed in the oven to dry completely and weighed to determine ash. After that, the bones were ashed (600°C in muffle furnace) and the ash weight was recorded. The percentage of ash was determined relative to the dried weight of tibia. The Zn content in tibia was estimated by using AAS (Atomic Absorption Spectroscopy).

Statistical analysis

The experimental data obtained were analyzed statistically (Snedecor and Cochran (1994) as a completely randomized design by analysis of Variance (ANOVA) by using SPSS. The treatment mean differences were compared using Duncan's Multiple Range Test.

RESULTS AND DISCUSSION

Performance parameters

The BWG, FC, FCR, and EFPR of broiler chicken supplemented with inorganic and organic Zn is depicted in Table 3. The organic Zn-supplemented broilers (T2 and T3) had comparable BWG than ZnS-supplemented broilers. The significantly ($p<0.05$) higher FC was observed in T1 followed by T2 and T3 group. Significantly ($p\leq0.05$) better FCR was recorded in T2 (1.65±0.00) and T3 (1.66±0.00) than T1 (1.71±0.01). The EPEF was significantly ($p\leq0.05$) higher in T2 than T3 while T1 and T_2 were comparable. The results indicated that the organic Zn (ZnGly and ZnPro) supplementation was beneficial in improving feed efficiency (FCR) than inorganic Zn (ZnS) with significantly better EPEF by ZnGly. These results correlate with the Liu *et al.* (2011), El-Husseiny *et al.* (2012), Winiarska-Mieczan and Kwiecieñ (2015) and De Marco *et al.* (2017).

Carcass characteristics

The dressing parameters evaluated at 35th day are depicted in Table 4. The supplementation of inorganic and organic Zn did not affect the percentage yield of evisceration, giblet, dressing and fat pad in broilers. In corroboration with our results, Kwiecien *et al.* (2016)

Table 3: Effect of inorganic and organic forms of Zn on overall (0-5 weeks) performance parameters of broiler chickens (Mean±SE)

Groups	Body weight gain (g)	Feed consumption (g)	FCR	EPEF	Livability (%)
T1	1723.23±10.38	2941.13^{b}±14.83	1.71^{b}±0.01	286.53^{ab}±1.58	96.67
T2	1748.86±26.61	2887.23^{ab}±40.69	1.65^{a}±0.00	305.50^{b}±1.02	98.33
T3	1691.62±8.11	2811.91^{a}±9.23	1.66^{a}±0.00	268.75^{a}±12.54	90.00
SEM	11.917	22.682	0.010	6.445	
p-value	0.139	0.032	0.008	0.034	

Means bearing different superscripts within the column differ significantly (p≤0.05)

Table 4: Effect of inorganic and organic forms of Zn on carcass characteristics of the broiler chickens (Mean±SE)

Groups	Evisceration %	Giblet %	Dressing %	Fat pad %
T1	63.39±0.60	4.92±0.21	68.32±0.50	2.38±0.09
T2	63.69±1.45	5.07±0.12	68.75±1.44	1.84±0.23
T3	62.70±0.94	5.08±0.13	67.88±0.85	2.06±0.23
SEM	0.583	0.089	0.556	0.121
p-value	0.837	0.759	0.832	0.201

found that organic Zn below recommended level did not affect the slaughter yield adversely.

Antioxidant profile

The Antioxidant profile is illustrated in Table 5. Results revealed that significantly (p≤0.05) lower MDA lipid peroxidation level (nmol/mgHb) in blood in T2 group as compared to T1 group, while T3 was comparable with T1 and T2. Liver MDA lipid peroxidation level among various treatment groups was non-significant. The T2 group blood GSH-Px level was significantly (pd"0.05) higher than T1, while T3 was comparable with T1 and T2. The total plasma antioxidant capacity (µmol/ml) was numerically highest in T2 (606.46±45.59) than T1 (453.01±61.43) and T3 (479.54±38.68) groups. These results correlate with Sahin *et al.* (2005), Sun *et al.* (2012), Zhang *et al.* (2017) and Zhu *et al.* (2022) indicated that ZnGly and ZnPro supplementation at lower dose level than ZnS improve the antioxidant profile and protect the broilers from oxidative stress.

MT gene expression

The fold MT gene expression relative to endogenous control gene expression for various tissue is illustrated in Table 6. The fold MT gene expression in blood was significantly (p<0.05) higher in groups T2 than T1 while T3 was comparable with T1 and T2 groups. In liver and duodenum tissues, a significantly higher MT fold gene expression was recorded in groups T2 and T3 than in T1.

In accordance with our findings, significantly higher hepatic mRNA gene expression of ZnGly than ZnS was reported by Zhu *et al.* (2022). Wang *et al.* (2019) found significantly higher MT concentrations in broiler liver by organic Zn supplementation than control. Cao *et al.* (2002) also observed that the organic Zn slightly upregulated the MT protein level broiler intestine than inorganic Zn.

Table 6: Effect of inorganic and organic forms of Zn on fold metallothionein gene expression of broiler chickens in different tissues (Mean±SE)

Groups	Blood	Liver	Duodenum
T1	0.23^{a} ±0.05	0.41^{a} ±0.1	7.24^{a} ±1.22
T2	15.92^{b} ±4.8	18.94^{b} ±7.01	41.67^{b} ±6.06
T3	8.66^{ab} ±1.02	17.04^{b} ±5.8	34.32^{b} ±5.55
SEM	8.27±2.19	12.12±3.51	27.74±4.44
p-value	0.005	0.049	0.000

Means bearing different superscripts within the column differ significantly (p≤0.05)

Table 5: Effect of inorganic and organic forms of Zn on antioxidant profile of broiler chickens (Mean±SE)

Groups	Blood Lipid peroxidation/MDA levelnmol/mg Hb	Liver Lipid peroxidation/MDA levelnmol/g	Blood Glutathione peroxidase U/mg Hb	Plasma total antioxidant capacity µmol/ml
T1	2.15^{a} ±0.40	632.88±60.44	17.37^{a} ±2.82	453.01±61.43
T2	1.08^{b} ±0.17	479.67±48.50	27.17^{b} ±2.01	606.46±45.59
T3	1.52^{ab} ±0.12	527.49±68.03	25.92^{ab} ±3.63	479.54±38.68
p-value	0.038	0.210	0.061	0.096

Means bearing different superscripts within the column differ significantly (p≤0.05)
MDA: Malonaldehyde

Tibia bone parameters

The tibia bone parameters are depicted in Table 7. The tibia Zn content (ppm) was significantly (pd"0.05) higher in T2 (173.59±6.87) and T3 (166.11±7.47) groups than T1 (144.59±6.06) group. However, tibial ash percentage and weight did not differ significantly among different groups. The addition of ZnGly significantly increased the Zn accumulation in bone (Kwiecien *et al.*, 2016).

Table 7: Effect of inorganic and organic forms of Zn on tibia bone parameters of broiler chickens (Mean±SE)

Groups	Tibia Zn (ppm)	Tibia ash (%)	Tibia bone weight (g)
T1	144.59[a]±6.06	37.06±1.13	7.16±0.27
T2	173.59[b]±6.87	39.82±1.37	6.66±0.16
T3	166.11[b]±7.47	37.24±1.51	6.81±0.39
SEM	4.753	0.790	0.162
p-value	0.023	0.294	0.558

Means bearing different superscripts within the column differ significantly (p≤0.05)

CONCLUSION

Supplementation of the organic Zn as Zinc Mono Glycinate (ZnGly) @ 27.5 g/ton and Zn Proteinate (ZnPro) @ 30.0 g/ton of feed was beneficial in improving (p≤0.05) feed efficiency (FCR), MT mRNA gene expression in liver and duodenum and tibia Zn content without affecting body weight gain and dressing parameters.

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Conflict of Interest: All authors declare that they do not have any conflict of interest.

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