Effect of substituting increasing levels of organic Zn for inorganic Zn on performance, hematological and serum biochemical constituents, antioxidant status and immune response in rat

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Summary

The effect of replacing dietary Zn supplemented from inorganic ($ZnCO_3$) source with organic Zn (Zn methionine; Zn-met) was investigated in 72 rats (98.42 ± 1.483 g) by randomly allotting to 4 diets (6 replicates/diet, 3 rats/replicate). Basal diet was prepared with purified ingredients without Zn. The control diet (AIN-76A) contained 12 ppm of Zn from $ZnCO_3$ (100-I). In the other diets $ZnCO_3$ was replaced with Zn-met at the rates of 50 (50I:50O), 75 (25I:75O) or 100% (100-O). Weekly body weight and daily feed intake were recorded for 14 weeks. Blood was collected by retro-orbital puncture on the 70th and 80th day to determine haematological and various serum biochemical constituents, and antioxidant enzyme activities in haemolysate, respectively. Rats were antigenically challenged with sheep RBC on day 73 to assess humoral immune response (HIR), and on day 95 for cell mediated immune response (CMIR) and rats were sacrificed at the end of rearing period to collect liver, muscle, pancreas and kidneys for Zn estimation and oxidative stress markers in liver. The data were analysed using completely randomized design. Weight gain and feed intake, hematological and serum biochemical constituents, Zn content in organs (except liver) were not influenced by replacing $ZnCO_3$ with Zn-met. Zinc concentrations in the serum and liver were higher ($P<0.05$) with 50% replacement of $ZnCO_3$ with Zn-met compared to 0 or 100% replacement. Lower ($P<0.05$) lipid peroxidation and higher ($P<0.05$) glutathione peroxidase and glutathione reductase activities were observed with 50 and 75% replacement of $ZnCO_3$ with Zn-met compared to 0 or 100% replacement. Protein carbonyls and reduced glutathione in liver were not affected, while TBARS decreased ($P<0.05$) with substituting Zn-met (50-100%) for $ZnCO_3$. The HIR and CMIR increased with increasing Zn-met supplementation and the highest response was observed with 75-100% replacement of $ZnCO_3$ with Zn-met. It is concluded that replacement of 50 or 75% of $ZnCO_3$ with Zn-met increased antioxidant and immune response in rats with no effect on growth.

Key words: Antioxidant status, Immunity, Inorganic Zn, Organic Zn, Performance

Introduction

Zinc (Zn) influences various biological functions by being a cofactor for more than 300 metalloenzymes (Prasad, 1991). Zinc is essential for growth (Coleman, 1992), humoral (Rink and Gabriel, 2000) and cell mediated (Saha *et al*., 1995) immune responses, and plays an important role in antioxidant defense system (Prasad, 1991). Most of the mineral requirements including Zn have been worked out considering growth as principal criterion (Ammerman *et al*., 1995), the relevance of which is questionable for the current rapidly growing breed and strains of animals, which are more prone to stress. In addition, weight gain may not be an ideal index to evaluate trace mineral requirements, and mineral dependent enzyme activities are considered as sensitive criteria to determine their requirements (Ammerman *et al*., 1995). The mineral requirements of animals are generally supplied using dietary inorganic sources of minerals. The inorganic sources of minerals are generally low in bioavailability and hence higher levels of minerals are included in diet to ensure the dietary adequacy of minerals and to enhance immune response. However, excess mineral (e.g., Zn) supplementation could interfere with absorption and metabolism of other minerals (e.g., Cu), resulting in more faecal excretion of mineral (Ao *et al*., 2009) and causing environmental pollution. To avoid such problems, concept of organic minerals was developed in which mineral is in chemically inert form, more stable and less prone to interactions and has greater bioavailability (Ao *et al*., 2009). An enhanced bio-availability of mineral source may reduce the mineral excretion and increase the retention (Ao *et al*., 2011). Several researchers conducted experiments on complete replacement of inorganic Zn with organic Zn and observed higher mineral status, health and immunity (Ao *et al*., 2009; Ao *et al*., 2011; Nagalakshmi *et al*., 2014). Due to the higher bioavailability and cost of organic minerals compared with inorganic sources, there is a

need to determine the extent of replacing inorganic mineral with organic source at which maximum beneficial effect of organic minerals could be achieved. Thus, the present study was undertaken to investigate the effect of replacing inorganic Zn at graded levels with organic Zn (Zn-methionine; Zn-met) on performance, hematological and serum biochemical constituent, antioxidant status and immune response in rat.

Materials and Methods

Feeding and housing management

Seventy-two weaned female Sprague Dawley rats (98.42 ± 1.483 g), after 10 d acclimatization, were randomly allotted to 4 dietary treatments, with 6 replicates of 3 rats each, in a completely randomized design. The purified diets were prepared with purified ingredients as per the modified formulae of AIN-76A (casein was replaced with EDTA treated soybean meal to minimize Zn contribution from dietary ingredients) that varied in Zn supplementation (Table 1). The Zn supplementation in these diets were 12 ppm, supplemented from inorganic ($ZnCO_3$) and organic (Zn-met) Zn combinations of 100:0, 50:50, 25:75 or 0:100, respectively. The diet was analyzed for crude protein, ether extract and crude fiber (AOAC, 2012). The rats were reared according to guidelines of Institutional Ethics Committee under hygiene conditions with controlled temperature (22-23°C) and photoperiod (12 h/d) in polypropylene cages in the Animal House (College of Veterinary Science, Hyderabad, India). The rats received diets *ad libitum* to ensure 5% of orts, and provided wholesome clean deionized water in nipple fitted polypropylene bottles to minimize Zn contamination throughout the experimental duration of 14 weeks. All the rats were weighed at the beginning and at the end of the experimental period and at weekly intervals before the morning offering of feed and water. The average daily gain was calculated for each rat by subtracting the initial body weight from the final one and divided by the experimental days. The rats were offered measured quantities of respective diets *ad libitum* and the left over residue was weighed the next day to determine the daily feed intake.

Haematological and serum biochemical constituents

Blood was collected from all the rats at 70th day of experiment aseptically through retro orbital puncture in two sets, first set in a clean sterilized heparin coated vacutainers for hematology. For second set, blood was collected in clean sterilized glass tubes to collect serum which was stored in micro-tubes at -20°C for analysis of total protein (Reinhold, 1953), albumin (Gustafsson, 1976), glucose (Cooper and McDaniel, 1970), cholesterol (Wybenga *et al*., 1970) and alkaline phosphatase (Kind and King, 1954). Immediately after blood collection, the samples were analyzed for hematological constituents i.e., white blood cell count (WBC, 100/µL), red blood cell count (RBC, 100/µL), hemoglobin (Hb, g%), mean corpuscular volume (MCV, FI), lymphocyte, monocyte and granulocyte (%) using automatic whole blood analyzer (Huma Count, Med Source Ozone Biochemical Pvt. Ltd., India).

Table 1: Ingredients and chemical composition of modified purified diet (AIN-76A)

Ingredient	Proportion (g/kg diet DM)
Sucrose	500.0
EDTA-treated soybean protein	250.0
Corn starch	100.0
Vegetable oil	50.0
Cellulose	50.0
Mineral mixture[1*]	35.0
Vitamin mixture[2*]	10.0
DL-methionine	3.0
Choline chloride	2.0
Chemical composition (%, on DM basis)	
Dry matter	90.26
Organic matter	93.17
Crude protein	16.96
Ether extract	5.10
Crude fiber	4.92
Zinc (ppm)	0.08
Metabolizable energy (kcal/g)[#]	3.66

[*] Mineral mixture and vitamin mixture was prepared as per specifications for AIN-76A, without Zn supplement. [1] Mineral premix provided (per kg): Ca, 5.2; P, 4.4; Na, 1.1; K, 3.8; Mg, 0.5; Fe, 34.25 mg; Mn, 59.34 mg; Cu, 6.73 mg; Co, 0.02 mg; I, 0.21 mg. [2] Vitamin premix provided (per kg): Vitamin A, 4000 IU; Vitamin D_3, 1000 IU; Alpha-Tocopherol, 64240 IU; Vitamin K, 0.50 mg; Thiamine, 5.90 mg; Riboflavin, 6.29 mg; Niacin, 30.15 mg; Pantotheinc acid, 15.26 mg; Choline, 1040.00 mg; Pyridoxine, 7.12 mg; Folic acid, 2.10 mg; Biotin, 0.21 mg; Vitamin B_{12}, 10.10 µg. [#] Calculated value

Oxidative stress markers and antioxidant enzyme activity

Haemolysate

On day 80 whole blood was collected from all the rats aseptically through retro orbital puncture in heparinized vacutainers and immediately centrifuged at 2000 rpm for 15 min at 4°C to separate buffy coat and erythrocyte pellets, then the leftover erythrocytes were washed thrice with phosphate buffer saline (PBS) (PH ~ 7.4). Equal volume of PBS was added to the obtained packed RBC and then diluted as per required with distilled water. The prepared haemolysate was stored in micro tubes at -20°C for analysis of lipid peroxidation (LPx) (Placer *et al*., 1966), RBC catalase (Bergmeyer, 1983), glutathione peroxidase (GPx) (Paglia and Valentine, 1967) and glutathione reductase (Carlberg and Mannervik, 1985) enzyme activities. The protein and Hb concentrations in haemolysate were estimated colorimetrically according to procedures of Lowry *et al*. (1951) and Cannan (1958), respectively.

Liver

At the end of experiment, all the rats were sacrificed and livers were collected and immediately perfused with normal saline (0.9%) to reduce RBC contamination. The liver samples were then fixed in liquid nitrogen and

stored at -20°C for estimating thiobarbituric acid reacting substances (TBARS) (Balasubramanian *et al*., 1988), reduced glutathione (GSH) (Moron *et al*., 1979) and protein carbonyls (Levine *et al*., 1990).

Immune response

Blood was withdrawn from the jugular vein of sheep and RBCs were preserved in Alseveir solution. It was then suspended in phosphate buffered saline for later use. After 72 days of the experiment, all the rats were antigenically challenged twice, at one week intervals with sheep RBC (0.5×10^9 cells/100 g, i.p.). The blood was withdrawn from retro-orbital plexus of all antigenically challenged rats after one week of primary and secondary challenge. Twenty-five µL of serum was serially diluted with 25 µL of phosphate-buffered saline. Sheep RBC (0.025×10^9 cells) was added to each of these dilutions and incubated at 37°C for 1 h. The rank of minimum dilution that exhibited hemagglutination was considered as the antibody titer. On the 95th day of the experiment, cellular immune response (CMI) was assayed by footpad reaction method in rats. The increase of the paw volume induced by an injection of sheep RBC (0.025×10^9 cells) in the sub-plantar region of right hind paw, was assessed after 24 and 48 h. The mean percent increase in paw volume was considered as delayed type of hypersensitivity reaction, and considered as an index of cell-mediated immunity. The volume of the left hind paw, injected similarly with phosphate buffered saline served as control.

Estimation of Zn

Approximately 1 g of tissue sample (liver, muscle, kidney and pancreas) or 1 ml of serum was wet digested by diacid method in which samples were kept overnight with 10 ml of concentrated HNO_3, the next day again 10 ml of HNO_3 and 2-3 ml of perchloric acid were added to sample and digested on hot plate at 180-200°C till the dense white colour fumes appeared. The digested sample was then transferred to a 50 ml volumetric flask by washing several times with double distilled water through Whatman filter paper No. 42 and final volume was made to 50 ml. These processed samples were transferred to separate sterilized plastic vials till analysis with an atomic absorption spectrophotometer (Varian AA 240) with standard solution of different concentrations of Zn in order to estimate the final concentration of Zn in the organs. The concentration was expressed as parts per million (ppm).

Statistical analysis

The obtained data were analyzed statistically by one way ANOVA as completely randomized design using statistical package for social sciences (SPSS) 16th version. A polynomial contrast was used to test the linear or quadratic effects of different levels of Zn-met on measured traits. The means were compared by the Duncan's multiple range test.

Results

Body weight, feed intake, haematological and serum biochemical constituents

Weekly and average body weight changes, average daily gain (ADG) (Table 2), feed intake (FI) (Table 3), haematological and serum biochemical constituents (Table 4) were not affected in rats fed diets supplemented with various combinations of inorganic (I) and organic (O) Zn sources (50I:50O, 25I:75O and 100% organic), except for the quadratic response on feed intake during the 4th to 6th week with partial replacement (50 and 75%) of $ZnCO_3$ with Zn-met. The feed intake increased with replacement of 50 and 75% of $ZnCO_3$ with Zn-met during the 4th ($P<0.05$), 5th ($P<0.01$) and 6th ($P<0.05$) weeks, but the intake with 100% Zn-met was comparable to 100% $ZnCO_3$.

The linear regression equation calculated for body

Table 2: Body weight changes (g) in rats fed zinc supplemented diets from various combinations of inorganic and organic sources

Day	100-I	50I:50O	25I:75O	100-O	SEM	P-value	
						Linear	Quadratic
Initial	98.83	98.83	97.06	98.94	1.48	0.92	0.76
7	143.2	140.4	141.1	142.6	1.68	0.94	0.55
14	158.1	158.4	158.4	158.6	1.66	0.93	0.98
21	174.8	173.3	172.0	174.2	1.76	0.85	0.62
28	179.5	177.4	179.7	177.2	1.95	0.80	0.97
35	188.3	187.9	189.8	188.4	2.05	0.95	0.76
42	203.9	204.2	209.3	207.1	2.03	0.77	0.50
49	221.2	222.8	226.9	225.8	2.15	0.89	0.69
56	236.1	235.6	238.9	240.4	2.42	0.95	0.79
63	247.4	245.1	254.1	248.1	2.58	0.50	0.25
70	248.8	246.7	260.7	249.0	2.67	0.15	0.074
77	256.8	255.7	270.2	254.9	2.70	0.075	0.054
84	262.5	264.9	277.6	261.5	2.65	0.058	0.086
91	271.3	272.1	284.2	267.5	2.61	0.060	0.075
98	273.9	278.2	288.8	273.8	2.57	0.071	0.15
Average	200.6	210.8	208.8	197.7	3.10	0.70	0.090
Average daily gain (g/d)	1.785	1.837	1.956	1.785	0.030	0.653	0.061

SEM: Standard error of the means, O: Organic, and I: Inorganic

Table 3: Daily feed intake (g) in rats fed zinc supplemented diets from various combinations of inorganic and organic sources

Week	100-I	50I: 50O	25I: 75O	100-O	SEM	P-value	
						Linear	Quadratic
1	14.67	15.00	15.17	15.00	0.175	0.49	0.50
2	14.76	15.11	15.61	15.46	0.183	0.13	0.50
3	14.83	14.60	14.89	14.85	0.207	0.86	0.84
4	12.85^{b}	13.19^{a}	13.43^{a}	11.67^{b}	0.258	0.17	0.047
5	9.38^{b}	11.37^{a}	11.75^{a}	9.45^{b}	0.282	0.001	0.001
6	14.67^{ab}	14.91^{ab}	15.70^{a}	13.47^{b}	0.290	0.23	0.024
7	17.92	17.34	17.76	17.53	0.378	0.83	0.83
8	16.41	17.19	16.76	15.98	0.275	0.50	0.18
9	19.04	18.18	19.49	19.02	0.395	0.75	0.82
10	18.80	18.13	19.75	18.54	0.381	0.80	0.73
11	21.73	20.07	21.50	20.79	0.608	0.81	0.71
12	21.46	20.64	20.60	22.77	0.398	0.26	0.063
13	21.17	20.16	19.82	22.67	0.441	0.26	0.067
14	20.55	20.29	19.34	21.56	0.354	0.50	0.080
Average	17.02	16.87	17.26	17.06	0.117	0.64	0.923

SEM: Standard error of the means, O: Organic, and I: Inorganic. ^{ab} Means with different superscripts in a row differ significantly ($P<0.05$)

Table 4: Haematological and serum biochemical constituents in rats fed zinc supplemented diets from various combinations of inorganic and organic sources

Attribute	100-I	50I:50O	25I:75O	100-O	SEM	P-value	
						Linear	Quadratic
Haematological constituent							
WBC ($\times10^3$/cu mm)	12.17	10.68	10.15	10.71	0.494	0.29	0.32
RBC ($\times10^6$/cu mm)	9.08	9.49	9.26	9.16	0.226	0.99	0.60
Haemoglobin (%)	15.02	15.47	15.08	14.90	0.285	0.79	0.61
HCT (%)	59.35	62.42	59.77	59.30	1.314	0.82	0.53
MCV (%)	65.30	65.83	64.50	65.00	0.360	0.49	0.99
MCH (%)	16.48	16.39	16.12	16.32	0.147	0.59	0.65
Lymphocytes (%)	79.17	79.50	77.72	82.03	1.004	0.46	0.34
Monocytes (%)	4.67	3.96	3.58	3.73	0.563	0.56	0.73
Granulocytes (%)	15.95	16.15	18.68	13.80	0.735	0.53	0.079
Serum biochemical constituent							
Glucose (mg/dl)	85.23	63.66	66.81	68.24	3.538	0.12	0.17
Cholesterol (mg/dl)	149.02	149.78	148.55	148.27	5.702	0.95	0.97
Alkaline phosphatase (IU/L)	64.50	66.76	64.42	58.77	3.236	0.53	0.57
Total protein (g/dl)	6.91	6.97	6.52	6.56	0.231	0.50	0.99
Albumin (g/dl)	4.27	3.97	4.21	3.77	0.095	0.14	0.71
Globulin (g/dl)	2.64	3.00	2.31	2.79	0.256	0.92	0.91

WBC: White blood cells, RBC: Red blood cells, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, SEM: Standard error of the means, O: Organic, and I: Inorganic

weight gain (g) with days was $Y=3.519-0.032X$ with R^2 of 0.414 and was significant ($P<0.01$). Similarly, the quadratic regression equation of body weight gain (g) with days was $Y=4.696-0.095X+0.001X^2$ with R^2 of 0.710, which was significant ($P<0.01$).

Zinc status, oxidative stress markers and anti-oxidant enzymes

The Zn concentration in serum and liver increased quadratically with replacement of increasing levels of $ZnCO_3$ with Zn-met (Table 5). The serum concentration of Zn was highest ($P<0.05$) by replacing 50% of $ZnCO_3$ with Zn-met. The Zn content of liver increased ($P<0.05$) with feeding of 50I:50O and 25I:75O Zn combination diets compared to sole supplementation of Zn either from inorganic or organic sources. Organic Zn supplementation had no effect ($P>0.05$) on Zn concentration in muscle, kidneys and pancreas (Table 5).

A quadratic response was observed for lipid peroxidation, glutathione reductase and peroxidase activities in haemolysate with replacement of $ZnCO_3$ with Zn-met at graded levels (Table 6). The lipid peroxidation reduced quadratically ($P<0.05$) with replacement of 50 or 75% of $ZnCO_3$ with Zn-met, while the lipid peroxidation in 100% Zn-met group was comparable to 100% $ZnCO_3$ group. The GPx activity ($P<0.01$) improved with 50I:50O and 25I:75O combination than 100I:0O. No effect of Zn-met supplementation was observed on RBC catalase activity, while the glutathione reductase activity reduced ($P<0.01$) with feeding of 100% Zn-met compared to 100% $ZnCO_3$, with no effect of partial replacement.

Table 5: Concentration of zinc in serum and organs of rats fed diets supplemented with combinations of organic and inorganic zinc sources

Attribute	100-I	50I:50O	25I:75O	100-O	SEM	P-value	
						Linear	Quadratic
Serum (mg/L)	1.117[b]	2.617[a]	1.486[b]	1.421[b]	0.184	0.87	0.017
Liver (µg/g dry weight)	148.4[b]	176.7[a]	177.7[a]	148.3[b]	5.950	0.99	0.016
Muscle (µg/g dry weight)	78.19	71.90	76.84	84.34	5.073	0.63	0.53
Pancreas (µg/g wet weight)	23.08	17.71	26.98	18.51	1.933	0.80	0.69
Kidneys (µg/g dry weight)	119.6	117.6	119.8	140.4	5.222	0.18	0.29

SEM: Standard error of the means, O: Organic, and I: Inorganic. [ab] Means with different superscripts in a row differ significantly (P<0.05)

Table 6: Oxidative enzyme activities in haemolysate and liver of rats fed diets supplemented with combinations of organic and inorganic zinc sources

Attribute	100-I	50I:50O	25I:75O	100-O	SEM	P-value	
						Linear	Quadratic
Lipid peroxidation (nM MDA/g Hb)	67.19[a]	54.48[b]	56.73[b]	67.88[a]	2.394	0.82	0.013
RBC catalase (µM/min/Hb)	5.40	4.47	4.74	3.79	0.288	0.085	0.99
Glutathione peroxidase (µM/mg protein)	1.48[b]	3.04[a]	3.14[a]	2.66[ab]	0.196	0.009	0.003
Glutathione reductase (µM/mg protein)	25.03[a]	25.48[a]	24.93[a]	17.23[b]	1.032	0.003	0.002
Liver							
TBARS (nM MDA/mg protein)	0.0133[a]	0.0102[b]	0.0088[b]	0.0087[b]	0.00083	0.043	0.35
Protein carbonyls (nM/mg protein)	0.773	0.860	0.889	0.897	0.0390	0.28	0.63
Reduced glutathione (µM/mg protein)	23.68	29.25	13.76	11.71	1.712	0.38	0.89

TBARS: Thiobarbituric acid, SEM: Standard error of the means, O: Organic, I: Inorganic. [ab] Means with different superscripts in a row differ significantly (P<0.05)

Table 7: Humoral and cell mediated immune response in rats fed diets supplemented with combinations of organic and inorganic zinc sources

Attribute	100-I	50I:50O	25I:75O	100-O	SEM	P-value	
						Linear	Quadratic
Humoral immune response, HA (log_2 titres)							
Primary response	4.00[b]	6.00[a]	6.67[a]	6.17[a]	0.285	0.005	0.006
Secondary response	6.83[b]	7.33[ab]	8.17[a]	6.33[b]	0.1949	0.59	0.017
Cell mediated immune response (% increase in paw volume)							
24h	2.13	2.36	2.38	1.99	0.124	0.57	0.16
48h	4.03[c]	5.57[b]	6.56[b]	7.28[a]	0.315	0.001	0.46

SEM: Standard error of the mean, HA: Haemagglutination, O: Organic, and I: Inorganic. [abc] Means with different superscripts in a row differ significantly (P<0.05)

Dietary inclusion of Zn-met linearly lowered (P<0.05) the TBARS concentration in liver, while the protein carbonyls and reduced glutathione content in liver remained unaffected by replacement of $ZnCO_3$ with Zn-met (Table 6).

Immune response

The primary (P<0.01) and secondary (P<0.05) humoral immune responses increased quadratically with replacement of $ZnCO_3$ with Zn-met (Table 7). The primary immune response was higher (P<0.01) with Zn-met, with no effect of level of replacement of $ZnCO_3$. The secondary immune response was highest with 75% replacement of $ZnCO_3$ with Zn-met. The cell mediated immune (CMI) response at 24 h post immunization was not affected by Zn-met supplementation, and was comparable among the four dietary treatments. At 48 h, CMI response improved linearly (P<0.01) by increasing the concentration of Zn-met in diet (Table 7).

Discussion

No effect of replacing $ZnCO_3$ with Zn-met on weekly body weight changes, ADG and FI in current study was in agreement with the findings of Nagalakshmi *et al*. (2012), who observed optimum growth and feed intake in rats with 12 ppm Zn supplementation as $ZnCO_3$ and no further beneficial effect was observed on weight gain and feed intake with increase in the Zn supplementation more than 12 ppm (24-48 ppm). This clearly indicated that 12 ppm Zn supplemented from $ZnCO_3$ was sufficient to achieve optimum growth performance in rats. Similarly, Nagalakshmi *et al*. (2014) observed no effect of total replacement of $ZnCO_3$ (12 ppm) with Zn-met and other organic sources (Zn proteinate or Zn propionate) on weekly body weight, ADG and FI in rats. While El-Husseiny *et al*. (2012) reported improvement in growth performance in broilers with partial replacement (50%) of inorganic minerals (ZnO, MnO, $CuSO_4$) with

organic sources improved growth performance in broiler chicken.

The haematological and serum biochemical constituents were comparable among dietary treatments and the values obtained for various haematological and biochemical parameters were in normal range as suggested by Hrapkiewicz and Medina (2013). The results are in accordance with the findings of Nagalakshmi *et al*. (2013), who observed comparable haematological and serum biochemical constituents among the rats supplemented with different levels (12, 24 and 36 ppm) of Zn from $ZnCO_3$, indicating that 12 ppm Zn supplementation from $ZnCO_3$ (inorganic Zn) in rats was sufficient to maintain the various haematological and serum biochemical parameters balance. Similarly, Nagalakshmi *et al*. (2014) observed no effect of 100% replacement of $ZnCO_3$ with various sources of organic Zn (Zn methionine, Zn proteinate or Zn propionate) on haematological and serum biochemical constituents. The comparable serum concentrations of glucose, cholesterol, alkaline phosphatase, total protein, albumin and globulin between inorganic and organic Zn supplemented at various levels (50-100%) were corroborated with findings of earlier studies on guinea pigs (Shinde *et al*., 2006).

Zinc status, oxidative stress markers and antioxidant enzymes

Several researchers observed higher Zn retention in the body when Zn was supplemented in organic form compared to inorganic form (Ao *et al*., 2009; Ma *et al*., 2011). The improvements of Zn concentrations in serum and liver with supplementation of $ZnCO_3$ and Zn-met (50I:50O; 25I:75O) compared to supplementation of Zn solely from either organic or inorganic clearly indicate the higher bioavailability of Zn through combination diets than Zn supplementation solely from either organic or inorganic sources. Organic minerals usually utilize peptide or amino acid uptake pathway rather than normal mineral ion uptake pathway of inorganic ion in the small intestine, so supplementation of mineral in combination of inorganic and organic sources might provide more Zn to target tissue by utilizing both routes (Power and Horgan, 2000). The GPx and malondialdehyde (MDA) levels are considered as oxidative stress markers because they are involved in the antioxidant defense system (Meister, 1988; Del Rio *et al*., 2005). In current study, Zn-met replacing 50 or 75% of $ZnCO_3$ increased the GPx activity, and lowered the MDA levels in haemolysate (lipid peroxidation) and liver (TBARS). The antioxidant status was better in rats supplemented with Zn as either 50I:50O or 25I:75O combination compared to 100% supplementation of Zn either from organic or inorganic sources (Table 6). Higher bioavailability of Zn (Table 5) in rats fed diets supplemented with combination of $ZnCO_3$ and Zn-met (50I:50O; 25I:75O) might have protected the cells from the oxidative damage as indicated by lower LPx levels in haemolysate and TBARS concentration in liver (Table 6) or by improving the Zn dependent antioxidant enzymes activity (Meister, 1988; Peerapatdit and Sriratanasathavorn, 2010).

Immune response

Zinc is essential for maintenance of natural killer cells activity and phagocytosis of macrophages and neutrophils (Rink and Gabriel, 2000) and is necessary for the activity of thymic hormone, thymulin (Bach, 1983), which induces differentiation in immature T-cells (Saha *et al*., 1995). In this experiment, the improved humoral and cell mediated immune response by dietary incorporation of Zn-met might be due to the higher bioavailability of Zn from organic source (Ao *et al*., 2009) resulting in the higher antioxidative status in rats (Table 6). This could have protected the immune cells from damage by free radicals and improved the activity of immune cells (Ao *et al*., 2009; Osaretin and Gabriel, 2009). Similarly, Nassiri Moghaddam and Jahanian (2009) observed better immune response in broilers by replacing 75% of $ZnSO_4$ or ZnO with Zn-met compared to 100% replacement of inorganic source with organic source. Feng *et al*. (2010), working with broilers, observed improvement of immunoglobulin levels (IgA, IgM and IgG) and immune organs index (thymus, spleen and bursa of fabricius) with dietary replacement of $ZnSO_4$ (120 mg/kg) with Zn-glycinate (90 or 120 mg/kg).

Overall, the results indicated that dietary replacement of 50 or 75% of $ZnCO_3$ with Zn-met improved the Zn concentration in serum and liver, antioxidant enzyme activities and immunity, without affecting the performance of rats compared to Zn supplementation from 100% of $ZnCO_3$ or Zn-met. There was no difference in animal response with 50 or 75% replacement of the Zn sources. Thus, 50% of Zn supplemented from $ZnCO_3$ could be replaced by Zn-met with higher Zn status and immune response in rats.

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Conflict of interest

Authors declare that they have no conflicts of interest.

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