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Effect of dietary zinc nanoparticle supplementation on mineral balance, tissue minerals status and immune response in Guinea pigs

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ABSTRACT

The present study was designed to examine the effect of dietary supplementation of zinc nanoparticles on mineral balance, tissue minerals status and immune response in guinea pigs. Depending upon the source of Zn, 30 weaned male guinea pigs were randomly allocated to five different groups having 6 animals in each group in CRD. Animals under group T_1 (control) were fed with basal diet with inorganic Zn ($ZnSO_4$), whereas, animals were fed with organic Zn (Zn methionine) in group T_2, commercial zinc nanoparticle in group T_3 and two other zinc nanoparticles prepared in our laboratory in groups T_4 and T_5, respectively. They were supplemented with 20 mg Zn/kg feed on dry matter basis for 90 days. Results revealed that intake and balance of nitrogen and minerals (calcium, phosphorus, Zn and Cu) was not affected (P>0.05) by supplementation zinc nanoparticles. The level of Zn in liver and testes was significantly higher (P<0.05) in the zinc supplemented groups, when compared to that of control group, however, the mean values of Cu, Fe and Mn were comparable (P>0.05) among different groups, irrespective of the source of Zn. The mean antibody titre was significantly (P<0.05) higher in the commercial nanoparticle supplemented group as compared to other groups. As evidenced in our study spanning 90 days of trial, zinc nanoparticles can be safely supplemented up to 20 ppm level in the diet of guinea pigs.

Keywords: Guinea pig, Humoral immune response, Nanoparticle, Zinc

Zinc (Zn) is an important component of various metalloenzymes and activator of more than 300 enzymes (Wang *et al*. 2010; Bun *et al*. 2011; Salim *et al*. 2012), thereby, plays an important role in metabolic activity in animals. Zinc also plays an important role in thyroid metabolism (Gupta *et al*. 1997) and regulation of gene expression (Tsai *et al*. 2016), which in turn regulates body metabolism (Suttle 2010). So far, the major dietary source of Zn for animals has been its inorganic salts, such as zinc sulphate, zinc oxide and zinc chloride (Mohanta and Garg 2014). However, bioavailability of Zn from inorganic sources has been reported to be reasonably low (Zhao *et al*. 2014). Therefore, researchers have always been searching different mineral sources with higher bioavailability. Among such alternate sources, organic minerals particularly metal-amino acid complex like Zn-methionine and metal proteinates have been extensively studied (Mohanta and Garg 2014). However, it has been demonstrated that nanoparticles of minerals have higher bioavailability as compared to their normal sized particles due to their novel characteristics such as greater specific surface area, higher surface activity, high catalytic efficiency and stronger adsorbing ability (Rajendran *et al.* 2013; Sheikh *et al*. 2016; Swain *et al*. 2016). Nanoparticle also showed enhanced gene expression (Zhang *et al*. 2016) and newer transport and uptake mechanisms leading to higher absorption efficiencies (Liao *et al.* 2010; Albanese *et al*. 2012; Rajendran *et al.* 2013). However, so far, very little information is available on the suitability and efficacy of Zn nanoparticles in the diet of the animals. Therefore, present experiment was carried out to study the effect of supplementation of Zn nanoparticles on nutrient digestibility, balance of minerals in blood and tissues and immune response in guinea pigs.

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MATERIALS AND METHODS

Synthesis of Zinc nanoparticles: Zinc nanoparticles were synthesized by two different methods, i.e. direct precipitation method (Becheri *et al.* 2008) using $ZnCl_2$ as starting material and alkali as precipitating agent and Chitosan/ZnO method (AbdElhady 2012) using chitosan and ZnO as starting materials and alkali as precipitating agent.

Characterization and quantification of Zinc: Particle size of synthesized products (zinc nanoparticles) was measured using transmission electron microscope (JEOL JEM-1400) at ICAR-National Institute of High Security Animal Diseases, Bhopal. For this purpose, a small amount of synthesized nanoparticles were suspended in 750 µL of triple distilled water in a micro-centrifuge tube. Then sample suspension was poured on copper grid and left for drying. When the sample was completely dried, it was loaded in the electron microscope for viewing. Concentration of zinc in the synthesized product was measured using atomic absorption spectrophotometer (Model 4141, Electronic Corporation of India Limited, Hyderabad, India).

Animals, feeding and management: Thirty weaned healthy male guinea pigs (*Cavia porcellus*) were procured from Laboratory Animal Research Section of ICAR-Indian Veterinary Research Institute, Izatnagar and divided into five groups of six each on the basis of body weight (289±3.5 g) following completely randomized design. Experimental animals were housed in a well-ventilated room adopting similar management and hygienic practices throughout the experiment. Clean drinking water was provided *ad lib*. The experiment was approved by Institutional Animal Ethics Committee (IAEC) and conducted under guidelines prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Experimental feeding was similar in all five groups, except for the source of zinc, which was zinc sulphate in group T_1, organic zinc (Zn-methionine) in group T_2, commercial zinc nanoparticles (SRL chemicals) in group T_3, zinc nanoparticles prepared by the method of Becheri *et al.* (2008) in group T_4 and by the method of AbdElhady (2012) in group T_5. The weighed amount of the basal diet [20% ground maize (*Zea mays*) hay, 32% ground maize grain, 22.5% bengal gram (*Cicer arietinum*), 9% soybean (*Glycine max*) meal, 9% wheat (*Triticum aestivum*) bran, 6% fish meal, 1% mineral mixture (without zinc), 0.45% common salt and 0.05% vitamin C, 20 ppm zinc along with about 10–20 g green maize fodder daily (to meet their vitamin A requirement)] was offered daily at 09.30 h to meet the nutrient requirement (ICAR 2013). The amount of feed was regularly revised at weekly interval as per body weight of the animals during entire experimental feeding period of 90 days.

Estimation of minerals in tissue: Concentration of trace minerals (Zn, Cu, Fe, Mg, Co) in serum and tissue samples were estimated by atomic absorbance spectrophotometer (Model no. 4141, Electronic Corporation of India, Hyderabad, India) at the end of experiment (90 days)

Chemical Analysis: The feed and fodder were dried in a hot air oven until a constant weight is achieved and then ground to pass 1 mm sieve and stored in airtight polythene bags for further analysis. Analyses of proximate principles (AOAC 2012), fibre fractions (Van Soest *et al.* 1991; AOAC 2012), calcium (Talapatra *et al.* 1940) and phosphorus (AOAC 2012) were done using standard procedures. Ashed feed and faeces samples were used to prepare a hydrochloric acid (HCl) extract after dissolving the ash in 0.1 N HCl. Urine samples were ashed after drying the urine in silica crucibles on a water bath and an HCl extract was prepared and used for estimation of minerals. 1 ml serum was wet digested in triple acid and the extract is then diluted to 25 ml with triple distilled water for estimation of minerals. Calcium (Ca) content in feed, faeces and urine samples were analysed by the method of Talapatra *et al.* (1940), while phosphorus (P) was determined colorimetrically (AOAC 2012). Zn, Cu, Fe, Co and Mn were estimated in an air-acetylene flame on an atomic absorption spectrophotometer (Model no. 4141, Electronic Corporation of India, Hyderabad, India).

Humoral immune response: To assess the humoral immune response, animals were sensitized with *Salmonella pullorum* antigen and serum antibody production was monitored on 0, 7, 14, 21 and 28 days post sensitization using standard tube agglutination test (STAT). Serial two fold dilutions of serum samples were made in normal saline. 0.5 ml of plain antigen of which the O.D. (optical density) was adjusted to brown opacity tube 2 was added to 0.5 ml of diluted test samples in test tubes. The tubes were shaken well and overnight incubation was done at 37°C. STAT titre was expressed as the reciprocal of the highest dilution showing 50% clearing.

Statistical Analysis: The data generated in the present study were analysed as a randomized block design with the General Linear Model procedure using SPSS 20.0 based on the statistical mode. Means were tested using Duncan's multiple range tests (Snedecor and Cochran 1994). Significant difference among the treatments was established at $P<0.05$.

RESULTS AND DISCUSSION

The chemical compositions of the concentrate mixture and green maize used for feeding of guinea pigs were similar to the values reported by ICAR (2013) and were adequate to meet the nutrient requirement of the guinea pigs at the quantity determined (Table 1).

Intake and balance of nitrogen and minerals: The analysis of the data on intake and balance of nitrogen and minerals (calcium, phosphorus, Zn and Cu) revealed no significant ($P>0.05$) difference in the intake, outgo and balance of nitrogen and minerals among five groups (Table 2). Intake and balance of N, Ca, P, Zn and Cu was positive and comparable ($P>0.05$) in all experimental groups (Table 2) indicating that supplementation of zinc either through organic source (Zn methionine) or as its nanoparticles at 20 ppm level in the diet had no effect on retention of these nutrients. Contrary to our observations,

Table 1. Chemical composition (% DMB) of basal diet and green fodder

Nutrient	Basal diet	Green maize
Organic matter	92.51	92.24
Crude protein	20.29	4.90
Ether extract	2.35	2.24
Neutral detergent fibre	48.77	70.69
Acid detergent fibre	20.08	39.67
Calcium	2.17	0.85
Phosphorus	1.20	0.58
Zinc (ppm)	18.00	7.75
Copper (ppm)	14.98	11.42
Iron (ppm)	50.20	10.60
Manganese (ppm)	39.72	17.11
Cobalt (ppm)	1.77	0.36

Table 2. Intake and balance (g/d) of nitrogen and minerals in guinea pigs

Attribute	T_1	T_2	T_3	T_4	T_5	SEM	P value
Nitrogen							
Intake	0.72	0.75	0.78	0.78	0.79	0.02	0.884
Feces outgo	0.21	0.21	0.21	0.22	0.23	0.01	0.619
Urine outgo	0.43	0.46	0.46	0.45	0.48	0.01	0.859
Balance	0.07	0.09	0.11	0.10	0.08	0.01	0.828
Calcium							
Intake	0.49	0.51	0.53	0.53	0.54	0.01	0.890
Feces outgo	0.36	0.37	0.37	0.38	0.41	0.01	0.858
Urine outgo	0.09	0.08	0.10	0.09	0.07	0.01	0.431
Balance	0.04	0.06	0.07	0.06	0.05	0.01	0.718
Phosphorus							
Intake	0.28	0.30	0.31	0.31	0.31	0.01	0.890
Feces outgo	0.22	0.21	0.20	0.24	0.22	0.01	0.791
Urine outgo	0.04	0.05	0.07	0.03	0.06	0.01	0.155
Balance	0.02	0.04	0.04	0.04	0.03	0.01	0.887
Zinc							
Intake	0.83	0.87	0.91	0.91	0.92	0.02	0.890
Feces outgo	0.39	0.34	0.34	0.34	0.38	0.02	0.923
Urine outgo	0.04	0.04	0.07	0.05	0.06	0.01	0.425
Balance	0.40	0.49	0.50	0.52	0.48	0.03	0.818
Copper							
Intake	0.35	0.37	0.38	0.38	0.39	0.01	0.890
Feces outgo	0.19	0.20	0.21	0.21	0.22	0.01	0.943
Urine outgo	0.02	0.03	0.06	0.04	0.04	0.01	0.831
Balance	0.14	0.13	0.12	0.14	0.12	0.01	0.943

Table 3. Tissue mineral concentration (mg/kg fresh basis) in guinea pigs

Attribute	T_1	T_2	T_3	T_4	T_5	SEM	P valve
Copper							
Liver	24.9	28.7	31.7	32.0	34.5	1.15	0.166
Spleen	13.1	14.1	13.5	14.5	13.0	0.67	0.964
Kidney	10.6	8.9	9.6	7.9	9.0	0.43	0.547
Testes	3.0	4.0	3.9	3.7	3.2	0.24	0.717
Hair	12.2	10.7	11.9	14.0	13.0	1.20	0.932
Zinc							
Liver*	14.1^{a}	22.4^{b}	23.4^{b}	23.2^{b}	22.8^{b}	0.96	0.048
Spleen	39.6	42.3	44.2	42.2	43.0	1.56	0.966
Kidney	22.5	24.7	24.1	23.3	24.5	0.53	0.809
Testes*	16.2^{a}	23.4^{b}	24.1^{b}	22.7^{b}	22.7^{b}	0.77	0.039
Hair	108.3	111.8	103.3	107.9	105.2	1.40	0.335
Iron							
Liver	44.6	45.8	44.1	46.1	43.3	0.42	0.098
Spleen	517.2	513.3	519.1	516.4	521.1	4.22	0.986
Kidney	45.3	42.0	47.0	45.5	47.6	1.08	0.503
Testes	66.4	66.0	62.1	64.3	65.0	1.56	0.941
Hair	4.8	4.0	4.2	4.8	4.3	1.10	0.888
Manganese							
Liver	4.7	4.8	4.8	5.3	5.1	0.13	0.585
Spleen	38.8	39.4	36.2	40.7	41.1	1.25	0.768
Kidney	6.7	6.9	6.7	7.3	7.3	0.16	0.619
Testes	7.8	7.9	8.1	8.2	8.2	0.18	0.968
Hair	2.0	2.6	2.4	2.1	2.9	0.31	0.362

*Mean bearing different superscript in a row differ significantly ($P<0.05$).

Table 4. Anti-*Salmonella pullorum* log_{10} STAT titre of guinea pigs in different groups

Group	Period (days post vaccination)						P value		
	0 d	7 d	14 d	21 d	28 d	Group mean	G	P	G×P
T_1	ND	1.60	1.90	2.51	1.90	1.98^a	0.045	<0.001	0.040
T_2	ND	1.60	1.90	2.58	2.05	2.03^a			
T_3	ND	1.68	1.98	2.66	2.13	2.11^b			
T_4	ND	1.60	1.98	2.51	2.05	2.03^a			
T_5	ND	1.53	1.90	2.43	1.90	1.94^a			
Period mean	ND	1.60^A	1.94^B	2.54^C	2.02^B				

^{ab}Mean with different superscripts in a column differ significantly ($P<0.05$); ^{ABC}Mean with different superscript in a row differ significantly ($P<0.01$).

in an experiment, Garg *et al*. (2008) reported an adverse effect on Ca balance in 20 ppm organic Zn supplemented lambs. However, most of the studies did not show any adverse effect on intake and balance of minerals and nitrogen with different sources of Zn. Nitrogen balance was not affected in kids supplemented organic zinc (Kala 2009; Garg *et al*. 2008). Mandal *et al*. (2007) also did not observe any difference in balance of Ca, P and nitrogen in bulls supplemented 35 ppm organic Zn as compared to control group. Aliarabi *et al.* (2015) did not find any difference in Zn and Cu balance in lambs supplemented 20–40 ppm of organic Zn or $ZnSO_4$. Supplementation of 20 ppm Zn either through Zn methionine or $ZnSO_4$ did not affect balance of Zn in goats (Jia *et al.* 2009). Similar results were also obtained in kids (Kala, 2009) and bulls (Mandal *et al*. 2007) supplemented 5–40 ppm Zn either from organic or inorganic sources.

Tissue mineral profile: The effect of dietary supplementation of different sources of Zn on tissue mineral concentration in guinea pig presented in Table 3. The level of Zn in liver and testes was significantly higher ($P<0.05$) in the zinc supplemented groups, when compared to that of control. But when compared among Zn treatments there was no significant difference among the groups with different source of zinc. However, there was no such difference in levels of Zn in other tissues/organs. The mean values of Cu, Fe and Mn were comparable ($P>0.05$) among different groups, irrespective of the source of Zn. Comparable ($P>0.05$) Cu, Fe, and Mn content in liver, spleen, kidney, testes and hair among different groups indicated that supplementation of Zn either through organic source (Zn methionine) or as its nano particles at 20 ppm level in the diet had no effect on tissue and hair minerals status in guinea pigs except that of Zn. Shinde *et al.* (2006) also reported that supplementation of 20 ppm organic Zn or $ZnSO_4$ had no effect on Cu, Fe, Mn and Co content in liver and spleen. Similar results were observed in piglets fed 50–3,000 ppm organic Zn or ZnO (Wang *et al*. 2010).

Similarly, comparable ($P>0.05$) values of Zn in kidney, spleen and hair of guinea pigs indicated that different sources of Zn supplementation had no effect on Zn status in kidney, spleen and hair. However, level of Zn was significantly ($P<0.05$) higher in the liver and testes of guinea pigs in the Zn supplemented groups as compared to control group. It indicated that supplementation of Zn either through organic source (Zn methionine) or as its nano particles at 20 ppm in the diet increased the levels of Zn in liver and testes. It appears that smaller particles could translocate via the lymphatic system to the liver and spleen (Jani *et al*. 1990). Smaller particles that are capable of being taken up by the villus epithelium may directly enter the blood stream and are then predominantly deposited in the liver resulting in higher liver mineral level (Hillery *et al.* 1994). Contrary to our observations, Jahanian and Rasouli (2015) observed significant higher liver Zn level in broiler on replacement of 40 ppm inorganic Zn from Zn methionine. Sridhar *et al*. (2015) also reported significantly higher concentration of Zn in liver of broilers supplemented 30 ppm organic Zn as compared to 40 ppm $ZnSO_4$. It suggested that bioavailability of Zn from organic sources and as its nanoparticles was higher as compared to $ZnSO_4$ (inorganic source), which could be the possible reason of higher Zn content in liver and testes in our experiment. However, similar effect across groups irrespective of source of Zn also gives an idea that process of preparation and form of nano-mineral has also certain effect on bioavailability and performance.

Humoral immune response: The serum antibody production against *Salmonella pullorum* vaccine antigen in guinea pigs at different days post vaccination (DPI) as measured by STAT is presented in Table 4. The mean antibody titre was significantly ($P<0.05$) higher in the commercial nanoparticle supplemented group as compared to other groups. Need for Zn is more in the highly proliferating cells in the body, especially the immune system (Suttle 2010; Maares and Haase 2016). Zinc depletion causes decrease in immune cell functions (Maares and Haase 2016), e.g. all functions are impaired (monocytes), cytotoxicity is decreased (natural killer cells), and phagocytosis is reduced (neutrophils). The normal functions of T cells are impaired and B cells undergo apoptosis. An adequate Zn supplementation can reverse the impaired immune functions caused by Zn deficiency (Ibs and Rink 2003). Significantly ($P<0.05$) higher STAT titer in T_3 as compared to other groups indicated that supplementation of commercial grade Zn nano particles at 20 ppm level in the diet improved the immune status of the guinea pigs. However, the other nano Zn supplemented groups (T_4 and T_5) had no such effect. There appears to be very few reports on the effect of Zn nano particle supplementation on immune response of the animals. Similar to our observation, Roy *et al.* (2014) observed that levels of immunoglobulin (IgG and IgE) in serum were significantly higher in mice administered Zn nano particles at 0.2–3 mg as compared to control. In conclusion, supplementation of 20 ppm commercial Zn nanoparticles improved humoral immune response in guinea pigs. The liver and testes zinc level was higher irrespective of source of zinc. Furthermore, it was evident that dietary supplementation of Zn nanoparticles can be done safely up to 20 ppm level in guinea pigs as observed in the experiment for 90 days duration.

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