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Effect of Replacing Inorganic Zinc with Lower Levels of Organic Zinc on Zinc Retention and Follicular Population in Rats

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ABSTRACT

An experiment of 10 weeks duration was conducted on 48 weaned female rats (285.2 ± 1.95 g) of strain Sprague Dawley to study the effect of replacing dietary Zn (12 ppm) supplementation from inorganic ($ZnCO_3$) to organic (Zn nicotinate; Zn-nic) source at lower (6 or 9 ppm) or equal (12 ppm) levels on Zn retention and ovarian follicular population. Higher Zn concentration (on day 42) in serum (P<0.01) and liver (P<0.05) was noticed with 9 and 12 ppm Zn supplementation as Zn-nic compared to other dietary treatments. Zn deposition in pancreas, muscle and kidney was comparable among the dietary treatments. In comparison to 12 ppm inorganic Zn, RBC catalase and glutathione peroxidase activities (42nd d) improved (P<0.05) with 9 and 12 ppm organic Zn. Significantly (P<0.05) highest and lowest serum progesterone concentration was observed with 9 or 12 ppm Zn as Zn-nic and 6 ppm as Zn-nic supplementation, respectively. Regular estrous cycle was observed with 9 or 12 ppm Zn supplementation as Zn-nic, while 30% rats fed on other dietary treatments showed irregular estrous cycle. The proportion of primary follicles was lowest (P<0.01) and that of corpus luteum was highest (P<0.01) with 12 ppm Zn supplementation from Zn-nic, compared to other dietary treatments. The study indicated that Zn concentration in diets could be reduced by 75% (9 ppm) when supplemented as Zn nicotinate without affecting estrous cycle and follicular population. In addition, replacement of 12 ppm inorganic Zn with 12 ppm organic Zn significantly improved its retention and follicular population.

Keywords: Ovarian follicular population, Rats, Zinc retention, Zinc nicotinate

Zinc (Zn) is component of more than 300 metalloenzymes and influences various biological functions including reproduction (Chasapis*et al.* 2012). In addition to that, it plays a vital role in antioxidant defense system (Eide, 2011). The role of Zn in male reproductive system is well established and its deficiency leads to failure of spermatogenesis and lowers the testosterone secretion (Roy *et al.* 2013). Similarly, Zn plays a vital role in female reproductive system and necessary for normal ovulation and fertilization. Brown and Pendland (2007) stated that Zn is essential for progesterone synthesis. Female reproductive system is negatively influenced by oxidative stress and could be overcome by dietary supplementation of antioxidant nutrients such as Zn (Agarwal *et al.* 2012). Follicular population study provides important information about ovarianfunction,

especially the relationship between follicular number and the factors that regulate the survival and maturation of follicles at any stage of their development (Myers *et al.* 2004).

Recently concept of organic minerals is gaining importance as source of minerals supplementation in diets. In organic sources, mineral is in a chemically inert form, more stable and less prone to mineral and nutrient interactions, soabsorbed and circulated to target tissues very efficiently (Swiatkewicz *et al.* 2014). Some researchers in mineral nutrition have shown that lower levels of Zn supplementation in organic form is sufficient to meet the requirements (Moghaddam and Jahanian, 2009; Feng *et al.* 2010; Ao *et al.* 2011) of minerals. Many organic sources of Zn are available i.e., Zn proteinate, Zn amino acid complex, Zn poly saccharide, Zn methionine, Zn glycinate and their efficiency has been tested in many livestock species in terms of growth, immunity and reproduction. Zn nicotinate is an organic source of Zn with nicotinic acid (vitamin) as the ligand and the literature on supplementing Zn-nic as source of Zn in diets is not available. Hence, the present experiment was carried out to study the effect of replacement of inorganic zinc with graded levels of zinc nicotinate (organic source) on Zn retention, estrous cycle and follicular populationin rats.

MATERIALS AND METHODS

Feeding and housing management

Forty eight female rats of Sprague Dawley strain (SD) with an average body weight of 285.20 ± 1.947 g were housed in polypropylene cages in the Animal House of College of Veterinary Science, Hyderabad. Rats were managed under hygienic conditions with controlled temperature (22-23^{O}C) and photoperiod (12 h/d) for an experimental duration of 10 weeks. The rats were reared as per the guidelines of Institutional Animal Ethics Committee of the college. The rats were randomly distributed to 24 replicates with 2 rats in each and these 24 replicates further randomly allotted to 4 dietary treatments. The rats were offered respective diet *ad libitum* with provision of free access to wholesome clean deionised water through polypropylene bottles having nipples. A basal diet (BD) was prepared with purified ingredients but without Zn (Table 1) as per the modified formulae of AIN- 76A (casein was replaced with EDTA treated soybean meal to minimize Zn contribution from dietary ingredients. To the basal diet supplied 12 ppm Zn was added from inorganic source (Zn carbonate) (control diet). The three experimental diets were BD supplemented with Zn nicotinate (Zn-nic) so as to supply Zn at concentration of 6, 9 and 12 ppm. Blood was collected on day 42 in two sets by retro-orbital puncture to analyse the progesterone and Zn concentration in serum and antioxidant enzymes in haemolysate. At the end of experiment all rats were sacrificed to collect the organs (liver, pancreas, muscle, kidneys and ovaries). The collected organs (liver, pancreas, muscle and kidneys) and serum were stored in deep freezer(-20^{O}C) and thawed to room temperature at the time of analysis of Zn. The ovaries were fixed in buffered formalin for follicular populationstudy.

Table 1. Ingredient composition of Purified diet (AIN-76A)

Ingredient	Proportion, g/kg diet
Sucrose	500.0
Casein	250.0
Corn starch	100.0
Oil	50.0
Cellulose	50.0
Mineral mixture*	35.0
Vitamin mixture*	10.0
DL-methionine	3.0
Choline chloride	2.0

*Mineral mixture and vitamin mixture was prepared as per specifications for AIN-76A without Zn supplement.

Antioxidant enzymes activity

The blood collected in clean heparinized vacutainers was centrifuged at 2000 rpm for 15 minutes at 4^{O}C to separate buffy coat and erythrocyte pellet. The erythrocytes were washed thrice with phosphate buffer saline (PH 7.4). The packed RBC obtained was mixed with an equal volume of phosphate buffer saline and then diluted as per requirement with distilled water. The oxidative enzymes viz., RBC catalase and glutathione peroxidase in haemolysate were estimated as per the procedures of Bergmeyer (1983), Paglia and Valantine (1967) respectively. The haemoglobin and protein concentration in haemolysate were estimated colorimetrically as per the procedure described by Cannan(1958) and Lowry *et al.* (1951), respectively.

Estimation of Zn

Approximately 1 g of sample (liver, pancreas, muscle and kidneys) and 1 ml of serum was wet digested by diacid method in which samples were kept for overnight with 10 ml of concentrated HNO_3, next day again10 ml HNO_3 and 2-3 ml perchloric acid was added to sample and digested on hot plate at 180-200^{O}C till the dense white colour fumes appeared. The digested sample was then transferred to a 50ml volumetric flask by several

washing with double distilled water through Whatman filter paper No. 42 and final volume was made to 50ml. These processed samples were transferred to a separate sterilized plastic vials till analysed with Atomic Absorption Spectrophotometer (Varian AA 240) with standard solution of different concentrations of elements in order to estimate the final concentration of Zn in the organs. The concentration was expressed as parts per million (ppm).

Progesterone concentration, vaginal smear study and quantification of follicles

Serum progesterone was estimated using commercial ELISA kit (Omega diagnostics, pathozyme, progesterone, Scotland, UK). A vaginal smear study was conducted for two consecutive estrous cycles after 56 days of experiment (Marcondes *et al.* 2002). The ovarian tissue fixed in buffered formalin was embedded in paraffin (58.6^{O}C). The sections of the paraffin blocks were cut by a rotator microtome (5ì) and sections were stained by eosine and haematoxylin and observed under a compound microscope. The quantification study of follicles was performed according to Patil *et al.* (1998). Diameters and morphologies of the follicles were used to classify the follicles.

Statistical analysis

The data was subjected to one way analysis of variance. The differences between the means were tested for significance using Duncan's multiple range test (Duncan, 1955). All the statistical procedures were carried out as per the methods described by Snedecor and Cochran (1994) by programming and processing in computer.

RESULTS AND DISCUSSION

Zinc retention and antioxidant enzyme activities

In present study, Zn deposition in pancreas, muscle and kidney was not affected with reducing Zn supplementation by 50% (6 ppm) as Zn-nic compared to 12 ppm Zn supplementation as $ZnCO_3$. Also to this significantly (P<0.05) higher Zn concentration was noticed in serum and liver with 9 and 12 ppm Zn supplementation as Zn-nic compared to other dietary treatments (Table 2). Organic trace minerals being stable in digestive tract, prevents formation of complexes with other dietary components and thus, results in greater absorption of minerals (Ao *et al.* 2011). This might be the reason for higher Zn concentration in serum and liver with supplementation of 9 ppm (lower) or 12 ppm Zn as Zn-nic compared to 12 ppm Zn as $ZnCO_3$. Similarly, Wang *et al.* (2010) observed comparable Zn deposition in kidney, pancreas, spleen and *Longismussdorsi* muscle in pigs between 100 mg/kg Zn as Zn-gly and 3000 mg/kg as ZnO supplementation.

Zn plays an important role in the antioxidant system in two ways: first is the protection of proteins and enzymes against free radical attack, or oxidation (Osaretin and Gabriel, 2009) second is through the prevention of free radical formation by other metals, such as iron and copper (Prasad, 2014). Glutathione peroxidase (GPx) and catalase (CAT) are involved in the antioxidant defense system and protects from potential oxidative damage (Flohe, 2009; Peerapatdit and Sriratanasathavorn, 2010). In this experiment supplementation of 12 ppm Zn as Zn- nic significantly (P<0.05) increased the activities of CAT and GPx (Table 3) compared to others. The antioxidant enzymes activity was comparable between rats fed with 6 ppm Zn as Zn-nic or 12 ppm Zn as $ZnCO_3$ clearly indicating Zn requirement could be reduced by 50% if supplemented from organic source. Bun *et al.* (2011) observed improvement (P<0.01) in GPx activity with Zn supplementation in dose dependent manner in broilers (0, 20, 40 and 60 ppm). Reducing the Zn supplementation from 12 to 9 ppm, as Zn-nic, still had higher activities of GP_X (P<0.01) and catalase (by 23.13%) than those with 12 ppm Zn supplementation from inorganic source. Our results were in similar to the findings of Ma *et al.* (2011) who observed improvement

Table 2.Concentration of zinc in serum (µg/ml) and various organs (µg/g weight) in rats fed organic zinc supplemented diets at varied concentration

Attribute	12-I	6-O	9-O	12 -O	SEM	P value
Serum	1.179^{c}	1.004^{d}	1.332^{b}	1.411^{a}	0.027	0.001
Liver	26.84^{ab}	26.26^{b}	27.84^{a}	27.79^{a}	0.196	0.050
Pancreas	22.85	24.69	29.07	29.41	2.252	0.685
Muscle	20.29	20.76	20.92	23.97	2.034	0.923
Kidney	26.71	30.41	31.47	30.14	1.118	0.481

^{ab}Means with different superscripts in a row differ significantly: P<0.05; P<0.01.

Table 3. Antioxidant enzyme activities in haemolysate in rats fed organic zinc supplemented diets at varied concentration

Attribute	12-I	6-O	9- O	12 -O	SEM	P value
Glutathione peroxidase (µmole/mg protein)	5.22[c]	2.77[c]	10.27[b]	15.29[a]	1.112	0.001
RBC catalase (µmole/min/Hb)	8.04[ab]	5.14[b]	9.90[a]	10.98[a]	0.650	0.002

[ab]Means with different superscripts in a row differ significantly: P<0.05; P<0.01.

Table 4. Serum Progesterone concentration and number of different follicles (%) in ovary of rats fed organic zinc supplemented diets at varied concentration

Attribute	12-I	6-O	9- O	12 -O	SEM	P value
Progesterone (nmol/L)	84.85[b]	53.32[c]	91.86[a]	92.75[a]	2.506	0.001
Follicular stage (%)						
Primary follicles	41.11[a]	36.18[a]	42.12[a]	30.55[b]	1.042	0.001
Secondary follicles	19.36	18.78	20.64	23.50	0.936	0.293
Tertiary follicles	16.07	18.43	13.02	17.03	0.750	0.066
Graafian follicle	13.21	16.37	12.30	13.33	0.689	0.175
Corpus luteum	10.22[b]	10.23[b]	11.91[b]	15.59[a]	0.489	0.001

[ab]Means with different superscripts in a row differ significantly: P<0.05; P<0.01.

in activity of GPx enzymes in liver with 90 mg Zn/kg diet supplementation as Zn-gly compared to broiler chicks fed 120 mg Zn/kg diet as $ZnSO_4$.

Serum progesterone levels and follicular population

In current study higher (P<0.05) serum progesterone levels was noticed with 9 and 12 ppm levels of Zn supplementation as Zn-nic followed by those with 12 ppm Zn supplemented as $ZnCO_3$ and lowest progesterone concentrationwas observed in rats supplemented with 6 ppm Zn as Zn-nic (Table 4). Zn is needed for progesterone synthesis (Brown and Pendland, 2007). Also several researchers (Al-Daraji and Amen, 2011; Nagalaksmi *et al.* 2013) observed increase in serum progesterone levels with increasing the level of dietary Zn supplementation. In present study significantly (P<0.05) higher serum progesterone levels was observed with 9 and 12 ppm (75 and 100% of control Zn) of Zn supplementation from organic source compared to12 ppm Zn supplementation from inorganic sourceand no difference was observed between 9 and 12 ppm Zn supplemented from Zn-nic.

Zn plays an important role in estrous cycleby affecting the release of gonadotropic hormones (Keen and Hurely, 1989) and in ovarian follicle growth and ovulation. The deficiency of Zn leads to failure of follicle rupture and thereby affects the corpus luteum (CL) formation (Tian and Diaz, 2011). In vaginal smear study for two consecutive estrouscycle, a regular cycle of 4 days with no irregularities in estrous cycle was observed in rats supplemented with either 9 or 12 ppm Zn as Zn-nic. While inrats fed diets supplemented with 12 ppm Zn as $ZnCO_3$ or 6 ppm Zn as Zn-nic, about 30% of rats showed irregularities in estrous cycle.

In present study primary follicles were lower (P<0.05) in rats supplemented with 12 ppm Zn as Zn-niccompared to rats supplemented with 6 or 9 ppm Zn from the same source or 12 ppm Zn from $ZnCO_3$ but the percentage of CL formation which is an indication for maturation and rupture of follicles, was significantly (P<0.01) higher with 12 ppm Zn supplemented as Zn-nic as compared to other dietary treatments (Table 4). Also the formation of CL formation was not affected (P>0.05) with reducing the dietary Zn supplementation by 50 (6 ppm) or 75 (9 ppm)per cent added as Zn-nic (Table 4). Oocyte maturation, ovulation and leuteolysisare affected by reactive oxygen species (ROS) caused due to oxidative stress at cellular level, but antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase counteracts ROS and reduces the oxidative damage onactively growing follicles in ovaries (Agarwal *et al.* 2012). Supplementation of 12 ppm Zn as Zn-nic significantly (P<0.05) increased the activities of catalase and glutathione peroxidase (Table 2), clearly indicative of

the effect of organic Zn supplementation in reducing oxidative stress and improving the follicular development and serum progesterone concentration in ovaries in rats.

Based on the results, it could be concluded that replacement of 12 ppm inorganic Zn with 12 ppm organic Zn significantly improved the reproductive efficiency and dietary Zn concentration can be reduced by 75% (9 ppm) as Zn nicotinate without affecting estrous cycle and follicular population compared to 12 ppm Zn from inorganic source.

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