IMPACT OF DIETARY REPLACEMENT OF INORGANIC ZINC WITH ORGANIC ZINC ON OVARIAN FOLLICULOGENESIS

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ABSTRACT

Seventy-two weaned Sprague Dawley female rats (98.4±1.5 g) were randomly distributed to four dietary groups with six replicates in each and reared in polypropylene cages for 14 weeks. Dietary treatments were prepared by adding 12 ppm Zn to the basal diet (formulated with purified ingredients without Zn) from inorganic source ($ZnCO_3$, control) and replacing inorganic Zn (Zn_i) at 0% (100_i), 50% (50_i:50_o), 75% (25_i:75_o) and 100% (100_o) with organic Zn (Zn_o, Zn methionine). All the rats were sacrificed at the end to collect the ovaries and the liver to study ovarian folliculogenesis and thiobarbituric acid reacting substances (TBARS), respectively. The dietary incorporation of Zn_o irrespective of level of replacement of Zn_i lowered ($p<0.05$) TBARS in liver. The highest ($p<0.05$) ovarian weight was observed after feeding 100% Zn_o compared to 100% Zn_i and other Zn combinations. Serum progesterone (on 70th d) was increased ($p<0.05$) after Zn_o supplementation compared to sole Zn_i supplementation. The regular estrus cycle was observed following Zn_o supplementation at either combination, while 30% Zn_i supplemented rats had irregular estrus cycle. The number of preantral follicles in ovaries were comparable ($p>0.05$) among the dietary groups, while the percentage of graafian follicles and corpus luteum was though comparable, the number improved by 53.2% and 26.7% with 75% replacement and, 60% and 65.14% with 100% replacement of Zn_i with Zn_o, respectively. In brief, Zn_o replacing 75% or 100% of Zn_i improved the follicular development and its maturation in ovaries of rats.

Keywords: Ovarian folliculogenesis, Rats, TBARS, Zinc methionine, $ZnCO_3$

INTRODUCTION

Zinc (Zn) plays a vital role in female reproductive system and is necessary for progesterone synthesis, normal ovulation and fertilization (Brown and Pentland, 2007). The negative influence of oxidative stress on female reproductive system could be overcome by dietary supplementation of antioxidant nutrients such as Zn (Agarwal *et al.*, 2012). The study of folliculogenesis provides important information about ovarian function, especially the relationship between follicular number and the factors that regulate the survival and maturation of follicles at any stage of development (Myers *et al.*, 2004).

The source of mineral supplementation also affects the bioavailability of mineral in body and hence its function. Organic minerals are chemically inert, more stable and less prone to mineral and nutrient interactions, hence, they are absorbed and circulated to target tissues very efficiently (Swiatkewicz *et al.*, 2014). Based on this concept other researchers have conducted experiments considering the performance and immunity as main criteria and reported beneficial effect of mineral supplementation in organic form (Ao *et al.*, 2011). However, few trials have been conducted to investigate the effect of Zn_o on female reproduction. Hence, the present work was carried out to study the effect of Zn supplementation from organic source replacing inorganic source at graded levels on ovarian folliculogenesis in rats.

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MATERIALS AND METHODS

Seventy-two weaned female Sprague Dawley rats (98.4±1.5 g), after 10 d acclimatization, were randomly distributed into 24 replicates with 3 rats per replicate. These replicates in turn were allotted to 4 dietary treatments. The diets were purified diets 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 casein), varying in Zn supplementation, Table 1). The Zn supplementation in these diets were 12 ppm, supplemented from combination of inorganic (Zn_i, $ZnCO_3$) and organic Zn (Zn_o, Zn methionine) in ratio of 100:0, 50:50, 25:75 and 0:100. The rats were reared according to guidelines of Institutional Ethics Committee under hygiene conditions with controlled temperature (22-23^{0}C) and photoperiod (12 h/d) in polypropylene cages in Animal House. The rats were allowed to feed their respective diets *ad libitum* and provided wholesome clean deionized water from polypropylene bottles to minimize Zn contamination, throughout the experimental duration of 14 weeks. Blood was collected on 70th day by retro-orbital route for serum collection and then stored at -20^{O}C for further estimation of progesterone concentration. At the end of experiment all rats were sacrificed to collect the liver and ovaries. The ovaries were fixed in buffered formalin for follicular study. For measuring thiobarbituric acid reacting substances (TBARS), liver were collected from the sacrificed rats and immediately perfused with normal saline (0.9%) to reduce RBC contamination. Then the samples were fixed in liquid nitrogen and stored at -20^{0}C for the estimations of TBARS (Balasubramanian *et al*., 1988).

Table 1: Ingredient composition (g/kg) of purified diet (Modified AIN-76A)

Ingredient	Proportion, g/kg diet
Sucrose	500
Casein	250
Corn starch	100
Oil	50
Cellulose	50
Mineral mixture*	35
Vitamin mixture*	10
DL-methionine	3
Choline chloride	2

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

Serum progesterone concentration was estimated using commercial ELISA kit (Omega diagnostics, pathozyme, progesterone, Scotland, UK). A vaginal smear study was conducted for two consecutive estrus 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 (5µ) and sections were stained by eosine and haematoxylin and observed under a compound microscope. The quantification study of folliculogenesis was performed and the size, number of layers of granulosa cells around the oocyte and presence of antrum were recorded (Patil *et al.*, 1998). 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.

RESULTS AND DISCUSSION

The highest ($p<0.05$) ovarian weight was observed following feeding of 100% Zn_o compared to 100% Zn_i and other Zn combinations (50_i:50_o; 25_i:75_o; Table 2). Serum progesterone increased ($p<0.05$) following replacement in diet with Zn_o (91.53-94.06 nmol/L) compared to Zn_i supplementation (82.65 nmol/L), with no effect of the level of replacement (50-100%, Table 2). Other researchers had observed increase in serum progesterone with the increasing level of dietary Zn supplementation (Nagalaksmi *et al.*, 2013). In the present study, higher ($p<0.05$) serum progesterone was observed following dietary incorporation of Zn_o even though all the rats were supplemented with equal amount of Zn (12 ppm), which could be due to greater bioavailability of Zn_o (Ao *et al.,* 2011). Several researchers observed higher Zn retention in the body

Table 2: Serum progesterone, different type of follicles (%) and corpus luteum in the ovary of rats fed with combinations of organic and inorganic zinc

Attribute	**100_i**	**50_i:50_o**	**25_i:75_o**	**100_o**
Ovarian weight, % b wt	0.0529[b]	0.0596[b]	0.0610[b]	0.0983[a]
Progesterone, nmol/L	82.65[b]	94.06[a]	93.72[a]	91.53[a]
Follicular development				
Preantral follicles	53.92	49.86	62.17	54.22
Graafian follicle	13.64	12.49	20.90	21.82
Corpus luteum	8.98	8.03	11.38	14.83

Means with different superscripts in a row differ significantly, p<0.05

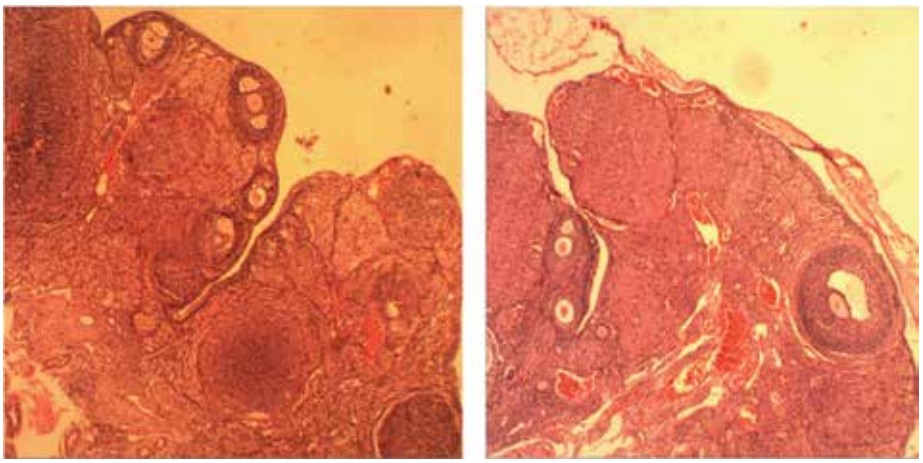

Figure 1: Photomicrograph of rat ovary fed with 25i:75o Zn (left) or 100o Zn (right) showing graafian follicles and corpus luteum

when Zn was supplemented as organic form compared to inorganic form (Ma *et al.*, 2011).

Zinc can influence estrus cycle by affecting release of gonadotropic hormones (Keen and Hurely, 1989) and subsequent ovarian follicle growth and ovulation. The deficiency of Zn leads to failure of follicle rupture and thereby affects the corpus luteum formation (Tian and Diaz, 2011). In vaginal smear, the study for two consecutive estrus cycles, revealed a regular cycle of 4 days with no irregularities in rats supplemented with Zn_o, while 30% rats with 100% Zn_i had irregular estrus cycle. The dietary incorporation of Zn_o had no impact (p>0.05) on follicular development in ovaries, but the percentage of graafian follicles and corpus luteum in ovaries improved following dietary replacement of Zn_i with Zn_o (Table 2, Figure 1).

Oocyte maturation, ovulation and luteolysis are affected by reactive oxygen species (ROS) produced due to oxidative stress at cellular level, but the higher bioavailability of Zn from organic sources counteracts ROS by improving the Zn dependent antioxidant enzyme activities, thereby, actively protecting growing follicles in ovaries from oxidative damage and improves the follicular growth, ovulation and luteolysis (Agarwal *et al.*, 2012). The concentration of TBARS (nM MDA/mg protein) in liver was lowered (p<0.05) following Zn supplementation in combination of inorganic and organic sources at 50_i:50_o (0.0096), 25_i:75_o (0.0093) and 100_o (0.0087) compared to sole supplementation from inorganic source (0.0133). The lower TBARS

concentration is indicative of better antioxidant status (Del Rio *et al.,* 2005). In addition, organic Zn supplementation (75 or 100%) might be the reason for higher ($p>0.05$) CL formation as Zn is an important factor for maturation of follicle (Tian and Diaz, 2011).

Thus, it could be concluded that dietary replacement of 75 or 100% of inorganic Zn with organic Zn in diet improved the follicular development with more number of graffian follicle and corpus luteum in ovaries of rats compared to Zn supplementation from 100% inorganic source.

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