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Comparative evaluation of organic zinc supplementation as proteinate with inorganic zinc in buffalo heifers on health and immunity

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ABSTRACT

Graded Murrah buffalo heifers (18) were randomly allotted to 3 dietary groups varying in source and level of Zn supplementation in concentrate mixture to study the effect of organic (O) Zn (Zn proteinate; Zn-prot) supplementation (80 or 140 ppm) compared to inorganic Zn (I) ($ZnSO_4$) (140 ppm) on serum biochemical parameters, antioxidant status and ovarian folliculogenesis. Mineral and biochemical constituents in serum and antioxidant enzyme activities in haemolysate were measured on 90th d of experiment. Antibody titres (log_2) against *Brucella abortus* S_{19} and chicken RBC antigens was measured in serum at 7, 14, 21 and 28th d post sensitization (humoral immunity) and cell mediated immunity was assessed (120 d) by *in-vivo* delayed type hypersensitivity (DTH) against phytohemagglutinin-P (PHA-P). After 60 days of feeding, ovarian folliculogenesis study was made daily with ultrasound scanner in all the animals for next 60 days. Highest Zn concentration in serum without affecting the retention of other minerals (Cu, Mn and Fe) was observed with 140 ppm Zn supplementation as Zn-prot and mineral concentrations was comparable between 80 ppm Zn as Zn-prot and 140 ppm Zn as $ZnSO_4$. Alkaline phosphatase, total protein, globulin, and glucose concentrations in serum increased with organic Zn supplementation. Organic Zn lowered lipid peroxidation (140O<80O<140I) and improved RBC catalase, glutathione peroxidase and superoxide dismutase (140O>80O>140I) activities. Antibody titres against *B. abortus* and chicken RBC and *in vivo* DTH response improved with organic Zn supplementation. Similarly, irrespective of the dose, organic Zn supplementation significantly increased the number of large follicle with greater follicular size in ovary. The study indicated that 140 ppm Zn supplementation as Zn-prot resulted in better antioxidant status, immune response and folliculogenesis in ovaries than inorganic source and the Zn supplementation could be reduced from 140 to 80 ppm as Zn-prot without any adverse effect in buffalo heifers.

Key words: Antioxidant status, Buffalo heifers, Immune response, Ovarian folliculogenesis, Serum biochemical parameters, Zn proteinate

Zinc (Zn) influences various biological functions by being a co-factor for more than 300 metalloenzymes (Chasapis *et al.* 2012). Besides this, Zn is essential for humoral and cellular immune responses (Gruber and Rink 2013) and also plays an important role in antioxidant defense system (Oteiza 2012). Similarly, Zn is required for female reproductive system and necessary for normal ovulation and fertilization (Tian and Diaz 2011). It protects the female reproductive system from reactive oxygen species by lowering the oxidative stress (Agarwal *et al.* 2012). In addition to this, several researchers have noticed beneficial effect of higher levels of Zn supplementation than recommendations on antioxidant status, immunity (Shinde *et al*. 2007 and Nagalakshmi *et al*. 2009 a) and reproduction (Laar and Jongbloed 2010 and Nagalakshmi *et al*. 2014).

Zn is deficient in most parts of soils in India (Gowda *et al*. 2009 and Nagalakshmi *et al.* 2009b) and due to its importance in various functions, it is a common practice by farmers and feed manufacturers to supplement with a large safety margin to avoid deficiency. But it's over supplementation could lower the absorption and increase the mineral excretion of other minerals such as Cu (Spears 1996), hence it causes mineral imbalance which could adversely affect the animal performance. To avoid this problem, the concept of organic minerals was developed in which mineral is in a chemically inert form, more stable and less prone to interactions, so absorbed and circulated to target tissues very efficiently (Spears 1996) thereby, decreasing the mineral excreted and hence lowering the requirement. Based on this issue, present study was conducted to investigate the effect of Zn proteinate

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supplementation at 80 or 140 ppm levels on serum biochemical parameters, antioxidant status, immune response and ovarian follicular development in buffalo heifers compared to 140 ppm Zn as $ZnSO_4$.

MATERIALS AND METHODS

Animal and feeding management

The experiment of 120 days duration was conducted at Dairy Experimental Station, College of Veterinary Science, Rajendranagar, Hyderabad. Graded Murrah buffalo heifers (18; 20–28 months age) with average body weight of 279.2±7.10 kg were randomly divided into 3 groups of 6 animals each in completely randomized design. The concentrate mixture was prepared from the locally available feed ingredients (Table 1) and to this, calculated amount of Zn was added from various sources during its preparation. The dietary treatments varied in the source and level of Zn supplementation in the concentrate mixture. Control concentrate mixture contained 140 ppm Zn as $ZnSO_4$, whereas experimental concentrate mixtures were prepared by adding 80 or 140 ppm Zn from Zn proteinate (Zn-prot). All the animals were fed with their respective concentrate mixture to meet 80% of protein requirements and *ad lib.* para grass (*Bracharia mutica*) to meet the nutrient requirements (ICAR 2013) during the entire experimental period. The concentrate mixture in all groups was fed 2 times a day i.e., at 9.30 AM and 2.30 PM and fresh drinking water was made available at all times. All the experimental animals were housed in a well-ventilated animal shed with the provision for individual feeding and watering. Strict hygienic and management practices were followed throughout the experiment. Deworming was done for both endo and ecto parasites before start of the experiment.

Blood collection: Blood was collected aseptically from jugular vein with the help of sterilized needles in clean sterilized glass tubes and heparin coated vacutainers from all animals on day 90 of feeding trial for serum and haemolysate preparation, respectively. The serum was stored at –20°C for estimation of various biochemical constituents and Zn content.

Table 1. Ingredient composition (%) of concentrate mixture

Ingredient	Kg/100kg
Maize	50
Soybean meal	21
Red gram husk	14
Molasses	9
Urea	2
Lime stone powder	2.13
Salt	1
Mono calcium phosphate	0.787
Trace mineral and vitamin premix[1]	0.35

[1]Trace mineral and vitamin premix provided (mg/kg): iron, 41; manganese, 21; copper, 10; cobalt, 0.1; Iodine, 0.27; selenium, 0.3. Vitamin A, D and E were provided to supply 2927 IU; 1097 IU and 39 IU per kg diet, respectively.

Haemolysate (RBC lysate) preparation: The blood collected in clean heparinized vacutainers was centrifuged at 2000 rpm for 15 min at 4°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 for estimation of lipid peroxidation and antioxidant enzyme activities.

Serum biochemistry and antioxidant enzyme activities: Serum alkaline phosphatase (ALP) activity, total protein, albumin and glucose concentration were determined by spectrophotometer (Labomed, UVD–3000) as per Kind and King (1954), Reinhold (1953), Gustafsson (1976) and Cooper and Mc Daniel (1970), respectively. The globulin was determined by subtracting albumin from total protein content. The minerals concentration in serum was analysed by atomic absorption spectrometer (AAS) according to the procedure described by Arenza *et al*. (1977).

The lipid peroxidation activity in haemolysate was estimated as per the method of Placer *et al.* (1966)**.** To 200 µl of RBC lysate (1:20 dilution) taken in a clean dry test tube, 1.3 ml Tris KCl buffer and 1.5ml TBA reagent were added. After cooling, 3 ml pyridine butanol mixture and 1ml 1N NaOH was added and optical density (OD) was measured at 548 nm against the blank. The antioxidant enzymes viz. glutathione peroxidase and RBC catalase (Bergmeyer 1983) activities were estimated in haemolysate and the activities of these enzymes were expressed as units per mg hemoglobin (Hb). The glutathione peroxidase activity was estimated by taking 2 ml phosphate buffer saline (PBS) (0.1 M; pH 7.4), 100 µl of haemolysate (1:20 dilution) in a clean dry test tube followed by 100 µl of reduced glutathione and 100 µl of H_2O_2 buffer. Tubes were incubated at room temperature for 5 minutes, following which 100 µl NADPH solution was added and OD was recorded at 320 nm for 5 minutes at 60 seconds interval (Paglia and Valentine 1967). For measuring RBC catalase activity, 2 ml haemolysate (1:500 dilution) was taken into cuvette and 1ml of hydrogen peroxide solution was added and OD was recorded at 240 nm against the blank at 15 second interval (Bergmeyer 1983). The Hb concentration in RBC lysate was estimated colorimetrically by using Drabkin's solution as per the procedure described by Cannan (1958).

The superoxide dismutase (SOD) activity was measured in serum. Microtiter plates (96 well) were used for assay of SOD activity. In test wells, 15 µl of 100 µM pyrogollol, 6 µl of 1.25 mM 3-(4,5–dimethylthiazol-2-yl)-2–5-diphenyl tetrazolium bromide (MTT) and 100 µl serum was added. In control wells, 6 µl MTT and 100 µl serum was added and the volume in all wells was made up to 150 µl with PBS. Freshly prepared pyrogollol was added after addition of all other reagents and then incubated for 5 min at room temperature (30°C). The reaction was terminated with addition of 150 µl of dimethyl sulfoxide (DMSO), which helps to arrest the reaction and dissolve the MTT formazan

crystals formed. Plates were shaken for a few minutes and read on a microtiter plate reader at 630 nm (Madesh and Balsubramanian 1998).

Immunity

Humoral immune response: Humoral immune response was assessed by using heat killed *Brucella abortus* S_{19} (procured from Indian Veterinary Research Institute, Izatnagar) and chicken RBC as immunogens. Heat killed phenolised suspension of *Brucella abortus* S_{19} was centrifuged at 10,000 rpm for 15 min. The sediment was resuspended in normal saline and was repeated three times to match the turbidity of the cell suspension to McFarland tube number 4 (*i.e*. 1.2×10^9 cells/ml). For preparation of chicken RBC, freshly collected chicken whole blood was freshly collected in Elsevier's solution prepared in laboratory in sterilized conditions. The collected blood was centrifuged at 2500 rpm for 5 min and cell pack was resuspended with sterile physiological saline to make 20% suspension. Prior to sensitization, all the heifers were screened for Brucellosis with the help of Rose Bengal Plate Test (RBPT).

After 60 days of experimental feeding, all heifers were sensitized with 5 ml heat killed *Brucella abortus* S_{19} and 20% chicken RBC cell suspension, administered intramuscularly and a booster dose of antigens were given after 15 days. The serum was collected from sensitized heifers on 7, 14, 21 and 28th day post sensitization to assess antibody titres against the antigens.

The antibody response against *Brucella abortus* antigen was measured by standard tube agglutination test (STAT) (Alton *et al*. 1975). Two fold serial dilutions of sera were made in 0.5 ml volumes using phenolic saline in agglutination tubes. Then equal volume i.e., 0.5 ml of *Brucella abortus* antigen was added to all dilutions of sera. Antigen control was kept by mixing volumes of saline and antigen. The tubes were incubated at 37°C for 24 h. Antigen control was observed for absence of autoagglutination and the reciprocal of highest dilution of serum showing positive agglutination was taken as STAT titer of antibodies. The titers were expressed as $\log_2$ of the reciprocal of the highest dilution showing agglutination.

The immune response against chicken RBC was measured by direct haemagglutination assay (DHA) (Wegmann and Smithies 1966). To each well of a 96 well microtiter plate 0.05 ml normal saline (pH 7.2) was added. The initial well in each row received 0.05 ml serum (complement in the serum was heat inactivated at 56°C for 30 min), which was then serially, diluted two fold. Then 0.05 ml of 1% chicken RBC was added to each well. The RBC control was kept by adding equal volumes (0.05 ml) of normal saline solution and chicken erythrocytes. Microtitre plates were rocked well and incubated for 30 min at 37°C. The agglutination titers were expressed as $\log_2$ of the reciprocal of the highest dilution showing agglutination of chicken RBC.

Cell mediated immune response: The cell mediated immune (CMI) response was assessed at the end of experiment in all animals by *in vivo* delayed type hypersensitivity (DTH) reaction against phytohaemagglutinin phosphate (PHA-P). The skin on both sides of the neck was cleaned and shaved with help of sterile razor, 24 h prior to injection so that any inflammation set during shaving or due to abrasion may subside. Next day, 150µg of PHA-P in 100 µl of Phosphate buffer saline (PBS) (pH 7.4) was injected intra-dermally into shaved area on one side of neck and 100µl of PBS was injected on other side of neck as a control. The injected sites were measured in millimeters using vernier calipers prior to injection and at 24, 48 and 72 h post injection. Inflammatory response was measured as a change in skin thickness as per the procedure described by Quist *et al*. (1997).

Ovarian follicular study: After 60 days of feeding, ovarian folliculogenesis study was done in all animals daily for next 60 days using ultrasound scanner equipped with 7.5 M Hz transducer designed for trans rectal placement. The follicle sizes in both ovaries were measured against in-built centimeter scale displayed on screen. Based on size, the follicles were grouped as large (>8 mm), medium (5–8 mm) and small (3–5 mm).

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

RESULTS AND DISCUSSION

The serum Zn concentration (ppm) was higher ($P<0.01$) in heifers fed diets supplemented with 140 ppm Zn as Zn-prot compared to those fed diets with 80 ppm Zn as Zn-prot and 140 ppm Zn as $ZnSO_4$ (Table 2). Reducing the Zn supplementation approximately by 50% (80 ppm) as Zn-prot had no effect on serum Zn concentration in buffalo heifers compared to animals fed on diets supplemented with 140 ppm Zn as $ZnSO_4$. Jia *et al.* (2009) observed reduction in Cu concentration in plasma with increase in plasma Zn concentration due to supplementation of Zn as $ZnSO_4$. However, in current investigation increased serum Zn concentration with organic Zn supplementation did not affect the retention of other minerals (Cu, Mn and Fe) (Table 2), which could be due to higher bioavailability of organic minerals that are in chemically inert form and less prone to

Table 2. Effect of organic zinc supplementation on serum mineral status of buffalo heifers

Mineral	Inorg-140	Org-80	Org-140	SEM	P value
Zinc (ppm)	1.89^a	1.99^a	2.28^b	0.046	0.001
Copper (ppm)	0.78	0.86	0.91	0.026	0.124
Manganese (ppm)	0.83	0.84	0.82	0.015	0.861
Iron (ppm)	4.22	4.04	4.14	0.073	0.633

^{ab} Means with different superscripts in a row differ significantly: $P<0.01$; SEM, standard error mean.

interaction with other minerals (Spears 1996). Similarly, Garg *et al.* (2008), Wang *et al.* (2013) and Mallaki *et al.* (2015) observed higher Zn concentration in the serum of lambs supplemented with organic Zn compared to those fed an equal amount of Zn from inorganic source. In concurrence to our findings, Cope *et al.* (2009) observed comparable serum Zn levels in dairy cows supplemented with either recommended level (60 ppm) of Zn from ZnO or lower levels (36 ppm) from chelated Zn.

The ALP activity, which can be used as an indicator of animal Zn status (Miller *et al*. 1965) significantly (P<0.05) improved with organic Zn (80 or 140 ppm) supplementation compared to heifers fed 140 ppm inorganic Zn (Table 3). Similarly, total protein and glucose concentrations increased (P<0.05) with organic Zn supplementation compared to inorganic Zn. Significantly (P<0.01) highest globulin concentration was observed with 140 ppm Zn supplementation as Zn-prot compared to other dietary treatments. Similarly, Nagalakshmi *et al.* (2009 a) and Devi *et al.* (2014) observed higher serum ALP activity in lambs and kids, respectively, with organic Zn supplementation compared to inorganic Zn supplementation

Lipid peroxidation in haemolysate, indicative of oxidative stress was significantly (P<0.01) lowered (Table 4) with Zn-prot supplementation compared to $ZnSO_4$ supplementation and comparison between the organic Zn supplemented groups, the LPx significantly (P<0.01) reduced with 140 ppm Zn compared to 80 ppm Zn. Glutathione peroxidase (GPx) and catalase (CAT) are involved in the antioxidant defense system and protects the cells from potential oxidative damage (Prasad 2014). In the present study, organic Zn supplementation significantly increased the activities of CAT (P<0.01) and GPx (P<0.10) (Table 4) compared inorganic Zn. Zn is a co-factor for superoxide dismutase (SOD) enzyme (Marklund *et al*. 1982) and also considered as marker for Zn bioavailability (Paik *et al. 1*999). This enzyme activity significantly (P<0.01) increased in heifers receiving 140 ppm Zn as Zn-prot, followed by 80 ppm as Zn-prot and lowest activity was observed with 140 ppm Zn supplemented as $ZnSO_4$. Higher bioavailability of Zn from organic source (Spears 1996) might have improved the antioxidant status of the animals though they received lower Zn (80 ppm) compared to animals supplemented with 140 ppm Zn from inorganic source. Similarly, Nagalakshmi *et al.* (2009 a) and Ayman Ahmed *et al.* (2011) observed increase in antioxidant enzyme activities with organic Zn supplemented at equal levels compared to inorganic Zn supplementation in sheep.

Zn is essential for maintenance of natural killer cells activity and phagocytosis of macrophages and neutrophils (Gruber and Rink 2013). Antibody titres (log_2) on 7, 14, 21 and 28 d of post immunization against *B. abortus* and SRBC were used to assess the humoral immune response. The titres against *B. abortus* antigen on 7 and 14 d of post immunization was comparable among the dietary treatments but on 21 and 28 d of post immunization the titres significantly (P<0.05) improved in organic Zn supplemented animals (Table 5). Similarly, titres against chicken RBC increased (P<0.05) with Zn-prot

Table 3. Effect of organic zinc supplementation on serum biochemical constituents in buffalo heifers

Constituent	Inorg-140	Org-80	Org-140	SEM	P value
ALP (U/L)	91.14[a]	98.44[ab]	107.55[b]	2.628	0.026
Total protein (g/dl)	5.46[a]	6.01[ab]	6.74[b]	0.204	0.023
Globulin (g/dl)	3.81[a]	3.23[a]	5.05[b]	0.249	0.003
Glucose (mg/dl)	34.62[a]	48.66[b]	45.50[b]	1.704	0.001

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

Table 4. Effect of organic zinc supplementation on lipid peroxidation and antioxidant enzyme activity in buffalo heifers

Attribute	Inorg-140	Org-80	Org-140	SEM	P value
Lipid peroxidation (µmol MDA/mg Hb)	2.03[c]	1.60[b]	1.26[a]	0.096	0.001
Glutathione peroxidase (µmole NADPH oxidized/g Hb/min)	6.52[a]	7.26[ab]	7.91[b]	0.255	0.076
RBC Catalase (mmol/mg Hb)	3.26[a]	4.32[ab]	4.76[b]	0.224	0.009
Serum SOD (IU/mg protein)	0.116[a]	0.149[b]	0.188[c]	0.008	0.001

[abc] Means with different superscripts in a row differed significantly: P<0.05; SEM, standard error mean.

Table 5. Effect of organic zinc supplementation on immune response in heifers

Days of post sensitizationnn	Inorg-140	Org-80	Org-140	SEM	P value
Humoral immune response (log_2 titres)					
B. abortus					
7	6.84	7.33	7.00	0.206	0.628
14	4.83	5.67	5.68	0.200	0.146
21	7.17[a]	8.00[b]	8.16[b]	0.173	0.028
28	3.50[a]	4.33[ab]	5.00[b]	0.226	0.014
Chicken RBC					
7	5.00[a]	6.33[b]	6.67[b]	0.291	0.036
14	4.67	5.18	5.50	0.227	0.341
21	5.16[a]	6.17[b]	6.66[b]	0.214	0.006
28	3.00[a]	4.00[b]	4.69[b]	0.241	0.008
Cell mediated immune response (against PHA-P, increase in skin fold thickness, mm)					
Hours post sensitization					
24h	2.19[a]	3.01[b]	3.14[b]	0.135	0.002
48h	1.13[a]	2.04[b]	2.26[b]	0.144	0.001

[abc] Means with different superscripts in a row differed significantly: P<0.05; SEM, standard error mean.

Table 6. Average number of different follicles (%) and their average follicle size (mm) in ovaries of heifers fed organic Zn supplemented diets

Follicle	Inorg-140	Org-80	Org-140	SEM	P value
Right ovary					
Small	52.22[a]	38.28[b]	35.97[b]	2.772	0.023
Medium	38.17	50.12	49.20	2.464	0.080
Large	9.60	11.60	14.83	1.590	0.424
Left ovary					
Small	38.56[ab]	34.21[b]	47.82[a]	2.393	0.049
Medium	55.04[a]	49.57[a]	38.66[b]	2.223	0.002
Large	6.40[b]	16.22[a]	13.52[a]	1.623	0.027
Follicular size					
Right ovary	5.8	5.6	5.3	0.010	0.186
Left ovary	5.5[b]	5.9[a]	5.6[ab]	0.007	0.040
Average	5.6	5.8	5.5	0.007	0.164

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

supplementation on 7, 21 and 28 d of post immunization compared to inorganic Zn. Similar to humoral immune response, the CMI against PHA-P (24 and 48 h of post sensitization) significantly (P<0.01) increased with lower (80 ppm) or equal (140 ppm) levels of organic Zn supplementation compared to inorganic Zn supplemented at 140 ppm. Higher Zn availability and antioxidative enzyme activities in heifers with organic Zn supplementation could have protected the immune cells from free radicals damage resulting in higher immune response (Osaretin and Gabriel 2009). Similarly, Nagalakshmi *et al.* (2009 a) and Wang *et al.* (2013) observed better immune response with organic Zn supplementation compared to inorganic Zn supplementation in lambs and dairy cows, respectively.

The effect of organic Zn supplementation on ovarian follicular development is presented in Table 6 and Figures 1–3. The average number of small follicles were higher

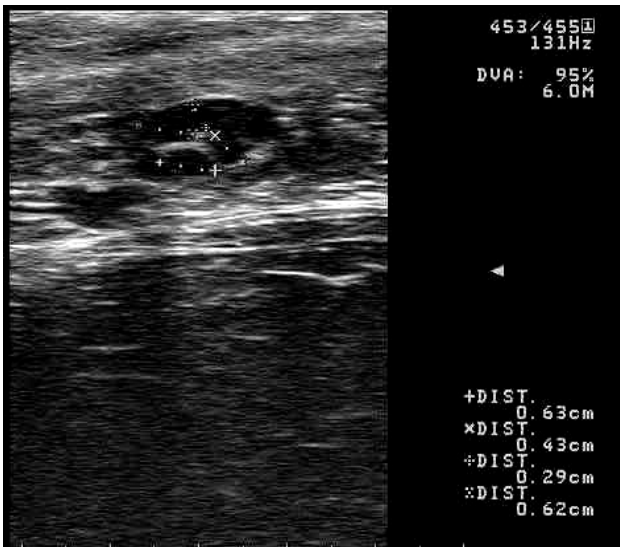

Fig. 1. Ovaries of heifers fed diets supplemented with 140ppm Zn as $ZnSO_4$.

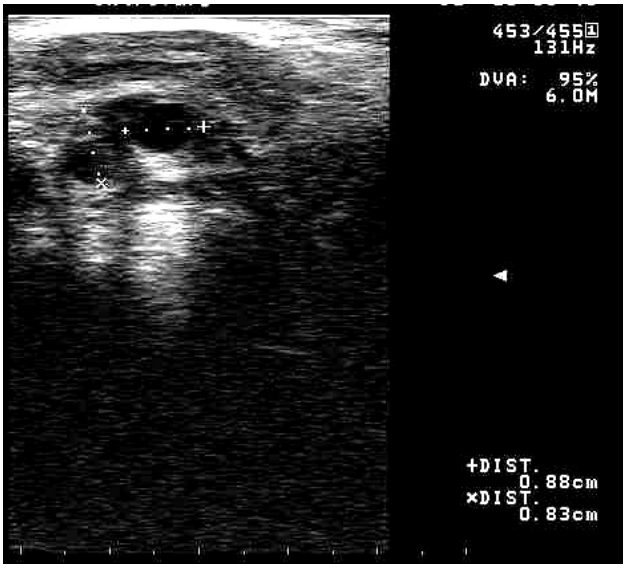

Fig. 2. Ovaries of heifers fed diets supplemented with 80ppm Zn as Zn-prot.

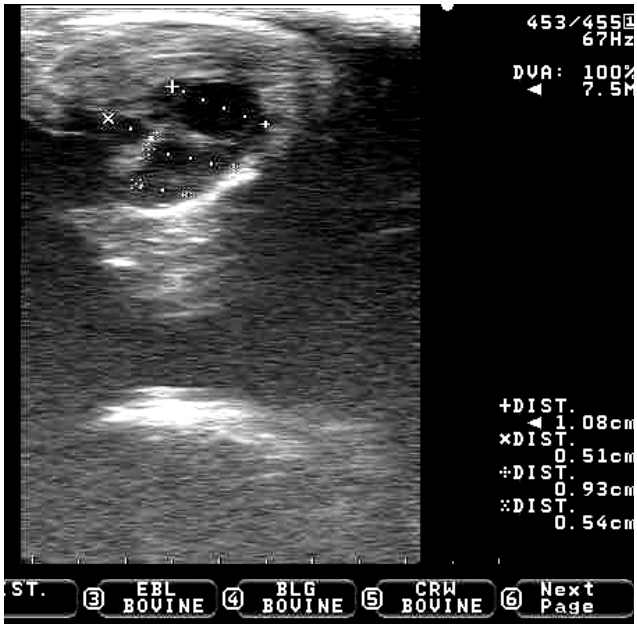

Fig. 3. Ovaries of heifers fed diets supplemented with 140ppm Zn as Zn-prot.

(P<0.05) in right ovary in heifers fed diets supplemented with 140 ppm Zn as $ZnSO_4$ than those fed organic source of Zn. The number of large follicles was higher by 20.83% and 64.77% in 80 and 140 ppm Zn supplementation from Zn-proteinate group than 140 ppm Zn from $ZnSO_4$, respectively. In left ovary, the number of small sized follicles were higher (P<0.05) and medium sized follicles was lower (P<0.01) in 140 ppm Zn supplemented as Zn-prot than those fed 80 ppm Zn from Zn-prot and 140 ppm Zn from $ZnSO_4$. The number of large sized follicles was higher with organic Zn supplementation compared to inorganic source of Zn and no difference was observed between the two doses of organic Zn supplementation.

Monem and El-Shahat (2011) noticed increase in number of large follicles with increasing the level of Zn supplementation. Similarly, Nagalakshmi *et al*. (2014) observed increase in follicular size and their number with increasing the level of Zn supplementation (0<80<140 ppm) as $ZnSO_4$ in buffalo heifers. However in the present study, size and number of large follicles in ovaries increased with organic Zn (80 or 140 ppm) supplementation compared inorganic Zn. In addition, reducing Zn supplementation when supplemented as Zn-prot had no adverse effect on folliculogenesis in ovaries, which could be attributed to lower oxidative stress with organic Zn supplementation protecting the rapidly growing ovarian follicles form reactive oxygen species (Agarwal *et al*. 2012).

The study indicated that 140 ppm Zn supplementation as Zn proteinate resulted in better antioxidant status, immune response and folliculogenesis in ovaries than from inorganic source and the Zn supplementation could be reduced from 140 to 80 ppm when supplemented as Zn proteinate without any adverse effect on antioxidant status, immune response and ovarian follicular development.

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