The Indian Journal of Animal Reproduction; 29(1): 42-49; June 2008

Research Article

Effect of inorganic and organic zinc supplementation on semen quality in Murrah buffalo (*Bubalus bubalis)* bulls

S.IMAM, M.R. ANSARI[1], RIPU SUDAN KUMAR, VISHAL MUDGAL, V.P. VARSHNEY[2] AND R.S.DASS

Mineral and Vitamin Nutrition Laboratory,
Centre of Advanced Studies in Animal Nutrition
Indian Veterinary Research Institute, Izatnagar - 243 122, India
rsd@ivri.up.nic.in

ABSTRACT

The present study was conducted to elucidate the effect of inorganic (zinc sulphate) and organic (zinc propionate) zinc supplementation on *in vitro* semen fertility and semen quality in buffalo bulls. Twelve buffalo bulls of about 2 years of age and 302.0±1.6 kg average body weight were divided into 3 equal groups. All the animals were fed on wheat straw and concentrate mixture to meet their nutrient requirement. Animals in-group I were treated as control, whereas animals in group II and III were experimental and were supplemented with 70 ppm and 35 ppm zinc through zinc sulphate and zinc propionate, respectively. This feeding practice lasted for 6 months. Bulls were trained to donate semen into artificial vagina. After 3 months of experimental feeding, 6 ejaculates from each bull were collected to study the effect of zinc supplementation on sperm fertility and semen characteristics. Statistical analysis of the data revealed that there was no effect of zinc supplementation on semen pH and semen volume; however, sperm concentration, mass motility and individual motility differed significantly ($P<0.01$) among the groups and were higher in zinc-supplemented groups as compared to control. Similarly, reaction time (second), live sperm (%) and acrosomal integrity (%) were significantly ($P<0.05$) different in 3 groups. *In vitro* fertilizing ability test HOSST (%) and BCMPT (distance in mm) revealed significantly ($P<0.05$) higher fertilizing ability of sperm in zinc-supplemented groups as compared to control group. Results further revealed that live sperm (%), acrosomal integrity (%) and *in-vitro* fertilizing ability of sperm (HOSST, %) and BCMPT (distance in mm) were significantly ($P<0.05$) higher in zinc propionate supplemented group as compared to zinc sulphate supplemented group. It may be concluded that supplementation of zinc as zinc propionate improved semen quality as well as *in-vitro* sperm fertilizing ability as compared to zinc sulphate.

Key words: Buffalo bull, Semen, Zinc sulphate, Zinc propionate, Semen characteristics,

INTRODUCTION

Zinc plays a significant role in male reproduction as it influences prostate, epididymal and testicular functions (Ebisch *et. al.*, 2003). Zinc has been reported to enhance the process of spermatogenesis (Wong *et. al.*, 2002), controls sperm motility (Wroblewski *et. al.*, 2003), stabilizes sperm membrane (Kendall *et. al.*, 2000), preserves the ability of sperm nuclear chromatin to undergo decondensation and modulates sperm functions (Suruki *et. al.*, 1995). Zinc is also essential for the development of primary and secondary sexual characters (Davies, 1985). Low zinc intake by young males of several species, including humans hampered their normal sexual development (Apgar, 1985). Experimental zinc deficiency in humans led to reduce sperm counts, combined with reduced serum testosterone concentration (Abbasi *et. al.*, 1980). Hypozinkemia led to gonad dysfunction,

[1]Artificial Insemination Laboratory, Division of Animal Reproduction [2] Nuclear Research Laboratory, Division of Physiology and Climatology

decreased testicular weight, atrophy of seminiferous tubules, and complete cessation of spermatogenesis in lambs (Martin *et. al.*, 1994). Zinc is present in high concentration in male reproductive tract as well as in semen (Chia *et. al.*, 2000; Massonyi *et. al.*, 2004). The recommended level of zinc in the diet of cattle is 35-40 ppm (NRC,1989), and is sufficient for normal body functions, but for enhanced immunity higher level of zinc has been found beneficial (Mandal *et. al.*, 2006). Organic/ chelated form of zinc has been found more bioavailable to animals as compared to inorganic sources (Spears *et. al.*, 1991; Engle *et. al.*, 1995; Droke *et. al.*, 1998; Mandal *et. al.*, 2007). Literature is scarce on the effect of zinc supplementation, especially the organic Zn on semen quality of buffalo bulls. The objective of the present study was to elucidate the effect of organic (zinc propionate) and inorganic (zinc sulphate) zinc supplementation on semen quality in buffalo bulls.

MATERIALS AND METHODS

Animals, their feeding and management

This study was conducted on twelve buffalo bulls (2 years old, 302.0±1.6 kg mean body weight), divided into 3 groups (I, II and III) of four animals in each. Bulls were maintained in Animal Nutrition Division of Indian Veterinary Research Institute, Izatnagar and fed on wheat straw and concentrate mixture in the ratio of 1:1 to meet their dry matter and crude protein requirement (Kearl, 1982). The basal diet (wheat straw and concentrate mixture) had 32.54ppm zinc. Concentrate mixture was comprised of crushed maize grain (30%), soybean meal (25 %), wheat bran (42 %), mineral mixture (2 %) and common salt (1 %). In addition, the animals in-group II and III were given 70 and 35ppm zinc through zinc sulfate and zinc propionate, respectively; whereas group I was not supplemented any additional Zn and was control group. All the bulls were kept in a well-ventilated shed having cemented floor and arrangements for individual feeding with sufficient drinking water. Body weights of all the animals were recorded at an interval of 15 days for the formulation of diet and calculation of zinc to be supplemented in the diet of each animal. This feeding practice lasted for 6 months.

Collection of semen

Before starting the experimental feeding, three ejaculates of semen from all buffalo bulls were collected in artificial vagina over a dummy to assess semen characteristics of bulls at the start of experiment. In last 3 months of experimental feeding 6 ejaculates from each bull were collected to study the effect of Zn supplementation on semen quality and *in vitro* sperm fertilizing ability.

Evaluation of semen

Ejaculate volume (ml) of semen was directly recorded in a graduated glass tube. Semen pH was noted immediately after collecting the semen using digital pH meter (Century, India). Mass motility of semen was graded from 0-5 scale, based on appearance of waves and swirls created by sperm movement when visualized by keeping one drop of semen on a glass slide, without cover slip, under low power magnification (10x) of microscope (Salisbury *et. al.*, 1985). The individual motility of freshly diluted semen was assessed after covering a semen drop on a glass slide with a thin cover slip at 37°C, under high power magnification (40 x). The individual motility was recorded as percentage of progressive motile sperms. The concentration of sperm (millions/ ml) in the fresh semen was determined using a haemocytometer (Salisbury *et. al.*, 1985). Sperm livability (percentage) was calculated by using Eosin-Nigrosin stain. Percent intact acrosome was assessed by staining the semen smears with Giemsa stain (Watson, 1975). Bovine cervical mucus penetration test (BCMPT) was carried out by following the procedure described earlier (Matousek *et. al.*, 1989), in which distance (mm) traveled by progressive sperm was measured under high power magnification (40x) of microscope, by allowing sperm to travel in a capillary tube, filled

with cervical mucus of a cyclic buffalo, at 37°C for 60 minutes. Percentage of hypo-osmotic swollen sperms was observed by incubating semen with hypo-osmotic solution of fructose and sodium citrate (100mg/ml) at 37°C for 60 minutes and examined swelling of sperm tail under high power magnification (40x) of microscope for hypo-osmotic sperm swelling test (Jeyendran *et. al.*, 1984).

Statistical analysis

Data collected during period of study were analyzed (Snedecor and Cochran, 1989), using one-way analysis of variance. Significant means were compared using Duncan's multiple range test (Steel and Torrie, 1980)

RESULTS AND DISCUSSION

Semen characteristics of buffalo bulls at the start of experiment:

The mean values of semen characteristics viz., ejaculate volume (ml), pH, mass motility (0-5 scale), individual motility (%), concentration of spermatozoa (million /ml), livability (%), acrosomal integrity (%), BCMPT (distance in mm) and HOSST (%) of buffalo bulls at the start of experiment in three groups were, 1.67±0.16, 7.25±0.08, 1.43±0.25, 30.33±4.58, 480.83±89.20, 64.42±3.13, 70.50±2.82, 14.083±1.66 and 46.66±4.39, respectively. Results revealed no significant difference in any of these parameters, in three groups at the start of experiment.

Semen characteristics of bulls after 6 months of zinc supplementation:

Mean values of semen characteristics of different groups of buffalo bulls after completion of six months of zinc supplementation have been presented in Table 1. The mean ejaculate volume (ml) in different groups was 1.4, 1.8and 1.6 in Imam *et. al.*
group I, II and III, respectively, which indicated a nonsignificantly ($P>0.05$) higher ejaculate volume in-group II as compared to other groups. The mean sperm concentration (million/ml) in buffalo bulls ejaculates was 736.36, 977.82 and 1040.9 in three groups, respectively, which was significantly ($P<0.0001$) higher in Zn supplemented groups as compared to unsuppleemnted control. It has been reported that zinc supplementation had a beneficial effect on spermatogenesis (Saleh *et. al.*, 1992; Tharwat, 1998; Kendal *et. al.*, 2000; Wong *et. al.*, 2002), as zinc acts as a cofactor in mitotic and meiotic cell division and also helps in encoding a transcript factor involved in spermatogenesis (Nagamine *et. al.*, 1990). Most important enzymes involved in process of spermatogenesis are sorbitol dehydrogenase (SoDH) and lactate dehydrogenase (LDH), which are zinc metalloprotease enzymes (Bedwall and Bahuguna, 1994).

The mean pH values of the semen ejaculates were 7.30, 7.27 and 7.20 in group I, II and III, respectively. No significant difference in semen pH was noted between the Zn supplemented and control group. These results are contradictory to the findings of Saleh *et. al.* (1992) and Kumar *et. al.* (2006), who reported increased and decreased semen pH values in Zn supplemented bucks and crossbred cattle bulls, respectively, which may be due to difference in animal species and duration of Zn supplementation in the diet.

Mean mass motility of spermatozoa in different groups (0-5 scale) was 3.3, 3.9 and 4.1 in-group I, II and III, respectively, which was significantly ($P<0.0001$) higher in Zn supplemented groups as compared to control. These observations are similar to previous reports in men (Wong *et. al.*, 2002), sheep (Kendal *et. al.*, 2000), rabbit (Tharwat, 1998) and cattle (Kumar *et. al.*, 2006) supplemented with Zn. The mean individual sperm motility (%) was 65.4, 73.5and 78.9 in-group I, II and III, respectively, which indicated significantly ($P<0.0001$) higher individual sperm motility in-group II and III as compared to control group. It is documented that for the movement of sperm flagella energy is needed and zinc controls the motility of spermatozoa by controlling energy utilization through ATP

Table I. Semen chara cteristics of buffalo bulls after zinc supplementation

Attribute	Group			SEM
	I	II	III	
Reaction time (Sec)*	72.38^{b}	70.81^{ab}	69.27^{a}	0.47
Semen volume (ml)	1.4	1.8	1.3	0.11
pH	7.30	7.27	7.20	0.032
Mass motility****	3.3^{a}	3.9^{ab}	4.1^{b}	0.07
Individual motility (%)****	65.40^{a}	73.54^{ab}	78.95^{b}	1.29
Sperm concentration (million/ml)****	736.36^{a}	977.82^{ab}	1040.9^{b}	29.29
Live sperm (%)*	70.27^{a}	79.63^{b}	85.13^{c}	1.20
Acrosomal integrity (%) *	73.63^{a}	84.72^{b}	89.40^{c}	1.23
HOSST (%)*	56.22^{a}	64.63^{b}	70.13^{c}	1.17
BCMPT (distance in mm)*	23.81^{a}	31.40^{b}	35.68^{c}	0.88

Significance levels: *(p<0.05), **(p<0.01), ***(p<0.001),**** (p<0.0001)

system (El-Masry *et. al.*, 1994; Mohan *et. al.*, 1997). It is postulated that zinc supplemented group had an increased concentration of zinc containing enzymes *viz.* sorbitol dehydrogenase (SoDH) and lactate dehydrogenase (LDH), which play significant role in sperm motility (Bedwal and Bahuguna, 1994). Zinc also acts as an antioxidant, as it destroys the free oxygen radical and protects sperm from oxidative damage and lipid peroxidation by inhibiting phosphatase (Eggert Kruss *et. al.*, 2002). It is postulated that antioxidant action of zinc may also be responsible for improved motility of spermatozoa in zinc-supplemented groups.

The mean sperm livability (%) was 70.2, 79.6 and 85.1 in group I, II and III, respectively and was significantly (P<0.05) higher in Zn supplemented groups as compared to control group. Increased livability percentage due to zinc supplementation is in accordance with El-Masry *et. al.* (1994) and Omu *et. al.* (1998), who observed improved live sperm percentage by zinc supplementation in rabbit and men, respectively. Improved livability of sperms may be due to the membrane stabilizing action of zinc by virtue of which it prevent leakage of enzymes and other vital component of the spermatozoa and thus extends the sperm livability. Zinc also stabilizes the ribosome, lysosomes, DNA and RNA, which increase the livability and normal function of spermatozoa. It also protects the spermatozoa from free radicals by its antioxidant properties and thus improves the sperm livability. Bires *et. al.* (1997) reported that as a constituent of large number of metalloenzymes, zinc is involved in carbohydrate, protein, lipid and nucleic acid metabolism, which may improve the sperm livability. It also has been reported that zinc is a primary cofactor responsible for production of an antibacterial substance from prostrate gland in semen, which might have increased the livability of spermatozoa in zinc-supplemented groups.

The mean acrosomal integrity (%) was 73.6, 84.7 and 89.4 in-group I, II and III, respectively. It was significantly (P<0.05) higher in Zn supplemented groups as compared to control. Results further revealed that acrosomal integrity (%) was significantly (P<0.05) higher in zinc propionate supplemented group as compared to zinc sulphate supplemented group. Similar higher acrosomal integrity (%) was reported in cattle bulls given different dietary levels of zinc (Kumar *et. al.*, 2006). Increased acrosomal integrity (%) in zinc-supplemented groups may be due to antioxidant property and membrane

stabilizing action of zinc. Zinc has also been found to stabilize various acrosomal enzymes like acrosin, acid phosphatase, phospholipase etc, which may improve the acrosomal integrity (%). Acrosomal part of spermatozoa is derived from lysosomes during spermiogenesis, and zinc has been reported to stabilize the lysosomes (Gavella and Lipovac, 1998).

BCMPT (Distance in mm):

The mean BCMPT (mm) was 23.818, 31.409 and 35.682 in group I, II and III, respectively, indicating significantly ($P<0.05$) higher values in zinc supplemented groups as compared to control; results further revealed that sperm penetration distance (mm) was significantly ($P<0.05$) higher in zinc propionate supplemented group as compared to zinc sulphate supplemented group. Similarly, Kumar *et. al.* (2006) also observed significantly ($P<0.01$) higher BCMPT values in the semen of Zn supplemented crossbred cattle bulls. Intrinsic sperm motility plays a crucial role in the penetration of mucus (David *et. al.*, 1979). Improved values of BCMPT in Zn supplemented groups may be due to improved sperm motility and livability, as sperm penetration distance depends on number of motile and viable sperms (Dev *et. al.*, 1996). Our findings also support the above report that BCMPT was correlated with sperm motility (%). The significant positive correlation of BCMPT with sperm motility in present study was in agreement with Okuda *et. al.* (1988). One of the most important factors influencing the sperm penetration through cervical mucus is presence of anti-sperm antibody in seminal plasma, which reduces normal progression of sperm through cervical mucus. Kremer and Jager (1980) observed that zinc reduces the level of anti-sperm antibody in semen, which caused increased penetration distance in zinc-supplemented groups.

HOSST (%):

The mean HOSST (%) was 56.22, 64.63 and 70.13 in group I, II and III, respectively, which was significantly ($P<0.05$) higher in groups supplemented with zinc. This observation is in consonance with the findings of previous researchers who observed a significant ($P<0.05$) increase in HOSST (%) after zinc supplementation in men (Omu *et. al.*, 1998), sheep (Kendal *et. al.*, 2000) and cattle (Kumar *et. al.*, 2006). HOSST (%) is directly correlated with the sperm membrane integrity, and zinc supplementation has been reported to elicit the membrane stability by inner coating with some functional group of intrinsic component of sperm membrane. Zinc also helps in formation of stable mercapeptides by reacting with -SH group of membrane protein, which changes the fluidity and stabilizes the membrane (Eggert Kruss *et. al.*, 2002). Therefore, it is postulated that increase in HOSST (%) in the present study is attributed to increase in motility and viability of sperms in zinc supplemented groups.

Conclusion:

It may be concluded that dietary zinc supplementation improved the semen quality of buffalo bulls and zinc propionate had shown better results in comparison to double dose of zinc sulphate supplementation.

ACKNOWLEDGEMENT

Authors are grateful to Director, Indian Veterinary Research Institute, Izatnagar for providing the necessary facilities for carrying out this research work. We are thankful to M/s Kemin, Chennai for providing a free sample of zinc propionate for carrying out this study. This work was carried out under AP Cess Scheme of Indian Council of Agricultural Research, Krishi Bhawan, New Delhi.

REFERENCES

Abbasi, A.A., Prasad, A.S.and Rabbani, P. (1980). Experimental zinc deficiency in man: effect on testicular function. Journal

of Laboratory Clinical Medicine, **96**: 544-50.

Apgar, J. (1985). Zinc and reproduction. Annual Review of Nutrition, **5**: 43-68.

Bedwal, R.S. and Bahuguna, A. (1994). Zinc, copper and selenium in reproduction. Experentia, **50**: 625-640.

Bires, J., Bartko, P. and Huska, M. (1997). Distribution of risk element in the organism of sheep after industrial intoxication with zinc. Spectroscopic Letter **30**:1263-1277.

Chia,S.E., Ong, C.N., Chua, L.H., Ho, L.M. and Tay, S.K. (2000). Comparison of zinc concentration in blood and seminal plasma and the various sperm parameters between fertile and infertile men. Journal of Andrology, **21**(1): 53-57.

David, M.P., Amit, A., Bergman, A., Yedwab, G., Paz, G.F. and Homonnal, Z.T. (1979). Sperm penetration *in vitro*: correlation between parameters of sperm quality and penetration capacity. Fertility and Sterility, **32**: 676.

Davies, S. (1985). Zinc, nutrition and health. In: 1984-85 Yearbook of Nutritional Medicine (Ed; J. Bland), Keats Publishing, New Canaan, Conn, pp 113-152

Dev, S., Pangawkar, G.R., Sharma, R.K. and Matharoo, J.S. (1996). Sperm mucus penetration and its relation to semen quality of buffalo bulls. Indian journal of Animal Sciences, **66**(7): 713-715.

Droke, E.A., Gengel bach, G.P. and Spears, J.W. (1998). Influence of level and source (inorganic vs. organic) of zinc supplementation on immune function in growing lambs. Asian Australasian Journal of Animal Science, **11**: 139-144.

Ebisch, T.M.W., Van Heerde, W.L., Thomos, C.M.G., Vander Put, N., Wong, W.Y. and Steegers Theunissen, R.P.M. (2003). C677T methylene tetrahydrofolate reductase polymorphism interferes with effect of folic acid and zinc sulphate on sperm concentration. Fertility and Sterility, **80**:1190-1194.

Eggert Kruss, W., Zwick, E.M., Batschulat, K., Rohr, G., Armbruster, F.P., Petzoldt, D and Strowitzki, T. (2002). Are zinc levels in seminal plasma associated with seminal leukocyte and other determinant of semen quality Fertility & Sterility, **17**(2): 260-269.

El-Masry, K.A., Nasr, A.S. and Kamal, T.H. (1994). Influences of season and dietary supplementation with selenium and vitamin E or zinc on some blood constituents and semen quality of NewZealand white rabbit males. World Rabbit Science, *2(3): 79-86.*

Engle, T.E., Nockels, C.F., Kimberling, C.V., Weaben, D.L. and Johnson, A.B. (1995). The effect of feeding organic and inorganic zinc on biochemical parameters in zinc deficient calves. Proceedings of Western Section of American Society of Animal Science, **46**: 471-474.

Gavella, M. and Lipovac, V. (1998). *In vitro* effect of zinc on oxidative changes in human semen. Andrologia, **36(6)**: 317-323.

Jeyendran, R.S., Vanderven, L.J.D., Perezpelaez, H.H. M., Crabo, B.G. and Zaneveld, L.J.D. (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. Journal of Reproduction and Fertility,**70**: 219-228.

Kearl, L. C. (1982). Nutrient Requirement of Ruminants in Devoloping Countries. International Feedstuffs Institute, Utah Agriculture Station, Utah State University, Logan, Utah (USA).

Kendall, N.R., McMullan, S., Green, A. and Rodway, R.G. (2000). Effect of zinc, cobalt and selenium soluble glass bolus on trace element status and semen quality of ram lambs. Animal Reprod. Science, **62(4)**: 277-283.

Kremer, J. and Jager, S. (1980). Characterization of anti-sperm antibodies responsible for shaking phenomenon with special regard to immunoglobulin class and antigen reactive sites. Int. Journal of

Andrologia, **3:** 143.

Kumar, N., Verma, R.P., Singh, L.P., Varshney, V.P. and Dass, R.S. (2006). Effect of different levels and sources of zinc supplementation on seminal attributes, testosterone level with special reference to *in vitro* fertility tests in crossbred bulls. Reproduction Nutrition Development (Accepted)

Mandal, G. P., Dass, R.S., Isore D. P., Garg A. K. and Ram, G.C. (2007). Effect of zinc supplementation through different sources on growth, nutrient utilization and immune response in male crossbred cattle (Bos indicus x Bos taurus) calves. Animal Nutrition and Feed Technology **138**:1-12.

Martin, G.B., White, C.L., Markey, C.M. and Blackberry, M.A. (1994). Effect of dietary zinc deficiency on the reproductive system of young male sheep: testicular growth and the secretion of inhibin and testosterone. Journal of Reprod. and Fertility, **101:** 87-96.

Massonyi, P., Tomon, R., Trandzik, J., Nad, P., Skalicka, M. and Korenekova (2004). Concentration of copper, zinc, iron, cadmium, lead and nickel in bull, ram, boar, stallion and fox semen. Trace Element and Electrolyte, **21** (1): 45-49.

Matousek, J., Riha, J., Srean, V., Veselsky, L. and Launda, F. (1989). Penetration of cervical mucus and other body fluids by bull sperm in capillary tube. Animal Reproduction Science, **18:** 161-166.

Mohan, H., Verma, J., Singh, I., Mohan, P., Marwah, S. and Singh, P. (1997). Interrelationship of zinc levels in serum and semen in oligospermic infertile patients and fertile males. Indian Journal of Pathology and Microbiology, **40**(4): 451-455.

Nagamine, C.M., Chan, K., Hake, L.E. and Lau, Y.F.C. (1990). The two-candidate testis determining Y genes (Zfy-1 and Zfy-2) are differentially expressed in fetal and adult mouse tissues. Genes and Development ,**4:** 63-74.

NRC (1989). Nutrient Requirement of Dairy Cattle. National Academy Press, Washington, DC,

Okuda, K., Murase, T., Sato, K., Matsuzaki, S., Iwane, S. and Iwane, N. (1988) Penetration ability of bull spermatozoa in to cervical mucus. Proceeding of the 11th International Congress on Animal Reproduction and Artificial Insemination, Dublin, **3:** 279-281.

Omu, A.E., Dashti, H. and Al-Othman, S. (1998). Treatment of asthenozoospermia with zinc sulphate: andrological, immunological and obstetric outcome. European Journal of Obstetrics and Gynecology Reproduction Biology, **79:** 179-184.

Saleh, A.M., Ibrahim and Yousri, R.M. (1992). The effect of dietary zinc, season and breed on semen quality and body weight in goat. International Journal of Animal Science **7**(1): 5-12.

Salisbury, G.W., Van Denmark, N.L.and Lodge, J.R. (1985). Physiology of Reproduction and Artificial Insemination of cattle (2nd edn), CBS Publishers and Distributors, Sahadra, Delhi, India.

Snedecor, G.W. and Cochran, W.G. (1989). Statistical Method, 6th edition. The Iowa State University Press, Ames, Iowa, USA.

Spears, J.W., Harvey, R.W. and Jr. Brown, T.T. (1991). Effect of zinc methionine and zinc oxide on performance, blood characteristics and antibody titer response to viral vaccination in stressed feeder calves. Journal of American Veterinary Medical Association, **199**:1731-1733.

Steel, R.G.D. and Torrie, G.H. (1980). Principles and Procedures of Statistics: A Biometrical Approach, 2nd Ed, McGraw Hill, New York, pp 633.

Suruki, T., Nakajima, K., Yamamoto, A. and Yamanaka, H. (1995). Metallothionein binding zinc inhibits nuclear chromatin decondansation of human spermatozoa. Andrologi, **27**: 161-164.

Tharwat, E.E. (1998). The use of zinc sulphate to improve semen characteristics and fertility of New Zealand White Rabbit buck during hot season. Annals of Agricultural Sciences (Cairo), 3: 765-770.

Watson, P.F. (1975). Use of Giema stain to detect changes in acrosome of frozen ram spermatozoa. Veterinary Record, 97: 12-15.

Wong, W.Y., Merkus, H.M., Thomas, C.M., Menkveld, R, Zielthuis, G.A. and Steegers-Theunissen, R.P. (2002). Effect of folic acid and zinc sulphate on male factor sub fertility, a double blind, randomized placed controlled trial. Fertility and Sterility, 77(3): 491-498.

Wroblewski, N., Schill, W.B. and Henkel, R. (2003). Metal chelators change the human sperms motility pattern. Fertility and Sterility, 79 (Suppl. 3): 1584-1589.

The Indian Journal of Animal Reproduction
(The Indian Society for Study of Animal Reproduction)
(Regd. No. Bom. 153/78)
AUTHOR'S DECLARATION CERTIFICATE

Article entitled :

1. **The first author is life member of ISSAR. His life membership no.....** It is mandatory for the first author to be life member of the ISSAR for the publication in IJAR.
2. The article has been seen by all the authors (signatures given below) who are satisfied with its form and content and are responsible for the technical details and ethical maters of the paper.
3. Due credit of authorship has been given to every scientist who has made a notable contribution to the paper and are satisfied with sequence in which the authors names appear in the byline.
4. The by-line of the article does not include any name of the scientist who has not made a notable contribution to the paper.
5. The name of institute appearing below the by-line is that of the institute where the research was conducted and not of the institute where the first author (or the author who has sent the paper) is currently employed.
6. Two copies of the article along with two copies of tables and all illustrations have been submitted.
7. Article has been not been published or sent simultaneously for publication to any other journal.
8. The article has not been rejected for publication in any other journal. Rejection elsewhere does not necessarily disqualify the paper for publication in the *The Indian Journal of Animal Reproduction* but please attach a copy of the reasons given for rejection.

Signature of all authors, their designation and present correspondence address.

Note: Please enclose this Certificate at the time of Submission of the Manuscript, without which the article maynot be processed or considered.

President ISSAR