Effect of organic zinc supplementation in hens on fertility from cryopreserved semen

Shanmugam Murugesan.*, Alagarsamy Kannan and Ramkrishna Mahapatra

ICAR-Directorate of Poultry Science, Rajendranagar, Hyderabad-30, India

* Corresponding author email id: physioshan@gmail.com; Shanmugam.murugesan@icar.gov.in

Abstract

Organic zinc supplementation in hen has been reported to improve fertility. The current study evaluated the effect of organic zinc supplementation in hens on fertility after insemination with cryopreserved semen. White Leghorn rooster semen was cryopreserved using 4% dimethylsulfoxide (DMSO) in 0.5ml French straws. Different semen parameters and fertility were assessed in post-thaw samples. White Leghorn hens were divided into 5 groups with 30 birds in each group. Each group was further divided into six replicates of five birds each. The control group was fed basal diet, other groups were fed with basal diet supplemented with 40, 60, 120 and 160 mg/kg organic zinc (zinc proteinate). After two weeks of feeding insemination was done in hens per vagina using thawed semen (200 million sperm/0.1 ml). Basal group hens were inseminated with fresh or cryopreserved semen and served as control groups. Sperm motility, live sperm, and acrosome intact sperm parameters were significantly ($p < 0.05$) lower in post-thaw semen samples. Fertility from cryopreserved semen was significantly ($p < 0.05$) lower and organic zinc supplemented hens had fertility similar to that of cryopreserved semen inseminated into basal diet group hens. In conclusion, organic zinc supplementation in hens does not improve fertility after insemination with 4% DMSO cryopreserved semen.

Key Words: Chicken; cryopreservation; fertility; semen; zinc

1. INTRODUCTION

Zinc is an important trace mineral in poultry that is involved in various biological and metabolic processes. Zinc is required for growth, normal functioning of reproductive and immune systems in chicken (Huang et al., 2019). Dietary supplementation of zinc in diet produced beneficial effects on laying performance, egg quality and antioxidant capacity in laying hens (Li et al., 2019). Zinc deficiency has been shown to affect hatchability in chicken (Blamberg et al. 1960). Zinc supplementation in chicken feed either had no effect on fertility (Stahl et al., 1986; Durmuş et al., 2004) or improved fertility and hatchability in addition to reduced embryonic mortality (Amen and Al-Daraji, 2011; Zhang et al., 2017; Li et al., 2019).

Fertility from cryopreserved semen is influenced by different factors such as breed/line of bird, cryoprotectant, cryopreservation protocol and presence of additives in the cryopreservation mixture (Donoghue and Wishart, 2000).

Studies have evaluated factors that improve fertility outcome from cryopreserved semen giving attention to male reproductive system. In chicken after insemination semen is stored in the sperm storage tubules (SST) up to three weeks and sperm are released from this storage site periodically so that the sperm move up the reproductive tract and fertilize the released ovum. The storage and release mechanisms of sperm are not fully deciphered (Sasanami et al., 2013). Turkey hens have been shown to influence the sperm penetration of inner perivitelline membrane and fertility and this effect is independent of sire (Christensen et al., 2006). Considering the foregone information it is not known whether manipulation of female reproductive system will improve fertility from cryopreserved semen. The aim of this study was to assess whether zinc supplementation in layer hens improve the fertility after insemination with cryopreserved semen.

2. MATERIALS AND METHODS

48 2.1 Experimental birds and husbandry

49 The experiment was carried out at the poultry farm of ICAR- Directorate of Poultry
50 Research located in Hyderabad, India. The White Leghorn layers (IWH line) used in the
51 experiment was housed in individual cages in an open-sided house. Feed and water were
52 provided *ad libitum*. The experiment protocols were approved by the Institutional Animal
53 Ethics Committee (IAEC/DPR/18/8).

54 2.2 Experiment

55 For the study, 150 White Leghorn layer hens of 38 weeks of age were selected and
56 divided into 5 groups with 30 birds in each group. Each group was further divided into six
57 replicates of five birds each. The five dietary treatments were Basal diet, Basal diet
58 supplemented with 40, 80, 120 and 160 ppm organic zinc as zinc proteinate. The basal diet
59 consisted primarily of corn and soybean meal (Table 1). Birds were subjected to 14 hrs of
60 light per day. All hens were kept under the same managerial conditions. The supplemental
61 trial period was for 10 weeks duration.

62 2.3 Semen collection and processing

63 Fifteen White Leghorn roosters aged 39 weeks were earlier trained to respond to
64 abdominal massage technique (Burrows and Quinn, 1937) for collection of semen. Semen
65 was collected randomly from the roosters on a day, pooled and kept on ice during the
66 experiment. Collected semen was brought to the laboratory over ice in a covered thermocol
67 box, evaluated and processed for cryopreservation. In the laboratory a portion of semen was
68 diluted in a semen diluent (D (+)-glucose - 0.2 g, D (+)-trehalose dehydrate- 3.8 g, L-
69 glutamic acid, monosodium salt- 1.2 g, Potassium acetate- 0.3 g, Magnesium acetate
70 tetrahydrate - 0.08 g, Potassium citrate monohydrate - 0.05 g, BES- 0.4 g, Bis-Tris-

0.4 g in 100 ml distilled water, pH 6.8; Sasaki et al., 2010) and was used for evaluation of semen quality parameters.

The pooled semen samples were initially evaluated for sperm concentration. The samples were diluted with cryoprotectant free diluent such that the sperm concentration was arrived at 4 million/µl. The samples were equilibrated at 5°C for 30 minutes and were diluted in 1:1 proportion with diluent containing 8% dimethyl sulfoxide (DMSO) so that the final concentration of DMSO was 4% and the final sperm concentration was 2 million/ µl in each treatment. The semen mixed with DMSO was immediately loaded into 0.5 ml French straws and sealed with polyvinyl alcohol powder. The filled straws were placed 4.5 cm above the liquid nitrogen (LN_2) on a Styrofoam raft floating on LN_2 in a thermocol box. The straws were exposed to nitrogen vapours for 30 minutes, plunged into LN_2 and stored at -196°C until further use. Semen straws were stored for a minimum of seven days before evaluation. Cryopreserved semen after thawing at 5°C for 100 sec in ice water (Sasaki et al., 2010) was evaluated on nine different occasions for progressive sperm motility, live and abnormal sperm and intact sperm acrosome.

2.4 Sperm motility

Sperm motility was recorded as percentage of progressively motile sperm by placing a drop of diluted semen on a Makler chamber and examining under 20 x magnification. The percentage of sperm with normal, vigorous, and forward linear motion was subjectively assessed and scored.

2.5 Live and abnormal sperm

Percent live and abnormal sperm were estimated by differential staining technique using Eosin-Nigrosin stain (Campbell et al., 1953). Semen smear was prepared by mixing one

drop of semen with two drops of Eosin-Nigrosin stain and air dried. Slides were evaluated under high power (100x) objective lens. All full and partially pink stained sperm were considered dead and unstained sperm as live. The percentage of live sperm was determined by counting at least 200 sperm. The same slides were used for estimating the abnormal sperm percent that were showing different morphological abnormalities.

2.6 Intact sperm acrosome

The intactness of sperm acrosome was assessed according to Pope et al. (1991). In brief, 10 µl of semen was mixed with 10 µl of stain (1% (wt/vol) rose Bengal, 1% (wt/vol) fast green FCF and 40% ethanol in citric acid (0.1 M) disodium phosphate (0.2 M) buffer (McIlvaine's, pH 7.2-7.3) and kept for 70 sec. On a clean glass slide a smear of the mixture was made, dried and examined under high magnification (1000x). The acrosomal caps were stained blue in acrosome-intact sperm and no staining in the acrosome region of acrosome reacted sperm. A minimum of 200 sperm were counted in each smear sample and the percent acrosome intact sperm was calculated.

2.7 Fertility trial

Fertility trial was conducted using cryopreserved semen. After two weeks of feeding trial in hens insemination was carried out. In fresh semen insemination group and DMSO control groups 15 hens/treatment were inseminated whereas in all other zinc supplemented groups 20 hens/treatment were inseminated. Insemination was done twice at five days interval. The semen straws were thawed at 5°C for 100 sec in ice water (Sasaki et al., 2010) and inseminated into hen per vagina with sperm concentration of 200 million sperm/0.1 ml. For cryopreservation control the basal diet group hens were inseminated with cryopreserved semen. Freshly collected semen was inseminated into basal diet group hens with 100 million sperm/0.1 ml dose. Eggs were collected from second day of first insemination and stored at

118 15°C until incubation. The number of eggs incubated in different treatments ranged from 84
119 to 128. The eggs incubated at standard conditions in an automatic setter were candled on 18th
120 day of incubation for embryonic development. Infertile eggs were broken open to confirm
121 absence of embryonic development.
122
123 2.8 Statistical analysis

124 Data were analyzed using SPSS 16 software and $p < 0.05$ was considered significant.
125 Percentage data were arcsine transformed and analyzed. Statistical analyses of semen
126 parameters and fertility were done by one-way ANOVA with Tukey's post hoc test.

127 **3. RESULTS**

128 The progressive sperm motility, live sperm and acrosome intact sperm parameters
129 were significantly ($p < 0.05$) lower in post-thaw semen samples (Table 2). Fertility from
130 cryopreserved semen was significantly ($p < 0.05$) lower in comparison to fresh semen
131 insemination (Fig. 1). Fertility obtained in organic zinc supplemented hens was similar to that
132 from cryopreserved semen inseminated in hens fed basal diet. No fertile eggs were obtained
133 from 120 ppm organic zinc supplemented group.

134 **4. DISCUSSION**

135 Fertility after insemination with cryopreserved semen in chicken is variable and
136 research is undertaken to improve it by manipulating the cryopreservation protocols. The
137 present study evaluated a novel way to improve fertility from cryopreserved semen through
138 organic zinc supplementation in hens. The National Research Council recommends inclusion
139 of zinc at 50 and 65 mg/kg diet for optimum productive and reproductive performance
140 respectively (NRC 1994). A corn soybean diet should contain about 72 mg Zn/Kg of diet for

obtaining good fertility and hatchability (Kidd et al., 1993). Hens fed corn-soy diet with 30 mg Zn/kg for 29 weeks resulted in decreased fertility and hatchability (Anshan, 1990). Thus a corn soybean diet should contain sufficient amount of zinc for optimum reproductive performance. The sperm egg penetration test and fertility was higher in zinc (100 mg/kg) supplemented broiler breeder hens (Amen and Al-Daraji, 2011). The reasons attributed for the higher results due to zinc supplementation were improvement in storage of sperm in SST and sperm motility. Zinc supplementation as zinc glycinate has been shown to improve fertility in comparison to basal diet and as well as with hens supplemented with zinc sulphate (Zhang et al., 2017). In addition to fertility zinc glycinate supplementation improved the antioxidant status in the birds. Li et al. (2019) had also reported higher fertility and hatchability in Chinese yellow feathered chicken supplemented with zinc (48-120 mg/kg). Cobb 500 broiler breeder hens supplemented with zinc oxide (60-120 mg/kg) had higher fertility at the later phase of laying period (Sharideh et al., 2016). These studies indicated that the supplemental zinc as well as source of zinc has an effect on fertility. However, few studies have reported no effect of dietary zinc supplementation on fertility. Zinc supplemented either as zinc oxide or zinc methionine did not improve fertility or hatchability in broiler breeders (Kidd et al. 1993). No effect of zinc supplementation either as zinc carbonate (40 mg/kg) or zinc sulphate (2000 mg/kg) on fertility was observed in White Leghorn layers (Stahl et al., 1986; 1990). Similarly no effect of zinc supplementation up to 210 mg on fertility was reported in brown egg layers (Durmuş et al., 2004). In the present study fertility from cryopreserved semen was not affected by organic zinc supplementation. This may be due to the source of zinc or the breed used in the study. Though zinc supplementation had no effect in the present study, future research should evaluate fertility from cryopreserved semen using minerals or compounds that has been shown to improve fertility in hens under normal conditions. Thus the unique reproductive physiology of female

166 birds should be taken into consideration for improving the fertility outcome from
167 cryopreserved semen in conservation programs of avian species.

168 In conclusion, organic zinc supplementation in the hen diet does not improve fertility from
169 cryopreserved semen.

170 **CONFLICT OF INTEREST**

171 None of the authors have any conflict of interest to declare.

172

173 **AUTHOUR CONTRIBUTIONS**

174 Experiment was designed, data were analysed, interpreted and manuscript prepared by all
175 three authors. Feeding trials were conducted by A. Kannan and semen cryopreservation and
176 fertility trials were conducted by M.Shanmugam.

177 **REFERENCES**

178 Amen, M.H.M. and Al-Daraji, H. J. (2011). Effect of dietary supplementation with different
179 level of zinc on sperm egg penetration and fertility traits of broiler breeder chicken.
180 *Pakistan Journal of Nutrition*, 10, 1083-1088.

181 Anshan, S. (1990). Effects of zinc and calcium levels in hen diets on fertility and hatchability
182 of the egg and their newborn chicks. *Scientia Agricultura Sinica*, 23, 82-86.

183 Blamberg, D. L. , Blackwood, U. G., Supplee, W. C. and Combs, G. F. (1960). Effect of
184 zinc deficiency in hens on hatchability and embryonic development. *Proceedings of*
185 *the Society for Experimental Biology and Medicine,* 104, 217-228.

186 Burrows, W.H. and Quinn, J. P. (1937). The collection of spermatozoa from the domestic
187 fowl and turkey. *Poultry Science,* 16, 19-24.

188 Campbell, R.G., Hancock, J.L. and Rothschild, L. (1953). Counting live and dead bull
189 spermatozoa. *Journal of Experimental Biology*, 30, 44-49.

190 Christensen, V. L., Fairchild, B. D. and Ort, D. T. (2006). Dam and sire effects on sperm
191 penetration of the perivitelline layer of eggs produced by two strains of commercial
192 turkeys. *Journal of Applied Poultry Research,* 15, 82-88.
193 Donoghue, A.M. and Wishart, G.J. (2000). Storage of poultry semen. *Animal Reproduction*
194 *Science*, 62, 213–232.
195 Durmuş, İ., Ataşoğlu, C., Mizrak, C., Ertaş, S. and Kaya, M. (2004). Effect of increasing zinc
196 concentration in the diets of brown parent stock layers on various production and
197 hatchability traits. *Archiv fuer Tierzucht Dummerstorf*, 47, 483-489.
198 Huang, L., Li, X., Wang, W., Yang, L. and Zhu, Y. (2019). The role of zinc in poultry
199 breeder and hen nutrition: an update. *Biological Trace Element Research*, 192, 308-
200 318.
201 Kidd, M.T., Anthony, N.B., Newberry, L.A. and Lee, S.R. (1993). Effect of supplemental
202 zinc in either a corn-soybean or a milo and corn-soybean meal diet on the
203 performance of young broiler breeders and their progeny. *Poultry Science*, 72, 1492-
204 1499.
205 Li, L., Abouelezz, K. F. M., Gou, Z., Lin, X., Wang, Y., Fan, Q., Cheng, Z., Ding, F., Jiang,
206 S. and Jiang, Z. (2019). Optimization of dietary zinc requirement for broiler breeder
207 hens of chinese yellow-feathered chicken. *Animals*, 9, 472.
208 https://doi.org/10.3390/ani9070472
209 NRC (ed). (1994). Nutrient Requirements of Poultry, 9th revised edn. National Academy
210 Press, Washington, DC.
211 Pope, C.E., Zhang, Y.Z. and Dresser, B.L. (1991). A simple staining method for evaluating
212 acrosomal status of cat spermatozoa. *Journal of Zoo and Wildlife Medicine*, 22, 87-
213 95.

214 Sasaki, K., Tatsumi, T., Tsutsui, M., Niinomi, T., Imai, T., Naito, M., Tajima, A. and Nishi,
215 Y. (2010). A method for cryopreserving semen from Yakido roosters using n-
216 methylacetamide as a cryoprotective agent. *The Journal of Poultry Science*, 47, 297-
217 301.
218 Sasanami, T., Matsuzaki, M., Mizushima, S. and Hiyama, G. (2013). Sperm storage in the
219 female reproductive tract in birds. *Journal of Reproduction and Development*, 59,
220 334–338.
221 Sharideh, H., Zhandi, M., Zaghari, M. and Akhlaghi, A. (2016). Dietary zinc oxide and 6-
222 phytase effects on fertility rate in old broiler breeder hens. *Journal of Agricultural*
223 *Science and Technology*, 18, 327–336.
224 Stahl, J.L., Cook, M.E. and Sunde, M.L. (1986). Zinc supplementation: its effect on egg
225 production, feed conversion, fertility, and hatchability. *Poultry Science*, 65, 2104-
226 2109.
227 Stahl, J.L., Greger, J.L. and Cook M.E. (1990). Breeding-hen and progeny performance when
228 hens are fed excessive dietary zinc. *Poultry Science*, 69, 259-263.
229 Zhang, L., Wang, Y.X., Xiao, X., Wang, J.S., Wang, Q., Li, K.X., Guo, T.Y. and Zhan, X.A.
230 (2017). Effects of zinc glycinate on productive and reproductive performance, zinc
231 concentration and antioxidant status in broiler breeders. *Biological Trace Element*
232 *Research*, 178, 320–326.
233

234 **TABLE 1** Composition and nutrient levels of basal diet

Ingredient	%
Maize	61.46
Soyabean meal	24.74
Deoiled rice bran	0.42
Stone grit	10.90
Di-calcium Phosphate	1.50
Salt	0.50
DL-Methionine	0.10
Trace minerals[A]	0.10
Vitamin premix[B]	0.02
Vitamin B Complex	0.02
Choline chloride	0.10
Toxin binder	0.10
Tylosine	0.05
Total	100
Calculated values	
Metabolizable energy, MJ/kg	11.33
Crude Protein, %	17.22
Crude fibre, %	3.32
Lysine,%	0.84
Methionine, %	0.37
Methionine and cystine , %	0.58
Calcium, %	4.56
Nonphytate P, %	0.47
Zinc, mg/kg	52

235

236 [A] Supplied (mg/kg diet):Mn, 24.2; Zn, 22; Cu, 5; Fe, 23.1; Se, 0.68; I, 1.9; Co, 0.21.

237 [B]Supplied (mg/kg diet): thiamin 1; pyridoxine, 2; cyanocobalamine, 0.01; niacin, 15;
238 pantothenic acid, 10; a tocopherol, 10; riboflavin, 10; biotin, 0.08; menadione, 2; retinol
239 acetate, 2.75; cholecalciferol, 0.06; choline, 650.

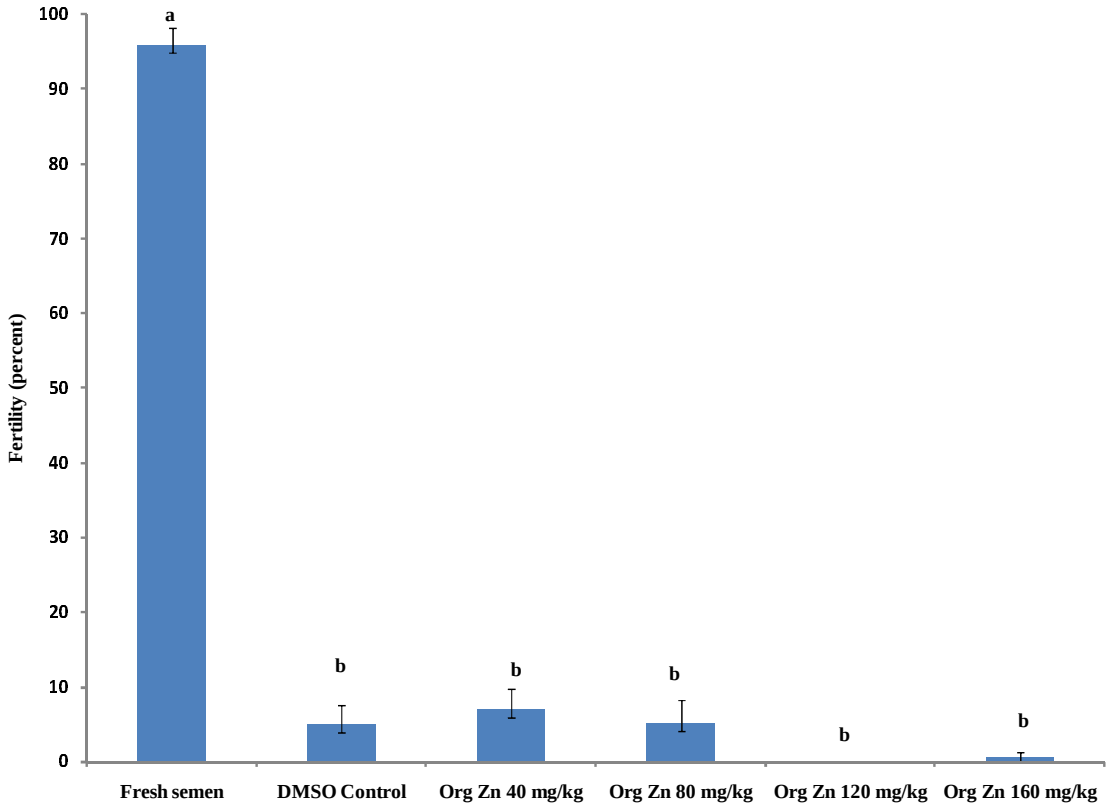
240

TABLE 2 In vitro semen parameters in fresh and post-thaw White Leghorn semen cryopreserved using Sasaki diluent and 4% dimethyl sulfoxide.

Parameters	Fresh semen	4% DMSO
Progressive sperm motility (%)	65.56 ± 1.6[a]	16.11 ± 1.62[b]
Live sperm (%)	77.16± 2.8 [a]	21.28 ± 1.9 [b]
Abnormal sperm (%)	2.10 ± 0.17	2.19 ± 0.31
Acrosome intact sperm (%)	93.56 ± 1.4 [a]	87.91 ± 2.42 [b]

Values are mean±SE obtained from nine independent evaluations.
Figures bearing different superscripts in a row differ significantly ($p < 0.05$).

Figure 1. Fertility after insemination of cryopreserved semen in organic zinc supplemented White Leghorn hens



Bars bearing different superscripts differ significantly ($p < 0.05$).