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Effect of Organic Zinc Supplementation on Fertility of White Leghorn Hens Inseminated with Cryopreserved Semen

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Abstract

Organic zinc supplementation in hens 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-thawed 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 a basal diet, other groups were fed with a 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.

Keywords: chicken, cryopreservation, fertility, semen, zinc

Introduction

Zinc is an important trace mineral in poultry that is involved in various biological and metabolic processes. Zinc is required for the growth, and normal functioning of reproductive and immune systems in chickens (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 chickens (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). Higher fertility result in hens was obtained after zinc supplementation that had supposedly resulted in improvement in the storage of sperm in sperm storage tubules (SST) and sperm motility (Amen and Al-Daraji, 2011). 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 of the birds. Thus zinc supplementation in hens might help in improving fertility after insemination.

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). Cryopreservation causes a drastic reduction in sperm ATP concentration and the resulting fertility varies between the pedigreed lines (Long, 2006). Furthermore, the sperm undergoes irreversible damage during cryopreservation that leads to reduced motility and fertility.

Studies have evaluated factors that improve fertility outcomes from cryopreserved semen giving attention to the male reproductive system. In chickens after insemination semen is stored in the sperm storage tubules (SST) for 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 the 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 the 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 of cryopreserved semen.

Materials and Methods

Experimental birds and husbandry

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

Experiment

For the study, 150 White Leghorn breeder hens of 38 weeks of age were selected and divided into 5 groups with 30 birds in each group. Each group was further divided into six replicates of five birds each. The five dietary treatments were a basal diet, a basal diet supplemented with 40, 80, 120 and 160 mg/kg organic zinc as zinc proteinate. The basal diet consisted primarily of corn and soybean meal (Table 1). The analyzed zinc concentration in the basal diet was 49.33 mg/kg. Birds were subjected to 14 hrs of light per day. All hens were kept under the same managerial conditions. The supplemental trial period was for 10 weeks duration.

Semen collection and processing

Fifteen White Leghorn roosters aged 39 weeks were used for semen collection by abdominal massage technique (Burrows and Quinn, 1937). Semen was collected randomly from the roosters on a day, pooled and held on ice during the experiment. The semen was brought to the laboratory in a thermocol box, evaluated and cryopreserved.

Table 1. Composition and nutrient levels of basal diet

Ingredient	%	*Calculated values*	
Maize	61.46	Metabolizable energy, kcal/kg	2707
Soyabean meal	24.74	Crude Protein, %	17.22
Deoiled rice bran	0.42	Crude fibre, %	3.32
Stone grit	10.90	Lysine,%	0.84
Di-calcium Phosphate	1.50	Methionine, %	0.37
Salt	0.50	Methionine and cystine , %	0.58
DL-Methionine	0.10	Calcium, %	4.56
Trace minerals[A]	0.10	Nonphytate P, %	0.47
Vitamin premix[B]	0.02	Zinc, mg/kg	52
Vitamin B Complex	0.02		
Choline chloride	0.10		
Toxin binder	0.10		
Tylosine	0.05		
Total	100		

[A] Supplied (mg/kg diet): Mn, 24.2; Zn, 22; Cu, 5; Fe, 23.1; Se, 0.68; I, 1.9; Co, 0.21.
[B]Supplied (mg/kg diet): thiamin 1; pyridoxine, 2; cyanocobalamine, 0.01; niacin, 15; pantothenic acid, 10; a tocopherol, 10; riboflavin, 10; biotin, 0.08; menadione, 2; retinol acetate, 2.75; cholecalciferol, 0.06; choline, 650.

A portion of semen was diluted in a semen diluent [D (+)-glucose (0.2g), D (+)-trehalose dehydrate (3.8g), L-glutamic acid, monosodium salt (1.2g), Potassium acetate (0.3g), Magnesium acetate tetrahydrate (0.08g), Potassium citrate monohydrate (0.05g), BES (0.4g), Bis-Tris (0.4g) in 100 ml distilled water, pH 6.8; 12) and used for evaluating semen parameters.

The pooled semen was evaluated for sperm concentration and was diluted using diluent without cryoprotectant to arrive at a concentration of 4 million sperm/µl. The samples were equilibrated for 30 minutes at 5°C and then diluted in 1:1 ratio with a diluent containing 8% dimethyl sulfoxide (DMSO). The final DMSO concentration was 4% and the sperm concentration was 2 million/ µl in each treatment. The semen mixed with DMSO was quickly loaded into 0.5 ml French straws and sealed. In a thermocol box, the straws were placed 4.5 cm above the liquid nitrogen (LN_2) on a floating Styrofoam raft. The straws were exposed to nitrogen vapours for 30 minutes, plunged into LN_2 and stored at -196°C. Semen straws were held for a minimum of a week before evaluation. The semen straws were thawed at 5°C for 100 sec in ice water (Sasaki et al., 2010) for evaluation of progressive sperm motility, live and abnormal sperm and sperm acrosome integrity. The procedure of cryopreservation and thawing was repeated nine times and data was collected.

Sperm motility

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

Live and abnormal sperm

Percent live and abnormal sperm were estimated by differential staining technique

using Eosin-Nigrosin stain (Campbell et al., 1953). One drop of semen was mixed with two drops of Eosin-Nigrosin stain and a semen smear was prepared. The smears were evaluated under high power (100x) objective lens. All full and partially pink stained sperm were considered dead and unstained sperm as live. A minimum of 200 sperm were counted for calculating the percentage of live sperm. Abnormal sperm that were showing different morphological abnormalities were calculated in the same slides.

Intact sperm acrosome

The sperm acrosome intactness was assessed as per Pope et al. (1991). In short, 10 μl each of semen and 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) was mixed and kept for 70 sec. A smear of the mixture was examined under high magnification (1000x). The acrosomal caps were stained blue in acrosome-intact sperm and had no staining in the acrosome region of acrosome-reacted sperm. About 200 sperm were counted in each sample for calculation of the percent acrosome intact sperm.

Fertility trial

A fertility trial was conducted using cryopreserved semen. After two weeks of feeding trial in hens, insemination was carried out. In the 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. The group of hens inseminated with fresh semen served as a reference group. Insemination was done twice at five days intervals. 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 a 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 the second day of the first insemination and stored at 15°C until incubation. The number of eggs incubated in different treatments ranged from 84 to 128. The eggs incubated at standard conditions were candled on the 18th day of incubation for the presence of an embryo. The infertile eggs were broken to confirm absence of any embryonic development.

Statistical analysis

Data were analyzed in SPSS 16 software and $P < 0.05$ was considered significant. Percentage data were arcsine transformed and analyzed. Statistical analyses of semen parameters were done by t-test and fertility from the cryopreservation treatments was done by one-way ANOVA.

Results

The progressive sperm motility, live sperm and acrosome intact sperm parameters were significantly ($P < 0.05$) lower in post-thaw semen samples (Table 2). The fertility obtained after fresh semen insemination was 96%. Fertility obtained in organic zinc supplemented hens were similar ($P > 0.05$) to that from cryopreserved semen inseminated in hens fed basal diet (Fig. 1). No fertile eggs were obtained from the 120 ppm organic zinc supplemented group.

Discussion

Cryopreservation is a stressful event for sperm and post-thaw its functionality is reduced. In the present study, the in vitro sperm parameters declined after cryopreservation.

Table 2. *In-vitro* semen parameters in fresh and post-thaw, White Leghorn semen cryopreserved using Sasaki diluent and 4% DMSO.

Parameters	Fresh semen	Post-thaw
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 ($P < 0.05$)

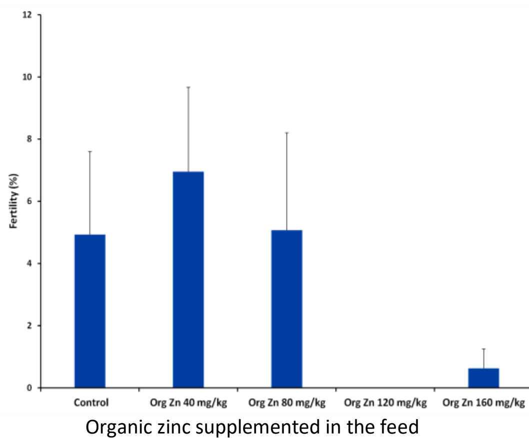

Figure 1. Fertility after insemination of DMSO cryopreserved semen into organic zinc supplemented White Leghorn hens

Chicken semen cryopreservation using DMSO has been reported in different studies with varying fertility 92.8% (Van Voorst *et al*., 1995), 38.8% (Hanzawa *et al*., 2010), 1.9% (Shanmugam *et al*., 2018) and 78.14% (Pranay Kumar *et al.* 2019). In the present study, fertility of 4.93% was obtained using DMSO alone. The variation in the fertility between the studies may be due to different breeds/lines of chicken or diluent employed.

Fertility after insemination with cryopreserved semen in chicken is variable and research is undertaken to improve it by manipulating the cryopreservation protocols. The present study evaluated a novel way to improve fertility from cryopreserved semen through organic zinc supplementation in hens. The National Research Council recommends the inclusion of zinc at 50 and 65 mg/kg diets for optimum productive and reproductive performance 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 a corn-soybean diet with 30 mg Zn/kg for 29 weeks resulted in decreased fertility and hatchability (Anshan, 1990). Thus, a corn-soybean diet should contain a sufficient amount of zinc for optimum reproductive performance. The sperm-egg penetration test and fertility were higher in zinc (100 mg/kg) supplemented broiler breeder hens (Amen and Al-Daraji, 2011). Li et al. (2019) 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 the laying period (Sharideh et al., 2016). These studies indicated that supplemental zinc as well as a source of zinc affects 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 have been shown to improve fertility in hens under normal conditions. Thus, the unique reproductive physiology of female birds should be taken into consideration for improving the fertility outcome from cryopreserved semen in conservation programs of avian species.

Conclusion

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

Conflict of Interest

The authors declare that they have no conflict of interest.

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