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## Effect of zinc supplementation during cryopreservation on post-thaw chicken semen parameters and fertility

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**ABSTRACT:** The present study evaluated supplementation of zinc from different sources during chicken semen cryopreservation on post-thaw semen quality and fertility. Adult White Leghorn chicken semen was cryopreserved using 4% dimethyl sulfoxide (DMSO) in Sasaki diluent (SD). In the semen cryomixture zinc oxide (6.25 and 12.5  $\mu\text{M}$ ) and zinc sulphate (100 and 200  $\mu\text{M}$ ) were added before freezing process. The plastic semen straws were thawed at 5°C for 100 sec and samples were evaluated for sperm motility, live, abnormal and acrosome intact sperm as well as lipid peroxidation levels in the seminal plasma. The semen cryopreservation and evaluation were repeated on six occasions. Fertility of the zinc supplemented semen was evaluated by inseminating into White Leghorn hens. Zinc supplementation did not affect any of the post-thaw sperm parameters. The lipid peroxidation and fertility were similar between the treatments. In conclusion, supplementation of zinc as zinc oxide or zinc sulphate during chicken semen cryopreservation does not affect post-thaw semen parameters or fertility.

**Keywords:** Chicken, Fertility, Semen cryopreservation, Zinc oxide, Zinc sulphate

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## INTRODUCTION

Conservation of chicken through semen cryopreservation is a management tool. The semen cryopreservation protocol is almost well standardized in cattle, however, similar consistent fertility results could not be achieved in chicken because of reasons such as line or breed variability and uniqueness of the chicken sperm (Long, 2006). Different studies have evaluated compounds added during the cryopreservation process to improve the fertility outcome from post-thaw chicken semen (Zhandi et al., 2017; Pranay Kumar et al., 2019).

Zinc is an important mineral having role in testicular development and steroidogenesis (Hamdi et al., 1997). In sperm zinc acts as a second messenger having role in motility and fertility (Chu, 2018). Zinc is an important component of metalloproteins and has role in sperm homeostasis and fertilizing ability (Kerns et al., 2018). The human sperm chromatin got stabilized and DNA damage prevented when zinc is added prior to cryopreservation (Kotdawala et al., 2012). Zinc inhibited the in vitro generated superoxide anion in human semen (Gavella and Lipovac, 1998). Zinc supplementation in bull semen cryopreservation mixture improved sperm motility, viability and total antioxidant capacity (Dorostkar et al., 2014). Recently, zinc oxide supplementation in chicken semen during freezing has been shown to improve post-thaw in vitro tested semen parameters (Zhandi et al., 2020). However, this study has not reported on the effect on fertility. Furthermore, the effect of zinc sulphate supplementation during chicken semen cryopreservation is not known. Thus, the present study evaluated the effect of zinc in cryopreservation media on post-thaw semen quality and fertility in chicken.

## MATERIALS AND METHODS

Semen from White Leghorn (IWK line) roosters aged 45 weeks was collected by dorso-abdominal massage method (Burrows and Quinn, 1937), pooled and cryopreserved with 4% DMSO. The birds were maintained in individual cages in an open-sided house at the institute poultry farm. The experimental protocol was approved by the Institutional Animal Ethics Committee. The pooled semen sample was equilibrated for 30 min at 5°C and then diluted using Sasaki diluent (D (+) -glucose- 0.2 g, D (+) - trehalose dehydrate- 3.8 g, L-glutamic acid, monosodium salt- 1.2 g, Potassium acetate- 0.3 g, Magnesium acetate tetrahydrate- 0.08 g, Potassium citrate monohydrate- 0.05 g, BES- 0.4 g, Bis-Tris- 0.4 g in 100 ml distilled water,

final pH 6.8; Sasaki et al., 2010). The effect of zinc during semen cryopreservation was evaluated using zinc oxide at 6.25 and 12.5 µM, and zinc sulphate at 100 and 200µM. The zinc levels were selected based on the earlier published reports (Ghallab et al. 2017; Zhandi et al. 2020). Samples with only cryoprotectant served as control. After mixing diluent containing different concentration of zinc the samples were maintained at 5°C for 30 min. The semen cryomixture was then loaded into 0.5 ml French straws with the final sperm concentration 2000 x 10<sup>6</sup>/ml and placed 4.5 cm over liquid nitrogen for 30 min after which they were plunged and stored in liquid nitrogen. The procedure of cryopreservation and post-thaw evaluation was repeated six times. The straws were stored for a minimum of a week and thawed at 5°C for 100 sec for evaluation and insemination. The thawed samples were assessed for progressive sperm motility, live sperm, abnormal sperm, and intact sperm acrosome. Insemination in 47 weeks old White Leghorn hens (8 hens/treatment) with thawed semen (200 million sperm/0.1 ml semen) was done three times at four days interval. The eggs collected were incubated under standard incubation conditions. The fertility was determined by candling the eggs on 18<sup>th</sup> day of incubation and expressed as percent fertility (Total number of fertile eggs/Total number of eggs incubated) x 100.

The progressively motile sperm in each sample was scored subjectively by evaluating a drop of semen on a Makler chamber under 20x magnification.

The live and abnormal sperm were assessed using Eosin-Nigrosin stain (Campbell et al., 1953). A semen smear was prepared after mixing a drop of semen and a drop of stain, air dried and examined under high (1000x) magnification. The live membrane intact sperm that were clear in appearance were counted and percent live sperm calculated. A total of 200 sperm were counted in each slide. The abnormal sperm percent assessed based on morphological abnormalities were also estimated in the same slides.

The intact sperm acrosome was evaluated as per Pope et al. (1991). Semen (10 µl) was mixed with equal volume 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 (Mc Ilvaine's, pH 7.2-7.3) ] and left for 70 sec. A smear of the mixture was made, dried and evaluated at high magnification (1000x). The sperm having intact acrosome was identified by the blue stained acrosomal

caps while no stained cap could be observed in the acrosome reacted sperm. The acrosome intact sperm percent was calculated by counting a minimum of 200 sperm in each sample.

The lipid peroxidation was evaluated by the thiobarbituric acid method (Hsieh et al., 2006) and expressed as the malondialdehyde (MDA) concentration in seminal plasma. The seminal plasma was separated by centrifuging semen samples at 3000 x g for 5 min and stored until analysis. The assay was performed by mixing 0.9 ml of distilled water and 0.1 ml seminal plasma in a glass tube followed by addition of 0.5 ml of thiobarbituric acid reagent. The tubes were kept in a boiling water bath for an hour. The cooled content of the tubes was measured for absorbance against blank at 540 nm in a colorimeter.

The data is presented as mean  $\pm$  SE. Data were analyzed in SAS 9.2 and  $P < 0.05$  was considered significant. The different zinc treatments were compared by one-way ANOVA with Tukey's post hoc test. The data were arcsine transformed where appropriate before analysis.

## RESULTS

The sperm motility, live and abnormal sperm and acrosome intact sperm values were similar between the treatments (Table 1). There was no difference in lipid peroxidation levels between the treatment groups. The fertility from zinc supplemented treatments was similar to that of the control cryopreservation treatment.

## DISCUSSION

Semen cryopreservation is a stressful event for the sperm where it undergoes structural as well as functional damages. Few reports have indicated beneficial

effects of zinc addition during semen cryopreservation. In the present study addition of zinc during semen cryopreservation was evaluated. Two salts of zinc, zinc oxide and zinc sulphate, were supplemented at concentrations reported elsewhere and post-thaw semen parameters and fertility studied.

In the present study zinc oxide addition in the cryopreservation extender had no effect on post-thaw semen parameters. This is in contrast to other reports in chicken (Zhandi et al., 2020) and human (Kotdawala et al., 2012) where addition of zinc prior to freezing improved the post-thaw sperm motility. The chicken sperm motility and mitochondrial membrane potential were improved only in the 1  $\mu$ g/ml treatment and higher level had no effect (Zhandi et al., 2020). However, the beneficial effects of addition of zinc on fertility was not reported. In the present study zinc oxide inclusion did not improve the fertility from cryopreserved semen. The zinc oxide concentration used in the present study was similar to Zhandi et al. (2020), however, no positive changes on semen parameters were observed. These differing results may be due to the different chicken breeds used in the studies. Furthermore, the differing results may also be due to the different freezing medium and straw size used in the studies. In vitro addition of zinc to human semen has been shown to reduce lipid peroxidation level (Gavella and Lipovac 1998). Zinc is a component in antioxidant enzymes that reduce the superoxide anions generated during oxidative stress which in turn results in lower lipid peroxidation level. In the present study the post-thaw seminal plasma MDA concentration was unaffected by supplementation of zinc salts. There are no reports of zinc inclusion during chicken semen cryopreservation on post-thaw semen lipid peroxidation for comparison of the results of the present study.

**Table 1.** Effect of zinc supplementation in cryopreservation medium on post-thaw semen parameters and fertility

Parameters	4% DMSO	Zinc oxide (6.25 $\mu$ M)	Zinc oxide (12.5 $\mu$ M)	Zinc sulphate (100 $\mu$ M)	Zinc sulphate (200 $\mu$ M)
Progressive sperm motility (%)	15.0 $\pm$ 1.29	13.33 $\pm$ 1.67	20.0 $\pm$ 2.24	17.5 $\pm$ 1.71	15.83 $\pm$ 1.54
Live sperm (%)	25.8 $\pm$ 0.57	26.78 $\pm$ 1.28	28.72 $\pm$ 1.63	30.57 $\pm$ 0.84	28.63 $\pm$ 1.71
Abnormal sperm (%)	2.82 $\pm$ 0.18	2.8 $\pm$ 0.25	2.6 $\pm$ 0.23	2.65 $\pm$ 0.16	3.12 $\pm$ 0.23
Acrosome intact sperm (%)	89.67 $\pm$ 0.42	91.0 $\pm$ 1.21	89.0 $\pm$ 2.0	83.83 $\pm$ 2.43	85.83 $\pm$ 2.21
Seminal plasma Lipid peroxidation (nM MDA/ml)	0.88 $\pm$ 0.08	1.03 $\pm$ 0.08	0.89 $\pm$ 0.03	0.93 $\pm$ 0.02	0.91 $\pm$ 0.05
Fertility (%)	5.61 $\pm$ 2.95	7.12 $\pm$ 3.79	3.33 $\pm$ 2.25	1.81 $\pm$ 1.81	0
Number of eggs incubated	52	60	65	54	66

Inclusion of zinc sulphate at 200µM during stallion semen cryopreservation has been shown to improve motility, viability, plasma membrane integrity and acrosome status (Ghallab et al., 2017). Supplementing 0.288 mg/L zinc sulphate in bull semen extender during cryopreservation improved the sperm motility and total antioxidant capacity, however, higher levels were found to be deleterious (Dorostkar et al., 2014). In the present study zinc sulphate was used at 100 and 200 µM concentrations. In the earlier studies in other species similar or higher concentrations have produced beneficial effects. This difference in result may be due to the species difference.

In conclusion, addition of zinc as zinc oxide or zinc sulphate during chicken semen cryopreservation does not affect the post-thaw semen parameters or fertility.

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#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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