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Effect of Tempol in Sasaki Diluent on the Quality and Fertility of Cryopreserved Chicken Semen

Short Communication

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ABSTRACT

Sperm are exposed to oxidative stress during cryopreservation leading to damage of membrane lipids. In the present study, the effects of adding Tempol during cryopreservation of chicken semen on post-thaw semen parameters and fertility were investigated. Adult PD-1linesemen were cryopreserved using 4% dimethyl sulfoxide (DMSO) in Sasaki diluent (SD). Tempol (1 and 5 mM) was added to the semen-cryopreservation mixture at final concentrations. The semen with additives was filled in 0.5 mL French straws and exposed to liquid nitrogen vapours for 30 min and then stored in liquid nitrogen. The semen straw was thawed at 5 ˚C for 100 seconds and evaluated for sperm motility, live, abnormal, and acrosome intact sperm. The seminal plasma was evaluated for lipid peroxidation. The fertilization potential of the cryopreserved sperm was evaluated after insemination in the PD-1line hens. Sperm parameters after thawing were significantly ($P<0.05$) lower in the cryopreserved groups compared to fresh semen. Lipid peroxidation was significantly ($P<0.05$) higher in cryopreserved groups. Fertility was significantly ($P<0.05$) lower in all the cryopreserved groups. In conclusion, the addition of template cryo diluent did not improve the post-thaw semen parameters or fertility.

KEY WORDS acrosome reaction, dimethyl sulfoxide, lipid peroxidation, PD-1 Chicken.

INTRODUCTION

Cryopreservation of chicken semen is a low-cost management tool for conservation. The desired fertility results in chickens similar to those of cattle are still difficult to achieve for various reasons such as line or breed variability (Long, 2006). The concentration of polyunsaturated fatty acids in chicken sperm is high and is prone to lipid peroxidation (Surai *et al.* 2001). The antioxidant system in the semen consists of enzymatic antioxidants (superoxide dismutase, catalase, and glutathione peroxidase) present in the cytoplasm of sperm and nonenzymatic antioxidants (vitamin A, tocopherol, and ascorbic acid).

The limited antioxidant system present in semen is overwhelmed during the cryopreservation process during which high levels of reactive oxygen species are produced, resulting in greater damage to the sperm membrane by lipid peroxidation (Partyka *et al.* 2012). The cryopreservation process results in decreased superoxide dismutase (SOD) activity, while other antioxidant enzymes catalase and glutathione peroxidase are not affected in chicken sperm (Partyka *et al.* 2012). Therefore, the addition of antioxidants to the semen cryo-diluent will help reduce the damage that occurs during freezing and thawing. Tempol (4-hydroxy 2, 2, 6, 6-tetramethylpiperidine-1-oxyl) is a low molecular weight cyclic nitroxide compound that has SOD

enzyme mimetic activity. It has very good cell permeability and has been used in human and alpaca semen cryopreservation (Santiani *et al.* 2013; Bateni *et al.* 2014; Azadi *et al.* 2017).To our knowledge, there is no report on the use of tempol during chicken semen cryopreservation. Therefore, the present study aimed to evaluate the addition of tempol in cryo diluent and determine its effect on semen quality and fertility of chickens after thawing.

MATERIALS AND METHODS

The experimental procedure was performed at the poultry farm of ICAR-Directorate of Poultry Research, Hyderabad. Adult PD-1 chickens were housed individually in cages in an open-sided house. Feed and water were available *ad libitum* during the experimental period. The experimental procedures were approved by the Institutional Animal Ethics Committee.

Semen collection, cryopreservation, and insemination

PD-1 roosters at 40 weeks of age were used for semen collection by dorso-abdominal massage method (Burrows and Quinn, 1937). The collected semen was pooled and used for cryopreservation. An aliquot of pooled fresh semen was used to determine sperm motility, live, abnormal, and acrosome intact sperm. The semen was centrifuged at 3000X g for 5 min to separate the seminal plasma which was stored at -20 ˚C until analysis. Sasaki diluent (D (+)-glucose- 0.2 g, D (+)-trehalose dehydrates- 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 were dissolved in 100 ml distilled water, with a final pH of 6.8 (Sasaki *et al.* 2010) was used for cryopreserving the semen. The semen for cryopreservation was kept at 5 ˚C for 30 min and then mixed with an equal volume of the diluent containing DMSO resulting in a final concentration of 4% DMSO.

Tempol (CAS no.2226-96-2; Sigma-Aldrich Co., St. Louis, USA) was added to this mixture at a final concentration of 1 and 5 mM. The diluted semen was filled in 0.5 mL French straws and placed 4.5 cm above liquid nitrogen where it was exposed to the nitrogen vapors for 30 minutes after which the straws were stored in liquid nitrogen until further use. After a storage period of at least seven days, the straws were thawed at 5 ˚C for 100 seconds. The thawed semen samples were evaluated for progressive sperm motility, live sperm, abnormal sperm, and intact sperm acrosome.The seminal plasma of thawed semen was separated by centrifugation and analyzed for lipid peroxidation. Semen cryopreservation and post-thaw evaluation were performed six times and data collected. The fertility study was performed by inseminating thawed semen with 200 million sperm into 41 weeks old PD-1 hens (12 hens/treatment).

The insemination was repeated three times at four days interval. The group inseminated with fresh semen served as control. After insemination the eggs were collected and incubated under standard incubation conditions. On the 18th day of incubation, the eggs were candle-labeled to identify the fertilized eggs and the data were recorded.

***In vitro* semen evaluation**

Progressively motile sperm were assessed subjectively by placing a drop of semen on a Makler chamber and examining it under 200x magnification.

The live and abnormal sperm were examined using eosin-nigrosin stain (Campbell *et al.* 1953). A drop of semen was mixed with an eosin-nigrosin stain and a thin smear of this mixture was made on a glass slide. The smear was examined under 1000x magnification for live sperm that appeared unstained and the percentage was calculated. On the same slide sperm with detectable morphological abnormalities were counted and the percentage calculated.

Sperm with intact acrosome were assessed according to the previously described protocol (Pope *et al.* 1991). The diluted semen sample of 10 µL was mixed with an equal volume of stain solution (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 held for 70 seconds. A smear from the mixture was prepared on a glass slide, air dried and viewed under high magnification (1000x). The blue stained acrosomal caps were observed in acrosome-intact sperm while no such stained cap was seen in acrosome reacted sperm. The percentage of acrosome intact sperm was calculated after counting a total of 200 sperm in each sample.

Lipid peroxidation in seminal plasma was measured using the thiobarbituric acid method (Hsieh *et al.* 2006). To 100 µL seminal plasma, 900 µL of distilled water was mixed and then 500 µL of thiobarbituric acid reagent was added. The glass tubes containing the mixture were incubated in a boiling water bath for one hour. After incubation, the tubes were cooled and the absorbance of the tube contents against the blank was measured at 540 nm using a spectrophotometer.

Statistical analysis

All the data were analyzed by using SAS 9.2 (SAS, 2003) and $P < 0.05$ was considered significant. The fresh and cryopreservation treatments were compared by one-way ANOVA with Tukey's post hoc test. Percentage value data were arcsine transformed and then analyzed.

RESULTS AND DISCUSSION

All studied semen parameters and fertility were significantly ($P<0.05$) reduced after cryopreservation (Table 1).

Table 1 Effect of tempol addition during cryopreservation of chicken semen on *in vitro* semen parameters and fertility (mean±SE)

Parameters	Fresh semen	4% DMSO	4% DMSO + tempol 1 mM	4% DMSO + tempo l5 mM
Progressive sperm motility (%)	65.0±1.8^{a}	21.67±1.67^{b}	21.67±1.67^{b}	23.33±1.05^{b}
Live sperm (%)	77.58±2.7^{a}	30.98±1.86^{b}	28.58±1.17^{b}	30.15±2.26^{b}
Abnormal sperm (%)	1.8±0.29	1.8±0.18	1.8±0.27	2.0±0.14
Acrosome intact sperm (%)	93.0±0.93^{a}	79.83±4.39^{ab}	80.17±3.48^{ab}	76.13±4.48^{b}
Seminal plasma lipid peroxidation (nM MDA/mL)	1.97±0.32^{a}	6.75±1.23^{a}	4.41±0.34^{ab}	5.03±0.53^{a}
Fertility (%)	91.6±2.47^{a}	16.33±7.57^{b}	0^{b}	6.67±6.67^{b}
Number of eggs incubated	75	55	83	44

DMSO: dimethyl sulfoxide and MDA: malondialdehyde.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

Lipid peroxidation in seminal plasma was significantly (P<0.05) higher in cryopreserved groups. The addition of tempol during cryopreservation had no significant (P>0.05) effect on post-thaw sperm motility, seminal plasma lipid peroxidation, or fertility.

The addition of antioxidants to semen is useful in protecting or improving sperm functions. Enzymatic antioxidants (Partyka *et al.* 2013) or non-enzymatic antioxidants (Pranay Kumar *et al.* 2019) are useful in the cryopreservation of chicken semen. A previous report on liquid turkey semen has shown improvement in sperm parameters with the addition of Tempo an antioxidant compound (Donoghue and Donoghue, 1997). In the present study, a related compound Tempol has been investigated during semen cryopreservation.

The addition of SOD during cryopreservation of chicken sperm has been shown to benefit post-thaw sperm functions such as increased motility, higher plasma membrane integrity, and reduced apoptotic changes, however, there was no effect on sperm acrosome status (Partyka *et al.* 2013). The SOD mimetic tempol used in the present study did not improve sperm motility, plasma membrane integrity, or intact sperm acrosome. The deleterious effects of semen cryopreservation were observed in the present study in the form of higher lipid peroxidation levels in seminal plasma. The addition of tempol failed to reduce this increased lipid peroxidation level. Tempol a six-membered cyclic nitroxide and SOD mimetic reacts rapidly with superoxide anions and prevents the progression of Fenton reaction (Samuni *et al.* 1990). If tempol had neutralized superoxide anions in the present study, the extent of lipid peroxidation in the treatments to which it was added should have decreased, but it did not. Any positive effect would have resulted in higher motility and fertility in the treatment groups.

The use of tempol at a concentration of 1 mM during cryopreservation of alpaca semen was shown to improve sperm motility after thawing, functional integrity of the sperm membrane, and reduce sperm DNA fragmentation (Santiani *et al.* 2013). A low dose of 5 µM tempol improved sperm motility and viability while reducing sperm DNA fragmentation (Bateni *et al.* 2014; Azadi *et al.* 2017). During refrigerated storage of ram fluid semen, tempol at a dosage of 2 mM improved sperm motility and fertilization rate (Mara *et al.* 2005). Thus, the results of using tempol in semen storage indicate its ability to protect sperm cell during cryopreservation. In the present study, the amounts of tempol used in the cryopreservation medium may not have had any positive effects on sperm parameters after thawing. In future studies, tempol should be used at different concentrations in the cryopreservation of chicken semen to observe any beneficial effects.

CONCLUSION

The addition of tempol at concentrations of 1 and 5 mM during cryopreservation of chicken sperm had no effect on post-thaw sperm parameters or fertility.

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