Research

Evaluation of Semen Cryopreservation Protocols for Kadaknath Chicken

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ABSTRACT

Kadaknath is a black pigmented chicken breed found in the central India. *Ex situ in vitro* conservation of chicken is mainly done by semen cryopreservation. Two independent experiments were conducted to evaluate semen cryopreservation protocols for Kadaknath chicken. In the first experiment, Kadaknath semen was cryopreserved using 8% Ethylene Glycol (EG) in Sasaki diluent (SD), Lake and Ravie diluent (LR) or Red Fowl Extender (RFE). In the second experiment the semen was cryopreserved using 8% Ethylene Glycol (EG) in SD, 4% dimethyl sulfoxide (DMSO) in SD or 4% DMSO in LR. Semen was cryopreserved in 0.5 ml plastic straws which were thawed at 5°C for 100 sec or 37°C for 30 sec for assessment. The pre and post-thaw semen was assessed for sperm motility, live and abnormal sperm and acrosome integrity. The post-thaw semen was inseminated in adult hens to study fertility. The post-thaw sperm motility, live sperm and acrosome intact sperm were significantly lower ($P < 0.05$) in cryopreserved semen in both experiments. In experiment 1, the maximum post-thaw fertility (8.8%) was obtained from the 8% EG SD cryopreserved semen. In experiment 2, the maximum post-taw fertility (14.5%) was obtained from the 4% DMSO SD cryopreserved semen. In conclusion, Kadaknath chicken semen may be cryopreserved using 4% DMSO in SD and thawed at 5°C for 100 sec for obtaining better fertility.

Key words: Chicken, Cryopreservation, Fertility, Acrosome Reaction

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INTRODUCTION

Kadaknath is a native chicken breed reared by tribal communities in its breeding tract of Jhabua and Dhar districts in the western region of Madhya Pradesh and adjacent regions in the states of Rajasthan and Gujarat in India (Haunshi *et al.,* 2011). This is a dual-purpose breed with black coloured meat due to melanin pigmentation and is believed to possess aphrodisiac properties. Though this breed has poor production potential (Haunshi *et al.,* 2011; Haunshi *et al.,*2012) it is hardy in nature and can thrive under adverse climatic conditions. In recent times there is significant high demand for the products of this breed. Efforts are being undertaken to genetically improve the performance of this breed. Furthermore, Kadaknath breed is used to develop improved chicken varieties for backyard poultry farming. The general characteristics and production performance of this breed has been reviewed recently (Haunshi and Prince, 2021). In general, native breeds of chicken face an imminent threat of genetic dilution and erosion because of the propagation of high yielding varieties with the aim of increasing productivity of the backyard poultry production system. Thus, efforts are needed for conserving the Kadaknath breed for future genetic improvement programs.

The post-thaw sperm viability and fertilizing ability varies hugely between breed/line of chicken (Long, 2006, Blesbois, 2011)especially for at-risk populations. In contrast to domestic livestock species, cryogenic storage of poultry semen is not reliable enough for germplasm preservation. The relatively low fertilizing ability of frozen/thawed poultry sperm most likely results from physiological sensitivity to the cryogenic process coupled with the requirement for prolonged sperm functionality in the hen reproductive tract; however, the concept of defining these physiological challenges has been underemphasized. For example, alterations in membrane carbohydrate content and diminished energy production in frozen/thawed sperm have important implications for successful gamete interaction. Recent data suggests that both glycoconjugate content and adenosine triphosphate (ATP, therefore cryopreservation protocols need to be evaluated for each specific breed/line. Glycerol, due to its contraceptive effects in chickens as observed in the study by Hammerstedt and Graham (1992), must be eliminated before insemination through a process involving centrifugation and washing of the cryopreserved semen. These procedures impose extra stress on the sperm. Consequently, this study explored alternative cryoprotectants that do not require removal prior to insemination. Although the semen quality of

Kadaknath chicken at different ages has been reported earlier (Haunshi *et al.,* 2011, Haunshi *et al.,* 2012), there is no report on the semen cryopreservation protocol for this breed. Thus, the present study was undertaken to develop semen cryopreservation protocol for Kadaknath chicken.

MATERIALS AND METHODS

The experiment was performed at poultry farm of ICAR-Directorate of Poultry Research located in Hyderabad, India. Adult Kadaknath chicken randomly selected from a flock were reared in individual cages in an open-sided house. The birds were fed *ad libitum* and had free access to water. The experimental protocol was approved by the Institutional Animal Ethics Committee (Approval No: IAEC/DPR/18/8). Two independent experiments were conducted for evaluating different semen cryopreservation protocols.

Experiment 1

Kadaknath roosters of 28 weeks age (n=15) were trained for semen collection by abdominal massage (Burrows and Quinn, 1937) prior to the start of the experiment. Semen collected from individual males in glass container was pooled for evaluation and further processed for cryopreservation. The collected semen was placed on ice till the end of the experiment. For assessing the fresh semen parameters in the laboratory an aliquot from pooled semen was diluted four times in Sasaki diluent (SD) (Sasaki *et al.,* 2010)a chicken breed designated as a "Natural Monument" under the "Law for the Protection of Cultural Property" (Law No. 214, May 30, 1950 for subsequent use. The semen was cryopreserved with Ethylene Glycol (EG) as the cryoprotectant, in three semen diluents SD, Lake and Ravie diluent LR, (Lake and Ravie 1984) and Red Fowl Extender (RFE; 10).

The semen samples were equilibrated at 5°C for 30 minutes and then diluted at 1:1 ratio in the test diluents, such that the final EG concentration was 8% and the final sperm concentration was 2000 x 10^6/ml. The cryopreservative mixed semen was manually loaded into 0.5 ml plastic straws and sealed. The sealed straws were exposed to nitrogen vapours for 30 minutes by placing them 4.5 cm above the liquid nitrogen (LN_2) before immersing in LN_2 for longterm storage. After a storage period of minimum one week the semen straws were thawed and used for evaluation or insemination. The cryopreserved semen was thawed at 5°C for 100 sec in ice water and evaluated for sperm motility, live and abnormal sperm and acrosome integrity. The thawing temperature was selected on the basis of preliminary *in vitro* experiments. The entire procedure of semen cryopreservation and evaluation was repeated six times and data were collected.

Fertility percentage from thawed semen was calculated in experiments where 0.1 ml of thawed semen was inseminated into Kadaknath hens aged 29 weeks (12 hens/group) three times at four days interval. Freshly collected Kadaknath semen with sperm concentration of 200 million sperm in 0.1 ml semen volume was inseminated in one group of hens and was considered as control. One day after insemination eggs were collected, fumigated and stored at 15°C until incubation in an automatic setter/hatcher incubator. The fertility of incubated eggs was assessed on the 18th day of incubation by candling and observed for any embryonic development. The per cent fertility was calculated from the candling data and infertile eggs were broken open and confirmed for infertility.

Experiment 2

Semen was collected and cryopreserved from Kadaknath roosters of 30 weeks age (n=15) similar to experiment 1 with few modifications. The semen was cryopreserved using 8% EG SD, 4% DMSO SD or 4% DMSO LR with the final sperm concentration 2000 x 10^6/ml. After addition of cryoprotectants the mixture was kept at 5°C for 30 min and then filled in the plastic straws. Semen cryopreserved using 8% EG SD and 4% DMSO SD was thawed in ice water at 5°C for 100 sec whereas 4% DMSO LR semen was thawed at 37°C for 30 sec. The post-thaw temperature was selected based on the results of *in vitro* preliminary trials. The cryopreservation and thawing procedure were repeated six times and post-thaw samples were evaluated for sperm motility, live and abnormal sperm, and acrosome integrity. The seminal plasma from fresh and cryopreserved semen was separated by centrifugation at 3000 x g for 5 min and used for assessing lipid peroxidation.

Fertility trials were conducted similar to experiment 1 by inseminating the cryopreserved semen into Kadaknath hens aged 32 weeks (12 hens/group) three times at four days intervals and fertility data was obtained.

Semen quality assays

Sperm motility

Sperm motility was recorded as a percentage of progressively motile sperm. This was done by placing a drop of semen on a Makler chamber and examining under 200 x magnification. The sperm in the sample was subjectively assessed and scored based on normal, vigorous and forward linear motion.

Live and abnormal sperm

The percent live and abnormal sperm in the samples were estimated by differential staining technique using Eosin-Nigrosin stain (Campbell *et al.,* 1953).

Sperm acrosome integrity

The sperm acrosome integrity in the samples was assessed by a staining method (Pope *et al.,* 1991) only a modified triple-stain technique successfully differentiated the acrosomal reaction; however the complexity of the technique limited its usefulness. The single-step staining technique uses a solution of 1% fast green FCF, 1% rose bengal, and 40% ethyl alcohol in 0.1 M citric acid--0.2 M disodium phosphate buffer. Semen was diluted with 2.9% sodium citrate, and an equal volume of staining solution was added and incubated for 70 sec at room temperature. Ten microliters of the mixture then was pipetted onto a microslide, smeared with anther slide, and air dried at 37°C. Acrosomal status was evaluated using bright field microscopy (×1,000. Equal volume (10 µl) of diluted semen and 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) were mixed and kept for 70 sec. Then a smear was made on a glass slide, dried and examined under high magnification (1000x). The sperm having intact acrosomes had blue stained acrosomal caps that were counted and the percentage of sperm having intact acrosomes out of total sperm was calculated.

The seminal plasma lipid peroxidation was analyzed by the thiobarbituric acid method (Hsieh *et al.,* 2006) where malondialdehyde (MDA) a marker of lipid peroxidation is measured. In each tube 0.9 ml of distilled water and 0.1 ml of seminal plasma were mixed followed by the addition of 0.5 ml of thiobarbituric acid reagent. The contents of the tubes were mixed and incubated in a boiling water bath for one hour. The tubes were cooled and absorbance against blank at 540 nm was measured using a spectrophotometer.

Statistical analysis

The experimental data were analyzed using SAS 9.2 software and $P < 0.05$ was considered significant. Semen parameters and fertility were analyzed by one-way ANOVA. Percent value data were arcsine transformed before analysis.

RESULTS AND DISCUSSION

The current study evaluated different semen cryopreservation protocols for Kadaknath chicken. In experiment 1 the sperm motility, live sperm and acrosome integrity were significantly ($P < 0.05$) lower in the cryopreserved samples compared to fresh semen samples (Table 1). The fertility of eggs after frozen-thawed semen insemination was significantly ($P < 0.05$) lower compared to the control, however, 8% EG SD group gave higher fertility compared to other cryopreservation groups. Ethylene glycol has been used as a cryoprotectant in chicken semen cryopreservation (Miranda *et al.,* 2018; Svoradová *et al.,* 2018; Murugesan and Mahapatra 2020). The thawing temperature (5°C for 100 sec) used in the experiment was selected based on preliminary evaluations. Thawing temperature has a profound effect on the fertility outcomes of cryopreserved semen. It has been reported that on using EG as cryoprotectant no fertile eggs were obtained with semen thawed at 5°C. However, simply changing the thawing temperature to 37°C restored the fertility (Murugesan and Mahapatra, 2020).

In the present study, *in vitro* trials indicated better results with a thawing temperature of 5°C. The variation in thawing temperatures reported across studies may be attributed to differences in the chicken breeds used. Studies conducted at the institute further demonstrated that both breed and thawing protocol significantly influence fertility outcomes (Shanmugam and Mahapatra, 2021).

Ethylene Glycol (EG) was used with three different diluents in the current study. Previous reports have shown that the choice of diluent during cryopreservation may

Table 1: Post-thaw semen parameters and fertility of Kadaknath chicken in experiment 1.

Parameters	Control (Fresh semen)	8% EG SD	8% EG LR	8% EG RFE
Sperm motility (%)	66.4 ± 1.43^{a}	22.1 ± 1.48^{b}	22.9 ± 1.01^{b}	15.7 ± 1.30^{c}
Live sperm (%)	84.9 ± 1.97^{a}	32.4 ± 3.51^{b}	35.3 ± 3.37^{b}	31.9 ± 3.88^{b}
Abnormal sperm (%)	1.8 ± 0.23	2.1 ± 0.28	1.9 ± 0.28	2.2 ± 0.27
Sperm acrosome integrity (%)	98.4 ± 0.17^{a}	95.3 ± 0.75^{b}	95.1 ± 0.63^{b}	95.0 ± 0.62^{b}
Fertility (%)	97.3 ± 1.80^{a}	8.8 ± 2.50^{b}	2.0 ± 1.38^{c}	1.0 ± 1.01^{c}
No. of eggs incubated	71	104	108	90

Values are mean±SE.

^{a,b}Figures bearing different superscripts in a row differ significantly (P<0.05).

EG-Ethylene glycol, SD- Sasaki Diluent, LR- Lake and Ravie Diluent, RFE- Red Fowl Extender.

either affect (Thananurak *et al.,* 2017) or have no impact on (Murugesan and Mahapatra, 2020) post-thaw semen quality and fertility. In this study, semen cryopreserved with RFE exhibited lower sperm motility and fertility compared to SD, indicating that diluent composition significantly influences cryopreservation outcomes, consistent with earlier findings (Thananurak *et al.,* 2017).

The sperm motility, live sperm and acrosome integrity were significantly ($P < 0.05$) lower in experiment 2 (Table 2). The seminal plasma lipid peroxidation was significantly ($P < 0.05$) higher in 4% DMSO LR cryopreserved semen. The fertility from 4% DMSO SD was significantly ($P < 0.05$) higher than the other two cryopreservation groups. The cryoprotectant DMSO has been used for chicken semen cryopreservation in many studies at either 4 or 8% concentrations with reasonably better post-haw fertility results (Rakha *et al.,* 2018; Svoradová *et al.,* 2018; Kumar *et al.,* 2019; Murugesan and Mahapatra, 2022)8% ethylene glycol (EG. Using a lower concentration of DMSO at 2% level during cryopreservation did not produce any fertile eggs (Murugesan and Mahapatra, 2020). In experiment 2 after addition of cryoprotectant to the semen, the mixture was held at 5°C for 30 min and then exposed to LN_2 vapours. This incubation period was included for the cryoprotectant to penetrate and interact with cellular content for longer duration in bringing out more beneficial effects, if any. By adopting this protocol, which differed slightly from experiment 1, negligible fertility was obtained with semen cryopreserved in 8% EG SD. Thus, based on the results from the two experiments involving EG, it can be inferred that the additional incubation step introduced in Experiment 2 may have contributed to the reduced fertility observed in this group. The elevated lipid peroxidation in the seminal plasma of the 4% DMSO LR group could be attributed to the diluent composition or thawing temperature, potentially explaining the absence of fertile eggs in this treatment. Conversely, high fertility was achieved using 4% DMSO with SD in our laboratory.

Table 2: Post-thaw semen parameters and fertility parameters of Kadaknath chicken in experiment 2.

Parameters	Control (Fresh semen)	8% EG SD	4% DMSO SD	4% DMSO LR
Sperm motility (%)	63.3 ± 3.07[a]	25.0 ± 1.29[b]	15.8 ± 1.54[b]	14.2 ± 1.53[b]
Live sperm (%)	75.5 ± 3.19[a]	43.03 ± 3.89[b]	28.0 ± 1.41[b]	27.5 ± 1.31[b]
Abnormal sperm (%)	2.1 ± 0.26	1.6 ± 0.28	1.7 ± 0.34	1.68 ± 0.27
Sperm acrosome integrity (%)	96.0 ± 0.37[a]	89.2 ± 1.67[b]	87.5 ± 1.95[b]	80.5 ± 2.54[b]
Seminal plasma lipid peroxidation (nM MDA/ml)	0.67 ± 0.04[b]	0.64 ± 0.08[b]	0.90 ± 0.08[b]	1.51 ± 0.25[a]
Fertility (%)	93.2 ± 2.79 [a]	1.4 ± 1.4 [c]	14.5 ± 4.77 [b]	0
No. of eggs incubated	73	81	75	80

Values are mean±SE.

[a,b] Figures bearing different superscripts in a row differ significantly (P<0.05).

EG-Ethylene glycol, DMSO-Dimethyl sulfoxide, SD- Sasaki Diluent, LR- Lake and Ravie Diluent, MDA-Malondialdehyde

In conclusion, a cryopreservation protocol for Kadaknath chicken semen has been standardized using 4% DMSO in Sasaki diluent with thawing at 5°C for 100 sec. Studies in future may attempt to improve the protocol by incorporating additives that enhance the sperm fertilizing ability ultimately resulting in higher fertility.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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