Vol.65: e22210056, 2022
https://doi.org/10.1590/1678-4324-2022210056
ISSN 1678-4324 Online Edition

Article - Human and Animal Health

Effect of Permeable Cryoprotectants and Dextran in Cryopreserving Semen of Broiler Breeder Lines

Shanmugam Murugesan [1]*
https://orcid.org/0000-0003-0154-2859

Ramkrishna Mahapatra [1]
https://orcid.org/0000-0001-8571-3787

[1]ICAR-Directorate of Poultry Research, Rajendranagar, Hyderabad, India.

Editor-in-Chief: Alexandre Rasi Aoki
Associate Editor: Renata Marino Romano

Received: 25-Feb-2021; Accepted: 13-Sep-2021.

*Correspondence: shanmugam.murugesan@icar.gov.in; Tel.: +91-40-24015651 (S.M.).

HIGHLIGHTS

- Permeable cryoprotectants and dextran evaluated in broiler breeder semen.
- Permeable cryoprotectants produced post-thaw fertility in PB-2 breeder.
- Low fertility by ethylene glycol or dextran with dimethylacetamide in CB.

Abstract: The aim of this study was to assess permeable cryoprotectants and dextran during broiler breeder lines semen cryopreservation in two separate experiments. Semen of PB-2 broiler breeder line was cryopreserved with 8% Ethylene Glycol (EG) or 6% dimethylformamide (DMF) in Beltsville Poultry Semen Extender (BPSE). In another experiment semen of Control broiler (CB) breeder line was cryopreserved with 8% EG, 8.2% dextran 10 kDa + 9% dimethylacetamide (DMA) or 8.2% dextran 20 kDa + 9% dimethylacetamide (DMA) in Sasaki diluent (SD). Semen cryopreserved in 0.5 ml plastic straw was thawed either at 5°C for 100 sec or 37°C for 30 sec. Semen was assessed for motility, live sperm, abnormal sperm and acrosome intact sperm. Post-thaw semen was inseminated, eggs were collected and incubated for obtaining fertility and hatchability data. The cryopreserved samples had significantly ($p<0.05$) lower sperm motility, live sperm and acrosome intact sperm in both the breeder lines. Moderate fertility was obtained in PB-2 line with no difference between the two permeable cryoprotectants evaluated. The fertility was significantly ($p <0.05$) lower using 8% EG and 8.2% dextran 20 kDa + 9% DMA in CB line. Permeable cryoprotectants (8% EG or 6% DMF) produced acceptable fertility during PB-2 semen cryopreservation. The permeable cryoprotectant 8% EG or dextran in combination with DMA gave very low fertility in CB line.

Keywords: chicken; cryoprotectant; dextran; fertility; semen cryopreservation.

INTRODUCTION

Conservation of genetic lines developed for different economically important traits is essential so that they are not lost due to disease pandemics. Semen cryopreservation is the easiest and cheapest approach for long term *ex-situ* conservation [1]. The freezability and post-thaw sperm function of chicken semen differs among different genotypes, lines and individual males [2,3].

In semen cryopreservation the cryoprotectant employed highly influences the post-thaw parameters [2]. Permeable cryoprotectants are widely used for cryopreserving chicken semen, among which glycerol is an

effective one [4]. However, glycerol has to be removed before insemination as concentration above 1% results in contraception [5]. The processing for removal of glycerol may further result in damage to the sperm cells that may further affect the sperm functions. Cryoprotectants such as dimethylacetamide (DMA), dimethylformamide (DMF), dimethylsulfoxide (DMSO) and ethylene glycol were evaluated during chicken semen cryopreservation [6,7]. Semen cryopreserved using these cryoprotectants can be used for insemination without their removal.

Non-permeable cryoprotectants are high molecular weight compounds that do not cross the cell membrane and were used during semen cryopreservation. Disaccharides such as trehalose and sucrose are the reported non permeating sugars used in chicken or turkey semen cryopreservation [6,8–11]. The tonicity of extender is increased and cell membrane stabilized by non-permeating disaccharides [12]. Dextran a complex branched glucan polysaccharide has been used for turkey semen cryopreservation where dextran of molecular weight 10,000 and 20,000 was used at 10% concentration and has been shown to enhance preservation of post-thaw semen parameters and perivitelline membrane penetrability [13]. There is no report on the use of dextran in chicken semen cryopreservation.

The aim of the present study was to evaluate permeable cryoprotectants and dextran, a non-permeable cryoprotectant, during broiler breeder lines semen cryopreservation. Dextran of two molecular weights (10 and 20 kDa) was selected as non-permeable cryoprotectant for evaluation. *In vitro* evaluations showed that dextran of both the molecular weights used at 5 and 10% concentrations resulted in extremely low post-thaw live and motile sperm. Therefore, dextran was evaluated in combination with a permeable cryoprotectant. The thawing temperature influences the sperm functionality and fertility [7]. Thus, based on the preliminary trials the cryoprotectant and thawing temperature that gave better results for the individual experimental lines were selected.

MATERIAL AND METHODS

Experimental birds and husbandry

The trials were conducted at ICAR-Directorate of Poultry Research, Hyderabad, India with approval of experimental procedures by the Institutional Animal Ethics Committee. The birds were housed individually in an open-sided house. Restricted feeding was done as per the breeder manual and water was available *ad libitum* to the birds. The CB line is a pedigreed random bred broiler population and PB-2 line is a coloured broiler female line that is being selected for juvenile body weight and egg production for many generations.

Experimental procedures

Semen cryopreservation and artificial insemination in PB-2 line

Semen from PB-2 roosters (44 weeks age) was obtained by abdominal massage [14], pooled and used for cryopreservation. A portion of pooled fresh semen was used for evaluation of sperm concentration, progressive motility, live and abnormal sperm. Semen was cryopreserved with 8% EG or 6% DMF in Beltsville Poultry Semen Extender (BPSE)[15].

The fresh semen was equilibrated at 5°C for 30 minutes and then diluted at equal ratio with diluent containing the cryoprotectant. After mixing the semen and cryoprotectant the mixture was filled in 0.5 ml French straws and sealed. The sperm concentration in the cryopreserved sample was 2000 x 10^6/ml. The filled straws were kept 4.5 cm over liquid nitrogen (LN_2) and exposed to the vapours for 30 minutes. After exposure the straws were immersed in LN_2 and stored. The straws were stored for at least a week duration before evaluation. Semen cryopreserved using EG was thawed at 5°C for 100 sec whereas DMF cryopreserved semen was thawed at 37°C for 30 sec. The differing thawing temperatures for the two cryoprotectants were selected based on preliminary trials. The semen cryopreservation and evaluation were repeated six times for collecting data on in vitro parameters such as sperm motility, live sperm, abnormal sperm, and intact sperm acrosome. Post-thaw semen having a sperm concentration of 200 million was inseminated into 49 weeks old PB-2 hens (20 hens/group). A second insemination was done after four days of first insemination. The group with fresh semen insemination served as control. Post insemination eggs were collected and stored until incubation. The incubated eggs were candled on the 18th day for embryonic growth. Infertile eggs were broke open and confirmed the absence of any embryonic growth. Hatchability data were calculated based on chicks hatched at the end of incubation.

Semen cryopreservation and artificial insemination in CB line

Control broiler (40 weeks age) semen was cryopreserved following the procedure for PB-2 roosters with few alterations in protocol. Semen was cryopreserved using Sasaki diluent [16]. Semen was cryopreserved using 8% EG, 8.2% dextran 10 kDa + 9% DMA or 8.2% dextran 20 kDa + 9% DMA. The semen cryopreservation and evaluation were repeated on six occasions for collecting data on sperm motility, live and abnormal sperm, and intact sperm acrosome. Semen cryopreserved using EG was thawed at 37°C for 30 sec while dextran-DMA cryopreserved semen was thawed at 5°C for 100 sec. The concentration of cryoprotectants and thawing temperature was standardized during preliminary trials. After thawing the cryopreserved semen was inseminated into 42 weeks old CB hens (16 hens/group) with 200 million sperm. The birds were inseminated three times at four days interval. In the control group fresh semen was inseminated. The eggs were collected and incubated under standard conditions for obtaining data on fertility parameters.

Semen quality assays

Sperm motility

Sperm motility was scored subjectively as percentage of progressively motile sperm by placing a drop of diluted semen on a Makler chamber and examined under 20x magnification.

Live and abnormal sperm

The Eosin-Nigrosin staining procedure [17] was used to estimate percent live and abnormal sperm. Semen smear prepared by mixing a drop of semen with a drop of Eosin-Nigrosin stain was air dried and examined under high power (1000x) magnification. The unstained live sperm were counted and percent live sperm calculated after counting at least a total of 200 sperm. In the same slides the abnormal sperm percent was estimated.

Intact sperm acrosome

The sperm acrosome intactness was assessed as per the earlier reported procedure [18]. In short, 10 µl of diluted semen 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 (McIlvaine's, pH 7.2-7.3) and kept for 70 sec. On a clean glass slide a smear was prepared, dried and examined under high magnification (1000x). The acrosome-intact sperm had blue stained acrosomal caps whereas no staining could be observed in the acrosome reacted sperm. In each sample 200 sperm were counted for calculation of percent acrosome intact sperm.

Statistical analysis

Statistical analyses of data were done by one-way ANOVA using general linear models (GLM) procedure in SAS 9.2 software with Tukey's post hoc test. The percent value data were arcsine transformed and analyzed.

RESULTS

Cryopreservation of PB-2 semen

The post-thaw semen had significantly lower ($p < 0.05$) sperm motility and live sperm. The acrosome intact sperm were significantly lower ($p < 0.05$) in the post-thaw samples with 6% DMF cryopreserved sperm having the lowest values (Table 1). Fertility was significantly lower ($p < 0.05$) in 8% EG cryopreserved semen in comparison to control and there was no difference between the two cryoprotectants evaluated.

Table 1. Effect of ethylene glycol and dimethylformamide on PB-2 broiler breeder semen cryopreservation.

Parameters	Control	8% EG BPSE	6% DMF BPSE
Progressive sperm motility (%)	62.5 ± 1.12 [a]	21.7 ± 1.3 [b]	16.67 ± 1.67 [b]
Live sperm (%)	82.4 ± 1.78 [a]	43.38 ± 2.01 [b]	33.53 ± 4.45 [b]
Abnormal sperm (%)	1.93 ± 0.38	2.68 ± 0.61	2.95 ± 0.31
Acrosome intact sperm (%)	96.7 ± 0.42 [a]	84.5 ± 3.59 [b]	11.75 ± 2.65 [c]
Fertility (%)	70.10 ± 11.62 [a]	30.19 ± 7.37 [b]	46.58 ± 8.27 [ab]
Hatchability on FES (%)	87.14 ± 8.37	97.62 ± 2.38	92.71 ± 4.83
No. of eggs incubated	46	51	50

Values are Mean±SE.
Values with different superscripts in a row differ significantly ($p < 0.05$).
FES- Fertile egg set

Cryopreservation of CB semen

Semen cryopreservation significantly ($p < 0.05$) reduced sperm motility and live sperm. Similarly, cryopreservation significantly lowered ($p < 0.05$) the percent acrosome intact sperm (Table 2). Fertility obtained from cryopreserved semen was very low ($p < 0.05$) with no fertile eggs in 8.2% dextran 10 kDa + 9% DMA group.

Table 2. Effect of permeable and impermeable cryoprotectants in Control Broiler breeder semen cryopreservation.

Parameters	Control	8% EG SD	8.2% Dextran10 kDa 9% DMA SD	8.2% Dextran 20 kDa 9% DMA SD
Progressive sperm motility (%)	60.0 ± 2.24 [a]	18.33 ± 3.10 [b]	15.0 ± 1.29 [b]	11.67 ± 1.05 [b]
Live sperm (%)	67.9 ± 4.42 [a]	29.3 ± 3.10 [b]	30.10 ± 1.46 [b]	32.87 ± 2.40 [b]
Abnormal sperm (%)	1.75 ± 0.18	1.82 ± 0.10	2.10 ± 0.11	2.05 ± 0.14
Acrosome intact sperm (%)	98.33 ± 0.33 [a]	70.67 ± 6.37 [b]	9.70 ± 1.68 [c]	6.83 ± 1.90 [c]
Fertility (%)	55.86 ± 9.33 [a]	3.14 ± 1.69 [b]	0 [b]	2.34 ± 1.61 [b]
Hatchability on FES (%)	83.27 ± 5.0	100 ± 0	0	50 ± 50
No. of eggs incubated	102	90	98	94

Values are Mean±SE.
Values with different superscripts in a row differ significantly ($p < 0.05$).
FES- Fertile egg set

DISCUSSION

The permeable cryoprotectants EG and DMF were evaluated for cryopreserving PB-2 broiler breeder semen. The thawing temperatures for the cryoprotectants evaluated were different and were selected based on preliminary trials. In an earlier study it was reported that EG cryopreserved semen thawed at 37°C but not 5°C resulted in fertile eggs [7]. Similarly, DMF cryopreserved semen was also reported to produce fertile eggs when thawed at 5°C. In contrast to the earlier report, in the present study thawing EG cryopreserved semen at 5°C and DMF cryopreserved semen at 37°C resulted in producing fertility. In other studies semen cryopreserved with DMF was thawed at 5°C for 5 min that gave good fertility [19,20]. Similar to our study, Rakha and coauthors [21] have reported use of DMF at 8% concentration for cryopreserving Indian red jungle fowl with higher post-thaw sperm parameters and fertility. The authors have used 37°C as thawing temperature. Thus, different thawing temperatures in chicken semen cryopreservation protocol needs to be evaluated even for the same cryoprotectant depending on the breed or line of chicken for obtaining better fertility. The post-thaw acrosome intact sperm in the present study was different between the two cryoprotectants evaluated; however, there was no difference in the fertility.

The CB line semen cryopreserved with EG gave very low fertility. This result was different from that obtained in PB-2 line semen cryopreserved with EG. The thawing temperature was different between the lines, wherein thawing at 5°C of EG cryopreserved CB semen produced poor post-thaw parameters during preliminary trials. Therefore, thawing temperature of 37°C was selected. However, cryopreservation protocol with 37°C thawing temperature did not produce good fertility and may be due to difference in the diluent used or due to the effect of line itself.

The non-permeable cryoprotectant dextran 1000 was found to be toxic in turkey and dextran 10 and 20 kDa at 10% concentration retained post-thaw semen parameters and perivitelline membrane penetration in comparison with glycerol [13]. In the present study dextran 10 and 20 kDa were found to be toxic for chicken sperm when used at 5 and 10% concentrations (data not presented). During goat semen cryopreservation an inverse relationship between cryoprotecting potential and dextran molecular mass was observed [22]. Furthermore, it was observed that a combination of dextran and glycerol with their different mechanism of action gave higher cryoprotection than when used alone. Anticipating such a synergistic action, in the present study dextran of two molecular weights were combined with DMA and evaluated. The in vitro results that the combination of dextran and DMA did not produce any adverse or toxic effects on the post-thaw semen parameters in contrast to when dextran alone was used. However, this combination produced very low or no fertility depending on the cryoprotectant combinations. In rabbit semen cryopreservation inclusion of dextran (60-90 kDa) at 5 and 10% final concentration along with DMSO and acetamide improved the post-thaw acrosome integrity [23]. Dextran at 5% inclusion improved the reproductive performance when DMSO was used at 10.7% concentration. In our study the dextran evaluated was of low molecular weight compared to that used for rabbit semen cryopreservation. Dextran of higher molecular weights, either alone or in combination with other cryoprotectants may be assessed for the cryopreserving chicken semen in future.

CONCLUSION

Permeable cryoprotectants EG and DMF preserved sperm motility and yielded fertile eggs in PB-2 broiler breeder line. The permeant cryoprotectant EG, and a combination of non-permeable cryoprotectant dextran (10 and 20 kDa) and permeable cryoprotectant DMA use in control broiler breeder line semen cryopreservation resulted in very low or no fertile eggs. Thus, it can be inferred that the non-permeable cryoprotectant dextran at the tested concentrations may not be suitable for cryopreserving chicken semen either alone or in combination with DMA.

Funding: This research received funding from ICAR-Directorate of Poultry Research.
Acknowledgments: Maintenance of breeder line birds by Dr.B.L.N. Reddy and Dr.L.L.L.Prince is acknowledged.
Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

1. Silversides FG, Purdy PH, Blackburn HD. Comparative costs of programmes to conserve chicken genetic variation based on maintaining living populations or storing cryopreserved material. Br Poult Sci. 2012;53(5):599–607.
2. Long JA. Avian semen cryopreservation: what are the biological challenges? Poult Sci. 2006;85(2):232–6.
3. Long JA, Bongalhardo DC, Pelaéz J, Saxena S, Settar P, O'Sullivan NP, et al. Rooster semen cryopreservation: effect of pedigree line and male age on postthaw sperm function. Poult Sci. 2010;89(5):966–73.

4. Tselutin K, Seigneurin F, Blesbois E. Comparison of cryoprotectants and methods of cryopreservation of fowl spermatozoa. Poult Sci. 1999;78(4):586–90.
5. Lake P, Buckland RB, Ravie, O. Effect of glycerol on the viability of fowl spermatozoa-implications for its use in freezing semen. CryoLetters. 1980;1:299–304.
6. Mosca F, Madeddu M, Sayed AA, Zaniboni L, Iaffaldano N, Cerolini S. Combined effect of permeant and non-permeant cryoprotectants on the quality of frozen/thawed chicken sperm. Cryobiology. 2016;73(3):343–7.
7. Murugesan S, Mahapatra R. Cryopreservation of Ghagus chicken semen: effect of cryoprotectants, diluents and thawing temperature. Reprod Domest Anim. 2020;55:133–8.
8. Terada T, Ashizawa K, Maeda T, Tsutsumi Y. Efficacy of trehalose in cryopreservation of chicken spermatozoa. Jpn J Anim Reprod. 1989;35(1):20–5.
9. Madeddu M, Berlinguer F, Pasciu V, Succu S, Satta V, Leoni GG, et al. Differences in semen freezability and intracellular ATP content between the rooster (Gallus gallus domesticus) and the Barbary partridge (Alectoris barbara). Theriogenology. 2010;74(6):1010–8.
10. Blanco JM, Long JA, Gee G, Wildt DE, Donoghue AM. Comparative cryopreservation of avian spermatozoa: benefits of non-permeating osmoprotectants and ATP on turkey and crane sperm cryosurvival. Anim Reprod Sci. 2011;123(3–4):242–8.
11. Shanmugam M, Mahapatra R. Pellet method of semen cryopreservation: effect of cryoprotectants, semen diluents and chicken lines. Braz Arch Biol Technol. 2019;62:e19180188.
12. Crowe JH, Crowe LM, Carpenter JF, Aurell Wistrom C. Stabilization of dry phospholipid bilayers and proteins by sugars. Biochem J. 1987;242(1):1–10.
13. Gloria A, Toscani T, Robbe D, Parrillo S, De Amicis I, Contri A. Cryopreservation of turkey spermatozoa without permeant cryoprotectants. Anim Reprod Sci. 2019;211:106218.
14. Burrows WH, Quinn JP. The collection of spermatozoa from the domestic fowl and turkey. Poult Sci. 1937;16(1):19–24.
15. Rakha BA, Ansari MS, Akhter S, Hussain I, Blesbois E. Cryopreservation of Indian red jungle fowl (Gallus gallus murghi) semen. Anim Reprod Sci. 2016;174:45–55.
16. Sasaki K, Tatsumi T, Tsutsui M, Niinomi T, Imai T, Naito M, et al. A method for cryopreserving semen from Yakido roosters using N-Methylacetamide as a cryoprotective agent. J Poult Sci. 2010;47:297–301.
17. Campbell RG, Hancock, JL, Rothschild, L. Counting live and dead bull spermatozoa. J Exp Biol. 1953;30:44–9.
18. Pope CE, Zhang YZ, Dresser BL. A simple staining method for evaluating acrosomal status of cat spermatozoa. J Zoo Wildl Med. 1991;22(1):87–95.
19. Chuaychu-Noo N, Thananurak P, Chankitisakul V, Vongpralub T. Supplementing rooster sperm with Cholesterol-Loaded-Cyclodextrin improves fertility after cryopreservation. Cryobiology. 2017;74:8–12.
20. Thananurak P, Chuaychu-Noo N, Thélie A, Phasuk Y, Vongpralub T, Blesbois E. Different concentrations of cysteamine, ergothioneine, and serine modulate quality and fertilizing ability of cryopreserved chicken sperm. Poult Sci. 2020;99(2):1185–98.
21. Rakha BA, Ansari MS, Akhter S, Akhter A, Blesbois E, Santiago-Moreno J. Effect of dimethylformamide on sperm quality and fertilizing ability of Indian red jungle fowl (Gallus gallus murghi). Theriogenology. 2020;149:55–61.
22. Kundu CN, Chakrabarty J, Dutta P, Bhattacharyya D, Ghosh A, Majumder GC. Effect of dextrans on cryopreservation of goat cauda epididymal spermatozoa using a chemically defined medium. Reprod Camb Engl. 2002;123(6):907–13.
23. Viudes-de-Castro MP, Talaván AG, Vicente JS. Evaluation of dextran for rabbit sperm cryopreservation: Effect on frozen–thawed rabbit sperm quality variables and reproductive performance. Anim Reprod Sci. 2021;226:106714.