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Pellet Method of Semen Cryopreservation: Effect of Cryoprotectants, Semen Diluents and Chicken Lines

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HIGHLIGHTS

- Cryoprotectants, diluents and chicken lines effect in semen vitrification evaluated
- Dimethylformamide gave low fertility in Lake and Ravie diluent and no fertility in TES diluent
- Dimethylacetmide gave fertility up to 9.22 % and along with sucrose produced similar fertility
- Post thaw in vitro semen parameters similar between chicken lines

Abstract: The present study evaluated the effect of cryoprotectants, semen diluents and chicken lines during pellet method of semen cryopreservation. Three different experiments were conducted; Experiment 1 - semen was cryopreserved using dimethylformamide (DMF) at 6% and 9% concentrations in two semen diluents (Lake and Ravie diluent and TES/NaCl diluent), Experiment 2 - semen was cryopreserved using dimethylacetamide (DMA) at 6% and 9% with or without sucrose (100mM), Experiment 3- semen from two chicken lines (PD1 and PD6) was cryopreserved using DMA (6% and 9%). Semen was evaluated pre and post cryopreservation for progressive motility, live and abnormal sperm. Semen pellets were stored in cryovials for at least seven days before examination and insemination. Thawed semen was inseminated intravaginaly to study fertility. All the parameters studied were significantly lower ($p<0.05$) in cryopreserved semen. DMF in Lake and Ravie diluent gave very low fertility and TES/NaCl diluent no fertile eggs. DMA as cryoprotectant gave fertility up to 9.22 %. Addition of sucrose along with DMA produced fertility similar to other cryopreservation treatment groups. No difference in in vitro semen parameters between chicken lines was observed. There is difference in cryopreservation outcome due to semen diluent and type of cryoprotectant.

Keywords: chicken; diluent; fertility; pellet cryopreservation; semen.

INTRODUCTION

Semen cryopreservation is a strategy for long term ex situ conservation of genetic resources. With incidences of epidemics such as avian influenza conservation of chicken gene lines developed for specific traits has assumed greater importance. Glycerol is the least toxic and effective poultry semen cryoprotectant, however because of its contraceptive effect it has to be removed before insemination. Alternative cryoprotectants belonging to amide group such as dimethylacetamide, dimethylformamide and methylacetamide were used for cryopreserving poultry semen and the semen can be used for insemination without removal of the cryoprotectant.

Dimethylformamide (DMF) was used as cryoprotectant for chicken [1-3] and guinea fowl semen cryopreservation [4,5]. In these reports, the semen diluted with DMF was cryopreserved in plastic straws or in plastic vials using stepwise freezing protocols. Schramm [6] cryopreserved chicken semen using 6% DMF in a glass ampoule and programmable freezer. There is no report on chicken semen cryopreservation by pellet method using DMF as cryoprotectant.

The semen diluent or extender used for semen cryopreservation affects the post thaw semen parameters or fertility [4,7,8] due to difference in chemical composition.

Semen cryopreservation is carried out either as semen pellets or in plastic straws and each method has its own merits. The method of semen freezing and packing affects the outcome of chicken semen cryopreservation [9,10]. Pellet method of cryopreservation or vitrification is a process where a solution cooled very fast, the viscosity becomes so large and molecular diffusion is halted. At this state the sample is said to be a glass or vitreous solid [11]. High fertility from cryopreserved semen was obtained in chicken by using dimethylacetamide (DMA) as cryoprotectant and semen frozen by pellet method [9,12,13]. However, consistent result of high fertility using DMA and pellet method combination could not be achieved by others [10].

A combination of penetrating and non penetrating cryoprotectants was tested during semen cryopreservation in rooster and other species [14-17]. The penetrating cryoprotectants protect cells from cryoinjury by increasing membrane fluidity and reducing the intracellular ice crystal formation [18]. Non-permeating disaccharides such as trehalose and sucrose are suggested to protect cells by increasing the tonicity of the extender and stabilizing the cell membrane [19]. The earlier reports on use of sucrose in chicken semen cryopreservation had evaluated its efficacy through in vitro tests only and fertility studies using freeze/thawed semen was not performed.

The fertilizing ability and viability of cryopreserved sperm varies between chicken breeds necessitating breed or line specific sperm cryopreservation protocol [20,21]. The observed difference between breeds or lines may be due to differences in the sperm tolerance to the treatments which may be of genetic origin. Differences in seminal plasma proteins and sperm oxygen consumption rate were ascribed as reasons for resistant to freeze damage by sperm of some selected lines [22].

Based on the above information different experiments were carried to evaluate the effects of semen diluents, penetrating and non-penetrating cryoprotectants, and chicken lines during semen cryopreservation by pellet method.

MATERIAL AND METHODS

Experimental birds and husbandry

The experiment was carried out at the experimental poultry farm of ICAR-Directorate of Poultry Research located in Hyderabad, India. The study consisted of three separate experiments. PD1 and PD6 lines were used in these experiments. PD1 line was developed from Red Cornish and has been selected for shank length for ten generations. PD6 line was developed from multicoloured broiler population and has been selected for shank length for six generations. Both the lines are used as male lines and inseminations are done in two different female lines to produce commercial crosses. The birds were housed in individual

cages in an open-sided house. The experiments were carried out following the approval of the Institutional Animal Ethics Committee.

Experiment 1

Semen from ten adult PD1 males (39 weeks age) was collected by abdominal massage method [23], pooled and kept on ice throughout the experiment. An aliquot of semen was diluted four times in diluent and evaluated for different sperm parameters. The sperm concentration was estimated by optical density [24], sperm motility was assessed subjectively as percentage of progressively motile sperm by examining a drop of diluted semen on glass slide and covered with glass slip under high-power magnification (40x). Live sperm was determined by differential staining technique using eosin–nigrosin stain [25]. The slides were used for estimating the percentage abnormal sperm based on observable abnormalities. In each sample smear 200 sperm were counted in for calculating live and abnormal sperm.

Semen was cryopreserved by pellet method using DMF at 6% and 9% concentrations and in two diluents, Lake and Ravie (LR) diluent [26] (sodium glutamate 1.92 g, glucose 0.8 g, magnesium acetate $4H_2O$ 0.08 g, potassium acetate 0.5 g, polyvinylpyrrolidone [relative molecular mass (Mr) = 10 000] 0.3 g and double distilled water 100 ml, final pH 7.08, osmolality 343 mOsm/kg water) and TES/NaCl diluent [27] (NaCl 0.8 g; TES 1.374 g; 1 M NaOH 2.75 ml; glucose 0.6 g, dissolved and volume made to 100 ml with double distilled water, pH 7.4, osmolality 382 mOsm/kg water). The semen and cryodiluent mixture was equilibrated for 5 min at 5°C. Semen pellets were formed by dropping the DMF mixed semen directly into liquid nitrogen drop by drop from 1ml pipette and stored in plastic cryovials in liquid nitrogen for at least seven days before examination and insemination. Cryovials with semen pellets were thawed for 45 sec at 56°C in a water bath. Thawed samples were evaluated for sperm progressive motility, live and abnormal sperm. Semen was vitrified and evaluated for in vitro parameters on ten separate occasions. Immediately after thawing semen was inseminated into 27 weeks old PD2 line hens (15 hens/treatment) using a dose of 150 million sperm in 0.1ml volume. Insemination was repeated six times at three days interval. Freshly collected and inseminated semen served as control. Eggs were collected from second day after first insemination onwards and stored in cold chamber (15°C) till incubation. Eggs were candled on 18^{th} day of incubation for embryonic development. Infertile eggs were broke open for examination and confirmation of absence of embryonic growth.

Experiment 2

Semen collected from ten PD1 males (50 weeks age) by abdominal massage was pooled and processed on ice throughout the experiment for pellet cryopreservation. An aliquot of semen diluted in LR diluent was used for evaluating sperm concentration, sperm progressive motility, live and abnormal sperm as described in Experiment 1. Dimethylacetamide and sucrose were used as cryoprotectants for cryopreserving semen in LR diluent. The different treatments were DMA 6%, DMA 9%, DMA 6% + 100 mM sucrose and DMA 9% + 100 mM sucrose. In treatments containing sucrose diluent was supplemented with BSA at 0.5% final concentration. Semen samples mixed with cryoprotectants were equilibrated for 20 minutes at 5°C before pellet preparation [9]. Pellets were prepared and stored similar to the procedure described in Experiment 1. Semen was vitrified and evaluated on ten separate occasions for sperm progressive motility, live and abnormal sperm. Before insemination or evaluation pellets were thawed on hotplates constantly maintained at 60°C [9]. Soon after thawing semen was inseminated into 37 weeks old PD2 line hens (14 hens/treatment) using a dose of 200 million sperm in 0.1ml volume. Insemination was repeated five times at three days interval. Insemination using fresh semen served as control. Post insemination eggs were collected and fertility determined as in Experiment 1.

Experiment 3

Semen from PD1 (29 weeks age) and PD6 (35 weeks age) males was processed for cryopreservation similar to Experiment 2. Semen was diluted in medium [28] containing D (+) glucose (0.2 g), D (+)-trehalose dihydrate (3.8 g), L-glutamic acid, monosodium (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 at pH 6.8 and osmolality 360 mOsm/kg water. DMA at 6 and 9% was used as cryoprotectant. Pellets were prepared after equilibration for 20 minutes at 5°C [9]. Semen was vitrified and evaluated ten times for sperm progressive motility, live and abnormal sperm. The pellets were thawed on hotplate constantly maintained at 60°C [9]. PD1 semen was inseminated into 29 weeks old PD2 line hens (13 hens/treatment) and PD6 semen was inseminated into 32 weeks old PD3 line hens (13 hens/treatment). Insemination was done using a dose of 200 million sperm in 0.1ml volume. Insemination was repeated six times at three days interval. Fresh semen inseminated birds in each line served as control. Post insemination eggs were collected and fertility determined as in Experiment 1.

Statistical analysis

Data were analyzed using SAS 9.2 software and $p<0.05$ was considered significant. Statistical analyses of semen parameters were performed by one- or two-way ANOVA with Tukey's post hoc test. Percent value data were arcsine transformed before analysis.

RESULTS

Overall the parameters studied were significantly lower ($p<0.05$) in cryopreserved semen compared to fresh semen. In Experiment 1 semen diluted in LR diluent had significantly ($p<0.05$) higher post thaw motility and live sperm percentage than TES/NaCl diluent (Table 1). The interaction between diluent and DMF concentration was significant ($p<0.05$). Semen samples cryopreserved using TES/NaCl diluent had extremely low motility and no fertility. The fertility obtained from using LR diluent was significantly ($p<0.05$) higher than TES/NaCl diluent.

Table 1. Effect of DMF and semen diluents on post thaw semen parameters and fertility of semen cryopreserved by pellet method.

Parameters	Control	Lake and Ravie diluent		TES/NaCl diluent	
		6% DMF	9% DMF	6% DMF	9% DMF
Progressive sperm motility (%)	57.50 ± 2.34^{a}	18.33 ± 1.42^{b}	6.58 ± 0.77^{c}	3.50 ± 0.36^{d}	4.08 ± 0.48^{d}
Live sperm (%)	89.02 ± 1.79^{a}	28.31 ± 1.39^{b}	24.02 ± 1.64^{b}	17.79 ± 1.69^{c}	19.51 ± 2.30^{c}
Abnormal sperm (%)	2.53 ± 0.48	2.14 ± 0.27	1.72 ± 0.16	2.02 ± 0.24	1.91 ± 0.23
Fertility (%)	77.06 ± 3.95^{a}	1.19 ± 0.89^{b}	1.38 ± 0.82^{b}	0^{c}	0^{c}
No. of eggs incubated	332	374	332	355	388

Values given are mean±SE.

Figures bearing different superscripts in a row differ significantly ($p<0.05$).

In Experiment 2 though there was significant difference in sperm motility between the cryopreservation treatment groups no difference in the fertility was observed (Table 2). There were no fertile eggs from the cryopreservation treatment groups from eighth day after start of egg collection. When average fertility was calculated for the first seven days it ranged between 8-23% for different treatment groups.

Table 2. Effect of DMA and sucrose on post thaw semen parameters and fertility of semen cryopreserved by pellet method.

Parameters	Control	6% DMA	9% DMA	6% DMA+ 100 mM Sucrose	9% DMA + 100 mM Sucrose
Progressive sperm motility (%)	57.73 ± 1.56[a]	25.45 ± 1.42[b]	18.18 ± 1.39[c]	15.90 ± 1.32[c]	14.09 ± 0.90[c]
Live sperm (%)	93.27 ± 0.52[a]	40.23 ± 2.83[b]	39.62 ± 3.52[b]	39.62 ± 2.19[b]	41.81 ± 1.29[b]
Abnormal sperm (%)	2.18 ± 0.58	1.30 ± 0.33	1.14 ± 0.24	1.18 ± 0.26	1.16 ± 0.28
Fertility (%)	65.67 ± 4.15[a]	2.75 ± 1.26[b]	9.22 ± 4.58[b]	9.58 ± 4.31[b]	7.60 ± 3.48[b]
No. of eggs incubated	154	170	156	146	132

Values given are mean±SE.

Figures bearing different superscripts in a row differ significantly ($p<0.05$).

In Experiment 3 the progressive motility and fertility were significantly lower ($p<0.05$) in treatments compared to their respective controls (Table 3). Cryopreserved PD1 line semen produced negligible fertility whereas 6% DMA cryopreserved PD6 line semen produced comparatively higher fertility. There was no significant chicken line or line by treatment interactions for the parameters studied.

Table 3. Effect of chicken lines on post thaw semen parameters and fertility of semen cryopreserved by pellet method.

Parameters	PD1			PD6		
	Control	6% DMA	9% DMA	Control	6% DMA	9% DMA
Progressive sperm motility (%)	64.0 ± 2.45^{a}	30.0 ± 1.50^{b}	29.50 ± 1.57^{b}	62.50 ± 2.01^{a}	31.50 ± 1.67^{b}	27.80 ± 1.51^{b}
Live sperm (%)	86.74 ± 1. 20^{a}	34.74 ± 1.93^{b}	37.55 ± 1.62^{b}	86.95 ± 0.96^{a}	38.71 ± 2.03^{b}	36.64 ± 2.15^{b}
Abnormal sperm (%)	2.50 ± 0.33^{a}	1.34 ± 0.20^{b}	1.51 ± 0.23^{ab}	1.74 ± 0.17^{ab}	1.60 ± 0.21^{ab}	1.40 ± 0.30^{b}
Fertility (%)	66.02 ± 4.20^{a}	0^{c}	0.40 ± 0.40^{bc}	56.32 ± 5.02^{a}	9.81 ± 3.44^{b}	3.10 ± 1.71^{bc}
No. of eggs incubated	165	135	206	110	85	141

Values given are mean±SE.

Figures bearing different superscripts in a row differ significantly ($p<0.05$).

DISCUSSION

Cryopreservation is a stressful process for sperm, only few sperm survive this process and therefore lesser motile sperm in thawed samples. This is irrespective of the cryoprotectant or cryopreservation protocol applied [10,29]. Similar to many earlier reports, in the present study the sperm motility and live sperm were lesser in cryopreserved semen. Chicken semen mixed with DMF and methylacetamide, and cryopreserved in straws gave better post thaw motility parameters and fertility that ranged from 77 to 81% [2]. Chalah *et al.*[1] applied a step freezing programme for chicken semen cryopreservation with DMF as a cryoprotectant in plastic vials and obtained 79% fertility. Korean native chicken semen cryopreserved with 7% DMF gave higher live sperm, mitochondrial activity and lower acrosome damage [3]. The present study differs from the earlier reports where semen was vitrified using DMF as cryoprotectant. Though reasonable sperm motility was obtained in samples frozen using 6% DMF in LR diluent the fertility obtained in this, as well as other treatments, was almost negligible. Though DMF is comparatively less toxic to sperm, due to unexplained reasons low post thaw sperm parameters and fertility were obtained in this study. The reason for low fertility may be that DMF is not effective cryoprotectant when used in pellet cryopreservation or due to the effect of the chicken line used in this experiment. The composition of the diluent influences the outcome of cryopreservation process. In the present study LR diluent produced comparatively higher post thaw sperm parameters. Polyvinylpyrrolidone (PVP) is a component in LR diluent that acts as non permeable cryoprotectant and has been used as cryoprotectant at 6% in rooster semen cryopreservation [30]. PVP used at 6% produced comparable fertility with that of 3% DMSO during chicken and pheasant semen cryropreservation [30]. Recently, red jungle fowl semen cryopreserved with 6% PVP has been shown to produce higher fertility than semen

cryopreserved in glycerol [31]. In the present study the TES/Nacl diluent tested has only three constituents that provide buffering capacity and energy source. Therefore this diluent with minimum composition is not able to support the sperm during the potentially cell damaging process of freezing/thawing resulting in very lower post thaw sperm parameters and infertile eggs.

Dimethylacetamide has been reported to provide high fertility when used as cryoprotectant during chicken semen vitrification [1,9,12,13]. A moderate fertility of 25% was reported using 3% DMA and vitrification method [10]. Chicken semen cryopreserved using 6% DMA and inseminated intravaginally gave the lowest fertility [32]. The present study employed the protocol described by Tselutin *et al.* [9], however, a low fertility was obtained despite comparatively good sperm motility. Assuming that low sperm concentration might be one of the factors for low fertility in Experiment 1 the sperm insemination concentration was increased in the other experiments. In Experiment 2, 6% DMA alone gave higher sperm motility than other treatment combinations; however, the fertility was similar to other treatments. The reason for obtaining no fertile eggs from eighth day till end of experiment from the groups inseminated with cryopreserved semen is not known.

The non permeable cryoprotectants trehalose and sucrose were studied in chicken semen cryopreservation [14,15,17]. Preliminary studies in our laboratory to vitrify chicken semen using sucrose (up to 300 mM) alone as cryoprotectant did not produce any post thaw viable sperm. Sexton [33] has reported that sucrose at different concentrations (4, 8, 12%) reduced the chicken sperm motility and fertilizing capacity. It was suggested that combining penetrating and non-penetrating cryoprotectants may provide added protection to sperm in withstanding damage during freeze-thaw process. Trehalose when combined with glycerol gave higher post thaw sperm motility and oxygen consumption rates implying that both the compounds act synergistically in the cryoprotection of sperm [14]. However, Madeddu et al. [15] reported no effect of trehalose on post thaw sperm parameters when added along with penetrating cryoprotectant in rooster and partridge semen. Combining trehalose and sucrose with DMA and straw packing did not affect post thaw chicken sperm viability and progressive motility [17]. However, some sperm motility parameters were shown to be positively affected by non penetrating cryoprotectants, trehalose alone or in combination with sucrose. Our results are similar to the report by Mosca et al. [17] where sucrose combined with DMA had no influence on post thaw sperm parameters. Species difference in effect of sucrose and trehalose was observed where these osmoprotectants improved post thaw sperm motility in turkey but not in crane [16].

The fertility obtained by inseminating cryopreserved semen was different between lines [34,35]. Furthermore, the fertility outcome from cryopreserved semen varies with the lines/breeds of females inseminated [35] as well as individual males from which semen was cryopreserved [36]. The fertility after insemination (intravaginal or intrauterine) with DMA cryopreserved semen was different between the inbred/specialized strains of chicken; intravaginal insemination in a particular line did not produce any fertile egg [37]. Roushdy et al.[38] reported difference in fertility between lines tested after insemination with semen cryopreserved using 3% DMA. In Experiment 3, the fertility obtained in PD1 line is negligible and the result obtained in Experiment 2 using the same line could not be achieved. In Experiment 3, younger PD1 males and diluent reported by Sasaki et al. [28] were used.

Different in vitro parameters especially membrane fluidity measured on fresh semen samples was correlated with fertility after cryopreserved semen insemination [36]. Furthermore, these parameters were suggested to have predictive value for success rate of chicken semen cryopreservation. In the present study, post thaw sperm motility of 25-30% was obtained, however, the fertility rate was found to be highly variable. The hypothetical reasons for this highly variable fertility may be change in the sperm membrane proteins during and after cryopreservation or any other molecular process/structures that affect sperm functions in female reproductive tract. It should be noted that the fertility obtained after cryopreserved semen insemination differs between females of different lines [35] indicating a role played by recipient female reproductive physiology.

CONCLUSION

In conclusion, DMF appears to be a poor semen cryoprotectant for preserving PD1 chicken semen cryopreservation by pellet method and TES/NaCl diluent a simple semen diluent is not useful for semen cryopreservation. DMA as cryoprotectant in pellet method of semen cryopreservation produced low fertility levels. Non penetrating cryoprotectant sucrose does not provide any additional advantage when combined with penetrating cryoprotectant in improving post thaw semen parameters and fertility. There was no difference in post thaw semen parameters and fertility between the chicken lines studied.

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