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Research Article

CRYOPRESERVATION OF ROOSTER SEMEN USING N-METHYLACETAMIDE AS CRYOPROTECTIVE AGENT

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Abstract- An attempt has been made to cryopreserve PD6 rooster semen using N-methylacetamide (MA) as a cryoprotective agent (CPA). Semen was collected from roosters, and pooled semen sample was diluted such that the concentration was arrived at 4 million/µl, followed by equilibration at 5°C for 30 minutes. Then the samples were diluted in 1:1 proportion with diluent containing 12%, 18% and 24% MA so that the final concentration of MA was 6%, 9% and 12% respectively and the final sperm concentration was 2 million/µl in each treatment. Immediately semen was loaded into 0.5 ml straws, sealed and kept 4.5 cm above liquid nitrogen (LN_2) and exposed to nitrogen vapours for 30 minutes and subsequently immersed into $LN_2$and stored in LN_2 container. Frozen semen was thawed in ice water. Semen was evaluated before and after cryopreservation for progressive sperm motility, live and abnormal sperm percent and seminal plasma lipid peroxidation. Fertility trial was conducted by inseminating PD-3 line hens per vagina (200 million sperm in 0.1 ml semen dose) six times at three days interval. The progressive sperm motility percent in 6% MA, 9% MA and 12% MA treatments were 30.91, 34.55 and 27.73 respectively. The live sperm percent in 6% MA (31.82), 9% MA (35.63) and 12% MA (29.91) was similar between the treatments. Abnormal sperm percent was comparable between treatments. The percent fertility achieved with 6%, 9% and 12% MA cryopreserved semen were 1.5%, 0% and 5.3%, respectively, while control group inseminated with fresh semen produced 94.3% fertility. The percent hatchability on fertile eggs set (FES) for 12% MA treatment was 62.5% that was significantly ($P<0.05$) lower than the control group 93.82%. There was zero hatchability on FES in 6% MA and 9% MA groups.

Keywords- cryopreservation, fertility, genetic resources, N-methylacetamide, rooster, semen.

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Introduction

Semen cryopreservation is one of the most effective and feasible method for preservation of genetic resources in avian species. This method enables storage of a very large number of samples in a relatively short time and is non-invasive for both donors and recipients. A cost comparison study pertaining to preservation of avian genetic resources had shown that cryopreservation of semen to be approximately ninety percent less expensive than the total cost of maintaining live birds [1]. The high vitellus content of megalecithal egg in avian species makes cryopreservation of oocyte and embryo difficult and the low efficacy of reconstitution and high cost associated with blastodermal or primordial germ cell preservation makes these approaches prohibitive [2].

Many freezing methods with slow and rapid freezing procedures, using variety of cryoprotective agents such as Glycerol, Dimethylacetamide (DMA), Dimethyl sulphoxide (DMSO), N-methylacetamide (MA), Sucrose, Trehalose and Polyvinyl pyrrolidone (PVP) and different types of sperm packaging methods (pellets in plastic/glass ampoules and French straws) have been explored to cryopreserve rooster sperm [3-9]. Gramapriya is a multi-coloured layer type chicken variety developed at ICAR-Directorate of Poultry Research, Hyderabad, for free range farming in rural and tribal areas and for rural backyard rearing.PD6 developed from multicoloured broiler population has been selected for shank length for six generations and is used as male line, and PD3 used for production of coloured germplasm for free range farming is used as female line to produce rural poultry cross “Gramapriya”. The rural and tribal farmers of many states of India are being profited by this variety. Considering the importance of the PD6 (male line) experiments are needed for determining the optimal cryopreservation protocol for freezing semen and for studying the fertility with frozen-thawed semen.N-methyl acetamide is a permeating cryoprotective agent that reduces the intracellular ice crystallization and curtails damaging effects of cryopreservation. Recent studies have investigated use of MA as cryoprotective agent for cryopreservation of rooster sperm with promising results [7, 10]. Thus, the present study was designed to test the efficacy of N-methylacetamide as cryoprotectant for cryopreservation of PD-6 rooster semen by evaluating post-thaw semen quality, fertility and hatchability.

Materials and Methods

Ethical approval

This study was conducted after approval by the Institutional Animal Ethics Committee.

Experimental animals

PD6 males (30 weeks) and PD3 females (28 weeks) were used in this experiment. Birds were reared in individual cages in open sided poultry sheds for the entire period of study and were provided with diet containing 2600 kcal/kg ME and 16% CP, with water available *ad libitum* throughout the experimental period.

Semen diluent

The chemical composition of the semen diluent was used in the present study was

as follows (per 100 mL): D (+)-glucose 0.2 gm, D (+)-trehalose dehydrate 3.8 gm, L-glutamic acid monosodium salt 1.2 gm, Potassium acetate 0.3 gm, Magnesium acetate tetrahydrate 0.08 gm, Potassium citrate monohydrate 0.05 gm, BES 0.4 gm, Bis-Tris 0.4 gm.

Semen cryopreservation

The roosters were trained to respond to abdominal massage technique [11] for collection of semen samples. Collected semen was immediately transported over ice in a covered Styrofoam box to the laboratory. The pooled semen samples were initially evaluated for sperm concentration. The samples were diluted with cryoprotectant free diluent such that the sperm concentration was arrived at 4 million/µl. The samples were equilibrated at 5°C for 30 minutes. Then samples were diluted in 1:1 proportion with diluent containing 12%, 18% and 24% MA so that the final concentration of MA was 6%, 9% and 12% respectively and the final sperm concentration was 2 million/µl in each treatment. Immediately semen was loaded into 0.5 ml French straws and sealed with polyvinyl chloride powder. The straws were frozen in liquid nitrogen vapours by placing 4.5 cm above the level of liquid nitrogen (LN_2) on a Styrofoam raft and exposed to nitrogen vapours for 30 minutes, followed by plunging into LN_2 [Fig-1]. Fresh and cryopreserved semen after thawing were evaluated on Eleven different occasions for progressive sperm motility, live and abnormal sperm and seminal plasma lipid peroxidation.

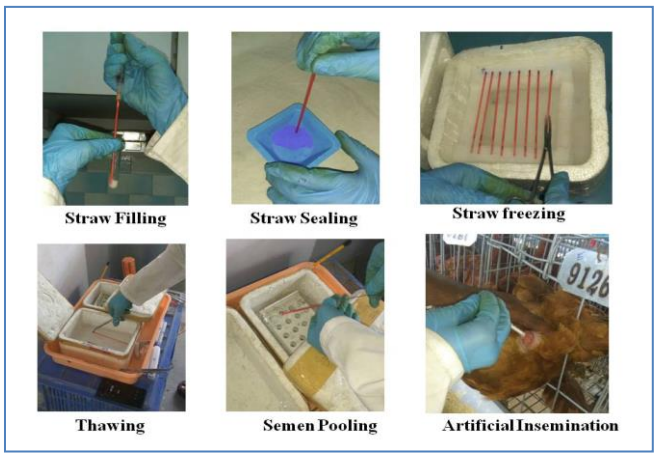

Fig-1 Semen Cryopreservation Procedure

Fertility trail

Fertility trial was conducted by inseminating PD-3 line hens six times at three days interval. Forty eight PD-3 line females were divided equally into four groups with 12 hens per group. The semen straws were thawed at 5°C for 100 sec in ice water and inseminated into hen per vagina with sperm concentration of 200 million sperm/0.1 ml. The group inseminated with freshly collected and diluted semen (200 million sperm/0.1 ml dose) served as control. Eggs were collected from second day of first insemination and stored at 15°C until incubation. The eggs were incubated at standard conditions in automatic setter. The eggs were candled on 18th day of incubation for embryonic development and fertile eggs were transferred into setter compartment. The chicks hatched on 21st day of incubation were counted for calculating hatchability. The infertile eggs were broken open to confirm absence of embryonic development.

Statistical analysis

The data analysis was carried out by one way ANOVA using SAS 9.2. Differences between means were tested using Tukey's test with significance level set at $P<0.05$.

Results

The progressive sperm motility percent in 6% MA, 9% MA and 12% MA treatments were 30.91, 34.55 and 27.73 respectively which were comparable among the treatments but was significantly ($P<0.05$) lower than that of the control group (75.91%). The live sperm percent in 6% MA (31.82 ± 1.74), 9% MA (35.63 ± 1.66) and 12% MA (29.91 ± 0.90) was similar between the treatments. However, live sperm percent in cryopreservation treatments was significantly ($P<0.05$) lower than the control (81.95 ± 2.54). The abnormal sperm percent was highest in 12% MA (5.36 ± 0.80) but was comparable with other treatment groups namely 6% MA and 9% MA with values 3.77 ± 0.41 and 3.68 ± 0.50 respectively. The control group had lowest abnormal sperm percent of 2.41 ± 0.38 that was significantly ($P<0.05$) lower than 12% MA but similar to 6% MA and 9% MA. The seminal plasma lipid peroxidation measured as Malondialdehyde (nM/ml) levels was comparable between cryopreservation treatment groups (6% MA, 9% MA and 12% MA) having values of 7.18 ± 1.21, 8.22 ± 1.31, and 8.48 ± 1.46 respectively which were higher than control (2.33 ± 0.36) [Table-1].

The fertility percent resulted in 6% MA, 9% MA and 12% MA cryopreservation treatment groups was 1.55 ± 1.08,0 and 5.38 ± 1.80 respectively. Though the fertility percent results were statistically similar among the MA treatment groups, numerically high fertility % was observed in MA 12% treatment group. The control group inseminated with fresh semen had significantly ($P<0.05$) highest fertility percent. The percent hatchability on fertile eggs set (FES) for 12% MA group was (62.5 ± 18.30) significantly ($P<0.05$) lower than the control group (93.82 ± 2.33). There was zero hatchability on FES in 6% MA and 9% MA groups [Table-1].

Table-1 *Effect of N-methylacetamide on post thaw semen parameters, fertility and hatchability of cryopreserved semen*

Parameters	Control (fresh semen)	6% MA	9% MA	12% MA
Progressive sperm motility (%)	75.91 ± 1.62^{a}	30.91 ± 1.13^{bc}	34.55 ± 1.06^{b}	27.73 ± 1.04^{c}
Live sperm (%)	81.95 ± 2.54^{a}	31.82 ± 1.74^{b}	35.63 ± 1.66^{b}	29.91 ± 0.90^{b}
Abnormal sperm (%)	2.41 ± 0.38^{b}	3.77 ± 0.41^{ab}	3.68 ± 0.50^{ab}	5.36 ± 0.80^{a}
Seminal plasma lipid peroxidation (MDA nM/ml)	2.33 ± 0.36^{b}	7.18 ± 1.21^{a}	8.22 ± 1.31^{a}	8.48 ± 1.46^{a}
Fertility (%)	94.35 ± 2.46^{a}	1.55 ± 1.08^{b}	0^{b}	5.38 ± 1.80^{b}
Hatchability on FES (%)	93.82 ± 2.33^{a}	0^{c}	0^{c}	62.5 ± 18.30^{b}
No. of eggs incubated	108	95	132	145

Values given are mean±SE.
Figures bearing different superscripts (a,b,c) in a row differ significantly ($P<0.05$).

Discussion

Despite the significant research into rooster semen cryopreservation, the post-thaw viability and fertility of the cryopreserved sperm was low and fertility is highly variable ranging from 0 to 90% with an average of 60% [12], which may be attributed to the accumulated cellular injuries that arise throughout the cryopreservation process.In the present study, post thaw progressive sperm motility obtained using different concentrations of MA (6%, 9% and 12%) ranged from 27 to 34%which was similar to that reported by Ehling *et al* [13] who obtained 32.7% post thaw motility using 6.5% MA. However, the progressive sperm motility percent obtained was lower compared to that observed in fresh semen. One probable reason for this reduction in motility might be due to reduction in mitochondrial function and the resultant loss in availability of adenosine triphosphate (ATP) for the sperm functions. After cryopreservation the extent of loss of ATP was severe, furthermore, the sperm ATP concentrations vary between commercial and research lines [14]. A reduction of 30to 60% in poultry sperm motility after the freeze/thaw cycle has been reported [14]. Cryopreservation is a

stressful process and many cells do not survive during the process. Thus, the live sperm in cryopreserved samples were lower in this study ranging from 29-35%. In the present study, sperm viability was similar among the three different concentrations of MA used. The present study findings were in agreement with that of the other reports where there was significant reduction in live sperm after cryopreservation [15-17]. A study reported that semen cryopreservation resulted in 34% and 30% sperm viability for 9% and 12% MA concentrations in Korean Oge chicken [15]. The MA concentration did not influence the sperm viability in Korean Oge chicken line. However, in White Leghorn chicken MA concentration was found to influence the sperm viability with 9% MA giving 38% viable sperm while 12% MA levels resulted in 17% live sperm, which may be attributed to the breed specific variation in sperm freezing ability [15]. Behaviour of the sperm of certain species/breed in a particular extender at low temperatures will affect its preservability [18-19]. The abnormal sperm percent was non-significant (P>0.05) among the treatment groups.

Lipid Peroxidation (LPO) is a chain reaction with the formation of lipid peroxides and ultimately the formation of cytotoxic aldehydes [20]. LPO has been considered to be one of the main mechanism responsible for sperm damage caused by reactive oxygen species (ROS), leading to impaired sperm function [21]. In semen, peroxidation of lipids has critical consequences. Oxidation reactions in biomembranes lead to amplification of reactive oxygen species (ROS), change in membrane fluidity, loss of compartmentalization and plasma-membrane integrity, disturbance of ion-gradients, impairment of lipid-protein interactions, modification of DNA and proteins [22]. In our study, higher seminal plasma lipid peroxidation level in cryopreserved samples was similar to that of an earlier report in chicken [23]. A balance between free radical level and antioxidant capacity of semen is maintained under normal circumstances. Any change in this balance may affect the semen quality thus the higher seminal plasma lipid peroxidation in this study might have contributed to reduced post thaw sperm parameters and fertility.

It is little questionable, whether the subjective assessment of parameters related to the functional and morphological characteristics of sperm, would increase the predictability of the fertilizing potential of cryopreserved semen [23]. Hence in the present study a fertility trial was carried out to evaluate the quality of frozen-thawed semen. The fertility from cryopreserved samples was very low which was similar to the reports of Tucker (2014) where only 3% fertility was obtained using similar freezing protocols [24]. In our study with use of 9%MA there was no fertile eggs. The result is in contrary to other reports where MA was used as cryoprotectant [7,10, 15-17,25]. However, the high fertility (>70%) reported in some studies [7,10] could not be replicated in other studies where different breeds of chicken were utilized [15-17]. Ehling *et al.* (2012) reported a moderate fertility percent (39.8%) using MA (6.5%) as single cryoprotectant whereas combination of MA and Dimethyl formamide (DMF) led to very high fertilization results ranging from 75 to 80 % [13]. An apparent variation in fertility after using MA as cryoprotectant due to difference in breeds of chicken could be observed from the literature. The low fertility obtained in this study may be due to the breed/line effect. The sperm fertilizing ability following a freeze/thaw cycle has been reported to vary among the poultry lines and between individual males [14]. The difference in hatchability in the cryopreserved treatment groups may be due to the very low or nil fertility obtained.

Conclusion

The present investigation demonstrates that the use of MA as CPA at 6, 9 and 12% concentrations for cryopreservation of PD6 semen has resulted in very low fertility requiring research into elucidation of the cellular and molecular changes that occur during cryopreservation is key for developing strategies to circumvent cryopreservation challenges in poultry species.

Application of research: Developing rooster semen cryopreservation techniques with better fertility results would enable ex-situ conservation of poultry genetic resources and facilitate sharing of these genetic resources among counties through shipping of cryopreserved semen.

Research Category: Poultry reproduction

Abbreviations:

CPA	Cryoprotective agent
DMA	Dimethylacetamide
DMSO	Dimethyl sulphoxide
MA	N-methylacetamide
PVP	Polyvinyl pyrrolidone
ICAR	Indian Council of Agricultural Research
BES	N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
˚C	degree Celsius
%	Per cent
AI	Artificial Insemination
ANOVA	Analysis of variance
LPO	Lipid peroxidation
LN2	Liquid nitrogen
MDA	Malondialdehyde
ME	Metabolisable energy
CP	Crude protein
SAS	Statistical Analysis System
DMF	Dimethyl formamide

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