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Original Research Paper**Dimethyleacetamide improves the cryosurvivability of Indian red jungle fowl (*Gallus gallus murghi*) sperm**

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Running Title: Cryopreservation of Indian red jungle fowl sperm with DMA

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ABSTRACT

It was hypothesized that dimethyleacetamide (DMA) can be used as an alternate to glycerol for cryopreservation of Indian red jungle fowl semen. Four concentrations of DMA (4%, 6%, 8% and 10%) in extender were compared with previously optimized cryopreservation protocol based on 20% glycerol (control) for Indian red jungle fowl. Sperm motility, plasma membrane integrity, viability, and acrosome integrity were assessed at the stage of post-dilution, cooling, equilibration, and freeze-thawing. The whole experiment was repeated/replicated for five times independently. Sperm motility, plasma membrane integrity, viability and acrosome integrity were recorded highest ($P<0.05$) at post-dilution, cooling, equilibration, and freeze-thawing in extender having 6% DMA compared to control and other experimental extenders. The highest ($P<0.05$) recovery rates of all aforementioned parameters were also recorded in extender having 6% DMA; thus, 6% DMA was further compared with control (20% glycerol) for fertility after artificial insemination. Eggs were collected for five days after artificial insemination with semen cryopreserved in extender containing 6% DMA and control. The higher no. of fertilized eggs, fertility, no. of hatched eggs, hatch (%) and hatchability were recorded with semen cryopreserved in extender having 6% DMA compared to control. It is concluded that 6% DMA maintained higher post-thaw quality and fertility of Indian red jungle fowl semen and is a better replacement of glycerol.

Keywords: Cryo-damage; Motility; Fertility; Acrosome; Sperm viability; Indian red jungle fowl

1. Introduction

Cryopreservation provides safety net against the stochastic events of nature; continuously at work and playing role in the deletion of valuable genetic resource [1]. During the last few decades, increasing human population has resulted in extreme exploitation and selection pressure in poultry birds that pushed indigenous or local species/breeds in the background, disposing of valuable genes [2,3]. According to Domestic Animal Diversity Information System, about 50% of the poultry species have been enlisted in endangered category [4]. The Indian red jungle fowl is a wild sub-species of genus *Gallus*, and is considered as one of the ancestor of domestic chicken [5-10]. The population of Indian red jungle fowl is facing a notable threat in its native range due to habitat destruction, poaching, inbreeding and genetic hybridization [11]. The cryo-banking has great potential for application in *ex situ in vitro* conservation of this precious *Gallus* sub-species [12]. Semen cryopreservation is the most feasible method in birds as cryopreservation of oocyte or embryo is not possible because of large size, high lipid content and polar organization [13], and alternative methodologies such as primordial germ cells methodologies are too invasive for at risk populations [14, 15].

It is generally accepted that avian sperm has low surface to volume ratio and elongated tail that makes it more vulnerable to osmotic, chemical, thermal and physical stresses during cryopreservation process compared to mammalian sperm [16,17]. Freeze-thawing process not only causes irreversible damage to mitochondria, mid-piece and acrosome of sperm [18], but also induces physical and chemical changes that alter physiological processes. Sperm may lose ATP due to energy metabolism [19] and glycoproteins or glycolipids necessary for transport and maturation [16,20].

The species-specific differences in sperm and their response to freeze-thaw process are important determinants of fertility and need the development of successful freezing

protocol, identification of novel cryoprotectants and diluents [21]. Of all cryoprotectants available, glycerol and dimethyleacetamide (DMA) are considered more adequate [14,22]. Glycerol is least toxic and more effective cryoprotectant for low fertility lines of poultry [14]. Nevertheless, due to its contraceptive properties removal of glycerol is needed prior to artificial insemination that makes it difficult to use [7, 23-25]. Glycerol is also known to interact with the metabolism of sperm, alter lipid-bilayer that possibly decreases the efficiency of acrosome reaction [26, 27].

The DMA is a permeable cryoprotectant, is not contraceptive and does not need to be removed prior to artificial insemination; thus may be helpful in reducing damage due to cryoprotectant removal [14]. Furthermore, DMA may offer an alternative since high levels of fertility have been obtained with this cryoprotectant in chicken [28,29]. DMA has the capacity of penetration by increasing plasma membrane fluidity through the rearrangement of lipids and proteins that cause reduction in formation of ice-crystallization at low temperature [21,30]. DMA has been used as cryoprotectant for sperm in houbara bustards [31], fowls [29,32], eagles, and peregrine falcons [33,34], ducks [35], landese ganders [36], sandhill cranes and turkeys [37], Japanese quail [38], and emus [39]. However, the intensity of cryo-induced changes are highly species-specific [30,40,41]. One major disadvantage of DMA is the toxic effect associated with its concentration [34]. In a previous study, we showed that glycerol could be an efficient cryoprotectant for red-jungle fowl sperm cryopreservation [9]. However, considering the importance of semen cryo-banking for conserving valuable genetic resources of Indian red jungle fowl, and in order to simplify the cryopreservation process of red-jungle fowl sperm, the present study was planned to evaluate the use of different concentrations of DMA on quality and fertility of cryopreserved Indian red jungle fowl sperm.

2. Materials and methods

All experimental procedures and animals used in this study were approved by the ethical committee of the Department of Wildlife Management, PMAS-Arid Agriculture University Rawalpindi, Pakistan.

2.1. Experimental animals

Eight sexually mature cocks (age: 5 years) of Indian red jungle fowl (*Gallus gallus murghi*) were selected and raised at Avian Research Station, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi-Pakistan. The birds were housed in single sand floor pen (8 m²) with roof cover and kept under natural photoperiod and temperature conditions. They were fed commercial feed containing corn 61.0%, rice polish 4%, corn gluten 1%, canola meal 5%, rapeseed meal 2%, soybean meal 13%, sunflower meal 4%, limestone 8%, DL-Methionine 0.10%, soda bicarb 0.10%, salt 0.30%, vitamin and min. premix 0.40% and MDCP 1%. Water was available over the experimental period *ad libitum*.

2.2. Experimental design

Semen was collected from eight mature cocks twice a week (October 2015-March 2016). A total of 40 ejaculates (5 ejaculates/cock) were processed for semen analysis. Ejaculates (whole) having >60% motility were pooled and aliquoted for dilution in to five experimental extenders. Experiment involved the addition of DMA at 4%, 6%, 8% and 10% of extender and 20% glycerol as a control. Semen was cryopreserved and quality was assessed at post-dilution, post-cooling, post-equilibration and post thawing. The whole experiment was repeated/replicated for five times independently. Ten pools of semen with 6% DMA and 20% glycerol were used to inseminate 50 hens. A total of 80 ejaculates (10 ejaculates/cock) were frozen for each extender separately. Five hens per extender were inseminated with each pool of semen and fertility was estimated through the fertilized and unfertilized eggs collected up to 5 days following insemination.

2.3. Semen collection and dilution with freezing extender

Semen was collected twice a week as described by Burrows and Quinn [42] in a graduated plastic tube using massage technique. Qualifying ejaculates from eight cocks were pooled on each replicate. Each pool was immediately diluted 1:1 (v/v) using diluent comprised of D-fructose (1.15g), sodium glutamate (2.1g), polyvinylpyrrolidone (0.6g), glycine (0.2g) potassium acetate (0.5g) and distilled water (100mL) to final pH (7.0) and osmolarity 380mOsm/kg [7]. Precaution of the temperature shock was taken, and thus the tubes containing diluents were kept in water bath at 37°C. The extender was divided into 5 experimental extenders containing respectively 4%, 6%, 8%, 10% DMA, and 20% glycerol (control). All diluents and media were prepared in the laboratory using analytical grade chemicals purchased from Sigma-Aldrich, Co., 3050 Spruce street, St Louis, USA.

2.4. Cryopreservation and thawing of semen

The semen (mean concentration 1200×10^6 sperm/mL) diluted in freezing extenders were immediately cooled to 4 °C in two hours ($0.275 \text{ }^\circ\text{C min}^{-1}$) and equilibrated for 10 min at 4°C. Cooled semen was filled in 0.5 mL French straws (IMV, L'Aigle, France), kept over liquid nitrogen vapors for 10 minutes and plunged into liquid nitrogen for storage. After 24 hours, the straws were thawed for 30 sec in water bath at 37 °C and held at 37 °C to assess motility, plasma membrane integrity, viability, and acrosomal integrity. For fertility measurements, semen frozen with 6% DMA and 20% glycerol were thawed as previously described and used in artificial insemination. Glycerol was removed after thawing by following stepwise dilution protocol as suggested by Purdy et al. [43].

2.5. Semen quality assays

2.5.1. Motility

The percentage of motile sperm and the quality of sperm were assessed subjectively as described by Santiago-Moreno et al. [16]. Semen sample was placed on a pre-warmed (37°C) glass slide and observed under a phase contrast microscope (400x).

2.5.2. Plasma membrane integrity

Plasma membrane integrity at different stages of cryopreservation was assessed by using hypo-osmotic swelling test (HOST) as described by Santiago-Moreno et al. [44]. The HOS solution composed of sodium citrate (1g) and distilled water (100 mL). Previously diluted 25 µl semen was mixed with 500 µl of a HOS solution (100 mOsm/kg) and incubated at 37°C for 30 minutes. The slides were fixed in buffered 2% glutaraldehyde. The plasma membrane integrity of sperm was scored on the basis of swollen heads, swollen and coiled tails. The sperm that respond to HOS solution become swollen and coiled were classified as normal sperm having intact plasma membrane. Nevertheless, sperm that did not respond to HOS solution remained straight were classified as sperm with damaged plasma membrane. A total of 200 sperm were counted at four separate fields under a phase-contrast microscope (1000x with oil immersion).

2.5.3. Sperm viability

Viability (% Live / total sperm) at different stages of cryopreservation was examined by adding eosin-nigrosin to the lake glutamate solution. Lake's glutamate solution [45] was prepared by adding sodium glutamate (0.01735g), potassium citrate (0.00128g), sodium acetate (0.0085g) and magnesium chloride (0.000676g) in 100 mL distilled water. Water soluble nigrosin (5g) and water soluble Eosin-bluish (1g) were added into Lake's glutamate solution. Twelve drops of stain were mixed with 1 drop of semen. A smear was made on a glass slide, fixed and air dried. A total of 200 sperm were assessed per slide under a phase-contrast microscope (1000x with oil immersion). The mixture provides a clear background in

the smear to enhance the contrast of white, unstained “live” sperm or the pinkish stained “dead” sperm.

2.5.4. *Acrosomal integrity*

The percentage of sperm having intact acrosome was determined by phase contrast microscopy, examining 200 Giemsa blue-stained cells under the magnification of 1000x as described by Jianzhong and Zhang [46]. Giemsa staining involved spreading of 5µl of diluted semen on to a glass slide, allowing them to dry and then fixed in neutral formal-saline (5% formaldehyde) for 30 min followed by air-drying. Fixed slides were kept in Giemsa stain for 1.5 h and washed with distilled water and air-dried. Sperm with hooked, swollen, thinned, unstained and missing acrosome were classified as sperm having damaged acrosome. However, evenly stained spermatozoa were classified as normal sperm having intact acrosome. A total of 200 sperm were observed at least in four separate fields. All variables were analyzed by the same person.

2.6. *Recovery rate for semen quality parameters*

The recovery rate of sperm motility, plasma membrane integrity, viability and acrosomal integrity rate were calculated through following formula [7,36];

$$\text{Recovery rate} = \frac{\text{Value of a given parameter in frozen semen}}{\text{Value of a given parameter in fresh semen}} \times 100$$

2.7. *Artificial insemination*

The fertilization capacity of sperm was estimated from the results of two consecutive (at one day interval) intravaginal artificial inseminations involving 300 million sperm/female at each insemination. A total of 50 hens of Indian red jungle fowl were inseminated using 6% DMA and 20% glycerol. All inseminations were performed between 12:00 and 14:00 h as described by Burrows and Quinn [47]. Eggs were collected from day 2 after the first

insemination up to 6 days, and there were a mean of 50 eggs/treatment. Fertility (%fertile/incubated eggs) was determined by candling of eggs on day 7 of incubation.

2.8. Statistical analysis

The data were presented as means (\pm SEM). The effect of different stages of cryopreservation and the cryoprotectant concentration were analyzed by two factor ANOVA using (MSTAT-C® ; version 1.42, Michigan State University, East Lansing, MI, USA). When F-ratio was found significant ($P<0.05$), post hoc comparison between the means was done through Fisher's protected LSD test. Student t-distribution under the notion of comparing two independent groups (6% DMA vs 20% glycerol) was used to test the influence of cryoprotectant on fertility rate.

3. Results

The data on effect of DMA on motility, plasma membrane integrity, viability and acrosome integrity of Indian red jungle fowl spermatozoa are presented in Figure 1. Sperm motility, plasma membrane integrity, viability and acrosome integrity of Indian red jungle fowl spermatozoa was recorded higher ($P<0.05$) in extender having 6% DMA compared to control and other experimental extenders with 4%, 8%, 10% DMA at all stages of cryopreservation. Nevertheless, cryopreservation have significantly ($P<0.05$) negative effect on motility, plasma membrane integrity, viability and acrosome integrity of Indian red jungle fowl spermatozoa irrespective of experimental extenders.

The data on recovery rates of motility, plasma membrane integrity, viability and acrosome integrity of Indian red jungle fowl spermatozoa after cryopreservation are given in Table 1. The recovery rates of all aforementioned parameters were recorded higher ($P<0.05$) in extender having 6% DMA compared to control and other experimental extenders.

The results of fertility obtained after artificial insemination of frozen-thawed semen with 6% DMA or 20% glycerol (Control) are given in Table 2. The highest ($P<0.05$) no. of fertilized eggs, percent fertility, no. of hatched chicks, percent hatch and hatchability of fertilized eggs were recorded after AI with 6% DMA compared to 20% glycerol.

4. Discussion

Gamete cryopreservation methodology has been developed for a wide range of species over the last sixty-five years [47]. The success of sperm cryopreservation depends on many factors including the type of cryoprotectant, packaging method (straws and pellet), freezing and thawing rates for avian species [13,17, 29] that are responsible for the alteration of structure and function of sperm [48]. Cryoprotectants help to prevent enzyme denaturation, protein destabilization, and ice crystal formation during freeze-thaw process [49]. The most commonly used cryoprotectant for avian sperm are glycerol [50] and DMA [51]. It has been demonstrated that 20% glycerol can be used in cryopreservation of Indian red jungle fowl sperm [7], while information on use of DMA is lacking in published literature. The previous studies on chicken and turkey have reported that sperm cryopreservation with DMA/straws produced less fertility compared to sperm cryopreserved with glycerol [51]. It is believed that low fertility with DMA/straws based cryopreservation has been attributed to deleterious interaction of plastic straws and the DMA. Moreover, this type of interaction is more deleterious and spectacular to sperm viability in the species having low fertility. The sperm cryopreservation with DMA is recommended for species having high fertility compared to glycerol, as high technical skills are needed to remove glycerol before artificial insemination [14]. The present study showed that 6% DMA efficiently protect Indian red jungle fowl sperm during cryopreservation and yielded higher fertility rate compared to glycerol.

It is well known that stages of the cryopreservation (post-dilution, post-cooling, post-equilibration, before freezing and after freezing, post-thawing) do affect sperm survival and are highly dependable on the diluent and cryoprotectant used. In our study, damages to sperm motility occurred first from post-dilution to post-cooling and were much higher with 10% and 4% DMA (10% decrease), intermediate with 8% DMA (6% decrease) and control 20 % Glycerol (6%) and very less in extender having 6% DMA (3.7%). The second part of damage occurred from post-cooling to post equilibrium stage of cryopreservation; very less at 6% DMA (5%) compared to 13-21% damage at other concentrations of DMA. Finally, the freezing-thawing itself was more deleterious as it cumulated the combined effects of the huge temperature and osmotic stress of the freezing that induced membrane and metabolic injuries at thawing such as potential re-crystallization of ice crystals imposed by the disruption of proteins that may cause further cell damage [52,53]. However, in our study, the freezing-thawing stages induced significantly less damage of sperm (22% for sperm motility with the dose of 6% DMA) compared to control 20% glycerol (38%) and 4%, 8%, 10% DMA (23-27%). The damage reported during cryopreservation was very high in the previous studies on chicken [54,55] and duck [35]. It is relevant to mention that higher concentrations of DMA are considered very toxic to rooster sperm [22].

It is relevant to discuss that Tselutin et al. [29] recommended the use of DMA in a sperm cryopreservation process in pellets achieved by dropping diluted sperm samples directly into liquid nitrogen. Rapid freezing is usually employed with DMA [56], while slow freezing using nitrogen vapour in combination with packaging straws are usually used with dimethyl sulphoxide [57] and glycerol [50]. A cryopreservation method with semen packaged in straws was chosen in our study because the sperm freezing in nitrogen vapour was usually used in our lab and other labs working with avian spermatozoa. Contrary to previous reports,

our findings revealed higher fertility rates than previous reports in chickens employing both DMA in straws [43] and pellets [12].

As DMA is toxic to spermatozoa [15,22], the concentration used is therefore very important. It should be noted that the sperm of native Mediterranean breed of chicken are not very tolerant of DMA, and concentrations lower than 6% are recommended [12]. Although a similar condition could be expected in the Indian red jungle fowl [12], the present results showed that DMA 6% provided the greater cryoprotective activity. Moreover, DMA 6% improved the fertility results regarding to previously used glycerol 20% protocol for Indian red jungle fowl sperm.

For fertility, higher glycerol concentration was kept as control because in our previous study, higher glycerol concentration gave better results [7]. Both glycerol and DMA are permeable cryoprotectants that provide intra and extracellular protection, dehydrating the sperm by an osmotically driven flow of water and thus avoid ice crystal formation during cryopreservation process [23,32]. Permeable cryoprotectants improve membrane fluidity and thus increase ability to survive post-thawing [21]. However, many permeable cryoprotectants such as DMA may have toxic effect on sperm, that is directly related to cryoprotectant concentration used and time to cell exposure [58,59]. Thus, the dose balances between a positive cryoprotectant effect and a negative toxic effect is important. In the present study, the optimum evolved concentration of DMA was 6%. The previous work on Houbara bustard [31] and chicken [60] would confer our results that sperm cryopreserved in an extender having 6% DMA can retain their viability, motility and fertility even after cryopreservation and suggest that lower concentration (6%) of permeable cryoprotectant is more favorable to sperm during cryopreservation process [12,29,35]. In our study, it has been observed that mean fertility rates [56.4% (control), 66.8% (6% DMA)] were higher compared to previous studies in ducks [35] and turkeys [54] using same cryoprotectant in different diluents.

It is concluded that the cryopreservation (pre-freeze steps and freezing-thawing) process affects the quality of red jungle fowl sperm. Moreover, 6% DMA could be efficiently used as cryoprotectant for the cryopreservation of Indian red jungle fowl sperm. To our knowledge, this is the first report on the use of DMA as cryoprotectant for Indian red jungle fowl sperm.

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1 Table 1: Recovery rate (%) of semen quality parameters of Indian red jungle fowl semen after
 2 freezing-thawing (n = 40; 5 ejaculates /8 cocks).

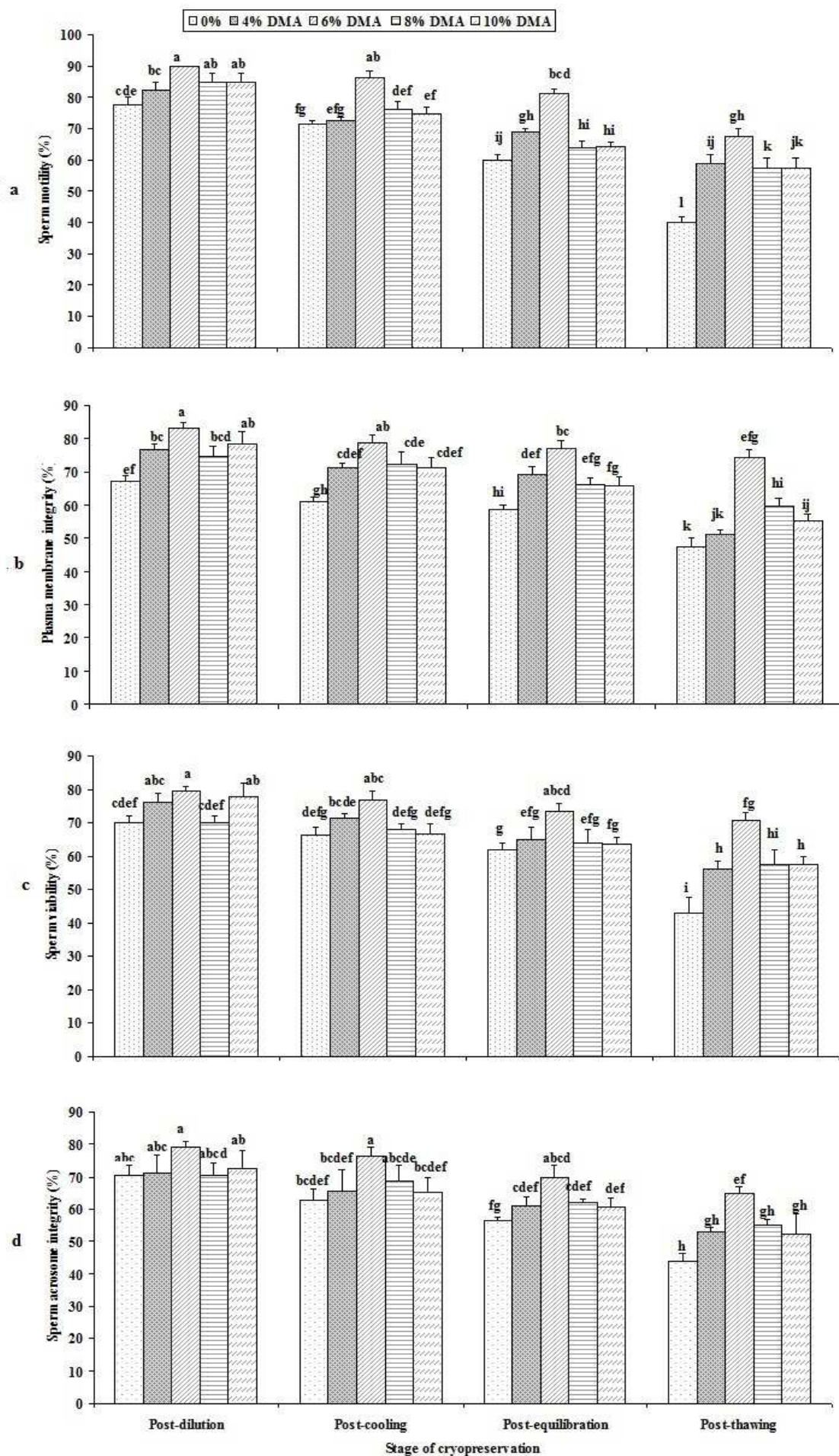
Extender	Recovery rate (%)			
	Motility	Plasma membrane Integrity	viability	Acrosome integrity
20% Glycerol	51.8 ± 3.1 ^c	64.8 ± 1.6 ^c	61.9 ± 7.4 ^b	62.6 ± 5.2 ^b
4% DMA	71.2 ± 3.0 ^a	64.2 ± 1.5 ^c	67.7 ± 4.4 ^{ab}	68.0 ± 2.7 ^{ab}
6% DMA	75.0 ± 2.8 ^a	79.4 ± 1.9 ^a	79.6 ± 2.0 ^a	75.3 ± 2.4 ^a
8% DMA	62.0 ± 2.7 ^b	75.0 ± 3.8 ^{ab}	71.2 ± 4.2 ^{ab}	67.1 ± 5.9 ^{ab}
10% DMA	67.9 ± 4.3 ^{ab}	69.9 ± 4.1 ^{bc}	67.4 ± 3.5 ^b	67.3 ± 5.1 ^{ab}

3 The values (mean ± SE) with different superscript in a column differ significantly (P<0.05).
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Table 2: Fertility and hatchability obtained with frozen-thawed sperm using as cryoprotectant glycerol 20% or DMA 6% (n = 80; 10 ejaculates/8 cocks).

Items	Cryoprotectant	Day after insemination					Mean \pm SE
		2	3	4	5	6	
Number of laid eggs	Glycerol 20%	50	45	47	48	45	45.0 \pm 1.4
	DMA 6%	45	47	48	40	45	47.0 \pm 0.9
Number of fertile eggs	Glycerol 20%	30	32	34	36	28	25.4 \pm 1.1 ^b
	DMA 6%	27	22	24	28	26	32.0 \pm 1.4 ^a
Fertility (%)	Glycerol 20%	60.0	46.8	50.0	70.0	57.8	56.9 \pm 4.1 ^b
	DMA 6%	60.0	71.1	72.3	75.0	62.2	68.1 \pm 3.0 ^a
Number of hatched chicks	Glycerol 20%	22	18	20	20	20	20.0 \pm 0.6 ^b
	DMA 6%	28	30	28	30	25	28.2 \pm 0.9 ^a
Hatch (%)	Glycerol 20%	48.9	38.3	41.7	50.0	44.4	44.7 \pm 2.2 ^b
	DMA 6%	56.0	66.7	59.6	62.5	60.1	60.1 \pm 2.1 ^a
Hatchability of fertile eggs (%)	Glycerol 20%	93.3	93.8	82.4	83.3	89.3	81.5 \pm 2.2 ^b
	DMA 6%	81.5	81.8	83.3	71.4	76.9	88.4 \pm 2.4 ^a

Different letters (a,b) between diluents, for each item, indicate significant differences (P<0.05).



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Figure 1: Effect of Dimethylacetamide (0%, 4%, 6%, 8% and 10%) on motility (a), plasma membrane integrity (b), viability (c) and acrosome integrity (d) of Indian red jungle fowl sperm at different stages of the freezing-thawing process. The results are expressed as mean \pm SEM, n= 40 ejaculates (5 ejaculates/8 cocks). Different superscripts indicate statistically significant differences ($P < 0.05$).

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Highlights

- Glycerol have contraceptive properties and must be replace with alternate cryoprotectant
- DMA was evaluated for cryopreservation of Indian red jungle fowl sperm
- DMA improve the sperm motility, PMI, viability and acrosome integrity
- DMA improve the fertility rate of frozen thawed Indian red jungle fowl semen

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