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

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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.

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

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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 maintained at Avian Research Station, Pir Mehr Ali shah Arid Agriculture University Rawalpindi-Pakistan. The birds were housed in single sand floor pen (8 m^2) 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 (1.15 g), sodium glutamate (2.1 g), polyvinylpyrrolidone (0.6 g), glycine (0.2 g) potassium acetate (0.5 g) and distilled water (100 mL) 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 °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 min and plunged into liquid nitrogen for storage. After 24 h, the straws were thawed for 30 s 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 (1 g) 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 min. 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