EFFECT OF TWO DIFFERENT PERMEABLE CRYOPROTECTANTS ON FREEZABILITY OF EXOTIC STALLION AND JACK SEMEN

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ABSTRACT

Semen was collected from the exotic stallions and exotic jacks (n=5 each) using an artificial vagina. The fresh semen was treated with primary extender and centrifuged to remove seminal plasma. Sperm pellet obtained after centrifugation was equally divided to receive secondary extender containing same amount (5%) of different permeable cryoprotectants, either glycerol (Gly) or dimethyl formamide (DMF) before submitting for cryopreservation. Various sperm parameters were assessed at pre-freeze and post-thaw stage which revealed differences ($p<0.05$) in motility, liveability, plasma membrane integrity and acrosome integrity of stallion as well as Jack semen cryopreserved with DMF or Gly. A variation ($p<0.05$) existed between individual stallions and jacks extended either with DMF or Gly. In brief, cryoprotectant DMF for stallion and Gly for jack semen were suitable for retaining the plasma and acrosome integrity of spermatozoa.

Keywords: Cryopreservation, Di-Methyl Formamide, Glycerol, Jack, Semen, Stallion

INTRODUCTION

Equine semen is a one of the most difficult in industry to cryopreserve efficiently without causing damage to membrane or apoptosis. The successful use of cryopreserved sperm largely depends on cryosurvival rates, which show large variation among species and individuals of the same species (Vidament *et al*., 2009; Wu *et al*., 2015). In fact, only 20% of fertile stallions produce sperms that survive the freezing and thawing processes (Tischner, 1979). Glycerol (Gly) is a preliminary cryoprotectant that has not only beneficial cryoprotective effects (Hoffman *et al*., 2011), but also toxic effects on spermatozoa (Alvarenga *et al*., 2005), despite with contraceptive effects in mare (Vidament *et al*., 2009). Glycerol lowered the fertility of equine semen when included in extenders for fresh, cooled, and frozen semen (Wu *et al*., 2015). Other cryopritectant like Dimethyl formamide (DMF) were less toxic and yielded similar or superior results as compared to glycerol (Alvarenga *et al*., 2005). Therefore, the present study aimed to assess the effects of two permeable cryoprotectants viz. Glycerol (GLY) and Dimethyl Formamide (DMF) on the freezability of exotic stallion and jack semen.

MATERIALS AND METHODS

Fiive healthy exotic (Thoroughbred) stallions and five exotic Jacks (Poitou breed), aged 6-10 yr, maintained in well-ventilated boxes on a standard diet (5 kg concentrate with mineral mixture, salt and 15 kg fodder - green: dry in 3:1 ratio) and *ad lib* fresh drinking water were used in the present study during the breeding season.

The semen from all the animals were collected using artificial vagina (Colorado model) equipped with a disposable liner as per the standard method (Talluri *et al*., 2017). Semen collection, evaluation and processing for freezing were done according to described methods (Talluri *et al*., 2017). Immediately after semen collection, seminal parameters like appearance, volume, colour, consistency, pH were recorded by visual observation. The gel portion of fresh semen was sieved through a sterilised gauze filter and volume of total and gel free

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semen was calculated visually through graduated sterile semen collection bottle. For calculating total gel volume, the gel free semen volume was deducted from fresh semen collected before sieving. After performing macroscopic and microscopic evaluation of fresh semen, the semen samples were processed, extended and frozen according to the described methods (Talluri *et al*., 2012a).

Semen samples having progressive motility >60% were processed for cryopreservation. Gel free semen was mixed with modified Citrate-EDTA primary extender in the ratio of 1:1 and centrifuged at 550 g for 3 min. The supernatant was discarded and sperm pellet was extended with Glucose-EDTA-lactose secondary extender having cryoprotectant agents either 5% GLY or 5% DMF. The diluted semen (100×10^6 sperm cells/ml) was kept in semen cooling cabinet at 4ºC for 2 h as equilibration period. Semen samples were again assessed for pre-freeze seminal characteristics. Equilibrated semen was manually filled in 0.5 ml straws using vacuum pump. The straws were sealed using PVC powder and were cooled for 30 min at 4ºC. The straws were spread over freezing racks, 4 cm above liquid nitrogen (LN_2) in a traditional styrofoam box for exposure to LN_2 vapours for 10-12 min and then plunged directly into LN_2. Later, the semen straws were transferred to LN_2 cryocans. The microscopic evaluation of frozen thawed sperm was done at least 24 h after storage. The straws were thawed in 37ºC water bath for 30 sec. Each frozen thawed semen sample was evaluated for determining the post-thaw motility, live and dead percentage, plasma membrane integrity through hypo-osmotic swelling (HOS) test and acrosome integrity as per the standard procedures.

As repeated collection of semen was done on same stallion/jack at different time intervals, a repeated measure ANOVA was done to partition the variability attributable to differences between treatments and individual variation among stallion/jack/subjects in treatment groups. Statistical analyses were performed using the SPSS 20.0 statistical software package. One-way ANOVA was used to test statistical differences between different treatment groups. Pair wise comparisons (or *post hoc* test) were performed using the T-method (Tukey's honestly significant difference method).

RESULTS AND DISCUSSION

The fresh semen collected from exotic stallions and jacks was white to creamy white and consistency was variably thick and viscous as observed earlier (Talluri *et al*., 2018). The fresh semen volume collected from stallions ranged from 25-125 ml and in jacks from 45-83 ml (Table 1). The total volume of stallion semen varies between 30-250 ml (Ricketts, 1993). In the current study, average total semen volume, gel free semen and gel in semen was recorded less than reported for indigenous stallions (Talluri *et al*., 2012a and 2012b). The pH of stallion semen ranged from 6.92-7.21 and of jacks was 6.52-7.10 (Table 1). The pH range was in correlation with earlier reports for stallions (Talluri *et al*., 2018) and Jacks (Rabindra Kumar *et al*., 2018).

The sperm concentration in fresh semen was $185.7\text{-}294.7\times10^6$ ml^{-1} in stallions and $281.7\text{-}341.1\times10^6$ ml^{-1} in jacks. with a mean of $318.29\pm16.29\times10^6$ ml^{-1} (Table 1). The variation in sperm concentration between individual stallions and jacks was simular ($p<0.05$), however, between stallions and jacks was different ($p<0.05$). The sperm concentration for exotic stallions and jacks observed in this study was higher compared to earlier reports for Stallions (Talluri *et al*., 20018) and jacks (Rabindra Kumar *et al*., 2018). Total sperm motility in gel fractioned semen was 79.3-88.2% in stallions and was 80.9 to 91.1% for jacks (Table 1), which was higher than observed for Marwari stallions (Pal *et al*., 2009) and Poitou jacks (Rabindra Kumar *et al*., 2018). The progressive sperm motility in gel free semen was 70.2-79.9% for stallions and 76.3-84.7% for jacks (Table 1). The stallion semen exhibiting >60% progressive sperm motility can be considered appropriate for cryopreservation.

Table 1: Fresh seminal characteristics of exotic Stallion and Poitou Jack (n=40 each).

Seminal attribute	Stallion	Jack
Total Volume, ml	45±6.7	53.8±8.3
Gel Volume, ml	9±1.18	9.6±0.81
Final volume, ml	36±5.7	45.2±8.3
pH	7.03±0.01	6.82±0.06
Sperm Concentration, $x10^6$	205.4±12.6	389.0±15.5
Total Initial Motility, ml	81±5.3	85±2.7
Progressive Motility, ml	74.7±1.70	80±2.73

A major difference was observed in pre-freeze and post-thaw motility of stallion spermatozoa cryopreserved with either DMF or Gly (Fig. 1). The stallion semen extended with DMF had better ($p<0.05$) progressive motility compared to Gly both at pre-freeze and post-thaw stages. Furthermore, at pre-freeze and post-thaw stages, a difference ($p<0.05$) existed in sperm motility between individual stallions treated with DMF or Gly (Fig. 1). In case of jacks, the semen cryopreserved with Gly had better ($p<0.05$) motility both at pre-freeze and post-thaw stages. The jack semen treated with Gly exhibited superior ($p<0.05$) motility over DMF or Gly treated stallion semen with at both stages (Fig. 1).

At post-thaw stage, the liveability was better ($p<0.05$) for stallion and jack semen cryopreserved with DMF but not for semen extended with Gly (Fig. 1). The functional integrity of plasma membrane as determined by HOS test was different ($p<0.05$) in spermatozoa extended with DMF or Gly at pre-freeze stage but not the post-thawed stallion semen. The jack semen extended with Gly had good plasma membrane integrity than semen extended with DMF and the same was *vice versa* for the stallion semen (Fig. 1). A highly significant difference ($p<0.05$) was observed in acrosome integrity of stallion spermatozoa extended with DMF or Gly at pre-freeze stages only.

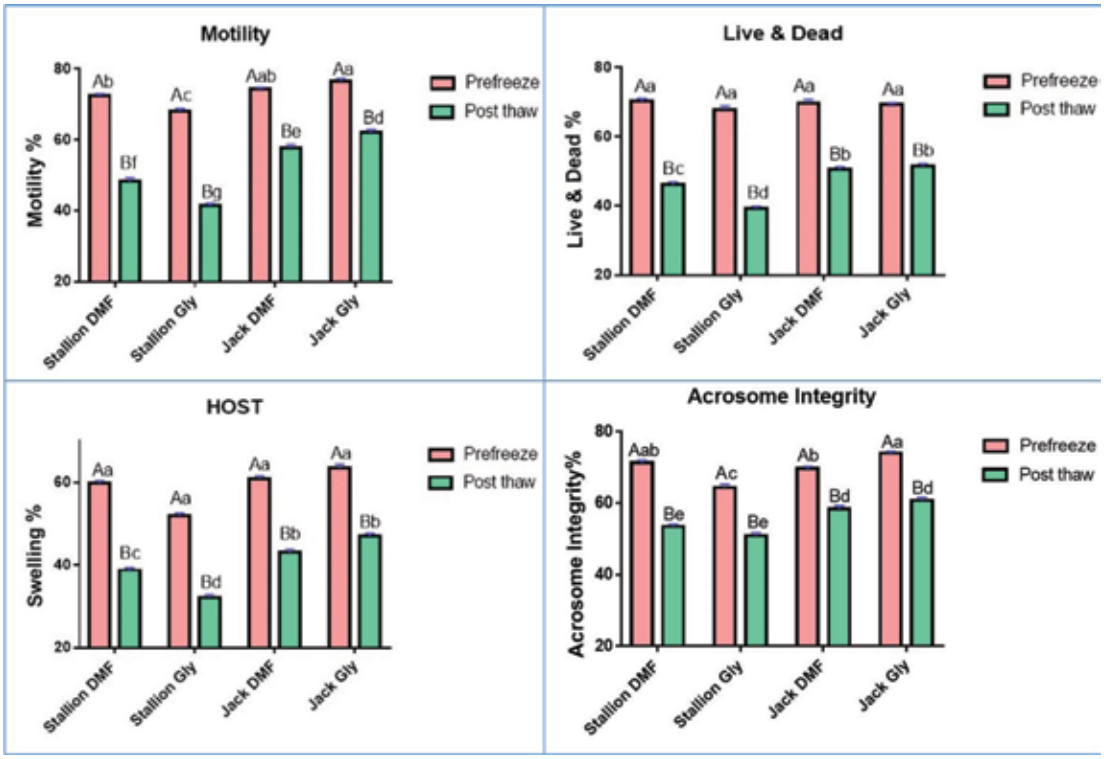

Fig. 1: Pre-freeze and post-thaw seminal characteristics of exotic Stallion and Jacks (n= 40 each group). ^{A,B}p<0.05 - within a group (between pre-freeze and post thaw). $^{a-g}$p<0.05 - across the groups.

The stallion semen treated with DMF had higher ($p<0.05$) percentage of sperms with acrosome integrity than that of Gly at both pre-freeze and post-thaw stages. Furthermore, the difference ($p<0.05$) in acrosome integrity was observed between individual stallion semen treated with DMF or Gly at post-thaw stage only (Fig. 1). In jacks, the semen extended with either DMF or Gly had difference in acrosome integrity at post-thaw stage ($p<0.05$), with the semen extended with Gly had higher acrosome integrity than DMF (Fig. 1). The freezability of semen from exotic horses and exotic jacks was 90% (20 ejaculates out of total 22 ejaculates) and 95% (21 ejaculates out of total 22 ejaculates), respectively.

In brief, the stallion and jack spermatozoa may be damaged by glycerol, but the toxic effect of glycerol is more obvious for the stallions. Thus, DMF can be used as an alternative to Gly as cryoprotectants for stallions and Gly can be used for jack semen to obtain better post-thaw motility and integrity of sperm membranes.

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