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Effect of thawing temperature and duration on post thaw seminal characteristics in exotic donkeys

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Abstract
Advances in artificial insemination (AI) techniques and semen handling have led to an increased rate of successful pregnancies and increased efficiency in large-scale breeding programs. AI with frozen Jack's semen is an important aspect in production of superior mules. Various studies in other species indicated that thawing temperature and duration play significant role in post-thaw motility. The following study aims to determine the optimal temperature for thawing extended cryopreserved ejaculated semen and a trial was conducted on different thawing temperatures and time durations to study its effect on post-thaw sperm motility. Five exotic jacks maintained at Equine Production Campus, NRCE, Bikaner were utilized for semen collection and its preservation. Citrate- EDTA and Lactose glucose-EDTA–Egg yolk were used as primary and secondary extenders, respectively for semen cryopreservation. Ejaculated jack semen collected by means of an AV and cryopreserved in 0.5ml straws and thawed at different temperatures. PTM was observed as 43.48, 51.4 and 47.8% for thawing of frozen semen straws at 37 °C for 30 sec, one min and two min, respectively. At 50 °C water bath temperature for 15 sec, 30 sec and one min, post thaw motility was 32.6, 23.2 and 18.6% respectively. Similarly, at 70 °C water bath temperature for 5 sec, 10 sec and 15 sec, the post thaw sperm motility observed was 44.40, 18.75 and 17.4%, respectively. It is concluded that the optimum post thawing temperature for cryopreserved jacks' semen is 37 °C for one min. However, it can also be thawed satisfactorily at 37 °C for 30 sec or 2 min and at 70 °C for 5 sec only as there was no significant differences ($p \leq 0.05$) were observed in liveability and motion characteristics. The present study infers that the thawing temperature and its duration does affect the motility of cryopreserved jack semen.

Keywords: Poitou donkey, semen, sperm, thawing, viability, motility

Introduction
Equines hold special position in livestock both for civil and military purpose in view of its multifaceted utility in hilly trains and non-metal road [1]. Sperm cryopreservation facilitates storage and transport for use in assisted reproduction technologies. Equine semen is a one of the most difficult in the industry to cryopreserve efficiently without causing damage to the membrane or apoptosis [2]. The first study on freeze-thawing of jack semen was carried out by Polge and Minotakis in the year 1964. Cryopreservation of semen from equines, and particularly from donkeys, is still a challenge. Sperm quality in donkeys after freezing and thawing is still considered lower than that from other animals, including horses. Several factors can influence the sperm longevity and viability of cooled semen, among them the extender used, cooling protocols, methods of transport, physical, morphological, biochemical, and metabolic characteristics inherent to sperm and membranes, as well as individual animal variability.

AI with frozen Jack's semen is an important aspect in production of superior mules [3]. However, in general, fertility rates with frozen semen are lower compared to those obtained with fresh or cooled semen [4, 5]. This apparently occurs due to damages to structural, biochemical and biophysical integrity of spermatozoa plasma membrane during spermatozoa cryopreservation [6]. Therefore, strategies to improve the physicochemical factors that affect the spermatozoa survival during freezing and thawing processes are needed to reach greater viability and fertility results in horses [7]. Thawing of frozen semen is very important in the process of AI. Straw thawing temperature and duration play vital role in post-thaw motility and fertility of semen. The easiest method available for thawing semen is to place the straws in a warm water bath. The frozen semen straws may be thawed in a variety of ways, either at a

lower temperature for a longer period or at a higher temperature for a shorter period [8-10]. The study was carried out to optimize thawing protocol of cryopreserved jack semen to get optimum post-thaw sperm motility.

Materials and Methods

Animals

Apparently five healthy exotic Jacks (Poitou breed), aged between 6 to 10 yrs and being maintained at Equine Production Campus, Bikaner were used for the present study. These jacks were kept in well-ventilated boxes with feeding and water trough. The jacks were daily fed a standard diet that included 5 kg of concentrate with mineral mixture, salt and 15 kg fodder (green: dry in 3:1 ratio). Fresh drinking water was allowed *ad lib*. The current experiments were conducted during the breeding season.

Semen collection and Cryopreservation

Semen was collected from Poitou jacks using Colorado model of artificial vagina (AV) equipped with a disposable liner as per the standard method at five different occasions. The jacks were kept at distance for visual stimuli to get proper erection before mounting for ejaculation in the AV. An estrus jenny was used as dummy. Soon after the semen collection, the semen was passed through sterilized gauze filter to remove the gel. The microscopic and macroscopic analyses were performed. The semen having progressive motility of more than 60% was processed for cryopreservation. Gel free semen was mixed with modified Glucose-EDTA primary extender as per the method described by Cohran *et al.*, (1984) [8] in the ratio of 1:1 and centrifuged at 2000 rpm for 4-5 min at 8-10 °C. The supernatant was aspirated off and the sperm pellet was dissolved in the modified secondary extender and added glycerol @ 3% of the total volume. After filling the diluted semen in 0.5 ml French straws containing 3% glycerol were given 1hour of equilibration time. The semen-filled straws were frozen in the programmable Bio-med Planner and plunged into liquid nitrogen (LN_2), and kept stored in LN_2 as described by Pal and Legha (2008) [1]. Frozen straws were thawed at 37 °C for 30 sec, one min and two min, at 50 °C for 15 sec, 30 sec and one minute and at 70 °C for 5, 10 and 15 sec in water bath and emptied into a clean vial maintained at 37 ^{0}C and evaluated for post-thaw motility. The sperm morphology and live/dead status was determined according to Bloom's method (eosin/nigrosine) [11]. The smears of a drop of semen with stain were made and observed using a phase contrast microscope at 40X and oil immersion magnifications.

Statistical Analysis

Statistical analyses were performed using the SPSS 20.0 statistical software package. One-way ANOVA was used to test statistically significant differences between the means of different treatment groups. The minimum significant range of confidence was evaluated at 0.05 and 0.01 level. All data was expressed as mean±SEM.

Results

Effect of thawing temperature and duration on post-thaw sperm motility has been presented in table 1. The cryopreserved straws were thawed in water at 37 ^{0}C for 30 sec, one min and 2 min, at 50 ^{0}C for 15 sec, 30 sec and 1 min, at 75 ^{0}C for 5 sec, 10 sec and 15 sec. PTM of semen straws thawed in water at 37 ^{0}C for 1 min was significantly (P<0.05) higher than thawing in water at 37 ^{0}C for 30 sec and 2 min. PTM of semen straws thawed in water at 50 ^{0}C for 15 sec was significantly (P<0.05) higher than thawing in water at 50 ^{0}C for 30 sec and 1 min. PTM of semen straws thawed in water at 70 ^{0}C for 5 sec was significantly (P<0.05) higher than thawing in water at 70 ^{0}C for 10 sec and 15 sec. PTM of semen straws thawed in water at 37 ^{0}C for 1 min was significantly (P<0.05) superior than other methods studied. PTM of semen thawed at 37 ^{0}C for 30 sec, at 37 ^{0}C for 2 min and at 70 ^{0}C for 5 sec was non-significantly different.

Discussion

Post-thaw motility is a good indicator of how well the sperm have withstood the stresses of being frozen and thawed and one should be cautious about using frozen semen with less than acceptable post-thaw motility. A significant reduction in progressive motility after thawing is indicative of sperm damage and there may even be sub-lethal damage to sperm that remain motile after thawing that would reduce fertility. Equine semen is very less tolerant to the freezing and thawing processes than bull semen. In order to get maximum reproductive efficiency from the use of frozen jack semen, it is essential that the semen be frozen, stored and thawed correctly. In present study the progressive motility of spermatozoa were fond comparatively higher than to the report of [12] in exotic (Poitou) donkeys. The mean value of live sperms count recorded for Poitou donkeys overall mean of 91.84±0.32%. Non-significant difference was found among donkeys for live sperm count. Our finding indicated that live sperm (%) of Poitou donkey was higher (P<0.01) than Indian jacks while, live sperm (%) in Indian jacks was higher (P<0.01) than the live sperm concentration in exotic (Poitou) jack semen, reported by Gupta *et al*., 2008 [12]. Cochran *et al*. (1984) [4] also demonstrated that immersion of 0.5ml straws in a water bath at 75 ^{0}C for 7 sec followed by immediate immersion in a water bath at 37 ^{0}C for longer than 5 sec, resulted in better PTM than immersion in a 37 ^{0}C water bath for 30 sec. In similar type of study, Pal and Legha (2012) [13] reported faster thawing rate (45 ^{0}C for 15 sec) as superior for obtaining maximum post-thaw motility. It is concluded that the optimum thawing temperature for cryopreserved jacks' semen is 37 °C for one min. However, it can also be thawed satisfactorily at 37 ^{0}C for 30 sec or 2 min and 70 °C for 5 sec only. The viability of cryopreserved semen using the conventional or fast-freezing techniques were not affected by the slow (37 °C 30 s^{-1}) and fast (75 °C7s^{-1}) thawing. Although several studies [4, 8, 10] have reported greater spermatozoa viability after semen thawing at 75 °C for 7 s, the maintenance of spermatozoa under high temperatures for longer periods than recommended levels may result in severe spermatozoa damages making its use more doubtful on the field. It could be inferred that the percentage of live spermatozoa will be influenced by the temperature of thawing and that jack semen thawed at 35"C for 30 sec will help obtaining satisfactory percentage of live spermatozoa. Temperature transitions associated with semen fast chilling or freezing are in fact well known for their production of deleterious effects on sperm survival and consequently, lower conception rates following artificial insemination [12]. In addition, it has been reported [14] that spermatozoa damages may occur after fast thawing protocols due to reduced spermatozoa efflux of cryoprotective agents out of the spermatozoa. Therefore, based on our results, we recommend the use of the slow thawing protocol (37 °C for 1 min) as it may reduce the risk of spermatozoa damages due to

prolonged duration during thawing.

Table 1: Effect of different thawing temperature and duration on jack seminal characteristics (Row values with different superscripts differ significantly ($P \leq 0.05$) (n=25)

Thawing temp (ºC)	Duration (time)	Seminal parameters	
		Post-thaw sperm motility (%)	Liveability (%)
37 °C	30 sec	43.48±2.07^{a}	44.73±0.29^{b}
	1 min	51.40±1.31^{b}	58.21±1.27^{a}
	2 min	47.80±1.13^{ac}	42.46±1.70^{ab}
50 °C	15 sec	32.60±0.94^{a}	34.37±2.96^{c}
	30 sec	23.20±1.05^{b}	28.30±0.07^{c}
	1 min	18.60±0.87^{c}	25.41±0.97^{bc}
70 °C	5 sec	44.40±1.03^{a}	48.39±2.14^{a}
	10 sec	18.75±0.79^{b}	24.48±2.01^{bc}
	15 sec	17.40±0.89^{b}	20.10±1.82^{abc}

Conclusion

Cryopreservation of the male gamete has proved to be the most successful arsenal for the genetic conservation and propagation of elite germplasm through successful application of AI in breeding programmes. Present study would be helpful in developing AI and semen cryopreservation protocol for genetic improvement of Poitou jacks and production of good quality mules.

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