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Cryopreservation and freezability of epididymal and ejaculated stallion spermatozoa

TR TALLURI[1✉], DINESH JHAMB[2], NILENDU PAUL[3], S C MEHTA[1], JITENDAR SINGH[1], R K DEDAR[1], R A LEGHA[1] and YASH PAL[1]

ICAR-National Research Centre on Equines, Equine Production Campus, Bikaner, Rajasthan 334 001 India

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ABSTRACT

The aim of the present study was to compare the seminal attributes and freezability of the epididymal sperm recovered from castrated stallions and ejaculated semen collected through artificial vagina from the same stallions previously. The seminal quality parameters viz., viability, sperm morphology, plasma membrane functional integrity, acrosome integrity and mitochondrial membrane potential were studied, and recorded immediately after fresh semen collection/retrieval, before freezing, and after cryopreservation. Similar protocols were followed for freezing the epididymal and ejaculated semen. From the current study, it was observed that the sperm concentration of the epididymal recovered was significantly higher than that of fresh ejaculation. Other seminal parameters in the fresh sample, like total and progressive sperm motility, sperm membrane functional integrity, acrosome integrity and kinematic parameters like VSL, STR, and linearity were similar without any significant differences between both. However, mitochondrial membrane potential, BCF and linearity of the spermatozoa differed significantly between the groups after cryopreservation. From the current study, it can be concluded that the protocols used for cryopreserving the fresh ejaculates can be adopted for freezing the stallion epididymal spermatozoa without causing significant damage and through this method; elite stallion germplasm can be safely cryopreserved and conserved.

Keywords: Castration, Cryopreservation, Epididymal sperm, Ejaculated semen, Mitochondrial membrane potential

After the death of an animal, spermatozoa in the testis will be live for a period of time (Yoon *et al.* 2016). The retrieval of sperm from the epididymis may be the last chance to obtain valuable genetic material when sudden death or serious injuries occur in elite stallions (Monteiro *et al.* 2013). Since billions of fertile spermatozoa can be harvested from the epididymis and ductus deferens of stallions, this sperm reservoir opens possibilities for applications for further use in assisted reproductive biotechnologies (Roels *et al.* 2014). In general, sperm cryopreservation is performed on ejaculated semen. Cryopreservation of epididymal sperm is a useful technique for preserving the genetic potentials of elite and precious deceased males or desired stallions that need to be castrated or euthanized after severe catastrophic injury or any other event that makes semen collection impossible (Papa *et al.* 2008). Mature sperm capable of fertilization can be collected from the caudal epididymis of particular stallions by retrograde flushing of the convoluted tubule of the epididymis. The harvest of sperm from the epididymal cauda in stallions was reported previously and has proven to be an efficient means of recovering viable spermatozoa (Tiplady *et al.* 2002, Bruemmer 2006, Melo *et al.* 2008, Papa *et al.* 2008). However, the fertility of frozen thawed epididymal sperm has also been shown to be lower than that of ejaculated sperm (Morris *et al.* 2002). The recent successful fertility rates achieved in horses using sperm recovered from the cauda epididymis highlight the importance of studying the effects of cryopreservation on these cells (Papa *et al.* 2008, Monteiro *et al.* 2011). Such studies become even more relevant when it is considered that the harvesting of sperm from cauda epididymis may allow for a final opportunity to obtain semen from deceased stallions.

It is speculated that the variation in morphology and function between epididymal sperm contribute to differences in membrane stability, cold shock susceptibility and resistance to osmotic stress. It is also postulated that methods used for cryopreservation of ejaculated sperm might not be appropriate for epididymal sperm (Hewitt *et al.* 2001). There are very meagre studies on the comparison of the fresh semen collected versus epididymal sperm reserve from the same stallions and their freezability. The limitations experienced in the freezing procedures for ejaculated sperm are more exaggerated and less developed for epididymal sperm (Perez-Osorio *et al.* 2018). With this background, the present study was undertaken to compare

Present address: [1]ICAR-National Research Centre on Equines, Bikaner, Rajasthan. [2]College of Veterinary and Animal Sciences, Navania, Udaipur, Rajasthan (RAJUVAS, Rajasthan). [3]Central Institute for Research on Buffaloes, Nabha, Punjab. ✉Corresponding author email: raotalluri79@gmail.com

the semen quality parameters and freezability of ejaculated equine sperm collected using an artificial vagina, with the sperm recovered from the epididymal cauda immediately after orchiectomy.

MATERIALS AND METHODS

Experimental animals: The present study was conducted on the same animals (Marwari breed) for both fresh ejaculations and the stallions were later used for castration and recovery of the epididymal spermatozoa. Semen was collected from three adult stallions of 8 to 10 years that are being maintained at Equine Production Campus (EPC), ICAR-National Research Centre on Equines (ICAR-NRCE), Bikaner, Rajasthan, India. No separate provisions were made for light or feed for the stallions involved in this study. Epididymides of these stallions were collected immediately after standardized routine castration under general anesthesia. Ultrasonographic and macroscopic examination of the testes and epididymides did not reveal any abnormalities. All the experiments conducted in the current study were previously approved and performed according to the guidelines laid down by the Institute animal ethics committee (IXX14589, ICAR-NRCE).

Recovery of epididymal spermatozoa: Epididymal sperm were collected from the epididymides of Marwari stallions (aged 8–10 years old) following routine castration. Castration was performed using 10% xylazine hydrochloride, 0.5 mg/kg IV, combined with 0.05 mg/kg 1% acepromazine maleate IV for sedation. Local anaesthesia was performed by intratesticular injection of 10 mL of 2% lidocaine hydrochloride with epinephrine. Immediately after castration, the cauda epididymides were isolated from each testis. The spermatic cord was ligated and the testicles/epididymides were then transported immediately to the laboratory. The cauda epididymis excluding the vas deferens was dissected from extraneous tissues. Epididymis from each testis of each stallion were processed using the modified retrograde flushing technique for sperm harvesting (Granemann 2006). Briefly, after separating the testis-epididymis complex and removing the surrounding connecting tissues covering the cauda epididymis, the epididymis was straightened. The epididymis was cut into three to four fragments to facilitate easy flushing for the entire duct. Retrograde flushing was performed at room temperature (27°C) using pre-warmed citrate-EDTA, which is usually used as the primary extender in the process of stallion semen cryopreservation. For sperm flushing, an intravenous catheter (22G) was gently inserted into the lumen of the convoluted tubule of the fragments (cauda epididymis) are then placed vertically and flushing was performed by the injection of semen extender in the upper portion of lumen until sperm cells were recovered at the lower extremity.

Semen processing and cryopreservation: The ejaculated semen samples were collected directly into a clean dry graduated bottle attached to the latex cone of the AV. The tubes containing semen were marked and placed in a water bath at 37°C immediately after collection. Total ejaculate volume of the semen was recorded as visible from graduated collection bottle, filtered through sterilized gauze and gel free semen volume was noted. Gel free semen was extended with primary extender (Citrate- EDTA extender) in equal ratio, and divided into five equal aliquots. These tubes were centrifuged at 600g for 5 min to obtain sperm rich pellet fraction. The supernatant containing seminal plasma was discarded and obtained sperm pellet was extended further with secondary semen extender (Glucose- Lactose-EDTA, with Dimethylformamide (DMF) @ 5% as cryoprotectant) to obtain a final concentration of 250×10^6/ml. The semen recovered from the epididymis and fresh ejaculates were processed for freezing in the similar manner.

French medium straws of 0.5 ml capacity were filled with extended semen by automatic straw filling and sealing machine (IMV, France) and kept at 4°C for 2 h equilibration in cooling cabinet. After equilibration and before freezing (Pre-freeze stage of cryopreservation), once again all the samples were analyzed for the seminal attributes. After equilibration, the straws filled with diluted semen were laid horizontally onto a wired net and lowered into a styrofoam box containing two-inch level of liquid nitrogen for 10-12 min before plunging into liquid nitrogen. After 24 h of its storage, straws from each group were thawed at 37°C for 30 sec for post-thaw evaluation.

Semen evaluation

Assessment of motility and livability: The total and progressive motility parameters were evaluated using a Computer Assisted Semen Analyzer (CASA) (HTB CEROS II, Version 1.3, Hamilton Thorne Research, Beverly, MA, USA,) equipped with a thermostage (MiniTherm®, Hamilton Thorne Inc. Beverly, MA, USA) maintained at 37˚C. For determining the total and progressive motility of the spermatozoa, 5 µl diluted aliquots of semen sample (50 µl of semen sample dissolved in 1 ml of 2.94% Sodium citrate diluting fluid to make a 1:20 dilution) was loaded in disposable chambers with a 20 µm chamber depth (Leja® Standard Count 8 Chamber Slide, 20 µm, Leja® Products BV, Netherlands). Total motility (%), progressive motility (%), straight linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s) and other parameters like linearity LIN%, beat cross frequency (BCF) (Hz) and amplitude of lateral head displacement (ALH) were recorded. The CASA analysis was set up at 60 Hz (frame per second), 45 frames, minimum contrast of 70, and minimum cell size of four pixels. Cells were considered progressive with at least 50 mm/second average path velocity and 75% straightness. All experimental procedures were done all stages of semen processing. A minimum of 10 microscopic fields were analysed for each assessment, which included at least 200 cells. Concentration of the spermatozoa was estimated using a hemocytometer chamber.

Stained sperm smear was prepared in duplicate, by using eosin-nigrosin staining and 200 sperm per slide were

evaluated. One drop (5 µL) of semen was placed together with one drop (5 µL) of dye on a slide, and a smear was made after 2-3 min of incubation on a stage warmer at 37°C. The sperms classified as the intact membrane (viable) were not stained by eosin, while the non-intact membrane (non-viable) showed pink-red stained nuclei. From each one sample, 200 sperm cells were counted under the common optical microscope.

Assessment of plasma membrane functional integrity: The hypo-osmotic swelling test (HOS test) was used to assess the functional integrity of the spermatozoa plasma membranes. The HOS test was performed by incubating 30 µl of semen with 300 µl of a 150 mOsmol Fructose-base hypo-osmotic solution at 37˚C for 60 min. After incubation, 20 µl of the mixture was spread on a warm slide with a cover slip, subsequently, the sperms were analysed by counting 200 sperm cells from each sample in phase contrast microscope (Nikon Instech Co. Ltd., Japan) (400×). The number of swollen spermatozoa out of 200 was counted; swelling was characterised by a coiled tail, indicating that the plasma membrane is intact. The cells were classified by the degree of coiling at the tail region (Neild *et al.* 1999).

Sperm acrosome integrity: Giemsa stain was used to assess the acrosome integrity of jack spermatozoa as per the protocols described previously by Watson (Watson 1975). Briefly, diluted semen drop was kept on clean grease free slide and thin smear was prepared. After air drying, the smear slide was fixed in methanol for 15 min and then after washing, the fixed slide was kept in working solution of Giemsa for 90 min. Excess stain was removed by washing under gentle stream of water. It was dried in air and examined under the bright field 100×oil immersion objective of phase contrast microscope. Around 300 spermatozoa were assessed in different fields of a slide and the same were expressed in percentage.

Mitochondrial membrane potential: Evaluation of mitochondrial membrane potential was done by cationic dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide) as previously described and modified by Talluri *et al.* (Talluri *et al.* 2020) .

Semen was diluted to a concentration of 25–50×10^6 sperm/ml in primary extender. In a glass test tube, 20 µl JC-1 (working solution) and 500 µl of diluted sperms were added. The sample was incubated in a 37°C incubator for 20 min. JC-1 stained sample (250 µl) was added to 250 µl primary extender in a separate glass test tube and mixed gently. The slide was read using a fluorescent microscope using filter 590 and 530 nm. Two slides were analysed with a minimum of 200 cells counted per slide under 400×. Two populations of sperm were visualized. Sperm with the mitochondrial midpiece fluorescing intense orange at 590 nm (high mitochondrial potential) and cells with the mitochondrial midpiece fluorescing intense green at 530 nm (low mitochondrial potential). Percent sperm with high mitochondrial membrane potential were calculated.

Statistical analysis: All the results are presented as mean±standard error (SE). The variability of sperm characteristics between stallions were tested using the Kolmogorov–Smirnov normality test. All the epididymal sperm samples were also compared with the fresh ejaculates. Student's t-test was performed to assess differences in various seminal parameters, among the epididymal sperm as well as fresh ejaculates during fresh, and post thaw stage. These comparisons were performed using a Univariate General Linear Model (GLM), in which stallion was considered a random factor. Differences were considered significant when $P \leq 0.05$. Statistical analysis was performed using GraphPad Prism version 8.4.3.

RESULTS AND DISCUSSION

The collection and cryopreservation of epididymal spermatozoa from stallions has been proposed (Bruemmer *et al.* 2002, Tiplady *et al.* 2002, Bruemmer 2006) to allow the conservation of semen from stallions with high genetic value that had died suddenly or become permanently unable to reproduce. The retrograde flushing technique reported by Garde *et al.* (1994), Brummer *et al.* (2002) was rapid and efficient in recovering sperm from the epididymal cauda of horses. In the current study, the retrograde method of flushing of the epididymal spermatozoa was effective in retrieving most of the viable spermatozoa. In equids, retrograde flushing of the epididymal tail is the preferred technique because of the perceived higher recovery and less blood contamination than the slicing float-up technique (Bruemmer 2006, Ellerbrock *et al.* 2017).

Seminal parameters of freshly isolated epididymal spermatozoa and fresh ejaculates: The qualitative and kinematic parameters of spermatozoa from the epididymal and freshly ejaculated spermatozoa are summarised and presented in Table 1.

In present study, mean sperm concentration in the epididymis of the stallions was 6.96±284.73×10^9 spermatozoa/ml. This result is in corroboration with the results that are previously reported by Naden *et al*. (1990) in 2 year old colts (6.49×10^9 sperm/ml) and found to be significantly lesser than that was observed by Amann *et al.* 1979 in 9-year-old stallions (25.8×10^9 sperm/ml). Another study demonstrated that the number of spermatozoa recovered from the cauda epididymis is higher than the one recovered from artificial vagina on a single collection (Muradás *et al.* 2006). Our results corroborated the hypothesis that cauda epididymis should be considered mainly a storage site for stallion spermatozoa, however, sperm concentration in the epididymis could depend on factors such as sexual rest, season, age of the stallion and frequency of semen collection.

In the current study, we observed no significant differences in the seminal parameters like total motility, progressive motility, sperm abnormalities, functional membrane integrity and mitochondrial membrane potential in the spermatozoa of the both the groups at fresh stage of collection or isolation. In contrast to the present study, Stout

Table 1. Seminal parameters of freshly isolated epididymal sperm and freshly ejaculated spermatozoa (Mean±SE).

Seminal parameters (No of stallions=3)	Epididymal spermatozoa	Fresh ejaculated spermatozoa
Sperm concentration ($\times 10^6$/ml)	6960.66 ± 284.73^B	344.41 ± 28.99^A
Total motility (%)	89.51 ± 2.54^a	85.33 ± 0.58^a
Progressive motility (%)	76.66 ± 2.10^a	74.07 ± 2.45^a
Sperm viability (%)	90.76 ± 2.12^b	82.15 ± 2.00^a
Sperm abnormalities (%)	15.63 ± 2.03^a	11.10 ± 1.69^a
HOST (%)	67.99 ± 1.83^a	68.47 ± 1.50^a
Acrosome integrity (%)	89.1 ± 2.24^b	79.31 ± 0.99^b
MMP (%)	88.4 ± 2.40^a	77.04 ± 4.72^a
VAP (µm/s)	121.35 ± 2.05^b	109.59 ± 8.88^a
VCL (µm/s)	216.23 ± 8.52^B	154.27 ± 8.68^A
VSL(µm/s)	111.67 ± 4.53^a	104.24 ± 3.18^a
STR (%)	78.98 ± 3.74^a	87.34 ± 5.45^a
LIN (%)	62.25 ± 5.58^a	67.75 ± 6.86^a
ALH (µm)	15.78 ± 1.54^a	15.73 ± 1.86^a
BCF (Hz)	48.74 ± 4.16^a	68.11 ± 4.79^b

Mean values bearing different superscripts differ significantly (*$P\leq0.05$; ** $P\leq0.01$). In the same row, values with different letters in superscript (a/b, $p<0.05$; A/B, $p<0.01$) differ significantly.

et al. (2000) reported, poor motility in stallion epididymal sperm, which was probably due to the lack of exposure to activating factors present in the seminal plasma.

The CASA kinematic parameters like VAP, VCL and BCF and the seminal parameters like viability and acrosome integrity parameters were significantly ($P<0.05$) differing between both the groups (Table 1). An interesting and unprecedented observation was the significant ($P<0.05$) decrease in viability and acrosome integrity in the freshly ejaculated sperm in comparison to the epididymal spermatozoa. These data suggest that ejaculation or the mixture with the seminal plasma resulted in a selective change of the environment in which spermatozoa were submerged (Gloria *et al.* 2011, Monteiro *et al.* 2013).

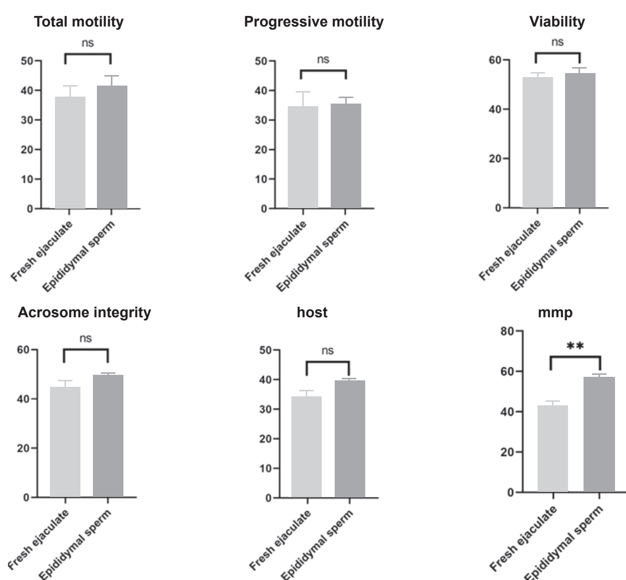

Fig. 1. Seminal parameters of the epididymal and fresh ejaculated spermatozoa after freezing and thawing. (HOST: Hypo-osmotic swelling test; MMP: Mitochondrial membrane potential). (Note: ns; non-significant; **highly significant ($P\leq0.01$)).

The kinematic parameters like VAP, VCL ($P<0.01$) and BCF were significantly ($P<0.05$) differed from the epididymal sperm to freshly ejaculated sperm. This suggests that the spermatozoa, once differentiated in the testis, remain quiescent in epididymal fluids until they are released by ejaculation (Monteiro *et al.* 2011). Dilution of the epididymal fluid surrounding the spermatozoa, either with seminal plasma or with a semen extender, allows the initiation of sperm motility and metabolism (Turner and Reich 1985). Another hypothesized reason for variation in the sperm quality is, high levels of Na^{++} and K^+ concentration in the seminal plasma which can induce spontaneous lipid peroxidation in the sperm's membrane (Alvarez and Storey 1982). Therefore, the electrolytes in the seminal plasma might have contributed to the decrease in kinematic parameters and membrane integrity of the ejaculated sperm in this study. The sperm morphology did not differ among the epididymal and fresh sperm samples after collection. The present reports are in acceptance with the results reported in different breeds of stallions (Monteiro *et al.* 2011). However, studies on bulls have reported a greater occurrence of morphological defects in epididymal sperm than in ejaculated sperm (Silva *et al.* 2003, Martins *et al.* 2007).

Seminal parameters of isolated epididymal spermatozoa and fresh ejaculates at post thaw stage: The sperm recovered from the epididymis, fresh ejaculates were cryopreserved in a similar manner, and same cryoprotectant was used for the freezing the sperm from both the groups. The differences in the observed seminal quality parameters and kinematic are presented in Fig. 1 and 2 respectively. At post-thaw stage, there were no significant differences observed in the seminal quality parameters viz., total and progressive motility, viability, acrosome integrity and HOST (Fig. 1). After thawing, no differences are seen between total and progressive motility of epididymal semen compared

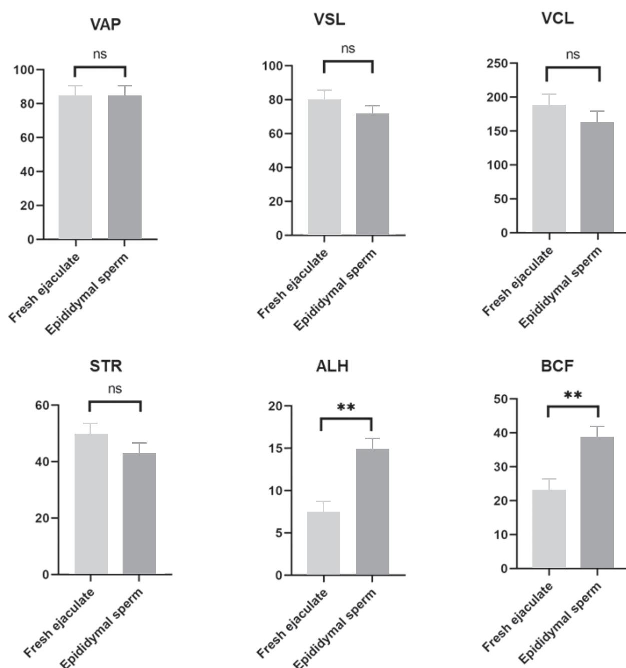

Fig. 2. Kinematic parameters of the epididymal and fresh ejaculated spermatozoa after freezing and thawing. VAP: Average path velocity; VSL: Straight line velocity; VCL: Curvilinear velocity; STR: Straightness; ALH: Amplitude of lateral head; BCF: Beat cross frequency. (Note: ns; non-significant; **highly significant (P≤0.01)).

with ejaculated semen (Weiss *et al.* 2008). There was a highly significant (P<0.01) difference in mitochondrial membrane potential between both groups and it was higher in epididymal spermatozoa (Fig. 1). The kinematic parameters like VAP, VSL, VCL and STR observed at post-thaw stages for epididymal sperm and freshly ejaculated sperm were not significantly different. On the other hand, the kinematic parameters of epididymal sperm like ALH and BCF differed significantly (P<0.01), while that of LIN differed significantly (P<0.05) from that of fresh ejaculates (Fig. 2). However, were no differences in ejaculated sperm compared with epididymal sperm were observed when conventional freezing procedures were used.

From the current study, we have found that DMF is a suitable cryoprotectant for equine epididymal as well as ejaculated spermatozoa freezing without significantly deteriorating the quality of the semen. It was observed that the presence of DMF in the freezing extender restored the motility percentages as well as preserved the rest of epididymal sperm quality parameters with acceptable values during cryopreservation. Alvarenga *et al*. (2005) reported that amides protected stallion sperm from cryodamage and could improve fertility. These beneficial effects may be due of relative lower viscosity and molecular weight of the amides likely favoured an enhanced permeability of these compounds into the plasma membrane, resulting in a reduction osmotic damage on stallion spermatozoa.

Moreover, it was also evident from the current study that the freezing protocols that were followed for cryopreserving the fresh ejaculates may also be applied for epididymal spermatozoa with similar freezability rates. This observation is in agreement with the earlier studies where it was also found that freezing of epididymal spermatozoa could be performed according to standard freezing techniques that are followed for fresh ejaculates (Squires 1999). The ability of epididymal spermatozoa to withstand cryodamage matches the ability of ejaculated spermatozoa to withstand cryodamage within a given individual (Bruemmer *et al.* 2004).

Optimizing epididymal sperm harvesting can have a profound monetary and biological impact on breeding and conservation efforts, the present study aligns with this goal. Sperm recovered from the epididymal cauda of stallions are equally resistant to the process of cooling, and the kinetic parameters and seminal parameters of epididymal sperm are in line with the values that are of ejaculated sperm which concludes, that even after the death of a stallion, it is possible to preserve genetic material through the process of cryopreservation.

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