Indian Journal of Animal Sciences **94** (9): 786–791, September 2024/Article
https://doi.org/10.56093/ijans.v94i9.145538

Effect of lyophilized heterologous seminal plasma supplementation on Marwari stallion epididymal spermatozoa during 24 hours cool storage

VACHNA RAM[1], DINESH JHAMB[1], MITESH GAUR[2], NAVEEN KUMAR[2], THIRUMALA RAO TALLURI[3]✉, OMPRAKASH SOLANKI[2], ROHIT JUNEJA[2], KALPESH KUMAR PARGI[2], DEVKI NANDAN[2], SHARAD ARORA[4] and HIMANSHU VYAS[4]

College of Veterinary and Animal Science, Navania, Vallabhnagar, Udaipur, Rajasthan 313 601 India

Received: 22 November 2023; Accepted: 2 August 2024

ABSTRACT

A study was carried out to investigate the effect of storage of testes-epididymis complex at 5°C for 24 h and supplementation of lyophilized heterologous seminal plasma (LHSP) at different concentrations on the sperm quality during cool storage. Six pairs of testicles were collected immediately after orchiectomy from six stallions. One testes-epididymis (F1) was immediately processed for sperm retrieval and second testes-epididymis (F2) complex of each stallion was stored at 5°C for 24 h. Spermatozoa were harvested from the epididymis by flotation technique and evaluated. Highly significant difference was observed among mean values of progressive motility (%) of epididymal sperm after 0 and 24 h of testicular-epididymal complex storage at 5°C, while no significant difference was observed among mean values of live sperm, membrane integrity and acrosomal integrity (%). Both epididymal sperm suspensions were supplemented with skim milk glucose semen extender fortified with different concentrations (0, 2, 5 and 10 mg/ml) of LHSP and cooled for 24 h. Sperm suspension evaluation at 0, 12 and 24 h of cool storage revealed that addition of 2 mg/ml LHSP has beneficial effect as compared to control on progressive motility and HOST in both epididymal sperm suspension at 12 h and 24 h of cool storage at 5°C. From the current study, it was inferred that the sperm quality was not deteriorated even after the sperm retrieval after 24 h from the epididymis on storage at 5°C. Further, LHSP supplemented @2 mg/ml originated from good fertility stallion is the suitable concentration for cool-storage of equine epididymal sperm suspension.

Keywords: Castration, Cool sperm, Equine, Epididymal sperm, HOST, Seminal plasma

Unexpected death, calamitous injury or castration can prematurely terminate a stallion's reproductive life. Breeders ask for a final semen collection as an alternative to preserve the genetic value of a stallion. Epididymal sperm extraction has been developed to collect the sperm after terminal cases. The timing and often location of the castration or post-mortem tissue recovery are not always appropriate for sperm handling and preservation. Equine testicles can be safely stored at 5°C in ice or equitainer used for shipping for 24 h (Mislei *et al*. 2016), while preserving sperm motility and viability. Stallion sperm recovered from the epididymal cauda either immediately after castration or after 24 h storage at 4°C in the epididymis have similar fertility capacity as ejaculated sperm (Monteiro *et al*. 2011a).

Seminal plasma (SP) is a complex mixture of proteins and non-protein substances such as ions and organic substances of low molecular weight that include free amino acids, monosaccharides, lipids, polyamines, prostaglandins and steroid hormones. SP constituents are considered to play an important role in sperm physiology and provide natural environment for spermatozoa to act as vehicle for sperm transportation, influence uterine inflammatory response to insemination and affect gamete interaction (Wood *et al*. 2016). Various workers have reported beneficial effect of SP supplementation on epididymal sperm characteristic on preservation.

This study investigated the effect of storage of equine epididymis at 5°C *in situ* on the quality of harvested sperm and keeping quality of cooled epididymal spermatozoa in skimmed milk glucose extender fortified with different concentrations of LHSP was evaluated.

MATERIALS AND METHODS

Experimental animals: Healthy adult stallions reported for routine castration at Veterinary Polyclinic, Udaipur were utilized for study. Further study was conducted at Semen lab, Department of Veterinary Gynaecology and Obstetrics, CVAS, Navania, Udaipur, Rajasthan, India. Age, breed and breeding history of stallion reported for routine

Present address: [1]Veterinary Hospital, Pali, Rajasthan. [2]Department of Veterinary Gynaecology and Obstetrics, CVAS, Navania, Vallabhnagar, Udaipur, Rajasthan. [3]Equine Production Campus, ICAR-National Research Centre on Equines, Bikaner, Rajasthan. [4]Veterinary Polyclinic, Udaipur, Rajasthan. ✉Corresponding author email: raotalluri79@gmail.com

castration was recorded. Twelve testicles were collected immediately after orchiectomy. Harvesting of sperm was performed from both epididymis (Roels *et al.* 2014). The spermatic cord was ligated with silk to avoid sperm loss and to ensure closure of the ductus deferens. The ligature was performed the farthest possible from epididymal cauda, so that viable sperms can be obtained from this region. The testicles and epididymis were rinsed using an isotonic solution such as a normal saline solution to remove blood and contaminants. The testicles and epididymis were packed in gloves filled with 0.9% NaCl solution to prevent tissues from desiccation and sent to semen laboratory in a styrofoam box with ice packs as soon as possible. Upon arrival at the semen laboratory, the testis was removed from the epididymis. The epididymis spermatozoa were harvested by flotation method. Twelve to fifteen incisions were made along the epididymis before placing in a petri dish filled with approximately 20 ml of warmed semen extender (Fig. 1). During ten minutes, spermatozoa were allowed to swim out of the epididymis. Thereafter, the remnants of the epididymis were removed and the extender with the semen were collected for further processing. For preparation of skim milk glucose extender, non-fat dry milk (2.4 g) and glucose (4.9 g) were poured in a beaker. The distilled water was added (92 ml) and mixed until milk and glucose were dissolved. The sodium bicarbonate (2 ml) and gentamycin sulfate (2 ml) were added. *p*H was measured and adjusted as needed to 7.0 by adding either an acid (HCl) or a base (NaOH) (Mccue 2014).

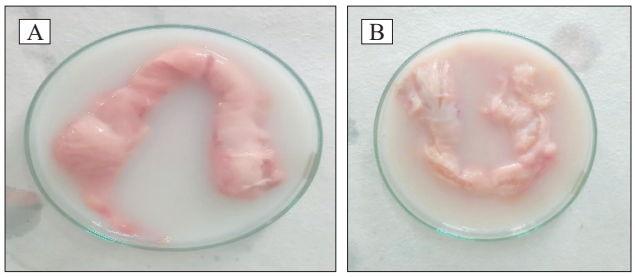

Fig. 1. Flotation technique for sperm retrieval (A) Epididymis in petridish with media; (B) 10 to 12 incision by scalpel blade on epididymis.

Fresh sperm suspension 1 (F1) from first epididymis and sperm suspension II (F2) from second stored epididymis were evaluated for microscopic parameters, sperm concentration, progressive motility (PM), viability, plasma membrane integrity and acrosome integrity immediately after harvesting.

The sperm concentration in sperm suspension sample mixed with diluting fluid was estimated by haemocytometer according to Kant (2016) with some modification. Diluting fluid used was prepared by dissolving 0.05 g Eosin Y (water soluble) and 1 g sodium chloride (NaCl) in 100 ml distilled water. 50 µL of sperm suspension sample was extended in 2 ml of diluting fluid in a micro-centrifuge tube to make a 1:40 dilution. The diluted sperm suspension sample (1:40) was dispensed carefully to avoid spillage from the side of cover slip placed over the haemocytometer. The semen sample was allowed to settle for 2 min. The total 5 counting squares (4 corners and 1 center) were counted for presence of sperm under 40× objective. Then sperm concentration was calculated as per the following formula:

$$\text{Sperm concentration (million/ml)} = (\text{Dilution Factor} = 40)\ (\text{Count in 5 secondary squares})\ (0.05 \times 10^6).$$

The progressive motility of the sperm was subjectively examined by placing 10 µL of the sperm suspension onto a pre-warmed glass microscopic slide at 37°C and visualized under a light microscope at 400× magnification. Motility was expressed as the percentage of progressively motile sperm (Pamornsakda *et al.* 2011).

For sperm viability, 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 incubation on a stage warmer at 37°C (Soni *et al*. 2019). The spermatozoa classified as intact membrane (viable) were not stained by eosin, while the non-intact membrane (non-viable) showed the pink-red stained nuclei. From each one, a sample of 200 sperm cells were counted under common optical microscope.

Evaluation of the plasma membrane/functional integrity of sperm membrane was done by hypo-osmotic swelling test (HOST) 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 sperm were analysed by counting 300 sperm cells from each sample in phase contrast microscope (40×) (Talluri *et al*. 2012). The number of swollen spermatozoa out of 300 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 and hypo-osmotic stress test-positive cells (Fig. 2) in each sample were calculated.

To examine the acrosome integrity, diluted semen drop was kept on a clean grease-free slide, and a thin smear was

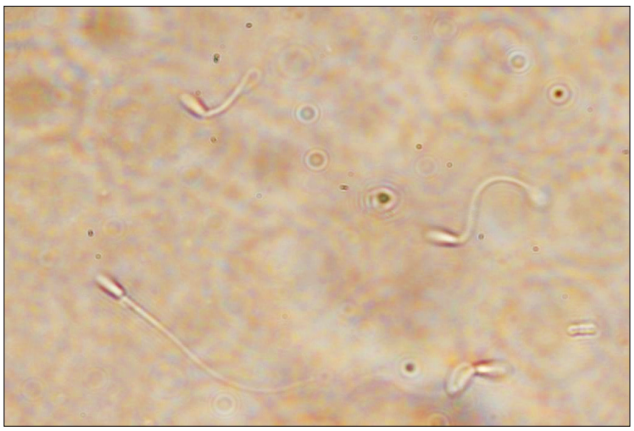

Fig. 2. Positive and negative response of spermatozoa for sperm plasma membrane integrity (HOST) (400×).

prepared. After air-drying the smear, the 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 (Kumar *et al.* 2019). 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 the phase-contrast microscope. Around 300 spermatozoa were assessed in different fields of a slide, and the same were expressed in percentage.

Semen from a proven fertile stallion was collected and seminal plasma (SP) from that stallion was isolated and lyophilized. For LHSP preparation, immediately after semen collection, the gel portion of the semen was removed through a sterilized gauze filter. The remaining gel-free semen was immediately centrifuged at 3000× g for 30 min to separate the SP from the spermatozoa. The harvested SP was re-centrifuged at 3500× g for 20 min to obtain sperm free SP and stored at –20°C for a minimum of 24 h before lyophilisation. Lyophilisation of SP was carried out using a modified protocol described by Gianaroli *et al.* (2012). Samples were put inside a Freeze Dry System (Labconco, Freezone, USA) and exposed to a 30 h lyophilisation cycle with a condenser temperature of –50°C and vacuum of 25×10^{-3} mbar. Lyophilized samples were mixed and stored at room temperature.

The epididymal sperm suspension was supplemented with extender fortified with different concentrations of lyophilized heterologous seminal plasma (LHSP) keeping final concentration of 50 million sperm/ml (Monteiro *et al*. 2013a) and final LHSP concentration as groups Control group (C_1) without LHSP, T_1 with LHSP at 2 mg/ml, T_2 with LHSP at 5 mg/ml and T_3 with LHSP at 10 mg/ml concentration. Usuga *et al.* (2020) and Mehra (2021) used nearly similar concentrations of SP. Epididymis sperm of all four groups (C_1, T_1, T_2 and T_3) were evaluated for PM, viability, membrane integrity and acrosomal integrity at immediate (0 h), 12 h and 24 h of cooling at 5°C.

Completely randomized mixed models were fitted for statistical analyses of data. Data normality was assessed with the Shapiro-Wilk test and means were compared using Tukey's test with significance level $P<0.05$. Statistical analyses were conducted using SAS version 9.2 software (SAS Inst. Inc., USA).

RESULTS AND DISCUSSION

The mean of left and right epididymal sperm output ($\times10^9$) was 12.13±0.76 and 11.25±0.72, respectively which was non-significantly ($P>0.05$) different. Similar observations were recorded by Monteiro *et al.* (2011b) and Mislei *et al*. (2016) but lower value of total epididymal sperm output/epididymis was reported by Heise *et al.* (2011) in warm blooded Welsh and Boerperds breed stallions aged 4-5 yrs and Guimarães *et al*. (2012) in Peruvian Paso Fino and Lusitano stallions aged 3-10 yrs and higher value of total epididymal sperm output/ epididymis was reported by Guasti *et al*. (2013) in Brazilian Jumping horses aged 3 to 5 years and Monteiro *et al.* (2013b) in Brazilian Jumping, Quarter, Mangalarga Marchador and Lusitano aged 4-6 years old. These variations can probably be due to differences in age, breed of the stallions and season.

The flotation method applied for sperm harvesting in present study is rapid and performed with ease. Flotation method yielded less sperm per epididymis as compared to retrograde flush, but flotation technique is selective for more functional sperm that are able to swim out of epididymis while flushing method is more traumatic to individual spermatozoa (Falomo *et al.* 2016, Carneiro *et al.* 2017 and Rana 2017). In contrast to the flotation technique, retrograde method of flushing was also tried in the previous studies, which was also found to be more effective than the flotation technique (Talluri *et al.* 2023a and Talluri *et al.* 2023b).

Mean values of PM (%) of epididymal sperm after 0 (F1) and 24 h (F2) of testicular-epididymal complex storage at 5°C was 24.25±1.13 and 19.92±1.02, respectively, which was highly significant ($P\leq0.01$). Mean PM (%) of F1 and F2 supplemented with different concentrations of LHSP at 0 h (immediate), 12 h and 24 h of cool storage (5°C) are mentioned in Tables 1 and 2. It was observed that there was an increase in PM on addition of skimmed milk glucose extender with different concentrations of LHSP (0, 2, 5 and 10 mg/ml) immediately and there was reduction during storage at 5°C in all groups at 12 and 24 h of storage.

The 2 mg/ml LHSP increased ($P\leq0.05$) PM as compared to control, while 5 mg/ml had intermediate effect and 10 mg/ml LHSP addition reduced PM non-significantly as compared to control. Present results of cooling epididymal sperm are supported by Guimarães *et al.* (2012), Brogan *et al.* (2015), Neuhauser *et al.* (2018) and Miro *et al.* (2020). The findings are consistent with the hypothesis by Monteiro *et al*. (2011b) that regardless of the inferiority of the initial epididymis parameters, after the addition of the diluent, there was a significant enhancement in the sperm parameters.

The present results are in concurrence with Stout *et al.* (2000), Heise *et al.* (2011) and Morrell *et al.* (2014). Ellerbrock *et al.* (2017) rationale behind adding SP to flushing techniques is the need for SP proteins to initiate motility in live, but quiescent sperm, which corroborate with findings of this study. Several molecules showing motility regulatory effects on spermatozoa have been identified and located in epididymis which initiate motility of spermatozoa (Dungdung *et al.* 2016)

The results of reduced PM by 10 mg/ml LHSP supplementation may be caused by excess antioxidant compounds and therefore, a pro-oxidant effect (Usuga *et al*. 2020). Contrary, Tiplady *et al.* (2002) documented non-significant improvement in PM in similar studies.

Mean values of live sperm (%) of epididymal sperm after 0 and 24 h of testicular-epididymal complex storage at 5°C were 85.67±1.69 and 82.75±1.11, respectively which is non-significant ($P>0.05$) difference. Mean viability of F1 and F2 sperm suspension supplemented with different concentrations of LHSP at 0 h (immediate), 12 h and

Table 1. Mean values of various parameters of first epididymal sperm suspension (F1) supplemented with different concentrations of LHSP at 0 h (immediate), 12 h and 24 h of cool storage (5°C)

	Progressive motility (%)			Viability (%)			Membrane integrity (%)			Acrosome integrity (%)		
F1	0 h	12 h	24 h	0 h	12 h	24 h	0 h	12 h	24 h	0 h	12 h	24 h
C1	32.09^{a}±0.83	27.34^{a}±0.74	22.75^{a}±0.92	83±1.53	79±1.53	75±1.53	69^{a}±1.39	66.84^{a}±1.09	61.5^{a}±1.79	72±1.26	72±1.26	70.5±1.23
T11	36^{b}±1.23	31.09^{b}±1.22	27.59^{b}±1.25	83.50±1.47	79.83±1.53	75.42±1.52	75.92^{b}±2.56	73.25^{b}±1.44	69.09^{b}±2.34	74.25±1.26	74.25±1.26	72.59±1.25
T12	32.17^{a}±1.15	28.17^{ab}±1.17	24.17^{ab}±1.44	82.08±1.53	78.25±1.58	74.75±1.57	72.59^{ab}±1.22	67.5^{b}±1.44	62.34^{a}±1.18	73.09±1.29	73.09±1.29	71.75±1.37
T13	31^{a}±1.22	26.92^{a}±1.16	21.75^{a}±1.19	81.83±1.58	78±1.6	73.75±1.57	68.67^{a}±1.19	65.09^{a}±0.97	60.92^{a}±1.22	71.5±1.23	71.5±1.23	70.5±1.23

Mean values with different superscripts (a, b, c) within column differ significantly (P≤0.05).

Table 2. Mean values of various parameters of second epididymal sperm suspension (F2) supplemented with different concentrations of LHSP at 0 h (immediate), 12 h and 24 h of cool storage (5°C)

	Progressive motility (%)			Viability (%)			Membrane integrity (%)			Acrosome integrity (%)		
F2	0 h	12 h	24 h	0 h	12 h	24 h	0 h	12 h	24 h	0 h	12 h	24 h
C2	30.92^{a}±1.57	25.92^{a}±1.52	22.92^{a}±1.52	80.17±1.18	75.5±1.19	70.58±1.10	70.75^{ab}±1.60	65^{a}±1.88	61.09^{a}±2.49	71.09±0.78	69.59±0.84	68±0.78
T21	35.75^{b}±1.79	30.25^{b}±1.76	26.25^{b}±1.76	81.25±1.17	76.17±1.17	71.42±2.40	75.59^{b}±1.97	73.09^{b}±3.52	69.84^{b}±3.39	72.25±0.78	70.25±0.78	69±0.78
T22	33^{ab}±1.22	28^{ab}±1.15	24.59^{ab}±1.10	78.83±1.06	74.83±1.23	70.17±1.27	71^{ab}±1.61	66.25^{a}±1.53	63.25^{a}±1.53	71.34±0.74	69.84±0.82	68.42±0.76
T23	29.84^{a}±1.49	23.84^{a}±1.43	19.84^{a}±1.43	78.25±1.13	74±1.28	70.48±1.27	68.09^{a}±1.59	64.09^{a}±1.59	59.17^{a}±2.72	70.09±0.78	67.92±0.81	67±0.78

Mean values with different superscripts (a, b, c) within column differ significantly (P≤0.05).

24 h of cool storage (5°C) are mentioned in Tables 1 and 2. There was a decrease in viability on addition of skimmed milk glucose extender with different concentrations of LHSP (0, 2, 5 and 10 mg/ml) immediately and there was reduction during storage at 5°C in all groups at 12 and 24 h of storage. These findings proved non-significant beneficial effect of 2 mg/ml LHSP supplementation, but non-significant detrimental effect of 5 mg/ml and 10 mg/ml LHSP supplementation for both F1 and F2 epididymal sperm suspensions.

Similarly, studies by Brogan *et al.* (2015), Guimarães *et al.* (2012) and Miro *et al.* (2020) also support findings of the study, that viability is retained to satisfactory (90, 83.5 and 83.8% respectively) level by cooling stallion epididymal sperm for 24 h and more. Brogan *et al.* (2015) documented that 5°C is most common chilling storage temperature in practice, cooling spermatozoa results in lowered membrane lipid fluidity and associated membrane alterations, due to which most enzymatic process are slowed. Similarly, Stout *et al.* (2000) and Mislei *et al.* (2016) also reported non-significant improvement in sperm viability after addition of heterologous SP in epididymal sperm preservation.

Mean values of HOST (%) of epididymal sperm after 0 and 24 h of testicular-epididymal complex storage at 5°C were 73.92±1.54 and 70.25±1.59, respectively, which is non-significantly (P>0.05) different. Mean membrane integrity of F1 and F2 sperm suspension supplemented with different concentrations of LHSP at 0 h (immediate), 12 h and 24 h of cool storage (5°C) are mentioned in Tables 1 and 2. There was reduction in HOST positive spermatozoa in all groups at 12 and 24 h of storage.

Results prove beneficial (P≤0.05) effect of 2 mg/ml LHSP supplementation as compared to control. 5 mg/ml LHSP had intermediate beneficial effect and 10 mg/ml LHSP supplementation had non-significant detrimental effect on HOS reactive sperm in both F1 and F2 epididymal sperm suspension at 0, 12 and 24 h of cool storage. The current study results are concurrent with similar findings by Monteiro *et al.* (2011 a, b) and Usuga *et al*. (2020).

Deleterious effect of higher concentration is supported by Kareskoski *et al.* (2006) and Monteiro *et al.* (2013b) who stated that there is no exposure of epididymal sperm to SP; supplemented SP has a greater *p*H and greater Na^{+} and Cl^{-} content, which are detrimental to sperm survival during the conservation process. Deleterious effect of SP on sperm quality is due to biochemical changes that increase membrane permeability (Lozano *et al.* 2011) which cause greater susceptibility to cooling

damage on the sperm plasma membrane.

Mean values of acrosome integrity (%) of epididymal sperm after 0 and 24 h of testicular-epididymal complex storage at 5°C were 72.92±1.23 and 71.83±0.73, respectively which is non-significant (P>0.05). Mean acrosome integrity of F1 and F2 sperm suspension supplemented with different concentrations of LHSP at 0 h (immediate), 12 h and 24 h of cool storage (5°C) are mentioned in Tables 1 and 2. There was reduction in mean acrosome integrity in all four groups for both F1 and F2 epididymal sperm samples after 24 h of cooling. It proves the beneficial effect of 2 mg/ml LHSP supplementation as compared to control, though non-significant. 5 mg/ml LHSP had intermediate effect and 10 mg/ml had deleterious effect on acrosome integrity, both non-significant.

Reduction in acrosome integrity in all four groups by cooling supports the statement that sperm exhibit a decreased percentage of post-thaw quality parameters as compared to fresh, which is a characterization of the cold stress (Papa *et al.* 2015). Other studies by Morrell *et al.* (2014) also support these results. Contrary, Mehra (2021) reported detrimental effects on acrosomal integrity of cooled semen by supplementation of SP (2 mg/ml) in semen extender. Results of other researchers showing acceptable quality of epididymal sperm parameters by preservation through freezing or vitrification also strengthen these results (Papa *et al.* 2015, Gloria *et al.* 2016, Carneiro *et al.* 2017, Guasti *et al.* 2017, Álvarez *et al.* 2019, Neuhauser *et al.* 2019). These results on cooling of stallion epididymal sperm are encouraging and supported by Atroshchenko *et al.* (2017) who stated that confirmation of the high safety of epididymal spermatozoa, as well as their resistance to cryopreservation, allows us to make a promising forecast for the development and application of the preservation technology for epididymal sperm of stallions.

ACKNOWLEDGEMENT

The authors acknowledge the help received from Director (Research), RAJUVAS, Bikaner for financial support.

REFERENCES

Álvarez C, González N, Luño V and Gil L. 2019. Ejaculated compared with epididymal stallion sperm vitrification. *Animal Reproduction Science* **211**: 106205.

Atroshchenko M M, Kalashnikov V V, Bragina E E and Zaitsev A M. 2017. Comparative study of the structural integrity of spermatozoa in epididymal, ejaculated and cryopreserved semen of stallions. *Sel'skokhozyaistvennaya Biologiya [Agricultural Biology]* **52**(2): 274–80.

Brogan P T, Beitsma M, Henning H, Gadella B M and Stout T A E. 2015. Liquid storage of equine semen: Assessing the effect of D-penicillamine on longevity of ejaculated and epididymal stallion sperm. *Animal Reproduction Science* **159**: 155–62.

Carneiro J A M, Monteiro G A, Martin I, Maziero R R D, Sancler-Silva Y F R, de Paula Freitas-Dell'Aqua C and Dell'Aqua Jr J A. 2017. Heterologous oviductal cells binding capacity of cryopreserved equine ejaculated and epididymal spermatozoa. *Journal of Equine Veterinary Science* **59**: 40–48.

Dungdung S R, Bhoumik A, Saha S, Ghosh P, Das K, Mukherjee S and Majumder G C. 2016. Sperm motility regulatory proteins: A tool to enhance sperm quality. *Intech Open: Rijeka, Croatia* 161–77.

Ellerbrock R E, Prell M J, Stewart J L, Bojko M S, Lima F S and Canisso I F. 2017. Comparison of centrifugation and non-centrifugation methods to cryopreserve stallion epididymal semen. *Journal of Equine Veterinary Science* **50**: 27–32.

Falomo M E, Rossi M and Mantovani R. 2016. Collection, storage and freezability of equine epididymal spermatozoa. *Italian Journal of Animal Science* **15**(3): 386–89.

Gianaroli L, Magli M C, StanghelliniI, Crippa A, Crivello A M, Pescatori E S and Ferraretti A P. 2012. DNA integrity is maintained after freeze-drying of human spermatozoa. *Fertility and Sterility* **97**: 1067–73.

Gloria A, Carluccio A, Petrizzi L, Noto F and Contri A. 2016. Characteristics of frozen epididymal spermatozoa from stallions that died 12 to 36 hours after colic surgery. *Theriogenology* **85**(2): 345–50.

Guasti P N, Monteiro G A, Maziero R R D, Carmo M T, Dell'Aqua Jr J A, Crespilho A M and Papa F O. 2017. Pentoxifylline effects on capacitation and fertility of stallion epididymal sperm. *Animal Reproduction Science* **179**: 27–34.

Guasti P N, Monteiro G A, Maziero R R, Martin I, Avanzi B R, Dellaqua Jr J A and Papa F O. 2013. Effects of pentoxifylline on equine epididymal sperm. *Journal of Equine Veterinary Science* **33**(12): 1153–56.

Guimarães T, Lopes G, Ferreira P, Leal I and Rocha A. 2012. Characteristics of stallion epididymal spermatozoa at collection and effect of two refrigeration protocols on the quality of the frozen-thawed sperm cells. *Animal Reproduction Science* **136**(1-2): 85–89.

Heise A, Thompson P N and Gerber D. 2011. Influence of seminal plasma on fresh and post-thaw parameters of stallion epididymal spermatozoa. *Animal Reproduction Science* **123**(3-4): 192–201.

Kant S. 2016. 'Effect of caffeine as an additive in semen extender to improve frozen thawed semen quality of Marwari horses and exotic donkeys.' M.V.Sc. Thesis, (Deemed University) Indian Veterinary Research Institute, Izatnagar, U.P.

Kareskoski A, Reilas T, Andersson M and Katila T. 2006. Motility and plasma membrane integrity of spermatozoa in fractionated stallion ejaculates after storage. *Reproduction in Domestic Animals* **41**: 33–38.

Kumar P, Kumar R, Mehta J S, Chaudhary A K, Ravi S K, Mehta S C, Ansari M M, Legha R A, Tripathi B N and Talluri T R. 2019. Ameliorative effect of ascorbic acid and glutathione in combating the cryoinjuries during cryopreservation of exotic jack semen. *Journal of Equine Veterinary Science* **81**: 102796.

Lozano D, Álvarez C and Gil L. 2011. Efecto de la adición de plasma seminal en el semen equino descongelado. *Rev. Sanid. Milit.* **67**: 284–90.

Mccue P. 2014. Semen extenders and sperm media, pp. 406 *Equine Reproductive Procedures.* (Eds) Dascanio J J and McCue P. 1st Edition. Wiley-Blackwell. Commerce Place, 350 Main Street, Malden 02148, MA USA._https:// doi.org/10.1002/9781118904398.ch125

Mehra R. 2021. 'Effect of addition of lyophilized heterologous seminal plasma and colostrum to semen extender on cooled and post thaw stallion semen quality.' M.V.Sc Thesis, Rajasthan University of Veterinary and Animal Sciences,

Bikaner, Rajasthan.

Miro J, Morato R, Vilagran I, Taberner E, Bonet S and Yeste M. 2020. Preservation of epididymal stallion sperm in liquid and frozen states: Effects of seminal plasma on sperm function and fertility. *Journal of Equine Veterinary Science* **88**: 102940.

Mislei B, Merlo B, Asta D, Iacono E, Love C C and Mari G. 2016. Effects of two different cooling devices for testicles transport on stallion epididymal sperm quality. *Journal of Equine Veterinary Science* **46**: 64–68.

Monteiro G A, Freitas-Dell'Aqua C P, Guasti P N, Landim-Alvarenga F C, Dell'Aqua Junior J A, Alvarenga M A and Papa F O. 2011a. Comparison of apoptotic index between cryopreserved ejaculated and epididymal sperm in stallions, pp. 226. *AAEP, 57th Annual Convention of American Association of Equine Practitioners. San Antonio, USA*,

Monteiro G A, Guasti, P N, Hartwig F P, Dellaqua Jr J A, Alvarenga M A and Papa F O. 2013a. Cooling of ejaculated and epididymal stallion sperm. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia* **65**: 681–86.

Monteiro G A, Guasti P N, Rocha A S, Martin I, Sancler-Silva Y F R, Dell'Aqua C P F and Papa F O. 2013b. Effect of storage time and temperature of equine epididymis on the viability, motion parameters, and freezability of epididymal sperm. *Journal of Equine Veterinary Science* **33**(3): 169–73.

Monteiro G A, Papa F O, Zahn F S, Dellaqua Jr JA, Melo C M, Maziero R R D and Guasti P N. 2011b. Cryopreservation and fertility of ejaculated and epididymal stallion sperm. *Animal Reproduction Science* **127**(3-4): 197–201.

Morrell J M, Georgakas A, Lundeheim N, Nash D, Morel M D and Johannisson A. 2014. Effect of heterologous and homologous seminal plasma on stallion sperm quality. *Theriogenology* **82**(1): 176–83.

Neuhauser S, Gosele P and Handler J. 2018. The effect of four different commercial semen extenders on the motility of stallion epididymal sperm. *Journal of Equine Veterinary Science* **62**: 8–12.

Neuhauser S, Bollwein H, Siuda M and Handler J. 2019. Effects of different freezing protocols on motility, viability, mitochondrial membrane potential, intracellular calcium level, and DNA integrity of cryopreserved equine epididymal sperm. *Journal of Equine Veterinary Science* **82**: 102801.

Papa P M, Papa F O, Oliveira L A, Guasti P N, Castilho C and Giometti I C. 2015. Different extenders in the cryopreservation of bovine epididymal spermatozoa. *Animal Reproduction Science* **161**: 58–63.

Pamornsakda T, Pojprasath T, Suwimonteerabutr J and Tharasanit T. 2011. Effects of cholesterol-loaded cyclodextrins on the quality of frozen–thawed equine epididymal sperm. *Cryobiology* **63**(2): 90–95.

Rana R. 2017. 'Characterization of bubaline epididymal spermatozoa stored at 5°C *in-situ* and their storage resistance in modified citrate dilutors.' Doctoral dissertation, G.B. Pant University of Agriculture and Technology Pantnagar, U.S. Nagar (Uttarakhand), India.

Roels K, Leemans B, Ververs C, Govaere J, Hoogewijs M and Van Soom A. 2014. Collection and freezing of equine epididymal spermatozoa. *Vlaams Diergeneeskundig Tijdschrift* **83**(6): 321–25.

Soni Y, Talluri T R, Kumar A, Ravi S K, Mehta J S and Tripathi B N. 2019. Effects of different concentration and combinations of cryoprotectants on sperm quality, functional integrity in three Indian horse breeds. *Cryobiology* **86**: 52–57.

Stout T A E, Morris L H A, Li X and Allen W R. 2000. The effect of seminal plasma on the motility and cryopreservability of horse epididymal sperm. Proceeding of first meeting of European Equine Gamete Group, 5-8 September 1999 Poland. *Havemeyer Foundation Monograph Series* **1**: 5–6.

Talluri T R, Jhamb D, Paul N, Singh J, Dedar R K, Mehta S C, Legha R A and Pal Y. 2023a. Effect of isolation protocols and cryoprotectants on freezing of stallion epididymal spermatozoa. *Cryo Letters* **44**(3): 134–41.

Talluri T R, Jhamb D, Paul N, Mehta S C, Singh J, Dedar R K, Legha R A and Pal Y. 2023b. Cryopreservation and freezability of epididymal and ejaculated stallion spermatozoa. *The Indian Journal of Animal Sciences* **93**(7): 691–96.

Talluri T R, Arangasamy A, Ravi S K and Pal Y. 2012. Hypo-osmotic Swelling test for quality evaluation of fresh and frozen semen quality in horses. *Indian Veterinary Journal* **89**(11): 68–70.

Tiplady C, Morris L H A and Allen W R. 2002. Stallion epididymal spermatozoa: pre-freeze and post thaw motility and viability after 3 treatments. *Theriogenology* **58**(2): 225–28.

Usuga A, Rojano B and Restrepo G. 2020. Lyophilized seminal plasma can improve stallion semen freezability. *The Indian Journal of Animal Sciences* **90**(2): 171–75.

Wood P L, Scoggin K, Ball B A. Troedsson M H and Squires E L. 2016 Lipidomics of equine sperm and seminal plasma: Identification of amphiphilic (O-acyl)-ω-hydroxy-fatty acids. *Theriogenology* **86**(5): 1212–21.