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Effect of Storage Time and Temperature of Equine Epididymis on the Viability, Motion Parameters, and Freezability of Epididymal Sperm

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ABSTRACT

The recovery of sperm from the epididymal cauda may be the last chance to obtain genetic material when sudden death or serious injuries occur in valuable stallions. However, the lack of technical knowledge regarding the storage and transportation of the epididymis often prevents the preservation of the sperm. Therefore, the aim of this study was to compare sperm parameters of sperm obtained immediately after orchiectomy with sperm recovered from epididymal cauda at different times after storage at 5°C and at room temperature (RT). For that, 48 stallions of different breeds were used. In group 1 (control group), eight stallions were used, and the harvest of the epididymal sperm was performed immediately after orchiectomy. In group 2, 40 stallions were used, which were divided into five groups according to the storage time of the epididymis after orchiectomy (6, 12, 18, 24, or 30 hours), making a total of eight stallions per group. One epididymis of each stallion was stored at 5°C, and the contralateral epididymis was stored at RT, both for the same period. The sperm parameters of total motility, progressive motility, progressive linear velocity, curvilinear velocity, percentage of rapid sperm, and plasma membrane integrity were evaluated in all the groups after sperm recovery, resuspension in a sperm freezing diluent, and thawing. In conclusion, the storage of the testis-epididymis complex at 5°C provided better preservation of epididymal sperm than the storage at RT, and regardless of the temperature, the progressive motility is the sperm parameter that is most sensitive to storage time.

1. Introduction

The harvest of sperm from the epididymal cauda in stallions has proven to be an efficient means of recovering viable sperm cells [1-7]. Pregnancies by use of epididymal sperm have been reported in several species [8]. However, the lack of technical knowledge about the storage and transportation of the epididymis often results in post-mortem tissue degeneration that prevents the preservation of the sperm [2].

Studies have evaluated the sperm viability as a function of the storage time of the epididymis, thus enabling the definition of the best methods for packaging and transporting the material to a breeding center capable of recovering and cryopreserving these cells [2,9-11].

James et al. [2] evaluated the sperm viability after storage of the epididymis of horses at 5°C for up to 96 hours. In this study, there was no difference in the percentage of progressive motility (PM) and membrane integrity after thawing. Additionally, studies in cats, dogs, goats, pigs, and cattle have shown that most of these species demonstrated superior seminal viability when the epididymides were stored between 4°C and 5°C compared with those kept at room temperature (RT) [12].

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At RT, Granemann [9] demonstrated that the sperm quality in stallions declined according to the time of storage, and the limit of viability was close to 24 hours postorchiectomy. This decrease can be explained by both the metabolic depletion of the sperm and the process of postmortem tissue degeneration. Lower temperatures slow the process of degeneration and decrease sperm metabolism, keeping the sperm viable for a longer time.

The goal of the present study was to compare the sperm viability of sperm harvested from the epididymal cauda immediately after the procedure of orchiectomy with those obtained from epididymides stored at 5°C or RT for 6, 12, 18, 24, or 30 hours after orchiectomy.

The hypothesis is that the refrigeration of the epididymis during storage is better for sperm viability and longevity compared with maintenance at RT.

2. Material and Methods

For this experiment, 48 stallions of different breeds (34 Brazilian Jumping horse, eight Quarter horse, four Mangalarga Marchador, and two Lusitano) aged 4 to 6 years were used.

In group 1 (control group), eight stallions were used, and the harvest of epididymal sperm was performed immediately after orchiectomy (time 0). In this group, only one epididymis of each stallion was included (a total of four right epididymides and four left epididymides).

In group 2, a total of 40 stallions were divided into five groups based on the storage time and temperature and then subjected to orchiectomy. After the orchiectomy, one epididymis from each stallion was stored at 5°C for 6, 12, 18, 24, or 30 hours, corresponding to the 5°C-6h, 5°C-12h, 5°C-18h, 5°C-24h, and 5°C-30h groups, respectively; the contralateral epididymis was stored at RT (18°C-24°C) for 6, 12, 18, 24, and 30 hours after orchiectomy in groups RT-6h, RT-12h, RT-18h, RT-24h, and RT-30h, respectively. Thus, the harvesting of epididymal sperm was performed after the storage time, totaling four right epididymides and four left epididymides per group (n = 8).

The packaging of the epididymides at 5°C was performed in passive refrigeration containers (Botutainer, Botupharma, Botucatu, Brazil) with a cooling rate of 0.05°C/min until stabilization at 5°C. The epididymides stored at RT were kept in a controlled-temperature laboratory between 18°C and 24°C.

The harvesting of the sperm from the epididymal cauda was performed using the technique of retrograde flushing described by Garde et al. [13] and Bruemmer et al. [14]. The epididymal cauda was isolated from the testis. The connective tissue was carefully dissected, and the epididymal cauda was straightened. A 10-mL pipette tip was attached to a 10-mL syringe, and the epididymal cauda was flushed using 30 mL of a skim milk-based extender (Botu-Semen, Botupharma) per epididymis. The flushing fluid from both epididymides of each animal was recovered in a 200-mL beaker.

After recovery, the samples were centrifuged at 600 × *g* for 10 minutes. The pellet was resuspended in a freezing egg yolk-based extender (Botu-Crio, Botupharma) at a final concentration of 200 million viable sperm/mL. The semen samples were packed into 0.5-mL straws, maintained at 5°C for 20 minutes in a commercial refrigerator (Minitub do Brasil LTDA™, Porto alegre, Brazil) and subsequently frozen in liquid nitrogen vapor, 6 cm above the surface of liquid nitrogen, for 20 minutes. The straws were immersed in liquid nitrogen and stored at −196°C until analysis. After thawing at 46°C for 20 seconds [15], the sperm were transferred to a 1.5-mL plastic tube and maintained in a dry block at 37°C for semen evaluation.

The seminal samples were analyzed immediately after sperm recovery, after dilution in the freezing extender and after thawing. The samples were adjusted to a concentration of 50 × 10^6 sperm/mL and incubated at 37°C for 10 minutes before analysis. Five fields per sample were selected for the evaluation of the motility parameters by computer-assisted semen analysis (HTM-IVOS 12, Hamilton Thorne Research, MA). The computer-assisted semen analysis setup is described in Table 1. The plasma membrane integrity (PMI) was evaluated using the fluorescent probes carboxyfluorescein diacetate and propidium iodide, as described by Harrison and Vickers [16].

The data were analyzed by an analysis of variance followed by the Tukey test to compare the treatments. The significance level was set at $P < .05$ (SAS Institute Inc., Cary, NC).

3. Results

The average number of sperm recovered from the epididymal cauda by the retrograde flushing technique was 16.2 ± 7.79 × 10^9 sperm/epididymis.

The sperm parameters from the samples were assessed immediately after recovery (Table 2), after resuspension in the freezing diluent (Table 3), and after thawing (Table 4).

As shown in Tables 3 and 4, there was no difference in the total motility (TM) in either the prefreezing or the postthawing between the control group sample (0 hour) and the samples stored at 5°C (5°C-6h, 5°C-12h, 5°C-18h, 5°C-24h, and 5°C-30h). However, a significant decrease in the TM was observed in the samples stored at RT after 12 hours of storage (RT-12h, RT-18h, RT-24h, and RT-30h).

There was no difference in the PM in either the prefreezing or the postthawing between the control group sample (0 hour) and the samples stored at 5°C for up to 12 hours (5°C-6h and 5°C-12h). After 18 hours of storage at

Table 1
Methodologies of CASA for equine sperm analyzer

Characteristic	Adjusted to
Number of frames	30
Minimum contrast	60 pixels
Minimum cell size	3 pixels
Contrast to static cells	30 pixels
Straightness	80%
Average path velocity cutoff	30.0 μm/s
Minimum VAP to progressive cells	70.0 μm/s
VSL cutoff to slow cells	20.0 μm/s
Static head size	0.62-2.98
Static head intensity	0.24-1.19
Static head elongation	100-0
Magnification	1.95×
Temperature	37°C

CASA, computer-assisted semen analysis; VAP, average path velocity; VSL, straight-line velocity.

Table 2
The mean values and standard deviations of the sperm parameters TM, PM, VSL, VCL, RAP, and PMI evaluated immediately after recovery of the samples stored at 5°C (5°C-6h, 5°C-12h, 5°C-18h, 5°C-24h, and 5°C-30h) and at room temperature (control-0h, RT-6h, RT-12h, RT-18h, RT-24h, and RT-30h)

Groups (n = 8)	TM (%)	PM (%)	VSL (μm/s)	VCL (μm/s)	RAP (%)	PMI (%)
Control-0h	29.4 ± 20.80[a]	9.8 ± 7.90[ab]	68.2 ± 8.83[ab]	205.0 ± 20.10[ab]	20.1 ± 17.31[ab]	72.1 ± 10.28[a]
5°C-6h	44.0 ± 21.98[a]	17.6 ± 12.11[a]	70.6 ± 9.46[a]	175.9 ± 21.16[a]	32.9 ± 18.12[a]	69.9 ± 17.33[ab]
5°C-12h	30.0 ± 30.61[ab]	10.1 ± 12.41[ab]	75.4 ± 11.44[ab]	181.0 ± 34.78[a]	21.9 ± 28.70[ab]	68.1 ± 9.28[ab]
5°C-18h	32.9 ± 20.61[ab]	11.7 ± 10.48[ab]	71.5 ± 6.82[ab]	171.1 ± 18.30[a]	22.5 ± 18.10[ab]	62.0 ± 9.30[ab]
5°C-24h	26.4 ± 18.73[ab]	8.6 ± 10.50[ab]	59.3 ± 13.81a[ab]	162.6 ± 34.62[abc]	7.1 ± 8.66[b]	66.0 ± 10.66[ab]
5°C-30h	36.5 ± 26.95[a]	13.0 ± 10.58[ab]	77.8 ± 13.12[a]	185.3 ± 29.88[a]	26.25 ± 19.41[a]	68.8 ± 14.62[ab]
RT-6h	32.89 ± 26.95[ab]	12.5 ± 9.69[ab]	64.4 ± 6.95[ab]	156.5 ± 28.52[abc]	22.9 ± 18.54[ab]	69.4 ± 12.23[ab]
RT-12h	1.1 ± 1.25[b]	0.25 ± 0.46[b]	31.6 ± 27.98[b]	86.8 ± 72.97[abc]	0.25 ± 0.46[b]	65.3 ± 13.48[ab]
RT-18h	0.6 ± 1.06[b]	0.25 ± 0.46[b]	18.4 ± 25.66[b]	39.5 ± 54.52[abc]	0.1 ± 0.35[b]	62.0 ± 14.32[ab]
RT-24h	1.7 ± 3.11[b]	0.25 ± 0.71[b]	38.8 ± 36.02[b]	90.8 ± 81.00[bc]	3.5 ± 5.29[b]	48.5 ± 20.06[b]
RT-30h	1.0 ± 0.76[b]	0.5 ± 0.53[b]	30.0 ± 36.48[b]	66.3 ± 72.19[c]	0.2 ± 0.46[b]	50.9 ± 19.77[ab]

PM, progressive motility; PMI, plasma membrane integrity; RAP, rapid sperm; TM, total motility; VCL, curvilinear velocity.
Control-0h, sperm recovered from the epididymal cauda 0 hour after castration; 5°C-6h, sperm recovered from the epididymal cauda 6 hours after castration and storage at 5°C; 5°C-12h, sperm recovered from the epididymal cauda 12 hours after castration and storage at 5°C; 5°C-18h, sperm recovered from the epididymal cauda 18 hours after castration and storage at 5°C; 5°C-24h, sperm recovered from the epididymal cauda 24 hours after castration and storage at 5°C; 5°C-30h, sperm recovered from the epididymal cauda 30 hours after castration and storage at 5°C; RT-6h, sperm recovered from the epididymal cauda 6 hours after castration and storage at room temperature; RT-12h, sperm recovered from the epididymal cauda 12 hours after castration and storage at room temperature; RT-18h, sperm recovered from the epididymal cauda 18 hours after castration and storage at room temperature; RT-24h, sperm recovered from the epididymal cauda 24 hours after castration and storage at room temperature; RT-30h, sperm recovered from the epididymal cauda 30 hours after castration and storage at room temperature.
[a,b,c]Different letters in the columns indicate significant differences ($P < .05$).

5°C (5°C-18h, 5°C-24h, and 5°C-30h) and in all the samples stored at RT (RT-6h, RT-12h, RT-18h, RT-24h, and RT-30h), a significant decrease in the PM was observed. The greatest decrease occurred after 18 hours of storage at RT (Table 4).

There was no difference in the straight-line velocity (VSL) in either the prefreezing or the postthaw between the control group sample (0 hour) and the samples stored at 5°C (5°C-6h, 5°C-12h, 5°C-18h, 5°C-24h, and 5°C-30h). However, a significant decrease in the VSL was observed in the groups stored at RT after 12 hours of storage (RT-12h, RT-18h, RT-24h, and RT-30h).

As shown in Table 3, a decrease in the curvilinear velocity was observed in the prefreezing sample when the epididymis was stored at RT for more than 24 hours. In the postthaw sample, the decrease in this sperm parameter was observed only in the group stored at RT for 30 hours compared with the group in which recovery of the epididymal sperm was performed immediately after orchiectomy (control-0h).

In the prefreezing sample, the percentage of rapid sperm (RAP) demonstrated a significant decrease only in the groups in which the epididymides were stored for 12 hours or more at RT (RT-12h, RT-18h, RT-24h, and RT-30h), as described in Table 3. In the postthaw sample, there was a decrease in the RAP in the groups stored at 5°C for 18 and 30 hours (5°C-18h and 5°C-30h) and in all the groups stored at RT compared with the control group (Table 4).

In the prefreezing sample analysis for the PMI, there was no difference between the control group (0 hour) and any of the groups stored at 5°C (5°C-6h, 5°C-12h, 5°C-18h, 5°C-24h, and 5°C-30h). However, a decrease in the PMI was observed in the sample stored for 24 hours at RT (RT-24h) (Table 3). In the postthaw sample, there was no difference between the groups (Table 4).

4. Discussion

In many cases, the distance between the stud farm and a specialized equine reproduction center is presented as an obstacle in the preservation of epididymal sperm. Thus, it is necessary to evaluate the sperm viability at different

Table 3
Mean values and standard deviations of the sperm parameters TM, PM, VSL, VCL, and RAP evaluated after centrifugation and dilution in Botu-Crio, freezing extender of the samples stored at 5°C (5°C-6h, 5°C-12h, 5°C-18h, 5°C-24h, and 5°C-30h) and at room temperature (control-0h, RT-6h, RT-12h, RT-18h, RT-24h, and RT-30h)

Groups (n = 8)	TM (%)	PM (%)	VSL (μm/s)	VCL (μm/s)	RAP (%)
Control-0h	84.0 ± 5.96[a]	46.4 ± 6.86[a]	83.0 ± 5.40[a]	184.0 ± 15.62[ab]	73.9 ± 8.40[a]
5°C-6h	77.4 ± 11.29[a]	33.1 ± 8.92[ab]	80.5 ± 8.83[a]	201.5 ± 27.80[a]	66.2 ± 16.43[a]
5°C-12h	76.6 ± 7.72[a]	31.4 ± 3.70[ab]	77.4 ± 6.99[ab]	197.0 ± 22.96[a]	63.6 ± 8.89[a]
5°C-18h	71.4 ± 14.00[a]	27.6 ± 9.24[bc]	75.4 ± 8.58[ab]	198.4 ± 22.46[a]	60.1 ± 17.16[a]
5°C-24h	68.4 ± 18.73[a]	27.4 ± 13.72[bc]	71.4 ± 11.13[abcd]	181.1 ± 24.96[abc]	53.4 ± 24.24[a]
5°C-30h	70.4 ± 21.86[a]	29.6 ± 13.11[b]	76.9 ± 9.17[ab]	194.9 ± 21.43[a]	60.0 ± 23.84[a]
RT-6h	69.5 ± 16.88[a]	27.6 ± 9.71[bc]	74.3 ± 9.25[abc]	170.4 ± 50.02[abc]	54.7 ± 20.51[a]
RT-12h	39.9 ± 17.54[b]	13.0 ± 7.11[cd]	64.4 ± 4.34[bcde]	161.9 ± 19.39[abc]	23.9 ± 12.64[b]
RT-18h	24.9 ± 22.76[bc]	6.9 ± 7.43[d]	60.0 ± 6.46[de]	160.8 ± 17.77[abc]	15.8 ± 18.60[b]
RT-24h	29.8 ± 24.42[bc]	8.5 ± 8.00[d]	61.9 ± 7.20[cde]	150.2 ± 23.31[bc]	18.6 ± 20.27[b]
RT-30h	4.3 ± 2.66[c]	0.9 ± 0.83[d]	56.4 ± 6.70[e]	141.9 ± 19.95[c]	1.9 ± 1.25[b]

[a,b,c,d,e]Different letters in the columns indicate significant differences ($P < .05$).

Table 4
Mean values and standard deviations of the sperm parameters TM, PM, VSL, VCL, RAP, and PMI evaluated after thawing of the samples stored at 5°C (5°C-6h, 5°C-12h, 5°C-18h, 5°C-24h, and 5°C-30h) and at room temperature (control-0h, RT-6h, RT-12h, RT-18h, RT-24h, and RT-30h)

Groups (n = 8)	TM (%)	PM (%)	VSL (μm/s)	VCL (μm/s)	RAP (%)	PMI (%)
Control-0h	72.4 ± 6.86[a]	36.2 ± 8.44[a]	78.7 ± 7.2[a]	178.7 ± 13.92[a]	59.2 ± 10.69[a]	41.4 ± 5.94[a]
5°C-6h	56.9 ± 13.57[ab]	24.9 ± 10.11[ab]	74.6 ± 10.28[ab]	173.9 ± 24.49[ab]	42.1 ± 15.72[ab]	46.6 ± 9.94[a]
5°C-12h	51.4 ± 11.84[abc]	24.0 ± 9.65[abc]	73.5 ± 8.29[ab]	175.2 ± 11.71[ab]	38.5 ± 13.40[abc]	40.8 ± 4.59[a]
5°C-18h	40.1 ± 20.78[abcd]	17.6 ± 11.48[bcde]	65.9 ± 11.59[abcd]	159.3 ± 26.10[ab]	28.1 ± 17.75[bcde]	45.3 ± 12.44[a]
5°C-24h	52.8 ± 21.86[ab]	21.0 ± 13.11[bc]	68.0 ± 12.61[abc]	162.2 ± 32.70[ab]	36.5 ± 23.26[abc]	45.0 ± 12.42[a]
5°C-30h	45.8 ± 23.80[abc]	18.7 ± 9.68[bcd]	69.7 ± 11.96[abc]	169.4 ± 29.66[ab]	34.8 ± 21.10[bc]	40.4 ± 8.86[a]
RT-6h	49.0 ± 13.65[abc]	19.6 ± 6.65[bc]	70.6 ± 6.62[abc]	166.2 ± 26.06[ab]	33.6 ± 13.43[bcd]	43.5 ± 7.95[a]
RT-12h	25.4 ± 15.60[cde]	10.0 ± 6.97[cdef]	63.5 ± 8.16[bc]	155.1 ± 24.69[ab]	16.7 ± 12.80[cdef]	40.1 ± 3.87[a]
RT-18h	12.4 ± 16.59[e]	3.6 ± 5.58[ef]	51.9 ± 9.41[d]	145.8 ± 19.24[ab]	7.4 ± 11.86[ef]	44.8 ± 10.14[a]
RT-24h	18.7 ± 15.43[de]	4.8 ± 5.17[de]	61.8 ± 7.79[bc]	144.3 ± 22.55[ab]	9.5 ± 11.19[def]	39.6 ± 9.72[a]
RT-30h	2.25 ± 2.19[e]	0.4 ± 0.51[f]	54.8 ± 5.44[c]	139.7 ± 22.47[b]	0.9 ± 1.13[f]	29.9 ± 13.17[a]

[a,b,c,d,e,f]Different letters in the columns indicate significant differences ($P < .05$).

storage temperatures, aiming for a more efficient transport of this material.

Studies in stallions reported the effect of storage time on the epididymal sperm viability [9,12], but none of these studies compared epididymides processed immediately after orchiectomy with epididymides stored at 5°C and at RT. Furthermore, this study evaluated the sperm viability after the freezing process to maintain viable sperm cells for future use as a genetic reserve.

After the processes of centrifugation and resuspension in the freezing diluent, the samples showed higher sperm kinetic parameters compared with sperm that were harvested immediately after orchiectomy. This difference can be explained by the absence of accessory gland secretions, which, in addition to the transport function, also improve the initial motility after ejaculation. Spermatozoa, once differentiated in the testis, remain quiescent in epididymal fluids until they are released by ejaculation.

The 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 [17]. The presence of immobilin in the epididymal cauda increases the viscosity of the epididymal fluid and reduces the motility of the sperm cells [18], which substantiates the findings in the present study. Moreover, Heise et al. [19] found that the initial spermatozoal motility of fresh epididymal sperm plus seminal plasma was higher than that of fresh epididymal spermatozoa mixed with freezing medium. This suggests that the seminal plasma, besides causing the dilution of the fluid surrounding the epididymal sperm, also has the capacity to stimulate a higher initial motility of epididymal spermatozoa.

Regarding the present study, the motility parameters of epididymal sperm improved considerably after centrifugation and the addition of freezing extender (Botu-Crio, Botupharma). These findings are consistent with the results obtained by Monteiro et al. [11], which demonstrated that, despite the inferiority of the initial sperm parameters of cells recently harvested from the epididymis, after the addition of the freezing diluent (Botu-Crio, Botupharma), there was a significant improvement in the sperm parameters, achieving results comparable with those obtained with sperm collected from the ejaculate.

The comparative analysis of the sperm parameters of cells recovered immediately after orchiectomy in the groups kept at 5°C showed similarity in the TM, VSL, RAP, and PMI until 30 hours of storage. These results are consistent with the findings described by James [12], who observed similar values in the sperm obtained from the epididymis stored at the same temperature for up to 96 hours. These data are consistent with those reported by Monteiro et al. [11], who showed that the fertilizing capacity of epididymal sperm stored at 5°C for 24 hours did not differ from that of sperm recovered immediately after orchiectomy or of ejaculated sperm, supporting the hypothesis that the epididymal sperm recovered after the storage of the epididymis at 5°C remain viable, enabling the storage and transportation of the epididymis for a period of 24 hours.

The PM was the sperm parameter that was most sensitive to the storage of the epididymis, showing a significant decrease after 12 hours at 5°C and 6 hours at RT, both in the prefreezing and postthawing analysis. In cattle, the PM was positively correlated with fertility after thawing [20]. However, the fertilizing capacity has no absolute correlation with sperm motility [21]; thus, studies are needed to assess the fertility of these cells.

According to the data presented in Table 3, there was no significant difference in the TM or RAP between the control group (0 hour) and sperm recovered after the storage of the epididymis for up to 12 hours at RT. These data are similar to the results of Granemann [9], who reported a significant decrease in motility and vigor after 12 hours of storage.

In the analysis of PMI, assessed by epifluorescence microscopy [16], no significant differences were observed among 11 groups after thawing, indicating that this parameter was not influenced by the storage of the epididymis up to 30 hours, regardless of temperature. These findings corroborate the results obtained by James [12].

In the present study, the storage of epididymides at 5°C in passive transport containers contributed to the decline in sperm metabolism, providing a better sperm preservation during storage for up to 30 hours and a decrease only in the PM in the groups with more than 12 hours of storage. However, the sperm stored at RT showed a gradual and significant decrease in the parameters of TM, PM, VSL, and RAP, both pre- and postfreezing, after 12 hours of storage.

The viability of the spermatozoa present in the epididymis proved to be satisfactory until 24 hours of storage at 5°C, allowing the safe shipment of the epididymis to a breeding center for collection and cryopreservation of epididymal sperm.

In addition, the evaluation of the sperm from the epididymides stored at RT showed that the faster the testes and epididymides are removed and cooled to 5°C, as in cases of sudden death, the better the sperm parameters and sperm viability.

5. Conclusion

This study confirmed the hypothesis that epididymis refrigeration during storage is better for sperm viability and longevity when compared with maintenance at RT.

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