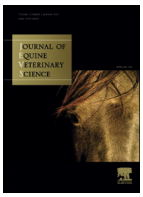
Journal of Equine Veterinary Science 33 (2013) 552-556

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com



Original Research

Comparison of Apoptotic Cells Between Cryopreserved Ejaculated Sperm and Epididymal Sperm in Stallions

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ARTICLE INFO

Article history:
Received 21 December 2011
Received in revised form
23 July 2012
Accepted 16 August 2012
Available online 3 November 2012

Keywords:
Epididymal
Sperm
Apoptosis
Cryopreservation

ABSTRACT

The development of a reliable technique to freeze epididymal semen would provide a unique opportunity to preserve valuable genetic material from unexpectedly lost stallions. The aim of this study was to compare the apoptotic indices of sperm obtained from ejaculate, sperm recently recovered from the epididymides (EP), and sperm recovered from epididymides stored at 5°C for 24 hours (EP-stored). For the first category, two ejaculates from seven stallions were collected and then submitted to cryopreservation using an egg yolk-based extender. One week after the last semen collection, the stallions were submitted to bilateral orchiectomy, and sperm from one of the cauda epididymis was harvested immediately after castration (EP). The remaining testicle was stored in a passive refrigeration container at 5°C for 24 hours before the cauda epididymal sperm was harvested (EP-stored). Sperm harvesting from the epididymis for EP and EP-stored was performed by retrograde flushing of the caudal portion of the epididymis using a skim milk-based extender. The recovered sperm was then cryopreserved using the egg yolk-based extender. Sperm motility parameters were studied by computer-assisted semen analysis, and apoptosis was estimated by measuring caspase activity and membrane phospholipid translocation using epifluorescence microscopy. The samples were evaluated immediately (0 hour) and 8 hours after thawing. At 0 hour, no differences in sperm parameters were observed among the groups, but after 8 hours, significant statistical differences were observed in sperm motility parameters and plasma membrane integrity among the treatment groups. In addition, viable cells with no apoptotic signs were more prevalent in EP and EP-stored, suggesting that epididymal sperm is less sensitive to the cold shock caused by sperm cryopreservation.

1. Introduction

Unexpected death due to gastrointestinal disorders or traumatic accidents often ends the reproductive life of stallions with high genetic value. In such cases, the recovery of epididymal sperm has been an alternative for preserving the genetic material of these animals.

Recent reports on the fertility of stallion epididymal sperm [1,2] have demonstrated the importance of maximizing the cryopreservation protocols for these cells. Such studies have become even more relevant given that the harvesting of sperm from the cauda epididymis is, in most cases, the last chance to preserve sperm from castrated stallions or even from stallions after their deaths.

The first pregnancy obtained using frozen equine sperm was reported by Barker and Gandier [3] using

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http://dx.doi.org/10.1016/j.jevs.2012.08.010

cauda epididymal sperm. Despite knowing the fertilizing ability of such sperm for more than five decades, most studies have focused on the cryopreservation of ejaculated sperm.

However, the growing interest in the preservation of semen from endangered species and sperm from farm animals with high genetic value that die unexpectedly or that are unable to mate or deliver semen by conventional methods has intensified the amount of research related to the recovery and cryopreservation of viable sperm from the cauda epididymis [1,4-8].

Recently, phenomena similar to apoptosis were identified by sperm cell analysis [9-11]. These phenomena affect cell death and inflict different degrees of damage on spermatozoa structures that are important for longevity [12,13]. During the initiation of apoptosis, cells lose plasma membrane integrity (PMI). Phosphatidylserine (PS), which is normally present inside the membrane of a healthy cell, is translocated and expressed on the exterior. This process can gradually lead to membrane damage [14]. It has been shown that PS translocation can be used as an early marker of membrane deterioration in frozen–thawed human [15], bovine [9], and equine spermatozoa [16,17].

A number of authors have demonstrated the existence of caspase (Casp)-dependent factors in the apoptosis pathway in human ejaculate [18,19]. These enzymes are considered to be major transducers and effectors within different apoptosis signaling pathways in somatic cells. The enzymes belong to a highly specific family of proteases that contain the amino acid cysteine within their active sites [18]. From a functional point of view, the caspases involved in apoptosis can be classified as initiators (caspases 8, 9, and 10) or effectors (caspases 3, 6, and 7) [20].

Active caspases 3, 7, and 9 have been detected in stallion spermatozoa [12], indicating that the intrinsic pathway of apoptosis had been triggered. The higher incidence of apoptosis in semen from subfertile men, and evaluation of the Casp activity was indicated as a diagnostic marker for semen quality, thus providing a more accurate parameter to andrological exams [21,22].

Apoptosis is defined as programmed cell death induced by intra- and extracellular stimuli [18,20]. Apoptosis is a physiological process in the testicular parenchyma that is partially responsible for controlling the number of germ cells, and it eliminates defective cells that reach the stage of spermiation [23,24].

Apoptotic cells have also been determined to have low sperm motility after thawing, as a result of changes arising from the cryopreservation process. Furthermore, these apoptotic cells have a reduced longevity in the female reproductive tract [12]. This represents a particular problem in equids because mares have long estrus periods, and thus, it is difficult to determine the best time to inseminate with respect to ovulation. Despite numerous studies comparing ejaculated and epididymal sperm [2,6,7], none of these studies has evaluated the apoptotic cells after thawing. Thus, the aim of this study was to compare kinetics parameters, PMI, and apoptotic rates in ejaculated sperm (EJ), recently recovered cauda epididymal sperm (EP), and sperm obtained from stored epididymides at 5°C for 24 hours (EP-stored).

2. Materials and Methods

2.1. Sperm Harvest

For this experiment, seven stallions, aged between 4 and 6 years, were used. The breed distribution was three Brazilian Show Jumpers, two Lusitano, and two Mangalarga Marchador. Sperm from each animal served as its own control. Initially, three ejaculates from each stallion were collected with an interval of 2 days to eliminate possible damaged cells from cauda epididymis and to stabilize the sperm parameters. In EJ (the control group), each stallion was submitted to two semen collections using an artificial vagina, with an interval of 1 week between collections. Ejaculates from the second semen collection were diluted at a ratio of 1:1 with skim milk-based extender (Botu-Sêmen, Botupharma, Botucatu, Brazil) and were centrifuged at 600 × *g* for 10 minutes. The supernatant was removed, and the pellets were resuspended with egg yolk-based extender (BotuCrio, Botupharma) to a final concentration of 200 million motile sperm/mL.

The semen was packaged in 0.5 mL straws and then cooled in a Minitub (Minitub do Brasil LTDA, Porto Alegre, Brazil) refrigerator at 5°C for 20 minutes. Subsequently, a 45-L isothermal box was filled with liquid nitrogen up to 3.5-cm height, and the straws were placed horizontally 6.0 cm above the liquid nitrogen for 20 minutes. After this time, the straws were immersed in nitrogen and stored [1].

One week after the semen from the second collection was processed, the stallions were submitted to bilateral orchiectomy. Immediately after castration, recovery of sperm from one epididymal cauda of each stallion was performed (EP). The remaining testis, with the epididymides attached, was stored in a passive refrigeration container (Botutainer, Botupharma) at 5°C for 24 hours before epididymal sperm was harvested (EP-stored).

Recovery of cauda epididymal sperm was performed using the retrograde flush technique reported by Garde et al. [25] and Bruemmer et al. [26]. After recovery, the samples were processed for cryopreservation using the same aforementioned protocol for ejaculated sperm.

2.2. Seminal Analysis

All semen analyses were performed immediately after thawing at 46°C for 20 seconds (0 hour) and after incubation at 20°C for 8 hours.

PMI, PS translocation, and Casp activity were estimated using an epifluorescence (Leica Microsystems DMLB, Wetzlar, Germany) microscope at 1000× magnification as described in the following text. A total of 200 cells were counted for each sample.

2.2.1. Sperm Motion

After thawing at 46°C for 20 seconds, the samples were transferred to a 1.5-mL plastic tube and incubated at 37°C during evaluation. Five fields per sample were selected for the evaluation of the sperm parameters—total motility, progressive motility, average path velocity (VAP, μm/s), straight linear velocity (μm/s), and the percentage of rapid sperm (%RAP)—by computer-assisted semen analyzer (HTM-IVOS 12, Hamilton Thorne Research).

Table 1
Methodologies of computer-assisted semen analysis 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

The computer-assisted semen analysis setup is described in Table 1.

2.2.2. Plasma Membrane Integrity

The percentage of sperm with intact plasma membranes (%PMI) was analyzed from each sample using propidium iodide (PI) and carboxyfluorescein diacetate by epifluorescence microscopy, according to Harrison and Vickers [27].

2.2.3. PS Translocation

Evaluation of PS translocation was performed using an Annexin V–FITC Kit II for apoptosis estimation (556570; BD Bioscience Pharmingen, Franklin Lakes, NJ), according to the producer's recommendations. Semen aliquots were diluted in an Annexin V buffer solution (10 m*M* hydroxyethyl piperazineethanesulfonic/NaOH, pH 7.4, 140 m*M* NaCl, and 2.5 m*M* $CaCl_2$) to a concentration of 1×10^6 spermatozoa/mL. Aliquots of 100 μL (final concentration of 1×10^5 spermatozoa/mL) of the samples were placed in Eppendorf cryotubes, and 5 μL of Annexin V-FITC, 5 μL of PI (50 μg/mL), and 2 μL of Hoechst 33342 (H342, 40 μg/mL) were added. The samples were then homogenized and incubated for 15 minutes. The different patterns of cells found during the analysis were classified as viable cells (An−/PI−), viable cells with translocation of PS (An+/PI−), damaged cells with translocation of PS (An+/PI+), and dead cells (An−/PI+) [13].

2.2.4. Activated Casp

The association of FITC–VAD–FMK (G7462, Promega, Madison, WI) was used as a marker of Casp activated in situ, according to the producer's recommendations. One milliliter of phosphate-buffered saline and 1×10^6 spermatozoa were added to 1 μL of 5 m*M* FITC–VAD–FMK, following which the samples were homogenized and incubated at room temperature for 20 minutes in the dark. After incubation, this solution was centrifuged ($200 \times g$ for 5 minutes) and resuspended in phosphate-buffered saline at the initial concentration. Next, 5 μL of PI (50 μg/mL) and 2 μL of Hoechst 33342 (H342, 40 μg/mL) were added. The final reading was taken after 5 minutes. The different patterns of cells found during the analysis were classified as viable cells (Casp−/PI−), viable cells with activated Casp (Casp+/PI−), damaged cells with activated Casp (Casp+/PI+), and dead cells (Casp−/PI+) [13].

2.3. Statistical Analysis

All parameters were analyzed by a one-way analysis of variance (SAS Institute Inc., Cary, NC), followed by Tukey test to identify significant differences. Differences between treatments were considered significant for $P < .05$.

3. Results

As shown in Table 2, there were no differences in sperm motility parameters and sperm PMI when the sperm was analyzed immediately after thawing. However, 8 hours after thawing, all parameters in Table 2 showed higher values in the sperm recovered from the cauda epididymis just after harvest (EP), compared with the ejaculated sperm (EJ). In addition, the motility characteristics VAP, RAP, and PMI were also higher in the epididymal sperm obtained from epididymides cooled at 5°C for 24 hours (EP-stored), compared with those characteristics in the ejaculated sperm.

As shown in Table 3, no difference was found among the groups in the translocation of immediately after thawing (0 hour). However, 8 hours after thawing, the ejaculated sperm (EJ) had a lower percentage of viable spermatozoa and a higher percentage of damaged cells with translocation of membrane phospholipids when compared with the groups of epididymal sperm (EP and EP-stored).

In the analysis of Casp, no difference was found at the 0-hour time point among the groups; however, by 8 hours after thawing, a smaller percentage of viable cells was observed in the ejaculated sperm (EJ) compared with the epididymal sperm from EP and EP-stored. Moreover, the

Table 2
Mean (±SD) values for percentage of total motility (%TM), percentage of progressive motility (%PM), average path velocity (VAP) in μm/s, straight linear velocity (VSL) in μm/s, percentage of rapid sperm (%RAP), and percentage of plasma membrane integrity (%PMI) in EJ, EP, and EP-stored immediately after thawing 0 hour and after incubation for 8 hours at 20°C

Groups	TM (%)	PM (%)	VAP (μm/s)	VSL (μm/s)	RAP (%)	PMI (%)
EJ (0 hour)	62.3 ± 12.87^{A}	31.6 ± 9.16^{A}	95.9 ± 7.82^{A}	76.9 ± 5.24^{A}	49.3 ± 14.33^{A}	45.6 ± 9.11^{A}
EP (0 hour)	72.6 ± 8.40^{A}	35.3 ± 10.32^{A}	101.0 ± 9.47^{A}	79.3 ± 8.56^{A}	59.7 ± 13.59^{A}	52.0 ± 9.54^{A}
EP-stored (0 hour)	61.4 ± 15.76^{A}	29.1 ± 11.4^{A}	92.7 ± 11.44^{A}	75.1 ± 7.80^{A}	45.4 ± 15.55^{A}	49.7 ± 8.73^{A}
EJ (8 hours)	26.0 ± 21.59^{b}	6.1 ± 6.4^{b}	66.1 ± 15.87^{b}	50.7 ± 10.59^{b}	13.7 ± 14.85^{b}	25.6 ± 4.65^{b}
EP (8 hours)	54.7 ± 12.23^{a}	17.4 ± 8.54^{a}	91.9 ± 12.43^{a}	67.9 ± 8.78^{a}	37.6 ± 14.15^{a}	34.7 ± 2.56^{a}
EP-stored (8 hours)	49.0 ± 22.80^{ab}	16.7 ± 8.73^{ab}	88.4 ± 17.58^{a}	66.1 ± 15.18^{ab}	36.3 ± 17.95^{a}	30.4 ± 2.64^{a}

EJ, ejaculated sperm; EP, epididymal sperm recovered immediately after orchiectomy; EP-stored, sperm obtained from epididymis stored at 5°C for 24 hours. Values with different uppercase superscript letters represent significant difference from the analysis at 0 hour after thawing. Values with different lowercase superscript letters in the same column represent differences ($P < .05$) from the analysis at 8 hours after thawing.

Table 3
Mean (±SD) values from epifluorescence microscopy analysis for the assessment of translocation of membrane phospholipids (An+), as An−/PI− = % viable cells, An+/PI− = % viable cells with translocation of phospholipids, An+/PI+ = % damaged cells with translocation of phospholipids, and An−/PI+ = % dead cells in EJ, EP, and EP-stored, immediately after thawing 0 hour and after incubation for 8 hours at 20°C

Groups	An−/PI−	An+/PI−	An+/PI+	An−/PI+
EJ (0 hour)	25.1 ± 3.89^{A}	10.8 ± 3.53^{A}	22.86 ± 5.11^{A}	41.14 ± 7.49^{A}
EP (0 hour)	28.7 ± 4.15^{A}	13.0 ± 3.11^{A}	21.0 ± 4.79^{A}	37.3 ± 7.91^{A}
EP-stored (0 hour)	29.4 ± 4.16^{A}	12.4 ± 3.51^{A}	17.8 ± 5.96^{A}	40.3 ± 7.50^{A}
EJ (8 hours)	6.4 ± 2.07^{b}	11.7 ± 1.80^{a}	30.8 ± 5.72^{a}	51.0 ± 7.46^{a}
EP (8 hours)	12.1 ± 3.76^{a}	14.0 ± 3.26^{a}	24.6 ± 5.83^{b}	49.3 ± 6.78^{a}
EP-stored (8 hours)	12.57 ± 2.99^{a}	11.7 ± 1.70^{a}	22.7 ± 7.20^{b}	53.4 ± 8.30^{a}

Values with different uppercase superscript letters represent significant difference from the analysis at 0 hour after thawing. Values with different lowercase superscript letters in the same column represent differences ($P < .05$) from the analysis at 8 hours after thawing.

percentage of dead cells (Casp−/PI +) in EP was lower than in the ejaculated sperm (Table 4).

4. Discussion

Sperm motility parameters evaluated at 0 hour did not differ among the groups, which is in agreement with the results previously reported by Monteiro et al. [7], who reported similar motility characteristics of frozen–thawed, ejaculated, and epididymal sperm. However, in the present report, 8 hours after thawing, epididymal sperm collected immediately after orchiectomy showed greater total motility, progressive motility, straight linear velocity, VCL, RAP, and PMI, compared with ejaculated sperm. Moreover, the values of the parameters VAP, RAP, and PMI were also higher in sperm cells obtained from stored epididymides at 5°C for 24 hours, compared with EJ. These results demonstrate that the epididymal sperm seemed to be more resistant to the process of freezing and thawing. These findings support the hypothesis that epididymal sperm is less sensitive to cold shock and, consequently, is more resistant to freezing [2,28] and cooling processes [29].

A similar trend was observed in sperm apoptotic status immediately after thawing (0 hour). However, when the sperm was stressed by incubating it for 8 hours at 20°C after thawing, the ejaculated samples showed a lower percentage of viable spermatozoa and a higher percentage of damaged cells with membrane phospholipid translocation, compared with epididymal sperm. In addition, the evaluation of Casp activation demonstrated a smaller percentage of viable sperm and a greater percentage of dead cells in the ejaculated sperm, compared with the epididymal sperm from EP. The results are supported by studies that have demonstrated that a higher incidence of apoptosis may be a diagnostic marker of sperm quality [12,23], as shown by the higher staining of Casp-3 in infertile men [30].

The epididymal sperm was not exposed to seminal plasma, which may be a possible explanation for the better viability of the cauda epididymal sperm when compared with ejaculated sperm after 8 hours of incubation. This explanation is supported by studies showing a deleterious effect of seminal plasma on sperm viability, which results in biochemical changes that increase membrane permeability [31,32], leading to a decrease in sperm motility parameters and an increase in morphological defects in sperm tails [2]. These biochemical changes in the plasma membrane caused by contact with the seminal plasma are the result of increased membrane permeability, which leads to the membrane changes associated with freezing damage [9], thus explaining the smaller percentage of viable sperm and the greater percentage of damaged cells with membrane phospholipid translocation in the ejaculated sperm analyzed 8 hours after thawing.

The superior sperm viability of epididymal sperm demonstrated in this study is particularly important in horses, because mares have long estrus periods and therefore require greater attention to inseminate them closer to ovulation. This fact becomes even more relevant with the use of cryopreserved sperm, as this sperm has lower viability and longevity compared with fresh sperm [33]. The higher values of sperm motility parameters and PMI without apoptosis markers of epididymal sperm when compared with ejaculated sperm may indicate the possible higher fertility of epididymal sperm. Such higher fertility is supported by the results reported by Monteiro et al. [7], who showed that epididymal sperm immediately after orchiectomy tended to have a higher conception rate,

Table 4
Mean (±SD) values from epifluorescence microscopy analysis to assess the activation of caspase (Casp+), as Casp−/PI− = % viable cells, Casp+/PI− = % viable cells with activated caspase, Casp+/P+ = % damaged cells with activated caspase, and Casp−/PI+ = % dead cells, in EJ, EP and EP-stored, immediately after thawing 0 hour and after incubation for 8 hours at 20°C

Groups	Casp−/PI−	Casp+/PI−	Casp+/PI+	Casp−/PI+
EJ (0 hour)	25.7 ± 4.71^{A}	13.14 ± 3.72^{A}	27.4 ± 6.29^{A}	34.6 ± 6.29^{A}
EP (0 hour)	27.6 ± 2.51^{A}	13.0 ± 3.51^{A}	23.7 ± 3.15^{A}	36.6 ± 3.10^{A}
EP-stored (0 hour)	28.4 ± 3.05^{A}	12.4 ± 3.69^{A}	23.4 ± 5.32^{A}	35.7 ± 4.07^{A}
EJ (8 hours)	5.86 ± 2.67^{b}	9.14 ± 1.57^{b}	29.3 ± 3.86^{a}	55.7 ± 5.88^{a}
EP (8 hours)	12.9 ± 2.54^{a}	13.6 ± 4.04^{a}	24.4 ± 5.09^{a}	49.1 ± 2.91^{b}
EP-stored (8 hours)	10.9 ± 2.79^{a}	12.8 ± 3.62^{a}	25.4 ± 4.20^{a}	50.8 ± 4.74^{ab}

Values with different uppercase superscript letters represent significant difference from the analysis at 0 hour after thawing. Values with different lowercase superscript letters in the same column represent differences ($P < .05$) from the analysis at 8 hours after thawing.

compared with ejaculated sperm (92.3% and 61.5%, respectively).

5. Conclusion

On the basis of these results, we can conclude that frozen–thawed cauda epididymal sperm has similar or higher motility parameter values than ejaculated sperm after thawing. In addition, incubating sperm at 20°C for 8 hours after thawing resulted in higher motility parameter values, better PMI, and lower apoptotic status of the epididymal sperm. This finding suggests that epididymal sperm is more resistant to the cold shock caused by sperm cryopreservation.

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