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Cryopreservation and fertility of ejaculated and epididymal stallion sperm

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ABSTRACT

The cryopreservation of epididymal sperm is important to preserve genetic material from valuable deceased males. This study evaluated the viability of sperm samples from eight stallions under three conditions: (1) collected using an artificial vagina (EJ-0 h), (2) recovered from the epididymal *cauda* immediately after orchiectomy (EP-0 h), and (3) recovered from the epididymal *cauda* after 24 h of storage at 5 °C (EP-24 h). To obtain EJ-0 h sperm, two ejaculates were collected from each stallion. After 1 week, the stallions were submitted to bilateral orchiectomy, and one of the removed epididymides was flushed to obtain EP-0 h sperm. The contralateral epididymis was stored at 5 °C for 24 h before being flushed to obtain EP-24 h sperm. The sperm samples were analyzed at three different times: immediately after sperm recovery, after dilution in the freezing extender, and post-thawing. A fertility trial was performed using 39 estrous cycles. After ovulation induction with 1 mg of deslorelin acetate (i.m.), mares were inseminated with 800×10^6 sperm. The total number of sperm recovered was $7.8 \pm 4.7 \times 10^9$ for EJ-0 h sperm, $12.9 \pm 9.2 \times 10^9$ for EP-0 h sperm and $12.0 \pm 8.0 \times 10^9$ for EP-24 h sperm. The sperm motility, evaluated by total motility, progressive motility and the percentage of rapid cells, was similar among the samples before and after freezing ($P > 0.05$). However, the plasma membrane integrity was different between EJ-0 h and EP-0 h pre-freezing and between EJ-0 h and EP-24 h post-thawing ($P < 0.05$). The conception rates were similar between groups inseminated with sperm recovered from the epididymal cauda immediately after orchiectomy (EP-0 h), after 24 h of storage at 5 °C of the epididymal cauda (EP-24 h) and with ejaculated sperm (EJ-0 h) ($P > 0.05$). In conclusion, the viability and fertility of cauda epididymal sperm are similar to those of ejaculated sperm.

1. Introduction

Sudden death, catastrophic injury, castration, obstructive processes or any other event that precludes semen collection or mating may prematurely terminate a stallion's reproductive life. In these cases, horse owners have requested a final sperm collection to preserve the genetic value of an animal.

Studies on the preservation of epididymal sperm for future use have been intensified to prevent genetic losses. These investigations have described techniques for epididymal sperm collection (Cary et al., 2004; Bruemmer, 2006), laboratory procedures such as the use of different extenders for flushing and cryopreservation (Melo et al., 2008; Papa et al., 2008; Guasti et al., 2009), the addition of seminal plasma (Morris et al., 2002; Tiplady et al., 2002; Moore et al., 2005; Pasquini et al., 2008; Heise et al., 2010),

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insemination (Morris et al., 2002) and assisted reproduction techniques, such as intracytoplasmic sperm injection (Herrera et al., 2006).

Protocols describing the storage of the testis/epididymis after an orchiectomy procedure or post-mortem have been developed (Bruemmer et al., 2002; James et al., 2002; Monteiro et al., 2009). The purpose of these past studies was to determine the most desirable type of transport container in which to store the testis–epididymis complex without affecting its viability until the sperm can be processed and cryopreserved in specialized laboratories.

After removal of the epididymis, sperm remain viable until affected by tissue decomposition (Bruemmer et al., 2002; Muradás et al., 2006; Monteiro et al., 2009). Sperm viability is maintained for longer periods when the epididymis is stored at 5 °C (Bruemmer et al., 2002; James et al., 2002; Monteiro et al., 2009), although little information about fertility after this type of storage is available.

Considering the promising use of epididymal sperm for reproduction purposes, the present study aimed to compare the viability and fertility of ejaculated sperm obtained using an artificial vagina with cauda epididymis sperm collected immediately after castration or after 24 h of storage at 5 °C.

2. Materials and methods

Eight stallions of different breeds (four Brazilian Jumping Horses, two Lusitano and two Mangalarga Marchador) aged 4–6 years were used. Initially, three ejaculates from each stallion were collected with an interval of 2 days to eliminate possible damaged cells from epididymal cauda and to stabilize the sperm parameters. The same animals provided the sperm samples to the control samples (EJ-0 h) and the EP-0 h and EP-24 h samples.

For the control samples (EJ-0 h), two ejaculates were collected from each stallion over a 1-week interval using an artificial vagina. Both ejaculates were diluted 1:1 (v/v) in a skim milk-based extender (Botu-Semen™, Botupharma, Botucatu, Brazil) and centrifuged at 600 × *g* for 10 min. The pellet was re-suspended in a freezing egg yolk-based extender (Botu-Crio™, Botupharma, Botucatu, Brazil) 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 min in a commercial refrigerator (Minitub™) and subsequently frozen in liquid nitrogen vapor, 6 cm above the surface of liquid nitrogen, for 20 min. The straws were immersed in liquid nitrogen and stored at −196 °C until analysis. After thawing at 46 °C for 20 s (Dell'aqua et al., 2001), the sperm were transferred to a 1.5 mL plastic tube and maintained in a dry block at 37 °C for semen evaluation.

One week after the second semen collection, the stallions were submitted to bilateral orchiectomy. Immediately after castration, one epididymal cauda of each stallion was flushed for sperm recovery (EP-0 h). The contralateral epididymis was stored for 24 h at 5 °C in a refrigerated transport container (Botutainer™, Botupharma, Botucatu, Brazil). After 24 h of cooling, epididymal sperm were recovered (EP-24 h).

Table 1
Methodologies of computer-assisted semen analysis (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

In the EP-0 h and EP-24 h samples, the epididymal cauda was isolated from the testis. The connective tissue was carefully dissected, and the epididymal cauda was straightened. A 10 μL pipette tip was attached to a 10-mL syringe, and the epididymal cauda was flushed using 30 mL of Botu-Semen™ per epididymis. The flushing fluid from both epididymides of each animal was recovered in a 200 mL beaker. The cryopreservation procedure for epididymal sperm was similar to that described for the ejaculated samples (EJ-0 h; control).

Sperm from ejaculate (EJ-0 h), sperm recovered from the epididymis immediately after castration (EP-0 h) and sperm recovered after the storage of the epididymis for 24 h at 5 °C (EP-24 h) were analyzed immediately after sperm recovery, following dilution in the freezing extender and post-thawing. Five fields per sample were selected for the evaluation of the motility parameters by CASA (HTM-IVOS 12, Hamilton Thorne Research, USA). The CASA setup is described in Table 1. The plasma membrane integrity was evaluated using the fluorescent probes carboxyfluorescein diacetate and propidium iodide (CFDA/PI) as described by Harrison and Vickers (1990). The sperm morphology was determined by evaluating smears stained by the modified Karras method (Papa et al., 1988).

For the fertility evaluation, a crossover trial was conducted with 13 mares (39 estrous cycles) aged 3–8 years old. For each estrous cycle, a sperm sample from a different sample (EJ-0 h, EP-0 h and EP-24 h) was used. The mares were monitored daily by transrectal ultrasonography; when follicles reached 35 mm, ovulation was induced with 1 mg (i.m.) of deslorelin. Twenty-four hours after ovulation induction, the mares were monitored every 6 h until ovulation was detected. Then, insemination was performed in the tip of the uterine horn ipsilateral to the ovulation using a flexible pipette[1] containing 800×10^6 viable frozen-thawed sperm, corresponding to a pool of eight straws from different stallions. Pregnancy was diagnosed by ultrasonography between 12 and 15 days after ovulation and was interrupted with an application of 5 mg (i.m.) of dinoprost tromethamine.

[1] Minitub do Brasil Ltda, Porto Alegre-RS, Brasil.