Journal of Equine Veterinary Science 46 (2016) 64–68

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research

Effects of Two Different Cooling Devices for Testicles Transport on Stallion Epididymal Sperm Quality

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ARTICLE INFO

Article history:
Received 22 March 2016
Received in revised form 20 June 2016
Accepted 16 July 2016
Available online 25 July 2016

Keywords:
Epididymal sperm
Stallion
Testicle
Storage

ABSTRACT

This study evaluates the effects of two cooling devices and temperature for testicles storage on epididymal sperm quality after 24 hours; different levels of seminal plasma (0% and 10%) were evaluated on sperm after recovering. Testicles from six stallions were recovered immediately after castration (2) or at the slaughterhouse (4); of the same animal, one testicle was placed in Equitainer (+8°C), the other in a styrofoam box with ice (+3°C). After 24 hours, the temperature of parenchyma was measured, and testicles and epididymal were weighted. Sperm were flushed from the cauda epididymides with Kenney extender, total sperm number recorded and motility and viability evaluated immediately after flushing (T0) with or without 10% SP (G1 Eq 0%, G2 Eq 10%, G3 Ice 0%, G4 Ice 10%). Motility and viability were evaluated after 24 hours and 48 hours of storage at +4°C. Temperature of the parenchyma was lower in testicles stored in ice compared to Equitainer (3.2 ± 0.6°C and 8.6 ± 2.5°C, respectively; $P < .05$). Motility and viability at T0 were similar ($P > .05$) in G1 and G3, whereas addition of SP after recovery significantly improved motility only in samples stored in Equitainer (G2). Viability was higher ($P < .05$) in G2 than in G4. At T24 and T48, no differences ($P > .05$) in sperm quality were found between storage methods or samples with or without SP. In conclusion, equine testicles can be safely stored either at lower (+3°C) or higher (+8°C) temperature than +5°C. This can be useful, especially when testicles are shipped in a hot climate, where devices cannot guarantee optimal refrigeration conditions.

1. Introduction

Flushing of tail of the epididymis permits recovery and preservation of sperm from stallions that die prematurely, due to acute or chronic illness, as well as following routine castration.

The equine *cauda epididymis* contains a high number of spermatozoa with normal fertilizing ability and resistance to cold shock [1]. It has been demonstrated that epididymal sperm has similar progressive motility [2] and fertility [3] when compared to ejaculated sperm collected by artificial vagina.

Location of castration or recovery of testicles postmortem may be remote to the laboratory for sperm handling and preservation; therefore, transport or shipment of the testicles to the site of semen processing may require a long period of time (24–48 hours). In these cases, testicles and epididymis are generally sent refrigerated to the laboratory [4].

Previous studies have reported the effect of epididymal storage at +4–5°C following various periods of storage [3–8]. In these studies, the method of storage varied, but

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http://dx.doi.org/10.1016/j.jevs.2016.07.005

comparison between storage devices was not performed, as well as the temperature of the testicular parenchyma after storage was not recorded. Only James [6] stored one testicle on ice, at +4°C, and shipped it in a styrofoam cooler box for 24 hours before processing; the other one was stored at +4°C for 48 hours, 72 hours, and 96 hours.

Brinsko [9], in a very detailed study, showed that stallion semen may be able to tolerate a wider range of cooling rates and storage temperatures than previously thought.

In addition, different authors already showed that temperatures between +4°C and +15°C are suitable for preserving the fertilizing capacity of stallion semen stored for 22 hours or more in INRA 96 extender [10], and more important that temperature between +8–10°C can be more suitable than +4°C for "bad cooler" stallions [11].

The motility of epididymal sperm is often low, probably due to the lack of exposure to activating factors present in the SP [12], and effect of SP on the quality of epididymal sperm is unclear as several studies have not shown any improvement in freezability or fertility with or without added SP [2,10,13].

Different studies investigated the amount of SP to add to epididymal sperm. Tiplady [14] showed that addition of SP marginally improve the initial total and progressive motility after equilibration at +4°C, but they did not calculate the SP level they used because SP was used for flushing the *cauda epididymis*. Neuhauser [15], in a study about post-thaw addition of SP to epididymal sperm, showed that motility improved in a dose-dependent manner at concentration of 20% and 50% but did not further increase at 80%. They concluded that there seems to be a threshold level, above which there is no further improvement in most of sperm motion characteristics. Five to 20% SP was beneficial for sperm motion characteristics for up to 96 hours of storage and are usually recommended for preservation [15–23].

Based on these assumptions, the aims of this study were: (1) to evaluate motility and viability of epididymal sperm recovered after storage of testicles for 24 hours at different temperatures, especially to evaluate if temperature higher than previously used can effect semen quality; for this purpose, two different cooling environments (Equitainer with one coolant can and ice) were used; (2) to evaluate sperm motility and viability immediately after recovering and after 24 and 48 hours of refrigeration; (3) to evaluate the effect of adding 10% seminal plasma.

2. Materials and Methods

2.1. Samples Collection

Testicles and epididymides from six healthy stallions of different breeds, between 3 and 5 years, were recovered immediately after castration (2) or at the slaughterhouse (4). There was no history of previous ejaculation, and castration was performed because owners wanted to eliminate stallion behavior. The *vas deferens* was ligated, and each testicle from one stallion was placed in a plastic bag and stored, one in Equitainer (Hamilton-Thorne Research, Danvers, MA, USA), containing one frozen coolant can, the other one immersed in a styrofoam box with ice.

After 24 hours of storage, the temperature of each testicle was measured by placing the probe (Hanna Checktemp1, Hanna Instruments, Italy) into the testicular tissue. After carefully dissecting the connective tissue, epididymis and *vas deferens* were isolated from the testis, and the weights of epididymis and testis were measured. A retrograde flushing of the tail of epididymis was performed using a cat urinary catheter (Arnolds 96 1.3 mm OD 4f × 30.5 cm.) connected with an AI syringe (Luer; 20-mL Norm-Ject) containing 20 mL of Kenney extender [24].

2.2. Semen Dilution and Evaluation

Spermatozoa were recovered in a sterile 50-mL Falcon tube, and the volume of recovered fluid was measured. A NucleoCounter SP-100 (Chemometec, Denmark) was used for evaluating sperm concentration and viability.

For concentration, 50 μL of the sample were diluted with 5000 μL of Reagent S100 (Chemometec, Denmark), aspirated in SP1- Cassette (Chemometec, Denmark) and inserted in the NucleoCounter.

For viability, a second sample diluted with 5000 μL of PBS was used to evaluate non-viable sperm; the percent of viable sperm was calculated using the following formula:

$$\frac{Concentration - Non\ viable\ \ (\%)}{Concentration}$$

From each treatment (ice and Equitainer), 300 × 10^6 spermatozoa were diluted in 10 mL of Kenney extender (30 × 10^6/mL) with or without 10% seminal plasma (SP) and stored in a fridge at +4°C (Tube 10 mL100 × 16PP, SARSTEDT, Australia).

For motility evaluation, at each time point (T0, T24, and T48), 1 mL was incubated at 37°C for 10 minutes, and a pre-warmed slide (Leja Standard Count four Chamber Slide 20 micron; Leja Products B.V., Nieuw Vennep, The Netherlands) was used for CASA evaluation (IVOS Vers.12, Hamilton Thorn Biosciences), with standard equine setup (Table 1). Sperm motility endpoints assessed were total motility (TM); progressive motility (PM); curvilinear velocity (VCL); straight line velocity (VSL); average path velocity (VAP); amplitude of lateral head displacement (ALH); straightness (STR); linearity (LIN); and beat cross frequency (BCF).

2.3. Harvesting of Seminal Plasma

Seminal plasma was obtained by centrifuging at 3000 g for 30 minutes semen collected from a fertile stallion

Table 1
Standard equine setup for CASA.

Characteristic	Adjusted to
Frames per second	60
Number of frames	45
Minimum contrast	70 pixels
Minimum cell size	4 pixels
Straightness	75%
Minimum VAP to progressive cells	30 μm/second
VSL cutoff to slow cells	1.0 μm/second
VAP cutoff to slow cells	20 μm/second
Temperature	37°C

Abbreviations: CASA, computer-assisted sperm analysis; VAP, average path velocity; VSL, straight-line velocity.