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Original Research

# Comparison of the Effects of Four Freezing Methods on Motility Characteristics, Morphology, and Viability of Postthaw Stallion Epididymal Sperm

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ABSTRACT

Semen cryopreservation is of growing interest in the horse breeding industry, and collecting epididymal sperm might provide the chance to preserve genetic material from valuable stallions after severe injury or death. In case of an unexpected emergency, there may not always be an adequate laboratory nearby. Therefore, we compared fast and slow freezing methods using either a programmable freezer or a styrofoam box filled with liquid nitrogen. Epididymides of 10 stallions were collected immediately after routine castration under general anesthesia. Epididymal spermatozoa were evaluated before and after the freeze-thaw process for motility, viability, morphological, and kinematic parameters. Neither postthaw motility nor kinematic values differed among the four freezing protocols. Morphological abnormalities after freezing and thawing differed among epididymal segments. However, there were significantly more nonviable spermatozoa after the freeze-thaw process using the fast freezing process in the styrofoam box filled with liquid nitrogen compared with all other freezing processes. According to the results of this study, freezing in nitrogen vapor should be considered as an alternative to the programmable freezer only in combination with a prolonged cooling period.



## 1. Introduction

Semen cryopreservation has been established for horses since the late 1950s [1] and is of growing interest in horse breeding industry because an increasing number of breed associations allowed registration of foals born after insemination with frozen-thawed semen [2,3]. Many studies investigating different extenders and freezing procedures were carried out with the aim at improving fertility rates with frozen-thawed stallion semen [4]. In contrast to bull semen, the postthaw semen quality varies considerably among individual stallions, which in fact makes it difficult to find one particular freezing protocol useful and successful for all stallions [5].

Preservation of epididymal sperm is useful in endangered species, and the domestic horse can serve as a model for threatened wild equids [6,7]. Furthermore, in case of unexpected injury, which will end the breeding career, or cause death of valuable sires, collecting epididymal sperm might be the last chance to preserve their genetic material. In these cases, it is very important to use an optimal cryopreservation procedure because only a limited amount of epididymal sperm from a particular stallion will be available.

Every step of the preservation process influences sperm parameters. Damage of spermatozoa mostly occurs during

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the freezing and thawing process [8,9]. Resistance to cryopreservation injuries depends to a great extent on the composition of the plasma membrane [10]. During maturation along the epididymis, spermatozoa become fertile and motile, but as a consequence of remodeling of the plasma membrane, they also become more susceptible to cold shock [10]. The contact with seminal plasma (SP) during ejaculation initiates a further maturation process, leading to reduced resistance against cooling and freezing injury [9,11]. Therefore, epididymal spermatozoa may need different cooling and freezing protocols than ejaculated sperm.

To preserve spermatozoa, special equipment is necessary, but in case of an unexpected emergency, there may not always be an adequate laboratory nearby. Then, a styrofoam box with liquid nitrogen ($LN_2$) could serve as an easy, cost effective, and valuable way to cryopreserve gametes.

In this study, we compared four freezing methods using a programmable freezer and a styrofoam box filled with $LN_2$ and a floating rack. We investigated the differences in motion characteristics, morphology, and viability of frozen-thawed sperm from three different segments of the cauda epididymidis.

## 2. Material and Methods

### 2.1. Experimental Animals

Epididymides of 10 healthy stallions of different breeds (Warmblood, Lusitano, Thoroughbred, Trotter, and American Quarter Horse) were collected immediately after standardized routine castration under general anesthesia. Stallions were between 3- and 14-year-old and without history of previous ejaculation. Castration was performed because owners wanted to stop stallion behavior; therefore, it was not possible to compare data of ejaculated and epididymal sperm. Macroscopic examination of the testes and epididymides did not reveal any abnormalities. A 9-year-old healthy Shetland pony stallion with poor sperm quality was used as SP donor.

### 2.2. Collection of Epididymal Sperm

The cauda of both epididymides was dissected into three segments (E9—most caudal, E8—middle, and E7—most cranial segments; [12]). Spermatozoa were harvested by retrograde flush (E9) or mincing and incubation for 10 minutes at 37°C (E7 and E8). After recovery and incubation using 10-mL Dulbecco phosphate buffered saline (BioWhittaker; Lonza, Verviers, Belgium), semen was centrifuged (600 × g; 10 minutes), and the sperm pellet was extended using a commercial skim-milk extender to a concentration of 800 × $10^6$ sperm/mL (E9) and 80 × $10^6$ sperm/mL (E7 and E8) (EquiPro; Minitube, Tiefenbach, Germany). Then samples were diluted using an egg-yolk extender with 5% glycerol (Gent freezing extender; Minitube) to a concentration of 400 × $10^6$ sperm/mL (E9) and 40 × $10^6$ sperm/mL (E7 and E8). Final dilution of sperm comprised 2.5% of glycerol. Thereafter, semen was filled into 0.5-mL straws.

### 2.3. Freezing Protocols

Freezing was performed using either a programmable freezer or a styrofoam box filled with $LN_2$ and a floating rack. For both methods, we compared a fast and a slow freezing protocol. A total of four different freezing protocols were investigated (Table 1). Using the programmable freezer, for the fast and slow freezing processes, cooling rates of −1.0 and −0.1°C/min between 20°C and 4°C were performed, respectively. From 4°C to −140°C, a freezing rate of −60°C/min was applied. Then all straws were plunged into $LN_2$. For freezing in nitrogen vapor, straws were placed onto the floating freezing rack 5 cm above $LN_2$ surface for 20 minutes, either immediately after packaging at room temperature (fast) or after an equilibration time of 150 minutes at 4°C (slow). Thereafter, the straws were also plunged into $LN_2$.

Four aliquots of each segment of each epididymis were randomly allocated to one of the freezing processes. Therefore, the protocols could be compared among stallions and among the three segments of the cauda epididymidis.

After at least 1 month of storage in $LN_2$, one straw per epididymal segment per stallion per freezing protocol was thawed at 38°C for 20 seconds. Homologous SP was added to the thawed semen sample resulting in an 80% proportion of SP in the final volume. Then, motion characteristics were analyzed on a prewarmed microscope slide at 37°C. Seminal plasma was collected from a Shetland pony stallion, centrifuged, filtered (Millex GP Filter Unit; Millipore Express PES Membrane 0.22 μm; Millipore, Carrigtwohill, Ireland), stored at −20°C, thawed at room temperature, and prewarmed to 38°C before addition to the semen sample.

**Table 1**
Freezing protocols

| Protocol 1 | Protocol 2 | Protocol 3 | Protocol 4 |
|---|---|---|---|
| Programmable freezer | Programmable freezer | Nitrogen vapour | Nitrogen vapour |
| Fast freezing process | Slow freezing process | Fast freezing process | Slow freezing process |
| 20° C (-1.0° C/min) 4° C | 20° C (-0.1° C/min) 4° C | - | Refrigerator at 4° C for 150 min |
| 4° C (-60° C/min) -140° | 4° C (-60° C/min) -140° | 5 cm above $LN_2$ for 20 min | 5 cm above $LN_2$ for 20 min |
| $LN_2$ for at least 1 month | $LN_2$ for at least 1 month | $LN_2$ for at least 1 month | $LN_2$ for at least 1 month |
| Thawing: 38° C for 20 sec. | Thawing: 38° C for 20 sec. | Thawing: 38° C for 20 sec. | Thawing: 38° C for 20 sec. |
| Addition of homologous seminal plasma (80%) | Addition of homologous seminal plasma (80%) | Addition of homologous seminal plasma (80%) | Addition of homologous seminal plasma (80%) |

$LN_2$ means liquid nitrogen; values in brackets are cooling rates.