Cryopreservation of Equine Semen Loaded in Cryovials*

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ABSTRACT

Background: Over the past twenty years the assisted reproduction techniques reached a rapid advance in domestic species of economic interest. By the time the male gamete has provided to be the most successful tool used to improve animal breeding programs. Freezing and stock of semen is a safe procedure to preserve reproductive potential of animals with superior genetic heritage. In the horse industry, unlike observed in ruminants, the development of sperm cryopreservation techniques is very slow but despite the technical barriers the artificial insemination with frozen semen is growing.

Materials, Methods & Results: This experiment was divided in three steps. The first one compared the use of different diluents and freezing techniques. The other two stages were the use of cryogenic tubes for storing semen and the determination of mare pregnancy rates that were artificially inseminated with frozen semen packaged in cryogenic tubes. Six stallions were submitted to semen collection and twelve mares were artificially inseminated at first estrus of the breeding season. The samples were diluted in INRA82 or Nagase, containing 5% glycerol, with a concentration of 200 x 10^6 sperm/mL. A fraction of the diluted semen was stored in straws (0.5 mL), some of those straws were immediately frozen. The remaining straws were cooled from room temperature (± 24°C) to 5°C before freezing. The samples were thawed in a water bath at 37°C during 30 s. The same protocol with cooling prior to freezing was performed with samples filled into cryogenic tubes, and these samples were thawed in a water bath at 50°C during 100 s. The in vivo efficiency of cryopreserved semen was determined through artificial insemination (AI). The pregnancy diagnosis was performed after 20 days by ultrasound examination. After analyzing the data it was found that there was no significant difference in sperm motility and vigor among the tested diluents. Differences (P = 0.05) were observed when the diluted semen was previously cooled or frozen directly. Sperm motility obtained with semen diluted into INRA82 was 43.32% and 26.66%, and using Nagase solution the sperm motility was 41.08% and 24.44%, respectively. There were no differences regarding the vigor, motility and sperm defects among the diluents and the semen containers. The mares inseminated with semen loaded in cryovials showed 50% (3/6) pregnancy rate, and 16.66% (1/6) was observed in the mares inseminated with semen loaded in straws. All pregnant mares delivered health foals.

Discussion: The fertilizing capacity of frozen semen is influenced by several factors including stallion, semen characteristics, freezing technique, insemination dose, as well as status and management of the mare. Glycerol is the most commonly cryoprotectant used to preserve equine spermatozoa. We first investigate the efficiency of two semen extenders in promoting the survival of frozen spermatozoa. After that the use of two cryopreservation procedures, one cooling previously the semen samples at 5°C before freezing and the other by exposing the semen directly to liquid nitrogen vapor. The statistic analysis revealed no differences among the tested cryoprotectant solutions in provide sperm survival assessed by motility and vigor. Moreover, comparison of tested cryopreservation procedures revealed significant differences in sperm survival rates, taking into account both used semen extenders (INRA82 or Nagase). The fertilizing capacity of cryopreserved semen was checked by the AI of mares at the first estrus of the breeding season, with only one insemination performed close to ovulation. The sperm motility rates were higher when the semen was previously cooled, independent of the used semen extender. The survival rate of sperm samples loaded into straws or cryovials was similar. The semen stored in cryovials is a viable alternative for the use on the equine AI.

Keywords: semen, diluents, equine, freezing techniques, cryopreservation, cryovial.
INTRODUCTION

By the time the male gamete has provided to be the most successful tool used to improve animal breeding programs. In horses, unlike that obtained with cattle, semen cryopreservation progress slowly, but despite the barriers employment of equine AI has been growing [10]. The average results of sperm motility obtained with frozen/thawed semen vary from 30 to 50%, showing that semen cryopreservation is not yet an established technology in equine reproduction [27].

Usually stallion semen is stored in straws of 0.5 mL [27], however there are alternatives that use higher volumes, for example: big straws of 4 mL [12], glass macro tube of 12 mL [27], aluminum macrotube of 25 mL [25] and sachet of 15 mL [8]. The cryopreservation of equine semen packaged in 3 or 6 mL cryovials was already described [11]. On the other hand the comparison between 0.5 mL straw and 2 mL cryovial, as well as AI using frozen semen loaded into 2 mL cryovial has not been documented in the equine species.

The objectives of the experiment were to determine the rates of sperm motility and vigor of semen samples frozen immediately after collection or previously cooled at 5°C, diluted into INRA82 or Nagase extenders. Second, determine the sperm survival rates of samples that were diluted into Nagase extender and loaded into 2 mL cryovials. Third, determine the pregnancy and parturition rates of mares artificially inseminated with semen samples diluted in Nagase extender and loaded into cryovials.

MATERIALS AND METHODS

The salts and reagents when not identified in the text were supplied by Sigma Aldrich Ltda. The semen extenders solutions used in the experiments were prepared at the Laboratory of Embryology and Biotechnics of Reproduction, Faculty of Veterinary Medicine, Universidade Federal do Rio Grande do Sul (UFRGS).

The experiment was divided into three steps. The first step was held at Haras Warszawsky located in Porto Alegre, latitude 30° and longitude 51°, were it was compared the efficiency of two semen extenders and two freezing protocols. The data obtained allowed the definition of the protocol for cryopreservation of semen samples to be used in other experiment steps. In a farm of the Brazilian Army, Coudelaria de Rincão, located in São Borja, latitude 28° and longitude 56°, in state of Rio Grande do Sul were realized the other two steps, where it was tested in vitro and in vivo, utilization of cryovials for storage of cryopreserved semen.

The animals used in the experiments belonged to the Crioulo, Quarter Horse and Brasileiro de Hípismo breeds. Six stallions, with a mean age of five years, were selected after undergoing evaluation of potential breeding soundness and showed to have sperm motility above 70%. Twelve mares were available for the in vivo experiment step, with age between 4 and 17 years, and were artificially inseminated at first estrus of the breeding season.

A mare in estrus, properly restrained, was used as a dummy and semen collection performed with the aid of artificial vagina (Hannover model). The ejaculates, in number of three for each stallion were collected with intervals of 48 h.

The collected semen was evaluated for appearance, odor, color, volume, motility, vigor, pH, sperm morphology and concentration. Before the assessment the semen was filtered through sterile gauze to separate the gel fraction. Motility was evaluated from 0 to 100% and the vigor on a scale of 0 to 5, with the aid of a light microscope (x200) using a drop of semen between slide and cover slip preheated to 37°C. Two samples were identified and separated for further analysis: one sample was reserved for evaluation of sperm concentration using a Neubauer chamber containing diluted semen in formalin-saline solution (1:20), and another sample was used for analysis of sperm morphology by wet mount and evaluation by phase contrast microscopy (x1000). The latter was performed classifying the sperm in major and minor defects [3].

The skimmed milk extender [9] was used for semen centrifugation after collection, in the proportion of one part semen to one part of diluent. After the extender addition the diluted semen was transferred to eight plastic conical tubes and then centrifuged at 400 x g for 10 min [24]. The supernatant was removed and then the pellets were resuspended with the freezing extender, adjusting the concentration of the samples to 200 x 10^6 sperm/mL. Semen samples were diluted into the INRA82 [19] or by Nagase [16], both containing 5% glycerol.
The diluted semen of each ejaculate was loaded into eight straws (0.5 mL), four of which were immediately frozen by exposure to nitrogen vapor (-120°C) for 15 min, after plunged into liquid nitrogen and finally stored in a cryogenic container. The remaining four straws were previously cooled from room temperature (24°C) at 0.25°C/min to 5°C in a glass tube submerged in alcohol 70ºG, and maintained in domestic refrigerator. The straws, 15 min after reaching the 5°C, were subjected to the same freezing process described above. The sperm samples were thawed in a water bath at 37°C for 30 s. The freezing protocol of cryovial loaded sperm samples was the same as described above. The cryovials were thawed at 50°C for 100 s.

The heat resistance test (HRT) was performed after sperm samples were thawed and placed into 2 mL eppendorfs tubes, that were maintained in one incubator at 38°C during different times. Sperm motility and vigor were evaluated after 30, 60, 90 and 120 min. The in vivo efficiency of cryopreserved semen was determined through AI after follicular dynamics control by ultrasound (every 6 h). In females that presented ovarian follicle with a diameter equal to or greater than 40 mm was administered 1.500 UI (1.0 mL) of hCG intravenously [23], and the mares were inseminated as close as possible to ovulation. After receiving hCG the mares were randomly divided into two groups and subjected to one AI with the contents of four straws of 0.5 mL or a cryovial of 2 mL, with total sperm concentration of 400 x 10⁶. AI was performed by conventional method (in which the gloved hand of inseminator guided the pipette until the passage of the cervix), and semen is deposited in the body of the uterus. The pregnancy diagnosis was performed 20 days after AI by ultrasound and confirmed on further examination at 90 days.

The data obtained were statistically analyzed by analysis of Variance for Repeated Measures of the SAS program of the Department of Statistics in Institute of Mathematics, UFRGS.

**RESULTS**

The Table 1 presents the seminal evaluation findings of the stallions performed immediately after collection. Values represent the mean of three ejaculates from each stallion and meet the standards recommended by the Brazilian College of Animal Reproduction [5] for cryopreservation of equine semen.

The Table 2 shows that the sperm motility average was higher in samples frozen after previously cooling. Concerning the sperm vigor was no difference among the samples frozen immediately after collection or previously cooled.

The Table 3 shows the sperm characteristics examined and no differences between the data on thawed semen samples loaded in cryovials or in straws were observed.

<table>
<thead>
<tr>
<th>Stallion</th>
<th>Motility (%)</th>
<th>Vigor (0 – 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>73.3</td>
<td>4.0</td>
</tr>
<tr>
<td>B</td>
<td>83.3</td>
<td>3.6</td>
</tr>
<tr>
<td>C</td>
<td>83.3</td>
<td>3.3</td>
</tr>
<tr>
<td>D</td>
<td>75.0</td>
<td>3.3</td>
</tr>
</tbody>
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**Table 1.** Means of sperm motility and vigor of fresh collected equine semen.
The statistic analysis of sperm vigor shows no difference among the semen extenders, freezing protocols and tested store devices (Tables 2 and 3). The sperm samples loaded into cryovials or straws were submitted to the HRT (120 min) and shows similar sperm survival rates among the stallions D, E and F. The pregnancy rate obtained through AI using sperm samples loaded into straws (control group) was 16.6% (1/6), and 50% (3/6) with the use of sperm samples loaded into cryovials. All pregnant mares delivered health foals.

**DISCUSSION**

The fertilizing capacity of frozen semen is influenced by several factors including stallion, semen characteristics, freezing technique, insemination dose, as well as status and management of the mare [21]. Glycerol is the most commonly cryoprotectant used to preserve equine spermatozoa [1,15]. We first investigate the efficiency of two semen extenders in promoting the survival of frozen spermatozoa. After that the use of two cryopreservation procedures, one cooling previously the semen samples at 5°C before freezing and the other by exposing the semen directly to liquid nitrogen vapor. The statistic analysis revealed no differences among the tested cryoprotectant solutions in provide sperm survival assessed by motility and vigor (Table 2). Moreover, comparison of tested cryopreservation procedures revealed significant differences in sperm survival rates, taking into account both used semen extenders (INRA82 or Nagase) (Table 2). In others experiments [7,26] similar sperm motility rates were observed after thawing of semen previously cooled (46.7% and 47%) and without cooling (25.20% and 23%).

In assessing the survival of semen through the HRT, it was observed that regardless of the type of store device was a sperm motility decrease and
Vigor throughout the test. This reduction on sperm viability was observed in other experiments [7,17], using only straws for sperm filling. Moreover, it was reported for semen loaded into straws 10% motility and vigor 2 after 120 min sperm incubation at 38°C [27], different of our observations 5.55% of motility and vigor 1 for semen loaded into straws, and 5.11% of motility and vigor 1 for semen loaded into cryovials.

An experiment [11] comparing different semen store devices described similar results (39.3% sperm motility for semen loaded into 0.5 mL straws, and 31.7% sperm motility for semen loaded into 3.6 mL cryovials) as observed in our study for semen loaded into 2.0 mL cryovials (41.9%). These authors also did not achieve significant differences among the types of tested semen store devices. A comparison of three semen store devices: mini-straw (0.25 mL), french straw (0.5 mL) and macrotube (4 mL), showed that the mini-straws promoted better sperm survival rates [6]. These data differs from our observation, where sperm motility rates were similar when the semen was loaded into 2 mL cryovials or 0.5 mL straws. However, it can take into account the observation that cell damage is minimized when the semen is frozen in large volumes [2]. This observation was confirmed by other experiment [21] were the highest post-thaw progressive motility was obtained when the semen was loaded into 12 mL cryovials (50%).

A single insemination requires the use of multiple straws [20], on the other hand the advantage of employing the cryovials for AI is the need of only one cryovial. It was highlighted that the use of multiple straws increases the risks of failures, since they need to be thawed, dried, cut, have the contents emptied and subsequently loaded again together into one insemination dose [11]. The insemination dose divided among several straws increases the risks of contamination, damage, loss or technical error [21]. Cryovials were easy to filling and to identifies, and it is internationally accepted as a benchmark of health security [4]. The quality of post thaw semen loaded into cryovials is acceptable and allows to filling the entire insemination dose [11]. Thus, we observed that the handling of cryovials are easy, fast, safe, and can optimize the storage capacity of the cryogenics container, and moreover allows the use of the entire insemination dose filled into a single cryovial, thus facilitating the AI technical procedure.

The fertilizing capacity of cryopreserved semen was checked by the AI of mares at the first estrus of the breeding season, with only one insemination performed close to ovulation. The mares inseminated with cryovial showed pregnancy rate of 50% (6/3), however, these data were not analyzed statistically because of the low sample number. Two others experiment also working with only one insemination, reported an average of 42.2% [23] and 45% [14] pregnancy rate. This result was similar to the 54% pregnancy rate described in mares inseminated in the first season estrous cycle with semen loaded in straws [26]. On the other hand we observed only 16.6% (1/6) pregnancy rate of mares inseminated with sperm loaded into straws (control group), similar to the 11.9% [18] also using straws. No pregnancies were reported [13] working with the same protocol after the artificial insemination of 15 mares.

CONCLUSIONS

The sperm motility rates were higher when the semen was previously cooled, independent of the used semen extender. The survival rate of sperm samples loaded into straws or cryovials was similar. The semen stored in cryovials is a viable alternative for the use on the equine AI.

SOURCES AND MANUFACTURERS

1Sigma Aldrich Ltda., São Paulo, SP, Brazil.
2Profasi® - Intervet do Brasil, Cotia, SP, Brazil.

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