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## Pregnancy outcomes using stallion epididymal sperm stored at 5 °C for 24 or 48 hours before harvest

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### ABSTRACT

The cryopreservation of epididymal sperm can be useful in a variety of circumstances for ensuring genetic preservation of a valued stallion. Although early studies have reported pregnancy rates significantly lower than those achieved with cryopreserved ejaculated sperm, two recent studies report over 60% one-cycle pregnancy rates with epididymal sperm stored for 24 hours at 5 °C before harvest and cryopreservation. The aims of this study were to: (1) attempt to replicate the one-cycle pregnancy rate of over 60% using epididymal sperm cooled and stored within the epididymis for 24 hours before harvest and cryopreservation and (2) evaluate pregnancy outcome with sperm cooled and stored within the epididymis for 48 hours before sperm harvest and cryopreservation. Testicles were obtained from 13 stallions undergoing routine castration. The epididymides were stored at 5 °C for either 24 or 48 hours before sperm harvest and cryopreservation in an egg yolk and dimethylformamide-based freezing extender. Thirteen mares were bred on one cycle with cryopreserved epididymal sperm stored for 24 hours before harvest, and 10 of those 13 mares were also bred on a previous or subsequent cycle with samples from the same stallion that had been stored for 48 hours before harvest. Pregnancy occurred in 7 of the 13 inseminations of sperm stored for 24 hours before harvest, and in 4 of the 10 inseminations of sperm stored for 48 hours before harvest. The pregnancy rate using epididymal sperm stored for 24 hours before harvest is consistent with that of previous reports. In addition, these results provide evidence that pregnancies can be achieved when the epididymides are cooled and stored for 48 hours before sperm harvest and cryopreservation.

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### 1. Introduction

Pregnancies resulting from artificial insemination with cryopreserved epididymal stallion sperm were first reported in 1957 [1], and the technique is now used clinically as a means of preserving the genetics of valuable stallions that have died unexpectedly, require castration of one or both testicles, or are otherwise affected by a condition that

renders semen collection difficult or impossible. Since 1957, refinement of the technique for harvesting epididymal sperm has resulted in a substantial increase in the number of sperm harvested and a resultant increase in the number of insemination doses produced [2]. This has made the process more cost-effective. In addition, it has been shown that epididymal sperm can be stored within the epididymis for up to 24 hours at 5 °C before harvest and cryopreservation without a significant loss in post-thaw motility [2–5].

To date, only four studies have reported on pregnancy rates in mares bred with cryopreserved stallion epididymal sperm. The first two studies used a variety of different sperm processing methods, breeding dosages, and

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insemination techniques, and reported pregnancy rates ranging from 7% to 30% [6,7]. These pregnancy rates are lower than those typically reported for cryopreserved ejaculated sperm [8,9]. The remaining two studies used a recently developed stallion freezing extender (Botu-Crio; Botupharma Biotechnology, Botucatu, Brazil) that contains egg yolk and dimethylformamide, among other proprietary ingredients, along with the same sperm processing and insemination techniques [10,11]. Their post-thaw per-cycle pregnancy rates ranged from 61% to 92%, which are not only above the published minimum pregnancy rates for cryopreserved ejaculated sperm, but represent an improvement in these rates. The most recent of these studies also reported identical pregnancy rates for mares bred with frozen-thawed ejaculated sperm (61.5% or 8 of 13 mares bred) and frozen-thawed epididymal sperm cryopreserved after 24 hours of epididymal storage at 5 °C (61.5% or 8 of 13 mares bred) obtained from the same stallion after castration [11]. As both of these studies were performed in a single laboratory, and similar pregnancy rates have not yet been reported by others, our first objective was to attempt to replicate a per cycle pregnancy rate of 60% or higher using epididymal sperm stored at 5 °C within the epididymis for 24 hours before harvest and cryopreservation.

Despite these findings, there are still limitations with regard to the feasibility of the procedure in the clinical setting, particularly with regard to shipping of epididymides for processing within 24 hours of castration. The ability to achieve acceptable pregnancy rates after storage times of longer than 24 hours before processing would ameliorate many of the limitations associated when weekend or international shipments are necessary. Although fertility has not been evaluated in stallion epididymal sperm that has been stored at 5 °C for longer than 24 hours, many studies across multiple species have confirmed that the post-thaw motility of cryopreserved epididymal sperm remains unchanged after epididymal storage periods of 24 hours and longer [3,4,12–15]. Considering these data, it is reasonable to speculate that stallion sperm cryopreserved following well over 24 hours of cooled storage within the epididymis may maintain good post-thaw motility and result in acceptable pregnancy rates. Our second objective, therefore, was to evaluate pregnancy outcome in mares bred with sperm stored at 5 °C within the epididymis for 48 hours before sperm harvest and cryopreservation.

## 2. Materials and methods

### 2.1. Animals

Animal procedures for this work were approved by the Animal Care and Use Committee of the University of Pennsylvania. Thirteen light horse and small pony stallions, aged 2 to 12 years, were used. The testicles and epididymides were obtained via routine castration of pony stallions from the Havemeyer Equine Behavior Lab ( $n = 3$ ), horse stallions presented for routine castration at New Bolton Center's Widener Hospital for Large Animals ( $n = 9$ ),

and a single stallion from which testicles were recovered after humane euthanasia ( $n = 1$ ). All testicles were grossly normal. The three ponies and two of the horses presented for castration had sired offspring. For the remaining eight stallions, breeding history was unavailable.

Thirteen light horse mares of various breeds, ranging in age from 3 to 12 years, that were maintained by the University of Pennsylvania School of Veterinary Medicine's teaching or embryo recipient programs were used for the breeding trials. All mares were reproductively normal with histories of good fertility.

### 2.2. Epididymal storage

After castration, a ligature was placed around the cut end of the vas deferens to prevent leakage of sperm. Each epididymis was dissected free from the associated proper ligament of the testis, and a second ligature was placed around the body of the epididymis to isolate the sperm within the epididymal tail between the two ligatures. Each epididymis was then transected through the body just distal to the second ligature and was placed in an individual Whirl-Pak bag (Teel Classic, Baraboo, WI, USA) with 1.0 mL of sterile lactated Ringer's solution. Paired epididymides were stored at 5 °C, with one epididymis from each pair being stored for 24 hours and the other for 48 hours before flushing. As Botupharma products were not commercially available in the United States at the time of this study, a delay in the import of products from Brazil necessitated that the first four sets of epididymides be flushed with INRA 96 (IMV International Corp., Maple Grove, MN, USA) semen extender and stored in Equitainers (Hamilton Research Inc., Ipswich, MD, USA), whereas the remaining nine sets were flushed with Botu-Semen (Botupharma, Botucatu, Brazil) semen extender and stored in Botutainers (Botupharma, Botucatu, Brazil).

### 2.3. Sperm harvest and cryopreservation

After 24 or 48 hours of storage at 5 °C, sperm were harvested from the epididymides using the retrograde flush method described by Monteiro et al. (2011). After harvest, the entire suspension of epididymal sperm and extender was transferred to a 50-mL polypropylene conical-bottomed tube (Corning Inc. Life Sciences, Tewksbury, MA, USA) and underlain with 1 mL of iodixanol density gradient medium (OptiPrep, Sigma-Aldrich, St. Louis, MO, USA). Samples were centrifuged at  $1000 \times g$  for 20 minutes at ambient temperature. Immediately after centrifugation, as much of the supernatant and iodixanol medium were removed as was possible without disruption of the sperm pellet. The sperm pellet was then resuspended to a concentration of 400 million cells/mL with temperature-matched freezing media (Botu-Crio, Botupharma) and loaded into 0.5-mL polyamide-polyethylene straws (IMV Technologies, L'Aigle Cedex, France). Straws were individually sealed using an ultrasonic sealer (Ultraseal 21, MTG Medical Technology, Bruckberg, Germany) and then incubated at 4 °C for 20 minutes. After cooling, packaged sperm were frozen in liquid nitrogen

vapor by placing the straws on a rack suspended 3 cm over liquid nitrogen for 20 minutes. Frozen straws were then immediately plunged into the liquid nitrogen. Straws were thawed in a 46 °C water bath for 20 seconds before evaluation and artificial insemination.

#### 2.4. Sperm motility and viability

Sperm motility was determined by computer-assisted sperm analysis (IVOS; Hamilton Thorne, Beverly, MA, USA) using the settings described by Monteiro et al. (2011). Total and progressive sperm motilities were measured after final dilution in the cryopreservation extender (pre-freeze) and again at 5 minutes after thawing. Membrane integrity was evaluated at the same time points based on exclusion of propidium iodide as measured by a fluorescence-based automated cell counting machine (NucleoCounter SP-100; ChemoMetec, Allerød, Denmark).

#### 2.5. Breeding trials

In a crossover design, mares were bred on two separate estrous cycles with cryopreserved–thawed epididymal sperm from the same stallion that had been stored for either 24 (St-24h) or 48 hours (St-48h) before sperm harvest and cryopreservation. The first seven mares were bred with St-24h samples on the first cycle, followed by St-48h samples on the second cycle. The remaining six mares were to be bred first with St-48h and then with St-24h; however, three of the St-48h samples did not contain sufficient numbers of motile sperm to meet our predetermined insemination dose. Therefore, these three mares were only bred with St-24h samples.

For breeding management, mares were administered cloprostenol sodium (Estrumate 250 µg i.m.) to induce luteolysis. Each mare's reproductive tract was monitored daily by transrectal palpation and ultrasonographic examination. For ovulation induction, hCG was administered (Chorulon 2500 IU i.v.) on the first day that the dominant follicle measured 35 mm. A two-dose insemination technique was used to breed all mares. The first insemination was performed before ovulation at 30 hours after hCG administration, and the second was performed after ovulation at 42 hours after hCG administration. If ovulation had not occurred by 42 hours after hCG administration, the mare was reexamined every 8 hours until ovulation was confirmed, and the second breeding dose was inseminated at that time. All inseminations were performed using a deep-horn technique with a flexible 96-cm pipette (MOFA Global, Verona, WI, USA), using the lowest number of straws necessary to deliver a breeding dose containing a minimum of 800 million motile sperm cells. Pregnancy evaluation was performed using transrectal ultrasonography at 13 to 17 days after ovulation, and 250 µg of cloprostenol was administered by intramuscular injection at this time to shorten the interestrus interval for rebreeding.

#### 2.6. Management of intrauterine fluid

Mares were evaluated by transrectal ultrasonography for intrauterine fluid development immediately before and

after inseminations in addition to evaluation at 24-hour intervals after the second insemination. Fluid was measured at the largest pocket identified within the uterine body. Mares that had more than 3 cm of fluid at the time of insemination received a uterine lavage with 2 liters of sterile lactated Ringer's solution immediately before insemination. Mares that had 1 to 3 cm of fluid at any point after the second insemination received 1 mL of oxytocin (20 IU/mL) administered intravenously every 8 hours over the subsequent 48-hour period. Mares that had greater than 3 cm of fluid at any point after the second insemination received a uterine lavage with 2 liters of sterile lactated Ringer's solution followed by 1 mL of oxytocin (20 IU/mL) administered intravenously every 8 hours over the subsequent 48-hour period.

#### 2.7. Statistical analysis

Normality of the continuous data and equality of variances were assessed using the Shapiro–Wilk and Levene's tests, respectively. Comparison of the proportion of pregnancy after 24 hours versus 48 hours of epididymal storage was done using the McNemar test for paired proportions. The adjusted Wald method was used to calculate the 95% confidence intervals (CIs). Epididymal sperm pre-freeze and post-thaw motility and viability variables of samples that resulted in pregnancies versus samples that did not were compared using the Student's *t* test. Epididymal sperm motility and viability variables of St-24h versus St-48h were compared using the paired *t* test. For all analyses, *P* value less than 0.05 was considered significant.

### 3. Results

The one-cycle proportion of pregnancy observed for the breeding trial was 7 of 13 (53.8%; 95% CI: 29.1%–76.8%) for St-24h samples, and 4 of 10 (40%; 95% CI: 16.7%–68.8%) for St-48h samples. Differences in the proportions of pregnancies between the St-24h and St-48h groups were not statistically significant (*P* = 0.500).

When bred with St-24h, 7 of the 13 mares were diagnosed pregnant (53.8%; 95% CI: 29.1%–76.8%); and when bred with St-48h samples, 4 of the 10 mares were diagnosed pregnant (40%; 95% CI: 16.7%–68.8%). Differences in the proportion of pregnancies between the St-24h and St-48h groups were not statistically significant (*P* = 0.500); however, the power was insufficient to detect a difference, and therefore, we cannot conclude that these proportions are similar.

Table 1 summarizes progressive motility (PM) and viability of the St-24h and St-48h samples, and Table 2 summarizes PM and viability for St-24h and St-48h samples that resulted in pregnancy and those that did not. Although pre-freeze and post-thaw PMs were significantly higher for St-24h samples than for St-48h samples, there was no difference in PM or viability in those samples that resulted in pregnancies and those that did not. No further statistically significant differences were identified; however, resulting variation provided insufficient power to meaningfully conclude that no differences existed.

**Table 1**

Pre-freeze and post-thaw motility and viability (mean  $\pm$  standard deviation) of 24 hour- and 48 hour-stored samples (n = 10).

	24 h	48 h	P value
Pre-freeze progressive motility (%)	48.5 $\pm$ 13.1	28.6 $\pm$ 9.4	<0.001
Pre-freeze viability (%)	84.6 $\pm$ 11.7	89.4 $\pm$ 7.0	
Post-thaw progressive motility (%)	18.7 $\pm$ 6.8	10.7 $\pm$ 4.9	0.001
Post-thaw viability (%)	38.9 $\pm$ 13.7	38.3 $\pm$ 10.2	

#### 4. Discussion

In this study, the one-cycle proportion of pregnancy for mares bred with cryopreserved epididymal sperm stored at 5 °C for 24 hours before processing was 7 of 13. This is similar to the pregnancy rates for 24-hour cooled, stored epididymal sperm that were previously reported by Papa et al. [10] in 2008 (12 of 18) and Monteiro et al. in 2011 (8 of 13) [11]. The Monteiro study also reported a remarkable per-cycle pregnancy rate of 92% (12 of 13) for mares bred with epididymal sperm that was cryopreserved immediately after recovery from the epididymis [10]. All these studies were performed at the same institution. We now have replicated these pregnancy rates at a different institution with a different group of researchers following this previously published protocol. Taken together, these data provide compelling evidence that the pregnancy rates using cryopreserved epididymal sperm can equal or exceed those achieved using cryopreserved ejaculated sperm [8,9]. These findings render the cryopreservation of epididymal sperm a much more practical and likely cost-effective process than previously considered. This is likely to impact owners' decisions on whether to preserve epididymal sperm from a stallion at the time of castration or euthanasia.

Although multiple studies have estimated the fertility of stallion epididymal sperm based on various sperm parameters, only four studies to date have reported pregnancy rates after breeding mares with cryopreserved epididymal sperm [6,7,10,11]. When compared to these studies, the pregnancy rates reported in this study, along with those of Monteiro et al. and Papa et al., are notably higher than those reported by Morris et al. (2002; 17%–30%) and Heise et al. (2010; 6.7%–27.8%) [6,7,10,11].

**Table 2**

Pre-freeze and post-thaw motility and viability (mean  $\pm$  standard deviation) of the epididymal sperm samples that did, and that did not result in pregnancy.

St-24h	Pregnancy	No pregnancy
	n = 6	n = 7
Pre-freeze progressive motility (%)	47.4 $\pm$ 14.5	49.8 $\pm$ 13.8
Pre-freeze viability (%)	89.1 $\pm$ 7.6	79.3 $\pm$ 14.0
Post-thaw progressive motility (%)	16.3 $\pm$ 7.0	21.5 $\pm$ 5.7
Post-thaw viability (%)	37.9 $\pm$ 13.2	40.2 $\pm$ 15.4
St-48h	n = 4	n = 6
Pre-freeze progressive motility (%)	26.0 $\pm$ 9.9	28.0 $\pm$ 8.3
Pre-freeze viability (%)	93.8 $\pm$ 0.5	87.2 $\pm$ 8.5
Post-thaw progressive motility (%)	9.0 $\pm$ 2.5	10.5 $\pm$ 5.3
Post-thaw viability (%)	35.3 $\pm$ 11.9	42.0 $\pm$ 8.8

One similarity between the present study and the other two “high pregnancy rate” studies [10,11], is that the same commercially available freezing extender was used (Botu-Crio), whereas the two “low pregnancy rate” studies used different skim milk or lactose/egg yolk/glycerol-based freezing extenders [6,7]. As Botu-Crio extender has been shown to result in significant improvements in post-thaw epididymal sperm measures when compared to selected other available cryopreservation extenders [10], the inclusion of this extender in the freezing protocol is one potential contributing factor to the improved pregnancy rates.

It is also worth noting that at least two of the three “high pregnancy rate” studies (this study and [11]) used deep-horn insemination technique with 800 million motile or viable sperm, as compared to a body insemination with 200 million progressively motile sperm by Heise et al., and either hysteroscopic or body insemination with 6 to 115 million progressively motile sperm by Morris et al. [6,7]. It is therefore possible that the higher pregnancy rates are at least in part attributable to the greater number of progressively motile sperm in the breeding dose and the insemination technique used.

The four previous studies reporting pregnancy rates using cryopreserved stallion epididymal sperm involved methodological variations that could have impacted pregnancy rates [6,7,10,11]. These variations include: epididymal flush technique, sperm processing technique, freezing protocol, thawing protocol, mare cycle management, and timing of insemination with relation to administration of the ovulation induction agent.

The present study clearly documents for the first time that pregnancy can result when mares are bred with epididymal sperm stored at 5 °C for 48 hours before sperm harvest and cryopreservation, with 4 of 10 (40%) mares achieving pregnancy. There is a possible physiologic basis for our finding that epididymal sperm is capable of resulting in pregnancies despite longer storage times. Although epididymal sperm are capable of motility while still within the epididymal cauda, they are maintained in an immotile, metabolically quiescent state until ejaculation [16–18]. These adaptations are believed to preserve sperm viability in the epididymis for longer periods of time compared to ejaculated sperm. Therefore, it seems intuitive that sperm maintained in the epididymis could survive longer storage times than ejaculated sperm, at least as long as epididymal somatic cells remain functional. In this regard, it has been shown that stallion sperm exhibit no loss of motility or viability when held in cooled storage within the epididymis for up to 96 hours post-mortem [4]. Although we only examined epididymal sperm stored for up to 48 hours before harvest and freezing, it would be very interesting to determine whether even longer storage times support sperm fertility.

Pre-freeze and post-thaw PM's were significantly lower in St-48 samples than in St-24 samples. These results are consistent with those of a previous study in which PM was the sperm parameter most adversely affected by longer epididymal storage times [19]. Certainly, further and larger studies will be required to adequately address the importance of lower PM (or other *in vitro* measures of sperm motility) and viability to pregnancy outcome [19–21].

In the present study, four stallions did not achieve a pregnancy with either the St-24h or St-48h samples, two stallions achieved pregnancy with the St-24h sample but not the St-48h sample, and all four of the stallions that achieved pregnancy with St-48h samples, also achieved pregnancy with St-24h samples. None of the stallions achieved a pregnancy with the St-48h sample and not with the St-24h sample. Although this study was not designed to critically examine variability in fertility of epididymal sperm among stallions, these findings do suggest that epididymal sperm from some stallions may be innately more tolerant of storage and cryopreservation than epididymal sperm from other stallions. To our knowledge, only one other study recorded information on the variability in “freezability” of epididymal sperm among individual stallions [22]. Similar to our study, these researchers reported increased odds of pregnancy resulting from breeding with cryopreserved epididymal sperm from some stallions compared to others. However, without an *in vitro* sperm parameter that can be used to predict the fertility of cryopreserved epididymal sperm, stallions that are “good epididymal freezers” can only be differentiated from “marginal” or “poor epididymal freezers” retroactively based on pregnancy rates.

In summary, we were able to closely replicate the one-cycle pregnancy rates reported by others [10,11]. These findings provide further evidence that breeding mares with cryopreserved epididymal sperm can result in pregnancy rates similar to those achieved by breeding mares with cryopreserved ejaculated sperm. Factors contributing to these higher pregnancy rates remain to be delineated but could be attributed to a variety of differences among study protocols, including the use of higher sperm numbers combined with deep-horn insemination, or the use of specific cryopreservation extender. Furthermore, these results indicate that pregnancies can be achieved using cryopreserved sperm harvested from stallion epididymides that have been stored at 5 °C for up to 48 hours. Thus, harvesting epididymal sperm from a valuable breeding stallion may be worthwhile even if circumstances prevent the delivery of the epididymides to a capable processing facility within 24 hours of castration.

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