Frozen semen: A breeding protocol that results in high fecundity

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Summary

Farrowing rate and number of pigs born alive were compared after use of either frozen semen or combined fresh and frozen semen matings in nulliparous gilts. Estrus was synchronized using Altrenogest (AT: Regu-Mate® Solution, Hoescht Roussel Vet, Warren, New Jersey) and detected with a mature boar. The farrowing rate was 74% for females inseminated with frozen semen, and 88% for females inseminated with a combination of fresh and frozen semen. There were 12.8 pigs born per litter when frozen semen was used, and 12.9 when a combination of fresh and frozen semen was used. These data suggest that when a managed system is used, breeding with frozen semen may result in acceptable production.

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The search for a successful cryopreservation protocol for semen can be traced back 200 years to the discovery that human, stallion, and frog spermatozoa rendered inactive by cooling in snow could be revived.[1,2] The subsequent finding by Polge et al,[3] that glycerol protects the sperm cell's structural integrity during freezing, opened the door to the development of cryopreservation procedures for semen and other cell types of a variety of species. The first successful freezing and thawing of swine semen was reported simultaneously by Crabo and Einarsson,[4] Graham et al,[5] and Pursel and Johnson.[6] While these original freezing protocols have undergone many changes, the farrowing rates obtained with the use of frozen semen today continue to average between 50% and 60%, with litter sizes of seven to 10 pigs.[7] These values are still appreciably lower than those observed when fresh semen is used. As a result, use of frozen semen in commercial breeding programs has been very limited.[7]

Acceptance of frozen semen as an alternative to fresh semen could be greatly enhanced if the fecundity associated with today's frozen/thawed semen could be increased. This was the objective of the current case study.

Preparation of frozen and fresh semen

Semen was provided by four boars that represented two genetic lines. Two of the boars were half siblings, and all were >1 year of age. Only one boar had proven fertility, i.e., had sired a litter prior to semen collection and freezing. Five months before beginning the estrus synchronization and insemination protocol described below, 240 doses of semen were frozen. Semen collection was performed not more than twice weekly on each boar. Semen was collected into a warmed thermos cup containing 50 mL of Androhep extender (Minitube, Verona, Wisconsin) that included 500 mg/mL of gentamicin sulfate. Motility was scored grossly at 40x and 100x magnification using the following scale: 0, dead or no sperm; 1, poor (sperm only wriggling); 2, fair; 3, good (clumping present); 4, good/very good (some clumping with some wave motion); 5, very good (wave motion present). Only ejaculates that received a score of at least 4 were extended for freezing or used as fresh semen for AI.

Before freezing, semen was extended by the addition of 1.5× its volume of Androhep extender at a temperature within 1°C of the semen. The plastic 250-mL bottle containing the extended semen was placed in a 1-liter beaker that contained 150 mL of extender at the same temperature as the semen. Extended semen was allowed to cool to room temperature (21°C to 23°C) over a 4-hour period. The beaker containing the bottle of extended semen was then placed in a refrigerator (17°C) and allowed to cool overnight. Semen in 5-mL macrotubes (5×10^9 sperm/macrotube) was frozen the next morning in liquid nitrogen vapor as described by Westendorf et al,[8] except that the prediluter used was Androhep extender. The freezing chamber was a Styrofoam box $41 \times 48 \times 30$ cm (length × width × depth), filled to a depth of 2 cm with liquid nitrogen.

At the end of each freezing trial, the proportion of live sperm in one thawed dose of semen was determined by supravital staining with a 2% solution of eosin G. One individual evaluated a total of 200 sperm in five fields on one side. The proportion of sperm that exhibited forward motility was assessed in a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). If motility was less than 15%, the entire complement of frozen doses was discarded. Macrostraws of frozen semen were transported to the breeding facility in a liquid nitrogen vapor "dry" shipping tank.

Fresh semen was collected from the same four boars 24 hours prior to inseminating the gilts. Semen was collected into a thermos containing 50 mL of Androhep extender warmed to 37°C. Additional extender was added to yield a final ratio of 1.5 parts extender to 1 part semen (vol. : vol.). Extended semen was aliquoted into 100-mL bottles (5×10^9 sperm per bottle), which were placed in a Styrofoam cooler. In order to maintain the temperature at 17°C, two partially thawed 250-mL bottles of acetic acid, which melts at approximately 17°C, were packaged with the semen bottles. The Styrofoam container was placed in a cardboard box and shipped overnight to the breeding farm. Upon arrival, the temperature of the semen in one

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bottle was measured, and a small sample of extended semen was removed, checked for gross motility, and scored as described. All semen bottles were placed in a refrigerator at 17°C, and were maintained at this temperature until used for insemination. Fresh semen was used within 24 hours of receipt.

Estrus synchronization

Altrenogest (AT: Regu-Mate® Solution, Hoescht Russel Vet, Warren, New Jersey) was fed (top-dressed) to 61 sexually mature gilts at a dosage of 10 mg per gilt per day for 20 to 24 days (AT is not labeled for use in swine). The last day of AT feeding was staggered so that different groups of 12 gilts had their last dose of AT over a period of 5 consecutive days, ensuring that estrus and insemination would be distributed over a 6- to 8-day period. This was necessary because caesarean sections (c-sections) were scheduled for all sows after 114 days of gestation, and it was calculated that a maximum of 10 c-sections could be performed daily. Twenty-four hours after the last feeding of AT, all gilts were treated with 10 mg of prostaglandin-$F_{2\alpha}$ (Lutalyse®, Pharmacia & Upjohn, Kalamazoo, Michigan). Sixty gilts expressed estrus and were bred.

Estrus detection and insemination

Estrus detection began 3 days after the last dose of AT was fed. Gilts were exposed to a sexually mature boar three times daily at 6-hour intervals. A gilt was considered to be in estrus if she exhibited a strong lordosis response in the presence of the boar, i.e., she allowed an individual to sit on her back. Beginning 30 hours after the onset of estrus, 34 gilts were inseminated with frozen semen only, two with fresh semen only, and 24 with alternating doses of frozen and fresh semen. Gilts inseminated with frozen semen only or with alternating doses of frozen and fresh semen were bred four times, 30, 36, 42, and 48 hours after the onset of estrus. Gilts inseminated with fresh semen only were bred three times, 30, 36, and 42 hours after the onset of estrus. The first day of insemination was designated day 0. Frozen semen was deposited in the female using sterile, disposable spiretes immediately after thawing. No gilts received frozen semen from the same ejaculate for all four inseminations.

Thawing protocol for frozen semen

Prior to thawing, the water temperatures of a double-chambered water bath were set at 50°C and 37°C. Androhep extender was prepared according to the manufacturer's instructions at least 1 hour prior to use, and 80-mL aliquots were dispensed into 100-mL insemination bottles. Extender bottles were placed in the 37°C water bath chamber and allowed to warm to that temperature. When the bottles had reached 37°C (after approximately 20 minutes), a timer was set for 40 seconds. A single straw was removed from the liquid nitrogen tank, plunged into the 50°C water bath, and gently swirled for 40 seconds. The straw was removed and wiped off with a paper towel, and one end was clipped with scissors. The clipped end was placed in a bottle of prewarmed Androhep extender, and the other end of the straw was clipped. The straw was then rinsed by aspirating and expelling a small amount of extender. The thawed semen was transported to the breeding area and deposited in the female within 5 minutes of thawing.

Cesarean derivation of piglets

C-sections were performed between days 115 and 117 of gestation, except for one that was performed on day 120. Nellor et al[9] (1975) demonstrated that when gestation was prolonged for 7–11 days by feeding 6-methyl-17-acetoxy-progesterone (MAP), there was no detrimental effect on survival of piglets derived by c-section from purebred Hampshire, Yorkshire, and Hampshire-Yorkshire crossbred gilts (control gestation, 114 days). We expected similar results with the pigs in this study, as the mean gestation length in the herd from which the gilts were obtained was also 114 days, and gestation was not prolonged more than 6 days. Natural parturition was delayed by feeding AT at a dosage of 20 mg per gilt per day, from day 100 of gestation until the day of the c-section. Sows were euthanized immediately after surgery by IV administration of pentobarbital, 80 mg/kg BW. (MAP is not labelled for use in swine.)

Statistics

The pregnancy rate and mean number of pigs born alive was similar for gilts bred with fresh semen alone and those bred with alternating frozen and fresh semen; therefore, these two groups were pooled into the Fresh treatment group (n=26). The Frozen treatment group (n=34) consisted of gilts bred with frozen semen only. The effect of semen type on pregnancy rate and proportion of mummified fetuses was analyzed by Chi square. The relationships between semen types, the mean day of gestation on which c-sections were performed, and the mean number of live born pigs were analyzed by T-test.[10]

Results

Semen was frozen from 25 collections made over a 3-month period (Table 1). Each freezing trial produced an average of 9.6 ± 0.7 doses. Sperm concentration was determined prior to freezing; after thawing, the average (± SE) motility was 28.2% ± 1.6 and the average (± SE) proportion of viable sperm was 32.2% ± 2.0. The mean (± SE) length of gestation prior to c-section was significantly greater (P=.027) for gilts in the Frozen group (116.1 days ± 0.3) than for those in the Fresh group (115.0 days ± 0.1). Pregnancy rate, mean number of live born pigs, and proportion of mummified fetuses did not differ significantly between the Frozen and Fresh treatment groups (Table 2).

Discussion

Our results show that artificial insemination with frozen semen may yield a mean number of live born pigs similar to that observed when fresh semen is used. The pregnancy rate did not differ by semen type. The protocol used in this study consisted of a "3 × 4 × 6" estrus detection and insemination protocol, i.e., estrus detection was carried out 3 times daily and gilts were inseminated with frozen-thawed semen a total of 4 times, once every 6 hours. This protocol may have been successful when combined with estrus synchronization

Table 1: Collection frequency and ejaculate sperm concentrations in semen frozen from four boars

Boar ID	A	B	C	D
Total number of ejaculates	2	9	12	2
Total number of frozen doses	15	84	121	20
Sperm concentration (range × 10^6 sperm/mL)	456–560	468–806	582–1350	491–820

Table 2: Effect of using frozen semen or alternating fresh and frozen semen on the pregnancy rate, mean number of pigs born alive, and proportion of mummified fetuses delivered in primiparous gilts

Semen type/protocol	Pregnancy rate (%)	Mean (±SE) number of pigs born live	Mummified fetuses born (%)
Fresh*	88 (23/26)	12.9 ± 0.7	1
Frozen	74 (25/34)	12.8 ± 0.7	3

* Fresh semen alone or used in an alternating schedule with frozen semen

using AT because both procedures allow multiple inseminations to be scheduled so that they are likely to occur near the time of ovulation.

When frozen semen is used, the ovum and sperm must be brought together in the oviduct as soon as possible after the semen has been thawed, because the freezing-thawing process causes partial decapitation of the sperm. Both fully and partially decapitated sperm are viable in the female tract for only a short period of time. According to Watson,[11] frozen/thawed sperm are "activated preparatory to meeting the oocyte, and if this does not occur within a short time, they will die." The swine practitioner must ensure that viable thawed sperm reach the oviduct as close to the time of ovulation as possible. Ovulation in swine is most likely to begin approximately 30–57 hours after the onset of estrus;[12,13] therefore, multiple inseminations should be scheduled throughout this 27-hour period.

In this study, the first insemination was performed 30 hours after the onset of estrus, followed by three additional inseminations at 6-hour intervals, in order to adequately cover the 27-hour window associated with the onset of estrus. The farrowing rate and average number of live born pigs were superior to those reported by several studies in which semen frozen in maxi-straws was used in a commercial breeding program.[14,15] Our results suggest that synchronization of estrus with AT, combined with application of the "3 × 4 × 6" insemination protocol, may have been successful in placing frozen/thawed sperm in the oviduct at or near the time of ovulation.

The "3 × 4 × 6" protocol is more labor-intensive than the standard program of twice-daily estrus detection and breeding with fresh semen 12 and 24 hours or 24 and 48 hours after the onset of estrus. Until the survival of thawed semen improves, this extra effort will continue to be necessary. Most swine semen is frozen using either the Beltsville pellet method or the Hulsenberg straw method.[7] A review of fertility results for frozen semen between 1970 and 1985 showed no significant differences in farrowing rate or litter size when these two methods were used.[16] Neither the farrowing rate (55–58%) nor the average litter size (8.3–9 pigs) was exceptional for semen frozen by either method. The time required for semen processing and freezing is significant for both methods. The Beltsville pellet method takes 5 hours from semen collection to freezing, while the Hulsenberg method requires 7–9 hours (or longer for the modified protocol used in this study).[7,15]

The preparation time and labor associated with the use of frozen semen can be reduced, particularly when a large number of females is to be inseminated, if estrus is synchronized using an induced abortion strategy or an orally active progestagen. Estrus synchronization allows efforts to be concentrated toward detecting estrus during a predictable time period, and shortens the time interval for performing multiple inseminations.

The amount of labor required to achieve the level of fecundity reported here is not trivial; however, the additional effort can make frozen semen an acceptable alternative to fresh semen. Hopefully, this protocol and development of equally effective breeding strategies in the future will increase the interest of the swine industry in the use of frozen semen.

Implications

- It is possible to use frozen boar semen in a program that results in farrowing rates and pigs born alive similar to those achieved with AI using fresh semen.
- Further research is required to determine how to reduce the high level of labor in the protocol reported here.
- This protocol may be developed to make the use of frozen semen a more attractive contingency plan for either health or production failures of boar studs.

References – refereed

1. Spallanzani L. Opuscoli di fiscal e vegetabile. Opuscolo II. Observazioni e esperienze intorno ai vermicelli spermatici dell' homo e degli animali. (Observations upon the spermatozoa of humans and animals). *Modena*, 1776. Cited by Watson PF, 1979.

2. Watson PF. The preservation of semen in mammals. In: Finn CA, ed. *Oxford Reviews of Reproductive Biology*. Oxford, UK: Clarendon Press. 1979;1:283–350.

3. Polge C, Smith AV, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperature. *Nature*. 1949;164:666.

4. Crabo BG, Einarsson S. Fertility of deep frozen boar spermatozoa. *Acta Vet Scand.* 1971;2:125–127.

5. Graham EF, Rajamannan AHJ, Schmehl MKL, Maki-Laurila M, Bower RE. Preliminary report on procedure and rationale for freezing boar semen. *AI Digest.* 1971;19:12.

6. Pursel VG, Johnson LA. Procedure for the preservation of boar spermatozoa by freezing. 1971. United States Department of Agriculture-Agricultural Research Service Bull. No. 44–227.

8. Westendorf P, Richter L, Treu H. Zur Tiefgefrierung von Ebersperma: Labor-und Besamungsergebnisse mit dem Hulsenberger Paillentten-Verfahren. *Dtsch Tierarztl Wschr.* 1975;82:261–300.

9. Nellor JE, Daniels RW, Hoefer JA, Wildt DE, Dukelow WR. Influence of induced delayed parturition on fetal survival in pigs. *Theriogenology.* 1975;4:23–31.

10. Snedecor GW, Cochran WG. *Statistical Methods.* 8th ed. Ames, Iowa: Iowa State University Press; 1989:53–58.

11. Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fertil Dev.* 1995;7:871–891.

12. Pope WF, Wilde MH, Xie S. Effect of electrocautery of nonovulated day 1 follicles on subsequent morphological variation among day 11 porcine embryos. *Biol Reprod.*1988;39:882–887.

13. Town SC, Almeida FRCL, Novak S, Foxcroft GR. Optimizing timing of artificial insemination in gilts [abstract]. *J Reprod Fertil.* 1999;(23):101.

14. Johnson LA, Aalbers JG, Willems CMT, Sybesma W. Use of boar spermatozoa for artificial insemination. I. Fertilizing capacity of fresh and frozen spermatozoa in sows on 36 farms. *J Anim Sci.* 1981;52: 1130–1136.

15. Almlid T, Clarke RN, Pursel VG, Johnson LA. Effectiveness of in vitro methods for predicting in vivo fertilizing capacity of boar spermatozoa cryopreserved with 2% or 4% glycerol. *Zuchthygiene.* 1989;24: 8–15.

16. Johnson LA. Fertility results using frozen boar spermatozoa 1970 to 1985. In: Johnson LA, Larson K, eds. *Deep freezing of boar semen.* Uppsala, Sweden: Swedish Univ Agric Sci; 1985:192–222.

References – nonrefereed

7. Johnson LA. Current developments in swine semen: Preservation, artificial insemination and sperm sexing. *Proc 15th IPVS Congr*, Birmingham, UK. 1998;225–229.