ONTARIO PORK RESEARCH PROPOSAL FINAL REPORT

Project Leader: R.N. Kirkwood

Project title: Use of controlled ovulation and seminal plasma on sow fertility to insemination of cryopreserved sperm.

Objective of Research Proposal: To develop a semen thawing protocol that will result in frozen sperm having fertility comparable to fresh sperm.

Executive summary:

Causes of poor fertility after insemination of frozen-thawed (FT) sperm include fewer live sperm at thawing and a shorter lifespan of surviving sperm in the female reproductive tract due to sub-lethal damage. The present studies examined the effect of incubating thawed boar sperm in seminal plasma (SP) on sperm viability and motility in vitro (experiment 1), in vivo sow fertility to double conventional AI (experiment 2), or single intrauterine AI (experiment 3). For experiment 1, FT sperm were thawed and incubated for 4-h in media containing 0%, 10%, or 50% SP. At 0, 1, 2, 3, and 4-h, sperm populations were examined for viability and motility. Each variable progressively decreased during the incubation period but incubation in 50% SP increased percentages of live sperm and sperm motility at all time points compared to incubation in either 0% or 10% SP. For experiment 2, multiparous Large White x Landrace sows (n = 82) each received 900 IU eCG at weaning and 750 IU hCG 80 h later to control time of ovulation. Sows were assigned on the basis of parity to be inseminated with pooled semen with or without SP. Sows received 3×10^9 live fresh-extended sperm or FT sperm thawed in 80 mL BTS extender or 3×10^9 live FT sperm thawed in 80 mL BTS containing 50% SP (FT-SP). Sows were inseminated at 36 and 42 h after hCG injection. Compared to sows receiving fresh semen, the pregnancy rate of FT inseminated sows tended to be lower with the FT-SP group being intermediate. Farrowing rates were not different. Inseminations with FT sperm were associated with a reduction in litter size, which was not evident in the FT-SP group. For experiment 3, multiparous Yorkshire sows (n=97) received 900 IU eCG at weaning and 5 mg pLH 80 h later to control time of ovulation. Sows were inseminated once intrauterine at 36 h after pLH with 3×10^9 live FT sperm with or without 50% SP, or 3×10^9 fresh sperm. Compared to controls, insemination of FT-SP decreased farrowing rate but litter sizes were not different from controls. In contrast, compared to controls, insemination of FT sperm without SP did not result in reduced farrowing rates but did reduce litter size. Taken together, these data confirm an adverse effect of inseminating FT sperm on sperm quality and sow fertility but suggest that thawing FT sperm in 50% SP may partially alleviate these adverse effects. The source of the seminal plasma may affect the results obtained.

Background:

Swine AI involves almost exclusively the use of liquid semen collected within the previous 3 to 7 days, although there is evidence to indicate that optimal fertility requires AI within 48 hours after collection and extension in both short- and long-term extenders (Johnson et al., 1982; Waberski et al., 1994a; Bennemann et al., 2005). In addition to removing time

constraints for sperm usage, cryopreservation would permit sperm to be stored longer to allow sufficient time for testing to ensure the absence of pathogens and so improve herd biosecurity.

The insemination of swine with cryopreserved sperm usually results in farrowing rates and litter sizes below those observed following insemination of fresh sperm (Johnson et al., 2000; Roca et al., 2003). This is believed to be a result of sperm cryo-injury including, but not limited to, a capacitation-like or premature aging effect (Bravo et al., 2005; Ortega Ferrusola et al., 2008), associated with an increase in DNA fragmentation (Fraser and Strzezek 2007). Capacitated spermatozoa do not form a functional sperm reservoir (Fazeli et al., 1999; Tienthai et al., 2004) and, similarly, cryopreserved sperm do not form a normal sperm reservoir (Abad et al., 2007a). Acceptable fertilization rates were observed only when insemination occurred within 4 hours of ovulation (Waberski et al., 1994b; Roca et al., 2003).

One potential means of improving the fertility of cryopreserved boar sperm is to ameliorate the capacitation-like or aging effect. Using chlortetracycline staining, it was demonstrated that incubation of fresh or cryopreserved sperm in media supplemented with 10% seminal plasma appeared to prevent, and possibly reverse, (cryo)capacitation (Suzuki et al., 2002; Vadnais et al., 2005a,b). However, when boar semen was supplemented with 0% or 10% seminal plasma and inseminated within 2 or 12 h of the predicted time of ovulation, there was no effect of timing of insemination or seminal plasma on sow fertility (Abad et al., 2007b). Interestingly, supplementing cryopreserved ram semen doses with 30% seminal plasma improves ewe fertility (Maxwell et al., 1999). Further, earlier workers observed that extension of cryopreserved boar sperm in 100% seminal plasma was beneficial to *in vivo* sperm longevity (Einarsson and Viring, 1973) and on gilt and sow fertility (Crabo and Einarsson, 1971; Einarsson et al., 1973). Given the evident *in vivo* benefits of extending cryopreserved boar sperm in relatively high concentrations of seminal plasma, the objective of the present study was to determine whether effects of seminal plasma are dose-dependant. We hypothesized that, compared to thawing sperm in 10% seminal plasma, thawing sperm in extender having 50% seminal plasma would enhance *in vitro* sperm quality and so subsequent sow performance.

Material and methods:

Experiment 1.

From May to November 2008, a total of 5 Large White boars of 12 to 35 months of age were selected based on documented fertility and normal semen quality. There was no pre-selection for semen freezability. All boars were kept in individual pens with the environmental temperature maintained at 18 to 22º C and a constant photoperiod of 12 h/d. The boars received 2.5 kg/d of a 13% protein diet and were allowed water *ad libitum*.

Processing of semen and seminal plasma

Each boar was allowed to mount a ‘dummy’ sow weekly and the sperm-rich fraction of the ejaculates collected. We used two ejaculates from each boar. Immediately after collection, the filtered ejaculates were transported to the laboratory. The volume of each ejaculate was measured on the basis of weight and sperm concentration estimated using a 60 µL aliquot of

semen in 3.0 mL of MR-A® extender in a photometer SDM-5 (Minitüb, Germany). Morphology and acrosome integrity were assessed and an initial analysis of sperm motility performed using a phase contrast microscope at 200x magnification. Only ejaculates with ≥80% motile sperm, ≥75% morphologically normal sperm and ≥95% normal acrosomes were used.

Semen was processed for cryopreservation according to the technique described by Eriksson and Rodriguez-Martinez (2000). Sperm were packaged at 5º C in 0.25 ml straws. The straws were thawed in circulating water at 50º C for 12 sec (Eriksson and Rodriguez-Martinez, 2000). From the same five boars used for semen collection, further ejaculates were collected and the seminal plasma harvested and stored at -80º C until required. Thawing of seminal plasma was done at 37º C in a water bath, at which time the seminal plasma was centrifuged a third time prior to use in the study and the absence of sperm confirmed using phase contrast microscopy under 400x magnification.

Sperm incubation and examination

Cryopreserved sperm from each of the five individual boars (two straws per ejaculate per boar; four straws total) and an additional sperm pool (of same five boars) were thawed and incubated in 50 mL centrifuge tubes at 37º C in a water bath for 4 h in 40 mL BTS extender (Beltsville Thawing Solution) containing 0%, 10%, or 50% autologous seminal plasma (individual boars) or pooled seminal plasma (sperm pool). The pooled sperm and seminal plasma were examined to confirm that responses observed were not the result of boar-to-boar variation. Sperm concentration was 25×10^6 sperm/mL. Sperm plasma membrane integrity and total motility were determined at approximately 10 min post-thaw (0-h) and at 1, 2, 3 and 4-h. These procedures were performed on three occasions.

Sperm plasma membrane integrity was evaluated using the method originally described by Harrison and Vickers (1990). For determination of percent sperm motility, a 4 µL aliquot of the sperm suspension placed in a pre-warmed (39°C) 10 µm deep Makler counting chamber and immediately transferred to the warm stage (39°C) of a Nikon Eclipse E-400 microscope with phase contrast optics at 200 x magnification. The video signal was acquired by a Basler A312F digital camera and associated CASA software. For each sample, six predetermined optical fields around the central reticulum of the chamber were used to count a minimum of 150 sperm per sample. The number of objects incorrectly identified as spermatozoa was minimized on the monitor by using the playback function.

Experiment 2

This study was conducted on a commercial 1,000-sow farrow-to-wean facility in Spain during September 2008 to April 2009. To stimulate post-weaning ovarian follicle growth and onset of estrus, 82 multiparous Yorkshire x Landrace sows received an intramuscular (IM) injection of 900 IU equine chorionic gonadotrophin (eCG; Folligon, Intervet/Schering, Boxmeer, the Netherlands). At 80 h after the eCG, sows received an injection (IM) of 750 IU human chorionic gonadotrophin (hCG; Chorulon, Intervet/Schering) to induce ovulation. Based on literature evidence ovulation was predicted to occur at 42 h after hCG injection (Soede and

Kemp, 1993; Hazeleger et al., 2000). Sows were housed in individual gestation crates and were provided nose-to-nose contact with a boar at the designated times of artificial insemination (AI), which were 36 h and 42 h after hCG injection. Semen and seminal plasma were pools derived from the same boars as used in Experiment 1. Sows were assigned on the basis of parity to one of three treatments and inseminated into the cervix with live fresh sperm (<48 h since collection; n = 30), 5×10^9 FT sperm thawed into 80 mL Beltsville Thawing Solution (BTS; n = 26), or FT semen thawed into 80 mL BTS supplemented with 50% seminal plasma (FT-SP; n = 26). Pregnancy status was assessed using real-time ultrasound at 28 d after insemination and the sows farrowed to permit determination of farrowing rates and subsequent litter sizes.

Experiment 3

This study was conducted at the MSU swine facility from January to September 2009. To stimulate a synchronous estrus and ovulation, 97 multiparous Yorkshire sows received 900 IU eCG at weaning and 5 mg pLH 80 h later. Predicted time of ovulation was 38 h after pLH. Sows were housed in individual gestation crates provided nose-to-nose contact with a boar at the designated time of AI, which was 36 h after pLH injection. Semen was commercially derived (IBS, Iowa) and seminal plasma collected from 10 Yorkshire and Landrace boars housed at a comercial farm in Michigan. Sows were assigned on the basis of parity to one of three treatments and inseminated once intrauterine with 3×10^9 live fresh sperm (<48 h since collection; n = 34), or 5×10^9 live FT from the same boars thawed into 80 mL BTS with (n=32) or without (n=31) 50% SP. The sows were allowed to farrow to permit determination of farrowing rates and subsequent litter sizes.

Statistical analysis

All data were analyzed using Statistical Analysis Systems software (Version 9, SAS Institute Inc., Cary, NC). Patterns of responses of sperm from the individual boars were very similar to each other and to the sperm pool. Therefore, for the purposes of analysis, the pool was considered as a sixth boar. Percentages of live sperm (i.e., intact plasma membranes) and percent sperm motility were measured at about 10 min post-thaw (0-h) and again at 1, 2, 3, and 4 h after thawing. Repeated measures ANOVA using PROC GLM was used to analyze the differences between treatment groups. Because of the observed non-normality in model residuals, the dependent variables were log-transformed. Two separate models were constructed for percentages of live sperm and percent sperm motility that included treatment group, boar, and repetition as between-subjects main effects. In addition, the interaction between boar and treatment was included in the model. Time was included as the repeated effect. Least squares means of the log-transformed variables were compared at all time points.

For experiments 2 and 3, differences in pregnancy and/or farrowing rates were compared using Chi square. Litter sizes (total born) were tested using GLM analysis of variance including treatment and parity as main effects in the model. Results are presented as mean ± SE and $P<0.05$ was considered significant.

Results:

Experiment 1

Treatment was a significant predictor in both models (P<0.001). The proportions of live sperm decreased progressively during the 4-h incubation period (Fig. 1a). Additionally, when sperm were incubated in 50% seminal plasma, least squares means of live sperm were greater at all time points examined when compared to sperm incubated in 0% or 10% seminal plasma (P<0.0001). There was no effect of 10% seminal plasma on percentage live sperm at any time point when compared to sperm incubated in 0% seminal plasma (Fig. 1a).

The percent sperm motility decreased progressively during the 4-h incubation period (Fig. 1b). Compared to the sperm populations incubated in 0% or 10% seminal plasma, when incubated in 50% seminal plasma the least square means of the percent sperm motility were greater (P<0.01) at 0-h and this level of significance was maintained at all time points examined except at 4-h, when the difference between 10% and 50% seminal plasma was at P<0.05 (Fig. 1b). The percentage motile sperm was higher at 2-h, 3-h and 4-h for those incubated in 10% seminal plasma compared to sperm incubated in 0% seminal plasma (P<0.01; Fig. 1b).

Experiment 2

Compared to sows receiving fresh semen, insemination of sows with FT boar sperm tended (P = 0.06) to reduce pregnancy rate, an effect ameliorated by thawing sperm into extender with 50% seminal plasma (Table 1). There was no significant effect of treatment on farrowing rate. Insemination with FT sperm was associated with a reduction in total born litter size (P<0.05), an effect not observed when sperm were thawed in 50% seminal plasma (Table 1).

Experiment 3

Compared to sows receiving fresh semen, insemination of FT sperm extended in 50% seminal plasma decreased farrowing rate (P<0.01) but litter sizes were maintained. In contrast, compared to sows receiving fresh seme, insemination of FT sperm without SP did not result in reduced farrowing rates but did reduce (P<0.05) litter size (Table 2).

Discussion

The present findings clearly indicate that seminal plasma can positively impact sperm quality in vitro, and that the effect appeared dose dependant. The effect of seminal plasma in vivo was not as obvious. Previous studies by our group have shown that, based on chlortetracycline staining patterns (Vadnais et al., 2005b) or merocyanine and peanut agglutinin lectin or propidium iodide and yo pro 1 (Gumbao, unpublished data), the capacitation-like effect due to freezing and thawing can be prevented by incubation of thawed sperm in 10% seminal plasma In contrast, we previously determined that extending thawed semen in 10% seminal plasma had no effect sperm's ability to form a functional sperm reservoir in the oviducts (Abad et al.,

2007a) nor on sow fertility if inseminated 2 or 12 h before predicted time of ovulation (Abad et al., 2007b). However, although speculative, given the improved pregnancy rate but not farrowing rate associated with 50% autologous seminal plasma noted in Experiment 2, it is possible that improved fertilization and subsequent pregnancy rates associated with thawing FT sperm in 50% seminal plasma were realized but that early pregnancy losses masked any effect on farrowing rate. That a disproportionate loss of pregnancy may occur after insemination of FT sperm is consistent with the concept of cryopreservation inducing a sperm aging effect with associated deleterious effects on sperm DNA integrity. Indeed, freezing greatly increased the percentage of ram sperm having a greater DNA fragmentation (Peris et al., 2007). Fertilization with sperm that were effectively older may then result in increased embryo mortality. Interestingly, inclusion of 50% seminal plasma depressed farrowing rate in experiment 3. The reason for this is unexpected result is unknown. However, we did use a pool of boars unrelated to the sperm donors and it is possible that when heterologous seminal plasma is used a detrimental effect may occur (Caballero et al., 2004).

In contrast to our previous study where we thawed FT sperm in 10% seminal plasma (Abad et al. 2007b), the present studies suggests a beneficial effect of thawing FT sperm in 50% seminal plasma on subsequent litter size. An improved efficacy of a greater seminal plasma inclusion rate on the ability to establish a pregnancy and maintain a greater number of fetuses to term may be a consequence of repair of sperm cryoinjury, or possibly increased antioxidant capacity limiting further sperm damage resulting in a larger viable sperm population. Alternatively, the effect may have been mediated by a volume-dependant interaction between seminal plasma constituents and the uterus. Seminal plasma may modulate uterine immune responses to sperm (Rozeboom et al., 1998) and to be involved in the regulation of early embryo development (O'Leary et al., 2004). However, the design of the present and of previous studies examining the fertility effects of seminal plasma, do not permit these potential effects to be separated.

Significance of findings for the pork industry

We conclude from the present data that extending cryopreserved boar sperm in 50% seminal plasma will significantly improve sperm viability and motility, with the possible net effect of extending the sperm's lifespan and positively impacting sow fertility. However, the effects of 50% seminal plasma were inconsistent, with a positive effect on pregnancy in one study and a negative effect in the other. This indicates that the composition, and so the effect, of seminal plasma is too inconsistent to warrant commercial use. However, with on-farm collection and so the ability to use autologous seminal plasma, the benefit to maintenance of pregnancy would be more easily realised. There was, however, a consistent positive effect on litter size. Clearly, more information on the potential mechanisms involved with the autologous seminal plasma effect on fertility is required.

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Fig. 1. Effects of incubation of thawed boar sperm for 4 h in media with 0%, 10% or 50% seminal plasma (SP) on mean ± SE for (A) percent live sperm, (B) percent motility. Each time-point represents the mean of 10 ejaculates, with each ejaculate split among the 3 treatments.

Within time, means having different letters differ; ab, $P<0.0001$; cde, $P<0.01$

Table 1. Effect of thawing FT boar sperm in 50% seminal plasma (SP) on sow fertility

	Fresh semen	FT plus SP	FT
No. sows	30	26	26
Pregnancy rate, %	86.7a	80.8ab	65.4b
Farrowing rate, %	83.3	69.2	65.4
Total born litter size	11.2±0.4c	12.5±0.5c	9.8±0.5d

Means followed by different letters differ; ab (P=0.06), cd (P<0.05)

Table 2. Effect of thawing FT sperm in 50% seminal plasma (SP) on sow fertility

	Fresh semen	FT plus SP	FT
No. sows	34	31	32
Farrowing rate, %	85.3a	53.1b	71.0ab
Total born litter size	10.9±0.7c	9.8±0.9cd	8.5±0.8d

Means followed by different letters differ; ab (P<0.01), cd (P<0.05)