ABSTRACT: Several laboratory assays have been designed to assess the fertility potential of a semen sample before insemination, but none have been consistent and accurate predictors of fertility. To determine whether zona-binding ability may be a useful fertility predictor, we validated and used an in vitro competitive assay to measure the ability of porcine sperm to bind to the zona pellucida. The zona-binding ability of sperm from 11 boars that exhibited a broad range in average litter size and farrowing rate was determined. Sperm from each boar were compared directly with sperm from eight other boars in a systematic, pairwise fashion. Sperm from two semen samples were labeled with fluorophores at concentrations that did not affect motility or zona-binding ability. An equal number of labeled sperm from each boar was coincubated with homologous oocytes. Least squares means from analysis of variance were used to rank boars based on zona-binding ability. The competitive assay was effective in establishing a ranking of the boars ($R^2 = 0.62$). Furthermore, there was a correlation between zona-binding ability and fertility when estimated by average litter size ($r = 0.64, P < 0.05$) but not when estimated by farrowing rate ($r = −0.28$). The explanation for this difference was that litter size and farrowing rate were poorly correlated ($r = 0.14$). In conclusion, a competitive zona-binding assay distinguished boars that sired either small or large litters. Competitive zona-binding ability may be useful for identifying boars with reduced fertility that produce smaller litters following insemination.

Key Words: Fertilization, Litter Size, Oocyte, Pig, Spermatozoa, Zona Pellucida

Introduction

The evaluation of sperm motility and morphology as well as the sperm concentration of a semen sample are common methods for estimating boar fertility. The usefulness of these measurements to accurately gauge the fertility of a semen sample is controversial (Braundmeier and Miller, 2001; Flowers, 1997). In several species, additional laboratory assays have been developed to measure the functional competence of sperm. Included among these are assays that examine sperm-oocyte interactions, such as the zona-free hamster oocyte penetration assay. This assay measures the ability of sperm to bind and fuse with the hamster oocyte membrane (Rogers, 1985). This assay was used to evaluate the fertility potential of boars (Berger and Parker, 1989). The value of the zona-free oocyte penetration assay to measure male fertility is controversial because it ignores the normal requirement of zona binding for fertilization (Yee and Cummings, 1988). Another assay used mostly in humans is the hemi-zona assay. The zona pellucida is bisected and the zona-binding ability of a semen sample is measured against a control semen sample in separate droplets (Oehninger et al., 1989). An advantage of this assay is that one-half of the zona pellucida can be used as an internal control; however, limited samples can be tested because of the requirement for zona bisection and only zona binding can be measured (Franken et al., 1989).

In this study, a competitive assay was used to assess zona-binding ability. Semen samples from two different boars were mixed together and then coincubated with oocytes. The advantage of a competitive zona-binding assay is that the binding of both sperm samples occurs with the same group of oocytes, thus controlling for variation in oocyte batches. The goal of these experiments was to determine whether 1) zona-binding ability is variable in boar sperm and 2) variation in zona binding is related to the fertility of boars in vivo.
Materials and Methods

Collection of Porcine Oocytes

Porcine ovaries retrieved from a commercial abattoir, Excel, Inc. (Beardstown, IL) were used for oocyte collection. Follicles ≥2 mm in diameter were aspirated with a 10-mL syringe and an 18-gauge needle. The follicular debris was separated from the follicular fluid by unit gravity sedimentation. The follicular debris was resuspended in 10 mL of dmTALP (100 mM NaCl, 3.1 mM KCl, 1.5 mM MgCl₂, 2.1 mM CaCl₂, 0.29 mM potassium phosphate, 10 mM sodium bicarbonate, 25 mM Hepes, 1 mM sodium pyruvate, 21.6 mM lactate, 6 mg/mL BSA, 100 units/mL penicillin, and 100 μg/mL streptomycin, pH 7.4). Oocytes were collected from the follicular debris (under a dissecting microscope) up to 24 h after aspiration of the follicles. The collected oocytes were vortexed at maximum speed for 5 min in 1 mL of dmTALP in a 1.5-mL microfuge tube to remove cumulus cells. The denuded oocytes were washed in Medium B (127 mM NaCl, 5.3 mM KCl, 18.2 mM Hepes, pH 7.2) and then fixed in Medium B containing 1.5% formaldehyde for 10 min. The fixed oocytes were washed through four droplets of Medium B and transferred to a storage droplet of dmTALP covered with embryo-tested mineral oil (Sigma Chemical Co., St. Louis, MO). The oocytes were stored at 5°C for no longer than 2 mo.

Preparation of Porcine Sperm

Historic fertility information was provided by Pig Improvement Company (PIC, Frankfort, KY) and was analyzed to select 11 boars with a wide range in fertility. Semen was collected from these boars by personnel at PIC using the gloved hand technique, and 3 billion sperm were extended in 80 mL of VitaBoar extender (IMV International, Maple Grove, MN) at 18°C. Extended semen was shipped overnight at 18°C and processed 24 h after collection. Extended semen was washed through a Percoll cushion containing 5.4 mL of Percoll (Sigma), 0.6 mL of 10× buffered saline (1.3 M NaCl, 40 mM KCl, 10 mM CaCl₂, 5 mM MgCl₂, 140 mM fructose, and 50 mg/mL BSA [fraction V; Sigma]), and 4.0 mL of dmTALP in a 15-mL conical tube. After centrifuging for 15 min at 800 × g, the supernatant was removed and the sperm pellet was suspended in 15 mL of dmTALP. After centrifuging for 5 min at 600 × g, the sperm pellet was resuspended in 300 μL of dmTALP. Sperm numbers were determined using a hemocytometer, and 8.5 × 10⁶ were added to a 1.5-mL microfuge tube, and the volume was adjusted to 300 μL with dmTALP. Sperm were incubated for 4 h at 39°C.

Validation of Sperm Staining

In order to determine the optimal concentration of dye to use for staining, sperm were incubated with several dye concentrations, and sperm motility, the percentage of sperm stained, and zona-binding ability were evaluated. Five million sperm were incubated with dyes for 1 h. The dyes used were 4-[4-(dihexadecylamino)styrlyl]-N-methylquinolinium iodide; an orange-red fluorophore (DiQ) and 3,3′-dihexadecyloxycarbocyanine perchlorate; a yellow-green fluorophore (DiOC₁₆; Molecular Probes, Eugene, OR). Each fluorescent lipophilic dye was prepared in 10 mM stock solutions, DiQ in dimethyl sulfoxide and DiOC₁₆ in dimethylformamide. The stock solutions were stored in aluminum foil at −20°C. Before use, an aliquot of dye or an equal volume of vehicle was thawed, sonicated, and centrifuged, and the supernatant was used to stain sperm. After labeling, sperm were centrifuged at 10,000 × g for 1 min, supernatant was removed, and pellet was resuspended with 1 mL of dmTALP. To estimate motility, 100 sperm were examined subjectively using a microscope with differential interference contrast optics. To determine the percentage of sperm stained, 100 sperm were counted for each dye concentration. These experiments were replicated three times using semen from three different boars. To determine the effect of staining on zona-binding ability, sperm were stained with 83 μM concentration of either dye for 1 h. Stained sperm were centrifuged and washed as previously described. Sperm from the same sample stained with either dye were then mixed in different ratios (25:75, 50:50, and 75:25 of the two dyes) in triplicate droplets containing 10 oocytes. Loosely adherent sperm were removed, and then the number of tightly bound sperm was counted. This experiment was replicated three times with semen from three boars.

Differential Staining of Sperm

Before Zona-Binding Assays

After capacitation, sperm were incubated with 83 μM of each dye for 60 min at 39°C in dmTALP. The sperm suspensions were centrifuged for 30 s at 10,000 × g, the supernatant was removed, and sperm were suspended in 300 μL of dmTALP. To estimate motility, 100 sperm were examined with a microscope using differential interference contrast optics and the percentage of motile sperm (progressive and nonprogressive) was calculated.

Competitive Sperm Zona-Binding Assay

Following sperm capacitation and staining, sperm were used in pairwise zona-binding competitions. In order to systematically balance the pairwise competitions among the 11 boars, each boar was randomly assigned a letter and a balanced assay schedule was arranged (Table 1). In this design, each boar was assayed against eight other boars throughout the course of this experiment, in which a different semen collection was used for each assay. Each individual assay, represented by an “X” in the table, consisted of two competitions in which dye assignment for the two samples was also reversed in order to minimize any effect of the dye on zona binding. Sperm labeled with each dye were then
Table 1. Competition arrangement for the 11 boars illustrating which boars were compared directly in the zona pellucida-binding assay

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*Each “X” represents a direct comparison in which sperm from two boars were mixed together and added to a droplet of oocytes. Each comparison of two boars includes a switch in fluorophore assignments to avoid any preferential effect of either fluorophore.

added to triplicate 25-µL droplets of dmTALP for a final total of 4.37 × 10^5 sperm in 50 µL. Each droplet contained 10 oocytes equilibrated to 39°C, for a total of 30 oocytes for each competition and a total of 60 oocytes after dye reversal. Gametes were coincubated for 15 min at 39°C to allow maximal binding. After incubation, sperm-bound oocytes were transferred to a 50-µL wash droplet of dmTALP by mouth pipette and then to a 25-µL droplet of 4% paraformaldehyde in PBS. These washing conditions were sufficient to remove any loosely adherent sperm from the oocyte. After washing oocytes, they were transferred to a 25-µL droplet of dmTALP on a microscope slide. The coverslip was prepared by placing a small amount of petroleum jelly on each corner to avoid crushing the oocytes and was placed gently over the droplet. The coverslip was sealed with nail polish and stored in aluminum foil until counting. Sperm were counted on a Zeiss Axioskop using fluorescence microscopy (Zeiss, Thornwood, NY). To detect DiOC16 fluorescence, the Zeiss 09 filter set, which has a band pass cutoff of 450 to 490 nm for excitation, a 510-nm beam splitter, and a long-pass 515-nm filter for emission, was used. For detecting DiQ fluorescence, the Zeiss 15 filter set, which has a band pass of 534 to 558 nm, a 580-nm beam splitter, and a 590-nm long-pass emission filter, was used.

Statistical Analysis

Least squares means from ANOVA using the GLM procedures of SAS (SAS Inst. Inc., Cary, NC) were used to accurately rank the boars on their ability to bind to the zona pellucida of the oocyte. The statistical model was

\[ nb = \text{assay} + \text{comp(assay)} + \text{boar} + b(\text{tb}) + \text{dye} + \text{dye} \times \text{boar} + \text{random error} \]

where nb is the number of sperm bound per oocyte per boar, assay is the effect of the assay, comp(assay) is the effect of each competition within each assay, boar is the male effect, tb is the total number of sperm bound per oocyte, b is the coefficient of regression of nb on tb, dye is the effect of each dye, and dye × boar is the interaction term. Boars were ranked by the calculated least squares means of the number of sperm bound per oocyte for each boar. This ranking was then compared to the fertility data of these boars obtained during the trial by determining correlation coefficients.

Artificial Insemination and Determination of Farrowing Rate and Litter Size

Artificial insemination was performed on PIC farms by PIC-trained technicians. All boars used were from the same genetic line. The total number of sows serviced for each boar ranged from 22 to 86. The average number of inseminations per estrus was 2.4, and the average parity of the group of sows to which each boar was bred ranged from 2.6 to 4.8 (SEM ± 1.0). All sows were from the same genetic line. Semen was collected and sows were bred during the months of October through February to minimize heat stress. Zona-binding data were obtained on the same samples and during the same months as when the AI was performed. Zona-binding assays were completed before the fertility data were obtained.

In vivo fertility data were collected by PIC and compared with binding data by linear regression. Farrowing rates were calculated for each boar as the percentage of sows inseminated that successfully farrowed. The average litter size for each boar was calculated as the total number of piglets born (live and dead) from each farrowing, averaged for all farrowings.

Results

Two fluorescent lipophilic dyes were used to identify sperm from each boar in the sperm mixtures used in the competitive zona-binding assay. To validate the sperm-staining procedure for this assay, the concentration of each dye that stained the majority of sperm with mini-
The effect of fluorophore concentration on sperm motility and the percentage of sperm stained. Upper panel: Effect of increasing 3,3′-dihexadecyloxacarbocyanine perchlorate (DiOC16) concentration on the percentage of motile sperm and sperm stained. Lower panel: Effect of increasing 4-[4-(dihexadecylamino)styryl]-N-methylquinolinium iodide (DiQ) concentration on the percentage of motile sperm and sperm stained.

Figure 1. The effect of fluorophore concentration on sperm motility and the percentage of sperm stained. Upper panel: Effect of increasing 3,3′-dihexadecyloxacarbocyanine perchlorate (DiOC16) concentration on the percentage of motile sperm and sperm stained. Lower panel: Effect of increasing 4-[4-(dihexadecylamino)styryl]-N-methylquinolinium iodide (DiQ) concentration on the percentage of motile sperm and sperm stained.

Mal effects on sperm motility was evaluated. Concentrations of DiQ less than 100 μM had minimal effects on motility and those greater than 75 μM stained more than 75% of sperm (Figure 1, upper). Concentrations of DiOC16 below 150 μM did not affect motility and those above 75 μM stained 60% of sperm (Figure 1, lower). Therefore, sperm were stained with 83 μM of either dye for the remaining experiments. This concentration was used to determine the effect of staining on zona-binding ability. Different ratios of stained sperm from the same semen sample were mixed in binding droplets and coincubated for 15 min. Although samples stained with DiOC16 bound to the zona slightly less, the percentage of stained sperm bound to the zona pellucida was proportional to the percentage of stained sperm added to the binding droplet (Figure 2).

Sperm from 11 boars were washed and capacitated before labeling with DiQ or DiOC16 fluorescent dyes. Equal numbers of sperm from each boar were incubated with oocytes in systematic pairwise competitions to determine zona-binding ability for each boar. The design of the comparisons allowed each boar to be compared directly with eight others (Table 1) and indirectly with the remaining two boars.

The 11 boars ranged from 8.4 to 20.5 sperm bound per oocyte (Figure 3). The pairwise competitive assay
Figure 4. Summary of farrowing rate and litter size data from the boars assayed. The boars are ranked 1 to 11 based on the zona-binding ability of their sperm. Upper panel: The 11 boars whose sperm were assayed for zona-binding ability had a wide range of farrowing rates (45 to 80%). Lower panel: These 11 boars also ranged in mean litter size from 6.3 to 11.5 pigs per litter.

was highly effective in ranking the 11 boars based on zona-binding ability; the model had a high $R^2$ value ($0.62; P < 0.05$; Figure 3).

In this study, a group of boars were selected that had a wide range of farrowing rates (45 to 80%) and litter size (6.3 to 11.5 pigs per litter) so that we could test the relationship of zona binding to fertility across a large fertility spectrum (Figure 4). The number of sperm bound per oocyte was positively correlated to average litter size ($r = 0.64, P < 0.05$); however, the number of sperm bound per oocyte was not correlated to the farrowing rates recorded for the 11 boars ($r = -0.28$) (Figure 5). The correlation between zona-binding ability and litter size but not farrowing rate indicated that litter size and farrowing rate were not correlated among the 11 boars. A comparison of litter size and farrowing rate for each boar confirmed the nonsignificant correlation ($r = 0.14; Figure 6$).

Figure 5. Relationship of zona-binding ability to least square means of litter size and farrowing rate. Upper panel: Using the competitive binding assay, a significant correlation was detected between average litter size and zona binding ability ($P < 0.05$). Lower panel: No significant correlation was detected between farrowing rate and zona-binding ability. LSMean = least squares mean.

Discussion

The fertilization process consists of several events that must all occur efficiently to achieve successful conception. Sperm that are defective in any of these events have reduced fertility. Laboratory analysis of semen samples can identify subfertile samples by evaluating sperm performance. This study focused on the first gamete interaction, sperm binding to the zona pellucida, using a competitive zona-binding assay. The principle of a competitive binding assay stems from the use of heterospermic insemination, in which semen samples from different males are mixed and used for insemination; phenotypic or genetic screening of the progeny is used to determine paternity. The use of heterospermic insemination provides a more accurate measurement of fertility than when a single insemination is used, particularly when there are few inseminations (Dziuk, 1996). Following the same principle, the advantage of a competitive zona-binding assay is that binding of both sperm samples occurs to the same group of oocytes, controlling for variation in oocyte batches. We found
that this assay ranked 11 boars by zona-binding ability and this ranking was correlated to average litter size but not farrowing rate. The difference in correlations may result from the fact that there was no relationship between litter size and farrowing rate data for the 11 boars.

Although zona binding is a required step for fertilization in vivo, we have found that it is related only to boar fertility when fertility is assessed by average litter size. In a previous study with cattle, we reported that zona binding was not significantly correlated with non-return-to-estrus rates (an estimate of conception rate), heterospermic insemination fertility index, or accessory sperm number (Braundmeier et al., 2002). One possible explanation for these results is that litter size in litter-bearing species may be a more accurate indication of fertility than conception rate (Xu et al., 1998). Therefore, one may be able to estimate fertility more accurately in swine, a litter-bearing species, than in cattle, a monotocous species. Another possible explanation is that, in cattle, low-fertility bulls are not selected for AI, narrowing the range of fertility among these bulls. Because subfertile bulls that may have zona-binding defects have been removed, there is less opportunity for a correlation between zona-binding ability and fertility. In swine, selection against subfertile boars is probably reduced because of the relatively short time during which AI has been practiced.

Maternal factors also influence the outcome of insemination. Parity, number of inseminations during each estrus, and breeding season all account for variation in farrowing rates (Clark et al., 1989). In that report, maternal influence on farrowing rate was reduced when boars were bred to sows with average parity of 2 to 5. Sows that were bred using two or more inseminations per estrus also had reduced variation in farrowing rates compared to sows inseminated less frequently. We minimized the effect of these maternal factors by using multiparous sows that were inseminated an average of 2.4 times per estrous period. Neither parity nor number of inseminations was correlated with either litter size or farrowing rate in this data set (data not shown). Pig Improvement Company-trained technicians performed AI to standardize insemination conditions and reduce any effect of technician skill. Finally, AI occurred during autumn and winter months to reduce heat stress.

Results herein demonstrate that litter size was positively correlated with zona-binding ability. Although zona-binding ability can be categorized as a compensable trait, litter size is related to both compensable and uncompensable traits (Xu et al., 1998). Compensable traits hinder the interaction of gametes but can be overcome by increasing sperm number in the insemination dose. Examples of these traits are low motility and tail defects. Uncompensable traits do not affect the interaction of gametes but impede fertilization and development. Consequently these traits cannot be overcome by increasing sperm number in the insemination dose. Examples of these traits are sperm with subtle morphological defects or nuclear vacuoles (Saacke et al., 2000). Uncompensable traits are more likely than compensable traits to be related with fertility when excessive sperm are inseminated. However, these results demonstrate that zona-binding ability, a compensable trait, is also related to fertility at a standard insemination dose.

Previous reports suggest that litter size may be a more accurate or sensitive measurement of fertility than farrowing rate. Xu et al. (1998) showed that insemination dose has a greater effect on litter size than farrowing rate. The closer relationship between litter size and insemination dosage held true over a wide range of insemination dosages (Flowers, 2001). A possible explanation is that farrowing rate measurements only give two possible outcomes (pregnant or not pregnant), whereas litter size measurements give a wide range of outcomes. This range may allow litter size to more accurately gauge the fertility status of boars than farrowing rate.

When considering how sperm traits may affect fertility, one would expect that most traits would affect fertility if the number of sperm with a specific defect would lie below a certain threshold (Amann and Hammerstedt, 1993). Semen samples with only minimal numbers of defective sperm would be expected to have normal fertility, particularly if high numbers of sperm are inseminated. Consistent with this notion, semen samples containing sperm with lower zona-binding ability produced smaller litter sizes after insemination. To our surprise, semen samples containing sperm with high zona-binding ability produced larger litters. This result suggests that many of the boars tested produced sperm with subthreshold zona-binding ability and that selection based on zona-binding ability may not only reduce the number of small litters but also increase the number of large litters.

Implications

Variation in zona-binding ability existed among sperm from 11 boars, and this variation was related
to litter size. Zona-binding data were obtained before fertility data, making this study a predictive study rather than a study correlating zona binding to historic fertility. Laboratory assays that evaluate zona-binding ability may be useful for removing lower fertility samples and thereby improving average litter size. Further studies with more animals are warranted to confirm this result. The development of laboratory assays that can accurately estimate important fertility traits will help pinpoint common defects in subfertile semen samples.

**Literature Cited**


