Journal of Andrology, Vol. 23, No. 5, September/October 2002

Examination of the Binding Ability of Bovine Spermatozoa to the Zona Pellucida as an Indicator of Fertility

ANDREA G. BRAUNDMEIER,* JILL M. DEMERS,* ROGER D. SHANKS,* RICHARD G. SAACKE,† AND DAVID J. MILLER*

*From the *Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana, Illinois; and the †Department of Dairy Science, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.*

ABSTRACT: Despite the development of many new techniques, laboratory assays still do not predict male fertility accurately. To identify targets for laboratory assessment, we first need to determine which steps in fertilization are most often defective in subfertile males. We developed a competitive in vitro fertilization assay in which spermatozoa from 2 different males, stained with different lipophilic dyes, are incubated together with oocytes in a droplet. By exposing mixed spermatozoa to the same oocytes, this assay controls for many of the variables of in vitro fertilization and should allow identification of the most common faulty steps in fertilization. The relationship of zona-binding ability to fertility is controversial. Therefore, as a first step, we determined if zona pellucida-binding ability, measured by this competitive assay, was related to bovine spermatozoal fertility. Fertility data were collected from 2 groups of bulls by 2 means of evaluation, nonreturn to estrus rates postinsemination and competitive insemination. In the nonreturn to estrus study, semen samples from 15 bulls were effectively ranked by zona-binding ability, using pairwise competitive in vitro zona-binding assays ($R^2 = 0.84$). However, this ranking was not significantly correlated with nonreturn rates ($r = -0.04$). In the competitive insemination study, semen samples from 8 bulls were effectively ranked by pairwise comparison using the competitive zona-binding assay ($R^2 = 0.67$). Again, this ranking was not significantly correlated to the competitive insemination index calculated for these bulls ($r = 0.29$). In the third study, we tested 3 bulls to determine if in vivo zona binding, assessed by the number of accessory spermatozoa, was correlated with in vitro zona binding. The number of accessory spermatozoa on oocytes recovered from cows after mating was not correlated with in vitro competitive binding of the spermatozoa. In conclusion, in vitro competitive zona binding was not correlated with bovine fertility or binding of accessory spermatozoa to oocytes in vivo.

Key words: Fertilization, oocyte, accessory, cattle, nonreturn, heterospermic.

J Androl 2002;23:645–651

The ability to identify low fertility semen samples prior to insemination is vital to maximize reproductive efficiency. For this reason, numerous laboratory assays have been examined as potential fertility tests. These laboratory fertility tests include assays of spermatozoal function (eg, motility or the ability to acrosome react) and assays of molecules that may have a function during fertilization (heparin-binding proteins, clusterin, acrosin, etc) (Marks and Ax, 1985; Shimizu et al, 1997; Ibrahim et al, 2000). There is also a report that intracellular calcium concentration in cryopreserved bovine spermatozoa may be related to fertility (Collin et al, 2000). However, none of these assays alone has been able to determine the fertility potential of all semen samples accurately (Flowers, 1997; Braundmeier and Miller, 2001). One likely reason is that these assays test for individual traits of spermatozoa. A defect in one of these traits may be sufficient to reduce fertility; therefore, all of these traits must be assayed to correctly determine where the defect occurs (Amann and Hammerstedt, 1993). This becomes an overwhelming task.

One approach to solving this problem is to use laboratory assays that test a multitude of traits. Some assays, such as successful in vitro fertilization, require that spermatozoa be motile, have normal morphology, have an adequate complement of zona pellucida and oocyte membrane receptors, be able to activate oocytes, etc. The relationship of in vitro fertilization rates to fertility has been variable, probably because of the normal variability between batches of oocytes, variation in semen handling, and inadequate internal controls. A second approach to solving this problem is to identify the traits that most often cause reduced fertility and make those part of the routine semen analysis. The hypothesis tested in this report was that binding to the oocyte's zona pellucida accounts for significant variation in fertility when assessing fertility by a nonreturn to estrus rate and a competitive index. We also used semen from bulls that differed in the number of accessory spermatozoa found on embryos and ova to compare in vivo zona binding with in vitro competitive zona binding.

When testing whether a trait is related to fertility, one

Correspondence to: Dr David Miller, 328 Animal Sciences Laboratory, 1207 W Gregory Dr, University of Illinois at Urbana-Champaign, Urbana, IL 61801 (e-mail: d-mille@uiuc.edu).

Received for publication November 14, 2001; accepted for publication March 20, 2002.

should consider fertility traits as divided into 2 components. One component includes defects that can be compensated for by inseminating more spermatozoal, so-called compensable traits (Saacke et al, 2000). Examples of these are spermatozoal viability, morphology, or factors that hinder the interaction of spermatozoa with the oocyte. If an animal has defects in compensable traits, reduced fertility is only observed when a less than optimal number of spermatozoa are inseminated. An excess of spermatozoa is usually inseminated in cattle, so laboratory assays that measure compensable traits are seldom highly correlated with bovine fertility. When excessive spermatozoa are inseminated, only the traits that cannot be compensated for are related to fertility; these traits are referred to as uncompensable. Examples of these traits are chromatin aberrations or morphological defects that do not affect sperm–oocyte interaction but that impair fertilization or embryogenesis once initiated. Although nonreturn rate is usually not well related to compensable traits, relative fertility based on the insemination of semen from 2 different males in competition would be affected by compensable traits (Saacke et al, 2000).

The goal of the experiments in this report was to determine if the first step in spermatozoa–oocyte interaction, zona binding, was related to fertility. This is considered a compensable trait because spermatozoa with an oocyte-binding defect would not be able to interact and fertilize an oocyte. Other normal spermatozoa in the sample could compensate for sperm defective in zona binding and could fertilize the oocyte. Previous studies aimed at determining if oocyte binding was related to fertility, assessed by a 56-day nonreturn rate, gave conflicting results (Fazeli et al, 1997; Zhang et al, 1998). The discrepancy may be due to the inability to control the variation in oocyte quality and spermatozoal preparation. Herein, we control this source of variation by using an in vitro competitive assay. Spermatozoa from 2 males were differentially stained, mixed, and allowed to bind to oocytes (Miller et al, 1998). This provides an internal control because spermatozoa bind to the same oocytes in the same droplet. Many samples can then be ranked by a series of pairwise comparisons. This principle has been used successfully with in vivo experiments to determine relative fertility when semen from 2 males was mixed and inseminated—so-called heterospermic insemination experiments (Saacke et al, 1980; Dziuk, 1996).

Materials and Methods

Preparation of Spermatozoa From Bulls With Nonreturn Rate Data

Cryopreserved bovine semen from 15 Holstein bulls greater than 5 years of age was obtained from Genex (Shawano, Wis). Semen was collected over variable period of times up to 1.5 years. The number of ejaculates used in this study ranged from 1 to 5. This semen was stored at −196°C in a commercial milk-based extender after freezing following routine procedures. Three straws of semen from each male were thawed for 1 minute in a 35°C water bath, and the semen was layered over a Percoll cushion containing 5.4 mL of Percoll (Sigma Chemical Company, St Louis, Mo), 0.6 ml of 10× HB saline (1.3 M sodium chloride [NaCl], 40 mM potassium chloride [KCl], 10 mM calcium chloride [$CaCl_2$], 5 mM magnesium chloride [$MgCl_2$], 140 mM fructose, and 50 mg/mL bovine serum albumin [BSA, fraction V; Sigma]), and 4.0 mL of dmTALP (100 mM NaCl, 3.1 mM KCl, 1.5 mM $MgCl_2$, 2.1 mM $CaCl_2$, 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 U/mL penicillin, and 100 μg/mL streptomycin, pH 7.4) in a 15-mL conical tube. After centrifuging for 15 minutes at 800 × *g*, the supernatant was removed, and the spermatozoa pellet was suspended in 15 mL of dmTALP. After centrifuging for 5 minutes at 600 × *g*, the spermatozoa pellet was resuspended in 300 μL of dmTALP. Depending on the experiment, from 3×10^6 to 9×10^6 spermatozoa were added to a 1.5-mL microfuge tube, and the volume was adjusted to 300 μL with dmTALP, with a final concentration of 10 μg/mL heparin to capacitate spermatozoa (Parrish et al, 1988). Spermatozoa were capacitated for 4 hours at 39°C.

Preparation of Semen From Bulls Whose Fertility Was Determined by Competitive Insemination

Cryopreserved bovine semen from 8 bulls (3 Hereford, 3 Angus, and 2 Simmental) was obtained from Clif Marshall at Select Sires (Plain City, Ohio). This semen had been prepared and used for a previous study involving 9 bulls; only 8 samples were still available (Saacke et al, 1980). Two or 3 ejaculates were collected in succession and pooled, providing sufficient semen doses for insemination and laboratory study. Semen was extended to 60×10^6 sperm/mL in egg yolk-citrate-glycerol, packaged in 0.5-mL French straws (Instruments de Médecine Vétérinaire, l'Aigle, France), and frozen to −196°C following the optimal procedure as described (Robbins et al, 1976). After plunging straws into 35°C water for 1 minute, equal numbers of spermatozoa from 2 bulls were mixed and reloaded into French straws for insemination. On the basis of the number (proportions) of calves sired competitively, a competitive index was derived for each of the 8 bulls (Saacke et al, 1980). For the in vitro competitive-binding assay, semen samples were thawed and capacitated as described above with 2 exceptions. Only 1 straw of semen was thawed from each male, and 1.5×10^6 spermatozoa were capacitated in 100 μL of dmTALP with heparin (Parrish et al, 1988).

Semen Collection and Processing for Quantification of Accessory Spermatozoa

Semen for this experiment was the same as that used in the study of Dalton et al (2001). Three Holstein bulls, ranging from 2 to 9 years of age, were selected on the basis of neat semen characteristics equal to or greater than 70% morphologically normal spermatozoa and 60% estimated progressive motility. Two ejaculates taken in succession from each bull were pooled and ex-

tended to 50×10^6 sperm/mL with clarified egg yolk-citrate-glycerol and cryopreserved in the same manner as the semen used in the heterospermic trial (above). For each bull, spermatozoa accessibility to the ovum in vivo was measured by quantifying accessory spermatozoa number in ova or embryos recovered nonsurgically 6 days postinsemination as described (Dalton et al, 2001). For the competitive zona-binding assay, semen samples were thawed and prepared as described above with the exception that 4.5×10^6 spermatozoa were capacitated in 300 μL of dmTALP with heparin (Parrish et al, 1988).

Differential Staining of Spermatozoa

Two fluorescent lipophilic dyes, DiQ (4-[4-(dihexadecylamino)styryl]-N-methylquinolinium iodide), an orange-red fluorochrome, and $DiOC_{16}$ (3,3′-dihexadecyloxacarbocyanine pechlorate), a yellow-green fluorochrome (Molecular Probes, Eugene, Ore), were prepared in 10-mM stock solutions. DiQ was dissolved in dimethyl sulfoxide and $DiOC_{16}$ in dimethylformamide. The stock solutions were stored in aluminum foil at −20°C. Prior to use, a 12.5-μL aliquot was thawed, sonicated for 10 seconds, and centrifuged at $15\,000 \times g$ for 1 minute, and the supernatant was used to stain sperm. After 3 hours of capacitation, spermatozoa were incubated with 83 μM of either dye for 60 minutes at 39°C in dmTALP. The sperm suspensions were centrifuged for 30 seconds at $10\,000 \times g$, the supernatant was removed, and the spermatozoa were suspended in 0.3 mL of dmTALP. To estimate motility, 100 spermatozoa were examined with a microscope using differential interference contrast optics.

Bovine Oocyte Collection and Preparation

Cumulus oophorus-free mature bovine oocytes were obtained from BoMed (Madison, Wis). These oocytes had been aspirated from 2- to 6-mm antral follicles and were processed in 114 mM NaCl, 3.2 mM KCl, 2 mM sodium bicarbonate, 0.4 mM monosodium phosphate, 10 mM lactate, 2.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES, and 100 IU/mL penicillin. Oocytes were stripped of cumulus cells by vortexing and shipped to Urbana, Ill, at 39°C by overnight mail. Oocytes were shipped in TC199 medium with Earle salts supplemented with bovine-luteinizing hormone, follicle-stimulating hormone, 0.22 mM sodium pryuvate, 25 μg/mL gentamicin, and 10% fetal calf serum. Upon arrival, they were washed in medium B (127 mM NaCl, 5.3 mM KCl, and 18.2 mM HEPES, pH 7.2) and then were fixed in 1.5% formaldehyde in medium B for 10 minutes. The fixed oocytes were washed through 5 droplets of medium B and were transferred to a droplet of dmTALP covered with mineral oil. They were stored in this droplet at 5°C for 1–25 days until use. Prior to use in competitive sperm-binding assays, oocytes were washed and placed in 25-μL drops of dmTALP under oil in groups of 10 oocytes per drop and equilibrated to 39°C.

Competitive Spermatozoa–Oocyte-Binding Assay

Following spermatozoa capacitation and staining, samples from 2 males were used for a competitive spermatozoa–oocyte-binding assay. An example of competition assignment is diagramed in the Table. This design allows indirect and direct comparisons of zona-binding ability. Each assay consisted of 2 competitions in which the fluorochromes used for each of the 2 spermatozoa samples were switched to minimize an effect of the fluorochromes. An equal number (from 1.25×10^5 to 3.75×10^5) of spermatozoa from each male labeled with either fluorochrome were mixed and added to triplicate 25-μL droplets of dmTALP containing 10 oocytes each equilibrated to 39°C. Gametes were coincubated for 15 minutes at 39°C to allow maximal binding. After incubation, spermatozoa-bound oocytes were transferred to a wash droplet of dmTALP by mouth pipette and then to a 25-μL droplet of 4% paraformaldehyde in phosphate-buffered saline. These washing conditions were sufficient to remove any loosely adherent spermatozoa on the oocyte. After the oocytes were washed, 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 at room temperature until counting. Spermatozoa were counted using a Zeiss Axioskop with fluorescence optics (Zeiss, Thornwood, NY) within 24 hours of preparation. To detect $DiOC_{16}$ fluorescence, the Zeiss 09 filter set was used that, for excitation, has a band-pass cutoff of 450–490 nm, a 510-nm beam splitter, and a long-pass 515-nm emission filter. For detecting DiQ fluorescence, the Zeiss 15 filter set was used that has a band pass of 534–558 nm, a 580-nm beam splitter, and a 590-nm long-pass emission filter.

Diagram of the assay competition schedule for semen from bulls with fertility data collected from competitive insemination; each bull was randomly assigned a letter, and an X denotes that an in vitro competitive zona-binding experiment was performed using semen from that pair of bulls

Bull	A	B	C	D	E	F	G	H
A		X	X	X				
B			X	X	X			
C				X	X	X		
D					X	X	X	
E						X	X	X
F	X						X	X
G	X	X						X
H	X	X	X					

Statistical Analysis

To rank the bulls accurately on their ability to bind to the zona pellucida of the oocyte, an analysis of variance (ANOVA) model to evaluate variables in the assay was used. The statistical model was as follows:

$$\text{nb} = \text{assay} + \text{comp(assay)} + \text{bull} + b(\text{tb}) + \text{dye} + \text{dye} \times \text{bull} + \text{random error}$$

where nb is the number of spermatozoa bound per oocyte, assay is the effect of the assay, comp(assay) is the effect of dye assignment for each competition within each assay, bull is the male effect, tb is the total number of spermatozoa bound per oocyte, b is the coefficient for regression of nb on tb, dye is the effect of each dye, and dye×bull is the interactive term. Bulls were ranked by the calculated least squares means of the number of spermatozoa bound per oocyte or bull. By calculating correlation

coefficients, this ranking was then compared to the ranking by 60- to 90-day nonreturn to estrus rate, the competitive index calculated from heterospermic insemination, and the number of accessory spermatozoa per ovum or embryo.

Calculation of Breeding Data

For 15 bulls, lifetime 60- to 90-day nonreturn to estrus data were obtained from a range of 1517 to 167855 services with an average of 84686 services per bull. For 8 bulls, a competitive index was calculated from a heterospermic insemination study (Saacke et al, 1980). For accessory spermatozoa, the mean number of accessory spermatozoa per 6-day-old ovum or embryo nonsurgically recovered after homospermic insemination to 1 of 3 bulls was obtained (n = 117, 39 ova or embryos per bull; Dalton et al, 2001). The nonreturn rate, competitive index, and accessory spermatozoa number were then used to assess the correlation of each with the ranking of the bulls by in vitro zona-binding ability.

Results

Relationship of Competitive Zona Binding to Fertility Calculated by Nonreturn Rate

Samples of spermatozoa from the 15 bulls with nonreturn rate fertility data were thawed, washed, and capacitated. DiQ or $DiOC_{16}$ was used to label spermatozoa during the last hour of capacitation in order to distinguish each bull's spermatozoa. At the concentration used, these dyes did not affect sperm motility or zona-binding ability (Miller et al, 1998). Stained spermatozoa from pairs of bulls were incubated with oocytes, and bound spermatozoa stained with each fluorochrome were counted. The bulls were randomly assigned a letter, and pairwise comparisons were performed between some pairs of bulls, as diagrammed in the Table.

Using ANOVA to analyze the binding data for each bull, the estimable least squares means for the number of spermatozoa from each bull that were bound to oocytes was determined. The pairwise competitive assay was able to effectively rank the 15 bulls on their ability to bind to the zona pellucida (R^2 = 0.84, Figure 1A). The 15 bulls used in this study had a relatively wide distribution (0.57–0.76) of nonreturn to estrus rates (Figure 1B). However, there was no significant correlation ($r = -0.04$, R^2 = 0.002) between the ability of a bull's spermatozoa to bind to the zona pellucida and the fertility of that bull on the basis of nonreturn rates (Figure 1C).

Relationship of Competitive Zona Binding to Fertility Calculated by Competitive Insemination

Although competitive zona-binding ability was not related to nonreturn rate, nonreturn rate only measures uncompensable traits because excessive numbers of spermatozoa are inseminated. A second limitation is that substandard

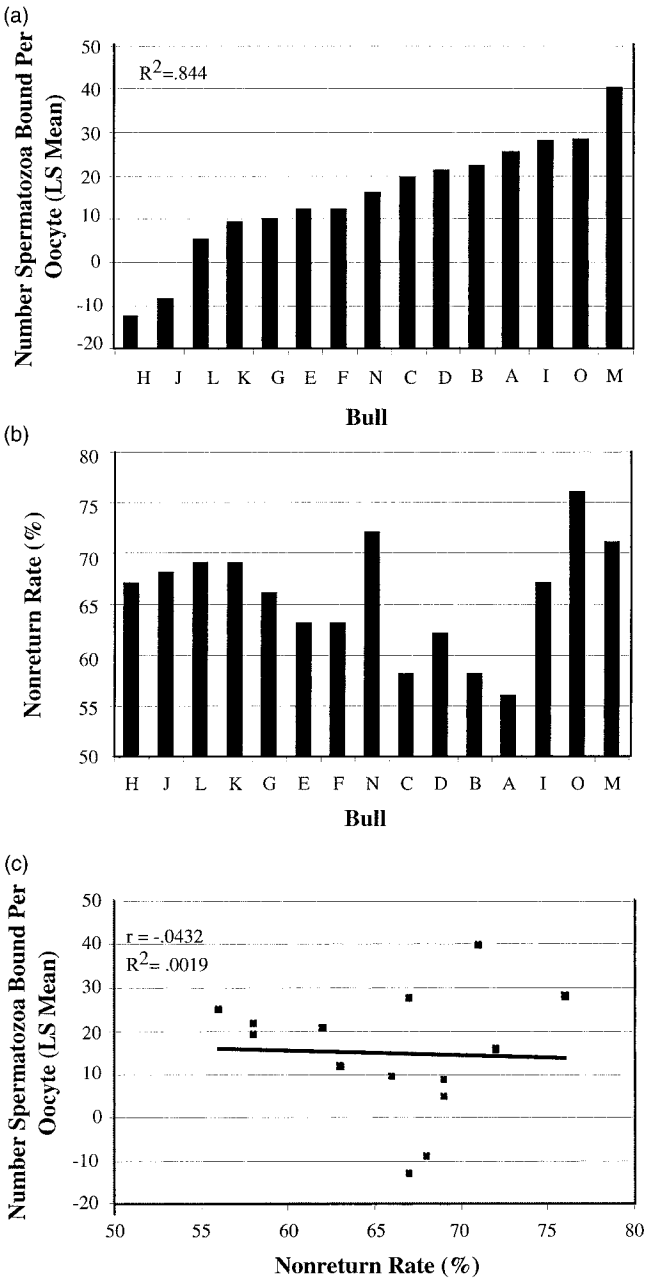

Figure 1. **(A)** Ranking of zona-binding ability of 15 bulls with historical nonreturn rate data. **(B)** Fifty-six-day nonreturn rate distribution of the 15 bulls used in the competitive zona-binding assay. **(C)** Correlation between the ranking of bulls on the basis of a competitive zona-binding assay and nonreturn rate (P = .89).

semen samples are not available commercially, thereby narrowing the range of semen quality available. In consideration of these facts, we performed a second study using semen from 8 bulls that was collected for previous experiments (Saacke et al, 1980). The fertility of these bulls was determined by competitive insemination (Saacke et al, 1980). Semen from pairs of bulls was inseminated, and paternity of offspring was determined by

genetic markers or blood typing. A competitive index was then calculated to determine relative fertility. Fertility determined in this manner would include the impact of both compensable and uncompensable seminal traits (Saacke et al, 2000).

Estimable least squares means of the average number of spermatozoa bound to the zona pellucida was calculated. Using a series of pairwise comparisons, bulls were ranked by zona-binding ability effectively (R^2 = 0.67, Figure 2A). These bulls also had a wide distribution of their heterospermic fertility competitive index (Figure 2B).

The zona-binding ability of spermatozoa was not correlated to fertility, as assessed by calculating the competitive index in a heterospermic study (r = 0.29, R^2 = 0.08, Figure 2C). Defects in zona binding were not frequent enough to allow the detection of a correlation between zona binding and fertility.

Relationship of Competitive Zona-Binding Ability to Accessory Spermatozoa

Despite the lack of correlation between zona-binding ability and fertility across a group of bulls, there may be occasional individual bulls with either a competitive advantage or a disadvantage in zona binding that may affect fertility. In this regard, one very interesting bull has been studied (Dalton et al, 2001). Significantly greater numbers of his spermatozoa are found as accessory spermatozoa on embryos after insemination than on embryos of control spermatozoa. We determined if more of his spermatozoa bound to oocytes in vitro when using a competitive assay.

Samples from the test bull and the 2 control bulls were compared by the in vitro competitive zona-binding assay (Figure 3A). These 3 samples were ranked effectively by zona-binding ability in the competitive assay (R^2 = 0.87) (Figure 3B). However, spermatozoa from the bull that had more accessory spermatozoa on embryos did not bind to the zona pellucida more frequently in vitro (Figure 3C).

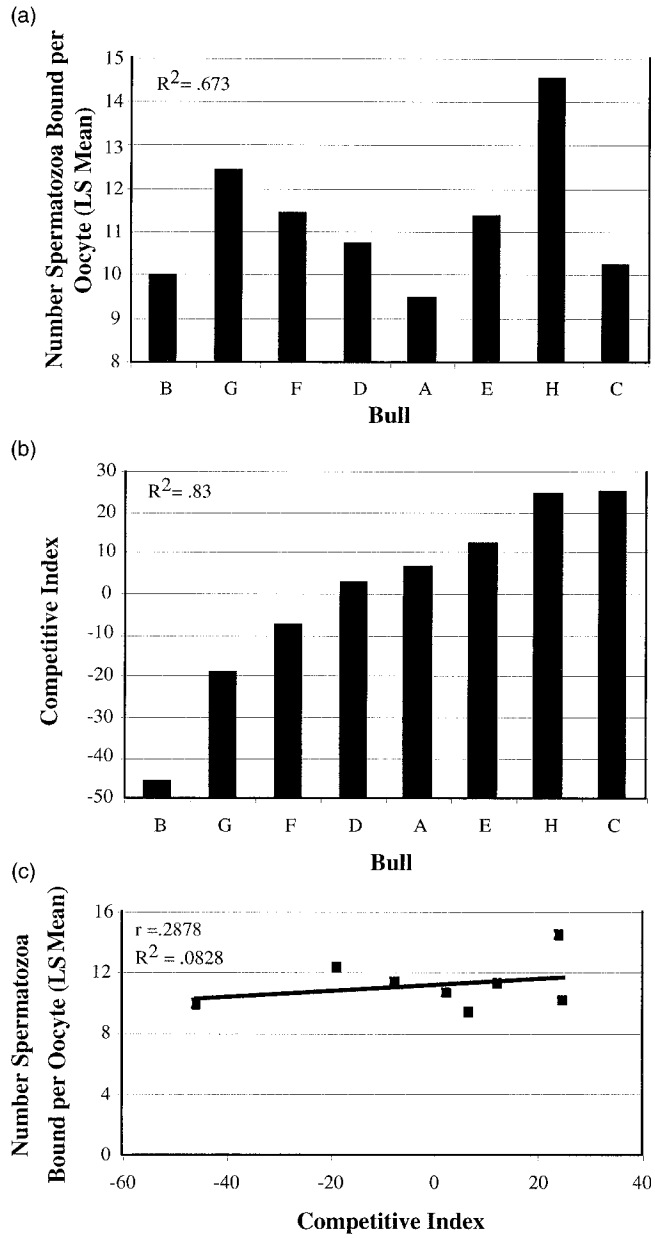

Figure 2. **(A)** Ranking of zona-binding ability of 8 bulls that had a competitive fertility index calculated by heterospermic insemination. **(B)** Distribution of the competitive fertility index for 8 bulls on the basis of heterospermic insemination. **(C)** Correlation between the ranking of bulls on competitive zona-binding ability and by in vivo heterospermic competitive index (P = .49).

Discussion

To design an accurate diagnostic assay of fertility, a thorough evaluation of the steps necessary for fertilization should be performed. These steps begin with the passage of spermatozoa through the female reproductive tract and to the site of fertilization and end with activation of the egg and initiation of the first mitosis. Spermatozoa that are defective in any of these steps are expected to have lower fertility. Since the first gamete interaction is zona binding, we determined whether this step was a common defect and was thereby related to bull fertility.

A competitive zona-binding assay was able to rank bulls efficiently on the basis of zona-binding ability. The ranking established from the competitive-binding assay was not correlated to either nonreturn to estrus rate or competitive index calculated from in vivo heterospermic inseminations.

The relationship between zona binding and fertility has been debated for some time. Two studies that tested whether zona binding was related to the 56-day nonreturn rate gave contrary answers (Fazeli et al, 1997; Zhang et al, 1998). One reason may be that in these studies, zona

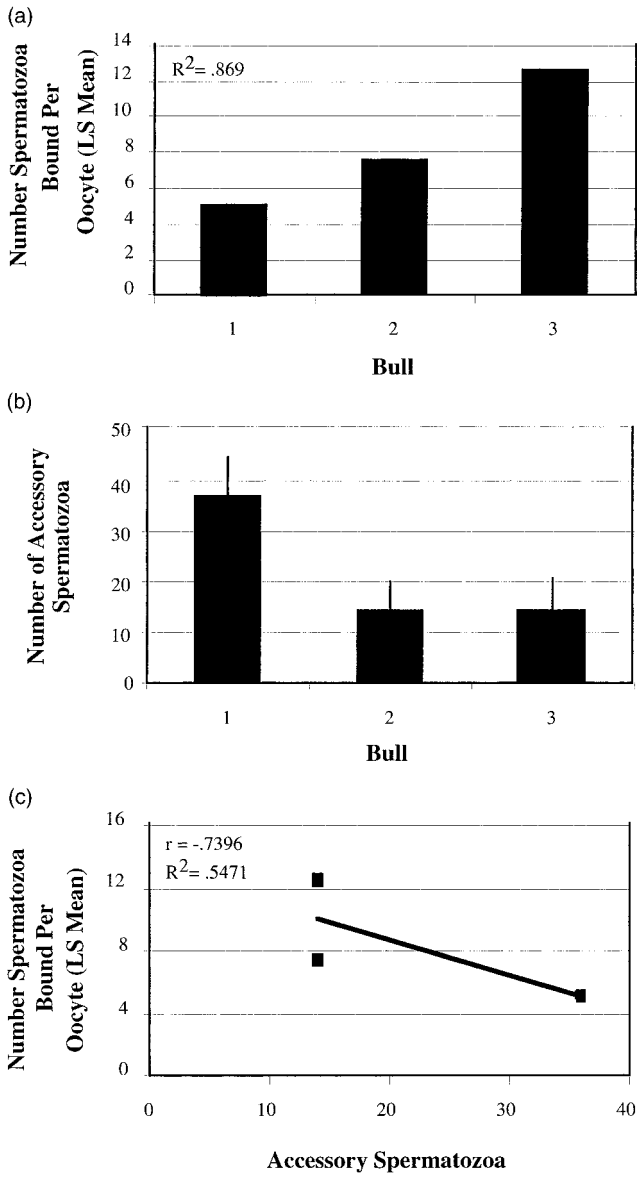

Figure 3. **(A)** Competitive zona-binding ability of spermatozoa from 3 bulls with historical differences in the number of accessory spermatozoa. **(B)** The number of accessory spermatozoa found on embryos after inseminating cows with semen from each of the 3 bulls. **(C)** Correlation between the number of accessory spermatozoa and the competitive zona-binding ability for sperm from 3 bulls ($P = .47$).

binding was calculated by 2 different methods. The Fazeli study used an index when recording zona binding, calculated by dividing the number of spermatozoa bound to oocytes for a test sample by the number of control spermatozoa bound to the oocytes, and did not detect any correlation between the index and nonreturn rate. In the Zhang study, both the absolute number of spermatozoa bound to oocytes and a binding index were recorded. Zhang et al (1998) found a correlation when calculating binding by absolute numbers but not when using an index. The studies described herein report zona-binding measurements as an average of each pairwise competition. Because we used a competitive assay, no correction for oocyte variability was needed. When using this method of estimating zona-binding ability, we did not detect a correlation between zona binding and 56-day nonreturn rates.

Other factors may account for the lack of correlation between zona binding measured by a competitive assay and nonreturn rate. One factor is that uncorrected nonreturn rates are influenced by many factors (ie, age of female and number of inseminations; Grossman et al, 1995). The influence of these factors has been corrected to yield the adjusted nonreturn rate values used in this study. Another factor to consider is that high dosages of spermatozoa are used for artificial insemination and that, in this situation, usually only bulls whose spermatozoa have uncompensable defects will have reduced fertility detectable by recording nonreturn rates. Considering this, it was important also to use a fertility measurement that could detect both uncompensable and compensable defects. In addition, analysis of a greater number of ejaculates may improve the relationship of nonreturn rate and zona binding.

In the second study, we assayed 8 bulls whose fertility was calculated from heterospermic inseminations, which detects uncompensable and compensable traits. Another advantage of measuring fertility by heterospermic insemination is that it is more reliable than nonreturn rates when using a limited number of inseminations (Robl and Dziuk, 1988). Fertility determined by this method was not correlated with in vitro binding. The nonsignificant correlation suggests that zona binding is not a step that frequently reduces bovine fertility. A later step in the fertilization pathway, such as zona penetration, membrane fusion, or oocyte activation, or an earlier step that does not influence zona binding may be defective.

Although we did not detect a significant correlation between zona binding and fertility, we also wanted to determine if there was a relationship between the number of accessory spermatozoa on the zona pellucida of embryos or ova and fertility. The number of accessory spermatozoa may be a reasonable indicator of spermatozoal transport, zona-binding ability in vivo, or both; however, spermatozoa from 1 bull that provided high numbers of accessory spermatozoa did not have a competitive advantage measurable by in vitro zona binding (Dalton et al, 2001). Spermatozoa from that bull may not have an advantage in zona binding, but instead, a higher proportion of his spermatozoa may be transported through the female reproductive tract to the site of fertilization. Transport through the female tract is an important variable in determining male fertility (Parrish and Foote, 1985). Indeed, in heterospermic insemination experiments, spermatozoa from 1 male can be predominant in compartments of the

female reproductive tract several hours after insemination (Overstreet et al, 1978).

In humans, zona binding is often defective in infertile males (Liu and Baker, 2000). This competitive-binding assay may be useful in human fertility. However, it is difficult to determine if zona-binding ability is related to human fertility due to the difficulty of collecting accurate fertility data.

Although a competitive zona-binding assay was accurate in ranking bulls on binding ability, zona binding was not correlated with fertility measurements or zona binding in vivo. In order to estimate the fertility potential of semen samples, spermatozoal transport and steps postzona binding need to be studied. Carefully designed studies should allow the identification of specific steps that are the most common defects that reduce male fertility.

Acknowledgments

This work was supported by the Illinois Agricultural Experiment Station and the Illinois Council for Food and Agricultural Research. The authors thank members of the Miller laboratory for advice in preparing the manuscript.

References

Amann RP, Hammerstedt RH. In vitro evaluation of sperm quality: an opinion. *J Androl.* 1993;14:397–406.

Braundmeier AG, Miller DJ. The search is on: finding accurate molecular markers of male fertility. *J Dairy Sci.* 2001;84:1915–1925.

Collin S, Sirard M, Dufour M, Bailey JL. Sperm calcium levels and chlortetracycline fluorescence patterns are related to the in vivo fertility of cryopreserved bovine semen. *J Androl.* 2000;21:938–943.

Dalton JC, Nadir S, Bame JH, Noftsinger M, Nebel RL, Saacke RG. Effect of time of insemination on number of accessory sperm, fertilization rate, and embryo quality in nonlactating dairy cattle. *J Dairy Sci.* 2001;84:2413–2418.

Dziuk PJ. Factors that influence the proportion of offspring sired by a male following heterospermic insemination. *Anim Reprod Sci.* 1996; 43:65–88.

Fazeli AR, Zhang BR, Steenweg W, Larsson B, Bevers MM, van den Broek J, Rodriguez-Martinez H, Colenbrander B. Relationship between sperm–zona pellucida binding assays and the 56-day nonreturn rates of frozen-thawed bull semen. *Theriogenology.* 1997;48:853–863.

Flowers WL. Management of boars for efficient semen production. *J Reprod Fertil.* 1997;52(suppl):67–78.

Grossman M, Koops WJ, den Daas JH. Multiphasic analysis of reproductive efficiency of dairy bulls. *J Dairy Sci.* 1995;78:2871–2876.

Ibrahim NM, Glibert GR, Loseth KJ, Crabo BG. Correlation between clusterin-positive spermatozoa determined by flow cytometry in bull semen and fertility. *J Androl.* 2000;21:887–894.

Liu DY, Baker HW. Defective sperm–zona pellucida interaction: a major cause of failure of fertilization in clinical in-vitro fertilization. *Hum Reprod.* 2000;15:702–708.

Marks JL, Ax RL. Relationship of nonreturn rates of dairy bulls to binding affinity of heparin to sperm. *J Dairy Sci.* 1985;68:2078–2082.

Miller DJ, Demers JM, Braundmeier AG, Behrens ML. The use of 2 fluorescent dyes to identify sperm in a competitive binding assay to oocytes. *J Androl.* 1998;19:650–656.

Overstreet JW, Cooper W, Katz DF. Sperm transport in the reproductive tract of the female rabbit. II. The sustained phase of transport. *Biol Reprod.* 1978;19:115–132.

Parrish JJ, Foote RH. Fertility differences among male rabbits determined by heterospermic insemination of fluorochrome-labeled spermatozoa. *Biol Reprod.* 1985;33:940–949.

Parrish JJ, Susko-Parrish JL, Winer MA, First NL. Capacitation of bovine sperm by heparin. *Biol Reprod.* 1988;38:1171–1180.

Robbins RK, Saacke RG, Chandler PT. Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in French straws. *J Anim Sci.* 1976;42:145–154.

Robl JM, Dziuk PJ. Comparison of heterospermic and homospermic inseminations as measures of male fertility. *J Exp Zool.* 1988;245:97–101.

Saacke RG, Dalton JC, Nadir S, Nebel RL, Bame JH. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. *Anim Reprod Sci.* 2000;60–61:663–677.

Saacke RG, Vinson WE, O'Conner M, et al. The relationship of semen quality and fertility: a heterospermic study. In: *Proceedings of the Eighth Technical Conference on Artificial Insemination and Reproduction.* Milwaukee, Wis. 1980:71–78.

Shimizu Y, Kodama H, Fukuda J, Tanaka T. Evidence of proacrosin molecule abnormality as a possible cause of low acrosin activity and unexplained failure of fertilization in vitro. *J Androl.* 1997;18:281–288.

Zhang BR, Larsson B, Lundeheim N, Rodriguez-Martinez H. Sperm characteristics and zona pellucida binding in relation to field fertility of frozen-thawed semen from dairy AI bulls. *Int J Androl.* 1998;21:207–216.