

EVALUATION OF BOAR SPERMATOZOA PENETRATING CAPACITY USING PIG OOCYTES AT THE GERMINAL VESICLE STAGE

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ABSTRACT

We evaluated the ability of immature pig oocytes (at germinal vesicle stage) to detect differences in the *in vitro* penetration rates of boar spermatozoa. In Experiment 1, immature and ovulated oocytes ($n=303$) were exposed to capacitated boar spermatozoa to determine if the penetrability of immature pig oocytes was comparable to that of ovulated oocytes. The percentages of penetrated oocytes and the mean number of spermatozoa per oocyte were similar for immature (88.82 and 7.42 ± 0.41) and ovulated oocytes (90.97 and 7.95 ± 0.34 , respectively). In Experiment 2, immature oocytes ($n=1230$) were inseminated with semen from 2 boars (A and B) with satisfactory semen characteristics to establish the variability of *in vitro* penetrating capacity between the boars. Semen was examined for motility, movement quality, acrosome integrity and plasma membrane integrity at various stages of the *in vitro* procedure. Although the sperm evaluation results were similar between boars, Boar A exhibited a significantly higher ($P < 0.001$) penetration rate (91.49%) and number of spermatozoa penetrated per oocyte (5.90 ± 0.25) than Boar B (52.87% and 2.03 ± 0.12 , respectively). Increasing the sperm concentration at insemination from 1×10^6 to 10×10^6 cells/ml resulted in an increased penetrating capacity for both boars, and the differences in the number of spermatozoa per oocyte between boars also increased. These results indicate that immature pig oocytes can be used in a homologous *in vitro* fertilization assay, and that despite similarities in semen characteristics a significant boar effect is evident for parameters of *in vitro* penetration of oocytes.

Key words: pig, spermatozoa, oocyte, *in vitro* fertilization, semen evaluation

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INTRODUCTION

The prediction of the fertilizing ability of boar semen is important in breeding herds, particularly when artificial insemination is used. Some subfertile boars appear fertile by routine semen analysis (1). There is no semen quality assay which has been shown to accurately predict male fertility. As fertilization is one of the key criteria which determines *in vivo* fertility, it has been hoped that development of *in vitro* fertilization (IVF) will result in a procedure to predict fertility *in vivo*. Yanagimachi et al. (2) proposed the use of zona-free hamster oocytes as a testing system for the assessment of the fertilizing capacity of human spermatozoa. Although the zona-free hamster ova bioassay can improve the *in vitro* assessment of fresh boar semen (1), it was hypothesized that a better prediction of fertility should be obtained by IVF of zona-intact homologous oocytes (3). The zona-free hamster ova bioassay evaluates only part of the fertilization process. It does not evaluate the initial steps of sperm binding to the zona pellucida or penetration of the zona pellucida. The use of zona-intact homologous oocytes may give more complete information on all stages of sperm penetration. However, the actual procedure of homologous IVF in pigs needs to be simplified, and the results correlated with *in vivo* fertility, before this test can become practicable.

There is high variability in the *in vitro* penetration rates of pig oocytes between laboratories or even assays within the same laboratory. These differences may be caused by variation in the fertilizing ability of spermatozoa among boars such as occurs in the bull (4-6). The influence of individual boars on homologous *in vitro* fertilization rates has been suggested by Yoshida (7) and Nagai et al. (8), but has not been clearly established.

The objectives of our study were 1) to determine if immature pig oocytes (at the germinal vesicle stage) can be penetrated as successfully as ovulated oocytes, in an effort to simplify the homologous test; and 2) to establish the variability of the *in vitro* penetrating capacity between boars with normal semen characteristics.

MATERIALS AND METHODS

Collection of Ovulated Oocytes

Ten (Landrace x Large White) prepubertal gilts were used in the study. The animals weighed 80 to 95 kg and were approximately 5.5 to 6 months old. Follicular growth and ovulation were induced by a single injection of 1250 IU, *i.m.* of PMSG (Intervet International B.V. Boxmeer, The Netherlands) followed 56 hours later by administration of 750 IU, *i.m.* of hCG (Intervet International). Freshly ovulated oocytes were surgically recovered 42 hours after hCG administration. Ovaries were exposed through a midline incision under general anesthesia. Whenever ovulation points were observed, ovulated ova were obtained by the flushing of oviducts. Each oviduct was flushed from the uterotubal junction towards the fimbria at least 2 times with 10 ml Dulbecco's phosphate buffered saline supplemented with 0.4% (w/v) bovine serum albumin (BSA: Fraction V, Sigma Chemical Co., St, Mo, USA), 0.34

mM pyruvate, 5.5 mM glucose, and 70 $\mu\text{g}/\text{ml}$ Kanamycin (DPBSm; 9) at 37°C. Ovulated oocytes were washed once in DPBSm and returned to the laboratory in a 10-ml test tube containing DPBSm. The oocytes were washed again 3 times in DPBSm at 37°C, transferred to 35-mm plastic dishes containing fertilization medium and were exposed to capacitated boar spermatozoa. The number of animals used each day ranged from 2 to 3. The collection of oocytes and preparation of cultures were completed within 50 and 60 minutes, respectively.

Collection of Immature Oocytes

Ovaries were obtained from prepuberal gilts weighing approximately 95 kg at a local abattoir just after of slaughter and were returned to the laboratory in DPBSm at 37°C within 1 hour. The oocytes were collected from medium-size antral follicles (3 to 5 mm diameter), by repeatedly puncturing the ovary with a sterile, 21-gauge needle, into DPBSm at 37°C. Oocytes with compact cumulus cells were washed twice with DPBSm and freed from cumulus and corona cells by repeated passage through a fine pipette. The denuded oocytes were washed again twice and placed in 35-mm plastic dishes containing fertilization medium for insemination with preincubated spermatozoa. Preparation of cultures was completed within 60 minutes.

Sperm Preparation

Experiment 1. To investigate if the penetrability of immature oocytes is comparable to that of ovulated oocytes, immature and ovulated oocytes were exposed to capacitated boar sperm following the method of Cheng et al. (10) modified by Mattioli et al. (9). The sperm-rich fractions were collected from 2 mature Landrace boars by the gloved hand method into prewarmed (37°C) thermos flasks. Immediately after collection, semen samples were diluted to 3×10^7 cells/ml in MR-A diluent (11) and were kept for about 24 hours at 16°C. Before use, the sperm suspensions from the 2 boars were pooled and centrifuged for 3 minutes at 50 g. Then, the supernatant was washed 3 times by centrifugation at 1,200 g for 3 minutes in 0.9% saline solution enriched with 1 mg/ml BSA (Fraction V, Sigma) at pH 7.2. The heterospermic pellet was subsequently diluted to 2×10^8 cells/ml in preincubation medium, consisting of TCM-199 with Earle's salts (Boehringer, Mannheim, Germany) at pH 7.8, supplemented with 12% heated fetal calf serum (Gibco, Grand Island, NY, USA), 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 2.92 mM calcium lactate, 50 IU/ml penicillin G, and 30 $\mu\text{g}/\text{ml}$ streptomycin sulphate. Semen samples were then preincubated for 40 minutes at 39°C in a tightly capped test tube.

Experiment 2. To establish the variability of the in vitro fertilizing capacity between boars, 2 mature Landrace boars (A and B) of similar age and sexual experience and with satisfactory semen characteristics in the 8 weeks preceding the experiment (total sperm per ejaculate $> 2 \times 10^{10}$, motility $> 70\%$, morphological abnormalities $< 15\%$ and acrosome abnormalities $< 10\%$) were selected as semen donors. In previous IVF experiments the boars were each used on separate days. Semen from Boar A exhibited high penetration rates in 12 assays, while semen from

Boar B exhibited low penetrability in 9 assays.

Nine ejaculates (sperm-rich fractions) were collected from each of the 2 boars within a period of 9 weeks. Shortly after collection, the semen was resuspended in MR-A diluent for a total of 3×10^7 cells/ml and was processed for the homologous in vitro penetration assay using immature oocytes, as described above, except that the sperm suspensions were not pooled.

Spermatozoa were evaluated for motility, movement quality, acrosomal integrity (Normal apical ridge: NAR), and plasma membrane integrity (PMI) at various stages of the in vitro procedure. For motility and movement quality evaluations, 3 sub-samples of each sample were placed on warm glass slides (39°C) and were examined under a light microscope (400x). Sperm motility was assessed by determining the percentage of spermatozoa showing any movement of the flagello. Movement quality of spermatozoa was determined using an arbitrary scale of 0 to 5 (0 = all spermatozoa are immotile; 1 or 2 = 10 to 25% or 25 to 50%, respectively, of the motile spermatozoa show progressive movement; 3 = 50 to 70% of the motile spermatozoa show progressive movement; 4 = 70 to 90% of the motile spermatozoa show progressive movement; 5 = all motile spermatozoa show progressive movement). The proportions of spermatozoa with NAR were measured by viewing wet mounts of semen fixed in buffered 2% glutaraldehyde solution (12) under phase-contrast microscopy (1000x). Two slides were counted for a total of 300 spermatozoa per sample. The PMI was examined using a fluorescent probe with carboxyfluorescein diacetate, as described by Harrison and Vickers (13). Next, 20 μl of a stock solution of carboxyfluorescein diacetate (Sigma) prepared in dimethylsulfoxide (DMSO) at a concentration of 0.46 mg/ml were added to 20 μl of a stock solution of formaldehyde (2.5 mg/ml in water) to achieve a final concentration of 1.7 mM-formaldehyde and 20 μM -carboxyfluorescein diacetate in 1 ml of MR-A medium. Samples of spermatozoa were diluted in aliquants of staining medium to a final concentration of 10^7 cells/ml and were then incubated for at least 10 minutes at 30°C . Then, 5 μl of the stained suspensions were observed under 400x magnification with epifluorescence illumination. Two slides were counted for a total of 300 spermatozoa per sample. The remaining of seminal characteristics (total sperm per ejaculated and normal morphology) were examined only in fresh semen, and standard laboratory techniques were used.

In Vitro Fertilization and Oocyte Examination

Each group of 10 ovulated or immature oocytes was coincubated with preincubated spermatozoa for 16 to 18 hours in 2 ml of fertilization medium (TCM-199, pH 7.4, with the same additives as the sperm preincubation medium except that it also contained caffeine 2 mM and calcium lactate 5.4 mM). A final sperm concentration of 1×10^6 cells/ml (Experiment 1), or 1×10^6 and 10×10^6 cells/ml (Experiment 2) was used. Cultures were maintained at 39°C in an atmosphere of 5% CO_2 in air. At the end of the culture period, excess spermatozoa were removed from the zona pellucida by pipetting. The oocytes were then washed in DPBSm, mounted on slides, fixed for 24 hours in acetic acid:ethanol (1:3, v/v) at room

temperature, and stained with 1% lacmoid (Sigma; 14). Each oocyte was evaluated under phase contrast microscopy (400x) for evidence of sperm penetration. Oocytes were considered to be penetrated when spermatozoa with swollen or unswollen heads or pronuclei and their corresponding sperm tails were found in the vitellus.

Statistical Analysis

Sperm evaluation parameters and the number of penetrating spermatozoa were represented as the mean \pm SEM and were analyzed using Student's t-test for paired samples. Penetration rates of the oocytes were analyzed by the Chi-square test (15).

RESULTS

Experiment 1

Of 10 prepubertal gilts used as donors of ovulated oocytes, 2 remained at the preovulatory phase, 1 had begun ovulation and the remaining 7 had ovulated at 42 hours after hCG administration. The number of ovulations estimated by the total number of mature follicles and ovulation points ranged from 8 to 65 (mean 19.30). Of the ovulated oocytes, 88.32% were recovered from tubal flushings, as determined after counting ovulation points.

The results of IVF are shown in Table 1. A total 303 ovulated and immature oocytes was examined; 90.97% of the ovulated oocytes were penetrated (121/133). This penetration rate was not significantly different from that observed in immature oocytes (88.82%, 151/170). The mean number of spermatozoa per ovulated or immature oocyte was similar (7.95 ± 0.34 vs 7.42 ± 0.41 , respectively) and ranged from 1 to 38 for ovulated oocytes and from 1 to 35 for immature oocytes. The incidence of polyspermy was consistent and remained high across all replicates.

Table 1. In vitro penetration of ovulated and immature pig oocytes

	Type of oocyte	
	Ovulated	Immature
No. of oocytes examined	133	170
No. of oocytes penetrated (%) ^a	121 (90.97)	151 (88.82)
Range of penetration among replicates (%) ^b	83.34-96.93	84.23-93.51
Mean No. of sperm/oocyte ^c	7.95 ± 0.34	7.42 ± 0.41
No. of polyspermic oocytes (%) ^d	118 (97.52)	144 (95.36)

^aOocytes were examined 16 to 18 hours after insemination.

^bExperiment was repeated 4 times.

^cEach value represents the mean \pm SEM.

^dPercentage of total number of oocytes penetrated.

After incorporation into the oocyte cytoplasm, the degree of sperm decondensation differed according to the source of the oocytes; 88.93% of the penetrating spermatozoa in ovulated oocytes showed nuclear decondensation or pronuclear formation. By contrast, in immature oocytes only 6.5% of the spermatozoa showed early stages of nuclear decondensation, while the remainder were unswollen.

Experiment 2

There were no differences between the boars in semen characteristics of each fresh ejaculate.

Table 2 shows the results of semen quality evaluation and the parameters of in vitro penetration using 1×10^6 sperm/ml at insemination. Motility, movement quality, NAR and PMI in diluted semen, and in stored (24 hours, 16°C) semen were satisfactory and did not differ between Boars A and B. Assessment immediately after preincubation treatment revealed a decrease for all parameters except for movement quality of spermatozoa which improved, and hyperactivated movement of spermatozoa was clearly visible. This pattern was similar for the two boars.

Although semen quality during all stages of the process was similar between boars, Boar A exhibited a significantly higher penetration rate (91.49%) and number of spermatozoa penetrated per oocyte (5.90 ± 0.25) than Boar B (52.87% and 2.03 ± 0.12 , respectively). Likewise, the number of polyspermic oocytes was different between the 2 boars (98.75% vs 60.25%).

Increasing the sperm concentration at insemination from 1×10^6 to 10×10^6 cells/ml resulted in an increased penetrating capacity for both boars, and the differences in the number of spermatozoa per oocyte between boars also increased (20.87 ± 0.75 vs 5.63 ± 0.33 ; Figure 1).

DISCUSSION

Nagai et al. (8) obtained a higher penetration rate of pig oocytes matured in vivo than those matured in vitro. However, oocytes matured under inadequate culture conditions are known to be less penetrable by spermatozoa. Thus, the co-culture of cumulus-enclosed oocytes with extroverted follicles produced mature oocytes with a higher rate of penetrability (9,16). Under these conditions, in vitro matured oocytes and immature oocytes showed similar penetrability (3,17). Our results are consistent with these earlier observations, and indicate that pig ovarian oocytes at the germinal vesicle stage and ovulated oocytes are equally penetrable by spermatozoa in vitro.

Pig oocytes differ from other species in that they undergo an exceptionally high incidence of polyspermy when fertilized in vitro (8,16,18,19). Ultrastructural studies by Cran and Cheng (20) have revealed that abnormal cortical granule exocytosis occurs during in vitro fertilization of in vivo-matured pig oocytes and this facilitates

Table 2. Semen quality evaluation and parameters of in vitro penetration (IVP) of ejaculates from 2 boars^a

Semen analysis	Boar A		Boar B	
	x±SEM	Range	x±SEM	Range
Diluted spermatozoa				
% Motility	76.00±1.21	71.7-81.7	73.00±1.42	66.7-78.3
Movement quality ^b	3.23±0.06	3.0-3.5	3.16±0.06	2.8-3.5
% NAR	92.00±0.54	90.7-94.3	89.63±0.87	85.7-93.3
% PMI	68.66±1.95	62.3-75.7	71.56±1.86	65.7-77.7
Stored spermatozoa				
% Motility	69.63±1.14	65.0-75.0	70.02±1.61	61.7-78.3
Movement quality ^b	3.10±0.11	2.8-3.5	3.10±0.11	2.8-3.5
% NAR	87.32±1.15	78.3-87.7	85.74±1.56	81.3-90.7
% PMI	65.74±1.76	60.7-71.0	63.40±1.66	58.3-68.7
Preincubated spermatozoa				
% Motility	55.33±4.32	31.7-70.0	65.00±2.12	51.7-73.3
Movement quality ^b	3.96±0.06	3.7-4.3	3.96±0.06	3.8-4.0
% NAR	66.57±2.19	60.3-71.7	61.42±2.85	52.3-71.0
% PMI	48.34±2.31	41.3-54.7	50.29±1.33	45.0-53.3
Parameters of IVP^c				
% oocytes penetrated (No. of oocytes examined)	91.49 ^d (247)	79.5-100 (53-56)	52.87 ^e (278)	18.7-71.4 (48-56)
x±SEM no. sperm/oocyte	5.90±0.25 ^d	3.9-7.3	2.03±0.12 ^e	1.2-2.6

^aEach value represents five replicates.

^bScale 0 to 5.

^cSperm concentration at coincubation was 1×10^6 cells/ml.

^{d,e}Different superscripts in the same row are significantly different ($p < 0.001$).

NAR = Normal apical ridge; PMI = Plasma membrane integrity.

polyspermy. In immature pig oocytes there seems to be no block to polyspermy, since the migration of the cortical granules beneath the oolemma takes place towards the end of maturation. In the present study, the same rates of polyspermy were obtained in ovulated and immature oocytes. This indicates that there is complete failure of the block to polyspermy in the in vitro fertilization system used.

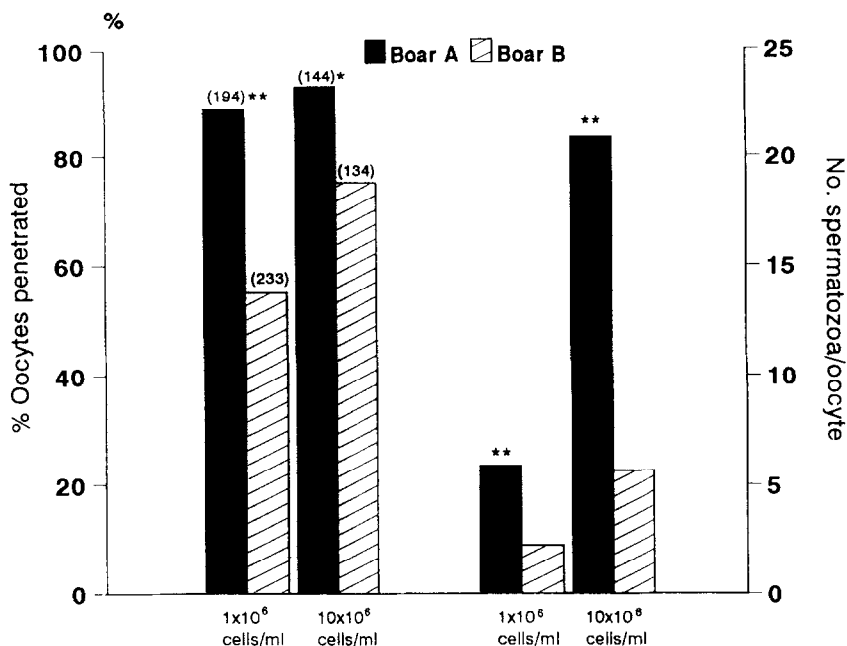


Figure 1. Effect of sperm concentration at coincubation on the parameters of in vitro penetration of ejaculates from 2 boars. Data were obtained from 4 replicates. There were at least 30 oocytes per group and replicate. Numbers in parentheses represent the number of oocytes examined. Asterisks indicate values are significantly different from that of other treatment groups at the same concentration of spermatozoa (* $P < 0.01$, ** $P < 0.001$).

The arrest of male pronuclear formation as a result of inadequate cytoplasmatic maturation of oocytes in culture is a typical phenomenon associated with in vitro matured oocytes (9,18,21-23). In our study, decondensing sperm heads were seldom observed within the cytoplasm of immature oocytes, whereas most of the spermatozoa incorporated into the vitellus of ovulated oocytes were transformed to male pronuclei. According to Mattioli et al.(3), such differences in the evolution of penetrated spermatozoa only reflect a different degree of cytoplasmatic maturation that is independent of the spermatozoa.

We consider that immature oocytes are ideal for testing the fertilizing ability of boar spermatozoa. These oocytes appear to yield fertilization frequencies comparable to those with ovulated oocytes, and large numbers can be obtained from

ovaries collected at the abattoir.

The data from Experiment 2 indicated variability in *in vitro* fertilization by different boars. This is not surprising in the light of previous findings with bulls (4-6). In the pig, little information is available concerning the influence of individual boars on *in vitro* fertilization rates. Only Berger and Parker (1), using zona-free hamster eggs, and Nagai et al. (8), using homologous IVF with frozen-thawed boar spermatozoa, showed variability among boars. It has been speculated that the time needed for capacitation may vary between the ejaculates from different boars (7). Thus, the variability in fertilization rates by different boars may be partly due to differences in the total number of capacitated or capacitating spermatozoa at the time of oocyte penetration. However, a correlation between the incidence of the acrosome reaction and *in vitro* fertilization has been not demonstrated. Berger et al. (24) showed that when the acrosome reaction was at a constant level, differences were observed among boars in rates of *in vitro* fertilization using zona-free hamster eggs. Using a triple stain technique (25), we have also observed that although the rate of true acrosome reactions after preincubation and throughout 2, 4 and 6 hours of coincubation was similar among 4 boars, there were significant differences in the *in vitro* fertilization rates using ovulated oocytes (26).

Clarke and Johnson (27) reported that the ability of spermatozoa to penetrate oocytes *in vitro* decreased when their motility after preincubation was lower. Nagai et al. (8), on the other hand, found that some spermatozoa were not able to penetrate oocytes despite having high motility after preincubation. Berger and Parker (1) also showed that motility and velocity of spermatozoa at insemination or after incubation was not correlated with variation between ejaculates from different boars in *in vitro* penetration of oocytes. Our study was limited by the low number of boars tested and because these boars were selected for differences in *in vitro* fertilization by their spermatozoa. Nevertheless, our results are consistent with those of previous studies, and suggest that the classical parameters accepted in assessing the viability of spermatozoa do not predict the penetrating ability of the spermatozoa *in vitro*. Other factors, unknown at present, may contribute to differences in the fertilizing potential of spermatozoa.

We conclude that regardless of semen evaluation data, for all parameters of *in vitro* fertilization (penetration rate and mean number of spermatozoa per oocyte) a significant boar effect is evident using immature pig oocytes. If confirmed on a larger number of boars, such an *in vitro* test would be valuable in evaluating the level of fertility of artificial insemination boars before they are used in the field. Large-scale insemination experiments are needed to correlate the results of this test with *in vivo* fertility of boars.

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