ELSEVIER

SPERM FACTORS RELATED TO IN VITRO PENETRATION OF PORCINE OOCYTES

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ABSTRACT

This study was designed to evaluate the relationship between sperm factors and penetration capacity in an in vitro system with immature porcine oocytes. The sperm parameters evaluated in 145 ejaculates were volume, sperm concentration, total cells in the ejaculate, ATP content, morpho-anomalies, percentage of motile sperm cells, forward progressive motility (FPM), acrosome status (NAR), hypo-osmotic swelling test (HOS), osmotic resistance test (ORT), eosin-nigrosin viability stain and sperm membrane integrity (DCF). Porcine oocytes (a total of 8736) were used to evaluate the capacity of the different sperm assays to predict penetration. Many parameters were found to be related to in vitro penetration ability; all conventional semen parameters, except sperm concentration and eosin-nigrosin staining, were significantly better in high (>75%) than in low penetration rates (<75%). When the ejaculates were preselected the number of significantly related parameters was lower.

When studying all conventional semen parameters through a stepwise multiple linear regression analysis of seminal measurements, up to 72.3% of total variance of the penetration rate could be predicted. However, as many as 4 parameters were needed (FPM in fresh semen, folded tail, NAR in post-treatment semen and DCF) for accurate prediction. On the other hand, the multiple logistic regression needed 7 parameters to discriminate 83.96% of the cases correctly.

In summary, the results from the present study showed that almost all studied parameters were significantly different for predicting penetration process attained or failed, but most of them were correlated together. These findings emphasize the complexity of sperm functions and the difficulty of assessing the fertilizing ability.

Key words: boar reproduction, in vitro penetration, spermatozoa, semen analysis

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INTRODUCTION

The binding and penetration of the zona pellucida is one of the most important barriers the spermatozoa must overcome in the fertilization process (12). Also, interaction with the oocyte plasma membrane appears to explain much of the variability in sperm fertilizing potential among fertile boars (4). So, assays that include study of gamete interaction might lead to a better way to predict male fertility than routine laboratory evaluation of semen (3).

In the pig some assays have been shown to be good tools for evaluating the fertilizing capacity of boar semen: sperm penetration assay (SPA) with zona-free hamster oocytes (4, 5, 11), sperm-zona binding to intact pig oocytes (15) or to stored pig oocytes (18), homologous in vitro fertilization using in vitro matured oocytes (7, 8, 35, 36) or using porcine zona-intact oocytes at the germinal vesicle stage (21, 23). The last named homologous in vitro penetration assay (hIVP) has been shown to be a good tool for evaluating the fertilizing capacity of fresh boar semen (10, 22) and for assessing stored boar semen (33).

An increasing interest in the conditions of porcine in vitro fertilization (IVF) has led to studies designed to evaluate the factors that support success in IVF of pig oocytes, such as maturation and fertilization media, culture conditions (reviwed in 9), the boar effect and semen dilution (34). Nevertheless, knowledge of sperm factors related to penetration capacity is limited.

The aim of this study was to evaluate the relation between sperm factors and penetration capacity in a hIVP system using porcine zona-intact oocytes at the germinal vesicle stage.

MATERIALS AND METHODS

Spermatozoa from 145 ejaculates from 47 Pietrain boars were evaluated by in vitro semen quality parameters and for homologous oocyte penetrating ability. Sperm-rich fractions were collected by an experienced operator using the gloved hand technique. At the time of collection, the ejaculate was filtered through a gauze to remove gel and immediately placed at 37°C in a temperature-controlled water bath. Each ejaculate was later diluted to a final concentration of 3×10^7 spermatozoa/mL in a commercial diluent for refrigerated semen (MR-A©, Kubus, Madrid, Spain) and stored at 15°C until assayed.

Routine Laboratory Evaluation of Semen

The volume of the sperm-rich fraction of the ejaculate was determined in a graduated cylinder, and sperm concentration was measured with a hemacytometer. To evaluate the motility and the forward progressive motility (FPM), 2 subsamples were placed on warm glass slides (39°C) and examined under a light microscope (x 400 magnification). The percentage of motile sperm cells was estimated subjectively to the nearest 5%, and the FPM was estimated using an arbitrary scale of 0 to 5 (20).

Wet mounts of semen fixed in buffered 2% glutaraldehyde solution were examined under a phase-contrast microscope (x 1000 magnification) to analyze morphology and acrosomes (25). The proportion of spermatozoa with a normal apical ridge (NAR) was determined on 2 slides per sample and a total of 200 spermatozoa per sample. Spermatozoa were categorized according to sperm morphology into those with normal morphology, cells with attached cytoplasmic droplets, folded tail, coiled tail and others (such as abnormal heads).

For eosin-nigrosin viability staining, a semen sample was diluted 1:1 with staining solution (5% yellow eosin, 10% nigrosin in a citrate solution, pH = 7.4) and smeared. Air-fixed stained spermatozoa were observed; 200 spermatozoa were evaluated per slide (2).

Sperm membrane integrity was assessed by incubation with carboxyfluorescein diacetate (DCF) (13) on at least 200 cells/sample using a microscope equipped with epi-fluorescence.

The osmotic resistance test (ORT) was carried out as described by Shilling and Vengust (28). The percentage of ORT was calculated by means of the percentages of intact acrosomes after the incubation in an iso-osmotic medium (300 mOsm/kg, 15 min, 39°C) and in an hypo-osmotic medium (150 mOsm/kg, 120 min, 39°C).

The hypo-osmotic swelling test (HOS) was performed as described by Jeyendran et al. (16) with the modifications of Vázquez et al. (32). An 0.1 mL aliquot of sperm suspension was mixed with 0.9 mL hypo-osmotic solution (150 mOsm/kg). The solution was then incubated for 60 min at 37° C. Two hundred spermatozoa per slide were counted under a phase contrast microscope at x 1000 magnification and the percentage of cells with swelling/coiling was determined. The proportion of coiled/swollen spermatozoa from a control sample (300 mOsm/kg) was subtracted from calculations.

Fresh semen was checked for ATP content using an enzymatic quantitative method (Sigma 366-UV), as described by Adams (1). The ATP concentrations were expressed in nmol /10^8 sperm cells.

Homologous In Vitro Penetration Test (hIVP)

Porcine oocytes were collected from fresh ovaries from prepubertal gilts weighing approximately 95 kg, just after slaughter at a local abattoir, and transported in less than 30 min to the laboratory in 0.9% (wt/vol) NaCl containing 100 µg/mL kanamycin sulphate at 30°C. The oocyte-cumulus complexes were collected from 2 to 5 mm diameter antral follicles by dissection of the ovarian surface with a single sterile blade in Dulbecco's phosphate-buffered saline (DPBS) supplemented with 4 mg/mL BSA (Fraction V, Sigma), 0.34 mM pyruvate, 5.54mM D-glucose, and 70 µg/mL kanamycin at 37°C. The COC were washed 3 times with modified DPBS before exposure to boar spermatozoa.

The sperm samples (diluted in MR-A extender and kept 24 h at 16°C) were pretreated by centrifugation at 50xg for 3 min and the subsequent concentration of the supernatants at 1200xg for 3 min. Resulting pellets of spermatozoa were diluted to 2 x 10^8 cell/mL in a preincubation medium consisting of Medium 199 with Earle's salts (Sigma) supplemented with 12% heat-inactivated fetal calf serum, 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 2.92 mM calcium lactate, 50 IU/L penicillin G and 30 µg/mL streptomycin sulphate (modified M-199) at pH 7.8. After that, sperm suspensions were used without preincubation (20) and were termed "post-treatment semen".

Each group of 15 immature oocytes was coincubated with spermatozoa (10^7/mL) for 16 to 18 h in a 35-mm plastic dish containing 2 mL of fertilization medium (modified M 199, pH 7.4, supplemented with 2 mM caffeine and 5.4 mM calcium lactate) at 39 °C under 5% CO_2 in air. At the end of the co-incubation period, oocytes were stripped of cumulus cells and

spermatozoa, mounted in slides and fixed for a minimum of 24 h with ethanol:acetic acid (3:1 v/v). They were later stained with 1% lacmoid and examined for evidence of sperm penetration under a phase contrast microscope (x 400 magnification). Immature oocytes were considered to be penetrated when spermatozoa with unswollen heads and their corresponding tails were found in the vitellus. The distinction between unswollen spermatozoa that had entered the oocyte cytoplasm and those remaining on the surface of vitelline membrane was based on the fact that the former had more intensive staining after lacmoid treatment than the latter and that the sperm tails inside the vitellus were straight and slightly separated from the heads (20, 24).

Experimental Design

In this study, 8736 oocytes were used to evaluate the capacity for penetration prediction of the different sperm assays. The ejaculates were grouped in 2 categories according to in vitro penetration rate (Low, <75%; high, >75%). Seminal parameters were evaluated and differences between penetration groups were investigated.

Statistical Analysis

Receiver-operating curves (ROC curves) were used to determine the overall discriminant power of penetration rate in the logistic regression model. An ROC curve was also used to calculate the breaking point (cutoff value for in vitro penetration rate in relation to a fertility trial of a previous study; 10).

The results (expressed as mean ± SEM) were analyzed by one-way ANOVA, using the multivariate general linear models of Systat, considering the penetration group as the main effect or by two-way ANOVA considering penetration group and kind of semen (fresh, diluted or post-treatment semen). When ANOVA revealed a significant effect, values were compared by the Tukey test.

Linear regression was used to further investigate relationships between the mean number of sperm per oocyte penetrated and measured semen parameters (Pearson correlation and multiple regression), and logistic regression was used to relate the dichotomous penetration rate data to the sperm parameters, as previously described by Holt et al. (14).

RESULTS

Semen Characteristics of the High and Low Success Penetration Group

The best cutoff value for in vitro penetration rate to forecast in vivo fertility was found to be 75% according to the ROC curve (Figure. 1). This cutoff resulted in a 74.17% true positive rate and 25.84% false positive rate in predicting in vivo fertilizing ability (Table 1).

Basic statistics of sperm parameters for the 2 groups of ejaculates according to penetration rate (more or less than 75%) are shown in Table 2. Most ejaculates were in the high penetration rate group (107 vs 38). The mean values obtained in the hIVP test using 6063 (Group 1) and 2673 oocytes (Group 2) for penetration rate (92.04±0.65 vs. 34.23±4.08) and the mean number of sperm cells per oocyte (23.01±1.81 vs. 2.89±0.54) were significantly different ($P<0.001$). All conventional semen parameters except sperm concentration and eosin-nigrosin staining were significantly better in high than in low penetration rates.

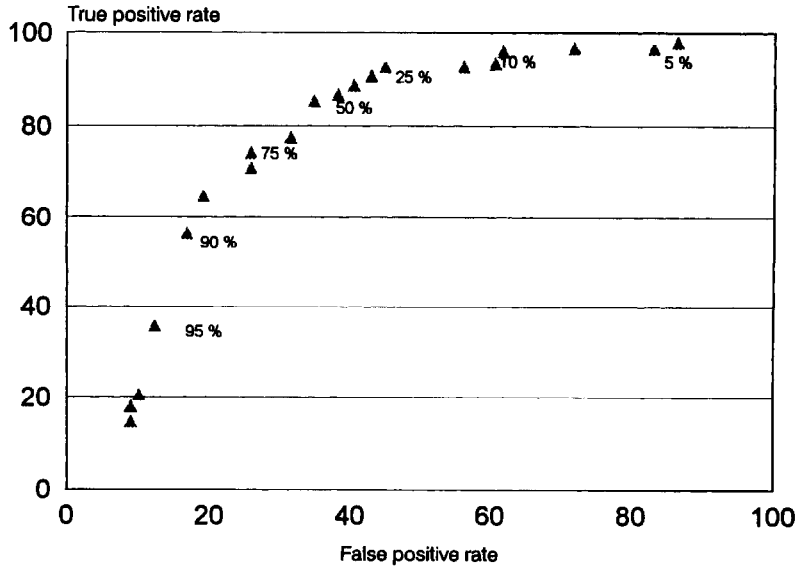

Figure. 1. Representative ROC-curve of the in vitro penetration rate and in vivo fertility (farrowing rate).

Table 1. Cross-tabulation that displays the relationship of penetration values on farrowing rate. Data obtained from a fertility trial of a previous study (10).

	Farrowing rate	
Penetration rate	No	Yes
<75%	66	39
>75%	23	112
Total	89	151

A significant effect of the type of semen evaluated were observed for motility, FPM, NAR and HOS, the highest values were obtained in the fresh semen. However, no significant differences were obtained between semen for ORT index and eosin-nigrosin staining. Anyway the interaction IVP group and semen type had not a significant effect.

Relation Between Sperm Characteristics and the In Vitro Penetration Assay

Linear and logistic regression analyses were used to relate the oocyte penetration rate data to the sperm parameters. In both analyses, all studied semen parameters were significantly related to the oocyte penetration rate except concentration (logistic and linear regression) and eosin-nigrosin staining in post-treatment semen (only linear regression) (Table 3).

Table 2. Measured sperm parameters for ejaculates allocated into two groups acording to oocyte penetration rate (mean ± SEM)

Parameter	Group 1(n=107) High penetration rate (>75%)			Group 2 (n=38) Low penetration rate (< 75 %)			Analysis of variance Sources of variation		
	Fresh semen	Diluted semen	Post-treatment semen	Fresh semen	Diluted semen	Post-treatment semen	IVP group	Semen type	Interaction
Volume (ml)	142.63±3.94 a			124.81±8.77 b			0.036		
Concentration (10^3sp/mm^3)	468.78±13.40			469.73±31.54			0.974		
Total cells (10^9)	64.97±2.21a			52.09±2.97b			0.002		
ATP (nmol/10^8 cells)	23.28±1.36a			19.09±1.38b			0.043		
Morpho-anomalies (%)		8.96±0.73a			31.21±3.99b		<0.001		
Cytoplasmic droplets (%)		4.94±0.61a			20.89±3.70b		<0.001		
Folded tail (%)		3.30±0.36a			9.18±1.19b		<0.001		
Swollen tail (%)		0.33±0.07a			0.76±0.20b		0.012		
DCF (%)		71.78±1.06a			65.57±1.45b		0.008		
Motility (%)	75.93±0.28a	71.32±0.57b	72.5±0.65b	70±1.37b	65±1.44c	62.64±1.87c	<0.001	<0.001	0.062
FPM	3.61±0.02a	3.07±0.04b	3.25±0.61b	3.09±1.02b	2.67±0.07c	2.69±0.10c	<0.001	<0.001	0.419
NAR (%)	88.05±1.23a b	90.39±0.41a	84.34±0.84b	83.25±2.23b c	83.57±2.23bc	78.33±1.53c	<0.001	<0.001	0.913
HOS (%)	51.79±1.98a	45.70±1.86a b		45.25±4.29a b	40.52±2.75b		0.027	0.041	0.794
ORT (%)	79.84±1.43a	79.33±1.05a		74.29±2.57a b	70.8±1.67b		<0.001	0.217	0.354
Eosin-nigrosin stain (%)		83.57±0.76	83.81±0.69		81.07±1.09	82.59±1.02	0.057	0.368	0.511
Penetration rate (hIVP) (%)			92.04±0.65a			34.23±4.08b	<0.001		
Sperm per oocyte			23.01±1.81a			2.89±0.54b	<0.001		

[a,b]: Numbers within rows with different superscripts differ (P<0.05); Tukey test.DCF: Sperm membrane integrity assessed with carboxyfluorescein diacetate. FPM: forward progressive motility. NAR: normal apical ridge. HOS: hypo-osmotic swelling test. ORT: osmotic resistance test. hIVP: homologous in vitro penetratation

Table 3. Linear and logistic regression of seminal measurements with average percentage of oocyte penetration

Parameter	Pearson		Logistic Regression		
	Correlation coeffient (r)	Significance (P)	Exp (B)	R	Significance (P)
Volume	0.165	0.047	1.009	0.146	<0.001
Concentration	0.053	0.522	1.000	0.000	0.504
Total cells	0.249	0.003	1.027	0.186	<0.001
ATP	0.375	0.032	1.143	0.253	<0.001
Morpho-anomalies	-0.659	<0.001	0.934	-0.360	<0.001
Cytoplasmic droplets	-0.553	<0.001	0.933	-0.306	<0.001
Folded tail	-0.536	<0.001	0.866	-0.331	<0.001
Swollen tail	-0.249	0.003	0.712	-0.139	<0.001
FRESH SEMEN					
Motility	0.602	<0.001	1.216	0.304	<0.001
FPM	0.669	<0.001	1.296	0.345	<0.001
NAR	0.546	0.001	1.160	0.342	<0.001
HOS	0.486	0.006	1.069	0.294	<0.001
ORT	0.452	0.011	1.125	0.331	<0.001
DILUTED SEMEN					
Motility	0.516	<0.001	1.125	0.306	<0.001
FPM	0.431	<0.001	1.167	0.277	<0.001
Eosin-nigrosin stain	0.178	0.033	1.033	0.108	<0.001
NAR	0.309	<0.001	1.073	0.173	<0.001
HOS	0.415	0.004	1.054	0.222	<0.001
ORT	0.603	<0.001	1.159	0.367	<0.001
DCF	0.291	0.005	1.068	0.212	<0.001
POST-TREATMENT SEMEN					
Motility	0.605	<0.001	1.144	0.355	<0.001
FPM	0.482	<0.001	1.132	0.286	<0.001
Eosin-nigrosin stain	0.119	0.164	1.020	0.055	<0.001
NAR	0.444	<0.001	1.082	0.256	<0.001

DCF: Sperm membrane integrity assessed with carboxyfluorescein diacetate. FPM: forward progressive motility. HOS: hypo-osmotic swelling test. NAR: normal apical ridge. ORT: osmotic resistance test

When studying all conventional semen parameters through a stepwise on multiple linear regression analysis of semen measurements, up to 72.3% of total variance of penetration rate could be predicted (Table 4). However, as many as 4 parameters were needed (FPM in fresh semen, folded tail, NAR in post-treatment semen and DCF) for accurate prediction. On the other hand, the multiple logistic regression needed 7 parameters to discriminate 83.96% correctly of the cases (Table 5).

For the mean number of spermatozoa per oocyte penetrated, significant correlations were obtained for volume, motility, FPM, ATP content and morpho-anomalies (Table 6). However, only NAR from fresh semen and ORT from diluted semen were related to the mean number of spermatozoa per oocyte, and the various values of membrane integrity were not

significantly correlated. Finally, a solid relationship was observed between the penetration rate and the number of spermatozoa per oocyte (r=0.55, P<0.001).

Table 4. Multiple linear regression of semen measurements with penetration rate (forward stepwise model)

Variable	Coefficient	Std. Error	t	P
Constant	-73.984	23.472	-3.152	0.002
FPM in fresh semen	3.583	0.391	9.151	<0.001
Folded tail	-1.703	0.324	-5.256	<0.001
NAR in post-treatment semen	1.107	0.264	4.189	<0.001
DCF	-0.794	0.256	-3.104	0.002

R^2=72.3%, P<0.001

DCF: Sperm membrane integrity assessed with carboxyfluorescein diacetate. FPM: forward progressive motility. NAR: normal apical ridge

Table 5. Multiple logistic regression of semen measurements with penetration rate (forward stepwise model)

Variable	B	Std. Error	Wald	P
Constant	5.375	0.981	30.013	-
Morpho-anomalies	-0.074	0.004	406.017	<0.001
Motility in diluted semen	0.057	0.010	30.655	<0.001
DCF	-0.033	0.006	31.971	<0.001
NAR in diluted semen	-0.057	0.009	38.422	<0.001
Concentration	-0.003	0.001	230.061	<0.001
Total cells	0.027	0.003	88.607	<0.001
FPM in fresh semen	0.032	0.014	4.988	0.025

R^2=83.96%; P<0.001

DCF: Sperm membrane integrity assessed with carboxyfluorescein diacetate. FPM: forward progressive motility. NAR: normal apical ridge

The stepwise linear regression for the mean number of spermatozoa per oocyte penetrated (Table 7) obtained a model that included volume, motility and functional integrity of the membrane (DCF), which explained 37.9% of the total variation.

Nevertheless, when the relationships between sperm characteristics and the in vitro penetration assay were studied in the different groups, only a few relationships were significant (Tables 8 and 9). For the high penetration rate group, the penetration rate was significantly related with FPM in fresh and post-treatment semen, motility of post-treatment semen, HOS of diluted semen and NAR in fresh semen (Table 8). However, for the low penetration group the relationship is significant for a higher number of semen parameters in all sorts of semen.

Table 6. Linear regression of semen measurements with average number of sperm per penetrated oocyte

Parameter	Correlation coeffient (r)	Significance (P)
Volume	0.224	0.007
Concentration	-0.161	0.054
Total cells	0.093	0.263
ATP	0.432	0.012
Morpho-anomalies	-0.245	0.003
Cytoplasmic droplets	-0.173	0.038
Folded tail	-0.279	0.001
Swollen tail	-0.178	0.033
FRESH SEMEN		
Motility	0.276	0.001
FPM	0.363	<0.001
NAR	0.435	0.014
HOS	0.123	0.510
ORT	0.294	0.108
DILUTED SEMEN		
Motility	0.372	<0.001
FPM	0.285	0.001
Eosin-nigrosin stain	0.051	0.545
NAR	0.137	0.101
HOS	-0.051	0.737
ORT	0.423	0.002
DCF	0.029	0.780
POST-TREATMENT SEMEN		
Motility	0.446	<0.001
FPM	0.383	<0.001
Eosin-nigrosin stain	0.468	0.123
NAR	0.167	0.087
Penetration rate (hIVP)	0.551	<0.001

DCF: Sperm membrane integrity assessed with carboxyfluorescein diacetate. FPM: forward progressive motility. HOS: hypo-osmotic swelling test. NAR: normal apical ridge. ORT: osmotic resistance test

DISCUSSION

Study of semen parameters is a routine procedure for assessing semen quality in artificial insemination centers; however, this is often not significantly correlated to fertility. In vitro fertilization of homologous, zona-intact oocytes is the most informative method for assessing sperm fertilizing ability in vitro (3). In fact, good results were obtained with in vitro matured oocytes (7,15,35,36). Moreover, the possibility of using zona-intact pig oocytes at the germinal vesicle stage (immature oocytes) in a hIVP assay of boar sperm fertility reduces the time required for the assay by saving time spent completing in vitro maturation (21, 23). This homologous in vitro penetration assay is a good method to predict fertilizing ability and is significantly correlated with fertility rate (10, 22).

When in vitro fertilization systems are employed, penetration rate is the commonly used marker of the boar sperm fertilizing ability (15, 21, 36). The average sperm number per penetrated oocyte does not reflect the normal fertilization events in vivo, but may provide a useful estimate of the spermatozoa with high fertilizing ability. In this paper, a strong correlation was found between the number of sperm per oocyte and penetration rate ($r=0.55$, $P<0.001$), nevertheless it is less important than the correlation obtained in a previous study ($r=0.71$, $P<0.001$), with a reduced number of boars (10).

Although a great number of studies have been carried out in the porcine IVF (revised by Funahashi and Day; 9), only a few of them are related with the sperm factors implied in the penetration ability. The literature regarding the influence of sperm factors on in vitro penetration success is confusing (5, 15, 21, 31). The contradictory results would be caused by great experimental differences, a few number of ejaculates or IVF trial analyzed, a high number of sperm per oocyte in the IVF system that could mask the relations or by a preselection of the ejaculates.

In human a great number of studies had the aim to detect a cut-off value of the in vitro penetration test as SPA (zona-free hamster ova sperm penetration) for the fertility prediction (19, 29). In the porcine no studies have determined the cutoff in the fertility prediction. In this study, the better cutoff value for in vitro penetration rate to forecast in vivo fertility was found to be 75% and assess one 74.16% true positive rate and 25.84% false positive rate in predicting in vivo fertilizing ability (Table 1).

Generally few relationships were found between the sperm parameters and the in vitro penetration ability and when they were significant, the regression coefficient was low (11). Few single sperm parameters appear to significantly correlate with in vitro penetration, specially when the semen samples are within acceptable ranges of normality. Also, it was suggested that the lack of correlation between conventional semen quality test and the sperm penetration assays suggest that these assays measure different aspects of the spermatozoa viability and fertilizing capacity. However, in this paper we have used a high number of ejaculates and we have not pre-selected them, so a great number of seminal parameters were found to be related with in vitro penetration ability, and when the ejaculates were pre-selected (Tables 8 and 9) the number of significant parameters related were lower.

Table 7. Multiple linear regression of semen measurements with mean number of sperm per penetrated oocyte

Variable	Coefficient	Std. Error	t	P
Constant	-69.882	22.619	-3.089	0.003
Volume	0.138	0.033	4.124	<0.001
Motility in fresh semen	0.645	0.291	2.212	0.029
FPM in diluted semen	1.553	0.356	4.363	<0.001
DCF	-0.338	0.194	-1.739	0.086

$R^2=37.9\%$, $P<0.001$

DCF: Sperm membrane integrity assessed with carboxyfluorescein diacetate FPM: forward progressive motility

Most of values studied were significantly related with in vitro penetration rate, except concentration and eosin-nigrosin staining. The ATP concentration in spermatozoa correlated

with the penetration rate and sperm per penetrated oocyte, however ATP measurement appears to have little diagnostic value in predicting the fertilizing capacity as evaluated by multivariate stepwise analysis, as previously described by Chang et al. (6). With regard to the sperm quality, progressive sperm motility seems to be a good indicator of sperm fertility and was highly correlated to oocyte penetration rates (8, 36) and with sperm number per penetrated oocyte (11). Also in this paper, these motility parameters appear in the multivariate models. However, in other studies no correlation was found between the penetration rate and sperm motility (21, 30).

Table 8. Linear regression of semen measurements with average percentage of oocyte penetration

Parameter	Group 1(n=107) High penetration rate (>75%)		Group 2 (n=38) Low penetration rate (< 75 %)	
	Correlation coeffient (r)	Significance (P)	Correlation coeffient (r)	Significance (P)
Volume	0.174	0.074	-0.053	0.750
Concentration	-0.161	0.098	0.288	0.079
Total cells	0.037	0.706	0.114	0.494
ATP	0.434	0.063	0.099	0.735
Morpho-anomalies	-0.028	0.773	-0.484	0.002
Cytoplasmic droplets	0.038	0.696	-0.381	0.018
Folded tail	-0.093	0.345	-0.402	0.012
Swollen tail	-0.160	0.101	-0.149	0.374
FRESH SEMEN				
Motility	0.124	0.203	0.536	0.001
FPM	0.244	0.011	0.657	<0.001
NAR	0.499	0.030	0.439	0.153
HOS	-0.224	0.357	0.727	0.007
ORT	0.224	0.356	0.376	0.229
DILUTED SEMEN				
Motility	0.127	0.196	0.612	<0.001
FPM	0.124	0.206	0.445	0.005
Eosin-nigrosin stain	0.051	0.607	0.217	0.190
NAR	0.143	0.143	-0.044	0.795
HOS	-0.602	0.001	0.783	<0.001
ORT	0.211	0.255	0.400	0.080
DCF	-0.217	0.080	0.408	0.038
POST-TREATMENT SEMEN				
Motility	0.348	0.001	0.466	0.005
FPM	0.296	0.005	0.415	0.015
Eosin-nigrosin stain	0.118	0.240	0.138	0.414
NAR	0.006	0.959	0.608	<0.001

DCF: Sperm membrane integrity assessed with carboxyfluorescein diacetate. FPM: forward progressive motility. HOS: hypo-osmotic swelling test. NAR: normal apical ridge. ORT: osmotic resistance test

Intactness of the spermatozoa plasma membrane is a prerequisite for suitable sperm metabolism and function. The different methods to assess the plasma membrane included

eosin-nigrosin staining, DCF and HOS. Eosin-nigrosin staining is a traditional method for assessing if the membrane is structurally intact or disrupted and was no correlated with the penetration rate. Carboxifluorescein diacetate evaluated the functional integrity of the plasma membrane and was better correlated with the penetration rates and it was present in all the multiple statistical models.

Table 9. Linear regression of semen measurements with average number of sperm per penetrated oocyte

Parameter	Group 1(n=107) High penetration rate (>75%)		Group 2 (n=38) Low penetration rate (< 75 %)	
	Correlation coeffient (r)	Significance (P)	Correlation coeffient (r)	Significance (P)
Volume	0.183	0.059	0.299	0.068
Concentration	-0.231	0.017	-0.067	0.689
Total cells	-0.050	0.612	0.249	0.132
ATP	0.332	0.165	0.373	0.189
Morpho-anomalies	0.172	0.078	-0.356	0.028
Cytoplasmic droplets	0.247	0.011	-0.259	0.116
Folded tail	-0.062	0.528	-0.367	0.024
Swollen tail	-0.128	0.190	-0.032	0.849
FRESH SEMEN				
Motility	0.088	0.370	0.233	0.165
FPM	0.224	0.020	0.312	0.060
NAR	0.321	0.180	0.418	0.177
HOS	-0.201	0.410	0.686	0.014
ORT	0.100	0.683	0.329	0.297
DILUTED SEMEN				
Motility	0.272	0.005	0.438	0.006
FPM	0.152	0.121	0.033	0.844
Eosin-nigrosin stain	-0.028	0.778	0.051	0.759
NAR	-0.077	0.430	-0.086	0.607
HOS	-0.513	0.006	0.531	0.019
ORT	0.071	0.703	0.550	0.012
DCF	-0.193	0.121	0.348	0.081
POST-TREATMENT SEMEN				
Motility	0.367	<0.001	0.365	0.034
FPM	0.263	0.012	0.395	0.021
Eosin-nigrosin stain	0.110	0.276	0.221	0.190
NAR	-0.025	0.829	0.246	0.191

DCF: Sperm membrane integrity assessed with carboxyfluorescein diacetate. FPM: forward progressive motility. HOS: hypo-osmotic swelling test. NAR: normal apical ridge. ORT: osmotic resistance test

Sperm morphology is another factor that plays an important role in the penetration process. This correlation was also pointed out by Rogers et al. (26) and Kruger et al. (17) in human IVF. In fact, the percentage of sperm with normal morphology also explained a large part of the variance in litter size born, indicating that morphological characteristics are a useful measure of semen quality (35).

The acrosome reaction is required for sperm penetration through the zone pellucida of the oocytes and subsequent fusion with the plasma membrane. However, no relationship was found between penetration rates of ovulated oocytes and maximal percentages of reacted acrosomes (PNA lectins and triple stain technique; 31). In the same way no relation was found by Lynham and Harrison (18) between strength of sperm-zone binding and acrosomal status assayed by fluorescein-conjugated peanut agglutinin. In these cases, perhaps the use of optimum assay conditions for sperm penetration resulted in a loss of sensitivity in detecting a specific correlation between sperm penetration and acrosome status. The NAR results in the present study were highly correlated with penetration rate and appeared in the stepwise multiple models. However the relationship with sperm number per oocyte was only significant for fresh semen and when the ejaculates were pre-selected no significant relation was found.

Finally, we have used different statistical models to analyze the relationship between penetration rate and seminal parameters. The logistic regression is a robust method to analyze categorical data (penetration rate) better than lineal regression of transformed data, but the former is difficult to manage with the odds ratio. However the result obtained was very similar, because the relationship between parameters was anyhow very closed. Both multiple regression stepwise models obtained explain a high percentage of the total variation with 4 (lineal) or 7 (logistic) variables. The results of the present study suggest that in vitro penetration failure o success cannot be explained by the alteration of any simple sperm characteristic although consideration of several characteristics in association may permit prediction of failure or success in the penetration events. At any rate, at the expense of copious counterintuitive or non-sense signs: DCF for the multiple linear regression (Table 4) and DCF, NAR in diluted semen and concentration for the multiple logistic regression (Table 5). Co-linearity among decisory variables may be the cause as previously described by Romar et al. (27) to apply the multiple discriminant analysis model for prognosis.

In summary, the results of the present study showed that almost every studied boar seminal parameters were significantly different between successes and failures, but most of them were correlated together. These findings emphasize the complexity of sperm functions and the difficulties to assess the boar fertilizing ability.

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