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Analysis of *In vitro* Fertilizing Capacity to Evaluate the Freezing Procedures of Boar Semen and to Predict the Subsequent Fertility

E Sellés, J Gadea, R Romar, C Matás and S Ruiz

Department of Physiology, Faculty of Veterinary Medicine, University of Murcia, Spain

Contents

A porcine *in vitro* fertilization (IVF) system and seminal quality parameters of frozen–thawed boar semen were used to assess the effectiveness of two different thawing rates of frozen boar semen, and to address the question of whether differences between fertility of ejaculates could be predicted in a limited field trial.

In the first experiment, two thawing procedures were analysed (37°C, 30 s; 50°C, 12 s) and no differences in sperm quality were found. However, when the procedure was 50°C, 12 s the IVF results showed a higher number of sperm per penetrated oocyte and a near 10 points higher rate of pronuclear formation.

In the second experiment, the fertility results obtained in the limited field trial show to be efficient enough for application in a commercial use, especially for three of the employed boars (fertility ≥80%). In this limited study, the conventional seminal parameters are not accurate enough to discriminate good and bad boars in relation to fertility. On the contrary, parameters of *in vitro* penetrability are more precise to predict subsequent fertilities.

As conclusion, the IVF fertilization system seems to be a good tool to evaluate the quality of frozen–thawed boar semen previous to its commercial way, to verify the bank semen storage quality and a good way to assay new sperm freezing procedures, as it is the more precise evaluating method in estimating the potential fertilizing ability.

Introduction

Despite almost 40 years of research in freezing of boar semen, the fertility results are not satisfactory enough for commercial use as it is in other domestic animals. However boar frozen–thawed semen is still a valuable tool as a complement to artificial insemination (AI) with fresh semen in some conditions. As it can be stored for a long time, it facilitates the supply of genetic material, as well as building up gene banks to encourage breeds or valuable individuals. In the last years, an increasing effort has been made to improve the fertility results mainly by to ways: first the design of better freezing methods in order to obtain acceptable semen quality (freezing procedures, diluents and cryoprotectants, packages, etc.) (reviewed by Bwanga 1991; Johnson et al. 2000), secondly with optimal routines for heat detection and timing of insemination close to the ovulation (Waberski et al. 1994).

Evaluation of the quality of frozen–thawed semen is an important goal and great deals of assays have been developed (Johnson et al. 1996). Besides, assays including the study of gamete interaction might lead to a better way of predicting male fertility than routine laboratory evaluation of semen (Gadea et al. 1998; Larsson and Rodriguez-Martinez 2000). Some of these assays have been shown to be good tools for evaluating the fertilizing capacity of diluted boar semen (Ivanova and Mollova 1993; Gadea et al. 1998; Xu et al., 1998). However, little information is available about frozen boar semen (Hammitt et al. 1989; Gadea et al. 2001; Pelaez et al. 2001), but it would be very useful to evaluate freezing procedures (Eriksson et al. 2000).

One of the most important factors related with the success of freezing procedures seems to be the thawing rate of semen (Johnson et al. 2000). So, the critical temperature range during thawing is an important factor affecting spermatozoan viability (Fiser et al. 1993). However, it has been demonstrated that the effectiveness of thawing rate also depends on the original rate of freezing (Mazur 1985). Different studies have previously described that when thawing rate is increased (in an optimum range), the motility and acrosome integrity are improved (Pursel and Johnson 1975; Fiser et al. 1993).

For these reasons the objectives of this study were to study the application of the *in vitro* fertilization (IVF) systems: (1) to assess the effectiveness of two different thawing rates of frozen boar semen, and (2) to address the question of whether differences between fertility of ejaculates could be predicted with semen quality parameters and with IVF fertilization systems in a limited field trial.

Material and Methods

Semen collection and freezing

Semen was regularly collected from five mature fertile boars (one Belgium Landrace and four Pietrain 18–30 month-old boars) using the hand method and a dummy. Sperm-rich fraction was collected in a pre-warmed thermos flask and the gel-fraction was held on a gauze tissue covering the thermos opening. The volume of the sperm-rich fraction of the ejaculate was measured in a graduated cylinder and sperm concentration measured with a haemacytometer (Neubauer, Brand, Wertheim, Germany) within 20 min after collection and prior to extension of the semen with isothermal Betsville thawing solution (BTS; Minitüb, Tiefenbach, Germany) extender at a ratio of 1 : 1. Semen was stored at 22°C for 2 h, and processed according to the straw-freezing procedure described by Westendorf et al. (1975) and Almlid and Johnson (1988). Briefly, diluted semen was placed at 15°C for 150 min (in a water bath placed into a freezer) and later centrifuged at 800 × ***g*** for 10 min. The supernatant was discarded and the semen pellet was re-suspended with lactose–egg yolk extender (LEY; 80 ml of 11% lactose and 20 ml egg yolk) to provide

1.5×10^9 spermatozoa per ml. Then, it was cooled at 5°C for 90 min (in a water bath placed into a freezer) and two parts of LEY extender semen were mixed with LEY extender with 9% glycerol and 1.5% Orvus Es Paste (Equex-Paste; Minitüb, Tiefenbach, Germany). The final concentration of semen to be frozen was 1×10^9 spermatozoa per ml and 3% glycerol. The cooled semen was leaded into 0.5 ml. straws (Minitüb, Tiefenbach, Germany) and sealed with polyvinyl alcohol. Straws were wiped dry and the air bubble was brought to the centre of the straw. The straws were placed in contact with nitrogen vapour about 3 cm above the liquid nitrogen level for 20 min in an expandable polystyrene box, plunged into the nitrogen tank and stored until use (1–3 months later). Straws were thawed in a circulating water bath at 50°C for 12 s or at 37°C for 30 s and immediately diluted in 10 ml BTS at 37°C.

Seminal parameters

Sperm motility and movement quality were determined placing two sub-samples on warm glass slides (39°C) and examined under a light microscope (100 × magnification). The percentage of motile sperm cells was subjectively estimated to the nearest 5% and the forward progressive motility (FPM) using an arbitrary scale of 0–5.

The proportion of spermatozoa with a normal apical ridge (NAR) was evaluated after fixed in buffered 2% glutaraldehyde solution and examined under a phase-contrast microscope (Leica DMR, Wetzlar, Germany) (1000 × magnification) to analyse acrosomes (Pursel et al. 1972). The NAR was determined on two slides per sample and a total of 300 spermatozoa per sample.

Eosin–nigrosin (EN) viability staining of sperm was also studied. It was diluted at a ratio of 1 : 1 a semen sample with staining solution (5% yellow eosin, 10% nigrosin in a citrate solution pH = 7.4) and smeared. After air-fixed stained spermatozoa were observed and evaluated 200 sperm per sample (Bamba 1988).

Sperm membrane integrity was evaluated applying a combination of the fluorophores carboxyfluorescein diacetate (DCF) and propidium iodide (Harrison and Vickers 1990) on at least 200 cells per sample using an epifluorescence microscope.

In vitro maturation and *in vitro* fertilization

Ovaries from pre-puberal gilts were obtained at a local slaughterhouse, and transported to the laboratory in saline (0.9% w/v NaCl) with 100 mg/l kanamycin at 35°C. Oocytes surrounded by cumulus cells, were slicing from 3 to 6 mm diameter follicles and washed twice in 35 mm plastic Petri dishes containing Dulbecco's phosphate-buffered saline modified supplemented with 1 mg/ml polyvinyl alcohol and 0.005 mg/ml red phenol. They were washed twice again in maturation medium previously equilibrated for a minimum of 3 h at 38.5°C under 5% CO_2 in maximally humidified air.

The medium used for oocyte maturation was Waymouth-supplemented as previously described (Coy et al. 1999) with 10 UI/ml pregnant mare serum gonadotrophin (PMSG), 10 UI/ml human chorionic gonadotropin (hCG), 1 μg/ml oestradiol-17b, 10% (v/v) foetal calf serum and 10% porcine follicular fluid (v/v). The maturation medium was disposed in three droplets of 100 μl covered with paraffin oil per dish, 20 oocytes per droplet and kept at 38°C under 5% CO_2 in air. After 20–22 h of culture in maturation medium the oocytes were transferred to fresh maturation medium without hormonal supplements, washed twice and cultured for an additional 20–22 h (Funahashi and Day 1993).

The *in vitro* fertilization medium was TCM199-supplemented as previously described (Coy et al. 1999) with 12% heat-inactivated foetal calf serum, 0.9 mM sodium pyruvate, 3.05 mM D-glucose, 8.75 mM calcium lactate, 0.68 mM penicillin G, 3.6 mM caffeine and 0.068 mM streptomycin sulphate at pH = 7.4.

After thawing, the sperm samples were centrifuged at 50 × ***g*** for 3 min and the supernatants at 1200 × ***g*** for 3 min. Resulting pellets of spermatozoa were diluted in TCM199-supplemented medium but without calcium lactate nor caffeine. A semen volume of 100 μl was introduced into Petri dishes containing 2 ml of fertilization medium (final concentration of 1×10^6 spermatoza/ml) and 20 *in vitro* matured oocytes previously washed twice in equilibrated fertilization medium. After 18 h the cultured oocytes were fixed in 3 : 1 ethanol : acetic acid for 24 h, stained with 1% lacmoid and examined under a phase-contrast microscope to asses penetration (PEN) rate, mean number of sperm per penetrated oocyte (S/O), monospermy (MON) rate and rate of male pronuclear formation (MPF).

Fertility trial

The fertility study was conducted on a commercial farm, using a total of 45 multiparous (two to seven pregnancies) crossbred sows. Oestrus was checked daily in the presence of a mature teaser boar. Occurrence of oestrus was defined by the standing reflex in front of a boar (back pressure test) and reddening and swelling of the vulva. The sows were inseminated immediately after thawing the semen at 50°C for 12 s and diluted with BTS to prepare insemination doses containing at least 5×10^9 spermatozoa in 80 ml. Insemination took place on 12 h after the diagnosis of oestrus and was repeated 12 h later, using disposable AI catheters.

Pregnancy diagnosis was performed 23–25 days after AI by ultrasonography. Fertility was measured for every ejaculate as the percentage of sows farrowing to AI. For each sow that farrowed, the number of dead and live piglets was counted and the sum was defined as the total number of piglets born.

Experimental design

Experiment 1. Thawing process

Five ejaculates from the same boar were frozen according to the methods described before and were thawed at each of the following test velocities resulting from immersion in water to 37°C for 30 s or at 50°C for 12 s. Seminal parameters and IVF capacity were assayed.

Experiment 2. Fertility trial

To evaluate the capacity for fertility prediction of different assays and to verify the quality of the frozen–thawed semen, ejaculates from five fertile boars were frozen–thawed and they were used both in an IVF system and in a field assay by AI with at least 5×10^9 sperm per dose. Seminal parameters were evaluated and differences between boars were investigated in blind fashion.

Statistical analysis

Data are presented as mean ± SEM. Data for all rates were modelled according to the binomial model of parameters and were analysed by two-way ANOVA; considering the thawing procedure and sperm batch as main effects in experience 1 and one-way ANOVA in experience 2. When ANOVA revealed a significant effect, values were compared by the Tukey test. Differences were considered statistically different at $p < 0.05$.

Linear regression was used to further investigate relationships between litter size and measured semen parameters (Pearson correlation and multiple regression), and logistic regression was used to relate the dichotomous farrowing rate data to the sperm parameters, as previously described by Holt et al. (1997).

Results

Experiment 1

The studied thawing velocities had no effect on the sperm quality (Table 1), but a significant difference on the motility and NAR ($p < 0.001$) was detected between batches. For both treatments the motility and membrane integrity (measured with EN or carboxifluorescein staining) were over 60%.

In relation to the *in vitro* fertilizing capacity of the sperm, thawed under two different procedures, the results obtained showed a higher number of S/O (3.91 vs 3.06, $p < 0.001$) (Table 2) and a near 10 points higher rate of MPF (75.47 vs 65.73, $p = 0.020$) when the procedure was 50°C for 12 s. Besides, the sperm batch had a significant effect on the PEN, number of S/O and MON rate ($p < 0.001$).

Experiment 2

The result of sperm assays showed a significant lower number of intact membrane (EN and DCF) and acrosomal integrity (NAR) for the frozen–thawed sperm from boar PI4 ($p < 0.001$, Table 3). However, all the four IVF parameters were significantly affected by the boar studied and showed a significant lower values for penetrability (PEN and S/O) in PI4 and PI779 boars against the other three (Table 4). In the same way, the MON was affected by boar and showed the highest values for the boars with less penetrability. The MPF was higher than 82% in all the boars studied and this parameter was not related with fertility (Tables 4 and 5).

The *in vitro* penetrability results are consistent with the limited data from the fertility field trial, as fertility was significantly affected by boar ($p = 0.019$), being those with lower penetrability (boars PI4 and PI779) these are with lower fertility (33%) than the other three

Table 1. Seminal parameters measured in boar semen thawed under two different procedures (mean ± SEM)

	Motility (%)	FPM (0–5)	EN (%)	NAR (%)	DCF (%)
Thawing velocity					
50°C for 12 s	62.27 ± 3.53	3.64 ± 0.15	76.55 ± 1.74	49.36 ± 4.34	66.64 ± 2.53
37°C for 30 s	60.45 ± 4.01	3.73 ± 0.14	72.36 ± 1.86	48.91 ± 3.57	63.45 ± 2.41
Source of variability					
Thawing velocity	0.462	0.671	0.058	0.961	0.117
Sperm batch	< 0.001	0.125	0.221	< 0.001	0.082
Interaction	0.459	0.658	0.132	0.850	0.053

FPM: forward progressive motility (0–5), EN: eosin–nigrosine stain, NAR: normal apical ridge, DCF: sperm membrane integrity assessed with carboxyfluorescein diacetate.

Table 2. The IVF results (mean ± SEM) for mature oocytes fertilized with frozen–thawed boar semen under two different procedures: rate of penetration (% PEN), mean number of spermatozoa per penetrated oocyte (S/O), monospermy rate (% MON) and rate of male pronuclear formation (% MPF)

	n	% PEN	S/O*	% MON*	% MPF*
Thawing velocity					
50°C for 12 s	230	69.13 ± 3.05	3.91 ± 0.27[a]	33.96 ± 3.77	75.47 ± 3.42[a]
37°C for 30 s	254	70.08 ± 2.88	3.06 ± 0.19[b]	34.83 ± 3.58	65.73 ± 3.57[b]
Source of variability					
Thawing velocity	0.840	< 0.001	0.641	0.020	
Sperm batch	< 0.001	< 0.001	< 0.001	0.654	
Interaction	0.949	< 0.001	0.519	0.019	

* Based on penetrated oocytes.
[a,b] Numbers within columns with different superscripts differ ($p < 0.05$).
n: number of oocytes.

Table 3. Seminal parameters measured in sperm thawed from five different boars used in the fertility trial (mean ± SEM)

	Motility (%)	FPM (0–5)	EN (%)	NAR (%)	DCF (%)
Boar					
BB	62.00 ± 2.00	3.95 ± 0.05	72.50 ± 2.88[a]	42.50 ± 3.49[a]	69.75 ± 3.17[a]
PI4	59.37 ± 2.17	3.75 ± 0.06	53.69 ± 2.51[b]	33.34 ± 2.48[b]	41.71 ± 3.78[b]
PI67	63.70 ± 1.55	3.88 ± 0.04	66.31 ± 1.40[a]	39.97 ± 1.44[a]	53.69 ± 3.48[ab]
PI779	58.92 ± 0.82	3.85 ± 0.04	70.73 ± 1.61[a]	46.76 ± 1.38[a]	57.41 ± 2.52[a]
PI89	59.30 ± 0.80	3.86 ± 0.03	69.20 ± 1.18[a]	51.16 ± 1.30[a]	56.47 ± 2.41[a]
Source of variability					
Boar	0.053	0.208	< 0.001	< 0.001	< 0.001

FPM: forward progressive motility (0–5), EN: eosin–nigrosine stain, NAR: normal apical ridge, DCF: Sperm membrane integrity assessed with carboxyfluorescein diacetate.
[a,b]Numbers within columns with different superscripts differ ($p < 0.05$).

Table 4. The IVF and *in vivo* fertility results (mean ± SEM) for mature oocytes fertilized with frozen–thawed semen from five different boars: rate of penetration (PEN), mean number of spermatozoa per penetrated oocyte (S/O), monospermy rate (% MON) and rate of male pronuclear formation (MPF)

	n	PEN (%)	S/O*	MON* (%)	MPF* (%)	Fertility (%)	Litter size
Boar							
BB	199	62.81 ± 3.43[cd]	2.78 ± 0.28[a]	48.80 ± 4.49[ab]	88.80 ± 2.83[ab]	80 (4/5)	11.5 ± 1.19
PI4	212	44.34 ± 3.42[b]	1.65 ± 0.13[b]	61.70 ± 5.04[ab]	82.98 ± 3.90[b]	33.33 (2/6)	6 ± 0
PI67	329	67.17 ± 2.59[d]	2.21 ± 0.11[ab]	40.72 ± 3.31[b]	87.33 ± 2.24[b]	84.62 (11/13)	9.18 ± 1.54
PI779	320	23.13 ± 2.36[a]	1.63 ± 0.13[b]	63.51 ± 5.63[a]	94.59 ± 2.65[ab]	33.33 (3/9)	10.67 ± 0.67
PI89	308	52.27 ± 2.85[bc]	2.54 ± 0.18[a]	50.93 ± 3.95[b]	98.14 ± 1.07[a]	83.33 (10/12)	7.3 ± 1.46
Source of variability							
Boar	< 0.001	< 0.001	0.001	< 0.001	0.019	0.405	

*Based on penetrated oocytes.
[a,b,c,d] Numbers within columns with different superscripts differ ($p < 0.05$).
n: number of oocytes.

Table 5. Logistic regression of seminal parameters with fertility result (farrowing rate)

Variable	Coefficient *B*	SE	*t*	Significance (p)
PEN	0.0208	0.0071	2.9402	0.0033
S/O	0.4384	0.1758	2.4935	0.0127
MPF	0.0063	0.0035	1.8046	0.0711
MON	0.0043	0.0050	0.8577	0.3910
Motility	0.0115	0.0051	2.2360	0.0253
FPM	0.1842	0.0821	2.2445	0.0248
EN	0.0107	0.0047	2.2654	0.0235
NAR	0.0164	0.0072	2.2870	0.0222
DCF	0.0135	0.0058	2.3482	0.0189

PEN: rate of penetration, S/O: mean number of spermatozoa per penetrated oocyte, MPF: rate of male pronuclear formation, MON: monospermy rate, FPM: forward progressive motility (0–5), EN: eosin–nigrosine stain, NAR: normal apical ridge, DCF: Sperm membrane integrity assessed with carboxyfluorescein diacetate, SE: standard error.

Table 6. Multiple logistic regression of seminal measurements with fertility result (farrowing rate)

Variable	Coefficient	SE	*t*	Significance (p)
Constant	−46.1846	24.9668	−1.8498	0.0643
PEN	0.3747	0.2228	1.6813	0.0927
MPF	0.8055	0.4882	1.6499	0.0990
Motility	−0.6857	0.5216	−1.3145	0.1887

McFadden's $R^2 = 0.492$; log likelihood: −27.373; SE: standard error.
Significance of statistical model $p < 0.001$.

with fertility rates over 80% (Table 4). No differences between boars were detected for litter size.

When the logistic regression was analysed between *in vitro* penetration and seminal parameters with fertility results, significant regression coefficients were found for PEN rate and S/O, and all the quality seminal parameters (motility, FPM, EN, NAR, DCF; Table 5, $p < 0.05$). Later, when studying all semen parameters through stepwise on multiple logistic regression forward, only three parameters (PEN, MPF and motility) were included (Table 6, $R^2 = 0.492$; $p < 0.001$).

In relation with litter size, significant Pearson correlation was found for all the IVF parameters (except MPF) and two quality parameters (FPM and DCF, Table 7). When a stepwise multivariate analysis was made a significant model ($p < 0.0001$) constructed only with fertilization parameters of penetrability (PEN and S/O) that may explain the nearly 80% of variability (Table 8).

Discussion

To improve the viability and fertilizing capacity of the boar frozen–thawed semen is necessary to optimize all the factors that have an effect in the freezing procedure. In this way we have focused our attention on one of them, the thawing process, and in the assessment methods for frozen semen evaluation.

Table 7. Linear regression of seminal measurements with average litter size[a]

Variable	Pearson correlation coefficient	Significance (p)
PEN	0.4461	0.0031
S/O	0.4356	0.0039
MPF	−0.1421	0.3692
MON	−0.4014	0.0084
Motility	0.2789	0.0635
FPM	0.3317	0.0260
EN	0.1968	0.1951
NAR	0.1181	0.4399
DCF	0.3450	0.0203

[a] Litter sizes included failed conceptions as zero values.
PEN: rate of penetration, S/O: mean number of spermatozoa per penetrated oocyte, MPF: rate of male pronuclear formation, MON: monospermy rate, FPM: forward progressive motility (0–5), EN: eosin–nigrosine stain, NAR: normal apical ridge, DCF: Sperm membrane integrity assessed with carboxy-fluorescein diacetate.

Table 8. Multiple linear regression of seminal measurements with litter size[a]

Variable	Coefficient	SE	*t*	p
PEN	0.0609	0.0356	1.7089	0.0952
S/O	1.4749	0.9276	1.5900	0.1197

[a] Litter sizes included failed conceptions as zero values.
$R^2 = 79.54\%$; $p < 0.0001$.
PEN: rate of penetration, S/O: mean number of spermatozoa per penetrated oocyte.

In the first experiment, we have found no differences in sperm quality with two thawing procedures near to the optimal conditions and previously accepted to thaw frozen boar semen in ministraws (Maxwell and Johnson, 1997; Erkisson et al. 2000). However, Fiser et al. (1993) found that after freezing at optimal rate, the percentage of motile spermatozoa and sperm with NAR increased gradually while increasing warming velocity. They studied a wide range of velocities, some of them out of optimal conditions and those results were related to cryoinjuries made during a slow thawing process by the re-growth of ice crystals (Mazur 1985). Besides this, another problem would be present in the rapid thawing process, as Bamba and Cran (1985) showed that boar spermatozoa might be also severely damaged by warm shock.

Concerning the *in vitro* PEN capacity only the number of S/O and the rate of MPF were significantly affected by the thawing temperature. The capacity of spermatozoa to penetrate oocyte might be assessed by PEN rate as well as S/O (Matttioli et al. 1988). In this study, we have obtained a higher number of S/O in the thawing method with the higher temperature. This situation might be caused by a better sperm function not correctly evaluated by classical sperm analysis (Gadea and Matás 2000). Apart from this, it has been reported that a fast velocity is associated to decrease the cellular damage, so that the effect of a fast velocity could minimize the damages associated to structure and DNA stability and a significant decrease on the growth of ice crystals (Mazur 1984). In the same way, the higher rate of MPF obtained with warmer temperature might be in relation with a less intense alteration of sperm chromatin decondensation (White 1993), or the alteration in the number of thiol groups during freezing process (Chatterjee et al. 2001). However, MPF has also been related with others factors such as the content of glutathione in oocyte (Funahashi et al. 1994), the maturation system (Coy et al. 1999) and boar (Xu et al. 1996).

Significant differences associated to batches (motility, NAR, PEN, S/O and MON) would be related to the use of manual methods (nitrogen vapour) difficulty repeatable. This trouble could be solved with the use of programmable freezing procedures and the optimal curve of freezing (Ruiz et al. 2002). Finally, it is obvious that to obtain the best viability after thawing is extremely important to adapt the thawing velocity to the freezing velocity and to the straw volume (Hofmo and Almlid 1990).

The fertility results obtained in the limited field trial (total 66.67%, 30/45) are efficient enough for application in a commercial use, specially for three of the employed boars (80, 83.33 and 84.32%). These results are comparable with those obtained by other authors (Almlid and Hofmo 1996; Bertani et al. 1997; Hofmo and Grevle 1999; Erkisson, 2000). However, the question of whether differences between fertility of ejaculates could be predicted with semen quality parameters and with IVF systems is not yet answered. A wide variability among boars in fertility rates obtained from their frozen semen have been demonstrated in several studies (reviewed by Johnson 1985), and recently a genetic basis of boar semen freezability have been demonstrated (Thurston et al. 2002). However, till now, no method of evaluating the quality of frozen–thawed semen is yet available when attempting to predict fertility.

In this limited study, the conventional seminal parameters are not enough efficient to discriminate good and bad boars in relation to fertility. Only one of the two bad freezer boars with a low fertility was detected by acrosome and membrane integrity assessment. On the contrary, parameters of penetrability (PEN and S/O), measured in an IVF system, are more precise to predict subsequent fertilities. The S/O does not reflect the normal fertilization events *in vivo*, but may provide a useful estimate of spermatozoa with high fertilizing ability.

The results obtained in the IVF system are consistent with *in vivo* fertility and significant logistic regression was found for PEN and this parameter was included in a multiple model. In this way, in a previous work using diluted fresh semen and a higher number of sows inseminated, a significant relationship was found between *in vitro* PEN rate and S/O with *in vivo* fertility and with a similar multiple logistic regression to fertility, where PEN rate and motility were included (Gadea et al. 1998). In this model the motility also appears, but surprisingly the motility change the sign from logistic regression (positive 0.0115, Table 5) to negative sign in multiple logistic regression (−0.6857, Table 6). These non-sense signs must be related with co-linearity among decisive variables as previously we detected in applications of multiple discriminant analysis model for prognosis of *in vitro* fertility (Gadea and Matás, 2000). The

logistic regression is a robust method to analyse categorical data such as fertility rate. It is better than lineal regression of transformed data, but the former is difficult to manage with the odds ratio.

On the other hand, a greater difficulty is presented to predict the litter size, probably related with the maternal and environment importance effects (ovulation rate, fertilization rate, relation insemination–ovulation, and so on). So, a fewer number of classical sperm parameters are related and only PEN and S/O are necessary to explain near 80% of variability.

In the present study the IVF is shown as a precise technique to assess freezing procedures of boar semen. In this way, a limited number of factors related with the freezing process have been evaluated by the use of an IVF system (holding time and type of package (Eriksson et al. 2000) or volume of straw (Cordova et al. 2001, 2002). As conclusion, we can determine that a fast thawing speed had a more positive effect on the fertilizing capacity *in vitro* of the frozen boar semen. Moreover, the IVF seems to be a good tool to evaluate the quality of frozen–thawed boar semen previous to its commercial way, to verify the bank semen storage quality and a good way to assay new sperm-freezing procedures, as it is the evaluating method more precise in estimating the potential fertilizing ability.

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Author's address (for correspondence): J Gadea, Dept. Fisiología, Facultad de Veterinaria, Universidad de Murcia, 30, 100 Murcia, Spain. E-mail: jgadea@um.es