



IN VITRO PENETRATION ASSAY OF BOAR SPERM FERTILITY: EFFECT OF VARIOUS FACTORS ON THE PENETRABILITY OF IMMATURE PIG OOCYTES

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Received for publication: *July 12, 1995*

Accepted: *January 3, 1996*

ABSTRACT

The present study was designed 1) to examine the influence of cumulus cells, ovary storage time and oocyte size on the penetrability of immature pig oocytes, and 2) to investigate the effect of 2 methods of treating the semen from different boars on the inter-assay variability of homologous in vitro penetration tests of boar sperm fertility. In Experiment 1, cumulus oocyte complexes, oocytes with spontaneous loss of the cumulus cells during collection, and oocytes mechanically stripped of cumulus cells were used. No differences were observed in oocyte penetrability among the 3 types of oocyte, although mechanical removal of the cumulus caused an increase ($P < 0.005$) in the degeneration rate compared with the other oocyte types. In Experiment 2, the oocytes were recovered from ovaries kept in PBS (30°C) for 2, 4 or 6 h after slaughter of prepuberal gilts. Ovary storage did not modify the penetrability of oocytes but increased ($P < 0.02$) their degeneration rates. In Experiment 3, the diameters of fresh oocytes were determined after co-incubation with spermatozoa. They were classified into 4 groups according to diameter: A) $< 105 \mu\text{m}$, B) $105\text{--}115 \mu\text{m}$, C) $116\text{--}120 \mu\text{m}$ and D) $> 120 \mu\text{m}$. Oocytes from Groups C and D exhibited higher ($P < 0.05$) penetrability than oocytes from the other groups. In Experiment 4, stored, diluted spermatozoa from 4 boars were pretreated by centrifugation at $50 \times g$ for 3 min and subsequent concentration of the supernatants at $1,200 \times g$ for 3 min. The pellets were treated (washed twice and preincubated for 40 minutes) before co-incubation with immature oocytes or used directly as untreated samples (unwashed and non-preincubated). A boar effect ($P < 0.001$) was evident for the parameters of in vitro penetration, independently of sperm treatment. When the oocytes were inseminated with untreated spermatozoa, the effects of the replicate and the boar-by-replicate interaction on the variability in oocyte penetrability were not significant. The results of this study indicate that the use of standardized immature pig oocytes and stored untreated, diluted spermatozoa can provide a useful method for optimizing the homologous in vitro penetration (hIVP) assay of boar fertility.

Key words: spermatozoa, immature oocyte, in vitro penetration test, boar fertility

Acknowledgments

The authors thank M. Iniesta for statistical advice, X. Lucas, S. Ruiz and P. Coy for technical support, staff of the Meat Inspection Office (El Pozo) for supplying the ovaries, and Dalland Hybrid España for the donation of boar semen. This research was supported by CICYT (AGF 92/0521), CDTI (94/0059), CARM (PIB 94/14) and IFRM (94/085).

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INTRODUCTION

Prediction of the fertilizing ability of boar spermatozoa is important in breeding herds, particularly when artificial insemination is used. Several investigators have suggested that *in vitro* fertilization of zona-intact homologous oocytes (hIVF) might lead to better prediction of male fertility than routine laboratory evaluation of semen (12,20,26). Differences between the semen of individual bulls (1,21,34,35) and boars (24,33,38,40,43) in penetration rates of hIVF have been demonstrated, and high correlations ($r=0.83$) between bovine oocyte penetration by bull spermatozoa with *in vivo* fertility have been observed (21). For this purpose, the actual procedure of hIVF in pigs needs to be simplified and standardized. It has been reported that zona-intact pig oocytes at the germinal vesicle (GV) stage (immature oocytes) show similar degrees of penetrability to those seen in *in vitro* matured (26) or ovulated oocytes (24). The possibility of using immature oocytes in a hIVP assay of boar sperm fertility would facilitate the collection of female gametes, thus shortening the time required for *in vitro* maturation (26). However, like hIVF, this procedure is subject to variability caused both by differences in the penetrability of oocytes and by variations between replicates using ejaculates from the same boars.

The objectives of our study were 1) to examine the influence of cumulus cells, ovary storage time from their collection until the recovery of oocytes, and oocyte size on the penetrability of immature pig oocytes, and 2) to investigate the effects of 2 treatments on semen from different boars on the inter-assay variability in the penetrability of immature oocytes to standardize the hIVP assay of boar fertility.

MATERIALS AND METHODS

Collection of Immature Oocytes

Ovaries were obtained just after slaughter from prepuberal gilts weighing approximately 95 kg and transported in less than 30 min to the laboratory in 0.9% (w/v) NaCl containing 100 $\mu\text{g/ml}$ kanamycin sulphate at 30°C. The immature oocytes were collected from medium-size antral follicles (2 to 5 mm in diameter) by dissection of the ovarian surface with a single sterile blade into Dulbecco's phosphate-buffered saline supplemented with 4 mg/ml BSA (Fraction V, Sigma Chemical Co., St. Louis MO, USA), 0.34 mM sodium pyruvate, 5.54 mM D-glucose, and 70 $\mu\text{g/ml}$ Kanamycin (modified DPBS) at 37°C. Immature oocytes were washed 3 times with modified DPBS before insemination with spermatozoa.

Sperm Preparation

The sperm-rich fractions were collected from mature Landrace boars, using the gloved-hand method. Immediately after collection, semen samples were diluted to 3×10^7 cells/ml in MR-A diluent (21) and kept for 24 h at 16°C (stored, diluted sperm). Sperm suspensions were pretreated by centrifugation at $50 \times g$ for 3 min and subsequent concentration of the supernatants at $1,200 \times g$ for 3 min. The pellets were washed twice at $1,200 \times g$ for 3 min in 0.9% saline solution enriched with 1 mg/ml BSA (Fraction V, Sigma) at pH 7.2. The spermatozoa were diluted to 2×10^8 cells/ml in preincubation medium consisting of Medium 199 with Earle's salts (Sigma) supplemented with 12% heated fetal calf serum (Sigma), 0.91

mM sodium pyruvate, 3.05 mM D-glucose, 2.92 mM calcium lactate, 50 IU/ml penicillin G, and 30 µg/ml streptomycin sulphate (modified M 199) at pH 7.8. Sperm suspensions were then preincubated for 40 min at 39°C in tightly capped test tubes.

In Vitro Penetration and Oocyte Examination

Each group of 15 immature oocytes was coincubated with spermatozoa 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) at 39°C under 5% CO₂ in air. A final concentration of 10 x 10⁶ spermatozoa/ml was used. At the end of the coincubation period, oocytes were stripped of cumulus cells and spermatozoa by pipetting, mounted on slides and fixed for a minimum of 24 h with ethanol:acetic acid (3:1 v/v). The fixed oocytes were stained with 1% lacmoid (5) and examined for evidence of sperm penetration under a phase contrast microscope (x 400 magnification). Oocytes were considered to be penetrated when spermatozoa with swollen or unswollen heads were found in the vitellus. Oocytes with a broken oolemma or abnormal looking cytoplasm were classified as degenerated.

Experimental Design

Experiment 1. To evaluate the influence of the cumulus cells on oocyte penetrability, intact cumulus-oocyte complexes (COC) and oocytes with spontaneous loss (partial or full) of the cumulus cells during collection (SDO) were obtained as described above. About one-half of the COC were mechanically stripped of cumulus cells (MDO) by vigorous and repeated pipetting. Subsequently, COC, SDO and MDO were inseminated with preincubated spermatozoa. The time elapsing between the arrival of the ovaries at the laboratory and initiation of coincubation period was 1 to 1.5 hours.

Experiment 2. This experiment examined the effects of ovary storage time from their collection until recovery of oocytes on oocyte penetrability. The ovaries were collected at a local slaughterhouse and transported to the laboratory as described above. The ovaries were washed 3 times with modified DPBS at 30°C and stored in a vacuum flask for 2, 4 or 6 h. The oocytes (COC and SDO) were recovered from each treatment groups under the same conditions as described above and inseminated with preincubated spermatozoa.

Experiment 3. The objective was to determine if oocyte size affects their penetrability in vitro. After coincubation with preincubated spermatozoa, oocytes (COC and SDO) were washed in modified DPBS, and excess spermatozoa and cumulus cells were easily removed by pipetting. Each oocyte was placed in a microdroplet of mDPBS on a slide (10 oocytes/slide), and the diameter (excluding the zona pellucida) was determined using an ocular micrometer under phase-contrast microscopy (x 400 magnification). The oocytes were then fixed and stained for evidence of sperm penetration. They were classified into 4 groups according to diameter: A) very small, <105 µm, B) small, 105-115 µm, C) medium, 116-120 µm and D) large, >120 µm.

Experiment 4. To investigate the influence of the spermatozoa treatment on the inter-assay variability in the in vitro penetration of immature oocytes, 4 mature Landrace boars with satisfactory semen characteristics were selected as semen donors. Stored, diluted sperm were

pretreated as described above. Then, the pellets were treated (washed twice and preincubated for 40 min) before coincubation with oocytes or used directly as untreated samples (unwashed and non-preincubated).

Statistical Analysis

Experiments 1, 2, 3 and 4 were conducted in 6, 4, 3 and 16 (4 times for each of the 4 boars) replicates, respectively. All the replicates in the same experiment (Experiments 1, 2 and 3) were carried out with semen from the same boar, although boars differed between experiments. The results are expressed as mean \pm SEM. Oocyte degeneration and penetration rates (the data were modelled according to the binomial model of parameters, as described by 13) and the number of spermatozoa per oocyte were analyzed by one-way (Experiments 1, 2 and 3) or two-way (Experiment 4) ANOVA, using the Multivariate General Linear Models of Systat (41). When ANOVA revealed a significant effect, values were compared by Tukey-test.

RESULTS

Experiment 1

The effects of the presence or absence of cumulus cells on the *in vitro* penetration of oocytes are shown in Table 1. No differences were observed between COC, SDO and MDO either in regard to the penetration rate or the mean number of spermatozoa per oocyte. However, mechanic removal of the cumulus cells caused a significant ($P < 0.005$) increase in the degeneration rate compared with that of the other oocyte types.

Table 1. Effects of the cumulus cells on the *in vitro* penetration of immature pig oocytes

Type of oocyte	Number of oocytes examined	Percentage of degenerated oocytes	Percentage of oocytes penetrated	Number of spermatozoa in penetrated oocytes
COC	349	21.5 \pm 1.4 ^a	83.9 \pm 2.6	8.2 \pm 0.4
SDO	315	24.8 \pm 1.6 ^a	92.0 \pm 3.1	8.5 \pm 0.7
MDO	395	42.3 \pm 1.9 ^b	89.0 \pm 3.2	8.1 \pm 0.5

COC: cumulus-oocyte complex. SDO: spontaneously denuded oocytes. MDO: mechanically denuded oocytes.

Values are mean \pm standard error of the mean from 6 replicates.

Values within columns with different superscripts differ ($P < 0.005$).

Experiment 2

The penetrability of immature oocytes recovered from ovaries stored at 30°C for different periods is shown in Table 2. The penetration rates of oocytes and mean numbers of spermatozoa penetrated per oocyte from ovaries stored for 2, 4 and 6 h were nearly identical.

The proportion of oocytes obtained from stored ovaries that degenerated during subsequent handling significantly increased with the length of storage.

Experiment 3

To determine oocyte penetrability in relation to oocyte size, a total of 1,029 oocytes was used, of which 11.9, 27.4, 36.0 and 24.7% were classified into Groups A, B, C and D, respectively. Degeneration rates did not differ among oocyte groups, and ranged between 28.35 and 31.54%. The mean \pm SEM diameter of oocytes was 95.0 ± 0.3 , 109.3 ± 0.1 , 119.1 ± 0.1 and 130.9 ± 0.1 μm for Groups A, B, C and D, respectively. The differences in the oocyte diameters between the 4 groups were significant ($P < 0.001$). Oocytes from Groups C and D exhibited a significantly ($P < 0.05$) higher penetration rate and number of spermatozoa penetrated per oocyte than oocytes from Groups A and B (Table 3).

Table 2. Penetrability of immature pig oocytes isolated from ovaries stored at 30°C for 3 different periods

Storage period (hours)	Number of oocytes examined	Percentage of degenerated oocytes	Percentage of oocytes penetrated	Number of spermatozoa in penetrated oocytes
2	939	18.2 ± 1.2^a	96.1 ± 1.4	13.7 ± 0.4
4	990	41.4 ± 3.1^b	96.9 ± 1.1	13.6 ± 0.5
6	909	47.1 ± 2.9^c	97.3 ± 0.8	14.1 ± 0.6

The ovaries of prepuberal gilts were collected at a local slaughterhouse, transported to the laboratory within 30 minutes and stored in a thermos.

Values are mean \pm standard error of the mean from 4 replicates.

Values within columns with different superscripts are significantly different, ($P < 0.02$ at least).

Table 3. Effects of oocyte size on the in vitro penetration of immature pig oocytes

Group/Diameter of oocytes (μm)	Number of oocytes examined	Percentage of degenerated oocytes	Percentage of oocytes penetrated	Number of spermatozoa in penetrated oocytes
A <105	122	29.5 ± 1.3	75.6 ± 3.1^a	4.8 ± 0.7^a
B 105-115	282	30.5 ± 1.1	76.7 ± 2.9^a	15.4 ± 1.1^b
C 116-120	371	31.5 ± 1.1	91.3 ± 1.8^b	19.2 ± 1.0^c
D >120	254	28.3 ± 1.0	95.6 ± 1.0^b	21.9 ± 1.2^c

Values are mean \pm standard error of the mean from 3 replicates.

Values within columns with different superscripts are significantly different, ($P < 0.05$ at least).

Experiment 4

Although semen quality was similar among boars, a boar effect ($P < 0.001$) was evident for in vitro penetration of oocytes, independent of treatment of spermatozoa. The penetration rates and mean number of spermatozoa per oocyte among replicates varied according to the spermatozoa treatment (Figure 1). When oocytes were inseminated with untreated spermatozoa, the effects of the replicate and the boar-by-replicate interaction on the variability in the penetration of oocytes were not significant. However, when oocytes were inseminated with treated spermatozoa, significant variability in both the penetration rate ($P < 0.05$) and the mean number of spermatozoa per oocyte ($P < 0.001$) among replicates was observed. The effect of the boar-by-replicate interaction on oocyte penetrability was also significant ($P < 0.001$). The overall variation of the penetration rates/mean number of spermatozoa per oocyte expressed by its extreme values among replicates was 7.8/1.0, 14.4/0.9, 9.1/0.7 and 7.9%/0.7 when oocytes were co-incubated with untreated spermatozoa and 17.2/9.0, 22.4/1.2, 26.2/3.6 and 28.4%/0.7 when oocytes were co-incubated with treated spermatozoa for Boars A, B, C and D, respectively.

DISCUSSION

Extensive research has demonstrated that the presence of cumulus cells during maturation is fundamental for the oocyte to undergo normal nuclear and cytoplasmic maturation and to maintain its penetrability in all domestic species studied (reviewed in 8,31), including the pig (16,25,27). In addition, the presence of cumulus cells around in vivo or in vitro matured oocytes during co-incubation was reported to be beneficial for fertilization of mouse (14,18,30), hamster (3), bovine (2,6,15,39) and porcine (7,19) oocytes. However, little information is available about the penetrability of immature oocytes with or without follicle cells. In contrast to findings for mature pig oocytes, the results of the current investigation provide evidence that the cumulus cells have no effect on sperm penetration of immature pig oocytes. There are clear differences between mature and immature oocytes with respect to the morphological and physiological characteristics of the cumulus cells. While an important aspect of oocyte maturation is the full expansion and mucification of the cumulus cells surrounding the oocyte (10,11), the oocytes at the GV stage are surrounded by tightly packed cumulus cells. As has been suggested (44), it is probable that some components of the mature cumulus positively affect penetration. Such components cannot be present in immature cumulus cells. The ability of spermatozoa to penetrate immature oocytes surrounded by cumulus cells may depend on the source of the spermatozoa being used. Wang et al. (41) reported that cumulus-free porcine oocytes at the GV stage and those undergoing maturation are equally penetrable by cryopreserved spermatozoa in vitro, but no penetration was observed when cumulus-intact porcine oocytes at the GV stage were used. As suggested, it may be difficult for frozen-thawed spermatozoa to traverse the intercellular matrix of the compacted cumulus mass of immature porcine oocytes. On the other hand, in our study, when oocytes were denuded by pipetting, the rates of degeneration of oocytes were higher than the rates for COC or SDO. Since enzymatic removal of the cumulus cells has not yet been found to be satisfactory (28), mature oocytes are generally denuded gently, after a culture period by means of repeated passage through a fine pipette. However, freshly obtained immature pig oocytes are surrounded by tightly adhering packed cumulus, and it was only after great effort that we could remove this mass of cells, which may have a detrimental effect on oocyte viability.

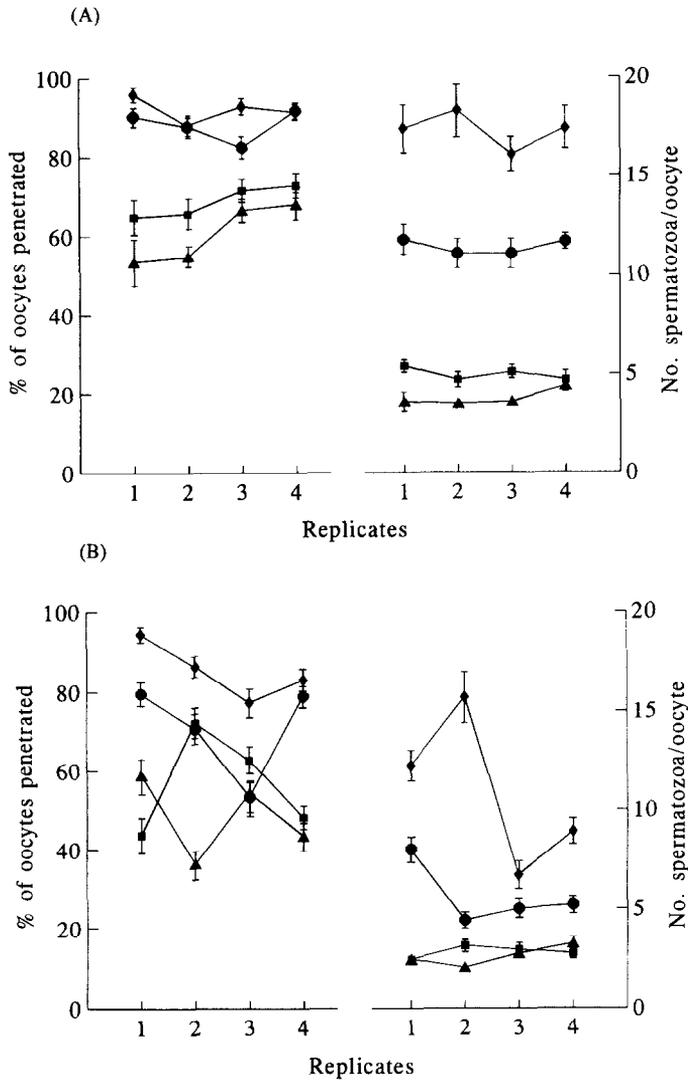


Figure 1. Variability in the parameters of in vitro penetration of ejaculates among replicates from 4 boars, using untreated (A) or treated (B) spermatozoa. Sperm concentration at co-incubation was 10×10^6 cells/ml. (Boar A: ◆-◆; Boar B: ▲-▲; Boar C: ●-●; Boar D: ■-■). Data were obtained from 4 replicates by each boar. The number of oocytes examined by boar and replicate ranged from 161 to 189. Each point represents the mean \pm standard error of the mean. Where no error bars are shown, they are shorter than the width of the symbol. See text for statistical detail.

For the *in vitro* production of embryos derived from oocytes collected from the ovaries of slaughterhouse animals, it is important to return the ovaries to the laboratory in a reasonably short period of time following slaughter (28). However, the effects of the ovary storage period on oocyte penetrability have not been determined. In Experiment 2, it was shown that oocytes from ovaries stored at 30°C for 2 to 6 h were penetrated at similar rates. In contrast, increased storage times led to higher degeneration rates. Therefore, from a practical standpoint, immature pig oocytes from ovaries stored at 30°C for up to 6 h can be used in an hIVP test for the assay of boar sperm fertility. However, when oocytes from ovaries stored more than 2 h are used, we recommend increasing the number of oocytes collected because of the increased rate of degeneration.

The quantity of oocytes which can be collected per hour is very important for efficient use of the hIVP assay. The collection of oocytes by follicle dissection or by aspiration methods is very labor intensive compared with puncturing or dissection of the ovarian surface. Besides, it has been demonstrated that bovine and caprine oocytes obtained by the dissection the ovarian surface may be matured, fertilized and cultured *in vitro* to produce blastocysts (4,37). The results of Experiment 3, using dissection of the ovarian surface, showed wide heterogeneity in oocyte size, which ranged from 90 to 134 μm in diameter and demonstrated a clear relationship between oocyte size and its penetrability. The penetration rates and mean numbers of spermatozoa per oocyte for oocytes with a diameter from 90 to 115 μm were significantly lower compared with those of oocytes with a diameter measuring 116 to 134 μm . Since the growth phase in pig oocytes is not completed until the antral follicle has reached a diameter of about 2.2 mm (32) and oocyte size from these follicles reaches up to 115 μm (29,32), our results suggest that the dissection of the ovarian surface was relatively indiscriminant, resulting, presumably, in the release of oocytes from small follicles (< 2 mm in diameter), and therefore of oocytes in the growth phase that were not highly penetrated. If this is so, because the penetrability of growing pig oocytes has not been examined, our results could coincide with the findings of Pavlok et al. (36), who demonstrated that the penetration rates in bovine oocytes derived from small follicles (< 2 mm diameter) were significantly lower than in oocytes derived from medium and large follicles. Since the zona pellucida appears during oocyte growth, increasing in width as oocytes increase in diameter (9), variations in their composition, sperm receptors or elastic and immunological properties might influence the penetrability of growing oocytes. Further reasearch is required to confirm this hypothesis.

The penetrability of oocytes varied widely among boars independently of sperm treatment. However, the hIVP system was found to be repeatable between replicates when stored untreated, diluted spermatozoa were used. In addition and confirming our previous studies (23), high rates of penetration of oocytes was achieved by untreated spermatozoa from all boars. While the differences in individual boars are probably due to variations in the *in vitro* penetrating capacity of the spermatozoa, the reasons for the differences in the penetration of oocytes between replicates using spermatozoa from the same boar are not known. When spermatozoa are washed, a relatively high centrifugal force is necessary for their sedimentation. Furthermore, the pellet is tightly packed and efforts to resuspend the spermatozoa may damage them (17). We have observed a decrease of 10 to 35% in plasma membrane integrity of washed and preincubated spermatozoa among replicates when ejaculates from the same boar were used (unpublished data). This may indicate that the boar sperm is influenced not only by the donor but also by the manipulation of the spermatozoa, and that ejaculates from the same boar

probably react differently to the same capacitation treatment. Since capacitation treatments may increase the susceptibility of spermatozoa of some boars to subsequent membrane damage, the use of unwashed and non-preincubated spermatozoa might help decrease variability among replicates.

In conclusion, the inter-assay variability encountered between ejaculates from the same boar in hIVP tests for assaying boar fertility can be decreased by standardizing the size of the immature oocytes to be used, and by co-incubation of these oocytes with stored, diluted spermatozoa which are unwashed and non-preincubated.

REFERENCES

1. Aoyagi Y, Fujii K, Iwazumi Y, Furudate M, Fukui Y, Ono H. Effects of two treatments of semen from different bulls on in vitro fertilization results of bovine oocytes. *Theriogenology* 1988; 30:973-985.
2. Ball GD, Leibfried ML, Lenz RW, Ax RL, Bavister BD, First NL. Factors affecting successful in vitro fertilization of bovine follicular oocytes. *Biol Reprod* 1983; 28:717-725.
3. Bavister BD. Evidence for a role of post-ovulatory cumulus components in supporting fertilizing ability of hamster spermatozoa. *J Androl* 1982; 3:365-372.
4. Carolan C, Monaghan P, Gallagher M, Gordon I. Effect of recovery method on yield of bovine oocytes per ovary and their developmental competence after maturation, fertilization and culture in vitro. *Theriogenology* 1994; 41:1061-1068.
5. Chang MC. Fertilizability of rabbit ova and the effects of temperature in vitro on their subsequent fertilization and activation in vivo. *J Exp Zool* 1952; 121:351-381.
6. Cox JF, Ormazábal J, Santamaría A. Effect of the cumulus on in vitro fertilization of bovine matured oocytes. *Theriogenology* 1993; 40:1259-1267.
7. Coy P, Martínez E, Ruiz S, Vázquez JM, Roca J, Gadea J. Environment and medium volume influence in vitro fertilisation of pig oocytes. *Zygote* 1993; 1:209-213.
8. Crozet N. Manipulation of oocytes and in-vitro fertilization. *J Reprod Fertil* 1991; 43(Suppl):235-243.
9. Dunbar BS, Wardrip NJ, Hedrick JK. Isolation, physicochemical properties and macromolecular composition of the zona pellucida from porcine oocytes. *Biochemistry* 1980; 19:356-365.
10. Eppig JJ. FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. *Nature* 1979; 261:483-494.
11. Eppig JJ. Regulation of cumulus oophorus expansion by gonadotropins in vivo and in vitro. *Gamete Res* 1980; 23:545-552.
12. First NL, Parrish JJ. In-vitro fertilization of ruminants. *J Reprod Fertil* 1987; 34(Suppl):151-165.
13. Fisz M. Some probability distributions. In: Krieger RE (ed), *Probability Theory and Mathematical Statistics*. Malabar, FL: John Wiley & Sons, Inc, 1980; 129-174.
14. Fraser LR. Albumin is required to support the acrosome reaction but not capacitation in mouse spermatozoa in vitro. *J Reprod Fertil* 1985; 74:185-196.
15. Fukui Y. Effect of follicle cells on the acrosome reaction, fertilization and developmental competence of bovine oocytes matured in vitro. *Mol Reprod Dev* 1990; 26:40-46

16. Galeati G, Modena S, Lauria A, Mattioli M. Follicle somatic cells influence pig oocyte penetrability and cortical granule distribution. *Mol Reprod Dev* 1991; 29:40-46.
17. Harrison RAP, White IG. Some methods for washing spermatozoa from bull, boar and ram: A comparison using biochemical and other criteria. *J Reprod Fertil* 1972; 29:271-284.
18. Itagaki Y, Toyoda Y. Factors affecting fertilization in vitro of mouse eggs after removal of cumulus oophorus. *J Mammal Ova Res* 1991; 8:126-134.
19. Kikuchi K, Nagai T, Motlik J, Shioya Y, Izaike Y. Effect of follicle cells on in vitro fertilization of pig follicular oocytes. *Theriogenology* 1993; 39:593-599.
20. Marquant-Le Guienne B, Humblot P. Tests de fertilité chez l'animal domestique par fécondation in vitro. *Ann Zootech* 1992; 41:361-370.
21. Marquant-Le Guienne B, Humblot P, Thibier M, Thibault C. Evaluation of bull semen fertility by homologous in vitro fertilization tests. *Reprod Nutr Dev* 1990; 30:259-266.
22. Martín S. How AI is progressing in Spain. *Pig International* 1984; May:24-28.
23. Martínez E, Matás C, Vázquez JM, Roca J, Gadea J, Ruiz S, Coy P. Effect of washing and preincubation on in vitro capacitation of boar spermatozoa. *Theriogenology* 1994; 41:248 abstr.
24. Martínez E, Vázquez JM, Matás C, Roca, J, Coy P, Gadea, J. Evaluation of boar spermatozoa penetrating capacity using pig oocytes at the germinal vesicle stage. *Theriogenology* 1993; 40:547-557.
25. Mattioli M, Galeati G, Bacci L, Seren E. Follicular factors influence oocyte fertilizability by modulating the intercellular cooperation between cumulus cells and oocytes. *Gamete Res* 1988; 21:223-232.
26. Mattioli M, Galeati G, Moretti M. Use of stored zonae pellucidae for the assessment of the fertilizing capacity of boar sperm. *Proc. 11th Intl Pig Vet Soc.* 1990; 478 abstr.
27. Mattioli M, Galeati G, Seren E. Effect of follicle somatic cells during pig oocyte maturation on egg penetrability and male pronucleus formation. *Gamete Res* 1988; 20:177-183.
28. McGaughey RW. In vitro oocyte maturation. In: Daniel JC Jr (ed), *Methods in Mammalian Reproduction*. New York: Academic Press, 1978; 1-19.
29. McGaughey RW, Montgomery DH, Richter JD. Germinal vesicle configurations and patterns of polypeptide synthesis of porcine oocytes from antral follicles of different size, as related to their competency for spontaneous maturation. *J Exp Zool* 1979; 209:239-254.
30. Miyamoto H, Chang MC. Fertilization in vitro of mouse and hamster eggs after the removal of follicular cells. *J Reprod Fertil.* 1972; 30:309-312.
31. Moor RM, Mattioli M, Ding J, Nagai T. Maturation of pig oocytes in vivo and in vitro. *J Reprod Fertil* 1990; 40(Suppl):197-210.
32. Motlik J, Crozet N, Fulka J. Meiotic competence in vitro of pig oocytes isolated from early antral follicles. *J Reprod Fertil* 1984; 72:323-328.
33. Nagai T, Takahashi T, Masuda H, Shioya Y, Kuwayama M, Fukushima M, Iwasaki S, Hanada A. In vitro fertilization of pig oocytes by frozen boar spermatozoa. *J Reprod Fertil* 1988; 84:585-591.
34. Ohogoda O, Niwa K, Yuhara M, Takahashi S, Kanoya K. Variations in penetration rates in vitro of follicular oocytes do not reflect conception rates after artificial insemination using frozen semen from different bulls. *Theriogenology* 1987; 29: 1375-1381.

35. Parrish JJ, Susko-Parrish JL, Leibfried-Rutledge ML, Critser ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology* 1986; 25:591-600.
36. Pavlok A, Lucas-Hahn A, Niemann H. Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. *Mol Reprod Dev* 1992; 31:63-67.
37. Pawshe CH, Totey SM, Jain SK. A comparison of three methods of recovery of goat oocytes for in vitro maturation and fertilization. *Theriogenology* 1994; 42:117-125.
38. Sirard MA, Dubuc A, Bolamba D, Zheng Y, Coenen K. Follicle-oocyte-sperm interactions in vivo and in vitro in pigs. *J Reprod Fertil* 1993; 48(Suppl): 3-16.
39. Tajik P, Niwa K, Murase T. Effects of different proteins supplements in fertilization medium on in vitro penetration of cumulus-intact and cumulus-free bovine oocytes matured in culture. *Theriogenology* 1993; 40: 949-958.
40. Vázquez JM, Martínez E, Roca J, Coy P, Pastor LM. Acrosome reaction of boar spermatozoa in homologous in vitro fertilization. *Mol Reprod Dev* 1993; 36:84-88.
41. Wang WH, Abeydeera LR, Okuda K, Niwa K. Penetration of porcine oocytes during maturation in vitro by cryopreserved, ejaculated spermatozoa. *Biol Reprod* 1994; 50:510-515.
42. Wilkinson H, Howe P. *Systat for Windows 5.0*. Evanston, IL: SYSTAT, Inc, 1992.
43. Xu X, Ding J, Seth PC, Harbison DS, Foxcroft GR. In vitro fertilization of in vitro matured pig oocytes: effects of boar and ejaculate fraction. *Theriogenology* 1995; 43:358 abstr.
44. Yanagimachi R: Mammalian fertilization. In: Knobil E, Neill J (eds), *The Physiology of Reproduction*. New York: Raven Press, 1988; 135-185.