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Oocyte Penetration by Fresh or Stored Diluted Boar Spermatozoa Before and After In Vitro Capacitation Treatments[1]

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ABSTRACT

This study examined whether washing and preincubation of boar spermatozoa was necessary to achieve high in vitro penetration rates of pig oocytes. In experiment 1, diluted, sperm-rich fractions, stored for 24 h at 16°C (stored diluted sperm) were used. After centrifugation once at 50 *g* for 3 min, the supernatant was concentrated at 1200 *g* for 3 min. The pellets were washed (1200 *g* for 3 min) 0, 1, or 2 times in saline-BSA solution and preincubated for 0, 20, or 40 min in modified Medium 199 before insemination of immature oocytes. In experiment 2, immature and ovulated oocytes were inseminated with untreated (unwashed and nonpreincubated sperm from concentrated pellet resulting from centrifugation of supernatant fraction obtained by initial low-speed centrifugation) or treated (washed 2 times in saline-BSA solution and preincubated for 40 min), diluted spermatozoa that had been stored and processed as described above. In experiment 3, freshly undiluted or stored diluted spermatozoa were untreated or treated and used to penetrate immature oocytes. In experiment 4, immature oocytes were exposed to freshly undiluted or stored diluted spermatozoa from untreated or treated samples, and, at various times after insemination, oocytes were examined for evidence of penetration. High penetrability rates were obtained when untreated, stored diluted spermatozoa were used. Type of oocyte (immature vs. ovulated) did not affect penetrability regardless of whether untreated or treated, stored diluted spermatozoa were used. Penetration rates and number of spermatozoa per oocyte were lower ($p < 0.05$) using stored diluted spermatozoa that were washed twice and preincubated 40 min than when freshly undiluted or untreated, stored diluted spermatozoa were used. First evidence of penetration of oocytes by untreated or treated spermatozoa (freshly undiluted or stored diluted) was observed 3 h after insemination. Results indicate that, under the in vitro conditions studied, boar spermatozoa undergoes capacitation and a true acrosome reaction during coincubation with oocytes even when not washed or preincubated.

INTRODUCTION

Spermatozoa are incapable of fertilizing eggs immediately after ejaculation [1, 2]. In most species, sperm acquires this capability after mating or after incubation in vitro in an appropriate medium in the absence of seminal plasma components [3]. However, the necessary events, collectively termed capacitation, are understood poorly in spite of their importance to sperm physiology.

Various in vitro systems have been proposed for sperm capacitation in the pig (for recent reviews see [4, 5]). In these systems, two features commonly used are sperm washing and holding for a period of sperm preincubation before coincubation of the gametes.

During the process of capacitation, an initial step is the removal of seminal plasma and certain coating proteins from the surface of spermatozoa. These are accumulated during epididymal transit and exposure to the seminal plasma [6] and are believed to be removed from pig spermatozoa by three washings with saline-BSA [7–15]. Generally, it has been accepted that sperm preincubation before coculture is an important variable in the procedure, although the mechanism by which preincubation time intervenes remains obscure. In 1985, Cheng [7] reported the first successful porcine in vitro fertilization, using ejaculated spermatozoa that were washed and preincubated for 4–5 h at 37°C or stored at 20°C for 16 h, washed, and preincubated only for 40 min. These procedures have been used by several laboratories with minor modifications. However, high in vitro penetration rates have also been obtained without preincubation. For example, Mattioli et al. [16] obtained high penetration rates, using Percoll-washed and nonpreincubated spermatozoa previously diluted in a commercial extender and stored for 24–48 h at 16°C. Likewise, acceptable in vitro penetration rates have been obtained with frozen-thawed [17] or fresh [18] spermatozoa washed in physiological medium but not preincubated.

The aim of the present study was to investigate whether washing and preincubation of boar spermatozoa is necessary to achieve high in vitro penetration rates of pig oocytes.

MATERIALS AND METHODS

Animals

Seven mature Landrace boars of similar age and sexual experience and with satisfactory semen characteristics in the 10 wk preceding the experiments (total sperm count per ejaculate $\geq 2 \times 10^{10}$, plasma membrane integrity $\geq$ 80%, forward progressive motility $\geq$ 3, acrosomal integrity $\geq$ 85%, and morphological abnormalities $\leq$ 15%) were selected as semen donors. Thirteen Landrace × Large White prepubertal gilts, aged 5.5–6 mo and weighing 80–95 kg each, were used as ovulated oocytes donors. The animals were housed in a well-ventilated building under natural conditions. Facilities are in the Department of Animal Pathology, University of Murcia. They were fed concentrates, and water was always available.

Chemicals and Supplements

Medium 199, BSA (fraction V), fetal calf serum, caffeine, kanamycin, penicillin G, streptomycin sulphate, lacmoid, dimethylsulfoxide, and carboxyfluorescein diacetate were obtained from Sigma Chemical Co. (St. Louis, MO); eCG and hCG were obtained from Intervet International B.V. (Booxmeer, Holland); all other chemicals were purchased from Merck (Darmstadt, Germany).

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Culture Media

The basic medium used for spermatozoa manipulation and oocyte fertilization was Medium 199 with Earle's salts supplemented with 12% heated fetal calf serum (v:v), 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 (mM199).

Collection of Ovulated Oocytes

The methods used for oocyte recovery were based on those described by Coy et al. [12]. Briefly, prepubertal gilts were induced to ovulate by a simple i.m. injection of 1250 IU eCG followed 56 h later by administration of 750 IU hCG. Freshly ovulated oocytes were recovered surgically 42 h after administration of hCG. The ovaries were exposed through a midline incision under general anesthesia (thiopental sodium; 6–10 ml, 100 mg/ml), and the oviducts were flushed with Dulbecco's PBS supplemented with 4 mg/ml BSA, 0.34 mM sodium pyruvate, 5.54 mM D-glucose and 70 μg/ml Kanamycin (mDPBS) at 39°C. The ovulated oocyte-cumulus complexes were washed once in mDPBS and returned to the laboratory in a 10-ml test tube containing mDPBS. The oocytes were washed again 3 times in mDPBS at 39°C before insemination.

Collection of Immature Oocytes

Ovaries were obtained from prepubertal gilts at a local abattoir just after slaughter and transported to the laboratory at 30°C in mDPBS. Within 1 h of slaughter, the medium-size follicles with a diameter of 3–5 mm were punctured with a sterile 21-gauge needle into mDPBS at 39°C. The oocyte-cumulus complexes were washed 3 times in mDPBS and exposed to boar's spermatozoa.

Experiment 1

In this experiment (3 × 3 factorial), immature oocyte-cumulus complexes were exposed to differently treated spermatozoa following the basic method described by Cheng [7]. Sperm-rich fractions were collected from four boars (A, B, C, and D), using the gloved-hand method. Immediately after collection, semen samples were diluted to 3×10^7 cells/ml in MR-A diluent [19] and kept for 24 h at 16°C (stored diluted sperm). Nine aliquots of each sperm suspension were pretreated by centrifugation at 50 *g* for 3 min and subsequent concentration of the supernatants at 1200 *g* for 3 min. Then, the pellets were washed 0, 1, or 2 times at 1200 *g* for 3 min in 0.9% saline solution enriched with 1 mg/ml BSA and diluted to 2×10^8 cells/ml in preincubation medium consisting of mM199 at pH 7.8. Then, sperm suspensions were preincubated for 0, 20, or 40 min at 39°C in tightly capped test tubes. Spermatozoa were evaluated for plasma membrane integrity (PMI), and forward progressive motility (FPM) at various stages of the in vitro procedure. The PMI percentage of spermatozoa was determined, using a fluorescent probe with carboxyfluorescein diacetate, as previously described [20]. The stained suspensions were observed, using an epifluorescence illumination at ×400, and two slides were counted for a total of 300 spermatozoa per sample. For FPM evaluations, 3 subsamples of each sample were placed on warm glass slides (39°C) and examined under a brightfield microscope at x200. The proportion of spermatozoa with FPM was determined, using an arbitrary scale of 0 to 5 (0, 1, 2, 3, 4, or 5 = 0–10%, 10–25%, 25–50%, 50–70%, 70–90%, or 90–100%, respectively, of the motile spermatozoa showing progressive movement). The remainder of the seminal characteristics were examined in fresh (total sperm per ejaculate, acrosomal integrity, and normal morphology) and stored (acrosomal integrity and normal morphology) semen, using standard laboratory techniques.

Experiment 2

In order to rule out confounding effects related to the source of oocytes, ovulated and immature oocytes were exposed to treated or untreated, stored diluted spermatozoa (2 × 2 factorial) collected from the same boars used in experiment 1. A total 161 ovulated oocytes were recovered from tubal flushing; 81.4% of the ovulated oocytes were matured and included in results. Before use, the sperm suspensions from the four boars were pooled. The final suspension was split into two aliquots, and each was pretreated as described above. The pellets were treated (washed twice and preincubated for 40 min) before coincubation with oocytes or used directly as untreated samples (unwashed and nonpreincubated).

Experiment 3

Since the requirement for sperm washing and preincubation may depend on the source of the spermatozoa being used, this experiment (2 × 2 factorial) was carried out to determine the effects of the washings and preincubation time on the in vitro penetrability of freshly undiluted or stored diluted sperm. Sperm-rich fractions of ejaculates were obtained from three boars (E, F, and G). Immediately after collection, 10-ml samples of each sperm-rich fraction (freshly undiluted sperm) were treated or untreated as described in experiment 2, except that sperm were not pretreated. Additional 10-ml samples of each sperm-rich fraction were diluted and stored for 24 h (stored diluted sperm) and pretreated as described in experiment 1. Then, stored diluted sperm were treated or untreated and used for insemination.

Experiment 4

To determine whether washing and preincubation of freshly undiluted or stored diluted spermatozoa can accelerate the time of penetration of oocytes, immature oocytes were exposed to treated or untreated spermatozoa (from boars E, F, and G), prepared as described in experiment 3. At various times after insemination (1, 2, 3, 4, 5, 6, 9, 12, and 23 h), oocytes were mounted, fixed, stained, and examined for evidence of sperm penetration.

In Vitro Fertilization and Oocyte Examination

Each group of 10–15 ovulated or immature oocytes was coincubated with spermatozoa for 16–18 h (except in experiment 4) in a 35-mm plastic dish containing 2 ml fertilization medium (mM199, pH 7.4, supplemented with 2 mM caffeine and 5.4 mM calcium lactate) at 39°C under 5% CO_2 in air. In experiments 1, 3, and 4, a final sperm concentration of 10×10^6 cells/ml was used. The volume of untreated sperm suspension (experiments 3 and 4) added to the fertilization medium ranged from 20 to 40 μl. In experiment 2, the final concentration of spermatozoa at insemination was 1×10^6 cells/ml. This count was used because most of the spermatozoa incorporated into the vitellus of ovulated oocytes show nuclear decondensation or pronuclear formation and a high number of penetrated sper-

matozoa is not easily evaluated. At the end of the coincubation period, the oocytes were stripped of cumulus cells and spermatozoa, 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 [21] and examined for evidence of sperm penetration under a phase contrast microscope at ×400. Ovulated oocytes with swollen heads or pronuclei and their corresponding sperm tails in the vitellus, and with two polar bodies, were considered to be matured and fertilized. Ovulated oocytes with the metaphase plate and one polar body were considered matured but unfertilized. Ovulated oocytes at the germinal vesicle stage or in the metaphase I to telophase I stages were classified as immature. Only mature oocytes from ovulated oocytes were included in the results of experiment 2. Immature oocytes at the germinal vesicle stage obtained from ovaries collected at the abattoir were considered to be penetrated when spermatozoa with unswollen heads and their corresponding sperm tails were found in the vitellus. The distinction between unswollen sperm 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 slowly separated from the heads [22, 23]. Oocytes with a broken oolemma or abnormal appearing cytoplasm were classified as degenerated.

Statistical Analysis

Experiment 1 was conducted in 16 replicates (4 times for each of the 4 boars). Experiments 2, 3, and 4 were conducted in 3 replicates, each on a different day. Data from each replicate were pooled. The results are expressed as mean ± SEM. Sperm evaluation parameters, oocyte penetration rates (the data were modeled according to the binomial model of parameters, as described earlier [24]) and the number of penetrating spermatozoa per oocyte were analyzed by two-way (experiments 1, 2, and 3) or one-way (experiment 4) analysis of variance (ANOVA), using the Multivariate General Linear Models of Systat [25]. When ANOVA revealed a significant effect, values were compared by Tukey-test. Differences were considered to be statistically different at $p < 0.05$.

RESULTS

Experiment 1

There were no differences between the boars as regards the semen characteristics of each fresh ejaculate. PMI, FPM, acrosomal integrity, and morphological abnormalities in stored sperm did not differ between boars, ranging from 81.5% to 87.2%, 2.9–3.3, 87.2% to 94.7%, and 8.5% to 13.7%, respectively. The results of the sperm analysis conducted immediately after each treatment are shown in Figure 1. PMI was affected ($p < 0.05$) negatively by the number of washings in all four boars. In contrast, the effect of preincubation on PMI varied according to the boar. There was a significant effect ($p < 0.001$) in boars A, C, and D, but not in boar B ($p = 0.14$). In general, the lowest PMI values were observed in spermatozoa washed twice and preincubated for 40 min. The PMI values ranged from 60.9 ± 1.6% (boar D) to 66.0 ± 7.1% (boar A). FPM in all boars showed a significant increase ($p < 0.005$) with the length of preincubation but was not affected by the number of washings. The FPM values were highest when the spermatozoa were washed twice and preincubated for 40 min, with values ranging from 3.7 ± 0.1 (boar C) to 4.1 ± 0.1 (boar D). The effect of the interaction number of washings × time of preincubation on the PMI and FPM values was not significant. On the other hand, within each treatment group no differences between boars were observed in PMI or FPM.

Data showing the effects of washing and preincubation on the penetrating ability of stored diluted spermatozoa are presented in Table 1. The percentage of oocytes penetrated and the number of spermatozoa in penetrated oocytes decreased as the number of washings increased from 0 to 2 times, in boars B, C, and D. In contrast, in boar A, although the mean number of spermatozoa per oocyte also decreased, no differences were observed in penetration rates among the different treatment groups. Effects of the length of preincubation and the washing × preincubation interaction on the penetrability of spermatozoa varied according to the boar. Nevertheless, a high penetrability was obtained in all boars by untreated spermatozoa.

Experiment 2

From the results of experiment 1, it is evident that, with the system used, washing and preincubation of stored diluted spermatozoa to stimulate in vitro sperm capacitation was not necessary for immature oocytes to be penetrated. The results of experiment 2 showed that the type of oocyte (immature vs. ovulated) did not affect the penetrating ability of spermatozoa (Table 2). However, spermatozoa treatment had, again, a negative effect on penetration rates ($p < 0.003$) and the mean number of spermatozoa per oocyte penetrated ($p < 0.001$).

Experiment 3

Data showing the effects of washings and preincubation time on the penetrating ability of freshly undiluted or stored diluted spermatozoa are presented in Table 3. The percentage of oocytes penetrated and the number of spermatozoa in penetrated oocytes were lower ($p < 0.05$) in stored diluted spermatozoa washed twice and preincubated for 40 min. No differences were observed between untreated and treated freshly undiluted sperm either as regards the penetration rates or the mean number of spermatozoa observed per oocyte.

Experiment 4

Freshly undiluted and stored diluted spermatozoa from untreated or treated samples started to penetrate oocytes 3 h after insemination (Table 4). Sperm penetration of the oocytes was complete 6–9 h after insemination, but the number of penetrating spermatozoa increased gradually with time after insemination. At 9–23 h after insemination, the penetration rates and the mean number of spermatozoa per oocyte penetrated were, again, significantly lower ($p < 0.05$) in stored diluted spermatozoa washed twice and preincubated for 40 min than in freshly undiluted or untreated stored diluted spermatozoa.

DISCUSSION

It has been reported that in vitro matured and immature cumulus-free pig oocytes show similar degrees of penetrability [26]. Moreover, we have shown that immature cumulus-free pig oocytes have penetration rates similar to those seen in ovulated cumulus-free oocytes [27]. However,

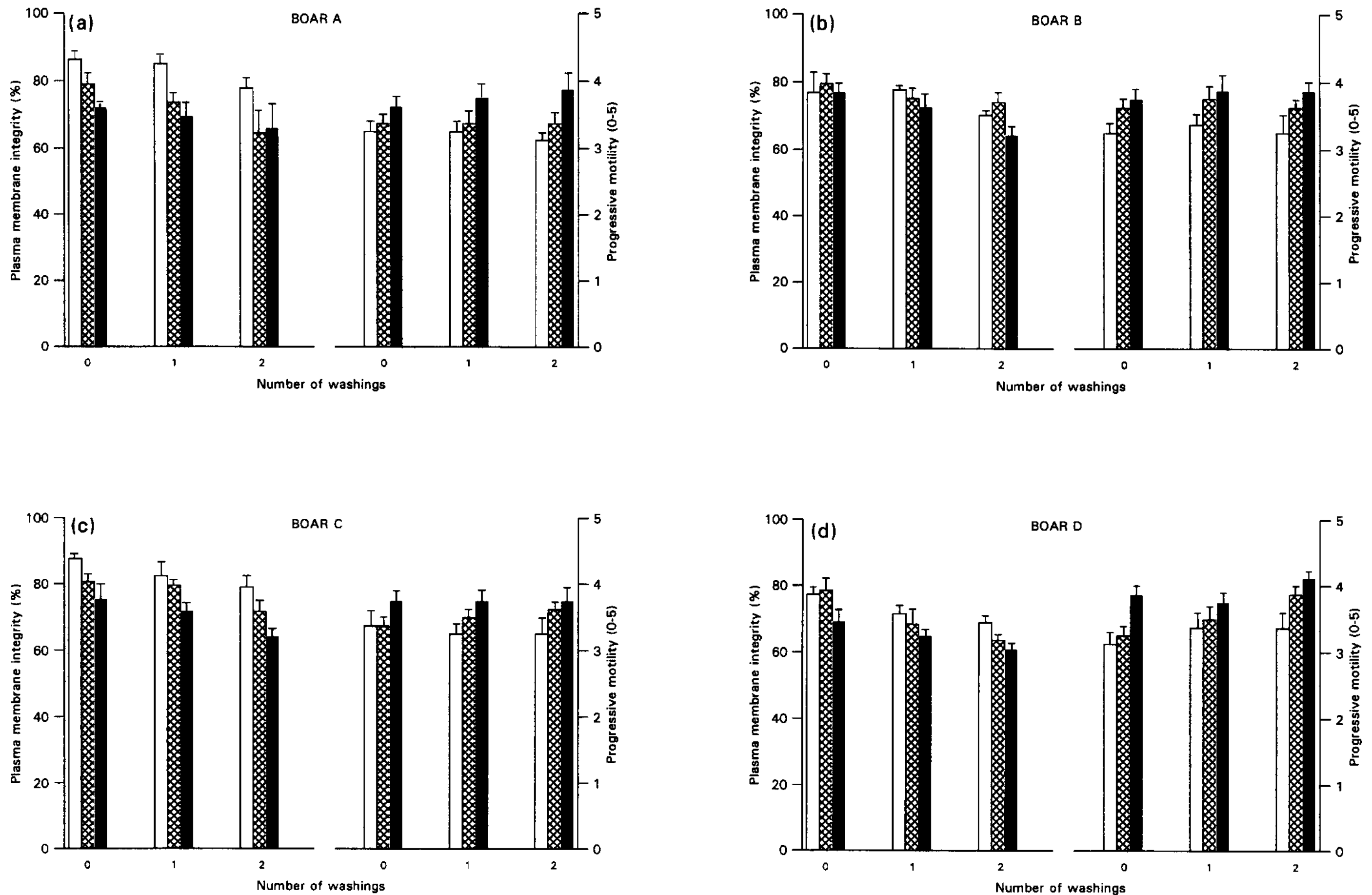

FIG. 1. Changes in plasma membrane integrity (PMI) and forward progressive motility (FPM) of stored diluted boar's spermatozoa washed 0, 1, or 2 times and preincubated for 0 (open bars), 20 (cross-hatched bars), or 40 (black bars) min. Data are means and SEM from four replicates for each boar. Analysis of variance showed that PMI was affected by the number of washings ($p < 0.05$) in all boars and by the time of preincubation ($p < 0.001$) in boars A, C, and D. FPM was affected ($p < 0.005$) alone by preincubation time in the four boars.

there are no reports to show the penetrability of immature pig oocytes surrounded by cumulus cells. The results provide evidence that cumulus-intact immature pig oocytes are penetrable by spermatozoa in vitro. The data from experiment 2 show that oocyte type (ovulated vs. immature) had no significant effect on the penetrating ability of spermatozoa, regardless of whether sperm cells were untreated or washed and preincubated. Because zona pellucida is probably the major obstacle encountered by sperm and because its penetration requires both an acrosome reaction and vigorous motility, we consider that the penetration of immature oocytes provides adequate information on sperm function and that this method may be useful for evaluating sperm-oocyte interaction during fertilization.

The data from experiment 1 show variations in the in vitro penetration by different boars. However, none of the sperm characteristics from each treatment group examined at insemination differed among boars. Our results concerning variability are consistent with those of previous studies [27–29] and indicate that the classical parameters accepted in assessing the viability of spermatozoa do not predict the penetrating ability of the spermatozoa in vitro.

As described in the *Introduction*, the classic methods for in vitro capacitation of boar spermatozoa involve preincubation of the sperm preceded by washings with saline-BSA. Generally, it is accepted that seminal plasma and decapacitation factors are removed by serial washing. Obviously, the decapacitation factors must be removed or modified before the acrosome reaction can occur, this being a major feature of capacitation. Various preincubation times such as 4 h [7, 8, 10, 28, 30, 31], 90 min [11, 13, 15, 32], or 30–40 min [7, 27, 33] have been used to promote pig in vitro fertilization, but no clear evidence has emerged to indicate the superiority of any given preincubation time. However, our results of experiment 1 (concerning in vitro penetration) indicated that washings in saline-BSA and preincubation of spermatozoa are unnecessary to obtain high penetration levels. It should be remembered that these results were obtained using stored diluted sperm-rich fractions of ejaculates and could be explained by the following. There is evidence that the storage of semen at room temperature enhances the capacitation of bull [34] and human [35] spermatozoa. Furthermore, it has been demonstrated in boar semen that extenders, preservation processes, and temperature changes have a strong influence on head plasma membrane fluidity and, therefore, the molecular organization of this type of membrane [36]. However, the results of experiment 3 demonstrated that high penetration levels can be obtained, using untreated, freshly undiluted spermatozoa. These findings suggest that the penetrating ability of boar spermatozoa is apparently acquired during gamete coincubation.

The efficiency of a capacitation procedure can be evaluated by the time required for egg penetration to occur after in vitro insemination [37]. However, few data exist on the timetable of sperm-egg interaction in the in vitro systems

TABLE 1. Effect of number of washings and length of preincubation of stored diluted spermatozoa obtained from 4 boars on their in vitro penetrating ability, using immature pig oocytes (mean ± SEM).*

	Boar A†			Boar B†			Boar C†			Boar D†		
Treatment Ws-PT	Oocytes examined (n)	Oocytes penetrated (%)	Spermatozoa in penetrated oocytes (no.)	Oocytes examined (n)	Oocytes penetrated (%)	Spermatozoa in penetrated oocytes (no.)	Oocytes examined (n)	Oocytes penetrated (%)	Spermatozoa in penetrated oocytes (no.)	Oocytes examined (n)	Oocytes penetrated (%)	Spermatozoa in penetrated oocytes (no.)
0-0	232	91.3 ± 2.0	21.5 ± 1.3[a]	222	65.4 ± 3.3[a]	4.3 ± 0.3[a]	241	85.8 ± 2.7[ab]	12.4 ± 0.9[a]	207	77.6 ± 3.6[a]	4.7 ± 0.4[a]
0-20	264	92.3 ± 2.0	18.9 ± 1.2[ab]	237	62.1 ± 3.1[ab]	3.8 ± 0.3[ab]	242	89.2 ± 2.7[b]	11.3 ± 0.7[a]	154	67.5 ± 4.1[ab]	4.1 ± 0.4[abc]
0-40	247	92.1 ± 2.0	18.0 ± 1.1[ab]	194	58.9 ± 3.5[ab]	3.3 ± 0.3[ab]	265	90.4 ± 2.6[b]	10.2 ± 0.5[a]	118	66.7 ± 4.7[ab]	4.5 ± 0.5[ab]
1-0	300	92.0 ± 1.8	14.8 ± 0.9[bc]	233	55.6 ± 3.2[ab]	4.4 ± 0.5[a]	225	74.1 ± 2.9[ac]	7.2 ± 0.6[b]	170	61.8 ± 4.0[ab]	3.7 ± 0.3[abc]
1-20	214	87.6 ± 2.2	16.5 ± 1.2[bd]	246	55.8 ± 3.1[ab]	2.7 ± 0.3[b]	214	76.2 ± 2.9[ac]	7.0 ± 0.5[b]	190	62.6 ± 3.8[ab]	3.7 ± 0.3[abc]
1-40	228	89.6 ± 2.1	13.1 ± 1.0[cd]	249	50.0 ± 3.1[b]	2.6 ± 0.2[b]	211	77.1 ± 2.9[ac]	5.1 ± 0.4[b]	133	65.0 ± 4.4[ab]	2.8 ± 0.2[bc]
2-0	202	89.5 ± 2.2	11.2 ± 0.7[c]	235	49.2 ± 3.1[b]	2.7 ± 0.2[b]	227	76.1 ± 2.8[ac]	5.9 ± 0.4[b]	199	67.0 ± 3.7[ab]	3.3 ± 0.3[bc]
2-20	212	84.8 ± 2.2	11.0 ± 1.0[c]	192	50.0 ± 3.5[b]	2.5 ± 0.2[b]	194	68.9 ± 3.1[cd]	5.6 ± 0.4[b]	192	51.0 ± 3.7[bc]	2.5 ± 0.2[c]
2-40	197	88.2 ± 2.3	12.5 ± 1.2[bc]	243	48.7 ± 3.1[b]	2.5 ± 0.2[b]	262	61.8 ± 2.6[cd]	5.3 ± 0.4[b]	113	37.5 ± 4.7[c]	2.9 ± 0.4[bc]
Probability												
Ws		NS	0.001		0.001	0.001		0.001	0.001		0.001	0.001
PT		NS	NS		NS	0.001		NS	0.002		0.001	NS
Ws × PT		NS	NS		NS	0.038		0.006	NS		0.004	NS

* Sperm concentration at coincubation was 10 × 10^{6} cells/ml. Experiments were repeated 4 times for each boar. Ws: number of washings; PT: preincubation time (min); Ws × PT: number of washing × preincubation time; NS: not significant.
† Values within columns with different superscripts differ ($p < 0.05$).

TABLE 2. Effect of oocyte type and in vitro capacitation treatment of stored diluted spermatozoa on oocyte penetrability (mean ± SEM).*

Type of oocyte	Treatment Ws-PT	Number of oocytes examined	Percentage of oocytes penetrated†	Number of spermatozoa in penetrated oocytes†
Ovulated	0-0	67	77.6 ± 2.4[a]	8.2 ± 1.0[a]
	2-40	64	60.9 ± 2.8[b]	2.9 ± 1.1[b]
Immature	0-0	114	84.5 ± 3.2[a]	7.1 ± 2.8[a]
	2-40	149	54.2 ± 1.9[b]	2.7 ± 2.1[b]
Probability				
Type of oocyte			NS	NS
Treatment			0.003	0.001
Type of oocyte × treatment			NS	NS

* Sperm concentration at coincubation was 1 × 10^{6} cells/ml. Experiments were repeated 3 times. Ws: number of washings; PT: preincubation time (min); NS: not significant.
† Values within columns with different superscripts differ ($p < 0.05$).

currently used. In the present study, the first evidence of penetration of the immature oocyte by untreated spermatozoa was observed 3 h after insemination, independently of the source of spermatozoa. Our own observations (unpublished data) show that the time of sperm penetration of in vitro-matured oocytes is similar to that of the penetration of immature oocytes. These results are in agreement with those described in the bibliography using pig [38] and bovine [39] oocytes matured and fertilized in vitro. Washing (twice) and preincubation of spermatozoa (40 min) did not accelerate the time needed to penetrate the oocytes, which implies that the spermatozoa are unable to interact immediately with oocytes. These results suggest that the sperm population that is capable of penetrating oocytes (penetrating spermatozoa) is decided during coincubation and that the acrosome reaction of the penetrating spermatozoa must be tightly synchronized with penetration through the egg vestments because acrosomal enzymes play an extremely important role in sperm penetration [3]. It is clear that environmental conditions of coculture are sufficient to induce sperm capacitation and true acrosome reaction. Therefore, the timing of the acrosome reaction might be of major importance for the penetrating spermatozoa.

In some boars, an increase in the rate of polyspermic fertilization has been recorded with increased preincubation periods [8]. By contrast, in our study, an increase in the

TABLE 3. Effect of source of spermatozoa and in vitro capacitation treatment of spermatozoa on oocyte penetrability (mean ± SEM).*

Source of spermatozoa	Treatment Ws-PT	Number of oocytes examined	Percentage of oocytes penetrated†	Number of spermatozoa in penetrated oocytes†
Freshly undiluted rich fractions	0-0	160	91.3 ± 2.2[ab]	23.4 ± 1.6[a]
	2-40	167	91.0 ± 2.2[ab]	20.7 ± 1.1[a]
Stored diluted rich fractions	0-0	178	93.8 ± 1.8[a]	22.3 ± 1.4[a]
	2-40	183	83.1 ± 2.8[b]	15.4 ± 0.9[b]
Probability				
Source of spermatozoa			NS	0.05
Treatment			0.05	0.001
Source of spermatozoa × treatment			0.05	NS

* Sperm concentration at coincubation was 10 × 10^{6} cells/ml. Experiments were repeated 3 times. Ws: number of washings; PT: preincubation time (min); NS: not significant.
† Values within columns with different superscripts differ ($p < 0.05$).

TABLE 4. Time of penetration in vitro of immature pig oocytes by freshly undiluted and stored diluted spermatozoa from untreated or treated samples (mean ± SEM).*

	Treatment of spermatozoa† (WS-PT)											
	Freshly undiluted rich fractions						Stored diluted rich fractions					
	0-0			2-40			0-0			2-40		
T.E.	Oocytes examined (n)	Oocytes penetrated (%)	Spermatozoa in penetrated oocytes (no.)	Oocytes examined (n)	Oocytes penetrated (%)	Spermatozoa in penetrated oocytes (no.)	Oocytes examined (n)	Oocytes penetrated (%)	Spermatozoa in penetrated oocytes (no.)	Oocytes examined (n)	Oocytes penetrated (%)	Spermatozoa in penetrated oocytes (no.)
1	69	0	0	58	0	0	77	0	0	61	0	0
2	72	0	0	66	0	0	69	0	0	70	0	0
3	150	14.7 ± 2.9	1.4 ± 0.1	141	19.1 ± 3.3	1.4 ± 0.1	163	15.3 ± 2.9	1.6 ± 0.1	148	17.1 ± 3.0	1.7 ± 0.1
4	162	62.3 ± 3.8	3.5 ± 0.3	158	60.8 ± 3.0	3.0 ± 0.2	147	66.7 ± 2.9	3.5 ± 0.2	140	56.8 ± 2.9	2.9 ± 0.1
5	146	69.2 ± 3.8	5.1 ± 0.4	144	71.5 ± 3.8	5.5 ± 0.5	133	70.8 ± 3.2	6.2 ± 0.4	161	62.4 ± 3.8	5.3 ± 0.3
6	124	83.9 ± 3.3	7.2 ± 0.5	125	81.6 ± 3.5	6.7 ± 0.5	146	84.5 ± 3.0	6.9 ± 0.4	153	69.0 ± 3.4	6.4 ± 0.3
9	133	92.5 ± 1.7[a]	15.6 ± 1.0[c]	136	90.2 ± 1.8[a]	16.9 ± 1.1[c]	155	89.1 ± 2.1[a]	14.5 ± 1.2[c]	149	77.1 ± 3.2[b]	8.5 ± 0.4[d]
12	151	91.4 ± 1.3[a]	21.4 ± 1.1[c]	145	93.4 ± 1.2[a]	19.4 ± 1.3[c]	144	92.9 ± 1.9[a]	18.4 ± 0.9[c]	152	75.4 ± 3.5[b]	12.5 ± 0.5[d]
23	146	93.8 ± 1.4[a]	22.5 ± 1.3[c]	139	91.9 ± 1.8[a]	23.5 ± 1.3[c]	165	95.8 ± 1.3[a]	24.1 ± 1.3[c]	163	80.5 ± 2.6[b]	16.8 ± 0.9[d]

* Sperm concentration at coincubation was 10 × 10^6 cells/ml. Experiments were repeated 3 times. T.E.: Time of examination (hours after insemination); Ws: number of washings; PT: preincubation time (min).
† a,b in the same row differ ($p < 0.05$); c,d in the same row differ ($p < 0.05$).

number of washings and the preincubation time led to a decrease both in penetration rates and number of spermatozoa per oocyte, when stored diluted spermatozoa were used. The reason for this decrease is not clear. Probably, stored diluted spermatozoa are less resistent to damage caused by the capacitation treatment than freshly undiluted spermatozoa. According to Harrison and White [40], when spermatozoa are washed in a physiological medium, a relatively great centrifugal force is necessary for their sedimentation. Furthermore, the pellet will be tightly packed, and efforts to resuspend the spermatozoa may damage them. In our study, the stored diluted spermatozoa that had been washed twice and preincubated for 40 min revealed a decrease of about 20% in PMI compared with untreated spermatozoa. The decrease in the number of viable spermatozoa observed might have been responsible for reducing the number of penetrating spermatozoa.

In summary, the freshly undiluted or stored diluted boar spermatozoa undergo capacitation and a true acrosome reaction during coincubation with oocytes even if they are not washed or preincubated. Moreover, washing and preincubation of sperm might have a detrimental effect on the sperm cells rather than a positive influence on their penetrating capacity when stored spermatozoa in extender are used.

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