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Influence of Sperm Pretreatment on the Efficiency of Intracytoplasmic Sperm Injection in Pigs

EMPAR GARCÍA-ROSELLÓ,* CARMEN MATÁS,* SEBASTIÁN CÁNOVAS,* PEDRO N. MOREIRA,† JOAQUÍN GADEA,* AND PILAR COY*

*From the *University of Murcia, Department of Veterinary Physiology, Faculty of Veterinary Science, Murcia, Spain; and the †Department of Animal Reproduction, Nacional Institute of Agricultural Research and Technology (INIA), Madrid, Spain.*

ABSTRACT: The purpose of this study was to determine the influence of sperm pretreatment on the efficiency of intracytoplasmic sperm injection (ICSI) in pigs. This was done by examining the effect of 1) the conservation method (fresh vs frozen); 2) the sperm treatment preinjection (resuspension in Dulbecco phosphate-buffered saline (DPBS) vs selection by a Percoll gradient); and 3) the acrosomal and live or dead status of the spermatozoa (by incubation with or without calcium ionophore, 1 μM and 5 μM). In vitro matured porcine oocytes were injected with treated spermatozoa according to each experiment. All the experiments were done with non–artificially activated oocytes. The percentages of activation and cleavage were higher (68% vs 43% and 63% vs 43%, respectively, $P < .05$) in oocytes injected with fresh vs frozen spermatozoa. The DPBS treatment allowed higher cleavage proportions than the Percoll treatment ($P < .05$). Moreover, a boar effect was observed in the percentage of developing blastocysts. None of the studied parameters was affected by the acrosomal or the live or dead status of the spermatozoa injected. In conclusion, the use of fresh semen is recommended for porcine ICSI, as well as careful selection of the boar; Percoll treatment is only recommended for poor-quality samples or for removing toxic agents, and no exogenous form of activation or induction of the acrosome reaction is necessary for porcine oocytes to develop a male pronucleus and cleave up to the 2-cell stage after ICSI, although experimental conditions to reach the blastocyst stage need to be investigated further.

Key words: Capacitation, sperm cryopreservation, acrosome reaction, early development.

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In spite of the different studies performed during recent years, the efficacy of in vitro fertilization (IVF) systems in pigs continues to be quite low, principally because of the high proportions of polyspermic fertilization (Coy and Romar, 2002; Kren et al, 2003). However, interest in the in vitro production of pig embryos is currently an unquestionable objective for many different reasons, including the following: the creation of transgenic animals for the production of pharmaceutical products; organ-tissue xenotransplants; in order to increase the output in animal production (Prather et al, 2003). Intracytoplasmic sperm injection (ICSI) is an assisted reproductive technique (ART), one that allows us to avoid the problem of polyspermy, since with this method, only 1 spermatozoon is injected into the oocyte. Recently it has been shown that it is possible to obtain viable embryos with this technique, and a few piglets have been obtained in different laboratories (Kolbe and Holtz, 2000; Martin, 2000; Lai et al, 2001; Nakai et al, 2003; Probst and Rath, 2003). However, it is evident from these studies that the conditions employed for the development of this technique in pigs are far from standardized, and the yield is still too low to be considered as a method ready for the commercial application.

According to Probst and Rath (2003), the current main problem arising from porcine ICSI is the low proportion of blastocysts obtained, and it has been suggested (Kren et al, 2003) that this low proportion is a consequence of the fact that porcine ICSI fertilized oocytes are unable to induce sperm head decondensation yielding functional male pronuclei (Lee et al, 2003). In addition to the contradictory results regarding the convenience of employing whole spermatozoa or isolated heads (Kwon et al, 2004; Lee and Yang, 2004), 3 considerations (related to sperm factor) should be evaluated for male pronuclear formation ability and subsequent normal fertilization post-ICSI. The first consideration is that cryopreservation procedures could induce DNA fragmentation of the sperm or damage to the sperm centriole, leading to failed fertilization and embryo cleavage (Billard, 1983; Kim et al, 2002; Baumber et al, 2003; Martin et al, 2004). Few data have been reported demonstrating differences after injection of fresh

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Correspondence to: Pilar Coy, Departamento de Fisiología, Facultad de Veterinaria, Universidad de Murcia, 30071 Murcia, Spain (e-mail: pcoy@um.es).

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or frozen ejaculated (the most available source of sperm cells) spermatozoa into in vitro matured (the most available source of oocytes) pig oocytes. Second, the use of purified samples of spermatozoa to ensure that only membrane-intact sperm cells are used could be an important point when an increase in the ICSI yield is the ultimate aim. In this case, the advantage of using a short and simple sperm treatment, compared to a complex procedure, to ensure membrane integrity should be pondered. Third, the impact of injecting the intact acrosome vesicle into the oocyte has been reported to be a factor causing delayed male chromatin decondensation and male pronuclear formation (Katayama et al, 2002a), but more categorical results are necessary before we can include the induction of the acrosome reaction in the porcine ICSI protocols.

The aim of this study was to evaluate the impact on porcine ICSI yield of 1) sperm cryopreservation, 2) sperm washing and selection procedure, and 3) the induction of artificial acrosome reaction with a calcium ionophore before the injection. Our experimental outcomes, in terms of oocyte activation, pronuclear formation, and embryo development postfertilization by ICSI, are presented here and discussed.

Materials and Methods

The protocols described herein were approved by the Bioethical Commission of the University of Murcia, where the experiments were conducted.

Media and Chemicals

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain). The oocyte maturation medium was NCSU-37 (Petters and Wells, 1993) supplemented with 0.57 mM cysteine, 1 mM dibutyryl cAMP, 5 μg/mL insulin, 50 μM β-mercaptoethanol, 10 IU/mL eCG (Folligon; Intervet International B.V., Boxmeer, Holland), 10 IU/mL hCG (Chorulon; Intervet International B.V.), and 10% porcine follicular fluid (vol/vol).

The medium used for embryo micromanipulation was Dulbecco phosphate-buffered saline (DPBS) supplemented with 10% fetal calf serum (FCS). After microinjection, oocytes recovered in TALP medium (Rath et al, 1999) consisting of 114.06 mM NaCl, 3.2 mM KCl, 8 mM Ca-lactate.$5H_2O$, 0.5 mM $MgCl_2.6H_2O$, 0.35 mM NaH_2PO_4, 25.07 mM $NaHCO_3$, 10 mL/L Na lactate, 1.1 mM Na-pyruvate, 5 mM glucose, 2 mM caffeine, 3 mg/mL bovine serum albumin (BSA; A-9647), 1 mg/mL polyvinylpyrrolidone (PVA), and 0.17 mM kanamycin sulfate.

The embryo culture medium was NCSU-23 containing 0.4% BSA (A-8022), 75 μg/mL potassium penicillin G, and 50 μg/mL streptomycin sulfate (Macháty et al, 1998).

Oocyte Collection and In Vitro Maturation

Within 30 minutes of slaughter, ovaries from prepubertal gilts were transported to the laboratory in saline (0.9% wt/vol NaCl) containing 100 μg/mL kanamycin sulfate at 37°C and were then washed once in 0.04% (wt/vol) cetrimide solution and twice in saline. Oocyte-cumulus cell complexes were collected from nonatretic follicles (3–6 mm in diameter), washed twice in 35-mm plastic Petri dishes containing DPBS supplemented with 4 mg/mL PVA, and then washed twice more in maturation medium previously equilibrated for at least 3 hours at 38.5°C under an atmosphere of 5% CO_2 and 100% humidity. Only oocytes harvested within 2 hours of slaughter (Matás et al, 1996) with a complete dense cumulus oophorus were matured. Groups of 50 oocytes were cultured in 500 μL maturation medium for 20–22 hours at 38.5°C under an atmosphere of 5% CO_2 and 100% humidity. Once cultured, oocytes were washed twice, transferred to fresh maturation medium without hormonal supplements or dibutyryl-cAMP, and cultured for an additional 20–22 hours (Funahashi and Day, 1993).

Sperm Collection and Treatments

Fresh semen was collected from stud boars of known fertility by the gloved-hand method. After collection, the sperm-rich fraction was immediately transported to the laboratory and diluted in Beltsville thawing solution (BTS) at 15°C. Fresh diluted spermatozoa were used on the same day of collection.

Semen samples were also cryopreserved using the straw freezing procedure described by Westendorf et al (1975), with minor modifications as indicated below. Diluted semen was held at 15°C for 2 hours and were later centrifuged at 800 × *g* for 10 minutes. The supernatant was discarded and the semen pellet was resuspended with lactose-egg yolk extender (LEY; 80 mL of 11% lactose and 20 mL egg yolk) to provide 1.5×10^9 spermatozoa/mL. After further cooling to 5°C over a 120-minute period, 2 parts of LEY-extender semen was mixed with a LEY-extender solution containing 1.5% Orvus Es Paste (Equex-Paste; Minitüb, Tiefenbach, Germany) and 9% glycerol. The final semen concentration to be frozen was 1×10^9 spermatozoa/mL in 3% glycerol. The diluted and cooled semen was loaded into 0.5-mL straws (Minitüb), sealed, transferred to a programmable freezer (Icecube 1800; Minitüb), and frozen horizontally in racks. The freezing rate was 1°C/min from 5°C to −4.5°C, holding for 1 minute at −4.5°C, and then 30°C/min from −4.5°C to −180°C. Frozen straws were stored in liquid nitrogen until use.

Thawing of cryopreserved spermatozoa was performed by straw immersion in a 52°C water bath for 11 seconds. Thawed semen was resuspended in BTS at 37°C and centrifuged at 100 × *g* for 10 minutes to remove the residual additive employed for cryopreservation.

The Percoll (Pharmacia, Uppsala, Sweden) sperm cell pretreatment used in all experiments of this study involved layering a 0.5-mL aliquot of semen on a discontinuous 45% and 90% (vol/vol) Percoll gradient (Parrish et al, 1995) and centrifuging at 700 × *g* for 30 minutes. Peletted cells collected from the bottom of the 90% fraction were washed in TALP medium by centrifugation at 100 × *g* for 10 minutes. This second sperm pellet was resuspended in DPBS supplemented with 10% FCS

(Biological Industries, Haemek, Israel) to give a final concentration of 5×10^5 spermatozoa/mL.

The DPBS sperm cell pretreatment used in the second experiment of this study involved the centrifugation of 10 mL of fresh semen at 1200 × *g* for 3 minutes, after which the pellet was resuspended in DPBS supplemented with 10% FCS to reach the final concentration of 5×10^5 spermatozoa/mL.

In the last experiment of this study, the acrosome reaction was induced artificially in sperm samples with a 15-minute incubation period in TALP medium containing 1 μM or 5 μM of calcium ionophore (A23187) at 38.5°C under an atmosphere of 5% CO_2 and 100% humidity. Aliquots of 100 μL from each sperm cell treatment were supplemented with 5 μL fluorescein isothiocyanate–labeled peanut agglutinin (FITC-PNA; 200 μg/mL) and 5 μL propidium iodide (PI; 200 μg/mL) and were maintained at 38°C for 5 minutes and finally fixed with 10 μL paraformaldehyde (1% vol/vol in saline solution). Spermatozoa were then examined under an epifluorescence microscope and divided into 4 categories according to their FITC-PNA/PI staining pattern, as follows: a) live acrosome intact spermatozoa, sperm cells with no FITC-PNA or PI staining; b) live acrosome-reacted spermatozoa, sperm cells with FITC-PNA acrosome staining; c) dead acrosome intact spermatozoa, sperm cells with PI nuclear staining; and d) dead acrosome-reacted spermatozoa, sperm cells with PI nuclear staining and FITC-PNA acrosome staining.

ICSI

Oocytes cultured for 44 hours in maturation medium were mechanically denuded by gentle aspiration with a pipette. Denuded oocytes were washed twice in supplemented DPBS medium and transferred to ICSI drops. ICSI was conducted on a heated microscope at 200× magnification using a Nikon Diaphot 300 inverted microscope with attached micromanipulators. Only fully matured MII oocytes were microinjected. The ICSI medium used was DPBS supplemented with 10% FCS. Prior to ICSI, oocytes were loaded on 4-μL microinjection drops placed onto the lid of a Petri dish (1 oocyte/drop). In total, 10 to 15 microdrops were placed in each lid surrounding central sperm drops, which resulted from a mixture of 4 μL of DPBS-FCS and 1 μL of the sperm suspension. Microdrops were covered with mineral oil (Sigma-Aldrich, M-8410). ICSI was performed as described by Probst and Rath (2003). Briefly, one single sperm was immobilized by crushing the midpiece with the tip of the injection pipette. The immobilized sperm was aspirated with the tail first. Thereafter, the injection pipette was moved into the drop containing the oocytes to be injected. A single oocyte was fixed by the holding pipette, positioning the polar body at the 6 or 12 o'clock position. The injection pipette was pushed through the zona pellucida and subsequently through the oolema into the cytoplasm at the 3 o'clock position. A small amount of ooplasm was aspirated into the injection pipette in order to ensure oocyte membrane penetration. Subsequently, the immobilized spermatozoon was released into the cytoplasm. The temperature was maintained at 38.5°C throughout the procedure using a heated microscopic stage. Injected oocytes were placed in TALP medium.

Zygotes were fixed with acetic alcohol 18–20 hours after microinjection, stained with 1% (wt/vol) lacmoid, and examined at 400× magnification under a phase contrast microscope. Oocytes with 1 pronucleus and 1 sperm inside, either decondensed or not, were classified as ''1PN & 1Sperm.'' Oocytes with 2 pronuclei were classified as ''2PN'' (Figure, a). Oocytes with only 1 pronucleus without sperm inside or with 3 pronuclei were designated as ''other'' (Figure, b).

Embryo Culture

After 18–20 hours in TALP medium, putative zygotes were washed 3 times in NCSU-23 previously equilibrated overnight, transferred to a 4-well Nunc multidish containing 500 μL of NCSU-23 per well, and incubated at 38.5°C under an atmosphere of 5% CO_2 and 100% humidity. At 48 and 144 hours postfertilization, the number of 2-cells (Figure, c) and blastocysts (Figure, d) were evaluated under a stereomicroscope and a sample was fixed and stained. Embryos developing to blastocyst stage were then placed on a slide, air-dried, and fixed in absolute ethanol for 24 hours. After staining with Hoechst 33342 (10 μg/mL in 2.3% sodium citrate), cell nuclei were counted under an epifluorescence microscope.

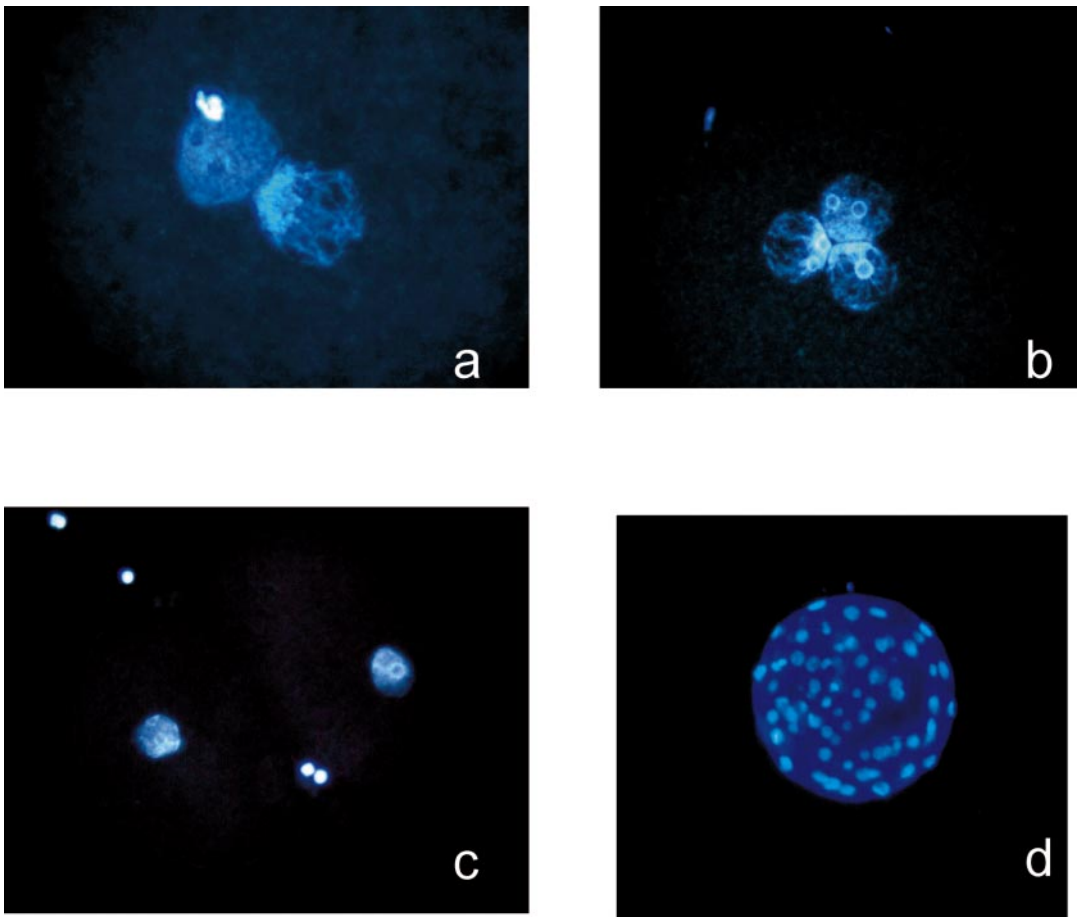
Statistical Analysis

In this study, data are presented as means plus or minus standard error of the mean (SEM) after being fitted to the binomial variable model. In our first and last experiments, data were analyzed by 1-way analysis of variance (ANOVA). In our second experiment, the 2-way ANOVA was used.

Results

The first experiment was designed to evaluate the impact of sperm cryopreservation on ICSI efficiency. For this experiment, only 1 sperm cell donor was used, and semen samples were pretreated by the Percoll gradient method. In a first assay, oocyte activation and fertilization were assessed. This evaluation was carried out in 4 replicates, and a total of 100 and 125 MII oocytes were microinjected with frozen-thawed and fresh spermatozoa, respectively. As shown in Table 1, 85 (68%) of the 125 oocytes microinjected with fresh spermatozoa resumed meiosis. This proportion of activated oocytes was significantly higher than the one obtained with frozen-thawed spermatozoa, in which only 43 (43%) of the 100 microinjected oocytes extruded the second polar body. Lower transition proportions through first mitosis were detected in our second assay with frozen-thawed spermatozoa (Table 2). Only 49 (43%) out of 114 oocytes microinjected with frozen-thawed spermatozoa advanced to the second cell stage, whereas this was accomplished by 70 (63%) of the 110 oocytes microinjected with fresh spermatozoa. In both groups, the number of zygotes that reached blastocyst stage was reduced, and, consequently, significant differences at blastocyst stage were not detected.

In our second experiment, the impact of the sperm treatment (DPBS vs Percoll) and sperm cell donor (2



Pig oocytes following intracytoplasmic sperm injection (ICSI), stained with Hoechst 33342 and observed under an epifluorescence microscope. **(a)** Zygote with 2 pronulcei, 20 hours postinjection; **(b)** Abnormal fertilization, classified as ''others,'' 20 hours postinjection: 3 pronuclei are visible; **(c)** Two-cell stage embryo, fixed at 48 hours postinjection; and **(d)** Blastocyst stage, 144 hours postinjection.

boars were used) on ICSI performance was evaluated. As shown in Table 3, when the ICSI performance was assessed by the in vitro embryo development post-ICSI, a sperm treatment effect, as well as a boar effect, was not detected. However, as shown in Table 4, the DPBS treatment allowed higher cleavage proportions than the Percoll treatment ($P = .0467$). Relative to the observed boar effect, significantly higher blastocyst formation proportions were obtained when ICSI was performed with spermatozoa collected from boar A ($P = .0003$). Independently of the treatment and sperm cell donor used, significant differences in blastocyst cell numbers were not detected.

Table 1. *The impact of intracytoplasmic sperm injection (ICSI) with fresh and frozen spermatozoa on oocyte activation and fertilization**

			Activated†		
Sperm Treatment	Oocyte (N)	Activated† (%)	1PN & 1 Sperm† (%)	2PN† (%)	Other† (%)
Frozen	100	43 (43)[b]	12 (28)	27 (63)	4 (9)
Fresh	125	85 (68)[a]	12 (14)	54 (64)	19 (22)
ANOVA		.0001	.0804	.8335	.0621

* Values with different superscript lowercase letters in the same column are significantly different ($P < .05$).

† Activated indicates oocytes resuming meiosis, with no metaphase plate visible, and with female pronucleus; 1PN & 1Sperm, oocytes with a female pronucleus and 1 sperm either decondensed or not; 2PN, oocytes with 2 visible pronuclei and 1 sperm tail; Other, oocytes with only 1 pronucleus without sperm inside or with 3 pronuclei; and ANOVA, analysis of variance.

Table 2. *The impact of intracytoplasmic sperm injection (ICSI) with fresh and frozen spermatozoa on embryo cleavage, blastocyst formation proportion, and blastocyst cell number**

Sperm Treatment	Oocytes (N)	Cleaved† (%)	Blastocyst‡ (% From N)	Cells/ Blastocyst
Frozen	114	49 (43)[b]	5 (4)	25.2 ± 3.3
Fresh	110	70 (63)[a]	2 (2)	41 ± 6
ANOVA		.0021	.0760	.0561

* Values with different superscript lowercase letters in the same column are significantly different ($P < .05$). ANOVA indicates analysis of variance.

† Proportion of cleavage 48 hours post-ICSI.

‡ Proportion of blastocysts 144 hours post-ICSI.

Table 3. *Effect of sperm treatment and boar on oocyte activation and fertilization after intracytoplasmic sperm injection (ICSI)*

Sperm Treatment	Boar	Oocytes (N)	Activated* (%)	Activated*		
				1PN & 1Sperm (%)	2PN* (%)	Other* (%)
DPBS	A	86	52 (60)	18 (35)	27 (52)	7 (13)
	B	91	62 (68)	11 (18)	42 (67)	9 (15)
Percoll	A	89	48 (54)	13 (27)	26 (54)	9 (19)
	B	87	65 (75)	20 (30)	37 (57)	8 (13)
ANOVA						
Treatment			.9600	.7211	.5857	.7569
Boar			.8308	.2727	.1854	.6400
Treatment × boar			.5245	.1084	.3786	.4317

* Activated indicates oocytes resuming meiosis, with no metaphase plate visible, and with female pronucleus; 1PN & 1Sperm, oocytes with a female pronucleus and 1 sperm either decondensed or not; 2PN, oocytes with 2 visible pronuclei and 1 sperm tail; Other, oocytes with only 1 pronucleus without sperm inside or with 3 pronuclei; DPBS, Dulbecco phosphate-buffered saline; and ANOVA, analysis of variance.

In order to determine whether the injection of acrosome-reacted spermatozoa would benefit our ICSI results, in a third experiment, fresh sperm samples collected from a single donor and treated by the Percoll gradient method were submitted to different concentrations (1 and 5 μM) of A23187, a calcium ionophore capable of artificially inducing acrosome reaction. In a first assay, the proportions of live and dead spermatozoa that retained or did not retain an intact acrosome after a 15-minute incubation with either concentration were determined and compared with unexposed control samples. A total of 2400 sperm cells were analyzed per treatment in 4 replicates. As we can see on Table 5, while the smallest of the concentrations of calcium ionophore enriched a population of live acrosome-reacted sperm cells (1445 out of 2400 treated cells, 60%), the highest concentration enriched a population of dead acrosome-reacted sperm cells (1385 out of 2400 treated cells, 58%). As expected, the majority of control spermatozoa (75%) retained their acrosome vesicle and were alive at the end of the incubation period.

In a subsequent assay we evaluated and compared the impact of both sperm cell treatments on oocyte activation and fertilization after ICSI. In Table 6, the outcomes are presented. In no case were the differences among treatments observed in the proportion of oocyte activation and fertilization significant. Despite these results, an evaluation was conducted of a possible later impact of both sperm cell calcium ionophore treatments on embryo development after ICSI. Again, significant differences were not detected (Table 7), and as in previous experiments, the proportion of blastocyst formation and number of cells per blastocyst were very poor.

Discussion

One of the main problems of the ART in pigs is the insufficient technical standardization and, consequently, the high variability in the results obtained. In order to improve the efficiency of the present ICSI procedure in porcine, this study focused specifically on identifying the best sperm treatment, providing the highest sperm head decondensation, pronuclear formation, oocyte activation

Table 4. *Effect of sperm treatment and boar on embryo cleavage, blastocyst formation proportion, and blastocyst cell number after intracytoplasmic sperm injection (ICSI)**

Sperm Treatment	Boar	Oocytes (N)	Cleaved† (%)	Blastocysts‡ (% From N)	Cells/Blastocyst
DPBS	A	116	74 (64)^{ab}	15 (13)^{a}	29.9 ± 4.8
	B	83	61 (74)^{b}	4 (5)^{ab}	33.7 ± 5.1
Percoll	A	112	62 (55)^{a}	11 (10)^{a}	20.9 ± 2.2
	B	114	72 (63)^{ab}	2 (2)^{b}	41 ± 6
ANOVA					
Treatment			.0467	.4175	.8877
Boar			.0636	.0003	.0651
Treatment × boar			.8401	.8722	.2014

* Values with different superscript lowercase letters in the same column are significantly different ($P < .05$). DPBS indicates Dulbecco phosphate-buffered saline; ANOVA, analysis of variance.

† Proportion of cleavage 48 hours post-ICSI.

‡ Proportion of blastocysts 144 hours post-ICSI.

Table 5. *Acrosomal and live-dead status of spermatozoa after a 15-minute period incubation in TALP medium without (control) and with 1 μM or 5 μM of calcium ionophore*

Ionophore Concentration	Time (min)	Sperm Cells (N)	Live With Acrosome (%)	Live Without Acrosome (%)	Dead With Acrosome (%)	Dead Without Acrosome (%)
Control	0	2400	1788 (75)	79 (3)	271 (11)	264 (11)
1 μM	15	2400	130 (5)	1445 (60)	230 (10)	595 (25)
5 μM	15	2400	43 (2)	605 (25)	368 (15)	1384 (58)

and fertilization, and subsequent development in vitro to blastocyst stage.

In our first experiment, the impact on the ICSI efficiency of fresh and cryopreserved sperm was evaluated and compared. Both types of spermatozoa were washed in a Percoll gradient prior to the fertilization process in order to ensure that only membrane-intact sperm cells were used in the ICSI procedure. We imposed these experimental conditions because our hypothesis was that possible outcome differences should be due to nuclear or cytoplasmic alterations (White, 1993) rather than membrane destabilization processes similar to capacitation (Maxwell and Johnson, 1997), both described during freezing/thawing procedures. Our ICSI results, which show a proportion of oocytes developing at least 1 pronucleus with fresh spermatozoa that was significantly higher than the proportion obtained with frozen-thawed spermatozoa, indicate that sperm cryopreservation in porcine induces cytoplasmic/nuclear alterations that compromise oocyte activation. By interpreting these results, taking into account previous reports in porcine describing a cytosolic sperm factor as the main factor responsible for oocyte activation (Macháty et al, 2000), it seems plausible that only spermatozoa without alterations in this factor could induce oocyte activation correctly. Cryopreservation and temperature shock may indeed denature this crucial factor for meiosis resumption. The lower cleavage proportion observed after ICSI with frozen-thawed spermatozoa indicates that sperm cryopreservation may also affect other nuclear/cytoplasmic factors required for transition through first mitosis. These factors could include 1) the sperm chromatin, leading to DNA fragmentation and failed fertilization and embryo cleavage, and 2) the sperm centriole, also leading to failed fertilization and embryo cleavage. With regard to sperm DNA fragmentation, several studies have reported that cryopreservation could result in increased sperm DNA fragmentation levels (Baumber et al, 2003; Martin et al, 2004). With regard to the sperm centriole, previous reports have suggested potential damage of the centriole after sperm cryopreservation (Billard, 1983; Kim et al, 2002).

These results confirm previous reports (Kolbe and Holtz, 1999) in which the injection of fresh ejaculated and frozen-thawed epididymal spermatozoa was compared and in which higher cleavage proportions were observed in the first case. In practical terms, our porcine ICSI results indicate that fresh spermatozoa provide better results than frozen spermatozoa. The use of fresh sperm cells will, however, not replace the use of frozen-thawed spermatozoa from gamete banks in laboratories that depend on sperm availability. Our porcine ICSI results also highlight important species differences, since they are in complete contrast with the results observed in rodent species, in which oocyte activation and progression through first mitosis are not compromised by the sperm freezing procedures frequently used before ICSI.

Sperm preparations for ICSI differ among laboratories. In our second experiment, we compared 2 sperm-washing and selection procedures frequently used. The first procedure comprised sperm wash and resuspension in DPBS medium supplemented with FCS, and the second com-

Table 6. *Effect of spermatozoa coincubation with calcium ionophore before intracytoplasmic sperm injection (ICSI) on oocyte activation and fertilization*

Sperm	Oocytes (N)	Activated* (%)	1PN & 1Sperm* (%)	2PN* (%)	Other* (%)
Control	76	51 (67)	11 (22)	38 (75)	2 (3)
1 μM	86	59 (69)	7 (12)	50 (85)	2 (3)
5 μM	64	40 (63)	6 (15)	32 (80)	2 (5)
ANOVA		.6801	.3808	.4135	.729

* Activated indicates oocytes resuming meiosis, with no metaphase plate visible, and with female pronucleus; IPN & 1Sperm, oocytes with a female pronucleus and 1 sperm either decondensed or not; 2PN, oocytes with 2 visible pronuclei pronuclei and 1 sperm tail; Other, oocytes with only 1 pronucleus without sperm inside or with 3 pronuclei; and ANOVA, analysis of variance.

Table 7. *Effect of spermatozoa coincubation with calcium ionophore before intracytoplasmic sperm injection (ICSI) on embryo cleavage, blastocyst formation proportion, and blastocyst cell number*

Sperm	Oocytes (N)	Cleaved* (%)	Blastocysts† (% From N)	Cells/ Blastocyst
Control	59	36 (61)	1 (2)	24
1 μM	59	38 (64)	5 (8)	25.6 ± 9.5
5 μM	63	41 (65)	5 (8)	25.6 ± 9.5
ANOVA		.886	.2500	.8814

* Proportion of cleavage 48 hours post-ICSI.
† Proportion of blastocysts 144 hours post-ICSI.

prised centrifugation through a Percoll gradient. The results obtained showed no significant differences in oocyte activation and fertilization proportions post-ICSI between treatments, and these proportions were similar to those previously reported under different conditions (Martin, 2000; Wu et al, 2001; Katayama et al, 2002b). However, when embryo in vitro development after ICSI was evaluated, a sperm treatment effect on the cleavage proportion and a strong boar effect on the proportion of blastocyst formation were detected. The reduced progression through first mitosis after ICSI with spermatozoa submitted to a Percoll treatment seems to indicate that in porcine, fewer embryo toxicity problems develop in response to ICSI when it is performed with DPBS-washed and selected sperm. In addition, the DPBS sperm-washing procedure requires less labor and is less expensive than the Percoll sperm treatment. The much higher proportion of blastocyst formation obtained with the sperm cells collected from boar A highlights the importance of careful selection of the sperm cell donor before ICSI in porcine. We considered it important to perform this evaluation, since we could not find any ICSI reference in pigs assessing this possible boar effect known in conventional IVF (Gadea and Matás, 2000). These results confirm previous evidence that regardless of the fertilizing ability of a particular sperm sample, the genetic background of the sperm cell donor modulates, to a great extent, the efficiency of ART.

In our third experiment, we tried to evaluate the impact on ICSI efficiency of the use of acrosome-reacted and non–acrosome reacted spermatozoa. The acrosome vesicle of a sperm cell has different enzymes, which can damage the oocyte when introduced in the ooplasm during ICSI (Tesarik and Mendoza, 1999). This does not happen in IVF, because only the acrosome-reacted sperm cells are able to fertilize. Some authors have observed that oocytes injected with acrosome-intact spermatozoa delay the onset of male chromatin decondensation and male pronucleus formation (Katayama et al, 2002a). We hypothesized that sperm pretreatment with calcium ionophore, an acrosome reaction inductor, could have a positive effect on ICSI outcomes. Two calcium ionophore concentrations were tested, enriching two significantly different sperm cell populations. The smallest of the concentrations enriched a population of live acrosome-reacted sperm cells and the highest concentration, a population of dead acrosome-reacted spermatozoa. However, neither of these 2 sperm cell populations were able to confirm our hypothesis. Differences in oocyte activation, pronuclear formation, and embryo development were not found between them or in relation to untreated control sperm samples after fertilization by ICSI. These results are in agreement with previous attempts in the porcine species, which used sperm heads from epididymal frozen-thawed spermatozoa (Nakai et al, 2003). However, in bovine (Goto et al, 1990), mouse (Lachan-Kaplan and Trounson, 1995), and sheep (Gómez et al, 1997), it was observed that the acrosome reaction or membrane alterations previous to ICSI were necessary to facilitate sperm head decondensation and fertilization. It is possible that porcine oocytes have a specific tolerance to acrosomal contents of the same species, as has been proposed (Sathananthan et al, 1997; Kimura et al, 1998). Considering all reported information and our experimental results, we conclude that the induction of the acrosome reaction before ICSI is beneficial depending on the species, but is probably not important in porcine. Moreover, from this experiment we also could observe that neither the live nor dead status of the spermatozoa affects the efficiency of ICSI in porcine. In particular, porcine behave like rodent species, in which it was shown that dead frozen and dehydrated sperm can be used successfully to generate offspring by ICSI (Wakayama et al, 1998).

In conclusion, the results of this study show that standardization and simplification of the ICSI procedure in pigs can be achieved by using fresh spermatozoa pretreated and selected with a single washing procedure in DPBS supplemented with FCS. They also indicate that sperm cell donor selection should be carefully performed for improved results. In addition, the results of this study also indicate that the acrosome reaction and live-dead status of the sperm cells used are parameters that do not affect the efficiency of the ICSI procedure in pigs. However, the relatively low percentage of blastocyst formation observed, although fertilization and cleavage proportions were always higher than 40%, underlines the problem that porcine ICSI zygotes still have limited ability to develop within current in vitro culture systems.

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