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Viability, acrosome morphology and fertilizing capacity of boar spermatozoa treated with strontium chloride

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Summary

The positive effect of strontium ions (Sr^{2+}) on sperm motility, capacitation and acrosome reaction has been demonstrated in the mouse, human, guinea pig and hamster. In the present study, we have evaluated the effect of Sr^{2+} on the viability and acrosome morphology of boar spermatozoa, and on the fertilization and development after the microinjection of Sr^{2+}-treated spermatozoa into porcine oocytes. Before incubation, 79% of spermatozoa were classified as propidium iodide (PI)-negative (live) using the LIVE/DEAD Sperm Viability Kit. After incubation with strontium chloride ($SrCl_2$), 39% (0 mM; no divalent cations), 25% (1.9 mM) and 24% (7.5 mM) of them were classified as PI-negative. The proportion of spermatozoa that had initiated the acrosome reaction was higher in Sr^{2+}-containing medium than in Sr^{2+}-free medium, when assessed by electron microscopy. There was no significant difference in percentage of spermatozoa initiating the acrosome reaction between Sr^{2+}-treated groups (1.9 mM: 22%, 7.5 mM: 33%, $p > 0.05$). After the microinjection of spermatozoa incubated with $SrCl_2$, 67% (1.9 mM) and 61% (7.5 mM) of injected oocytes were successfully fertilized, and then 43% (1.9 mM) and 41% (7.5 mM) contained a fully decondensed sperm head. Sham-injected oocytes were significantly activated at a lower rate than Sr^{2+}-treated groups (27%, $p < 0.05$). Next, after microinjection of spermatozoa incubated with 1.9 mM $SrCl_2$ ($n = 51$), 45% of injected oocytes cleaved on day 2, and 18% developed to blastocysts on day 7 (sham-injection, $n = 48$: 15% to cleavage and 0% to blastocyst). These results demonstrate that Sr^{2+} is likely to positively affect the fertilizing capacity of spermatozoa in the pig.

Keywords: Acrosome reaction, ICSI, Pig, Strontium, Viability

Introduction

Strontium (Sr^{2+}) treatment is well known as very effective in activation of the oocytes and thus used practically after nuclear transfer or microinjection of specific-stage spermatozoa such as round spermatids that are deficient or insufficient in their ability to activate oocytes. It has been reported that Sr^{2+} supports and/or positively affects the sperm-related physiological and morphological responses, such as the capacitation, acrosome reaction, hyper-activation, and penetration into the oocyte in the guinea pig, hamster, human, and mouse (Yanagimachi & Usui, 1974; Yanagimachi, 1978; Mortimer, 1986; Fraser, 1987; Mortimer *et al*., 1988; Stock & Fraser, 1989; Magnus *et al*., 1990), when substituted for calcium ions in the medium. There is, however, no report of how Sr^{2+} affects spermatozoa of domestic animals including the pig.

The pig would be an excellent model for certain human biomedical approaches, i.e. xenotransplantation, stem cell technologies, and so on (Prather *et al*., 2003). Of these, embryonic stem cell (ESc) technologies would be an important step to achieve the above purpose. Such research, however, requires a great number of high-quality embryos (i.e. blastocysts). For this purpose, *in vitro* production of embryos using *in vitro* fertilization

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is a convenient way for most laboratories. There are very few satisfactory protocols for *in vitro* production of embryos in this species, because of problems associated with polyspermy. For this reason, intracytoplasmic sperm injection (ICSI) that can result in monospermic fertilization is expected as a reliable and extensive application. Moreover, the ICSI procedure excludes some polyspermic oocytes that develop into blastocysts with abnormal chromosomes (McCauley *et al*., 2003). As reported thus far, the efficiently of ICSI in the pig is still not satisfactory and this approach requires certain improvements.

In the present study, we investigated whether Sr^{2+} affects the boar spermatozoa as in other species, i.e. the viability and acrosome morphology of spermatozoa incubated with Sr^{2+} were examined using the fluorescence staining and electron microscopy. Second, the spermatozoa treated with Sr^{2+} were injected into porcine oocytes matured *in vitro*, and the fertilization, cleavage, and subsequent development following sperm injection were examined.

Materials and methods

All chemicals and reagents were purchased from Sigma-Aldrich unless stated otherwise. For electron microscopy, all chemicals were purchased from Electron Microscopy Sciences, unless otherwise stated.

Sperm preparation

Sperm preparation was performed as described previously (Okada *et al*., 2006). The commercial extended boar semen (stored maximally for up to 5 days at 17–18 °C; Selko) was centrifuged at 210 *g* for 10 min. After centrifugation, the sperm pellet was loaded with some volume of extended medium onto a discontinuous gradient of 2 ml of 90% and 5 ml of 45% isotonic Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) prepared with Dulbecco's phosphate-buffered saline (PBS) in a 15 ml centrifugation tube and then centrifuged at 640 *g* for 15 min. The spermatozoa were recovered and then washed twice in PBS containing 0.01% polyvinyl alcohol (PBS-PVA) by centrifugation at 210 *g* for 10 min. The sperm pellet was resuspended in 0.5–1 ml of Ca^{2+}-free modified Tris-buffered medium (mTBM: 113.1 mM NaCl, 3 mM KCl, 20 mM Tris, 11 mM glucose, and 5 mM sodium pyruvate supplemented with 2 mg/ml BSA). The sperm suspension was diluted with 0.5 ml of each experimental medium to give a final concentration of 2×10^6 cells/ml, and spermatozoa were incubated for 1–2.5 h before use (see results).

Evaluation of sperm viability

The viability of boar spermatozoa was evaluated according to the method of Harayama (2003) using the LIVE/DEAD Sperm Viability Kit (Molecular Probes, Inc.). Briefly, after incubation with Sr^{2+} or Ca^{2+} for 1–2.5 h, 500 µl of sperm suspension (concentration: 2×10^6 cells/ml) was stained with 0.2 µM SYBR14 for 10 min and subsequently with 12 µM propidium iodide (PI) for 10 min at 38.5 °C. After staining, the suspension was centrifuged at 700 *g* for 5 min and the supernatant was removed to concentrate the cells (approximately 2×10^7 cells/ml). The evaluation was carried out under an epifluorescence microscope (BX61: Olympus Europa GMBH, Hamburg, Germany). One hundred spermatozoa from each sample were counted to determine the percentage of PI-negative (live) and positive (damaged or dead) spermatozoa.

Transmission electron microscopy

After incubation with Sr^{2+} or Ca^{2+} for 1 h, each sample of spermatozoa was fixed in 2.5% glutaraldehyde (pH 7.2) for 2 h and post-fixed in 1% OsO_4 containing 0.1 M cacodylate buffer for 60 min. The samples were then dehydrated through ethanol series, infiltrated in propylene oxide and embedded in epoxy resin (Durcupan® ACM Fluka). Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate. Electron micrographs were taken on a Zeiss EM-900 electron microscope (Carl Zeiss). One hundred sperm heads were assessed in each sample. According to Szollosi & Hunter (1978) and Stock & Fraser (1989), each cell was classified into one of the following four stages: Stage 1, intact acrosome: spermatozoa with the plasma membrane separated from the acrosome were classified as intact spermatozoa, as the membrane separation might be an artifact (Jones, 1973); Stage 2, initial stage of acrosome reaction (slight swelling of acrosome); Stage 3, advanced stage of acrosome reaction (swelling of acrosome and lacking of the acrosome contents); Stage 4, completed stage of acrosome reaction (exposed inner acrosome membrane except in the equatorial segment).

Oocyte collection and maturation

Pig ovaries were collected from prepubertal gilts at a local abattoir and transported to the laboratory in PBS-PVA at 30–35 °C. After three washes in PBS-PVA, healthy looking antral follicles that were 4–6 mm in diameter were dissected from ovaries using the technique described by Moor & Trounson (1977). The follicles were opened in HTF–HEPES medium (Cambrex Bio Science) supplemented with 2 mg/ml bovine serum albumin (BSA; essentially fatty acid free) and 50 µg/ml gentamicin (Gibco Invitrogen), and oocyte-cumulus-granulosa cell complexes (OCGCs) were isolated from follicles. Groups of 20–35 OCGCs were cultured in 500 µl of bicarbonate-buffered medium 199 supplemented with 4 mg/ml of bovine serum growth proteins (Sevapharma), 0.5 µg/ml FSH

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(from porcine pituitary), 0.5 μg/ml LH (from ovine pituitary), 40 μg/ml sodium pyruvate, 70 μg/ml L-cysteine, and 50 μg/ml gentamicin in 4-well dishes (Nunc) (Krylov *et al*., 2005). The OCGCs were cultured in a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 44–48 h. After culture, OCGCs were recovered and treated with 0.01% hyaluronidase to remove the cumulus cells. The oocytes were then denuded completely by pipetting with a small-bore pipette. Oocytes that showed a normal morphology with a polar body were selected and used for sperm injection.

Sperm injection and examination of fertilization

Microinjection was performed under an inverted microscope (IX71, Olympus Europa GMBH) equipped with Narishige micromanipulators (Narishige Co. Ltd) and the Cell Tram Oil microinjector (Eppendorf AG). For the manipulation, two 3 μl drops of Sydney IVF polyvinylpyrrolidone solution (10% PVP; COOK Australia) were placed on the lid of a plastic dish, and a 20 μl drop (for washing pipette) and several 10 μl drops (for injection) of HTF–HEPES medium surrounded the PVP drops. All drops were covered with paraffin oil (Carl Roth). Three microlitres of sperm suspension were then transferred into a PVP drop (final concentration of spermatozoa and PVP: 1×10^6 cells/ml and 5%, respectively). Ten to 15 oocytes were transferred to the medium drops and their manipulation was completed within 20 min. For sperm injection, a single motile spermatozoon was immobilized by hitting the midpiece with the injection pipette and the spermatozoon was then aspirated, tail first, into the pipette in a PVP drop. The pipette was moved to a drop of the HTF–HEPES medium, and the spermatozoon was microinjected conventionally into the oocyte (pipettes; Microtech IVF). Sperm injection was performed as described previously by Kolbe & Holtz (1999) and Katayama *et al*. (2005). As a control in all experiments, some oocytes were sham-injected using the same procedure but without a spermatozoon.

The injected oocytes were cultured in porcine zygote medium supplemented with 3 mg/ml BSA (PZM3: Yoshioka *et al*., 2002) for 18–20 h. Oocytes were then mounted on slides, fixed in an acetic acid–ethanol (1:3, v/v) solution for 36–48 h, and stained with 1% aceto-orcein. The rates of fertilization and the status of the sperm head were examined. Oocytes were defined as fertilized normally when they contained a fully decondensed male and female pronucleus with two polar bodies.

Development of injected oocytes

The injected oocytes were cultured as described previously (Okada *et al*., 2006). Briefly, after injection, any oocytes with signs of degeneration were excluded from subsequent culture and the remaining oocytes were cultured in 500 μl of PZM3 for 7 days. Embryos were transferred into a fresh medium on days 2 and 4. From days 4 to 7, PZM3 supplemented with 10% fetal bovine serum (Gibco Invitrogen), instead of BSA, was used. Cleavage of embryos and development to the blastocyst stage were evaluated on days 2 and 7, respectively. After culture, blastocysts were fixed in PBS–PVA containing 4% paraformaldehyde, stained with SlowFader Gold antifade reagent with DAPI (Molecular Probes Inc.) and the number of nuclei was counted under an epifluorescence microscope. In the present study, blastocysts with a cell number of more than 20 were considered normal.

Statistical analysis

The data shown in the tables were pooled from at least three experiments. Data for sperm assessments were analysed by one-way analysis of variance (ANOVA) and Tukey's test as a multiple comparison procedure using the Statcel program (OMS Publishing Inc.). Data for the fertilization and development after ICSI were analysed using the chi-squared test. A p value < 0.05 was considered statistically significant.

Results

Sperm viability and acrosome morphology after incubation with divalent cations

Before incubation, 79% of spermatozoa were classified as PI-negative (live) (Fig. 1, control). After incubation for 1–2.5 h in mTBM supplemented with 0 (no divalent cations), 1.9, and 7.5 mM $SrCl_2$, or 1.9 and 7.5 mM calcium chloride ($CaCl_2$), 39%, 25%, 25%, 24%, and 24% of incubated spermatozoa were classified as

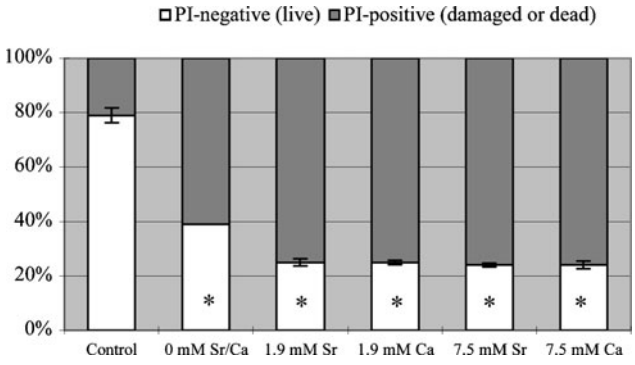

Figure 1 The viability of boar spermatozoa before and after incubation with divalent cations. In total, 300 spermatozoa were examined in each sample ($n = 3$). Propidium iodide (PI)-negative and -positive indicate live and damaged/dead spermatozoa, respectively. Control: non-incubated spermatozoa. Values are mean % ± SEM. $^*p < 0.05$ compared with control.

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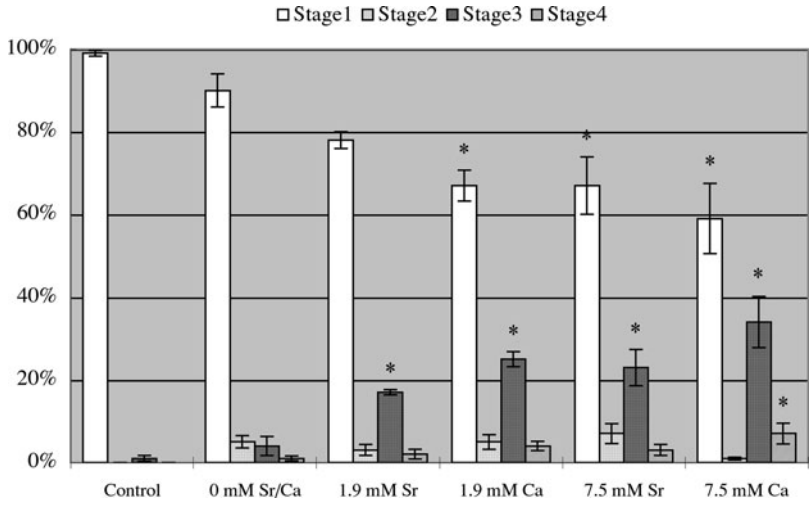

Figure 2 Transmission electron microscopy of the acrosome status of boar spermatozoa before and after incubation with divalent cations. In total, 300 spermatozoa were examined in each sample ($n = 3$). Stage 1: intact acrosome; Stage 2: initial stage of acrosome reaction; Stage 3: advanced stage of acrosome reaction; Stage 4: completed stage of acrosome reaction; Control: non-incubated spermatozoa. For a description and images of each stage, see Materials and methods, and Fig. 3, respectively. Values are mean % ± SEM. $^*p < 0.05$ compared with control.

PI-negative ($p > 0.05$), respectively. These values were significantly lower than the control ($p < 0.05$).

The occurrence of acrosome reaction evaluated by transmission electron microscopy (TEM) is summarized in Fig. 2. The total proportion of spermatozoa that had initiated the acrosome reaction (Fig. 3*b–d*: Stages 2–4) was lower in cation-free medium (0 mM, 10%) than in Sr^{2+}- or Ca^{2+}-containing medium after incubation. There was no significant difference in percentage of spermatozoa initiating the acrosome reaction (Stages 2–4) between divalent cations-treated groups (1.9 and 7.5 mM $SrCl_2$, 22% and 33%; 1.9 and 7.5 mM $CaCl_2$, 33% and 41%, respectively). When comparing the use of Sr^{2+} with Ca^{2+} at the same concentration, Ca^{2+} tended to promote the response for Stage 3 (1.9 mM $SrCl_2$ and $CaCl_2$, 17 and 25%; 7.5 mM $SrCl_2$ and $CaCl_2$, 23 and 34%, respectively) and Stage 4 (2 and 4%; 3 and 7%, respectively).

Microinjection of boar spermatozoa incubated with Sr^{2+} and their subsequent development

The spermatozoa incubated with Sr^{2+} were injected conventionally. Fifty (sham-injected control), 59 (Sr^{2+} 1.9 mM), and 52 (Sr^{2+} 7.5 mM) oocytes were injected and subsequently 48 (96%), 54 (92%), and 46 (89%) oocytes, respectively, survived. Following the injection of spermatozoa incubated with 1.9 or 7.5 mM $SrCl_2$, 67% and 61% of injected oocytes were fertilized, and subsequently 43% and 41%, respectively, contained a fully decondensed sperm head (Table 1). Sham-injected oocytes were activated at a significantly lower rate than the Sr^{2+}-treated groups (27%, $p < 0.05$). Thus, the 1.9 mM $SrCl_2$-treated group was chosen for the examination of development followed by ICSI.

After injection of spermatozoa treated with 1.9 mM $SrCl_2$, oocytes were cultured in PZM3 up

Table 1 Microinjection of the boar spermatozoa incubated with $SrCl_2$

Treatment	No. of oocytes examined	No. (%) of oocytes surviving after injection	No. (%) of oocytes fertilized[c]			
			Total[b]	FP + intact sperm head	FP + decondensing sperm head	FP + fully decondensed sperm head (MP)
Sham-injection[a]	50	48 (96)	13 (27)[d]	–	–	–
1.9 mM	59	54 (92)	36 (67)[e]	0 (0)	13 (22)	23 (43)
7.5 mM	52	46 (89)	28 (61)[e]	2 (4)	7 (15)	19 (41)

[a]Oocytes were injected in the same manner but without a spermatozoon.
[b]Value of sham-injection is the number of oocytes activated.
[c]Percentage value represents the number of oocytes that survived. FP: fully developed female pronucleus, MP: fully developed male pronucleus.
[d,e]Values within a column with different superscripts are significantly different ($p < 0.05$).

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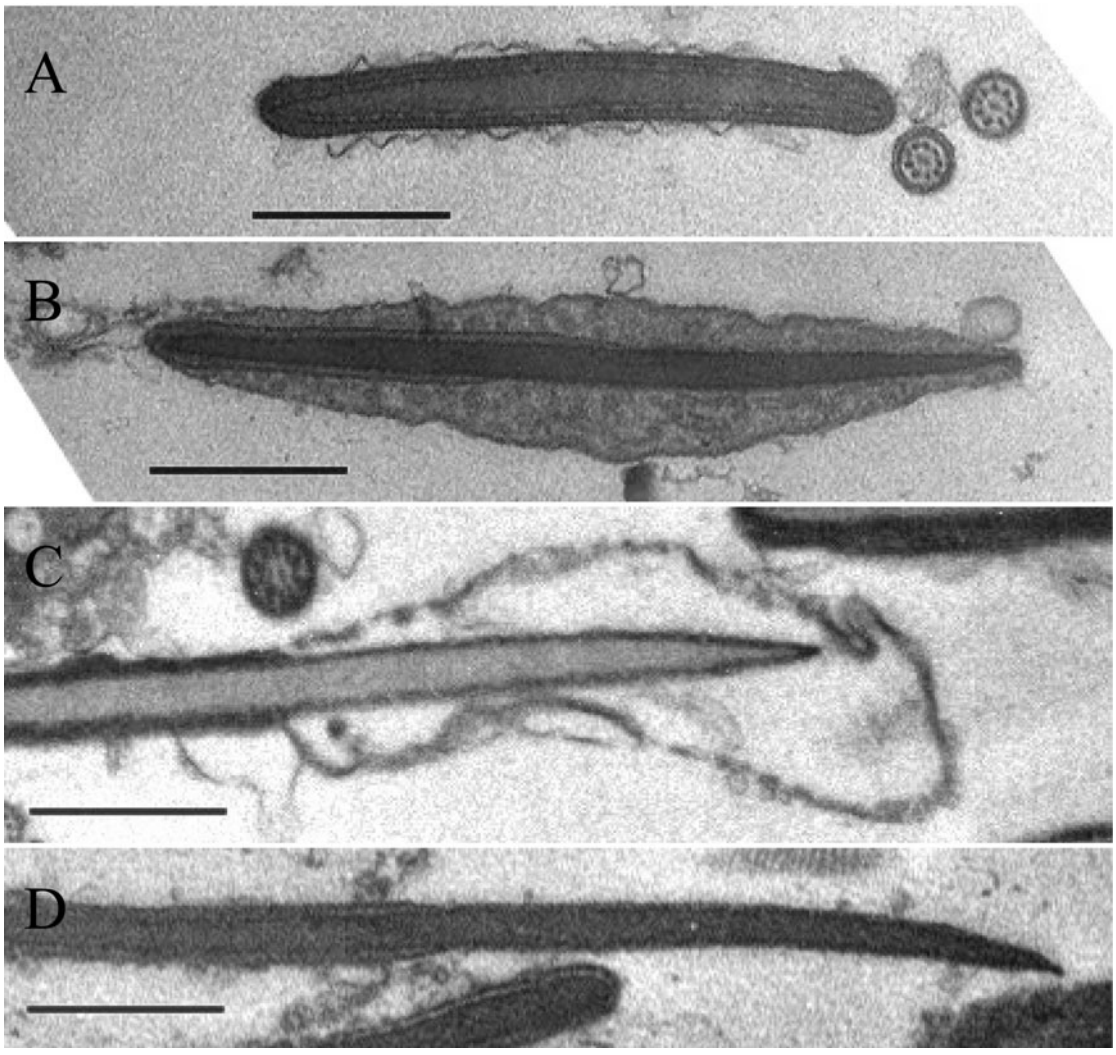

Figure 3 Transmission electron micrographs of extended boar spermatozoa before (*a*) and after incubation with 7.5 mM $SrCl_2$ (*b–d*) at different stages of acrosome reaction. (a) Stage 1: spermatozoa with intact acrosome; (*b*) Stage 2: initial stage of acrosome reaction; (*c*) Stage 3: advanced stage of acrosome reaction; (*d*) Stage 4: completed stage of acrosome reaction. Scale bars represent 1 μm. For the description of each stage, see Materials and methods.

Table 2 *In vitro* development of the oocytes injected with boar spermatozoon incubated with 1.9 mM $SrCl_2$

Treatment	No. of oocytes examined	No. (%) of embryos[b]			No. of blastocysts examined	Average cell no. of blastocysts[d]	Maximum cell no. of blastocyst
		Cleaved	Developed to blastocysts				
			Total	Hatching[c]			
Sham-injection[a]	48	7 (15)[e]	0 (0)	0 (0)	0	–	–
ICSI	51	23 (45)[f]	9 (18)	4 (8)	9	53 ± 7	83

[a]Oocytes were injected in the same manner but without a spermatozoon and then cultured.
[b]Cleavage of embryos and development to the blastocyst stage were evaluated on days 2 and 7, respectively.
[c]Others represent normal or expanding blastocysts.
[d]Mean ± SEM.
[e,f]Values within a column with different superscripts are significantly different ($p < 0.05$).

to 7 days. On day 2 after sperm injection, 45% of the injected oocytes cleaved (Table 2). These injected oocytes cleaved at a significantly higher rate than sham-injected oocytes (15%, $p < 0.05$). After 7 days, 18% (mean cell number: 53 ± 7) of the injected oocytes developed to the blastocyst stage. None of

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sham-injected oocytes developed to the blastocyst stage.

Discussion

In mammalian spermatozoa studied thus far, the presence of Sr^{2+} in Ca^{2+}-free medium supports sperm-related responses such as capacitation, acrosome reaction, penetration, and so on. It has been reported that when human spermatozoa were incubated in Sr^{2+}-containing medium for 20 h and then co-cultured with zona-free hamster eggs for 2–3 h, the penetration rate was significantly higher than with Ca^{2+} supplementation (Mortimer, 1986). Our experiments show that Sr^{2+} itself acts on boar spermatozoa in a manner similar to Ca^{2+}, as the number of living spermatozoa incubated in divalent cation-free medium (PI-negative, 39%) tended to be higher than for the Sr^{2+}-treated groups ($p > 0.05$). The results of our TEM study confirmed that the presence of divalent cations (7.5 mM $SrCl_2$, 1.9 and 7.5 mM $CaCl_2$) resulted in a significantly higher proportion of boar spermatozoa that initiate and/or undergo the acrosome reaction after the incubation than control group. Regarding the possibility that Sr^{2+} might be able to support the acrosome reaction, we noted variable results depending on the concentration of divalent cations. Our data provide the evidence that the percentage of acrosome reacting/reacted cells (Stages 2–4) was not significantly different between the groups treated with divalent cations, with the exception of 1.9 mM $SrCl_2$- and 7.5 mM $CaCl_2$-treated groups at Stage 3 (17% and 34%, respectively, $p < 0.05$). These results are in agreement with TEM observations in human spermatozoa (Stock & Fraser, 1989), who found that the proportion of acrosome-reacted cells in 1.8 mM $SrCl_2$ was slightly lower (6 h, 9.6%; 24 h, 12.5%) than that in 1.8 mM $CaCl_2$ (6 h, 12%; 24 h, 14%). Our result using a higher concentration (7.5 mM) of divalent cations shows that the percentage of acrosome reacting/reacted cells (Stages 2–4) in the Sr^{2+}-treated group (33%) was lower than in the Ca^{2+}-treated group (41%), although there was no significant difference between these groups. In the mouse, Sr^{2+} induces capacitation and acrosome loss efficiently with the same effectivity as Ca^{2+} (Fraser, 1987). Thus, the Sr^{2+} sensitivity of spermatozoa may be different among species, as mouse oocytes can be more effectively activated by Sr^{2+} than oocytes from other species. Although it is unclear how Sr^{2+} acts on mammalian spermatozoa (and oocytes also), it has been reported recently that the action of Sr^{2+} to induce Ca^{2+} oscillation is mediated through inositol 1,4,5-trisphosphate (InsP3) receptors. The activation of phospholipase C (PLC) is included in these processes in mouse oocytes (Zhang *et al*., 2005). On the other hand, the existence of a PLC-InsP3 receptor-signalling pathway has been reported, which generates an increase in intracellular free calcium ions in boar spermatozoa (Harayama *et al*., 2005). Taken together, Sr^{2+} may act on boar spermatozoa through such a signalling pathway to induce the capacitation and acrosome reaction.

The outcome of ICSI in the pig differs among research groups with approximately half of injected oocytes having a maternal and paternal pronucleus [52% (Kim *et al*., 1998); 31% (Katayama *et al*., 2002); 46.7% (Yong *et al*., 2003); 47.1% (Lee *et al*., 2003); and 64% (here '%' is from activated oocytes, Garcia-Rosello *et al*., 2006)], when ejaculated or cryopreserved spermatozoa were used with no artificial activation after injection. Values for the pretreatment of spermatozoa, however, differ among research groups. Thus, for example, a higher percentage of fully decondensed sperm head was achieved when progesterone was used for pretreatment of spermatozoa (64%; Katayama *et al*., 2002). In our study, 43% (1.9 mM) and 41% (7.5 mM) of oocytes injected with Sr^{2+}-treated spermatozoa contained fully decondensed sperm heads, indicating that Sr^{2+} has no detrimental effect on boar spermatozoa during their preincubation. Piezo-actuated manipulation might be powerful tool for the improvement of the ICSI procedure in boar, as it has been reported that fertilization following sperm injection was increased when compared with the conventional method in mouse, rat, human and bovine (Kimura & Yanagimachi, 1995; Dozortsev *et al*., 1998; Katayose *et al*., 1999; Yanagida *et al*., 1999). Katayama *et al*. (2005) have also demonstrated that the immobilization of boar spermatozoa by piezo-pulses enhances male pronucleus formation when compared with immobilization without piezo-pulse.

Unfortunately, development to the blastocyst stage following ICSI with no artificial activation is often low, as reported previously in pigs (4.6%, Lai *et al*., 2001; 17.5%, Yong *et al*., 2003; 8%, Lee *et al*., 2003; 4%, Garcia-Rosello *et al*., 2006), although early cleavage rates were relatively high (41.4%–63.3%). Conversely, Kim *et al*. (1998) have reported that 38% of injected oocytes formed blastocysts. In the present study, when Sr^{2+}-treated spermatozoa were microinjected, we found that both the rate of cleavage and the rate of development to blastocysts was be similar to that of the above-mentioned results (44% and 18%, respectively), as the use of Sr^{2+} may lead to a series of favourable sperm physiological responses during preincubation.

In conclusion, the present study is the first to report in the pig that $SrCl_2$ can affect sperm viability and induce a change in acrosome morphology. The study indicates

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that Sr^{2+} probably has a positive effect on the fertilizing capacity of boar spermatozoa.

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