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Analysis of different factors influencing the intracytoplasmic sperm injection (ICSI) yield in pigs

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Abstract

Intracytoplasmic sperm injection (ICSI) in pigs is a technique with potential application in diverse fields of animal production and biomedicine. Even though there are some cases of live offspring resulting from this technique, its yield is still quite low compared to other species. The aim of this study was to evaluate different factors affecting the ICSI performance. This was done by studying (1) the sequence of culture media for the oocytes after injection; (2) modifications in the *in vitro* maturation system (IVM) through meiotic inhibitors such as roscovitine, and changes in the IVM time; (3) oocyte activation through injection of inositol triphosphate ($InsP_3$) together with the sperm. *In vitro* matured oocytes were employed. All the ICSI experiments were performed with fresh ejaculated semen. Results showed that porcine ICSI zygotes give an improved proportion of two-cell embryos using the sequence IVF medium-embryo culture medium (NCSU-23) rather than transferring directly to NCSU-23. Pronuclear formation ability was not affected by prematuration, but a faster embryo development was observed in roscovitine treated oocytes. In relation to IVM times, oocytes matured for 36 h can achieve better fertilization percentages than oocytes matured for 44 h. These results were independent of the roscovitine treatment. Finally, no influence on embryo development was observed until the blastocyst stage with the use of the $InsP_3$ as an exogenous activating factor.
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Keywords: ICSI; Oocyte activation; Embryo culture; Roscovitine; *In vitro* maturation; Pig; Gamete biology; Reproductive technology; *In vitro* fertilization; Early development; Embryo transfer; Roscovitine; $InsP_3$

1. Introduction

Currently, there is considerable interest in generating large numbers of pig oocytes by *in vitro* maturation techniques and embryos by *in vitro* fertilization (IVF), to stimulate progress in both basic and biomedical research. The pig has become increasingly important as a potential organ donor for xenotransplants, as well as for using transgenic animals to produce specific proteins, based upon biological similarities with man. Attempts at cloning and producing transgenic pigs by means of pronuclear injections require mature oocytes and zygotes. However, obtaining oocytes and embryos from donor animals surgically is an expensive procedure, is time-consuming, and the quantity of biological material obtained is limited. Hence the effective use of ovaries from the abattoir to produce mature oocytes and embryos by means of *in vitro* techniques is crucial [1].

The overall objective of this study was to increase the effectiveness of *in vitro* embryo production in pigs, which scarcely reaches 15% with the present-day

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methodology [2]. The factors affecting this process are the following: complications associated with the *in vitro* maturation system, polyspermy, and inadequate embryo culture conditions. The polyspermy problem is solved by using the ICSI technique, since only one sperm is placed inside the oocyte. However, the already low blastocyst proportion obtained through traditional IVF after embryo culture [3], drops drastically after ICSI [4]. The reasons behind this failure that could be related to the oocyte, to the sperm or to the zygote development, need to be investigated.

With regard to sperm factors, the sperm together with all of its membranes is placed inside the oocyte during the ICSI technique, which does not occur in physiological fertilization. This aspect was previously studied in our laboratory when fresh semen *versus* cryo-preserved, acrosome reacted *versus* non-reacted and Percoll treated *versus* non-treated were used, and the differences observed mainly concerned both the boar and the preservation method used. However, the blastocyst rate only increased for one of the boars used [5].

Therefore the focus in this study was on both the oocyte and zygote, with the intention of increasing their developmental capacity. The first step was to optimize the sequence of the *in vitro* fertilization and embryo culture (IVF, EC) media that are employed in most of the laboratories. Pronuclear formation is produced in the TALP medium during traditional IVF [6,7] and it has been also shown that it takes place during the first 6 h after ICSI [8]. The fact that the zygotes are present in the IVF medium at this time, which is enriched with calcium, pyruvate and lactate, might facilitate the pronuclear formation, as could the early stages of embryo development. Maintaining these zygotes in this medium for a lengthy period of time (20 h) may also prove beneficial as this would provide sufficient time for the first embryo cleavages, as occurs in the oviduct. Then, our first objective was to corroborate this hypothesis.

Secondly, attempts were made to improve the *in vitro* maturation system by introducing meiotic inhibitors, such as roscovitine. Several ICSI studies conducted on pigs suggest incomplete terminal differentiation of the oocyte as the main cause for the low capacity of *in vitro* matured oocytes developed after fertilization [4,9]. To solve these maturation problems, different meiotic inhibitors which maintained the oocyte at the GV stage trying to mimic the *in vivo* conditions of the follicle, and increasing the cytoplasmic maturation period, have been used. Roscovitine [10] is one of the most effective metaphase promoting factor (MPF) inhibitors with fewer detrimental effects and our second objective consisted of studying the effect of this inhibitor on the ICSI yield. Furthermore, the optimal time for *in vitro* maturation was also studied by comparing three commonly used IVM times (36, 40 and 44 h).

Inositol 1,4,5-triphosphate ($InsP_3$) is one of the oocyte activation messengers [11]. Its mechanism of action consists of inducing an increase in calcium concentration liberated from the endoplasmic reticulum (ER). Amano et al. [12] demonstrated that the $InsP_3$ injection promotes oocyte activation, cleavage, and even blastocyst formation. In our experiments, we hypothesized that calcium liberation produced by the sperm injection was not similar in either its duration or intensity to that produced during normal fertilization because, although an oocyte activation was produced, further embryo development was defective. So a final experiment was designed in an attempt to evaluate the effect of $InsP_3$ on *in vitro* embryo development and to verify whether the problem actually lay in the necessity of artificially activating the oocyte–zygote during ICSI in order to facilitate calcium oscillations.

2. Materials and methods

2.1. Media and chemicals

Unless otherwise indicated, all chemicals and reagents were purchased from Sigma–Aldrich Química S.A. (Madrid, Spain). *R*-enantiomer of roscovitine (2-[*R*]-[1-ethyl-2-hydroxyethylamino]-6-benzylamino-9-isopropylpurine; R-7772) was solubilized in dimethyl sulphoxide before freezing at −20 °C as a 10 mM stock. The oocyte maturation medium was NCSU-37 [13] 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., Boxmeer, Holland), and 10% porcine follicular fluid (v/v).

The medium used for embryo micromanipulation was Dulbecco's phosphate buffered saline (DPBS) supplemented with 10% FCS. After microinjection, oocytes recovered in TALP medium [14] consisting of 114.1 mM NaCl, 3.2 mM KCl, 8 mM Ca-lactate·$5H_2O$, 0.5 mM $MgCl_2{\cdot}6H_2O$, 0.35 mM NaH_2PO_4, 25.1 mM $NaHCO_3$, 10 ml/l Na lactate, 1.1 mM Na-pyruvate, 5 mM glucose, 2 mM caffeine, 3 mg/ml BSA (A-9647), 1 mg/ml PVA and 0.17 mM kanamycin sulphate.

The embryo culture medium was NCSU-23 containing 0.4% BSA (A-8022), 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulphate [3].

Inositol 1,4,5 triphosphate ($InsP_3$) was diluted to 0.5 μM in a buffer solution (120 mM KCl, 20 mM Hepes and pH 7.4) before freezing at −20 °C until use [12].

2.2. Oocyte collection and in vitro maturation

Within 30 min of slaughter, ovaries from prepuberal gilts were transported to the laboratory in saline (0.9% (w/v) NaCl) containing 100 μg/ml kanamycin sulphate at 37 °C, and then washed once in 0.04% (w/v) cetrimide solution and twice in saline. Oocyte-cumulus cell complexes were collected from non-atretic follicles (3–6 mm diameter), washed twice in 35 mm plastic Petri dishes containing DPBS supplemented with 4 mg/ml PVA, and twice more in maturation medium previously equilibrated for at least 3 h at 38.5 °C under an atmosphere of 5% CO_2 and 100% humidity. Only oocytes harvested within 2 h of slaughter with complete dense cumulus oophorus were matured. Groups of 50 oocytes were cultured in 500 μl maturation medium for 20–22 h 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 a further 20–22 h [15]. Modifications in the IVM time was done by reducing the time in the medium without hormonal supplements or dibutyryl-cAMP. The oocytes pretreated with roscovitine were cultured in presence of 50 μM roscovitine in 500 μl of maturation medium without PMSG, HCG and dibutyryl-cAMP.

2.3. Hoechst staining

Oocytes were fixed for 15 min (2% (v/v) glutaraldehyde in PBS), stained for 15 min (1% (w/v) Hoechst 33342 in PBS) and finally washed in PBS containing 1 mg/ml polyvinylpyrrolidone and mounted on glass slides.

2.4. 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.

The DPBS sperm cell pretreatment involved the centrifugation of 10 ml of fresh semen at 1200 × *g* for 3 min, and pellet resuspension in DPBS supplemented with 10% FCS to reach a final concentration of 5×10^5 spermatozoa/ml.

2.5. Intracytoplasmic sperm injection (ICSI)

Oocytes cultured for 44 h 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 on to a lid of Petri dish (1 oocyte per drop). In total 10–15 micro-drops were placed in each lid surrounding central sperm drops which resulted from mixture of 4 μl of DPBS-FCS and 1 μl of the sperm suspension. Microdrops were covered with mineral oil (Sigma M-8410). ICSI was performed as described by García-Roselló et al. [5]. Briefly, one single sperm was immobilized by crushing the mid-piece 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 6 or 12 o'clock position. The injection pipette was pushed through the *zona pellucida* and subsequently through the oolemma 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 microscopical stage. Injected oocytes were placed in TALP or NCSU-23 medium, depending on the experiment.

For ICSI with $InsP_3$ one spermatozoon was aspirated into the injection pipette together with approximately 1.3 pl of the $InsP_3$ solution. In order to obtain a repeatable $InsP_3$ volume, a segment of $InsP_3$ of the length of a porcine sperm cells was aspirated into the injection pipette. The $InsP_3$ volume was estimated by the diameter of the injection pipette (5.5 μm) and the length of the sperm cell (55 μm) employing the formula $(5.5\ \mu m/2)2\pi \times 55\ \mu m$ [4].

At 22 hpi, a sample of oocytes was stained with Hoechst 33342 as described above and examined at 400× magnification for evidence of sperm penetration

and pronuclear formation under an epifluorescence microscope. Oocytes with at least one pronucleus (PN) were considered activated. Oocytes with two pronuclei were considered fertilized. Oocytes with only one pronucleus, with more than two pronuclei or with non-classifiable forms were designated as ‘‘other’’.

2.6. Embryo culture

After 20–22 h in TALP medium, putative zygotes were washed three 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 h post-fertilization, the number of two-cell embryos and blastocysts, respectively, were evaluated under a stereomicroscope. Embryos developing to blastocyst stage were then placed on a slide, air-dried, and fixed in absolute ethanol for 24 h. After staining with Hoechst 33342 (10 μg/ml in 2.3% sodium citrate), cell nuclei were counted under an epi-fluorescence microscope.

2.7. Experimental design

2.7.1. Experiment 1: impact of the culture media sequence on ICSI yield

COCs were matured following normal protocol of IVM during 44 h (see Section 2). Then, ICSI was performed and the injected oocytes were cultivated in TALP medium during different times: 0, 6 and 20 h and then were cultivated in NCSU-23. At 22 hpi some of the putative zygotes were processed to assess the ICSI variables and the remaining cells were introduced in the EC system to evaluated cleavage and blastocyst formation.

2.7.2. Experiment 2: impact of roscovitine prematuration and changes in IVM duration on ICSI yield

2.7.2.1. Experiment 2.1: impact of roscovitine prematuration on ICSI yield. Just after recovering, COCs were cultured in presence of 50 μM of roscovitine for 22 h. Following this period of time, COCs were washed and allowed to mature for 44 h under permissive conditions (ROS-IVM group). Other COCs were also collected and introduced in the IVM system at the same time than ROS-IVM group. Matured COCs from both groups were injected and 22 hpi samples of the putative embryos were processed to assess the ICSI variables.

2.7.2.2. Experiment 2.2: impact of IVM duration on ICSI yield. Just after recovering, COCs were cultured in presence of 50 μM of roscovitine. Then COCs were washed and allowed to mature for 36, 40 and 44 h. Matured COCs from three groups were injected and 22 hpi samples of the putative embryos were processed to assess the ICSI variables. In order to evaluate if the high results obtained in this experiment with 36 h of IVM was due or not to the roscovitine effect a new experiment was done with or without roscovitine and 36 h of IVM.

2.7.3. Experiment 3: impact of $InsP_3$ injection on in vitro embryo development

COCs were matured following the normal protocol of IVM. Four groups of injection were realized:

- *Control group*: sperm injected oocytes.
- *$InsP_3$ group*: sperm and *$InsP_3$* injected oocytes.
- *Sham-buffer group*: buffer injected oocytes. The buffer activation capacity was evaluated in this way.
- *Sham-$InsP_3$ group*: $InsP_3$ injected oocytes. The $InsP_3$ activation capacity without the sperm was evaluated in this way.

2.8. Statistical analysis

In this study, data are presented as means ± S.E.M. after being fitted to the binomial variable model. In our first and last experiment, data were analyzed by one-way ANOVA. In our second experiment the two-way ANOVA was used.

3. Results

The first experiment was designed to evaluate the impact of the culture media sequence on ICSI yield. In a first assay, oocyte activation and fertilization were assessed. This evaluation was carried out in six replicates and a total of 361 MII oocytes were microinjected. As shown in Table 1, more than 88% of microinjected oocytes resumed meiosis for three groups employed (direct transfer to NCSU-23 (or 0 h in TALP medium), 6 h in TALP medium and 22 h in TALP medium). Although a difference between the three groups in the proportion of activated oocytes that formed 2 pronuclei (considered normally fertilized), as well as a difference in sperm head decondensation was not observed, higher transition rates through first mitosis (% two-cell stage embryos) were detected in TALP groups (6 and 22 h, 9.7 ± 3.9 and 10.7 ± 3.1, respectively) in relation to the control group (0 h,

Table 1
Effect of different periods of culture in TALP medium on oocyte activation and fertilization following ICSI in pigs assessed 22 h post-injection

TALP	Oocytes (*N*)	Activated (%)	Activated			
			1PN and one sperm (%)	2PN (%)	Two-cell embryos (%)	Other (%)
0 h	91	91.2 ± 3.0	28.9 ± 5.0	55.4 ± 5.5	0 b	15.7 ± 4.0
6 h	80	90.0 ± 3.4	22.2 ± 4.9	59.7 ± 5.8	9.7 ± 3.9 a	8.3 ± 3.3
22 h	123	83.7 ± 3.3	26.2 ± 4.3	56.3 ± 4.9	10.7 ± 3.1 a	6.8 ± 2.4
ANOVA		0.2003	0.6393	0.8528	0.0096	0.1160

Values with different letters on the same column are significantly different ($P < 0.05$).

Table 2
Effect of different periods of culture in TALP medium on embryo cleavage, blastocyst formation and number of cells per blastocyst following ICSI in pigs

TALP	Oocytes (*N*)	Cleaved[a] (%)	Blastocysts[b] (% from cleaved)	Cells/blastocyst
0 h	77	54.5 ± 5.7	4.8 ± 3.3	50
6 h	90	64.4 ± 5.1	12.1 ± 4.3	41.7 ± 8.3
22 h	91	68.1 ± 4.9	4.8 ± 2.7	37.7 ± 8.7
ANOVA		0.1791	0.2424	0.8726

[a] Assessed 48 h post-injection.
[b] Assessed 144 h post-injection.

NCSU-23; $P < 0.05$). In the second assay, where the impact of the sequence of culture of ICSI oocytes was assessed in terms of *in vitro* embryo development, around 62% advanced to the second cell stage at 48 h post-injection for the three groups. In all groups, the number of zygotes that reached blastocyst stage was reduced and consequently significant differences at blastocyst stage were not detected (Table 2).

In the second experiment, the impact of roscovitine prematuration and changes in IVM duration (three IVM times were used) on ICSI yield was evaluated. As shown in Table 3, no effect was observed when the ICSI success was assessed by the oocyte activation and 2PN stage proportions. However, we observed that the percentage of embryos evaluated at 22 hpi, where oocytes prematured with roscovitine, reached the two-cell stage quicker than the control group (22.2 ± 4.4% and 7.1 ± 3.1%, respectively $P < 0.05$). In the second assay, the impact of IVM duration on oocyte activation and fertilization after ICSI was evaluated. This evaluation was carried out in seven replicates and 456 MII oocytes were microinjected. Although no difference between the three groups in the proportion of activated oocytes was detected, differences were observed in activated oocytes that formed 2PN (considered normally fertilized), and also in sperm head decondensation. The oocytes matured during 36 h achieved a higher proportion of 2PN stage than those with 44 h of IVM (75.5 ± 4.4 and 55.0 ± 4.8, respectively $P < 0.05$, Table 4). In order to evaluate if this previous effect was due to the roscovitine pretreatment, a third assay was designed. A total of 192 MII oocytes were microinjected with or without roscovitine pretreatment. No significant effect was detected for any of the analyzed variables (Table 5).

In order to determine whether the injection of $InsP_3$ together with the sperm would benefit the ICSI results, a third experiment evaluated and compared the impact of $InsP_3$ injection on *in vitro* embryo development. In Table 6, the results are presented. The differences among treatments observed in the proportion of cleaved oocytes were significant. Cleavage was higher for sperm injected oocytes (control and $InsP_3$) than for sham groups (buffer and $InsP_3$). In all groups, the number of

Table 3
Effect of roscovitine prematuration and 44 of IVM on oocyte activation and fertilization after ICSI, assessed 22 h post-injection

	Oocytes (*N*)	Activated (%)	Activated			
			1PN and one sperm (%)	2PN (%)	Two-cell embryos (%)	Other (%)
IVM	87	80.5 ± 4.3	35.7 ± 5.7	55.7 ± 5.9	7.1 ± 3.1 a	1.43 ± 1.43
ROS-IVM	112	81.3 ± 3.8	21.1 ± 4.3	48.9 ± 5.3	22.2 ± 4.4 b	7.8 ± 2.8
ANOVA		0.2946	0.0404	0.3945	0.0090	0.0683

Values with different letters on the same column are significantly different ($P < 0.05$).

Table 4
Effect of IVM time on oocyte activation and fertilization after ICSI assessed 22 h post-injection

IVM	Oocytes (*N*)	Activated (%)	Activated		
			1PN and one sperm (%)	2PN (%)	Other (%)
36 h	122	83.6 ± 3.4	18.6 ± 3.8 a	73.5 ± 4.4 a	7.8 ± 2.7
40 h	131	81.7 ± 3.4	29.9 ± 4.5 ab	61.7 ± 4.7 ab	8.4 ± 2.7
44 h	121	90.1 ± 2.7	41.3 ± 4.7 b	55.0 ± 4.8 b	3.7 ± 1.4
ANOVA		0.887	0.002	0.019	0.312

Values with different letters on the same column are significantly different ($P < 0.05$).

Table 5
Effect of roscovitine prematuration and 36 of IVM on oocyte activation and fertilization after ICSI, assessed 22 h post-injection

36 h IVM	Oocytes (*N*)	Activated (%)	Activated		
			1PN and one sperm (%)	2PN (%)	Other (%)
Control	77	79.2 ± 4.6	11.5 ± 4.1	72.1 ± 5.8	16.4 ± 3.6
ROS	94	79.8 ± 4.2	18.7 ± 4.5	72.0 ± 5.2	9.2 ± 2.8
ANOVA		0.928	0.252	0.987	0.145

Table 6
Effect of $InsP_3$ injection on embryo cleavage, blastocyst formation and number of cells per blastocyst

	Oocytes (*N*)	Cleaved (%)	Blastocysts (% from *N*)	Cells/blastocyst
Control	58	69.0 ± 6.1 a	13.8 ± 4.6 a	31.2 ± 5.3
$InsP_3$	54	59.3 ± 6.7 a	5.6 ± 3.1 ab	22.3 ± 7.9
Sham-buffer	66	9.09 ± 5.0 b	0 b	0
Sham-$InsP_3$	55	20 ± 5.4 c	1.8 ± 1.8 b	21
ANOVA		<0.001	0.008	0.610

Values with different letters on the same column are significantly different ($P < 0.05$).

zygotes that reached blastocyst stage was reduced and consequently significant differences at this stage were not detected.

4. Discussion

Given the growth in cloning techniques over recent years, the *in vitro* culture media used to develop zygotes remain inefficient in most mammalian species. As knowledge of the oviductal and uterine microenvironments increases, so it is agreed that there is a need to use sequential media within which the components change at the same time as the developing embryo requirements.

In pigs, NCSU-23 is the most widely used medium for embryo culture. When fertilization is performed by ICSI, several researchers directly transfer the zygotes to this medium [9,16–18]. The fact that the ICSI technique suppresses steps in the fertilization process related to gamete recognition, sperm-ZP binding, and the induction of the acrosome reaction or the oolemma fusion, does not mean that a recently injected zygote is found in a pronuclear state, or that its culture requirements are equivalent to those of a two-cell embryo. For this reason, we hypothesized that the culture in TALP medium before the transfer to NCSU-23 medium could improve the results. In previous studies, we have seen that pronuclear formation is produced in the TALP medium during traditional IVF [6,7] and it has been also shown that it takes place during the first 6 h after ICSI [8]. The fact that the zygotes are present in the IVF medium at this time, which is enriched with calcium, pyruvate and lactate, might facilitate the pronuclear formation, as could the early stages of embryo development. Maintaining these zygotes in this medium for a lengthy period of time (20 h) may also prove beneficial as this would provide sufficient time for the first embryo cleavages, as occurs in the oviduct.

The results from this work show that zygotes cultured for 6 or 20 h in TALP medium reached the two-cell stage more quickly than those zygotes transferred directly to NCSU-23. The moment of the first cleavage is an important indicator of further developmental capacity [19]. The fact that these first cleavages may be observed during the first 20–22 h post-injection indicates that the viability of these embryos would possibly improve, despite the fact that no significant effect was observed in the blastocyst proportion after 7 days of culture.

Due to the low blastocyst proportion obtained (below 10%), a second experiment was designed, which proposed the introduction of changes to the IVM system, initially by using meiotic inhibitors, and secondly through the changes made in the IVM time. Several ICSI studies conducted on pigs suggest incomplete terminal differentiation of the oocyte as the main cause for the low capacity of *in vitro* matured oocytes developed after fertilization [4,9]. To solve these maturation problems, different meiotic inhibitors which maintained the oocyte at the GV stage trying to mimic the internal *in vivo* conditions of the follicle, and increasing the cytoplasmic maturation period, have been used. Roscovitine [10] is one of the most effective metaphase promoting factor (MPF) inhibitors with fewer detrimental effects. In fact, we have previously demonstrated that live offspring can be obtained through IVM of oocytes precultured with roscovitine and *in vitro* fertilized before transfer to recipient sows [20].

Data from the present study reveal that the cleavage kinetics was higher in roscovitine treated oocytes, and that the cleavage proportions, assessed at 22 hpi, were higher in these oocytes. As far as we are aware, no references to pig oocytes that had been prematured with roscovitine and fertilized by ICSI exist. Recently, Franz et al. [21] worked with mare oocytes that had been prematured in roscovitine and fertilized by ICSI. They demonstrated that roscovitine is capable of increasing the development capacity when *cumulus*-enclosed oocytes are used, and a higher cleavage rate was also obtained. Thus, the hypothesis regarding the beneficial effect of a pre-maturation period is confirmed in our study, as the moment when the first cleavage takes place is, as referred to above, an important indicator of further embryo development capacity [19].

Another well-known critical factor for IVP of embryos is oocyte age, since aged oocytes have less fertilization ability than normal ones due to the lower H1kinase amount, and also to the lower quantity of activated MPF [22]. Funahashi et al. [23] demonstrated that around 47% of the oocytes reached MII at 36 h of IVM, so many oocytes are aged at the time of the *in vitro* fertilization when 44 h of maturation are used. Knowing that slaughterhouse oocytes kept with roscovitine for a few hours are able to develop to term [20], we decided to study the effect of IVM time on ICSI yield using roscovitine as a tool to modify the IVM times in batches of oocytes same-time collected.

Results showed that with an IVM period of 36 h (using roscovitine), the proportions of putative embryos were significantly higher than with 40 or 44 h. In fact, development to two-cell stage and male pronuclear formation were slower when oocytes underwent a maturation of either 40 or 44 h. No bibliographic references comparing this IVM duration time after ICSI fertilization were found. IVM times for ICSI vary by 42 and 50 h [9,16,18]. However, the putative embryo proportion in all these studies did not reach the proportions obtained in our study (73.5%). Most of the differences could be due to the different oocyte activation treatments involved, and also to both the oocyte quality and the IVM system (source of ovaries, distance to the slaughterhouse, aspiration or slicing of follicles to recover the oocytes, etc.).

In order to verify if the differences observed in our study were related to roscovitine prematuration, an additional experiment was designed in which the oocytes were matured either with or without roscovitine for 36 h. As the results showed, no significant differences were found for any of the variables analyzed. Furthermore, the putative embryo proportion reached 70% once more. This fact indicated that early embryo development was affected with a 44 h IVM period, regardless of the fact that an inhibitor, such as roscovitine, had been used or not. This was not observed in shorter IVM periods (i.e. 36 h), when the proportion of zygotes showing 2PN was higher than in the remaining groups after ICSI.

With the earlier ICSI results obtained, we can affirm that exogenous activation of the oocyte is not needed to obtain high percentages of putative embryos. The sperm stimulus is sufficient to both activate the oocyte and initiate embryo development. However, given the low number of embryos reaching the blastocyst stage, it is possible that the activation was inadequate, and therefore, the oocyte was incapable of further development to reach the blastocyst stage.

Inositol 1,4,5-triphosphate ($InsP_3$) is one of the oocyte activation messengers [11]. Its mechanism of action consists of inducing an increase in calcium concentration liberated from the endoplasmic reticulum (ER). Amano et al. [12] demonstrated that the $InsP_3$

injection promotes oocyte activation, cleavage, and even blastocyst formation. In our experiments, we hypothesized that calcium liberation produced by the sperm injection was not similar in either its duration or intensity to that produced during normal fertilization because, although an oocyte activation was produced, further embryo development was defective. So a final experiment was designed in an attempt to increase oocyte activation through an $InsP_3$ injection together with sperm.

The proportion of blastocyst obtained showed no improvement after the $InsP_3$ injection in comparison to the control group (sperm injected without $InsP_3$). As far as we are aware, the $InsP_3$ injection together with sperm has not been used to improve ICSI performance in pigs. Nonetheless, Kurokawa and Fissore [24] have demonstrated that ICSI fertilization modifies the start of calcium oscillations, and that it provokes a lower persistence and duration in these oscillations than during traditional IVF, this being the main cause of low embryo developmental capacity of ICSI zygotes in mice. The results could be explained as Banrezes et al. [25] proposed, since we did not properly mimic the calcium oocyte dynamics after fertilization, which consist of sequential activation and inhibition function series. Possibly, if we had sequentially injected the $InsP_3$ into either the oocyte or the medium in our study, we would have seen some improvement.

Nevertheless, research has still to explore the void that exists between the sperm injection and the initiation of all the appropriate mechanisms that lead to the zygote becoming a blastocyst.

Main findings from the present study reveal that the yield of the ICSI technique in pigs can be improved by (i) using TALP medium during the first hours post-injection, (ii) using a prematuration period in roscovitine, and (iii) using an *in vitro* maturation period of 36 h. Doing so, the proportions of two-cell stage embryos obtained are increased, although further development to blastocyst stage or to full term still remains to be solved.

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