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In vitro developmental competence of ICSI-derived activated ovine embryos

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Abstract

The present study was conducted to determine the necessity for activation after intracytoplasmic sperm injection (ICSI) in sheep. The effect of chemical stimulation with either 5 μM ionomycin (I) for 5 min or ionomycin + 2 mM 6-dimethylaminopurine (6-DMAP) for 3 h on the efficiency of ICSI, was compared in six experimental groups: (1) ICSI, (2) ICSI + I, (3) ICSI + I + 6DMAP, (4) Sham, (5) Sham + I, and (6) parthenogenetics (Sham and parthenogenetic groups were used as controls). In the present study, ovine oocytes needed additional chemical stimulation, after conventional ICSI, to activate (female pronucleous formation) and to form zygotes with male and female pronuclei (2PN). The percentage of cleaved embryos obtained and developed to blastocyst stage was higher ($P < 0.001$) for ICSI-derived zygotes, followed by activation (I and I + 6DMAP; 18.2 and 22.5%, respectively) than ICSI and Sham injection without activation (3.0 and 0.0%, respectively). There was, however, no significant difference between activation protocols I or I + 6DMAP. Furthermore, there was no significant difference among chemically activated, ICSI-derived zygotes in term of hatchability rate; however, the percentage was significantly higher in parthenogenetic and IVF groups than ICSI and Sham injection. In conclusion, neither sperm alone nor mechanical activation was sufficient for ovine oocyte activation and pronuclei formation. Therefore, in our study conditions for in vitro embryo development, chemical activation of oocytes must be considered an essential part of the ICSI procedure in sheep.

Keywords: ICSI; Activation; Parthenogenetic; Embryo; Ovine

1. Introduction

The intracytoplasmic sperm injection (ICSI) procedure bypasses sperm–egg binding, fusion and many upstream events associated with classical sperm–egg interactions, including plasma membrane and cortical region interactions. The ICSI procedure has expanded the possibilities of assisted reproduction technologies in both humans [1] and animals, and has provided an opportunity for investigation of fundamental aspects of fertilization such as mechanisms of gamete interaction, sperm-induced oocyte activation and first cell cycle control. A major application of this technique for animal production includes the use of genetically important male gametes for preserving both domestic and wild animals. Moreover, this technique can be used to extend the sperm vector for transgenic animal production and to use freeze-dried sperm where sperm motility is not required. Since the first report of ICSI in rodents [2], this technique was successfully used in several species; live births have been obtained in mice [3], rabbit [4], cattle [5], sheep [6,7], non-human primates [8], humans [1], horse [9], cats [10], pigs [11], and goats [12]. Fertilization failure after ICSI is

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caused by disruption of the normal sequence of events that lead to oocyte activation, sperm head decondensation, male pronucleus formation, fertilization and syngamy, or ejection of the spermatozoon from the oocyte. Furthermore, it is expected that sperm defects in microtubule function or centrosomal reconstitution will not be resolved by ICSI-assisted fertilization.

In several species, such as mice [3], hamsters [13], rabbits [14], humans [15] and horses [16], intracytoplasmic sperm injection by itself is sufficient to activate the oocyte for further embryonic development. It has also been reported that the use of piezo-electric actuator improves oocyte survival rate considerably in mouse ICSI [3]. Differences between species, however, in the response to ICSI, have been found. In sheep, there are controversial reports regarding the necessity of additional oocyte activation after the ICSI procedure. As reported, sheep [7] oocytes treated by ICSI without further activation, developed to blastocyst stage at a percentage lower than IVF (8 versus 18%, respectively). In another study, the same authors reported that the manipulation alone was inadequate to cause proper oocyte activation, unless calcium was also present and that the sperm may play no role in the early events of oocyte activation [17]. There is also evidence indicating that in sheep, sperm injection using a 3–4 micrometer ID pipette instead of a conventional 8–10 micrometer ID without sperm immobilization or additional activation, led to successful pregnancy [18].

Oocyte activation can be induced by a variety of stimuli. Artificial activation of oocytes aims to mimic the action of sperm cells during fertilization [19]. Some artificial activation treatments promote an increase in intracellular free calcium concentrations by the release of calcium from cytoplasmic stores, such as strontium chloride [20]; others promote influx of calcium from the extracellular medium, such as electrical stimulus and calcium ionophores; and there are also treatments that promote both effects, such as ethanol [21] and ionomycin [21]. These treatments are commonly associated with protein synthesis inhibitors (e.g. cycloheximide), which prevent cyclin synthesis, and phosphorylation inhibitors, such as 6-dimethylaminopurine [21–23], which prevent MPF activation. Studies with activation protocols demonstrated that in bovine and goat embryos, protocols with the best results were those that combined ionomycin with 6-dimethylaminopurine [24–26].

This study was designed to respond to the controversy regarding the necessity of oocyte activation after the ICSI procedure for normal embryo development in sheep. In the current study, Sham-injected oocytes and a parthenogenetic oocyte groups were considered as controls for mechanical and chemical activation, respectively.

2. Materials and methods

Except where otherwise indicated, all chemicals were obtained from the Sigma (St. Louis, MO, USA).

2.1. Oocyte collection

Adult and prepubertal sheep ovaries were collected during the non-breeding season (June to August) from a local abbatoir (Latitude: 32′17″ N; Longitude 50′51″ E; altitude 2049 m) and transported to the laboratory in saline (30–35 °C) in a thermos flask, within 1–3 h following collection. Ovaries were washed three times with pre-warmed fresh saline (37 °C), and all visible follicles with a diameter of 2–6 mm were aspirated using gentle vacuum (30 mmHg) via a 20 gauge short beveled needle connected to a vacuum pump. Prior to aspiration, the collecting tube was filled with 2 mL preincubated hepes-modified TCM, supplemented with 50 IU/mL heparin.

2.2. In vitro maturation

After aspiration, only oocytes surrounded by more than three layers of unexpanded cumulus cells (COCs: cumulus oocyte complexes) were recovered and selected for in vitro maturation (IVM). Before culturing, oocytes were washed in Hepes-buffered TCM199 (H-TCM199) supplemented with 5% FBS (Fetal bovine serum, Gibco 10270), and 2 mM glutamine. The oocyte culture medium (OCM) consisted of bicarbonate-buffered TCM 199 with 2 mM L-glutamine supplemented with 0.02 mg/mL cysteamine, 1 IU/mL hCG, 1 μg/mL E_2, 100 μL/mL penicillin, 100 μg/mL streptomycin, 10% FBS (Fetal bovine serum, Gibco 10270), and 0.2 mM Na-Pyruvate. The medium osmolarity was adjusted to 275 mOsm. The COCs were randomly distributed in maturation droplets (10 oocytes in 50 μL) and covered by sterile paraffin oil in a 60-mm Petri dish (Falcon 1008; Becton & Dickinson, Lincoln Park, NJ) and were then incubated under an atmosphere of 5% CO_2 and 95% air with 100% humidity at 39 °C for 24 h.

2.3. Experimental groups

Each experiment included at least six replicates. The in vitro matured oocytes were denuded and randomly allocated to six experimental groups, as follows:

Group 1: Denuded oocytes injected with immobilized sperm without activation
Group 2: Denuded oocytes injected with immobilized sperm and activated with 5 μm Ionomycin (5 min)
Group 3: Denuded oocytes injected with immobilized sperm and activated with 5 μm Ionomycin (5 min) + 1.9 mM 6DMAP (3 h)
Group 4: Denuded oocytes injected with < 5 pL of media (sham injection)
Group 5: Denuded oocytes injected with <5 pL of media (sham injection) and activated with 5 μm Ionomycin (5 min)
Group 6: Denuded oocytes activated with 5 μm Ionomycin (5 min; parthenogentic)
Group 7: In vitro fertilized oocytes (IVF)

2.4. Preparation of sperm and in vitro fertilization

Before transfer to fertilization drops, the oocytes were washed four times in H-SOF (HEPES-Synthetic Oviduct Fluid) and once in fertilization medium. The H-SOF and fertilization media were the same as used by Tervit et al. [27], with modifications for preparing H-SOF (20 mM of NaHCO3 was substituted with 20 mM HEPES; 10 mM free acid and 10 mM Na salt). Both media were supplemented with antibiotics.

Fresh semen was collected from a Lori-Bakhtiari breed ram of proven fertility. For swim up, 80–100 μL of semen was kept under 1 mL of BSA-HSOF in a 15 mL conical Falcon tube at 39 °C for up to 45 min. After swim up, the 700–800 μL of the top fluid were then added to 3 mL of BSA-HSOF, centrifuged twice at 200 × g for 3 min, and the final pellet resuspended with BSA-HSOF. Insemination was carried out by adding 1.0×10^6 sperm/mL to the fertilization medium. The fertilization medium was SOF enriched with 20% heated inactivated estrous sheep serum. A 5 μL aliquot of sperm suspension, containing 1×10^6 sperm/mL, was transferred into fertilization medium that included 10 oocytes per 45 μL fertilization drop. Fertilization was carried out by co-incubation of sperm and oocytes in an atmosphere of 5% CO_2 in humidified air at 39 °C for 22 h.

2.5. Intracytoplasmic sperm injection (ICSI)

ICSI was performed using an IX71-Olympus inverted microscope with Nomarsky optics (IX71 Olympus, Tokyo, Japan). After IVM, the oocytes were denuded and in a group of 15 oocytes were placed into a drop of 50 μL of injection medium (HSOF) covered with mineral oil. The spermatozoa used for ICSI was treated identically to the spermatozoa used for IVF. The prepared sperm was diluted 1:1 with 12% polyvinylpyrrolidone in PBS immediately, just before microinjection. One droplet of injection medium (50 μL) with two droplets (10 μL) of PVP diluted sperm was arranged in two columns on the lid of a 60 mm tissue culture dish (Falcon 1008; Becton & Dickinson, Lincoln Park, NJ, USA). The injection pipette had an inner diameter of 6 μm, and the holding pipette 20–30 μm. Sperm were individually immobilized by scoring the midpiece aspirated tail-first, and injected into the ooplasm through the zona pellucida. The first polar body was either in the 6 or 12 o'clock position, and the injection pipette was in the 3 o'clock position. During the injection, cytoplasm was aspirated to approve that the oolema was broken. The spermatozoon was injected into the ooplasm with a minimum volume of medium (<5 pL) at the 9 o'clock position. Sham injections were performed in a similar manner. After ICSI, oocytes were washed three times in HSOF containing 6 mg/mL BSA, and then activated.

2.6. Activation of ICSI ova

Within 1 h after injection, the injected oocytes (ICSI and Sham groups) were activated either by exposure to 5 μM ionomycin in H-SOF with 3% FBS for 5 min alone or cultured in IVF-SOF for 3 h to allow extrusion of the second polar body, and then exposed to 1.9 mM 6-dimethylaminopurine (DMAP) prepared with H-SOF at 39 °C, 5% CO2 in air for 3 h. Thereafter, oocytes were washed in HSOF and placed into 20 μL drops of IVC medium. A group of denuded IVM-oocytes was used as a control of the chemical activation (parthenogenetic group).

2.7. Nuclear staining

Sixteen hours after ICSI, the injected oocytes were transferred in ice-cold ethanol containing 10 mg/mL Hoechst 33342 for 15 min. The oocytes were directly mounted into the small droplet of glycerol on glass slide and examined under an epifluorescent microscope (IX71 Olympus, Tokyo, Japan). The nuclei appeared blue, caused by DNA labeling with the membrane permeable Hoechst 33342. The criteria used for defining fertilization versus parthenogenetic activation was the presence of both female and male pronuclei, and one or two polar bodies in the absence of sperm head within the oocyte or perivitelline space.

Table 1
Nuclear stage of injected sperm and chemically activated and not activated sheep oocytes at 20 h after ICSI (Data was collected over at least six replicates)

Nuclear stage	Treatment		
	ICSI	ICSI + I*	ICSI + I + 6DMAP
Total no. of oocytes analyzed	19	25	42
No. (%) oocytes degenerated	2	–	–
No. (%) oocytes not activated (oocytes at MII)	16 (84.2)[a]	3 (12)[b]	9 (21.4)[b]
No. (%) of oocytes activated (oocytes with female pronuclei)	1 (5.2)[a]	22 (88)[b]	33 (78.6)[b]
One female pronucleus	1	14	22
Two female pronuclei	–	3	6
Three female pronuclei	–	2	1
Ana-telophase II stage	–	3	4
Total no. (%) zygotes fertilized (oocytes with female and male pronuclei)	1 (5.2)[a]	14 (56)[b]	19 (45.2)[b]
Total no. (%) zygotes with intact sperm heads	–	8 (36.4)	14 (42.4)
Intact sperm + one female pronucleus	–	–	3
Intact sperm + two female pronuclei	–	3	6
Intact sperm + three female pronuclei	–	2	1
Intact sperm + Ana-telophase II stage	–	3	4

Numbers with different superscript letters (a, b) in the same row differ significantly ($P < 0.001$).
* Ionomycin.

2.8. In vitro culture

After IVF, presumptive zygotes were vortexed for 2–3 min to remove the cumulus cells and then washed in H-SOF to remove spermatozoa and cellular debris. They were then allocated to 20 μL culture drops (five or six embryos/drop) consisting of SOF supplemented with 2% (v/v) BME-essential amino acids, 1% (v/v) MEM-nonessential amino acids, 1 mM glutamine and 8 mg/mL fatty acid free BSA. The incubation conditions were 7% O2, 5%CO2, and 88% N2 at 39 °C in humidified air. On Days 3 and 5 of culture (Day 0 was defined as the day of fertilization) 10% charcoal stripped fetal bovine serum (FBS) was added to the medium. The culture was continued until 8 d post-fertilization. The ICSI and sham embryos were cultured similar to their counterpart IVF embryos. The osmolarity was maintained at 270–285 mOsmol. The percentage of cleaved embryos at Day 3 and the percentage of blastocysts at Day 7 were expressed on the basis of the number of oocytes at the onset of culture, and the percentage of hatched blastocysts at Day 8 expressed on the basis of the total number of blastocysts present at Day 7.

2.9. Statistical analysis

Data were collected over at least six replicates. All proportional data were subjected to an arc-sine transformation, and the transformed values were analyzed using one-way ANOVA. When ANOVA revealed a significant effect, the treatments were compared by Fisher LSD method. When an equal variance test failed, treatments were compared by Student-Newman-Keuls Method. When the normality test failed, the Kruskal–Wallis One Way Analysis of Variance on Ranks was applied. Chi-square and Fisher Exact Test was applied when qualitative evaluation was considered. All analyses were conducted with SPSS Version 11.5 (SPPS Inc., Chicago, IL, USA) and $P < 0.05$ was considered significant.

3. Results

The nuclear status of injected oocytes and sperm after injection are shown (Table 1). The total number of oocytes not activated was quite high in the ICSI group compared to the groups that received I or I + 6DMAP. Accordingly, the percentage of activated oocytes (oocytes with female pronuclei) was obviously low in the ICSI group. The pattern of percentage of fertilized oocytes followed the same pattern as activated oocytes (Table 1).

The cleavage rate in both ICSI and Sham groups was significantly lower than IVF and other chemically activated groups. However, among activated groups (including partenogenetic), the cleavage rate was not significantly different. The blastocyst rate followed the same pattern as cleavage, except that the blastocyst rate in ICSI + I and Sham + I groups was significantly lower than IVF. The rate of hatched blastocyst in IVF was comparable with other chemically activated groups,

Table 2
The effect of different activation methods on ICSI-derived ovine embryo development (at least six replicates)

Experimental groups	No. of oocytes manipulated	Embryonic developmental stages (%: mean ± S.E.M.)		
		Cleavage	Blastocyst	Hatched blastocysts*
ICSI	202	38(24.00 ± 8.5)[a]	5(3.00 ± 1.5)[a]	3(20.80 ± 14.0)[a,b]
ICSI + I**	269	215(80.50 ± 2.2)[b,c]	46(18.20 ± 3.6)[b]	20(41.10 ± 7.9)[a,c]
ICSI + I + 6DMAP	246	213(84.60 ± 3.5)[b,c]	52(22.50 ± 2.1)[b,c]	21(37.10 ± 6.3)[a,b,c]
Sham	160	12(7.30 ± 0.9)[a]	(0.00 ± 0.0)[a]	(0.00 ± 0.0)[b]
Sham + I	107	94(88.30 ± 4.4)[b,c]	19(18.10 ± 2.9)[b]	8(38.30 ± 12.1)[a,b,c]
Parthenogenetic	146	134(92.20 ± 1.8)[b]	30(21.40 ± 3.3)[b,c]	15(52.50 ± 8.1)[c]
IVF	177	132(74.00 ± 4.1)[c]	56(31.70 ± 1.3)[c]	29(52.20 ± 4.2)[c]

Within a column, numbers without a common superscript letters (a, b and c) differed ($P < 0.01$).
* The percentage of hatched blastocysts on Day 8 expressed on the basis of the total no. of blastocysts present at Day 7.
** Ionomycin.

although it was higher than the ICSI and Sham groups (Table 2).

4. Discussion

The ability of the spermatozoa to induce oocyte activation following injection is a crucial requirement for successful fertilization. If the sperm plasma membrane of a species is stable and the ability of an oocyte's cytoplasm to digest the sperm plasma membrane is low, the membrane will disintegrate slowly, or not disintegrate at all, within the oocyte [28]. Quick disintegration of the sperm plasma membrane within the oocyte is important, as oocyte activation depends on sperm-borne oocyte-activating factor (SOAF) [29].

In the present study, it was clear that sperm injection by itself was not sufficient to activate ovine oocytes. Higher percentages of oocyte activation and zygotes with male and female pronuclei and embryo development were obtained with ICSI plus chemical stimulation. The ICSI procedure per se resulted in oocyte activation only in 5.2% of cases, which was lower ($P < 0.001$) than ICSI-chemically activated oocytes (88.0 and 78.6% in ICSI + I and ICSI + I + 6DMAP, respectively). The proportion of fertilized zygotes (oocytes with female and male pronuclei) followed the same pattern (higher percentage in ICSI-chemically activated oocytes) as activated oocytes. These findings were consistent with those in cattle [14,24] and pigs [30], but contrasted with human [1], hamster [13], mouse [3], and a previous report in sheep that utilized a 3–4 μm ID injector pipette instead of a conventional 8–10 μm ID pipette [18].

As shown (Table 1), in the absence of chemical activation, only 1 of 19 injected oocytes was able to form a female pronucleus, leading to a zygote. In contrast, following chemical activation, 56.0 and 45.2% of injected oocytes displayed both female and male pronuclei (ICSI + I and ICSI + I + 6DMAP, respectively). In chemically activated oocytes 36.4 and 42.4% of injected sperm, were unable to form male pronucleus and remained condensed in ICSI + I and ICSI + I + 6DMAP, respectively. Several reasons could be proposed for relatively high proportion of residing intact sperm head in ovine injected oocytes. First; neither both two step swim up and presence of PHE during sperm preparation nor breaking sperm midpiece sufficiently exposed the injected oocytes to the SOAF. Horses and cattle have very stable sperm membranes that require cryopreservation or a substance with high potential to capacitate spermatozoa (e.g. ionomycin), which destabilizes the membranes to obtain fertilization [28,31]. In sheep, or in specific breeds of this species, whether the stability of sperm membrane could be a limiting factor has to be further investigated. When an intact spermatozoon was injected, SOAF would not be exposed to the oocyte's cytoplasm until the sperm plasma in the perinuclear theca in the postacrosomal region [32,33] and under the plasma membrane over the equatorial segment of the acrosome [34] disintegrated. Perhaps membrane disintegration may incompletely occur in some species, but not in others. Other contributing factors such as improper oocyte cytoplasmic maturation and the low contents of intracellular GSH concentration and consequently incorrect fertilization could be taken into account, although the measurement of GSH concentration was not done [35]. Therefore, both oocyte cytoplasmic integrity and the proper disintegration of sperm plasma membrane are crucial factors for successful fertilization following ICSI.

In terms of embryo development, the current results confirmed previous reports in, prepubertal goat oocytes, sheep, cattle, and pigs, indicating that mechanical

sperm injection into the ooplasm is not sufficient for proper embryo development [36,37]. Studies in adult goats, however, have obtained blastocysts by ICSI using the Piezo-driven procedure [12] or using frozen–thawed semen with broken tails [38]. In the current study, the mechanical stimulation and breaking sperm midpiece alone could only occasionally bring this about in sheep and in most cases this stimulus was insufficient.

Perhaps improper embryo development was related to the asynchronous development of pronuclei in ICSI-embryos [39]. In chemically activated oocytes, ionomycin as a histone kinase inhibitor closely mimiced fertilization by elevating oocyte intracellular Ca^{2+} concentrations and additionally preventing the reaccumulation of maturation promoting factor (MPF); this improves the efficiency of oocyte activation [22,40]. Moreover, addition of 6DMAP as a serine treonine kinase inhibitor acceleratef pronuclear formation, leading to more synchronous development of pronuclei and promotes mitosis [41]. As shown, chemically ICSI-activated oocyte with I or I + 6DMAP could significantly improve both cleavage and blastocyst rates. There is, however, a high probability that some percentages of blastocysts produced by ICSI + activation are parthenogenetically activated and/or abnormally fertilized (abnormal oocyte activation and pronuclear formation) into which developmental competence would be compromised.

In the current study, the cleavage rate in ICSI and sham groups was significantly lower than other groups, although the higher cleavage rate in ICSI (24%) compared to the sham group (7.5%) could be related to the partial effect of sperm on improvement of the cleavage rate.

Furthermore, the cleavage rate in chemically activated oocytes was comparable with IVF, though in parthenogenetic group the percentage was significantly higher than IVF. The relatively higher cleavage rate in parthenogenetic compared to the other ICSI-activated oocyte could be related to the probable harmful effect of ICSI procedure. Abnormalities found in fertilization after ICSI may be induced by the injection technique itself [7].

The percentage of blastocyst in IVF was higher than other groups. The difference, however, was insignificant from parthenogenetic and ICSI + I + 6DMAP groups. The significantly higher hatching rates in IVF and chemically activated groups once more indicated the beneficial effects of chemical activation on ICSI-derived ovine zygotes. There was no significant difference in terms of embryo development between two chemical activation protocols (I and I + 6DMAP).

In conclusion, following the ICSI procedure, neither sperm alone nor mechanical manipulation was sufficient for activation of injected oocytes and pronuclei formation in sheep. Therefore, based on our study, conditions for in vitro embryo development, chemical activation of oocytes must be considered an essential part of ICSI procedures in sheep.

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