Effects of Activation Timing on the Fertilization Rate and Early Embryo Development in Porcine ROSI Procedure

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Purpose: This study was undertaken to evaluate the optimal exposure time for nuclear envelope breakdown (NEBD) after injection of a round spermatid and to investigate the effect of oocyte activation timing on the fertilization rate and early embryo development in porcine round spermatid injection procedure.

Methods: Injected oocytes were fixed at 0.5, 1, 2, 3, and 4 h before activation, and NEBD state was examined. The three groups of oocytes were activated before and after the injection of spermatid using a single direct current pulse (100 V/mm, 50 μ s): group 1) at 2 h before the injection (pre), group 2) within 0.5 h after the injection (immediate), and group 3) at 2 h after the injection (post). Activated oocytes were cultured and pronucleus formation and blastocyst development was evaluated at 15–18 h and 7–8 days after the injection, respectively.

Results: The proportion of oocytes with NEBD significantly increased in the groups with over 2 h of exposure time ($p < 0.05$) and oocyte with premature chromosome condensation began to appear 3 h after the injection. Normal fertilization and development rate to the blastocyst stage were significantly higher in the post group than in those of the pre or immediate group ($p < 0.05$).

Conclusion: The optimal exposure time for NEBD is 2 h after the injection, and activation in 2 h after round spermatid injection improved the normal fertilization and early embryo development rate.

KEY WORDS: Activation timing; embryo development; NEBD; Porcine round spermatid; ROSI.

INTRODUCTION

The male gamete nucleus after its penetration into the oocyte cytoplasm undergoes a number of changes that lead to the formation of the male pronucleus. This process includes nuclear envelope breakdown (NEBD), decondensation of chromatin, a short stage of chromatin condensation, and reformation of a new

nuclear envelope. NEBD of a male gamete nucleus is prerequisite for the decondensation of the male gamete nucleus and subsequently for the formation of a male pronucleus (PN) (1–3). The capability of mammalian oocyte to induce NEBD of a male gamete disappears within a few hours after oocyte activation (4,5).

In ROSI (round spermatid injection) procedure, however, the cytoplasm of round spermatid impairs or delays the exposure of the round spermatid nuclear membrane to the oocyte cytoplasmic factors that induce NEBD (6). Moreover, a round spermatid from most mammalian species is lacking in oocyte activation factor; thus, an additional activation procedure is required to allow fertilization following spermatid injection (7–9). These facts suggest that the

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timing of spermatid injection and oocyte activation are critical for normal fertilization and embryo development when the conventional ROSI procedure is employed. Practically, round spermatids injected within 1 h after oocyte activation failed to undergo NEBD, resulting in a very low fertilization rate in a mouse (10), hamster (11), and rabbit (12). On the contrary, when round spermatid nuclei were left in nonactivated oocyte for an extended period of time, normal fertilization failed because of premature chromosome condensation (PCC) of spermatid nucleus (13). Therefore, the optimal exposure time for NEBD and the timing of oocyte activation for successful fertilization have not yet been fully elucidated in ROSI procedure. Under the hypothesis that prolonged exposure of a round spermatid to a non-activated oocyte until NEBD and oocyte activation at the time of NEBD are more efficient, this study was undertaken to evaluate the optimal exposure time for NEBD after injection and to investigate the effect of oocyte activation timing on fertilization and early development in porcine ROSI procedure.

MATERIALS AND METHODS

In Vitro Oocyte Maturation

Porcine ovaries were collected from a slaughterhouse and transported to the laboratory in normal saline (0.9% NaCl) at 30–35°C. Cumulus–oocyte complexes (COCs) were collected by aspiration from antral follicles 2–6 mm in diameter with 18-gauge needle and a 10-ml syringe. COCs were washed three times with Tyrode lactate-Hepes (TL-Hepes) (14). About 50 COCs were cultured in each 500 μ L of maturation medium, a North Carolina State University (NCSU)-23 (15) containing 10% porcine follicular fluid, 0.57 mM cysteine (Sigma, St. Louis, MO), 5.0 mM hypotaurin (Sigma), 10 IU/mL eCG, 10 IU/mL hCG in four-well dishes (Nunc Multidishes, Nalge Nunc International, Rochester, NY) for 20–22 h. They were subsequently cultured in the maturation medium without hormones for 24 h. The maturation culture was carried out at 39°C in a humidified atmosphere of 5% CO₂ in air.

Isolation of Round Spermatid

Testicular tissue was washed three times with sterilized physiological saline solution. After washing, testicular tissue was minced into small pieces with a pair of hand held scissors, and the cell in the seminiferous was released into the Dulbecco's Modified Eagle

Medium F12 (D-MEM/F12; Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco-BRL), 1% penicillin–streptomycin solution (Gibco-BRL) by repeated pipetting. The supernatant containing spermatogenic cells was centrifuged at 500 G for 5 min. The cell pellet was treated with 0.05% trypsin-EDTA (Gibco-BRL) and 1 μ L/mL soybean trypsin inhibitor (Sigma), 25 μ L/mL DNase (Sigma) in D-MEM/F12 for 15 min at 37°C with occasional stirring, respectively. After treatments, cell suspension was centrifuged at 500 G for 5 min, and the cell pellet was resuspended in the medium and filtered 3–5 times through a nylon mesh (pore size, 60 μ m). Round spermatids were identified by their size (9). The isolated cells could be stored for several hours at 4°C without losing their viability.

Intracytoplasmic Injection of Round Spermatid

After oocyte maturation, expended cumulus cells were removed by vortexing COCs in the presence of 0.1% hyaluronidase (Sigma). High-quality intact oocytes with a visible polar body were selected for further use. The injection of a round spermatid into the oocyte cytoplasm was performed using the method of Lee *et al.* (9) with slight modifications. Briefly, the injection needle used had a 9–10 μ m inner and 10–11 μ m outer diameter. The polar body was at 6 or 12 o'clock, and the point of injection was at 3 o'clock. An oocyte was penetrated by an injecting micropipette, a small amount of cytoplasm was drawn into the micropipette, and then the cytoplasm, together with the spermatid and a small amount of medium, was expelled into the oocyte. Immediately after ooplasmic injection, the injecting micropipette was withdrawn quickly, and the oocytes were released from the holding pipette to reduce the intracytoplasmic pressure exerted to the oocyte. Prior to the injection, roundspermatids were repeatedly aspirated and expelled into the PVP droplet (generally four or five times) through the injection pipette. After injection, oocytes were placed in NCSU-23 supplement with 4 mg/mL BSA prior to the activation.

Electrical Activation of Oocytes

The three groups of oocytes were activated by electrical stimulation before and after the injection of spermatid: group 1) at 2 h before injection (pre-activation), group 2) within 0.5 h after injection (immediate-activation), group 3) at 2 h after injection (post-activation). The electrical stimulation procedure was as follows. The oocytes were placed

between two 0.5-mm-diameter wire electrodes 1.0 mm apart, overlaid with 0.3 M mannitol solution containing 0.1 mM MgSO_4 , 1.0 mM CaCl_2 . Activation was induced with one DC pulse of 100 V/mm for

50 μs by an Electro Cell Manipulator 200 (BTX, San Diego, CA).

In Vitro Culture of ROSI Embryos

Activated oocytes were cultured in 50- μL drops of NCSU-23 supplemented with 4 mg/mL BSA overlaid with paraffin oil for 7 days at 39°C in 5% CO_2 in humidified air. The cleavage and development to the blastocyst stage were monitored on days 2 and 7, respectively.

Whole-Mount Preparation

Before activation, about 30 injected oocytes were fixed by whole-mount method at 0.5, 1, 2, 3, and 4 h after the injection of round spermatid, and stained with aceto-orcein to examine the induction of NEBD and PCC (Fig. 1). After activation, oocytes were fixed at

Fig. 1. Nuclear remodeling configuration. (A) intact-N; (B) NEBD; (C) PCC.

Fig. 2. Pronucleus formation of porcine oocytes 15–18 h after injection of a round spermatid. (A) 2PN + 2PB, (B) 1PN + sPN + 2PB.

Table I. Nuclear Remodeling Configurations According to the Injection Time of Porcine Round Spermatid

Time (h)	No. of Injected oocyte	Nuclear remodeling configurations (%)		
		Intact-N	NEBD	PCC
0.5	32	28 (87.5) ^a	5 (15.6) ^a	0 (0.0)
1	31	20 (64.5) ^b	11 (35.5) ^b	0 (0.0) ^a
2	37	13 (35.1) ^c	24 (64.9) ^c	0 (0.0) ^a
3	30	6 (20.0) ^d	20 (66.7) ^c	4 (13.3) ^{ab}
4	32	4 (12.5) ^d	20 (62.5) ^c	8 (25.0) ^b

Note. N: nuclear; NEBD: nuclear envelope breakdown; PCC: premature chromosome condensation. Different superscripts within column are significant difference ($p < 0.05$).

15–18 h after the injection of round spermatid to evaluate the PN formation. Oocytes with a second polar body (PB) or PN were estimated as the activated one. The PN formation was classified into three categories according to Kim *et al.* (16): 1) 2PN + 2PB, which was estimated as normal fertilization (Fig. 2A); 2) 1PN + sPN + 2PB (sPN: Condense or swelling male chromatin, Fig. 2B); 3) others (unidentified multiple pronuclei).

Statistical Analysis

All data were analyzed by Duncan's multiple-range test. Differences below the 0.05 probability level ($p < 0.05$) were considered statistically significant.

RESULTS

The proportion of oocytes with NEBD and PCC by the exposure time of an intact round spermatid into a nonactivated oocyte cytoplasm is shown in Table I. The proportion of eggs with NEBD was significantly increased as the exposure time prolonged to 2–4 h (64.0–66.7%) after the injection compared to 0.5–1 h after the injection ($P < 0.05$). PCC began to appear at

3 h after the injection and the proportion of eggs with PCC significantly increased at 4 h after the injection ($P < 0.05$). There was no difference in the activation rate between each activation timing; however, normal fertilization rate was significantly higher in the postactivation group (61.8%, $P < 0.05$) compared to those of the pre- (32.4%) or immediate (39.4%) activation groups (Table II). The development rate to the blastocyst stage was significantly higher in the postactivation group than in the pre- or immediate activation groups ($P < 0.05$). However, there was no significant difference between the pre- (10.9%) and immediate (9.1%) activation groups (Table III).

DISCUSSION

After fertilization, the male gamete rapidly undergoes NEBD and the chromatin is exposed to the oocyte cytoplasm, followed by decondensation of the male gamete nucleus and formation of a male PN. NEBD of male gamete nucleus is prerequisite for the transformation of the male gamete nucleus into a functional PN which is induced by a cytoplasmic factor termed the maturation promoting factor (MPF) (17). In mammalian oocytes, MPF activity is high at metaphase II stage (18,19) and gradually reduced by parthenogenetic activation (19,20). In ROSI procedure, however, the cytoplasm of round spermatid delays the exposure of the round spermatid nucleus membrane to the oocyte cytoplasmic factors, resulting in delayed or failed NEBD (6). Therefore, sufficient exposure time of a round spermatid into a nonactivated metaphase II oocyte cytoplasm may need to digest the cytoplasm layer of the round spermatid and induce NEBD. On the other hand, DNA of a round spermatid is lacking in protamine which is normally responsible for the compaction of sperm nucleus (21). The lack of protamine does not protect the spermatid nucleus from MPF of the oocyte, and

Table II. Activation and Male Pronucleus Formation of Porcine Oocytes at 15–18 h After Injection of a Round Spermatid

Activation timing	No. of oocyte examined	No. (%) of activated oocytes	No. (%) of oocytes with		
			2PN + 2PB	1PN + sPN + 2PB	Other
Pre	38	34 (89.5)	11 (32.4) ^a	19 (55.9) ^a	4 (11.8)
Immediate	38	33 (86.8)	13 (39.4) ^a	1 (42.4) ^{ab}	6 (18.2)
Post	39	34 (87.2)	21 (61.8) ^b	9 (26.5) ^b	4 (11.8)

Note. Pre: 2 h before injection; Immediate: within 30 min after injection; Post: 2–3 h after injection; sPN: condensed or swelling male chromatin; Other: unidentified multiple pronuclei. Different superscripts within column are significant difference ($p < 0.05$).

Table III. In Vitro Development of Porcine ROSI Embryos Cultured for 7–8 Days Following Round Spermatid Injection

Activation timing	No. of oocyte examined	No. (%) of survived oocytes	No. (%) of embryos developed to	
			2-cell	Blastocyst
Pre	74	64 (86.5)	44 (68.8) ^{ab}	7 (10.9) ^a
Immediate	77	66 (85.7)	43 (65.2) ^a	6 (9.1) ^a
Post	70	61 (87.1)	48 (78.7) ^b	12 (19.7) ^b

Note. Pre: 2 h before injection; Immediate: within 30 min after injection; Post: 2–3 h after injection. Different superscripts within column are significant difference ($p < 0.05$).

consequently PCC can occur (13). Furthermore, the spermatid chromosomes occurred PCC were either scattered in the oocyte cytoplasm or divided into two groups as the oocytes resumed meiosis, resulting in the formation of two small nuclear fragments resembling pronuclei after oocyte activation (10,11).

In this study, we examined the nuclear remodeling configuration following the injection of a round spermatid. The proportion of oocytes with NEBD significantly increased after 2 h of injection and PCC began to appear 3 h after the injection in this study. These results suggest that the optimal exposure time for the induction of NEBD of spermatid nucleus fertilizations is 2 h after the injection, and activation of oocytes at this time was considered to be more effective for fertilization and early development in ROSI procedure. Indeed, when oocytes were activated 2 h after the injection (postactivation group), normal fertilization and early embryonic development rates were significantly higher than oocytes activated within 0.5 h after the injection (immediate activation group) or at 2 h before the injection (preactivation group). Poor outcome of the immediate activation group might be due to the low proportion of oocytes with NEBD of round spermatid nucleus when activation pulse was applied. This result was similar to previous studies, in which low fertilization rates were obtained when oocytes were activated within 0.5 h after the injection of round spermatid in a rabbit (12) and a pig (16).

In our study, the preactivation groups showed low fertilization and early embryo development rates. This finding can be explained by the following observations. The level of active MPF was reported to decline significantly within 2 h of electrical activation of porcine oocytes, and remained depleted up to 10 h (22,23). Therefore, NEBD of round spermatid nucleus might not be induced in the preactivation group, which causes low rates of fertilization and early embryo development. In addition, it was also reported that round spermatids injected 1 h after oocyte activation failed to undergo NEBD and

their nuclei remained small (10,11). Finally, the increasing time interval between activation and insemination was known to dramatically decrease the ability of oocytes to promote the transformation of sperm nucleus into a fully formed male pronucleus in mouse (24) and pig (25), which may offer an additional explanation for the poor result in the preactivation group.

In conclusion, the optimal exposure time to induce NEBD of spermatid nucleus is 2 h after the injection, and activation at the time of NEBD of spermatid nucleus can enhance the fertilization and early embryo development rates in porcine ROSI procedure.

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