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Similar Time Restriction for Intracytoplasmic Sperm Injection and Round Spermatid Injection into Activated Oocytes for Efficient Offspring Production[1]

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ABSTRACT

The injection of male haploid germ cells, such as spermatozoa and round spermatids, into preactivated mouse oocytes can result in the development of viable embryos and offspring. However, it is not clear how the timing of intracytoplasmic sperm injection (ICSI) and round spermatid injection (ROSI) affects the production of offspring. We carried out ICSI and ROSI every 20 min for up to 4 h after the activation of mouse oocytes by Sr^{2+} and compared the late-stage development of ICSI- and ROSI-treated oocytes, including the formation of pronuclei, blastocyst formation, and offspring production. The rate of pronucleus formation (RPF) after carrying out ICSI started to decrease from >95% at 100 min following oocyte activation and declined to <20% by 180 min. In comparison, RPF by ROSI decreased gradually from >70% between 0 and 4 h after activation. The RPFs were closely correlated with blastocyst formation. Offspring production for both ICSI and ROSI decreased significantly when injections were conducted after 100 min, a time at which activated oocytes were in the early G1 stage of the cell cycle. These results suggest that spermatozoa and round spermatids have different potentials for inducing the formation of a male pronucleus in activated oocytes, but ICSI and ROSI are both subject to the same time constraint for the efficient production of offspring, which is determined by the cell cycle of the activated oocyte.

assisted reproductive technology, early development, gamete biology, sperm, spermatid

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is a reliable means of microinsemination by which viable offspring can be produced in several mammalian species, including rat, rabbit, human, sheep, horse, cattle, pig, and mouse [1, 2]. Viable offspring have also been obtained after round spermatid injection (ROSI) in mouse [3], rat [4], rabbit [5], and human [6]. Recently, these techniques have been demonstrated to be powerful tools for examining the developmental potential of spermatozoa and spermatids in mutants that lack the ability to become fertilized [7–11]. In addition, these techniques have made a variety of applications possible, such as freeze drying sperm [12], gene therapy [13, 14], metaphase II transgenesis for the production of transgenic animals [15], and research into sperm-induced oocyte activation [16, 17]. Thus, intracytoplasmic injection has opened new windows of opportunity within basic and applied reproductive research.

Successful ICSI and ROSI in mice was achieved after the Piezo-driven pipette system became available in 1995; before this, successful injection was difficult to achieve because mouse oocytes are relatively intolerant of intracytoplasmic injection [18, 19]. Typically, the protocols that have been used for ICSI and ROSI are different because round spermatids in mice have little or no capacity to activate oocytes [18]. In a standard ICSI protocol, a spermatozoon is simply injected into a metaphase II-arrested oocyte. In ROSI protocols, however, injected oocytes must be activated artificially before or after the injection of a round spermatid [18, 20]. The most efficient and most widely used protocol requires that ROSI is performed at the telophase II stage after the activation of the oocyte [18, 21]. Recently, in a protocol that resembles that of ROSI, ICSI of preactivated oocytes was reported to produce viable offspring [22]; we shall refer to this method of ICSI or ROSI as delayed. It would therefore appear that oocytes have the ability to accept either type of male germ cell, either before or after activation.

Round spermatids are immature haploid cells that have a decondensed nucleus; they transform slowly into elongated cells and finally become spermatozoa. During the last step, before becoming spermatozoa, most of the cytoplasm is lost from the elongated spermatid. The different protocols that are required for ICSI and ROSI to be successful (see above) are thought to be due to the different nuclear states of spermatozoa and round spermatids, which are condensed and decondensed, respectively [20, 23]. In addition, the nuclei of round spermatids reportedly transform into male pronuclei much faster than do spermatozoa in the oocytes of hamsters [21]. The present study was carried out to determine how the timing of ICSI and ROSI injections into activated mouse oocytes affects the production of pronuclei, blastocysts, and offspring.

MATERIALS AND METHODS

Animals

BDF1 mice (C57BL/6 × DBA/2) were used to prepare spermatogenic cells and also as oocyte donors. We used ICR females that were mated with vasectomized males of the same strain as surrogates for sperm- or spermatid-injected oocytes. All animals (obtained from SLC, Shizuoka, Japan) were maintained in accordance with the Animal Experiment Hand Book at the Center for Developmental Biology.

Collection of Oocytes

Mature oocytes were collected from the oviducts of 8- to 12-wk-old BDF1 females that had been induced to superovulate with 5 IU eCG, followed by 5 IU hCG 48 h later. Oocytes were collected from oviducts approximately 16 h after hCG injection, placed in CZB medium [24] supplemented with 5.56 mM D-glucose and 5 mg/ml bovine serum albumin

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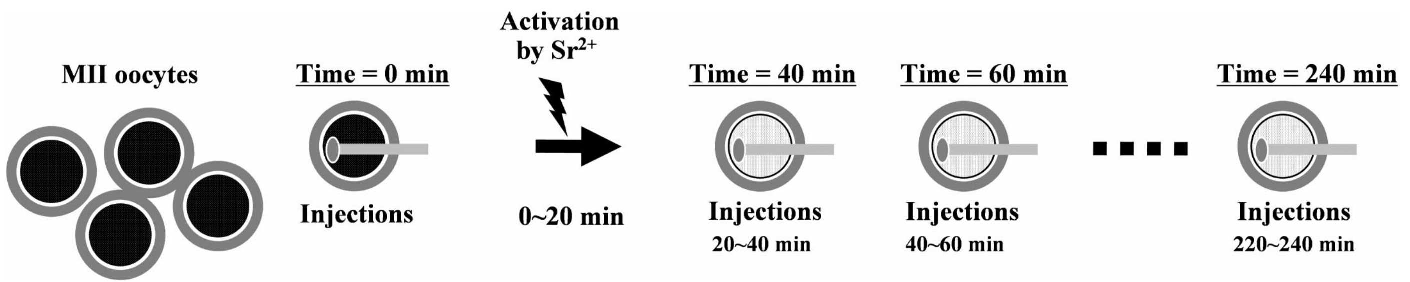

FIG. 1. Experimental procedures. For delayed intracytoplasmic sperm injection (ICSI) or round spermatid injection (ROSI), spermatozoa or round spermatids were injected into 10–15 activated oocytes within 15 min. For example, injections were carried out 20–40 min after activation for the period designated as Time = 40. For Time = 0, either ROSI or ICSI was carried out using unactivated oocytes that were subsequently activated by exposure to Sr^{2+}. MII, Metaphase II.

(BSA, fraction V), and treated with 0.1% hyaluronidase until the cumulus cells dispersed. The oocytes were then placed in synthetic oviductal medium enriched with potassium (KSOM) containing NEAA and EAA (KSOM AA; Specialty Media, Phillipsburg, NJ) supplemented with 1 mg/ml BSA, covered with paraffin oil (Nacalai Tesque, Kyoto, Japan), and stored at 37°C (5% CO_2:95% air).

Preparation of Epididymal Spermatozoa and Round Spermatids

A cauda epididymis was removed from a mature BDF1 male (8–12 wk old), and the caudal portion was excised using a pair of fine scissors. The tissue was compressed with forceps to release the dense mass of spermatozoa into a 1.5-ml polypropylene centrifuge tube; 200 μl of KSOM medium was then added to the tube. To collect spermatogenic cells, the seminiferous tubules of the testes from the same BDF1 male were minced as described previously [18], except that the cells were suspended in HEPES-buffered CZB medium. A 1-μl aliquot of the sperm or spermatogenic cell suspension was mixed with ~10 μl of HEPES-CZB medium containing 12% (w/v) polyvinylpyrrolidone (PVP; 360 kDa; Wako, Osaka, Japan) in a micromanipulation chamber.

Oocyte Activation and Microinsemination with Spermatozoa and Round Spermatids

ICSI and ROSI were carried out using a protocol that was described previously [18] except that our experiments were performed at room temperature. Briefly, for ICSI, the head of a spermatozoon was separated from the tail by applying pulses to the head-tail junction by means of a Piezo-driven pipette (Piezoelectric actuator; PrimeTech, Ibaraki, Japan). Only the sperm head was injected into each oocyte. For ROSI, a round spermatid (which was characterized by its small size [~10 μm] and a centrally located, distinct nucleolus) was injected into a single oocyte. For delayed ICSI or ROSI, oocytes that had been collected were placed into Ca^{2+}-free KSOM containing 5 mM $SrCl_2$ for 20 min [25]. After activation of the oocytes, spermatozoa or round spermatids were injected into 10–15 activated oocytes within 15 min of each of the time periods indicated in Figure 1. Injected oocytes were left for 10 min on the stage of the microscope at room temperature (24–26°C), after which they were transferred to KSOM medium before being placed within a CO_2 incubator. For ROSI of unactivated oocytes, oocytes were activated after the injection using Sr^{2+} in a similar manner to that described above. The injection of a round spermatid into the unactivated oocyte was carried out within 20 min.

Co-injection of Male Germ Cells

To co-inject a round spermatid and a spermatozoon simultaneously, sperm heads that had been removed from the tail (see above) were drawn into an injection pipette that contained spermatozoa within a drop of PVP; round spermatids in a different drop of PVP were then drawn into the same pipette. A round spermatid and a spermatozoon were injected simultaneously into an activated oocyte. In some cases (to distinguish the origin of the male pronuclei), round spermatids were injected first into a group of preactivated oocytes (8–10 oocytes) within 10 min; subsequently, spermatozoa that had tails were injected into the same oocytes. Each experiment was replicated at least twice until the total number of injections per experiment was greater than 25.

Examination of Pronucleus Formation and Hoechst Staining

Six hours after ICSI or ROSI, the number of pronuclei in the injected oocytes was examined using an inverted microscope with a relief contrast condenser (IX71; Olympus, Tokyo, Japan). A full-grown pronucleus in close proximity to the second polar body was assumed to be a female pronucleus. The injected oocytes were classified into three categories: oocytes with two pronuclei and one second polar body (2PN), oocytes with one pronucleus and one second polar body (1PN), and oocytes with three or no pronuclei and no second polar body (3PN). The rate of pronucleus formation (RPF) was calculated by dividing the number of oocytes in the first category (i.e., the number of 2PN oocytes) by the total number of oocytes in the first and second categories for each injection group (n = 10–15 oocytes); the resultant fraction was expressed as a percentage. To examine the status of the nucleus, oocytes were fixed 6 h after activation in a 2% glutaraldehyde solution for 2 min and were subsequently stained with 2 μg/ml Hoechst 33342 for 10 min.

Embryo Culture

Injected oocytes were cultured in KSOM medium for 24 or 96 h to examine their development in vivo and in vitro. For the data in Table 2, 1PN- or 2PN-containing zygotes were separated into different drops 6 h after injection. In all the other experiments, 1PN and 2PN oocytes were not separated.

Embryo Transfer

Injected oocytes that contained both 1PN and 2PN oocytes were cultured for 24–30 h in KSOM medium until the two-cell stage. Thereafter, 8–10 two-cell embryos were transferred to each oviduct of surrogate females on Day 1 of pseudopregnancy.

Mitosis-Promoting Factor Assay

To quantify the activity of mitosis-promoting factor (MPF), we used a MESACUP cdc2 kinase assay kit (MBL) according to the manufacturer's instructions. For each assay sample, 20 oocytes (either activated or unactivated) were drawn into a pipette and washed twice in phosphate-buffered PVA (PBS-PVA). The washed oocytes were then transferred into 2 μl PBS-PVA in a 1.5-ml centrifuge tube and stored at −80°C until the assay was performed. The cdc kinase activity in each well was detected using 492-nm excitation. The results were evaluated against a calibration curve (cdc kinase activity for 0, 1, 2, 4, 8, and 20 unactivated oocytes; $r > 0.95$). The assay was performed at least twice for each time period.

Statistical Analyses

Data for the rate of pronucleus formation were analyzed using the Welch *t*-test. Offspring production was compared using a Chi-squared test with Yates correction for continuity. A value of $P < 0.01$ was considered to be statistically significant.

RESULTS

Rates of Pronucleus Formation from Spermatozoa and Spermatids

To compare the developmental potential of spermatozoa and round spermatids injected into preactivated oocytes, we

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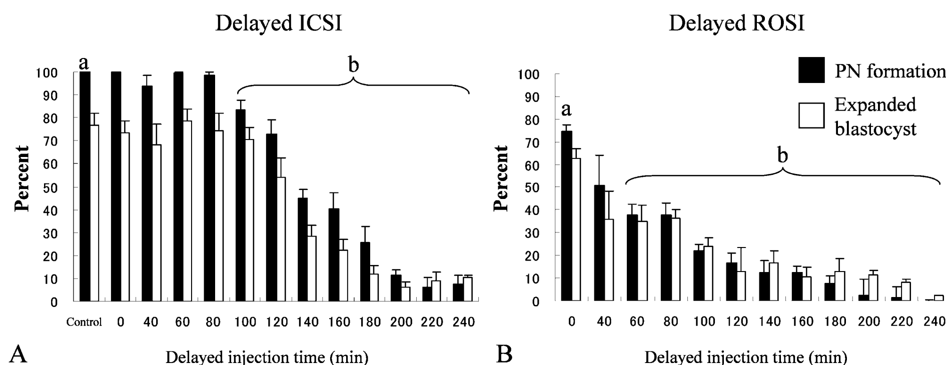

FIG. 2. Pronucleus formation and in vitro development after delayed ICSI and ROSI. The percentage (mean ± SEM) of injected oocytes that contained a second polar body, in which there were two pronuclei (2PN; closed bars), was calculated 6 h after delayed ICSI (**A**) or delayed ROSI (**B**). The percentage of 2PN oocytes represents the rate of pronucleus formation. Similarly, the rate of blastocyst formation after 96 h in culture is expressed as the mean percentage of expanded blastocysts (open bars). All data were obtained from at least three independent experiments for each time period (mean number of oocytes per time period = 60; range = 40–120). RPFs with different superscripts are significantly different ($P < 0.01$).

conducted delayed ICSI and ROSI at 20-min intervals for up to 240 min following activation (see Fig. 1). For both types of injection, the RPF and the in vitro developmental ability decreased gradually over time (Fig. 2). The RPF for delayed ICSI remained high until 80 min, but began to decline at 100 min, and fell to <20% by 180 min (Fig. 2A). By comparison, the RPF for delayed ROSI declined continuously between 40 and 240 min following activation (Fig. 2B). These results suggest that spermatozoa and round spermatids have different potentials for forming a pronucleus within activated oocytes.

Relationship Between Rate of Pronucleus Formation and Late-Stage Development

The RPF for both delayed ICSI and ROSI was closely related to the formation of an expanded blastocyst (Fig. 2). To examine in vivo development after delayed ICSI and ROSI, we transferred each group of two-cell-stage embryos from 1PN and 2PN oocytes into surrogate females. In contrast with blastocyst formation, the offspring production rate for both delayed ICSI and ROSI remained high for injections that had been carried out within 100 min of activation (Table 1). The rate of offspring production was significantly lower after 100 min following activation than before 100 min for both delayed ICSI and ROSI (Table 1). Interestingly, for both delayed ICSI and ROSI, in addition to the reduction in RPF (Fig. 1), the developmental ability of 2PN oocytes was lower for injections that were made after 100 min (Table 1) than for those made before 100 min. These results suggest that ICSI and ROSI should be carried out within 100 min of activation to obtain efficient full-term development.

Progression of Oocytes through the Cell Cycle after Activation by Sr^{2+}

After Sr^{2+} activation, the oocyte cell cycle was examined morphologically by staining the DNA (Fig. 3A). More than 80% of the Sr^{2+}-activated oocytes entered anaphase II/telophase II within 40 min of activation and entered G1 within 100 min (Fig. 3B). MPF activity was reduced to <40%

TABLE 1. In vivo development of mouse embryos following ICSI and ROSI.

Injection types	Postactivation (min)	Total no. of oocytes injected	No. (%) of oocytes with 2 PN	No. (%) of two-cell embryos	No. of two-cell transferred* (2PN+1PN)	No. of recipients (pregnant)	No. (%) of offspring	% of Offspring per 2PN
ICSI	No Ac	70	69 (99)	70 (100)	53 (51 + 2)	7 (7)	32 (60)[a]	63
	0	81	80 (99)	81 (100)	58 (56 + 2)	7 (7)	26 (45)	46
	40	69	68 (99)	66 (96)	51 (51 + 0)	6 (6)	27 (53)	53
	60	84	83 (99)	81 (96)	59 (58 + 1)	7 (7)	28 (47)	48
	80	94	87 (93)	90 (96)	73 (70 + 3)	9 (9)	30 (41)	43
	100	87	77 (89)	86 (99)	51 (49 + 2)	5 (5)	21 (41)	43
	120	68	49 (72)	64 (94)	46 (35 + 11)	5 (5)	12 (26)[b]	34
	140	80	44 (55)	76 (95)	65 (36 + 29)	6 (5)	12 (18)[b]	33
	160	61	28 (46)	55 (90)	57 (24 + 33)	6 (3)	5 (9)[b]	21
	180	103	27 (26)	101 (98)	88 (24 + 64)	8 (4)	6 (7)[b]	26
ROSI	0	211	167 (79)	210 (100)	105 (86 + 19)	11 (11)	30 (29)[c]	35
	40	118	48 (41)	117 (99)	79 (37 + 42)	7 (7)	18 (23)	49
	60	135	49 (36)	133 (99)	71 (28 + 43)	8 (8)	15 (21)	54
	80	74	24 (21)	74 (100)	52 (18 + 34)	4 (4)	10 (19)	56
	100	96	31 (32)	93 (97)	94 (31 + 63)	7 (6)	15 (16)	48
	120	97	25 (26)	97 (100)	75 (17 + 58)	7 (2)	2 (3)[d]	12
	140	47	11 (23)	46 (98)	46 (11 + 35)	5 (2)	2 (4)[d]	18
	160	56	5 (9)	56 (100)	56 (3 + 53)	5 (0)	0 (0)[d]	0
	180	38	2 (5)	37 (97)	37 (2 + 35)	4 (0)	0 (0)[d]	0

* Transferred two-cell stage embryos derived from both oocytes containing 2PN and 1PN.
[a–d] Significant χ^2 comparisons a versus b, and c versus d, $P < 0.01$.

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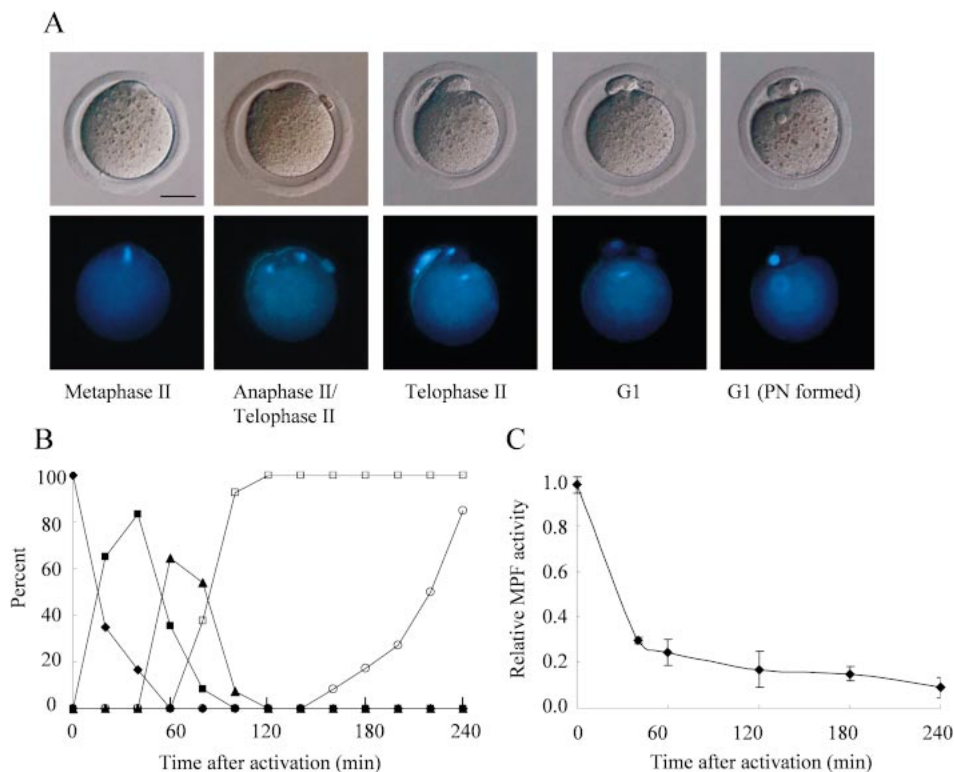

FIG. 3. Time course of oocyte cell cycle after activation by Sr^{2+}. **A**) Stages of the cell cycle in activated oocytes were categorized according to their morphology (upper panels) and the status of the pronucleus after staining with Hoechst 33342 (lower panels). Scale bar = 25 μm. **B**) For each time period, the percentage of activated oocytes (n = 15–20) in each stage of the cell cycle shown in (**A**) was calculated. Black diamonds, Metaphase II; black squares, anaphase II/telophase II; black triangles, telophase II; white squares, G1; white circles, G1 with pronucleus (PN). Data represent the mean value obtained from three independent experiments. **C**) Mitosis-promoting factor (MPF) activity (mean ± SEM) measured in activated oocytes. Note that MPF activity decreased rapidly to <40% within 40 min of activation.

within 40 min of activation (Fig. 3C). Therefore, oocyte activation by Sr^{2+} may be a suitable method for examining the relationship between the ability of activated oocytes to accept a male germ cell and the progression of activated oocytes through the cell cycle. Indeed, these results indicate that the apparent 100-min time limitation that is required to produce offspring efficiently (see above) corresponds with the entry of activated oocytes into the early G1 stage.

Formation of Male Pronuclei and In Vitro Development after Delayed Injections

To determine why the developmental ability differed for oocytes that were injected before or after 100 min following activation, the male pronuclei of zygotes were observed in detail. After delayed ICSI within 60 or even 180 min, spermatozoa became fully expanded male pronuclei with a de-

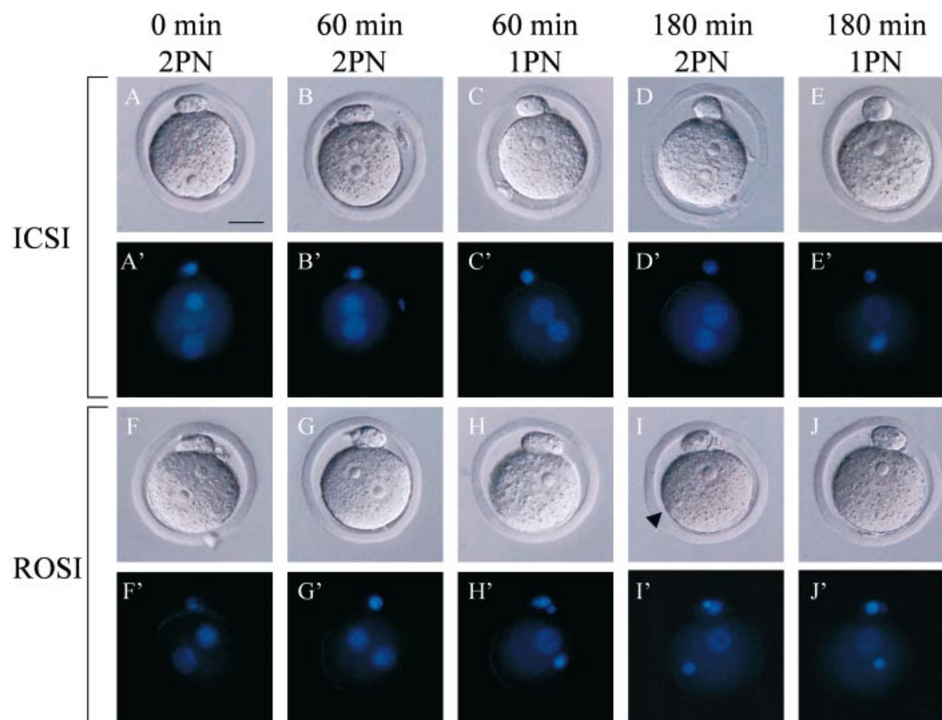

FIG. 4. Comparison of pronucleus formation after delayed ICSI and ROSI. **A–J**) Formation of a male pronucleus in injected nuclei, 6 h after ICSI or ROSI. The sperm- or spermatid-derived male pronuclei in the preactivated oocytes appeared to form normally, as did female pronuclei that were located close to the second polar body (regardless of whether a male pronucleus formed). Oocytes in which a male pronucleus failed to form contained a single pronucleus (1PN). A small but recognizable male pronucleus (I, arrowhead) was observed often after delayed ROSI. **A′–J′**) Chromosomes in the pronuclei of 2PN or 1PN oocytes after staining with Hoechst. Even in oocytes that contained 1PN after delayed ICSI, the injected spermatozoa were expanded partially; by contrast, spermatids in 1PN oocytes remained unexpanded. Scale bar = 25 μm.

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TABLE 2. Male pronucleus formation is essential for blastocyst formation.*

	States of oocytes for injection (min.)	No. of pronuclei	No. of oocytes cultured	After 72 h culture, % of embryos that developed to			After 96 h culture
				Morula	Blastocysts	Others	% of Expanded blastocysts
ICSI	Unactivated	2PN	73	68	16	16	75†
		1PN	—	—	—	—	—
	Preactivated	2PN	50	66	16	26	58†
	(80 min.)	1PN	—	—	—	—	—
	Preactivated	2PN	60	73	17	26	73†
	(120 min.)	1PN	46	33	0	67	0
ROSI	Postactivated	2PN	134	54	33	13	62†
		1PN	50	40	4	56	14
	Preactivated	2PN	64	50	36	14	73†
	(80 min.)	1PN	105	13	0	87	0
	Preactivated	2PN	43	44	35	21	67†
	(120 min.)	1PN	142	11	1	88	3
Parthenogenesis (haploid)		1PN	115	23	0	77	13

* Samples were separately cultured depending on the number of the pronuclei 6 h after injection.
† No significant differences were observed between different states of oocytes with 2PN in each delayed ICSI and ROSI.

finitive nucleolus; an identical result was observed in normal (nondelayed) ICSI 6 h after injection (Fig. 4, A, B, and D). Hoechst staining confirmed that those male pronuclei from spermatozoa were fully expanded (Fig. 4, A′, B′, and D′). Similarly, the formation of a male pronucleus after ROSI into postactivated or preactivated (delayed ROSI) oocytes was observed (Fig. 4, F, F′, G, and G′). In addition, as reported previously [18, 23], small pronuclei were often observed after delayed ROSI (Fig. 4, I and I′). Thus, even after 100 min, delayed injections still resulted in the production of fully expanded male pronuclei. As shown in Figure 2, the rate of expanded blastocyst formation was apparently correlated with the formation of the male pronucleus.

We further assessed the in vitro developmental ability of 2PN and 1PN oocytes that were cultured separately. Between 60% and 70% of the 2PN oocytes developed into expanded blastocysts within 80–120 min of delayed ICSI or ROSI, which is similar to the rate of blastocyst development in unactivated oocytes (Table 2). These data indicate that there was no significant difference in the rate of blastocyst formation for 2PN oocytes in which ICSI was carried out before or after 100 min; this suggests that recognizable male pronuclei support late development in vitro. Interestingly, Hoechst staining revealed that some of the 1PN oocytes that were subjected to delayed ICSI contained a partially expanded male pronucleus that was derived from the spermatozoon (Fig. 4, C, C′, E, and E′). However, these male pronuclei lacked nucleoli and could not support the formation of a blastocyst. In fact, the rate of blastocyst formation by 1PN oocytes was as poor as that of parthenogenetically derived haploid oocytes (Table 2). By contrast, round spermatids that failed to be transformed into recognizable male pronuclei remained unexpanded within the 1PN oocyte, even up to 6 h after injection (Fig. 4, H and J). The ability of such oocytes to develop into an expanded blastocyst was poor (Table 2). These data suggest that the formation of a male pronucleus is essential for the formation of a blastocyst. However, male pronuclei that are formed after delayed ICSI could support in vitro development regardless of the delay time.

Co-injection of Spermatozoa and Round Spermatids

The rate of pronucleus formation after delayed ICSI was higher than for delayed ROSI (Fig. 2). To examine whether the presence of spermatozoa can enhance the formation of a pronucleus following the injection of a round spermatid, a single spermatozoon and one round spermatid were injected simultaneously into an activated oocyte. This treatment failed to enhance the formation of a pronucleus from a round spermatid, although the rate of pronucleus formation by two spermatozoa within the same activated oocyte was the same as that of a single spermatozoon (Fig. 5A).

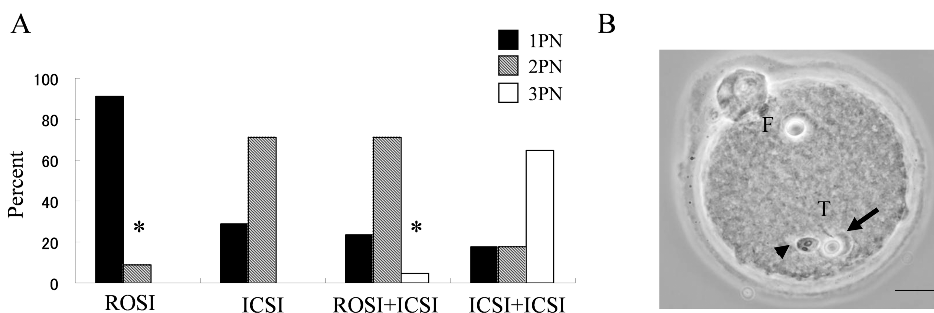

FIG. 5. Co-injection of spermatozoa and round spermatids. **A**) ICSI, ROSI, and co-injection (ROSI + ICSI and ICSI + ICSI) experiments were carried out 120 min after the activation of oocytes. The percentage of oocytes that contained a pronucleus was examined after aceto-orcein staining (total number of oocytes > 25 for each experiment). Asterisks indicate no significant difference. **B**) After delayed ROSI, a spermatozoon (with an intact tail) was injected into the same oocyte. A sperm-derived pronucleus (arrow) formed close to the tail (T), as well as a female pronucleus (F), whereas the spermatid remained unexpanded (arrowhead). Scale bar = 25 μm.

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Expanded male pronuclei were derived from spermatozoa. This was confirmed by using serial co-injections of spermatozoa with intact tails after delayed ROSI: in such oocytes, the expanded male pronuclei had a tail (Fig. 5B). Thus, the existence of a spermatozoon had no effect on the RPF following delayed ROSI.

DISCUSSION

This study investigated how the timing of the injection of sperm and round spermatids into preactivated oocytes affected in vivo and in vitro development. Our results indicate that, for both delayed ICSI and ROSI, the formation of a male pronucleus formation is essential for blastocyst formation (Table 2) and that the RPF decreases as the delay time increases (Fig. 2). Importantly, although spermatozoa and round spermatids have a differential ability to form a male pronucleus in preactivated oocytes, both types of injection must be carried out before the oocytes enter G1 to obtain an efficient rate of offspring production (Fig. 3 and Table 1). Therefore, it would appear that the time restriction that applies to ROSI, as reported previously [18, 21], is due not to the properties of the spermatid itself but, rather, is a reflection of a general limitation of the ability of haploid male cells to become incorporated into a zygote (Table 1).

Recently, it was reported that the nuclei of sperm cells that are injected up to 2 h after the activation of ovulated oocytes by ethanol can transform into fully grown male pronuclei that are capable of producing offspring [22]. Similarly, the results of our study revealed that a high rate of male pronucleus formation is maintained if injections are carried out within 100 min of the activation of oocytes by Sr^{2+} (Fig. 3A). In addition, we found that injecting spermatozoa into naturally activated anaphase II oocytes (which are often encountered during the collection of oocytes) resulted in the production of offspring (unpublished data). To date, mice that have been produced via the delayed ICSI method exhibit no abnormalities and reproduce normally. In addition, offspring are produced with a normal (1:1) Mendelian sex ratio (n = 25; 14 males, 11 females). Therefore, even in activated oocytes, sperm nuclei are efficiently transformed into male pronuclei that can support normal full-term development, irrespective of how the oocyte is activated.

In general, mouse oocytes (especially from inbred strains such as the C57BL/6) cannot tolerate the injection procedure. This explains, at least in part, why ICSI was not well established in the mouse before the application of the Piezo-driven pipette system [18]. However, the Piezo-driven pipette system requires a substantial investment of time to master the expertise that is required to produce offspring, and low survival rates are a major problem in cases where injections are made without using the Piezo-driven pipette system. The novel technique that we used in the present study, namely delayed ICSI, may solve this problem and may increase the rates of survival and offspring production to satisfactory levels because activated oocytes are more tolerant than unactivated oocytes of the mechanical stress that is involved in the injection procedure. Another positive attribute of delayed ICSI is that this method also makes it possible to inject spermatozoa without removing the tail of the spermatozoon, which is difficult to do when using MII oocytes (personal observation). It is imperative to determine whether delayed ICSI can be used in other species because, in many species, fragile oocytes or large sperm heads can be obstacles to performing ICSI successfully [26].

After delayed ROSI, the oocytes had a reduced ability to form a pronucleus. This begs the question: Do round spermatids require more time than spermatozoa to form a pronucleus? Ogura et al. [21] reported that round spermatids injected into hamster oocytes form male pronuclei more quickly (within 3–5 h) than do injected spermatozoa, but we have found no difference in the time course of pronucleus formation between round spermatids and spermatozoa in mouse oocytes (unpublished data). Furthermore, results from the co-injection experiment in the present study suggest that the presence of a spermatozoon in the same oocyte does not enhance the formation of a pronucleus by a round spermatid. Why delayed ROSI suffers from a lower rate of pronucleus formation is not yet known. However, our observation that delayed ROSI was associated with a decline in the RPF within 100 min of activation of the oocyte suggests that there are some active process(es) in unactivated oocytes that are not required for pronucleus formation by spermatozoa but that might be necessary to form a pronucleus from a round spermatid.

The formation of a male pronucleus is essential for the efficient production of offspring, as it is required for the formation of a blastocyst (see Table 2). We have noted that no pups produced via the transfer of 1PN oocytes after a delayed injection are carried to term (unpublished data). However, even though the RPFs of spermatozoa and round spermatids are different, the same time limitation exists for both types of injection in regard to the production of offspring. Our results suggest that the lower rate of offspring production when delayed ICSI or ROSI is carried out later than 100 min following activation may be attributed not only to lower RPFs but also to the lower developmental ability of 2PN oocytes, which is the result of the progression of these oocytes through the cell cycle. As shown in Table 2, there was no difference in the number of oocytes that developed into blastocysts after delayed ICSI at 80 and 120 min, which suggests that the male pronuclei that were formed as a result of delayed ICSI after 100 min were sufficient to support the formation of a blastocyst but could not support full-term development. Consequently, normal in vivo development would appear to require more than just the formation of a male pronucleus.

We observed a time limitation for injections in previous experiments in which the second polar body was exchanged with the female pronucleus in zygotes during very early developmental stages, and live offspring were obtained [27]. However, when late-stage zygotes were used as recipients, the zygote became fragmented after the exchange due to asynchronization of the cell cycle of the zygote and second polar body: the cell cycle of the second polar body remained fixed at the early G1 stage, as in the spermatid. Consequently, either of the haploid nuclei (male or female) must be transferred into activated oocytes within a limited period of time following the activation of the oocyte for the cell cycles of the activated oocyte and the transferred nucleus to be synchronized.

The results of the present study indicate that both ROSI and ICSI, carried out either before or after the activation of the oocyte, result in efficient offspring production. It has been suggested that somatic nuclear transfer requires reprogramming of the unactivated oocyte for full-term development to occur [28]. The differences between the outcomes of ICSI or ROSI and cloning suggest that the reprogramming of the unactivated oocytes that is required for cloning is not essential for embryonic development after ICSI or ROSI.

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The results of the present study suggest that male pronuclei that were formed following delayed ICSI after 100 min could support development in vitro but not in vivo. This raises the possibility that a specific amount of time is required for injected male nuclei to become male pronuclei that are capable of supporting in vivo development; this may reflect the need for the male pronuclei to be reprogrammed. To elucidate the importance of this critical time period, future studies will focus on epigenetic modifications within the male pronuclei, such as DNA methylation and histone acetylation.

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