

How Long Do Parthenogenetically Activated Mouse Oocytes Maintain the Ability to Accept Sperm Nuclei as a Genetic Partner?

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Purpose: Cytogenetic risk of intracytoplasmic sperm injection (ICSI) after artificial oocyte activation (post-activation ICSI) was evaluated in the mouse.

Methods: Mouse zygotes were produced by ICSI into eggs at various intervals after parthenogenetic exposure to strontium (Sr) for 30 min. Male pronucleus formation and the chromosome constitution were studied.

Results: Sperm nuclei injected into oocytes within 1 h after Sr exposure (from early through mid-telophase) transformed normally into male pronuclei, and the number of chromosome aberrations did not significantly increase in the resultant zygotes. When sperm nuclei were injected into eggs at intervals beyond 1 h after Sr exposure (from late telophase through the G₁ pronuclear stage), the rate of male pronucleus formation was significantly reduced. The incidence of chromosome aberrations increased with time between oocyte activation and ICSI.

Conclusions: ICSI into oocytes within 1 h after parthenogenetic activation produces cytogenetically competent embryos in the mouse.

KEY WORDS: Chromosomes; ICSI; mouse zygotes; oocyte activation.

INTRODUCTION

Parthenogenetic stimuli after intracytoplasmic sperm injection (ICSI) have been used to improve fertilization in bovine (1,2) and in human (3–7) oocytes. Electrical pulses after ICSI overcome sperm-borne oocyte activation failure and this procedure has resulted in the delivery of two healthy neonates (8). On the other hand, ICSI within a short time after parthenogenetic stimulation (post-activation ICSI) can also improve fertilization, because oocytes during meiosis II maintain the capability of transforming sperm nuclei to male pronuclei (9). For example, exposing golden hamster oocytes to a calcium

ionophore enhances the formation of male pronuclei (10). Furthermore, mouse embryos produced by ICSI after parthenogenetic stimulus with ethanol developed to full-term (11). Although the combination of ICSI and parthenogenetic activation is thus promising for assisted reproduction, the possibility of chromosome aberrations remains a concern.

The present study measures cytogenetic risk of post-activation ICSI by analyzing chromosomes of mouse 1-cell zygotes produced by ICSI at various intervals after parthenogenetic stimulation.

MATERIALS AND METHODS

Reagents and Media

All inorganic and organic reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan) unless specifically stated. Oocytes and embryos

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were cultured in CZB medium (12) supplemented with 5.56-mM D-glucose. Modified CZB (mCZB) contained 0.5% (w/v) of lipid-rich bovine serum albumin (AlbuMax, GibcoBRL, Auckland, New Zealand) instead of fraction-V albumin. Oocytes were collected and injected with sperm nuclei using mCZB containing 20-mM Hepes-Na, 5-mM NaHCO₃, and 0.1-mg/mL polyvinyl alcohol (cold water soluble PVA; Sigma, St. Louis, MO) instead of albumin (Hepes-mCZB). The pH value of Hepes-mCZB was adjusted to approximately 7.4 using 1-N HCl. The medium for incubating spermatozoa was TYH medium (13) supplemented with 0.4% AlbuMax. Sperm nuclei for ICSI were prepared in TYH containing 20-mM Hepes-Na, 5-mM NaHCO₃, 0.1 mg/mL PVA, and 10% (w/v) polyvinyl pyrrolidone (PVP-Hepes-TYH).

Collection and Parthenogenetic Activation of Oocytes

B6D2F1 female mice, 7–13 weeks of age, were injected with 8–10 IU of PMSG, followed by the same dosage of hCG 48 h later to induce superovulation. Mature oocytes collected from the oviducts 15–16 h later were freed from cumulus cells using 0.1% (w/v) of hyaluronidase (Type I-S, Sigma) in Hepes-mCZB. The oocytes were cultured in calcium-free mCZB supplemented with 5-mM SrCl₂·6H₂O for 30 min under 5% CO₂ in air at 37°C to induce parthenogenetic activation, washed with mCZB, and incubated in the same medium until sperm injection.

Meiosis II Progression of Oocytes

Before producing zygotes by post-activation ICSI, we estimated the morphological features of eggs and the time course of meiosis II following strontium (Sr) exposure. Cytological preparations of eggs were made at 30 min intervals after Sr exposure. Zona pellucidae were removed by digestion with 0.5% protease (Kaken Pharmaceuticals, Tokyo) in Dulbecco's phosphate buffered saline (PBS) at room temperature. The eggs were then washed with PBS containing 0.3% bovine serum albumin (PBS/BSA) and fixed with 4% formaldehyde in neutral buffered solution for at least 2 h at room temperature. After washing with PBS/BSA, the eggs were placed on poly-L-lysine coated glass slides and covered with Vectashield mounting medium (Vector, Burlingame, CA) containing 4 µg/mL of

4,6-diamidino-2-phenylindole (DAPI) for fluorescent microscopy.

Preparation of Spermatozoa and ICSI

A small amount of dense sperm mass was collected from the caudae epididymides of mature B6D2F1 males 8–14 weeks of age, transferred to a droplet (100 µL) of TYH medium that had been covered with paraffin oil (Merck, Darmstadt, Germany), and incubated under 5% CO₂ in air at 37°C. The incubation of spermatozoa in TYH medium never exceeded 1.5 h.

We applied ICSI as described by Kimura and Yanagimachi (14) with some modifications. A batch of 13–24 activated eggs was transferred into a small drop (15 µL) in a micromanipulation chamber on a microscope stage. A small amount (2–3 µL) of sperm suspension in TYH medium was transferred to a droplet (15 µL) of PVP-Hepes-TYH in the same chamber. A few piezo-pulses were used to separate heads from tails and then the heads were injected into activated eggs at 24–27°C. The injected eggs were returned to mCZB within 30 min, and another batch was placed in the injection drop for ICSI. Thus, ICSI was performed at intervals of approximately 30 min for up to 3 h after Sr exposure. Retention of spermatozoa in PVP-Hepes-TYH never exceeded 1 h to avoid any risk of sperm chromatin disintegration. When necessary, spermatozoa incubated in TYH medium were replenished in another drop of PVP-Hepes-TYH. Zygotes produced by ICSI into MII arrested oocytes served as a control.

Chromosome Preparation and Analysis

Injected eggs were cultured in mCZB at 37°C under 5% CO₂ in air. Approximately 8 h after the Sr exposure, eggs were transferred into mCZB containing 0.01 µg/mL vinblastine sulfate (Sigma) to stop development at the first cleavage metaphase. Between 19 and 20 h after Sr exposure, eggs were digested with 0.5% protease to loosen zona pellucidae. The eggs were then immersed in a hypotonic solution (1:1 mixture of 1% sodium citrate solution and 30% fetal bovine serum) for about 8 min at room temperature. Chromosome slides prepared by gradual-fixation/air-drying method (15) were stained with 2% Giemsa (Merck) in phosphate-buffered saline (pH 6.8) for 8 min for conventional chromosome analysis. Chromosomes were subsequently C-banded to distinguish

structural aberrations from aneuploidy as described elsewhere (16).

Statistical Analysis

Data from control and experimental groups were compared using Fisher's exact probability test and the chi-square test where appropriate. Differences at $P < 0.05$ were considered significant.

RESULTS

Meiotic Progression Following Sr Exposure

Nuclear stages of eggs at different time after Sr exposure were determined by examining 30–42 eggs. We subdivided the telophase stage into the following categories according to the degree of emission of a second polar body: 1) early telophase with two sets of chromosomes and no polar body, 2) mid-telophase with two sets of chromosomes and a prominence of the polar body, 3) late telophase with two sets of chromosomes and the complete polar body.

After Sr exposure for 30 min (defined as 0 h after exposure in this study), 88.1% of oocytes were at early telophase, and the remainder were at anaphase. At 0.5 h after Sr exposure, 75.0% of oocytes had proceeded to mid-telophase. At 1 h, 78.4% of oocytes had reached late telophase. At 1.5 h, eggs with a pronucleus at G₁ phase appeared, and the ratio of pronuclear eggs significantly increased with elapsed time (Fig. 1).

Development of Sperm Nuclei Injected into Activated Eggs

All sperm nuclei injected into oocytes within 0.5 h after the Sr exposure transformed to pronuclei, and

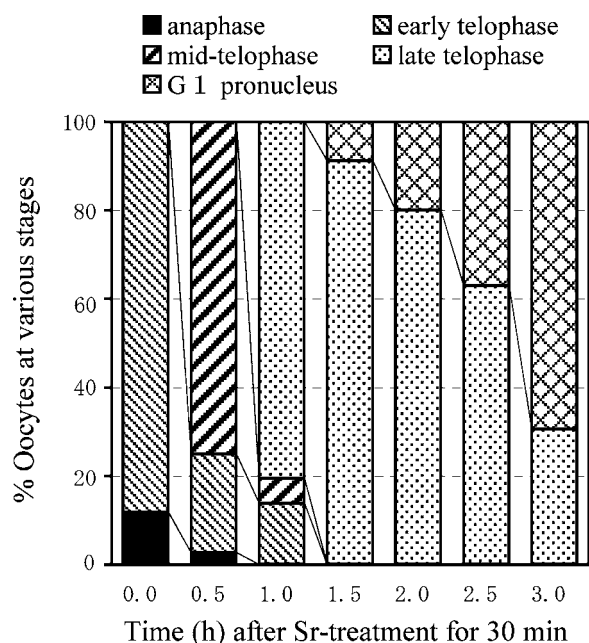


Fig. 1. Progression of second meiotic division of mouse oocytes following exposure to 5-mM strontium chloride for 30 min.

that 117 (97.5%) of them reached the first cleavage metaphase in synchrony with female pronucleus development (Table I). Similarly, 119 (99.2%) of 120 sperm nuclei injected into oocytes between 0.5 and 1.0 h after Sr exposure passed through the pronucleus stage and reached the first cleavage metaphase. However, the incidence of failure in male pronucleus formation significantly increased when sperm nuclei were injected into eggs from 1.0 h after Sr exposure. The chromatin of sperm nuclei in such eggs remained condensed, even though the egg nuclei reached the first cleavage metaphase (Fig. 2).

Table I. Development of Mouse Sperm Nuclei Injected into Eggs at Various Times After Exposure to Strontium (Sr) for 30 min and Examined at 19–20 h Thereafter

Interval (h) after exposure to Sr (no. of exp.)	No. of eggs analyzed	Developmental stages of sperm nucleus				Rate (%) of pronucleus formation ^a
		Intact head	Condensed chromatin	Pronucleus	First cleavage metaphase	
Control [5]	164	1	0	6	157	99.4
<0.5 [8]	120	0	0	3	117	100.0
0.5 ≤ <1.0 [7]	120	0	1	0	119	99.2
1.0 ≤ <1.5 [8]	123	1	8	0	114	92.7 [†]
1.5 ≤ <2.0 [8]	128	0	32	4	92	75.0*
2.0 ≤ <2.5 [6]	88	1	30	2	55	64.8*
2.5 ≤ <3.0 [6]	93	2	66	4	21	26.9*

^aCalculated using the sum of pronucleus and first cleavage metaphase as numerator.

[†] $P < 0.01$, * $P < 0.001$; determined by Fisher's exact probability test.

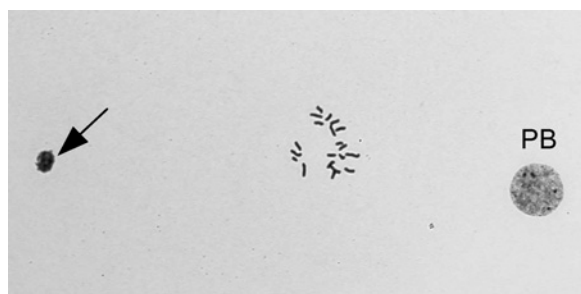


Fig. 2. Chromosome preparation of egg at the first cleavage metaphase shows egg metaphase chromosome spread and condensed sperm chromatin (arrow). PB, nucleus of second polar body.

Chromosome Analysis

Table II shows the incidences of structural chromosome aberrations, aneuploidy and triploidy in 1-cell zygotes produced by ICSI at various intervals after oocyte activation. Aberrations in zygotes produced by ICSI within 0.5 h and between 0.5 and 1.0 h after Sr exposure did not significantly increase. However, structural chromosome aberrations significantly increased in zygotes produced by ICSI from 1.0 h after Sr exposure. Almost all of the structural chromosome aberrations were of sperm origin. Most of the aberrations in zygotes produced by ICSI within 2.0 h after Sr exposure were breaks, fragments, and gaps of a chromosome-type, whereas exchanges of a chromatid-type markedly occurred in zygotes produced by ICSI from 2.0 h after exposure (Fig. 3).

DISCUSSION

The present study found that mouse sperm nuclei injected into oocytes within 1 h after exposure

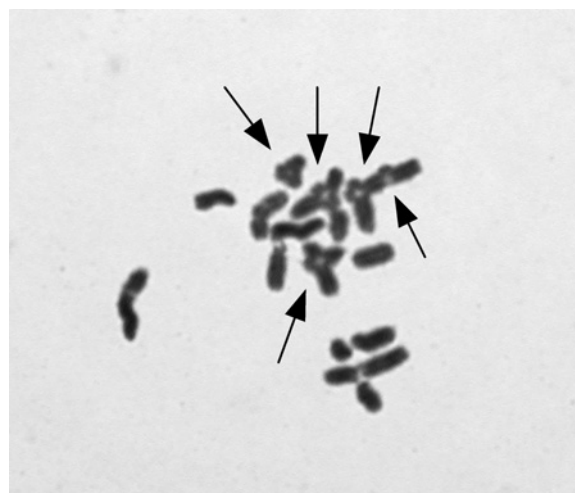


Fig. 3. Multiple chromatid exchanges (arrows) in paternal chromosomes of one-cell zygote produced by ICSI delayed between 2.5 and 3.0 h after oocyte activation.

to Sr for 30 min transformed into male pronuclei, and chromosome aberrations did not significantly increase in the resultant zygotes. Because nearly 90% of zygotes produced in this manner could develop to blastocysts (data not shown), ICSI into oocytes during anaphase through mid-telophase might produce cytogenetically and developmentally competent embryos in the mouse. If post-activation ICSI is intended for assisted mouse reproduction, sperm nuclei should be injected into oocytes within 1 h after parthenogenetic stimulation.

When ICSI was delayed at intervals beyond 1 h after Sr exposure (eggs at late telophase to G₁ phase), even though sperm nuclei transformed into pronuclei that appeared morphologically normal, the incidence of structural chromosome aberrations significantly increased. This result supports our previous finding

Table II. Chromosome Analysis at 1st Cleavage Metaphase of Mouse 1-Cell Zygotes Produced by ICSI into Eggs at Various Intervals After Parthenogenetic Stimulation with Strontium (Sr) for 30 min

Interval (h) after exposure to Sr	No. of zygotes analyzed	Normal (%)	Aberrations (%)		
			Structural aberrations	Aneuploidy	Triploidy
Control	157	144 (91.7)	6 (3.8)	5 (3.2)	2 (1.3)
<0.5	116	110 (94.8)	2 (1.7)	3 (2.6)	1 (0.9)
0.5 ≤, <1.0	119	110 (92.4)	6 (5.0)	2 (1.7)	1 (0.8)
1.0 ≤, <1.5	114	99 (86.8)	12 (10.5) [†]	1 (0.9)	2 (1.8)
1.5 ≤, <2.0	92	76 (82.6)	15 (16.3)*	1 (1.1)	0
2.0 ≤, <2.5	55	41 (74.5)	13 (23.6)*	0	1 (1.8)
2.5 ≤, <3.0	21	15 (71.4)	6 (28.6)*	1 ^a (4.8)	0

^aAccompanied by structural chromosome aberrations.

[†] $P < 0.05$, * $P < 0.001$; determined by χ^2 -test.

that the delay in sperm nuclear development behind egg development causes structural chromosome aberrations in male genomes of heterologous zygotes between Chinese hamster spermatozoa and golden hamster oocytes (17,18). The generation of chromosome damage may be associated with a disorder in sperm chromatin remodeling. During fertilization, highly condensed sperm chromatin quickly disperses, recondenses, and expands to form a pronucleus. These events are evidently supported by some ooplasmic factors, although the molecular mechanism is largely unknown. Mouse oocytes at late telophase maintain the ooplasmic factors that are required for transformation of a sperm nucleus into a male pronucleus [(9), the present study]. However, the morphological events for pronucleus formation at late telophase may not always be accompanied by complete DNA reorganization, thus resulting in chromosome damage.

Another concern is the cytogenetic evaluation of embryos produced by applying artificial stimuli onto oocytes that are unfertilized after ICSI due to sperm-borne oocyte activation failure. An electrical stimulus applied after ICSI has delivered healthy twins (8). However, Tesarik *et al.* (7) have reported that most human zygotes produced by treatment with the ionophore A23187 after ICSI undergo fragmentation during in vitro culture. In case of artificial oocyte activation after ICSI, decondensing sperm chromatin is exposed to parthenogenetic agents. To enhance the safety of artificial oocyte activation after ICSI for assisted human reproduction, the sensitivity of decondensing sperm chromatin to physical and chemical parthenogenetic agents should be explored.

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