Effect of Oocyte Preincubation and Intra-Ovarian Bursa Transfer on the Development of Oocytes Following Intracytoplasmic Sperm Injection in Rats

*Takashi Miyata[1, 2], Masao Ito[1], Gaku Shimoi[1], Yuki Fujita[1], Ryoichi Hashizume[1] and Yuichi Kameyama[1]**

[1]*Faculty of Bioindustry, Tokyo University of Agriculture, Hokkaido 099-2493, Japan*
[2]*Panapharm Laboratories, Kumamoto 869-0425, Japan*

Abstract: *Rat oocytes are known to be activated in vitro spontaneously. In the present study, we examined the effect of oocyte preincubation on the survival and development of oocytes after intracytoplasmic sperm injection (ICSI) in rats. Some presumptive oocytes produced by ICSI were transferred into the oviduct or ovarian bursa of recipient females to observe the development to term. When ICSI was performed without oocyte preincubation, the rate of oocyte survival was 90.5%, normally fertilized, 82.5%, and cleaved, 58.7%. These rates were reduced by oocyte preincubation for 3 or 5 h and the reduction could be related to the incomplete spontaneous activation of oocytes, which is known to result in no pronucleus formation and no cleavage. Both oviduct and ovarian bursa transfer allowed us to produce live offspring after ICSI (19.5 and 21.7% of transferred oocytes, respectively). Rat offspring could be produced by the ICSI protocol commonly used in mice. In rats, shortening of the period from oocyte recovery to sperm injection might be an important factor in the production of live offspring after ICSI. Intra-ovarian bursa transfer is technically easy and has been successfully applied to the production of rat offspring after various manipulations of oocytes, including ICSI.*

Key words: *Rat, Intracytoplasmic sperm injection, Ovarian bursa*

Introduction

Currently, several papers are available reporting the successful intracytoplasmic sperm injection (ICSI) in rats using epididymal sperm heads [1–5], testicular sperm heads [3], round spermatids [6, 7], sperm heads exposed to exogenous DNA [8, 9] and freeze-dried sperm heads [5]. To our knowledge, these reports are limited to only four laboratories, including our own, because of large sperm heads and fragile oocytes.

ICSI is a laborious and time-consuming procedure because each sperm head without a tail has to be injected into each oocyte. Mouse offspring were obtained from oocytes kept in CZB medium up to 3.5 h before sperm injection [10]. In human, oocyte preincubation within 9 h does not affect the survival, fertilization and cleavage rates of oocytes after ICSI [11]. Ovulated rat oocytes are known to be activated spontaneously but abortively during *in vitro* culture and the activated oocytes do not enter interphase but are arrested again at the next metaphase-like stage (MIII arrest) [12]. Spontaneous activation of oocytes is triggered immediately after euthanasia of donor rats and more than half the oocytes progress to anaphase-II/telophase-II stages at 70 min after euthanasia [13]. The relationship between oocyte incubation and fertilization after ICSI in rats is still not clear. On the other hand, all rat offspring after ICSI have been produced with oviduct transfer at the 1-cell or 2-cell stage [1–9]. Ogura *et al.* [14] reported the birth of offspring after intra-ovarian bursa transfer of Syrian hamster zygotes and they suggested the technique could be applied to other animals with ovarian bursae. Intra-ovarian bursa transfer is easy to perform and is applicable to rats for

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*To whom correspondence should be addressed.
e-mail: y-kameya@bioindustry.nodai.ac.jp

which embryo culture has not been fully established like Sylian hamsters. Therefore, we examined the influence of oocyte preincubation and intra-ovarian bursa transfer on the results of ICSI in rats.

Materials and Methods

Media

The medium for oocyte collection and ICSI was modified rat 1-cell embryo culture medium (mR1ECM) [15, 16] with modification in the supplementation of HEPES (15 mM) and reduction of $NaHCO_3$ (10 mM), referred to hereafter as HEPES-mR1ECM. The medium for sperm suspension immediately before ICSI was HEPES-mR1ECM supplemented with 12% (w/v) polyvinylpyrrolidone (PVP, average mol wt 360,000, SIGMA, St.Louis, USA). The original mR1ECM was used for the culture of oocytes before and after ICSI, and the transfer of the resulting embryos into recipient females.

Animals

Wistar-Imamichi and Brown-Norway rats were purchased from Japan SLC (Shizuoka, Japan). The rats were housed under controlled temperature (23 ± 2°C) and lighting (06:00 to 20:00). They were given free access to food and water.

Oocyte preparation

Female Wistar-Imamichi rats (2 to 3 months old) were injected subcutaneously with 30 IU PMSG (Serotropin, Teikoku-Zoki, Tokyo, Japan) and 30 IU hCG (HCG Mochida, Mochida Pharmaceutical, Tokyo, Japan) 48 h later. Oocytes were collected from the oviducts at 16 h after hCG injection and their cumulus cells were removed by pipetting in HEPES-mR1ECM containing 0.1% hyaluronidase (SIGMA, St. Louis, USA). Cumulus-free oocytes were placed in mR1ECM and kept in a CO_2 incubator (at 37°C in 5% CO_2 in air at 100% humidity) for 0, 3, or 5 h until ICSI.

Sperm preparation

Spermatozoa were squeezed out from the cauda epididymides of male Wistar-Imamichi or Brown-Norway rats (2 to 3 months old). Both strains were used to evaluate developmental ability to term after ICSI. The other experiments were conducted with spermatozoa from Wistar-Imamichi males only. The spermatozoa were allowed to disperse and to swim up in HEPES-mR1ECM for 5 to 15 min under 5% CO_2 in air at 37°C. A portion of the swim-up spermatozoa was transferred into HEPES-mR1ECM containing 12% PVP for ICSI.

ICSI procedure

ICSI was performed under an inverted microscope equipped with Hoffman modulation contrast and a piezo drive unit (Prime Tech, Tsukuba, Japan). A sperm head was injected into a denuded oocyte in HEPES-mR1ECM, as described previously in mice [10]. Briefly, a sperm head was separated from the tail by applying piezo pulses (intensity: 3–6, speed: 1) to the neck region at the blunt end of the injection pipette (external diameter: 5 μm, internal diameter: 4 μm). The injection pipette with the resulting sperm head penetrated the zona pellucida with several piezo pulses (intensity: 1–2, speed: 1) and advanced deeply through the ooplasm. The oolemma was punctured by applying a single piezo pulse (intensity: 1–2, speed: 1) and the sperm head was expelled into the ooplasm with the minimum amount of medium. Some oocytes were injected without a sperm head to observe parthenogenetic activation after sham injection.

Embryo culture

The injected oocytes were cultured in mR1ECM at 37°C in 5% CO_2 in air at 100% humidity. At 6 h after sperm injection, the numbers of surviving and fertilized oocytes were recorded under an inverted microscope with Hoffman modulation contrast. Oocytes with two pronuclei and a second polar body were considered to be normally fertilized and were selected for embryo transfer and continuous culture for up to 48 h.

Zygote transfer

Presumptive zygotes, oocytes with two pronuclei and second polar body, were transferred to the ovarian bursa or oviduct of recipient females on day 1 of pseudopregnancy. The recipients (Wistar-Imamichi, 3–4 months) had been mated during the previous night with vasectomized males of the same strain. Five to 10 oocytes were transferred into both sides of the ovarian bursa or oviduct in each recipient under anesthesia with fluothane. Some recipients were euthanized on day 3 of pseudopregnancy to evaluate the morphology of 4-cell embryos using lacmoid staining. The rest of the recipients were allowed to deliver naturally.

Cytological evaluation

Four-cell embryos produced *in vivo* or *in vitro* after ICSI were mounted between a slide and coverslip, fixed in 10% neutral-buffered formalin, and stained with lacmoid for cytological evaluation.

Table 1. Effect of oocyte preincubation on the survival, fertilization and *in vitr*o development of rat oocytes after ICSI

Oocyte preincubation period	Treatment	No. of oocytes injected	No. (%) of oocytes		No. (%) of oocytes developed to	
			survived	fertilized normally[d]	2-cell	4-cell
0 h	Sham	12	10 (83.3)[ab]	0 (0)	0 (0)	0 (0)
	ICSI	63	57 (90.5)[a]	52 (82.5)[a]	37 (58.7)[a]	11 (17.5)
3 h	ICSI	33	19 (57.6)[bc]	12 (36.4)[b]	4 (12.1)[b]	0 (0)
5 h	ICSI	73	38 (52.1)[c]	26 (35.6)[b]	11 (15.1)[b]	0 (0)

[a–c]Different superscripts within a column are significantly different ($p<0.05$). [d]Oocytes with two pronuclei and a second polar body were considered to be fertilized normally.

Statistics

Data were analyzed by chi-square test or Fisher's exact test, and differences with $p<0.05$ were considered statistically significant.

Ethical considerations of studies

All procedures in the present study were conducted according to the guidelines approved by the Animal Research Committee of Tokyo University of Agriculture.

Results

Oocyte viability, pronuclear formation and early development after ICSI

Oocyte incubation for 3 h and above prior to ICSI significantly ($p<0.05$) reduced the rates of oocytes surviving, being fertilized normally, and developing to the 2-cell stage (Table 1). When ICSI was carried out without oocyte preincubation, 90.5% of oocytes survived the ICSI procedure and 82.5% of injected oocytes were fertilized normally. The developmental rate of the oocytes to the 2-cell stage was 58.7% and to 4-cell, 17.5%; however, none of the oocytes developed to the 8-cell stage. Some of the sham-injected oocytes (4/12, 33.3%) formed a single pronucleus; however, no cleavage was observed after sham injection.

Morphology of 4-cell embryos produced in vivo and in vitro after ICSI

Nuclear fragmentation was found in the blastomere of 4-cell embryos produced *in vitro* after ICSI (11/63, 17.5%, Fig. 1b), and these embryos had started to degenerate. On the other hand, 4-cell embryos produced *in vivo* after ICSI were recovered from the oviduct on day 3 of pseudopregnancy, and looked morphologically normal without nuclear fragmentation (7/7, 100%, Fig. 1a).

Developmental ability to term

Twenty-five live offspring were produced by ICSI and the following zygote transfer into the ovarian bursa and oviduct (Table 2). Offspring from zygotes using Brown-Norway spermatozoa were identified by a brown coat. No significant difference in the live offspring rate was found between the site of zygote transfer and origin of spermatozoa (19.5–21.7%).

Discussion

Oocyte preincubation before sperm injection reduced survival, and fertilization and cleavage rates after ICSI in rats. Spontaneous activation in rat oocytes occurred rapidly; the proportion of oocytes progressing to anaphase-II/telophase-II was 5% at 10 min, 33% at 40 min, and 69% at 70 min after euthanasia of the donor [13]. In successful reports of rat ICSI, sperm heads were injected into denuded oocytes kept in a CO_2 incubator for less than 1 h [2–5, 8]. Injection of sperm heads into oocytes before spontaneous activation might be necessary for normal fertilization which leads to full-term development.

Preincubated oocytes were less resistant to the invasive technique of microinjection than oocytes without incubation. Incubation in mR1ECM, which includes PVA instead of bovine serum albumin (BSA), could lead to hardening of the oolemma as well as the zona pellucida [17]. Even in preincubated oocytes, injected sperm heads could contribute to normal fertilization. Spontaneous activation in rat oocytes is incomplete and no oocyte cleavage was observed at 34

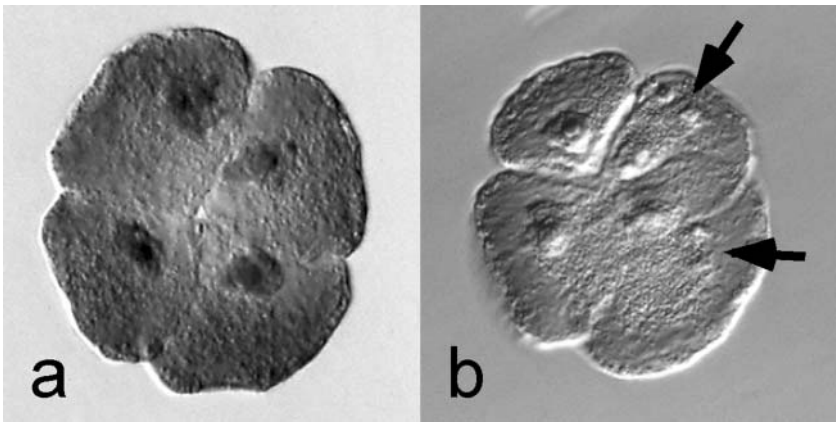

Fig. 1. Cytological observation of 4-cell embryo derived from ICSI and the following *in vivo* (a) or *in vitro* (b) culture. Presumptive zygotes were transferred into the oviduct on day 1 of pseudopregnancy and recovered on day 3 of pseudopregnancy (*in vivo* culture) or were cultured in mR1ECM for 48 h (*in vitro* culture). Arrowheads indicate fragmented nuclei in blastomeres.

Table 2. Production of rat offspring after ICSI and zygote transfer into ovarian bursa or oviduct

Site of zygote transfer	Origin of spermatozoa	No. of recepients pregnant/used	No. of zygote transferred to pregnant recepients	No. (%) of live offspring
Ovarian bursa	Wistar-Imamichi	2/2	35	7 (20.0)[a]
	Brown-Norway	3/7	46	10 (21.7)[a]
Oviduct	Brown-Norway	3/3	41	8 (19.5)[a]

[a]There was no difference among the three groups.

h of culture [13]. In the present study, sham-injected oocytes did not cleave even after 48 h of culture. Spontaneous activation could not only facilitate but could also interfere with the normal fertilization process with injected sperm. Further study is needed to clarify the mechanism of suppressed fertilization in spontaneously activated oocytes.

All oocytes injected with sperm heads ceased development at the 2-cell or 4-cell stage. The block and fragmented nuclei of our 4-cell embryos might be explained by the use of mR1ECM without BSA for culture media. Although mR1ECM can support the development of rat zygotes to the blastocyst stage, a two-phase culture system using K modified simplex optimized medium (KSOM) for 18 h followed by mR1ECM could greatly improve the efficiency [18]. Miyoshi *et al.* [19] reported that rat 1-cell embryos recovered from oviducts before pronuclear formation, or produced by *in vitro* fertilization, can develop to the blastocyst stage *in vitro* and that one or more factors in modified Krebs-Ringer bicarbonate solution (mKRB) are necessary to maintain their development in mR1ECM. Rat embryos attained high developmental ability to the blastocyst stage when embryos were fertilized in mR1ECM/BSA (mR1ECM including BSA instead of PVA) containing 110–130 mM NaCl and then cultured in mR1ECM [20]. To our knowledge, the best results for rat ICSI were reported by Hirabayashi *et al.* [9] who injected sonicated sperm heads (from SD rats) with foreign DNA into oocytes (from Donryu/LEW F1 rats) and cultured the injected oocytes in mR1ECM/BSA for 24 h and then in mR1ECM. More than 80% of the survived oocytes cleaved and 73.7% of the cleaved oocytes developed to the morula/blastocyst stage. The use of hybrid rats as oocyte donors and initial culture in KSOM or mR1ECM/BSA for 24 h might improve the efficiency of ICSI in rats.

We have demonstrated that intra-ovarian bursa transfer can be performed in rats as well as Syrian hamsters [14]. The transferred oocytes were naturally aspirated from fimbria into the oviduct, and then transported into the uterus and allowed to develop to full

term. Currently, the culture of rat zygotes up to the blastocyst stage does not seem to produce sufficient development as in mice. Intra-ovarian bursa transfer could be a powerful tool for producing live offspring after various reproductive techniques have been applied to rat oocytes. Furthermore, there was no difference in the rates of live offspring between the origins of spermatozoa used in the present study. Kato *et al.* [21] observed hybrid vigor in the production of blastocysts from zygotes cultured in mR1ECM and the births of offspring after the following embryo transfer into the uterus. Combinations of strains could affect hybrid vigor in embryo development.

A piezo-driven pipette enabled us to produced live rat offspring after ICSI by the same methodology as in mice [10]; however, further studies are needed to improve the overall efficiency of ICSI in rats. Efficiency might be improved by attention to the following points. First, the sperm head should be injected as fast as possible to precede the spontaneous activation of oocytes. For this purpose, all procedures from euthanasia of the donor animal to the end of sperm injection should be refined. For example, if several superovulated rats are used for oocyte donors, the timing of hCG injection should be adjusted in each animal for time difference of oocyte recovery, and the sonication of sperm prior to injection would help to save time by cutting the sperm tail without impairing the development of injected oocytes [9]. The addition of MG132, a protease inhibitor that blocks the first meiotic metaphase-anaphase transition in the rat [22], to handling medium for ICSI might another option for inhibiting spontaneous activation reversibly [23]. Second, the presumptive zygotes should be transferred to *in vivo* conditions as soon as possible after the confirmation of pronucleus formation. For this purpose, intra-ovarian bursa transfer is technically much easier than oviduct transfer and should facilitate the efficiency of ICSI in rats.

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