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**Technology Report**

# Lower developmental potential of rat zygotes produced by ooplasmic injection of testicular spermatozoa versus cauda epididymal spermatozoa

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Abstract. Intracytoplasmic sperm injection (ICSI) is clinically used to treat obstructive/nonobstructive azoospermia. This study compared the efficacy of ICSI with cauda epididymal and testicular sperm in Wistar (WI) and Brown-Norway (BN) rats. The transfer of ICSI oocytes with cryopreserved epididymal and testicular WI sperm resulted in offspring production of 26.2% and 3.7%–4.7%, respectively ($P < 0.05$). Treatments for artificial oocyte activation (AOA) and acrosome removal improved pronuclear formation in BN-ICSI oocytes; however, only AOA treatment was effective in producing offspring (3.7%–6.5%). In the case of ICSI with testicular sperm (TESE-ICSI), one offspring (0.6%) was derived from the BN-TESE-ICSI oocytes. The application of AOA or a hypo-osmotic sperm suspension did not improve the production of TESE-ICSI offspring. Thus, outbred WI rat offspring can be produced by using ICSI and less efficiently by using TESE-ICSI. Challenges in producing offspring by using ICSI/TESE-ICSI in inbred BN strain require further investigation.

Key words: Artificial oocyte activation, Cauda epididymal sperm, Rat ICSI, Testicular sperm

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*In vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are essential tools for producing transferable embryos in domestic animal breeding [1] and human infertility therapy [2]. Human spermatozoa retrieved from the cauda epididymis and testicular seminiferous tubules are used to treat obstructive/nonobstructive azoospermia, abbreviated as microsurgical epididymal sperm aspiration (MESA)-ICSI [3] and testicular sperm extraction (TESE)-ICSI [4], respectively. Under general physiological conditions, full maturation of sperm, such as motility and oocyte-binding ability (theoretically inessential in ICSI), occurs during migration from the seminiferous tubules to the cauda epididymis [5]. While the quality of human embryos produced by MESA-ICSI and TESE-ICSI is comparable in preimplantation genetic tests [6], some retrospective reports have indicated that the pregnancy rate following the transfer of TESE-ICSI embryos is lower than that of MESA-ICSI embryos (18.2% *vs*. 36.3% [6]; 23.1% *vs*. 31.3% [7]; 24.5% *vs*. 38.9% [8]). Because these pregnancy outcomes were derived from sperm donors with different spermatogenetic potentials in azoospermia, little is known about the efficacy of MESA- and TESE-ICSI in the same population.

The production of ICSI offspring is difficult in small rodents because of the hook-like shape of the sperm head. The fragility of the oocyte membrane can be overcome by applying piezo-driven micromanipulators [9, 10]. The ICSI technique using cauda epididymal spermatozoa has been used to maintain infertile transgenic lines [11], produce live offspring from freeze-dried spermatozoa [12, 13], and generate transgenic rodents through co-injection of spermatozoon and foreign DNA into an oocyte [14, 15]. In addition, the rodent ICSI model offers an opportunity to ensure transgenerational normality of human ICSI babies beyond the risk of miscarriage and malformation. Recently, Kanatsu-Shinohara *et al.* [16] reported that behavioral abnormalities detected in ICSI-derived mouse offspring were transmitted to the F2 generation in an atavistic manner, requiring the establishment of a rat model capable of a higher learned behavior test. Acrosome (AR) removal with Triton X-100 or lysolecithin treatment has been reported to improve the efficacy of rat ICSI (offspring rate 19.0%−20.3% *vs*. 7.6% in non-treated controls) [17]. Furthermore, artificial oocyte activation (AOA) with ionomycin treatment contributed to an increase in the production of rat ICSI offspring (17.8% *vs*. 5.6% in non-treated controls) [18]. Very limited information is available for rat TESE-ICSI and ICSI, except for an earlier study describing offspring rates of 1.7% and 3.9%, respectively [19]. In this study, we conducted a comparative study on the developmental abilities of ICSI and TESE-ICSI embryos in outbred Wistar (WI) and inbred Brown-Norway (BN) rat strains.

Crlj:WI and BN-EGFP rat sperm heads were separated from the tail by sonication and cryopreserved until ICSI, and oocytes were retrieved from superovulated Crlj:WI and BN/SsNSlc juvenile females immediately before ICSI. Response of oocyte donors to equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) was different between the two rat strains, as the proportion of responding WI donors (92.0%) and mean number of oocytes retrieved per responding donor (48.5) were higher than those of BN donors (responding donor 60.8%−62.7%; oocyte retrieval 20.4−26.2). The

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poor response of BN rats to eCG-hCG administration, as previously described [20], is one of the factors influencing ICSI performance because rat oocytes spontaneously activate once they are retrieved from the oviductal ampullae [21, 22]. We should note that a transgenic line carrying cytomegalovirus early enhancer element/chicken β-actin gene/rabbit β-globin gene (CAG)-enhanced green fluorescent protein (EGFP) gene in BN background, which had been generated and managed in Kyoto University (Kyoto, Japan), was used as sperm donors in the present study, even though no harmful effect of the CAG-EGFP transgene was known for production of the next generation.

The results of ICSI/TESE-ICSI using cauda epididymal and testicular WI sperm are summarized in Table 1. Oocyte survival rates at 6 h post-ICSI were comparable with those post-TESE-ICSI, but pronuclear formation rates in ICSI oocytes were higher than those in TESE-ICSI oocytes, suggesting different potentials for oocyte activation/sperm head remodeling between epididymal and testicular spermatozoa. Oviductal transfer into foster mothers of surviving WI- ICSI oocytes, including a few cleaved embryos (22 h post-ICSI), resulted in offspring rates of 26.2% and 4.7%, respectively ($P < 0.05$). The use of cryopreserved sperm heads in rat ICSI may be more favorable than fresh sperm heads. A higher likelihood of ICSI offspring rates using frozen-thawed sperm heads has also been observed in the Sprague-Dawley (SD) rat strain [15]. Testicular cell suspensions contain cytoplasm-rich somatic and spermatogenetic cells (*e.g.*, Sertoli cells, Leydig cells, spermatids, spermatocytes, and spermatogonia), which may disturb the pick-up of testicular spermatozoa; these cells were dramatically reduced by hypo-osmotic treatment during the TESE process (due to membrane rupture induced by rapid influx of large amounts of water). Testicular sperm heads morphologically similar to their epididymal counterparts were selected for TESE-ICSI. It was confirmed that the application of × 1/20 PBS (−) to facilitate testicular sperm recovery had no adverse effect on the pronuclear formation (57.6% *vs*. 48.8%) and offspring production (3.7% *vs*. 4.7%) in the WI-TESE-ICSI experiment. Offspring rates in outbred rat ICSI vary considerably from 6.6% [23] and 10.9% in WI [24] to 35.0% in SD [13]. Information on offspring production by rat TESE-ICSI is limited to an earlier study [19], which allows a direct comparison of ICSI offspring rates using testicular, caput epididymal, and cauda epididymal WI spermatozoa at 1.7%, 3.8%, and 3.9%, respectively.

The results of ICSI/TESE-ICSI using cryopreserved BN sperm are summarized in Table 2. Despite the relatively high oocyte survival post-ICSI, the formation of two pronuclei in BN-ICSI oocytes was significantly delayed when compared to WI-ICSI oocytes (7.4% *vs*.

**Table 1.** ICSI/TESE-ICSI outcome using WI rat spermatozoa

| Sperm source | Sperm cryopreservation | Sperm suspension | Injected | Survived (%) | Pronuclei-formed (%) | Transferred | Offspring (%) |
|---|---|---|---|---|---|---|---|
| Epididymal | − | Iso-osmotic | 210 | 172 (81.9 ± 4.5) | 121 (70.3 ± 6.2) [a] | 126 | 18 (14.3 ± 3.4) [ab] |
| | + | Iso-osmotic | 110 | 83 (75.5 ± 4.2) | 65 (78.3 ± 3.9) [a] | 65 | 17 (26.2 ± 11.3) [a] |
| Testicular | + | Iso-osmotic | 96 | 80 (83.3 ± 3.9) | 39 (48.8 ± 7.9) [b] | 43 | 2 (4.7 ± 6.0) [b] |
| | + | Hypo-osmotic | 186 | 137 (73.7 ± 1.8) | 79 (57.6 ± 2.8) [b] | 82 | 3 (3.7 ± 3.5) [b] |

%: Mean ± SEM. [a, b] Different superscripts indicate significant differences at $P < 0.05$.

**Table 2.** ICSI/TESE-ICSI outcome using cryopreserved BN rat spermatozoa; effect of ionomycin and/or Triton X-100 treatment

| Sperm source | Sperm suspension | Ionomycin | Triton X-100 | Injected | Survived (%) | Pronuclei-formed (%) | Transferred | Offspring (%) |
|---|---|---|---|---|---|---|---|---|
| Epididymal | Iso-osmotic | − | − | 129 | 108 (83.7 ± 5.7) | 8 (7.4 ± 4.2) [c] | 87 | 0 (0) |
| | Iso-osmotic | + | − | 109 | 92 (84.4 ± 3.4) | 62 (67.4 ± 10.0) [ab] | 54 | 2 (3.7 ± 3.3) |
| | Iso-osmotic | − | + | 85 | 62 (72.9 ± 4.5) | 22 (35.5 ± 13.9) [bc] | 54 | 0 (0) |
| | Iso-osmotic | + | + | 80 | 69 (86.3 ± 3.8) | 65 (94.2 ± 2.6) [a] | 61 | 4 (6.5 ± 3.6) |
| Testicular | Iso-osmotic | − | − | 245 | 190 (77.6 ± 6.6) | 102 (53.7 ± 5.9) [b] | 170 | 1 (0.6 ± 0.4) |
| | Hypo-osmotic | − | − | 123 | 94 (76.4 ± 4.4) | 14 (14.9 ± 5.5) [c] | 81 | 0 (0) |
| | Hypo-osmotic | + | − | 114 | 99 (86.8 ± 2.5) | 32 (32.3 ± 6.3) [b] | 79 | 0 (0) |
| | Hypo-osmotic | − | + | 103 | 87 (84.5 ± 3.3) | 19 (21.8 ± 3.1) [bc] | 68 | 0 (0) |
| | Hypo-osmotic | + | + | 101 | 69 (85.1 ± 2.3) | 51 (59.3 ± 8.2) [ab] | 56 | 0 (0) |

%: Mean ± SEM. [a–c] Different superscripts indicate significant differences at $P < 0.05$.

78.3%, Table 1). Therefore, AOA removal with ionomycin [25] and AR removal with Triton X-100 [26] have been applied to BN-ICSI. AOA treatment and/or acrosome removal treatment had no adverse effect on post-ICSI survival but significantly improved pronuclear formation. Some normal female offspring were delivered (3.7%–6.5%) only when AOA treatment was applied. A positive effect of AOA supplementation on ICSI performance in rodents has also been reported [18, 27]. In contrast, AR removal did not affect the *in vivo* development of BN-ICSI zygotes, which is inconsistent with a previous report [17]. Multiple physical stresses, such as sonication during tail cutting and cryopreservation in TE buffer, may be harmful to the surfactant-treated BN sperm heads. The sensitivity of spermatozoa to various physical stressors varies among rat strains [28, 29]. Offspring rates reported in inbred rat ICSI ranged from 6.1% in BN (from an experiment on ICSI-mediated transgenesis) [24] to 23.1% in F344 [20]. In the first series of TESE-ICSI experiments conducted on BN spermatozoa, we produced only one BN offspring derived from a few hundred TESE-ICSI zygotes (0.6%). The reason for the relatively high pronucleus formation rate observed in this study is unknown. Successfully produced female BN rat offspring (female) appeared normal in size without any detectable abnormalities (Supplementary Fig. 1). Thereafter, we applied the AR removal treatment to BN testicular sperm retrieved from a hypo-osmotic suspension and/or AOA treatment to BN oocytes. However, none of the BN-TESE-ICSI zygotes developed into full-term offspring, even though AOA and/or AR removal improved the pronuclear formation rate to some extent.

In conclusion, outbred WI rat offspring can be produced by using ICSI, and less efficiently by using TESE-ICSI. Difficult offspring production by ICSI in the inbred BN strain could be partially overcome by using AOA treatment. However, the inability of TESE-ICSI requires further investigation, with accurate measurements of paternal factor (PLCζ) and maternal factors controlling post-fertilization responses (MPF, APC/C, CSF, and MAPK) in these rat strains.

## Methods

### *Animals*

All the animal experiments were reviewed and approved by the Animal Care and Use Committee of the National Institute of Natural Sciences (NINS; Aichi, Japan). Specific pathogen-free male and female Wistar (Crlj:WI; RGD ID:2312504) and female BN (BN/SsN Slc; RGD ID: 1302631) rats were purchased from Charles River Japan (Kanagawa, Japan) and Japan SLC (Shizuoka, Japan), respectively. These rats were housed under controlled lighting (14L:10D), temperature (23 ± 2°C), and humidity (55 ± 10%) with free access to a laboratory diet and filtered water in the NINS. The male WI rats were used as sperm donors at 7.5–13 weeks old. Original SD transgenic male rats carrying the EGFP gene under the control of the CAG promoter, SD-Tg(CAG-eGFP) (RGD ID: 2303761), were introduced from Japan SLC and repeatedly backcrossed to the BN strain. The BN;SD-Tg(CAG-eGFP) rat line was maintained under the same atmospheric conditions at the Graduate School of Medicine, Kyoto University, and used as BN sperm donors at 30–40 weeks old. A single sperm donor did not always serve as the source of both epididymal and testicular spermatozoa.

### *Chemicals and media*

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. The medium used for the collection, temporal incubation, and ICSI of rat oocytes was mR1ECM [30], with some modifications in the supplementation of Hepes (22 mM) and reduction of sodium bicarbonate concentration (5 mM), referred to as Hepes-R1ECM. The original mR1ECM was used for the culture of post-ICSI oocytes, collection of epididymal spermatozoa, and transfer of ICSI zygotes to surrogate mothers. The medium used to prepare testicular cell suspension was calcium- and magnesium-free phosphate-buffered saline, PBS (−). The medium used for freezing epididymal and testicular spermatozoa was TE buffer solution (pH 8.0; Nacalai Tesque Inc., Kyoto, Japan). The rat sperm heads were placed in Hepes-R1ECM supplemented with 8% (w/v) polyvinylpyrrolidone (PVP, 360 kDa; Fujifilm-Wako Pure Chemicals Industries, Osaka, Japan), defined as Hepes-R1ECM/PVP, immediately before ICSI.

### *Preparation of epididymal sperm*

Sperm donors were commercially available 7.5–13 weeks old WI male rats and 30–40 weeks old BN heterozygous transgenic male rats carrying the EGFP under the control of the CAG promoter. Spermatozoa from cauda epididymis were dispersed in 2 ml of mR1ECM, and the sperm suspension was incubated for 30 min at 37°C in 5% $CO_2$ in air. Swimming-up sperm were recovered from 1 ml supernatant of the supernatant of mR1ECM and concentrated by centrifugation for 5 min at 200 × *g*. The sperm pellet was washed and suspended in 1 ml TE buffer. Sperm heads were separated from their tails by sonication for 10 sec using 10% power output from an ultrasonic cell disruptor (Sonifier 250, Branson, Danbury, CT, USA) [31]. The sperm head suspension (100 µl per cryotube, Nunc; Roskilde, Denmark) was frozen in liquid nitrogen vapor and cryopreserved at −20°C until use for the ICSI [13]. When the AR removal treatment was applied to the BN rat spermatozoa, the sperm head suspension was treated with 0.02% Triton X-100 for 1 min with vortex mixing before cryopreservation.

### *Preparation of testicular sperm*

Testicular spermatozoa were recovered from the testicular cell suspensions. In brief, a pair of rat testes were isolated in PBS (−), and the tunica albuginea and blood vessels were removed from each testis. The seminiferous tubules were finely minced by using surgical scissors and tweezers and repeatedly aspirated into a 10 ml syringe connected to an 18-G needle to release the spermatogenic cells. The testicular cell suspension was filtered through a 100 µm pore-sized cell strainer (Falcon; Corning, NY, USA) and diluted 20 times with ultrapure water (hypo-osmotic treatment) to facilitate detection and recovery of testicular spermatozoa due to removal of contaminating cytoplasm-rich spherical somatic and spermatogenetic cells. Testicular sperm-rich fractions in hypo-osmotic buffer were sonicated for tail removal and cryopreserved as described above in epididymal spermatozoa. Otherwise, the PBS (−) was used to dilute the filtrated testicular sperm suspension (conventional iso-osmotic treatment), followed by sonication and cryopreservation.

### *Preparation of oocytes*

Juvenile WI and BN female rats at 4–6 weeks old were superovulated by using intraperitoneal injections of 300 IU/kg eCG (Serotropin; ASKA Animal Health, Tokyo, Japan) and hCG (Gonatoropin; ASKA Pharmaceutical, Tokyo, Japan) at 48 or 72 h intervals [32]. The cumulus-oocyte complexes were collected from the oviductal ampullae 17–19 h after hCG injection and freed from cumulus cells by treatment with 0.1% hyaluronidase for 5–10 min. The denuded oocytes were transferred to Hepes-R1ECM supplemented with 5 µM MG-132 at 37°C to suppress the incidence of spontaneous oocyte activation [33]. When AOA treatment was applied, the denuded oocytes were

washed three times with MG132-containing Hepes-R1ECM and incubated in mR1ECM supplemented with 5 µM ionomycin for 5 min at 37°C in 5% $CO_2$ in air [23]. Then, activated oocytes were incubated for up to 40 min at 37°C in 5% $CO_2$ in air before subjecting to the ICSI.

### *ICSI procedure*

Rat ICSI was performed according to the method described previously using a piezo-driven micromanipulator (PMM-150FU; Prime Tech, Ibaraki, Japan) at an ambient temperature (23 ± 2°C) [10]. In brief, 10–15 denuded oocytes and 0.8 µl of sperm suspension thawed in 37°C water bath were placed in an 8 µl microdrop of Hepes-R1ECM and Hepes-R1ECM /PVP, respectively. A single sperm head that hung on the blunt end of the 2−4 µm diameter injection pipette was released near the oocyte, which was aspirated with holding pipette to give a 6 or 12 o'clock position of the first polar body. After drilling the zona pellucida by using a few piezoelectric pulses, the sperm head was repositioned on the tip of the injection pipette and the pipette tip was mechanically advanced deep into the center of the oocyte, extensively stretching the oolemma (Supplementary Fig. 2). Upon application of positive light pressure and a piezoelectric pulse, the oolemma was punctured at the pipette tip, and the sperm head was ejected into the oocyte. The injection pipette was withdrawn quickly. The ICSI oocytes were transferred into 800 µl of Hepes-R1ECM and incubated at an ambient temperature for 10 min for recovery of the oolemma.

### *Culture and transfer of ICSI zygotes*

The ICSI oocytes were washed three times with mR1ECM and cultured in 100 µl microdrops of the same medium covered with mineral oil at 37°C in 5% $CO_2$ in air. Morphological survival and formation of both male and female pronuclei were assessed 6 h after completion of ICSI. The next day (22 h after ICSI), surviving oocytes were transferred into the oviductal ampullae of 8–13 weeks old WI recipients, which had been mated with a vasectomized male on the day of ICSI (defined as Day 0). The number of transferred oocytes per group varied depending on the availability of the recipient animals. Live offspring were delivered from the recipients by Caesarean section on Day 21.

### *Statistical analysis*

All experiments were repeated at least thrice. The ICSI/TESE-ICSI percentage data were arcsine-transformed and analyzed by using one-way analysis of variance (ANOVA). Tukey's honest significant difference test was used for multiple comparisons when the ANOVA showed a significant difference between the experimental groups. The statistical value of $P < 0.05$ was chosen as an indication of statistical significance.

**Conflict of interests:** The authors declare that there is no conflict of interest.

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