

# Viable rat offspring derived from oocytes intracytoplasmically injected with freeze-dried sperm heads

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## Summary

A 2 × 3 factorial designed experiment was conducted in order to examine whether freeze-dried rat spermatozoa can participate in full-term development following intracytoplasmic sperm injection (ICSI). A sperm suspension from cauda epididymides of Sprague–Dawley (SD) rats was prepared with or without ultrasonic treatment. The sonicated and non-sonicated sperm suspensions were processed for freeze-thawing (FT groups; 100 µl sample was cooled in liquid nitrogen vapor, stored for 1 day at –196 °C, and thawed in a 25 °C water bath) and freeze-drying (FD groups; 100 µl sample was frozen in liquid nitrogen for 20 s, lyophilized for 6 h, stored at 4 °C for 2 days, and rehydrated with 100 µl ultrapure water), or were subjected to immediate use for ICSI (fresh control groups). The sperm heads were microinjected into denuded SD oocytes using a piezo-driven micropipette 2–4 µm in diameter. The presumptive zygotes were transferred into oviducts of pseudopregnant Wistar female rats. Viable rat offspring were produced from all six experimental groups. Ultrasonic treatment of rat spermatozoa was effective in increasing the offspring rate (23.3% vs 6.7% in fresh control groups, 35.0% vs 7.6% in FT groups, 9.2% vs 2.5% in FD groups). The acrosomal region appeared to be intact even after ultrasonic FT and FD treatments as well as in the fresh controls, while the lateral dorsal region of the sperm membrane was more or less damaged in the sonicated, FT and FD samples. Thus, the successful participation of freeze-dried spermatozoa in full-term development was demonstrated by applying ICSI in the rat.

Keywords: Freeze-dry, ICSI, Rat oocytes, Sonication

## Introduction

Since freeze-dried spermatozoa can be stored at an ambient or refrigerated temperature, the costs required for the maintenance and shipping of spermatozoa would be reduced. To date, viable offspring in mice (Wakayama & Yanagimachi, 1998; Kusakabe *et al.*, 2001; Kaneko *et al.*, 2003a, b; Ward *et al.*, 2003) and rabbits (Liu *et al.*, 2004) have been produced by intracytoplasmic sperm injection (ICSI) using freeze-dried samples.

Blastocyst development after ICSI with freeze-dried sperm heads was also reported in large domestic animals including cattle (Keskintepe *et al.*, 2002) and pigs (Kwon *et al.*, 2004).

Spermatozoal centrosomes in rodents do not contribute to zygotic cleavage (Schatten *et al.*, 1991), while those in other mammalian species (e.g. rabbit, pig, cattle and human) serve as a microtubule-organizing centre in the zygotes (Yllera-Fernandez *et al.*, 1992; Palermo *et al.*, 1997). Therefore, in rodents, treatments to dissociate the long sperm tail/mid-piece from the spermatozoon, such as freeze-thawing or sonication (Kuretake *et al.*, 1996; Hirabayashi *et al.*, 2005), would facilitate the handling and microinjection of spermatozoa. The ultrasonic treatment of mouse spermatozoa had a negative impact (Kuretake *et al.*, 1996) or a negligible influence (Tateno *et al.*, 2000) on the developmental competence of ICSI oocytes. On the other hand, mouse, hamster and human spermatozoa microinjected into hamster oocytes easily underwent decondensation of the sperm heads, but

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the decondensation of rat and bovine spermatozoa was quite time-consuming (Perreault *et al.*, 1988).

As for rats, we have recently established the protocols for microinsemination, such as ICSI (Hirabayashi *et al.*, 2002a; Kato *et al.*, 2004a), elongating spermatid injection (ELSI; Kato *et al.*, 2004a), or round spermatid injection (ROSI; Hirabayashi *et al.*, 2002b; Kato *et al.*, 2004b). The offspring rate in rat ICSI (Kato *et al.*, 2004a) approaches the level achieved in practice by mouse ICSI (Ogura *et al.*, 2003). The objectives of the present study were to investigate whether freeze-dried rat spermatozoa are capable of participating in full-term development by ICSI, and whether the ultrasonic treatment of rat spermatozoa affects the production efficiency of the ICSI-derived offspring.

## Materials and methods

### Media

The medium used for the suspension, sonication, freeze-thawing and freeze-drying of the epididymal spermatozoa was 10 mM Tris-buffer (Nacalai Tesque, Kyoto, Japan) supplemented with 50 mM NaCl (Wako Pure Chemical Industries, Osaka, Japan) and 50 mM EGTA (Sigma-Aldrich, St Louis, MO), referred to hereafter as Tris/EGTA (Kusakabe *et al.*, 2001). The medium used for the collection and temporal incubation of the oocytes and for the culture and transfer of the ICSI oocytes was modified rat 1-cell embryo culture medium, mR1ECM (Oh *et al.*, 1998), containing 110 mM NaCl and 0.4% fatty-acid-free bovine serum albumin (BSA) (Sigma-Aldrich), referred to hereafter as mR1ECM/BSA. The mR1ECM/BSA was modified by the supplementation of Hepes (Wako) at 22 mM and the reduction of sodium bicarbonate to 5 mM, referred to hereafter as Hepes-R1ECM, and used for ICSI. Hepes-R1ECM supplemented with 12% polyvinylpyrrolidone (PVP, 360 kDa; ICN Pharmaceuticals, Costa Mesa, CA) was used for placing the sperm heads immediately before their injection.

### Animals

Specific pathogen-free Slc:SD 4- to 5-week-old female rats and Slc:SD 10-week-old male rats (Japan SLC, Shizuoka, Japan) were used as oocyte donors and sperm donors, respectively. Crj:Wistar female rats that were 8–13 weeks old (Charles River Japan, Kanagawa, Japan) were used as the surrogate mothers. The rats were housed in an environmentally controlled room with a 12 h dark to 12 h light cycle at a temperature of  $23 \pm 2^\circ\text{C}$  and humidity of  $55 \pm 5\%$ , and given free access to a laboratory diet (MF; Oriental Yeast, Tokyo, Japan) and filtered water.

### Sperm preparation

Spermatozoa from a pair of cauda epididymides were allowed to disperse in 2 ml of Tris/EGTA for 30 min and then collected in a 5 ml tube. The swim-up sperm in the upper 1 ml were divided into two groups. The first 0.5 ml of the sperm suspension was sonicated for 10 s using a 10% power output from an ultrasonic cell disrupter (Sonifier 250; Branson, Danbury, CT), while the remaining 0.5 ml was kept as the non-sonicated sample. Both the sonicated and non-sonicated sperm samples were allocated to one of three groups: freeze-thawed, freeze-dried or fresh control.

In each of the freeze-thawed groups, 100  $\mu\text{l}$  of the sperm suspension was transferred to a 1.0 ml cryotube (Nunc, Roskilde, Denmark), frozen in liquid nitrogen vapor for 1 min, and stored at  $-196^\circ\text{C}$  for 24 h. The sample was thawed in a  $25^\circ\text{C}$  water bath for 1 min.

In each of the freeze-dried groups, 100  $\mu\text{l}$  of the sperm suspension was transferred to a 1.5 ml polypropylene tube (Eppendorf, Hamburg, Germany), frozen in liquid nitrogen for 20 s, and lyophilized for 6 h by a connected freeze-drying apparatus (Yamato Scientific, Tokyo, Japan). After lyophilization, the tubes were sealed with parafilm under air and stored at  $4^\circ\text{C}$  for 2 days. The sample was rehydrated by adding 100  $\mu\text{l}$  of ultrapure water (Sigma-Aldrich) to the tube.

### Morphological evaluation of sperm membrane

The membrane and acrosomal status of the rat spermatozoa after sonication, freeze-thawing and freeze-drying was compared with that of the non-treated fresh spermatozoa, as previously described (Fazeli *et al.*, 1997). Briefly, a smeared sperm suspension (3  $\mu\text{l}$ ) on a microscope slide was air-dried and fixed with absolute methanol for 10 min. The smear was counterstained with a total of 5  $\mu\text{l}$  of 5  $\mu\text{g}/\text{ml}$  fluorescein isothiocyanate (FITC)-labeled peanut agglutinin (PNA, *Arachis hypogaea*) (FITC-PNA; Sigma-Aldrich) and 4  $\mu\text{M}$  ethidium homodimer-1 (EthD-1; Molecular Probes, Eugene, OR) for 30 min at  $37^\circ\text{C}$ . After being rinsed with phosphate-buffered saline (PBS) and mounted with coverslips, the slides were examined under an epifluorescence microscope (TE-300; Nikon, Tokyo, Japan) equipped with a DFT set of filters. Approximately 40–50 spermatozoa per treatment group were observed.

### ICSI

The ICSI experiments were performed for three successive days (fresh groups on day 1, freeze-thawed groups on day 2, freeze-dried groups on day 3) and replicated three times. Female rats were superovulated by intraperitoneal injections of 300 IU/kg equine chorionic gonadotropin (eCG; Nippon Zenyaku

Kogyo, Fukushima, Japan) and 300 IU/kg human chorionic gonadotropin (hCG; Sankyo Yell Yakuhin, Tokyo, Japan) at an interval of 48 h (Hirabayashi *et al.*, 2001). The cumulus-oocyte complexes were collected from the oviductal ampullae 14–17 h after the hCG injection and were freed from the cumulus cells by a 5 min treatment with 0.1% hyaluronidase (Sigma-Aldrich). The denuded oocytes were washed three times with mR1ECM/BSA and incubated for up to 45 min at 37 °C in 5% CO<sub>2</sub> in air.

The sperm heads were injected into the denuded oocytes at ambient temperature (23 ± 2 °C) using a piezo impact-driving unit (PMM-140FU; Prime Tech, Ibaraki, Japan) with a pulse controller (PMAS-CT140; Prime Tech), as previously described (Hirabayashi *et al.*, 2002a). Briefly, the sperm head that hung on the blunt end of the 2–4 µm diameter injection pipette was released near the oocyte. After zona drilling by a few piezo pulses, the sperm head was hung on the blunt end of the pipette again. The pipette tip was mechanically advanced deep into the oocyte, significantly stretching the oolemma. Upon application of a light positive pressure and a piezo pulse, the oolemma was punctured at the pipette tip and the sperm head was ejected into the oocyte. The injection pipette was then quickly withdrawn.

### Culture and transfer of ICSI oocytes

The ICSI oocytes were washed three times with mR1ECM/BSA and cultured in 100 µl microdrops of the same medium covered with mineral oil (Sigma-Aldrich) at 37 °C in 5% CO<sub>2</sub> in air. The morphological survival rates and cleavage rates were assessed at 6 and 24 h, respectively, after completing the ICSI. Oocyte activation rate was also assessed at 6 h after the ICSI. Presumptive zygotes at the 2-cell stage and non-degenerating 1-cell stage were harvested at 24 h post-ICSI, and then transferred into the oviductal ampullae of the recipient rats which had been previously mated with a vasectomized male rat. Embryo transfer to the pseudopregnant recipients (12–34 embryos per recipient) was performed on the day that the vaginal plug was detected. The pregnant recipients were allowed to deliver the offspring.

### Statistical analysis

The percentage data in each replicate were arcsin-transformed before being subjected to one-way analysis of variance. Significant differences were determined by Fisher's protected least significant difference test using the StatView program (Abacus Concepts, Berkeley, CA). A value of  $p < 0.05$  was chosen as an indication of statistical significance.

## Results

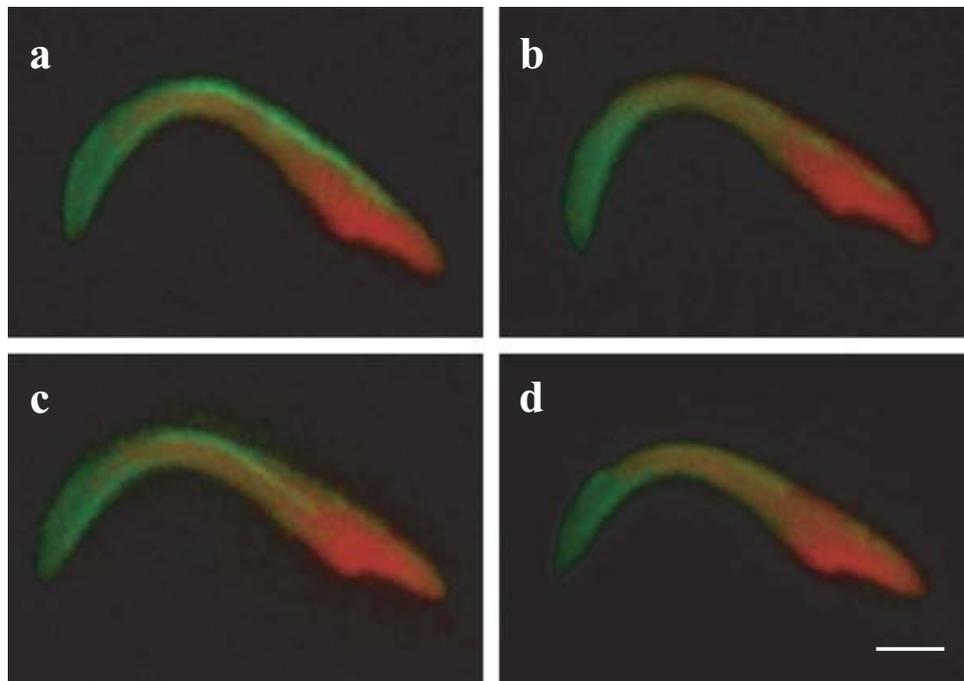
The ultrasonic treatment of the rat spermatozoa completely separated their tails/mid-pieces from the sperm heads, while less than 10% of the spermatozoa after freeze-thawing or freeze-drying were free of their tails/mid-pieces. Almost all spermatozoa in each treatment showed similar morphological characteristics in their acrosome and plasma membrane. As shown in Fig. 1, the staining of the sperm heads with FITC-PNA and EthD-1 demonstrated more damage to the sperm membrane in the sonicated, freeze-thawed and freeze-dried sperm samples than in the non-treated sperm samples. The sperm membrane in the lateral dorsal region of the sonicated sample (Fig. 1b) and the freeze-dried sample (Fig. 1d) appeared to be more disrupted than that in the freeze-thawed sample (Fig. 1c). Meanwhile, the acrosomal region (tip of sperm head) appeared to be intact irrespective of the type of sample (Fig. 1a–d).

The *in vitro* development of rat oocytes microinseminated with fresh, freeze-thawed and freeze-dried spermatozoa with or without ultrasonic treatment is shown in Table 1. There were no significant differences in the proportions of oocytes that survived ICSI among the six experimental groups (83.6–91.4%). Oocyte activation rates were also comparable among the groups (70.2–92.9%) except for non-sonicated/freeze-thawed group (33.6%). However, the oocytes microinseminated with sonicated sperm heads showed higher initial cleavage rates, especially in the fresh and freeze-thawed groups (35.4% and 48.6%, respectively).

The full-term developmental potential of the microinseminated rat oocytes is shown in Table 2. The oocytes microinseminated with sonicated sperm heads showed higher offspring rates in the fresh and freeze-thawed groups (23.3% and 35.0%, respectively). The ICSI oocytes with sonicated/freeze-dried sperm heads developed into live offspring at a significantly lower rate (9.2%) than those from the sonicated/freeze-thawed sperm heads (35.0%,  $p < 0.05$ ). The difference in the offspring rate between the sonicated/fresh group (23.3%) and sonicated/freeze-dried group (9.2%) was likely to be of statistical significance ( $p = 0.06$ ). Most of the rat offspring were born viable and appeared to be normal in size, except for one stillborn in the sonicated/freeze-dried group.

## Discussion

The ICSI-derived rat offspring obtained in the present study using freeze-dried spermatozoa demonstrate that the rat is the third species after the mouse (Wakayama & Yanagimachi, 1998) and rabbit (Liu *et al.*, 2004) whose freeze-dried spermatozoa can



**Figure 1** Acrosomal (green: FITC-PNA) and nuclear (red: EthD-1) status of the rat sperm heads. The acrosomal region (tip of sperm head) appeared to be intact irrespective of the treatments, while the lateral dorsal region of the sperm membrane was more or less damaged in the sonicated, the freeze-thawed and the freeze-dried samples. (a) Non-treated fresh control. (b) Ultrasonically treated. (c) Freeze-thawed. (d) Freeze-dried. Scale bar represents 2  $\mu\text{m}$ .

**Table 1** *In vitro* survival, activation and first cleavage of rat oocytes microinseminated with fresh, freeze-thawed and freeze-dried spermatozoa

Sperm storage condition	Ultrasonic treatment	No. (%) of oocytes			
		Injected	Survived	Activated	Cleaved
Fresh control	–	101	92 (91.1)	68 (73.9) <sup>a</sup>	14 (15.2) <sup>abc</sup>
	+	109	99 (90.8)	87 (87.9) <sup>a</sup>	35 (35.4) <sup>bc</sup>
Freeze-thawed	–	146	122 (83.6)	41 (33.6) <sup>b</sup>	9 (7.4) <sup>ab</sup>
	+	116	105 (90.5)	96 (91.4) <sup>a</sup>	51 (48.6) <sup>c</sup>
Freeze-dried	–	136	124 (91.2)	87 (70.2) <sup>a</sup>	0 (0.0) <sup>a</sup>
	+	139	127 (91.4)	118 (92.9) <sup>a</sup>	10 (7.9) <sup>ab</sup>

<sup>a–c</sup>Different superscripts within columns denote significant differences at  $p < 0.05$ .

**Table 2** Full-term development of rat oocytes microinseminated with fresh, freeze-thawed and freeze-dried spermatozoa

Sperm storage condition	Ultrasonic treatment	No. of oocytes transferred [Range per recipient]	No. of recipients pregnant/used	No. (%) of offspring
Fresh control	–	90 [16–34]	2/4	6 (6.7) <sup>a</sup>
	+	86 [14–28]	4/4	20 (23.3) <sup>bc</sup>
Freeze-thawed	–	105 [13–29]	4/5	8 (7.6) <sup>ab</sup>
	+	103 [20–34]	4/4	36 (35.0) <sup>c</sup>
Freeze-dried	–	119 [14–30]	2/5	3 (2.5) <sup>a</sup>
	+	120 [12–24]	4/6	11* (9.2) <sup>ab</sup>

<sup>a–c</sup>Different superscripts within columns denote significant differences at  $p < 0.05$ .

\*Including 1 stillborn offspring.

participate in full-term development after ICSI. In the mouse, the offspring rate after ICSI with freeze-dried spermatozoa was comparable to that with frozen-thawed spermatozoa (Kusakabe *et al.*, 2001; Ward *et al.*, 2003). However, a significantly lower offspring rate after ICSI with freeze-dried rat spermatozoa was noted (9.2% vs 35.0% in frozen-thawed group; Table 2), suggesting that chromosomal damage and deterioration of the sperm-borne oocyte-activating factor (SOAF) may be induced during the freeze-drying process of rat spermatozoa. Since more than 90% of freeze-dried rabbit spermatozoa carried a normal chromosome set, the impaired development of the rabbit embryos produced by ICSI with freeze-dried spermatozoa has been suggested to result from the deterioration of the SOAF molecules (Liu *et al.*, 2004). If so, assisting oocyte activation prior to ICSI may contribute to improving the offspring rate. Since the tube containing the freeze-dried sperm sample was sealed under air in the present study, the drying sperm may absorb moisture from the air and be oxidized. If cells are exposed to radical oxygen species, some DNA damage may be induced (Potts, 1994). The chromosomal integrity and SOAF activity of freeze-dried rat spermatozoa, the air-conditioning optimal for freeze-drying, as well as the possibility of long-term storage without a liquid nitrogen tank, remain to be investigated further.

In the present study, the ultrasonic treatment of rat spermatozoa contributed to improving the offspring rate after ICSI (Table 2), suggesting that the sperm membrane disruption induced by sonication may be favorable for the subsequent decondensation of sperm heads and the transformation into male pronucleus, because the ICSI procedure bypasses a series of physiological changes in the membranes. In addition, the oocyte activation rates shown in Table 1 suggest that accelerated interaction of SOAF molecules with ooplasm is associated with the ultrasonic treatment of the spermatozoa. During normal fertilization, the membrane-vesicle-associated membrane proteins (VAMP) are lost from the acrosome-reacted spermatozoa, and the nuclear matrix proteins (NuMA) appear in the decondensing sperm heads (Hewitson *et al.*, 1999). In the rhesus monkey, microinjection of acrosome-intact sperm heads resulted in a delay of sperm head decondensation (Sutovsky *et al.*, 1996). These authors suggested that persistence of VAMP in the sperm heads enclosed by perinuclear theca is responsible for this delay in nuclear decondensation. The PNA lectin-staining employed here (Fig. 1) was not fully effective for interpreting whether the complete removal of membrane-associated components plays an important role in improving the efficiency of ICSI, and further ultrastructural or cytochemical evidence is required.

An interspecies microinsemination assay showed that no rat or bovine spermatozoa decondensed in hamster oocytes even 1 h after ICSI, while the sperm heads from mouse, chinchilla, hamster and human enlarged within 1 h (Perreault *et al.*, 1988). In our preliminary study, decondensed rat sperm heads were not observed until at least 2 h after ICSI (data not shown), suggesting stable nuclear packaging in rat sperm heads. Perreault *et al.* (1988) reported that the treatment of rat spermatozoa with a disulfide-reducing agent, dithiothreitol (DTT), was partially effective in accelerating decondensation of the sperm heads. In cattle, the *in vitro* development of ICSI oocytes was improved by pretreatment of the spermatozoa with DTT (Rho *et al.*, 1998). Also in porcine ICSI, failed or abnormal decondensation of sperm chromatin occurs frequently. Treatment of boar spermatozoa with progesterone prior to ICSI was effective in inducing the acrosome reaction and promotion of male pronucleus formation (Katayama *et al.*, 2002). On the other hand, ultrasonic treatment of mouse spermatozoa had a negative impact (Kuretake *et al.*, 1996) or a negligible influence (Tateno *et al.*, 2000) on the developmental competence of ICSI oocytes. The difference in the effect of sonication between the rat and mouse may be due to the different profiles of p34<sup>cdc2</sup> kinase kinetics in the ovulated oocytes. The basal level of p34<sup>cdc2</sup> kinase activity in the rat oocytes was less than half that of the mouse oocytes and further decreased during the process of spontaneous activation, a unique characteristic in rat oocytes (Ito *et al.*, 2005). The ultrasonic treatment of rat sperm heads may destabilize the nuclear packaging as well as the membrane structures, and thus facilitate decondensation even in oocytes with a low p34<sup>cdc2</sup> kinase activity. In contrast, Kishigami *et al.* (2004) reported that pre-activated mouse oocytes with low p34<sup>cdc2</sup> kinase activity supported male pronucleus formation after ICSI. This means that mouse sperm head can decondense in conditions of low MPF activity of oocytes, suggesting a difference between mouse and rat.

It is concluded that viable rat offspring can be obtained by intracytoplasmic injection of freeze-dried and rehydrated spermatozoa, and that the ultrasonic treatment of spermatozoa, regardless of the subsequent freeze-thawing or freeze-drying, is effective in increasing the production of ICSI-derived offspring.

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