Offspring Derived from Intracytoplasmic Injection of Sonicated Rat Sperm Heads

*Michiko Nakai[1], Eri Saito[1], Akiko Takizawa[1], Yoko Akamatsu[1], Maiko Koichi[1], Shin Hisamatsu[2], Tomo Inomata[3], Masao Shino[1] and Naomi Kashiwazaki[1]**

[1]*Laboratory of Animal Reproduction, Graduate School of Veterinary Medicine,*
[2]*Laboratory of Environmental Chemistry, Graduate School of Environmental Health,*
[3]*Laboratory of Experimental Animal Science, Graduate School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa 229-8501, Japan*

Abstract: *The present study investigated the effect of separation of spermatozoa by sonication or Piezo-pulse on in vitro development of oocytes injected with sperm heads in the rat. We also examined development to term of rat oocytes injected with sperm heads. Rat frozen-thawed spermatozoa were separated into heads and tails by sonication for 10 sec or Piezo-pulse in KRB medium, and each treated sperm head was injected into an ooplasm. The oocytes were observed for formation of two pronuclei and development to 2-cell embryos. The percentages of formation of two pronuclei and development to the 2-cell stage did not significantly (P>0.05) differ between the two groups. Oocytes injected with sonicated sperm heads that reached the pronuclear stage at 10 h after injection of sperm heads were transferred into 7 recipients. Five recipients became pregnant, and 8 living pups were obtained. The results indicate that rat oocytes injected with sonicated sperm heads can develop to term in vivo. Furthermore, no difference was observed in the development in vitro between rat oocytes injected with sperm heads separated by sonication or by Piezo-pulse.*

Key words: *Intracytoplasmic injection of sperm heads, Rat, Sonication, Sperm head, Piezo-pulse*

Introduction

Intracytoplasmic sperm injection (ICSI) is an established technique in many mammals, including humans [1, 2], not only for generating normal offspring [3–6] when their spermatozoa lack motility, causing infertility, but also for understanding the complex fertilization process [7]. Rats are invaluable as experimental animals, and ICSI is a very useful tool for strain preservation and reproducing offspring derived from gene banks. However, as rat spermatozoa have long tails [8], sonicated sperm heads have been used for injection to improve injection efficiency [3, 9]. On the other hand, spermatozoa were reportedly damaged by sonication, since oocytes injected with sperm heads developed more poorly than those injected with whole sperm [5].

The present study investigated the effect of separation of spermatozoa by sonication or Piezo-pulse on development to the 2-cell stage *in vitro* of oocytes injected with sperm heads in the rat. We also examined development to term of the oocytes following intracytoplasmic injection with sonicated sperm heads.

Materials and Methods

Media

The medium used for the culture of oocytes was Krebs-Ringer bicarbonate solution (KRB) composed of 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 5.0 mM $NaHCO_3$, 5.56 mM glucose, 21.58 mM sodium lactate, 0.5 mM sodium pyruvate, 4 mg/ml BSA (A-9418; Sigma, St. Louis, MO), 75 mg/ml potassium penicillin G, 50 mg/ml streptomycin sulphate [10]. The medium used for manipulation of oocytes and spermatozoa was KRB supplemented with 20.0 mM Hepes and reduced $NaHCO_3$ (5.0 mM).

Received: April 20, 2005
Accepted: June 2, 2005
*To whom correspondence should be addressed.
e-mail: nkashi@azabu-u.ac.jp

Preparation of oocytes and spermatozoa

Wistar female rats, 3–4 weeks old, were each induced to superovulate by i.p. injection of 5.0 IU eCG (Teikoku Zohki, Tokyo, Japan) followed by 5.0 IU hCG (Sankyo, Tokyo, Japan) 48 h later. Oocytes were collected from oviducts at 16 h after the hCG injection. They were freed from cumulus by treatment with 0.1% (w/v) hyaluronidase from bovine testis (Sigma) in KRB-Hepes for 1 min. The cumulus free oocytes were washed three times in KRB, and kept in a CO_2 incubator (5% CO_2 in air at 37°C) for 1 h until intracytoplasmic injection.

Epididymides from Wistar male rats, (15–30 weeks old), were collected. Epididymal spermatozoa were then collected and frozen as described previously [11], and thawed in KRB-Hepes. The spermatozoa were then separated into heads and tails by sonication for 10 sec or Piezo-pulse (PMAS-CT150; Prime Tech Ltd., Tsuchiura, Japan). The sonication treatment was performed using 100% power output (300 W, 40 kHz) of an UT-305 ultrasonic cleaner (Sharp Manufacturing Systems Corporation, Osaka, Japan). After the sonication treatment, more than 80% of the spermatozoa were separated into heads and tails. The sonicated sperm heads were used for intracytoplasmic injection within 60 min after sonication. Separation of sperm heads by Piezo-pulse was performed using a pipette with an external diameter of 2–3 μm and a strong pulse (intensity 5, speed 5). One spermatozoon was first sucked into the pipette tail, and a strong pulse was then applied to cut the sperm tail when the head-tail junction was in the tip of the pipette [12].

All procedures in the present study were in accordance with the guidelines approved by the Animal Research Committee of Azabu University.

Intracytoplasmic injection of sperm heads

Intracytoplasmic injection of sperm heads was conducted with the aid of a pair of micromanipulators (MB-U; Narishige, Tokyo, Japan) on an inverted microscope (IX-70; Olympus, Tokyo, Japan), which was equipped with Hoffman modulation contrast. About 15 oocytes were transferred into 20 μl drops of KRB-Hepes. The solution containing oocytes was placed on the cover of a plastic dish (90 mm × 15 mm; Kanto Chemical, Tokyo, Japan). A small volume (0.5 μl) of the sperm heads suspension was transferred to 2 μl drops of KRB-Hepes supplemented with 12% polyvinylpyrrolidone (MW 400000; Sigma), which was prepared close to the drops for the oocytes. All the drops were covered with paraffin oil (Kanto Chemical). Microinjection of sperm heads into oocytes was performed using a Piezo-driven pipette that was prepared from borosilicate glass capillary tubes (Sutter Instrument Co., Novato, CA). The external diameter of the pipette tip was 4–5 μm. A single sperm head was aspirated into an injection pipette from the suspension and was moved to the drop containing oocytes. It was then injected into the ooplasm using a Piezo-actuated micromanipulator (PMAS-CT150; Prime Tech Ltd).

Development in vitro of oocytes injected with sperm heads

Oocytes with two pronuclei at 10 h after injection were investigated, and were observed for development to 2-cell stage embryos at 24 h of the culture under an inverted microscope. The oocytes were cultured in a 50 μl droplet of KRB in 5% CO_2 in air at 37°C.

Transfer of sperm head injected oocytes to recipients

The oocytes injected with a sonicated sperm head that reached the pronuclear stage at 10 h after injection were transferred into recipient female Wistar rats (8–15 weeks old). The females had been mated with vasectomized males of the same strain for pseudo-pregnancy induction on day-0. The oocytes were surgically transferred into the oviducts (10 per oviduct) of the recipients on day-1. The recipients were anesthetized by i.p. injection of 9 mg/head pentobarbital sodium (Nembtal: Dainippon Pharmaceutical Co., LTD). The recipient rats were sacrificed on day-20 after transfer, and pregnancy and the number of fetuses were confirmed. Obtained fetuses were fostered to lactating females.

Statistical analysis

All data were analyzed by the χ^2 test, and differences with $P<0.05$ were considered statistically significant.

Results

Development in vitro of oocytes injected with sperm heads

The percentages of formation of two pronuclei and development to the 2-cell stage after intracytoplasmic injection with sperm heads that were separated into heads and tails by Piezo-pulse or sonication are shown in Table 1. The percentages of formation of two pronuclei and development to the 2-cell stage were not significantly different ($P>0.05$) between the two groups.

Development in vivo of oocytes injected with sperm heads

The results of injected oocytes transferred to recipients are shown in Table 2. Five of seven recipients were found to be pregnant, and we obtained

Table 1. *In vitro* development of rat oocytes following intracytoplasmic injection of sperm heads which were separated into heads and tails by Piezo-pulse or sonication

Methods for separation into sperm heads and tails	No. (%) of oocytes		
	Injected	2PN [a]	2-cell [b]
Piezo-pulse	134	92 (68.7)	53 (40)
Sonication	174	112 (64.4)	55 (31.6)

[a] Oocytes with two pronuclei at 10 h after sperm head injection. [b] Development to the 2-cell stage at 24 h after sperm head injection.

Table 2. *In vivo* development of rat oocytes following intracytoplasmic injection of sperm heads which were separated into heads and tails by sonication

No. of transferred oocytes	No. of recipients	No. of pregnancies (%)	No. of offspring (%)
128	7	5 (71)	8 (6)

8 living pups, which looked morphologically normal. The pups were nursed by foster mothers, all survived until the weaning period.

Discussion

We confirmed that it is possible to produce rat offspring by intracytoplasmic injection with sonicated sperm heads. Hirabayashi *et al.* [3] and Said *et al.* [9] produced normal rat offspring from oocytes injected with sperm heads separated from tails by sonication, with 2–4% [9] and 2–10% [3] of oocytes transferred developing to term, respectively; we were also able to obtain normal offspring with equal rates (6%) in the present study.

In most mammals, the sperm provides a centrosome from its neck region, which is the source of the sperm aster and plays an important role in the completion of normal fertilization [13, 14]. However, in laboratory rodents (mice [15], hamster [16, 17], rat [18]), it is not the sperm centrosome but the centrosome of maternal origin which is important therefore the sperm centrosome is not important for the development of rat embryos.

Iritani and Hosoi [19] have reported that the fertilization rates of rabbit oocytes injected with a sperm head obtained by sonication are significantly lower than those of oocytes injected with one intact sperm. Furthermore, Goto [5] suggested that sonication of spermatozoa might cause the poor development of injected bovine oocytes. It was suggested that prolonged exposure of sonicated sperm nuclei to Na^+-rich medium is detrimental to sperm chromosomes, because the cytosolic concentration of K^+ is much higher than that of Na^+ in all cells [20]. The rates of development of oocytes *in vitro* to the 2-cell stage were not significantly different between the sonication group and the Piezo-pulse group, although Na^+-rich KRB was used for sonication and suspension of spermatozoa in the present study. The reason for this may be that we used sonicated sperm heads which were not exposed to KRB for long time. Martin *et al.* [21] and Rybouchkin *et al.* [22] also reported that a Na^+-rich medium might induce sperm chromosomal damage. Lopes *et al.* [23], in addition, reported a detrimental influence between the percentage of sperm DNA fragmentation and ICSI fertilization rate. A K^+-rich medium such as NIM medium [24] may improve developmental competence of the oocytes injected with sonicated sperm heads.

The sonicated sperm heads were used for a transfer test in the present study, because there were no significant differences between Piezo-pulse and sonication in the percentages of formation of two pronuclei and development to the 2-cell stage, and because sonication treatment can separate sperm heads from tails more easily than the Piezo-pulse treatment. However, further studies are needed to assess the effect of separation of spermatozoa by sonication or Piezo-pulse on *in vitro* and *in vivo* development of oocytes injected with sperm heads in rats.

In conclusion, rat oocytes injected with sonicated sperm heads can develop to term *in vivo*. In addition, no difference was observed in development *in vitro* between rat oocytes injected with sperm heads separated by sonication and by Piezo-pulse.

References

1) Van Steirtegham, A.C., Nagy, Z., Joris, H., Liu, J., Staessen, C., Smith, J., Wisanto, A. and Devroey, P. (1993): High fertilization and implantation rates after intracytoplasmic sperm injection. Hum. Reprod., 8, 1061–1066.
2) Tesarik, J. (1996): Fertilization of oocytes by injection spermatozoa, spermatids and spermatocytes. Rev. Reprod., 1, 149–152.
3) Hirabayashi, M., Kato, M., Aoto, T., Sekimoto, A., Ueda, M., Miyoshi, I., Kasai, N. and Hochi, S. (2002): Offspring derived from intracytoplasmic injection of transgenic rat sperm. Transgenic. Res., 11, 221–228.
4) Kimura, Y. and Yanagimachi, R. (1995): Intracytoplasmic sperm injection in the mouse. Biol. Reprod., 52, 709–720.
5) Goto, K. (1993): Bovine microfertilization and embryo transfer. Mol. Reprod. Dev., 36, 288–290.
6) Nakai, M., Kashiwazaki, N., Takizawa, A., Hayashi, Y., Nakatsukasa, E., Fuchimoto, D., Noguchi, J., Kaneko, H., Shino, M. and Kikuchi, K. (2003): Viable piglets generated from porcine oocytes matured in vitro and fertilized by intracytoplasmic sperm head injection. Biol. Reprod., 68, 1003–1008.
7) Nakai, M., Kikuchi, K., Koichi, M. and Kashiwazaki, N. (2004): Intracytoplasmic sperm injection in the pig: sperm-based approaches to improve viability of embryos generated by ICSI (in Japanese). J. Mamm. Ova. Res., 21, 185–191.
8) Clouthier, D.E., Avarbock, M.R., Maika, S.D., Hammer, R.E. and Brinster, R.L. (1996): Rat spermatogenesis in mouse testis. Nature, 381, 418–421.
9) Said, S., Han, M.S. and Niwa, K. (2003): Development of rat oocytes following intracytoplasmic injection of sperm heads isolated from testicular and epididymal spermatozoa. Theriogenology, 60, 359–369.
10) Toyoda, Y. and Chang, M.C. (1974): Fertilization of rat eggs in vitro by epididymal spermatozoa and the development of eggs following transfer. J. Reprod. Fertil., 36, 9–22.
11) Nakatsukasa, E., Inomata, T., Ikeda, T., Shino, M. and Kashiwazaki, N. (2001): Generation of live rat offspring by intrauterine insemination with epididymal spermatozoa cryopreserved at –196°C. Reproduction, 122, 463–467.
12) Yamauchi, Y., Yanagimachi, R. and Horiuchi, T. (2002): Full-term development of golden hamster oocytes following intracytoplasmic sperm head injection. Biol. Reprod., 67, 534–539.
13) Hewitson, L., Simerly, C. and Schatten, G. (2000): Cytoskeletal aspects of assisted reproduction. Semin. Reprod. Med., 18, 151–159.
14) Navara, C.S., Hewitson, L.C., Simerly, C.R., Sutovsky, P. and Schatten, G. (1997): The implications of a paternally derived centrosome during human fertilization: consequences for reproduction and the treatment of male factor infertility. Am. J. Reprod. Immunol., 37, 39–49.
15) Schatten, H., Schatten, G., Mazia, D., Balczon, R. and Simerly, C. (1986): Behavior of centrosomes during fertilization and cell division in mouse oocytes an in sea urchin eggs. Proc. Natl. Acad. Sci. USA., 83, 105–109.
16) Hewitson, L., Haavisto, A., Simerly, C., Jones, J. and Schatten, G. (1997): Microtubule organization and chromatin configurations in hamster oocytes during fertilization and parthenogenetic activation, and after insemination with human sperm. Biol. Reprod., 57, 967–975.
17) Shin, T.Y., Noguchi, Y., Yamamoto, Y., Mochida, K. and Ogura, A. (1998): Microtubule organization in hamster oocytes after fertilization with mature spermatozoa and round spermatids. J. Reprod. Dev., 44, 185–189.
18) Navara, C.S., Wu, G.J., Simerly, C. and Schatten, G. (1995): Mammalian model systems for exploring cytoskeletal dynamics during fertilization. Curr. Top. Dev. Biol., 31, 321–342.
19) Iritani, A. and Hosoi, Y. (1989): Microfertilization by various methods in mammalian species. In: Development of Preimplantation Embryos and Their Environment (Yoshinaga, K. and Mori, T., eds), pp. 145–149, Alan R. Liss, New York.
20) Tateno, H., Kimura, Y. and Yanagimachi, R. (2000): Sonication per se is not as deleterious to sperm chromosomes as previously inferred. Biol. Reprod., 63, 341–346.
21) Martin, R.H., Ko, E. and Rademaker, A. (1988): Human sperm chromosome complements after microinjection of hamster eggs. J. Reprod. Fertil., 84, 179–186.
22) Rybouchkin, A.V., De Sutter, P. and Dhont, M. (1996): Unprotected freezing of human spermatozoa exerts a detrimental effect on their oocyte activating capacity and chromosome integrity. Zygote, 4, 263–268.
23) Lopes, S., Sun, J.G., Jurisicova, A., Meriano, J. and Casper, R.F. (1998): Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertil. Steril., 69, 528–532.
24) Kuretake, S., Kimura, Y., Hoshi, K. and Yanagimachi, R. (1996): Fertilization and development of mouse oocytes injected with isolated sperm heads. Biol. Reprod., 55, 789–795.