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= Research Note =

Co-cultured sperm remaining after *in vitro* fertilization is developmentally competent to term by intracytoplasmic injection in rats

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ABSTRACT

Although *in vitro* fertilization (IVF) protocol using cryopreserved sperm has been quite recently established in rats, the developmental competence of ICSI (intracytoplasmic sperm injection) oocytes is lower in rats than that in mice. In several mammalian species, ICSI is a way to routinely obtain offspring derived from zygotes fertilized by cryopreserved sperm. If spermatozoa remaining after IVF can be used for ICSI, large numbers of offspring derived from the same male rat can be obtained by treatment combining IVF and ICSI. In the present study, we examined whether spermatozoa remaining after IVF can be utilized for ICSI in rats. Spermatozoa after IVF were recovered and then cryopreserved in cryotubes. After thawing, the spermatozoa were used for ICSI (IVF-ICSI group). As a control, spermatozoa were frozen-thawed in straws and then used for ICSI. In the control, the rates of pronuclear formation, two-cell, and blastocyst stages were 88.6 ± 4.8%, 63.8 ± 8.3%, and 25.1 ± 5.5%, respectively. In the IVF-ICSI group, these rates were 90.4 ± 4.6%, 73.2 ± 7.7%, and 36.4 ± 5.1%, respectively. In the IVF-ICSI group, offspring were obtained by embryo transfer. The present results show that co-cultured sperm remaining after IVF can be used for ICSI and that this procedure can probably be applied to generate offspring from banked sperm in rats.

Key words: intracytoplasmic sperm injection, *in vitro* fertilization, rat

INTRODUCTION

In vitro fertilization (IVF) is now an indispensable technology in various fields, including human clinical medicine [8]. Moreover, IVF is only the procedure for the efficient production of genetically valuable experimental animals, such as transgenic and knockout animals from preserved germ cells, since many oocytes can be fertilized at the same time. In mice, an IVF protocol using cryopreserved sperm was established in the 1990s [21,22,24]. In rats, on the other hand, IVF using cryopreserved sperm has only quite recently succeeded [18]. Although the developmental ability of rat IVF oocytes is still lower than that of mice and further improvements are needed, it is expected to become a routine technology in the near future. In IVF, some spermatozoa are fertilized with oocytes whereas others are not. In our rat IVF protocols, 5×10^5 sperm/ml was used to inseminate 40–50 oocytes in a 200 µl drop [18], which means that less than 1% of the spermatozoa could fertilize those oocytes. In the general IVF protocol, these unsuccessful spermatozoa are discarded after IVF.

As an alternative method to IVF, intracytoplasmic sperm injection (ICSI) is an established technology, having succeeded in a number of mammalian species, including rats [1,2,4,7,12,15,20,23]. In rodents, ICSI has

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been applied to new technologies, such as the generation of offspring using sperm derived from spermatogonial stem cells [19], embryonic stem cells, and induced pluripotent stem cells [5]. Also in rats, ICSI is used to obtain offspring routinely derived from genetic resources such as *N*-ethyl *N*-nitrosourea (ENU) mutagenesis [9]. For successful fertilization using a limited number of sperm, ICSI seems to be the most optimal technology. This leads us to the possibility that, if spermatozoa remaining after IVF can be used for ICSI, a much greater number of offspring derived from the same male rat will be obtained. Moreover, if IVF is unsuccessful for some unexpected reason, the remaining sperm could be easily preserved and then used for rescue by ICSI, which requires no cryoprotectants or complicated procedures [17]. For the production of genetically valuable rats, especially spontaneously mutated ones, the combination of IVF and ICSI will be a promising procedure. However, there have been very few studies in which these treatments were combined, at least in rats. Here we conducted a preliminarily whether or not spermatozoa remaining after IVF can be used for ICSI in rats.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. All procedures for the handling and treatment of the animals were conducted according to the guidelines established by the Animal Research Committee of Azabu University.

Animals

Wistar rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). The rats were housed in an environmentally controlled room with a 12-h dark/12-h light cycle at a temperature of 23 ± 2°C and humidity of 55 ± 5%, with free access to a laboratory diet and filtered water. Mature males (12–24 weeks old) and immature females (4–5 weeks old) were used as sperm and oocyte donors, respectively. Vasectomized male rats (15–30 weeks old) were used to induce pseudopregnancies. Although the vasectomized rats in our study were of the same strain as the other rats (Crlj: Wistar), we confirmed the sterility of the vasectomized male rats as part of this preliminary study.

Media

The culture medium for *in vitro* development of the ICSI oocytes was rat one-cell embryo culture medium (R1ECM) containing 76.7 mM NaCl, 3.2 mM KCl, 0.5 mM $MgCl_2$, 2.0 mM $CaCl_2$, 25.0 mM $NaHCO_3$, 7.5 mM D-glucose, 0.5 mM sodium pyruvate, 10.0 mM sodium lactate, 0.1 mM glutamine, 2% (v/v) minimal essential medium (MEM) essential amino acid solution (50x; Gibco BRL, Grand Island, NY, USA), 1% (v/v) MEM nonessential amino acid solution (100×; Gibco BRL) and 1 mg/ml PVA [10]. The base medium used for sperm collection and incubation was R1ECM-increased NaCl (110 mM) supplemented with 4 mg/ml of fatty-acid-free BSA instead of PVA (modified R1ECM, mR1ECM) [14]. For sperm cryopreservation, the freezing medium contained 23% (v/v) egg yolk, 8% (w/v) lactose monohydrate, antibiotics (1000 IU/ml penicillin G potassium, 1 mg/ml streptomycin sulphate), and 0.7% (v/v) Equex STM Paste (Nova Chemical Sales, Inc., Scituate, MA, USA) [13].

Oocyte collection and IVF

The females were superovulated by intraperitoneal injections of 30 IU equine chorionic gonadotropin (eCG; Nippon Zenyaku Kogyo Co., Tokyo, Japan) and 30 IU human chorionic gonadotropin (hCG; Asuka Pharmaceutical Co., Tokyo, Japan) at 48 h intervals, as reported by Fujiwara *et al*. [3]. The IVF protocol was carried out according to our previous report [18]. Fresh epididymal sperm (2 µl) was directly added in a paraffin-oil-covered 200 µl drop of mR1ECM (final sperm concentration: 5×10^5 sperm/ml) and cultured for 5 h. Twelve to 14 h after the hCG injection, the oviduct ampullae of the donor females were placed in oil, and cumulus-oocyte complexes (COCs) were collected from the oviductal ampullae. The COCs were transferred in the drops containing the sperm and co-cultured at 37°C under 5% CO_2 in air for 10 h. After the culture, COCs were transferred into a 100 µl drop of mR1ECM and cumulus cells were removed by being drawn up repeatedly into a fine pipette. The denuded oocytes were evaluated using an inverted phase-contrast microscope (IX70; Olympus, Tokyo, Japan). Oocytes having two pronuclei (2PN) and at least one sperm tail in the vitellus were considered fertilized. After IVF, 20 µl mR1ECM including spermatozoa (5×10^5 sperm/ml) were collected in a cryotube. The cryotube was just plunged into liquid nitrogen and stored until use.

ICSI

The spermatozoa in the straws were thawed in a 37°C water bath for 15 sec then diluted in 1 ml of

R1ECM containing 22 mM Hepes and 5 mM $NaHCO_3$ (Hepes-R1ECM). The spermatozoa were defined as the control. The sperm collection and freezing/thawing procedures were performed according to the methods described in our previous report [17] with some modifications. In brief, epididymal sperm were counted by a hemacytometer and then diluted to 5.0×10^7 sperm/ml with modified Niwa and Sasaki Freezing medium [13]. Sperm samples were loaded into 0.25-ml plastic straws (Fujihira Industry, Tokyo, Japan) and placed in a programmable freezer (Fujihira Industry) at 23°C. The straws were cooled to 5°C for 40 min (0.5°C/min). They were then exposed to liquid nitrogen vapor for 10–15 min, plunged into liquid nitrogen, and stored for at least 1 week. The preserved spermatozoa in the cryotube were thawed in a 37°C water bath for 15 sec then diluted in Hepes-R1ECM. This treatment was defined as the IVF-ICSI group. Spermatozoa in both groups were then separated into heads and tails by sonication for 10 sec at 25°C. The sonication treatment was performed using 100% power output (300 W, 40 kHz) of a UT-305 ultrasonic cleaner (Sharp Manufacturing Systems Corp., Osaka, Japan). More than 80% of the spermatozoa were separated into heads and tails by this sonication treatment, as described previously [17]. The spermatozoa were washed in Hepes-R1ECM by centrifugation (17,000 × *g* for 3 min) before ICSI.

The COCs were collected from the oviductal ampullae of the donor females using mR1ECM supplemented with 0.1% hyaluronidase. After the cumulus cells were removed, intracytoplasmic injection of sperm heads was carried out according to the method described in our previous study [17] with some modifications. In brief, about 10 oocytes were transferred into 20-µl drops of Hepes-R1ECM. A small volume (0.5 µl) of the sperm-head suspension was transferred to 2-µl drops of Hepes-R1ECM supplemented with 12% polyvinylpyrrolidone (MW 400,000), which was prepared close to the oil-covered drops for the oocytes. Microinjection of sperm heads into oocytes was performed using a Piezo-driven pipette (Prime Tech Ltd., Ibaraki, Japan) that was prepared from borosilicate glass capillary tubes (Sutter Instrument Co., Novato, CA, USA). The external diameter of the pipette tip was 2–4 µm. After the injection, the oocytes were cultured in mR1ECM up to 120 h. The rates of 2PN formation, cleavage, and blastocyst were evaluated at 6, 24, and 120 h, respectively. More than 70 oocytes were used in each group.

Embryo transfer

To evaluate the developmental competence *in vivo* of embryos derived from ICSI, one-cell embryos at 6–8 h after ICSI were transferred into the oviducts of recipients after the induction of pseudopregnancy as described previously [18]. Female recipient rats were mated with vasectomized males on day 0 to induce pseudopregnancy. On day 1, 9 or 10 of 2PN oocytes were transferred into each oviduct of the recipients. On day 22, the transferred females underwent Caesarean section to confirm pregnancy and the normality of the offspring.

Statistical analysis

Each experiment had at least three replicates. All percentage data were subjected to arcsine transformation before statistical analysis. Data were compared by one-way analysis of variance and Fisher's protected least significant difference test using StatView software (Abacus Concepts, Berkeley, CA, USA). A value of $P < 0.05$ was chosen to indicate statistical significance. Data are shown as means ± standard error of means (SEM).

RESULTS

In the control group, most (88.6 ± 4.8%) of the injected oocytes formed 2PN (Figure 1). More than half (63.8 ± 8.3%) of the embryos developed to the two-cell stage. Some (25.1 ± 5.5%) reached blastocyst stage. Also in the IVF-ICSI group, most (90.4 ± 4.6%) of the injected oocytes formed 2PN. The proportions of oocytes developed to the two-cell and blastocyst stages were 73.2 ± 7.7% and 36.4 ± 5.1%, respectively. There were no significant differences between the two groups at any developmental stage ($p > 0.05$).

Table 1 shows the *in vivo* development of ICSI oocytes. In the control group, 74 embryos were transferred to pseudopregnant recipients and six pups were delivered (12.7 ± 5.5%). On the other hand, in the IVF-ICSI group, 82 embryos were transferred to five recipients and six pups were obtained (9.4 ± 4.4%). There were no significant differences in developmental ability *in vivo* between the two groups ($p > 0.05$). These pups looked healthy and normal.

Table 1. *In vitro* development of ICSI oocytes after IVF

Treatment	No. of examined	No. of survived	No. of PN formed (%)	No. of 2 cell (%)	No. of blastocysts (%)
Control	93	80	73 (88.6 ± 4.8)	51 (63.8 ± 8.3)	19 (25.1 ± 5.5)
IVF-ICSI	78	45	41 (90.4 ± 4.6)	33 (73.2 ± 7.7)	16 (36.4 ± 5.1)

Table 2. *In vivo* development of ICSI oocytes after IVF

Treatment	No. of transferred embryos	No. of recipients	No. of offspring (%)
Control	71	5	6 (12.7 ± 5.5)
IVF-ICSI	80	5	8 (9.4 ± 4.4)

DISCUSSION

In the present study, we examined whether or not unfertilized spermatozoa after IVF can be used for ICSI. Our data showed high developmental ability in the oocytes injected with sperm remaining after IVF (IVF-ICSI group). Although there was no significant difference in developmental ability between the control and IVF-ICSI groups, the cleavage and blastocyst rates of the IVF-ICSI group were slightly higher than those of the control group. It has been demonstrated that the removal of the acrosomal membrane improves the developmental ability of ICSI oocytes in mice [11], rats [17], and pigs [6]. In this study, the spermatozoa in the IVF-ICSI group were totally cultured for 15 h (5 h for preincubation and 10 h for IVF) and then frozen-thawed before ICSI. Our previous study showed that incubation of frozen-thawed sperm for 5 h induces acrosome reaction in rats [16]. Therefore, incubation for 15 h seems to be sufficient to induce acrosome reaction, which may have contributed to the developmental ability of the oocytes in the IVF-ICSI group.

Our results showed that co-cultured sperm remaining after IVF could be utilized in ICSI in rats. Our results suggest that treatment with IVF and ICSI combined seems to be effective for the production of genetically valuable rats, especially those spontaneously mutated and stocked in gene banks.

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