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Improvement of the early developmental ability of *in vitro* matured MII oocytes using a spindle transfer technique

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Abstract: [Purpose] The early developmental ability of MII oocytes matured *in vitro* (IVM-MII oocyte) after *in vitro* fertilization (IVF) is lower than that of fresh MII oocytes matured *in vivo.* The low early developmental ability of IVM-MII oocytes is thought to be due to delayed cytoplasmic maturation rather than nuclear maturation. We compared the early developmental ability of IVM-MII oocytes with that of reconstructed MII oocytes generated by microinjecting the spindles of IVM-MII oocytes into the enucleated cytoplasts of fresh MII oocytes. [Methods] First, fresh MII oocytes and immature oocytes at the GV stage were collected from B6D2F1 female mice. The GV stage oocytes were maturated *in vitro*. Using the metaphase II spindle injection (MESI) technique, we generated reconstructed MII oocytes by injecting the spindles from IVM-MII oocytes into the cytoplasts of fresh MII oocytes. [Result] The percentage of oocytes reaching the expanded blastocyst and hatched blastocyst stages was 47.5% (95/200) in the reconstructed MII oocytes, a significantly higher percentage than the 18.3% (42/229) obtained with IVM-MII oocytes ($P < 0.001$). [Conclusion] These results support previous reports that the low early developmental ability of IVM-MII oocytes is due to an immature cytoplast.

Key words: *in vitro* maturation, IVM-MII oocyte, spindle transfer technique, reconstructed MII oocyte, early developmental ability

Introduction

In vitro maturation (IVM) is a reproductive technique used in the developmental stage in which mature metaphase II (MII) stage oocytes are obtained from immature oocytes, harvested from ovarian follicles at the germinal vesicle (GV) stage through *in vitro* cultivation. Oocyte maturation, resulting from both nuclear and cytoplasmic maturation during the first and second meiosis, is important for fertility and developmental ability. The first human pregnancy and delivery using IVM technology was reported in 1991 [1]. Recently, the IVM technique has been predominantly used as a treatment for polycystic ovarian syndrome (PCOS).

The embryonic development rate of IVM-MII oocytes after *in vitro* fertilization (IVF) is lower than that of fresh MII oocytes matured *in vivo* [2–4]. However, application of the IVM technique is expected to extend to patients with premature ovarian failure (POF), cancer, and advanced age.

Previous animal studies of the early developmental ability of IVM-MII oocytes matured in various mediums have reported that the rates of the blastocyst formation were 34–53% in bovine, and 18–46% in mice [5–9].

Two methods are available to improve the early developmental ability of IVM-MII oocytes. One method is improvement of the culture medium to promote the cytoplasmic maturation of IVM-MII oocytes, and the other method is the exchange of the immature cytoplast of IVM-MII oocytes with the mature cytoplast of fresh (*in vivo* matured) MII oocytes using the spindle transfer technique. Although the first method has been studied in human and animals using various compositions of the culture medium, significant improvements have not yet been reported. Therefore, the second method was investigated in this study.

Since it is inevitable that a small amount of the cytoplasm surrounding the spindle is co-transferred at the spindle transfer point in metaphase II spindle injection (MESI) [10], the reconstructed MII oocytes contain two different cytoplasm types of mitochondrial DNA (mtDNA). In a previous study of “mito-mice” carrying pathogenic

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mtDNA, an exogenous, -small amount of mtDNA did not affect the early development of the oocytes or ontogenesis. However, the proportions of exogenous mtDNA introduced by the spindle transfer increased during the post-partum development and aging of the mice [11–14]. Because the human life span is longer than that of mice, in the future, care must be taken with the co-transferred mtDNA when the spindle transfer technique is applied to human treatment.

In this study, we first quantified the amount of mtDNA co-transferred by spindle transfer using a real time PCR method.

Then, in order to determine the damage inflicted on oocytes by the MESI technique, we compared the early developmental ability of fresh MII oocytes with that of reconstructed MII oocytes created by microinjecting the spindles of fresh MII oocytes into the cytoplasts of other fresh MII oocytes.

Furthermore, we compared the early developmental ability of IVM-MII oocytes with that of reconstructed MII oocytes by microinjecting the spindles of IVM-MII oocytes into the cytoplasts of fresh MII oocytes. This study used B6D2F1 mice because we were only able to establish the MESI technique in B6D2F1 mice. In this study, the early developmental ability of the oocytes was examined by parthenogenetic activation.

Materials and Methods

Fresh MII oocytes were retrieved from the oviducts of superovulated 6- to 8-week-old B6D2F1 female mice following the subcutaneous administration of 10 IU of pregnant mare serum gonadotropin (PMSG) and 10 IU of human chorionic gonadotropin (hCG) 48 h apart. Approximately 14 h after the hCG administration, cumulus-oocyte complexes were collected from the oviducts and released from the cumulus cells using 0.5% hyaluronidase (Sigma Aldrich, St. Louis, USA) in HEPES medium (Quinn's Advantage™ Medium With HEPES, SAGE, Trumbull, USA). After collection, all oocytes were incubated in a KSOM medium (Ark Resource, Kumamoto, Japan) at 37 °C in a humidified atmosphere containing 5% CO_2 in air.

The fresh MII oocytes were treated with 1% cytochalasin B (CB, Sigma Aldrich, St. Louis, USA) in HEPES. We collected the MII spindles with a small amount of cytoplasm (the unpurified spindle) from fresh MII oocytes using a micropipette. The cytoplasm around the unpurified spindle was removed using a micropipette (the purified spindle). DNA was extracted from the fresh MII oocytes, the unpurified spindles, and the purified spindles using NucleoSpinR Tissue XS kit (Macherey-Nagel, Düren, Germany). The extracted and purified DNA was subjected to semi-quantification by real-time PCR using a Thermal Cycler Dice® Real Time System II (Takara Bio, Shiga, Japan). In brief, 12.5 μl of TB Green™ Premix Ex Taq™ II (Takara Bio, Shiga, Japan), 1.25 μl of each primer, and 2 μl of each DNA were added, and the total volume was increased to 25 μl with distilled water. The nucleotide sequences of the primer were forward 5′-TACTAGGAGACCCAGACAAC-3′, and reverse 5′-AGATGGAGGCTAGTTGGCC-3′. After initial activation at 95 °C for 15 sec, the PCR was conducted with 43 step cycles at 95 °C for 10 sec, 55 °C for 15 sec, and 72 °C for 45 sec [15]. The data were compared using Student's *t* test.

The method for reconstructing MII oocytes from two fresh MII oocytes using the MESI technique was as follows. The purified spindle and the enucleated cytoplast were obtained from two differentr fresh MII oocytes using a micropipette with 1% cytochalasin B in HEPES. The purified spindle was transferred to 7% polyvinylpyrrolidone (PVP, SAGE *in vitro* fertilization, Trumbull, USA), and then injected into the enucleated cytoplast of the other MII oocyte to generate a reconstructed MII oocyte in HEPES. The reconstructed MII oocytes were activated in an activation medium (4 mM $SrCl_2$, 1% CB, and Ca^{2+} free KSOM) for 2 h. After activation, the oocytes were transferred to a KSOM medium and incubated until blastocyst formation at 37 °C under 5% CO_2 in air. The numbers of oocytes reaching the 2-cell, 4-cell, 8-cell, morula, early blastocyst, expanded blastocyst, and hatched blastocyst stages were counted. The developmental rate is represented as the percentage of the number of oocytes reaching both the expanded and hatched blastocyst stages.

The method of reconstructing MII oocytes from the spindles of IVM-MII oocytes and the cytoplasts of fresh MII oocytes with the MESI technique was as follows. First, immature oocytes at the GV stage were collected as cumulus oocyte complexes (COCs) from the ovaries of 6- to 8-week-old B6D2F1 female mice. The COCs were then maturated *in vitro* in a medium for 20 h, followed by the collection of IVM-MII oocytes. The medium was composed of TYH (LSI Medience, Tokyo, Japan) and MENα (1x) + GlutaMAX™-I (Thermo Fisher Scientific, Massachusetts, USA) mixed in a 1:1 ratio and supplemented with 3 mg/ml BSA (Sigma Aldrich, St. Louis, USA), 0.12 μg/ml astaxanthin (Cayman Chemical, Ann Arbor, USA), 0.5 mM ascorbic acid (Wako Pure Chemical, Osaka, Japan), and 1.35 μM E-64 (Sigma Aldrich, St. Louis, USA) [16–20]. The purified spindles of the IVM-MII oocytes and the enucleated cytoplasts of fresh MII oocytes were

obtained as described above. Furthermore, the spindles obtained from IVM-MII oocytes were injected into the enucleated cytoplasts of fresh-MII oocytes in HEPES to generate reconstructed MII oocytes. The reconstructed MII oocytes were activated and incubated as described above, and their developmental ability was measured as described above. The data were compared using the chi-squared test.

Results

The semi-quantification of each mtDNA was carried out by real-time PCR to examine the amount of mtDNA in the cytoplasm carried by spindle transfer into the reconstructed MII oocytes. The amount of mtDNA in the unpurified spindle was approximately 4% of that in the MII oocyte ($P < 0.05$), whereas mtDNA in the purified spindle was approximately 0.9% of that in the MII oocyte ($P < 0.05$). The amount of mtDNA co-transferred in the spindle transfer technique was significantly less with the MESI technique using purified spindles that in the cell fusion using unpurified spindles (Table 1).

We also investigated the damage to the developmental ability of MII oocytes of the MESI technique. The developmental ability of reconstructed MII oocytes generated by microinjecting spindles of fresh MII oocytes into the enucleated cytoplasts of other fresh MII oocytes was 63.8% (37/58).

Furthermore, we generated reconstructed MII oocytes with spindles from IVM-MII oocytes and enucleated cytoplasts of fresh MII oocytes. The percentage of oocytes reaching the expanded blastocyst and hatched blastocyst stages was 47.5% (95/200) in these reconstructed MII oocytes. This was significantly higher than the 18.3% (42/229) of the IVM-MII oocytes ($P < 0.001$) (Table 2).

Discussion

There are two methods to generate reconstructed MII oocytes: the MESI technique, and cell fusion using chemical reagents, viruses, or electric fields. A previous study reported electrofusion success rates of 49–92% [21]. Spindle transfer by electric fusion requires an unpurified spindle, while spindle transfer by the MESI technique can use a purified spindle. Therefore, the amount of co-transferred mtDNA is reduced in spindle transfer using the MESI technique with a purified spindle.

We also examined the damage to the oocytes caused by the MESI technique. We compared the early developmental ability of fresh MII oocytes with that of reconstructed MII oocytes by microinjecting the spindles obtained from fresh MII oocytes into the cytoplasts of other fresh MII oocytes. The percentage of oocytes reaching the blastocyst stage in the parthenogenetic development of fresh MII oocytes is generally about 90% [22], while that of our reconstructed MII oocytes using the MESI technique was merely 60–70%, indicating that the MESI technique may damage the spindle and / or cytoplast. Since the manipulation of the spindle may induce chromosomal aberration, the MESI technique needs to be improved before it is used in human treatment. Nevertheless, the result of 60–70% of well-developed reconstructed MII oocytes indicates that the MESI technique could be used to generate reconstructed MII oocytes with spindles from IVM-MII oocytes and cytoplasts from fresh MII oocytes.

Table 1. The amounts of mtDNA in fresh MII oocytes, the unpurified spindle and the purified spindle semi-quantified by real-time PCR

	amount of mtDNA (n=3)			mean ±SD
Fresh MII oocyte	128.0	58.5	113.5	100.0 ± 29.9
Unpurified spindle	2.43	4.83	5.10	4.12 ± 1.20$^{\#}$
Purified spindle	1.08	0.53	1.04	0.885 ± 0.250$^{\#, \#\#}$

$^{\#}P < 0.05$ vs fresh MII oocyte, $^{\#\#}P < 0.05$ vs unpurified spindle.

Table 2. The developmental rates of reconstructed MII oocytes and IVM-MII oocytes. The reconstructed MII oocytes were created using the MESI technique of microinjecting the spindles of IVM-MII oocytes into the enucleated cytoplasts of fresh MII oocytes

	2 cell ~ early blastocysts	expanded and hatched blastocysts	total
reconstructed MII oocytes	105	95	200
IVM-MII oocytes	187	42	229

$P < 0.001$.

Furthermore, the early developmental ability of the reconstructed MII oocytes generated by microinjecting spindles from IVM-MII oocytes into enucleated fresh MII oocytes containing mature cytoplast was significantly better than that of IVM-MII oocytes. This result supports those of previous studies reporting that the low early developmental ability of IVM-MII oocytes is due to immature cytoplasm. This suggests that the development of oocytes is influenced by the quality of the cytoplasm.

The introduction of the MESI technique into clinical practice has the potential to improve the fertility of patients with POF, cancer, or advanced age.

Preserving the fertility of patients undergoing cancer therapy is a severe problem, as ovarian failure is often induced by chemo- or radiotherapy. Thus, the oocytes of such patients need to be collected prior to the start of therapy. The reproductive treatment of cancer patients is limited by age, marital history, and the time available prior to the start of therapy. In the case of women who are unmarried, pre-pubescent or with limited time prior to the start of cancer therapy, oocyte cryopreservation and ovary cryopreservation are viable options. With the rapid advance of oocyte cryopreservation techniques, over 1,500 babies have been born from cryopreserved oocytes, although the success rate remains low [23, 24]. Presently, MII oocytes are commonly used in oocyte cryopreservation. Although the spindle and chromosomes of MII oocytes are easily damaged by temperature and osmotic stress, cryopreservation of GV oocyte is expected to be used in the future [25, 26]. If the development of IVM-MII oocytes obtained from the cryopreserved GV oocytes is improved by the MESI technique of reconstructing MII oocytes, the success rate of oocyte cryopreservation may increase significantly.

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