Article

Metaphase II karyoplast transfer from human in-vitro matured oocytes to enucleated mature oocytes

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Abstract

Metaphase II karyoplast transfer is believed to be a useful method to rescue aged oocytes. This study attempted karyoplast transfer of in-vitro matured metaphase II (MII) oocytes, as a model of aged oocytes, into enucleated freshly ovulated metaphase II oocytes with visualization of their chromosomes under an inverted microscope. Recipient karyoplasts derived from immature oocytes were cultured in-vitro until first polar body extrusion. After 1–2 days culture, 52.1% extruded a polar body, 95.5% had PSC, aneuploidy was very low (4.5%) and none had structural aberrations. Donor oocytes were obtained from IVF or intracytoplasmic sperm injection (ICSI) patients. Chromosomes were easily confirmed in 92.3% and 95.0% of in-vivo and in-vitro matured oocytes respectively. Thirty-one karyoplasts were placed in the perivitelline space of enucleated donor oocytes, and 25 (80.6%) fused to form a reconstituted oocyte. Fertilization, cleavage and blastocyst formation rates following ICSI were 76.0%, 64.0% and 28.0% respectively for reconstructed oocytes and 59.2%, 48.0% and 3.1% respectively for control (in-vitro matured) oocytes. Chromosomal analysis of five embryos developed after karyoplast transfer and ICSI showed normal diploid sets of 46 chromosomes. In conclusion, this metaphase II karyoplast transfer technique can be applied to the solution of chromosomal abnormalities related to oocyte ageing.

Keywords: *aged oocytes, embryo development, karyoplast transfer, metaphase II, oocyte maturation*

Introduction

The main causes of repeated failure in assisted reproduction such as IVF-embryo transfer are believed to be ooplasmic deficiencies, abnormalities and ageing rather than nuclear deficiencies (Alikani *et al.*, 1995; Cohen *et al.*, 1997; Xia, 1997). It is a common phenomenon that pregnancy rates decrease, but miscarriages increase, as women grow older (Tietze, 1957; Keefe, 1998). Also, the percentage of fetal chromosomal abnormalities in miscarriages increases according to female age, reaching >90% when women are over 40 years old; surprisingly, about 90% of them are cases of autosomal trisomy (Hassold and Chiu, 1985; Fukuyama *et al.*, 2005). Such aneuploidy is mainly induced by the chromosomal pre-division, in which homologous chromosomes fail to pair during meiosis I and segregate before it is complete, resulting in disomic gametes (Angell, 1991, 1995, 1997; Vialard *et al.*, 2006).

Although there are some reports that present opposite opinions (Liu and Keefe, 2004; Cui *et al.*, 2005), restoring normal function to low-quality oocytes compromised

developmentally by using nuclear transfer may lead to the fundamental settlement of the issue. In this context, ooplasmic transplantation (Cohen *et al.*, 1997, 1998) and germinal vesicle (GV) transfer (Zhang *et al.*, 1999; Liu *et al.*, 2000; Takeuchi *et al.*, 2001) have lately become a focus of increasing research interest. However, in the case of the former, it is difficult to verify whether a small volume of injected cytoplasm has reversed any ooplasmic abnormalities. Concerning the latter, the developmental potentialities of oocytes reconstituted by GV transfer, that is, the success rates to complete meiosis and/or the developmental rates to blastocyst following ICSI, are in reality low. To overcome these disadvantages following GV transfer, serial karyoplast transfer must be performed between the in-vitro matured reconstituted oocytes and freshly ovulated (in-vivo matured) oocytes at the metaphase II (M-II) stage (Liu *et al.*, 2003). However, it is almost impossible to conduct nuclear transfer twice in succession in humans, due to the shortage of donated oocytes.

Recently, it was found that in-vitro matured oocytes and aged oocytes resembled one another in terms of a high incidence of sister chromatid pre-division (Watanabe, 2007). Later, a new technique was developed to visualize chromosomes of viable oocytes easily under conventional microscopic conditions (unpublished). In this study, using this technique and in order to evaluate the efficacy and safety (chromosomal normality) of M-II karyoplast transfer, karyoplast transfer was attempted at the M-II stage between in-vitro matured oocytes (recipient karyoplast) as a model of aged oocytes and freshly ovulated oocytes (donor ooplasm).

Materials and methods

Source of oocytes

Recipient karyoplasts were derived from immature oocytes at the GV stage (**Figure 1A**) or the metaphase I stage (**Figure 1B**), after ovarian stimulation (long protocol with GnRH agonist; Tan, 1994). They were matured by culture in human tubal fluid (HTF) medium (homemade: 101.60 mM NaCl, 4.69 mM KCl, 0.20 mM $MgSO_47H_2O$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_22H_2O$, 25.00 mM $NaHCO_3$, 2.78 mM D-glucose, 0.33 mM sodium pyruvate, 21.04 mM sodium lactate, 75 mg/l penicillin G potassium, 50 mg/l streptomycin sulphate, pH7.4) with 10% inactivated maternal serum for 1 or 2 days at 37 °C, 5% CO_2 in air until first polar body (1PB) extrusion was observed (**Figure 1C**). Using some of the in-vitro matured oocytes, chromosome aberrations were analysed according to the method described by Mikamo and Kamiguchi (1983a). Donor ooplasm was prepared from consenting IVF or ICSI patients from whom more than 20 matured oocytes at the M-II stage (**Figure 1D**) had been retrieved.

The research procedures, as well as the handling of patient material, were performed in accordance with a research protocol approved by the Japanese Organization of Obstetrics and Gynecology (approval date: 2006-04-12) and by the ethical committee of Saint Mother Hospital (approval date: 2004-08-23).

Preliminary experiment to visualize human M-II chromosomes

Initially, a polarized light (POL) microscope was employed, which offered a clear view of meiotic spindles (Oldenbourg, 1996), but unfortunately identification of the M-II spindle in living human oocytes was no more than 80%, and one-fifth of all M-II chromosomal localizations were impossible to identify (data not shown). In addition, it was difficult to micromanipulate the oocytes under the POL microscope. Therefore, as an alternative to this apparatus, an attempt was made to identify chromosomes directly under an inverted microscope without any special devices. Accordingly, using many of the in-vitro matured oocytes, M-II chromosomes were stained with Hoechst 33258, and their localization confirmed. Through this process, the experimenters were trained to distinguish the M-II chromosomes, and, as a result, it was possible to identify them in almost 100% of cases.

The key to finding the chromosomes is as follows: (i) use a made-to-order cover glass (Matsunami Glass Ind. Ltd., Osaka, Japan) that covers the bottom of a central depression in a dish; (ii) confirm the presence of the chromosome in the culture medium before putting it into cytochalasin B-containing medium, because shortly after putting it into the medium, chromosomes become difficult to see due to cytoplasmic clumping; (iii) handle the inverted microscope with extreme care, because the slightest change of focus can cause loss of view of the chromosomes and (iv) look for the chromosome lump centrally located in the transparent, round substance, which is the spindle.

Identification, removal and transfer of the M-II chromosome

Donor (freshly ovulated) and recipient (in-vitro matured) M-II oocytes were placed in a microdrop of HTF medium containing 80 IU/ml hyaluronidase, and their cumulus cells were denuded by pipetting. After that, they were placed in an HTF microdrop covered with mineral oil, their chromosomes were observed, and manipulations were performed with the aid of an inverted microscope (Nikon, TE300, Japan) equipped with a Normarski differential interference contrast system (**Figures 1–4**). Under this condition, an oocyte was held with the M-II chromosomes located in the 1–2 o'clock position (**Figure 2A**). The zona pellucida just above the M-II chromosome was breached with a glass micro-needle tangentially inserted into the perivitelline space, against the holding pipette (**Figure 2B**). Next, the oocytes with opened zona pellucida were placed in an HTF microdrop containing 5 μg/ml cytochalasin B. The M-II chromosome surrounded by a minimum amount of cytoplasm was aspirated, using a 10–12 μm inner diameter glass pipette that was inserted into the perivitelline space (**Figure 2C**), and the karyoplast was expelled into the surrounding medium (**Figure 2D**). The first PB was also removed using the same procedure. The M-II karyoplast

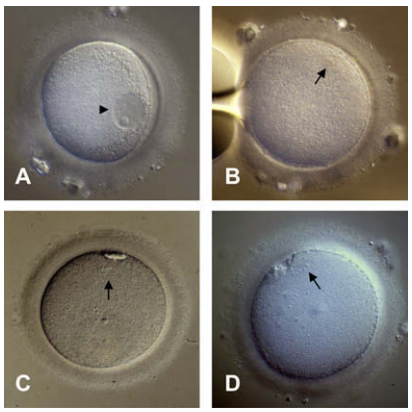

Figure 1. Human oocytes at the germinal vesicle (arrowhead, **A**), the metaphase I (arrow, **B**) and the metaphase II stages (arrows, **C** and **D**), (**C**) recipient (in-vitro matured), (**D**) donor (in-vivo matured and freshly ovulated) oocyte. All photographs were taken after removing the cumulus cells. Original magnification, 400×.

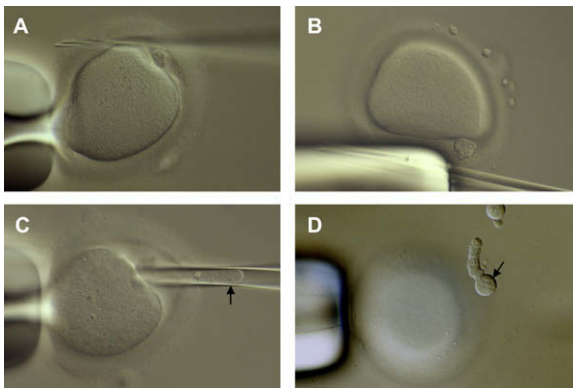

Figure 2. Removing a karyoplast from an in-vitro matured metaphase II (MII) human oocyte. Oocyte held with the MII chromosomes at the 1–2 o'clock position (**A**). Zona pellucida just above the MII chromosomes breached with a glass micro-needle (**B**). Aspiration of MII chromosomes surrounded by a minimum amount of cytoplasm (**C**). Karyoplast expelled into the surrounding medium (**D**). Arrows indicate MII chromosomes (**C** and **D**). Original magnification, 400×.

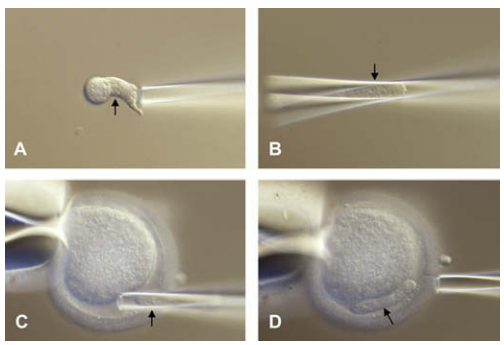

Figure 3. Inserting a karyoplast into the perivitelline space of an enucleated metaphase II human oocyte matured *in vivo*. Isolated karyoplast (**A**) was aspirated again into the pipette (**B**). The pipette was inserted (**C**) and the karyoplast transferred into the perivitelline space (**D**). Arrows indicate metaphase II chromosome. Original magnification, 400×.

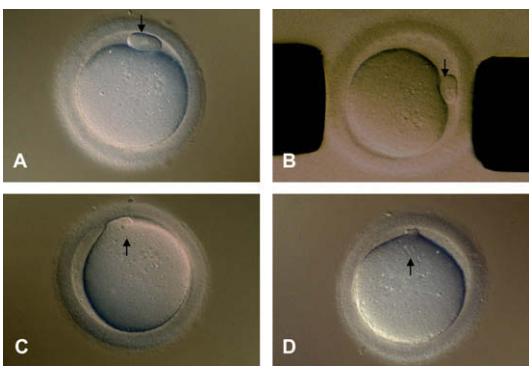

Figure 4. Electrofusion between a karyoplast and an ooplasm in human. Arrows indicate metaphase II chromosomes. Grafted oocyte (**A**) is placed between two microelectrodes (**B**). Fusing (**C**) and fused (**D**) karyoplast–cytoplast complex are shown after an electrical stimulation. Original magnification, 400×.

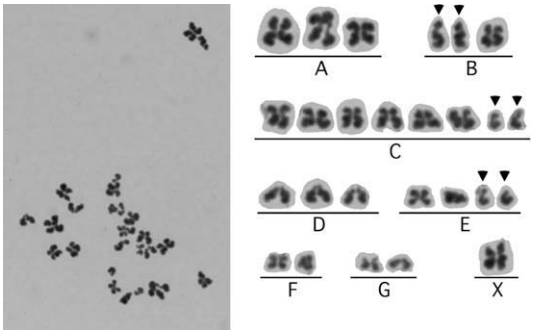

Figure 5. Chromosome plate of in-vitro matured oocytes (left). The karyotype was normal (23, X), but pre-divisions of sister chromatids were found in three chromosomes, one in each of **B**, **C** and **E** groups (arrows).

derived from the recipient (in-vitro matured) oocyte was aspirated again into the pipette, and then transferred into the perivitelline space of an enucleated donor (in-vivo matured) oocyte through the previously made slit in the zona pellucida (**Figure 3**). After insertion of the karyoplast, the grafted oocyte was washed and cultured for 30 min in HTF medium to remove cytochalasin B.

Oocytes in which the chromosomes could not be identified were fixed with Carnoy's solution and stained with aceto-orcein, and the nucleus was observed using a phase contrast microscope (Olympus BH-2, Tokyo, Japan).

Electrofusion

Membrane fusion between the ooplasm and M-II karyoplast was performed by electrofusion at room temperature (**Figure 4**). An LF201 generator (NEPAGENE Co. Ltd, Tokyo, Japan) was used to deliver electrical pulses. Each grafted oocyte was placed between two microelectrodes (ECF-100; Tokyo Rikakiki Co. Ltd, Tokyo, Japan) in 40 µl of Zimmerman cell fusion medium (Zimmermann *et al.*, 1984) and manually aligned to place the karyoplast perpendicular to the axis of the electrodes. The M-II karyoplast-cytoplast complex was fused with an alternating current pulse of 12 V/s, followed by a direct current pulse of 23 V/45 µs. After washing and culture for 60 min, the complexes were examined to confirm fusion and cell survival. To examine the effect of oocyte activation by electrofusion, the same electrostimulation was delivered to in-vitro matured oocytes and the number of normal sized pronuclei that developed was observed.

Embryonic development after ICSI

After fusion, the reconstituted oocytes were incubated in HTF medium for 2 h, following which ICSI was performed. The constituted oocytes were then cultured in sequential media (Quinn's Advantage Cleavage Medium and Blastocyst Medium, Cat.1029; In-vitro Fertilization, Inc., CT, USA) and their embryonic development observed until the blastocyst stage. To compare the embryonic potentiality of newly constructed oocytes, ICSI was performed using the in-vitro matured oocytes. Using some in-vitro matured oocytes and reconstituted embryos, chromosomal analysis was performed according to the method described by Mikamo and Kamiguchi (1983a).

Statistical analysis

Data were statistically analysed by Fisher's exact test.

Results

A total of 307 immature oocytes were cultured for in-vitro maturation, and 160 (52.1%) extruded a polar body. When 22 of these were cytogenetically analysed, pre-division of sister chromatids (PSC; **Figure 5**), which may result in an increase of aneuploidy at the second meiotic division, was found in the chromosome complements of 21 oocytes (95.5%). However, the incidence of aneuploidy was very low (4.5%; 1/22) in these oocytes, and none of them had structural aberrations (**Table 1**).

In both in-vitro matured oocytes (**Figure 1C**) and freshly ovulated oocytes (**Figure 1D**), the M-II chromosomes were easily recognized as a round transparent substance in which the chromosome body was centrally located, and they were usually beneath or adjacent to the 1PB. Although chromosomes tended to locate far from the 1PB in the in-vitro matured oocytes (71.1%) compared with the freshly ovulated ones (88.9%), it was still possible to confirm the chromosomes easily, so there was no significant difference between these two types of oocytes (92.3 versus 95.0%) in

Table 1. Chromosome aberrations in in-vitro matured oocytes.

No. of oocytes analysed	*No. of oocytes with PSC (%)*	*No. of oocytes with aneuploidy*		*No. of oocytes with structural aberrations (%)*
		Hypoploidy (%)	*Hyperploidy (%)*	
22	21 (95.5)	0 (0.0)	1 (4.5)	0 (0.0)

PSC, pre-division of sister chromatids.

Table 2. Identification of metaphase II chromosomes in in-vivo and in-vitro matured oocytes.

Type of oocytes	*No. of oocytes observed*	*No. of oocytes with visible chromosomes (%)*	*No. of oocytes with the spindle just beneath the 1PB (%)*
In-vivo matured[a]	39	36 (92.3)	32 (88.9)
In-vitro matured	40	38 (95.0)	27 (71.1)

1PB, first polar body.
[a]Freshly ovulated.

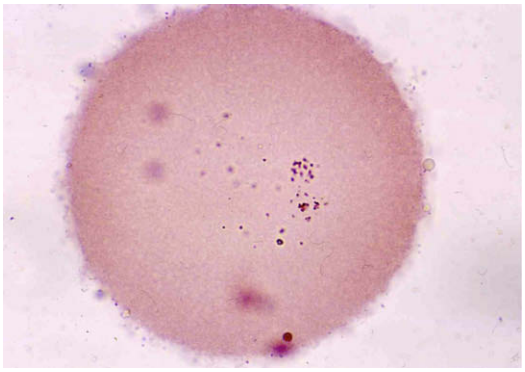

Figure 6. Scattered oocyte stained with aceto-orcein. The first polar body was out of focus. Original magnification, 400×.

Table 3. Development of reconstructed and in-vitro matured oocytes following intracytoplasmic sperm injection (ICSI).

Type of oocyte	*No. treated by ICSI*	*No. of oocytes fertilized (%)*	*No. of oocytes cleaved (%)*	*No. oocytes developed to blastocyst stage*
Reconstructed	25	19 (76.0)	16 (64.0)	7 (28.0)[a]
Control[b]	98	58 (59.2)	47 (48.0)	3 (3.1)

[a]Fisher's exact test, $P < 0.01$.
[b]In-vitro matured.

the percentages of identification of the M-II chromosome (**Table 2**). Five oocytes in which chromosomes could not be identified were stained with aceto-orcein, and it was found that most of them were scatters (**Figure 6**).

The M-II karyoplast was successfully removed in 33 (91.7%) of 36 of the donor oocytes and 31 (81.6%) of 38 of the recipient oocytes. When the removal of karyoplasts failed, it was because they were destroyed during the aspi-

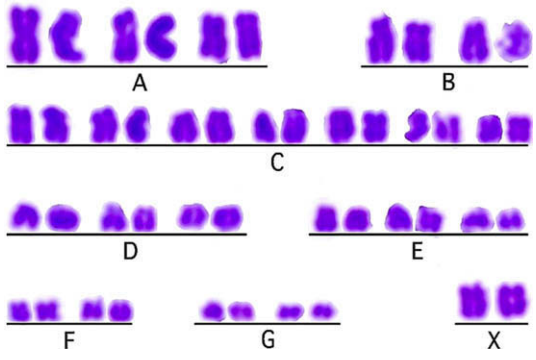

Figure 7. Karyotype of a blastomere derived from a reconstituted human oocyte.

ration procedure (**Figure 2C**). All of the 31 karyoplasts of recipient oocytes were replaced in the perivitelline space of enucleated donor oocytes and 25 of these (80.6%) were fused to form a reconstituted oocyte. Following electrofusion, the percentage of one pronucleus (1PN) occurrence, i.e. oocyte activation, was only 5% (1/20).

Table 3 shows the fertilization, cleavage and blastocyst formation rates following ICSI in reconstituted oocytes and control oocytes (in-vivo matured oocytes). In the reconstituted oocytes, all values were higher than those of the control oocytes, and significantly more reconstituted oocytes developed to the blastocyst stage ($P < 0.01$). When more than two blastomeres of five reconstituted embryos at the eight-cell stage were chromosomally analysed, all displayed 23 sets of normal diploid chromosomes (**Figure 7**).

Discussion

It is well known that the percentage of chromosomal anomalies in immature oocytes is considerably higher than that in mature ones, and that not all of them will develop into chromosomally normal mature (M-II) oocytes after in-vitro culture (Nogueira *et al.*, 2000). In in-vitro developed abnormal embryos, the appearance of mosaics, found in 20%, is generally attributed to chromosomal abnormalities that occur in mitosis during embryo development (Munné *et al.*, 2002). On the other hand, about 60% of trisomy 18 cases occurred at the meiotic II division stage (Fisher *et al.*, 1995). However, in the hamster, it was reported that there were no significant differences between the percentage of oocyte aneuploidy in the process of meiotic I and that in meiotic II stages (Mikamo and Kamiguchi, 1983b). Indeed, the incidence of aneuploidy in this study was very low even in in-vitro matured oocytes (4.5%, **Table 1**).

Nishino *et al.* (1994) has reported that the incidence of PSC in mature oocytes increased with the mother's age, suggesting that PSC should be considered as a factor affecting ageing-related early embryonic loss. In addition, Vialard *et al.* (2006) recently confirmed frequent PSC (80%) in the first polar bodies of fresh oocytes from women of advanced age. These findings suggest that PSC should be considered as a factor affecting age-related early embryonic loss. In this study, the incidence of PSC in in-vitro matured oocytes was extremely high (95.5%, **Table 1**) and the rate of blastocyst formation after ICSI was only 3.1% (**Table 3**). Therefore, the in-vitro matured oocytes seem to be very similar to oocytes from aged females. In contrast, 28.0% (**Table 3**) of enucleated freshly ovulated M-II oocytes grafted by means of electrofusion with in-vitro matured nuclei developed into blastocysts following ICSI. Furthermore, all chromosome complements were normal in five reconstructed oocytes that were cytogenetically analysed. These results demonstrate the reproducibility and safety of karyoplast transfer at the M-II stage and suggest that karyoplast transfer at this stage has considerable potential for the treatment of ooplasmic deficiency and abnormalities in oocytes from older patients.

Nuclear transfer at the GV stage is the expected method to eliminate chromosomal abnormalities, namely, non-disjunctions that appear at the first meiotic division. However, as of now, the cytoplasmic maturation of grafted oocytes following GV transfer has not been completed (Zhang *et al.*, 1999; Takeuchi *et al.*, 2001), and GV transfer does not seem to solve the fundamental problem of low-quality oocytes, including aged ones, because the rate of 1PB extrusion is high but the blastocyst formation rate is extremely low (Liu *et al.*, 2000). Liu *et al.* (2001, 2003) also reported that, embryonic development following karyoplast transfer of M-II oocytes was obviously superior to that of GV stage oocytes in the mouse. In humans, ooplasmic donation at the M-II stage into developmentally compromised oocytes restored normal growth (Cohen *et al.*, 1998).

Taking these points into consideration, the karyoplast transfer of in-vitro matured M-II oocytes was conducted into freshly ovulated M-II enucleated oocytes instead of GV transfer. In general, M-II karyoplast transplantations in humans are rarely practised because their chromosomes are invisible, even under an inverted microscope using Normarski optics. Therefore, initial examinations were made using a POL microscope (Oldenbourg, 1996), but this microscopic technique was unsatisfactory to identify and transplant the M-II karyoplast. A new alternative technique was therefore developed to identify the chromosomes directly under an ordinary inverted microscope. Using this technique, it was possible to identify the chromosomes in 92.3% and 95.0% of in-vivo and in-vitro matured oocytes respectively (**Table 2**). The majority (88.9%) of oocytes had M-II chromosomes directly beneath or adjacent to the 1PB; however, in in-vitro matured oocytes, about 30% were observed to be at some distance from the 1PB (**Table 2**). This sliding is considered to be attributable to the rotation of the oolemna itself or the transfer of the M-II chromosome itself as time elapses.

In this study, the electrical fusion rate between the karyoplast and ooplasm was 80%. Human oocytes have been reported to be relatively resistant to a wide variety of stimuli, unlike mouse oocytes (Abramczuk and Lopata, 1990; Winston *et al.*, 1991). In fact, in the current study a low percentage of oocyte activation (only 5%) was achieved through the electrofusion method, although another electric pulse or chemical activation was not added after fusion. The success rate will certainly be overcome by better techniques, resulting in improved fusion rates.

In conclusion, it has been demonstrated that oocytes constructed following the karyoplast transfer of in-vitro matured M-II oocytes into enucleated freshly ovulated M-II oocytes clearly had more efficient and chromosomally normal embryonic development than did in-vitro matured oocytes after ICSI. This new technique is promising for the rescue of low quality aged oocytes.

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