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SHORT COMMUNICATION

Aggregated chromosomes transfer in human oocytes

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Junko Otsuki obtained her PhD in embryology from Ochanomizu University in 2007. As a laboratory director of Nagai Clinic, she has investigated quality control problems related to mineral oil and human serum albumin in IVF and has defined two types of cytoplasmic abnormalities in human oocytes. Recent work involving molecular and morphological aspects of meiosis has discovered a phase of chromosome aggregation that precedes chromosome separation and also a novel mechanism that may be responsible for the formation of hermaphrodite individuals. Junko has also investigated cytokinesis in mouse and human oocytes. At the ESHRE conference in 2007, she won the prize for the best paper on assisted reproduction laboratory research.

Abstract Germinal-vesicle (GV) transfer, spindle–chromosome complex transfer in metaphase-II oocytes and two pronuclei transfer have been evaluated as possible treatments for patients who have mitochondrial diseases. However, GV transfers often lead to heteroplasmy while the other two methods are frequently associated with aneuploidy. The present study used a new method based on the transfer of aggregated chromosomes, which occurs in human oocytes, before the metaphase spindle is established. 

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Introduction

In women, the incidence of oocyte aneuploidy rises steadily with increasing age, from an abnormality rate of about 1 in 500 at the youngest maternal ages to about 1 in 20 at age 45 (Hook, 1981). This has been attributed to an age-dependent reduction in the number of mitochondria and copies of mtDNA in oocytes (May-Panloup et al., 2007) and/or an age-dependent decline in mitochondrial function. Such mitochondrial changes may impair nuclear spindle activity and chromosome segregation during the extrusions of the first and second polar bodies, possibly due to lower energy production in the affected oocytes.

Furthermore, mutations of mtDNA cause genetic diseases that are maternally inherited through the cytoplasm. For patients who have mitochondrial diseases, nuclear transfer or cytoplasmic transfer was introduced to prevent transmission of mtDNA mutations from the carrier women to their offspring. At this stage, three methods for nuclear transfer have been proposed. These include germinal vesicle (GV), metaphase-II spindle–chromosome complex and pronuclear transfers using donor oocytes and embryos. However, the latter two procedures are performed after first polar body extrusion, where a high rate of chromosome desegregation occurs depending on the quality of oocytes. Moreover, it has been reported that following spindle–chromosome complex transfer, a significant proportion of zygotes (52%)

showed abnormal fertilization as determined by an irregular number of pronuclei (Tachibana et al., 2013). It is for this reason that it may be better to perform nuclear/chromosome transfer before chromosome segregation starts during first meiosis. Moreover, the problem with GV transfer is that mitochondria are densely accumulated around the germinal vesicle.

This study group tried to overcome the above problems by injecting oocytes with aggregated chromosomes (AC). A phase during which meiotic human chromosomes become aggregated or assembled is a feature of human oocytes that was confirmed in a previous study (Otsuki and Nagai, 2007). In human oocytes, this unique characteristic, which appears to be chromosome aggregation, is visible from the first or second polar body extrusions to metaphase-II spindle formation. The aggregation is also visible from GV breakdown to metaphase-I spindle formation (Otsuki and Nagai, 2007). Following the completion of nucleolar breakdown, chromosomes become assembled into a single aggregation that heralds the start of nuclear membrane breakdown (Otsuki and Nagai, 2007).

Materials and methods

GV-stage oocytes

GV-stage human oocytes that were retrieved unexpectedly during follicular aspirations for IVF and intracytoplasmic sperm injection (ICSI) procedures were donated by patients who provided informed consent. The study was conducted at the Nagai Clinic after approval was obtained from the Ethics Committee (reference number Hum-9, approved 24 July 2012). Presence of the GV stage was confirmed using an inverted microscope after partial removal of granulosa cells.

Chromosome transfer

The GV-stage oocytes were cultured in HTF medium (InVitroCare, Japan) containing 1% human serum albumin (HSA; InVitroCare) in an incubator at 37°C with 5% CO₂ at most for 10 h until nucleolar breakdown and chromosome aggregation were observed. Then the oocytes at the AC phase were transferred to HTF HEPES medium (InVitroCare) containing 1% HSA and cytochalasin B (5 µg/ml; Sigma, Japan) in a glass-bottomed culture dish at 37°C immediately before the manipulation commenced to avoid oocyte membrane damage. The AC in an oocyte were extracted with ICSI injection pipettes, that had a tip internal diameter of 5–6 µm (MT-INJ30; Kitazato BioParma Co, Japan) or 6 µm (PINU06–25FT; Prime Tech, Japan) using a Piezo Micro Manipulator (PMM-150FU; Prime Tech) and an inverted microscope (Eclipse TE2000; Nikon). After the removal of the AC, the oocyte (oocyte A) was transferred to HTF medium (InVitroCare) containing 1% HSA and incubated at 37°C with 5% CO₂ for 1 h. After that, another oocyte (oocyte B) that attained the AC phase was placed into a drop of HTF HEPES medium containing 1% HSA and cytochalasin B (5 µg/ml) and the oocyte without AC (oocyte A) was placed in a different drop of HTF HEPES medium containing 1% HSA without cytochalasin B in the same culture dish.

Subsequently, the AC were extracted from oocyte B and injected into oocyte A. Then the AC-transferred oocytes were cultured in HTF medium containing 1% HSA for 1 day. As a control, the same amount of cytoplasm was extracted from 10 oocytes of the AC phase in the same conditions and cultured for a day.

Time-lapse observations

In order to observe cytoplasmic movements within a host oocyte that received AC transfer and to record the extrusion of the first polar body, time-lapse recordings were performed using a time-lapse monitoring system (Primo Vision, Vitrolife, Japan). The cytoplasmic images were captured every 5 min.

Results

The AC were tightly condensed (Figure 1A, F) and hardly become fragmented. The AC could be clearly observed and enucleation was easily confirmed under differential interference contrast (DIC) optics: i.e. it was as clear as spindle observation under a polscope (Figure 1B, G). The AC were consistently extracted with a small amount of cytoplasm (Figure 1C, H). The chromosome clump could be injected with no difficulty into the oocyte in which AC were extracted in advance (Figure 1D, D'). Polar body extrusion was observed by microscopy on the next day, approximately 10 h after the karyoplast transfer (Figure 1E).

In total, AC were successfully removed in 52 out of 54 (96.3%) oocytes and 25 AC transfers were performed (Table 1). Successful injections of AC were performed in 13 out of 25 oocytes (52.0%). In 13 oocytes containing transferred AC, polar body extrusion was confirmed in six oocytes (46.2%) approximately 10 h after the karyoplast transfer. In addition, there were no polar body extrusions from 27 enucleated oocytes. In contrast, all control oocytes in which aspiration of the same volume of cytoplasm was performed extruded polar bodies (Table 1). All 27 patients who donated GV-stage oocytes for this study also contributed sibling metaphase-II oocytes that progressed to normal embryo development after IVF and/or ICSI procedures.

Time-lapse observations revealed the dynamic changes of the cytoskeleton (Figure 1I–P) and the release of the metaphase-II polar body (Figure 1Q). In addition, the formation and location of the spindle were confirmed using DIC microscopy (Figure 1R, S).

Discussion

This study successfully extracted aggregated human chromosomes with only a minimal amount of ooplasm (Figure 2). This was probably a much smaller amount of ooplasm than in the previously reported three procedures. Since it has been reported very recently that there are no recipient mitochondria detected in embryonic stem cells derived from spindle–chromosome complex-transferred oocytes (Tachibana et al., 2013), the transfer of AC could be more promising in avoiding the concern about heteroplasmy by limiting the carry over of mtDNA. As the AC in human

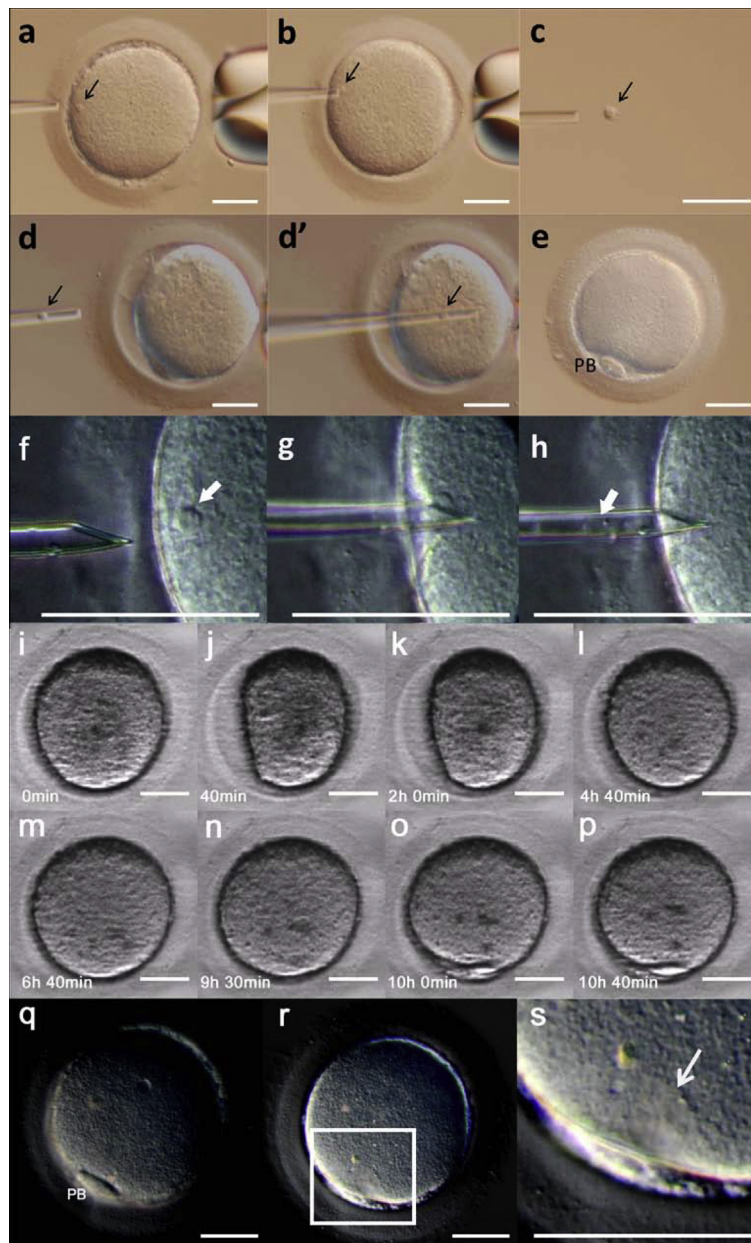


Figure 1 Transfer of aggregated chromosomes. (A, F) Chromosome aggregation approximately 1 h after GVBD (oocyte A; arrows). (B, G) Extraction of the chromosome aggregate (oocyte A). (C, H) Extracted aggregated chromosomes, with a tiny amount of surrounding material (oocyte A). (D, D') Injection of the extracted aggregated chromosomes (oocyte B). (E) Polar body extrusion after the chromosome transfer. (I–P) Time-lapse observations of an oocyte that received aggregated chromosomes. Time was measured from the start of the recording immediately after the aggregated chromosomes transfer. The oocyte membrane was indented towards the centre (J) of the oocyte but gradually recovered (K–M), and then approximately 3 h after the recovery the first polar body was extruded (N–P). (Q–S) The polar body (Q) and metaphase-II spindle (R, S) were confirmed using DIC microscopy. Inset in part R represents part S and shows the metaphase-II spindle with chromosomes aligned in the centre of the spindle. Bars = 50 μm.

oocytes can be clearly seen using DIC microscopy and the aggregate can be removed and injected using an ICSI injection pipette, damage of the ooplasm is minimal, as with ICSI. Among 27 enucleated oocytes that were used as recipients, there were no meiotic resumptions after AC extraction, suggesting that there was no residual AC left in the donor's oocytes. This work tried to aspirate the same amount of cytoplasm in control oocytes and found that all such oocytes extruded polar bodies.

Cytochalasin B was used to avoid lysis of cytoplasm in enucleated oocytes and to safely check polar body extrusion. However, if this procedure is applied clinically, it may be possible to avoid the use of cytochalasin B when AC are removed from both recipient's and donor's oocytes. In such cases, it is not necessary for the recipient's ooplasm to remain intact after the removal of AC and quality of the donor's oocytes could avoid lysis after simple AC extractions.

Table 1 Outcomes of aggregated chromosomes removal and transfer.

	<i>No. of oocytes attempted</i>	<i>No. of observations</i>
AC removal	54	52 (96.3)
AC transfer	25 ^a	13 (52.0)
PB extrusion	13	6 (46.2)
PB extrusion in enucleated oocyte	27	0
PB extrusion in control	10	10 (100)

AC = aggregated chromosomes; PB = polar body.

^aThe remaining two oocytes did not have a counterpart for the AC transfer due to failed AC removal in the corresponding oocytes.

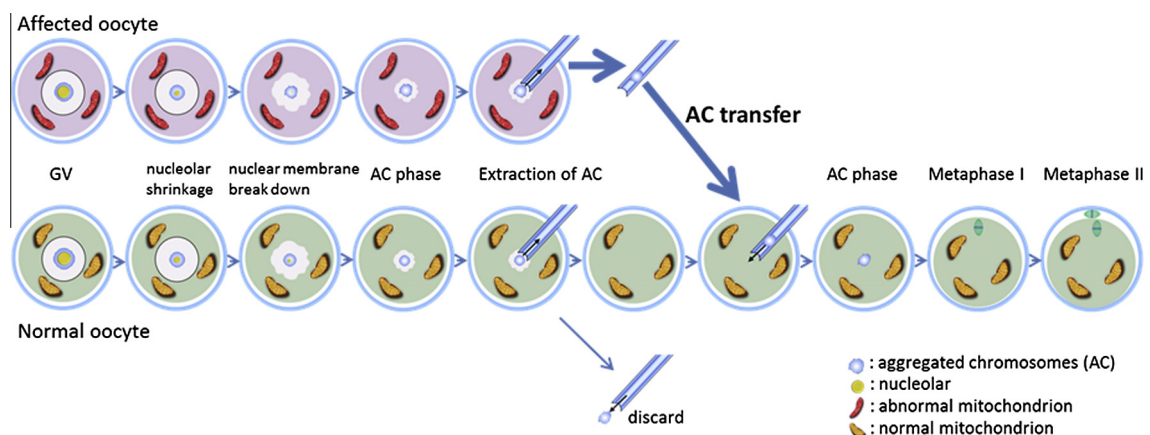


Figure 2 Schematic diagram of aggregated chromosomes transfer, depicting a proposed new chromosome replacement procedure, from oocytes that contain abnormal mitochondria into oocytes that contain normal mitochondria.

The reported studies on spindle and pronuclear transfer procedures were performed using donated eggs, while the present study used GV-stage oocytes that were retrieved with their normal sibling metaphase-II stage oocytes. In order to study normal fertilization and subsequent genetic analysis of embryos, immature oocytes should be retrieved about 20 h after human chorionic gonadotrophin administration while metaphase-II oocytes are retrieved 35–36 h after human chorionic gonadotrophin administration. However, it is considered unethical in Japan to obtain such healthy GV-stage oocytes for research purposes. Therefore, this paper proposes that AC transfer should be evaluated as an alternative method that could be an improvement on the existing spindle–chromosome complex transfer and pronuclear transfer.

Although this new technique needs to be improved, it introduces a possibly less harmful procedure and does not require electrical fusion or cell fusion using Sendai virus. The current study did not include genetic analyses, as these would have entailed an approval from both institutional ethical committees and the Japanese Academic Society of Obstetrics and Gynecology in addition to considerable expenditure. Further studies are required on genetic analyses of polar bodies and activation of good-quality oocytes consequently evaluating embryo development following the injection of aggregated meiotic chromosomes.

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