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SHORT COMMUNICATION

Pregnancy derived from human zygote pronuclear transfer in a patient who had arrested embryos after IVF

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Dr Zhang completed his medical degree in at the Zhejiang University School of Medicine, and subsequently received his Master's Degree at Birmingham University in the UK. In 1991, Dr Zhang earned his PhD in IVF, and, after studying and researching the biology of mammalian reproduction and human embryology for nearly 10 years, became the first Fellow in the Division of Reproductive Endocrinology and Infertility of New York University's School of Medicine in 2001. Dr. Zhang continues his research in minimal stimulation IVF, non-embryonic stem cell research, long-term cryopreservation of oocytes, and oocyte reconstruction by nuclear transfer.

Abstract Nuclear transfer of an oocyte into the cytoplasm of another enucleated oocyte has shown that embryogenesis and implantation are influenced by cytoplasmic factors. We report a case of a 30-year-old nulligravida woman who had two failed IVF cycles characterized by all her embryos arresting at the two-cell stage and ultimately had pronuclear transfer using donor oocytes. After her third IVF cycle, eight out of 12 patient oocytes and 12 out of 15 donor oocytes were fertilized. The patient's pronuclei were transferred subzonally into an enucleated donor cytoplasm resulting in seven reconstructed zygotes. Five viable reconstructed embryos were transferred into the patient's uterus resulting in a triplet pregnancy with fetal heartbeats, normal karyotypes and nuclear genetic fingerprinting matching the mother's genetic fingerprinting. Fetal mitochondrial DNA profiles were identical to those from donor cytoplasm with no detection of patient's mitochondrial DNA. This report suggests that a potentially viable pregnancy with normal karyotype can be achieved through pronuclear transfer. Ongoing work to establish the efficacy and safety of pronuclear transfer will result in its use as an aid for human reproduction.

KEYWORDS: cytoplasm, nucleus, oocyte, pronuclear transfer, zygote

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Introduction

Developmental arrest of embryos is a well-characterized phenomenon (Chi et al., 2000; Favetta et al., 2004; Hardy et al., 2001; Zamora et al., 2011). In humans, 50% of in-vitro produced embryos arrest during the first week of development (Hardy et al., 2001). Additionally, about 8% of embryos arrest at the two-cell stage when cultured *in vitro* after oocyte retrieval for IVF treatment (Chi et al., 2000). Although 48% of arrested embryos show chromosomal abnormalities (Almeida and Bolton, 1998; Benkhalifa et al., 2003), other reasons for this high rate of early developmental failure remain unclear.

Ooplasmic factors have been shown to be critical for the continued development of the zygote, particularly during the early cleavage stage (Cohen et al., 1998; Lanzendorf et al., 1999; Yao et al., 2014). A limited series of clinical ooplasmic transplantations were carried out nearly 20 years ago (Cohen et al., 1998; Lanzendorf et al., 1999). It was shown that, in couples that experienced repeated implantation failure as a result of poor embryo development, ooplasm transfer from donor oocytes at metaphase II (MII) stage into patient MII oocytes can be compatible with fertilization and pregnancy. These case series reports provided some interesting findings, but evidence of improved embryo development and implantation after ooplasmic augmentation remained elusive. Both nuclear and cytoplasmic deficiencies have been shown to be responsible for poor oocyte quality by contributing to meiotic defects and subsequent impaired embryo development (Fulka et al., 2001; Huang et al., 1999; Liu et al., 2000, 2003; Liu and Keefe, 2004; Moor et al., 1998). In assisted reproduction, it has been shown that the ooplasm of mature oocytes from young women could be applied to restore normal growth and viability in developmentally compromised embryos, where the underlying cause was attributed to ooplasmic deficiency (Fulka et al., 2001; Huang et al., 1999; Liu et al., 2000, 2003; Liu and Keefe, 2004; Moor et al., 1998). We have previously reported in humans (Zhang et al., 1999) and mice (Liu et al., 2000, 2003) that normal meiosis can occur after the transfer of germinal vesicle into an enucleated host oocyte. We have shown in mice that oocytes reconstructed by germinal vesicle transfer into a cytoplasm of the same developmental stage mature normally *in vitro* through the MII stage (Liu et al., 1999). Additionally, we have recently shown that germinal vesicle transfer can restore normal meiosis in meiotically arrested oocytes (Zhang and Liu, 2015). These data corroborate that a healthy cytoplasm is required for a normal nuclear function. We report herein a patient who had repetitive embryo arrest at the two-cell stage after two failed IVF cycles, and was subsequently able to conceive a normal pregnancy after pronuclear transfer into a donor cytoplasm.

Materials and methods

Clinical presentation

A 30-year-old nulligravida healthy woman had two failed IVF cycles characterized by all her embryos arresting at the two-cell stage. She was diagnosed with unexplained infertility after a complete work-up showing a normal ovarian reserve, normal semen analysis and patent fallopian tubes. The patient consented to the use of cytoplasm from a donated oocyte. The procedures used in this study were verbally approved by Sun Yat-Sen University Hospital Ethics Committee in China. Ovarian stimulation was then carried out and was synchronized for the patient and her oocyte donor so that their MII stage oocytes were retrieved within a 2-h period. After intracytoplasmic sperm injection, eight out of 12 patient oocytes and 12 out of 15 donor oocytes were fertilized with the sperm of the patient's partner. All pronuclei were then removed from the donor zygotes and discarded. The patient's (male and female) procnuclei were removed from each of her zygotes and transferred subzonally into the donor cytoplast (enucleated zygote).

Pronuclear karyoplast fusion

Electrofusion was then carried out as previously described (Zhang, 2015; Zhang et al., 2003, 2013). The donor's cytoplast with the male and female patient procnuclei were exposed to modified human tubal fluid medium with 10% serum supplemented with 7.5 μg/ml cytochalasin B (Sigma, St. Louis, MO, USA) for 15 min at room temperature to disrupt the microfilaments and increase plasma membrane flexibility before manipulation. The dish was then placed onto the stage of on Olympus IX71 inverted microscope equipped with micromanipulators. A slot was made in the zona pellucida of each oocyte by applying a sharp-tipped pipette that penetrates the zona while holding the oocyte against the wall of the holding pipette. This allowed the enucleation pipette to pass through the zona slot and approach the pronuclei before gently applying negative pressure to aspirate the procnuclei. Once a pronucleus was separated from the cytoplasm, it was transferred into the perivitelline space of the donor's enucleated cytoplast. The membrane fusion between both pronuclei and the donor's cytoplast was initiated by placing it into a fusion medium (0.3 M mannitol, 0.1 mM $CaCl_2$, and 0.05 mM $MgSO_4$) between platinum electrodes aligned in response to AC (6–8 V) current for 5–10 s before an electrical pulse (1.8–2.5 kV/cm DC for 50 μs) was delivered by a Model 2001 Electro Cell Manipulator BTX (Holliston, MA). The formed complexes were rinsed three times in modified human tubal fluid and then incubated in human tubal fluid medium at 5% CO_2 and 37°C. Membrane fusion usually occurred 30 min after the start of the electric pulse.

DNA extraction and mitochondrial genotype analysis

Nuclear and cytoplasmic DNA profiles were analysed in blood samples from the patient, the oocyte donor and the fetuses (after pregnancy loss). Nuclear DNA fingerprinting was carried out at 5 microsatellite loci with subtraction of the husband's genotype, as previously described (Epplen et al., 1997). Mitochondrial (mt) DNA was analysed by amplification and sequencing of a 524-bp segment in the D-loop region (16024–577). In brief, DNA was extracted from the embryos using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) according to the manufacturer's recommendation. The mtDNA

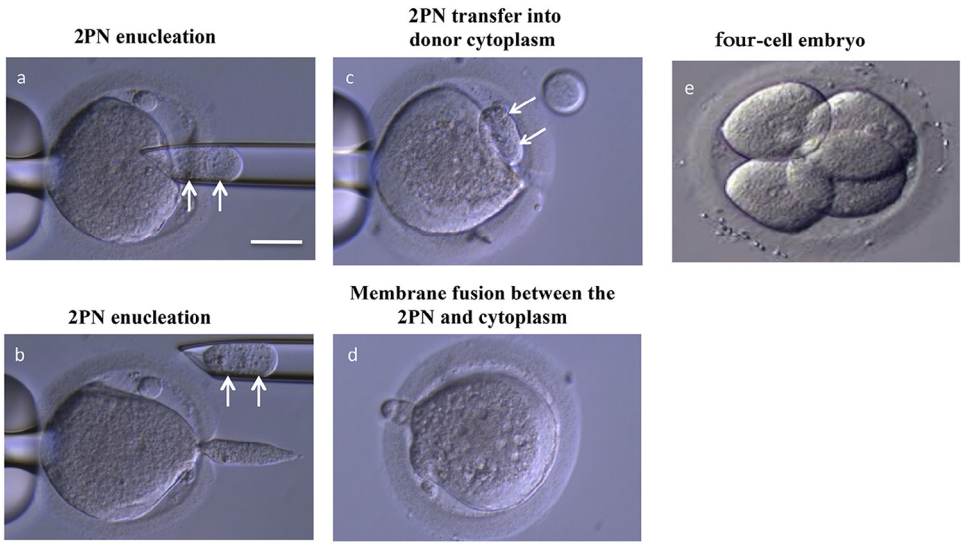

Figure 1 Transfer of pronuclei into the cytoplast of a donor's oocyte. (a,b) Enucleation of a zygote at the two pronuclei stage; (c) two pronuclei transfer into the perivitelline space of the donor's cytoplast; (d) membrane fusion between the two pronuclei and the cytoplast; (e) embryo at the four-cell stage. Arrow indicates pronucleus. Scale bar, 25 μm. 2PN, two pronuclei.

genotyping analysis was carried out using restriction fragment length polymorphism method, as previously described (Casali et al., 1999; Wong et al., 2002). Then, mtDNA was amplified by polymerase chain reaction (PCR) (Casali et al., 1999). The PCR products were then purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A 15 μl aliquot of the final product was digested overnight with the endonuclease *RsaI* (New England Biolabs, Ipswich, MA) and then electrophoresed on a 2.5% agarose gel and visualized by ethidium bromide staining under ultraviolet light.

Results

Electrofusion of the patient's karyoplast with the donor's cytoplast resulted in seven successfully reconstructed zygotes and one that degenerated (**Figure 1**). Upon the patient's request, given her poor history of embryo development, and given the poor embryo cryopreservation techniques at that time, the five reconstructed zygotes that cleaved to the four-cell stage at 48 h (after retrieval) were transferred to the patient's uterus. A triplet pregnancy with fetal heartbeats was achieved.

Fetal reduction to a twin pregnancy was performed transvaginally 33 days after transfer. At 24 weeks of gestation, Fetus B delivered as a result of premature rupture of membranes, and did not survive owing to respiratory distress. At 29 weeks of gestation, Fetus C was delivered after intrauterine fetal demise due to cord prolapse. Normal karyotypes were found in the embryonic tissue (46, XY), the 24-week (46, XX) and 29-week (46, XY) old fetuses. Nuclear genetic fingerprinting confirmed that the nuclear DNA from the 24-week and the 29-week old fetuses matched that of the patient's nuclear genetic fingerprinting. The mtDNA profiles of fetal red blood cells were identical to those of the donor's mtDNA with no detection of patient's mtDNA.

Discussion

Some of the reasons for early embryonic developmental arrest remain unclear. Early embryo arrest has been proposed as a protective mechanism for preventing further development of abnormal embryos because almost one-half of arrested human embryos shows chromosomal abnormalities (Almeida and Bolton, 1998). In rats, it has been reported that microtubule and microfilament distribution are involved in the developmental arrest of two-cell stage embryos (Fulka et al., 1998a; Matsumoto et al., 1998). Microtubules and microfilaments are indicators of embryonic normality because they represent the major cytoskeletal components and are associated with chromosomal condensation and formation of the mitotic spindle. Additionally, early developmental arrest in mouse embryos has been associated with elevated levels of free oxygen radicals, indicating that oxidative stress might be a reason for embryo arrest (Johnson and Nasr-Esfahani, 1994).

The interaction between the nucleus and the cytoplasm is important in the embryonic developmental process in mammals (Chiang et al., 2012; Cohen et al., 1998; Dekel and Beers, 1978; Fulka et al., 1998b; Heacox and Schroeder, 1981; Li et al., 2001; Liu et al., 1999, 2000; Willadsen et al., 1999; Zhang et al., 1999). Successful development of embryos reconstructed by nuclear transfer is dependent on a wide range of factors such as cell cycle coordination between the donor's and the recipient's oocytes, as in our case, in order to maintain normal ploidy (Campbell et al., 1996; Fulka et al., 1998a). Our case report further indicated that the structural and functional integrity of the reconstructed zygote was not compromised by the micromanipulation and electrofusion procedures themselves making them technically successful.

Another potential application of pronuclear transfer is the treatment of genetic diseases caused by mtDNA mutations (Barritt et al., 2001; Craven et al., 2010). Examples of these diseases include Leigh syndrome and MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like

episodes) (Leng et al., 2015). Therefore, the replacement of mutated mtDNA with wild-type haplotypes would potentially represent a cure for these genetic mutations. Some of the mutated mitochondria adjacent to the nucleus (or pronucleus), however, are likely to be carried over into the reconstructed oocytes during its transfer, thus creating mtDNA heteroplasmy (Samuels et al., 2013). To completely eliminate the risk of transmission of mutated mtDNA into the reconstructed oocytes, it is necessary to completely remove mtDNA from the patient carrying the genetic mutation when nuclear and pronuclear transfer is carried out. Interestingly in our case, the results of mtDNA analysis from the patient's blood, the donor's oocyte and the fetuses did not show any heteroplasmy using the pronuclear transfer technique.

In conclusion, pronuclei transfer in humans can produce a viable pregnancy with normal karyotype and minimal mtDNA heteroplasmy. This technique could potentially be used as a unique approach to prevent the transmission of mutated mtDNA from mothers to their children, and as an aid for women who produce embryos that arrest at the two-cell stage after IVF.

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