Original Article

Evaluation of Embryos Derived from *in vitro* Fertilized Oocytes Reconstructed by Meiosis-II Chromosome Transplantation from Aged Mice to Ooplasms of Young Mice

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Abstract

Background: To assess embryos derived by the transfer of meiosis-II chromosomes (M-II-t) from aged mice oocytes into ooplasms from younger mice to overcome the problem of age-related decline in female fertility.

Materials and Methods: The developmental capacity, karyotype, and ultrastructure of reconstructed oocytes derived from meiosis-II chromosome transplantation from aged mice into the ooplasms of young mice by piezo-micromanipulation were assessed.

Results: The survival rate of enucleated young oocytes was 54% and the percent of fertilized reconstructed oocytes was 23%. The rate of embryo development to the two-cell stage after cultivation was 40%. Since 82.4% of the analyzed embryos derived from reconstructed oocytes had condensed nuclei, it was not possible to analyze their chromosomal integrity. However, 17.6% of analyzable reconstructed old oocyte derived embryos (old-ODEs), had normal diploid sets of chromosomes. Major structural differences were not observed between young, old, and M-II-t derived two-cell embryos.

Conclusion: Our findings suggested that ooplasms from younger mice may overcome age-associated problems in older mice.

Keywords: Aging, Chromosome, Nuclear Transfer, Oocyte, Ultrastructure

Introduction

Age-related decline in female fertility is a common phenomenon in older women (1). Maternal age is shown to affect oocyte quality and early development (2) which may relate to aneuploidy in oocytes and embryos (3, 4). Segregation of chromosomes appears to be controlled by the meiotic spindle, but its components are largely supplemented by the ooplasm. Recently, attempts have been made to transfer a young oocyte cytoplasm into an old oocyte (5) or to transfer mitochondria and microtubules into an old oocyte cytoplasm to overcome these problems (6-9). Germinal vesicle (GV) transplantation is proposed as an approach for improving the oocyte quality of aged women (10, 11), assuming that cytoplasmic factor(s) in the ooplasm derived from younger women could reduce the incidence of oocyte abnormalities of older women. The placement of an aged GV nucleus into a younger ooplasm using micromanipulation and electrofusion reduces the occurrence of aneuploidy (11-13). In mice, it has been demonstrated that nuclear transplantation can be accomplished efficiently, and this technique appears not to impair subsequent oocyte maturation or increase the incidence of chromosomal abnormalities (10, 14). Maturation, fertilization, preimplantation and full term development in mice have been established using GV transplantation (15, 16). However, it is unclear whether transfer of meiosis-II chromosome transplantation (M-II-t) from aged mice to cytoplasts derived from oocytes of young mice could improve embryo development. The M-II-t technique enables nearly full genetic contribution of both parents to future embryos; it appears to be more flexible and efficient in boosting the implantation rate than oocyte cytoplasmic transfers (17). In the present study we examine, by M-II-t,

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the developmental capacity, chromosomal integrity, karyotype, and ultrastructure of reconstructed oocytes derived from aged mice.

Materials and Methods

Gamete collection

All animal care and surgical interventions were undertaken in strict accordance with the approval of the Royan Institutional Review Board and Institutional Ethics Committee. Female mice (NMRI strain, Razi Institute, Tehran, Iran) of various ages (old: 7-9 months; young: 6-8 weeks) were superovulated by a single i.p. injection with 7.5 IU pregnant mare serum gonadotrophin (PMSG, Intervet, Netherlands) followed 48 hours later by 7.5 IU human chorionic gonadotrophin (hCG, Intervet, Netherlands).

The oviduct was punctured 14-16 hours after hCG to obtain metaphase II (MII) stage oocytes. MII oocytes were harvested in KSOM medium supplemented with bovine serum albumin (BSA, 4 mg/ml, Sigma, A3311). Cumulus cells were removed by briefly exposing MII oocytes to medium containing 300 IU/ml hyaluronidase (Sigma). The denuded oocytes were washed three times in KSOM medium; then transferred and cultured in 20 µl drops of KSOM, under mineral oil, in 5% CO_2 and 95% air at 37 °C before micromanipulation.

Spermatozoa were obtained from the caudae epididymis of mature male mice (2-6 months) of the same strain and kept in KSOM medium for at least 1 hour for capacitation prior to injection.

Enucleation of MII oocytes

Two microdrops, each consisting of 3 µl KSOM-micromanipulation medium containing 3% sucrose, 20% fetal calf serum (FCS) and 7.5 µg/ml cytocalasin B (CB, Sigma, C6762) were placed on the bottom of a microinjection petri dish and covered with light mineral oil. Approximately 20 cumulus-free MII oocytes were placed respectively into two microdrops of the operation medium and treated for 5-10 minutes before micromanipulation. Observation of oocytes under an inverted microscope revealed a clear swelling around the metaphase chromosomes and the spindle itself appeared as a transparent area that distinguished it from the remainder of the cytoplasm.

The zona pellucida of the oocyte was slit with a piezo actuator (piezo pulse; speed: 6, intensity: 4, PiezoDrill; Burleigh Instruments, Inc., Fishers, NY, USA and PMMT.150FU, PrimeTech, Ibaragi, Japan) close to the position of the swelling. MII chromosomes from all recipients (young oocytes) were removed with an enucleation pipette. To ensure the efficiency of removal and transfer of the meiotic apparatus, a number of oocytes were stained with 5 µg/ml Hoechst 33342 (Sigma) and checked under an inverted microscope with a UV light source (18). After confirmation of enucleation, the experiments were conducted without Hoechst 33342 staining.

Nuclear transplantation and piezo-intra cytoplasmic sperm injection (ICSI)

The donor was obtained by aspiration of karyoplast containing MII meiosis apparatus or spindle from an old oocyte. The oocyte reconstruction was done by transferring an old oocyte donor MII meiosis apparatus into the cytoplasm of a recipient younger oocyte through the slit made by enucleation. Simultaneously, the reconstituted oocytes were injected with a single dissected sperm head by the aid of a piezo actuator, as previously reported (19). Briefly, a single spermatozoon was aspirated into an injection pipette of ~5 µm inner diameter. The sperm head and tail were separated by applying either a single or a few piezo pulses to the neck region. The head alone was injected. The fertilized eggs were cultured in KSOM. Therefore, there were three experimental groups including: embryos derived from young oocytes, old oocytes, and reconstructed old oocytes.

Embryos were collected approximately 24 hours after injection and their survival and developmental rates were evaluated.

Cytogenetic analysis

The zona-free zygotes derived from reconstructed oocytes were fixed for chromosome analysis. Zygotes were treated with 0.1 µg/ml colcemid 40 hours after fertilization. The embryos were then transferred into a 1% hypotonic trisodium citrate solution including 5mg/ml BSA for 15 minutes; swollen cells were transferred onto a clean slide and fixative I (methanol: acetic acid, 3:1, v/v) was added for 5 minutes. Fixative II (methanol: acetic acid, 1:1, v/v) and fixative III (methanol: acetic acid, 1:3, v/v) were subsequently added for at least 5 minutes and the slide air dried. For aging, the slides were maintained at 4 ºC for 24 hours and 28 hours at room temperature.

Finally, fixed blastomeres were stained with Giemsa in order to score the chromosomes.

Transmission electron microscopy

Embryos of old, young and reconstructed old oocytes were fixed for at least 1 hour in 2.5% glutaraldehyde (Sigma, USA) in 0.1 M cacodylate (Sigma, USA) buffer (pH 7.2), individually em-

bedded in 1% agarose, post-fixed for 1 hour in 1% aqueous osmium tetroxide, and dehydrated through a graded ethanol and isoamyl acetate series. Finally, the specimens were embedded in araldite (Sigma, USA). Samples were cut from pole to pole, alternating thin (70 nm) and thick (0.5 μ) sections. Thick sections were stained with toluidine blue and examined with a light microscope, whereas thin sections were stained with uranylacetate followed by lead citrate and observed under an electron microscope (Zeiss EM 900, Germany).

Results

Fertilization and embryo development

The survival rate of enucleated young oocytes was 54% (215/398). Of the reconstructed oocytes, 23% (50/215) were successfully fertilized after piezo/ICSI. Observation of the second polar body and pronucleus was conducted under an inverted microscope six hours after insemination. None of the unfertilized reconstructed oocytes had visible pronuclei. Therefore, we judged that they were not activated by piezo stimuli. The rate of embryo development in reconstructed oocytes to the two-cell stage (Fig 1A) was 40% (20/50). We did not assess further developmental rate of embryos due to a two-cell block in this strain of mice. The *in vivo* retrieved zygotes also did not pass the two-cell block as well. The perivitelline space was obvious around the blastomeres.

Cytogenetics of the fertilized reconstructed oocytes

One-cell stage embryos derived from reconstructed oocytes and old oocytes were processed for cytogenetic assessment. Normal karyotype was found in 93.3% (28/30) of the old-oocyte derived embryos (old-ODEs). Of the 6 (17.6%) analyzable reconstructed oocytes, all had a normal diploid set of chromosomes (Fig 3). However, 82.4% (28/34) of the analyzed embryos derived from reconstructed oocytes had a condensed nuclei and it was not possible to analyze them for karyotyping (Fig 1B and C). All embryos derived from young oocytes had normal karyotype.

Ultrastructure of two-cell embryos

We studied 15 two-cell embryos: 5 old-ODEs, 5 young ODEs and 5 reconstructed old-ODEs) by TEM (Fig 2). In all groups, by low magnification, the blastomers were uniformly covered with zona pellucida (Fig 2A, B, C). With higher magnification, the blastomeres were connected with the gap junction and covered with long, thin microvilli. At low magnification, the nuclei were located centrally in the blastomeres.

Ultrastructural examination was focused on the distinguishing features of the surface, cytoplasm, and nuclei of blastomeres in order to compare ultrastructural features in various groups.

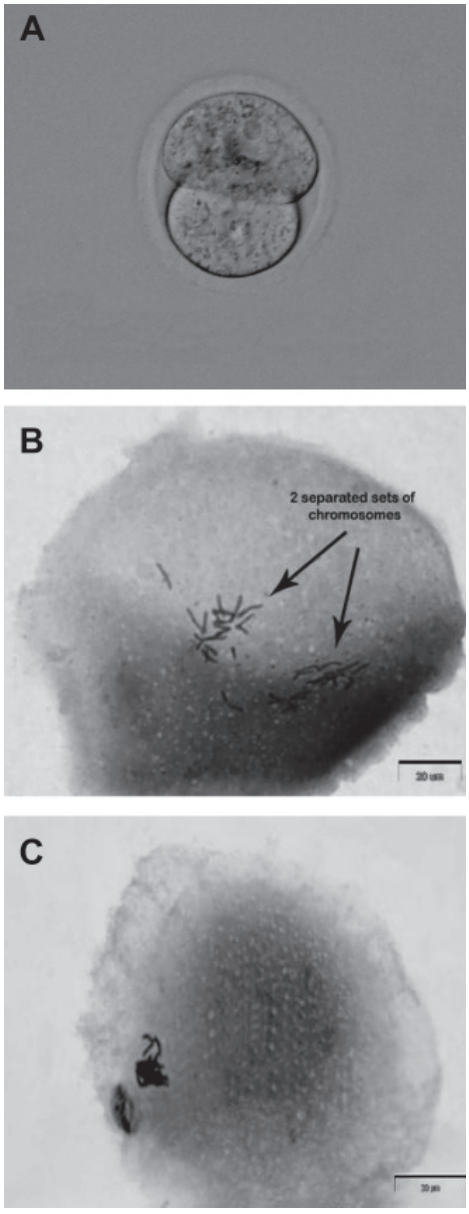

Fig 1: Embryo development and karyotype of a reconstructed ODE. (A) A two-cell stage embryo produced by piezo injection of a mature reconstructed oocyte. (B) Normal karyotype embryo. (C) Condensed nucleus of an embryo.

Blastomere surface

Plasma membrane invaginations were observed in embryos of all groups. Microvilli (MV) on the external surface, not in contact with other blastomeres, projected into the perivitelline space. Interdigitating MV were seen between

blastomeres in all embryos. As the contact points among blastomeres extended, electron-dense areas, possibly developing junctions, also appeared (Fig 2).

Cytoplasm

Lipid droplet vesicles containing flocculent material were seen in all groups. Occasionally cortical granules were found in the embryos. Cytoplasmic annulate lamellae were usually seen in the perinuclear area and terminated frequently in SER. Fibrogranular substances, aggregations of granules and round fibers, were observed. Phagosomes and lysosomes were rarely seen. Mitochondria were ovoid with a vaculated and/or hooded extremity.

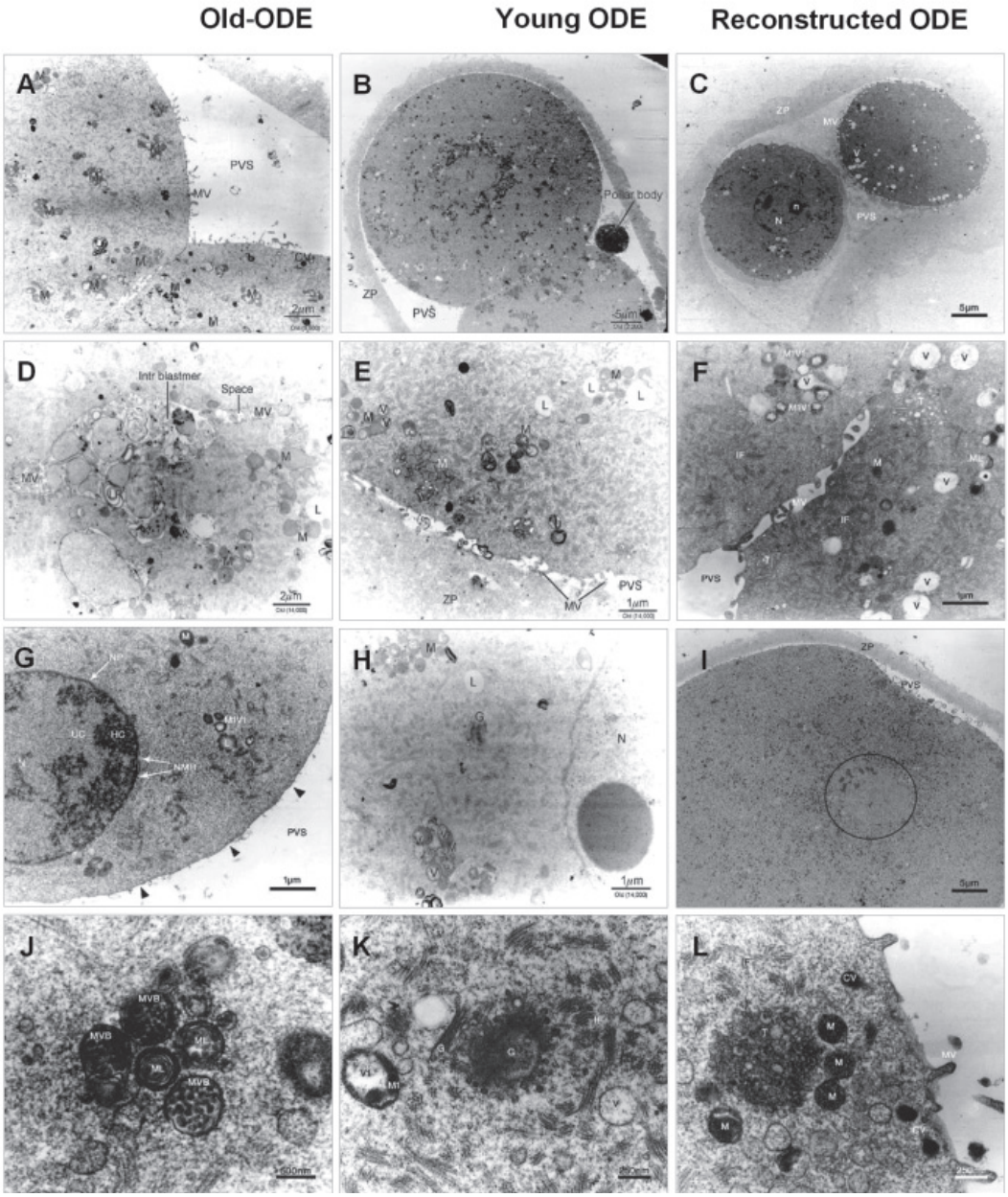

Fig 2: Electron micrograph of two-cell embryos derived from young, old and reconstructed oocytes. ODE: Oocyte derived embryo, CV: Cortical vesicle, G: Golgi apparatus, HC: Heterochromatin, IF: Intermediate filament, L: Lipid droplet, M: Mitochondrion, M1V1: Flattened crescent mitochondria encircling SER small vesicle, ML: Multilamellar body, MV: Microvilli, N: Nucleus, n: Nucleolus, PVS: Perivitelline space, ST: Sperm tail, T: Aggregates of tubules and vesicles, UC: Euchromatin, V: Vesicle, ZP: Zona pellucida.

The mitochondria matrix was dense. Mitochondrial cristae were mainly peripheral. One or more dark granules were also observed within some mitochondria. Intermediate filaments were observed as bundles. The Golgi apparatus with swollen cisternae were found in the nuclear region of all embryos. Abundant SER was observed in all embryos that usually was vesicular or slightly elongated, many of which were in contact with mitochondria and vacuoles. Polysomes were observed. Multivesicular and residual bodies were found in all embryos.

Nucleus

Pores were present in the nuclear envelope of all embryos. Small clumps of heterochromatin were observed which, in some nuclei, tended to be peripheral. The nucleoplasm was somewhat granular. Nucleoli, with or without a less electron-dense center was surrounded by granular components or with a vaculated center, were observed in all embryos.

Comparison of old-ODEs, young ODEs, and reconstructed old-ODEs

The two-cell embryos appeared similar under phase contrast. A slight difference in perivitelline space was more noticeable in the old-ODEs. Ultrastructural study of the embryos showed the periviteline spaces were more expanded and irregular in the old-ODEs than young and reconstructed old-ODEs. The cell organelles were homogeneously distributed throughout the cytoplasm in young and reconstructed old-ODEs, however in the old-ODEs, their density decreased peripherally. In the old-ODEs, mitochondria were seldom observed at the periphery of the cell but were more abundant around the nuclear envelope. Multivesicular and residual bodies were more abundant in old and reconstructed old-ODEs. Rough endoplasmic reticulum was seldom observed in the three groups. There were more lipid clusters and small droplet lipid in the old and reconstructed ODEs.

Discussion

Our primary finding is that although MII oocytes which were transferred from young to aged oocytes could undergo maturation and early cleavage; the young mice ooplasm could not possibly reduce age-associated problems of MII chromosomes from aged mice. Cui et al. also have reported that the transfer of GVs to ooplasms of young mice could not rescue age-associated chromosome misalignment in the meiosis of oocytes from aged mice (20). These observations may be related to the fact that the nuclear compartment plays a predominant role in the etiology of age-related meiotic defects. Researchers have shown that the behavior of chromosomal alignment and dispersal over the metaphase spindle is predominantly determined by the GV, not the ooplasm, in mice (20, 21). These observations are contrary to those reported in humans. It is reported that when the GV from oocytes of older women are transferred into enucleated immature oocytes of younger women, a normal (12, 13, 22) or abnormal (12) second meiotic metaphase chromosome complement is observed in the reconstructed oocytes. These findings in humans seem to support the idea that a young ooplasm does have a rescuing role in GV from older women. However, because the numbers of reconstructed oocytes examined are few in our study, it is hard to draw any definite conclusions. Whether there is a species difference of young ooplasms effect on aged GVs between mice and humans remains to be determined.

Moreover, it has been shown that transferring chromosomes from an *in vitro* matured oocyte into the cytoplasm of an enucleated oocyte matured *in vivo* (M-II-t) can improve subsequent embryonic development (23, 24). *In vitro* fertilization (IVF) of mouse oocytes reconstructed by M-II-t can result in live births, although the success rate of electrofusion of the karyoplast and cytoplast pairs from different oocytes and the fertilization rate of the fused oocytes is low (25). Chromosomal analysis of oocytes reconstructed with M-II-t followed by *in vitro* maturation, has been thoroughly studied in mice (25). Recently, Mitsui and Yoshizawa (26) reported that mice embryos produced by M-II-t have normal development and the incidence of chromosomal abnormalities of the M-II-t groups is similar to *in vitro* fertilization (IVF) derived embryos as previously reported (27). Additionally, Mitsui et al. reported that reconstructed oocytes which consisted of aged-karyoplasts and young-cytoplasts showed significantly improved embryonic development and development to term as compared with oocytes reconstructed from young karyoplasts and aged-cytoplasts (28). Generally speaking, in our study, at the ultrastructural level there is no significant difference between groups except the periviteline spaces in the old-ODEs are more expanded and irregular than young and reconstructed old-ODEs.

Conclusion

In summary, our results suggest that M-II-t from aged mice transferred to cytoplasts derived from MII oocytes of young mice may improve age-associated problems of oocytes.

Acknowledgements

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