Resumption of Meiosis-I After Transfer of Mouse Primordial Oocytes From Frozen-Thawed Ovarian Tissue to Enucleated Preovulatory Oocytes: A Preliminary Report

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Purpose: Ovarian tissue banking may be the best strategy to preserve female fertility. But optimal method to obtain viable mature oocytes remains challenging. In order to bypass the long in vitro oocyte growth period, we developed this study to test whether reconstruction of thawed primordial oocytes with enucleated preovulatory germinal vesicle (GV) oocytes could induce dictyate nuclei to undergo chromosomal condensation and meiotic maturation.

Methods: Isolated primordial oocytes from thawed mouse ovarian tissue were reconstructed with enucleated GV oocytes. After electrofusion and in vitro maturation, the reconstituted oocytes were assessed for first polar body extrusion, cytoskeleton configuration, and chromosome abnormalities.

Results: Primordial oocytes from thawed ovarian tissue showed a high survival rate. Following transfer and electrofusion, they could be fused with enucleated GV oocytes (35.6%, 36/101) and extruded a first polar body (52.8%, 19/36). These mature oocytes showed a normal spindle configuration and chromosome number.

Conclusions: We successfully established a mouse cell model to prove that omitting the whole growth and maturation period by transfer of primordial oocytes to developmentally older enucleated oocytes would bypass the long growth period required to the preovulatory stage. Polar body extrusion could also ensue after in vitro growth. This study provided an alternative approach for future investigations in oocyte maturation.

KEY WORDS: Cryopreservation; maturation; nuclear transfer; ovarian banking; primordial oocyte.

INTRODUCTION

The growth of clinical services for IVF has been a major stimulus for cryotechnology. It is well-recognized that the ability to cryopreserve sperms, oocytes or ovarian tissues, and embryos would make a significant contribution to infertility treatment. Although the cryopreservation of the sperms and embryos has

gained a satisfactory result, cryopreservation of the female gametes has met with little success (1,2). Because oocyte freezing has some inherited technical problems that would result in poor survival rates and in a low number of pregnancies (3–6). These difficulties are due to the biological features of the oocytes, including chromosome and meiotic spindle aberration (7), cytoskeleton abnormalities (8), premature release of cortical granules (9), hardening of zona pellucida (3), and parthenogenetic activation (10).

Ovarian tissue cryopreservation is an alternative approach because it contains multiple immature oocytes, predominantly primordial oocytes (11), and primordial oocytes appear less vulnerable to

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cryoinjury than growing or mature oocytes (12). This approach has been employed with some success in other species (13,14). Preliminary data on human ovarian tissue suggest that it may offer a clinical option in the future (15–17).

Following thawing of the ovarian tissue, the developmental stage may take place in vivo (e.g. organ transplantation) or in vitro (18). Considering the fact that ovarian tissue may serve as a reservoir for infections and diseases (especially for malignancy), it may also transmit that infection or disease to the recipients though they have already been free of the disease (19). This highlights the need to develop in vitro methods for primordial follicle growth and maturation. Although it has been achieved in the mouse (20), it poses a significant challenge in the human both at present and in the future (21,22), because human follicles grow very slowly and to much larger sizes than do mouse follicles.

Since ovarian tissue contains multiple primordial follicles, and nuclei of primordial oocytes are in the diplotene (dictyate) stage of meiotic prophase, which is in the same stage as the nuclei of preovulatory germinal vesicle (GV) oocytes. However, the GV of a preovulatory oocyte represents the final stage in the development of the dictyate nucleus that is achieved after more than 180 days of oocyte growth and differentiation (23). Previous investigations (24,25) proved that omitting the whole growth and maturation period did not affect the capability of bivalents to undergo chromosomal condensation and meiotic maturation, and transferred the diplotene nuclei to developmentally older oocytes did not alter the dynamics of meiosis. Clearly, such an approach would bypass the long period required to grow primordial follicles to the preovulatory stage.

Recent reports regarding nuclear transfer were mostly focused on animal cloning (26,27), transgenic animal models, gene therapy, and disease model investigation (28). In the field of human reproduction, the research is limited due to ethical issues involved in this kind of micromanipulation, especially for cloning. But GV nuclear transfer on human and mouse oocytes had been proved to be successful for further maturation and development (29,30). Our current research is also focused on GV nuclear transfer, and our preliminary results are encouraging. The reconstructed mouse oocytes could develop to blastocyst stage, and their ultrastructure and chromosome configurations are normal.

Currently, the technique of in vitro growth of primordial follicles in humans is far from being clinically

available (31). The aim of this study is to combine ovarian tissue cryopreservation and nuclear transfer techniques, in order to resolve the difficulties of post-thawed follicle growth, development, and maturation. Our ultimate goal is to establish this cell model as an alternative approach for oocyte in vitro growth and maturation.

MATERIALS AND METHODS

Source of Ovarian Tissue

To provide nearly pure primordial follicles, the newborn mouse ovaries which predominantly contain primordial follicles were used as a source of ovarian tissue. Female ICR mice, 3-day-old, were killed by decapitation and the ovaries were aseptically removed and placed in a 35 × 10-mm disposable petri dish (Falcon, Becton Dickinson, Lincoln Park, NJ, USA), containing 2 mL of M2 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT, USA) at room temperature. After dissecting from fat and adhering tissue, the ovaries were minced into pieces.

Freezing and Thawing

Pieces of ovaries were placed in 1.5 mol/L dimethylsulphoxide (DMSO) in M2 medium and held at room temperature (~20°C) for 20 min. A small volume of M2 + DMSO was drawn into a plastic freezing straw followed by an air bubble and finally ~0.2 mL of cell suspension (two or three minced ovaries per straw). The straw were sealed with hematocrit putty and placed in a programmable freezer (R202; Planer Biomed, Sunbury-on-Thames, UK) at 0°C and cooled at a rate of 2°C/min to –8°C. After 5 min, prechilled forceps were used to induce ice formation (seeding) in the fraction of M2 + DMSO at the top of the straw. After a further 5 min, the straws were cooled slowly at a rate of 0.3°C/min to –40°C and then rapidly cooled at 10°C/min to –150°C. The straws were then transferred into liquid nitrogen (LN₂) and stored for at least 72 h.

For thawing, the straws were removed from LN₂, held in air at room temperature for 40 s and then immersed in water at 30°C for 5–10 s. The contents of the straws were emptied and placed in 1–2 mL M2 at room temperature for 5 min, and then washed twice in M2 at room temperature (1–2 mL per wash). After 5 min, the ovaries were placed in M2 at 37°C for further manipulation.

Isolation of Primordial Oocytes From Ovarian Tissues: Used as Karyoplast Donor

The thawed ovarian tissues were incubated at 37°C for 20 min in M2 + 10% FCS containing 0.5 mg/mL each of testicular hyaluronidase (Type I-S, Sigma) and collagenase (Type Ia, Sigma). After incubation, the ovarian tissues were gently dissociated with 30-gauge needles. Free primordial oocytes were collected and transferred to drops of M16 + FCS medium under paraffin oil. They were stored at 37°C before further manipulations.

Recovery of Germinal Vesicle Oocytes

Female ICR mice (5–7 weeks old) were injected intraperitoneously with 5 IU pregnant mare serum gonadotrophin (PMSG; Sigma). Immature, germinal vesicle stage oocytes (GV oocytes) were collected 24 h later by puncturing the follicles with a needle and any attached cumulus cells were dissociated by repeated pipetting.

Before and during GV transfer all the immature oocytes were incubated in M2 + 10% FCS containing 50 μ g/mL 3-isobutyl-1-methylxanthine (IBMX; Sigma). This inhibitor of cyclic nucleotide phosphodiesterase prevents GV breakdown (GVBD).

Enucleated GV Oocytes: Used as Recipient Cytoplasm

GV oocytes were incubated in microdrops of M2 with 10% FCS and 7.5 μ g/mL cytochalasin B (CCB; Sigma) for 30 min at room temperature prior to enucleation. Following lancing of the zona pellucida with a sharp-tipped pipette, GV nuclei were removed from immature oocytes using a bevelled glass pipette (ID: 20–25 μ m). These procedures were performed using a micromanipulator attached to an inverted microscope.

Primordial Oocytes Transfer and Fusion

The isolated primordial oocyte (karyoplast, nucleus donor) was inserted subzonally into an enucleated GV oocyte. Membrane fusion between oocyte and karyoplast was accomplished by electrofusion at room temperature. Briefly, the complexes were transferred into a drop of fusion medium (0.3 M mannitol, 0.1 mM CaCl_2 , and 0.05 mM MgSO_4 in PBS) between the platinum electrodes of a fusion chamber. After alignment with an AC pulse (300 V/cm, 2 MHz) for

5–10 s, fusion of karyoplast–cytoplasm complexes was achieved with electrical pulses (2500 V/cm DC for 64 μ s) delivered by a cell fusion and gene transduction instrument (Model FI-700; Nippon Medical and Chemical Instruments Co., Osaka, Japan). Preliminary studies in our laboratory established that these are the minimal pulse parameters sufficient for membrane fusion.

In vitro Maturation of Reconstructed GV Oocytes

After electrofusion, the complexes were washed three times in M2 medium and then placed in M16 medium supplemented with 10% FCS for in vitro maturation. The primordial oocyte (karyoplast) that was successful in electrofusion was usually incorporated into the cytoplasm within 1 h. The reconstructed oocytes were monitored during a 24 h incubation period for evidence of maturation.

Cytoskeleton Study

Reconstructed oocytes with extruded first polar body within the 24 h incubation period were considered to be mature and were selected for immunocytochemistry study of the meiotic spindle, which was performed using prior published methodology (30). Each oocyte was fixed in 3.7% formaldehyde (Sigma) in 0.1 mol/L PBS (pH 4.7) for 40 min, and then permeabilized in PBS buffer containing 0.25% Triton X-100 (Sigma) for 25 min at room temperature. Free aldehydes were reduced by a 20 min rinse in 0.1 mol/L PBS containing 150 mmol/L glycine (Sigma). Microtubules were localized with a mouse monoclonal antibody to β -tubulin (Sigma). The primary antibody was detected using a fluorescence labeled goat anti-mouse IgG (Sigma). Each antibody was applied overnight at 4°C; oocytes were rinsed with PBS containing 0.25% bovine serum albumin (BSA, Fraction V; Sigma) between antibody applications. Chromosomes were simultaneously detected with Hoechst 33342 (5 μ g/mL in 0.1 mol/L PBS). The stained oocytes were mounted whole on glass slides and examined using light phase and fluorescence microscopy.

Analysis of Chromosome Abnormality in MII Oocytes

Additional oocytes extruding a first polar body were fixed for cytogenetic analysis as described (32). Briefly, each oocyte was transferred into a 1% hypotonic trisodium citrate solution for 15 min before

fixation with methanol: acetic acid (3:1) on a clean glass slide. Following gradual air-drying, the fixed chromosomes were spread and stained with 5 $\mu\text{g/mL}$ Hoechst 33342 (Sigma) in phosphate buffered solution (PBS). The number and the structure of the chromosomes were determined under fluorescence microscopy.

RESULTS

Survival of Primordial Oocytes After Thawing

After thawing, primordial oocytes with dictyate nuclei showed a good morphological characteristic after isolated from frozen-thawed ovarian tissue with hyaluronidase and collagenase (Fig. 1). Although still in the stage of primordial follicle, oocytes dimension differed because of different amount of ooplasm and developmental status. The isolated primordial oocytes were incubated at 37°C for at least 30 min before micromanipulation.

Primordial Oocyte Transfer, Electrofusion, and IVM

After transfer subzonally into an enucleated oocyte and electrofusion, membranes of primordial oocytes could be fused successfully with enucleated GV oocytes at the rate of 35.6% (36/101) (Fig. 2). Of them, 52.8% (19/36) of the reconstructed oocytes could be matured to metaphase II after in vitro culture.

Chromosome and Cytoskeletal Study

To determine whether meiosis progressed normally following reconstruction and maturation, nine mature

Fig. 1. Primordial oocytes isolated from frozen-thawed ovarian tissue. Oocyte dimension differed because of different amount of ooplasm and development stage.

Fig. 2. Preparation of enucleated cytoplasts from germinal vesicle (GV) stage, transfer and electrofusion of primordial oocytes, and the resulting metaphase II oocyte: removal of GV from GV stage oocyte (A); insertion of primordial oocyte to the perivitelline space of an enucleated GV oocyte (B); reconstructed oocytes during fusion (C); first polar body extrusion after in vitro maturation (D).

reconstructed oocytes (with extrusion of the first polar body) were stained with anti- β tubulin antibody followed by vital staining with Hoechst 33342. Two areas of fluorescence, one in the polar body and the other in the cytoplasm, were noted in each oocyte. Eight out of nine oocytes showed a bipolar spindle configuration and normal metaphase chromosome alignment on the spindle equator (Fig. 3).

Eight out of ten oocytes were successfully fixed for cytogenetic study; they all displayed a normal univalent chromosome number (Fig. 4).

Fig. 3. Matured reconstructed oocyte double stained with anti- β -tubulin antibody and Hoechst 33342. Normal bipolar spindle configuration and groups of chromosomes aligned on the equatorial plate of the spindle and in the polar body are shown. Bar = 10 μm .

Fig. 4. Chromosome spreads of mature reconstructed oocytes stained with Hoechst 33342. Bar = 10 μ m.

DISCUSSION

With the aim to solve the difficulties of postthawed follicular growth and maturation through combining cryopreservation of ovarian tissues and the technique of nuclear transfer, we first demonstrated a successful report of first meiosis after transfer and reconstruction of primordial oocytes, which were harvested from frozen-thawed ovarian tissue, with enucleated GV oocytes. Meanwhile, we proved that the capability of bivalents to undergo chromosomal condensation and meiotic dynamics were not affected. Apparently, these results are certain to have some clinical implications.

It has been suggested that preservation of the female gamete may be best achieved by storing pieces of ovarian tissue which predominantly contains primordial follicles (18). But at present, there are still uncertainties about the optimal use of stored ovarian tissue. Conventionally, there are three options for development of immature oocytes in cryopreserved ovarian tissue that can be applied to restore fertility, include autotransplantation (either orthotopic or heterotopic), xenotransplantation, and in vitro maturation.

Relatively, autotransplantation appears to be a simple and practical method. In addition to restoring ovarian function after transplantation of frozen-thawed ovarian tissue (33), it has also been reported that ovulation occurred in autografted human ovarian tissue (34). However, the expected relatively short life span of frozen-thawed ovarian graft and the potential risk of disease or malignant cell transmission are disputable concerns (19,35) that clinical applications may be limited. Xenotransplantation can eliminate the possibility of cancer or disease transmission, risk

of ovarian hyperstimulation syndrome, and can also bypass the difficulties of in vitro growth and maturation of primordial follicle (36), but the possibility of animal pathogens being transmitted to human tissue and the ethical concern should take into consideration. As for the feasibility of in vitro growth and maturation of human primordial follicles, it is still technically challenging. Although primordial oocytes can be matured and fertilized in animal models (20,37), and the two-step strategy for follicle and oocyte development was proposed (38), the ability to completely grow and mature human immature oocytes in vitro is not likely to be available in the near future.

As current protocols for in vitro growth are inadequate and the transplantation strategies have inherited limitations in their clinical applications, we developed this study in order to apply technique of nuclear transfer to bypass the long in vitro oocyte growth period. Followed by electrofusion and in vitro maturation of the reconstructed oocytes, we successfully generated competent oocytes and established an animal model by demonstrating first meiosis after transfer of frozen-thawed primordial oocytes into the perivitelline space of enucleated GV oocytes.

Previous study demonstrated that maturity of host oocyte cytoplasm determined meiosis after GV transfer (30). GV breakdown (GVBD) and first polar body extrusion could be better achieved if enucleated GV oocytes were used as cytoplasm donors. This is why we employed preovulatory GV oocytes, instead of mature metaphase II oocytes, which are routinely used in cloning animals (39), in our study. Noteworthy, the efficiency for electrofusion in the present study was relatively low (35.6%) compared with previous reports (29,30) and our own preliminary data in fresh GV transfer. The causes may be attributed to fragility of the cell membrane after freezing and the more disproportionate size between karyoplast (primordial oocyte) and cytoplasm (GV oocyte) (40). Following fusion, nonetheless, the reconstructed oocytes could respond normally to maturation/meiosis promoting factor (MPF), which is an important factor for the induction of GVBD, chromosome condensation, and final nuclear maturation. They resumed meiosis at the rate of 52.8%, and were comparable to others (29,30).

Clearly, the ability to undergo nuclear maturation is necessary but not sufficient for an oocyte to undergo full development after fertilization. Many oocytes probably undergo abnormal meiotic segregation even in morphologically normal cells (41). As a result, demonstration of chromosome number

and microtubule configuration in the reconstructed oocytes are very important. In this study, we delineated a normal microtubule configuration and chromosome number to ensure the success of first meiosis of thawed primordial oocytes after oocyte reconstruction and in vitro maturation.

As a recent report proved a normal embryogenesis following nuclear transfer of immature mouse oocytes from preantral follicles (42), it further justifies the role of nuclear transfer for accelerating oocytes in vitro maturation. Additionally, we extended the feasibility of nuclear transfer to primordial oocytes from thawed ovarian tissue. Besides resolving difficulties of ovarian tissue freezing, its implication is that the majority of follicles within human ovarian tissue are at the primordial or primary stages and preantral follicles are scarcer (43), which obviously will limit their future clinical application. Noticeably, the resumption of first meiosis may not guarantee that these oocytes are competent for full embryonic development and implantation (44), and the importance of genomic imprinting during oocyte growth has been demonstrated (25). In their studies, the developmental potential of the oocytes was compromised as a result of asynchronous nuclear and cytoplasmic maturation and changes in the methylation pattern of specific genes. Therefore, apart from this preliminary data of our study, it is extremely important to confirm a normal development of the embryos before going on clinical applications.

Our study confirmed that primordial follicles are less vulnerable to cryoinjury. Moreover, we proved that omitting the whole growth and maturation period by nuclear transfer to developmentally older enucleated oocyte did not affect the capability of bivalent chromosome to undergo condensation and meiotic maturation, even for a primordial oocyte from frozen-thawed ovarian tissue. Clearly, such an approach would bypass the long period required to grow postthawed primordial follicles to the preovulatory stage. Although further studies are necessary to determine whether normal offsprings can be produced using this protocol, our study successfully established a cell model to resolve the difficulty of in vitro growth and maturation of primordial follicles from frozen-banked ovarian tissue, which may also provide an alternative approach for future investigations.

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