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Effect of ovarian tissue vitrification method on mice preantral follicular development and gene expression

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ABSTRACT

Vitrification is considered a viable method for cryopreservation of ovarian tissue and selection of methods that minimize follicular damage is important. The objective of the present study was to evaluate the effects of two vitrification methods on ovarian tissue morphology, preantral follicles survival rate during *in vitro* culture, and relative expression of genes associated with oocyte maturation and cumulus expansion. Ovaries from 12-day-old mice were vitrified in media containing ethylene glycol, dimethyl sulphoxide, and sucrose. Before plunging in liquid nitrogen, ovaries were first loaded into an acupuncture needle (needle immersion vitrification [NIV]) or placed on a cold steel surface for 10 to 20 seconds (solid surface vitrification [SSV]). The integrity of the ovarian tissue was well-preserved after vitrification and was similar controls. Follicle viability in the SSV group was lower ($P < 0.05$) than in the control group after 6 days of culture and the NIV group after 10 day of culture. Follicle viability after 12 day of culture was 92.8%, 82.1%, and 58.4% in control, NIV, and SSV groups, respectively. *Bmp15*, *Gdf9*, *BmprII*, *Alk6*, *Alk5*, *Has2*, and *Ptgs2* gene expression patterns were similar among groups. However, the level of gene expression in the vitrification groups during Days 6 to 10 were higher compared with the control group. In conclusion, ovarian tissue morphologic integrity was well-preserved, regardless of the vitrification method. Vitrification using the needle immersion method resulted in greater follicular survival after 12 day of culture than the SSV method. Gene expression patterns during culture did not seem to explain the reduced survival rate observed in the solid surface group.

1. Introduction

Cryopreservation can be used to preserve oocytes and ovarian tissue to retain fertility in females with ovarian failure of various causes [1,2]. Because obtaining mature oocytes from prepubertal females is not possible, ovarian tissue cryopreservation could be a viable option [3,4]. Intra- and extracellular ice crystallization associated with low concentration of cryoprotectants and low cooling rate leads to reduced cell survival and low follicles survival rates when ovarian tissue is preserved using slow freezing methods [5,6]. Vitrification is considered a viable and efficient method for cryopreservation of ovarian tissue [7] and high follicle survival rates have been reported in rodents [8,9], domestic animals [10], primates [11], and humans [12].

An efficient ovarian tissue cryopreservation method preserves several follicles in different developmental stages [13]. Although live births of animals [14] and humans [15,16] have been reported after transplantation of cryopreserved ovarian tissue, widespread follicular fibrosis

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and risk of malignant cells transfer in autotransplantation procedure are problems that require attention [17]. When the transplantation procedure problems and live mice pup birth after culture of cryopreserved immature ovarian tissue-derived preantral follicles [18] are considered, it seems that follicle culture could be used to produce mature oocytes after ovarian tissue cryopreservation [7]. All molecular and cellular events that take place during *in vivo* development must also happen when *in vitro* culture is used to produce fertile oocytes [19].

Oogenesis and folliculogenesis involve complex interactions of endocrine, paracrine, and autocrine mechanisms among the oocyte and the somatic cells of the follicle [20]. Therefore, modification of gene expression caused by ovarian tissue vitrification can affect oocyte and follicular development. Growth differentiation factor 9 (*Gdf9*) and bone morphogenic protein15 (*Bmp15*) are the two most popular members of transforming growth factor β superfamily genes, which play important roles during folliculogenesis such as regulation of granulosa cell proliferation and differentiation [20]. Products of *Gdf9* and *Bmp15* genes act through specific receptors—activin receptor-like kinase 5 and 6 (*Alk5*, 6) and bone morphogenic protein receptor II (*BmprII*)—both of which are expressed in oocyte, granulosa, and theca cells [21]. In addition, other genes are regulated by *Gdf9* in cumulus and granulosa cells, such as hyaluronic acid synthase 2 (*Has2*) and prostaglandin-endoproxide synthase 2 (*Ptgs2*), which are responsible for cumulus expansion and follicle development [22]. Vitrification reduces the expression of *Gdf9* and *Bmp15* in sheep cumulus-oocyte complexes [21], and downregulation of *Mater* and *Hook1* and upregulation of *Sod1* in mature mouse oocytes [23].

The objective of the present study was to evaluate the effects of two vitrification methods on ovarian tissue morphology, preantral follicles survival rate during *in vitro* culture, and relative expression of genes associated with oocyte maturation and cumulus expansion.

2. Materials and methods

2.1. Animals and study design

Animals were handled according to the Ethical guidelines of the Royan Institute. Female adult NMRI mice (Pasteur Institute of Iran) were housed in a controlled temperature (20–25 °C) and lighting (12 hour light:12 hour dark) environment and provided food and water ad libtum. A total of 400 ovaries from 12-day-old female mice were randomly distributed into four experimental groups: Nonvitrified control, toxicity test, needle immersed vitrification (NIV), and solid surface vitrification (SSV). Experiments were replicated three times. *In vitro* follicular growth and gene expression were evaluated at 1, 6 (beginning of cumulus expansion), 10 (antrum formation), and 12 (antral follicle) days after culture.

2.2. Vitrification and histologic evaluation

Both ovaries, with intact tunica albuginea, were first equilibrated in 1.5 mL α-minimal essential medium (α-MEM; Gibco, Paisley, UK) supplemented with 7.5% ethylene glycol (Sigma, St Louis, MO, USA), 7.5% DMSO (Sigma), and 20% fetal bovine serum (FBS; Gibco) for 15 minutes at 4 °C in an ice bath. Ovaries then were transferred into 1.5 mL of vitrification solution (α-MEM supplemented with 15% ethylene glycol, 15% DMSO, 0.5 mol/L sucrose, and 20% FBS) for 30 minutes at 4 °C [18]. Before plunging in liquid nitrogen (LN_2), ovaries in the NIV group were first loaded into an acupuncture needle (Dong Bang; Boryeong, Korea) [24], whereas ovaries in the SSV group were placed on a cooled steel surface for 10 to 20 seconds; the steel surface had been placed into LN_2 for 15 minutes before this procedure and its temperature was approximately −150 to −180 °C. Ovaries were then transferred with only a thin film of vitrification medium into LN_2-filled cryotubes (Grenier bio-one, Frickenhausen, Germany) and stored in LN_2 for 1 week.

Upon removal from LN_2, vitrified ovaries were immediately immersed in warming solution I (α-MEM, 1 mol/L sucrose, and 20% FBS) at room temperature for 10 minutes, then transferred to warming solution II (α-MEM and 10% FBS) at 37 °C for 30 to 60 minutes. Ovaries in the toxicity test group were washed in the equilibration and vitrification solution without plunging in liquid nitrogen and then were transferred to the warming solutions to remove cryoprotectants. Ovaries were fixed in bouin's fixative for 24 hours, formaldehyde for 48 hours, dehydrated in ascending concentrations of ethanol, embedded in paraffin wax, sectioned (6 μm thicknesses), mounted, and stained with hematoxylin and eosin according to conventional techniques. Ovarian morphology was evaluated by light microscopy.

2.3. Follicle isolation and in vitro culture

Nonvitrified fresh (control group) and vitrified-warmed ovaries (NIV and SSV groups) were transferred into 50-μL droplets of α-MEM with 10% FBS and preantral (secondary) follicles with intact centrally located oocyte and two or more layers of surrounding granulosa cell (110 to 130 μm diameter) were isolated using a 29-ga needle. Follicles were individually cultured in 96-well plates (TPP, Trasadingen, Switzerland) for 12 days. Culture medium consisted of α-MEM supplemented with 5% FBS, 5 mg/mL insulin, 5 mg/mL transferrin, 5 ng/mL sodium selenite (Gibco), 10 mIU/mL recombinant FSH (Merck, Darmstadt, Germany) and 1 mIU/mL recombinant LH (Merck). It must be noted that recombinant LH was added only at the beginning of the culture period. Follicles were cultured in 75 μL culture medium under 45 μL mineral oil at 37 °C, 100% humidity, and 5% CO_2. Every 4 Day, 30 μL of culture medium was replaced with fresh medium. Follicle diameter was measured using an invert microscope (Nikon, Düsseldorf, Germany) every other day during the culture period. Follicles were considered viable when oocytes were clear, intact, and more than 50% of the granulosa cells were viable.

2.4. Evaluation of gene expression

For evaluation of gene expression, 1440 follicles (40 follicles/replicate) were used. Follicles from control and vitrification groups were pooled at different times after