Cryobiology xxx (2015) 1–8

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Three dimensional in vitro culture of preantral follicles following slow-freezing and vitrification of mouse ovarian tissue

Fatemeh Asgari [a], Mojtaba Rezazadeh Valojerdi [a, b, *], Bita Ebrahimi [b], Roya Fatehi [b]

[a] Department of Anatomy, Faculty of Medical Sciences, Tarbiat Modares University, P.O. Box14115-111, Tehran, Iran
[b] Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

ARTICLE INFO

Article history:
Received 15 September 2015
Received in revised form
6 November 2015
Accepted 9 November 2015
Available online xxx

Keywords:
Preantral follicle
Ovary cryopreservation
Three dimensional in vitro culture

ABSTRACT

To evaluate the effects slow-freezing and vitrification on three dimensional in vitro culture of preantral follicles, ovaries of 12–14 days old female NMRI mice were isolated and randomly assigned to fresh control, slow-freezing and vitrification groups. Slow-freezing was performed using programmable freezer. Vitrification was carried out in a medium consisting of ethylene glycol (EG) and dimethyl sulphoxide (Me_2SO) by needle immersion method. middle sized preantral follicles were mechanically isolated and cultured for 12 days in 0.7% sodium alginate gel. The follicles development and quantitative expression of oocyte specific genes (*Bmp15*, *Gdf9*, *Fgf8*) and the growth related genes (*Igf1*, *Kit*, *Kit-l*) were assessed after 1, 8 and 12 days of culture. Both cryopreserved groups showed reduction of follicular survival rates compared to the control group on days 8 and 12 of culture ($P < 0.05$). Antrum formation rates reduced in slow-freezing after 12 days of culture ($P < 0.05$). Evaluation of gene expression showed reduction of *Bmp15*, *Gdf9*, *Fgf8*, *Kit* and *Kit-l* during 12 days of culture ($P < 0.05$). *Kit* and *Kit-l* expression in slow-freezing group significantly reduced on day 8 of culture ($p < 0.05$). *Igf1* expression was lower in slow-freezing group on 1st day of culture than vitrification and control groups ($P < 0.05$). Finally, intergroup comparison showed same expression pattern of genes after 12 days of culture. Thus, cryopreservation of mouse ovaries by both methods can preserve most developmental parameters and expression of maturation genes. However, vitrification is a better method for cryopreservation of mouse ovaries due to greater antrum formation and expression of growth related markers.

1. Introduction

Chemotherapy and radiotherapy has improved life expectancy of patients with cancer, however these technique are gonadotoxic and may cause infertility [4,43]. To preserve fertility in females with ovarian failure, cryopreservation of ovarian tissue has been studied in different species including human [27], murine [16], rat [39], sheep [14], bovine [31], elephant [15] and rabbit [10]. Human ovaries were cryopreserved for the first time in 1996 by Hovatta [20]. Slow-freezing and vitrification are the two freezing techniques that are commonly employed in cryopreservation studies. Slow-freezing has long been used for cryopreservation of embryos, oocytes and ovarian tissues by a programmed freezer [18]. Despite its use for many years, the method suffers from drawbacks such as formation of intracellular ice crystals, cell damage and the need for expensive equipment [43]. Luyet was introduced vitrification method for tissue preservation in 1937 [23] and thereafter, Rall and Fahy were used this method to evaluate survival of mouse embryos. In their study, better survival rate of embryo was seen after vitrification because of no ice formation [34]. vitrification mentioned as a rapid, simple and economic method, which was associated with less cell damage than slow-freezing and no ice crystal formation [6]. Vitrification has the possible disadvantage of cell toxicity [33]. The main differences between these two cryopreservation methods are cryoprotectant concentration and cooling rate [33]. To improve the efficiency of cryopreservation, comparative studies have been performed between slow-freezing and vitrification methods to find the better freezing method for ovarian tissue [1,4,21].

Usually, human cryopreserved ovaries have been reused in two different methods; transplantation and in vitro culture. Due to the possibility of metastatic spread after transplantation [22,41],

* Corresponding author. Department of Anatomy, Faculty of Medical Sciences, Tarbiat Modares University, P.O. Box14115-111, Tehran, Iran and Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran.
E-mail addresses: mr_valojerdi@modares.ac.ir, mr_valojerdi@royaninstitute.org (M.R. Valojerdi).

http://dx.doi.org/10.1016/j.cryobiol.2015.11.001
0011-2240/

in vitro culture of follicles seems more attractive for achieving mature oocytes [38]. Nayadu used millicell hydrophobic insert to preserve the physical integrity of granulosa cells and oocyte [25]. Kreeger provided a three-dimensional structure using 1.5% alginate gel for the first time in 2005 and thus introduced the promotion and regulation of cell adhesion that lead to tissue development [22]. The follicle that was cultured three dimensionally by hydrogel, endured equal pressure in all directions with less stress on gap junctions so that structure was preserved, resulting in better follicle development [37,45].

To improve culture environment and oocyte quality, the efficiency of the cryopreservation methods can be evaluated by assessing the expression of folliculogenesis genes. Bone morphogenetic protein 15 (*Bmp15*), growth differentiation factor 9 (*Gdf9*) and fibroblast growth factor 8 (*Fgf8*) are oocyte-specific factors that are expressed within the oocytes [13]. They express during folliculogenesis, proliferation and differentiation of the granulosa cells [29]. Insulin-like growth factor 1 (*Igf1*), *Kit* and Kit ligand (*KitL*) are other growth-related genes [29] that are secreted by oocyte and granulosa cells respectively. They promote oocyte growth and maintenance of meiotic arrest in response to follicle-stimulating hormone receptor (FSHR) levels [32]. *Igf1* is expressed in granulosa, interstitial and theca cells at the end of folliculogenesis after increase of mouse ovarian size [17].

The objective of this study was to compare the effects slow-freezing and vitrification on preantral follicles development in mouse ovarian tissue and gene expression during 12 days of follicle culture in alginate gel.

2. Materials and methods

2.1. Animals and study design

The animals were handled according to the ethical guidelines of the Royan Institute. Female adult NMRI mice were housed in a controlled temperature (20–25 °C) and lighting (12 h light: 12 h dark) and provided food and water ad libitum. In order to enable pairing and birthing, pre pubertal female mice were selected. Ovaries of 12–14 days old female mice were isolated and randomly assigned to three experimental groups: fresh control, slow-freezing and needle immersed vitrification (NIV). In vitro follicular growth and gene expression were evaluated on first, 8th and 12th days of culture.

2.2. Slow-freezing

Ovaries were slowly frozen using a programmable freezer according to the protocol described by Min Xu [44]. The cryoprotectant was composed of α-minimal essential medium (α-MEM; Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Gibco), 0.1 mol/L sucrose (Sigma; Germany) and 1.5 mol/L Me_2SO (Sigma; Germany). Ovaries were first equilibrated in the cryoprotectant solution for 30 min at 4 °C with slow shaking. After that, one ovary was transferred into a 1.8 mL cryovial containing 1 mL cryoprotectant solution. The vials were cooled in a programmable freezer as follow: (1) cooled from 4 °C at−2 °C/min to −9 °C; (2) equilibrated for 6 min at −9 °C; (3) seeded manually; (4) held for 4 min at −9 °C; (5) cooled at −0.3 °C/min to −40 °C; (6) immersed into liquid nitrogen and stored for future use [44].

2.3. Thawing

The vials were removed from liquid nitrogen, left for 1 min at room temperature (22 °C), transferred to 37 °C water bath with slow shaking and removed as soon as they were thawed. The ovaries were transferred into dishes containing 3 mL of α-MEM supplemented with (1) 1 mol/L Me_2SO, 0.1 mol/L sucrose, 10% FBS for 5 min; (2) 0.5 mol/L Me_2SO, 0.1 mol/L sucrose, 10% FBS for 5 min; (3) 0.1 mol/L sucrose, 10% FBS for 5 min; (4) 10% FBS for 2.5 min. All thawing steps were carried out at room temperature. Finally, ovaries were incubated in α-MEM containing 10% FBS at 37 °C, 5% CO_2 before follicles isolation [44].

2.4. Vitrification

Ovaries were first equilibrated in 1.5 mL α-MEM supplemented with 1.35 mol/L EG (Sigma, St Louis, MO, USA), 1.05 mol/L Me_2SO, and 20% FBS for 15 min at 4 °C. They were then transferred into 1.5 mL of vitrification solution (α-MEM supplemented with 2.70 mol/L EG, 2.10 mol/L Me_2SO, 0.5 mol/L sucrose, and 20% FBS for 30 min at 4 °C. Finally, they were loaded with an acupuncture needle (Dong Bang; Boryeong, Korea) before plunging in liquid nitrogen [43].

2.5. Warming

Vitrified ovaries were immediately removed from liquid nitrogen and immersed in warming solution I (α-MEM, 1 mol/L sucrose and 20% FBS for 3 min, transferred to warming solution II (α-MEM, 0.5 mol/L sucrose and 20% FBS for 3 min and then transferred to warming solution III (α-MEM, 0.25 mol/L sucrose and 20% FBS for 3 min (every step was done at room temperature). Finally, the ovaries were incubated in α-MEM and 10% FBS at 37 °C for 30 min [46].

Table 1
Genebank accession numbers, primer sequence and product length.

Genes	Accession number	Primer Pair (5′ → 3′)	Product Length (bp)
Gdf9	NM_008110.2	F: CAAACCCAGCAGAAGTCAC R: AAGAGGCAGAGTTGTTCAGAG	164
Bmp15	NM_009757	F: AAATGGTGAGGCTGGTAA R: TGAAGTTGATGGCGGTAA	148
Kit	NM_001122733.1	F: GTCTACATCCGTGAACTCCA R: CAGAATCGTCAACTCTTGCC	148
Kit-l	NM_013598.2	F: AGGAATGACAGCAGTAGCAG R: GCAAAGCCAATTACAAGCGA	112
Igf1	NM_001111274.1	F: GGGACTTTCGTGACTGAG R: AGAGGCAAGTGACAGAGG	148
Fgf8	NM_001166361.1	F:TCATTGTGGAGACCGATAC R:TGTGAATATACGCAGTCCTT	118
GapDH	NM_008084	F: GACTTCAACAGCAACTCCCAC R: TCCACCACCCTGTTGCTGTA	125

Please cite this article in press as: F. Asgari, et al., Three dimensional in vitro culture of preantral follicles following slow-freezing and vitrification of mouse ovarian tissue, Cryobiology (2015), http://dx.doi.org/10.1016/j.cryobiol.2015.11.001