Sucrose affecting successful transplantation of vitrified-thawed mouse ovarian tissues

Jian-Min Zhang · Lin-Xia Li · Xue-Lian Liu ·
Yi-Xia Yang · Xiao-Ping Wan

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Abstract

Purpose The aim of this experiment is to detect effects of varying levels of sucrose on vitrified ovarian tissues.

Methods Ovarian tissues of mice were vitrified-thawed. Mice were randomly assigned to the fresh control group and experimental groups. According to different concentration of sucrose in vitrification solution, the experimental groups were randomly divided into Group I (0.2 M sucrose), Group II (0.4 M sucrose), Group III (0.8 M sucrose) and Group IV (1.6 M sucrose). Cytology was followed throughout the oophorectomy and transplantation period. Hormone levels and density of follicle were measured 1 month after transplantation.

Results The number of days before the resumption of estrous cycles in control group was significantly smaller than those in all of experimental groups. The serum estradiol levels of mice and the follicular density of ovarian grafts in control group were significantly higher than those in all of experimental groups. In addition, the number of days before the resumption of estrous cycles in Group II and Group III were smaller than those in Group I and Group IV. The serum estradiol levels of mice and the follicular density of ovarian grafts in Group II and Group III were significantly higher than those in Group I and Group IV. However, no difference was observed in the number of days before the resumption of estrous cycles and the serum estradiol levels between Group II and Group III. A similar follicular density was also observed in Group II and Group III.

Conclusion Sucrose concentration of 0.4 M or 0.8 M in cryoprotective media is suitable for vitrifying mouse ovarian tissues.

Keywords Vitrification · Ovarian tissues · Sucrose · Concentration · Allotransplantation

Introduction

Advances in the diagnosis and treatment of childhood, adolescent, and adult cancer have greatly enhanced the life expectancy of premenopausal women with cancer. As a result, there is a growing population of adolescent and adult long-term survivors of childhood cancer [1]. Cryopreservation of embryos is a possible method of fertility preservation, but is usually inappropriate in young girls waiting for chemotherapy or radiotherapy. Cryopreservation of ovarian tissues is a potential alternative for sustaining fertility in women at risk of premature ovarian failure [2].

Vitrification is an effective, simple and inexpensive protocol for cryopreservation of the human ovarian tissues. Until now, vitrification has been adopted to cryopreserve embryos [3, 4], oocytes [5–9] and ovarian tissue [10–13]. Kuwayama reported that vitrification, compared with slow cooling, resulted in high survival rates for all stages of embryo development [14]. The first human live birth following oocyte vitrification was reported in 1999 [15]. Vitrification of in vitro-matured mouse oocytes results in high survival rates, normal meiotic spindle and chromosome alignment, and no increased incidence of aneuploidy [16]. Although cryopreservation of ovarian tissues is still at an experimental stage, it is now considered promising.

J.-M. Zhang · L.-X. Li · X.-L. Liu · Y.-X. Yang · X.-P. Wan (✉)
Department of Obstetrics and Gynecology, First People's Hospital
Affiliated to Shanghai Jiao Tong University,
Haining Road 100,
Shanghai 200080, China
e-mail: zhang_1974217@163.com

Factors affecting the efficiency of vitrification include type and concentration of cryoprotective agents (CPA), as well as temperature and exposure time to CPA. There have been many studies involving CPA [17]. Sucrose, as non-permeating cryoprotectants, has been used in the cryopreservation. As early as in 1993, mouse embryos were vitrified with 2.75 M dimethyl sulfoxide and 2.75 M propylene glycol supplemented with 1.0 M sucrose after a 0.5-min exposure, which exhibited a significantly higher in vitro survival rate (82%) than the solution without 1.0 M sucrose (44%) [18]. Furthermore, it has been reported that vitrification solution with added sugars could significantly improve the survival rate of the vitrified bovine blastocysts [19]. In a study of cryopreservation of human oocytes, it was clearly suggested that sucrose concentration of 0.1 M or 0.2 M in the cryoprotectant solution improve the post-thaw survival rate of oocytes [20]. Investigators reported that sucrose concentration influenced the rate of human oocytes with normal spindle and chromosome configurations after slow-cooling cryopreservation [7, 9]. A low concentration of sucrose (0.1–0.5 M) has been used to increase the viability of early-stage follicles in mouse [11] and human [21].

To date, there are few reports published about the optimal concentration of sucrose for vitrifying ovarian tissues. Therefore, the present experiment was initially designed to detect the effects of varying levels of sucrose on vitrified mouse ovarian tissues, thereby exploring the suitable concentration of sucrose for vitrifying mouse ovarian tissues.

Materials and methods

Animals and ovarian tissue

Chemicals were purchased from Sigma-Aldrich Company (St. Louis, MO, USA) unless otherwise indicated.

Four-week-old female mice (ICR) were used in this study ($n=60$). The mice used were all healthy and were sourced from Shanghai Animal Center. The mice were housed under temperature-controlled conditions ($22\pm 2^\circ\text{C}$). Food and water were available at all times under a photoperiod of 12 h of light and 12 h of dark. Ovaries were cut into 1 mm $\times$ 1 mm $\times$ 1 mm slices free of fat and mesentery. Approval for this study was obtained from the Animal Research Ethical Committee of the Shanghai Jiao Tong University.

Vitrification and thawing

Cryopreservation solution was prepared in modified phosphate-buffered saline (DPBS) medium supplemented with

15% heated-inactivated fetal calf serum (Sijiqing Co., Hangzhou, China).

1. Preparation of the equilibration solution and the vitrification solution.

The mice were randomly assigned to fresh control group and treatment groups (**I, II, III, IV**). For Group I, the equilibration solution was prepared with 10% (v/v) ethylene glycol (EG) + 10% (v/v) 1,2-propanediol (PROH) in DPBS; The vitrification solution was prepared with 20% (v/v) EG + 20% (v/v) PROH + 0.2 M sucrose in DPBS. For Group II, the equilibration solution was prepared with 10% (v/v) EG + 10% (v/v) PROH in DPBS; The vitrification solution was prepared with 20% (v/v) EG + 20% (v/v) PROH + 0.4 M sucrose in DPBS. For Group III, the equilibration solution was prepared with 10% (v/v) EG + 10% (v/v) PROH in DPBS; The vitrification solution was prepared with 20% (v/v) EG + 20% (v/v) PROH + 0.8 M sucrose in DPBS. For Group IV, the equilibration solution was prepared with 10% (v/v) EG + 10% (v/v) PROH in DPBS; The vitrification solution was prepared with 20% (v/v) EG + 20% (v/v) PROH + 1.6 M sucrose in DPBS.

2. Equilibration and vitrification.

Ovarian tissues (1 mm $\times$ 1 mm $\times$ 1 mm) were equilibrated in the equilibration solution at 4°C for 20 min, then were placed in vitrification solution at 4°C for 10 min. Finally the ovarian tissues were placed in 0.25-ml plastic straws (L'Aigle, France). Straws were partially filled with a 1 cm column of cryoprotectant medium (three slices per straw). After sealed with a plastic plug, the straws were plunged immediately into liquid nitrogen for storage.

3. Thawing procedure.

The ovarian tissues were cryopreserved for 30 days. The straws were warmed at room temperature for 20 s and then placed in a 25°C water for 20 s. The contents of each straw were expelled into 1 mL of descending concentrations of sucrose (1 M, 0.5 M, 0.25 M) at room temperature for 5 min at each dilution. Finally, the warmed strips were rinsed three times in the base medium and put into an incubator at 37°C with an atmosphere of 5% CO₂ and high humidity for 30 min for allotransplantation.

Allotransplantation

All mice were ovariectomized. The mice in control group received fresh ovarian allotransplants, while the mice in experimental groups received frozen-thawed ovarian allotransplants. The kidney was exteriorized through a dorsal-horizontal incision. A small hole was torn in the kidney capsule using fine watchmaker's forceps under aseptic conditions. Ovarian tissue was inserted under the kidney

capsule through the small hole. Both sides of kidneys of each mouse received grafts, and three grafts were allo-transplanted to each mouse. The transplantation process was performed at room temperature. The duration of the grafting process was ~30 min in each experimental trial.

Vaginal smear examination

Vaginal smears were taken daily, starting on day 5 post-surgery, from all mice in groups ($n=60$). Using sterile pipettes and sterile normal saline, the vagina of each mouse was flushed gently and the cells were smeared onto a slide. The stage of the estrous cycle was determined from the cell types observed in the smear [22]. The appearance of cornified epithelial cells (>15%) was considered as initiation of estrous cycle. The survival and functional recovery of grafts were defined as at least two consecutive 4-day estrous cycles showing.

Estradiol assay

At 30 days after surgery, serum estradiol levels of mice ($n=30$) were measured by RIA and by chromatography. The double antibody RIA for estradiol was performed in duplicate using reagents supplied by ICN Biomedicals (Beijing North Biotechnology Institute, Beijing, China). The procedures were performed according to the instruction of Estradiol Detection KIT. The interassay and intra-assay coefficients of variation were 16% and 7%, respectively.

Measurement of follicular density

At 30 days after surgery, the whole ovarian grafts ($n=30$) were removed and fixed in 3.5% paraformaldehyde for 4–24 h at 4°C. All samples were fixed in Bouin's solution, embedded in paraffin wax, cut into 5- μ m sections, and stained with haematoxylin and eosin. The sections were performed at 120- μ m intervals. All follicles were counted

Fig. 1 The number of days before the resumption of estrous cycles in each group

Fig. 2 The serum estradiol levels in each group (pmol/L)

from these serial sections, and each large follicle was counted only once. To calculate follicle density all sections of the whole slice of tissue were analyzed by light microscopy (BX 40; Olympus, Hamburg, Germany) at $\times 400$ magnification. Follicular densities were estimated as previously reported. The volume of the ovarian tissue analyzed (V) was calculated by the formula: $V (\text{mm}^3) = S \times 0.06$, where S corresponds to the sum of all the sections areas (mm^2) and 0.06 mm the thickness of the tissue analyzed. For each slice, the follicular density was expressed as the number of follicles per mm^3 of ovarian cortex [23, 24].

Statistical analysis

Statistical comparisons were carried out using analysis of variance (ANOVA). Values were considered significant when $P < 0.05$. SAS version 8.1 software (SAS Institute, Cary, NC, USA) was used for all statistical analysis.

Results

Vaginal smear examination and estradiol assay

Figures 1 and 2 show resumption of the estrous cycle and serum estradiol level in each group after surgery. The number of days before the resumption of estrous cycles of

Fig. 3 The follicular density of grafts in each group (n/mm^3)

Fig. 4 The follicle in graft (↓), the kidney tissue (→) (HE ×400)

mice in control group (6.2 ± 0.6) was smaller than those in experimental groups. The number of days before the resumption of estrous cycles of mice in Group II (0.4 M sucrose) and Group III (0.8 M sucrose) were smaller than those in Group I (0.2 M sucrose) and Group IV (1.6 M sucrose) (8.1 ± 0.8 and 7.8 ± 1.0 versus 11.8 ± 1.5 and 12.3 ± 0.9 , respectively, $P < 0.05$). But no difference was observed in the number of days before the resumption of estrous cycles between Group II (0.4 M sucrose) and Group III (0.8 M sucrose). The serum estradiol level in control group (34.23 ± 3.52 pmol/L) was higher than those in experimental groups. The serum estradiol levels in Group II (0.4 M sucrose) and Group III (0.8 M sucrose) were significantly

higher than those in Group I (0.2 M sucrose) and Group IV (1.6 M sucrose) (26.09 ± 2.61 and 25.83 ± 2.75 pmol/L versus 15.70 ± 2.15 and 16.37 ± 2.55 pmol/L, respectively, $P < 0.05$). However, no significant difference was observed in the serum estradiol levels between Group II (0.4 M sucrose) and Group III (0.8 M sucrose).

Follicular density after freezing-thawing

The results of the follicular density (mean $\pm$ SD; number of follicles per square millimeter of ovarian tissues) are shown in Figs. 3 and 4. The follicular density of ovarian grafts in control group ($1.01 \pm 0.25/\text{mm}^3$) was significantly higher than those in experimental groups, the follicular density of ovarian grafts in Group II (0.4 M sucrose) and Group III (0.8 M sucrose) were significantly higher than those in Group I (0.2 M sucrose) and Group IV (1.6 M sucrose) (0.75 ± 0.19 and $0.77 \pm 0.15/\text{mm}^3$ versus 0.51 ± 0.16 and $0.49 \pm 0.12/\text{mm}^3$, respectively, $P < 0.05$). A similar follicular density was observed in Group II (0.4 M sucrose) and Group III (0.8 M sucrose).

Discussion

In the management of young adults and children before intensive chemo- and/or radiotherapy, three main strategies are offered for female fertility preservation: embryo freezing, oocyte freezing, and ovarian tissues cryopreservation. The advantages and disadvantages of different methods of fertility preservation in females are shown in

Table 1 Advantages and disadvantages of different methods of fertility preservation in females

Method	Advantages	Disadvantage
Embryo cryopreservation	(i) Established method (ii) Offered by reproductive centres world-wide	(i) Not feasible for pubertal girls (ii) Spermatozoa required (iii) Possible religious and moral objections (iv) Requires an expensive programmable freezing machine
Oocyte cryopreservation	(i) Spermatozoa not required (ii) Avoids quandaries related to the storage and disposal of embryos (iii) Useful for prepubertal girls	(i) ICSI required (ii) Possible risks of meiotic spindle damage (iii) Currently experimental
Ovarian tissue cryopreservation	(i) Spermatozoa not required (ii) Avoids quandaries related to the storage and disposal of embryos (iii) Useful for prepubertal girls (iv) Ovarian cortex contains thousands of primordial follicles (v) Preserves endocrine function (vi) Can be done independent of the menstrual cycle (vii) Primordial follicles are less cryosensitive	(i) Involves two operations (ii) Loss of 60% of all follicles (iii) Grafts has a limited lifespan (iv) Experimental only

Table 1 [25–27]. Cryopreservation of ovarian tissues is a promising option for fertility preservation.

Cryoprotectants are an critical factor determining the success of cryopreservation. Generally, cryoprotectants are divided into two types: membrane-permeating (e.g. ethylene glycol, dimethyl sulphoxide) and membrane non-permeating (e.g. sucrose, Ficoll). The membrane-permeating cryoprotectants decrease the freezing point of the solution and prevent damage from high electrolyte concentrations [28]. The protective action of the membrane non-permeating cryoprotectants is very complex, attributable to a number of their special properties. Due to their large molecular size, sucrose could cause an osmotic gradient across the cell membrane, which enhances dehydration of the cell before freezing of extracellular water. In addition, it is suggested that sucrose is capable of preserving the structural and functional integrity of cell membranes at low water activities [29]. Sucrose, as non-permeating cryoprotectants, has been used in the vitrification of ovarian tissues. Marsella et al [30, 31] verified that increased sucrose concentration enhances morphological features of cryopreserved human ovarian tissues, whereas few study exists about optimal concentration of sucrose for vitrifying ovarian tissues. Therefore, the present study attempted to evaluate the effects of varying levels of sucrose on the ovarian tissues in order to seek the suitable concentrations of sucrose for vitrifying ovarian tissues.

In this study, the number of days before the resumption of estrous cycles of mice in Group II (0.4 M sucrose) and Group III (0.8 M sucrose) was smaller than those in Group I (0.2 M sucrose) and Group IV (1.6 M sucrose), whereas no difference was observed in the number of days before the resumption of estrous cycles between Group II (0.4 M sucrose) and Group III (0.8 M sucrose). Furthermore, the serum estradiol levels in Group II (0.4 M sucrose) and Group III (0.8 M sucrose) were significantly higher than those in Group I (0.2 M sucrose) and Group IV (1.6 M sucrose). A similar serum estradiol level was observed in Group II (0.4 M sucrose) and Group III (0.8 M). Analyzing the results, we conclude that adding sucrose at concentration of 0.4 M or 0.8 M can exert beneficial effects on ovarian tissues, whereas the addition of 1.6 M sucrose to the vitrification solution proved detrimental. We postulate that adding sugars to the vitrification solution could enhance viscosity, whereby incubation of cells in this solution before vitrification helps withdraw more water from the cells and reduce exposure of cells to the toxic effects of the cryoprotectants. A 0.2 M sucrose concentration was not sufficient to allow suitable cell dehydration before freezing. On the other hand, increased sucrose concentration (1.6 M) imposed a great osmotic stress and increased the likelihood of damage to the tissues. It may

explain why 0.4 M or 0.8 M sucrose is appropriate for vitrifying mouse ovarian tissues.

Measurement of follicular density of grafts has been used as a tool to evaluate the functional restoration of grafted ovary [23, 32]. The results of present study also showed that the follicular density of ovarian grafts in Group II (0.4 M sucrose) and Group III (0.8 M sucrose) were significantly higher than those in Group I (0.2 M sucrose) and Group IV (1.6 M sucrose), whereas there was no statistical difference in follicular density of ovarian grafts between Group II (0.4 M sucrose) and Group III (0.8 M sucrose). The results were consistent with above findings, thus it confirmed the postulate.

In conclusion, the addition of sucrose at concentration of 0.4 M or 0.8 M to solution can be beneficial to vitrified ovarian tissues, whereas the addition of sucrose at concentration of 1.6 M to solution proved detrimental to vitrified ovarian tissues. Therefore we conclude that sucrose concentration of 0.4 M or 0.8 M is suitable for vitrifying mouse ovarian tissues. These results should encourage further work to perfect the conditions for human ovary cryopreservation in order to restore the endocrine and reproductive functions of female patients suffering from cancer.

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Conflict of interest statement The authors declare that there are no conflicts of interest.

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