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Short communication

Experimental study on the vitrification and xenotransplantation of human ovarian tissue

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ABSTRACT

Background: Cryopreservation of ovarian tissue is a promising method for preserving fertility. Transmission electron microscopy (TEM) is an evaluation system for cryo-injury during the cooling and warming process which is very laborious and needs to be optimized.
Objective: In this study, we evaluated that serum 17β-oestradiol (E2) may be used as an indicator of vitrified ovarian tissue.
Methods: Immunodeficient nude mice were used as hosts for xenografting of vitrified-warmed human ovarian tissues. A total of 54 mice were divided into two group: vitrified ovarian xenotransplant (VOX) group (n = 45) and non-transplant control group (n = 9). The transplanted mice were grouped into vitrified/warmed grafted-4 weeks (VOX-4w, n = 15), vitrified/warmed grafted-6weeks (VOX-6w n = 15) and vitrified/warmed grafted-12 weeks (VOX-12w n = 15) according to the time after transplantation.The viable and functional recovery of grafted ovarian tissue was assessed by light microscopy, transmission electron microscopy, and hormone (E2) assays.
Results: Serum E2 concentration was significantly higher in VOX-6w (group (21.07 pg/ml) than that of VOX-12w group (15.59 pg/ml). VOX-12w group showed a lower value (12.61 pg/ml) for E2 concentration. The trend for E2 concentration was consistent with the morphological identification of the grafts.
Conclusion: In vivo serum hormone E2 released by cortical biopsies can be used as a functional marker for xenotransplanted vitrified-warmed human ovarian tissue reserve.

1. Introduction

Thousands of reproductive-age females are diagnosed with cancer every year. However, most of them could survive after chemotherapy or radiotherapy[1] and they have to suffer years of hormone replacement therapy as well as the loss of reproductive fitness, either as a result of cancer or its gonad toxic treatments.[2] With the advance in reproductive medicine and cryobiology, some fertility preservation methods have been established in the past decades.[3] Cryopreservation of ovarian tissue is a promising method for preserving fertility, which can be expediently performed without delay in cancer treatment and is the only option for adolescents and premenstrual girls. Cryopreservation of ovarian tissue has been developed as one of the options to preserve fertility.[4] More than 130 successful births worldwide after cryopreserved ovarian tissue

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auto-transplantation and the mean duration of ovarian endocrine function after transplantation is about 5 years.[5,6] Cryopreservation of ovarian tissue, still in its infancy as a therapeutic option, needs to be optimized. At present, transmission electron microscopy (TEM) is the best-known method used to evaluate cryo-injury during the cooling and warming process.[7,8] However, the sample processing/staining for electron microscopy is highly laborious. Furthermore, the analysis for electron microscope is conducted on a per-cell basis and only a fraction of cells within each sample can be studied. Therefore, TEM cannot be used for routine determinations, especially in conditions for assessing the functional recovery of the transplanted ovarian tissue.

Serum levels of estradiol, inhibin B, follicle-stimulating hormone, and antimullerian hormone (AMH) are important markers for ovarian reserve.[9] Estrogen synthesis is an essential characteristic of healthy ovaries in reproductive females.[10] Invitro study revealed an increase in the level of 17β-oestradiol (E2) at the end of the culture period in comparison with the beginning of culture is correlated with the increase in the proportion of growing follicles.[11]

In the present study, we hypothesized that serum E2 can be used to predict the functional recovery of the grafts that are available to optimize the cryopreservation procedure of human ovarian tissues, which will be a valuable tool for indicating cryopreservation effects and hopefully help to find better fertility preservation strategies.

2. Materials and METHODS

2.1. Ethics statement

Animal experimental protocols were approved by the Peking University Laboratory Animal Administration Committee, and experiments were performed according to the Peking University Guidelines for Animal Experimentation. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

The study was performed under a protocol approved by the institutional review boards from the Peking University Shenzhen Hospital. All the study participants provided written informed consent at enrolment.

2.2. Animal

54 female nude mice (BALB/C nude mice, 6–7 weeks of age) were obtained from the experimental animal center of Southern Medical University. The animals were housed in a high-efficiency particulate air-filtered positive pressure room. Cages were filter topped and animals had free access to food and water, under12 h light and 12 h darkness conditions. Groups of 5–6 mice were housed in one cage. Upon arrival from the breeding company, the mice were allowed to get acclimated for one week. All procedures, tests, and injections were performed under a laminar flow hood in a positive pressure room.

2.3. Tissue preparation

Ovarian tissue was obtained from 4 informed patients with various forms of the benign ovarian disease: the mean age of the women was 29.6 ± 1.5 years. Normal ovarian tissues rather than diseased parts were collected and transported to the laboratory within 24 h in sterile 15 ml Corning tubes, which contained about 5 ml Leibovitz-15 medium supplemented with penicillin/streptomycin (0.1 mg/ml) and 10% human serum albumin (HSA), and were pre-warmed to 37 °C. After removal of the medulla, the cortex was sliced into pieces 1–2 mm thick and 10–20 mm long with a scalpel blade under a stereomicroscope.

2.4. Vitrification-warming protocol

The vitrification protocol was based on a two-step method in an open carrier. Ovarian samples were placed in a 60 mm dish containing 15 ml vitrification solution I (VSI) consisting of 10% dimethyl sulfoxide (DMSO), 10% ethylene glycol (EG), 0.1 M sucrose, 0.1 M trehalose, 6% HSA, M199 medium, and Hepes at room temperature for 15min. Subsequently, the samples were transferred into a second 60 mm dish containing 15 ml vitrification solution II (VSII) consisting of 20% DMSO, 20%EG, 0.3 M sucrose, 0.1 M trehalose, 6%HSA, M199 medium, and Hepes at room temperature for 10min. Then the ovarian slices were transferred onto a sterile cryotissue straw or self-made simple cryotissue solid grids. The straws or grids were immediately plunged into liquid nitrogen and transferred into cryotubes for storage.

The tissues were warmed at room temperature for 30s and immediately into thawing solution (TS) I (2.5%EG + 2.5%DMSO + 0.5 M sucrose + 0.2 M trehalose + 6% HSA + M199) at 37 °C for an additional 1 min. The tissues were then washed stepwise in thawing solution II, III and IV, TS II (0.5 M sucrose +0.2 M trehalose + 6% HSA + M199) for 3 min, TS III (0.5 M sucrose + 6% HSA + M199) for 5 min, TS IV (0.25 M sucrose + M199) for 5 min. Finally, the warmed samples were rinsed in M199 medium.

2.5. Transplantation into nude mice

Fifty-four recipient mice were used for the experiment and all they underwent oophorectomy, which was performed under narcosis with ketamin irrespective of the stage of the oestrus cycle. During surgery, mice were kept on a warming plate. The incision site was disinfected with 75% alcohol and covered with a one-way sterile towel. Both ovaries were removed by a small body wall incision that was sutured with absorbable thread.

54 mice were divided into two group: vitrified ovarian xenotransplant Group (VOX, n = 45) and non-transplant control group (n = 9). Xenografting of the ovarian cortex was performed 14 days after oophorectomy. The ovarian tissue pieces were placed in an intramuscular pocket of the back muscle.

The transplanted mice were grouped into vitrified/warmed grafted-4 weeks (VOX-4w, n = 15), vitrified/warmed grafted-6weeks (VOX-6w, n = 15) and vitrified/warmed grafted-12 weeks (VOX-12w, n = 15) according to the time after transplantation.

2.6. Gonadotropin stimulation

Mice received every other day i.p. injections of human menopausal gonadotropin (hMG, Menogon, Ferring, Kiel; 2 IU Follicle-stimulating hormone (FSH)/2 IU luteinizing hormone (LH) per animal/two days) from day 14 after transplantation until 4 weeks, 6 weeks, or 12 weeks. The no transplanted group received the hormonal treatment for the same period as the transplanted group.

2.7. Oestrus cycle stage determination

Vaginal smears were taken once a week from all mice starting at day 14 after transplantation using sterile pipettes, to examine if follicular tissues survived the transplantation and could produce enough estrogens to cornify the vaginal epithelium. Vaginal cells were left to dry after being smeared on a microscopic slide and were then stained with methylene blue. The epithelium cells were classified into one of the four following categories: a) pro-estrous, b) estrous, c) metestrous, d) diestrous. The animals were weighed and killed respectively at 4 weeks, 6 weeks, and 12 weeks after transplantation, and serum was collected for E2 measurement by E2 ELISA Kit purchased from Elabscience Biotechnology with a sensitivity of 9.38 pg/L.

Uterus was collected after its periphery tissues were carefully trimmed and were weighted after being fixed in 4% paraformaldehyde. The uterus/weight ratio was used to evaluate the grafts function recovery. Grafts were recovered and fixed in Paraformaldehyde for 24 h.

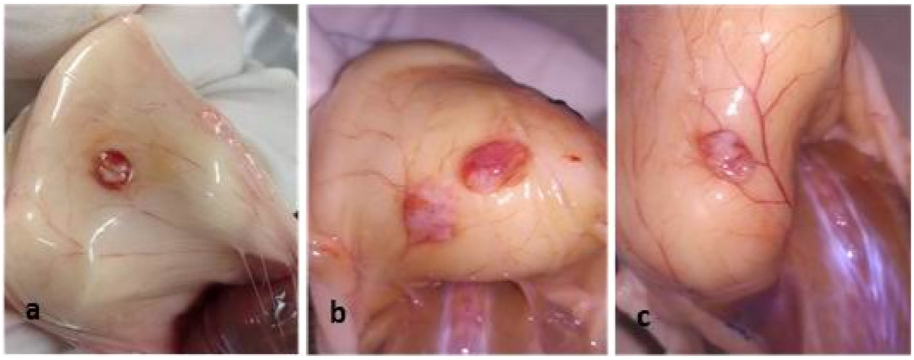

Fig. 1. Vitrified-warmed ovarian cortical after xenotransplantation: (a) VOX-4w, (b): VOX-6w, (c) VOX-12w.

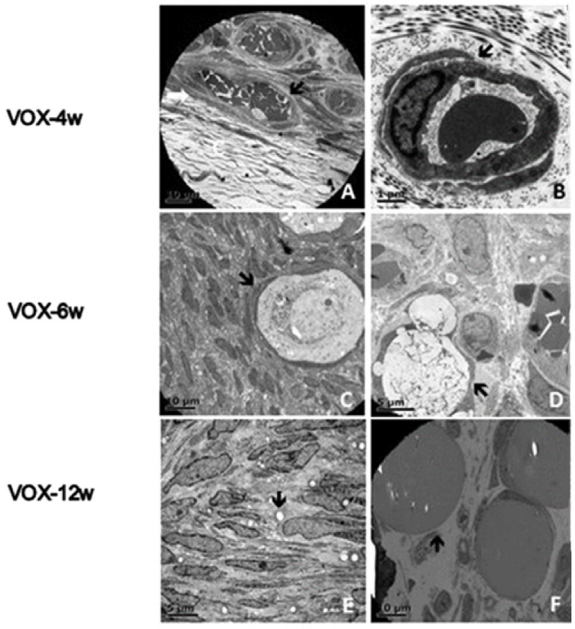

Fig. 2. TEM morphological comparison of ovarian tissues before and after xenotransplantation: Arrow shown in (A) and (B): new vessel, (C) normal primary follicle, (D) injured primary follicle, (E) vacuole, (F) steatosis.

2.8. Morphological evaluation

Vitrified-warmed samples were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 6 h at 4 °C for light microscopic (LM) evaluation and some samples with high numbers of follicles were selected for TEM. For ovarian cortical pieces from VOX-4w, VOX-6w, and VOX-12w, they were also fixed respectively in 2.5% glutaraldehyde in PBS (pH 7.4) for 6 h at 4 °C for LM and some typical samples were picked for TEM.

For LM, after routine paraffin embedding, the samples were serially sectioned (4 μm) and every tenth section was stained with hematoxylin and eosin and examined microscopically. The developmental stages of the follicles were evaluated according to the regulation defined by Gougeon.[9]

For TEM, the fixed samples were then post-fixed with 1% osmium tetroxide in the same buffer for 2 h. After dehydration in an ascending series of ethanol, specimens were placed in propylene oxide and embedded in LX-112, then cut into 0.5 μm sections and stained with toluidine blue. Ultra-thin sections (60 nm) were contrasted with uranyl acetate and lead citrate and examined by using a transmission electron microscope. The ultrastructural analysis was performed according to subcellular criteria reported by Hreinsson J.[7]

2.9. Statistical evaluation

SPSS 22.0 was used for data evaluation. Nominal data were compared by using the *t*-test and it was considered statistically significant when the p value was less than 0.05.

3. RESULTS

3.1. Morphological change

After 4, 6, and 12 weeks of xenografting, human ovarian tissue specimens were recovered respectively. Some connecting grafted human tissues with mouse subcutaneous were observed. The vessels were established macroscopically in grafts at 4, 6, and 12-weeks, especially well at 6 weeks (Fig. 1).

3.2. Morphological change

Light microscopy showed that the vitrified-warmed human ovarian cortical tissues and different transplanted time point ovarian tissues all displayed normal morphologic characteristics, especially the primordial and primary follicles was well preserved with normal morphologic characteristics, but the number of follicles decreased in the transplanting period while that of fibrosis increased in the samples.(see Fig. 2).

TEM of transplanted ovarian tissues showed that in VOX-4w group, the new vessels had formed between the host and graft as well as in the graft (3A, 3B). In VOX-6w group, the oocytes and granulosa cells showed regularly shaped nuclei with nucleoli and finely dispersed chromatin (3C), and the apoptosis follicle was also observed with a damaged shape (3D). In- VOX-12w group, both the stromal intercellular vacuolization (3E) and steatosis (3F) had formed.

The transplantation groups showed changes in vaginal cytology characteristics of oestrus cycles at days 14–18, 24–28 after surgery. These changes were evident through the presence of cornified epithelial cells (Fig. 3).

The result showed that the average E2 level for VOX-4w, VOX-6w and VOX-12w group were 12.61, 21.07, and 15.59 pg/ml, respectively. Compared with the no transplanted group, there were significant differences between VOX-6w with the other three groups: $F = 9.871$ ($P < 0.05$). (Fig. 4).

4. Discussion

The cryopreservation of ovarian tissues aims to preserve primordial and primary follicles, for these viable small follicles constitute the follicular reserve after retransplantation. It has shown that the vitrified-warmed human ovarian cortical tissue produces and releases oestradiol in the serum of nude mice after xenotransplantation. The average oestradiol level was the highest for VOX-6w group compared to VOX-12w group, followed by the VOX-4w group. These results were also

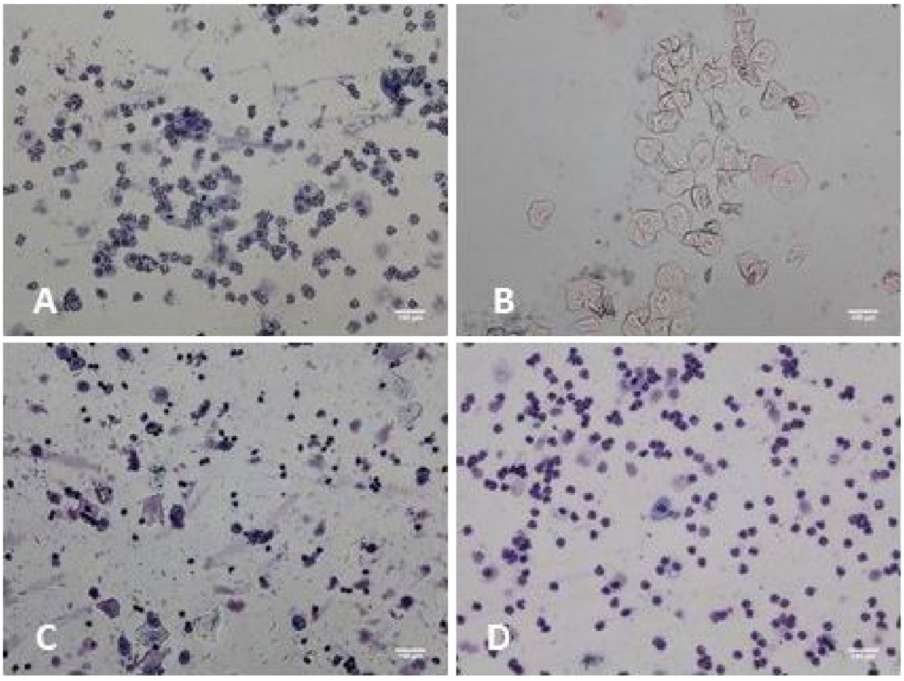

Fig. 3. Vaginal cytology characteristics in nude mice 6 weeks after xenotransplantation: (A) Proestrus, (B) Oestrus, (C) Metoestrus, (D) Diestrus.

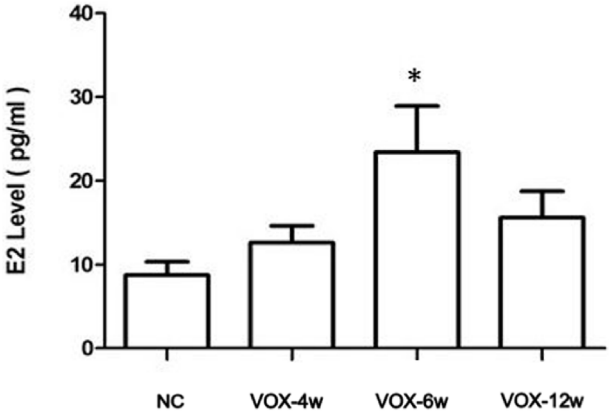

Fig. 4. The E2 level in serum of nude mice at different time after human ovarian tissue transplantation (X±SD, *P < 0.05 compared with NC).

observed in the mice uteruses collected after grafting. 4 weeks after transplantation, vessels could be seen. And the vessels were established macroscopically in grafts especially well at 6w. Light microscopy showed that compared with VOX-4w and VOX-6w group, the number of follicles decreased, and fibrosis increased in VOX-12w group, which is in accordance with findings under TEM.

During the process of folliculogenesis (more specifically, secondary and antral folliculogenesis), both thecal and granulose cells are involved in cell-specific estrogen synthesis.[12] The outcome of this assay may be an indication in theory for the number of secondary and antral follicles which we didn't find yet is present in the collected grafts after transplantation.

This study does not directly measure the survival of the target population of primordial and primary follicles for quantities reasons. To what extent these follicles grow after transplantation could be a focus of work in the future.

5. Conclusion

The results show that the trends of E2 are consistent with morphological identification in the grafts and can be used as an indicator of the functional damage sustained by the ovarian tissue during the freeze/thawing and transplant procedure.

6. Limitation

Bilateral oophorectomy was done before transplantation in this study but in a real application, not all women who undergo cryopreservation of ovarian tissues have the same basal E2 level. How to use E2 to determine the effect or predict ovarian reserve after grafting in women patients with different hormone levels is still a problem to solve. Thus, additional studies are needed.

Content to participate

All participants aged 18 and above were given information about the study, and they were asked for their voluntary participation. Written informed consent was administered to each participant; all participants read and signed written consent forms before being enrolled in the study.

Consent for publications

All authors have confirmed authorship and are approved for publication.

Contributions

WR select the topics and evaluate the design, TH, ZJ and GY designed and conceptualized the study, worked on experiments and data analysis, LJ interpreted and revised data, drafted the initial manuscript, and approved the final manuscript. We thank ZL, DJ, DH, LH, HF for providing human ovarian tissue; SM, YX for doing the transmission electron microscopy (TEM) assay; WC for doing some light microscopic (LM) evaluation; DY, as head of BGI for this project, for co-ordinating the

other BGI authors doing the work.

Conflict of interest

No potential conflict of interest was reported by the authors.

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References

1. Demeestere I, Simon P, Englert Y, et al. Preliminary experience of ovarian tissue cryopreservation procedure: alternatives, perspectives, and feasibility. *Reprod Biomed Online*. 2003;7(5):572–579. https://doi.org/10.1016/s1472-6483(10)62074-3.
2. Morgan S, Anderson RA, Gourley C, et al. How do chemotherapeutic agents damage the ovary? *Hum Reprod Update*. 2012;18(5):525–535. https://doi.org/10.1093/humupd/dms022.
3. Practice Committee of the American Society for Reproductive Medicine. Electronic address: asrm@asrm.org. Fertility preservation in patients undergoing gonadotoxic therapy or gonadectomy: a committee opinion. *Fertil Steril*. 2019;112(6):1022–1033. https://doi.org/10.1016/j.fertnstert.2019.09.013.
4. Hoekman EJ, Louwe LA, Rooijers M, et al. Ovarian tissue cryopreservation: low usage rates and high live-birth rate after transplantation. *Acta Obstet Gynecol Scand*. 2020;99(2):213–221. https://doi.org/10.1111/aogs.13735.
5. Donnez J, Dolmans MM, Pellicer A, et al. Restoration of ovarian activity and pregnancy after transplantation of cryopreserved ovarian tissue: a review of 60 cases of reimplantation. *Fertil Steril*. 2013;99(6):1503–1513. https://doi.org/10.1016/j.fertnstert.2013.03.030.
6. Donnez J, Dolmans MM. Fertility preservation in women. *N Engl J Med*. 2017;377(17):1657–1665. https://doi.org/10.1056/NEJMra1614676.
7. Hreinsson J, Zhang P, Swahn ML, Hultenby K, Hovatta O. Cryopreservation of follicles in human ovarian cortical tissue. Comparison of serum and human serum albumin in the cryoprotectant solutions. *Hum Reprod*. 2003;18(11):2420–2428. https://doi.org/10.1093/humrep/deg439.
8. Martinez-Madrid B, Camboni A, Dolmans MM, et al. Apoptosis and ultrastructural assessment after cryopreservation of whole human ovaries with their vascular pedicle. *Fertil Steril*. 2007;87(5):1153–1165. https://doi.org/10.1016/j.fertnstert.2006.11.019.
9. Gougeon A. Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr Rev*. 1996;17(2):121–155. https://doi.org/10.1210/edrv-17-2-121.
10. Cui J, Shen Y, Li R. Estrogen synthesis and signaling pathways during aging: from periphery to brain. *Trends Mol Med*. 2013;19(3):197–209. https://doi.org/10.1016/j.molmed.2012.12.007.
11. Ramezani M, Salehnia M, Jafarabadi M. Short term culture of vitrified human ovarian cortical tissue to assess the cryopreservation outcome: molecular and morphological analysis. *J Reproduction Infertil*. 2017;18(1):162–171.
12. Hsueh AJ, Kawamura K, Cheng Y, et al. Intraovarian control of early folliculogenesis. *Endocr Rev*. 2015;36(1):1–24. https://doi.org/10.1210/er.2014-1020.