Effects of vitrification cryopreservation on follicular morphology and stress relaxation behaviors of human ovarian tissues: sucrose versus trehalose as the non-permeable protective agent

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STUDY QUESTION: Is sucrose more effective than trehalose in human ovarian tissue cryopreservation?

SUMMARY ANSWER: The effect of sucrose as a cryoprotective agent (CPA) was not significantly different from that of trehalose in human ovarian tissue cryopreservation.

WHAT IS KNOWN ALREADY: Sugars have the ability to keep the cell membrane intact and can decrease the toxicity of CPAs. Sucrose is the most commonly used non-permeable CPA, while trehalose is rarely used in human ovarian tissue cryopreservation. Although various methods are utilized to evaluate the efficiency of human ovarian tissue cryopreservation, few studies have evaluated the effect of cryopreservation from the viewpoint of biomechanics.

STUDY DESIGN, SIZE, DURATION: A total of 15 ovarian tissue samples were collected from 15 patients (20–41 years old) with benign ovarian tumors or malignancies, and each was dissected into six slices. Two slices were taken as the fresh control group. The remaining four slices were vitrified using different vitrification protocols. After warming, samples in each group were either fixed for histological evaluation or destined for stress relaxation test.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The CPA solutions for the control and vitrified groups were composed of EDS and EDT (E, ethylene glycol; D, dimethylsulphoxide; S, sucrose; T, trehalose). The stress relaxation experiments were carried out at room temperature using a dynamic mechanical analyzer. Ovarian tissue samples were assessed for both their morphology and viscoelasticity. Stress relaxation data (SRD) were calculated as a percentage, representing the ability to maintain the initial stress after stretching. The percentage of morphologically normal follicles was compared between groups, which was represented by morphologic preservation ratio.

MAIN RESULTS AND THE ROLE OF CHANCE: The morphologic preservation ratio of the primordial follicles in the fresh control group (87.58%) was higher than that in group S (72.33%) (P = 0.000) and group T (79.56%) (P = 0.002). Although not statistically significant, compared with the S group, vitrification with T suggested a trend toward a higher morphologic preservation ratio of the primordial follicles. The SRD in the fresh control group (0.6433 ± 0.7233) was significantly different from that in group S (0.5200 ± 0.8331, P = 0.000) or in group T (0.5667 ± 0.6415, P = 0.000). However, no significant difference was found between groups S and T.

LIMITATIONS, REASONS FOR CAUTION: Experimental samples were directly exposed to the air, which will result in a discrepancy in the viscoelastic properties between experimental tissues and in vivo tissues.

WIDER IMPLICATIONS OF THE FINDINGS: Our study suggested a trend toward a higher morphologic preservation ratio of the primordial follicles after vitrification in trehalose compared with sucrose, which may provide a basis for further optimizing human ovarian tissue vitrification. In addition, it was possible to evaluate the effect of ovarian tissue cryopreservation from a biomechanics perspective.
Introduction

Due to the improvements in diagnosis and treatment, the life spans of cancer patients, including children, adolescents and adults, have been lengthened. However, radiotherapy or chemotherapy can lead to deprivation of ovarian function and loss of fertility with consequent premature ovarian failure (Jeong et al., 2012). Hence, the life quality of cancer survivors merits attention. Currently, the established fertility preservation methods include oocyte and embryo banking, ovarian tissue cryopreservation and in vitro maturation (Ross et al., 2014). Ovarian tissue cryopreservation is the only therapeutic option for saving fertility in prepubertal children (Sarnacki, 2014) and is the only feasible option for patients who cannot delay treatment (Ross et al., 2014). According to the data published, at least 30 pregnancies after orthotopic reimplantation of frozen-thawed ovarian cortex slices have been proclaimed throughout the world (Donnez and Dolmans, 2013), even though ovarian cortex cryopreservation is deemed experimental.

The conventional slow-freezing is commonly regarded to inevitably introduce ‘two-factor’ injuries to the cells, i.e. the injuries caused by intracellular ice formation and the elevated concentration of the residual solution (Mazur et al., 1972). Vitrification has been shown to overcome these problems, owing to the fact that vitrification solution could be ultra-rapidly cooled and transformed into a glassy state (Fahy et al., 1984). Nevertheless, vitrification requires a high cooling rate or a high concentration of cryoprotective agent (CPA) (Zhang et al., 2009; Zhao et al., 2014). The commonly used CPAs for vitrification cryopreservation of ovarian tissues include both permeable [ethylene glycol (EG), propane-diol (PROH) and dimethyl sulfoxide (DMSO)] and non-permeable (sucrose, trehalose and ficoll) agents. To decrease the toxicity of the high concentration of CPAs used in vitrification is a popular topic in the field of cryopreservation (Pegg, 2005). Sugars have the ability to keep the cell membrane intact at low water activities and can decrease CPA toxicity as they stand in a line at the outer membrane by formation of hydrogen bonds with membrane phospholipids (Amorim et al., 2011a,b). Sucrose is the most commonly used non-permeable CPA for various species, such as humans (Herraiz et al., 2014), bovines (Lucci et al., 2004) and murines (Youn et al., 2014). However, Neto et al. found that trehalose appeared to be more conducive to preserve the follicles upon the experimental results on cryopreservation of doe rabbit ovarian tissues (Neto et al., 2008). In addition, Amorim et al. found that vitrification cryoprotectants containing EG, trehalose and fetal bovine serum had no deleterious effect on the morphology of human pre-antral follicles (Amorim et al., 2011a,b).

During the cooling and thawing processes, the exposure to CPAs could induce physical and chemical disturbances to biological samples. For instance, osmotic stress, toxicity, mitochondrial dysfunction and oxidative stress have been observed with consequent changes in structural and functional properties of the tissues (Ahn et al., 2002; Pegg, 2007; Fahy, 2010; Fabbri et al., 2014). Furthermore, Jung et al. showed that there was negligible impact on the mechanical properties of the patellar tendon tissue following eight freeze–thaw cycles (Jung et al., 2011). Even if the morphological structure remains intact, the frozen tissues may differ from the fresh ones in macroscopic mechanical properties, and little research has been done at this level. Ovarian tissue, as a biological soft tissue, shows typical viscoelasticity. From the viewpoint of modeling, soft tissue viscoelastic properties could be well described via stress relaxation tests, and in which the relaxation modulus is often determined (Troyer et al., 2012). Stress relaxation tests are capable of supplying an approach of quantifying and comparing the time-dependent behavior of tissues by matching the relaxation data to an appropriate viscoelastic model (Pai and Ledoux, 2011). Fung’s quasi-linear viscoelastic (QLV) theory (Fung, 1993) is widely used in the viscoelastic models, and it has been further improved over the years for more feasible implementation (Pai and Ledoux, 2011).

The objective of the study was to investigate the effects of vitrification cryopreservation with two different non-permeable CPAs (sucrose and trehalose) on both the morphology of pre-antral follicles and the viscoelastic properties of human ovarian tissues. We tried to set up a viscoelastic evaluation guideline for optimization of cryopreservation protocols of human ovarian tissues from the biomechanics perspective. Both the viscoelastic behaviors of the fresh and frozen-thawed ovarian tissues were examined using the QLV theory.

Materials and Methods

All reagents were purchased from Sigma-Aldrich Co. (USA) unless otherwise stated. The study was performed in the Reproductive Medical Center (The First Affiliated Hospital of Anhui Medical University, China) and Laboratory of Cryo-Biomedical Engineering (University of Science and Technology of China, China), and was approved by Biomedical Ethics Committee of Anhui Medical University (Anhui, China).

Ovarian tissue collection

Ovarian tissue was acquired from 15 female patients (20–41 years old, mean ± SD age 29.40 ± 8.02 years) with benign ovarian tumors (e.g. Teratoma and巧克力囊肿) or malignancies (e.g. breast cancer and cervical cancer) after signing written informed consent.

Tissue sample preparation

Since Isachenko et al. had shown the possibility of hypothermic storage of human ovarian cortex at suprazero temperatures for long-distance transport without a decrease in viability (Isachenko et al., 2009), we carefully followed Isachenko et al.’s approach to eliminate the possible influences of pre-freezing handling conditions on cryopreservation outcome of ovarian tissues. Ovarian tissue was placed in aseptic 15 ml BD Falcon tubes containing 10 ml Dulbecco’s phosphate-buffered saline (DPBS) and immediately transported on ice to the laboratory within 0.5 h. Then the tissue was transferred

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Key words: ovarian tissue / vitrification / cryoprotective agents / viscoelastic / stress relaxation
to a sterile culture dish (BD, USA) containing 10 ml HEPES (SAGE IVF, USA) and 10% human serum albumin (HSA) (SAGE IVF, USA). The medulla part was removed within 10–15 min. Ovarian cortex slices were dissected with ophthalmic scissors into six samples, which were c. 2.5–7.0 mm in length, 1.4–5.2 mm wide, 0.4–1.2 mm thick.

One slice of ovarian tissue was promptly fixed in 10% formalin for histological evaluation and was taken as the fresh control. Another fresh slice one was put in a sterile culture dish containing 2 ml DPBS for the stress relaxation test (group F). The remaining four cortical tissue pieces were frozen using two different methods, described as follows. Each tissue was stored in liquid nitrogen for 1 month. After warming, the piece in each group was either fixed for histological evaluation or destined for stress relaxation test.

Vitrification and warming protocol
All the liquid used in the experiment was percolated by a microporous filter of 0.2 μm diameter (Millipore, France). The base media were composed of M199, supplemented with 12% HSA, 100 U/ml Penicillin and 100 μg/ml streptomycin. The base media were composed of M199, supplemented with 12% HSA, 100 U/ml Picillin and 100 μg/ml streptomycin. The tissue pieces were first incubated for 5 min at room temperature in 3 ml base medium, then incubation for 5 min at room temperature in 3 ml vitrification solution, composed of 2.0 mol/l dimethylsulphoxide (DMSO) and 0.1 mol/l sucrose or trehalose in base medium, followed by incubation for 5 min at room temperature in 3 ml vitrification solution, composed of 2.0 mol/l DMSO, 0.2 mol/l sucrose (group S) or trehalose (group T) in base medium with 2.0 mol/l ethylene glycol (EG). After the last step, the remaining solution was absorbed via a sterile transfer pipette. The tissues were removed to two stainless steel barrels that were partially immersed in liquid nitrogen. Once vitrified, the pieces were put into precooled cryovials (CORNING, USA) and stored in liquid nitrogen.

To thaw, the cryovials were air warmed for 30 s then immersed in a water bath at 37°C until the ice melted. The ovarian tissues were submerged into warming solution 1 (WS1) containing 0.5 mol/l sucrose or trehalose in base medium, then transferred to WS2 (0.25 mol/l sucrose or trehalose), WS3 (0.125 mol/l sucrose or trehalose) and WS4 (0 mol/l sucrose or trehalose). Each bath was performed at room temperature for 5 min.

Histological evaluation
After warming, one piece from each experimental group was fixed in 10% formaldehyde solution. The samples were paraffin-embedded and sliced into 5 μm thick sections. Every 10th section was collected and stained with hematoxylin and eosin. To avoid double-counting, each follicle was followed through adjacent sections, and counted only once under light microscope (Olympus, Japan). The number of follicles at different developmental stages was classified according to Gougeon (1996): (i) primordial follicles: oocyte surrounded by a single layer of flat granulosa cells; (ii) primary follicles: oocyte surrounded by a complete single layer of cuboidal granulosa cells; (iii) secondary follicles: oocyte surrounded by two layers or more of cuboidal granulosa cells; and (iv) atretic follicles: oocyte with eosinophilic cytoplasm, cell disorganization were observed frequently (Fig. 1).

Stress relaxation test
The length, width and thickness of each piece of ovarian tissue were measured by a caliper prior to the stress relaxation test. The cross section area was acquired by calculating. The stress relaxation experiments were carried out at room temperature using a computer-controlled electronic universal testing machine (DMA, CMT 8501, China). The experimental processes were as follows: the two ends of a slice of tissue were mounted onto the upper and the nether clamps of the DMA, respectively, and then axial strain the sample was increased at a speed of 1 mm/min (at a strain rate of 0.2%/min), and then the constant strain 2% was held for 10 min. The maximum loading stress is set to 0.4 N. During the whole experiment, the stress versus time data were collected automatically by a computer through the DMA software.

Quasi-linear viscoelasticity
Historically, the QLV theory has been a popular model due to its relative simplicity. The QLV formulation catches the well-established elastic nonlinearities of soft tissues, and assumes linearity of the temporal aspect and the elastic and time-dependent properties are separable. The resultant stress is then determined by a linear combination of these non-linear terms (Troyer et al., 2012):

$$\sigma(t, \varepsilon) = \int_0^t G(t - \tau) \frac{\partial \sigma^\varepsilon(\tau)}{\partial \varepsilon} d\tau$$

(1)

where $G(t)$ (normalized, $G(0) = 1$) is the reduced relaxation function, $t$ represents time, $\tau$ is a time variable of integration, $\sigma^\varepsilon(\tau)$ represents the instantaneous elastic stress and $\varepsilon(t)$ is the applied strain history.

Stress also can be expressed as:

$$\sigma(t) = G(t) \cdot \sigma^\varepsilon(\varepsilon), G(0) = 1$$

(2)

All stress relaxation data (SRD) of both the fresh and the two cryopreserved groups were indicated by the value expressed as a percentage, which means the ability to maintain the initial stress after stretching.

Statistical analysis
All statistical analyses were performed using the Statistical Package for the Social Sciences version 16.0 software (SPSS, Inc., Chicago, IL, USA). The proportions of the morphologically normal follicles between multiple independent samples before and after cryopreservation were compared using Chi-square test ($\chi^2$), and then Chi-square test ($\chi^2$) was conducted between any two groups. The SRD values within each group were compared using one-way analysis of variance followed by the Student–Newman–Keuls test. Unless otherwise stated, the differences were considered to be significant when $P < 0.05$. The value of SRD was expressed as mean ± SD.

Results
Histological evaluation
A total of 1242 follicles (from 45 pieces of fresh and vitrified/warmed ovarian tissues) were analyzed by light microscopy to evaluate the morphology at different developmental stages (Table I). The morphologic preservation ratio of the primordial follicles in the fresh control group (87.58%) was higher than that in group S (72.33%) and group T (79.56%), and the differences were statistically significant, respectively ($P < 0.0167$, based on statistical methods, the level of statistical significance in this step was set at $P < 0.0167$). However, there was no statistically significant difference between group S and group T ($P > 0.0167$, according to the chi-square cutting principle, $P < 0.0167$ was used to define statistical significance). Accompanied with the changes in morphology, shrinkage of the cytoplasmic compartment in the follicular cells and granulosa cell disorganization were observed frequently (Fig. 1).

Stress relaxation test
In the fresh control group, after stretching the ovarian tissues, the value of SRD (0.6433 ± 0.7233) was significantly higher than that in group S.
Table I  Number of human follicles in fresh and vitrified/warmed ovarian tissue identified by light microscopy in the three study groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Primordial follicles</th>
<th>Primary follicles</th>
<th>Secondary follicles</th>
<th>Total follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Morphologically normal</td>
<td>Abnormal</td>
<td>Morphologically normal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>Group F</td>
<td>423</td>
<td>60</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Group S</td>
<td>311</td>
<td>119</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Group T</td>
<td>253</td>
<td>65</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

Group F: the fresh control group; Group S: the sucrose group; Group T: the trehalose group. The morphologic preservation ratio represented the percentage of morphologically normal follicles in the total number of follicles. The morphologic preservation ratio of the primordial follicles in group F (87.58%) was higher than that in group S (72.33%) ($P=0.000$) and group T (79.56%) ($P=0.002$). The proportions of the morphologically normal follicles between multiple independent samples before and after cryopreservation were compared using Chi-square test ($\chi^2$), and then Chi-square test ($\chi^2$) was conducted between any two groups.

Figure 1  Light microscopy images of human ovarian cortex before and after cryopreservation. (A–C) Human primordial follicles with normal morphological appearance stained by hematoxylin and eosin (HE) sequentially from the fresh control group (group F), the trehalose group (group T) and the sucrose group (group S): follicle spherical in shape with even distribution of a layer of flat granulosa cells (high morphological quality). (D–E) Primordial follicles with abnormal morphological appearance from group T and group S, respectively: the granulosa cells arranged in incomplete layers and nuclear chromatin condensation (low morphological quality). (F–H) Primary follicles with normal morphological appearance, respectively, from group F, group T and group S: the oocyte is surrounded by a regular layer of cubic granulosa cells. (I–J) Secondary follicles with normal morphological appearance from group F and group T, respectively: The oocyte is surrounded by two or more cubic granulosa cell layers. (K–L) Secondary follicles with abnormal morphological appearance from group T and group S, respectively: nuclear chromatin shrinkage and irregularly distribution of granulosa cells.
Our study showed that the proportion of morphologically abnormal primordial follicles was 12.42% in the fresh control group. Fabbri et al. showed that women with Hodgkin’s disease have an unexpected frequency of subtle follicular damage by histological examination of the fresh ovarian cortex tissues before chemo/radiotherapy treatment for the first time (Fabbri et al., 2011). The mechanism of morphologic damage in fresh ovarian tissues has not yet been fully understood. Raffaella et al. considered that some factors associated with benign and malignant patients were participated in the expression of damage (Fabbri et al., 2014).

CPAs have been widely investigated in vitrification of the ovarian cortex tissues (Herraiz et al., 2014; Youm et al., 2014). EG and DMSO are two of the most commonly used permeable CPAs for two traditional approaches to the cryopreservation of biological material, slow freezing and vitrification (Herraiz et al., 2014; Youm et al., 2014). Rall et al. reported that membrane-permeating CPAs could prevent damage from high electrolyte concentrations and decrease the freezing point of a solution (Rall et al., 1984). As a poor glass-forming agent (Mullen and Critser, 2007), EG appears to have a low toxicity and high diffusivity into cell (Orief et al., 2005). DMSO, an effective glass-forming agent (Fahy et al., 2004), could contribute to reducing the viscosity of a solution (Mochida et al., 2013). Youm et al. indicated that using the solution consisting of EG, DMSO and sucrose and gradually increasing their concentrations could achieve successful dehydration and vitrification of ovarian tissues (Youm et al., 2014). Accordingly, vitrification solutions containing EG and DMSO were employed in our study.

As the most commonly used non-permeable CPA, sucrose has been proven to increase human oocytes survival after cryopreservation and improve human ovarian tissue survival after thawing (Marsella et al., 2002). Trehalose is generally used in cryogenic storage of spermatozoa (McGinnis et al., 2005), oocytes (Eroglu et al., 2009) and embryos (Yavin et al., 2009), but not in ovarian tissue cryopreservation. Trehalose is one kind of disaccharide with special biological properties, which could effectively protect the activity of proteins and other macromolecules in dry, dehydration, frozen and other harsh conditions. In this study, the proportion of morphologically normal follicles after vitrification was decreased with trehalose or sucrose. Compared with the sucrose control group, vitrification with trehalose suggested a trend toward a higher morphologic preservation ratio of the primordial follicles, even though there was no significant difference in the proportion.

At present, mechanisms of trehalose action are described as follows: (i) water replacement hypothesis (Crowe et al., 1992). Crowe et al. proposed that under dry and frozen conditions, trehalose and other biological macromolecules were connected by hydrogen bonding, forming a protective film in place of the lost structural water film to govern the stabilization of living cells. (ii) glassy state hypothesis (Timasheff, 1993). Timasheff deemed that the efficient bio-protective effect of trehalose was associated with the formation of a glassy state, which could help biomolecules to maintain a certain spatial structure. (iii) priority exclusion hypothesis (Sola-Penna and Meyer-Fernandes, 1998). Sola and Meyer suggested that sugars could not directly interact with the spatial structure of a protein, but preferentially bind with water molecules around protein surface, then the radius of the solvent layer was reduced, and the phenomenon was conducive to withstand the impact from extreme environments. Furthermore, Chen and Haddad demonstrated that trehalose could withstand effects of low osmotic pressure, chemical interaction, hypoxia and other factors on cells via osmo-regulation.

**Discussion**

Ovarian tissue cryopreservation is an alternative strategy for female fertility preservation because surgical removal of ovarian tissue causes no delay in cancer treatment and the extracted tissue yields a plentiful supply of primordial follicles (Chung et al., 2013). Human ovarian tissue includes different types of cells (oocytes, granulosa cells) and fibrous stroma, which may influence the cryostability of the tissue and make vitrification more challenging. Given that primordial follicles are more resistant to cryoinjury, they are the primary target of successful cryopreservation (Hovatta, 2005).

To evaluate the quality of follicles in ovarian tissues, many methods are developed, including histological evaluation, cell proliferation by immunohistochemistry staining, estradiol concentration analysis and specific gene expression analysis. Among them all, histological evaluation is the primary approach, which is used in follicular density assessment, diameters of oocyte and follicle measurement, and layers of granulosa cells count (Smits et al., 2010).

![Figure 2](https://academic.oup.com/humrep/article-abstract/30/4/877/614618)  
*Figure 2* Experimentally determined relaxation modulus $G(t)$ in the fresh group and the two cryopreserved groups (Data are mean ± SD, n = 15). All stress relaxation data (SRD) of both the fresh and the two cryopreserved groups were indicated by the value expressed as a percentage, which means the ability to maintain the initial stress after stretching. In the fresh control group, the value of SRD (0.6433 ± 0.7233) was significantly higher than that in the sucrose group (group S) (0.5200 ± 0.8331) and in the trehalose group (group T) (0.5667 ± 0.6415), and the difference was statistically significant, respectively ($P < 0.05$). The SRD values within each group were compared using one-way analysis of variance followed by the Student-Newman-Keuls test.

(0.5200 ± 0.8331) and group T (0.5667 ± 0.6415), and the difference was statistically significant, respectively ($P < 0.05$). However, no significant difference was found between group S and group $T$ ($P > 0.05$), despite the fact that the vast majority of the SRD values of the Group T were higher than Group S in three experimental groups. In general, the stress relaxation response of both fresh and vitrified/warmed ovarian tissues was similar and exhibited a very rapid relaxation within the first 20 s, with a much slower relaxation after 300 s (Fig. 2).
temperatures during the freezing/thawing process (Holt and North, 1994).

In the process of collecting tissues, we found that the ovarian volume was increased significantly owing to benign or malignant ovarian tumors. According to Holt and North, the plasma membrane endures excessive chemical and physical stress due to high salt concentrations and low temperatures during the freezing/thawing process (Holt and North, 1994). Therefore, we conjecture that the increasing of the ovarian volume could perhaps influence the biomechanical properties of ovarian tissues.

In the present study, the DMA measurements of stress relaxation on human ovarian tissues implied that the viscoelastic behaviors of both the fresh and the cryopreserved samples can be well described by using the QLV model. The fresh group can maintain $\sim 60-70\%$ of the original stress after relaxation of 600 s, while the vitrification groups can only keep $\sim 50\%$ of the original stress. Compared with group S, group T seems more excellent in maintaining the initial stress. These results are consistent with the experimental data acquired from histological evaluation, indicating that the assessment method is theoretically feasible. Additionally, when comparing different vitrification protocols, a stress relaxation curve can visually present the freezing effects. Given so many variables, results obtained from vitrification are discrepant within different individuals. Stress relaxation curve can intuitively figure out which method is more suitable for a person, and the cryogenic storage program should then be individualized.

Our study reports for the first time an evaluation of fresh and cryopreserved human ovarian tissues from the viewpoint of biomechanics. Certainly a limitation of the present study is that experimental samples are directly exposed to the air, which leads to the generation of discrepancy in viscoelastic properties of ovarian tissues compared that in vivo growth environment. So in the future work, we can simulate human physiological environment to conduct mechanics experiment.

In summary, this study showed that nonpenetrating CPAs are capable of improving the cryopreservation of follicles with advantages for trehalose, which is important in optimizing human ovarian tissues vitrification. Meanwhile, this study indicates that a vitrification protocol that may be chosen based on histological evaluation and other methods, will have a substantial effect on the biomechanical behaviors of human ovarian tissues.

**Authors’ roles**

Each author contributed to the work in the following ways: T.T.: conception and design, experiments, collection of samples, statistical analysis and interpretation of data, manuscript drafting and revision of the article. G.Z.: design, analysis and interpretation of data, revision of the article critically for important intellectual content. D.H.: collection of samples and acquisition of data. K.Z.: design, experiments, analysis and interpretation of data. D.C.: revision of the article. Z.Z.: conception and design, revision of the article, and final approval of the version to be published.

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**Conflict of interest**

The authors declare that there is no conflict of interests regarding the publication of this original paper.

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Holt WV, North RD. Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of plasma membrane
Vitrification protocol for human ovarian tissue