

# Chapter 2

## Utility of Animal Models for Human Ovarian Tissue Cryopreservation

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### Abstract

Success in cryopreservation of ovarian tissue (OT) in animal models has led to develop efficient cryotechnologies for human ovarian tissue. In this chapter, cryopreservation protocols developed for animal experiments are described.

**Key words** Ovarian tissue, Cryopreservation, Slow freezing, Vitrification, Fertility preservation

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### 1 Introduction

Although the technology of cryopreservation of ovarian tissue (OT) is still at a relatively experimental stage, it seems to be a promising strategy for fertility preservation with encouraging results. Indeed, it is the only available option for fertility preservation in pre-pubertal girls. In the last decade, a wealth of information has been collected through studies using different animal models, and this has led to the development of new technologies and optimization of existing ones. Success in cryopreservation of OT in animal models has allowed its application in humans. Today, the results of those studies still continue to form the basis of OT cryobiology.

The most commonly used animal models in developing the principles of OT cryopreservation for application in humans are sheep and bovine models. Especially bovine ovaries have become useful due to the similarities in the ovary size and composition as well as length of folliculogenesis. Nevertheless, the rodent model has contributed more than any other animal models to scientific experiments. Although establishing the optimal cryopreservation protocol is one of the most important steps to improve OT survival and many laboratories have developed various protocols, cryopreservation of OT has not been fully optimized (especially in humans).

Cryopreservation of OT can be done by either slow freezing or vitrification. To date, the slow freezing method has been considered a standard technique for OT cryopreservation in clinical settings. Recently, vitrification of OT has been studied by multiple investigators; however, the results are still controversial. In theory, vitrification of OT should provide better results than those of slow freezing; however, it will take some time to perfect the vitrification technology for OT.

Cryoprotective agents (CPAs), by increasing the total concentration of solutes in the system, reduce the amount of ice formation at any given temperature; however, to be biologically acceptable, they must be able to penetrate into the cells and have low toxicity. CPAs commonly used in OT vitrification are dimethyl sulfoxide (DMSO), propanediol (PrOH), and ethylene glycol (EG) as permeating CPAs and sucrose, trehalose, and Ficoll as nonpermeating CPAs. Some studies suggested that the combination of two or more CPAs can reduce the toxicity and may be more effective than a single type of CPA. Nevertheless, the efficacy of different types and concentrations of CPA should be further investigated.

Here, we describe slow freezing and vitrification protocols for mouse, sheep, and bovine OTs.

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## 2 Materials

### 2.1 Mouse Ovarian Tissue Cryopreservation [1, 2]

#### 2.1.1 Vitrification

From 4- to 7-week-old B6D2F1 female mice were housed under controlled conditions as follows: lighting (12-h light/dark cycle), temperature (20–22 °C), humidity (40–60 %) and fed ad libitum.

1. Basic medium (BM): 20 % fetal bovine serum (FBS) in Dulbecco's phosphate buffered saline (D-PBS).
2. Equilibration solution (ES): 7.5 % (v:v) Ethylene glycol (EG), 7.5 % (v:v) Dimethylsulfoxide (DMSO) in BM.
3. Vitrification solution (VS): 20 % (v:v) EG, 20 % (v:v) DMSO, 0.5 M sucrose in BM.
4. Warming solution: 1 M, 0.5 M, 0.25 M, and 0 M sucrose in BM.
5. Four-well dish.
6. Sterilized filter paper or gauze.
7. Liquid nitrogen (LN2).
8. Cryovial.
9. Cane.
10. Electron microscopic grid (IGC400)
11. Sterile hood.

### 2.1.2 Slow Freezing

1. Basic medium (BM): 20 % FBS in D-PBS.
2. Slow freezing solution: 1.5 M DMSO, 0.1 M sucrose in BM.
3. Warming solution: 0.5 M, 0.25 M, 0 M sucrose in BM.
4. Four-well dish.
5. LN2.
6. Cryovial.
7. Forceps/cotton tips.
8. Sterile hood.
9. Slow freezing machine: Kryo 360, Cryologic etc.

## 2.2 Sheep Ovarian Tissue Cryopreservation [3–5]

Sheep ovaries immersed in cold (0–4 °C) D-PBS were transported from a local slaughterhouse to laboratory within 2 h on ice (*see Note 1*). Prepare the ovarian cortex into thin cortical slices (1 mm thick).

### 2.2.1 Vitrification

1. Basic medium (BM): 20 % FBS in L-15 medium.
2. Equilibration solution (ES): 7.5 % (v:v) EG, 7.5 % (v:v) DMSO in BM.
3. Vitrification solution (VS): 20 % (v:v) EG, 20 % (v:v) DMSO, 0.5 M sucrose in BM.
4. Warming solution: 1 M, 0.5 M, 0.25 M, and 0 M Sucrose in BM.
5. Sterilized filter paper or gauze.
6. LN2.
7. Curved scissors.
8. Forceps.
9. 35 mm and 60 mm cell culture dishes.
10. Sterile hood.
11. Water bath.

### 2.2.2 Slow Freezing

1. Basic medium (BM): 20 % FBS in L-15 medium.
2. Slow freezing solution: 1.5 M DMSO + 0.1 M sucrose in BM.
3. Thawing solution: TS1: 1.0 M DMSO + 0.1 M sucrose, TS2: 0.5 M DMSO + 0.1 M sucrose, TS3: 0.1 M sucrose
4. Sterilized filter paper or gauze.
5. LN2.
6. Cryovial.
7. Cane.
8. Curved scissors.
9. Forceps/cotton tips.
10. 35 mm and 60 mm cell culture dishes.

11. Sterile hood.
12. Water bath.
13. Slow freezing machine: Kryo 360, Cryologic etc.

**2.3 Bovine  
Ovarian Tissue  
Cryopreservation [6, 7]**

**2.3.1 Vitrification**

1. Basic medium (BM): 20 % FBS in L-15 medium.
2. Equilibration solution (ES): 7.5 % (v:v) EG, 7.5 % (v:v) DMSO in BM.
3. Vitrification solution (VS): 20 % (v:v) EG, 20 % (v:v) DMSO, 0.5 M sucrose in BM.
4. Warming solution: 1 M, 0.5 M, 0.25 M, and 0 M Sucrose in BM.
5. Sterilized filter paper or gauze.
6. LN2.
7. Curved scissors.
8. Forceps.
9. 35 mm and 60 mm cell culture dishes.
10. Sterile hood.
11. Water bath.

**2.3.2 Slow Freezing**

1. Basic medium (BM): 20 % FBS in L-15 medium.
2. Slow freezing solution: 1.5 M DMSO + 0.1 M sucrose in BM.
3. Thawing solution: TS1: 1.0 M DMSO + 0.1 M sucrose, TS2: 0.5 M DMSO + 0.1 M sucrose, TS3: 0.1 M sucrose
4. Sterilized filter paper or gauze.
5. LN2.
6. Cryovial.
7. Cane.
8. Curved scissors.
9. Forceps/cotton tips.
10. 35 mm and 60 mm cell culture dishes.
11. Sterile hood.
12. Water bath.
13. Slow freezing machine: Kryo 360, Cryologic etc.

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## 3 Methods

Carry out all procedures at room temperature unless otherwise specified and use the sterile hood when processing.

### 3.1 Mouse Ovarian Tissue Cryopreservation [2]

The whole ovary of the BDF1 female mice (*see Note 2*).

#### 3.1.1 Vitrification of Mouse Ovarian Tissue

##### Vitrification procedure

1. Expose intact ovaries to the equilibration solution for 10 min.
2. Expose to a vitrification solution for 3–5 min (*see Note 3*).
3. Load each of the ovaries onto the EM grid and excess vitrification solution be removed using sterilized filter papers (*see Note 4*).
4. Immediately plunge the ovaries on the EM grids into LN2 for 30 s (*see Note 5*).
5. Place the vitrified ovaries attached to the EM grids into 1.5 mL cryovials filled with LN2 and store.

##### Warming procedure

1. Hold cryovials for 20 s in air at room temperature and then fill with warming solution (1.0 M sucrose in BM) at 37 °C for 30 s. Move the ovaries with the grids to the 4-well dishes and wash in warming solutions in a stepwise manner (0.5 M, 0.25 M, and 0 M sucrose in BM, 3 min each).
2. Further incubate the ovaries detached from the grids for 10 min in BM.

#### 3.1.2 Slow Freezing of Mouse Ovarian Tissue

##### Freezing procedure

1. After washing in the isolation medium, transfer ovarian tissue to the dish containing the slow freezing solution.
2. Place each ovary into the cryovial containing a small volume of slow freezing solution (*see Note 6*).
3. After 20 min exposure to the cryoprotectant at room temperature for equilibration, place the cryovials in a programmable freezer precooled to 4 °C.
4. Cool at 2 °C/min to –7 °C and hold for 5 min (*see Note 7*).
5. Induce an ice formation (seeding) manually by touching the cryovial at the level of the meniscus with cotton tip/precooled scissors/forceps. Hold 5 min after seeding before further cooling.
6. Further cool down to –40 °C at a rate of –0.3 °C/min and then cool to –100 °C at –10 °C/min.
7. Plunge directly into LN2 for storage.

##### Warming procedure

1. Hold cryovials for 20 s in air at room temperature and place the cryovials in a water bath at 37 °C for 30–60 s.

2. Remove the cryoprotectant by serial dilutions of the cryoprotectants in medium stepwise, which could avoid rapid osmotic changes in the cryopreserved tissue.
3. After removing cryoprotectant, wash the thawed ovaries repeatedly in L-15 medium containing 10 % (v:v) FBS.

### **3.2 Sheep Ovarian Tissue Cryopreservation [8, 9]**

#### *3.2.1 Vitrification of Sheep Ovarian Tissue*

##### Vitrification procedure

1. Equilibrate prepared ovarian cortical tissue ( $5 \times 5 \times 1$  mm) in equilibration solution for 15-min at room temperature.
2. After the equilibration period, transfer tissues into the vitrification solution and equilibrate for 10–15 min at room temperature.
3. Place the tissues on a piece of gauze to remove the residual vitrification medium.
4. Place each ovarian tissue in a cryovial.
5. Plunge the cryovial with ovarian tissue into LN2. And then, close the cover, and place in a LN2 tank for storage.

##### Warming procedure

1. Hold cryovials for 20 s in air at room temperature and fill with 37 °C pre-warming solution (1.0 M sucrose in BM) for 2 min.
2. Move the ovaries to the 35 mm dishes and wash in warming solutions for 3 min each in a stepwise manner (0.5 M, 0.25 M, and 0 M sucrose in BM).
3. Wash in BM (x2–3).

#### *3.2.2 Slow Freezing of Sheep Ovarian Tissue*

##### Freezing procedure

1. After washing in the isolation medium, transfer ovarian tissue to the dish containing slow freezing solution. Place each section of ovarian tissue into the cryovial containing a small volume of slow freezing solution (1 ml).
2. After 30 min exposure to the cryoprotectant at room temperature for equilibration, place the cryovials into the programmable freezer precooled to 4 °C.
3. Cool at 2 °C/min to –7 °C and hold for 5 min.
4. Induce seeding manually by touching the cryovial at the level of the meniscus with precooled cotton tips/forceps. Hold 5 min after seeding before further cooling.
5. Further cool down to –40 °C at a rate of –0.3 °C/min and then cool to –100 °C at –10 °C/min.
6. Plunge directly into LN2 for storage.

### Warming procedure

1. Hold cryovials for 20–30 s in air at room temperature.
2. Then place into 37 °C water bath for 2–3 min and agitate vigorously.
3. Thaw stepwise using prepared thawing solutions (TS1: 1.0 mol/L DMSO with 0.1 mol/L sucrose, TS2: 0.5 mol/L DMSO with 0.1 mol/L sucrose, TS3: 0.1 mol/L sucrose), 3 min each.
4. Wash thawed tissue in L-15 medium (x2–3).

### **3.3 Bovine Ovarian Tissue Cryopreservation**

#### *3.3.1 Vitrification of Bovine Ovarian Tissue*

### Vitrification procedure

1. Equilibrate prepared ovarian tissue (5 × 5 × 1mm cortex) in equilibration solution for 15-min at room temperature.
2. After equilibration period, transfer ovarian tissue into the vitrification solution and equilibrate for 10–15 min at room temperature.
3. Place ovarian tissue on a piece of gauze to remove the residual vitrification medium.
4. Place each ovarian tissue in a cryovial.
5. Plunge the cryovial with ovarian tissue into LN2. And then, close the cover and place in a LN2 tank for storage.

### Warming procedure

1. Hold cryovials for 20 s in air at room temperature and then fill with 37 °C pre-warming solution (1.0 M sucrose in BM) for 2 min.
2. Move ovarian tissue to the 35 mm cell culture dishes and wash in warming solutions for 3 min each in a stepwise manner (0.5 M, 0.25 M, and 0 M sucrose in BM).
3. Wash in BM (x2–3).

#### *3.3.2 Slow Freezing of Bovine Ovarian Tissue*

### Freezing procedure

1. After washing in the isolation medium, transfer ovarian tissue to culture dishes containing slow freezing solution.
2. Place each section of ovarian tissue into the cryovial containing a small volume of slow freezing solution (1 ml).
3. After 30 min exposure to the cryoprotectant at room temperature for equilibration, place the cryovials in a programmable freezer precooled to 4 °C.
4. Cool at 2 °C/min to –7 °C and hold for 5 min.
5. Induce seeding manually by touching the cryovial at the level of the meniscus with precooled cotton tips/forceps and hold 5 min more.

6. Further cool down to  $-40\text{ }^{\circ}\text{C}$  at a rate of  $-0.3\text{ }^{\circ}\text{C}/\text{min}$  and then cool to  $-100\text{ }^{\circ}\text{C}$  at  $-10\text{ }^{\circ}\text{C}/\text{min}$ .
7. Plunge directly into LN2 for storage.

#### Warming procedure

1. Hold cryovials for 20 s in air at room temperature.
2. Place the cryovials in a water bath at  $37\text{ }^{\circ}\text{C}$  for 2–3 min and agitate vigorously.
3. Wash tissue stepwise using prepared thawing solutions for 3 min each (TS1: 1.0 mol/L DMSO with 0.1 mol/L sucrose, TS2: 0.5 mol/L DMSO with 0.1 mol/L sucrose, TS3: 0.1 mol/L sucrose).
4. Wash thawed ovarian tissue (x2–3) in L-15 medium.

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## 4 Notes

1. The effect of transport temperature on ovaries is unclear and different temperatures ( $0\text{--}4\text{ }^{\circ}\text{C}$ ,  $22\text{--}25\text{ }^{\circ}\text{C}$  and  $35\text{--}38\text{ }^{\circ}\text{C}$ ) are used. And D-PBS or 0.9 % saline can be used as a storage solution during transportation. We prefer transporting tissue at  $4\text{ }^{\circ}\text{C}$  in D-PBS or L-15 solution.
2. Because of the small dimension ( $\sim 2 \times 2 \times 2\text{ mm}^3$ ) of the mouse ovary, we vitrified whole organs instead of ovarian slices.
3. Keep ES and VS vials at the room temperature ( $25\text{ }^{\circ}\text{C}\text{--}27\text{ }^{\circ}\text{C}$ ) at least 1 hour before vitrification.
4. Only cryovials or cryotubes without EM grids can be used. However, EM grids offer good performance for cooling due to their very high thermal conductivity.
5. The transparent glassy appearance should be maintained during cooling and warming.
6. Various kinds of carriers are being used (plastic straw, cryovial, etc.).
7. Different cooling rates are used depending on the laboratory.

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