Optimizing cryoprotectant perfusion conditions for intact ovary: a bovine model

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Abstract

Purpose The aim of this study was to detect the effects of different perfusion pressure and different length of perfusion period on whole ovarian cryopreservation

Methods Bovine whole ovaries were vitrified-warmed. The ovaries were divided into the experimental groups according to different perfusion pressure and different length of perfusion period. Follicular viability was assessed using the trypan blue test; the percentage of morphologically normal primordial follicles and the 17- β estradiol level in the culture supernatants were measured.

Results When perfusion pressure was 100 mmHg, and the length of perfusion period was 40 min, the viability of ovarian tissues in bovine whole ovarian cryopreservation were higher than other protocols.

Conclusion Protocol IIB (the perfusion pressure was 100 mmHg, and the length of perfusion period was 40 min) was appropriate for bovine whole ovarian cryopreservation.

Keywords Perfusion pressure · Length of perfusion period · Cryopreservation · Bovine whole ovaries

Introduction

Survival rates of cancer patients are on the increase because of constant improvements in the diagnosis and treatment of the disease [1]. However, patients requiring chemotherapy and /or radiotherapy for cancer or other benign pathologies are likely to suffer premature ovarian failure [2, 3]. Cryopreservation of ovarian tissue offers the possibility to circumvent gonadotoxic injury.

Human ovarian cortical tissue strips have been successfully cryopreserved, thawed and autotransplanted, and to date, at least 28 healthy children have been born worldwide as a result of transplanted ovarian tissue [4–8]. However, many follicles are lost during revascularization and the key factor responsible for follicular loss is ischemia [9, 10], although it was reported that the function of transplanted ovarian cortical strip in three women continued up to 7 years [11].

In theory, cryopreservation of intact ovary, followed by restoration of reproductive function after autologous transplantation using microvascular anastomosis, could achieve an immediate blood supply [12]. Spontaneous pregnancies after transplantation of an intact ovary with microvascular anastomosis have occurred in rat, ewe, and human [12–14]. It shows that this technique is promising.

In large mammals and humans, cryopreserving a large, intact ovary may prove more problematic than in small animals because of the difficulty of adequate diffusion of

Capsule Bovine whole ovarian cryopreservation.

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cryoprotective agents into large tissue masses. With the aim of increasing cryoprotectant penetration of the medullar compartment and avoiding intravascular ice formation, cryoprotective medium was perfused through the ovarian artery at a constant pressure [15–24].

To our knowledge, few studies have reported the optimal perfusion pressure and perfusion length for cryopreservation of whole ovaries. The present experiment was therefore designed to perform a comparative investigation of several different protocols using different perfusion pressure and perfusion length and to improve the protocol for cryopreservation of whole ovaries.

In this study, we choose bovine as a model in this experiment. The reasons are: (i) the ovaries of bovine are comparable in size with human ovaries; (ii) only one or two follicles mature each cycle as the maturation of multiple follicles greatly influences the ovarian volume and therefore the outcome of freezing process; (iii) the bovine has a monthly cycle [22].

Materials and methods

Chemicals

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

Species

Approval for this study was obtained from the Animal Research Ethical Committee of Shandong University. We collected one hundred ovaries with ovarian pedicles from bovines aged 5 ± 0.4 years at the Shandong Hypor Liuhe Breeding Farm. All the selected ovaries were in follicular phase.

Ovarian perfusion and cryopreservation of ovary

The vessel was transected 10 cm under the ovarian hilus. Next, a cannula (0.75 mm OD) was inserted into the vessel and secured with sutures. The ovary was then placed in a sterile perfusion tray and immersed in cryopreservation media. The ovary was initially perfused, via the cannula, with cold, sterile, heparinised (100 IU/mL) Ringer's solution for 10 min at a rate of 1 ml/min before being perfused with the cryopreservation media. The canule was connected to a pump (Jianyuan Co., Changsha, China) (Fig. 1).

According to different perfusion pressure, samples were randomly divided into group I (50 mmHg), group II (100 mmHg), and group III (150 mmHg). According to different length of perfusion period, samples were randomly divided into group a (20 min), group b (40 min), and group

Fig. 1 The illustration of perfusion apparatus. The cryopreservation media in the syringe (*downwards arrow*) was perfused into artery at constant pressure with the pump (*rightwards arrow*) via canule (*leftwards arrow*)

c (60 min), so the experimental groups include group Ia, group Ib, group Ic, group IIa, group IIb, group IIc, group IIIa, group IIIb, group IIIc (Table 1). The one hundred ovaries were randomly divided into fresh control group and experimental groups (ten ovaries for each group).

The ovaries were perfused via the ovarian artery with the cryopreservation media. The procedure was carried out according to the protocol that we previously reported [24]. The cryoprotective mixture contained phosphate-buffered saline medium (PBS), 25 % (v/v) dimethyl sulfoxide (DMSO), 25 % (v/v) propylene glycol (PROH) and 15 % heated-inactivated fetal calf serum. To ensure osmotic balance, the cryoprotective mixture was introduced in two steps of increasing concentration: 12.5 % (v/v) DMSO+12.5 % (v/v) PROH and 25 % (v/v) DMSO+25 % (v/v) PROH. After perfusion at 4 °C, the ovaries were transferred into liquid-nitrogen-proof ethyl vinyl acetate cryobags containing the cryoprotective mixture. The ovaries were vitrified by plunging the bags into liquid nitrogen.

Table 1 The experimental groups with different perfusion pressure and different length of perfusion period

Group	Perfusion pressure (mmHg)	Perfusion period (min)
Control group		
Group Ia	50	20
Group Ib	50	40
Group Ic	50	60
Group IIa	100	20
Group IIb	100	40
Group IIc	100	60
Group IIIa	150	20
Group IIIb	150	40
Group IIIc	150	60

Warming of ovary

One month later, the ovaries were removed from the Dewar and held in air at room temperature for 2 min before plunging in a water bath at 37 °C for another 2 min with gentle shaking. To remove the cryoprotectant, the ovaries were bathed and perfused in 3 steps of 10 min each at room temperature with a reversed sucrose concentration gradient (1.0 M, 0.5 M and 0.25 M sucrose) in Leibovitz L-15 medium to prevent osmotic injury. The perfusion pressure was 100 mmHg.

Evaluation of ovarian follicular viability

The cryopreserved ovarian tissue was evaluated in term of plasma membrane function and structural integrity by trypan blue exclusion test. The method was used as described by Fauque et al. [25]. The ovarian fragments were thinly sectioned in Leibovitz L-15 medium supplemented with 1 mg/ml (200 IU/ml) type 1 collagenase, incubated at 37°C for 2 h and pipetted at intervals of 30 min. Collagenase activity was inhibited by using 50 % FCS. The suspension was filtered using a 70- μ m nylon filter (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and centrifuged at 400g for 5 min. The precipitate was diluted with 50 μ l of Leibovitz L-15 medium and kept in a water bath at 37°C. Trypan blue (0.4 %; 20 μ l) was added to the suspension containing the follicles, deposited on a glass slide and examined under inverted microscope. For each group, 100 intact follicles were examined, and the partially or completely denuded oocytes were excluded. The number of stained follicles and the total number of follicles were counted. The percentage of viable follicles was determined by calculating the percentage of unstained cells.

Histological examination

Ovaries were fixed in Bouin's solution for light microscopic evaluation. Serial 5- μ m sections were prepared; every 10th section of each ovary was mounted on a glass slide, and stained with hematoxylin and eosin.

Follicular morphology was examined by microscope (magnification, $\times 400$). For each ovary, 100 primordial follicles were counted in sections where the oocyte nucleus was visible, and their morphology was recorded. Normal follicles had a complete layer of flattened pregranulosa cells, oocytes with cytoplasm, and a normal nucleus. Abnormal follicles were classified as follows: pyknotic nucleus, and both nuclear and cytoplasmic damage.

Culture of frozen ovarian tissue

An in vitro culture system was used as described by Scott and colleagues [26]. The strips from the thawed ovaries

were immersed in PBS, cut into small pieces (1 mm $\times$ 1 mm $\times$ 1 mm), and placed into 24-well culture dishes (Corning, USA). Millicell culture plate inserts (Millipore, Sundbyberg, Sweden) coated with 100 μ l pre-diluted Matrigel extracellular matrix (Becton Dickinson, Sttokholm, Sweden) were put into each well to support the growth of the ovarian tissues. Every insert contained 2 pieces of ovarian tissue. The culture medium comprised α -MEM, 0.5 IU/ml follicle-stimulating hormone, 1 % ITS (10 μ g/ml insulin, 5.5 μ g/ml transferrin and 7.6 ng/ml sodium selenite), 50 μ g/ml vitamin C, 0.47 mmol/l pyruvate acid, 2 mmol/l L-glutamine, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. Initially, 150 μ l culture medium was added inside the insert and 550 μ l outside it. The tissues were cultured in a humidified incubator at 37 °C with 5.5 % CO₂ for 14 days. Every other day, 400 μ l of the culture medium outside the inserts was replaced by fresh medium.

Hormone assays

At 14 th day after culture, the spent medium was collected and stored at -80 °C for later analysis. The levels of 17- β estradiol (minimum detection limit: 5.0 pg/mL) were measured using a heterogeneous competitive magnetic separation immunoassay (LRW, Shenzhen, Guangdong, China).

Statistical analysis

Follicle viability and the percentage of morphologically normal primordial follicles were compared using chi-square test analysis. The hormone levels were compared by 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

Ovarian follicle viability

The follicle viability in the fresh control group (87.4 % $\pm$ 5.2 %) was significantly higher than those in all experimental groups ($P < 0.05$). The follicle viability in group IIb (75.9 % $\pm$ 3.9 %) was the significantly highest in all experimental groups ($P < 0.05$). With regard to group I, the follicle viability in group Ib (67.3 % $\pm$ 4.7 %) was significantly higher than those in group Ia (53.6 % $\pm$ 4.1 %) and group Ic (56.1 % $\pm$ 4.5 %) ($P < 0.05$). With regard to group III, and the follicle viability in group IIIb (65.2 % $\pm$ 4.1 %) was significantly higher than those in group IIIa (54.6 $\pm$ 3.6 %) and group IIIc (53.1 $\pm$ 2.9 %) ($P < 0.05$) (Fig. 2).

Fig. 2 **a** Follicle considered to be alive after trypan blue staining. **b** Follicle with a blue oocyte considered to be dead (Bar = 10 μ m)

Histological examination of primordial follicles

The percentage of morphologically normal primordial follicles in fresh control group ($89.5 \% \pm 8.5 \%$) was significantly higher than those in all experimental groups ($P < 0.05$). The percentage of morphologically normal primordial follicles in group IIb ($80.3 \% \pm 7.1 \%$) was the highest in all experimental groups ($P < 0.05$). With regard to group I, the percentage of morphologically normal primordial follicles in group Ib ($65.3 \% \pm 5.1 \%$) was significantly higher than those in group Ia ($54.1 \% \pm 5.2 \%$) and group Ic ($56.5 \pm 5.4 \%$) ($P < 0.05$). With regard to group III, the percentage of morphologically normal primordial follicles in group IIIb ($63.3 \% \pm 4.5 \%$) was significantly higher than those in group IIIa (55.6 ± 3.5) and group IIIc (54.5 ± 4.5) ($P < 0.05$) (Fig. 3).

Hormone assay

17- β estradiol concentration in fresh control group (395 pg/ml) was significantly higher than those in all experimental groups respectively ($P < 0.05$). 17- β estradiol concentration in group IIb (339 pg/ml) was the highest in all experimental groups ($P < 0.05$). With regard to group I, 17- β estradiol concentration in group Ib (268 pg/ml) was significantly higher than those in group Ia (201 pg/ml) and group Ic (198 pg/ml) respectively ($P < 0.05$). With regard to group

III, 17- β estradiol concentration in group IIIb (259 pg/ml) was significantly higher than those in group IIIa (207 pg/ml) and group IIIc (212 pg/ml) respectively ($P < 0.05$).

Discussion

Transplantation of whole ovaries with vascular anastomosis has been considered as a promising strategy to restore fertility in cancer patients. Thus far, five reports have described fresh whole ovary grafts with vascular anastomosis in human, and one successful pregnancy was recorded [14, 27–31]. In the past few years, attempts at freezing and grafting whole ovaries in animal have yielded encouraging results. The first case of restoration of fertility in rats after whole frozen-thawed ovary transplantation was described by Wang et al. in 2002 [13]. They described successful vascular transplantation of frozen-thawed rat ovaries and reproductive tract in 4 out of 7 (57 %) transplants. These ovaries survived for 60 days or more, and resulted in one pregnancy. Chen et al. showed that frozen-thawed rabbit ovaries remained functional for at least 7 months after microvascular transplantation in 13 out of 15 (86.7 %) animals [15]. However, rat and rabbit ovaries are smaller than human ovaries and thus easier to evenly freeze and thaw. In sheep studies, most follicular population was lost because of vascular complications after transplantation with vascular

Fig. 3 **a** Morphologically normal primordial follicle: follicle spherical in shape with even distribution of pregranulosa cells, spherical oocyte (rightwards arrow). **b** Morphologically abnormal primordial follicle showing shrinkage of oocyte and pyknotic nucleus (leftwards arrow) (Bar = 50 μ m)

anastomosis [16, 17, 23], although a successful pregnancy and an ovarian function of 6 years were reported after whole ovary cryopreservation and autotransplantation in sheep [12, 32]. In cow study, the results were also not encouraging [24, 33]. To date, it has not been possible to perform whole ovary transplantation after cryopreservation in humans because of technical difficulties in cryopreservation.

The major technical problems with whole ovary freezing is the larger volume of tissue that causes slow penetration of the cryoprotectant and increases the risk of breaking the structure of cells. Imhof et al. showed that perfusion of porcine ovaries with DMSO as a cryoprotective agent, resulted in improved protection from damage induced by the cryopreservation process, compared with only submerging the ovary in the cryoprotective solution [22]. Martinez-Madrid et al. showed that perfusing the ovary via its afferent vessel increased the survival and condition of the ovarian blood vessels which was crucial for successful reperfusion after transplantation [20]. However, vascular perfusion can impose a great osmotic stress and chemical toxicities, then increase the likelihood of damage to the tissue. Also, changes in cell volume during perfusion can increase vascular resistance in ways that compromise the perfusion process itself [20].

Histological examination is an important tool to evaluate the efficiency of ovarian cryopreservation. Follicle morphology is usually analyzed through several criteria: (i) eosinophilia and vacuolations, which are the most commonly used criteria for oocyte cytoplasm; and (ii) contraction and clumping of nucleus chromatin material, which are usually considered signs of atresia [34–37]. However, in our study, we noticed that: (i) oocyte cytoplasm staining frequently varied from one slice to another, independent of any follicular damage; and (ii) oocyte cytoplasm vacuolations were also observed on fresh ovarian tissue. Previous studies have also reported that the vacuolization of cytoplasm in oocytes appeared in both the fresh and the frozen-thawed groups analyzed by HE staining [38, 39]. It seems that this phenomenon occurs during the fixation and the stain procedures. As a consequence, we have chosen to evaluate oocyte morphology considering only the chromatin material aspect. Because the trypan blue test provides a means to estimate the follicular viability after cryopreservation, it has been used as a tool to assess the efficiency of the cryopreservation procedure [16–18, 25]. Besides, hormone production in culture is a sign of follicular growth in culture and thus an evaluation marker of viability, as hormone can be secreted only from secondary stages onwards, therefore the hormonal activity of thawed and cultured tissue is a criterion for the effectiveness of the cryopreservation protocol [40].

In this study, follicle viability, the percentage of morphologically normal primordial follicles, and hormone levels in group IIb (the perfusion pressure is 100 mmHg, and length

of perfusion period is 40 min) were the highest in all the experimental groups. It suggests that protocol IIb is more effective than the other protocols in restoring ovarian viability after cryopreservation of bovine whole ovaries. Low perfusion pressure and short length of perfusion period may not ensure a complete saturation of the tissue, then the intracellular and extracellular ice form, which will cause physical damage to the cells. Whereas, high perfusion pressure and long length of perfusion period may result in vessel rupture and put cells to the risk of excessive chemical toxicity. This may be the reason that protocol IIb is more suitable for cryopreservation of bovine whole ovaries.

In summary, protocol IIb (the perfusion pressure is 100 mmHg, and length of perfusion period is 40 min) is more effective than the other protocols in restoring ovarian viability after cryopreservation of bovine whole ovaries; therefore protocol IIb is suitable for cryopreservation of bovine whole ovaries. Nevertheless, it is a preliminary study; we should make further efforts to explore the optimal protocol for cryopreservation of human whole ovaries, thereby restoring the endocrine and reproductive functions of female patients with cancer [41].

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

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