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Cryopreservation of whole bovine ovaries: comparisons of different thawing protocols

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ABSTRACT

Objective: The aim of this study was to perform a comparative investigation of several different thawing protocols and to determine an appropriate protocol for thawing whole bovine frozen ovaries.
Study design: Bovine ovaries were slowly frozen and then thawed by applying different protocols. Ultrastructural change, follicle viability, and the hormone levels of culture supernatant were measured.
Results: The percentage of morphologically normal primordial follicles and the hormone levels of culture supernatant in group D (two-step, thawing in water at 39 °C) were significantly higher than those in any other group. Moreover, the ultrastructural alteration of oocyte in group D (two-step, thawing in water at 39 °C) was slighter than those in any other group.
Conclusions: The two-step protocol involving short-term exposure to water at a moderately high temperature (39 °C) proved to be a suitable for thawing bovine whole ovaries.

Introduction

Human ovarian cortical tissue strips have been cryopreserved, thawed, and autotransplanted [1,2]. In these fertility preserving procedures, the retransplanted ovarian cortical tissues typically start to show the signs of spontaneous estradiol production after 2–8 months, but a cessation of the function is seen in most cases within 6–9 months after retransplantation [3,4]. Studies suggest that many follicles are lost during revascularization, which affects long-term graft functionality [5,6].

Cryopreservation of intact ovaries in vitro, followed by restoration of reproductive function after autologous transplantation using microvascular anastomosis, could achieve an immediate blood supply in vivo to maximize graft survival [7]. The first live birth from whole ovary transplantation of fresh ovary between two monozygotic twins was reported in 2008 [8]. Besides, orthotopic microvascular reanastomosis of whole cryopreserved ovine ovaries has been resulted in pregnancy and live birth [9]. These results show that this technique is promising.

Efforts have been made to optimize the freezing of whole ovaries [10,11]. However, the thawing procedure has not been systematically investigated. Thawing of an entire organ is much more complicated than warming ovarian cortical strips, suspended cells (oocyte), or group of cells (embryo). The major technical problems associated with whole ovary thawing are large volume of tissue and diversity of cell types, which cause slow heat transfer and outflow of the cryoprotectant. Whole frozen ovaries were usually thawed by applying two main protocols: (1) One-step protocol: the cryovials or cryobags were directly transferred from the liquid nitrogen to a water bath, where they were immersed until the ice melted [12–17]; (2) Two-step protocol: first, the cryovials or cryobags were removed from the liquid nitrogen and held in air. Second, the cryovials or cryobags were plunged in a water bath with gentle shaking [18–20]. However, to date, standard thawing protocols for frozen whole ovaries have not been established. We therefore perform a comparative investigation and determine an appropriate protocol for thawing whole frozen 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 [21].

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Materials and methods

Sample preparation

Chemicals were purchased from Sigma–Aldrich Company (St. Louis, MO, USA), unless otherwise indicated. Approval for this study was obtained from the Animal Research Ethical Committee of People's Hospital of Laiwu City. 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

The procedure was carried out according to the protocol that we previously reported [22]. 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).

Cryopreservation of ovary

The procedure of cryopreservation was carried out according to the protocol that we previously reported [23]. The ovaries were perfused via the ovarian artery with the cryopreservation media. The perfusion pressure was 100 mmHg, and the length of perfusion period was 40 min. 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.

Thawing of the ovaries

The frozen ovaries were thawed 1 month later. Based on the different thawing protocols (A, B, C, D, E, and F), the fifty-six ovaries were randomly assigned to fresh control group, group A, group B, group C, group D, group E, and group F.

Protocol A: the ovaries were directly plunged into a water bath at 37 °C for 3 min. Protocol B: the ovaries were held in air for 30 s at room temperature before being plunged in a water bath at 37 °C for 3 min. Protocol C: the ovaries were directly plunged into a water bath at 39 °C for 3 min. Protocol D: the ovaries were removed from liquid nitrogen and held in air for 30 s at room temperature before being plunged in a water bath at 39 °C for 3 min. Protocol E: the ovaries were directly plunged into a water bath at 41 °C for 3 min. Protocol F: the ovaries were held for 30 s at room temperature before being plunged in a water bath at 41 °C for 3 min.

To remove the cryoprotectant, the ovaries were perfused through the ovarian artery with Leibovitz L-15 medium at 37 °C supplemented with decreasing sucrose concentrations (0.25, 0.125, and 0 M) at 3 ml/min for a total of 30 min (10 min for each step).

Ultrastructural assessment

Electron microscopy is one of the best assessments to find damage due to cryopreservation procedures [24]. For numerical analysis of the cryodamage, mean size of vacuoles per 100 μm^2 of cytoplasm of oocyte was counted and compared among the groups [24]. For each group, 10 preantral follicles were examined.

After thawing, samples of the cortex were taken for ultrastructural evaluation. The tissue was fixed in 2.5% glutaraldehyde in phosphate-buffered saline for 2 h, and postfixed 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 Epon 812. Ultrathin sections (80 nm) were contrasted with uranyl acetate and lead citrate and examined by transmission electron microscopy.

Ovarian tissue culture and histological examination

Ovarian tissue culture was based on an established method as previously described [25,26]. The thawed ovarian cortical tissue from whole ovary was cut into pieces. The pieces were immediately cultured at 37 °C in a humidified atmosphere containing 5% CO_2, in 0.5 mL Dulbecco's Modified Eagle's Medium with Glutamax supplemented with human serum albumin, glutamine, follicle-stimulating hormone, insulin-transferrin-selenium, and antibiotic/antimycotic. The culture medium was changed daily. The cultured ovarian tissue pieces were fixed in Bouin's solution for light microscopic evaluation after seven days. 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 [27–29].

Culture of ovarian tissue and hormones assays

Estradiol production in culture is a sign of follicular growth in culture and thus an evaluation marker of viability, as estradiol can be secreted only from secondary stages onwards [30]. An in vitro culture system was used as described by Scott and colleagues [31]. The strips from the thawed ovaries were immersed in the base medium, cut into small pieces, and placed into 24-well culture dishes. Millicell culture plate inserts coated with 100 μL pre-diluted Martrigel extracellular matrix 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, insulin, follicle-stimulating hormone, penicillin, and so on. The tissues were cultured for 14 days. Every other day, 400 μL of the culture medium outside the inserts was replaced by fresh medium.

At 14th day after culture, the spent medium was collected and stored at −80 °C for later analysis. The level of 17-β estradiol was measured using a heterogeneous competitive magnetic separation immunoassay (LRW, Shenzhen, Guangdong, China).

Statistics

The percentage of morphologically normal primordial follicles was compared using X^2 analysis. Mean size of vacuoles per 100 μm^2 of cytoplasm of oocyte and the hormone levels of culture supernatant were compared 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 analyses.

Results

Ultrastructureal assessment

The mean size of vacuoles per 100 μm^2 of ooplasm of oocyte in group D (two-step, thawing in water at 39 °C) was the significantly lest in all the experimental groups ($P < 0.05$). The mean size of