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Original Article

An research on the isolation methods of frozen-thawed human ovarian preantral follicles

Fang-Li Dong[1*], Li Ma[2*], Sen-Lin Shi[1], Shan-Jun Dai[1], Xin-Gui Liu[1], Ying-Chun Su[1], Yi-Hong Guo[1], Fang Wang[1], Ying-Pu Sun[1]

[1]*Center for Reproductive Medical, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450000, China;* [2]*Zhengzhou People's Hospital, Zhengzhou 450000, China.* [*]*Equal contributors.*

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Abstract: Objective: To explore the effective isolation method for preantral follicles from human frozen-thawed ovarian tissue. Methods: The ovarian cortical tissue was frozen by direct cover vitrification (DCV). The frozen-thawed ovarian tissue was used for isolation of preantral follicles with collagenase combined with mechanical method and mechanical method alone, respectively. Results: 1. There was no statistical difference in the survival rates of follicles in various stages between before and after freezing ($P > 0.05$). 2. The survival rate of secondary follicles was higher, but the survival rate of primordial follicles was lower in mechanical method alone than in collagenase combined with mechanical method (all $P < 0.05$). 3. The diameters of follicles were larger and E_2 levels were higher in mecha-nical method alone than that in collagenase combined with mechanical method (all $P < 0.05$). Conclusion: After the frozen-thawed ovarian tissue was cultured for 6 days, compared with collagenase combined with mechanical method, mechanical method alone can obtain higher survival rate of secondary follicles, greater follicular diameter and higher E_2 level, which are conducive to follicular subsequent development.

Keywords: Ovarian tissue, follicle isolation, preantral follicles, calcium alginate three-dimensional culture

Introduction

With the development of radiotherapy and chemotherapy, the survival rate of patients with cancer is gradually growing, and the female with the loss of ovarian function is also increasing [1, 2]. Therefore, how to prevent their fertility has become an important issue. However, the severe shortage of mature oocytes is the biggest obstacle to resolve this problem. Human ovarian tissue contains rich preantral follicles, and primordial follicles account for 99%. In the women's lifetime, only a few primordial follicles undergo maturation and ovulation, and most of primordial follicles become atresic [3]. Primordial follicles have strong ability to withstand cryodamage due to small size and the absence of cortical granule and zona pellucida [4]. Therefore, the better combination of in vitro growth technique, maturation technique and cryotechnique for human oocytes will obtain rapid development of human reproductive medicine.

The frozen-thawed ovarian tissue may be used for ovarian tissue transplantation, in vitro culture of ovarian tissue or ovarian follicles. The possibility of reintroducing tumor cells into cancer patients by autografting of ovarian tissue can not excluded and xenotransplantation of ovarian tissue is limited by ethics and animal-borne infectious diseases. The ovarian follicles isolated from the frozen-thawed ovarian tissue are cultured in vitro into mature follicle to obtain embryos, which avoids above problems.

The aim of in vitro culture for ovarian tissue is to incubate primordial follicles accounting for 99% of the total follicles in the ovarian cortex into mature follicles, obtaining offspring by in vitro fertilization. However, exploring a simple and effective method to isolate follicles from ovarian tissue is important. At present, the more common methods to isolate follicles include collagenase combined with mechanical method and mechanical method alone. Any method will cause damage on the surrounding matrix of

preantral follicles, affecting in vitro development of preantral follicles.

To explore a simple and effective method to isolate human ovarian follicles, we compared the survival rates of follicles in different developmental stages, the diameters of post-cultured preantral follicles and the levels of post-cultured preantral follicle-secreted E_2 between the two methods. This study provides a theoretical basis for clinical application.

Materials and methods

All study methods were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All the subjects enrolled into the study gave written formal consent to participate.

Research desgin

Experiment 1: Partial fresh ovarian cortical tissue was used for isolation of preantral follicles with collagenase combined with mechanical method, and the other ovarian cortical tissue underwent direct cover vitrification (DCV). Partial thawed ovarian tissue was used for isolation of preantral follicles with collagenase combined with mechanical method. These isolated follicles in various stages were all stained with trypan blue to assess their survival rates. The survival rates of follicles in various stages were compared between before and after DCV.

Experiment 2: The thawed ovarian tissue was cultured for 6 days in vitro, and then was used for isolation of preantrol follicle with collagenase combined with mechanical method and mechanical method alone, respectively. The obtained preantral follicles were cultured in calcium alginate three-dimensional culture system for 10 days. When the preantral follicles were cultured for 6, 8 and 10 days, E_2 levels were determined. When the preantral follicles were incubated for 6 and 10 days, the follicle diameters were measured. When the preantral follicles were incubated for 10 days, the survival rates of follicles were evaluated after trypan blue staining. The estradiol (E_2) level, follicle diameter and survival rate of follicles were compared between the two methods.

Subjects

Ovarian cortical tissue was obtained from 23 patients undergoing ovarian benign tumor resection by laparoscopes or surgery in our hospital between March, 2010 and March 2011. The remaining normal ovarian tissue after pathological examination was used in this study. These patients were diagnosed with ovarian benign tumor by postoperative pathology. They were (29.2 ± 3.9)-year-old (range: 23-35). In recent 6 months, these patients had regular menstruation, but had no related history of oral hormone, endocrine disorder, radiotherapy and chemotherapy.

Sample collection and grouping

On a super clean bench, ovarian tissue was washed with DPBS 2 or 3 times followed by removal of medullary substance and blood vessels. The obtained ovarian cortex was washed with frozen base fluid 2 or 3 times, and then cut into about pieces of 0.5 mm × 0.5 mm × 1 mm.

In each patient, 5 pieces of fresh ovarian tissue were used to isolate preantral follicles with collagenase combined with mechanical method before DCV, the other fresh ovarian tissue underwent DCV. Five pieces of thawed ovarian tissue were directly used to isolate preantral follicles with collagenase combined with mechanical method, another 10 pieces of thawed ovarian tissue were incubated in vitro for 6 days. Of the 10 pieces of thawed ovarian tissue, 5 pieces were used to isolate preantral follicles with collagenase combined with mechanical method, and another 5 pieces were used to isolate preantral follicles with mechanical method alone.

CDV and thawing for ovarian tissue

In this study, freezing and thawing were performed according to the method described by Chen et al. [6] and Zhao et al. [7].

Isolation of preantral follicles

Collagenase combined with mechanical method: Ovarian tissue pieces and 1 mg/ml of type I collagenase were placed in a centrifuge tube, and then the tube was placed in 37°C water for 60 min. DPBS containing 10% of FBS was added into the tube followed by centrifugation at 4°C for 5 min at 50 g. After the removal of the supernatant, fresh medium was added. The sediments were collected by centrifugation, and then preantral follicles were isolated with 1 ml-syringe under an invert microscope. These

Table 1. The effects of the freezing and isolating process on the follicular survival rate ($\bar{x}$ ± S.D, n = 6 for each method)

	Primordial follicles (n%)	Primary and Second follicles (n%)
The survival rate before freezing (%)	74.26 ± 10.15	69.61 ± 29.60
The survival rate after freezing (%)	76.10 ± 4.64	65.56 ± 9.53

isolated follicles were washed with DPBS 2 or 3 times to remove collagenase.

Mechanical method alone: Preantral follicles were isolated with 1 ml-syringe under an invert microscope from ovarian tissue.

In vitro culture of preantral follicles

Preparation of calcium chloride solution: Calcium chloride (0.20 g) was added into 10 ml of distilled water to make 2.0% of calcium chloride solution. The calcium chloride solution was disinfected at 120°C for 30 min, and then stored at 4°C for future use.

Preparation of sodium alginate solution: 0.5% of sodium alginate solution was made with sodium alginate. The sodium alginate solution was disinfected at 120°C for 30 min, and then stored at 4°C for future use.

Preparation of calcium alginate three-dimensional culture system: The sodium alginate hydrogel containing 2 preantral follicles was slowly dropped into calcium chloride solution. After standing, spherical calcium alginate condensation was formed with follicles in its center. The spherical calcium alginate condensation was washed with medium three times, and then transferred to 4-well plate. Each well contained 400 μl of medium and 4 spherical calcium alginate condensations. The 4-well plate was placed in a 37°C incubator in an atmosphere of 5% CO_2 for 10 days. A half of medium was changed every 2 days during incubation duration.

Trypan blue staining for follicles

The isolated follicles were placed in 10-20 μl of trypan blue solution for 10 min. The follicle that its oocyte was not stained and only less than 10% of granule cells were stained was regarded as a normal follicle. The follicle that more than 10% of granule cells or its oocyte was stained was regarded as an abnormal follicle.

E_2 levels

E_2 levels were determined by electrochemiluminescence immunoassay (ECLIA). E_2 kit was purchased from Roche Company (Germany). ECLIA analyzer was purchased from Roche Company (Switzerland).

Measurement of follicle diameters

The longest and shortest diameters of a follicle were measured under invert microscope with OCTX Eyeware image analysis system. The average of the longest and shortest diameters was served as the diameter of the follicle.

Statistical analysis

Data were treated with SPSS17.0 software. Measurement data were expressed as $\bar{x}$ ± s. *t* test was used for the comparison of mean. x^2 test was used for the comparison of rates. Statistical significance was established at $P < 0.05$.

Results

Effects of cryopreservation on survival rates of follicles

Follicles were isolated from human ovarian tissues before and after DCV with collagenase combined with mechanical method, and then the survival rates of follicles were evaluated by trypan blue staining. Our results indicated that there were no statistical differences in the survival rates of follicles in various stages between before and after DCV ($P > 0.05$), suggesting that DCV failed to affect the survival rates of follicles in various stages (**Table 1**).

Effects of different isolation methods on diameters of follicles cultured in three-dimensional culture system

After ovarian tissue was cultured in vitro for 6 days, follicles were isolated with collagenase combined with mechanical method or mechanical method alone. These isolated follicles were cultured in three-dimensional culture system. The diameters and diameter growth of follicles cultured in three-dimensional culture system

Table 2. The effects of different isolation methods on the preantral follicles diameters (μm) in vitro culture ($\bar{x}$ ± S.D, n = 171 for enzymatic digestion-mechanical isolation and n = 84 for mechanical isolation)

	Preantral follicles diameters (μm) on 6th day	Preantral follicles diameters (μm) on 10th day	Diameter increase from 6th day to 10th day
① Enzymatic digestion-mechanical isolation	84.9 ± 4.3	101.7 ± 4.5	15.5 ± 3.9
② Mechanical isolation	95.5 ± 8.8*	116.2 ± 5.8*	22.7 ± 4.4*

Note: *P < 0.05, compared with group ①.

Table 3. The effects of different isolation methods on the E_2 level (pg/ml) secreted by the preantral follicles in vitro culture ($\bar{x}$ ± S.D, n = 171 for enzymatic digestion-mechanical isolation and n = 84 for mechanical isolation)

	E_2 level (pg/ml) on 6th day	E_2 level (pg/ml) on 8th day	E_2 level (pg/ml) on 10th day
① Enzymatic digestion-mechanical isolation	66.8 ± 2.9	79.2 ± 7.2	92.1 ± 3.8
② Mechanical isolation	85.8 ± 2.3*	93.9 ± 6.3*	109.3 ± 2.9*

Note: *P < 0.05, compared with group ①.

Table 4. The effects of different isolation methods on the survival rate of the preantral follicles *in vitro* culture ($\bar{x}$ ± S.D, n = 6 for each method)

	Primordial follicles (n%)	Primary follicles (n%)	Second follicles (n%)
① Enzymatic digestion-mechanical isolation	41.07 ± 4.51	43.78 ± 2.08	15.13 ± 1.72
② Mechanical isolation	27.90 ± 4.90*	46.81 ± 4.85	25.28 ± 6.61*

Note: *P < 0.05, compared with group ①.

for 6 and 10 days were respectively greater in mechanical method alone than in collagenase combined with mechanical method (all P < 0.05) (**Table 2**).

Effects of different isolation methods on E_2 levels secreted by follicles cultured in three-dimensional culture system

E_2 levels secreted by follicles cultured in the three-dimensional culture system for 6, 8 and 10 days were respectively higher in mechanical method alone than in collagenase combined with mechanical method (all P < 0.05) (**Table 3**).

Effects of different isolation methods on survival rates of follicles

Preantral follicles obtained with different isolation methods all were cultured in the three-dimensional culture system. When the follicles were cultured for 10 days, the survival of second follicles was higher in mechanical method alone than in collagenase combined with mechanical method (P < 0.05), but the survival rate of primordial follicles was lower in mechanical method alone than in collagenase combined with mechanical method (P < 0.05, **Table 4**).

Discussion

Effects of DCV on follicular development

The cryopreservation of human ovarian tissue was first reported by Newotn [8] in 1996. Vitreous cryopreservation of ovarian tissue is that the ovarian tissue is placed in liquid nitrogen after balanced osmosis with cryoprotectants of high concentration to minimize ice crystal-induced damage on oocytes. Vitreous cryopreservation is characterized by rapid cooling, simple procedures, short time-consuming and no requirement of expensive instrument, so it is superior to the traditional program frozen. In 2006, Chen et al [6] first reported DCV. DCV is better than other vitreous cryopreservation methods because its cooling rate can

reach 15000°C/min with marked cryopreserved effect, simple procedures and short time-consuming.

In this study, DCV failed to affect the survival rates of follicles in various stages. At present, there are different reports on the effect of DCV on survival rate of follicles. Sibeezhnrubl et al. [9] have found that the survival rate of primordial follicles is decreased after freezing. Gook et al. [10] have reported that there is no statistical difference in the survival rate of follicles between pre- and post-freezing, which is consistent with our results. The majority of the ovarian cortex is primordial follicles. Primordial follicles are more resistant to the damage caused by freezing due to its small size, lower metabolic rate, less organelles and absence of freeze-sensitive cortical granule and zona pellucida. Primary and secondary follicles are more sensitive to freezing because their volume is larger and the number of mitochondria is more than those of primordial follicles, so their survival rates after freezing are decreased [11]. In this study, there were no statistical differences in the survival rates of follicles in various stages, demonstrating that DCV could minimize ice crystal-induced damage on follicles, and is suitable to preservation of human preantral follicles.

Effects of different isolation methods on the development of preantral follicles

At present, the methods to isolate follicles include mechanical method alone and enzymatic digestion combined with mechanical method. The majority of follicles embedded in human ovarian tissue are primordial follicles with diameters of 30-60 μm. It is difficult to isolated primordial follicles with mechanical method alone from uncultured ovarian tissue because human ovarian tissue is dense and contains many collagen fibers which are in direct proportion to the age. In 1997, Abir et al. [12] first isolated single follicles with diameters ≥ 200 μm, and these follicles were cultured in vitro for 18 days and developed into antral follicles. Mechanical method is conducive to late development of follicles, because it can avoid enzyme-produced damage on follicles. In this study, the ovarian tissue became loose after 6-day culture and the diameters of partial primordial and primary follicles increased in a relatively stable environment, which provided a basis for isolating follicles with mechanical method alone. Therefore, the follicles we isolated are more than that from uncultured ovarian tissue.

Another common method to isolate follicles is collagenase combined with mechanical method. Although the application of collagenase can isolate more follicles compared with mechanical method alone, collagenase endotoxin-produced damage on follicles affects the viability and development of follicles. Therefore, as long as collagenase is used, in vitro development of follicles is affected; and with the increase in the concentration of collagenase, the adverse effects of collagenase on follicles become strong.

In this study, the survival rates of primordial follicles were (41.07 ± 4.51)% and (27.90 ± 4.90)% and the survival rates of secondary follicles were (15.13 ± 1.72)% and (25.28 ± 6.61)% in collagenase combined mechanical method and mechanical method alone, respectively, all with $P < 0.05$. From the results above, we can see that the survival rate of secondary follicles was lower in colagenase combined with mechanical method. This may be that collagenase destroys the basement membrane and granular cells of follicles, which is not conducive to the signal transduction between granular cells and oocytes, absorption of nutrient substance and removal of metabolites, affecting the late development of follicles. The diameters of partial primordial and primary follicles increased in a relatively stable environment after the ovarian tissue was cultured in vitro for 6 days, which provided a basis for isolating follicles with mechanical method alone. The integrity of the follicles to isolate with mechanical method alone is better, which is beneficial to follicle development. Therefore, the survival rate of secondary follicles was higher in mechanical method alone. From **Tables 2** and **3**, we could seen the diameter of follicles was greater and follicle-secreted E_2 level was higher in mechanical method alone than in collagenase combined with mechanical method, demonstrating that the follicles isolated with mechanical method alone had better growth and development potential in three-dimensional culture system than that with collagenase combined with mechanical method.

In summary, CDV can well preserve human ovarian follicles in various stages, and may be

used for cryopreservation of human ovarian tissue. The method, by which human ovarian tissue is cultured in vitro for 6 days followed by mechanical follicles isolation, can obtain more viable primary and secondary follicles, which is conducive to late growth, development and maturation of follicles.

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Disclosure of conflict of interest

The authors declare that there are no conflicts of interest.

Address correspondence to: Ying-Pu Sun, Center for Reproductive Medical, The First Affiliated Hospital of Zhengzhou University, No. 1 Constructive East Road, Zhengzhou, China. Tel: +86-371-67966166; Fax: +86-371-67966166; E-mail: syp2008@vip.sina.com

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