"Should we isolate human preantral follicles before or after cryopreservation of ovarian tissue?"

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Abstract

To evaluate the survival and growth potential of human preantral follicles isolated before and after cryopreservation.

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Should we isolate human preantral follicles before or after cryopreservation of ovarian tissue?

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Objective: To evaluate the survival and growth potential of human preantral follicles isolated before and after cryopreservation.
Design: Pilot study.
Setting: Gynecology research unit in a university hospital.
Patient(s): Six women aged 27 to 32 years.
Intervention(s): Six ovarian biopsy samples were cut into two equal parts, half subjected to slow-freezing followed by follicle isolation (cryo-iso group) and alginate-matrigel embedding, and half immediately processed for follicle isolation and alginate-matrigel embedding followed by slow-freezing (iso-cryo group) or used as fresh controls (fresh group).
Main Outcome Measure(s): Follicle number, viability, diameter, and morphology.
Result(s): After 1,134 preantral follicles had been isolated from fresh biopsy samples and 1,132 from frozen specimens, the three groups were compared before and after 7 days of in vitro culture (IVC) in alginate-matrigel beads. No statistically significant differences in viability were found between the three groups before or after IVC, but follicle diameter increased in all three groups after IVC. Morphology analysis revealed well-preserved follicles in both the iso-cryo and cryo-iso groups after IVC.
Conclusion(s): Human preantral follicles can be successfully cryopreserved before or after isolation without impairing their ability to survive and grow in vitro. This could lead to development of new protocols for follicle cryopreservation, IVC, and grafting in clinical and research settings for fertility preservation. (Fertil Steril® 2013;99:1363–8. ©2013 by American Society for Reproductive Medicine.)
Key Words: Alginate, cryopreservation, follicle culture, follicle isolation, slow-freezing

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Advances in the diagnosis and treatment of cancer have greatly increased the life expectancy of premenopausal women, but this has also resulted in a growing population of women who, having survived childhood malignancy, find themselves at high risk of infertility secondary to premature ovarian failure caused by their cancer treatment (1). Methods such as embryo or oocyte cryopreservation are currently available to preserve fertility before cancer therapy (1, 2). Unfortunately, these two options cannot be applied to patients of prepubertal age, women with no partner, or those unable to undergo a cycle of ovarian stimulation (3–5). For these patients, cryopreservation of ovarian cortex followed by transplantation can be performed. This technique, which is the only option to date to have restored both endocrine function and fertility, has led to 20 live births so far (5–10). Despite the promising results obtained with this technique, there is a risk of reintroducing malignant cells in case of certain types of cancer (11, 12). A safer alternative could be grafting of a specific number of isolated preantral follicles.

The follicle isolation procedure is usually performed after thawing of frozen ovarian cortical strips (11, 13–18).

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However, cryopreserving isolated preantral follicles could have some advantages over tissue freezing. It could prevent technical problems related to cryopreservation of heterogeneous cell types such as those found in ovarian cortex (19) and improve the cryoprotective agent effect by smaller diffusion lengths. Moreover, the literature shows high follicular loss after ovarian tissue transplantation caused by the ischemic process (20), and difficulty in quantitatively and qualitatively evaluating the follicular population in ovarian fragments (21, 22) because distribution of preantral follicles in human ovarian cortex is uneven (23, 24).

Results already obtained from isolated follicle studies have been encouraging. Indeed, several investigations into cryopreservation of isolated follicles have been performed in mice (17), rats (25), sheep (22, 26–29), goats (30–33), dasyurid marsupials (34), cats (35), and monkeys (36). Live birth of mouse pups was also achieved by Wang et al. (18), who in vitro cultured secondary follicles isolated from vitrified murine ovarian tissue. In our team, Dolmans et al. (37) isolated small human preantral follicles from fresh ovarian tissue and grafted them to SCID mice for a period of 5 months, at the end of which antral-stage human follicles were obtained. All this demonstrates the ability of both fresh and frozen isolated follicles to survive and develop in vitro and in vivo.

In this study, we isolated human preantral follicles using our recently developed procedure with Liberase DH (Dispase High) (38). To facilitate handling and maintain their three-dimensional structure during cryopreservation and in vitro culture (IVC) procedures, we encapsulated the follicles in an alginate-matrigel matrix (39). We determined whether isolating human preantral follicles before freezing could improve follicle quality compared with isolation after tissue cryopreservation. For this purpose, we compared the follicles' viability, growth ability, and morphology before and after the cryopreservation procedure. Follicle analyses were performed before and after 7 days of IVC to show any cryodamage more accurately.

MATERIALS AND METHODS

Collection of Ovarian Tissue

Use of human tissue for this study was approved by the institutional review board of the Université Catholique de Louvain. Ovarian tissue biopsy samples were collected from six patients (between 27 and 32 years of age) after obtaining written informed consent. The patients were all undergoing laparoscopic surgery for benign gynecologic disease.

Tissue was transported within 5 minutes of surgery from the operating theater to the research laboratory in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered modified Eagle's medium (HEPES-MEM, ref 42360–024; GIBCO) on ice. The medullar part was removed from the samples using surgical scissors. The cortical biopsy samples ranged between 85 mm^3 and 351 mm^3 in size.

Tissue Treatment, Follicle Isolation, and Cryopreservation

Experimental design. Six series of experiments were performed with ovarian biopsy samples from six patients. As detailed in Supplemental Figure 1 (available online), a small fragment from each sample was fixed in formol to serve as a fresh control (control). The remaining tissue was cut into two equal parts: one-half for preantral follicle isolation and alginate-matrigel encapsulation followed or not by cryopreservation (iso-cryo or fresh groups) and the other half for tissue cryopreservation followed by follicle isolation and alginate-matrigel embedding (cryo-iso group). Encapsulated follicles from all three groups were in vitro cultured for 7 days (fresh-IVC, iso-cryo-IVC, and cryo-iso-IVC). At each stage of the procedure, follicles were analyzed for viability, and their diameter was recorded. A sample was also taken for morphological analysis of the follicles (see Supplemental Fig. 1).

Enzymatic Digestion of Ovarian Tissue

The enzymatic protocol used to isolate preantral follicles was previously described by Vanacker et al. (38). Briefly, small pieces of fresh tissue or the frozen-thawed half of each cortical biopsy were incubated in 10 mL of Dulbecco's phosphate-buffered saline (DPBS) medium with calcium and magnesium (ref BE17–513F; Lonza, BioWhittaker) supplemented with 0.28 Wünsch unit/mL of Liberase DH (Dispase High) (ref 05401054001; Roche) at 37°C with gentle agitation for 75 minutes.

Recovery of Isolated Follicles

After enzymatic digestion, the resulting suspension was centrifuged at 50 × *g* for 10 minutes at 4°C. The supernatant was then discarded, and the pellet was transferred to plastic Petri dishes and investigated for preantral follicles under a stereomicroscope (Leica, Van Hopplynus Instruments). The follicles were picked up using a 135 μm-diameter stripper tip (ref MXL3–135 μm; Mid-Atlantic Diagnostics) linked to a tubing set with a sterile filter (ref H-903513; Swemed Laboratories). Care was taken to avoid picking up stromal cells.

Isolated Follicle Embedding

The protocol used was a modification of that described by Amorim et al. (16). A 1% (w/v) solution of sodium alginate (alginic acid, ref 72138; Sigma) was prepared and autoclaved. Isolated follicles were transferred to droplets (20 μL) of alginate solution containing 10% growth factor-reduced extracellular matrix (GFR Matrigel, ref 356230; BD Biosciences) (39). To form the beads, the droplets were slowly released into a small Petri dish containing a solution of $CaCl_2$ (0.1 M). Beads containing follicles (approximately 15/bead) were removed from the dish and then washed in DPBS without calcium and magnesium supplemented with 10% fetal bovine serum (FBS, ref 10437028; GIBCO).

Freezing and Thawing of Ovarian Tissue or Embedded Isolated Follicles

For each patient, one fresh half of each cortical biopsy sample and a number of alginate-matrigel beads (2 to 17 depending on the patient) containing isolated follicles were frozen according to the method described by Van Eyck et al. (20). The tissue was cut into cortical strips of 2 × 5 mm. The strips

or beads were placed in a Petri dish containing HEPES-MEM supplemented with 10% dimethylsulfoxide (ref. D2650; Sigma) and 2% human serum albumin (ref RVG 16910; Sanquin). They were then transferred to cryovials containing 800 μL of this freezing medium. The cryovials were cooled in a programmable freezer (CL-8800i, CryoLogic) using the following program: [1] cooled from 0°C to −8°C at −2°C/min; [2] seeded manually by touching the cryovials with forceps prechilled in liquid nitrogen; [3] cooled to −40°C at −0.3°C/min; and [4] cooled to −150°C at −30°C/min and transferred to liquid nitrogen (−196°C) for storage.

For thawing and cryoprotective agent removal, the cryovials were exposed to room temperature for 2 minutes and immersed in a water bath at 37°C for 2 minutes. To remove the cryoprotective agent, the ovarian tissue or beads with follicles were transferred from the cryovials to Petri dishes containing HEPES-MEM, where they were washed three times (5 minutes per bath).

In Vitro Culture of Ovarian Follicles

For each patient, alginate-matrigel beads containing isolated follicles were in vitro cultured, as described by Amorim et al. (16). The beads were placed in a 4-well or 24-well culture dish (ref 176740 or 150628; Nunc) fitted with Millicell-CM inserts (12 mm in diameter, 0.4 μm pore size, ref PICM01250; Millipore). One bead was cultured per well. Culture medium was added to each well: 250 μL was pipetted into the inserts and 400 μL into the well outside the inserts. The isolated follicles in the alginate-matrigel beads were cultured for 7 days at 37°C in a 95% air and 5% CO_2 humidified environment. Every second day, 150 μL of culture medium was removed and replaced with fresh medium in the inserts.

Follicle Evaluation before and after IVC

Number of follicles recovered from fresh and frozen ovarian tissue. The number of follicles isolated from fresh tissue and the frozen-thawed parts of each biopsy sample was counted immediately after enzymatic isolation to ensure that the two groups were comparable.

Viability testing. Follicle viability was assessed as described by Dolmans et al. (40). One or two alginate-matrigel beads containing isolated follicles were transferred to 20 μL of DPBS containing 2 μmol/L calcein-AM and 5 μmol/L ethidium homodimer-I (ref L-3224; Molecular Probes). The follicles were incubated with the fluorescent dyes for 30 minutes at 37°C in the dark (41). They were then classified into four categories depending on the percentage of dead granulosa cells (GCs): V1, live follicles = follicles with the oocyte and all GCs viable; V2, minimally damaged follicles = follicles with <10% of dead GCs; V3, moderately damaged follicles = follicles with 10% to 50% of dead GCs; V4, dead follicles = follicles with both the oocyte or >50% GCs dead.

Follicle diameter evaluation. Before fixation, each follicle tested for viability was photographed with a Leica DFC320 camera at ×40 magnification. The follicle diameter was measured from the basement membrane using ImageJ, an image processing and analysis program developed at the U.S. National Institutes of Health.

Morphological analysis. Isolated follicles embedded in alginate-matrigel beads were fixed for light microscopy and processed according to the procedure reported by Dolmans et al. (40). They were first fixed in 1.5% glutaraldehyde in DPBS, then postfixed with 1% osmium tetroxide (Agar Scientific) in DPBS and embedded in small blocks (width: 5 mm; height: 1 mm) of 1% agar (UltraPure agarose; GIBCO). They were then dehydrated through an ascending series of ethanol, immersed in propylene oxide (solvent substitution), embedded in Epon 812 (Agar Scientific), and sectioned using a Leica EM UC7 ultramicrotome. Semithin sections (1 μm thick) were stained with toluidine blue and examined by light microscopy (Zeiss Axioskop).

Statistical Analysis

The number of follicles recovered from fresh and frozen-thawed tissue were compared using Student's t test. The mean diameter of isolated follicles was compared between the three groups (fresh, iso-cryo, and cryo-iso) before and after 7 days of culture using the Mann-Whitney test. Comparisons between percentages of viable follicles found in control samples (controls 3 to 5) and after IVC were analyzed by the chi-square test. $P \leq .05$ was considered statistically significant.

RESULTS

Follicle Count after Isolation from Fresh and Frozen-Thawed Biopsy Samples

As shown in Table 1, the size of the biopsy samples ranged from 85 mm^3 to 351 mm^3. A total of 1,134 preantral follicles were isolated from fresh biopsy halves and 1,132 from frozen halves. No statistically significant differences were observed between the number of follicles obtained from fresh and frozen-thawed tissue ($P>.05$), which means results in fresh, cryo-iso, and iso-cryo groups could be compared. It also indicates that freezing and thawing of ovarian tissue do not appear to alter the yield of follicles recovered after enzymatic isolation.

Follicular Viability

Altogether in the three groups, a total of 1,588 follicles were analyzed for viability before and after IVC using calcein-AM and ethidium homodimer-1. As in our previous study

TABLE 1

Follicle count in both halves of each biopsy sample.

Experiment	Patient age (y)	Biopsy size (mm^3)	No. isolated follicles	
			Fresh half	Frozen half
1	29	135	180	120
2	28	285	97	135
3	30	130	180	135
4	29	85	70	60
5	28	209	322	297
6	25	351	285	385
Total			1,134	1,132

Note: Fresh = control.

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(40), we grouped V1 and V2 together because they represented follicles with high viability. Before IVC (Table 2), no statistically significant difference was found between the freshly isolated follicles (fresh group), cryopreserved isolated follicles (iso-cryo group), and follicles isolated from frozen-thawed tissue (cryo-iso group) within the highly (V1 and V2) and poorly (V3 and V4) viable follicle categories ($P>.05$).

We performed IVC to reveal any possible serious cryoinjury that could be underestimated if viability was only analyzed soon after the isolation procedure. The IVC step confirmed the results obtained after enzymatic isolation: no statistically significant difference was observed between the three groups in the proportions of highly (V1 and V2) and poorly (V3 and V4) viable follicles (Table 3).

Follicle Diameter

As detailed in Table 4, the initial follicle diameters were similar in freshly isolated follicles (control 3) (40.3 ± 7.8 μm), frozen-thawed isolated follicles (iso-cryo group) (43.6 ± 12.1 μm), and follicles isolated from frozen-thawed tissue (cryo-iso group) (43.15 ± 11.0 μm).

After 7 days of IVC, follicles were found to have increased in size in all three groups. However, we also noted that of the 15 follicles encapsulated in each of the alginate beads, 2.4% to 3.6% had a similar diameter on day 0 and day 7. Follicle diameter statistically significantly increased to 50.7 ± 14.4 μm, 52.5 ± 18.5 μm, and 55.8 ± 18.8 μm for freshly isolated follicles, frozen-thawed isolated follicles, and follicles isolated from frozen-thawed tissue, respectively ($P<.001$). However, follicles isolated from cryopreserved tissue and in vitro cultured for 7 days (cryo-iso-IVC group) showed a statistically significantly greater diameter than the freshly isolated and frozen-thawed isolated follicles (control 6 and iso-cryo-IVC groups, respectively) ($P<.05$). By contrast, no statistically significant difference was observed between freshly isolated and frozen-thawed isolated follicle diameters after culture (control 6 and iso-cryo-IVC groups, respectively).

Follicle Morphology

As shown by light microscopy pictures in Supplemental Figure 2 (available online), both the general morphology and three-dimensional structure of freshly isolated follicles, cryopreserved isolated follicles, and follicles isolated from frozen-thawed ovarian tissue were maintained before and after IVC (see Supplemental Fig. 2A–2D).

TABLE 2

Viability before 7 days of in vitro culture.

	Fresh	Iso-cryo	Cryo-iso
V1	96.4% (106/110)	90.6% (116/128)	85% (148/174)
V2	2.7% (3/110)	9.4% (12/128)	14.4% (25/174)
V3	0	0	0
V4	0.9% (1/110)	0	0.6% (1/174)

Note: Cryo-iso = slow-freezing followed by follicle isolation and alginate-matrigel embedding; fresh = control; iso-cryo = follicle isolation and alginate-matrigel embedding followed by slow-freezing.

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TABLE 3

Viability after 7 days of in vitro culture.

	Fresh IVC	Iso-cryo IVC	Cryo-iso IVC
V1	82.4% (154/187)	72.5% (272/375)	64.65% (397/614)
V2	17.1% (32/187)	25.1% (94/375)	33.9% (208/614)
V3	0.5% (1/187)	1.3% (5/375)	0.65% (4/614)
V4	0	1.1% (4/375)	0.8% (5/614)

Note: Cryo-iso = slow-freezing followed by follicle isolation and alginate-matrigel embedding; fresh = control; iso-cryo = follicle isolation and alginate-matrigel embedding followed by slow-freezing; IVC = in vitro culture.

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A total of 16 isolated follicles were analyzed by light microscopy on semithin sections from the three groups before and after 7 days of IVC. Before IVC, freshly isolated follicles (fresh group) and follicles isolated from cryopreserved tissue (cryo-iso group) were found to be round and well preserved when observed at ×1,000 magnification. The GCs were cuboidal with a large nucleus, and very few vacuoles were encountered.

After IVC, isolated follicles were retracted, even in the fresh group, and vacuoles were also observed. Nevertheless, isolated follicles from the iso-cryo and cryo-iso groups were found to be well preserved (see Supplemental Fig. 2E and 2F), showing normal GCs and oocytes and no sign of swelling. Only a few vacuoles were visible. Preantral follicles with more than one layer of cuboidal GCs (secondary follicles) were observed in both groups.

DISCUSSION

For cancer patients who cannot benefit from transplantation of cryopreserved ovarian tissue due to the risk of transmission of malignant cells, an alternative is isolation of preantral follicles for further in vitro culture or transplantation. In vitro procedures need to be developed to allow complete folliculogenesis from isolated primordial follicles in humans. Promising results have already been achieved in this field. Outcomes after culture of single-layered follicles embedded in alginate (16) are encouraging, and multilayered follicles matured in vitro within cortical strips (42) or isolated from cryobanked tissue and embedded in alginate (17) were shown to reach the antral stage. In vivo, isolated human primordial and primary follicles were found to reach the antral stage after

TABLE 4

Follicle diameter in all three groups before and after 7 days of in vitro culture.

Size (μm)	Freshly isolated (n = 227)	Iso-cryo (n = 421)	Cryo-iso (n = 538)
Initial	40.3 ± 7.8 (control 3)[a]	43.6 ± 12.1[a]	43.15 ± 11.0[a]
Final	50.7 ± 14.4 (control 6)[b,c]	52.5 ± 18.5[b,c]	55.8 ± 18.8[b,d]

Note: Cryo-iso = slow-freezing followed by follicle isolation and alginate-matrigel embedding; fresh = control; iso-cryo = follicle isolation and alginate-matrigel embedding followed by slow-freezing.

[a,b] Values with different superscripts differ significantly between rows ($P<.05$).

[c,d] Values with different superscripts differ significantly between columns ($P<.05$).

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xenotransplantation to immunodeficient mice (37) Alginate is currently being tested as a biodegradable scaffold to transplant isolated preantral follicles and ovarian cells (39) with the ultimate goal of recreating an artificial ovary.

Regardless of the approach (in vitro or in vivo development), isolated preantral follicles need to be cryopreserved, so our study investigated when follicles should be isolated. In this study, we compared freshly isolated follicles, cryopreserved isolated follicles, and follicles isolated from frozen-thawed ovarian tissue in terms of viability, growth capacity, and morphology before and after 7 days of IVC to determine whether isolating human preantral follicles before freeze-thawing could improve follicle quality compared with isolation after tissue cryopreservation. Our results showed that follicle viability was comparable before and after IVC between freshly isolated follicles, cryopreserved isolated follicles, and follicles isolated from frozen-thawed ovarian tissue, showing that cryopreservation before or after isolation does not appear to affect follicle viability.

We observed an increase in size in all three groups after 7 days of IVC. Surprisingly, we noted a greater increase in diameter in in vitro cultured follicles isolated from frozen-thawed ovarian tissue than in freshly isolated or frozen-thawed isolated follicles. Such an increase in follicle diameter after IVC may be due to typical development of follicles during culture or a consequence of swelling of GCs caused by osmotic influx after thawing. Follicular morphology analysis by light microscopy showed that this increase in size was not due to swelling but rather to real growth, as some follicles were found to be at the secondary stage and no cell swelling was observed. Based on the appearance of well-preserved isolated follicles after IVC, we can assume that human preantral follicles can be successfully cryopreserved before or after isolation without impairing their ability to survive and grow in vitro.

It is interesting that we were able to isolate a similar number of follicles from the fresh and frozen halves of each biopsy. This was quite unexpected as, in our experience, isolating follicles from cryopreserved ovarian tissue is more challenging than from fresh tissue, and ovarian stromal cells are known to be damaged by current slow-freezing protocols. Gosden et al. (43) suggested that stromal cells are more vulnerable to cryoinjury than follicular cells because of considerable water diffusion distance within tissue due to the density of ovarian stroma. Thus, leaking intracellular content of these injured stromal cells could be toxic to surrounding follicles. However, in our study, follicles were successfully isolated from cryopreserved tissue, were viable right after the isolation procedure, and were able to survive 7 days of IVC. We believe that these successful results were due to the isolation protocol we developed with Liberase DH (38). This enzyme blend contains dispase, which does not cleave laminin (44), the main constituent of the basal membrane of primordial and primary follicles (45). Furthermore, Liberase DH contains only negligible levels of endotoxins, avoiding any lot-to-lot variations and thus yielding very reproducible results.

Our study demonstrated that it is now conceivable to isolate follicles from frozen-thawed tissue when tissue transplantation is excluded, as in case of leukemia patients (11). Indeed, female fertility preservation banks worldwide are currently made up of cryopreserved ovarian tissue fragments, so for these patients development of new clinical protocols for in vitro growth and grafting of human preantral follicles is vital. On the other hand, the fact that human preantral follicles can also be successfully cryopreserved after isolation is of particular importance for experimental research. Indeed, cryopreservation of isolated follicles allows qualitative evaluation of the follicle population and freezing of a known number of follicles per bead. Moreover, freezing of isolated follicles within alginate beads would facilitate follicle handling and transportation to centers not performing follicle isolation, thereby increasing material exchange among laboratories all over the world.

In conclusion, this study demonstrates that human preantral follicles can be successfully cryopreserved before or after isolation without impairing their ability to survive and grow in vitro, although follicles from frozen-thawed ovarian tissue appear to grow faster than cryopreserved isolated follicles. Further experimental studies are therefore warranted to assess the functionality of cryopreserved isolated follicles after xenotransplantation. This could lead to the development of new protocols in clinical and experimental research settings to restore endocrine activity and fertility in cancer patients. Keeping follicles in the cortex is clearly the best clinical approach, if appropriate; this will enable application of new technologies that will emerge in the future. However, for patients at high risk of ovarian metastasis, freezing preantral follicles both ways (enclosed in ovarian tissue and after isolation) may be advised to maximize their options.

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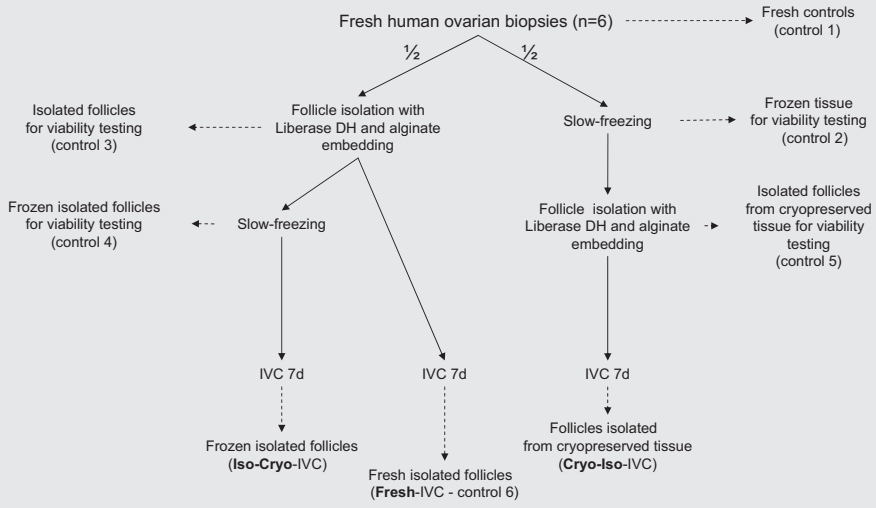
SUPPLEMENTAL FIGURE 1



Experimental design of the study.

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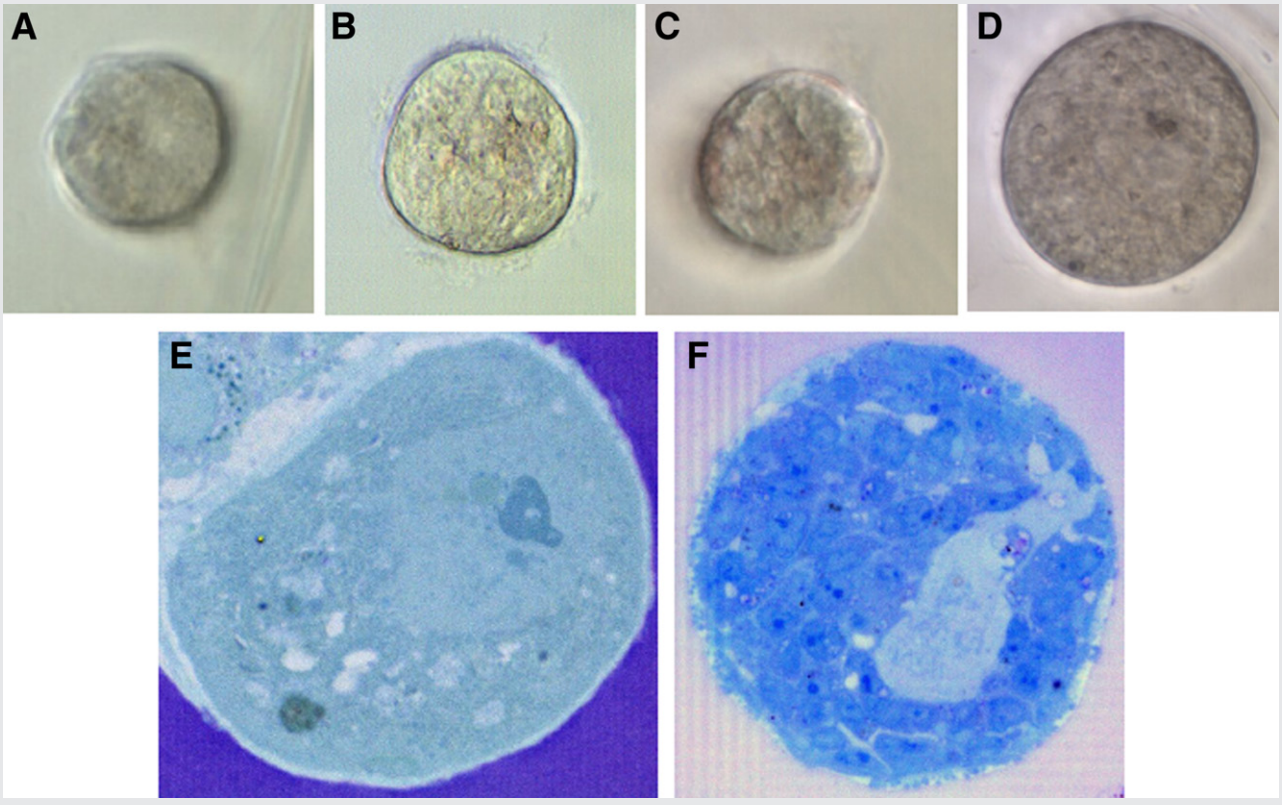
SUPPLEMENTAL FIGURE 2



Light microscopy pictures of (A) a freshly isolated follicle before IVC, (B) a freshly isolated follicle after IVC, (C) a cryopreserved isolated follicle after IVC, (D) and a follicle isolated from frozen-thawed ovarian tissue after IVC encapsulated in an alginate-matrigel bead at ×40 magnification. Semi-thin sections of (E) a cryopreserved isolated follicle and (F) a follicle isolated from frozen-thawed ovarian tissue encapsulated in an alginate-matrigel bead after IVC at ×1,000 and ×400 magnification, respectively.

Vanacker. Follicle isolation and cryopreservation. Fertil Steril 2013.