Journal of Reproduction and Development, Vol. 59, No 3, 2013

—Original Article—

Vitreous Cryopreservation of Human Preantral Follicles Encapsulated in Alginate Beads with Mini Mesh Cups

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Abstract. To completely avoid ice crystal formation and thus get a higher survival rate, vitrification methods have been commonly used for cryopreservation of oocytes and embryos. However, currently used vitrification methods for oocytes and embryos are not suitable for the cryopreservation of preantral follicles (PFs). In the present study, stainless steel mesh was fabricated into mini mesh cups to vitrify isolated PFs. Moreover, isolated follicles were encapsulated and then subjected to vitreous cryopreservation to facilitate *in vitro* culture/maturation of follicles after warming. The results showed that the percentages of viable follicles did not differ significantly between the vitrification group and fresh group soon after warming (81.25% *vs.* 85.29%, P>0.05) and after a 7-day culture period (77.78% *vs.* 83.33%, P>0.05). No difference in mean follicular diameter was observed between cryopreserved and fresh follicles when cultured *in vitro*. Transmission electron microscopic analysis revealed that vitreous cryopreservation could maintain the ultrastructure of follicles in alginate beads. In conclusion, the present vitrification method could efficiently cryopreserve isolated human ovarian follicles encapsulated by calcium alginate, which could be put into immediate use (*in vitro* culture/ maturation) after warming. However, more follicles and some detailed biochemical analyses are required to further investigate the effects of vitrification on the long-term growth of human encapsulated PFs.

Key words: Alginate, Cryopreservation, *In vitro* culture, Preantral follicle, Vitrification

(**J. Reprod. Dev. 59:** 288–295, 2013)

Advances in the treatment of female cancers have contributed to increased patient survival rates but can cause infertility in young women. Therefore, preserving fertility before cancer treatment is necessary for many of these patients [1, 2]. Although oocyte/ embryo freezing can be offered to patients [3–5], some patients do not get the chance to have mature oocytes/embryos cryopreserved for later use (*in vitro* fertilization/ embryo transfer), as radiotherapy and chemotherapy cannot always be delayed to get mature oocytes through ovarian stimulation [6]. Therefore, the freezing of a relatively large quantity of immature oocytes enclosed in preantral follicles (PFs) has emerged as a promising alternative to safeguard fertility for cancer patients in recent years [7, 8].

PFs can be frozen as isolated follicles or *in situ* (ovarian tissue cryopreservation) [7–9]. Cryopreservation of isolated ovarian follicles has been attempted in several species [10–12]. The most successful experiments, carried out in mice, showed that it is possible to obtain normal offspring after isolated follicle cryopreservation and *in vitro* culture [13]. However, only a few studies have reported the cryopreservation of isolated PFs from large mammals and humans because of the relatively large size, fragile architecture, and difficulties of *in vitro* culture.

In recent years, there has been great progress with respect to the *in vitro* culture of isolated ovarian follicles. Follicle encapsulation within alginate hydrogels, a common tissue engineering scaffold, mimics the ovary by providing the appropriate three-dimensional (3D) context while supporting somatic cell and egg interactions to optimize oocyte development [14–18]. If a 3D scaffold is provided to the freshly isolated human PF and the encapsulated follicles are cryopreserved, the isolated ovarian follicles could be ready for use at any time, i.e., put into direct culture or transplanted after thawing without a long and complicated process of follicle preparation.

Slow-freezing methods are most commonly used for the cryopreservation of cells and tissues [19]. However, it is extremely difficult to preserve the intactness and integrity of cells and scaffolds, as the formation of ice crystals would destroy the complicated 3D constructs [20]. Vitrification is defined as glass-like solidification and/or complete avoidance of ice crystal formation during cooling and warming [21–23]. Therefore, cryopreservation of 3D constructs could be more efficient if the cryopreservation solutions enclosed in constructs and cells were ice-free during the entire procedure [24–26].

In the present study, we investigated the prospect of vitreous cryopreservation of isolated human PFs encapsulated with calcium alginate. The viability, growth, and ultrastructure of the ovarian follicles were compared with those of fresh controls that were encapsulated but without cryopreservation.

Received: October 14, 2012
Accepted: February 13, 2013
Published online in J-STAGE: March 14, 2013

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Materials and Methods

Collection of human ovarian tissue

This study was approved by the Ethics Committee of The First Affiliated Hospital of Sun Yat-Sen University. Ovarian tissues were obtained from 27 women aged 22 to 38 years (28.52 ± 4.90), who had undergone laparoscopic surgery or laparotomy for nonovarian benign gynecological disease, such as myomas and tubal ligation. Ovarian tissue (about 5×5×3 mm to 10×10×3 mm) obtained from each woman was placed into a sterile glass cup incubated with HEPES-buffered modified Eagle's medium (HEPES-MEM, Lonza Walkersville, Walkersville, MD, USA) and transported to the laboratory on ice. All patients recruited into the study provided written informed consent.

PFs isolation

Human PFs were isolated from ovarian tissue according to the protocol of Dolmans *et al.* [27]. Briefly, the ovarian tissue was cut into 1×1×1-mm fragments using a tissue chopper (McIlwain Tissue Chopper, The Mickle Laboratory Engineering, Guildford, UK) adjusted to yield 1-mm serial sections. The tissue fragments were put in PBS solution containing 0.07 mg/ml Liberase enzyme (Roche, Indianapolis, IN, USA) and 20 U/ml DNA enzyme (Sigma-Aldrich, Carlsbad, CA, USA) in 14-ml test tubes and incubated in a water bath at 37 C for 1 h with gentle agitation. During incubation, the ovarian tissue was blown and aspirated by Pasteur pipettes every 15 min to facilitate the digestion of tissue. Digestion was terminated by the addition of an equal volume of HEPES-MEM at 4 C supplemented with 10% human serum albumin (HSA; SAGE In Vitro Fertilization) and centrifuged at 50 *g* for 10 min at 4 C. The supernatant was discarded, and the pellet was transferred to culture dishes for investigation of PFs under a stereomicroscope (Leica, Wetzlar, Germany). Morphologically normal PFs, with two or three layers of granulosa cells and centrally located spherical oocytes, were washed three times in Dulbecco's phosphate-buffered saline (D-PBS) supplemented with 10% HSA and used in the present study.

Calcium alginate embedding

A 1.5% (w/v) solution of sodium alginate (55–65% guluronic acid, FMC BioPolymer, Philadelphia, PA, USA) in PBS was prepared and autoclaved [28]. The isolated follicles were transferred with a micropipette to droplets (2 µl) of alginate solution. To form beads, the droplets were slowly released into a small beaker containing a solution of $CaCl_2$ (0.1 M). The droplets immediately gelled to form beads. Beads containing individual follicles were then immediately removed from the beaker using glass pipettes 2 min after adding $CaCl_2$ and then washed three times in culture medium.

Experimental design

The encapsulated follicles from each patient were randomly distributed into two groups. In group 1, the encapsulated follicles were vitrified, stored in liquid nitrogen for 4 h and then cultured *in vitro* for 2 h or 7 days after warming (vitrification group). In group 2, the follicles were placed directly into culture without cryopreservation. In both groups, we assessed the diameter and survival rate of part of the follicles after 2 h of *in vitro* culture, while other follicles were allowed to grow *in vitro* for 7 days. The diameter, viability and ultrastructure of PFs and the proliferation of granulosa cells were compared between the two groups after 7 days of *in vitro* culture.

Vitrification and warming procedures

A small piece of stainless steel mesh (mesh size, 50 µm; Zhenxing Hardware Sifting Screen Factory, Guangzhou, China) was carefully molded into a cup shap (d = 0.6–1 mm; h = 0.2–0.4 mm). This homemade cryo-container was then sterilized and ready for use [29]. The encapsulated preantral follicles to be vitrified were placed into the cup-shaped stainless steel mesh (Fig. 1), immersed in an equilibration solution consisting of 10% ethylene glycol (EG; Sigma-Aldrich) in Dulbecco's phosphate-buffered saline (D-PBS) with 10% HSA for 3 min, transferred to 25% EG in D-PBS for 3 min (vitrification solution 1, VS1), and then immersed in a vitrification solution consisting of 40% EG (v/v), 0.6 mol/l sucrose, and 20% HSA in D-PBS (VS2) for 3 min. After the final step, the stainless steel mesh loaded with encapsulated PFs was submerged immediately into liquid nitrogen. The vitrification procedures were carried out at room temperature (23–25 C).

After 4 h of cryopreservation in liquid nitrogen, the cryopreserved follicles were taken out of the liquid nitrogen box and warmed immediately. The five-step cryoprotectant dilution method was performed to warm the encapsulated PFs. Briefly, the mesh cup loaded with encapsulated preantral follicles was placed in a dish containing 10% HSA and 1 mol/l sucrose in D-PBS for 1 min at 37 C. After incubation, the preantral follicles were transferred sequentially to 0.75 mol/l, 0.5 mol/l, 0.2 mol/l, and 0.1 mol/l sucrose at 23–25 C and then washed twice in D-PBS. Finally, the follicles were picked out of the mesh cup and equilibrated for 15 min in the culture medium at 37 C in a 5% CO_2 environment before culture. From this point on, these encapsulated follicles were handled the same way as the control nonfrozen encapsulated follicles.

In vitro culture of encapsulated follicles

The follicles were grown individually in 96-well plates (1 follicle per well) in 100 µl culture medium at 37 C in a humidified atmosphere of 5% CO_2 [30]. The culture medium consisted of alpha MEM (Sigma) supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 10% HSA, ITS (insulin 5 µg /ml, transferrin 5.5 µg/ml, selenium 5 ng/ml), 0.23 mM pyruvate, 2 mM glutamine and 2 mM hypoxanthine). The medium was exchanged every 2 days.

Follicles were cultured at 37 C in 5% CO_2 for 7 days. Every 2 days, half of the media volume was exchanged, and the follicles were examined for survival and size measurements after 2 h and 7 days of *in vitro* culture. Two diameters were measured for each follicle, and selected images were captured. The integrated measuring tools in the ImageJ software were used in the present study (Java-based image processing program developed at the National Institutes of Health) [31].

Assessment of follicle viability

The viability of morphologically normal encapsulated follicles was analyzed using live (calcein-AM) and dead (ethidium homodimer-1) markers. The vitrified/warmed and fresh follicles were transferred to PBS containing 2 µmol/l of calcein-AM and 5 µmol/l of ethidium homodimer-1 (Molecular Probes, Leiden, The Netherlands). They

were incubated with the fluorescent dyes for 20 min at 37 C in the dark. Then the follicles were washed in PBS and observed under an inverted fluorescence microscope (Leica). The encapsulated follicles were classified into three categories depending on the percentage of dead granulosa cells and oocytes, as follows: live follicles (follicles with oocytes and all the granulosa cells viable), partially damaged follicles (follicles with viable oocytes and some dead granulosa cells) and dead follicles (follicles with dead oocytes or most of the granulosa cells dead; Fig. 2).

Ultrastructure

The ultrastructure of cryopreserved and fresh encapsulated follicles was assessed as described by Matos *et al*. [32, 33]. Briefly, 61 ovarian follicles embedded in calcium alginate were fixed in 1.5% glutaraldehyde in PBS solution containing 5% BSA (29 cryopreserved PFs and 32 fresh PFs). After fixation for 2 to 5 days at 4 C, the samples were rinsed in PBS, postfixed with 1% osmium tetroxide (Agar Scientific, Elektron Technology UK, Stansted, Essex, England) in PBS and rinsed again in PBS. The samples were dehydrated through increasing concentrations of ethanol, immersed in propylene oxide (solvent substitution), embedded in Epon 812 and sectioned using a Reichert-Jung Ultracut E ultramicrotome (Leica Mikrosysteme GmbH, Vienna, Austria). Ultrathin sections (60–80 nm) were cut with a diamond knife, mounted on copper grids and contrasted with saturated uranyl acetate followed by lead citrate. They were examined and photographed using Zeiss EM109 and Zeiss EM 10 electron microscopes at 80 kV.

The following ultrastructural alterations of the selected cell organelles and structures of encapsulated follicles were recorded: (1) reduced abundance of microvilli, (2) reduction of the rough endoplasmic reticulum, (3) existence of swollen mitochondria, with few or no crista, (4) changes of granulosa cells, (5) rupture of the oolemma, (6) fracture or delamination of the zona pellucida and (7) enlarged perivitelline space. We recorded the ultrastructural modifications one by one, calculated the proportions and compared them between the cryopreserved and fresh follicles groups respectively.

^{3}H-thymidine incorporation capability of granulosa cells

After 7 days of *in vitro* culture, culture media were exchanged and replaced with media supplemented with 0.4-Ci methyl-^{3}H-thymidine (PerkinElmer, Boston, MA, USA; 1 μci/100 μl) per well. After 16 h, 4 follicles in beads were collected for each replicate (3 replicates), washed twice with D-PBS, and then dissolved in 10 mM EDTA. Next, ^{3}H-thymidine incorporation was assayed as described previously [34–37]. Nonspecific incorporation was determined using empty alginate gels.

Statistical analysis

The follicular viability was compared using the chi-square test. The diameters of the follicles were expressed as means ± SD and compared using the paired sample t-test. The counts per minute of ^{3}H-thymidine incorporation in granulosa cells were converted by log, expressed as means ± SD and compared using the paired sample t-test. Differences were considered significant when $P<0.05$.

Results

Viability of encapsulated preantral follicles after vitrification/warming and in vitro culture

The majority of the human preantral follicles were intact after isolation and encapsulation, with a central oocyte and surrounding layers of granulosa cells. The damaged follicles or follicles with partially injured granulosa cells detached from the follicle, likely a result of the mechanical isolation procedure, were excluded from the study. The number of follicles isolated from each patient sample ranged from 0 to 16 (Table 1).

A total of 144 encapsulated human preantral follicles were examined for their viability based on the live (calcein-AM) and dead (ethidium homodimer-1) markers (Fig. 2). The influence of the cryopreservation procedure on the viability of encapsulated follicles is demonstrated in Table 2. The vitrification group had a viability of 81.2% immediately after warming and 2 h of *in vitro* culture, which was not significantly different from that of the fresh group (85.3%, $P>0.05$). The percentage of viable follicles did not differ significantly between the vitrification and fresh groups after a 7-day culture period (77. 8 *vs.* 83.3%, $P>0.05$).

Follicle diameter and granulosa cell proliferation

The diameters and functions of vitrified and noncryopreserved encapsulated follicles were recorded over 7 days of continuous culture. Table 3 demonstrates that the diameter of encapsulated follicles from both groups increased after the continuous *in vitro* culture period. The mean follicle diameter of vitrified/warmed human preantral follicles in the study was 96.5 ± 8.3 μm at the onset of the culture period and 123.1 ± 10.5 μm after the 7-day culture period. Oocyte diameter also increased from 66.3 ± 1.5 μm to 94.7 ± 1.6 μm in the vitrification group. There was a significant increase in follicular and oocyte diameter after 7 days of *in vitro* culture in both groups. There was no significant difference in mean follicular diameter between the cryopreserved follicles and those cultured without cryopreservation at the beginning and end of the 7-day culture period.

Moreover, vitrification/warming did not significantly affect the proliferation of granulosa cells. After the 7-day culture period, the mean counts per minute (converted by log) of ^{3}H-thymidine incorporation in granulosa cells in the fresh group and vitrification group follicles were 3.4 ± 0.3 and 3.3 ± 0.5 cpm, respectively, with no significant difference found between the two groups ($P>0.05$).

Ultrastructure of encapsulated human preantral follicles after vitrification and warming

By transmission electron microscopy, most of the cryopreserved follicles appeared healthy looking, presenting normal ultrastructural features of human follicles immediately after warming (Fig. 3): intact nuclear and cellular membranes, normally arranged chromatin, abundant amount of rough endoplasmic reticulum and normal cristae and an electron-dense matrix in most mitochondria. Some minor ultrastructural alterations were observed, and these alterations were different from follicles after 7 days of *in vitro* culture. For example, Fig. 3A and 3C show an enlarged space between follicular cells and oocytes, which was observed mainly in the follicles immediately after warming. On the other hand, a vacuolated cytoplasmic organelle

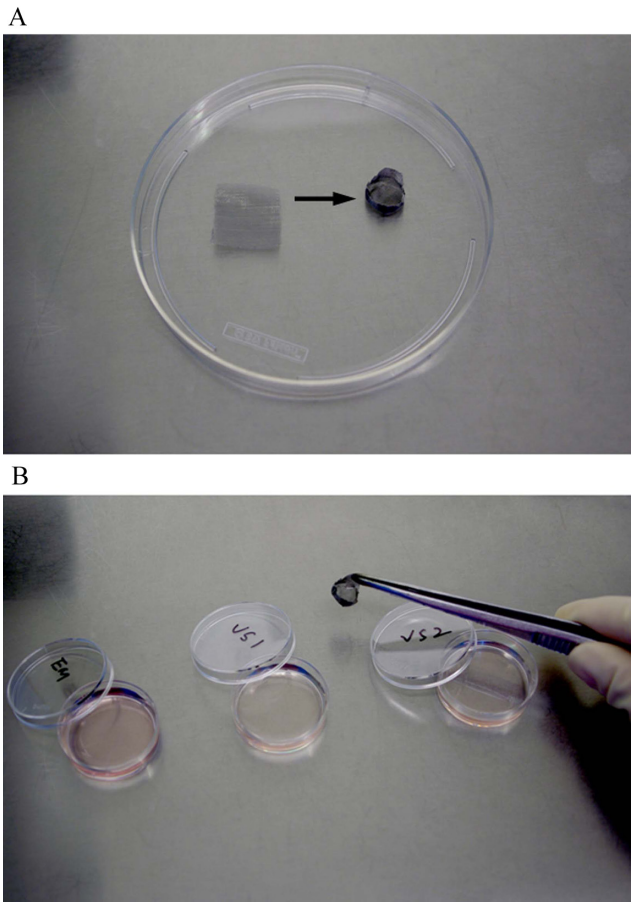

Fig. 1. Fabrication of the vitrification carrier and the manipulation procedures for encapsulated human preantral follicles. A: A small piece of stainless-steel mesh (mesh size, 50 μm) was molded into a cup shape. B: Encapsulated human follicles loaded in the cup-shaped stainless-steel mesh could be easily transferred from one vitrification medium to another with a pair of tweezers.

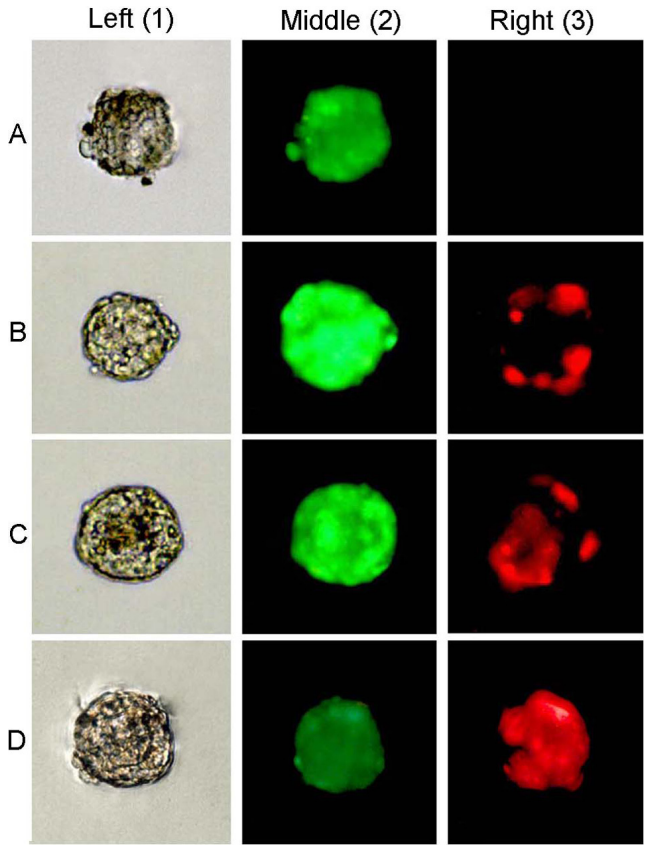

Fig. 2. The viability classification of the isolated follicles. Encapsulated follicles were stained with calcein-AM, which indicated the viability of the cell cytoplasm, and with ethidium homodimer-1 to show nuclei of dead cells. The left column (1) shows a follicle in a bright field; the middle column (2) shows images obtained for the same follicle with a filter to visualize calcein-AM staining; and the right column (3) shows ethidium homodimer-1 staining. Follicles were classified into three categories depending on the viability of granulosa cells (GCs) and oocytes. Live follicles (A): follicles with the oocyte and all the GCs viable. Partially damaged follicles (B, C): follicles with the oocyte viable and <50% of dead GCs. Dead follicles (D): follicles with both the oocyte or >50% GCs dead, as photo D3 shows using the filter to visualize red fluorescence. 200×.

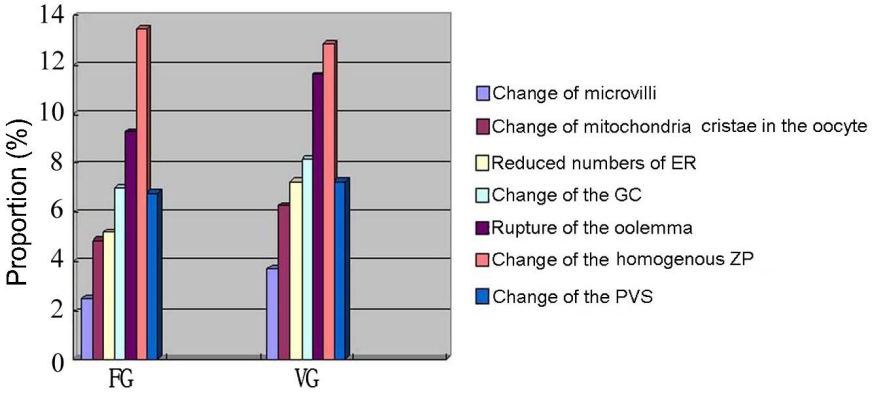

Fig. 4. Proportions of ultrastructural alterations of follicles in the vitrification Group (VG) and fresh Group (FG) after 7 days of *in vitro* culture. The proportions of alterations (change of microvilli, change of mitochondria cristae in the oocyte, reduced numbers of ER, rupture of the oolemma, change of the homogenous zona pellucida (ZP), change of the perivitelline space (PVS), change of the GC) in follicles were analyzed in VG and FG after 7 days of *in vitro* culture. No significant difference was found between the two groups.

Table 1. Secondary follicle isolation from each patient

Patient	Age	Diagnosis	Methods of collection	VG (n)	FG (n)	Total (n)
Pat**1**	23	Myomas	Laparotomy	5	7	12
Pat**2**	26	Tubal ligation	Laparoscopic	5	6	11
Pat**3**	31	Myomas	Laparotomy	4	5	9
Pat**4**	34	Tubal ligation	Laparoscopic	4	6	10
Pat**5**	27	Myomas	Laparotomy	0	0	0
Pat**6**	23	Myomas	Laparotomy	4	6	10
Pat**7**	38	Tubal ligation	Laparoscopic	3	5	8
Pat**8**	35	Tubal ligation	Laparoscopic	0	0	0
Pat**9**	24	Myomas	Laparotomy	5	7	12
Pat**10**	26	Myomas	Laparotomy	3	6	9
Pat**11**	28	Tubal ligation	Laparotomy	0	0	0
Pat**12**	34	Tubal ligation	Laparoscopic	6	5	11
Pat**13**	22	Myomas	Laparotomy	7	6	13
Pat**14**	23	Myomas	Laparotomy	0	0	0
Pat**15**	25	Tubal ligation	Laparoscopic	7	6	13
Pat**16**	33	Myomas	Laparotomy	3	3	6
Pat**17**	29	Tubal ligation	Laparoscopic	6	5	11
Pat**18**	26	Myomas	Laparotomy	5	5	10
Pat**19**	24	Myomas	Laparotomy	9	5	14
Pat**20**	28	Tubal ligation	Laparoscopic	0	0	0
Pat**21**	27	Myomas	Laparotomy	7	6	13
Pat**22**	28	Tubal ligation	Laparoscopic	0	0	0
Pat**23**	23	Myomas	Laparotomy	5	5	10
Pat**24**	34	Tubal ligation	Laparoscopic	6	7	13
Pat**25**	38	Myomas	Laparotomy	0	0	0
Pat**26**	27	Myomas	Laparotomy	8	8	16
Pat**27**	34	Tubal ligation	Laparoscopic	4	6	10

VG, human preantral follicles subjected to vitreous cryopreservation after encapsulation by calcium alginate; FG, encapsulated follicles directly subjected to *in vitro* culture without cryopreservation.

Table 2. Viability of encapsulated preantral follicles of the vitrification group after freezing/warming and *in vitro* culture compared with the fresh group without cryopreservation

	2 h of *in vitro* culture		7 days of *in vitro* culture	
	VG	FG	VG	FG
No. of encapsulated follicles	32	34	36	42
Live follicles	26	29*	28	35*
Partially damaged follicles	5	4	6	4
Dead follicles	1	1	2	3

VG, vitrification group after 2 h or 7 days of *in vitro* culture; FG, fresh group after 2 h or 7 days of *in vitro* culture. * No significant differences between the vitrification and fresh groups after 2 h and 7 days of *in vitro* culture.

Table 3. Comparison of diameter (μm) of encapsulated preantral follicles between the vitrification group and fresh group before and after *in vitro* culture

Group	No. of follicles	Before culture		After 7 days of *in vitro* culture	
		Diameter of follicle	Diameter of oocyte	Diameter of follicle	Diameter of oocyte
VG	36	96.5 ± 8.3^{a}	66.3 ± 1.5^{b}	$123.1 \pm 10.5^{*,c}$	$94.7 \pm 1.6^{*,d}$
FG	42	93.2 ± 9.5^{e}	69.7 ± 1.8^{f}	$131.5 \pm 12.3^{*,g}$	$96.2 \pm 12.3^{*,h}$

VG, vitrification group after 7 days *in vitro* culture; FG, fresh group after 7 days *in vitro* culture. * Significantly higher than their initial size ($P<0.05$) respectively in VG and FG after 7 days of *in vitro* culture. There were no significant differences between the $VG^{a,b}$ and $FG^{e,f}$ groups before culture ($P>0.05$). There were also no significant differences between the $VG^{c,d}$ and $FG^{g,h}$ groups after *in vitro* culture for 7 days ($P>0.05$).

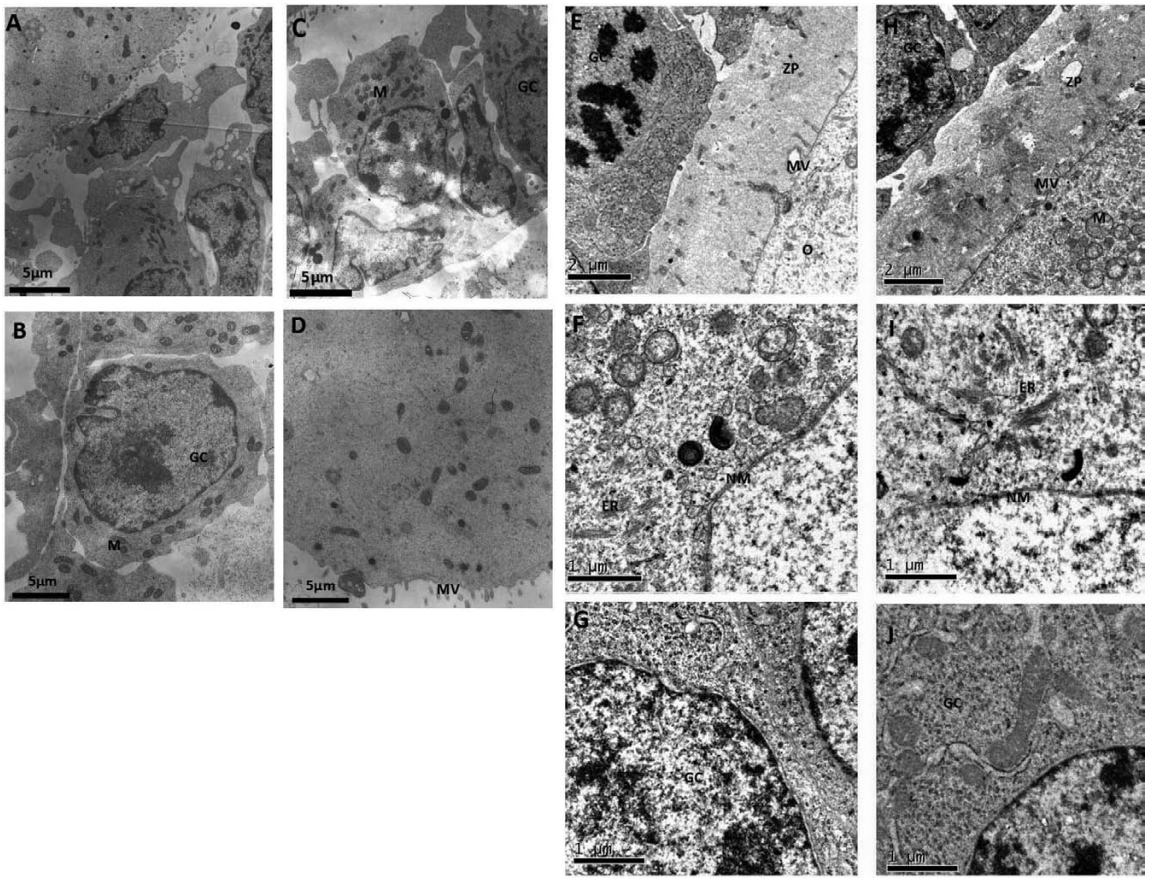

Fig. 3. Transmission electron microscopic assessment of vitrified human encapsulated follicles after 2 h (A–D) and 7 days (E–J) of *in vitro* culture. A continuous layer of flattened/irregular cuboid follicular cells surrounds the oocyte. The membrane of the nucleus is intact and surrounded by a perinuclear cluster of cytoplasmic organelles including mitochondria, electron-dense lipid bodies and endoplasmic reticulum (ER). GC, granulosa cells; M, mitochondria; NM, nuclear membrane; BM, base membrane; MV, microvilli.

in the oocyte cytoplasm was only observed after 7 days of *in vitro* culture (Fig. 3F) in both cryopreserved and fresh follicles, which might be related to the nonoptimal *in vitro* culture conditions.

After 7 days of *in vitro* culture, a regularly structured zona pellucida was observed at the oocyte-follicular cell interface in both groups, and some interdigitations between oocyte microvilli and granulosa cell prolongations were also observed (Fig. 3E, H). However, more ultrastructural alterations were detected after 7 days of *in vitro* culture than in follicles immediately after warming. A relatively higher trend of ultrastructure alterations was observed than in the corresponding fresh follicles after 7 days of *in vitro* culture, although no statistical difference was observed (Fig. 4).

Discussion

Compared with isolated PFs, ovarian tissue is a complex structure that needs a longer period of exposure to the cryoprotectants for vitrification. However, to decrease the toxic effects of a high concentration of cryoprotectant on cells and tissues, equilibration with the vitrification medium must be strictly controlled (for example, 1 min in vitrification medium and less than 30 sec for being loaded on cryocontainers for oocytes and embryos). Inadequate permeation of vitrification solutions might affect the number of normal follicles that survive [38–40]. Therefore, it is logical to speculate that vitreous cryopreservation of isolated, individual PFs might be more effective than vitrification of small sections of ovarian tissues.

Quickly and efficiently transferring embryos/cell constructs among different vitrification solutions to get them adequately permeated and minimizing the time for loading them on/into cryocontainers are critical for successful vitrification, especially for the cryopreservation of tens of encapsulated PFs, and this is closely related to the type of cryocontainer used and the efficiency of manipulation. In the present study, small pieces of stainless steel mesh were carefully molded into a cup-shaped container, and PFs were loaded into the cryocontainer in the first procedure and kept in it during the whole process of vitrification/warming. It was thus not necessary to use Pasteur pipettes to manipulate PFs among vitrification/warming solutions, and the loading procedure was also omitted. Therefore, the mesh cup behaved here not just as a cryocontainer itself but also as a transfer vehicle during the vitrification/warming process, which

makes this vitrification method easier to carry out than currently used vitrification methods.

Encapsulation would reduce the cooling and warming rates a little because of the slightly increased volume of encapsulated follicles compared with "naked" follicles without encapsulation. However, encapsulation of follicles could theoretically protect fragile human follicles from some mechanical damage when they are manipulated during vitrification/warming and *in vitro* culture. Moreover, storage of encapsulated (pre-cultured) PFs could solve the problems related to labor, required techniques and time constraints when cryopreserved follicles are put into clinical use. In the present study, the percentage of viable follicles did not differ significantly between the vitrification and fresh groups soon after warming and after a 7-day culture period. No difference in mean follicle diameter was observed when they were cultured *in vitro*. These results demonstrated the effectiveness of the present vitrification method. However, transmission electron microscopic analysis revealed a slightly higher trend of ultrastructure changes in the vitrification group than in the fresh group. More follicles are required to further investigate any possible damage resulting from vitrification in encapsulated follicles. On the other hand, scaffold integrity is a primary concern for the preservation of encapsulated follicles at low temperatures [24]. Cracks in the scaffold can affect the subsequent culture and growth of follicles [41]. Although the microstructure of the scaffold was not investigated here, the increased diameters of oocytes/follicles and proliferation of cumulus cells indicated the effectiveness of the scaffold after vitrification and warming.

Alginate beads are reported to have a high porosity range and to limit the diffusion of only large proteins. Substrates with a molecular weight below 2×10^4 can diffuse freely into and from calcium alginate beads at approximately the same rate as in water. Thus, encapsulation of the follicles did not theoretically affect the diffusion of cryoprotectant throughout the follicles and thus the viability of cryopreserved follicles [42, 43]. However, high-molecular-weight proteins cannot diffuse freely into calcium alginate beads [44], possibly affecting follicular growth when subjected to *in vitro* culture after warming. Inclusion of hormones or other proteins in the scaffold might solve this problem.

In conclusion, the present vitreous cryopreservation method could effectively maintain the viability of encapsulated human ovarian follicles, which could be put into immediate use (*in vitro* culture or transplantation [9]) after warming. More follicles are required to further evaluate the effects of vitrification on the *in vitro* growth of cryopreserved encapsulated human preantral follicles.

Acknowledgement

This study was supported by grants from the Key Laboratory of Guangdong Province and the National Natural Science Foundation of China (81100472).

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