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Title: Effects of isolation method and pre-treatment with ethylene glycol or raffinose before vitrification on in vitro viability of mouse preantral follicles

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Running head: Mouse follicle vitrification

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## ABSTRACT

Effects of isolation and vitrification protocols on follicular survival after warming were examined. Mouse preantral follicles enzymatically or mechanically isolated from ovaries of 12-day-old mice were exposed either to 2 M ethylene glycol (EG) for 2 or 5 min, or to ascending concentrations (0.15 then 0.3 M) of raffinose for 2 or 5 min each (2-2 and 5-5 min). They were then exposed to a vitrification solution (VS) composed of 6 M EG and 0.3 M raffinose for 0.5, 1, or 2 min before vitrification. Mechanically isolated follicles showed higher survival than enzymatically isolated follicles, regardless of periods of exposure to EG or raffinose and subsequent exposure to VS. After 10 days of culture, follicular growth and maturational ability of oocytes derived from vitrified follicles exposed to 2 M EG for 5 min and to VS for 1 min were higher than those from follicles exposed to raffinose solutions for 2-2 min and to VS for 1 min. Histological evaluation revealed that exposure of preantral follicles to raffinose solutions caused cytoplasmic vacuolation in granulosa cells which could be due to cellular shrinkage during dehydration; whereas, exposure to 2 M EG induced morphological alterations in follicles only to a lesser extent.

Oocytes derived from antral follicles are commonly used for in vitro embryo production (IVP), in spite of the fact that number of preantral follicles in the ovary is much greater than that of antral follicles. If we could preserve and use these preantral follicles for IVP, the efficiency of IVP will be improved dramatically to the levels that would enable production of embryos using preserved ovarian tissues of endangered animals and for female cancer patients undergoing chemotherapy and/or radiotherapy. Recently, live offspring were produced from frozen-thawed and vitrified-warmed mouse ovarian tissues after transplantation to live mice (4,17,23). However, the xenografting and autografting techniques of endangered animal organs are not established. For human use, xenografting may pose ethical problems, and autografting may carry a risk of reintroduction of malignant cells to patients recovered from cancer. Therefore, in vitro culture system for preantral follicles combined with cryopreservation technology of preantral follicle seems to be well-suited to these purposes.

Culture technology for mouse preantral follicles is now available for obtaining offspring (7,9-11,22). Preantral follicles obtained from mouse ovaries by either mechanical or enzymatic isolation can be grown in vitro by individual spherical or by group non-spherical follicle culture (12). The group culture of enzymatically dissociated preantral follicles embedded in a collagen gel (24), agar (21), or collagen insert (11) could produce large numbers of fertile oocytes. However, the production of pups using oocytes derived from cryopreserved preantral follicles have met a limited success (2-4,9,16,23).

Vitrification provides a simple and economical alternative to the conventional slow freezing and avoids the potential risk of cellular injury caused by the formation of intracellular ice. However, chemical toxicity and osmotic damage to follicles may result

from exposure to the high concentrations of cryoprotectants required for vitrification. Recently, raffinose has been found to modify the vitrification properties of ethylene glycol (EG)-based solution by raising the glass transition point to a greater extent compared with other sugars (14). In mouse metaphase II (MII) oocytes, exposure to a low concentration of permeable cryoprotectants (13,25) or to ascending concentrations of sucrose (19) before exposure to a high concentration of cryoprotectants are important procedures to minimize chemical toxicity. The exposure of mouse MII oocytes to a low concentration of EG or to raffinose solutions before exposure to a vitrification solution (VS) composed of EG and raffinose increases their post-warming viability (8). In our previous study (9), the pups were obtained from oocytes derived from mechanically isolated preantral follicles which had been vitrified using EG pre-exposure treatment; however, the efficiency of IVP in this study with vitrified preantral follicles was lower than that with non-vitrified preantral follicles. In the previous study (9), we did not try to use the pre-treatment with raffinose solutions. This treatment may improve the post-warming viability of preantral follicles similar to the results obtained in oocytes (8).

The main aim of the present study was to investigate whether both enzymatically and mechanically isolated mouse preantral follicles could survive and maintain their developmental ability after vitrification, and to determine the effects of pre-exposure to a low concentration of EG or to raffinose solutions on follicular survival. Likewise, the morphological characteristics of vitrified and non-vitrified preantral follicles will be evaluated.

## MATERIALS AND METHODS

Experimental Animals. Female C57BL/6J and male CBA mice purchased from Japan SLC Inc. (Shizuoka, Japan) were housed and bred to produce F1 offspring in the animal housing facilities in the Graduate School of Veterinary Medicine, Hokkaido University following the guidelines set by the university. They were kept in light- and temperature-controlled conditions (12 h light: 12 h dark photoperiod, 22±2°C) and given chow pellets and water ad libitum. Female F1 offspring, 12 days of age were used as follicle donors. They were killed by cervical dislocation and the ovaries were collected into Leibovitz's L-15 medium (L-15; Gibco BRL, Grand Island, NY, USA) supplemented with 4 mg/mL BSA (Fraction V; Sigma Chemical Co., St. Luis, MO, USA), 75 µg/mL penicillin and 50 µg/mL streptomycin (L-15+BSA) at 37°C.

Isolation of Preantral Follicles. Mouse preantral follicles were enzymatically (11) or mechanically (5) collected from the ovaries as described previously. Briefly, to isolate preantral follicles enzymatically, the whole ovaries were treated with L-15+BSA containing 2 mg/mL collagenase (Wako Pure Chem. Industries, Ltd., Tokyo, Japan) for 30 min at 37°C, and dissociated into small pieces by repeated pipetting in fresh L-15+BSA without collagenase. For mechanical isolation, preantral follicles were dissected using 25 gauge needle attached to 1-mL syringe into L-15+BSA at 37°C. After these treatments, round-shaped preantral follicles with 2-3 layers of granulosa cells and the spherical oocyte centrally located within the follicle were adjudged as morphologically normal and used in the experiments.

Vitrification and Warming. The vitrification procedure for mouse MII oocytes described in our previous work (8) was adopted for the vitrification of mouse preantral follicles. Briefly, preantral follicles were first exposed to 2 M EG or 0.15 then 0.3 M raffinose in L-15+10% fetal calf serum (FCS, Gibco) (L-15+FCS), and then exposed to a VS

composed of 6 M EG and 0.3 M raffinose in L-15+FCS. The samples were initially rinsed in a 100- $\mu$ L drop of VS before transfer to a 40- $\mu$ L droplet of VS at room temperature (22-25°C). The drop containing approximately 30 follicles was drawn into a 0.25-mL French straw (I.M.V., L'Aigle, France). For loading the straw, approximately 100  $\mu$ L of L-15+BSA with 1 M sucrose was filled into the straw followed by a short column of air and approximately 10  $\mu$ L of VS. The 40  $\mu$ L of VS containing the follicles was aspirated and separated by an air space on each side. The remainder of the straw was filled with L-15+BSA with 1 M sucrose. The straws were stored into liquid nitrogen for 1 to 60 days. For warming and dilution, the straws containing the preantral follicles were directly immersed into 20°C water and held for 20 sec. Then the contents of the straw were expelled into 1 mL of 1 M sucrose in L-15+BSA, and kept for 10 min at room temperature. The follicles were transferred to 3 mL of L-15+BSA at 37°C for 5 min and held in 3 mL of the same medium for 10 to 15 min before culture during which the follicles were checked for survival.

In Vitro Culture of Preantral Follicles. The follicle culture system developed for the enzymatically isolated preantral follicles (11) was used with a slight modification. In brief, approximately 115 preantral follicles, which were isolated mechanically and treated with collagenase before culture (9), were cultured for 10 days at 37° C in an atmosphere of 5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub> in Transwell-COL membrane inserts (3.0  $\mu$ m pore size, 12 mm diameter; Corning Costar Corp. Cambridge, MA, USA) fitted in Costar 12-well cluster dish with 2 mL of follicle culture medium: Waymouth medium (Gibco) supplemented with 5% FCS, 2 mM hypoxanthine (Sigma), 0.23 mM sodium pyruvate and 50  $\mu$ g/mL gentamicin sulfate. Medium change was done every 48 h by exchanging 1 mL of fresh medium for the same volume of the spent medium. Immediately before

culture, both fresh and vitrified mechanically isolated preantral follicles were treated with 2 mg/mL collagenase in L-15+BSA for 10 min at 37°C and washing through 3 changes in 2 mL of L-15+BSA.

In Vitro Maturation of Oocytes. After 10 days of follicle culture in vitro, morphologically normal oocyte-granulosa cell complexes (OGCs) were subjected to oocyte maturation culture in vitro. They were cultured in a 100 µL-drop of Waymouth medium supplemented with 5% FCS, 1 IU/mL porcine FSH (Antorin R; Denka Pharmaceutical Co. Ltd., Kanagawa, Japan), 10 ng/mL human recombinant EGF (Sigma), 0.23 mM sodium pyruvate and 50 µg/mL gentamicin sulfate at 37°C in a humidified atmosphere of 5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub>. After 14-16 h of incubation, the oocytes were fixed and stained with 1% aceto-orcein, and their nuclear statuses were examined under a phase contrast microscope.

Histological Evaluation of Preantral Follicles. Fresh and vitrified-warmed preantral follicles were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffer (PB), post-fixed in 1% osmium tetroxide in 0.1 M PB, dehydrated through an ethanol series and embedded in Epon 812 (Okenshoji Co, Ltd., Tokyo, Japan). Serial sections were made through the follicles. Semi-thin sections (0.3 µm) taken from the oocyte equatorial region where the nucleolus was visible, were stained with 1% toluidine blue (Kanto Chemical, Tokyo, Japan) for light microscopy.

Experimental Studies. In the first experiment, the effects of periods of exposure to EG or raffinose and exposure to VS before cooling on the survival of enzymatically or mechanically isolated preantral follicles were determined. Preantral follicles were pretreated with either (1) 2 M EG for 2 or 5 min or (2) ascending concentrations (0.15 then 0.3 M) of raffinose for 2 or 5 min each, hereafter, referred to as 2-2 and 5-5 min

raffinose exposure. The pre-treated samples were subsequently exposed to VS for 0.5, 1 or 2 min and then vitrified. Survival of the follicles just after warming was assessed by morphological appearance.

In the second experiment, the in vitro viability of vitrified-warmed preantral follicles was determined. Mechanically isolated preantral follicles were exposed either to 2 M EG for 5 min or to raffinose solutions for 2-2 min and then to VS for 1 min before vitrification. After 10 days of in vitro culture, the maturational competence of oocytes derived from vitrified preantral follicles was determined. Non-vitrified preantral follicles were also cultured as control.

In the third experiment, the histological changes induced by vitrification procedures in preantral follicles were characterized. Vitrified-warmed preantral follicles collected by mechanical or enzymatical isolation and pre-exposed to 2 M EG for 5 min or to raffinose solutions for 2-2 min were examined. The vitrified-warmed follicles, which were adjudged as survived after warming, and the non-vitrified follicles were processed for light microscopic evaluation (5 follicles each).

Statistical Analysis. Comparisons of viability among the periods of exposure to VS under each pre-treatment procedure were analyzed using one-way ANOVA followed by Tukey-Kramer's HSD. Comparisons of viability between enzymatically and mechanically isolated preantral follicles and between pre-treatment procedures under the same VS exposure condition were made using Student's  $t$ -test. The data on nuclear status of oocytes were analyzed using Chi-square test. All analysis was performed using software (JMP version 5; SAS Institute Inc., Cary, NC, USA).

## RESULTS

First Experiment. After warming, the vitrified follicles showed various percentages of survival and degeneration among the follicles treated with different protocols (Table 1). Mechanically isolated preantral follicles which showed morphological irregularities such as the formation of clear space between the granulosa cells and oocyte or within the granulosa cell mass, and the collapse of oocyte within the follicle were considered as degenerated. The enzymatically isolated preantral follicles which showed partial or complete detachment of granulosa cells with the expulsion of denuded oocytes were also considered as degenerated.

There were significant interactions among the effects of the method of follicle collection, periods of exposure to EG and to VS on the survival of follicles ( $P < 0.01$ ), as shown in Table 1. Under the same pre-exposure to EG and exposure to VS conditions, mechanically isolated preantral follicles showed higher survival than enzymatically treated ones ( $P < 0.01$ ). The 5 min pre-exposure to EG showed higher survival rates than 2 min pre-exposure when follicles were subsequently exposed to VS for 0.5 and 1 min ( $P < 0.01$ ) regardless of the method of follicle collection. The survival rate of enzymatically isolated follicles exposed to EG for 2 min was not affected by the periods of exposure to VS; while the mechanically isolated follicles exposed to 2 M EG for 2 min showed higher survival with prolonged exposure to VS ( $P < 0.05$ ). When the mechanically isolated follicles were exposed to EG for 5 min, subsequent exposure to VS for 0.5 and 1 min showed the highest survival rates ( $P < 0.01$ ). No significant interaction was observed between the effects of periods of exposure to raffinose and to VS on follicular survival (Table 2). The mechanically isolated preantral follicles showed higher survival rates than enzymatically isolated follicles regardless of the periods of exposure to raffinose and to VS ( $P < 0.01$ ). The 2-2 min exposure to raffinose showed

higher survival than 5-5 min exposure under the same period of exposure to VS ( $P<0.05$ ). With 2-2 min exposure to raffinose, 1 min exposure to VS showed the highest survival rate ( $P<0.05$ ). There was no significant difference in terms of the highest survival rates of vitrified-warmed preantral follicles pre-exposed either to EG ( $84.3\pm 4.1$  and  $80.8\pm 8.2\%$  in Table 1) or raffinose ( $75.0\pm 3.6\%$  in Table 2).

Second Experiment. The proportions of morphologically normal follicles after collagenase treatment did not differ between the vitrified and fresh preantral follicles isolated mechanically (Table 3). The follicles which were pre-exposed to EG before exposure to VS showed higher survival than those pre-exposed to raffinose ( $P<0.01$ ) after 10 days of culture. However, the survival rates of vitrified follicles exposed to EG ( $P<0.05$ ) or raffinose ( $P<0.01$ ) were lower than those of the fresh follicles.

The proportion of oocytes that reached the MII stage derived from the follicles exposed to EG before exposure to VS was higher than that of follicles exposed to raffinose ( $P<0.01$ ), as shown in Table 4. This was similar to that of the fresh preantral follicles.

Third Experiment. Histological evaluation revealed that the non-vitrified fresh follicles isolated mechanically had granulosa cell layers circumscribed by basal lamina and flattened theca cells (5/5, Fig. 1a). The non-vitrified enzymatically isolated preantral follicles, however, did not have intact basal membrane (5/5, Fig. 1b), and the granulosa cells and ooplasm were more swollen compared with those of the follicles isolated mechanically. Meanwhile, the vitrified preantral follicles isolated mechanically and pre-exposed to 2 M EG exhibited a morphological appearance similar to that of the fresh preantral follicles (5/5) after warming. On the other hand, only one out of 5 mechanically isolated preantral follicles pre-exposed to raffinose showed normal

structure, while the 4 remaining follicles were observed to have extensive cytoplasmic vacuolations in granulosa cells (Fig. 1c). Two out of 5 enzymatically isolated follicles pre-exposed to EG showed darkly stained granulosa cells and oocytes (Fig. 1d), while granulosa cell layers were partially or completely detached from the oocyte in 1 follicle. Three out of 5 enzymatically isolated preantral follicles pre-exposed to raffinose possessed a darkly stained cytoplasm, while the other 2 follicles showed extensive cytoplasmic vacuolations.

## DISCUSSION

The mechanically isolated preantral follicles showed higher survival rates than enzymatically isolated follicles when they were exposed either to 2 M EG or to raffinose solutions, regardless of periods of exposure to VS. The presence of basement membrane in the mechanically isolated follicles could have provided structural support throughout the vitrification process. Enzymatic isolation with collagenase removed theca cells and basal membrane (11) leading to the eventual collapse of some follicles during dilution. The damage to the plasma membrane by enzymatic treatment could also account for the lower survival or disintegration of some of isolated preantral follicles.

In our previous study (8), the exposure of the mouse MII oocytes to 2 M EG for 2 min and then to VS for 1 min, or to 2 M EG for 5 min and then to VS for 0.5 min resulted in a high survival; however, prolonged (2 or 5 min) exposure of oocytes to VS after 2 or 5 min exposure to EG reduced their developmental capacity. In the present study, exposure of mouse preantral follicles to 2 M EG for 2 min resulted in low survival regardless of period of exposure to VS; while exposure to 2 M EG for 5 min followed by exposure to VS for 0.5 or 1 min yielded a high follicular survival. The difference in the

cryobiological characteristics between preantral follicles and MII oocytes could be attributed to their morphological and functional differences. As compared with the oocyte which is a single unit, the follicle is a complex structure comprising the granulosa and theca cells, basal lamina and the oocyte, which may affect the ability of the cryoprotectant to permeate to the center of the follicle, the movement of water during cellular dehydration and rehydration, and the removal of the cryoprotectant during dilution. Moreover, cell permeability may change as the follicles develop (1), so that the coefficient of permeability of growing oocytes in the preantral follicles may be different from that of fully grown or mature oocytes in the antral follicles. In this study, 5 min pre-exposure to 2 M EG could have allowed enough dehydration and initial permeation of EG into the follicles so that a brief (0.5 or 1 min) exposure to VS might have induced small amount of EG influx and further dehydration which concentrate the intracellular solutes producing a cytoplasm capable of vitrification (20). The degree of cryoprotectant permeation and dehydration achieved with 2 min pre-exposure to 2 M EG and subsequent exposure to VS may be inadequate to induce vitrification of mouse preantral follicles.

The exposure of follicles collected enzymatically and mechanically to raffinose solutions for 2-2 min showed higher survival than 5-5 min exposure under the same periods of exposure to VS. The present result is in contrast to our previous findings (8), wherein, exposure of mouse MII oocytes to raffinose solutions for 5-5 min resulted in higher viability than 2-2 min exposure. In the present study, the exposure of preantral follicles to raffinose for 5-5 min could have produced a greater degree of follicular shrinkage probably due to damaging the connections between the oocyte and granulosa cell layer; while the 2-2 min exposure could have reduced the shrinkage with minimal

effect on oocyte-granulosa cell junctions. The most remarkable morphological alterations found in preantral follicles exposed to raffinose were the vacuolations of the cytoplasm in granulosa cells. The reasons by which raffinose exerts this effect are unclear. However, it was reported that the cryopreservation of cultured kidney cells induced swelling of mitochondria with damage to their membranes and cristae (15). The same phenomenon was reported in freezing bovine embryo cells (18) and follicular cells in vitrified-warmed ovarian tissue (4). There is a possibility that mitochondria were damaged by dehydration immersion of follicles in pre-treatment solution with high concentration of raffinose. Further investigation is therefore warranted to clarify this assumption.

After 10 days of culture, the *in vitro* viability of mechanically isolated preantral follicles pre-exposed to raffinose and the maturational competence of their oocytes were lower compared to those of follicles pre-exposed to EG. It is most probable that the connections between the oocyte and granulosa cells were lost due to the shrinkage of oocyte during exposure to raffinose even if the follicles showed normal morphological appearance. The maturation capacity of the oocytes derived from vitrified follicles pre-exposed to EG in the present study is comparable with that of preantral follicles cryopreserved by slow freezing (6). Histological observations demonstrated that preantral follicles pre-exposed to EG before vitrification were not different morphologically from those of the fresh samples.

In conclusion, the present results indicate that the mechanically isolated preantral follicles survived through the vitrification process better than the enzymatically isolated ones which were affected adversely by vitrification. Exposure of preantral follicles to raffinose before vitrification induced severe morphological modifications, resulting in low

survival after warming and culture in vitro. On the other hand, exposure of preantral follicles to EG did not compromise their morphological integrity nor the ability of their oocytes to grow to maturity in vitro. The present work demonstrated the fundamental cryobiological characteristics of preantral follicles which determined their viability after vitrification and warming.

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Table 1 The effects of isolation method, pre-exposure to ethylene glycol (EG) and exposure to vitrification solution (VS) on the survival of vitrified-warmed mouse preantral follicles

Pre-exposure to EG <sup>a</sup> (min)	Exposure to VS (min)	% of follicles survived (No. of follicles examined)	
		Enzymatic	Mechanical
2	0.5	19.2±3.5 <sup>c</sup> (78)	36.2±1.4 <sup>c*</sup> (80)
	1	22.1±2.9 <sup>c</sup> (81)	39.3±1.2 <sup>c*</sup> (89)
	2	19.5±0.5 <sup>c</sup> (77)	44.6±0.8 <sup>c*</sup> (101)
5	0.5	48.9±7.0 <sup>b</sup> (85)	84.3±4.1 <sup>b*</sup> (102)
	1	56.9±6.3 <sup>b</sup> (95)	80.8±8.2 <sup>b*</sup> (97)
	2	25.0±5.8 <sup>c</sup> (80)	40.5±5.1 <sup>c*</sup> (79)

% values are means±SD of 3 replicates.

% survival was based on the no. of follicles recovered.

<sup>a</sup> Follicles were pre-exposed to 2 M EG for 2 or 5 min before exposure to VS composed of 6 M EG and 0.3 M raffinose.

<sup>b, c</sup> Values with different superscripts within a column differ significantly (P<0.05).

\* Mechanically collected follicles showed higher survival rates than enzymatically isolated follicles (P<0.05).

Table 2 The effects of isolation method, pre-exposure to raffinose and exposure to vitrification solution (VS) on the survival of vitrified-warmed mouse preantral follicles

Pre-exposure to raffinose <sup>a</sup> (min)	Exposure to VS (min)	%of follicles survived (No. of follicles examined)		
		Enzymatic	Mechanical	Total
2-2	0.5	45.8±1.2 (85)	58.9±4.9 (90)	52.4±7.8 <sup>bc*</sup> (175)
	1	51.9±5.1 (80)	75.0±3.6 (92)	63.5±13.3 <sup>b*</sup> (172)
	2	34.9±4.3 (83)	46.7±3.4 (88)	40.8±7.3 <sup>c*</sup> (171)
5-5	0.5	29.7±0.9 (74)	44.1±8.7 (94)	36.9±9.6 <sup>b</sup> (168)
	1	22.7±2.8 (79)	37.5±2.3 (88)	30.1±8.4 <sup>bc</sup> (167)
	2	13.5±11.8 (82)	25.5±4.3 (86)	19.5±10.3 <sup>c</sup> (170)
Total		33.1±14.2 <sup>d</sup> (483)	47.9±16.7 <sup>e</sup> (538)	

% values in each experimental group are means±SD of 3 replicates.

<sup>a</sup> Follicles were pre-exposed to 0.15 and 0.3 M raffinose for 2 or 5 min each (2-2 and 5-5 min) before exposure to VS composed of 6 MEG and 0.3 M raffinose.

<sup>b-c</sup> Values with different superscripts within a column differ significantly (P<0.05) in same pre-exposure to raffinose.

<sup>d, e</sup> Values with different superscripts within a row differ significantly (P<0.05).

\*Follicles pre-exposed to raffinose for 2-2 min showed higher survival rates than follicles treated with raffinose for 5-5 min (P<0.05).

Table 3 Viability of vitrified-warmed mechanically isolated preantral follicles after collagenase treatment and culture in vitro

Treatments of follicles		No. of follicles (replicates)	% of follicles survived after	
			Collagenase*	Culture**
Vitrified	EG <sup>a</sup>	461 (3)	93.6±3.1	72.0±0.7 <sup>c</sup>
	Raffinose <sup>b</sup>	348 (3)	91.6±3.0	41.0±1.3 <sup>d</sup>
Non-vitrified		425 (3)	96.0±0.9	81.3±5.7 <sup>e</sup>

\* Based on the no. of morphologically normal follicles after vitrification and warming.

\*\*Based on the no. of morphologically normal follicles after collagenase treatment.

<sup>a</sup> Follicles were pre-exposed to 2 M EG for 5 min and exposed to VS for 1 min.

<sup>b</sup> Follicles were pre-exposed to 0.15 and 0.3 M raffinose for 2 min each (2-2 min) and exposed to VS for 1 min.

<sup>c-e</sup> Values with different superscripts within a column differ significantly (P<0.05).

Table 4 In vitro maturation of oocytes originated from mechanically isolated preantral follicles

Treatment of follicles		No. of oocytes	% of oocytes at				
			GV	GVBD	MI	AI/II	MII
Vitrified	EG <sup>a</sup>	103	20.4 <sup>c</sup>	16.5	7.8	3.9	51.5 <sup>c</sup>
	Raffinose <sup>b</sup>	83	39.8 <sup>d</sup>	21.7	7.2	3.6	27.7 <sup>d</sup>
Non-vitrified		94	13.8 <sup>c</sup>	21.3	4.3	3.1	57.4 <sup>c</sup>

<sup>a</sup> Follicles were pre-exposed to 2 M EG for 5 min and exposed to VS for 1 min.

<sup>b</sup> Follicles were pre-exposed to 0.15 and 0.3 M raffinose for 2 min each (2-2 min) and exposed to VS for 1 min.

<sup>c, d</sup> Values (pooled from 2 replicates) with different superscripts within a column differ significantly (P<0.01).

GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; AI/II: anaphase/telophase I; MII: metaphase II.

## Figure legend

Fig. 1 Histological characteristics of preantral follicles before and after vitrification

a) Non-vitrified follicle collected mechanically having granulosa cell layers circumscribed by basal lamina and theca cells. b) Non-vitrified enzymatically isolated preantral follicle not having intact basal membrane. c) Vitrified-warmed mechanically isolated preantral follicle pre-exposed to raffinose showing extensive cytoplasmic vacuolations. d) Vitrified-warmed enzymatically isolated follicle pre-exposed to EG showing darkly stained granulosa cells and oocytes. Bar = 50  $\mu\text{m}$ .

Fig. 1

