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Optimal Vitrification Protocol for Mouse Ovarian Tissue Cryopreservation

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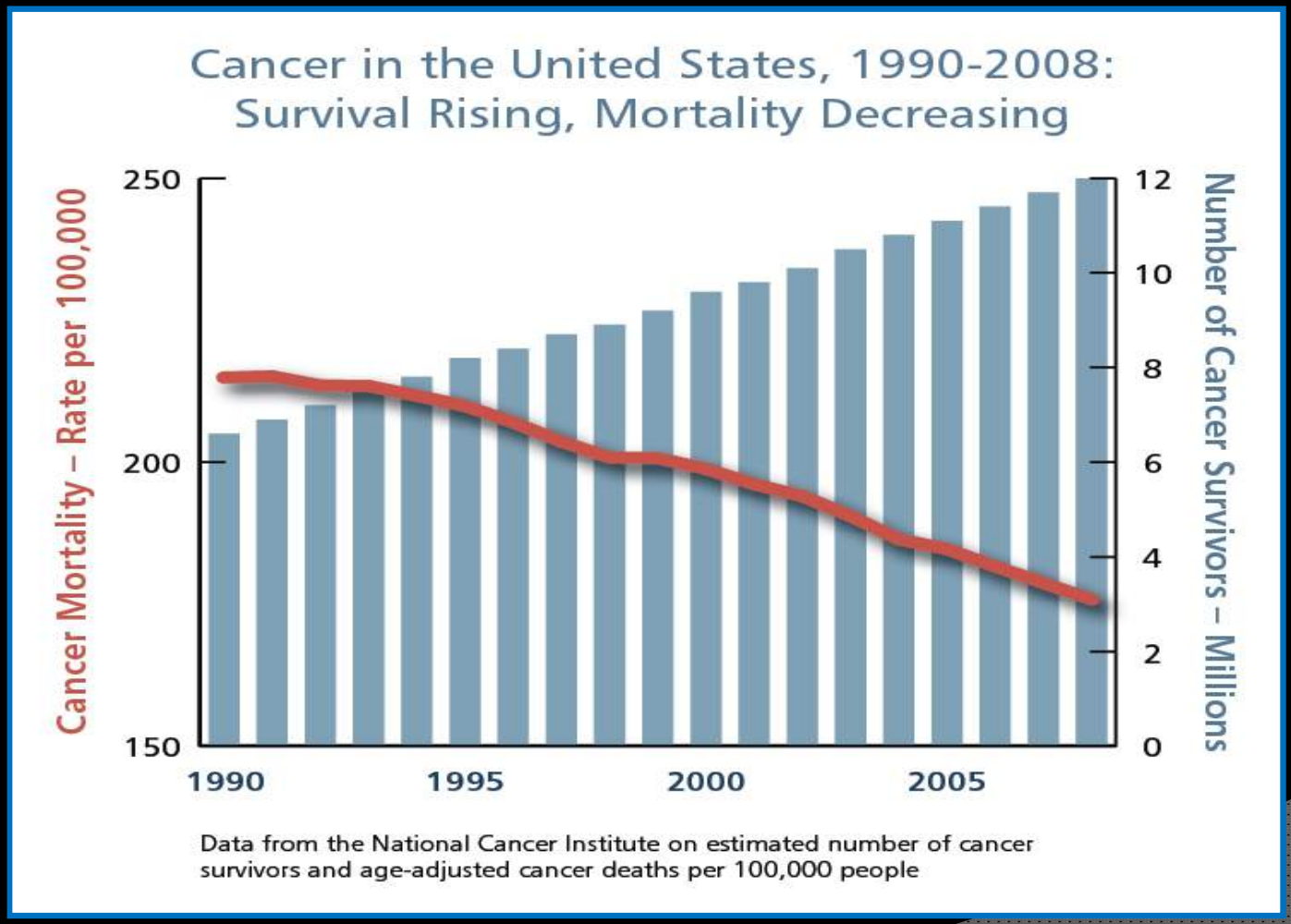
염 혜 원

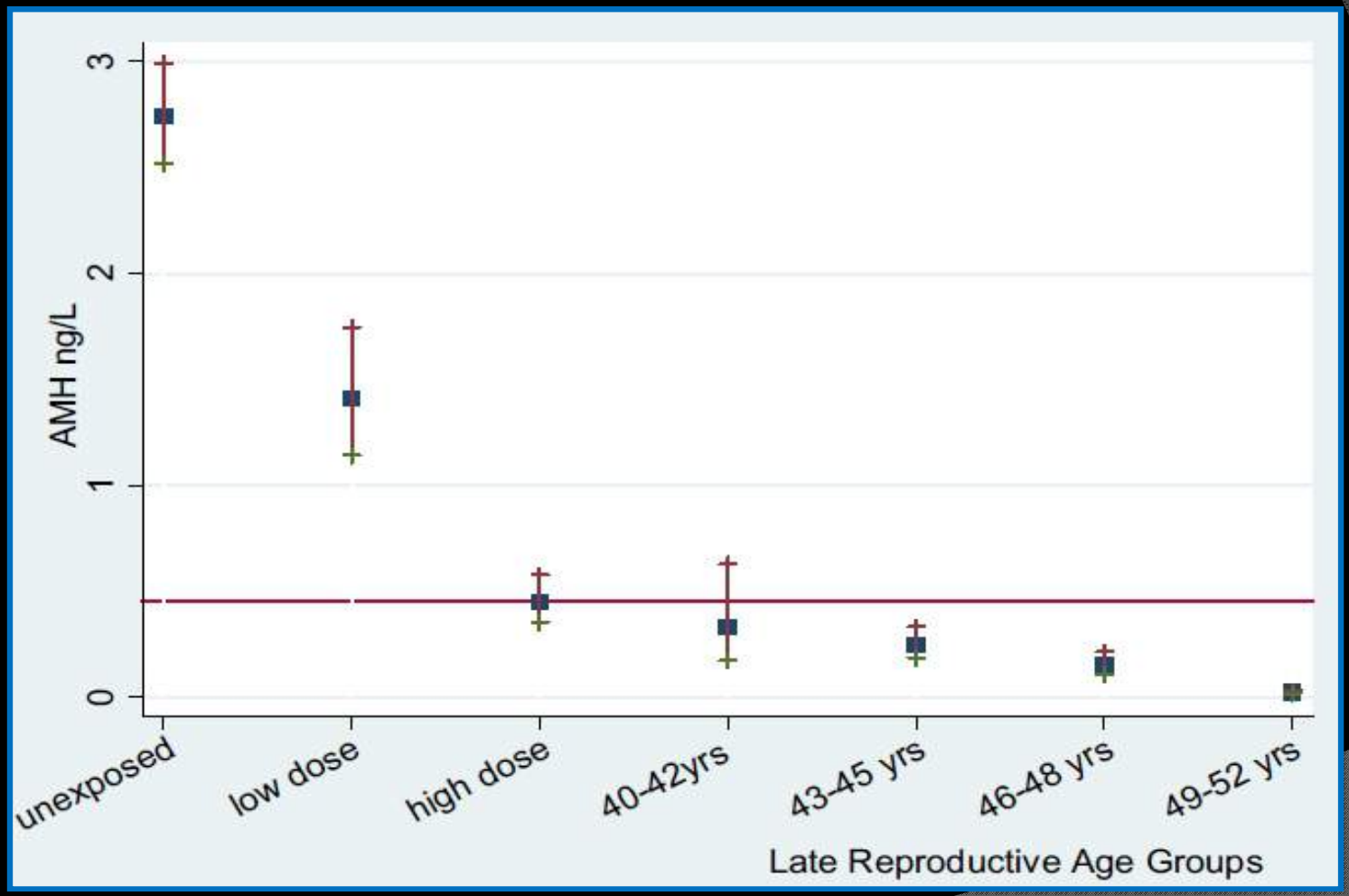
Increased Cancer Survival Rate



National cancer institute, 2008

Increase in the life expectancy of women survived from cancer

Ovarian Reserve after Cancer Therapy



Gracia et al., Fertil Steril. 2012

Fertility Preservation Options

- Ovarian protection (GnRH-a, Oophoropexy)
- Oocyte / Embryo freezing
- **Ovarian tissue cryopreservation**

  → autotransplantation, in vitro culture

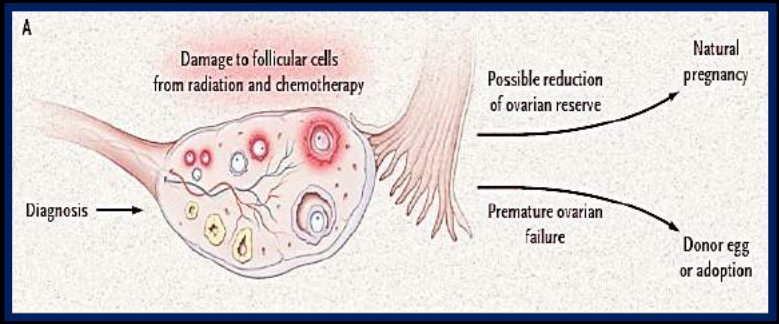
Limitation

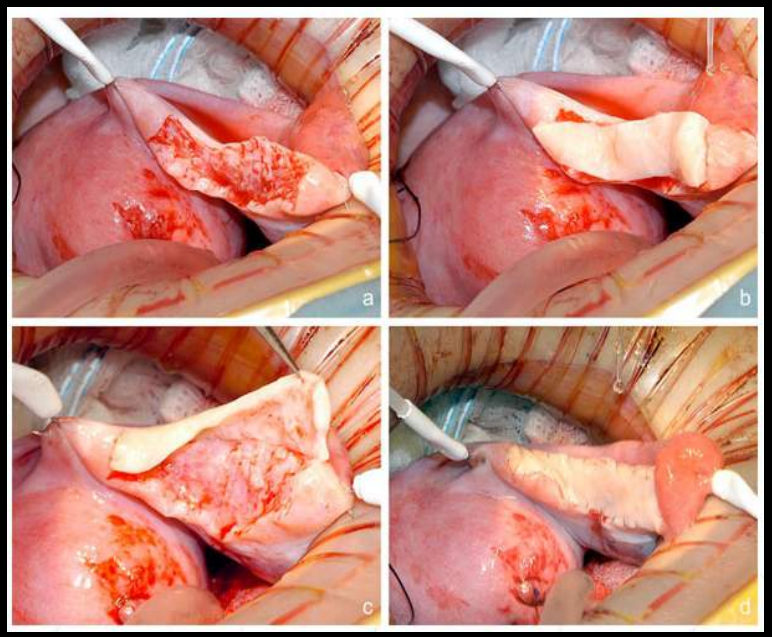
- Cryoinjury : many different cell types → difficult to optimize conditions
- Ischemic injuries after transplantation → no vascular anastomosis

Ovarian Cortex

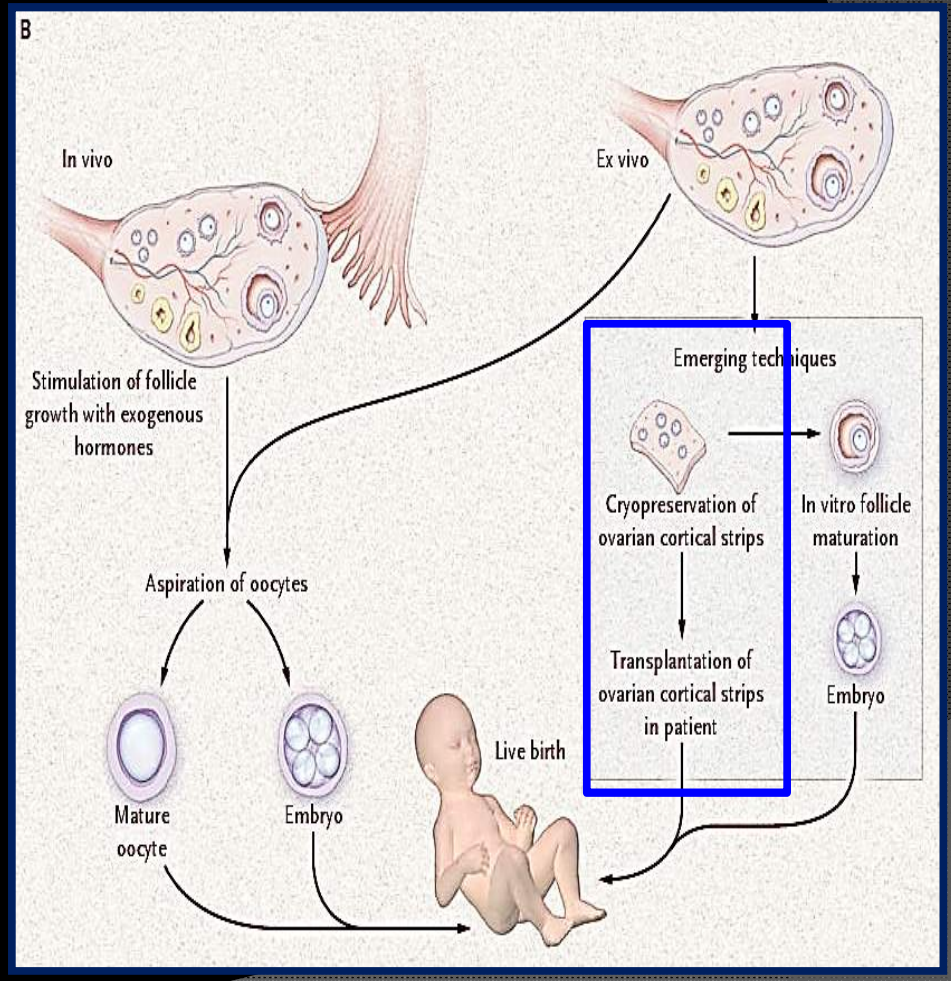
→ the 1mm outer layer of ovary
→ contain more than 90 % of the primordial follicles
→ allows effective cryopreservation of follicles
→ active follicles constitute the organ function

Chemotherapy Damage





Silber et al., Fert Steril. 2010.



Jeruss et al., NEJM. 2009.

Worldwide Frozen Ovarian Cortex Transplantation Pregnancy

TABLE 2

Series of 20 live births after transplantation of frozen-thawed ovarian cortex.

References	Cryopreservation procedure	Graft site	Live birth: Spontaneous	Live birth: IVF
Donnez et al., 2004 (9), 2008 (20), 2011 (4,32)	SF	Peritoneal window (2 steps)	+	+ (+)*
		Ovarian medulla	++ (+)*	
Meirow et al., 2005 (17)	SF	Beneath the ovarian cortex	–	+
Demeestere et al., 2007 (24)	SF	Ovarian and peritoneal windows (2 steps)	++	–
Andersen et al., 2008 (18); Ernst et al., 2010 (33); Schmidt et al., 2011 (29)	SF	Subcortical ovarian pocket	+	+
		Ovarian medulla	+	+
Silber et al., 2008 (34), 2010 (35)	SF	Ovarian medulla	+	–
			+	
Piver et al., 2009 (25); Roux et al., 2010 (11)	SF	Ovarian and peritoneal windows (1 and 2 steps)	+	–
			+	
Sanchez-Serrano et al., 2010 (36)	SF	Ovarian medulla	–	++ (twins)
Revel et al., 2011 (37)	SF	Peritoneal window	–	+
Dittrich et al., 2012 (38)	SF	Ovarian medulla	–	+
Revelli et al., 2012 (39)	SF	Ovarian medulla	+	

* Parentheses indicate ongoing pregnancy at the present time.

Donnez. Live birth after bilateral oophorectomy. Fertil Steril 2012.

Slow Freezing

Mainly used for OT freezing
→several successes

Low CPA concentration

Limitation : ice crystal

→ destroy cell interactions

→ lead to cell death

CPA: cryoprotectant

Vitrification

Recently used with many successful reports.

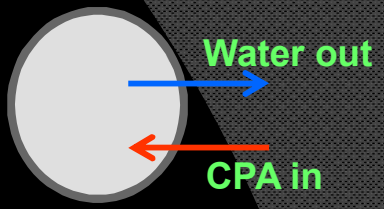
Only one live birth in Japan (PNAS 2013)

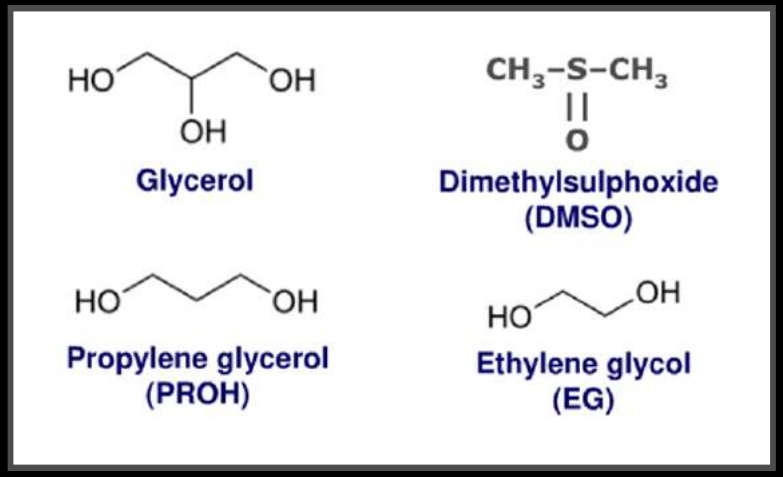
No ice crystal → glassy condition

Limitation : high CPA concentration
→ cause osmotic stress & cellular damage

Already used in sperm, oocyte, embryo, testis

Cryoprotectant (CPA)



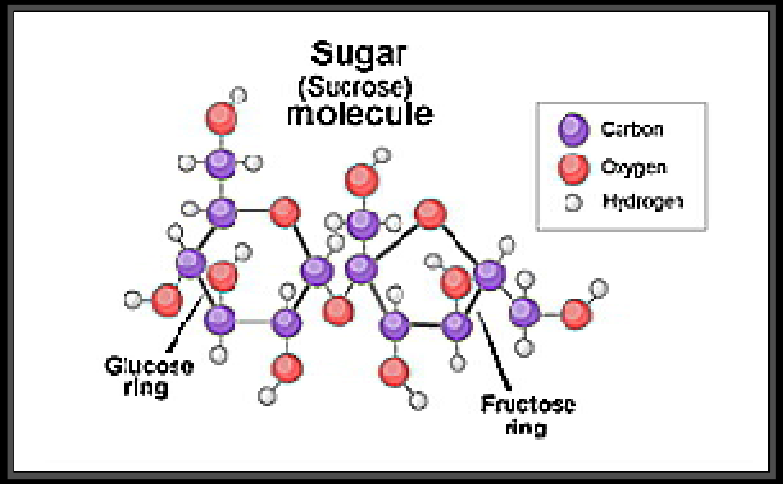


Permeating-CPA

Penetrate into the cytoplasm and exchange the water to CPA.

It is too concentrated to make ice crystal nucleation.

→ 'Glassy' state



Non-Permeating-CPA

Hypertonic soln.

Required to prevent swelling and shrinkage of cells.

Chilling Injury

WCFC

Addition of CPA

CPA acts like antifreeze agent

→ lower freezing temperature and increase viscosity

→ protective action

CPA toxicity and permeation ability are different with different CPAs.

CPA mixture has lower toxicity.

Purpose

To optimze vitrification protocols for mouse OT : comparing 8 different CPA solutions

To evaluate the damage of vitrification using short-term in vitro culture (IVC) system

To confirm the recovery of ovarian function after OT transplantation

Materials and Methods

CPA compositions

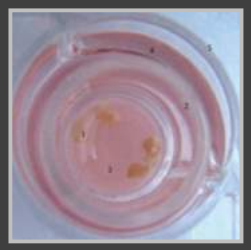
CPA	Vitrification protocols
EDS	7.5% EG + 7.5% DMSO : 10min 20% EG + 20% DMSO + 0.5M sucrose : 5min
ES	38% EG + 0.5M sucrose : 5min
ED	7.5% EG + 7.5% DMSO : 10 min 20% EG + 20% DMSO : 5 min
EPS	10% EG + 10% PROH : 10min 20% EG + 20% PROH + 0.5M sucrose : 5min
EF	20% EG for 10min 40% EG + 18% Ficoll : 5min
EFS	20% EG : 10min 40% EG+18% Ficoll + 0.3M sucrose : 5min
E	38% EG : 5min
EP	10% EG + 10% PROH : 10min 20% EG + 20% PROH : 5min

Materials and Methods

Exp. I.

Ovary → Vitrification → Warming → In vitro Culture (~4h) → Sample Analysis

Control + 8 Exp. groups



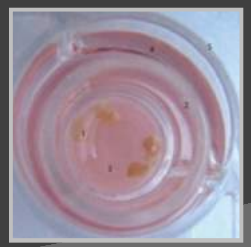
H&E staining

TUNEL assay

Exp. II.

Ovary → Vitrification → Warming → In vitro Culture (~4h) → Transplantation → Sample Analysis

Control + 8 Exp. groups



H&E staining

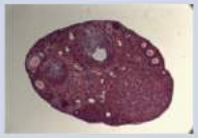
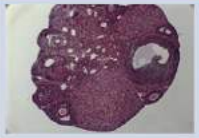
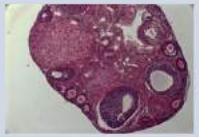
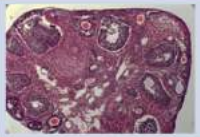
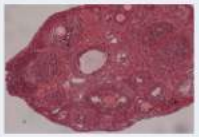
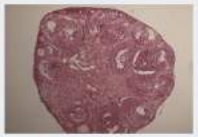
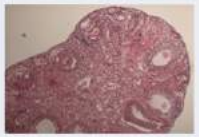
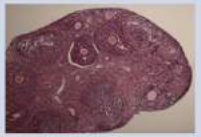
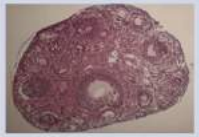
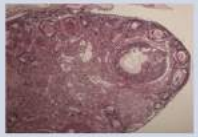
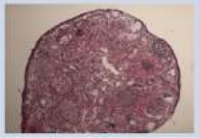
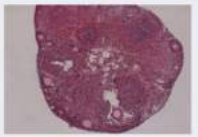
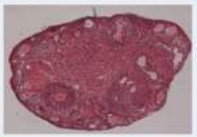
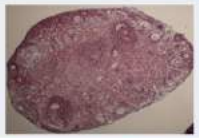
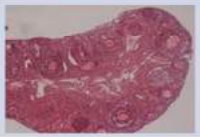
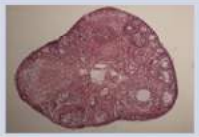
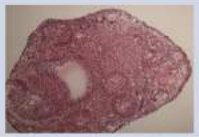
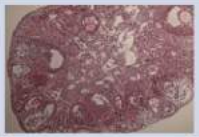
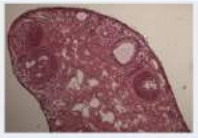
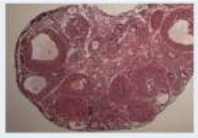
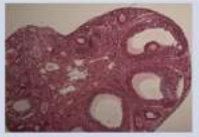
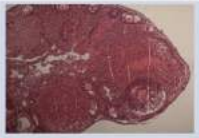
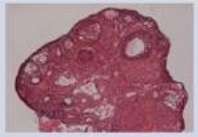
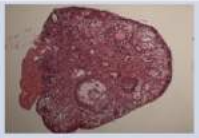
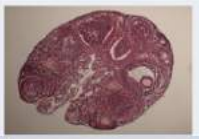
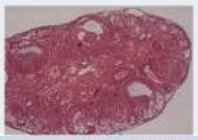
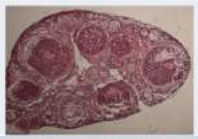
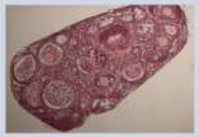
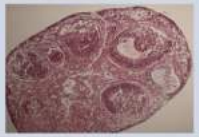
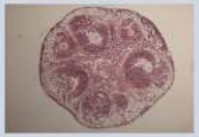
TUNEL assay

Ki 67 IHC

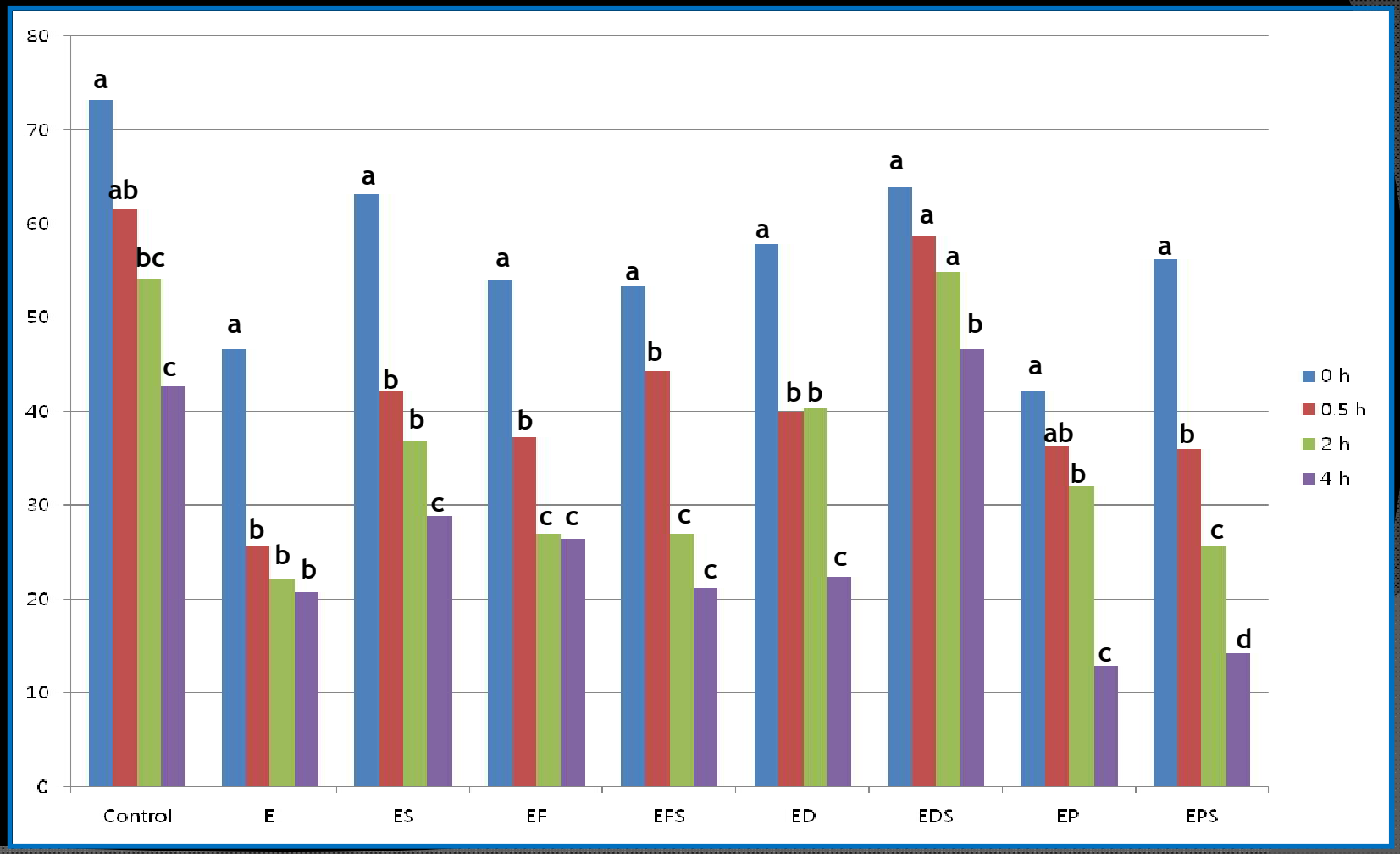
FSH ELISA

Result

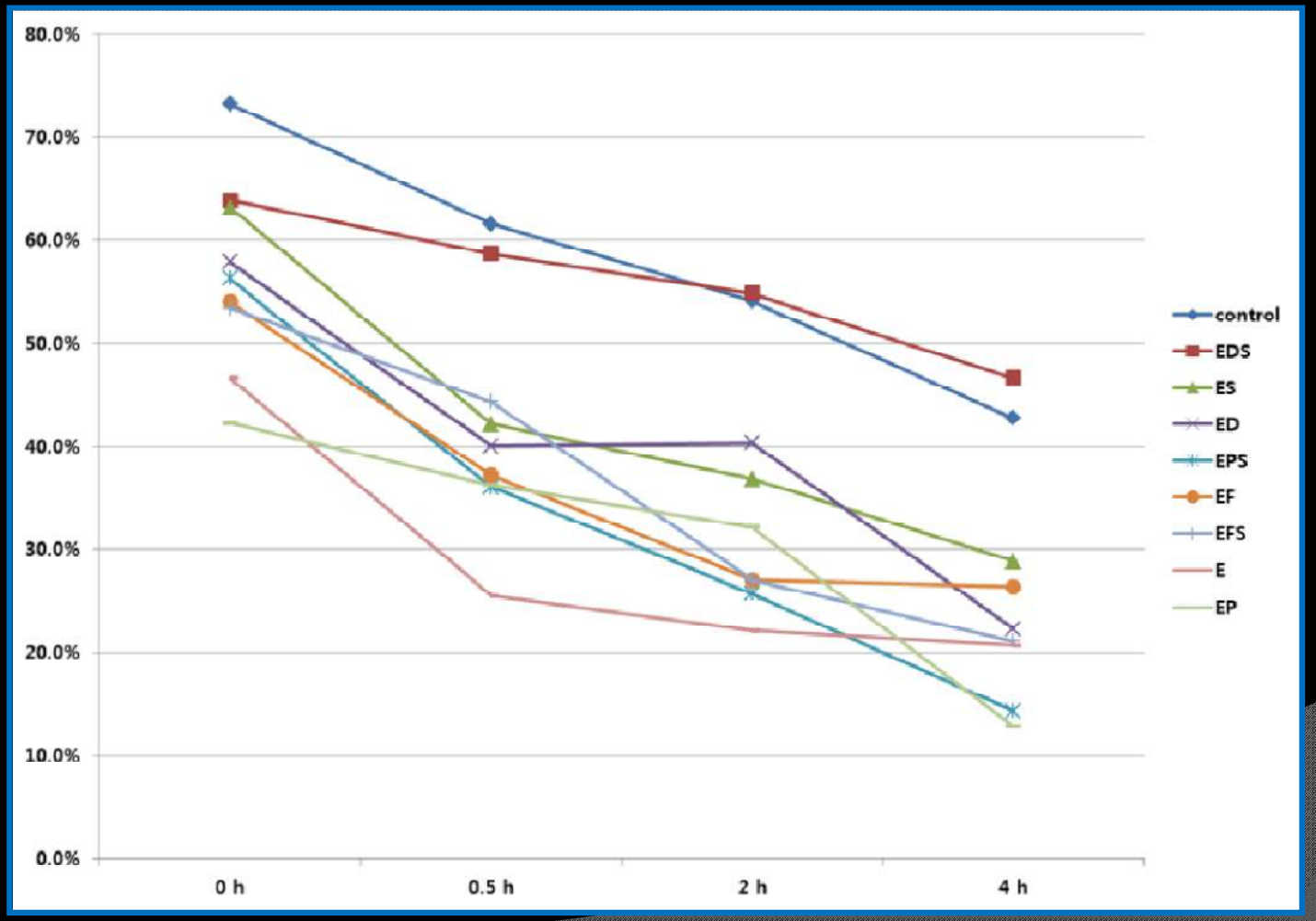
Comparison of G1 Follicles after Vitrification

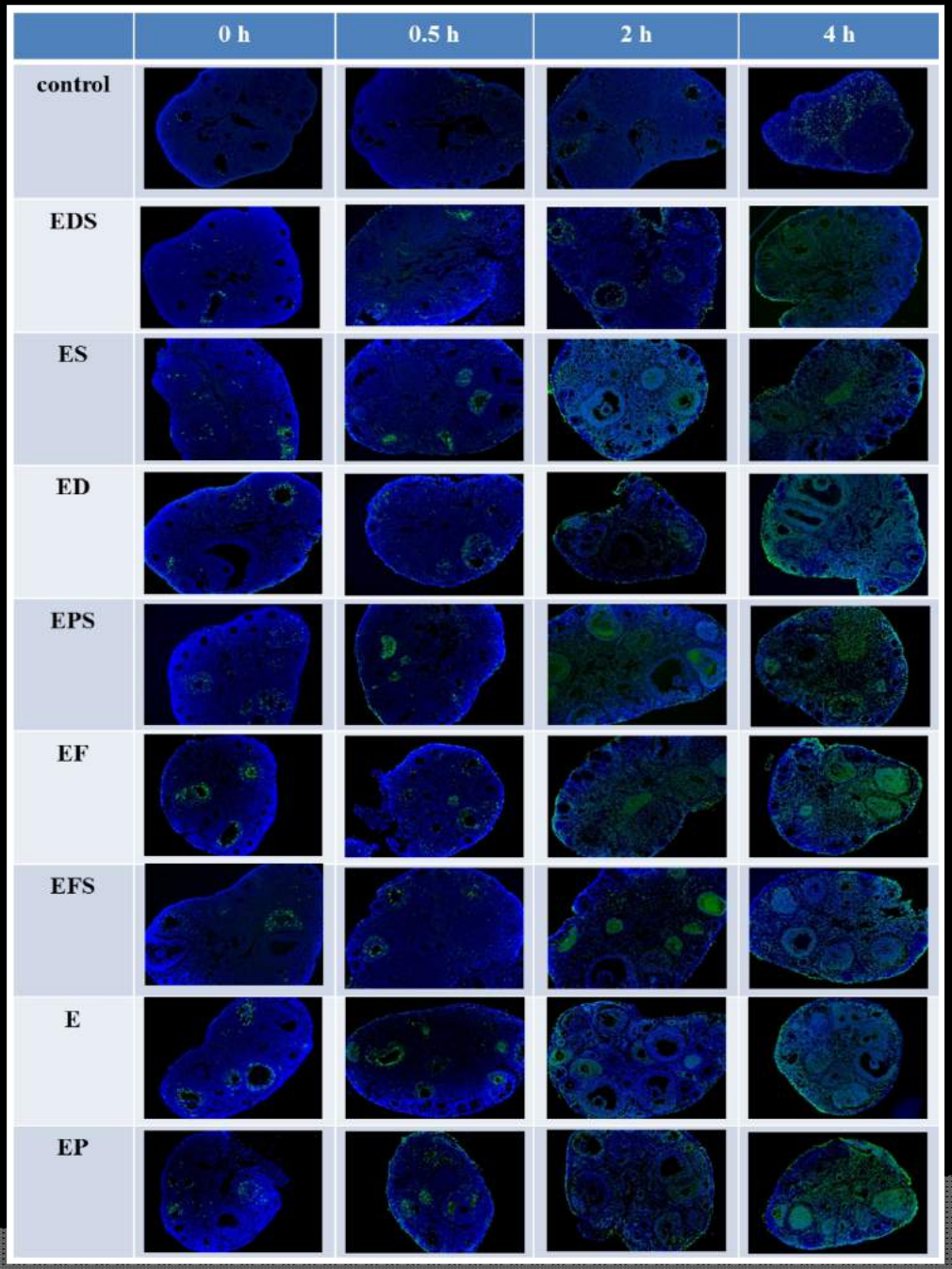
	0h	0.5h	2h	4h
control				
EDS				
ES				
ED				
EPS				
EF				
EFS				
E				
EP				

Comparison of G1 Follicles after Vitrification



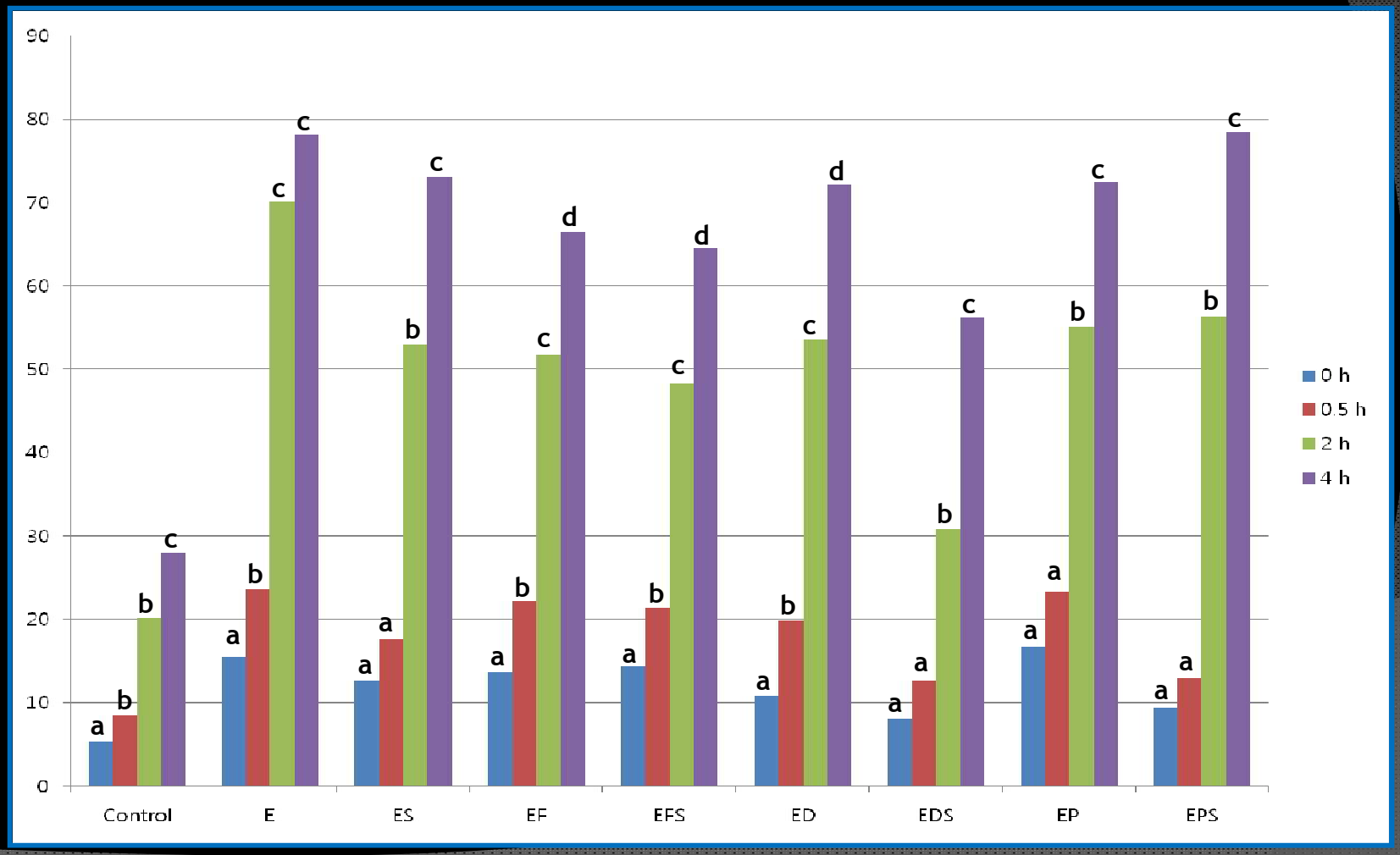
Comparison of G1 Follicles after Vitrification



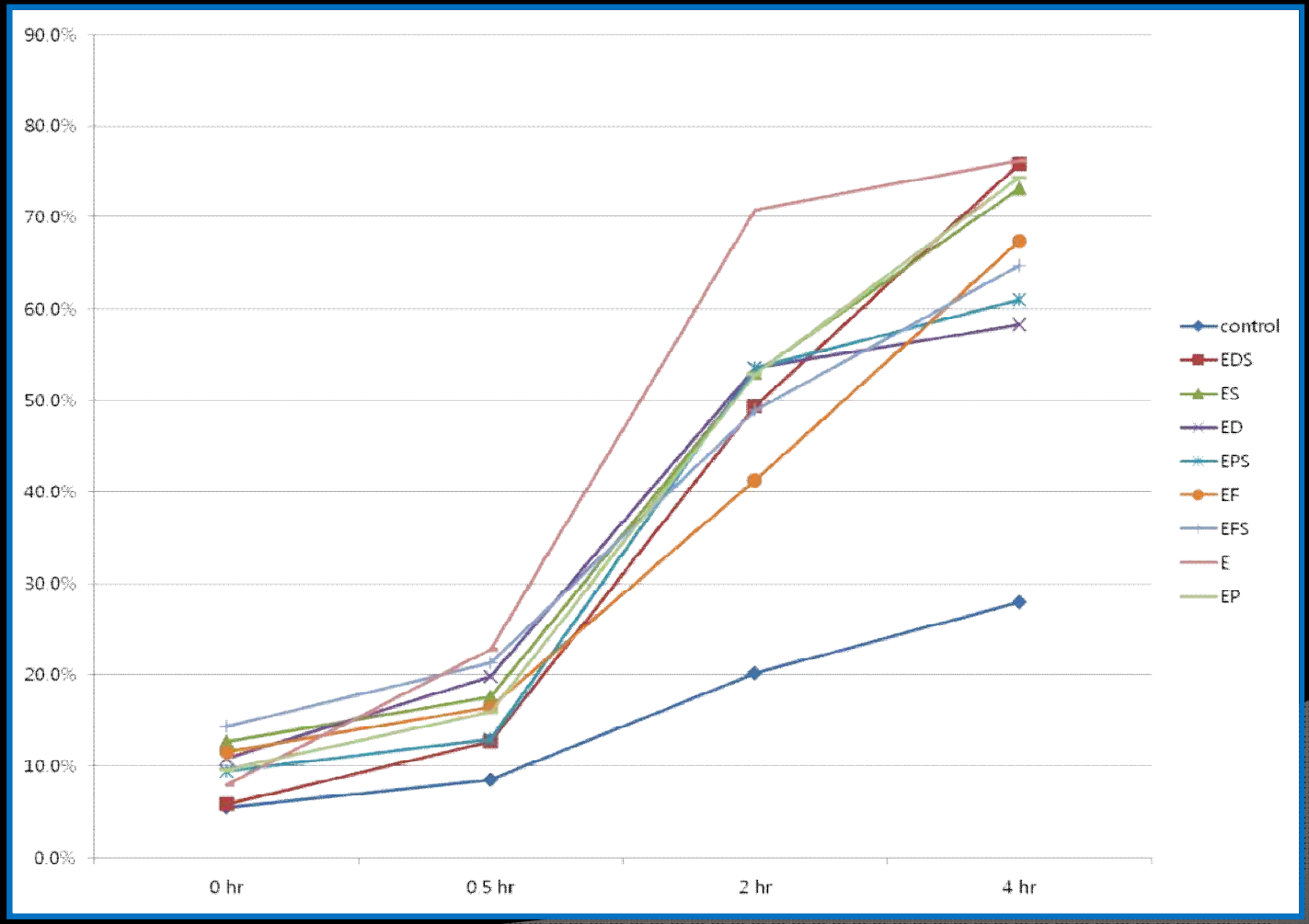


Comparison of Apoptosis after Vitrification

Comparison of Apoptosis after Vitrification



Comparison of Apoptosis after Vitrification



Conclusion (I)

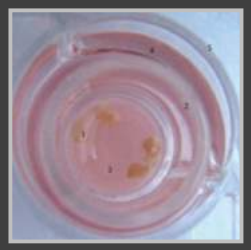
- All the 8 vitrification groups showed significant decreases in GI follicles and increases in apoptotic follicles as IVC duration progressed.
- The type of CPA and sucrose addition influences on OT survival crucially.
- EDS was the best among the 8 vitrification protocols.
- Exp.II : EDS & ES groups → autotransplantation → 3 weeks → sacrifice

Materials and Methods

Exp. I.

Ovary → Vitrification → Warming → In vitro Culture (~4h) → Sample Analysis

Control + 8 Exp. groups



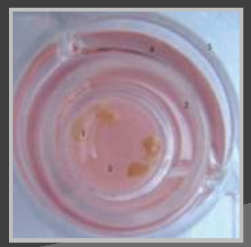
H&E staining

TUNEL assay

Exp. II.

Ovary → Vitrification → Warming → In vitro Culture (~4h) → Transplantation → Sample Analysis

Control + 8 Exp. groups

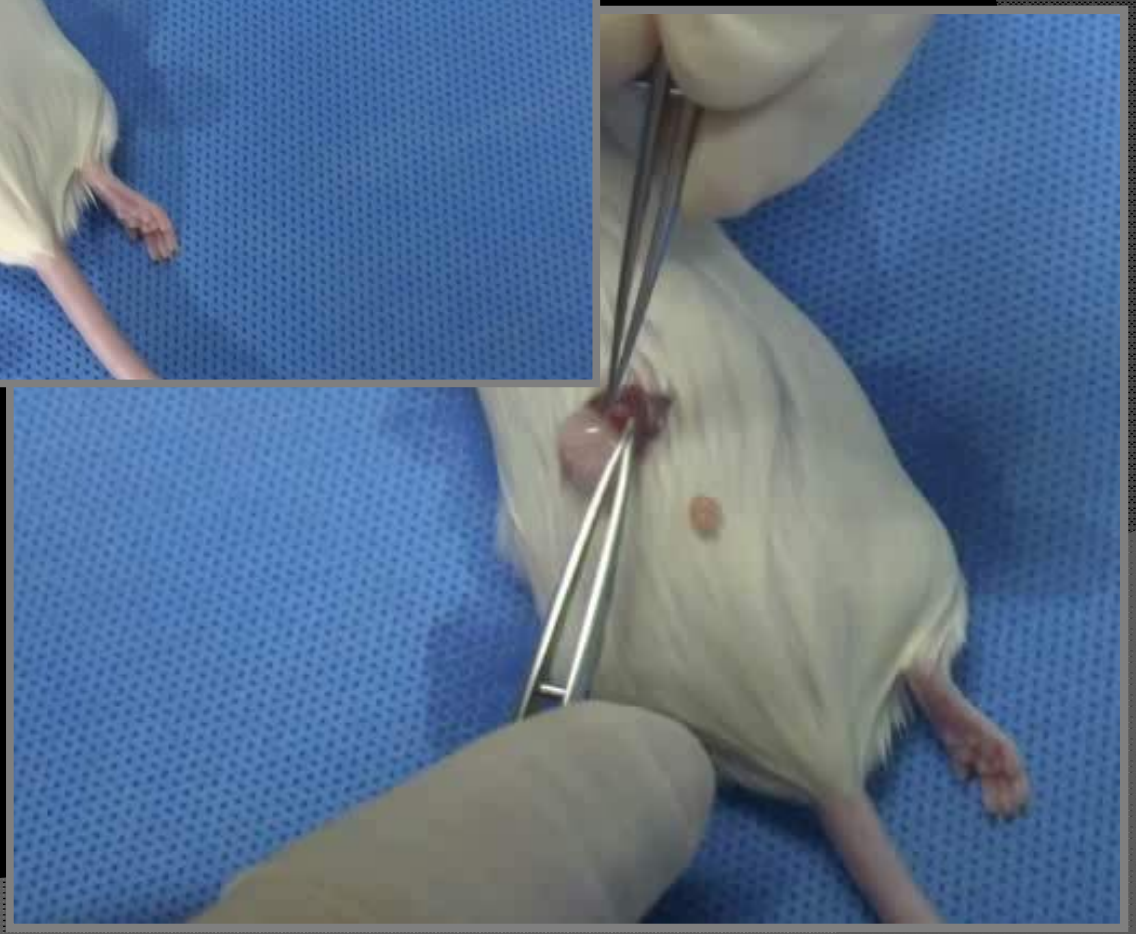


H&E staining

TUNEL assay

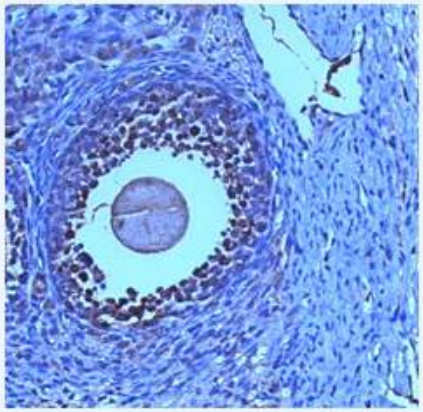
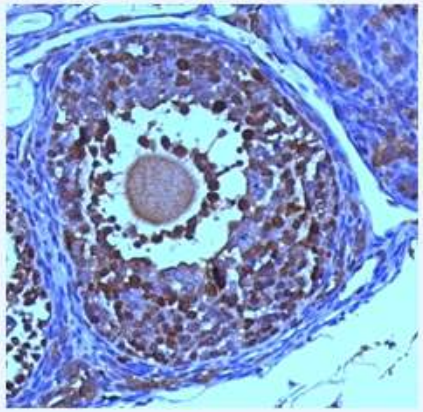
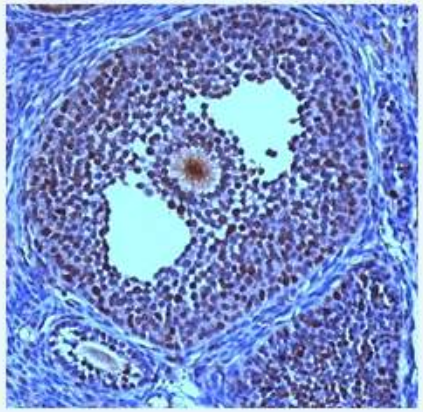
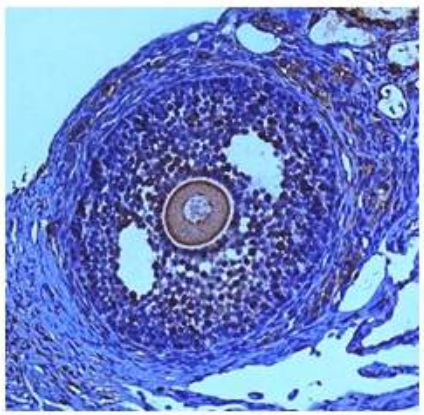
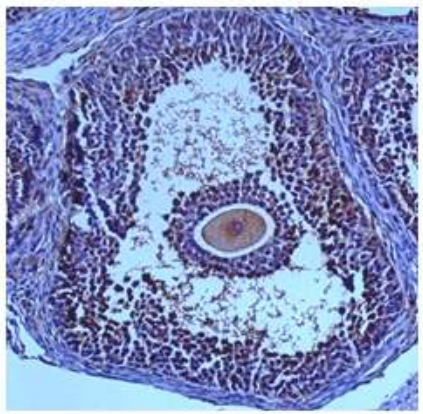
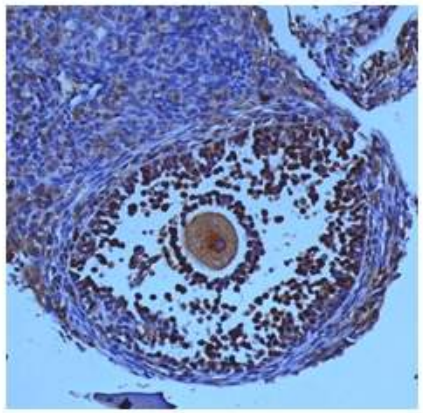
Ki 67 IHC

FSH ELISA



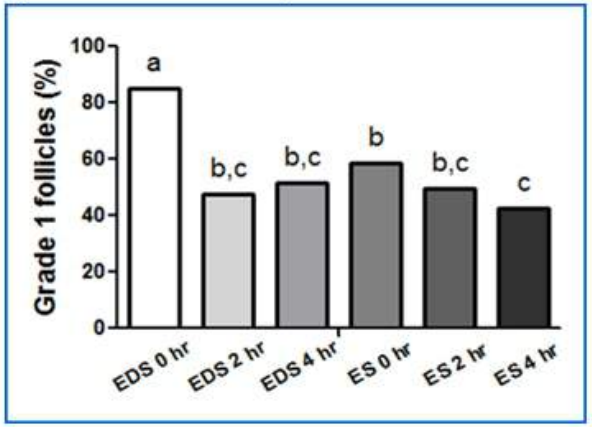
Transplantation into the Kidney Capsule

Ki67 (+) Follicles in OT Grafts

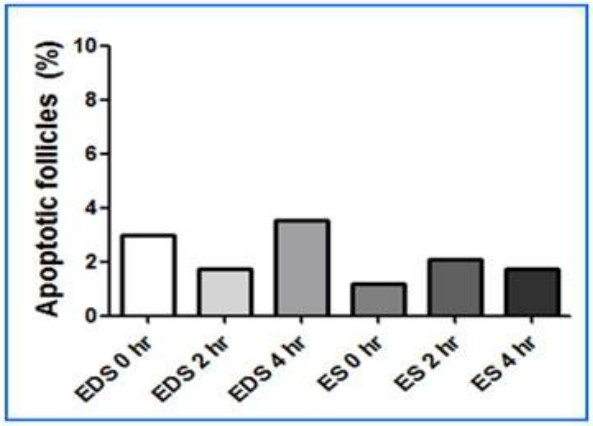
	0 h	2 h	4 h
EDS			
ES			

OT Transplantation (EDS & ES Groups)

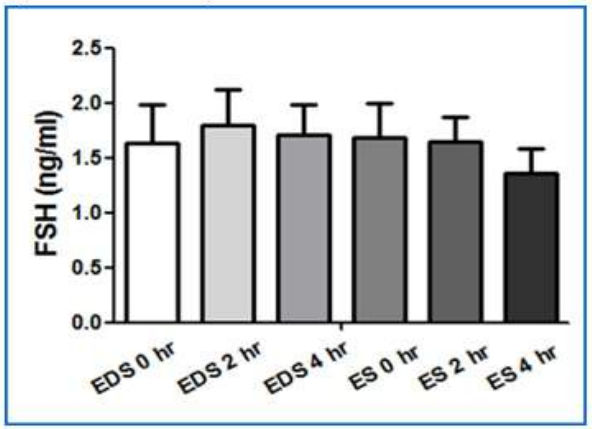
A (Grade 1 follicles)



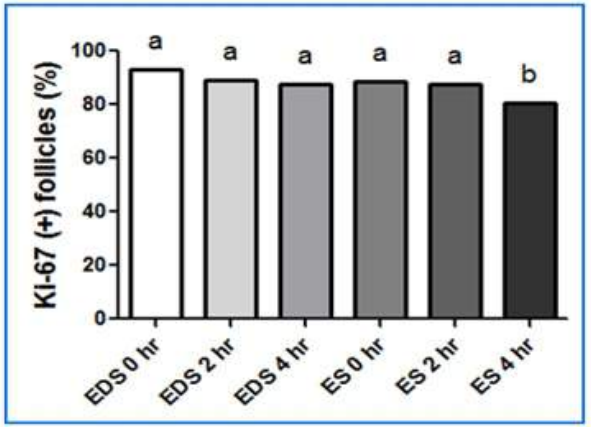
B (Apoptosis follicles)



C (FSH levels)



D (Ki-67 positive follicles)



Conclusions (II)

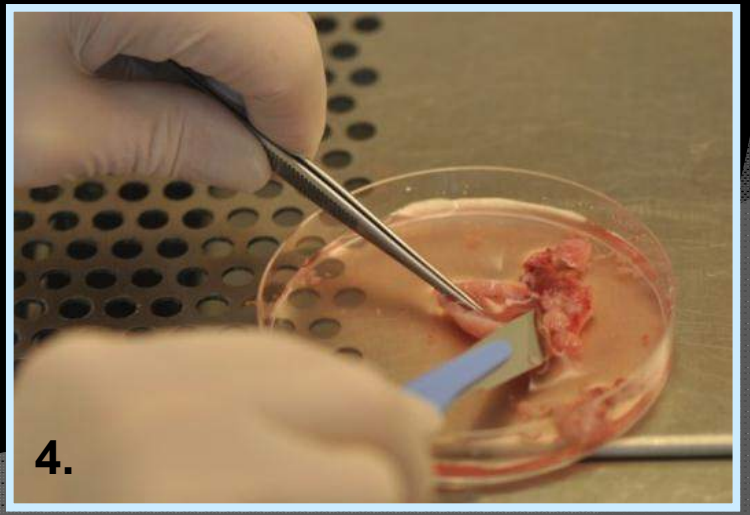
- Similar to the IVC results, the **EDS** was the most effective protocol in this study.
- **Short-term IVC** or **transplantation** are essential to evaluate the quality of vitrified OTs.
- The damaged follicles after vitrification & IVC were not restored after grafting.
- This data will help improve OT vitrification for human as a **basic protocol.**

OT Preparation


1.


2. Human oocyte


3.


4.

Vitrification of Human OT

For Further Study

Cryoinjury

optimize cryopreservation method

supplementation of protective agents

Ischemic injuries

optimal transplantation site

supplementation of angiogenic factors

Thank you.

Introduction

Why mouse..

limited availability of human OT

the mice : an effective model

to assess the risk of malignant recurrence

for the protocols before clinical application

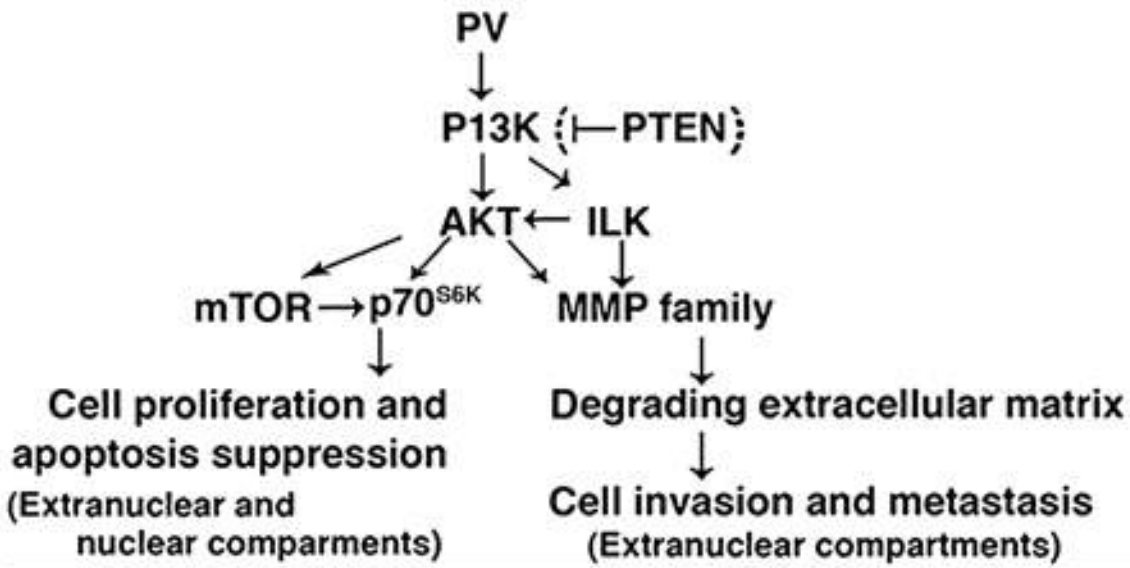
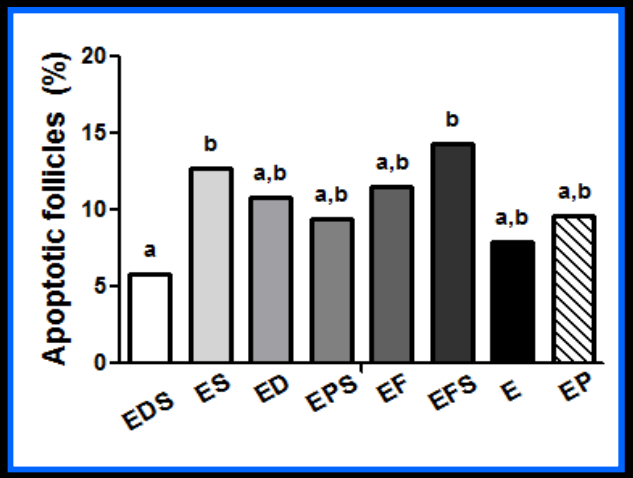


Fig. 6.

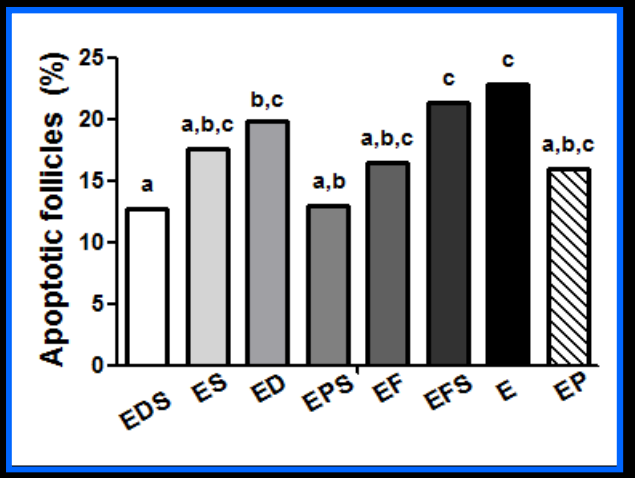
Activation of PI3K signaling by PV. The physical interaction of PV with p85α results in the activation of two PI3K downstream pathways: the AKT–ILK–MMP pathway and the AKT–mTOR–$p70^{S6K}$ pathway. The activation of the former leads to the degradation of the extracellular matrix involved in cell invasion and metastasis, and the activation of the latter results in increased cell proliferation and suppression of apoptosis. The bracket indicates that PV did not have an effect on the expression of PTEN, suggesting that PV-induced activation of PI3K is not mediated by the repression of PTEN.

Apoptotic follicles according to CPA and IVC

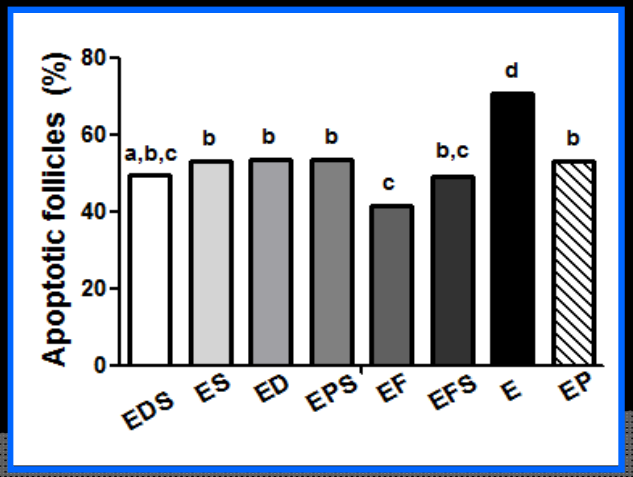
A (0 hr)



B (0.5 hr)



C (2 hr)



D (4 hr)

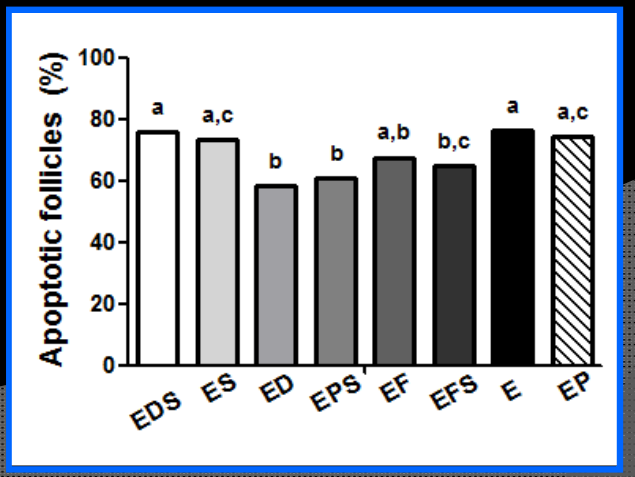


Table 1.2. The number and proportion of morphologically intact follicles (G1) after vitrification and in vitro culture.

IVC duration	0 h			0.5 h			2 h			4 h		
CPA	Follicle No.	G1 No.	G1 %	Follicle No.	G1 No.	G1 %	Follicle No.	G1 No.	G1 %	Follicle No.	G1 No.	G1 %
Control	362	265	73.2^{a}	309	190	61.5^{ab}	292	158	54.1^{bc}	239	102	42.7^{c}
EDS	323	206	63.8^{a}	295	173	58.6^{a}	314	172	54.8^{a}	305	142	46.6^{b}
ES	333	210	63.1^{a}	311	131	42.1^{b}	299	110	36.8^{b}	267	77	28.8^{c}
ED	275	159	57.8^{a}	285	114	40.0^{b}	258	104	40.3^{b}	238	53	22.3^{c}
EPS	361	203	56.2^{a}	328	118	36.0^{b}	292	75	25.7^{c}	328	47	14.3^{d}
EF	211	114	54.0^{a}	223	83	37.2^{b}	248	67	27.0^{c}	322	85	26.4^{c}
EFS	268	143	53.4^{a}	325	144	44.3^{b}	278	75	27.0^{c}	242	51	21.1^{c}
E	221	103	46.6^{a}	220	56	25.5^{b}	326	72	22.1^{b}	179	37	20.7^{b}
EP	223	94	42.2^{a}	326	118	36.2^{ab}	299	96	32.1^{b}	148	19	12.8^{c}

IVC: in vitro culture, CPA: cryoprotective agents, E: Ethylene Glycol, D: Dimethyl sulfoxide (DMSO), P: Propanediol (PrOH), S: sucrose, and F: Ficoll

* Different superscript letters indicate statistically significant differences ($p<0.05$).) and the superscripts were used for each group separately.

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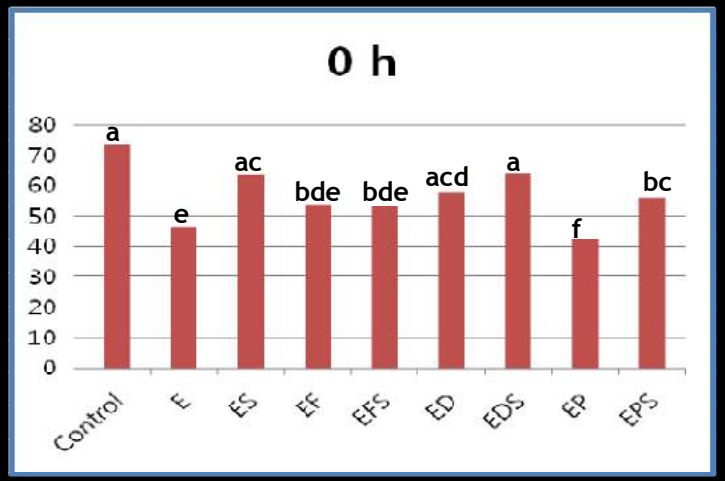
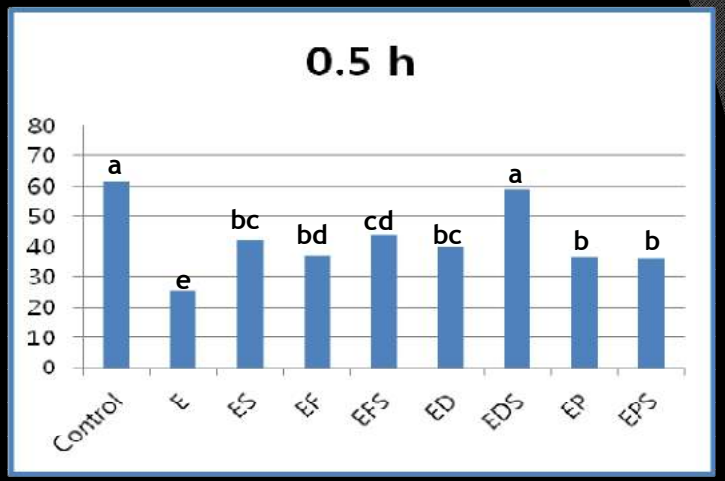
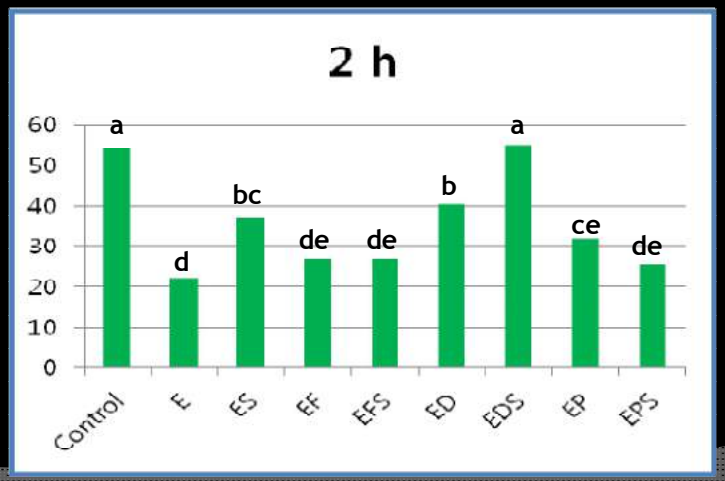
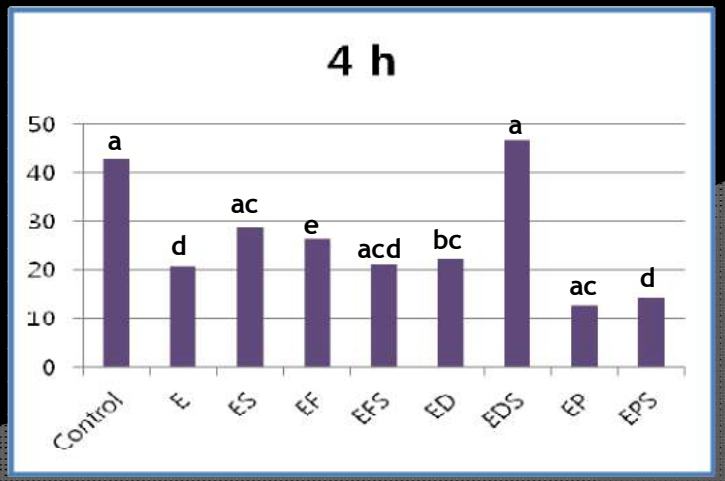
Comparison of G1 follicles after vitrification

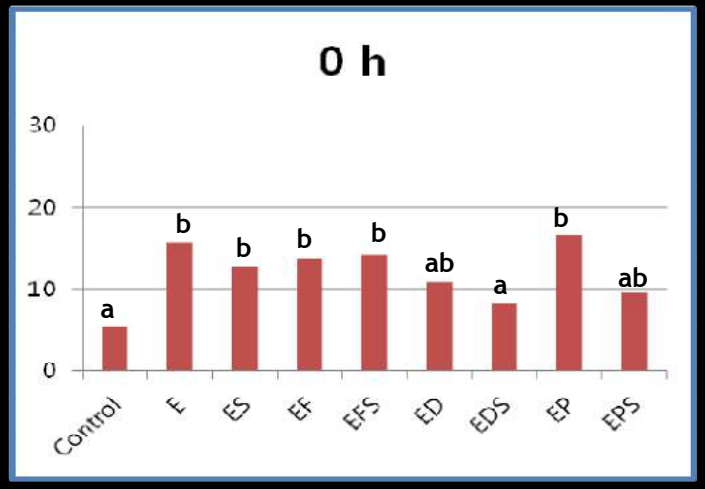


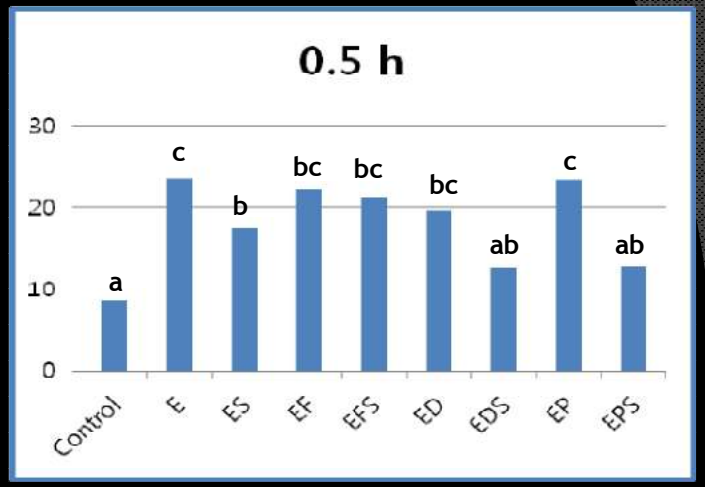


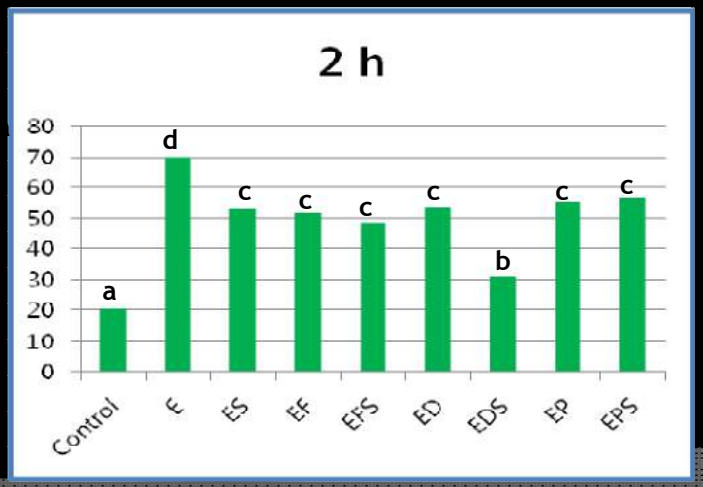


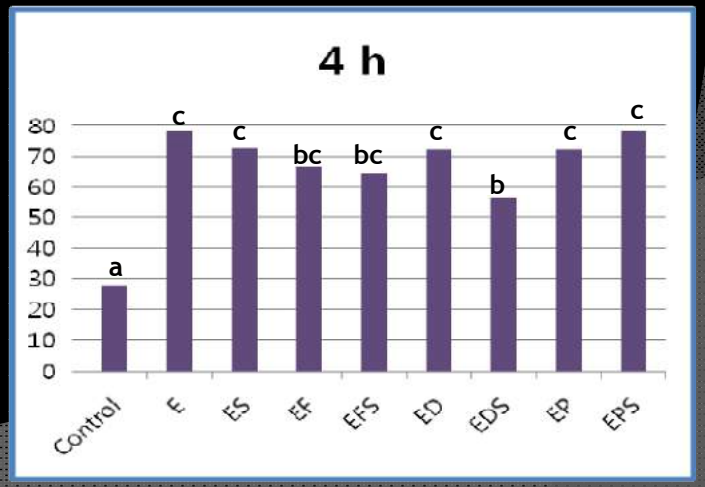


Comparison of apoptosis after vitrification









Introduction

Ovarian tissue (OT)

Cryopreservation & transplantation

→ restore the fertility of cancer patient

→ almost 30 live births in the world

→ a promising alternative to preserve fertility

Slow freezing vs. vitrification

	Slow Freezing	Vitrification
CPA concentration	Lower: 1.5M	Higher: 3-6M
Procedure time	Longer: ~2h	Shorter: ~20min
Technique	Easier	More clinical expertise
LN2 contact	Closed system	Open & Closed system
Freezing machine	Need	No need
Ice Crystal	Yes	No (glassy state)
Cooling Rate	Lower: 0.3C/min	Higher: 15000-30000C/min

Freezing Methods for Reproductive Cells

1) **Slow freezing**

cryoprotectants : 1.0-1.5 M and low

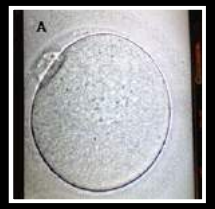
cooling rates : low (0.3~2ºC/min)

2) **Vitrification**

cryoprotectants : 3M and high

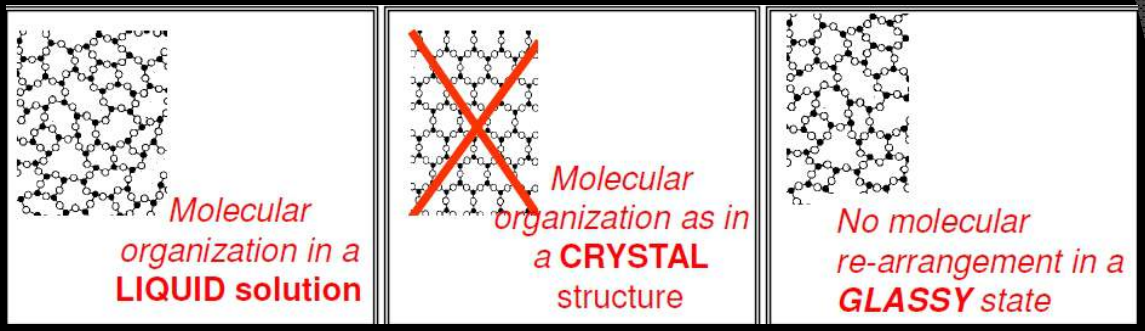
cooling rate : high (over 20,000ºC/min)

Chilling Injury



There are two potential sources of cryo-damage.

1. Ice crystal
2. Dehydration



Large ice crystal → cellular dehydration → cytoplasm: high concentrations of some solutes to toxic levels → stresses & damage on the cell

A little extracellular ice could be tolerable, however any increase in intracellular ice is fatal.

Cryoprotective Dehydration

Many of these damage can be reduced by using CPA.

If oocytes are sufficiently dehydrated by CPA, they can survive in LN2.

- Require of high concentrated osmotic compounds (CPAs)

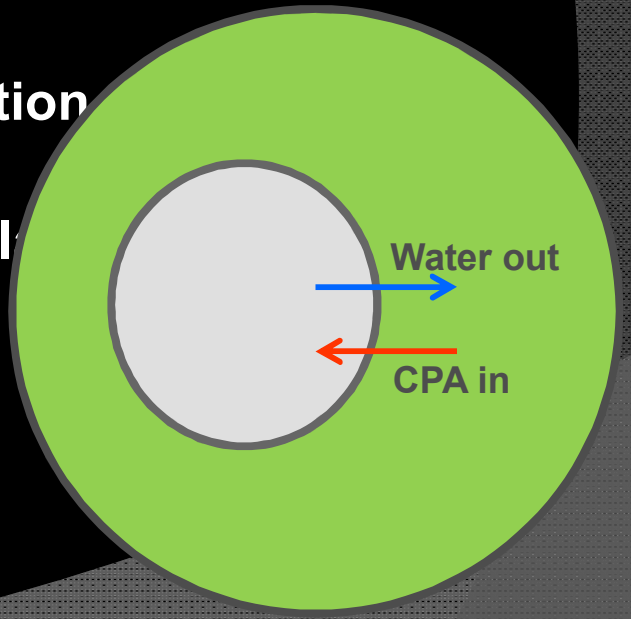
- Reduce more water to prevent ice formation

- Increases the osmotic pressure of cytopl

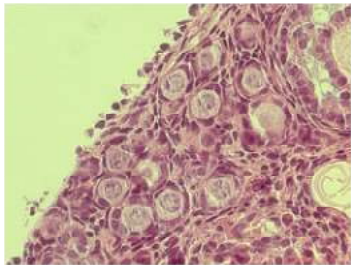
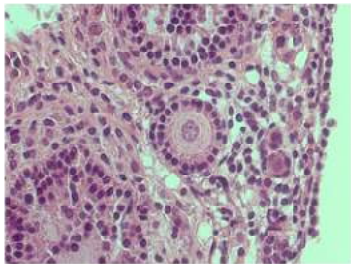
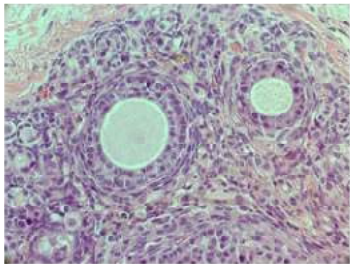
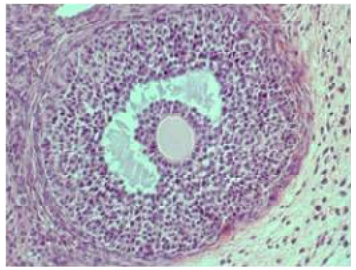
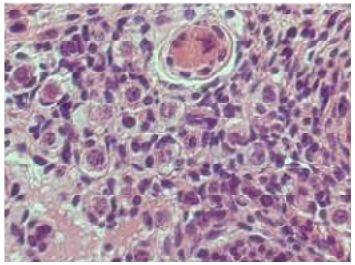
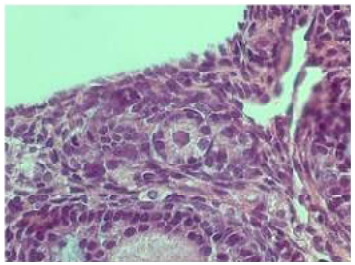
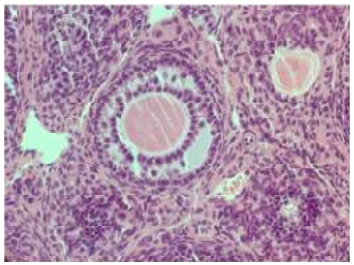
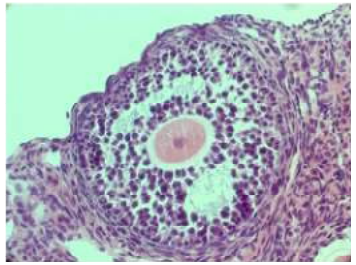
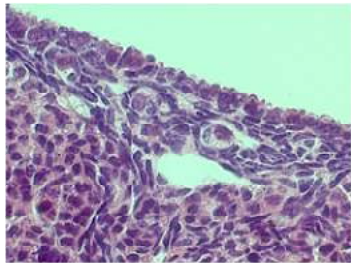
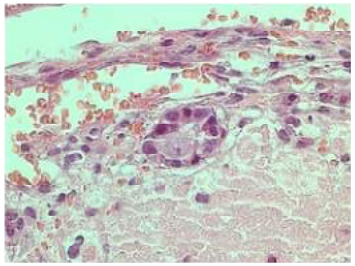
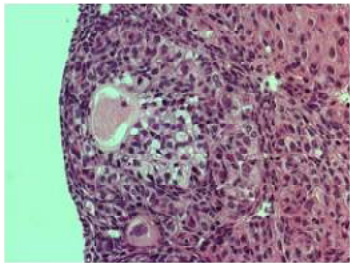
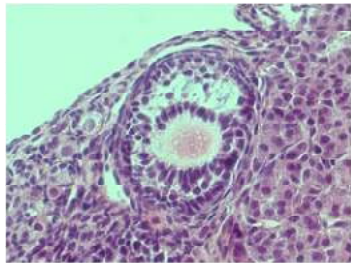
→ Depress the freezing temperature

→ Promote vitrification

→ Inhibit intracellular ice formation

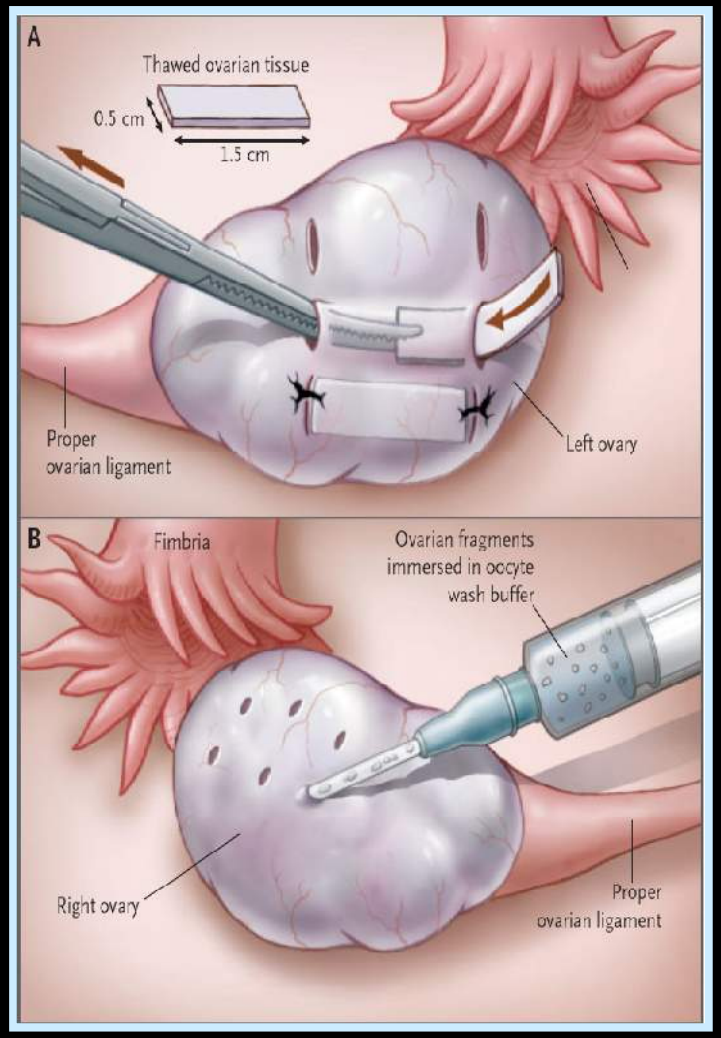


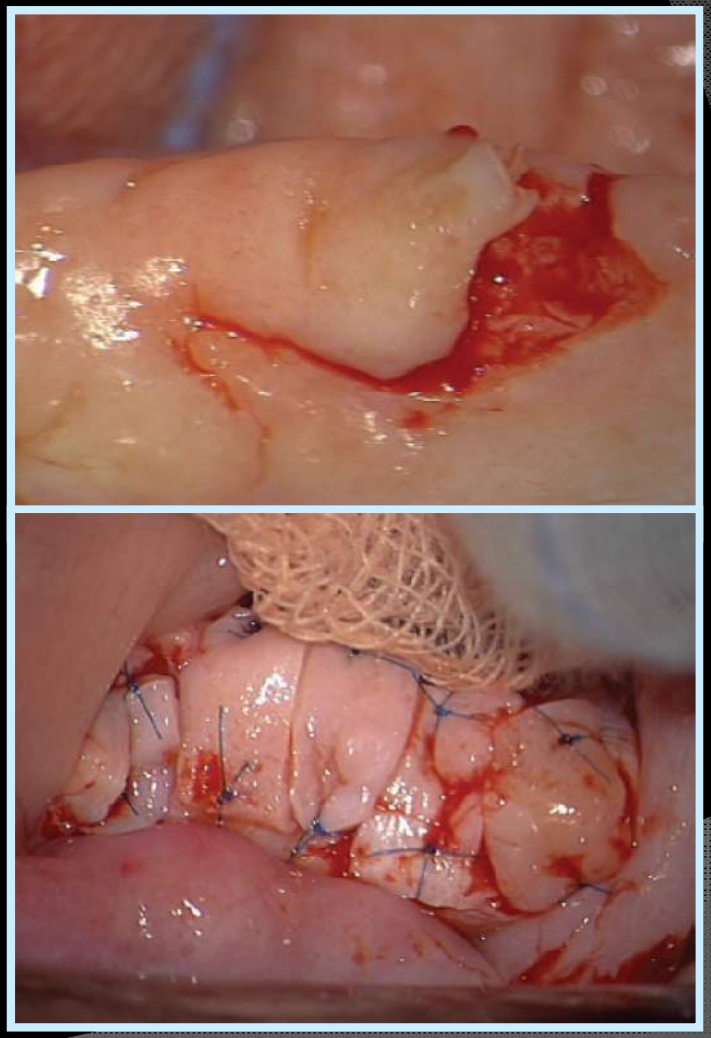
Morphological classification

	Primordial	Primary	Secondary	Antral
G1				
G2				
G3				

Slow Freezing of Ovarian Cortex

Transplantation of Human Ovarian Tissue

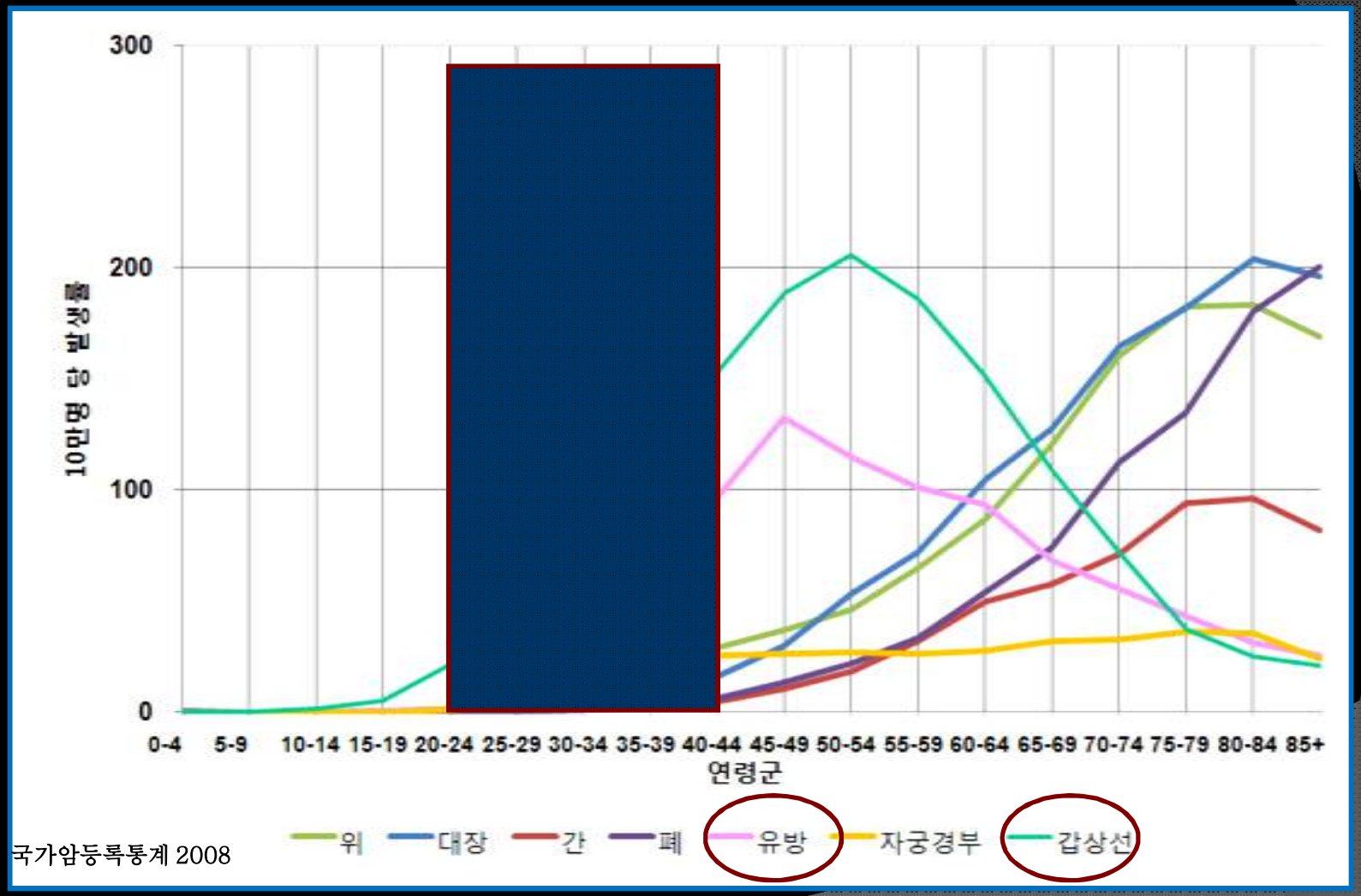




Meirow et al., NEJM 2005;353:318

Donnez et al., Hum Reprod Update 2006;12:519

Female Cancer Incidence According to Age



국가암등록통계 2008

Cryopreservation: Affecting Factors

- Chilling injury
- CPA (toxicity and temperature)
- Osmotic injury
- Speed of freezing and thawing

→ Good Result