Conference Paper

Ovarian Tissue Vitrification as a Method for Ovarian Preservation in Women with Cancer: An Analysis of Granulose Cell Apoptosis

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Abstract

The aim of this study was to obtain the effective method of ovarian function preservation with granulose cell apoptosis assessment. Ovarian tissue vitrification became a method for ovarian function preservation in women with cancer. This technique can be done anytime without delay on cancer therapy, in prepubertal and unmarried patient. It also can store many primordial follicles. Ovarian tissue vitrification study is still limited to animal test and there was no data about apoptosis assessment after ovarian vitrification in human ovary. This is a quasi experimental study which was held in Department of Obstetrics and Gynecology Faculty of Medicine Universitas Indonesia - Dr. Cipto Mangunkusumo General Hospital and Fatmawati Hospital Jakarta from March 2012 to May 2015. Ovaries from thirteen women between 31-37 years of age who underwent oophorectomy with gynecological indication were examined. There were no difference morphologically between follicles from fresh and warmed-vitrified ovaries. The mean protein Bax expression on the fresh ovaries which assessed in the form of H-score was 1,66 ± 0,14 compared to 1,68 ± 0,13 on the warmed-vitrified grup (p = 0,165). The mean protein Bcl-2 expression on the fresh ovaries which assessed in the form of H-score was 1,73 ± 0,10 compared to 1,71 ± 0,10 on the warmed-vitrified grup (p = 0,068). As a conclusion, it was shown that vitrification did not affect Bax and Bcl-2 expression on human ovary.

Keywords: Apoptosis, Bax, Bcl-2, ovarian tissue vitrification

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1. Introduction

Over the past three decades, there has been a remarkable improvement in the survival rates due to progress in cancer treatment, more than 650.000 new female cancer cases are estimated to be diagnosed in 2003 in the USA [1]. Advances in the diagnosis and treatment of childhood, adolescent and adult cancer have greatly enhanced the life expectancy of premenopausal women with cancer. As a result, there is a growing population of adolescent and adult long-term survivors of childhood cancer [2]. Ovaries, which are endowed with an irreplaceable number of follicles, are extremely sensitive to cytotoxic drugs that induce an irreversible gonadal damage [1,3].

Ovarian cortex vitrification is one of the promising freezing techniques in order to maintain reproductive function. But freezing procedure is a process that can cause

follicle damage due to its potential to increase apoptosis process. Assessment of apoptosis by expression of pro and anti-apoptotic proteins can be conducted to determine biomolecular processes occurring intracellular that has been indicated to precede morphological changes. Ovarian cortex vitrification research is still limited in animal experiments and there has been no data to assess the incidence of apoptosis of the granulosa cells after vitrification of human ovarian cortex that can be seen from the expression of apoptosis related genes [1,3,4,5].

2. Methods

All experimental procedures were approved by the Ethical Research Committee of the Faculty of Medicine, Universitas Indonesia After obtaining written informed consent, ovaries were obtained from six women between the ages of 30 and 37 years who underwent oophorectomy due to cervical or breast cancer. Surgeries were performed in the Acute Tertiary Care Hospital in Jakarta during the period of March 2012 to April 2015.

3. Ovarian Tissue Vitrification

Ovarian tissue was suspended in 37°C phosphate buffered saline (PBS) and transferred to the laboratory within 15 minutes. A tissue slicer (Square Measure, Kitazato, Shizuoka, Japan) was used to cut the ovarian cortex into pieces measuring 10 × 10 × 1 mm [6,7].

Two chosen section of ovarian cortexes were divided into two groups. The first group is a control group for assessing Bax and Bcl-2 protein expression using immunohistochemical method. The second group is an experiment group that were frozen by vitrification technique and followed to thawing process, then Bax and Bcl-2 protein expression were assessed with the same immunohistochemical method to be compared with the first group. After being excised, ovarian cortexes were placed in 15 mL of α-MEM *(minimum essential medium)*. Ovarian cortexes were initially equilibrated in 7,5% ethylene glycol (EG); 7,5% dimethyl sulfoxide (DMSO) for 25 minutes, followed by a second equilibration in 20% EG, 20% DMSO, 0,5 mol/L sucrose liquid for 15 minutes. Ovarian cortexes were placed in a minimum volume of solution onto a thin metal strip and submerged directly into liquid nitrogen [7].

4. Immunohistochemical (IHC) Staining of Bax and Bcl-2 Protein in Fresh and Vitrified Human Ovarian Tissue

Bax and Bcl-2 protein staining using IHC were done in fresh and vitrified ovarian cortex from each patient. IHC staining was performed using 3 steps polymer detection system: Starr Trek Universal HRP Detection System (BioSB Santa Barbara, CA, USA). The negative control groups were stained using the same method, with the first antibody substituted by PBS. Rabbit anti-human monoclonal Bax and mouse anti-human monoclonal Bcl-2 antibodies were used (BioSB). All paraffin-embedded samples were

Characteristic	n (%)	Mean ± SD
Age (year)	-	33 ± 1,6
Body mass index (kg/m2)	-	23,6 ± 4,7
Parity		
Nuliparity	3 (23,1%)	-
Primiparity	4 (30,8%)	
Multiparity	6 (46,2%)	
AMH level (ng/mL)	-	2,9 ± 1,6

Table 1: Subject characteristics.

deparaffinized and rehydrated. Sections were incubated in 0,5% H_2O_2 to block endogenous peroxides, and then incubated with blocking background sniper to reduce nonspecific binding. The primary antibody was then applied and the sections kept at room temperature overnight. Universal link (secondary monoclonal antibody from rabbit and mouse) were put on for 15 minutes. After incubating with TrekAvidin-horseradish peroxidase to bind antibody with chromogen, sections were stained with DAB (Diamino benzidin tetraacetic acid) for 5-10 minutes.

The expression of Bax and Bcl-2 proteins was identified as diffuse brown cytoplasmic staining. Ovarian cortex apoptosis were assessed semi-quantitatively with Bax and Bcl-2 protein expression in primordial follicle, primary follicle, and secondary follicle. The number of granulosa cells stained (Pi) in each follicle were counted and the mean Pi from all follicles examined was calculated. The color intensity (i) was scored as weak (1), moderate (2), or strong (3) and the final H-score was calculated with the algorithm: H-score = $\sum$ Pi (i+1).

5. Statistical Analysis

Statistical analyses were performed using Statistical Program for Social Sciences (Chicago, IL, USA) version 20.0. Saphiro-Wilk test was used to assess normality. Comparison between H-score were done using paired T-test.

6. Result

6.1. Sample Characteristics

Samples obtained from thirteen patients between 30-37 years of age who underwent oophorectomy due to cervical cancer (stage IB) or breast cancer (Table 1). The mean level of anti-mullerian hormone (AMH) in these patients was 2.9 ± 1,6 ng/mL.

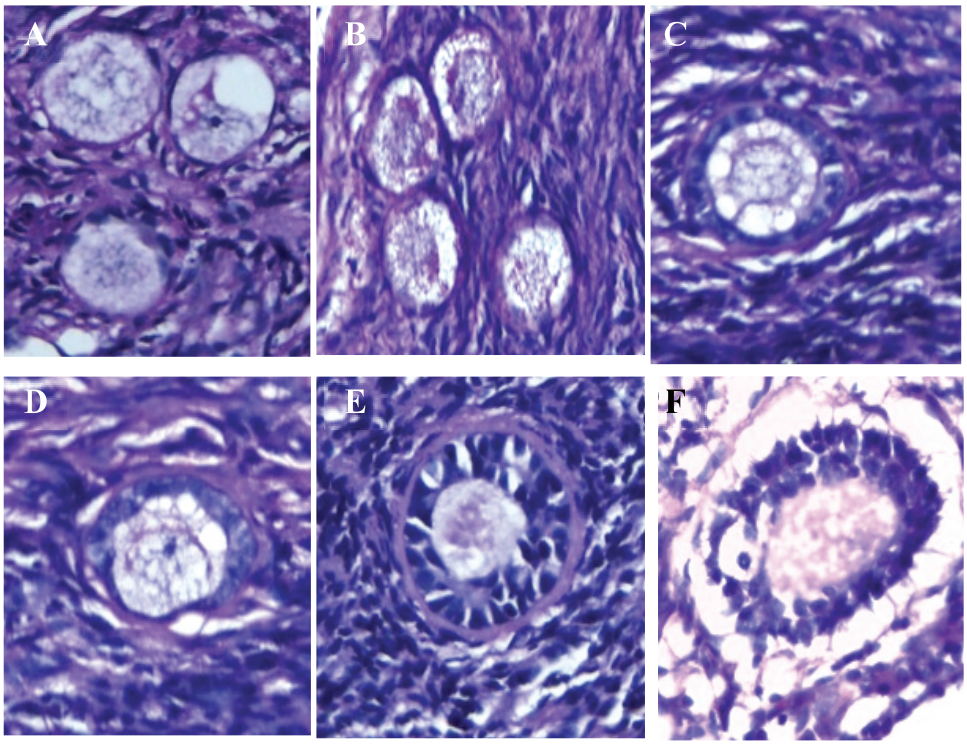

Figure 1: Follicles from fresh and warmed-vitrified ovaries. Primordial follicles (A and B), primary follicles (C and D), secondary follicles (D and E). There were no significant morphological differences between follicles from fresh and warmed-vitrified ovaries based on examinatons of basal membrane, granulosa cells, and oocyte. A, C, E: follicles from fresh ovaries; B, D, F: follicles from warmed-vitrified ovaries.

6.2. Evaluation of Morphology and Apoptosis of Follicles from Fresh and Warmed-Vitrified Ovaries

There were no significant morphological differences between follicles from fresh and warmed-vitrified ovaries based on examinatons of basal membrane, granulosa cells, and oocyte (Figure 1). This findings also supported by the result of IHC staining of Bax and Bcl-2 protein expression, which also showed no changes (Figure 2 and 3). The mean H-score for Bax on fresh ovaries was 1.66 ± 0.14 versus 1.68 ± 0.13 on warmed-vitrified ovaries ($p = 0.165$). The mean H-score for Bcl-2 on fresh ovaries was 1.73 ± 0.10 versus 1.71 ± 0.10 on warmed-vitrified ovaries ($p = 0.068$).

7. Discussion

In this study, vitrification of human ovarian tissue was performed according to the method by Kagawa who used DMSO and EG as a cryoprotectants [7]. No significant difference was found in terms of follicles morphology (basal membrane integrity of follicles, and oocyte) between fresh and warmed-vitrified ovarian tissue. Apoptosis can be initiated by the extrinsic or intrinsic pathways. Bax and Bcl-2 were expressed in granulosa cells, oocytes, and stroma of both fresh and warmed-vitrified ovaries. However, vitrification did not increase apoptosis via the intrinsic pathway in follicles.

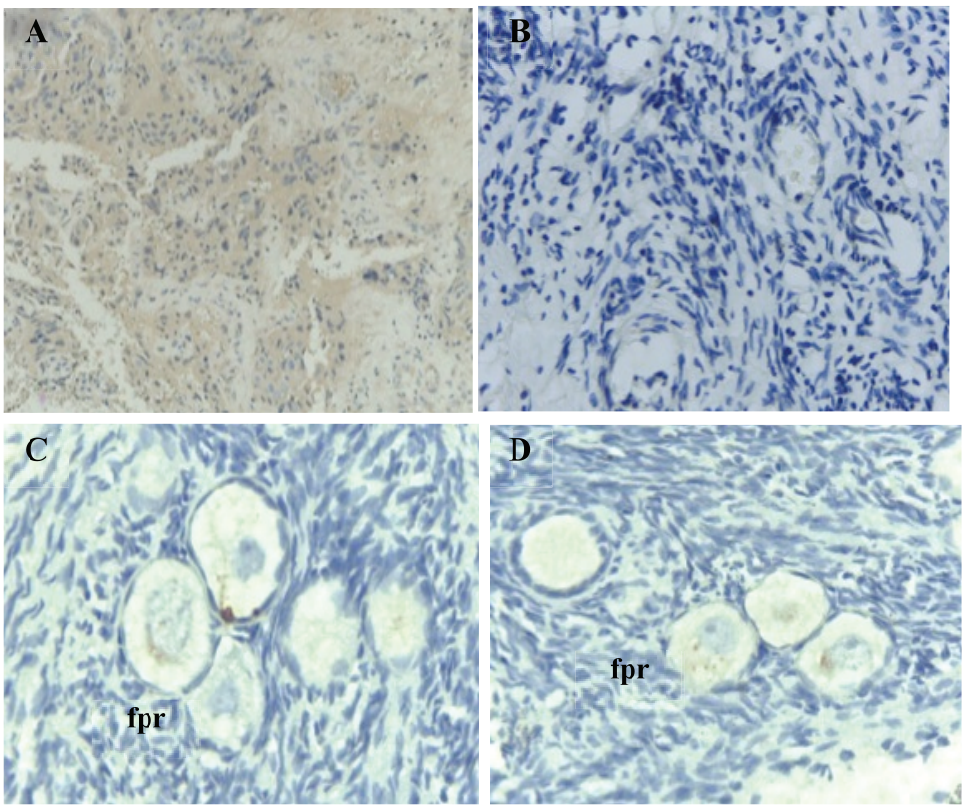

Figure 2: Bax expression on ovarian tissue. A. Bax expression on breast cancer tissue (positive control). B. No visible Bax expression on stromal ovarian tissue (negative control). C. Bax expression on granulosa cells and oocyte with low intensity in fresh ovarian tissue. D Bax expression on granulosa cells and oocyte with low intensity in warmed-vitrified ovaries.
fpr: primordial follicles

Numerous studies also reported no ultrastructural changes in oocytes, ovarian follicles, and stromal cells in post-vitrification ovarian tissue. Sheiki et al found that vitrification is the best method for ovarian tissue cryopreservation. Vitrification liquid that were used on that experiment was ethylene glycol as cryoprotectant. There were also no significant difference among oocytes ultrastructure, granulosa cells, and stromal cells from electron microscope observation [8].

A non-randomized study comparing slow freezing to vitrification was reported on 20 ovarian biopsies. There was no significant difference in the number and morphology of follicles between two groups [9]. Kagawa, Silber and Kuwayama also reported the successful technique using Cryotissue method with the results of oocyte viability and no significant difference in morphology of ovarian cortex between fresh ovary and warmed-vitrified ovaries. This method used ethylene glycol, DMSO and sucrose as a cryopretectants in ovarian cortex vitrification technique [7].

Assesment of ovarian tissue apoptosis can be performed based on morphology, level of apoptosis and protein expression related apoptosis in granulosa cells. With a good assesment, we can understand biomolecular process that happened intracellular. Protein expression changes precede morphological changes, so that not only morphological changes were assessed but it is also impontant to determine protein expression changes in ovaries after vitrification. The expression of Bax and Bcl-2 were

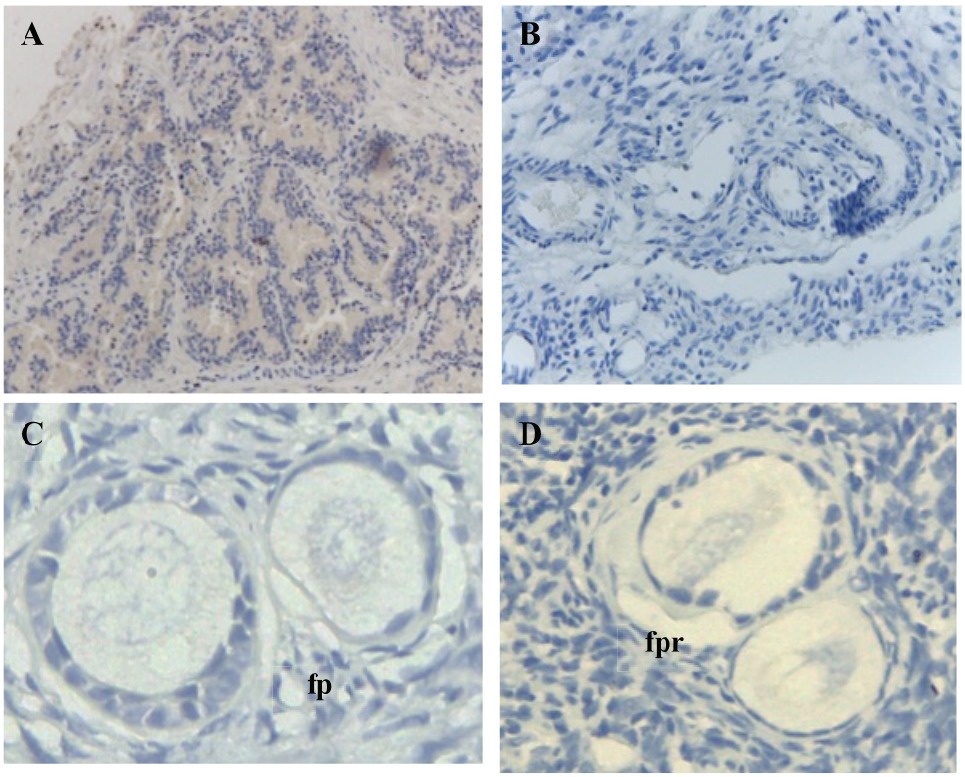

Figure 3: Bcl-2 expression on ovarian tissue. A. Bcl-2 expression on breast cancer tissue (positive control). B. No visible Bcl-2 expression on stromal ovarian tissue (negative control). C. Bcl-2 expression on granulosa cells and oocyte with low intensity in fresh ovarian tissue. D Bcl-2 expression on granulosa cells and oocyte with low intensity in warmed-vitrified ovaries.
fpr: primordial follicles, fp: primary follicle

evaluated because vitrification has been reported to initiate the intrinsic apoptosis pathway [6,10,11].

Apoptotic process were regulated by various cell signaling process, both intrinsic and extrinsic pathway. Extrinsic pathway was initiated by increasing level of death receptor on cell surface [12,13]. Death receptor send apoptotic signal, such as Fas, tumor necrosis factor receptor (TNFR), interferon (IFN), and TNF-related apoptotis-induce ligand (TRAIL) [12]. Intrinsic pathway were initiated by mitochondria permeability and release of pro-apoptotic molecule into cytoplasm without initiation from death receptor. Anti-apoptotic protein such as Bcl-2 and Bcl-x contribute as apoptosis regulator, found in mitochondria membrane and cytoplasm. DNA damage can cause Bcl-2 and Bcl-x loss from mitochondria membrane and replaced by pro-apoptosis protein, such as Bax and Bak. Decrease of Bcl-2 and Bcl-x level were followed by increase of mitochondria permeability, causing cytochrome C released from mitochondria. Cytochrome c will bind to Apaf-1 protein (Apoptotic protease activating factor-1) and caspase-9, and later activate caspase cascade and induce cell death [12,13].

This study showed that vitrification did not affect the morphology of pre-antral follicles and that the expression of Bax and Bcl-2 on both ovarian cortex was unchanged by this process. Wiweko B also reported that pre-antral follicle vitrification did not increase caspase-3 and FasL expression [6]. No significant changes between pro-apoptosis mRNA gen (FasL, Bax, p53, and caspase-3) and anti-apoptosis gen (Bcl-2) in both

fresh and warmed-vitrified ovary also reported by Abdollahi et al [14]. In conclusion, vitrification did not increase apoptosis process in ovary, both via intrinsic and extrinsic pathways.

References

[1] M. Sommezer and K. Oktay, Fertility reservation in female patients, *Human Reproduction Update*, **10**, no. 3, 251–266, (2004).

[2] J. Donnez and S. Bassil, Indications for cryopreservation of ovarian tissue, *Human Reproduction Update*, **4**, no. 3, 248–259, (1998).

[3] HJ. Chang and CS. Suh, Fertility preservation for women with malignancies: current developments of cryopreservation, *J Gynecol Oncol*, **19**, no. 2, 99–107, (2008).

[4] I. Demeestere, P. Simon, S. Emiliani, A. Delbaere, and Y. Englert, Fertility preservation: Successful transplantation of cryopreserved ovarian tissue in a young patient previously treated for Hodgkin's disease, *Oncologist*, **12**, no. 12, 1437–1442, (2007).

[5] P. Jadoul, M.-M. Dolmans, and J. Donnez, Fertility preservation in girls during childhood: Is it feasible, efficient and safe and to whom should it be proposed? *Human Reproduction Update*, **16**, no. 6, Article ID dmq010, 617–630, (2010).

[6] B. Wiweko, *Upaya preservasi fungsi ovarium dengan melakukan vitrifikasi korteks dan folikel pre-antral*, Universitas Indonesia, Jakarta, 2014.

[7] N. Kagawa, S. Silber, and M. Kuwayama, Successful vitrification of bovine and human ovarian tissue, *Reproductive BioMedicine Online*, **18**, no. 4, 568–577, (2009).

[8] M. Sheikhi, K. Hultenby, B. Niklasson, M. Lundqvist, and O. Hovatta, Clinical grade vitrification of human ovarian tissue: An ultrastructural analysis of follicles and stroma in vitrified tissue, *Human Reproduction*, **26**, no. 3, 594–603, (2011).

[9] V. Keros, S. Xella, K. Hultenby, K. Pettersson, M. Sheikhi, A. Volpe, J. Hreinsson, and O. Hovatta, Vitrification versus controlled-rate freezing in cryopreservation of human ovarian tissue, *Human Reproduction*, **24**, no. 7, 1670–1683, (2009).

[10] T. Mazoochi, M. Salehnia, S. Pourbeiranvand, M. Forouzandeh, S. J. Mowla, and E. Hajizadeh, Analysis of apoptosis and expression of genes related to apoptosis in cultures of follicles derived from vitrified and non-vitrified ovaries, *Molecular Human Reproduction*, **15**, no. 3, 155–164, (2009).

[11] H.-C. Liu, Z. He, and Z. Rosenwaks, Mouse Ovarian Tissue Cryopreservation Has only a Minor Effect on In Vitro Follicular Maturation and Gene Expression, *Journal of Assisted Reproduction and Genetics*, **20**, no. 10, 421–431, (2003).

[12] M. R. Hussein, Apoptosis in the ovary: molecular mechanisms, *Hum Reprod Update*, **11**, no. 2, 162–178, (2004).

[13] F. Lumongga, (2008)., Apoptosis. Medan: USU.

[14] M. Abdollahi, M. Salehnia, S. Salehpour, and N. Ghorbanmehr, Human ovarian tissue vitrification/warming has minor effect on the expression of apoptosis-related genes, *Iranian Biomedical Journal*, **17**, no. 4, 179–186, (2013).