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

Budi Wiweko¹, Huthia Andriyana², and Achmad Aulia²

¹Department of Obstetrics and Gynecology, Faculty of Medicine Universitas Indonesia, Dr. Cipto Mangunkusumo General Hospital, Jakarta 10430, Indonesia
²Department of Histology, Faculty of Medicine Universitas Indonesia, Jakarta 10430, Indonesia

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 group (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 group (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

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 cortices 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 cortices were placed in 15 mL of $\alpha$-MEM (minimum essential medium). Ovarian cortices 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 cortices 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
### Table 1: Subject characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (%)</th>
<th>Mean ± SD</th>
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</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>-</td>
<td>33 ± 1.6</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>-</td>
<td>23.6 ± 4.7</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuliparity</td>
<td>3 (23.1%)</td>
<td></td>
</tr>
<tr>
<td>Primiparity</td>
<td>4 (30.8%)</td>
<td></td>
</tr>
<tr>
<td>Multiparity</td>
<td>6 (46.2%)</td>
<td></td>
</tr>
<tr>
<td>AMH level (ng/mL)</td>
<td>-</td>
<td>2.9 ± 1.6</td>
</tr>
</tbody>
</table>

5. Statistical Analysis

Statistical analyses were performed using Statistical Program for Social Sciences (Chicago, IL, USA) version 20.0. Sapiro-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.
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 examinations of basal membrane, granulosa cells, and oocyte (Figure 1). This finding is 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 $1.66 \pm 0.14$ versus $1.68 \pm 0.13$ on warmed-vitrified ovaries ($p = 0.165$). The mean H-score for Bcl-2 on fresh ovaries was $1.73 \pm 0.10$ versus $1.71 \pm 0.10$ 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 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.
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 cryoprotectants in ovarian cortex vitrification technique [7].

Assessment of ovarian tissue apoptosis can be performed based on morphology, level of apoptosis and protein expression related apoptosis in granulosa cells. With a good assessment, 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 important to determine protein expression changes in ovaries after vitrification. The expression of Bax and Bcl-2 were
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 apoptosis-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