

## The Role of Proteins in Apoptosis of Somatic and Germ Cells in the Mouse Ovary

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### Summary

In this study, to determine the mechanisms of cell death in developing follicles, we investigated whether expression of Bcl-2, p53 and Bax play a role throughout the growth of follicles in the mice. Ovarian tissues and oocytes were obtained from 30 Mus Musculus type mouse. The immunostaining of ovarian tissue sections and oocytes was performed using anti-Bcl-2, anti-Bax and anti-p53 antibodies and terminal deoxynucleotidyl transferase (TdT) assay (TUNEL) were also used for detection of internucleosomal DNA fragmentation. In ovarian tissue section evaluation, granulosa cells in antrum of secondary and Graaf follicles were positive with TUNEL staining. Immunoreactivity of Bcl-2 was moderate in primary and secondary follicles of granulosa cells. While immunostaining of p53 was moderate in granulosa cells of Graaf follicles, Bax immunoreactivity was moderate and strong in secondary and Graaf follicles, respectively. When granulosa cells were break off from zona pellucida, there were TUNEL positive cells. In unfertilised oocytes evaluation, TUNEL positive cells were observed in the granulosa cells which were free from zona pellucida. When the granulosa cells were attached with zona pellucida, they were TUNEL negative. While immunoreactivity of Bcl-2 was detected in both oocytes and granulosa cells which were attached with zona pellucida, immunostaining of p53 were only detected in granulosa cells which break off from zona pellucida. In conclusion, regulation of apoptosis in granulosa cells may be controlled by Bax expression and when the granulosa cells were not attached with zona pellucida, they may go into the apoptotic cascade. Therefore; we suggest that, the death of granulosa cells may control signals from intrinsic pathways in the Graaf follicle or from extrinsic pathways after ovulation. However, we consider of further studies to be necessary.

**Keywords:** *Development, Oocyte, Granulosa cell, Apoptosis*

## Fare Ovaryumunda Somatik ve Germ Hücrelerinin Apoptozisinde Proteinlerin Rolü

### Özet

Bu çalışmada gelişen foliküllerde hücre ölüm mekanizmasını belirlemek için büyüyen fare foliküllerinde Bcl-2, p53 ve Bax antikollarının ekspresyonu araştırılmayı amaçladık. Ovaryum dokuları ve oositler 30 adet Mus Musculus tip fareden elde edildi. Ovaryum doku kesitleri ve oositler immunhistokimyasal olarak anti-Bcl-2, anti-Bax ve anti-p53 antikolları ve DNA fragmentasyonunu belirlemek için terminal deoksinukleotidil transferaz (TdT) (TUNEL) yöntemi ile incelendi. Ovaryum kesitlerinin incelenmesinde sekonder folliküllerinin antrumunda ve Graaf folliküllerinin granuloza hücrelerinde TUNEL pozitif hücreler gözlemlendi. Bcl-2 immunreaktivitesi primer ve sekonder folliküllerinde orta şiddetteydi. p53 immunboyanması Graaf folliküllerinin granuloza hücrelerinde orta şiddetteyken, Bax immunreaktivitesi sekonder folliküllerde orta, Graaf folliküllerinde ise şiddetli olarak gözlemlendi. Zona pellusidadan ayrılan granuloza hücreleri ise TUNEL pozitif boyanma gösterdi. Fertilize olmamış oositlerin değerlendirilmesinde, zona pellusidadan ayrılan granuloza hücrelerinde TUNEL pozitif hücreler gözlemlendi. Zonaya yapışık hücreler TUNEL negatif özellikliydi. Bcl-2 immunreaktivitesi oosit ve granuloza hücrelerinin her ikisinde de zona pellusidaya yapışık granuloza hücrelerinde gözlenirken, p53 immunboyanması sadece zona pellusidadan ayrılan granuloza hücrelerinde gözlemlendi. Sonuç olarak, granuloza hücrelerinde apoptozisin düzenlenmesinde zona pellusidadan ayrılan granuloza hücrelerinin apoptotik kaskada uğrayabileceği ve bunun Bax ekspresyonu ile kontrol edilebileceği düşünülmüştür. Bu nedenle, granuloza hücre ölümünün Graaf follikülünde intrinsic pathway, ovulasyondan sonra ise extrinsic pathway ile düzenlendiğini düşünmekteyiz. Bu sonuçlara ek olarak daha ayrıntılı çalışmalara ihtiyaç olduğunu düşünmekteyiz.

**Anahtar sözcükler:** *Gelişim, Oosit, Granuloza hücresi, Apoptozis*

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## INTRODUCTION

Programmed cell death (PCD) or apoptosis is very important mechanism in the embryonic development and tissue homeostasis <sup>1</sup>. Apoptosis is responsible for balancing cell proliferation and maintaining constant cell numbers in tissues undergoing turnover. In addition, during development, apoptosis plays a key role by eliminating unwanted cells from a variety of tissues <sup>2</sup>.

There are at least two broad pathways that lead to apoptosis, an "extrinsic" and an "intrinsic" pathway. The extrinsic pathway begins outside a cell, when conditions in the extracellular environment determine that a cell must die. The intrinsic apoptosis pathway begins when an injury occurs within the cell. The injury could result in necrosis and produce an inflammatory response, but the apoptotic mechanism is in place to ensure that the damaged cell is packaged and removed cleanly, in order to prevent inflammation.

The fetal human ovary contains millions of germ cells, with a peak number observed at fifth month of pregnancy ( $6.8 \times 10^6$  germ cells) <sup>3</sup>. However, at birth, the number of germ cells decreases dramatically, the number of germ cells enclosed in primordial follicles at birth being less than 20% of its peak number <sup>3</sup>. After birth, the store of primordial follicles decreases with time, finally ending in the menopause as the consequence of the exhaustion of the pool of primordial follicles.

In the development; the flattened granulosa cells around primordial follicles become cuboidal, and the oocyte grows as the follicle matures to become a primary follicle <sup>4,5</sup>. The granulosa cells lining the growing follicles acquire receptors for follicle-stimulating hormone (FSH) and proliferate to form two or three layers in secondary follicles <sup>4,5</sup>. Although follicle growth continues in the absence of circulating FSH during the preantral stage, FSH is necessary for the development of follicle antrum <sup>6,7</sup>.

Generally, two major stages of degeneration can be in the ovary: Degeneration of germ cells (attrition), which accounts for the largest loss of oocytes <sup>8</sup> and occurs mainly prenatally, and follicle degeneration (atresia), which occurs during postnatal reproductive life. Ovarian follicular degeneration or atresia is a hormonally controlled apoptotic process, whereby degenerating follicles are eliminated in a coordinated fashion <sup>9</sup>. In the ovary, the mechanisms underlying decisions of life or death involve interaction between pro-apoptotic and anti-apoptotic molecules. Apoptosis is found in ovarian follicles throughout fetal and adult life. During fetal life, apoptosis localized to the oocytes whereas in adult life, it is detected in granulosa cells of

secondary and antral follicles <sup>10</sup>.

Many of members of the Bcl-2 family have been detected in the ovary. Bax, Bcl-2 and Bcl-X<sub>L</sub> expression is highly restricted to the ovary <sup>11</sup>. In rats, ovarian granulosa cell survival promoted by gonadotropin hormones is correlated with a reduction in the level of Bax expression in the absence of any change in Bcl-2 or Bcl-X<sub>L</sub> levels <sup>12</sup>. Subsequent studies of rat <sup>13-16</sup> and human <sup>17</sup> ovary, increased Bax expression has been unceasingly correlated with granulosa cell demise. Developing mouse ovary has shown that apoptosis of fetal germ cells is as well correlated with increased levels of Bax accumulation in the absence of any change in Bcl-2 levels <sup>18</sup>.

The oocyte also undergoes degeneration by apoptosis during atresia. Morphological alterations corresponding to apoptosis have been described in granulosa cells of atretic rat follicles <sup>19-20</sup>. Similar to most cell types apoptotic granulosa cells demonstrate chromatin margination, cytoplasmic vacuolization, cytoplasmic blebbing, both cytoplasmic and nuclear condensation and fragmentation. Biochemical assessments have supported the assignment of apoptosis in granulosa cells by demonstration of internucleosomal DNA fragmentation <sup>19-22</sup>. However, there are several factors that may control apoptosis of granulosa cells. In each stage of the cycle about 50% of the large preantral and antral follicles are in the process of apoptotic death <sup>23</sup>. For example, p53 tumor suppressor protein is transcriptional regulators play in the process of granulosa cell apoptosis. Nuclear accumulation of p53 in rat granulosa cells destined for apoptosis <sup>24</sup>. Furthermore, over-expression of p53 in rat granulosa cells has been reported to trigger a rapid onset of apoptosis <sup>25</sup>. p53 is detectable only in follicle population that concomitantly express high levels of Bax <sup>24,26</sup>. These data therefore suggest that the Bax death gene may in fact be a target for p53 trans-activation in the ovary <sup>27,28</sup>.

In the present study, we investigated the distribution of Bcl-2, Bax and p53 in mouse developing follicles and unfertilized oocytes to reveal the controlling pathway for apoptosis in ovary.

## MATERIAL and METHODS

### Animals

All experiments were performed according to the institutional guidelines for animal experimentation at Celal Bayar University Faculty of Medicine (Manisa, Turkey). We obtained approval from the research ethics board of The University of Celal Bayar for the study. Mus musculus type female mice (n=10), weighing 30 g and 6 weeks of age, were used. The mice were kept in our

laboratory under controlled temperature conditions and a 12 h light/dark regimen (lights on from 7:00 am to 7:00 pm). Mice were used after cycle phase determined with vaginal smear for determination of menstrual phase. They were superovulated with 5 IU/ml single dose of recombinant FSH at estrus phase and sacrificed after 48 h of injection.

### ***Collection of Ovaries***

Left ovaries from all mice were removed and fixed in 10% formaline before embedding in paraffin for light microscopic evaluation. The 5 micron sections from ovaries were stained with hematoxylin-eosin (H&E) for histochemical investigation. The rest of the sections were used for detection of apoptotic cells using both TUNEL and indirect immunohistochemical techniques.

### ***Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End-Labeling (TUNEL) Assay for Ovary***

Immunohistochemical detection of cells undergoing DNA fragmentation was carried out using a terminal deoxynucleotidyltransferase-biotin nick end-labelling (TUNEL) method with a commercial in situ apoptosis detection kit (Dead End Colorimetric TUNEL system, Promega G7130). The TUNEL staining technique labels broken strands of DNA which arise mainly from apoptosis but also from necrosis. Five micrometer thick sections were cut from the paraffin blocks and they were deparaffinized in xylene. They were then incubated with 20 mg/ml proteinase K for 10 min, and rinsed in distilled water. Endogenous peroxidase activity was inhibited by 30 min incubation in 3% hydrogen peroxide in methanol at room temperature, and the sections were then washed several times in phosphate buffered solution (PBS). The sections were then incubated with equilibration buffer for 1 min and TdT-enzyme for 60 min in a humidified atmosphere at 37°C. They were incubated with streptavidin-peroxidase for 45 min. Each step was separated by careful washing in PBS. Diaminobenzidine was used as a chromogen and counterstaining was performed using Mayer's hematoxylin. All steps were performed according to kit instructions. Stained sections examined by light microscopy (Olympus BX 50, Tokyo, Japan).

### ***Immunohistochemistry Assay for Ovary***

After deparafination at 60°C overnight, sections were held in xylene for 1 h. After washing with serial concentrations of ethanol (95%, 80%, 70%, and 60% for 2 min each), the sections were washed with distilled water and PBS for 10 min. They were held in 2% trypsin in Tris buffer at 37°C for 15 min, and then washed in PBS (three 5-min washes). The limits of sections were drawn with a Dako pen (S-2002; Dako, Carpinteria, CA) and

incubated in 3% hydrogen peroxidase for 15 min to inhibit the endogenous peroxidase activity. The tissues were then given three 5-min washes in PBS. The primary antibodies anti-Bcl-2 in a 1/100 dilution (MS-123-P, Neomarkers, Fremont, CA), anti-p53 in a 1/100 dilution (MS-186-P, Neomarkers, Fremont, CA), anti-Bax in a 1/100 dilution (RB-1486, Neomarkers, Fremont, CA) were incubated for 18 h at 4°C. They were then given an additional three 5-min washes in PBS, followed by incubation with biotinylated anti-mouse IgG and administration of streptavidin peroxidase (Histostain Plus kit Zymed 87-9999; Zymed, San Francisco, CA). After washing the secondary antibody with PBS three times for 5 min, the sections were stained with DAB Substrate system containing diaminobenzidine to detect the immunoreactivity, then stained with Mayer's hematoxylin for counterstaining. They were covered with mounting medium (1012; Signet Laboratories, Dedham, MA) and observed with light microscopy (Olympus BX-40, Tokyo, Japan). Control samples were processed in an identical manner, but in the absence of the primary antibody. Serial sections were examined and immunolabelling patterns were compared.

### ***Collection of Oocytes***

Right ovaries were transferred in Gamete™-20 medium at 37°C and approximately 60 oocytes with cumulus cells were collected.

### ***TUNEL Assay for Oocytes***

The oocytes were fixed 4% paraformaldehyde in PBS for 15 min at room temperature, washed twice in PBS and permeabilize for 5 min in 0.01% Triton X-100. In Situ Cell Death Detection Kit (1684817, Roche, Germany) is used<sup>29</sup>. Permeabilized oocytes were washed three times in PBS for 15 min each wash and transferred to the TUNEL-labeling solution containing terminal deoxynucleotidyl transferase and fluorescein-dUTP in reaction buffer for 1 night at +4°C. The oocytes were then washed three times in PBS for 15 min each. Stained oocytes examined by fluorescein microscopy (Olympus BX 50, Tokyo, Japan). Immediately following, oocytes were washed PBS and treated horse radish peroxidase converter POD for 30 min at 37°C. And then oocytes were washed PBS and stained DAB solution. Stained oocytes examined by light microscopy (Olympus BX 50, Tokyo, Japonya).

### ***Immunohistochemical Assay for Oocytes***

Rest of the oocytes were blocked with 10% sheep serum for overnight and incubated with anti-Bcl-2 in a 1/100 dilution and anti-p53 2 in a 1/100 dilution, antibodies for 18 hours +4°C. The oocytes were washed and incubated with anti-mouse FITC secondary antibody (F-

9137, Sigma, USA) conjugated with fluorescein +4°C for overnight. Samples were then washed and evaluated under Olympus BX 50 microscopy.

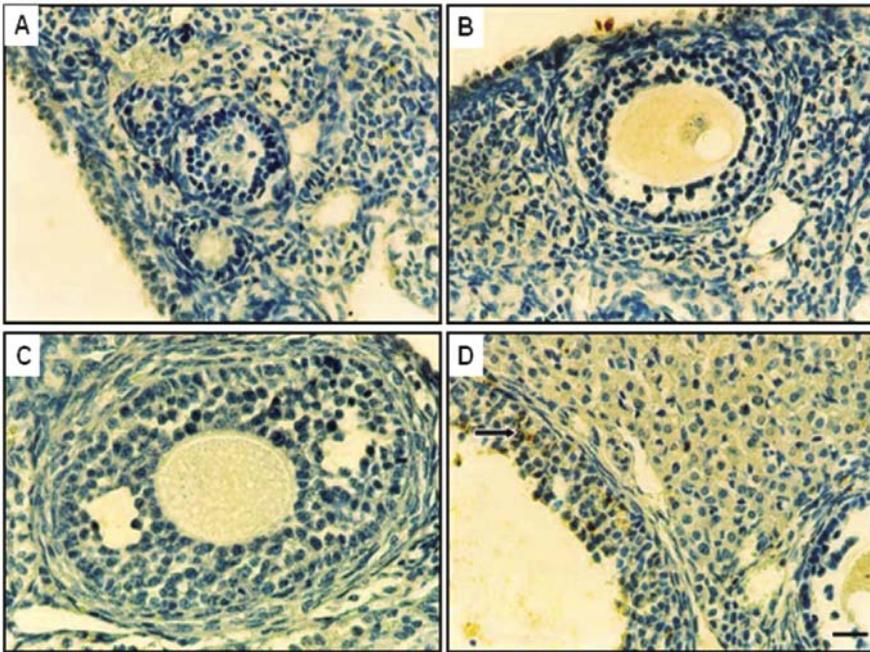
## RESULTS

### Ovaries

After histochemical evaluation of ovaries, cortex including developing of ovarian follicle in periphery side and medulla in central side were observed. After TUNEL staining, TUNEL positive cells were not detected primordial, primary and secondary follicles (Fig. 1 A-C), the apoptotic

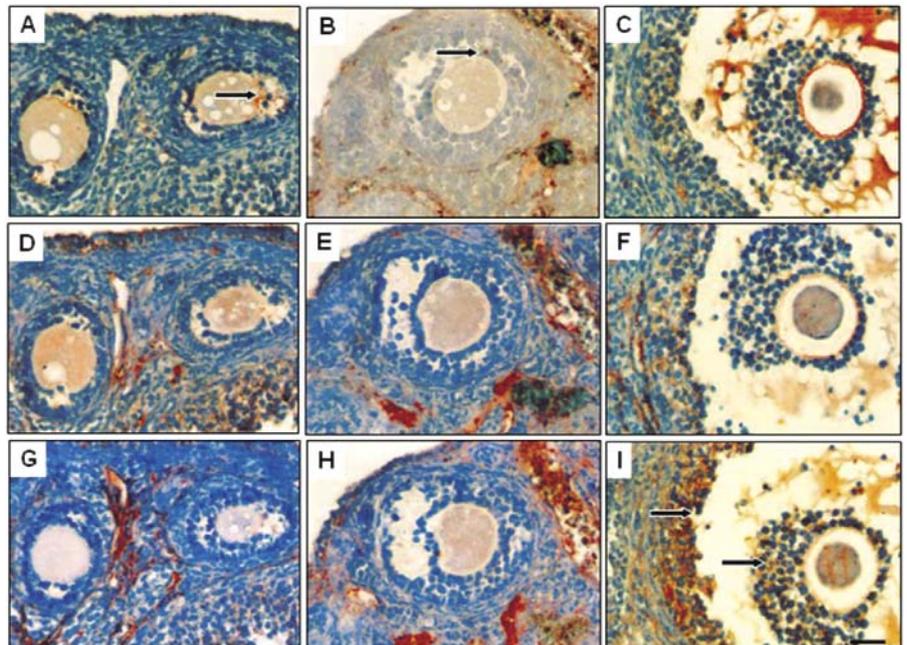
cells were detected in granulosa cells in graafian follicles and these cells were not attached the zona pellucida (Fig. 1D).

While the immunoreactivity of Bcl-2 were detected as a mild in granulosa cells in primary and secondary follicles (Fig. 2A and B, Fig. 2C has not positive cell), p53 immunoreactivity were not detected in any granulosa cells of any follicles (Fig. 2D,E,F,G,H). In addition, strong immunoreactivity of Bax were detected in granulosa cells of graafian follicles (Fig. 2I), this immunoreactivity was absent in granulosa cells of primary and secondary follicles (Fig. 2E).



**Fig 1.** TUNEL staining of primordial (A), primary (B), secondary (C) and graafian follicles (D) Scale Bar: 150 µm. Arrow shows TUNEL positive cell

**Şekil 1.** Primordial (A), primer (B), sekonder (C) ve Graaf (D) follüküllerinde TUNEL boyanması. Scale bar: 150 µm. Ok TUNEL pozitif hücreyi göstermekte



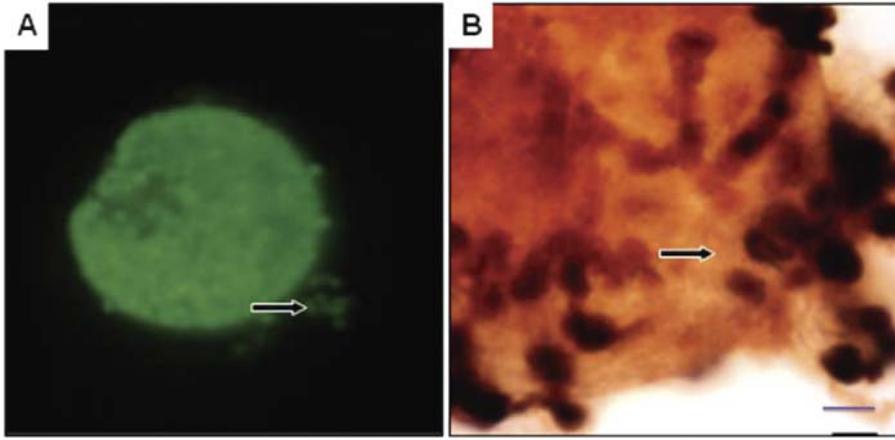
**Fig 2.** Distribution of Bcl-2 (A,B,C), p53 (D,E,F) and Bax (G,H,I) in primary, secondary (A,D,G,B,E,H) and Graafian (C,F,I) follicles. Scale Bar: 150 µm

**Şekil 2.** Primer, sekonder (A,D,G,B,E,H) ve Graaf (C,F,I) follüküllerinde Bcl-2 (A,B,C), p53 (D,E,F) ve Bax (G,I,H) dağılımı. Scale bar: 150 µm

### Oocytes

After stained of unfertilised oocyte, TUNEL positive cells were observed in the granulosa cells which were free from zona pellucida (Fig. 3A, 3B). When the granulosa cells were attached with zona pellucida, they were TUNEL negative.

After immunohistochemical evaluation, positive immunoreactivity of Bcl-2 was observed in both oocyte and granulosa cells which connected with zona pellucida (Fig. 4A). However, the granulosa cells which free from zona pellucida were only p53 positive (Fig. 4B).

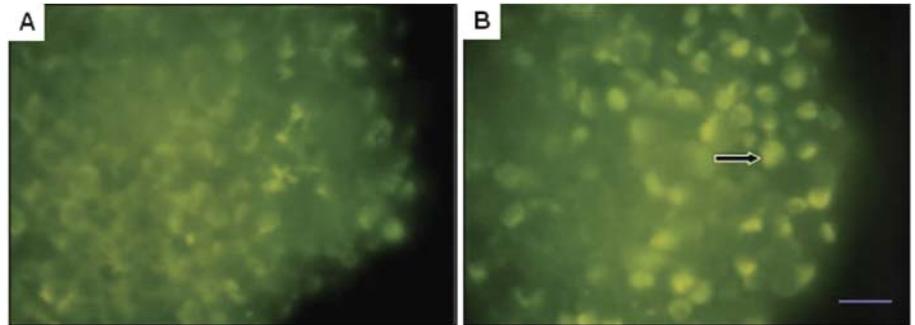


**Fig 3.** TUNEL staining of oocyte with fluorescein (A) or antfluorescein +Conventer-POD+DAB conjugate (B). Arrows indicated TUNEL positive cells. TUNEL positive cells were observed in the granulosa cells which were free from zona pellucida. Scale Bars 150 µm

**Şekil 3.** Oositlerin floresan boyamalı (A) ve floresan olmayan dönüştürücü POD+DAB ile boyanan (B) TUNEL boyanması. Oklar TUNEL pozitif hücreleri göstermekte. TUNEL pozitif hücreler zona pellusidadan ayrılan granuloza hücrelerinde gözlenmekte. Scale bar: 150 µm

**Fig 4.** Immunoreactivity of Bcl-2 and p53 in oocyte with granulosa cells. Arrows indicated immunopositive cells. Scale Bars 150 µm

**Şekil 4.** Granuloza hücreli oositlerde bcl-2 ve p53 immunreaktivitesi gözlenmekte. Oklar immun pozitif hücreleri göstermekte. Scale bar 150 µm



## DISCUSSION

The control mechanism of apoptosis in different tissue is still not well understood. Apoptosis has been demonstrated to be a sign of atresia of follicles in the various growth stages<sup>30</sup>. The mechanism responsible for depletion of the stockpile of germ cells in quiescent primordial follicles throughout adult life has not yet been clarified. In addition, granulosa cell express several proteins for survive of oocyte and they die after apoptotic pathway.

Ovarian follicle is mediated via apoptosis, a process that can be initiated either within ovarian germ or follicle somatic cells depending upon the stage of follicle organization<sup>31</sup>. Apoptosis mediated via several intrinsic

and extrinsic pathways<sup>32,33</sup>. During follicular development, while some granulosa cells will still survive, the others receive cell death signals and they go into apoptotic pathway<sup>34</sup>.

Raffaella et al. demonstrate that Bcl-2 was expressed in the granulosa cells of secondary but not of primordial follicles, and that there is a significant positive correlation between Bcl-2 and the absence of apoptosis<sup>35</sup>. They hypothesize those quiescent follicles, after entering the growing follicle pool, acquire anti-apoptosis protection factors, and that Bcl-2 could be one of these factors. Therefore, follicular cells expressing Bcl-2 may be the

viable cells that will be available for ovulation<sup>35</sup>. Similarly in our study, immunoreactivity of Bcl-2 was detected as a mild in granulosa cells in primary and secondary follicles and both oocyte and granulosa cells which connected with zona pellucida. But, p53 immunoreactivity was not detected in any granulosa cells of any follicles. However, the granulosa cells which free from zona pellucida were only p53 positive.

The apoptosis-suppressing protein Bcl-2 has been overexpressed in ovarian cells under the control of the inhibin-promoter/enhancer<sup>36</sup>. Using an *in vivo* gonadotropin treatment protocol<sup>19</sup>, these animals showed decreased apoptosis of ovarian follicles compared with wild-type animals. Following gonadotropin treatment, ovulation is enhanced; indicating that Bcl-2 overexpression can rescue follicles that otherwise would become atretic<sup>36</sup>.

Bcl-2 overexpression in somatic follicular cells also increases the frequency of germ cell tumors in aging animals, suggesting that prolonged survival of somatic cells may stimulate tumorigenesis of germ cells<sup>36</sup>. Alternatively, follicle atresia may also serve a protective function in normal animals by deleting follicles containing a defective oocyte with tumorigenic potential.

We have shown that, the strong immunoreactivity of Bax was detected in granulosa cells of graafian follicles. But, there was not immunoreactivity of Bax in secondary or primary follicles. The role of Bax in mediating granulosa cell demise has been reinforced by histological analysis of ovaries collected from mice deficient in functional Bax protein<sup>37</sup>. These data have indicated knock-out of Bax gene renders granulosa cells resistant to normal induction of apoptosis in follicles destined for atresia<sup>37</sup>. In contrast, Greenfeld et al. have shown that proapoptotic Bax is an important regulator of follicle survival. They observed that while Bax deficiency led to a reduction in average litter size, and also a reduction in the number of oocyte ovulated in response to exogenous gonadotropins, bax deficiency did not alter follicle atresia<sup>38</sup>.

In addition to apoptotic pathway, many otocrin/paracrine factors also effect of developing follicles. In addition to pituitary endocrine hormones, locally produced growth factors such as insulin-like growth factor-I (IGF-I) may regulate folliculogenesis. Granulosa cells express receptors for IGF-I, and IGF-I synergizes with gonadotropins to promote granulosa cell differentiation<sup>39,40</sup>. Furthermore, similar to the action of gonadotropins, IGF-I suppresses follicle apoptosis<sup>41</sup>. Estrogens are indispensable for the growth and maturation of follicles. Treatment with estrogen increases the division of granulosa cells and increases ovarian weight<sup>42</sup>. Atretic follicles exhibit decreased estrogen production and a lower estrogen/androgen ratio in the follicular fluid, suggesting the importance of local estrogens for the maintenance of healthy follicles<sup>43</sup>. Interleukin-1 (IL-1), a cytokine initially identified as a regulator of the immune system, also affects ovarian functions<sup>44</sup>. In many cell types, treatment with IL-1 increases the synthesis of nitric oxide (NO), which, in turn, activates soluble guanyl cyclase<sup>45</sup>. In the pre-ovulatory follicle culture, treatment with IL-1 increases NO production and inhibits apoptosis<sup>46</sup>.

In the future studies to study both hormonal regulation of ovarian follicle and the apoptotic mechanism will provide a better understanding of the molecular process underlying ovarian cell demise.

In conclusion, our result demonstrated that, while granulosa cells were survive during follicle development, initiation of apoptosis of granulosa cells were observed

in graafian follicles with increased expression of Bax. In addition, after ovulation, when the granulosa cells were attached the zona pellucida, they were survive. Regulation of apoptosis in granulosa cells was controlled with Bax expression and when the granulosa cells were not attached with zona pellucida, they may go to apoptotic cascade. Therefore, the death of granulosa cells may control signals from intrinsic pathways in graafian follicle or from extrinsic pathways after ovulation. However, immunohistochemical assays alone are not sufficient to decide this issue and further experimentation is necessary.

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