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Apoptosis-related genes expression in primary in vitro culture of human ovarian granulosa cells

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Abstract

Ovarian granulosa cells (GCs) play a crucial role in oocyte maturation, creating a favorable microenvironment around the oocyte. Therefore, enhanced apoptosis and GCs loss may negatively affect the intrafollicular milieu and compromise the oocyte quality, reducing pregnancy chances. Based on the RT-qPCR method, the present research revealed the differential expression of apoptosis-related genes (*BCL2*, *BAX*, *p53*, *CASP9*) during the seven days of primary *in vitro* culture of GCs isolated from patients undergoing *in vitro* fertilization (IVF) procedure. Individual gene expression changes may reflect the GCs survival and/or apoptotic status at different time points.

Running title: Apoptosis-related genes expression in granulosa cells *in vitro*

Keywords: apoptosis-related genes, granulosa cells, primary culture

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Introduction

The balance between cell survival and pro-apoptotic factors defines eukaryotic cell lifespan *in vivo* and under the conditions of *in vitro* culture [1–3]. The cells undergo apoptosis either due to the activation of specific apoptotic signaling pathways and/or via the inactivation of survival anti-apoptotic pathways. Tissue-specific apoptosis plays a significant role in tissue remodeling and development programs [4,5]. The apoptosis of the granulosa cells has been shown to play a decisive role in maintaining the number of maturing follicles within the ovary in each species of animals [6]. Granulosa cells participate in follicular development, the corpus luteum formation, and function [7]. The granulosa cell fate appears to be determined by the complex signaling network involving distinct paracrine, autocrine, and endocrine pathways [8,9]. Understanding the mechanisms regulating granulosa cell survival and apoptosis is of great clinical importance as impaired signaling in any of these pathways is likely to contribute to the development of pathophysiological conditions of female reproductive tract, such as granulosa cell tumours, cystic ovaries or infertility.

The BCL-2 proteins family has been identified to play a significant role in activating the apoptotic cascades. The observation showing that BCL-2 is involved in targeting Raf-1 to mitochondria and Raf-1, in turn, can phosphorylate pro-apoptotic BAD *in vitro* suggested the possible involvement of these proteins in transducing the reduction in Raf-MEK-ERK pathway into apoptotic signals [10]. The expression profiles of BAX, BCL-2, and BCl-XL are modulated according to the proliferative and apoptotic status of granulosa cells suggesting the vital role of these apoptotic factors [11]. Furthermore, it has been demonstrated that *p53* is highly expressed in apoptotic granulosa cells and directly modulates the transcriptional activity of *BCL2* and *BAX* genes [12,13]. Additionally, the scientific reports showing that the inhibition of caspases attenuates the apoptosis of the granulosa cells underline the role of the caspases in this process [14].

Considering this background, the presented research was aimed to determine the expression of apoptosis-related genes – B-cell lymphoma 2 (*BCL2*), *BCL2* Associated X (*BAX*), tumor protein *p53* gene, and Caspase 9 (*CASP9*) – in primary *in vitro* culture of human ovarian granulosa cells (GCs).

Materials and Methods

Patients Characteristics

Human GCs were obtained from 3 infertile female patients (mean age 33.67 years ± 1.46 (SEM) undergoing *in vitro* fertilization (IVF) procedure at the Centre of Diagnosis and Treatment of Infertility at Division of Infertility and Reproductive Endocrinology, Poznan University of Medical Sciences. Controlled ovarian hyperstimulation was performed using recombinant human follicle-stimulating hormone (rhFSH; Gonal F, Merck sp. z o.o, Poland or Puregon, MSD Poland sp. z o.o, Poland) and highly purified human menopausal gonadotropin (hMG; Menopur, Ferring Pharmaceuticals Poland sp. z o.o, Poland) in individualized doses. Gonadotropin-releasing hormone (GnRH) antagonist protocol (Cetrotide, cetrorelix 0.25 mg, Merck sp. z o.o, Poland or Orgalutran, ganirelix 0,25 mg, MSD Poland sp. z o.o, Poland) was used to suppress the function of the pituitary gland. Ovulation was stimulated with rh chorionic gonadotropin (rhCG; Ovitrelle, 250 ug, Merck sp. z o.o, Poland) to induce oocyte maturation on days 9 to 12 after initial administration of gonadotropin. Follicular fluid containing GCs was collected using transvaginal ultrasound-guided oocyte pick-up 36 h after rhCG injection.

Patients with diagnosed tubal infertility factors were selected for the study. The selected patients had no history of ovarian surgery, polycystic ovarian syndrome (PCOS), endometriosis, nor other chronic or endocrine diseases and were characterized by a BMI < 30 kg/m^2.

Isolation and primary GCs culture

The procedure of GCs isolation was performed as described previously [15,16]. Briefly, the follicular fluid was washed twice with supplemented DMEM and centrifuged for 10 min at 200 g at room temperature to separate and collect GCs. The medium was changed every 48 hours of culture.

Total RNA isolation and RT-qPCR analysis

Total RNA was isolated at 7 time points, corresponding to 7 days of *in vitro* cultivation. The improved Chomczyński-Sacchi method was used for RNA isolation [17]. The GCs were collected from the culture vessels and suspended in 1 ml of a monophase solution of guanidine thiocyanate and phenol (TRI Reagent®, Sigma; Merck KGaA). The chloroform was then added, with the samples centrifuged to obtain three separate phases. Total RNA was located in the upper, aqueous phase. RNA was then precipitated with 2-propanol (Sigma; Merck KGaA, catalog number I9516), added in an amount adequate for 1 ml of TRI-reagent, and washed twice with 75% ethanol. RNA quantity and purity were examined spectrophotometrically (Epoch, Biotek, Bad Friedrichshall, Germany). Reverse transcription was performed according to the protocol provided by the manufacturer — SABiosciences (RT2 First Stand kit-330401). RT-qPCR was carried out using LightCycler (Roche Diagnostics GmbH, Mannheim, Germany). The amplification process was carried out using 2 µL of cDNA solution, 18 µL of QuantiTect® SYBR® Green PCR (Master Mix Qiagen GmbH, Hilden, Germany) and primers (**Tab. 1**).

TABLE 1 Sequences of primers used in RT-PCR for analysis of gene expression

GENE	PRIMER SEQUENCE	
BCL2	F:	ATGTGTGTGGAGAGCGTCAA
	R:	GAAATCAAACAGAGGCCGCA
BAX	F:	TGACATGTTTTCTGACGGCA
	R:	CACCCTGGTCTTGGATCCA
*p*53	F:	GCTGAATGAGGCCTTGGAAC
	R:	TTATGGCGGGAGGTAGACTG
CASP9	F:	TGATGTCGGTGCTCTTGAGA
	R:	CGCAACTTCTCACAGTCGAT
ACTB	F:	AAAGACCTGTACGCCAACAC
	R:	CTCAGGAGGAGCAATGATCTTG

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 8 software (USA). One-way analysis of variance (ANOVA) with Tukey's post hoc test was used to determine differences between the groups. The results are presented as mean ± standard deviation (SD), and $P < 0.05$ was considered statistically significant.

Ethical approval

The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance with the tenets of the Helsinki Declaration. It has been approved by the authors' institutional review board or equivalent committee. Poznan University of Medical Sciences Bioethical Committee approval no. 1290/18.

Informed consent statement

Informed consent has been obtained from all individuals included in this study.

Results

The obtained results revealed the differential expression of apoptosis-associated genes. *BCL2* mRNA levels fluctuated during the seven days of *in vitro* culture (**Fig. 1**). There was an initial decrease on day 2 ($p < 0.001$), after which *BCL2* expression leveled off during the next days. Finally, *BCL2* transcript levels increased significantly on day 6 and 7 ($p < 0.001$).

The expression of the *BAX* gene remained unchanged during days 1-4, followed by a significant drop on day 5 ($p < 0.001$). However, during the next two days (D6, D7), the expression level achieved the initial values and continued to rise ($p < 0.01$) (**Fig. 2**).

The expression of the *p53* gene grew on day 2, remained constant during the next days, and decreased significantly on day 5 ($p < 0.001$). Next, the transcript level of *p53* rose substantially on day 6 ($p < 0.001$) . (**Fig. 3**).

There was a gradual decrease in *CASP9* expression until day 5 ($p < 0.01$), followed by a slight growth on day 6, which became significant on day 7 ($p < 0.001$) (**Fig. 4**).

Discussion

Despite having approximately 400,000 to 500,000 follicles in different development stages within the human ovary, there are only very few in each cycle that fully mature, while others become eliminated by atresia, a process exhibiting both morphological and molecular features of programmed cell death [18–20]. Moreover, in each reproductive cycle, a new corpus luteum is formed, and the old one degenerates through the luteolysis, which by nature represents the programmed cell death process [21,22].

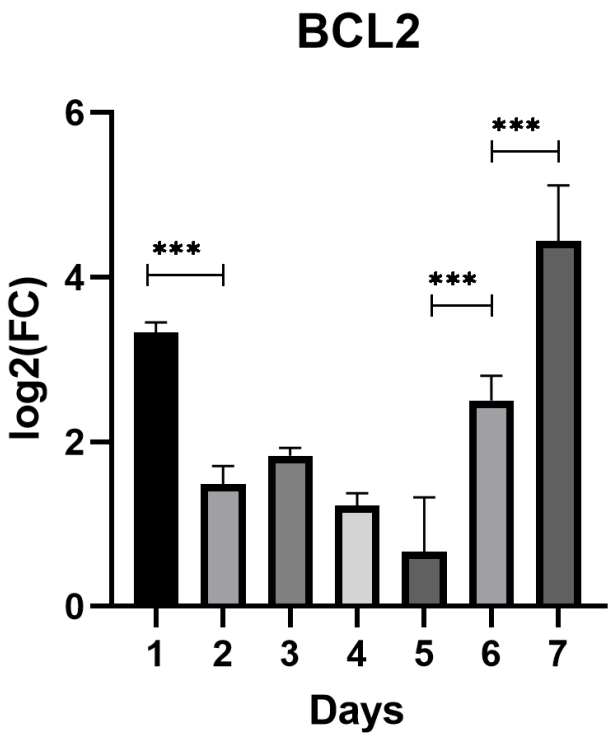

FIGURE 1 *BCL2* gene expression levels in GCs primary culture. Results are shown as mean ±SD. *p <0.05, ** p <0.01, *** p < 0.001

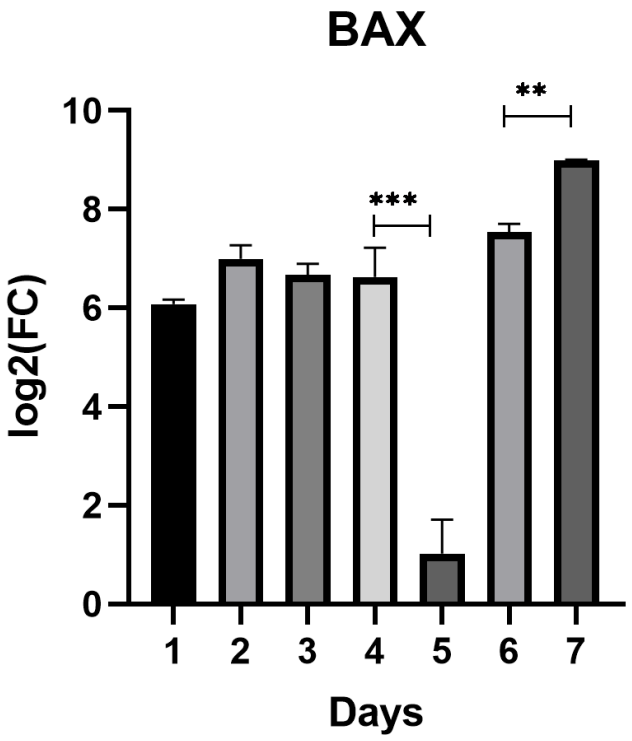

FIGURE 2 *BAX* gene expression levels in GCs primary culture. Results are shown as mean ±SD. *p <0.05, ** p <0.01, *** p < 0.001

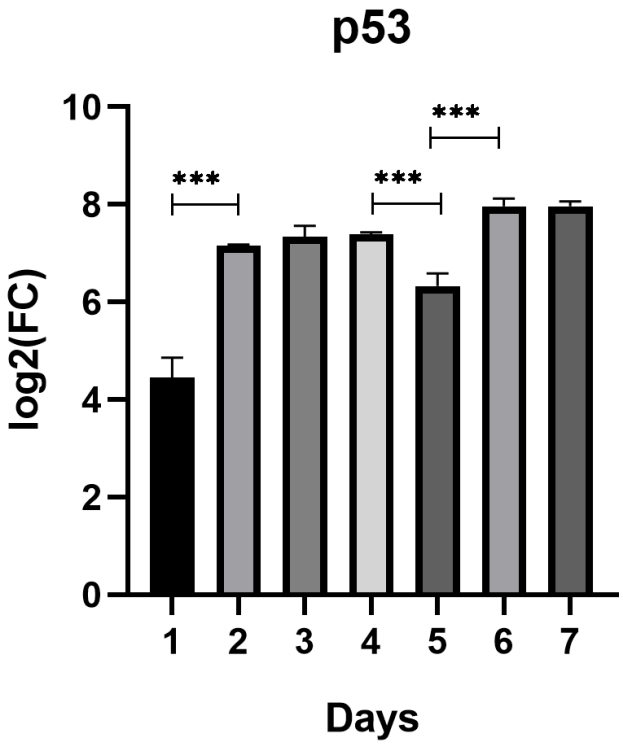

FIGURE 3 *P53* gene expression levels in GCs primary culture. Results are shown as mean ±SD. *p <0.05, ** p <0.01, *** p < 0.001

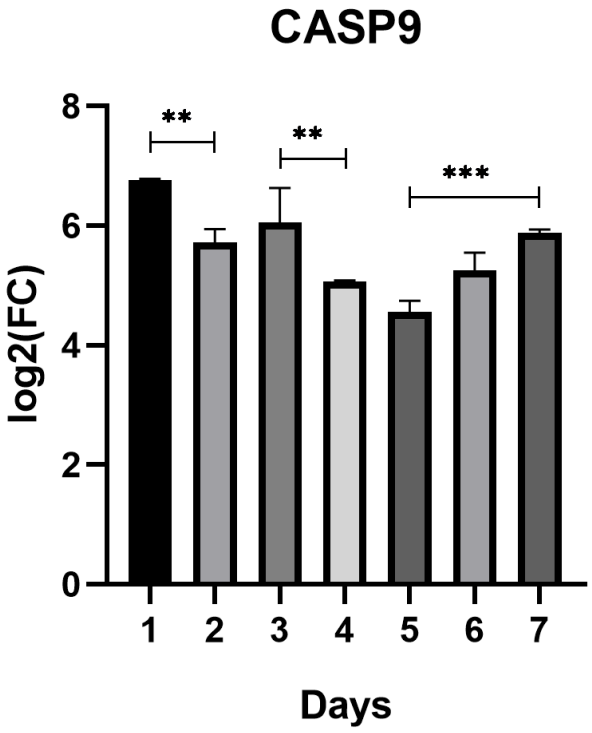

FIGURE 4 *CASP9* gene expression levels in GCs primary culture. Results are shown as mean ±SD. *p <0.05, ** p <0.01, *** p < 0.001

Apoptosis is considered to represent a key mechanism of germ cell loss in newborn ovaries. Later, after menarche, approximately 1000 follicles are lost monthly, and after 35 years of age, this rate increases [23].

The expression and balance of the apoptotic-related genes *BCL2* and *BAX* are considered to regulate the apoptosis of germ cells [24,25]. In ovarian GCs, the gonadotropin-mediated inhibition of apoptosis has been reported to be associated with changes in the expression of several cell death-related genes, including members of the BCL-2 family (*BCL2*, *BCL-X*, and *BAX*) [13].

The immunohistochemical analysis of rat ovarian sections revealed that ovary expression of Bcl-2 and BAX proteins was identified mainly in GCs [26]. Primordial and primary follicles of newborn rat ovary showed intensive nuclear staining for BAX but faint staining for Bcl-2. Adult and one-month-old rat GCs showed increased staining both for Bcl-2 and BAX proteins. Taking into consideration the apparent link between the expression of the apoptosis-regulating proteins Bcl-2 and BAX and follicular development, the authors suggested Bcl-2 and BAX involvement in rat ovarian GCs demise.

The *in vitro* study conducted by Gebauer *et al*. indicated that *BAX* protein levels in isolated GCs remained unchanged until 72 h of culture, followed by a small change from 96 h onwards. At the same time, the Bcl-2 level showed little or no change until 96 h with a decrease starting from 108 h onwards [27]. In the present study, we have examined the mRNA levels of both *BAX* and *BCL2* genes. Noticeably, *BCL2*/*BAX* ratio was improved on days 5-7, which may indicate the switch between the apoptotic and survival status of cultured cells.

Interestingly, Lobach *et al*. investigated whether the expression of apoptosis-related genes in human ovarian GCs can be useful for follicular quality assessment and prediction of IVF outcomes [28]. The authors have found that Caspase-8, *BCL2*, and *BAX* were not related to ovarian response to controlled ovarian stimulation. By contrast, a positive correlation between Caspase-3 (CASP3) gene expression and the dose of gonadotropins required for ovarian stimulation were reported. Furthermore, there was a negative correlation between CASP3 expression and the number of preovulatory follicles, collected, mature and fertilized oocytes, and the number of viable embryos.

In our study, we investigated the expression of *CASP9* in order to find out whether the *in vitro* conditions could modulate the apoptotic signaling in GCs. By contrast to Caspase-3, which serves as an effector caspase, Caspase-9 represents the canonical initiator caspase in the intrinsic mitochondrial pathway [29]. In general, mRNA levels of *CASP9* did not correlate with *BCL2*/*BAX* gene expression at most time points. However, on day 5, there was a downregulation of both *CASP9* and *p53* gene expression accompanied by a change in *BCL2*/*BAX*, which might favor GCs survival over apoptosis.

Conclusions

Ovarian GCs play a pivotal role in oocyte maturation, creating a favorable microenvironment around the oocyte. Therefore, enhanced apoptosis and GCs loss may negatively affect the intrafollicular milieu and compromise the oocyte quality reducing the chances of pregnancy. The present research revealed the differential expression of apoptosis-related genes during the seven days of primary *in vitro* culture of GCs isolated from patients undergoing IVF. The changes in individual gene expression may reflect the GCs survival and/or apoptotic status at different time points.

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Conflict of interest statement

The authors declare they have no conflict of interest.

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