Experimental
&
Clinical Article

Reproductive Medicine; *Endocrinology and Infertility*

Does the Apoptosis Value of Cumulus Cells Play a Role in Rescue Oocyte in Vitro Maturation?

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ABSTRACT

OBJECTIVE: In this study, we aimed to investigate the role of the cumulus cell's apoptosis parameter in the maturation of immature rescue oocytes.

STUDY DESIGN: In this experimental study, donated immature germinal vesicle oocytes were cultured for, in vitro maturation, embryo development in matured germinal vesicle oocytes were compared with apoptotic properties of cumulus cells.

RESULTS: In all of the immature oocytes after oocyte in vitro maturation, the maturation rate has been observed as 56.1% and 2PN rate as 63.0%. Afterin vitro maturation of germinal vesicle oocytes, there was no difference in apoptosis rates of the cumulus cells between mature and immature oocytes (p> 0.05). The ratio of 2PN in matured germinal vesicle oocytes showing embryo development was 35.4%. A positive correlation was found between luteinizing hormone values on day 3 and E_2 values during HCG days during oocyte maturation and embryo development (p=0.021, p=0.020). In addition, it has been observed that the germinal vesicle oocytes, which have completed their maturation and developed into embryos, have high E_2 values during HCG days (p=0.020).

CONCLUSION: In our study, it has been demonstrated that in vitro maturation in rescue oocytes from stimulated cycles, embryo development potential could not be explained by the apoptosis parameter.

Keywords: Apoptosis, Cumulus cells, In vitro oocyte maturation human, Tunel assay

Gynecol Obstet Reprod Med 2021;27(3):223-241

Introduction

Use of gonadotropins in *in-vitro* fertilization (IVF) applications results in a large number of follicles and it is observed that oocytes obtained from the follicles did not complete maturation by nearly 20%. This rate is higher in patients with polycystic ovary (1).

In-vitro maturation (IVM) is one of the favorable techniques for immature oocytes to mature under laboratory conditions and is often preferred in unstimulated cycles as another option to IVF therapy in patients with polycystic ovary syndrome (PCOS) (2). IVM is not only for patients with the polycystic ovary, at the same time it may be a good treatment option for giving poor ovarian response patients (3). There are limited publications on in vitro maturation of oocytes in the germinal vesicle (GV) phase or metaphase 1 (M1) phase in stimulated cycles. It is observed that the cases which are responded poorly to gonadotropins are also responded poorly to the rescue IVM treatment in the same cycle (3). Rescue in vitro maturation (IVM) is currently not a routine procedure in association with IVF. Mehrdad Mir Farsi and colleagues' study has shown that 46% of the GV oocytes obtained from the stimulated cycles are matured, and about 25% of them are meiosis-associated anomalies (4).

Apoptosis means genetically programmed cell death and it has an important role in the physiological events of the mammals. It is well known that ovarian follicles in the verte-

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Submitted for Publication: 13.03.2020 Revised for: Publication 09.04.2020
Accepted for Publication: 02.05.2020 Online Published: 15.06.2020

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Quick Response Code:	Access this article online
	Website: www.gorm.com.tr e- mail: info@gorm.com.tr
	DOI:10.21613/GORM.2020.1087

How to cite this article: *Irez T. Ercan S. Ciraci E. Aksoyer SB. Toprak MS. Dayioglu N. Guralp O. Does the Apoptosis Value of Cumulus Cells Play a Role in Rescue Oocyte in Vitro Maturation? Gynecol Obstet Reprod Med 2020;27(3):234-241*

brates may be exposed to apoptosis by a procedure called atresia and this apoptosis is initiated in the granulosa cell layer. Apoptosis has been shown to be involved in the uterine adaptation process, implantation, and pathological conditions as well as follicular atresia (5). In many studies, the rate of blastocyst development in embryos obtained from oocytes of atretic follicles was found below. It is suggested that apoptosis is a very important part of the abnormal follicular development process that causes a pathological condition in the ovaries in the name of oocytes and it is also the mechanism that provides the luteolysis of the corpus luteum (5). Granulosa cells are the first cells to be affected by apoptosis. According to previous studies, the lack of gonadotropins leads to strong atresia in the ovary. In the primordial phase of follicular development, the first cell to be affected by apoptosis is the oocyte. Apoptosis performs many functions, including the formation of the blastocyst cavity, before implantation of the embryo (6). Although the rate of apoptosis is not precise in the cumulus cells, it may be a sign of embryo development potential. Cumulus cells are thought to play an important role in oocyte maturation and fertilization, with signaling and regulation of functions (3). Many studies have shown us that oocyte quality and fertilization potential are related to the rate of granulosa cell apoptosis in women receiving IVF treatment (7). It has also been shown that granulosa and cumulus cells play an important role in oocyte maturation due to cell-cell relationship, therefore the emergence of apoptosis or necrosis in granulosa cells may be associated with future embryo quality and poor IVF/intracytoplasmic sperm injection (ICSI) outcomes. (8-10). It was reported that lower apoptosis degree correlated with oocyte development potential in cumulus cells in cattle and human studies (8-11).

In this study, we aimed to investigate the maturation levels and fertilization of GV oocytes obtained from stimulated IVF cycles, embryo development capacities, related to whether it is associated with cumulus apoptosis. Although the developmental potentials most after maturation of the GV oocytes obtained from stimulated cycles were not found, it is aimed to investigate the possibility that high potential developmental oocytes may be predictable by studying apoptosis from these potentially potent influencing factors.

Material and Method

This work was approved by the ethics committee of Zeynep Kamil Hospital approved with 116 number dated 6/12/2013 the study and written informed consent was obtained from patients before oocyte collection.

Oocyte collection :In this study, 98 germinal vesicles (GV) stage immature oocyte and cumulus cells of 30 volunteers (below 35 years old and endometriosis cases not included) were evaluated after controlled ovarian hyperstimulation and oocyte pick up. Under standard culture conditions (37 °C and 5% CO_2 in the air), it was cultured in 50 mL Quinns' Advantage Fertilization medium (Sage IVF, Biocare Europe) for 2-4 hours. After incubation of hyaluronidase (40 IU/mL; Sage IVF, Biocare Europe), the cumulus cells were removed by pipetting. The cumulus free oocytes were classified as mature (MII) or immature, the latter having no polar body (PB) or nuclear structure (MI, metaphase I), or those with a germinal vesicle structure without PB in the perivitelline area.

The cumulus cells of MII and GV oocytes were spread on the slide and left to dry and fixed with 96% ethanol for at least one hour (for apoptotic evaluation).

Oocyte in vitro maturation, Intracytoplasmic sperm injection and embryo development: In this prospective study, oocytes in the germinal vesicle stage from the oocyte retrieval procedures at IVF center were cultured in culture medium containing 75 mIU/mL follicle-stimulating hormone (FSH) and 100 mIU/mL HCG for 24 hours (IVM medium-Origin, USA), and then assessed for extrusion of the first polar body The embryonic growth was achieved by conducting ICSI with matured oocytes and embryo development was followed up today 5.

Evaluation of apoptosis: To determine the presence of apoptosis-specific DNA strand breaks, fixed smears were treated for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using the Cell Death Detection Kit (Boehringer, Mannheim, Germany) containing fluorescein isothiocyanate (FITC) - labeled dUTP. This method adds FITC-labeled nucleotides to the exposed ends of multiple DNA fragments resulting from apoptosis-induced internucleosomal DNA breakage; this produces a fluorescent signal in place in the respective cell nuclei. Preliminary experiments were performed including supravital staining of cell suspensions with propidium iodide according to the manufacturer's instructions for distinguishing between apoptotic and necrotic cells.

At the end of analysis were evaluated with Green stained apoptosis-positive (+), with blue stained apoptosis negative (-), and blue-Green stained partial apoptosis (Figure 1a, b, c).

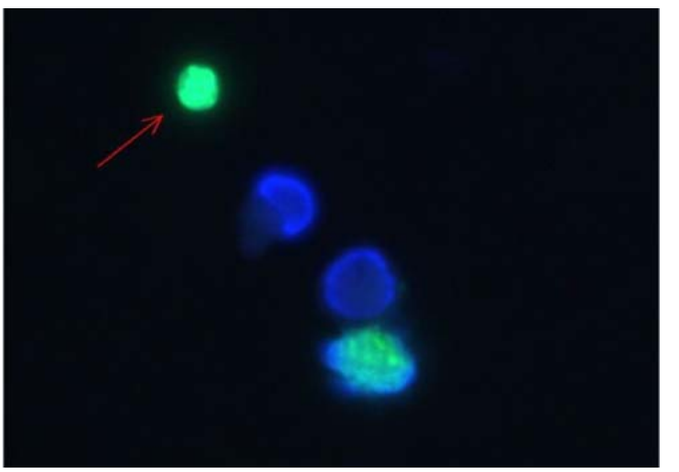

Figure 1a: *Tunel display green apoptosis positive (+)*

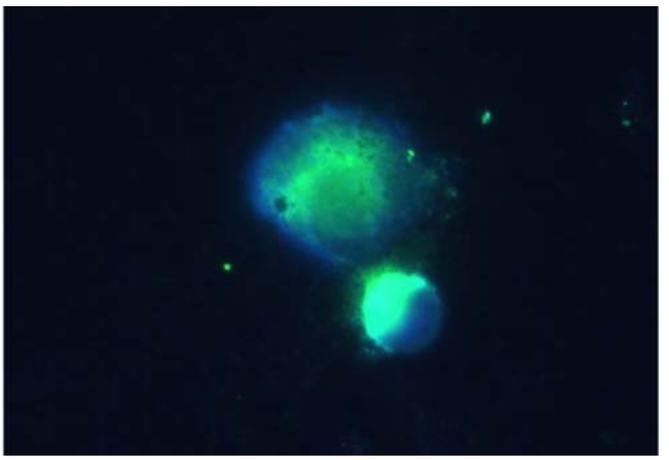

Figure 1b: *Tunel display blue-green partial apoptosis*

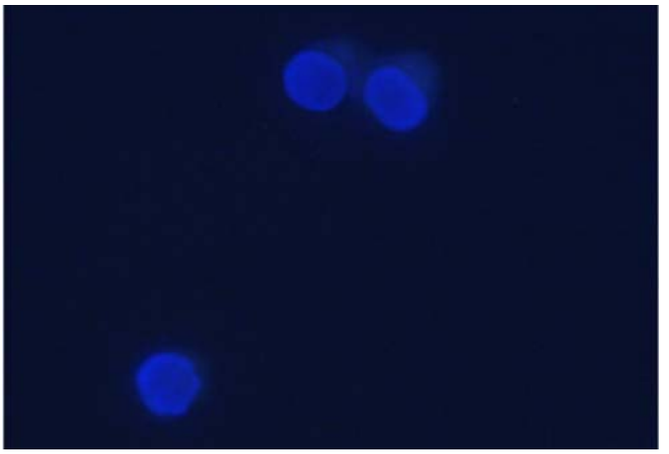

Figure 1c: *Tunel display blue apoptosis negative (-)*

Research plan and statistical analysis: IVM results were analyzed up to MII stage and fertilization, embryo development including blastocysts by C2 tests or variance analysis (ANOVA), where appropriate. Differences were considered statistically significant when the p-value was less than 0.05. All statistical analyzes were performed using Statistical Package for Social Sciences (SPSS) version 19.0.

Results

In this study, from 51 GV oocytes, which completed its maturation, 2PN developed in 29 after ICSI (56.1%); and 18 (39.1%) embryos at the 3-4-cell stage were obtained on the 2nd day, 20 (43.4%) embryos at blastomere stage were obtained on the 3rd day, 10 (21.7%) embryos were obtained at morula, and 3 (6.5%) embryos were obtained at blastocysts stage on the 5th day (Table I). The maturation was found as 52% and 2PN formation was found as 56% in all GVs to which was applied (Table I).

Table I: *The distribution of the total 456 oocyte, MII, and immature groups*

	Count	%
Total follicle count	532	
Total oocyte	456	85.7
MII	321	70.4
MI	37	8.1
GV	98	21.5
MII treated with ICSI	318	99.1
Fertilized MII	269	84.6
GV treated with IVM	98	100.0
GV treated with ICSI after 24 hours	51	52.0
GV in which 2PN formed 18 hours after CSI	29	56.9
Morula (%)	10	21.7
Blastocyst (%)	3	6.5

Cumulus apoptosis in ripened GV oocytes was not different in the maturing and immature group (p >0.05). In IVM-treated GVs, 2PN formation was observed in the embryo-developing group as 35.4% (Table 2). It was observed that there was a negative correlation between the percentage of GV oocyte and day 3 FSH level and a positive correlation between the GV maturation and basal luteinizing hormone (LH) value (Table II).

It was determined that the 3rd day LH value of the oocyte maturation and embryo development and the E_2 value of the HCG day showed a positive correlation (p =0.021, p =0.020) (Table II). However, it was understood that basal hormone values did not predict pregnancy (Table II). It was observed that the basal LH values were related to GV maturation and embryonal development (p=0.021). It was determined that the HCG day E_2 value was high in the GV oocyte group that completed the maturation and developed embryo (p=0.020) (Table II).

Table II: *Comparison of the groups with 3rd day hormone values and the average apoptosis rates*

	Maturation+emb+ (n=17) X±S	Maturation+emb- (n=8) X±S	Maturation-emb- (n=5) X±S	(Kruskal -Wallis Test)χ^2	p
Follicle-stimulating hormone	5.6±2.37	7.2±1.98	7.1±1.02	3.72	0.155
LH	**9.1±3.55 #**	6.50±2.5	4.7±1.16	7.68	**0.021**
E_2	48.5±17.9	40.9±23.1	36.4±11.28	2.25	0.324
HCG day E_2	**3829.4±1439.1 #**	2754.3±1383.8	1664±345.6	7.82	0.020
GV apoptosis rate	0.53±0.10	0.48±0.10	0.58±0.09	2.85	**0.239**
Apoptosis green + suspicious apoptosis rate	0.66±0.10	0.63±0.08	0.69±0.12	0.89	0.941

The apoptosis in the cumulus cells obtained from the M2 oocytes was found to be lower than the apoptosis of the GV cumulus (p <0.0001) (Table III). A statistically significant difference was found between the average GV apoptosis rate of the total group and the average M2 apoptosis rate. It was determined that the apoptosis rate was lower in the M2s (p = 0.0005) (Table III).

There was no statistically significant difference between apoptosis rates of immature GVs and apoptosis levels of mature GVs (Table IV). There was no statistically significant difference between the mean apoptosis rates of fertilized GVs and unfertilized GVs (Table IV).

No statistically significant differences were detected between the average apoptosis rates of the mature fertilized GVs which reached blastomere less than 10 on the 5th day and the GV's which reached 10 and more blastomere (Table IV). The apoptosis rates do not show significant differences between the mature GVs which were not fertilized and the groups which were fertilized and developed embryo (Table V).

No relation was determined between the GV apoptosis rate and GV apoptosis + GV suspicious apoptosis rate and hormone levels and other variables. Since the number of cases was not adequate, Spearman's Rho Correlation analysis, which is a non-parametric method, was applied (Table VI)

Table III: *Comparison of the average apoptosis rate in germinal vesicles treated with in vitro maturation and the average apoptosis in matures*

	Mean	*t* (paired samples test)	*p*
GV apoptosis rate (n=30)	**87** 0.52±0.10	6.25	**0.0005**
M2 apoptosis rate (n=30)	0.33±0.15		

Apoptosis rate = green / (blue + green + blue - green)

Table IV: *Comparison of the apoptosis rates in matured and immature germinal vesicles and the average apoptosis rates in germinal vesicles which were and were not fertilized and comparison of the average apoptosis rate in germinal vesicles according to the number of blastomeres on the 5th-day embryos*

	Immature GV (n=8) X±S	**Matured GV (n=22) X±S**	**Mann-Whitney U Test**	
			z	***p***
GV apoptosis rate	0.54±0.09	0.51±0.10	0.71	0.48
Apoptosis green + Suspicious apoptosis rate	0.68±0.09	0.64±0.10	0.92	0.36
	Non-fertilized GV (n=13) X±S	**Fertilized GV (n=17) X±S**	**Mann-Whitney U Test**	
			z	***p***
GV apoptosis rate	0.51±0.10	0.52±0.10	0.25	0.802
Apoptosis green + Suspicious apoptosis rate	0.52±0.10	0.66±0.10	0.23	0.818
	Cell count<10 (n=11) X±S	**Cell count>=10 (n=6) X±S**	**Mann-Whitney U Test**	
			z	***p***
Apoptosis green / Total cell	0.49±0.08	0.58±0.10	1.71	0.087
Apoptosis green + Suspicious apoptosis rate	0.66±0.10	0.66±0.10	0.35	0.725

GV: Germinal vesicle

Table V: *Apoptosis rates between the mature germinal vesicles which were fertilized and the groups which developed embryos*

	Fertilization negative (n=13) X±S	Fertilized and on the 5th day<10 cell (n=11) X±S	Fertilized and on the 5th day>10 cell (n=6) X±S	Mann-Whitney U Test	
				z	*p*
Apoptosis green / Total cell	0.51±0.10	0.49±0.08	0.58±0.10	2.56	0.278
Apoptosis green + Suspicious apoptosis rate	0.65±0.096	0.66±0.10	0.66±0.10	0.11	0.948

Table VI: *Correlation analysis with the germinal vesicle apoptosis rate and germinal vesicle apoptosis +germinal vesicle suspicious apoptosis rate (Spearman's Rho Correlation)*

		FSH 23	LH	E_2	HCG day E_2	MII fert. rate	GV fert. rate	2PN on the 1st day/IVM-GV count
GV apoptosis rate	r	-0.20	0.12	0.04	0.09	-0.09	0.01	0.01
	p	0.278	0.536	0.828	0.648	0.637	0.986	0.986
	n	30	30	30	30	30	30	30
GV apoptosis+Suspicious rate	r	0.01	-0.05	0.01	-0.29	-0.24	0.17	0.17
	p	0.951	0.778	0.984	0.126	0.2	0.368	0.368
	n	30	30	30	30	30	30	30

FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, HCG: Human choriogonadotropic, MII: Mature, fert.: Fertilitazion, GV: Germinal vesicle, PN:Pronuclear, IVM: In-vitro maturation, GV: Germinal vesicle

Discussion

In vitro maturation was first applied in 1935 by Pincus and Enzmann in rabbit oocytes (9). In 1965, Edwards tested in mice, bovine, bovine oocytes, and human oocytes (10,11). In 1991, for the first time, pregnancy was obtained from IVM oocytes from the study of Cha et al. (12). In 1994, Trounson obtained pregnancy with IVM from an untreated patient with PCOS (13). IVM can be performed in patients with OHSS risk, patients with OHSS history, patients with gonadotoxic therapy, or patients with hormone-sensitive tumors, which may be harmful to E_2 (5).

In PCOS patients, the oocyte maturation rate was found to be between 45-65% and the fertilization rate was 66% as a result of IVF application (8). In our study, 52% of immature oocytes completed maturation, 56% of which were fertilized after ICSI, 39.1% on day 2 in 3-4 cell stage, 43.4% on day 3 in 5-8 blastomere, 21.7% reached morula and reached the blastocyst was 6.5%. This result shows that similarity in oocyte maturation and embryo development in stimulated and untreated IVF cycles. However, in the study of Virant-Kline et al., immature oocytes, mostly derived from normal IVF cycles, were found to be abnormal (14). Jones, Strassburger, and Nogueira et al. showed that abnormal gene expression and chromosome contents and aneuploidies in embryos developed after IVM in stimulated cycles (15-17). In 2010, Reichman and colleagues examined the in vitro post-maturation fertilization potentials, embryo development, and pregnancy outcomes of immature oocytes obtained from stimulated cycles and 263 immature oocytes, and 234 mature oocytes were obtained from the same cycles (2). When the fertilization rates were examined, it was 62.1% of oocytes put into IVM medium and 64% in mature oocytes. In the 2nd-day embryo evaluation, 28.3% of the embryos in the IVM group and 54.3% of the embryos in the M2 group reached four cells. When the fragmentation rates were examined, 65.2% of the embryos in the IVM group and 30% of the embryos in the M2 group were observed. As a result, debates have arisen as to whether the use of GV oocytes with cumulus peeled and 24 h IVM-affected and their status in terms of meiotic maturation is questionable (2).

Shirasawa et al. and previous researchers have indicated the necessity of implementing the IVM method as a fertility protector (18). In 2013, Yalçınkaya and colleagues presented a treatment using an in vitro maturation technique in a patient given the decision to cancel cycles (4). As a consequence of this event, it has been suggested that oocyte in vitro maturation may be a good treatment choice not only for patients with polycystic ovarian disease but also for patients with a poor response (4). In 2013, Shin et al. conducted a study involving 463 cycles to assess the fertilization potential of immature oocytes acquired from controlled ovarian stimulation cycles (19). In the group matured from MI oocytes, fertilization rates were 37%, while M2 oocyte was 72.3%. The embryos developed in the MI-MII group were transferred to only five patients. Biochemical pregnancy was observed and terminated (19).

In our study, the maturation and 2PN formation rates were 56.1% and 63.0% in all GV applied IVM. This finding is also close to the findings obtained in the literature. When the fertilization rate was examined in general, it was observed as 60% in GV oocytes and 67.2% in MI oocytes. From the second day onwards, the rate of fragmentation of embryos increased. According to Alvarez et al., the fertilization rate of MII oocytes was 70.7% and the fertilization rate of MI oocytes incubated in the IVM medium was 55.7% (20). In the same study, the clinical pregnancy rate obtained from MII oocytes was 33.1% and for MI oocytes incubated in the IVM medium was 12.4%. The low rate was observed in the group with MI oocytes incubated in the IVM medium. While it is known that the cumulus-oocyte complex affects maturation and implantation in normal maturation, it is accepted that pregnancy rates are lower because the IVM application is performed with denudated oocytes. However, more than two thousand children without malformations were born with in vitro maturation (5).

In this study, apoptosis in the cumulus cells of MII oocytes was found to be lower than that of ovarian cumulus cell apoptosis in the germinal vesicle stage obtained from the stimulated cycles. This value is statistically significant. According to the report of Mikkelsen et al., when the ratio of apoptosis of MII and immature oocytes in controlled ovarian stimulations is ex-

amined, the rate of apoptosis in immature oocytes is high in granulosa cells. This result confirms our findings (21). According to the study performed by Host et al., the relationship between the apoptosis of cumulus cells, the change in the thickness of the zona pellucida, and the effect on oocyte maturation and fertilization was investigated (22). In this retrospective study, in a study performed on 50 patients in a special clinic, apoptosis of cumulus cells, zona pellucida thickness and oocyte fertilization, maturation and embryo quality. No association was observed between embryo quality and zona pellucida thickness and cumulus cells apoptosis. In our study, the apoptosis level of cumulus cells was higher in GV oocytes than in MII oocytes. However, no statistically significant difference was observed in the level of apoptosis in GV oocytes that completed the maturation with embryo-developing or non-embryonic GV oocyte groups and administered ICSI. These results indicate that the apoptosis level is insufficient in the future behavior of GV oocytes placed in vitro maturation. For this reason, it is understood that only the apoptosis parameter is inadequate to predict the development of IVM oocytes.

Some studies have been done on GV maturation in stimulated cycles. Sifer and his colleagues conducted research on IVM on 22 cancer patients. GV oocytes obtained from the cycles were frozen by crypto vitrification, post-freeze-thawed and left in culture for 24 hours. MII oocytes from healthy control patients were frozen, thawed and left in culture. The survival rate in the IVM group was 88%, in the stimulated group (COH) 86.3% and in the control group was 88.1%. As a result, although the viability rates are similar, the meiotic progression rates of GV oocytes in the IVM medium are significantly lower than those of the stimulated control group. (16.5% and 56.8%, p =0.018). In our study, the meiotic development of GV oocytes in 51 of 98 GV oocytes was 52%. This result seems to be similar to the study performed by Sifer et al in the stimulated cycles (23).

In our study, there was a correlation between oocyte maturation and embryo development day 3 LH value and day HCG E_2 value. LH plays an important role in the steroidogenesis of the ovary. Normal ovulation occurs with LH elevation (24). Many studies have been carried out on the role of LH in folliculogenesis. As is known, germinal vesicle breakdown (GVBD) begins with the induction of LH. After metaphase I, anaphase 1 and telophase 1 occur, resulting in secondary oocyte and a first polar body. The 2nd meiosis begins without interfacing. A second pause in the metaphase-II phase of the second meiosis division occurs and ovulation occurs during this time. With the penetration of the spermatozoon, the meiosis division is completed. Ovulation events must occur synchronously and harmoniously. Ripening regulatory factor (MPF) plays an important role at this point (24).

In the ovary, two cell groups are independently stimulated by LH and FSH, producing egg steroids (25). The production of androgens from cholesterol and without release during folliculogenesis depends on the stimulation of theca cells by LH, and together with FSH, the key drivers of ovarian follicle growth and maturation. (25)

LH elevation initiates a series of critical changes for ovulation in ovarian follicles in the middle of the cycle Normal ovulation plays a role in many critical changes such as steroidogenesis, cumulus cell growth, and oocyte maturation. LH stimulation leads to re-initiation of the meiosis by ovulation induction, accompanied by the expression of mRNA and proteins, and the secretory properties of the cumulus cells of the oocyte change. The effect of luteinizing hormone (LH) on GV maturation in stimulated cycles is shown by Cha et al. (26). In this study, it was shown that GV oocytes obtained from stimulated cycles with and without stimulation with GV oocytes had earlier germinal vesicle destruction in stimulated cycles during IVM administration. This effect was interpreted as the realization of GVBD by stimulating the cumulus complex of LH (27,28). In our study, we also found that oocyte maturation and embryo development correlated positively with day 3 LH. This result confirms the possible effects of LH. Therefore, normal follicular development is the result of the complementary effect of FSH and LH.

In our study, oocyte in vitro maturation and embryo development were investigated using apoptosis parameters. It is known that there are many factors affecting oocyte maturation in stimulated IVF cycle. The effect of various chemical agents, growth factors added to culture media is shown above in the development of rescue oocytes (29). In this case, it is necessary to develop new methods in addition to the methods present in the maturation of immature oocytes of stimulated cycles. Therefore, it was found that granulosa cell apoptosis is not the only factor in the in vitro maturation process, but many other factors should be examined and folliculogenesis is a prolonged process. The transfer of blastocysts obtained in our study could not be done because there are ethical limitations in this regard. However, our study shows that GV oocytes obtained in cases with poor responders may have embryos that can be brought up to the blastocyst stage after maturation.

Conflict of interest statement: The authors declare no conflict of interest.

Acknowledgment: None. Funding: None

Author contributions: TI: Study design, writing manuscript. SE: Laboratory procedures. EC:, SBA:, MST: Methodology of manuscript. ND: Statistics of the study. OG: Correction of english writing of manuscript

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