ORIGINAL RESEARCH ARTICLE

Analysis of anti-apoptotic protein (Bcl-xl) levels and mRNA expression in infertile patients

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Abstract

In assisted reproductive technology (ART), researchers have tried to predict *in vitro* fertilization (IVF) outcomes depending on follicular cells apoptosis. Previous reports suggested the B-cell lymphoma-extra-large (BCL-XL) as an apoptosis inhibitor in mammalian ovaries. Therefore, the present research aimed to correlate BCL-XL expression in blood and concentration in follicular fluid (FF) with some outcomes of IVF. A prospective case-control study on infertile women (n=80, mean age= 31.18± 6.04 years) who underwent an IVF program at Kamal Al-Samarai Hospital (Baghdad/Iraq), July 2021- January 2022. All women were split based on pregnancy outcome into two groups: the non-pregnant group (n=40) and the pregnant group (n=40). Samples of FF and blood were assembled at oocyte retrieval time. The BCL-XL mRNA expression was assessed with the Real-Time Quantitative Reverse Transcription–polymerase chain reaction (Real-Time qRT-PCR) technique, whereas the BCL-XL concentration in FF was investigated by sandwich enzyme-linked immunosorbent assay (ELISA). The BCL-XL concentration in FF from pregnant group (3.90 ng/ml ±1.16) raised significantly (*P* = 0.00) compared with non-pregnant group (1.90 ng/ml ± 0.63). Similarly, the BCL-XL mRNA expression in blood from pregnant group (0.73 ±0.31) raised significantly (*P* = 0.00) compared with non-pregnant group (0.34± 0.12). In addition, FF BCL-XL concentration associated significantly with fertilization rate (*r*= 0.399, *P*=0.048). The research proposed that the elevation of anti-apoptotic BCL-XL might assist in diminished apoptosis in pregnant women who underwent IVF treatment. *(Afr J Reprod Health 2022; 26[10]: 63-71).*

Keywords: *In vitro* fertilization, apoptosis, BCL-XL, follicular fluid, pregnancy

Résumé

Dans le domaine de la technologie de procréation assistée (ART), les chercheurs ont tenté de prédire les résultats de la fécondation in vitro (FIV) en fonction de l'apoptose des cellules folliculaires. Des rapports antérieurs ont suggéré le lymphome à cellules B extra-large (BCL-XL) comme inhibiteur de l'apoptose dans les ovaires de mammifères. Par conséquent, la présente recherche visait à corréler l'expression de BCL-XL dans le sang et la concentration dans le liquide folliculaire (FF) avec certains résultats de la FIV. Une étude prospective cas-témoins sur des femmes infertiles (n = 80, âge moyen = 31,18 ± 6,04 ans) ayant suivi un programme de FIV à l'hôpital Kamal Al-Samarai (Bagdad/Irak), juillet 2021-janvier 2022. Toutes les femmes ont été réparties sur la base sur l'issue de la grossesse en deux groupes : le groupe non enceinte (n=40) et le groupe enceinte (n=40). Des échantillons de FF et de sang ont été assemblés au moment de la récupération des ovocytes. L'expression de l'ARNm de BCL-XL a été évaluée avec la technique de réaction en chaîne par polymérase de transcription inverse quantitative en temps réel (Real-Time qRT-PCR), tandis que la concentration de BCL-XL dans FF a été étudiée par dosage immuno-enzymatique sandwich (ELISA) . La concentration de BCL-XL dans le FF du groupe enceinte (3,90 ng/ml ± 1,16) a augmenté de manière significative (P = 0,00) par rapport au groupe non enceinte (1,90 ng/ml ± 0,63). De même, l'expression de l'ARNm de BCL-XL dans le sang du groupe enceinte (0,73 ± 0,31) a augmenté de manière significative (P = 0,00) par rapport au groupe non enceinte (0,34 ± 0,12). De plus, la concentration de FF BCL-XL était significativement associée au taux de fécondation (r = 0,399, P = 0,048). La recherche a proposé que l'élévation du BCL-XL anti-apoptotique pourrait aider à diminuer l'apoptose chez les femmes enceintes qui ont subi un traitement de FIV. *(Afr J Reprod Health 2022; 26[10]: 63-71).*

Mots-clés: Fécondation in vitro, apoptose, BCL-XL, liquide folliculaire, grossesse

Introduction

The distinct role of apoptosis in female mammals reproduction has been documented[1,2]. Organizing ovulation, folliculogenesis, and luteal phase follicles depend on pro-apoptotic and anti-apoptotic agents balance to organize cell survival[3]. While pro-apoptotic agents dominate over anti-apoptotic,

apoptosis speeds up, ovarian reserve falls, and ovarian aging takes place[4,5]. In assisted reproductive technology (ART), researchers have made efforts to predict *in vitro* fertilization (IVF) outcomes depending on the apoptosis of cumulus and granulosa cells[6-9]. Hence, markers of apoptosis have been proposed for oocyte and embryo quality election. Researchers have documented that poor oocyte and embryo quality might correlate to apoptosis[10,11]. Additionally, other research reported the association between granulosa cell apoptosis and poor oocyte quality, fertilization, embryo fragmentation, pregnancy, and live birth rate[12-16].
Apoptosis existence in female germ cells, granulosa cells, oocytes, follicular fluids (FFs), and cumulus cells have been determined[17-19]. In granulosa cells, progression of apoptosis in mitochondria occurs via stimulation of the B-cell lymphoma-2 (Bcl-2) proteins superfamily (intrinsic pathway apoptosis)[20,21]. The Bcl-2 superfamily has been split into: apoptotic (BID and BIM), pro-apoptotic (BAX and BAK), and anti-apoptotic (Bcl-2 and B-cell lymphoma-extra large BCL-XL)[22]. The progression of the apoptosis pathway initiates as follows: The BID and BIM may stimulate BAX and BAK that cause the outer mitochondrial membrane damage, inner membrane remodeling, cytochrome c liberation, caspase cascade stimulation, and DNA fragmentation induction[20,23,24]. The BCL-XL may defend cells from death by segregating the pro-apoptotic analogs[22]. The BCL-XL have been suggested as apoptosis inhibitor in rat ovary[25,26], mouse ovary[27], hen ovary[28,29], baboon ovary[30], and human ovary[30,31]. It is worth mentioning that there have been no published articles on BCL-XL concentrations in FF at oocyte pickup. Hence, the present research is the first to study the BCL-XL level in the FF and correlate it with the outcomes of IVF. Therefore, the present research aimed to analyze the BCL-XL mRNA expression in blood and the protein concentration in FF at oocyte pickup and correlate it with some outcomes of IVF (oocyte maturation, cleavage, fertilization, and pregnancy rate).

Methods

Subjects

A prospective case-control study was carried out on 80 infertile women who underwent an IVF program with tubal cause infertility (n=24), unexplained infertility (n=23), and male factor infertility (n=33) at Kamal Al-Samarai Hospital (Baghdad/Iraq), July 2021- January 2022. The mean age of the women was 31.18± 6.04 years. All women signed written informed consent. The research experiments were accomplished by relying on the principles of the Helsinki declaration. The ethical committee of the research comprising human subjects Al-Nahrian University approved the research protocol according to the reference number: MB-1-11-2022. The patients have undergone a complete history, entire gynecologic examinations, hormonal profile (serum E2, FSH, LH), and transvaginal ultrasound (to reveal the uterus thickness, antral, and follicles number and size).

Polycystic ovarian syndrome, endometriosis, and poor responders women were excluded. All women were split, relying on pregnancy outcomes (beta hCG pregnancy test, which is accomplished 14 days post embryo transfer), into two groups: the non-pregnant group (n=40) and the pregnant group (n=40).

Controlled ovarian hyperstimulation

The GnRH antagonist protocol was utilized. The protocol initiates with Gonal-F® injection (recombinant FSH) (150-225 IU) at cycle day 2. When follicles were achieved (12-14mm), the Cetrorelix (GnRH antagonist) (0.25 mg) was utilized every day. Cetrorelix and Gonal-F® have been utilized with each other till 2-3 follicles are achieved (17-18 mm). Then, Ovitrelle® (6500 IU) (recombinant human chorionic gonadotropin hCG) was utilized.

Oocyte retrieval, FF, and blood sampling

After (34-36) hours, oocytes were aspirated utilizing a needle and transvaginal ultrasound. The FFs were gathered at the oocyte retrieval period, centrifuged (3000×g, 10 min, lab temperature), and saved at (-20ºC) in tubes. Venous blood was gathered at the oocyte retrieval period.

Oocyte morphology, oocyte maturation, and intracytoplasmic sperm injection (ICSI) procedure

The maturation of oocytes was guided by recognizing the first polar body. Morphology of

oocytes was determined by the oocyte maturation status (metaphase II (MII) oocyte)[32,33]. The denudation was accomplished by utilizing a buffered medium of 80 IU/ml hyaluronidase to remove cumulus and corona cells. The denuded oocytes were checked for maturation. Then, oocytes were washed and incubated for ICSI[34]. After oocyte retrieval of 3-5h, ICSI was accomplished by selecting MII oocytes[32,33].

Fertilization and embryonic development

Fertilization was assessed by the occurrence of two polar bodies and two pronuclei. Then 24 hours later, cleavage was achieved. Grading of embryos was accomplished morphologically[34]. Furthermore, the transfer of embryos was achieved on day 2 of embryonic progression (two-three embryos). After oocyte retrieval, luteal phase support was achieved by 1500 IU hCG injection. In addition, 200mg micronized progesterone was administered till pregnancy test day[35]. On day 12, after embryo transfer, a serum hCG test was accomplished.

IVF outcomes

Oocyte maturation rate= mature oocytes number/ all oocytes number[36].
Cleavage rate= informed embryos number/ fertilized oocytes number[37].
Fertilization rate= zygotes (2 pro nucleus) number/ mature oocytes (MII) number[38].

Total RNA isolation and qRT-PCR

Extraction of total RNA utilizing the TRIzol LS Reagent depends on the manufacturer's procedure (Trizol LS Reagent, 2012). The Quantus Fluorometer was utilized to reveal the RNA concentration. The cDNA sequences of Testis Enhanced Gene Transcript (TEGT) were got from the NCBI GenBank database. The qRT-PCR primers were designed utilizing Primer Premier 3 software and equipped from Macrogen Company, with melting temperature of 58-62oC, primer length of 18-23 nucleotides, and PCR amplicon length of 75-150 base pair. The primer sequences are shown in (Table 1). Gene expression analysis was accomplished by qRT-PCR(Mic qPCR Cycler).

The PCR reactions were achieved for 40 cycles that involved: the denaturation step (95 °C, 20 seconds), annealing step (58 °C, 20 seconds), and extension step (72 °C, 20 seconds). The results were calculated by the relative quantification method (folding =2-ΔΔCT)[39].

ELISA detection

Human BCL-XL concentration in FF was assessed by human BCL-XL ELISA Kit, utilizing sandwich enzyme-linked immunosorbent assay technique (ELISA) according to LifeSpan
BioSciences, Inc., America. Intra-assay precision is CV< 5.66 %, and Inter-assay precision is CV<5.26 %. The concentrations of BCL-XL were expressed in ng/ml.

Statistical analysis

The results were analyzed utilizing SPSS version 26 (SPSS Inc. Chicago, IL, United States). Shapiro–Wilk test of normality was employed. Results were shown as mean ± standard deviation (SD). The student's t-test was employed to examine variances between the groups. Moreover, the Pearson correlation test determined correlation coefficients (*r*). Results counted as statistically significant when a *P*-value < 0.05[40].

Results

BCL-XL concentration in FF and mRNA expression in the blood of all women

In all studied patients, the BCL-XL concentration in FF was 2.86 ng/ml ±1.02, whereas BCL-XL mRNA expression in blood was 0.53 ± 0.22 (Figure 1).

Correlation analysis of BCL-XL concentration and expression with IVF outcomes

As shown in (Table 2), there was a significant positive correlation between FF BCL-XL concentration with fertilization rate (*r*= 0.399, *P*=0.048).

Table 1: The qRT-PCR primers

Primer Name	Seq.	Annealing Temp °C
TEGT-F	5`-TGCTGGATTTGCATTCCTTACA-3	58
TEGT-R	5`-ACGGCGCCTGGCATAGA-3`	58
BCL-XL-F	5`-CGTGGAAAGCGTAGACAAGGA-3`	55
BCL-XL-R	5`-CAAGGCTCTAGGTGGTCATTCA-3`	55

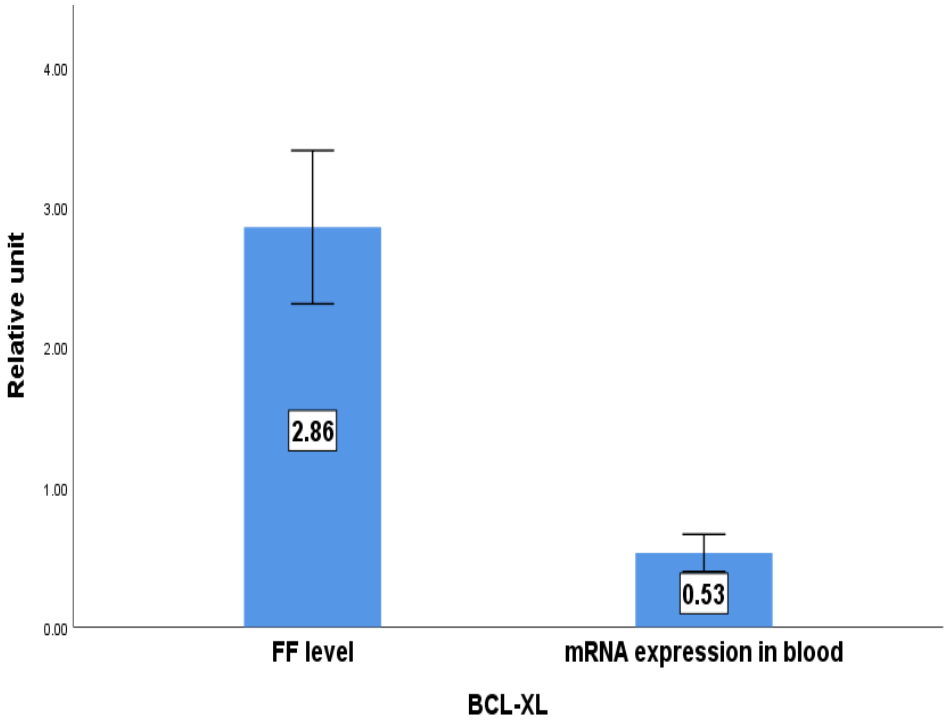

Figure 1: Mean value ± SD of BCL-XL concentration in FF and mRNA expression in the blood of the studied patients

Table 2: Correlation analysis of BCL-XL concentration and expression with IVF outcomes

		FF. BCL-XL (ng/ml)	BCL-XL folding
maturation rate%	*r*	0.035-	0.01-
	P	0.86	0.95
Fertilization rate%	*r*	**0.399**	0.24
	P	**0.048**	0.24
Cleavage rate%	*r*	0.28	0.16
	P	0.16	0.42

BCL-XL concentration and expression according to pregnancy state

The BCL-XL concentration in FF from pregnant group (3.95 ng/ml ±1.16) raised significantly (*P* = 0.00) compared with non-pregnant group (2.00 ng/ml ± 0.63). Similarly, the BCL-XL mRNA expression in blood from pregnant group (0.71 ±0.32) raised significantly (*P* = 0.01) compared with non-pregnant group (0.39± 0.20) (Figure 2 and Figure 3).

Discussion

Previous reports have documented the capacity of BCL-XL expressed in ovarian tissues of humans and various species to prevent cell death. In the present research, the anti-apoptotic BCL-XL mRNA expression and concentration were detected in blood and FF, respectively, from IVF treatment patients. By Northern blot analysis, researchers analyzed the BCL-XL gene from human granulosa-lutein cells from patients who underwent ART[30]. Moreover, others analyzed BCL-XL in hen granulosa cells, and its levels are directly associated with the viability of follicles (*in vivo*) and resistance

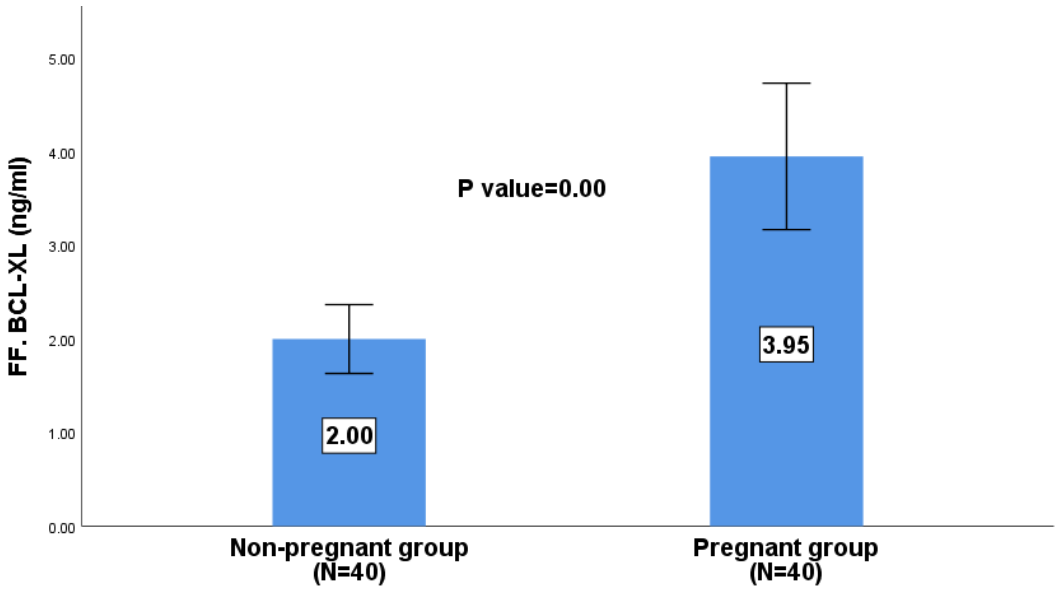

Figure 2: The BCL-XL concentration in FF according to pregnancy state

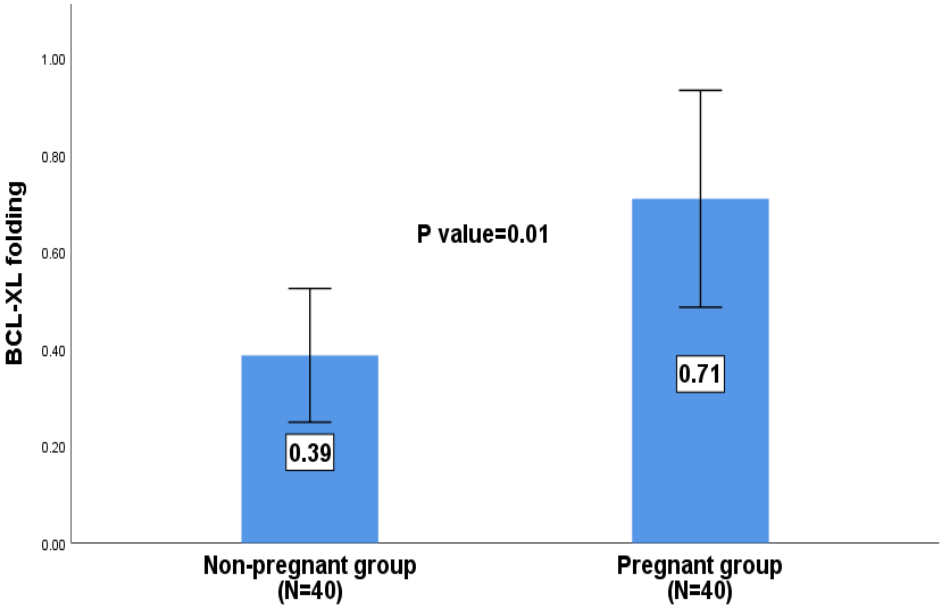

Figure 3: The BCL-XL mRNA expression in blood according to pregnancy state

to apoptosis (*in vitro*). They proposed that diminished BCL-XL mRNA expression in follicles prone to undergoing atresia is comparable to diminished porcine mRNA expression for LH, FSH receptor, and cytochrome P450 aromatase in atretic follicles[41]. They proposed that the diminish in BCL-XL mRNA expression for a reason for cellular nucleic acids degeneration[28]. Similarly, another report on hen granulosa cells observed that follicles expressed higher BCL-XL mRNA levels and higher inhibitors of T-cell apoptosis (ita) mRNA levels. They suggested that follicle recruitment might take

place when granulosa cells express raised ita levels and are consequently resistant to apoptosis[42]. Supportively, in cultured rat follicles, BCL-XL mRNA expression falls during granulosa cell apoptosis. They indicated that the absence of gonadotropic assistance to cultured follicles elevated BAX and reduced BCL-XL mRNA expression in granulosa cells. They proposed that gonadotropins diminished BAX expression in granulosa cells to enhance growing follicles' survival[26]. In hen granulosa cells, researchers documented that elevated BCL-XL levels take place via the cAMP pathway and are associated with granulosa cells' resistance to apoptosis (in vitro) and follicles' resistance to atresia (*in vivo*)[29]. It is worth mentioning that there is no relation between BCL-XL mRNA expression and follicle growth in the theca cell layer[43]. Because FF content is a production of transport of blood plasma components and secretion of theca and granulosa cells[44], therefore, the present research hypothesized that BCL-XL concentration in FF may derive from BCL-XL gene expression of granulosa cells as well as blood, subsequently may reflect the anti-apoptotic state of these cells. However, the present research is the first to study the BCL-XL concentration in the FF.

Confirming that the apoptosis could affect IVF outcomes[4,7,19,45,46], we next analyzed the effect of anti-apoptotic BCL-XL expression and transcript concentration in FF on some IVF outcomes (maturation rate, fertilization rate, cleavage rate, pregnancy rate). However, the present research documented associations between BCL-XL concentration in FF with fertilization and pregnancy rates. Apoptosis stimulated adaptive elevates of anti-apoptotic expression to return the balance between cell survival and death, thus enhancing the success of IVF[45,46]. Another report suggested that cumulus cell apoptosis may be utilized in predicting the outcome of IVF[7]. Similarly, apoptosis rates elevation of granulosa cells has been correlated with lower retrieved oocyte number, oocyte, and embryo quality. They speculated that ovarian stimulation protocols impact on apoptosis of granulosa cells[19]. It would appear from the present research that the expression of BCL-XL and its concentration in FF does not affect oocyte maturity. Consistent with our result, others documented that BCL-XL expression in cumulus cells does not correlate with the oocyte maturity stage[31]. However, the present research succeeds in finds an association between BCL-XL concentration in FF and fertilization rate. Others have also shown that the oocytes may fail to fertilize due to elevating incidence of cumulus cell apoptosis[7]. In support of the present research, one study from mature human oocytes reported that cumulus cells' apoptosis declined the fertilization rate; this may happen because of apoptotic signals transport from cumulus cells to oocytes, which impacts oocyte growth[47]. In contrast, others failed to find a correlation between BCL-XL expression levels in granulosa cells and fertilization rate in polycystic ovary syndrome women who underwent IVF treatment[48]. Other reports observed elevation of DNA damage for cumulus cells from inseminated oocytes (fertilized) and showed no change in the BCL-XL expression of fertilized and unfertilized oocytes. They interpreted that DNA damage has happened through the insemination because of the beginning of cumulus cells apoptosis. After fertilization, cumulus cells are eliminated from the oocyte, and BCL-XL would not elevate as BCL-XL enhances cell survival[31]. Different samples, techniques, and patient selection must be taken into account to reconcile these evident disagreements.

The present study demonstrated a significant increase in mRNA expression and FF concentration of BCL-XL in pregnant compared to nonpregnant women who underwent IVF treatment. Similarly, pregnant women significantly reduced apoptotic bodies incidence in mural granulosa cells[13]. Our results reveal that the apoptosis process stimulates adaptive augmentation of the anti-apoptotic gene expression to decline apoptosis, maintain cell survival, and subsequently may promote the outcome of IVF[49]. In supporting our results, the diminished apoptosis of the granulosa-lutein cells of pregnant women following IVF treatment has been determined[14]. Furthermore, diminished cumulus cells apoptosis of fertilized oocytes and pregnant women has been determined[7]. That can be interpreted as follows: a high incidence of apoptosis in cumulus cells may diminish the supporting ability of cumulus cells, causing poor oocyte growth, fertilization, and pregnancy[50]. The

present research failed to find associations between BCL-XL expression and FF concentration with cleavage rate. Hence, we postulated that the embryo cleavage rate might not be linked to the anti-apoptosis status in FF, which could invert the anti-apoptosis status of follicular cells·

Conclusion

The present research reported the association between BCL-XL level in FF with fertilization rate and pregnancy rate for the first time. The research proposed that the elevation of anti-apoptotic BCL-XL might assist in diminished apoptosis in pregnant women who underwent IVF treatment. However, future research is demanded to investigate the BCL-XL mRNA expression at the follicular cell level to realize the relation between follicular apoptosis and outcomes of IVF.

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