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# Analysis of Caspase-9 enzyme and Cytochrome C levels in infertile patients

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**Abstract**

Granulosa cell (GC) apoptosis has impaired oocyte maturation and reduced pregnancy rates. Apoptotic markers have been investigated and are supposed to be reliable predictors of *in vitro* fertilization (IVF) outcomes. The present research aims to compare caspase-9 and cytochrome C levels in follicular fluid (FF) and their gene expression (*CASP9* and *CYCS*) in blood in pregnant and non-pregnant women. Further, to investigate if the apoptotic markers can be used in predicting IVF pregnancy outcomes. Two groups of women were recruited: the non-pregnant group (n=40) and the pregnant group (n=40). The *CASP9* and *CYCS* expressions were inspected with the Quantitative Reverse Transcription–polymerase chain reaction (qRT-PCR), whereas their protein levels were examined by enzyme-linked immunosorbent assay (ELISA). A significantly lower gene expression and protein levels of caspase-9 and cytochrome C were observed in pregnant women compared to non-pregnant women. Besides, a significant inverse correlation between pregnancy outcomes and apoptotic markers was detected. It is considered that caspase-9 had the best predictive value of pregnancy outcomes compared to cytochrome C. The findings have indicated that an elevated apoptotic rate of GCs may adversely impact oocyte growth, reduce pregnancy rates, and cause IVF failure.

**Keywords:** *in vitro* fertilization (IVF), follicular fluid, apoptosis, pregnancy, caspases.

# تحليل مستويات انزيم كاسباس9 والسيتوكروم سي في مرضى العقم

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**الخلاصة**

يؤدي موت الخلايا المبرمج للخلايا الحبيبية (GC) إلى إضعاف نضوج البويضات وانخفاض معدلات الحمل. وقد تم التحقيق في علامات موت الخلايا المبرمج ويفترض أنها تنبئ بنتائج التلقيح الصناعي (IVF). يهدف البحث الحالي إلى مقارنة مستويات كاسباس-9 وسيتوكروم سي في السائل الجريبي (FF) وتعبيرهما

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الجيني (*CASP9* و *CYCS*) في الدم لدى النساء الحوامل وغير الحوامل. علاوة على ذلك، للتحقيق فيما إذا كان من الممكن استخدام علامات موت الخلايا المبرمج للتنبؤ بنتائج الحمل في التلقيح الصناعي. تم تجنيد مجموعتين من النساء: مجموعة النساء غير الحوامل (n = 40) ومجموعة النساء الحوامل (n = 40). تم فحص تعبيرات *CASP9* و *CYCS* باستخدام تفاعل البوليميراز المتسلسل العكسي الكمي (qRT–PCR) بينما تم فحص مستويات البروتين الخاصة بهما بواسطة اختبار الممتز المناعي المرتبط بالإنزيم (ELISA). لقد لاحظنا انخفاضا كبيرا في التعبير الجيني ومستويات بروتين الكاسبيز–9 والسيتوكروم سي لدى النساء الحوامل مقارنة بالنساء غير الحوامل. بالإضافة إلى ذلك، اكتشفنا وجود علاقة عكسية كبيرة بين نتائج الحمل وعلامات موت الخلايا المبرمج. ويُعتقد أن الكاسبيز–9 كان له أفضل قيمة تنبؤية لنتائج الحمل مقارنة بالسيتوكروم سي. وقد أشارت نتائجنا إلى أن ارتفاع معدل موت الخلايا المبرمج للخلايا الجرثومية قد يؤثر سلبًا على نمو البويضة ويقلل من معدلات الحمل ويسبب فشل التلقيح الصناعي.

## 1. Introduction

Researchers have evidence that apoptosis has a distinguished function in female reproduction [1-3]. Apoptosis is a cellular system that participates in luteal regression and follicular atresia [4-6]. Cumulus granulosa cells, a cluster of linked granulosa cells (GCs) that encircle and sustain oocytes, perform a crucial role by secreting various growth factors as well as proteins, which are expressed and produced during oocyte and embryonic growth [7, 8]. It has been demonstrated that the death of GCs and cumulus cells (CCs) is implicated in the maturation of oocytes, fertilization, and embryonic growth in both *in vitro* and *in vivo* [9-13]. Furthermore, reduced GC apoptosis may be associated with successful pregnancy [14], whereas elevated CC apoptosis has impaired oocyte maturation and fertilization and reduced pregnancy [12,15]. Hence, apoptotic markers have been extensively investigated and are supposed to be a reliable predictor of *in vitro* fertilization (IVF) outcomes [13]. A previous report proved that apoptotic elements in human CCs might be utilized as markers for oocyte competence [16]. Likewise, another report displayed that apoptotic genes of human CCs were suggested to predict pregnancy and embryo selection [17]. In assisted reproductive technologies (ART), the pregnancy and live birth rates following IVF are always low [18]–[20]. Indeed, follicular fluid (FF) surrounding the oocyte has participated in follicular maturation, oocyte growth, and developmental competence [21,22]. Consequently, FF might act as a dependable source of markers that might be utilized as predictive tools in IVF [23 - 25].

The cellular request to initiate apoptosis can be activated and disseminated via various pathways comprising essential regulatory genes and their products, including the caspase protein family [26]. Caspases are cellular cysteine proteases with potential roles in vertebrates' initial and final apoptotic phases [27]. In the mitochondrial apoptotic pathway, mitochondria may be stimulated to liberate cytochrome C by several stress signals, B-cell lymphoma 2 (Bcl-2) proteins, apoptosome production, or following caspase stimulation by surface receptors [28]. The releasing of mitochondrial cytochrome C into cytoplasm could stimulate the caspase pathways, which are the cell death mediators [11]. Then, the interaction between cytochrome C in the cytosol and the protease activating factor-1 (Apaf-1) converts the pro-caspase-9 to caspase-9, which forms the axis of the mitochondrial apoptotic process [29]. The fundamental role of caspase-9 is the stimulation of its downstream effector, caspase-3, which stimulates DNA fragmentation [11,30].

The mitochondria-dependent pathway corresponds with the triggering of GC apoptosis [11]; however, comprehending the pathways controlling GC survival and apoptosis is clinically significant, as disrupted signalling in these pathways is probable to impact oocyte

growth and subsequent IVF potential. Major apoptotic mediators, including caspase-9 and cytochrome C, have been expressed in the GCs of humans and animals, and their protein levels in FF [31-35]. Follicular atresia was associated with the overexpression of *CASP9* in the GCs of mice [32] and pigs [33]. Additionally, *CASP3* and *CASP9* were overexpressed in GCs from women with low response compared to those with a normal response, indicating potential involvement of apoptosis in the aetiology of poor response to ovarian stimulation [34]. Western blot study indicated elevated expression of *CYCS* and *CASP3* in human GCs, implying that elevated cytochrome C production from mitochondria can stimulate caspase-3, which is crucial for apoptosis-related luteal activity [35]. Correspondingly, this investigation was interested in the contribution of caspase-9 and cytochrome C in initiating apoptosis of GC via mitochondrial pathway. There is no report on the effect of *CASP9* and *CYCS* on oocyte development or the significance of these markers in predicting IVF pregnancy outcomes. Therefore, it is hypothesized that upregulation of *CASP9* and *CYCS*-induced apoptosis in GCs may increase their protein secretions to the corresponding FFs and negatively impact oocyte development and subsequent IVF failure. Two significant questions may arise: Can these markers utilize to establish strategies to inhibit GC apoptosis? Does this approach improve oocyte developmental potential, facilitate the selection of high-quality oocytes for IVF, and boost embryo quality and development, ultimately leading to a higher success rate in IVF? Accordingly, the current research aims to compare the apoptotic caspase-9 and cytochrome C levels in FF and their expression in blood in pregnant and non-pregnant women following IVF treatment. Further, to investigate if the apoptotic markers can be used to predict pregnancy outcomes.

## 2. Materials and methods

### *2.1. Patients*

The prospective research was conducted between February 2023 and October 2023 in the Kamal Al-Samarai IVF Centre (Baghdad/ Iraq). Two groups of women (age=33.04± 6.03 years) who underwent IVF cycles were enlisted: the non-pregnant group (n=40) and the pregnant group (n=40). The inclusion criteria consisted of the following: (1) women aged under 35 years; (2) tubal cause infertility; (3) idiopathic infertility; (4) male factor infertility; (5) women receiving a gonadotropin-releasing hormone antagonist protocol (GnRH antagonist); and (6) women with regular menstrual cycles and normal levels of sex hormones. All women underwent gynecologic examination, hormonal analysis, and transvaginal ultrasound. Laparoscopy and hysterosalpingography were utilized to diagnose women who had tubal infertility [36]. Ovulation confirmation, regular tubes and uterine cavity (confirmed by laparoscopy or hysterosalpingography), and standard semen analysis, as reported by the World Health Organization (WHO), were utilized to diagnose women with idiopathic infertility [37].

Women undergoing IVF for male factor infertility were identified by examining semen param eters based on macroscopic and microscopic tests by the fifth edition of the WHO's recomme ndations [38]. The exclusion criteria consisted of women with the following conditions: (1) age exceeding 35 years; (2) obesity; (3) diabetes mellitus; (4) metabolic syndrome; (5) premature ovarian failure; (6) ovarian tumour; (7) polycystic ovarian syndrome; (8) poor ovarian responders; (9) uterine diseases; and (10) endometriosis. The present research was conducted according to the principles of the Helsinki Declaration. The ethical committee of the Biotechnology Research Center, Al-Nahrian University, accepted the research protocol. All women gave written informed consent.

*2.2. In vitro fertilization procedure*

The GnRH antagonist protocol was utilized to stimulate all women. On menstrual cycle day 2, 150-225 IU of the recombinant human follicle-stimulating hormone injection (rhFSH, Gonal-F®; Serono, Germany) was given. Later, when follicles had grown to 12-14mm, 0.25mg of the GnRH antagonist (Cetrorelix, Cetrotide; Asta Medica AG, Germany) was prescribed every day till 2-3 follicles had developed to at least 17-18 mm. Subsequently, 6500 IU of the recombinant human chorionic gonadotropin (rhCG, Ovitrelle®; Merck Serono, Italy) was administered. Then, 34-36 hours later, oocytes were collected through a needle-guided aspiration during the transvaginal ultrasound. After retrieval, 200mg micronized progesterone inside the vagina till pregnancy test day was provided luteal phase support. Embryo transfer of 2–3 embryos was accomplished on days 2-3 of embryo progress.

*2.3. Follicular fluid and blood sampling*

During oocyte retrieval, FF from follicles was gathered and separated at laboratory temperature (3000xg, 10 min). The FF supernatant was maintained at -20 °C for investigation. On oocyte pickup day, venous blood samples were taken, and mRNA expressions were evaluated in peripheral blood using the Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR).

*2.4. CASP9 and CYCS gene expression analysis via PCR*

The RNA was extracted using TRIzol LS Reagent, (Trizol® LS Reagent, Catalog 10296028; Thermo Fisher Scientific, USA), depending on the manufacturer's procedure. The Quantus Fluorometer was employed for estimating the RNA concentration. In addition, the cDNA sequence of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (as a housekeeping gene) was determined from the NCBI GenBank (GeneID: 2597). The qRT-PCR primers were generated using Premier 3 software (Macrogen Inc., Seoul, South Korea) (Table 1). The gene expression analysis was performed using qRT-PCR (Mic qPCR Cycler). The thermal profile was used to program the cycling protocol, which consisted of a single cycle of denaturation or enzyme activation at 94 ◦C for 5 min, followed by forty cycles of denaturation at 94 ◦C for 15 s, annealing at 55 ◦C for 25 s, and extension at 72 ◦C for 30 s. The dissociation step was conducted at 1 min/95 ◦C-30 s/55 ◦C-30 s/95 ◦C. The relative quantification method (folding =2-ΔΔCT) was utilized to calculate the gene expression of *CASP9* (GeneID: 842) and *CYCS* (GeneID: 54205)[39].

**Table 1:** The qRT-PCR primers.

| Gene Name | Forward primer | Reverse primer | Product size (bp) | Melting temp °C |
|---|---|---|---|---|
| *GAPDH* | GAAATCCCATCACCATCTTCCAGG | GAGCCCCAGCCTTCTCCATG | 164 | 64 |
| *CASP9* | GTCCAGGGCTAGTGACTTGT | GAAGACGCGTTACTGGCATT | 169 | 59.2 |
| *CYCS* | CCCAAGCAGCAATCATTCCA | CTACCTCCGAGTCTTGCGTA | 182 | 58.81 |

*2.5. Analysis via ELISA*

Levels of cytochrome C and caspase-9 were quantified in human FF by the human Caspase-9 and Cytochrome C ELISA Kit, utilizing sandwich enzyme-linked immunosorbent assay technique (ELISA) based on the Abcam, Inc., UK methodology. Sensitivity, Intra- and Inter-assay precision of caspase-9 are 0.4 ng/mL, 6.6 %, and 9.0 %, whereas cytochrome C are 0.05 ng/mL, 6.0 % and 4.0 %. The caspase-9 and cytochrome C concentrations in FF were expressed in ng/mL.

*2.6. Pregnancy outcome*

Biochemical pregnancy can be explained as a serum HCG test conducted 15 days post-oocyte retrieval. HCG positive is classified as the hormone levels exceeding 20 mIU/mL.

*2.7. Statistical analysis*

Data analysis was performed using IBM SPSS, version 26 (SPSS Inc., United States). The Shapiro-Wilk and Kolmogorov-Smirnov tests were utilized to investigate data normality. The findings are displayed as median and interquartile range (IQR). The Mann–Whitney U test determined median differences between groups. Further, graphics are displayed as box plots. The Spearman correlation test was employed to determine the association between variables using Spearman's rank correlation coefficient ($r$). The area under the curve (AUC) and the marker's predictive efficiency for pregnancy at a selected level were derived from the Receiver-operating characteristic (ROC). In addition, specificity and sensitivity were determined at the appropriate cut-off value. Differences were assessed to be significant at $P \leq 0.05$.

## 3. Results

*3.1. Caspase-9 and cytochrome C levels in the FF and mRNA expression in blood:*

Follicular fluid and blood samples from 80 women were analysed. The pregnant women group (N=40) was compared to the non-pregnant women group (N=40) following IVF treatment. Results revealed that the gene expression of *CASP9* and *CYCS* in blood samples of pregnant women was significantly lower than in non-pregnant women ($P < 0.05$) (Figure 1). Similarly, significantly lower levels of caspase-9 and cytochrome C were found in the FFs of pregnant women than in non-pregnant women ($P < 0.05$) (Figure 2). These results indicate that the pregnancy outcomes of IVF correlate with *CASP9*, *CYCS*, and their protein levels in FF.

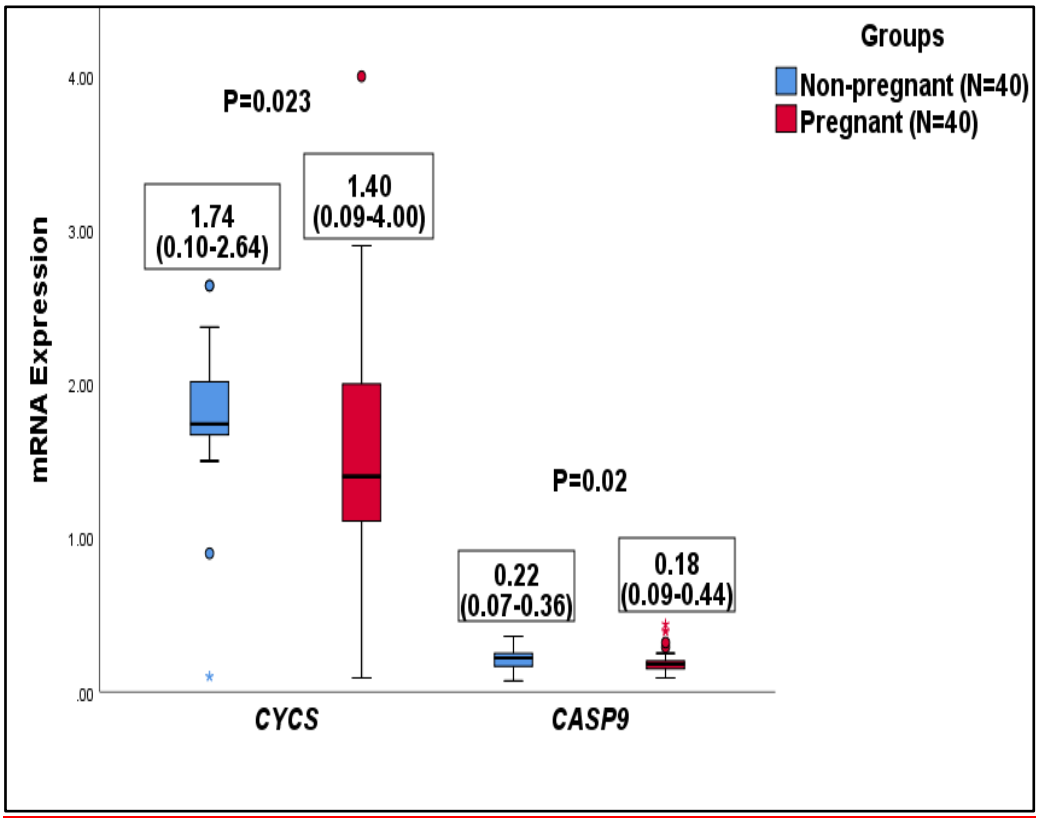


**Figure 1:** The CASP9 and CYCS gene expression in the blood of pregnant and non-pregnant groups.

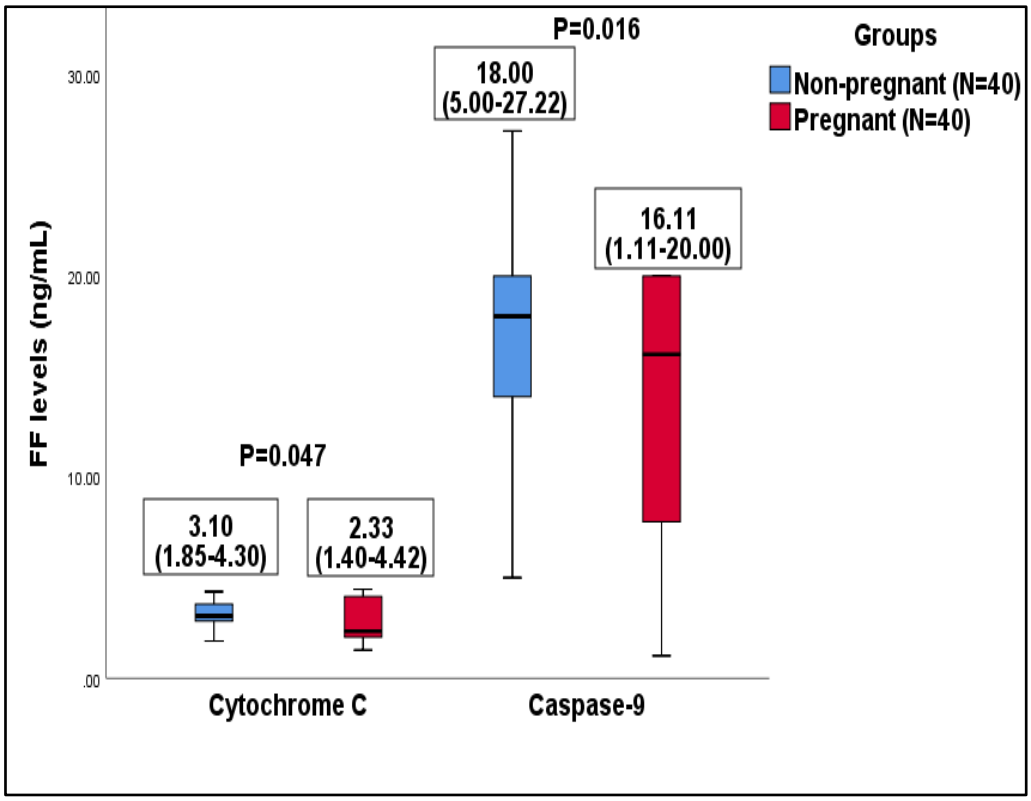


**Figure 2:** The levels of caspase-9 and cytochrome C of pregnant and non-pregnant groups in FF.

*3.2. Spearman correlation between IVF pregnancy outcomes with caspase-9 and cytochrome C levels in the FF and mRNA expression in blood:*

The Spearman correlation between the expression of these genes, their protein levels in FF, and pregnancy outcomes of IVF were analysed. As shown in Figure 3, the results exhibited a significant inverse correlation ($r < 0$, $P < 0.05$) between pregnancy outcomes of IVF and *CASP9* expression, *CYCS* expression, FF caspase-9 levels, and FF cytochrome C levels.

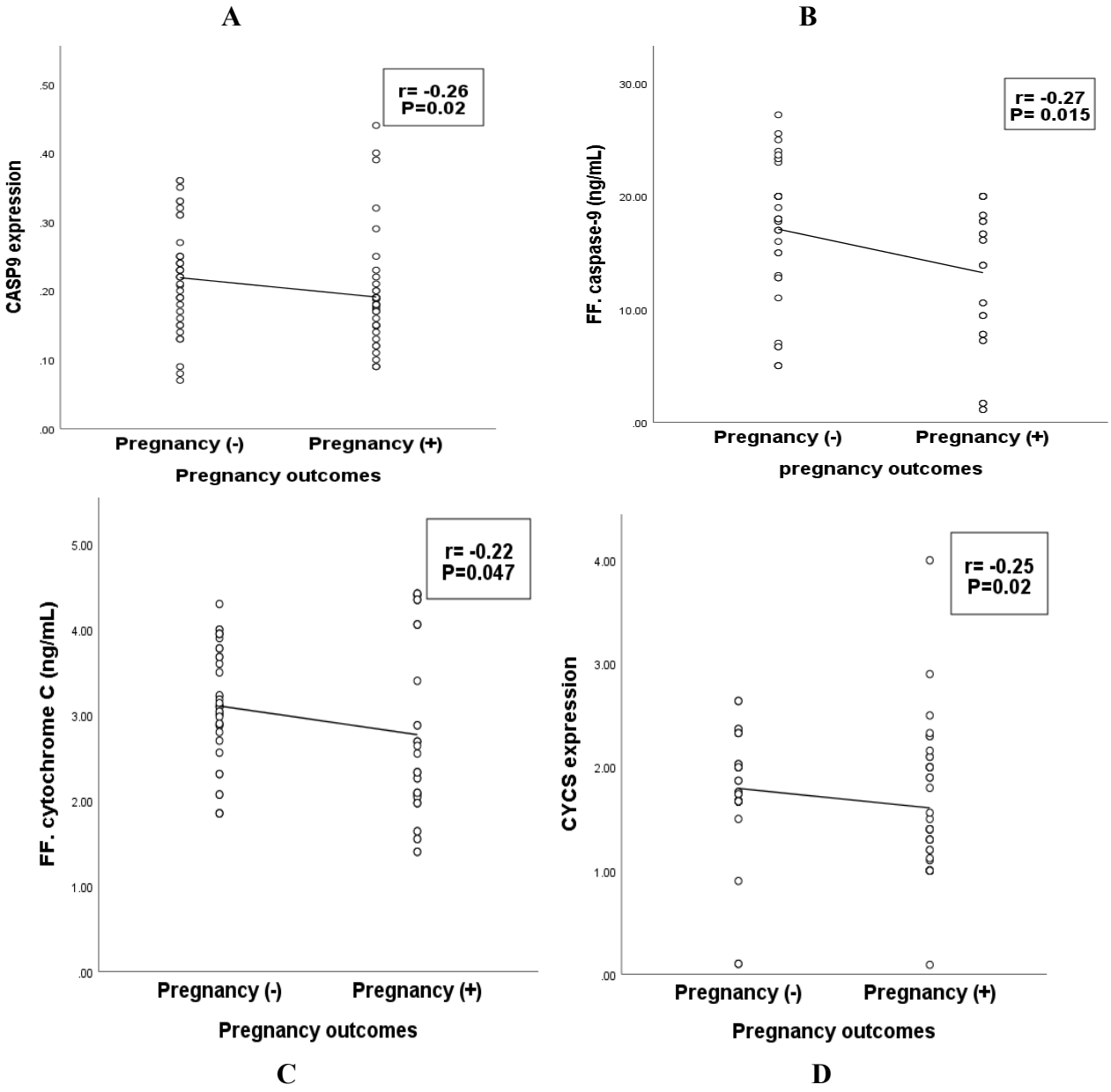


**Figure 3:** Scatter plots represent the correlation between IVF pregnancy outcomes and *CASP9* mRNA expression (A), caspase-9 levels in FF (B), *CYCS* mRNA expression (C), and cytochrome C levels in FF (D).

*3.3. Receiver-operating characteristic curve of caspase-9 and cytochrome C to predict pregnancy outcomes:*

The receiver-operating characteristic curve (ROC) demonstrates that caspase-9 levels in FF and mRNA expression were more reliable predictors for pregnancy than cytochrome C (Table 2, Figure 4).

**Table 2:** The ROC analysis findings of the apoptotic markers to predict pregnancy outcomes.

| Markers | AUC | *P* value | cut-off | Specificity % | Sensitivity % |
|---|---|---|---|---|---|
| *CASP9* expression | 0.70 | 0.02 | 0.19 | 70 | 60 |
| FF. caspase-9 | 0.70 | 0.016 | 17.39 (ng/mL) | 60 | 60 |
| *CYCS* expression | 0.65 | 0.023 | 1.71 | 53 | 60 |
| FF. cytochrome C | 0.63 | 0.047 | 2.89 (ng/mL) | 70 | 70 |

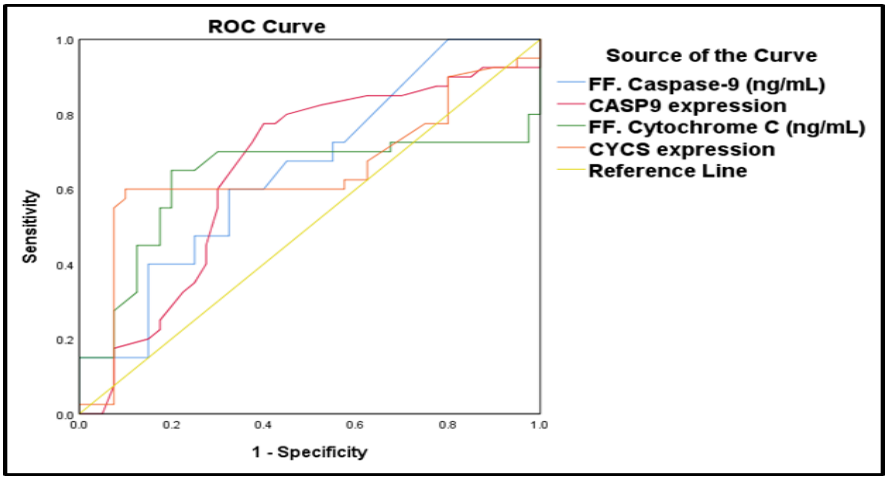


**Figure 4:** The ROC curve for the apoptotic markers to predict pregnancy outcomes.

## 4. Discussion

Apoptosis within the GCs of the maturing ovarian follicle is a marker for poor oocyte quality, one of the most critical factors affecting pregnancy outcomes after the IVF cycle [40], [41]. Therefore, an optimal follicle environment is crucial for common folliculogenesis and oocyte growth [42]. Evaluating gene expression in GCs may indicate changes in the follicle environment, thereby enabling the indirect evaluation of both oocyte quality and embryonic developmental potential [43,44]. Therefore, searching for molecular and protein markers with predictive values of pregnancy outcomes may provide new insights to improve the IVF success rate. Accordingly, the present research has related the mRNA expression of two genes that participate in apoptosis (*CASP9* and *CYCS*) and their protein levels in FF to pregnancy outcomes. It has also investigated whether these markers can be beneficial in predicting pregnancy outcomes following IVF treatment. The findings demonstrate that FF contains information about the apoptotic protein levels associated with IVF pregnancy outcomes. The current study revealed a significant lowering in mRNA and FF levels of caspase-9 and cytochrome C in pregnant women compared to non-pregnant women. In addition, significant negative relationships between the expression of *CASP9* and *CYCS* genes and their protein levels in FF with pregnancy outcomes of IVF were observed. Herein, we can speculate that apoptosis rates of GCs were reduced in the pregnant women group. These findings may provide the starting line for identifying the ideal FF levels and gene expression of these apoptotic markers that might be utilized as potential markers to predict IVF pregnancy outcomes.

Apoptosis, a continuing mechanism during follicle growth, organizes follicle growth and oocyte maturation in a particular manner. Specifically, GC death may impact the connection between the oocyte and GCs [45]. The concept that lowering apoptotic GCs and CCs in patients undergoing IVF cycles is related to good IVF outcomes seems well confirmed. It had been observed that the diminished apoptosis rate of the mural GCs had a relationship with mature oocytes and better embryo yield [12]. Likewise, another report displayed a lower DNA fragmentation index (DFI) of CCs of transferred blastocysts than arrested embryos[16]. Perhaps lower DFI levels of CCs may be due to survival pathway stimulation and could be associated with oocyte maturation and good outcomes of IVF [46,47].

Follicular fluid originates from the transition of blood plasma ingredients and from the secretion of GCs and theca cells, providing a crucial milieu for oocyte development [48].

Correspondingly, the apoptotic protein levels in FF may be utilized as markers of IVF outcomes in our study. It is worth mentioning that, inside the ovarian follicle, GCs exhibit a greater susceptibility to apoptosis than CCs or theca cells [14]. Hence, we speculate that the existence of apoptotic caspase-9 and cytochrome C in FF are transported from blood plasma and secreted from GCs, which could reflect the GC apoptotic state.

The results of qRT-PCR and ELISA experiments indicated elevated *CASP9* and *CYCS* mRNA expression and protein levels in non-pregnant women compared to pregnant women after IVF, suggesting a higher apoptosis rate of GCs and lower the development of oocytes, which negatively impacts pregnancy and IVF failure. Notably, we found affirmations of our hypothesis. A prior study utilizing flow cytometry identified overexpression of *CASP3* and a greater apoptotic rate of cumulus GCs in non-pregnant women compared to pregnant ones [49]. As a principal executor, caspase-3 participates in many apoptotic pathways, and its activated form triggers apoptosis [30].

Furthermore, it has been proved that an elevated apoptosis rate of cumulus GCs may negatively influence IVF pregnancy outcomes [50]. Perhaps the explanations of the current findings are that: I) Elevated GC apoptosis could diminish intracellular junctions and microvilli, subsequently resulting in abnormal morphology, impaired development, and reduced maturation of oocytes, as well as IVF pregnancy failure [50]. II) It was reported that a protein known as rapamycin may stimulate rapamycin complexes, intracellular P70-S6 protein Kinase1, and serine/threonine kinase (Akt), which inhibits caspase-9 via phosphorylation [51]. Subsequently, caspase-9 inhibition may inhibit caspase-3 executor, suppress apoptosis, enhance protein synthesis, and favourably boost cell division [30]. Accordingly, our study implies the possible involvement of rapamycin and the reduction of the Akt signalling pathway into the intrinsic apoptotic signals, which may induce GCs apoptosis. Others can emphasize our hypothesis. Interestingly, Gong *et al*. observed elevated apoptotic rates, abnormal expression and levels of caspase-3 and caspase-9 in GCs, and diminished phosphoinositide 3-kinases (PI3K)/Akt pathway among women who underwent IVF [52]. III) Furthermore, a study revealed that elevation of caspase-3 and cytochrome C levels in FF with positive antinuclear antibodies (ANA) patients are involved in the lowest fertilization, lowest pregnancy rates, and highest miscarriage rate, suggesting the possible involvement of local autoimmunity in imbalanced apoptosis and bad IVF outcomes [53]. IV) Interestingly, researchers have reported that in the mitochondrial apoptotic pathway, mitochondria as energy sources could not produce energy which is fundamental for oocyte maturation [54,55]. Clarifying the mechanism underlying the apoptosis of GCs could be beneficial in developing strategies that suppress GC apoptosis, hence boosting oocyte developmental potential, improving pregnancy outcomes, and raising IVF success rates.

A study on fetal mouse ovaries revealed a negative relationship between caspase-9, caspase-3, and the X-linked inhibitor of apoptosis; the authors noted that elevated apoptosis inhibitor and reduced caspase-9 resulted in diminished oocyte loss [56]. Furthermore, the *in vitro* investigation by Kocherova *et al*. proved that *CASP9* expression in isolated human GCs was downregulated, potentially promoting GC survival over apoptosis [57]. A previous investigation revealed that *CASP3* expression in human GCs adversely impacts the number of preovulatory follicles, mature and fertilized oocytes, and viable embryos [58]. Recently, Bódis *et al*. assessed *CASP8* and *CASP3* expression in GCs and FF patients who underwent IVF treatment. Their study has displayed a non-significant difference in *CASP3* and *CASP8* expression and FF levels of women with and without pregnancy. However, their study was deemed secondary proof of apoptosis impacts on IVF outcomes [59].

Additionally, they were inconsistent with the current findings regarding *CASP9* expression in blood and FF levels. However, these disagreements may be interpreted due to different patient selections, methodologies, stimulation protocols, and samples. Other researchers in human GC have revealed that *CASP8* expression has impacted pregnancy rate negatively, whereas *CASP3* expression has not [60]. It has been observed that *CASP3* and *CASP8* are correlated to the Fas apoptotic pathway [61,62]. In the cell membrane, the Fas ligand binds to Fas, which activates caspase 8. Then, caspase 8 transports the signal to caspase 3, considered the major protease that causes the degeneration of caspases to start the apoptotic pathway [60]. In the endometrium, a comparison of *CASP8* and *CASP10* expression of women who accomplished a successful pregnancy and those who did not after failed IVF treatments has proved to differ significantly [63]. Blastocysts may trigger a paracrine death response regulated by the Fas pathway. Indeed, the embryonic organization of apoptosis in endometrial epithelial cells during the implantation was confirmed [64]. Researchers have observed decreased secretomes caspase-3 levels from good quality blastocysts leading to pregnancy; in contrast, increased caspase-3 levels of poor quality and arrested embryos [65]. Embryos of bad quality exhibit high DNA fragmentation levels triggered by the excuter caspase-3, and arrested embryos during growth might result from defects in cell division or apoptosis [66-68].

The expression of *CYCS* in mice ovaries was convincingly evidenced, as its overexpression causes modified mitochondrial dynamics, accelerated apoptosis, cellular loss, reduction of the ovarian follicular reserve, and hastened reproductive ageing [69]. Nevertheless, proof of *CYCS* expression in human GCs and the protein level in FF on pregnancy outcomes of IVF is absent in the scientific literature. However, this study is the first to correlate *CYCS* expression in blood and their levels in FF with pregnancy outcomes after IVF treatment.

Research has proved that GCs generate FF that facilitates oocyte development and growth [8,70]; therefore, GC gene expression and FF proteins may serve as non-invasive indicators of oocyte development and subsequent pregnancy outcomes of IVF [43,71]. Accordingly, using ROC analysis, it is considered that caspase-9 had the best predictive value of pregnancy outcomes compared to cytochrome C. The study findings displayed that *CASP9* mRNA expression >0.19 and FF level >17.39 (ng/mL) were reliable predictors of pregnancy outcomes after IVF treatment. Interestingly, this might be the first research to exhibit caspase-9 as a predictive method for people undergoing IVF treatment.

The expressed apoptotic genes of GCs elucidated the impact of apoptosis on the follicular milieu, oocyte maturation, and subsequent IVF potential. Accordingly, the methodology applied to assess apoptosis had technical limitations. The apoptotic genetic markers for GCs from ovarian follicles cannot be accomplished, and were replaced by blood samples. Additional limitations of this study include the study's restriction to a single reproductive centre, heterogeneity of the included women regarding infertility diagnoses, and the relatively small sample size.

## 5. Conclusion

Ovarian GCs are crucial for oocyte maturation, developing an appropriate milieu surrounding the oocyte. Therefore, apoptosis and the depletion of GCs may adversely impact the intrafollicular environment, diminish oocyte quality, and lower the likelihood of pregnancy. Considering the obvious relationship between apoptotic caspase-9 and cytochrome C and pregnancy rate, our study proposed caspase9 and cytochrome C involvement in oocyte development. Briefly, *CASP9* and *CYCS* expression in blood and their

protein levels in FF exhibited an inverse correlation with pregnancy outcomes, indicating that an elevated apoptotic rate of GCs may adversely impact oocyte growth, reduce pregnancy rates, and subsequently cause IVF failure. Specifically, *CASP9* expression and FF protein level may serve as indirect indicators of apoptosis in ovarian follicles and are potentially predictive of pregnancy failure after IVF. The expression of *CASP9* and *CYCS* in human GCs warrants consideration as the quantifiable caspase-9 and cytochrome C in human FF, potentially aiding in the assessment of IVF pregnancy outcomes. Extensive investigations may be required to eliminate the practical limitations and establish the current findings.

**Ethical clearance**

This research was ethically approved by the Research Ethical Committee of both the Ministry of Higher Education and Scientific Research and the Ministry of Health and Environment in Iraq.

**Conflict of interest:**

The authors declare that they have no conflict of interest.

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