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Cytochrome C in in vitro fertilization: a promising tool for distinguishing spent embryo culture fluids of normoresponder, diminished ovarian reserve, and polycystic ovarian syndrome

Duygu Kütük[1], Nergis Özlem Kılıç[1], Çağrı Öner*[2], Ertuğrul Çolak[3]

[1]*Department of Histology and Embryology, Faculty of Medicine, Maltepe University, İstanbul, Turkiye*
[2]*Department of Medical Biology, Faculty of Medicine, Kırklareli University, Kırklareli, Turkiye*
[3]*Department of Biostatistics, Faculty of Medicine, Eskişehir Osmangazi University, Eskişehir, Turkiye*

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***Corresponding Author**: Çağrı Öner, cagrioner@klu.edu.tr

ABSTRACT

Aims: Cytochrome C (Cyt C) is a small heme protein located in the mitochondria, playing a dual and crucial role in cellular physiology as cellular respiration and apoptosis. This study aimed to investigate Cyt C gene expression in spent embryo culture fluid (SECF) as a non-invasive biomarker for distinguishing between normoresponder (NOR), early-diminished ovarian reserve (E-DOR), advanced-diminished ovarian reserve (A-DOR), and polycystic ovarian syndrome (PCOS) patients undergoing in vitro fertilization (IVF).

Methods: SECF samples were collected from patients at Bahçeci Health Group Umut IVF Laboratory, İstanbul, with informed consent and ethical approval (2023/19-22). Forty-eight samples from each group (NOR, E-DOR, A-DOR, PCOS) were analyzed. Ovarian stimulation followed an antagonist protocol, and embryos were cultured individually. On day 3, embryos were morphologically graded. Total RNA was isolated from SECF, converted to cDNA, and Cyt C gene expression was quantified using RT-PCR, with GAPDH as an internal control. Statistical analyses included Kolmogorov-Smirnov and Shapiro-Wilk tests for normality, Sidak for multiple comparisons, and Pearson correlation test for group relationships.

Results: Compared to NOR samples, Cyt C gene expression was significantly upregulated in A-DOR samples, while E-DOR samples showed downregulation ($p<0.001$). No statistical difference was observed between NOR and PCOS samples ($p>0.05$). Pearson correlation revealed a significant relationship only between NOR and A-DOR SECF samples ($r<0.01$).

Conclusion: These findings suggest that Cyt C expression in SECF can differentiate between early and advanced stages of diminished ovarian reserve, with age-related differences in apoptosis mechanisms. PCOS cases, characterized by a high quantity but low quality of oocytes, showed no significant variation in Cyt C expression, likely due to less active apoptotic pathways. Monitoring Cyt C in SECF may provide a valuable, non-invasive tool for assessing embryo quality and optimizing IVF outcomes. Further research is needed to validate these findings and establish clinical protocols.

Keywords: Cytochrome C, diminished ovarian reserve, in vitro fertilization, normoresponder, polycystic ovarian syndrome, spent embryo culture fluid

INTRODUCTION

Cytochrome C (Cyt C) is a small heme protein found loosely associated with the inner membrane of the mitochondrion. It plays a crucial role in the electron transport chain, which is a series of complexes that transfer electrons from electron donors to electron acceptors via redox reactions. This process is essential to produce ATP, the energy currency of the cell. Cyt C is also involved in the intrinsic pathway of apoptosis, where it is released from mitochondria into the cytosol in response to pro-apoptotic signals, leading to the activation of caspases and cell death.[1]

Spent embryo culture fluid (SECF) is the medium that surrounds embryos during in vitro culture. This fluid contains a variety of biomolecules, including proteins, metabolites, and nucleic acids, which are secreted by the embryos.[2] The analysis of SECF can provide valuable insights into embryo metabolism, viability, and developmental potential. It has been suggested that the composition of SECF can be used as a non-invasive biomarker for selecting the most viable embryos for transfer in assisted reproductive technologies (ART).[3]

Recent study has explored the presence and role of Cyt C in SECF. It has been hypothesized that Cyt C levels in SECF could be indicative of mitochondrial activity and embryo quality. For instance, a study by Smith et al.[4] found that higher levels of Cyt C in SECF were associated with better embryo development and higher implantation rates. This suggests that Cyt C could serve as a potential biomarker for embryo selection in ART.

This study aims to investigate the identification of the similarities and differences between NOR, early- and advanced- diminished over reserve and polycystic over syndrome patients as a perspective of Cyt C gene expression by using SECF samples.

METHODS

Human spent embryo culture medium samples donated to research were obtained with informed consent from patients undergoing IVF at the Bahçeci Health Group Umut IVF Laboratory, İstanbul. Forty-eight samples were collected from SECF for each NOR, early and advanced reduced response, and polycystic ovary syndrome. This study has been approved by the Ethics Committee of Maltepe University (Date: 09.11.2023, Decision No: 2023/19-22). All procedures were carried out in accordance with the ethical rules and the principles of the Declaration of Helsinki.

Ovarian stimulation (OS) was started on day 2 of the menstrual period by employing the antagonist protocol. The dosage of gonadotropins was individualized based on the cases' parameters (**Figure 1**). NOR, early- and advanced-diminished over reserve and polycystic over syndrome cases received 250 µg of human chorionic gonadotropin (hCG; Ovitrelle, Serono) or 0.2 mg of triptorelin (Gonapeptyl, Ferring) for the final oocyte maturation when at least two follicles reached 18 mm in diameter, and transvaginal ultrasound-guided follicle aspiration was performed for oocyte retrieval after 35 h. The oocyte retrieval, denudation, and ICSI procedures were performed as previously described.[5] After microinjection, oocytes were cultured individually in a unique, pre-equilibrated culture dish. Single-step media-

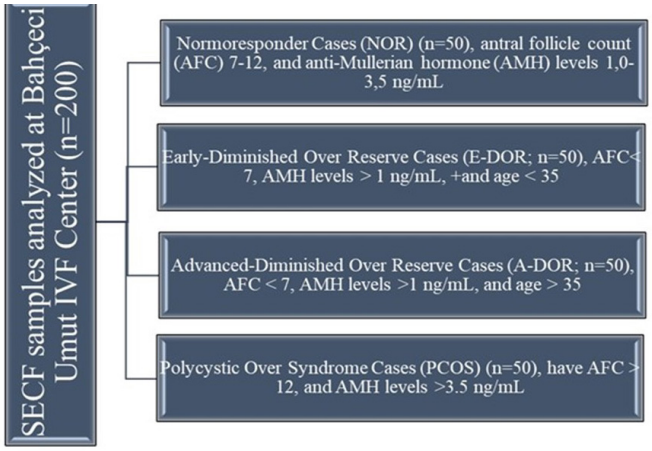

Figure 1. The properties of normoresponder, early- and advanced- diminished over reserve and polycystic over syndrome cases.

namely, single continuous culture complete (CSCM-C) with human serum albumin (Irvine Scientific, Santa Ana, CA, USA)-was used for the embryo cultures throughout the culture period in our study. All embryos were kept in benchtop incubators (MIRI, ESCO Medical, Singapore) and cultured until day 5 or 6 of embryo development. SECF materials were transferred into a PCR tube and kept frozen at 20° C until analysis.

On day 3 of embryo development, a division stage morphological score was assigned based on a three-point grading system using characteristics such as cell number, fragmentation, symmetry, and shape (**Figure 2**).

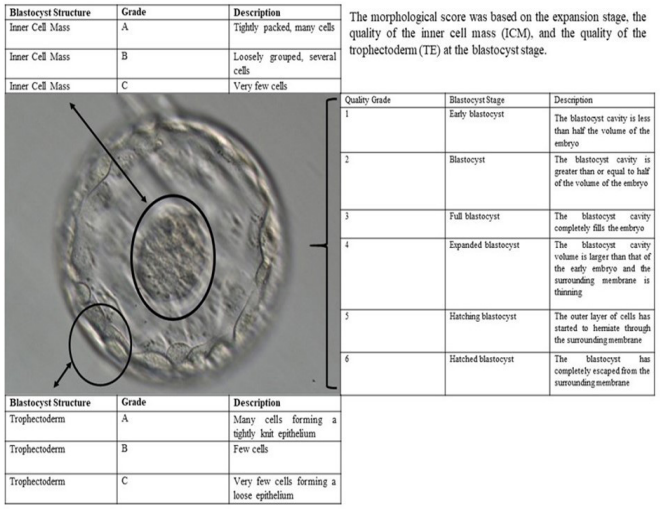

Figure 2. Morphological classification of normoresponder, early and advanced diminished over response, and polycystic over syndrome embryos

Total RNAs were isolated from the collected samples according to the manufacturer protocol (AnalytikJena, Germany). Total RNAs are converted to cDNA (Nucleogene, Turkiye) and then Cyt C; (BmLabosis, Turkiye) gene expression was detected by using real time- polymerase chain reaction (RT-PCR; Kogene, South Korea). As an internal control, glyseraldehide-3-phosphate dehydrogenase (GAPDH) was used. The primer sequences are shown in **Table 1**. ΔCT method was used to determine the Cyt C gene expression.[6] Kolmogorov-Smirnov and Shapiro-Wilk tests were used for normal distribution, and Sidak was used to determine multiple comparisons. *p<0.01; **p<0.05, and ***p<0.001 values were indicated as a result of statistical evaluations for gene expressions while ##r<0.01 value was used for correlations between experimental groups.

RESULTS

Compared to the NOR samples (-2.21±1.957), the gene expression of Cyt C was upregulated in the A-DOR samples (2.751±1.902) while the Cyt C gene expression of E-DOR (-4.969±3.69) was downregulated (p<0.001). There couldn't be observed any statistical difference between NOR and PCOS samples (-2.324±2.328; p>0.05). According to the Pearson correlation test, there is only a correlation between NOR and A-DOR SECF samples (r<0.01, **Figure 3**).

Table 1. Forward and reverse primer sequences used in RT-PCR

Gene	Forward sequence	Reverse sequence
Cyt C	5'-TCGTTGTGCCAGCGACTAAA-3'	5'-TCTTGTGCTTGCCTCCCTTT-3'
GAPDH	5′-CGAGGGGGGAGCCAAAAGGG-′3	3′-GAAACTGCGACCCCGACCGT-′5

Cyt C: Cytochrome C, GAPDH: Glyseraldehide-3-phosphate dehydrogenase

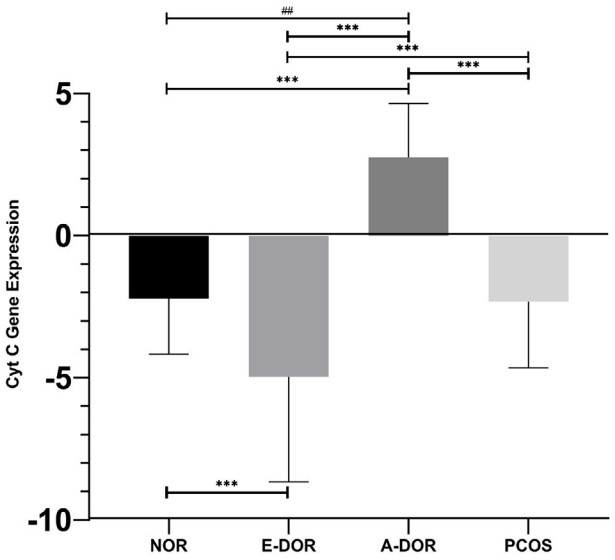

Figure 3. The Cyt C gene expression of normoresponder (NOR; p<0.001), early-diminished over response (E-DOR; p<0.001), advanced-diminished over response (A-DOR; p<0.001), and polycystic over syndrome (PCOS;p>0.05).

DISCUSSION

Cyt C is a mitochondrial protein that transfers electrons to the respiratory chain and sustains ATP production to perform life-supporting functions. Cyt C is also central to the intrinsic pathway of apoptosis. When cells receive pro-apoptotic signals, Cyt C is released from the mitochondria into the cytosol, triggering a cascade that activates caspases and leads to controlled cell death. This process is vital for tissue homeostasis, development, and the elimination of damaged or abnormal cells.[7]

Recently, embryo waste culture fluid has been used to analyze various parameters, especially biochemical and genetic. In contrast to trophectoderm biopsy, Shitara et al.[8] discovered that cell-free DNA (cf-DNA) in spent culture medium accurately reflects the chromosomal condition of embryos after culturing past implantation.In a study, to help choose a viable embryo with high implantation potential, metabolic analysis of spent embryo culture medium may be a useful supplement to morphological characteristics in assessing the embryonic viability and attachment potential of preimplantation embryos.[9] Furthermore, Zhang et al.[10] predicted embryo development potential by using digital PCR detection of mtDNA/gDNA ratio in SECF. It was reported that granulosa cell (GC) apoptosis is a physiological phenomenon in the follicle and may cause a decrease in follicle number by inducing follicular atresia.[7,11]

Limitations

The detection of Cyt C in SECF could also provide insights into the metabolic state of the embryo. Mitochondrial dysfunction is known to impair embryo development and is a common cause of infertility. By monitoring Cyt C levels in SECF, clinicians could potentially identify embryos with compromised mitochondrial function and avoid their transfer, thereby improving the success rates of ART procedures. This study has some limitations, one of these being that the number of samples taken represents only a single population, there is a possibility that the sample is not representative of the entire population. Furthermore, given the resources available to us, fund, only a single gene could be examined. Therefore, further genetic studies could be conducted for the same study group.

In the study, SECFs from advanced-diminished ovarian reserve (A-DOR) patients showed significantly upregulated Cyt C expression, suggesting higher apoptosis and potentially lower viability (p<0.001). Early-diminished ovarian reserve (E-DOR) embryos had downregulated Cyt C, indicating less apoptosis and possibly better viability (p<0.001). NOR and polycystic ovarian syndrome (PCOS) embryos showed no significant difference in Cyt C levels, suggesting similar apoptotic activity and viability.

The absence of variation in Cyt C within the PCOS group, coupled with the divergent expression profiles of DOR throughout early and late ages, indicates that SECF may serve as a valuable tool for differentiating between early and late stages of DOR. PCOS cases are characterized by a high quantity of oocytes, although their quality is inadequate. In such a situation, apoptotic pathways would not be effective and therefore Cyt C expression would be low, or no difference would be expected. According to our findings, cases with low Cyt C expression indicate E-DOR, while cases with high Cyt C expression are A-DOR individuals. One of the main reasons is thought to be the age of the cases. E-DOR individuals are younger than A-DOR individuals. Therefore, it is known that the apoptosis mechanism normally seen in various tissues and cells of these individuals is more active than in young individuals. In addition, the apoptosis rate in A-DOR cases was significantly higher than in E-DOR cases. It is thought that one of the reasons for the number of oocytes being less than 7 in the A-DOR cases may be that Cyt C exits from the mitochondria to the cytosol and initiates the apoptotic cascade.

In the context of in vitro fertilization (IVF), Cyt C levels in SECF can reflect the metabolic and apoptotic status of embryos. Elevated or reduced expression of Cyt C may indicate mitochondrial dysfunction or altered apoptosis, which can affect embryo viability and developmental potential. Furthermore, Monitoring Cyt C in SECF offers a non-invasive method to assess embryo quality, helping clinicians select embryos with higher implantation potential and optimize IVF outcomes. It can also help differentiate between ovarian response types, such as NOR, diminished ovarian reserve, and PCOS, providing insights into

underlying cellular mechanisms. Balanced Cyt C levels are associated with healthy mitochondrial function and controlled apoptosis, both of which are crucial for embryo viability. Abnormal levels, either too high (excessive apoptosis) or too low (mitochondrial dysfunction), can negatively impact embryo development and reduce the likelihood of successful IVF. Thus, Cyt C measurement in SECF is a promising tool for optimizing embryo selection and improving reproductive outcomes.

CONCLUSION

As a result, Cyt C is a vital protein involved in both cellular respiration and apoptosis. Its presence in SECF offers a promising avenue for non-invasive assessment of embryo quality and viability in ART. By identifying embryos with compromised mitochondrial function or abnormal apoptotic activity, Cyt C measurement may improve the success rates of ART and contribute to personalized treatment strategies. Further research is needed to fully understand the implications of Cyt C levels in SECF and to develop reliable protocols for its use in clinical practice.

ETHICAL DECLARATIONS

Ethics Committee Approval

This study has been approved by the Ethics Committee of Maltepe University (Date: 09.11.2023, Decision No: 2023/19-22).

Informed Consent

Written informed consent was obtained from all individual participants prior to their inclusion in the study. Participants were fully informed about the study's aims, procedures, potential risks and benefits, and their rights-including the right to withdraw at any time without consequence. All participants voluntarily signed a written informed consent form.

Peer Review Process

This manuscript was subject to external peer review.

Conflict of Interest

The authors declare no conflicts of interest related to this study.

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Author Contributions

Concept: D.K., N.Ö.K., Ç.Ö.; Design: D.K., N.Ö.K., Ç.Ö.; Control: D.K., N.Ö.K., Ç.Ö.; Resources: D.K., N.Ö.K., Ç.Ö.; Materials: D.K., N.Ö.K., Ç.Ö.; Data Collection and/or Processing: D.K., N.Ö.K., Ç.Ö., E.Ç.; Analysis and/or Interpretation: E.Ç., Ç.Ö.; Literature Review: D.K., N.Ö.K., Ç.Ö.; Writing The Article: D.K., N.Ö.K., Ç.Ö., E.Ç.; Critical Review: E.Ç., Ç.Ö.

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