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Analysis of Cell-Free DNA (as a genetic index for embryo quality) in an Iranian Women Undergoing IVF

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A B S T R A C T

1) Background: In vitro fertilization (IVF) is offered as a treatment for infertility in women. . The Cell free DNA in plasma/follicles were suggested as a biomarker for embryo quality in IVF. Higher the levels of Cell-free DNA indicate poorer embryo quality in the follicular fluid during IVF. Therefore, this study was designed to examine the cell-free DNA (cfDNA) in plasma and Fluid-follicle of women undergoing IVF-embryo transfer. 2) Methods: The study examined 100 women undergoing IVF treatment in an IVF unit in Yas Hospital of Tehran University of Medical Sciences in Tehran-Iran. Cell-free DNA was extracted from plasma and follicular fluid of patients using the NucleoSpin® Extraction Kit. The total cfDNA was examined by qPCR for *ALBUMIN* and *GAPDH* genes. 3) Results: we did not find a statistically significant association among the variables such as CT-plasma, CT-follicular fluid, delta-CT and average of these data between two groups (including patients who had a successful IVF cycle and those who had a failed IVF cycle). 4) Conclusion: Plasma and follicular fluid cfDNA may reflect the presence of factors which interfere with embryo implantation. Further research is required to determine the usefulness of cfDNA as a biomarker of IVF outcome and to examine the underlying pathologies as potential sources for increased plasma/follicular fluid cfDNA concentrations.

Introduction

In vitro fertilization (IVF) accounts for 99% of assisted reproductive technology (ART) procedures. IVF is known as an expensive and invasive method of promoting fertility, and is often used as a last resort after all other treatment options have been exhausted. One of the main challenge of IVF is to select the single best embryo with the greatest implantation potential and the greatest chance of developing full-term baby(Rule, Chosed et al. 2018). Nevertheless, the subjective observation of embryo morphology to predict a successful pregnancy shows limitations(Aydiner, Yetkin et al. 2010).

Therefore, many recent studies have focused on the identification of new non-invasive biomarkers based on the analysis of the oocyte microenvironment to improve the accuracy of embryo selection.

In some studies, follicular fluid (FF) components derived from plasma or secreted from granulosa cells, were investigated as potential biomarkers (Borowiecka, Wojsiat et al. 2012, Lédée, Gridelet et al. 2012).

Cell-free DNA as DNA fragments released into the body fluids from apoptosis or necrotic processes is usually considered as a biomarker(Swarup and Rajeswari 2007). The presence of cell-free DNA (cfDNA) in the circulation was first documented in adult serum in 1948 (Mandel 1948). The cfDNA has been found in the plasma of both healthy and diseased populations.

Elevated concentrations have been identified in several medical conditions, such as certain types of cancer and inflammatory conditions (Duvvuri and Lood 2019, Fettke, Kwan et al. 2019), stroke (Arnalich, Maldifassi

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et al. 2010) and preeclampsia (Rafaeli-Yehudai, Imterat et al. 2018). Also, it has been demonstrated that cfDNA analysis has high sensitivity and extremely low false positive rates for common autosomal trisomies in pregnancy across quantitation platforms (Stokowski, Wang et al. 2015). The preliminary results from E. Scailci's et al.'s study demonstrated that cell -free DNA in follicles may serve as a biomarker of embryo quality in IVF. High levels of Cell-free DNA indicate the poor embryo quality in the follicular fluid during IVF and vice versa(Scalici, Traver et al. 2014).

The emergence of non-invasive prenatal testing (NIPT), based on fetal cfDNA detection in the maternal blood, is a promising approach for obstetrics and gynaecology. It has been believed that high levels of cell-free DNA may lead to apoptosis in the follicles, which in turn affects the quality of the embryo. As has been reported previously, qPCR is highly sensitive and high-throughput method to measure the cell free DNA in serum and a properly designed primer set has dramatically increase the sensitivity of size-dependent DNA measurement(Umetani, Kim et al. 2006). Despite new findings about the predictive role of cfDNA level in embryo and pregnancy outcomes of women undergoing IVF, there have been no reports regarding cfDNA level in Iranian women undergoing IVF; therefore, this study aimed to examine cfDNA in plasma and the follicular fluid of individual follicles from patients undergoing conventional in vitro fertilization (IVF) using qPCR.

Materials and Methods

Subjects

One hundred patients who were candidate for IVF were selected from Yas hospital, affiliated of Tehran University of Medical Sciences-Iran, between Jan 2017 and Jul 2018. The inclusion criteria for the study were as follows: patients <40 years old; no previous history of spontaneous abortion and without a history of autoimmune diseases, and with proper endocrine functions. After the conventional ovulation induction treatment, follicular fluid was collected from>16 mm diameter follicles (Broekmans, Verweij et al. 2014). On the day of oocyte retrieval, blood samples also were collected for further analysis. The mean age of the participants was 34 ± 5 years. The study exclusion criteria were specified as follows: male factor as the leading cause of infertility, uterine anomalies and polycystic ovary syndrome. The study protocol was approved by local Ethics Committee (IR.TUMS.MEDICINE.REC.1395.1072) and all specimens were collected from patients who had provided the written consent.

Cell-free DNA extraction and quantification by qPCR

Cell-free DNA was separated and extracted from plasma and follicular fluid using the NucleoSpin® Extraction Kit (MACHEREY-NAGEL GmbH & Co., Düren, Germany) according to manufacturer's instructions and stored at −80 °C. The quality of extracted DNA was observed by a 2% agarose gel electrophoresis. Moreover, The extracted cfDNA was quantified by Nanodrop. The total cfDNA was examined by qPCR using primer sets, generating 350-bp (*ALBUMIN* primers) and 75-bp (*GAPDH*) amplicons. For each qPCR reaction, 1 µl of DNA was added to a reaction mixture (final volume: 15 ml) containing 1 µL of forward and reverse primers, 7.5 µl of 2X SYBR Green master mix (high ROX, Amplicon) and 4.5 µl H_2O. Each sample was repeated three times and the average value was calculated. The details of primers are presented in table 1.

Statistical analysis

All results were presented as mean ± SEM. Statistical analysis was performed using SPSS software. The mean values of total cfDNA were compared among the groups using the Student's t-test. $P<0.05$ was considered as statistically significant.

Results

Cell-free DNA was successfully extracted from plasma and follicular fluid. Our results showed proper ratio for cfDNA extracted from plasma and follicular fluid of patients (Pure nucleic acids typically yield a 260/280 ratio of ~1.8).

After two weeks, it was determined that Only 13 of the total patients had a successful IVF cycle and became pregnant. The amplification of *ALBUMIN* and *GAPDH* genes was carried out by CyberGreen's Real-Time PCR to investigate the molecular characteristics of cfDNA in both groups including patients who a successful IVF cycle and those who had a failed IVF cycle. The number of broken DNA fragments was examined in the plasma and follicular fluid of two groups (including patients who a successful IVF cycle and those who had a failed IVF cycle). The Real-Time PCR results showed that *ALBUMIN* gene did not amplify in samples with no product (Figure 1). The *ALBUMIN* gene replication curve was derived from CT analysis, melt curve and positive control examination approved the given data. Our results also showed that *GAPDH* gene was amplified normally and the product (75bp) was observed (Figure 2).

The results of cell-free DNA detection in the plasma and follicular fluid samples showed that there were no long DNA fragments (350 bp) in all samples and no significant difference was observed between two groups (including patients who had a successful IVF cycle and those who had a failed IVF cycle). The data suggested that cell-free DNA was mainly originated from the cellular apoptotic events with no necrosis process. Table 2 presents the X^2 results of the variables between patients who had a successful IVF cycle and those who had a failed IVF cycle.

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Table 1 - Primers Information

Gene	Primer sequence	Product size	Annealing Temp (°C)
GAPDH	Forward primer 5-' ACACAGGGACGGGATTGTCT -3' Reverse primer 5'- GCACGGAAGGTCACGATGT- 3'	75	62
ALBUMIN	Forward primer 5-' GCCCTCTGCTAACAAGTCCTAC -3' Reverse primer 5' – GCCCTAAAAAGAAAATCCCCAATC- 3'	350	62

Table 2- X^2 Results of the Variables between Patients who had a Successful IVF Cycle and those who had a Failed IVF Cycle

	CT-Plasma	CT-Follicular fluid	Delta CT	AVERAGE: $(CT_p+CT_f)/2$
P-Value	0.433	0.456	0.336	0.433

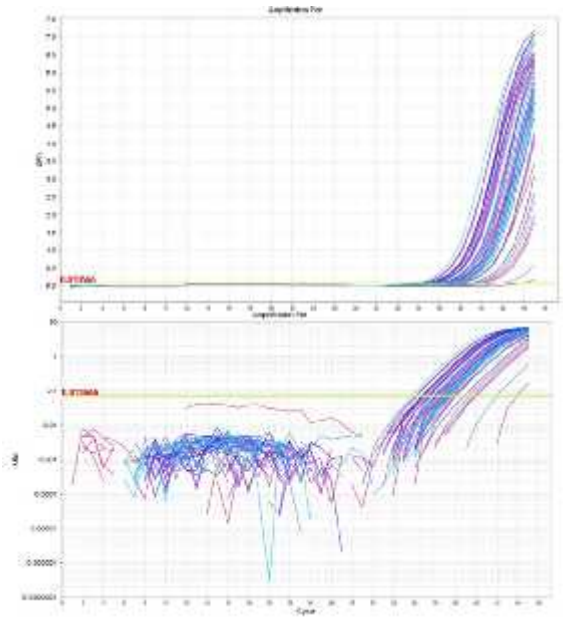

Fig. 1- The Amplification Plot of *ALBUMIN* Gene

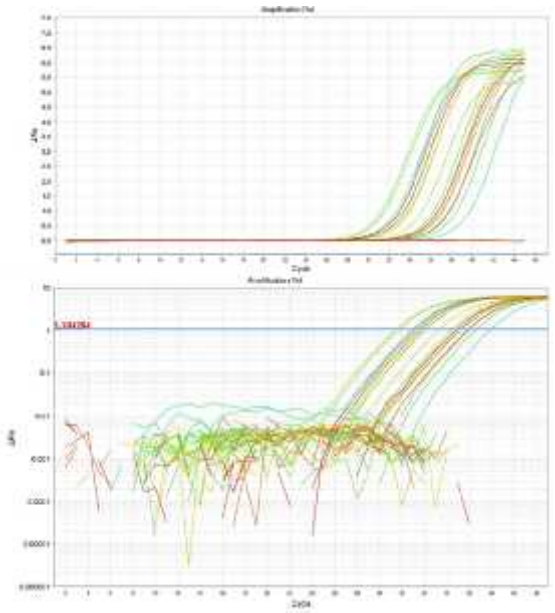

Fig. 2- The Amplification Plot of *GAPDH* Gene

Discussion

Cell-freeDNA (cfDNA) fragments are resulted from apoptotic or necrotic processes and can be found in bloodstream(Ossandon, Agrawal et al. 2018). The released nuclear and mitochondrial DNA levels in the blood are then phagocytized by macrophages in healthy individuals, in whom the basal cfDNA levels remains low. CfDNA can be actively secreted by cells, leading to an increase in cfDNA levels of some serious disorders. For this reason, cfDNA can be served as non-invasive diagnostic and/or prognostic biomarker for some cancers and other severe pathologies(Scalici, Traver et al. 2014).

It has been believed that ovarian stimulation activates the apoptotic pathway in many recruited oocytes during the process of IVF stimulation. The higher the free DNA in the follicular fluid, the greater the apoptotic cascade will be, which in turn can result in either apoptotic oocytes or in limited number of oocytes.

The consequence of the above results, through the high free DNA concentrations, seems to affect directly embryo development and subsequently embryo quality and implantation potential(Liu and Li 2010).

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DNA released from necrotic cells varies in size, whereas DNA released from apoptotic cells is uniformly truncated to shorter fragments with 185–200 bp(Umetani, Kim et al. 2006). Therefore, presence of long DNA fragment (such as *ALBUMIN*-350bp) represented cell-free DNA generated by necrosis process. Because the major source of free circulating DNA in healthy individuals is apoptotic cells, a preponderance of longer DNA fragments has been proposed as a marker for disorders detection(Jahr, Hentze et al. 2001). Accordingly, this study was designed to examine the presence of cfDNA in Iranian patients undergoing IVF. Our results showed no long DNA fragments (*ALBUMIN*-350 bp) in the plasma and follicular fluid of the patients who had a successful IVF cycle and those who had a failed IVF. We suggested that cell-free DNA could mainly originated from the cellular apoptotic events but not necrosis process. Also, we did not find any statistically significant association among the variables including CT-plasma, CT-follicular fluid, delta-CT and average of these data between two groups (including patients who had a successful IVF cycle and those who had a failed IVF).

Evidence for the association between several studies have provided evidence that IVF outcome can be associated with apoptotic processes. Liu and Li speculated that oxidative stress in granulosa cells had an effect on IVF-embryo transfer failure, and also connected the higher apoptotic rate to lower oocyte quality(Liu and Li 2010). Dı ´az-Fontdevila et al. found that cumulus cells of women with a diagnosis of endometriosis and those exposed to spermatozoa had higher rates of apoptosis. Cumulus cells of embryos with low quality had higher apoptotic rates than those with high quality(Díaz-Fontdevila, Pommer et al. 2009). Guan et al. showed that cell-free DNA could affect the levels of reactive oxygen species in the follicular fluid, which increased the apoptosis of granulosa cells. They found that this might be considered as the major factor where cell-free DNA affected the quality of embryos during IVF(Guan, Zhang et al. 2017). Rodgers et al. found that follicular fluid composition effects oocyte development, and has a strong influence on the quality of subsequent embryos (Rodgers and Irving-Rodgers 2010). High levels of cell-free DNA as main ingredients in the follicular fluid micro-environments, can affect the development of oocyte and the embryo, resulting in failure of IVF(Guan, Zhang et al. 2017).

Czamanski-Cohen et al. examined cfDNA concentrations in the plasma of peripheral blood of women undergoing IVF as a possible predictive factor affecting conception rates in IVF–embryo transfer treatment. Their results showed that the mean concentration of plasma cfDNA among all participants did not statistically significantly change(Czamanski-Cohen, Sarid et al. 2013). In addition, Hart et al. found no relationship between cfDNA concentrations in sera and conception rates in IVF patients(Hart, Patton et al. 2005). These results were consistent with our findings. The difference between studies might be due to the method used to measure cfDNA, different sample sizes and various inclusion and exclusion criteria.

The findings of the current study have to be seen in light of some limitations. The fact that women participating in the process of IVF treatment impeded our study ability to conclude whether increased cfDNA is related to the infertility phenomenon or the IVF treatment itself. Further research considering IVF populations during a non-stimulated menstrual cycle is required. Moreover, in the current study, the study groups were not match for size, only 13 women had had a successful IVF cycle, which can affect the comparison results. It is alsobeneficial to include control group in the study and use more primers to determine the apoptotic or necrotic origin of cfDNA (). In addition, further research is required to determine the usefulness of cfDNA as a biomarker of IVF outcome and to examine the underlying pathologies as potential sources for increased plasma and follicular fluid cfDNA concentrations. Future studies that include genetic sequencing of DNA fragments to determine the origin of the cfDNA would be beneficial to identify potential pathologies that affect fertility and prevent successful implantation after t IVF treatment.

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بررسی DNA آزاد سلولی (به عنوان مارکر ژنتیکی تعیین کیفیت جنین) در گروهی از زنان ایرانی تحت درمان با IVF

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چکیده

مقدمه: DNA آزاد سلول (cfDNA) در پلاسما یا فولیکول به عنوان بیومارکر برای سنجش کیفیت جنین در IVF (باروری خارج از لقاح در محیط غیرزنده) پیشنهاد شده است. مقدار زیاد cfDNA، کیفیت پایین تخمک ها را در مایع فولیکولی در طول IVF نشان می دهد. بنابراین این مطالعه برای بررسی cfDNA در پلاسما و مایع فولیکولی زنانی که برای IVF مراجعه نمودند طراحی گردید. روش کار: در این مطالعه ۱۰۰ زن تحت درمان با IVF در بیمارستان یاس دانشگاه علوم پزشکی تهران مورد بررسی قرار گرفتند. cfDNA از پلاسما و مایع فولیکولی بیماران با استفاده از کیت استخراج NucleoSpin استخراج شد. وجود cfDNA توسط qPCR برای ژنهای *ALBUMIN* و *GAPDH* مورد بررسی قرار گرفت. یافته ها: ارتباط معنی داری بین متغیرهای CT-پلاسما، CT- مایع فولیکولار، دلتا CT و میانگین این داده ها بین دو گروه مورد مطالعه (بیماران IVF موفق و ناموفق) مشاهده نشد. نتیجه گیری: cfDNA پلاسمایی و مایع فولیکولی ممکن است وجود عواملی را که در تکامل جنین دخیل هستند، شناسایی کند. بنابراین تحقیقات بیشتری برای تعیین نقش کاربردی cfDNA به عنوان بیومارکر پیش بینی کننده نتیجه IVF و بررسی پاتولوژی زمینه ای به عنوان منابع بالقوه برای افزایش غلظت cfDNA مایع پلاسما / فولیکولیک مورد نیاز است.

واژگان کلیدی: DNA آزاد سلول، مایع فولیکولی، پلاسما، IVF، *ALBUMIN*، *GAPDH*

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