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Cell-free DNA in Embryo Culture Media as Non-invasive Biomarker of the Quality of Embryo Cleavage

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Authors' contributions

This work was carried out in collaboration among all authors. Author MJ contributed to experimental work, samples and data collection, statistical work, manuscript writing and data interpretation. Author HD contributed to experimental work, design, conception, data analysis and statistical analysis. Author HJ contributed to experimental work. Author AK contributed to sample and data collection. Author ME contributed to sample and data collection. Author MZ contributed the creation of data resources. Author WS contributed to the creation of data resources. Author EMH contributed to the creation of data ressources. Author NL was responsible for overall supervision. Author RC contributed to overall supervision, study design, interpretation of data and manuscript revision. All authors read and approved the final manuscript.

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ABSTRACT

Background: The success of *in vitro*-fertilization (IVF) cycles is determined in large part by the quality of embryo cleavage, which in turn, is dependent on the quality of the embryo culture media (CM). Many factors can influence the quality of embryo CM, one of which is the levels of Cell Free *Deoxyribonucleic acid* (DNA). Understanding the association between Cell-free DNA levels in embryo CM and the quality of embryo cleavage could help improve the quality of IVF techniques.
Methods: This prospective study was conducted with 96 spent CM from patients undergoing IVF cycle, in order to determine relationships of Cell-free DNA levels in embryo CM with embryo cleavage quality on day 3. After intracytoplasmic sperm injection (ICSI), 48 embryos were evaluated on day 3 of their development, according to their cell number. Day 2 and day 3 CM corresponding to each one of the embryos was analyzed, by quantitative PCR, for estimation of Cell-free DNA levels.
Results: The results revealed a significant increase in Cell-free DNA levels on day 2 CM corresponding to 4 to 6 cell embryos compared to those corresponding to 7 to 8 cell embryos ($p=0.04$). As for day 3 CM, the results showed no significant difference between the Cell-Free DNA levels in CM of 7-8 and those of 4-6 cell embryos ($p=0.4$). Also, cell free DNA levels in embryo CM, were significantly higher on day 2 compared to day 3 for both 7-8 and 4-6 cell embryos ($p=0.03$; $p=0.04$).
Conclusion: We conclude that cell-free DNA levels in CM might be associated with delayed embryo cleavage.

Keywords: Cell-free DNA; embryo cleavage; embryo culture; in vitro fertilization.

1. INTRODUCTION

Among the essential components that affects the success of IVF results is the morphological quality of the embryo. Embryo morphology allows the evaluation of its growth, viability, and implantation capacity. Regular morphology, well-organized cells, and proper symmetry are characteristics of higher-quality embryos, which point to healthy development and higher rates of implantation. Low-quality embryos, on the other hand, frequently display morphological abnormalities, such as cell fragments, vacuoles, or asymmetry, which suggest aberrant development and a low chance of successful implantation [1,2,3]. Hence, the chances of IVF success can improved by selecting embryos of the best morphological quality. As an environment for *in vitro* embryos, CM can give insight into the quality of embryo cleavage kinetics. Therefore, analyzing its constituents can help identify the variables that have an impact on embryonic quality, either positively or adversely [4-6]. Previous research has demonstrated that the release of apoptotic-derived DNA fragments is associated with embryo quality [7-9]. “As a study has shown that cell-free DNA levels in follicular fluid samples corresponding to top quality embryos was significantly lower than in follicular fluid samples related to poor quality embryos” [10].

Cell-free DNA refers to free double-stranded DNA fragments that are released from cells in the process of apoptosis and necrosis. It can be found in human serum, follicular fluid, and plasma [11-13]. Due to its clinical applications and the expansion of non-invasive treatment options, the discovery of cell-free DNA in biological fluids has led to major advances in several medical specialties [14,15]. *In vivo* and in healthy individuals, macrophages can phagocytize DNA that has been passively released into the blood from apoptotic or necrotic cells, thereby maintaining a relatively low basal level [16-18]. *In vitro*, the cell-free DNA fragments released by apoptotic events in embryos are identified as contaminants in culture media [19].

Whether the quantity of cell-free DNA in CM can serve as a criterion for the integrity of embryo cleavage is still up for debate. This study's objective is to investigate the relationship between the release of apoptotic-derived cell-free DNA from embryos CM and the quality of embryo cleavage. By investigating this connection, we seek to better understand the connection between CM and embryo quality, and how this can be used to improve the success of IVF techniques.

2. MATERIALS AND METHODS

2.1 Study Participants

This prospective study was conducted at the IRIFIV fertility center, Casablanca (Morocco). It included 48 couples who were eligible for oocyte retrieval, with primary and secondary infertility and who underwent an intracytoplasmic sperm injection (ICSI) cycle between January 2022 and August 2022. Onlynormozoospermic semen samples in terms of numeration, mobility and motility were included, according to the World Health Organization (WHO) 2010 criteria (numeration >15 M/mL; progressive motility >32 %; typical morphology > 4%). Female patients over the age of 38 years were excluded from this study.

2.2 Ovarian Stimulation Protocol and Oocyte Collection

Women underwent controlled ovarian stimulation with the flexible gonadotrophin-releasing hormone (GnRH) antagonist protocol. A daily subcutaneously injection of recombinant follicle-stimulating hormone (rFSH; Gonal-F, Merck-Serono) was used alone or in combination with human menopausal gonadotrophin (HMG, Menopur; Ferring). The FSH dose was based on the women's age and AMH concentration, in addition to prior history of ovarian stimulation and adjusted according to usual parameters of follicle growth, determined by serum estradiol (E2) concentration and ultrasound monitoring.

A daily dose of GnRH antagonist (Cetrotide, Merck-Serono, or Orgalutran, MSD) was injected subcutaneously, starting from day 6 of FSH administration. The ovulation trigger was performed with 10 000 IU of human chorionic gonadotrophin (rHCG, Ovitrelle; Merck-Serono) and gonadotrophin-releasing hormone (Decapeptyl, Ferring), after obtaining follicles that reached dimensions of 17mm or greater in diameter and adequate serum E2 levels. Oocytes were retrieved 34-36 hours after hCG administration.

2.3 Oocyte and Sperm Preparation

The retrieved oocytes were isolated from the follicular fluid, rinsed and cultured in CM (SAGE 1-Step, Origio). Two to three hours after retrieval, the oocyte-corona-cumulus complexes were placed in a HEPES-buffered medium (Ferticult Flushing medium, Fertipro) containing hyaluronidase (Hyaluronidases in Ferticult Flushing medium, 80IU/mL, Fertipro) and were mechanically decoronated using a 20-200µL micropipette. The nuclear maturation grades were classified as metaphase II or non-metaphase II (Metaphase I or Prophase I) oocytes.

Sperm samples were collected from the male partner by masturbation in a sterile container, after 2-3 days of abstinence. At first, semen samples were evaluated for spermatic parameters (concentration, motility and morphology) based on the WHO (2010) recommendations, Motile spermatozoa were then selected using a discontinuous two-layer density gradient technique (Puresperm 80/40; SAGE).

All mature oocytes underwent ICSI after decoronisation. One micro-injected oocyte per patient was then randomly selected and placed in an oil-covered single drop of 100 µL of culture media (SAGE 1-Step, Origio), in a petri dish.

The embryos were transferred to a fresh medium on day 2 (42-46 after ICSI) and day 3(66-70h after ICSI). The spent CM from day 2 and day 3 were collected for cell-free DNA analysis.

2.4 Assessment of Embryo Quality on day 3

On day 3 only, embryo quality was evaluated according to the number of blastomeres. Embryos were divided into 2 groups: 7-8 cells embryos and 4-6 cells embryos.

The temperature inside the incubators (IVF-Cube AD3100, ASTEC; Thermo Scientific HeraCell 150) was controlled by a certified thermometer and remained at 37±0.2 °C. The oxygen level inside the incubators was at 5% and the cultivating medium pH was at 7.3 ± 0.02 with CO2 around 5.6%.

2.5 Cell-free DNA Extraction and Quantification

Day 2 and Day 3 CM, of the corresponding embryos, were collected for the quantification of Cell-free DNA. Free-DNA was extracted from culture media samples by the SaMag^{tm} STD DNA Extraction Kit according to the manufacturer's instructions. The total free-DNA was quantified by Qpcr, using ALU 115 primers (Unemati N et al., 2006). For each patient, 4 µl of CM are added to the reaction mixture of 0,25 µl of each ALU 115 5'CCTGAGGTCAGGAGTTCGAG-3' (forward) and 5'CCCGAGTAGCTGGGATTACA-3' (reverse) and 4 µl of Luna Universal qPCR Mix (containing the enzyme Taq DNA polymerase, nucleotides and free SybrGreenTM fluorescent intercalator). Cycling conditions were as follows: 95°C for 60s, then 40 cycles of 95°C for 15 s, 58 °C for 20 s and 60 °C for 30 s. All reactions were performed in duplicate on the Sacace biotechnologies. Cell-free DNA concentration in CM samples was determined using a standard curve obtained from a range of genomic DNA (genomic DNA was extracted using the phenol method chloroform as we indicated before). Negative and positive control was included in each series of quantitative PCR.

2.6 Statistical Analysis

The results are expressed as the mean ±Standard deviation. The comparison between the studied groups was carried out by the Student test (parametric test) for the comparison of two means. All of the statistical tests were carried out using the SPSS 2016 software (Statistical Package for the Social Sciences), the significance threshold retained is $p < 0.05$.

3. RESULTS

3.1 Association between Cell-free DNA Levels in CM and the Quality of Embryo Cleavage

All the embryos were divided into 2 groups according to their cell number (7-8 / 4-6 cells) on day 3. On day 2, the results showed a significant increase of Cell-free DNA levels in CM corresponding to 4-6 cells embryos compared to CM corresponding to 7-8 cells embryos (p=0.04). As for day 3 CM, the results showed no significant difference between the Cell-Free DNA levels in CM of these two groups (p=0.4) (Table 1).

Fluctuations of Cell-free DNA levels in CM between day 2 and day 3 within each group were analyzed. We noted that Cell-free DNA levels in embryo CM, were significantly higher on day 2 compared to day 3 (p=0.03; p=0.04), regardless of whether it's 7-8 or 4-6 cells embryos (Fig. 1).

Table 1. Comparison of Cell-free DNA in embryo CM according to the number of embryo cells on day 3

	7-8 cells embryos (n=25)	4-6 cells embryos (n=23)	P value
Cell-free DNA levels in CM on day 2	$9{,}56\times10^{-06} \pm 2{,}57\times10^{-05}$	$4{,}37\times10^{-05} \pm 0{,}0001$	0,04
Cell-free DNA levels in CM on day 3	$6{,}52 \times 10^{-07} \pm 3\times 10^{-06}$	$8{,}81\times10^{-07} \pm 1{,}58\times10^{-06}$	0,4

Values are reported as mean ±SD; (n): number of patients

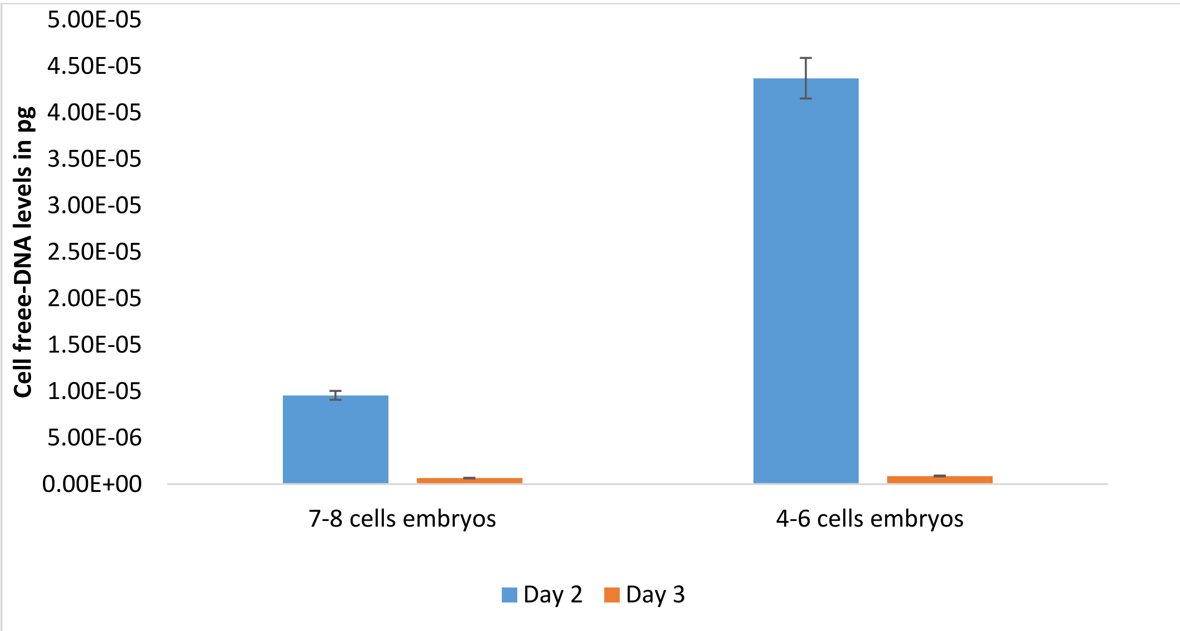

Fig. 1. Association of cell-free DNA levels in day 2 and day 3 CM with the quality of embryo cleavage on day 3

4. DISCUSSION

On day 2 after ICSI, our results revealed significantly higher Cell-free DNA levels in CM of 4-6 cells embryos compared to CM of 7-8 cells embryos. Initially, the presence of Cell-free DNA fragments in day 2 CM can be due to their release by the embryos following the physiological process of fertilization and the initiation of embryo cleavage. The quality of fertilization can influence the molecular mechanisms involved in the process of embryo cleavage. In fact, the regulation of DNA transcription that takes place during fertilization allows the progression of embryonic development. This involves the activation and deactivation of certain genes, which can lead to the release of apoptotic Cell-free DNA fragments into the embryo CM [20]. In addition, apoptotic events can occur during embryo cleavage for the elimination of cells that suffer irreparable damage to their DNA or cells that are no longer necessary for development [21-23]. These physiological processes are necessary for embryo growth and can explain the presence of Cell-free DNA levels in CM on day 2.

However, the significantly higher Cell-free DNA levels in day 2 CM of 4-6 cells embryos compared to those in day 2 CM of 7-8 cells embryos can highlight the association of excess levels Cell-free DNA in CM with the disturbance of embryo cleavage. As matter of fact, high rates of cell-free DNA can reflect abnormal apoptotic events [24,25]. These apoptotic events can be caused by a variety of factors, including oxidative stress and chromosomal abnormalities, that were previously shown by many studies to be associated with embryo quality [26]. Firstly, the many causes of embryo developmental delays are high degrees of chromosomal abnormalities as the timing of division can be affected by the normality of the nuclear condition. In fact, it has been shown that the majority of chromosomal aberrations were found in embryos that were either arrested or slowly dividing [27,28]. Multi-nucleation and mosaicism, two aberrations that have deleterious effects on the timing of cleavage, are the principal manifestations of these aberrations [29,30]. Secondly, the release of high levels of Cell-free DNA fragments into CM of 4-6 cells embryos may be associated with oxidative stress [31]. “Excessive levels of reactive oxygen species (ROS) can cause DNA damage, such as DNA chain breaks, base modifications and adductions, which can lead to the release of Cell-Free DNA fragments into CM” [32-34].

Cell-free DNA from day 3 CM originates from later stages of embryo cleavage, when the cell number normally lies between 5 and 8. In our study, Day 3 CM showed no significant difference between the Cell-Free DNA levels of 7-8 cells embryos and those of 4-6 cells embryos. Based on this result, we can speculate that the release of high levels of apoptotic cell-free DNA in CM might be related to the quality of embryo cleavage during the early stages of development rather than the later stages. Our data also demonstrated higher levels of cell-free DNA in day 2 CM, in comparison with day 3 CM, regardless of the quality of embryo cleavage. As we mentioned previously, cell-free DNA from day 2 CM originates from the process of fertilization (day 1) and the initiation of embryo cleavage (day 2). The higher levels of cell-free DNA fragments in day 2 CM, compared to day 3 CM may be due to their accumulation after the chaining of the mechanisms of these two processes. The lower cell-free DNA levels in day 3 CM can be due to embryo culture renewal as on day 2, CM was renewed by transferring the embryo to a new dish with a fresh CM. According to these results, it might be more beneficial to transfer the embryo to a fresh CM on day 1, namely just after the establishment of fertilization and right before the process of cleavage initiation. Transferring the embryos to fresh culture media on day 1 can help minimize the accumulation of cell-free DNA fragments in CM. These cell-free DNA fragments can be toxic for the developing embryos and interfere with normal cellular processes such as DNA replication and repair [35,36]. Therefore, reducing the levels of cell-free DNA in CM can help create a more favorable embryo environment and mitigate embryonic deletions. Furthermore, fresh media contains all the necessary nutrients and growth factors needed for developing embryos, which can help, provide them a more optimal environment for their development [37,38].

5. CONCLUSION

Our study demonstrated that high levels of cell-free DNA in CM could be associated with delayed embryonic cleavage at day 3. In addition, we were able to demonstrate that the release of cell-free DNA fragments by embryos, in relation with the quality of embryo cleavage, could occur during the early stages of embryonic cleavage.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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