Original Research Article

Assessment of Cell Free-DNA in embryo culture media: Association with the quality of embryo cleavage

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Abstract:

Background: The quality of embryo cleavage is one of the most important criteria for the success of IVF cycles. The quality of embryo culture media (CM) can have a significant impact on the quality of embryo cleavage. Many factors can influence the quality of embryo CM, including the levels of Cell Free-DNA. Understanding the relationship between Cell Free-DNA levels in embryo CM and the quality of embryo cleavage could help improve the quality of IVF techniques.

Methods: In this study, we investigated the association between embryo cleavage quality on day 3 and the levels of Cell Free-DNA in embryo CM. After intracytoplasmic sperm injection (ICSI), 48 embryos were evaluated, on day 3 of their development, according to their cell number. Embryo CM from day 2 and day 3 corresponding to each one of the embryos were analyzed, by quantitative PCR, for estimation of Cell Free-DNA levels.

Results: The results revealed a significant increase of Cell Free-DNA levels in day 2 CM corresponding to 4-6 cells embryos compared to those 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 7-8 and those of 4-6 cells embryos ($p=0.4$). Also, Cell Free-DNA levels in embryo CM, were significantly higher on day 2 compared to day 3 ($p=0.03$; $p=0.04$), regarding 7-8 and 4-6 cells embryos.

Conclusion: We conclude that the increase of Cell Free-DNA levels in embryo CM might originate from the accumulation of disturbances in the mechanisms of fertilization and embryo cleavage.

Keywords : Embryo culture; Embryo cleavage, Cell Free-DNA, In Vitro Fertilization.

INTRODUCTION

Among the essential components of 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 Hence, the chances of IVF success can increase by choosing embryos of the best morphological quality. As an environment for in vitro embryos, culture media can give insight into the quality of embryo cleavage kinetics. Therefore, analyzing its constituents can help identify the either elements that have an impact on embryonic quality, positively or negatively [1-3]. Prior research has demonstrated that the release of apoptotic-derived DNA fragments from preimplantation embryos can impair embryo viability [4-6]. Cell Free-DNA refers to free double-stranded DNA fragments that cells release after going through processes like apoptosis and necrosis. It can be found in human serum, follicular fluid, and plasma [7-9]. Due to its clinical applications and the expansion of non-invasive treatment options, the discovery of free DNA in biological fluids has led to major advances in several medical specialties [10,11]. In vivo and in healthy individuals, macrophages can phagocytize DNA that has been passively released into the blood from apoptotic or necrotic cells, keeping a relatively low basal level [12-14]. In vitro, the free DNA fragments released by apoptotic events in embryos are iendtified as contaminants in culture media [15].

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 evaluate the relationship between the release of apoptotic-derived Cell Free-DNA from embryos in CM and the quality of embryo cleavage. By investigating this connection, we seek to better understand the connection between CM and embryo quality.

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MATERIAL AND METHODS

Patients

This study prospectively included 48 couples, with primary/secondary infertility and undergoing oocyte retrieval cycles of assisted reproduction, between January 2022 and August 2022. Only normozoospermic 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. All couples signed informed consent before the IVF cycle.

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 was 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.

Oocyte and sperm preparation

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The retrieved oocytes were isolated from follicular fluid, rinsed and cultured in CM (SAGE 1-Step, Origio). 2-3 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 WHO (2010) recommendations, Motile spermatozoa were then selected using a discontinuous two-layer density gradient technique (Puresperm 80/40; SAGE) as described by Aboulmaouahib et al. [16].

All matures 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 medium was renewed on day 2 (42-46 after ICSI) and day 3(66-70h after ICSI).

Assessment of embryo quality on day 3

On day 3, 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. Oxygen level inside the incubators was at 5% and the cultivating medium pH at 7.3 ± 0.02 with CO2 around 5.6%.

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Cell Free-DNA extraction and quantification

The spent CM of the corresponding embryos (day 2, day 3) 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 intructions. 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 Ul 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). A negative and positive control was included in each series of quantitative PCR.

Statistical analysis

The results are expressed as the mean ±Standard deviation or percentage of total. Data was obtained with the student's t-test using SPSS (Statistical Package for the Social Science). Statistical significance was defined as $p < 0.05$.

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RESULTS

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, 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. (**Figure 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	$9{,}56 \times 10^{-06} \pm 2{,}57 \times 10^{-05}$	$4{,}37 \times 10^{-05} \pm 0{,}0001$	0,04
Cell Free-DNA levels in CM on day 3	$6{,}52 \times 10^{-07} \pm 3 \times 10^{-06}$	$8{,}81 \times 10^{-07} \pm 1{,}58 \times 10^{-06}$	0,4

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

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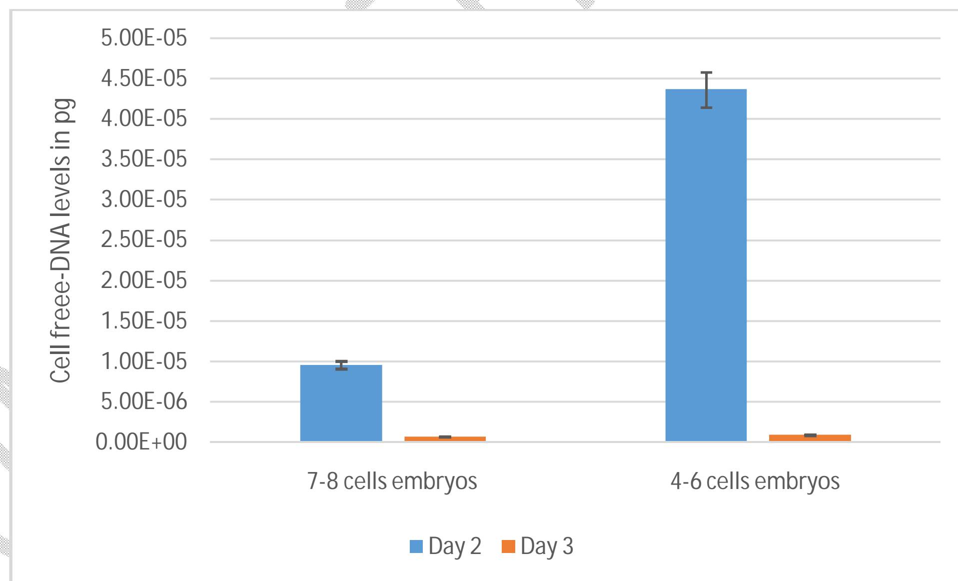

Figure 1: Association between Cell Free-DNA levels in CM on day 2 and day 3 and the quality of embryo cleavage.

DISCUSSION

The quality of embryo cleavage is one of the key indicators of preimplantation embryo quality [17-19]. Embryo cleavage is the process by which cells in the embryo divide to form blastomeres [1]. This process play a major role in the following cell organization, namely blastulation [20, 21]. In normal conditions, the blastomeres get organized in an orderly and symmetrical manner, which is necessary for the healthy development of the embryos [22, 23]. However, when embryo cleavage is abnormal or delayed, it can lead to chromosomal abnormalities and disturbances in cell differentiation, thus compromising embryo quality and its implantation competence [24, 25].On day 2 after ICSI, our results revealed significantly higher Free Cell-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 CM on day 2 can be explained by the release of Cell Free-DNA fragments by the embryos following the physiological process of fertilization and the initiation of the process of embryo cleavage. The release of Cell- Free-DNA fragments by embryos during normal fertilization can be explained by different mechanisms. 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 Cell Free-DNA fragments into the embryon CM [26]. In addition, the release of Cell Free-DNA fragments can also be associated with events related to apoptosis. Cells which suffer irreparable damage to their DNA or which are no longer necessary for the development of the embryo can be eliminated by apoptosis and lead to the release of Cell Free-DNA fragments into the embryo CM [27-29]. Those processes that are normal and necessary for embryo development can explain the Cell Free-DNA levels in CM on day 2.

The significantly higher day 2 Cell Free-DNA levels in CM of 4-6 cells embryos compared to those in CM of 7-8 cells embryos can highlight the association of Cell Free-DNA with the disturbance of embryo cleavage. On day 2, the CM may contain Cell Free-DNA fragments originating from the process of fertilization, the process of embryonic segmentation or both. The quality of fertilization can influence the molecular mechanisms involved in the process of embryo cleavage. Proteins and genes involved in embryo cleavage are regulated by molecular signals depending on the state of chromosomes and genes after fertilization. Chromosomal and

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genetic abnormalities can disrupt these signals and lead to abnormal cleavage [30-32]. All these events can explain the release of large quantities of Cell Free-DNA into CM following the altered process of fertilization and embryo cleavage.

The release of high levels of Cell Free-DNA fragments into CM of 4-6 cells embryos may be associated with oxidative stress [33]. 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 [34,35]. On the other hand, delay in embryo cleavage increases the risk of chromosomal abnormalities, which can lead to increased release of Cell-Free DNA fragments in CM. These chromosomal abnormalities can be caused by distribution errors during cell division or by disruption of DNA repair processes [36, 37].

On day 3, the embryo CM showed no significant difference between the Cell-Free DNA levels of 7-8 cells embryos and those of 4-6 cells embryos. This result can highlight the possible main origin of the Cell Free-DNA fragments, which is the process of fertilization. In agreement with this result, our data also demonstrated higher levels of Cell Free-DNA in CM on day 2, in comparison with day 3, regardless of the quality of embryo cleavage. Knowing that the process of fertilization and the initiation of embryo cleavage takes place between day 0 and day 2 after ICSI, the high levels of Cell Free-DNA fragments in day 2 CM may simply reflect their accumulation by the embryos due to the chaining of the mechanisms of these two processes. This result can highlight the importance of renewing embryo CM on day 1, namely just after fertilization, in order to minimize the accumulation of Cell Free-DNA fragments as contaminants in CM.

Conclusion

To conclude, this study demonstrated that the increase of Cell Free-DNA levels in embryo CM might originate from disturbances in the mechanisms of fertilization. In addition, the accumulation of Cell Free-DNA fragments in CM may be associated with the quality of embryo cleavage. These observations may encourage the improvement of embryo culture conditions by favoring CM renewal after fertilization and before the initiation of embryo cleavage. This CM renewal system could help decrease the possible disruptions of embryo cleavage mechanisms.

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CONSENT

All couples signed an informed consent before the IVF cycle.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

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