Original Research Article

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

Abstract:

Background: The success of invitro-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). ~~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~~one of which is the levels of Cell Free-DNA. Understanding the~~association~~~~relationship~~between Cell Free-DNA levels in embryoCM 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 in~~of~~Cell Free-DNA levels o~~in day~~n day 2 CM corresponding to 4 to 6~~-6-~~cell~~s~~embryos compared to those corresponding to 7 to 8~~-7-8-~~cell~~s~~ 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~~and those of 4-6 cell~~s~~ embryos(p=0.4). Also, Cell Free-DNA levels in embryo CM, were significantlyhigher on day 2 compared to day 3 for both~~(p=0.03; p=0.04), regarding~~ 7-8 and 4-6 cell~~s~~ embryos(p=0.03; p=0.04).

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.

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Comment [A1]: This title does not demonstrate what was done in the study. It should read more like...'To access the association between day 2 and day 3 post fertilisation embryo cell count and the quantity of cell free DNA in the embryo culture media'.

Comment [A2]: All abbreviations should be defined before subsequent use. Please do this all the rest in the text.

Comment [A3]: Briefly discuss your data handling and analysis in this section.

Comment [A4]: Your conclusion must be based on your results. This conclusion appears to be an inference which is not supported by the results provided in the abstract. Please rephrase.

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

INTRODUCTION

Among the essential components that impact~~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 be~~increase~~ improved by ~~choosing~~ selectingembryos 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 ~~th~~the~~e eitherelements~~ variablesthat have an impact on embryonic quality,either positively or ~~negatively~~adversely [1-3]. Pr~~evious~~~~ior~~ 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 are released from cells~~cells release~~in the process of ~~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~~ thereby maintaininga relatively low basal level [12-14]. In vitro, the free DNA fragments released by apoptotic events in embryos are ~~iendtified~~identified as contaminants in the 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~~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

Comment [A5]: Provide a reference

Comment [A6]: Would be good to briefly state the findings from these studies and quote any statistics thereof from the studies.

Comment [A7]: Having read the entire study, this study objective has not been demonstrated with the results you have provided.

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between CMand embryo quality and how this can be used to improve the success of IVF techniques.

MATERIAL AND METHODS

~~Patients~~ Study participants

This study prospectively included 48couples, 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~~ the participantssigned informed consent agreeing to the sudybefore the IVF cycle.Beneficience?

Comment [A8]: Why was 38years of age chosen for exclusion criteria?

Comment [A9]: How were they counselled and by who. Please state how the patients where recruited and how you arrived at your sample size

Comment [A10]: Is there is any benefit for the couple in using their embryos for the study.Is there any harm to their embryos in conducting this study?

Ovarian stimulation protocol and oocyte collection

Womenunderwentcontrolledovarian stimulation with the flexible gonadotrophin-releasing hormone (GnRH) antagonistprotocol. A dailysubcutaneously injection of recombinant folliclestimulating hormone (rFSH; Gonal-F, Merck-Serono) wasusedalone or in combination withhumanmenopausalgonadotrophin (HMG, Menopur; Ferring). The FSH dose wasbased on the women'sageand AMH concentration, in addition to priorhistory of ovarian stimulation and wasadjustedaccording to usualparameters of folliclegrowth, determined by serum estradiol (E2) concentration and ultrasound monitoring.

A daily dose of GnRH antagonist (Cetrotide, Merck-Serono or Orgalutran, MSD) wasinjectedsubcutaneously, startingfromday 6 of FSH administration. The ovulation trigger wasperformedwith 10 000 IU of humanchorionicgonadotrophin (rHCG, Ovitrelle; Merck-Serono)

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and gonadotrophin releasing hormone (Decapeptyl, Ferring), afterobtainingfolliclesthatreached dimensions of 17mm or greater in diameter and adequateserum E2 levels. Oocytes wereretrieved 34-36 hoursafter hCG administration.

Oocyte and sperm preparation

The retrieved oocytes were isolated from the 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.

Comment [A11]: Do not start a new sentence with numeric characters. Write in full and do same for all the rest in the text

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) 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

Comment [A12]: Did you also perform this detailed assessment for embryos on day 2?

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 correspondingembryosfor (day 2 and, day 3) werecollected for the quantification of Cell Free-DNA.Free-DNA was extracted from culture media samples by the SaMag^{tm} STD DNA Extraction Kit accordingKit according to the manufacturer's intructionsinstructions. The total free-DNA was quantified by Qpcr, using ALU 115 primers (Unemati N et al., 2006). For each patient, 4patient, 4µl of CM are added to the reaction mixture of 0,25 Ul ofUl of each ALU 115 5'CCTGAGGTCAGGAGTTCGAG-3' (forward) and 5and 5'CCCGAGTAGCTGGGATTACA-3' (reverse) and 4 µl of Luna Universal qPCR Mix (containing thecontaining 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

Comment [A13]: Your data handling and analysis is confusing. Please re-write this. You may consult a statistician or someone experienced in statistics

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 CM, the results showed no significant difference between the Cell-Free DNA levels in CMof 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 significantlyhigher 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-DNAlevels in CM on day 2	$9{,}56x10^{-06} \pm 2{,}57x10^{-05}$	$4{,}37x10^{-05} \pm 0{,}0001$	0,04
Cell Free-DNAlevels in CM on day 3	$6{,}52 \times 10^{-07} \pm 3x\,10^{-06}$	$8{,}81x10^{-07} \pm 1{,}58x10^{-06}$	0,4

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

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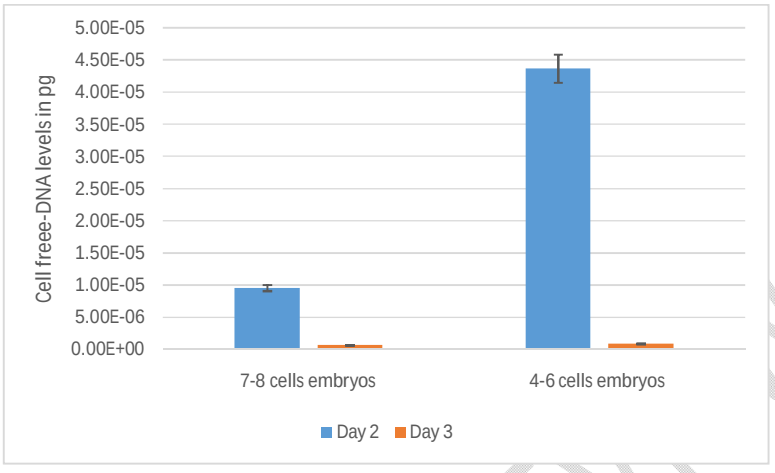

Figure 1: Association between Cell Free-DNA levelsin CM on day 2 and day 3and the quality of embryo cleavage.

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DISCUSSION

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 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~~

Comment [A14]: Overall your discussion doesn't seem to discuss your results. It is too abstract and theoretical. I expected you to be discussing the findings you had between days 2 and 3 embryo and the findings between 4-6 cell embryo and 7-8 cell embryo and their association and what explains your findings. This discussion needs to be looked at again. It would be good to compare your work with those of other scholars who have done similar studies.For now the discussion is quite abstract and more of theory.

~~release of Cell Free-DNA fragments by the embryos following the physiological process of fertilization and the initiationof the process of embryo cleavage.~~ The release of Cell- Free-DNA fragments by embryos during normal fertilization can be explained by a different mechanism~~s~~. 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 embryo~~n~~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~~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]. Th~~e~~~~o~~se physiological processes ~~that are normal and necessary~~are necessary for embryo development and ~~can~~ explains theraised 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~~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 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 inthe CM. These chromosomal abnormalities can be caused by distribution errors during cell division or by disruption of DNA repair processes [36, 37].

Comment [A15]: Why is there higher levels of cell free DNA in the 4-6 cell embryo more than 7-8 cell embryo. You have stated that cell free DNA is a bye product of cellular activity. Is not the case that a 7-8 cell embryo has undergone more cleavage than a 4-6 cell embryo and should have a higher level of by product (cell free DNA?)

Comment [A16]: This is confusing. You have not explained clearly why day 2 embryo has more cell free DNA than day 3 embryo. Neither is it clear why 4-6 cell embryo has more cell free DNA than 7-8 cell embryo. Please re-write this segment.

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On day 3, the embryoCM 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 placebetween 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.

Comment [A17]: Did you mean reviewing the culture media on day 2? What's the purpose of reviewing the culture on day 1 if fertilisation happens on day 2?

Conclusion

To conclude, this study demonstrated that the increase of Cell Free-DNA levels in embryo CMmight 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.

Comment [A18]: Your study did not show or check for this. You study concluded that day 2 embryo had significantly higher cell free DNA compared with day 3 embryo and 4-6 cell embryo also showed higher levels of 7-8 cell embryo. Your results or data had nothing on the quality of the embryo, you did not show us that your examined them and compared the features of a higher quality embryo stated in your introduction. You have not cited any literatures comparing the qualities of the embryo either. Alot is missing and your conclusion is not consistent with what you done. Conclusion is not for making inferences or assumptions but should stem from your results

Comment [A19]: What do you mean by renewal? If this is technical term then you should have a section for definition of terms in your methodology. Also there are many technical terms in your work, the work could be improved by providing some baseline definitions like what defines quality of embryo or cleavage?
Describe the process of fertilisation at ICSI and the process of stages and timings of cell division that follows

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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).

Declare your conflict of interest?

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Comment [A20]: Provide full citation

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