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Total And Mitochondrial Cell Free DNA Quantification in Day 5 Embryos Culture Media Reflect Embryos Quality

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ABSTRACT

Background: In vitro fertilization (IVF) technology still uses the morphological criteria as the main approach for selecting embryos of a certain quality, embryo fragmentation, blastomere size and cleavage rate. This group of tools is routinely used to grade cleavage stages of human embryos.

As a result of blastomere fragmentation, cell free mitochondrial DNA (cf mtDNA) is released into the embryo culture medium. Our study aims to confirm the presence of cell-free DNA (cfDNA) in embryos culture media by detecting a specific gene using PCR and to evaluate the correlation between two kinds of cf DNA (total cfDNA and cf mtDNA) content in blastocyst stage (Day 5 embryos) culture media and embryo grading.

Subject and Method: 40 spent culture media samples are collected; each blastocyst was morphologically graded. cfDNA is extracted from embryo culture media. Quality of cfDNA is checked by conventional PCR with specific primer then visualized by agarose gel. The cf mtDNA is profiled by isothermal PCR.

Results: Purified cfDNA from embryo culture media could be used to amplify specific genes by PCR. Further studies indicated that insignificant interdependent relationship is found when correlating the total cfDNA amount on day 5 and embryo grading. Similarly, the significant interdependent relationship is found when correlating cf mtDNA amount on day 5 and embryo grade. Notably, a significant correlation is noticed between cf mtDNA amount and blastocyst formation.

Conclusion: We confirmed the presence of cfDNA in embryo's culture media and noticed a significant correlation between the quality and embryos secrotome cf mtDNA levels.

Keywords: Blastocyst / cfDNA / Human embryos / cf mtDNA.

INTRODUCTION

Mitochondria are the main source of cellular energy; also play crucial roles in apoptosis, necrosis and cellular homeostasis including calcium regulation levels. During oocyte growth, mitochondrial number increases [1], which play vital roles in fertilization and embryonic development competence [2,3,4]. Embryo grade is important to the pregnancy rate results in IVF.

Any changes to the components of the culture medium could potentially affect the grade and quality of the embryos since it is the direct near environment to the embryos in vitro [5,6]. In vitro fertilization successful pregnancy and potential implanta

tion rates depend on the morphological criteria [7,8].

One of the main tools used for grading the human embryo and its potential implantation rate is the embryo fragmentation. Fragments of human embryo cells are the source of cell free mitochondrial DNA that passed into the embryo culture media (secretome), so the embryos with low quality contain high cfDNA/cf mtDNA levels in their spent culture media [9,10] as a result for cfDNA losing by physiological and apoptosis processes [11,12,13].

Previous studies reported that, the high implantation capacity is related to the blastocysts with low mitochondrial content [14,15,16] in contrast with Victor *et al* [18] and Victor *et al* [19]; who reported that cf mtDNA levels between all successful and unsuccessful embryos of their study revealed no significant differences.

The detection of cf mtDNA levels in spent culture media can be considered as a non-invasive technique [7,20,21,22] to assess the potential development of the blastocyst stage through the fragmentation of the embryo cells in the culture media [22,31].

For embryo selection and testing there are pre implantation genetic diagnosis (PGD) [25].

The use of these methods requires blastomere biopsy which may affect the quality of embryos on the future development. This method is not only invasive but also expensive in addition to mosaicism which leads to a false result [26,27].

In the present study, we hypothesized that fragmentation, should suggest that cell free DNA is passed into the human embryo spent culture media. Our study aimed to explore the presence of cfDNA/cf mtDNA in spent culture media of human embryos and to investigate that cfDNA levels were correlated with the morphological criteria of the embryos and with maternal age [28,29].

MATERIALS AND METHODS

Subjects:

12 patients (couples), were scheduled for intracytoplasmic sperm injection (ICSI) in Al Riyadh

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center for fertility and reproductive health, Cairo, Egypt. Average age of wives was 33 years, with body mass indices (BMIs) 28, base line of FSH, 6.6, LH 6.16 and prolactin 59.9.

The average age of husbands were 37 years, with semen count ranges from 15million/ml; motility: 35-60%; morphology: 98-96%. Wives underwent to ovarian stimulation Antagonist protocol (multiple dose protocol) according to Sitistatidis *et al.* [35]. Global total media for the culture and global sperm wash media for semen preparation are used during the ICSI procedure [34].

Sample collection

Embryos in spent culture media were investigated at D5. Spent culture media are collected after removing the embryos (including both arrested embryos at cleavage stage (32 embryos) and blastocysts stage (8 embryos)) for transfer or freezing (33 embryos were transferred, while the rest 7 embryos were cryopreserved. The spent culture media were collected in sterile DNA inhibitors free tube (Eppendorf) and maintained immediately at -20°C till purification. According to current system (**Current system cleavage includes**; **symmetry of blastomeres**: the cells should be about the same size, **Number of nuclei**: Each cell must have a single nucleus. When there is greater number of nuclei per cell, it can lead to errors in embryonic division, **Fragmentation:** Both the number of fragments and their distribution within the embryo are studied and thickness and **Appearance of the zona pellucida:** this layer should be neither too thin nor too thick. **Current system Blastocyst stage includes: Degree of expansion:** a scale from 1 to 5 is established, with grade 1 being the lowest degree of expansion of the embryo. **Appearance of inner cell mass (ICM):** number of cells, compaction, location. Its evaluation is done by assigning the letters A, B, C and D. A grade A blastocyst is considered an embryo of very good quality and with a high probability of implantation in the uterus.

Appearance of trophectoderm cells: number, location and shape [7].

Poor cleaved day 5embryos are morphologically scored according to the degree of fragmentation, number, size and equality of blastomeres (Grade C) [7]. Meanwhile, the blastocysts stage embryos are scored according to formation and distribution of trophectoderm and inner cell mass within zona pellucida (Grade A) [7].

Extraction of cfDNA from culture media:

The isolation of the cfDNA is carried out using Qiagen commercial kit specific for cfDNA extraction (QIAamp Circulating Nucleic Acid Kit) [5,31] following the instructions of the manufacturer.

By using nuclease free water the samples volume are adjusted to 1000µl. 100µl/sample of proteinase K was added followed by 800µl of buffer ACL which contains 1.0µg carrier RNA, then the mixture was incubated in water bath for 30minutes at 60°C. Then the ACB buffer was added (1.8 ml) and mixed for 15–30 seconds thoroughly by using pulse-vortexing. The mixture is then transferred to the columns of QIAamp Circulating Nucleic Acid and centrifuged at 14000 rpm/1 min. 600µl of ACW1 was added to the columns for wash once, then with 750µl of ACW2 buffer and finally washed by using absolute ethanol (750µl).

Centrifuged the column at 14000 rpm/5 min for drying and then left at 60 °C for 5 min to insure complete dryness. 50µl of AVE buffer was add to the column and centrifuged at 14000 rpm/1 min for elution of DNA. The DNA is measured by using Nano drop and stored at -20 °C until used.

PCR amplification:

The conventional PCR used for examination of cfDNA by universal vertebrate 16sr gene. Primer sequences are as the following: 16S Forward 5'ACTGTCTCTTACTCCCAA 3', Reverse 5' TTATATTCCGAGGTCACC 3'. PCR reactions takes place by using the Techne PCR mini thermal cycler using Green Master Mix with concentration (2X) containing DNA polymerase (DreamTaq), 0.4mM of each deoxyncleotide triphosphate (dNTP) and 4 mM Mgcl2. Green buffer (DreamTaq) is optimized for robust performance in PCR. In 0.2 ml Polypropylene PCR tubes; 12.5µl Green Master Mix, 1µl of each primer (10pmol/µl) and 5µl extracted DNA and by using deionized water the volume was adjusted to 25µl. The conditions of PCR for 16s primer is as follows: initial denaturation (1 cycle at 94°C for 3 mins), then 35 cycles, each cycle consisting of denaturation (30 seconds at 94°C), annealing (30 seconds at 53°C) and extension (1 min at 72°C), finally additional extension (5 min at 72°C). The PCR products were stored at 4ºC for electrophoreses.

Cell free Mitochondrial DNA amplification:

REPLI-g Mitochondrial DNA Kit used for cf mtDNA amplification following the manufacturer's instructions:

Firstly, purified DNA was thawed on ice; in PCR tube we mixed (27µl of reaction buffer, 2µl of the primer mix, 10µl of each sample and 10µl of nuclease free water) the mixture is then vortexed and briefly centrifuged.

The whole mixture was incubated at 75°C/5 min and then it was cooled to room temperature (15–25°C). Then each sample was mixed with 1µl REPLI-g Midi Polymerase and incubated in the thermal cycler for 8 hours at 33°C. The reaction mix incubated for 3 minutes at 65°C for inactivation of the enzymes and measured by Nano drop then kept at -20°C.

cfDNA and cf mtDNA quantification by NanoDrop Spectrophotometer:

Both of cfDNA and cf mtDNA concentration is determined by NanoDrop (Denovix, DS-11-Spectrophotometer). System is blanked by the use of TE elute buffer. 2.0 µl of sample are pipetted on to the end of a fiber optic cable.

The absorption at 260 nm is used to measure the concentration of DNA, and the sample's absorption at 280 nm is examined to make sure that proteins haven't contaminated the sample. For highly pure and high quality DNA, the 260/280 nm ratio is between 1.8 and 2.0.

Agarose gel electrophoresis:

Agarose powder, EDTA buffer (TBE, Ethidium Bromide (EthBr) stain. Agarose gel electrophoresis: Agarose powder (Fisher Scientific, UK), Tris Borate EDTA buffer (TBE): for 1 liter of working 1X TBE, 10.8gm Tris base, 5.5gm boric acid (United Co., Egypt), and 0.74gm of EDTA (WINLAB, USA) were dissolved and the volume is brought to 1 liter by ddH2O and Ethidium Bromide (EthBr) stain (Promega, USA) ready for use.

Statistical analysis

Our statistical analysis was performed by using the SPSS. Mean values are compared by two tailed Independent sample T-test. A *p*-value was considered significant if equal or less than 0.05.

Ethical approval:

This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans. Written informed consent form is obtained from each couple whose embryos were used in the present investigation. The study was done in the Al Riyadh center for fertility and reproductive health. The study was approved by the Ethics Board of Al-Azhar University.

All samples were taken by the physician of the center **(Doctor Hesham Elshaer; Professor of the Gynecology, obstetrics and infertility, faculty of medicin, Kasr El Aini, Cairo university)** and by the technicians of the embryo lab and the supervisors thank them for this. We have also been provided by photos of the embryos from their ways as well.

RESULTS

Patient characteristics:

The women's age mean is 33.5 ± 2.27 years and their body mass index (BMI) was 28.09 ± 1.06 kg/m2. Base line FSH (6.66±0.86 mIU/I). Base line LH (6.12±0.76 mIU/I). Base line prolactin hormone (59.88±25.0 mIU/I). Follow-up time of these data taken during 2020-2021. The hormonal baseline status is presented in in Table1.

Table 1: Characteristics of the study subjects.

Variables	Mean±SE
Wives	
Age (years)	33.5±2.27
BMI (kg/m2)	28.09±1.06
FSH (mIU/l)	6.66±0.86
LH (mIU/l)	6.12±0.76
Baseline prolactin hormone (mIU/l)	59.88±25.00
Follow-up time	From 2020:2021

3.2. Nano drop spectrophotometer for evaluation the integration of amount of total cfDNA and the embryo's parameters:

Samples are grouped into two groups according to the grade of embryos into; grade A and grade C represented by 8 (20 %) and 32 (80 %), respectively according to previous morphological criteria. Secondly, we checked the extracted DNA by targeting 16s cf mtDNA at 600 bp (Fig. 1). Total cfDNA amount were higher in the spent media of embryos with high morphological scores (Grade A) compared to those of poor grade embryos (Grade C) with statistically nonsignificant differences (*P*= 0.6) (Fig. 2).

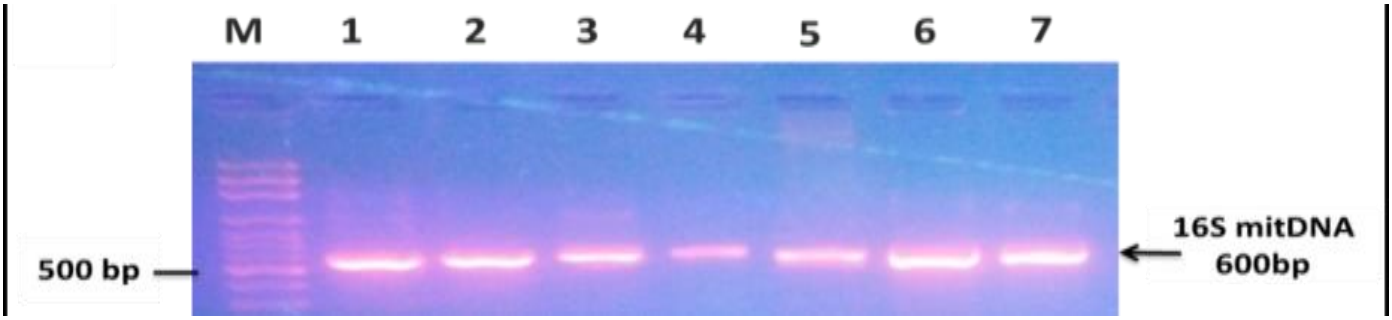

Fig.1. Agarose gel electrophoresis showing PCR products for 16S cf mtDNA (600 bp fragment).

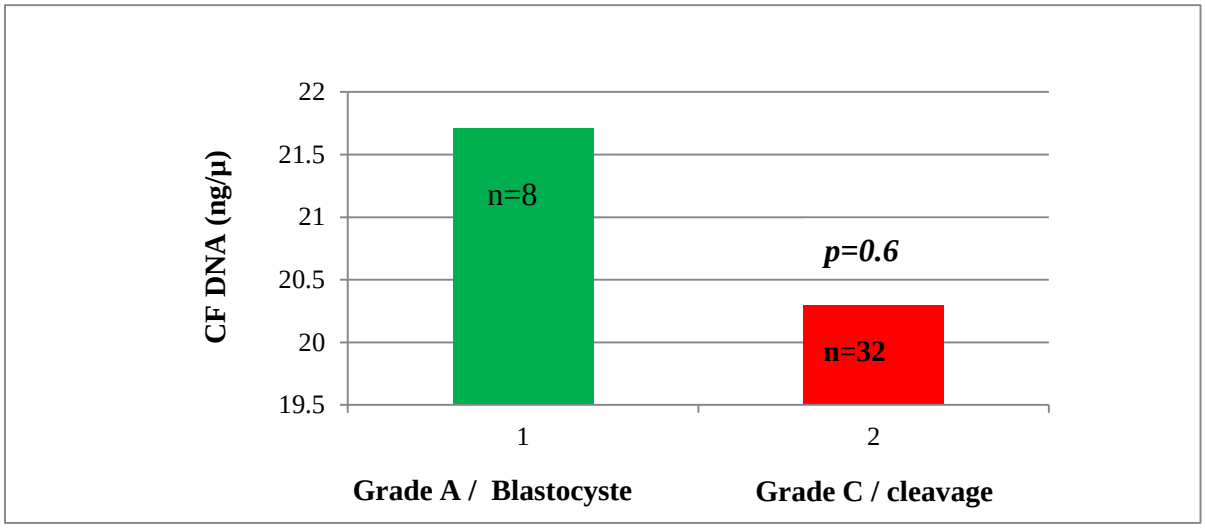

Fig.2. Amounts of the total circulating cfDNA in spent culture media for embryos that proceed to blastocyst stage (Grade A) or arrested at blastomere stage (Grade C). cfDNA of embryos with grade C is found to be lower than that of grade A. The data represent means±SEM, $P = 0.6$.

Integration of embryo's parameters and amount of cf mtDNA using Nanodrop spectrophotometer:

15 samples from the total collected spent culture media (40 samples) were amplified by using Repli-g mtDNA kit using isothermal PCR then quantified using Nanodrop. The samples are grouped into two groups (according to the previous criteria of embryos morphology); grade A and grade C represented by 7 (46.6%) and 8 (53.3%) respectively (Figure3)**.** Retrospectively; the current study defined correlations among cf mtDNA profiles and embryo quality. The cf mtDNA amounts appeared lower in spent culture media of embryos with high-grade (Grade A) compared with those of a poor grade morphological score (Grade C) with statistical significance ($P = 0.05$) (Fig. 4).

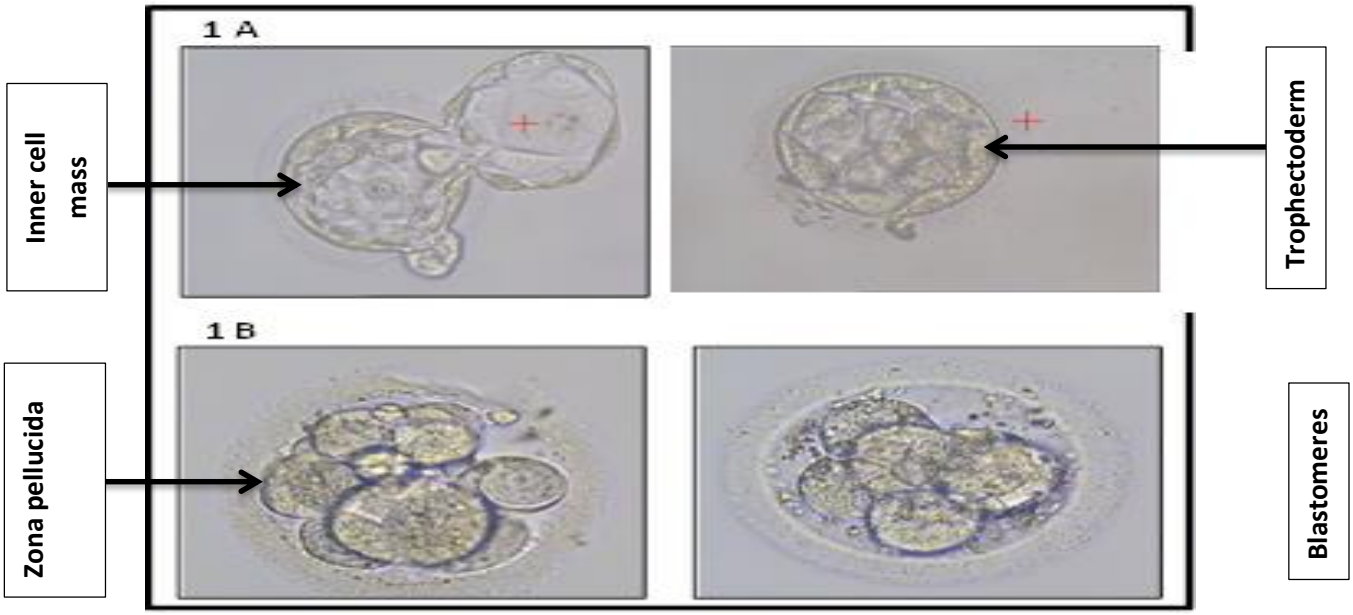

Fig.3. Morphological grading of blastocyst embryos in figure (1A) Grade A blastocysts on day 5 classified according to [8] in which embryos reached the blastocyst and hatching blastocyst stage using 3 quality scores included: the expansion, inner cell mass (ICM) and the trophectoderm (TE) of the blastocyst. In figure (1B) Grade C on day 5 is classified according to a current system [8] that all cells arrested at cleavage stage embryos, They morphologically still as 5,6 blastomeres per each embryo in day 5.

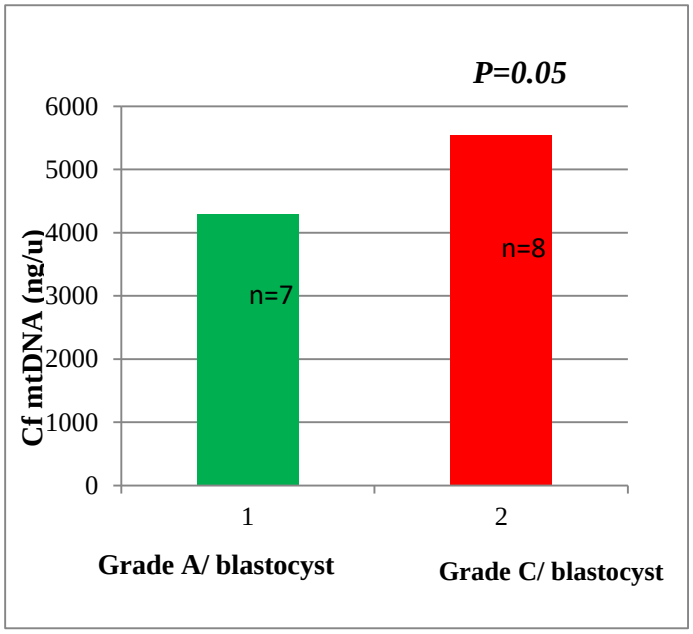

Fig.4. Charts comparing the amounts of cf mtDNA concentration in spent culture media using Nanodrop and embryo grades A and C. cf mtDNA of embryos with grade C are found to be significantly higher than that of grade A. The data represent means±SEM, *P = 0.05*.

Integration of DNA profiles and maternal age:

Seven embryos are derived from five women with an average age (26.66 years), a range of 19-22 and seven embryos from 7 women whose average age was 44 years old, a range of 43-45.

According to an analysis of cfDNA profiles based on maternal age, older women produced embryos with more cf mtDNA than younger women, with a statistically significant difference (p=0.03) (Fig. 5).

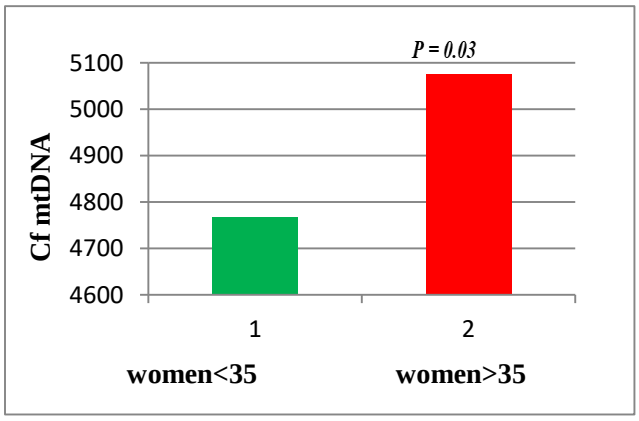

Fig.5. Charts comparing the amounts of cf mtDNA in spent culture media showing a significant correlation between embryos produced by women who are less than 35 or more than 35 years of age. According to an analysis of DNA profiles based on maternal age, older women produced embryos with more cf mtDNA than younger women. The data represent means±SEM, *P = 0.03*.

DISCUSSION

The cf mtDNA is mainly replicated during the early development and moved from primordial germ cells into oocytes then finally into embryo and fetus [1,32].

Recent research has demonstrated the possibility of using genetic testing from the embryos spent culture media, however the results from different groups have been variable [17,21].

Researchers hypothesize that cfDNA are released into spent culture media by the embryo cfDNA. are released from fragments or cells by the embryo as a kind of correction way for aneuploidies [21].

The cfDNA /cf mtDNA is a result of apoptotic processes in the cells of human embryos, so fragments of the human embryo cells are the main origin for cfDNA/cf mtDNA, which reflects the release of cf mtDNA into the human embryo spent culture medium which will be a positive correlation to embryo quality [11,12,13].

The result of the present study showed that cf mtDNA profile levels in human embryo spent culture medium are correlated with the embryos quality and grading , also cf mtDNA are correlated with blastocyst and non-blastocyst embryos formation which reflects that the good embryos contain low amounts of cf mtDNA in their spent culture media, and poor quality embryos contain high amounts of cf mtDNA in agreement with **stigliani *et al* (2014)**[24] and **Garden and Schoolcraft (1999)**[30]. Hence our study suggests that cf mtDNA can give us a stronger predictive for embryo grade and quality with embryo fragmentation and can be used in addition to current morphological criteria.

In correlation with high quality good embryos that reached the blastocyst stage had low cf mtDNA in their spent culture medium in agreement with the loss of cytoplasm from a blastomere being high in bad quality embryos and detrimental for embryo competence [4,33], and the presence of high levels of cf mtDNA is one of the causes of poor quality embryos.

Our study suggests the possibility of using cf DNA in human embryo spent culture medium for targeting a specific gene sequence as we can.

Our study suggests that instead of invasive gender selection and PGD method [23,25,31], we can provide non-invasive gender selection and PGD technique and this may facilitate the detection of the embryo gender by PCR amplifying and x -linked disorders.

CONCLUSION

We confirmed that cfDNA/ cf mtDNA is present in human embryo spent culture medium, we found the possibility of using cfDNA in human embryo spent

culture medium for targeting a specific gene sequence as we can which confirm the possibility of using culture medium as a new non-inavsive technique to detect the embryos gender and PGD for good healthy babies. We found that the best embryos contain a low amount of cf mtDNA in their culture media, and poor quality embryos contain high levels of cf mtDNA, so we can use the cf mtDNA profile levels in combination with morphological criteria in the selection of the high quality embryos.

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Author contribution: Authors contributed equally in the study.

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