In the name of GOD

The role of cell free DNA biomarkers in the results of assisted reproductive technique

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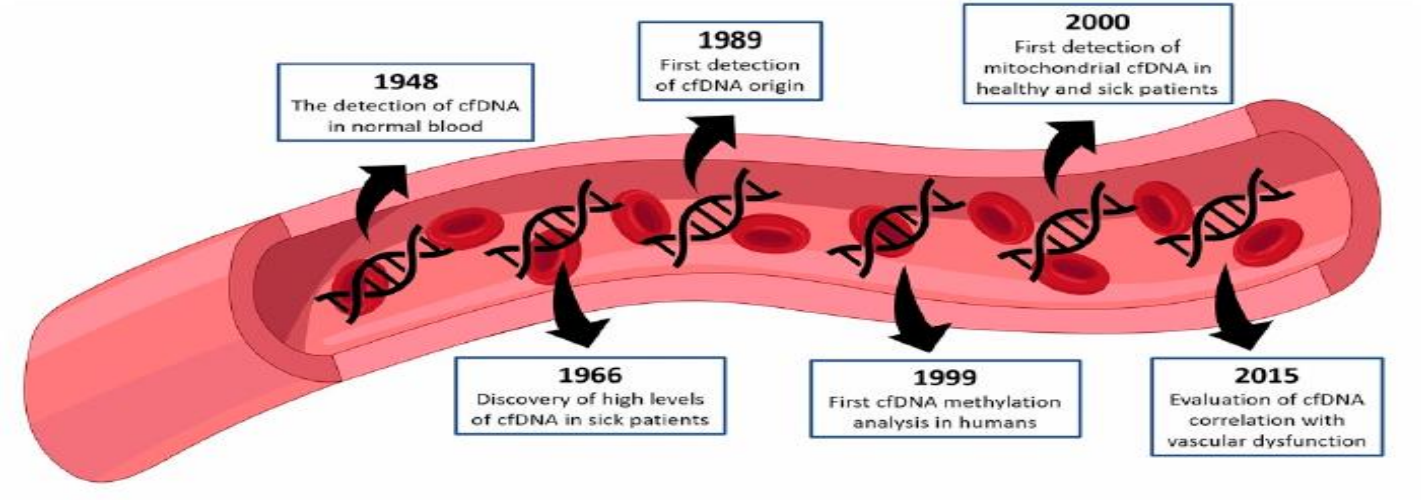
Cell free DNAs(cfDNA) : Historical Perspective

* Cell-free DNAs (cfDNAs) were initially discovered in human serum by Mandel & Metais, in 1948, that the level of cfDNA is significantly increased in the plasma of patients

* In 1977 found the presence of a small percentage of cfDNA originating from the fetus in the maternal plasma and serum, also existed in cancer patients

* cfDNA was discovered in the female & male reproduction field in 2004

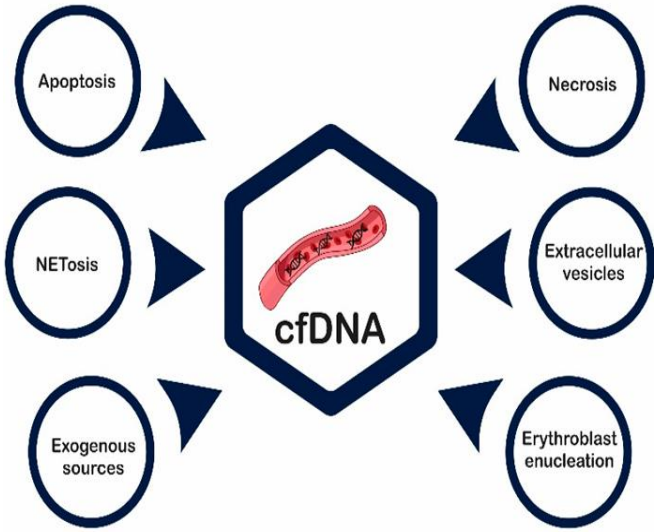


Source & Mechanism of Release

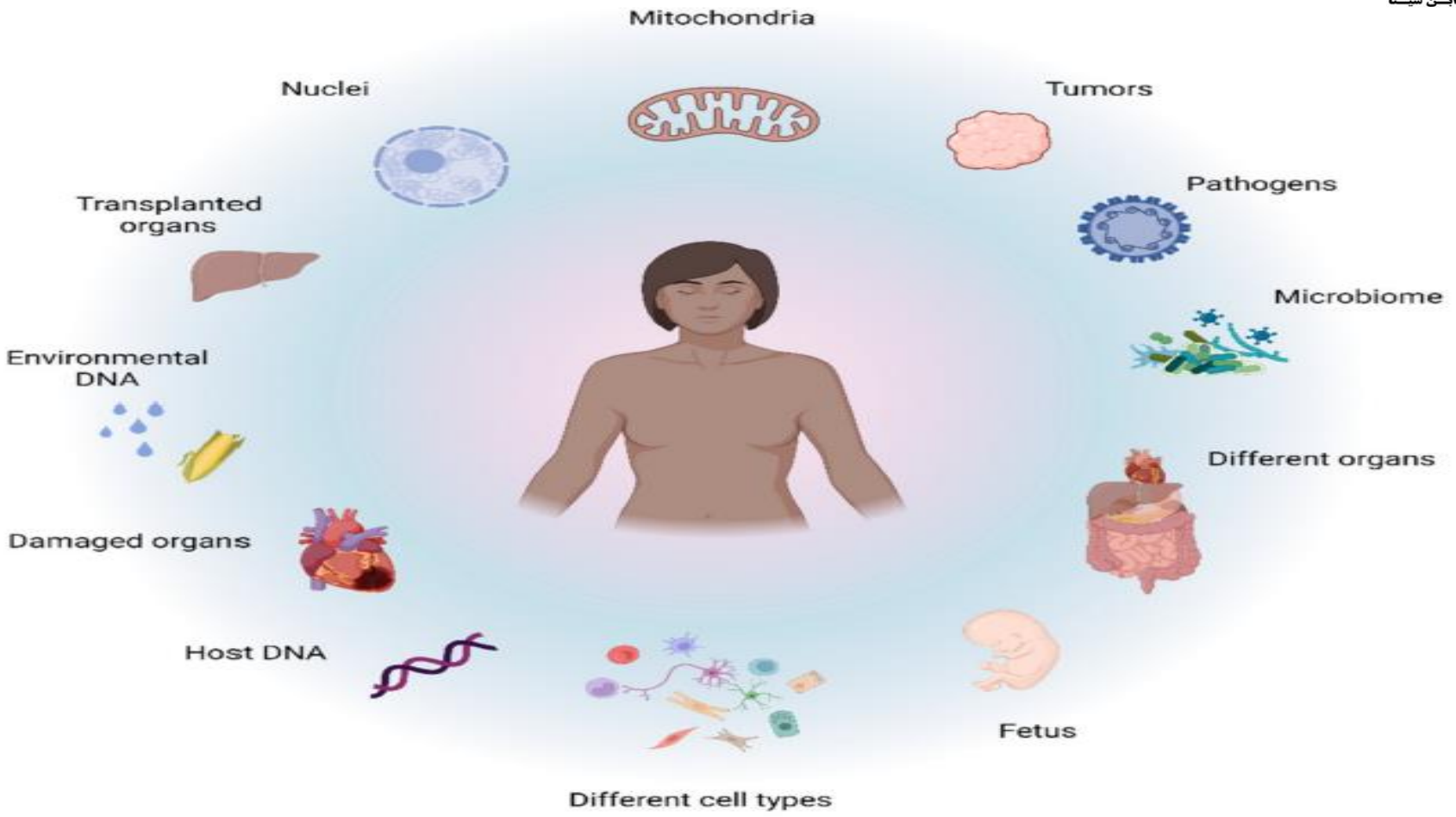
* cfDNA can be found in many body fluids, both in physiological conditions as well as in pathological disorders, Present in blood in low amount because of process of phagocytosis done by macrophages, their amount is increased in presence of other disease as cancers

* Different mechanisms allow the release of DNA fragments from the intracellular to the extracellular compartment. The release processes of DNA into the human blood circulation can originate from:

  - Apoptosis (healthy (lymphoid and myeloid cells)
  - Necrosis (oncological patients)
  - active DNA release
  - exogenous sources



The diverse possible origins of cfDNA in humans



Terminologies cell free DNA

- cfDNA (Any free DNA in serum)
- cffDNA (Cell free fetal DNA)
- ctDNA (Circulating tumor DNA)

Molecular Features

* The pathophysiological importance of cfDNA is also related to its molecular characteristics
* The cfDNA integrity (size) & its genetic and epigenetic profile and plasma concentration depend on its release mechanisms
* Two main types DNA fragments (the nucleus (cf-nDNA) and mitochondrial(cf-mtDNA)) with both types show different structural characteristics
* cf-nDNA is widely present in the physiological extracellular milieu, that exist in both free circulating form and extracellular vesicles (exosomes, apoptotic bodies & microvesicles) that has be in blood, urine, saliva, spinal fluid, semen, and follicular fluid
* cf-mtDNA is also detected in various body fluids and has some unique characteristics compared with nuclear DNA, including a short length, simple molecular structure
* cf-DNA is present in fluids in three different forms: *free, attached to proteins (HDL, LDL), or encapsulated /attached to extracellular vesicles (exosomes, microvesicles, apoptotic bodies)*

cfDNA Integrity (size) & Concentration

* cfDNA size can be evaluated by its fragmentation level, high & low molecular weight can be detected in different fluids (70 to 200bp)
*
* The differences in cfDNA *size can indicate origin*
  - *Physiological process (physical exercise and pregnant)*
  - *Pathological processes, (as inflammation, diabetes, tissue trauma,, myocardial infarction, and patients that received transplantations)*

* The total cfDNA level in cancer patients has a significant increase with a wide range (hundreds to thousands ng/mL in the blood) compared with the healthy controls (a relative level of 30 ng/mL and ranging between 0 and 100 ng/mL)

* cfDNA released on biological fluids contains the same genetic and epigenetic variations as nuclear and mitochondrial DNA from viable cells

cfDNA Concentration &Genetic and Epigenetic

* *Not only cfDNA concentration but also size distribution, genetic variation* (e.g., single nucleotide polymorphism, mutation, and ploidy status), and epigenetic pattern provide a wide spectrum of data about individuals that can be *subjected for screening, diagnosis, prediction of clinical outcome, response to treatment*

* All these plus cost-effectiveness, easy accessibility, and high stability of cfDNA have made it an appropriate biomarker in different fields of medicine

* Subsequently, many of research has been performed on extending the cfDNA utilization in various fields of medicine, such as oncology and transplantation, reproductive

Potential clinical applications of cfDNA

cf DNA was used applications in the diagnosis and prognosis of cancer ,infertility & prenatal diagnosis noninvasive prenatal including:

1-Prenatal

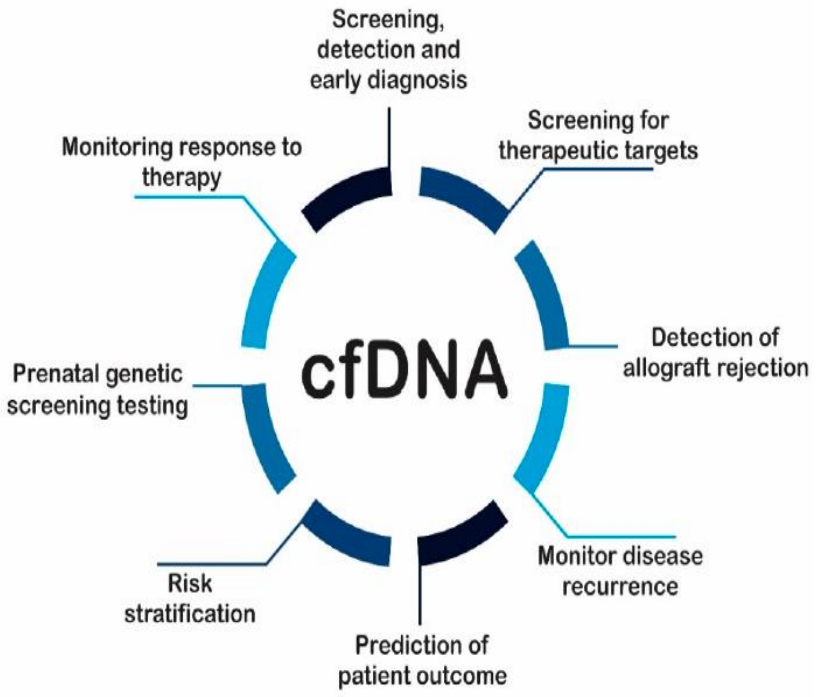
- Fetal RhD genotyping
- Fetal sex determination for sex linked disorders
- Chromosome aneuploidy detection
- Monogenic disorders

2-Oncology

- Cancer detection/ monitoring

3-Others

- Transplantation, Autoimmune diseases
- Male & female Infertility
- Quality oocyte and embryo



Evaluation cfDNA in ART outcomes

* Infertility is a problem in the worldwide that according to the statistics, 15-20 % couples

* various risk factors cause infertility, such as:
  - Anatomical defects and physiology
  - Hormone defect
  - Genetic agent
  - Diseases (cancer, immunology, infection, …)
  - Environmental (life style, Drug abuse, ROS …)

* Infertility severely affects the reproductive capabilities of mature partners, leading to increased application of assisted reproductive technology (ART)

* It is required to have biomarkers for prediction of ART outcome, as well as some non-invasive procedures for genetic/epigenetic assessments.

* cfDNA is an appropriate candidate for providing the both approaches in ART

cfDNA in ART outcomes

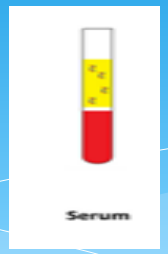
* Since cfDNA discovery in human blood plasma about 70 years ago, cfDNA has become an attractive subject of research as noninvasive disease biomarker

* cf-DNA fragments may constitute an easy to measure molecular tool for guiding the choice of care provided to infertile couples who benefit ARTs, that reliable applicable biomarkers as well as non-invasive diagnostic are highly required

* cf-DNA, either on genetic or epigenetic status, play roles not only *on fertility potential of individuals but also on developmental competence and disorders of the in vitro developed embryos*

cfDNA sources in ART

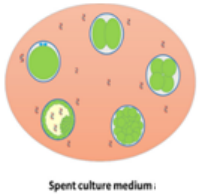
- Serum:
  - Biomarker of female subfertility
  - Biomarker of pregnancy



- Seminal plasma (SP)
  - Biomarker of male reproductive disorders
  - Biomarker of semen parameters
  - Biomarker of oxidative stress
  - Biomarker of sperm retrieval chance
  - Reflection of genetic/epigentic statues of reproductive tract

- Follicular fluid (FF)
  - Biomarker of female reproductive disorders
  - Biomarker of embryo quality and pregnancy

- Spent culture medium
  - Biomarker of embryo quality
  - Reflection of embryo genetic disorders



cfDNA in male reproductive system

* cfDNA was discovered in semen by Chou & colleagues, in 2004, cfDNA presence in seminal plasma (SP) (SP cfDNA) and categorized into *molecular weight fragments*
  - *low (1 kb)*
  - *high (12 kb)*

* Semen parameters (motility and morphology) and capacitation index were demonstrated, post wash hyper-activation was correlated with the amounts of low and high molecular weight fragments, respectively

* The *low molecular weight cfDNA fragment was introduced as a biomarker of semen good quality*

* This study new doors to male infertility research field which resulted in discovering SP cfDNA correlations with different *aspects of male reproduction*

Seminal plasma (SP) cfDNA

- SP cfDNA size distribution
- About 166 bp, contribute to apoptosis mechanism
- fragments > 10 kbp are ascribable to necrosis pathways
- Fragments derived from active releasing are appraised to be between 1000 and 3000 bp & the shortest fragment size, around 40–300 bp, belongs to cfmtDNA

* SP cfDNA size distribution, a variety of mechanisms are involved in SP cfDNA & different concentrations might be capable *to reflect pathological situations*

* SP cfDNA comparison azoospermia and normozoospermia showed higher SP cfDNA concentration in azoospermia semen, that *apoptosis of germ cells* may be considered as a mechanism of SP cfDNA secretion

The source of SP cfDNA

* Stress oxidative (OS) has been recommended as another source of SP cfDNA, treating semen by toxic compound causing oxidative stress that elevated levels of SP cfDNA, decreasing *sperms viability, motility & normal morphology, SP cfDNA concentration*

* mtDNA is the major source of ROS production, addition mtDNA content within the sperm (lower SP cf-mtDNA) triggers an increase in ROS level of the semen

* Sperm DNA fragmentation (SDF) was another source of SP cfDNA, the study compared its levels with different SDF rates. As a result, no association between SP cfDNA concentration and sperm SDF was identified

* SP cfDNA does not depend on extrusion of sperm's DNA fragments & therefore cannot reflect the sperm DNA damage

The main origin organ of SP cfDNA secretion

- The testis & epididymis as the main organs for SP cfDNA secretion

- SP cfDNA concentration in normozoospermia and vasectomized men were compared, Based on SP cfDNA concentration in normal semen was fourfold greater than vasectomized men

- Since vasectomized men semen does not contain testis and epididymis ejaculations, reduced SP cfDNA level in their semen represented that it is mainly originated from the desired organs

- Several studies focused on more applicable aspects of SP cfDNA, especially for biomarker utilization in reproductive medicine procedures

- The predictive value of SP cfDNA with ART outcome: a study assessed its correlation with embryo quality and pregnancy rate

The genetic abnormalities of male reproductive s

* In addition to concentration more characteristics of SP cfDNA have been assessed. The SP cfDNA can possibly be reflective of *the genetic abnormalities of male reproductive system, thus providing a non-invasive assessment for male infertility diagnosis*

* The studies were performed corroborated that *not only genetic abnormalities but also epigenetic patterns of male reproductive tract could be assessed by SP cfDNA analysis*

* Regarding SP cfDNA application in ART, *the biomarkers for some factors, capacitation index , but not SDF, embryo quality, and pregnancy outcome*

* Based on the biomarker utilization, *genetic/epigenetic status of SP cfDNA is more practical than the concentration*, since this characteristic can be applied *for diagnosis and prediction of success rate for sperm retrieval*

cfDNA and female reproduction

* The first report of cfDNA in the female reproduction field was the serum cfDNA value as a pregnancy marker, in 2004
*
* Serums of women at first week post embryo transfer were show that *the neither serum cfDNA concentration nor distributions size correlated with pregnancy success* thus as a biomarker in the female reproduction field could not be proved serum cfDNA biomarker applicability in the female reproduction field

* Elevated levels of serum cfDNA were correlated with *reduced pregnancy rate, serum cfmtDNA level as well to be associated with subfertility*

* The epigenetic status of serum cfDNA as the biomarker for polycystic ovarian syndrome (PCOS) disorder

Follicular fluid (F.F)

* Follicular fluid (F.F) derived from plasma & secreted from granulose cell

* Assessments on FFs of patients undergoing controlled ovarian stimulation (COS) show that larger number of retrieved oocytes were related to FFs with lower levels of FF cfDNA

* Ovarian stimulation has an impact on FF cfDNA level, that even among women undergoing COS, *stimulation duration as well as total dose of gonadotropins can influence the FF cfDNA level*

* FF cf-mtDNA might be more stable under conditions and therefore providing the more reliable biomarker in the female reproduction field

* Regarding FF cfDNA association with oocyte characterization, studies show that FF cfDNA level was not associated with oocyte's maturity stage (MII or MI)

Follicular fluid (F.F)

* FF cfDNA level *is not altered significantly through follicle developmental stages*

* In other words, it might be increase affected *by pathological rather than physiological conditions* that if this theory is accurate, the applicability of FF cfDNA biomarker in female reproductive medicine

* FF cfDNA with ART outcome failed to discover *any correlation with fertilization success rate*, but significant correlation with *day 3 embryo quality*

* The oocytes related to follicles with lower level of cfDNA developed to high quality embryos, they finally recommended FF cfDNA level as a biomarker of day 3 embryo quality

Follicular fluid (F.F)

* Recently reported that *FF cf-nDNA or FF cf-mtDNA levels* correlated with day 3 embryo quality

* Results indicated that *FF cf-mtDNA was lower*, whereas *FF cf-nDNA was higher* in follicles of the oocytes that *developed to blastocysts* than those that were not able to reach to the blastocysts. Only FF cf-mtDNA difference was significant, it was suggested as a biomarker of blastocyst developmental potential

* The positive correlation between *FF cf-nDNA and blastocyst developmental* competence was explained in this manner that, since granulosa cell apoptosis is a normal physiological event at follicle developmental stages that elevated level of FF cf-nDNA might represent the good quality of the oocyte

Follicular fluid (F.F)

* Apoptosis has been introduced as a source of FF cfDNA by several studies

* This theory was raised that the cause of *decreasing embryo quality in the presence of high levels of FF cfDNA is apoptosis of intrafollicular cells, which is increase the ROS levels*

* FF cfDNA presence and function might be due to combination of several mechanisms that all together influence the oocyte developmental ability

* Its *presence up to a specific level reflects oocyte's successful selection* and maturation, Therefore, investigations are needed *to define a normal cutoff point*

* FF cfDNA biomarkers in the ART, *significant association with pregnancy outcome*

* With the exception of one study ,all accepted a negative *correlation between FF cfDNA level and pregnancy*, thus recommended as a *highly sensitive and specific biomarker for prediction of pregnancy achievement*

Follicular fluid (F.F)

* Research on FF cfDNA has been performed based on two strategies:
  * *1- biomarker for ART outcome*
  2- Infertility disorder diagnosis

* In relation to ART outcome, most studies have confirmed its association with *oocytes' developmental ability and pregnancy rate*.

* The importance of biomarker use for these items is selection of *the high quality embryo via a reliable molecular method, estimation of the ART procedure outcome*

* There are some controversies for the exact type of *correlations (positive/ negative) between FF cfDNA level and embryo quality* that, it might be due to lack of the cutoff point definition

Eembryonic cfDNA

- Studies embryonic cfDNA are based on discovering two distinct applications:
  - Biomarker for embryo quality
  - Embryonic genetic assays
    - Preimplantation genetic testing for aneuploidy (PGT-A)
    - Preimplantation genetic diagnosis (PGD)

- Spent embryo culture medium *(SCM)* collection, *is strategy for embryonic cfDNA assessments*, SCM collection is completely a non-invasive method

- Numerous research has evaluated the accuracy and reliability of these methods for utilization of embryonic cfDNA in ART outcome

- the first clinical *application cfDNAin non-invasive prenatal testing (NIPT) for fetal sex determination and disorders through maternal blood*

Clinical Findings

* cfDNA both in plasma or serum has been studied as a potential *biomarker and noninvasive screening tool for many diseases, especially tumors and fetal genetic abnormalities*

* One of applying cfDNA was identifying *fetal cfDNA in maternal blood*, which enabled developing genetic tests in prenatal care. The *origin of the fetal cfDNA* found in maternal blood has described *from the placenta, fetal hematopoietic cells, and the fetus*

* Prenatal screening for *fetal aneuploidy tests using cfDNA had a lower false-positive rate in detecting trisomy 21 and 18, compared to the standard procedure*

* The use of cfDNA is well established for *fetal sex assessment, paternity testing, detection of aneuploidies and trisomies, diagnosis of monogenic diseases, fetal sex determination for sex-linked disorders & fetal RhD status*

Embryonic cfDNA

* With regard to biomarker *ability for embryo quality, for* the first time discovered both cf-nDNA and cf-mtDNA in SCMs of day 3 embryos

* All *SCMs contained* detectable *levels of cf-mtDNA*, but *not cf-nDNA*, they focused on analysis of cf-mtDNA biomarker potential

* *cf-mtDNA level was correlated with embryo fragmentation* rate
* *The higher cf-mtDNA/cf-nDNA ratio* was associated with blastocyst's developmental

* *Lower cf-mtDNA level* in SCM of *low-quality embryos* might reflect insufficient mtDNA reserve within regarded oocytes that resulted in impairment of its developmental ability

Embryonic cfDNA

* Studies on *oocyte and cumulus cells have revealed that higher mtDNA content did associate with embryo quality* , as well proved the same correlation for SCM cf-mtDNA level of day 3 embryos

* Since cf-mtDNA within SCM provides a non-invasive easy accessible biomarker, its combination with morphological assessment puts forward a practical molecular method to predict he day 3 embryo developmental competence, rather than merely morphological analysis

* Despite *cf-mtDNA's high potential at prediction of embryo's developmental* competence, *cf-nDNA can perfectly reflect embryo's genetic status*, thus providing PGT-A/PGD analysis tools

Embryonic cfDNA

* It has to be noticed that cfDNA application for *PTG-A/PGD* is in its initial way

* SCM cfDNA applicable potential for non-invasive PGD analysis: *detecting X-link disorders, alpha thalassemia , thalassemia mutation ,MTHFR C677T polymorphism and cystic fibrosis (CFTR) were diagnosed by SCM cfDNA assessments as well*

* *High levels of cfDNA* triggers *granulose cell apoptosis and influences oocyte maturation embryo development & pregnancy rates in IVF treatments.*

* cfDNA can be as a secondary criteria and predictive marker for the quality control of IVF embryo

Embryonic cfDNA

* Cell free fetal DNA (cffDNA) in maternal serum is used for noninvasive prenatal *screening testing (NIPS, NIPT) of fetal aneuploidies*

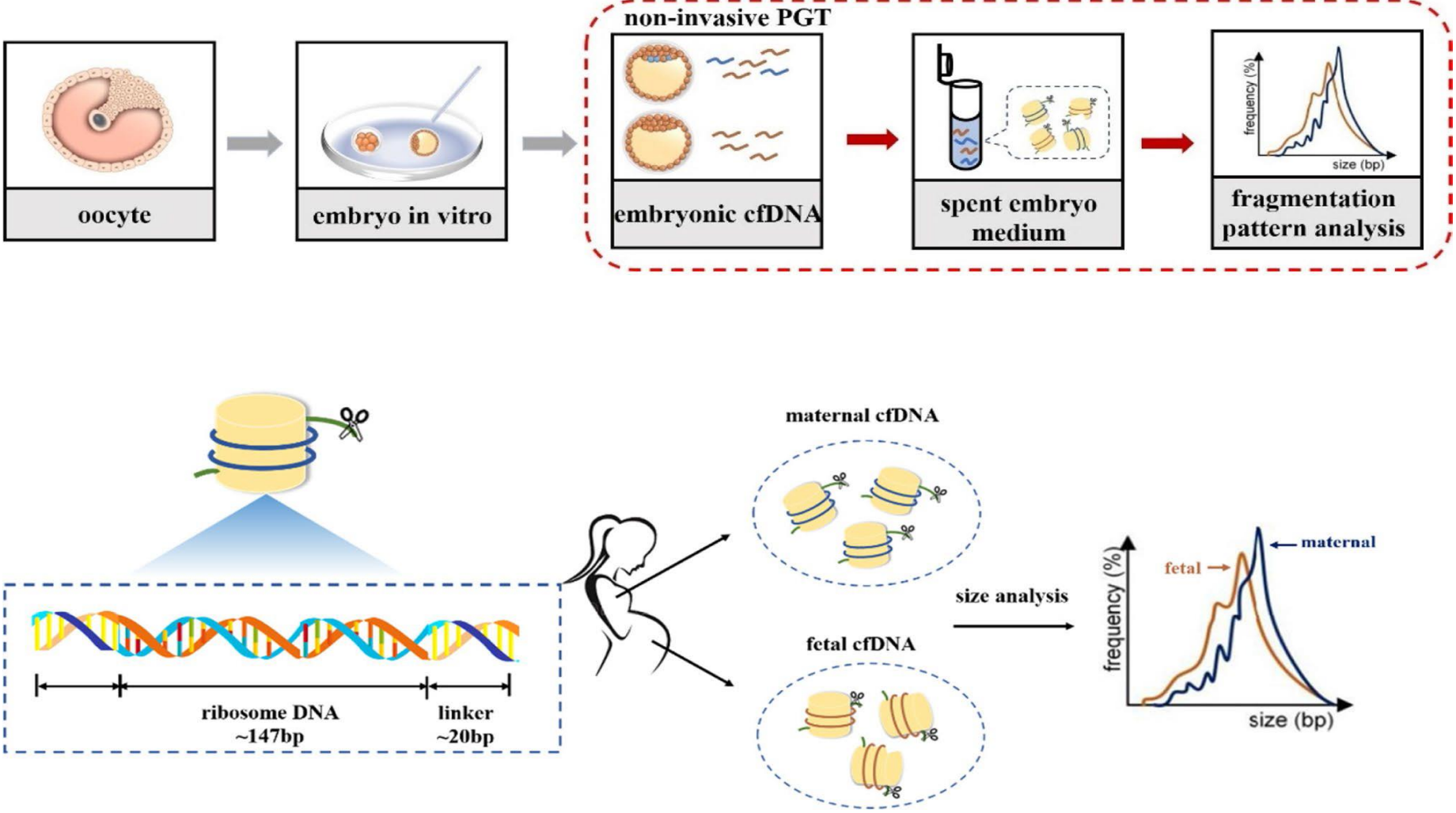
* SCM cfDNA, DNA contamination is one of those important challenges that can arise from external (plastic wares and medium) or maternal sources

* Polar bodies and cumulus cells are the sources of maternal DNA within the embryo culture media that interfere with embryonic cfDNA detection

* To solve the problem, scientists attempted to find solutions to minimize eliminate the maternal cfDNA in culture media

* One recommended resolution was transferring the day 4 or 5 embryo to a new individual culture media followed by collecting the SCM after 24 to 48 h, to decrease and increase the maternal DNA contamination and accuracy, respectively

Thank you for attention



* Non-invasive PGT for abnormal embryos based on fragmentation pattern of cfDNA in SEM. The modules in red dashed box show the strategy of non-invasive screening of embryos based on cfDNA fragment integrity analysis. B Size and fragmentation patterns of maternal and fetus-derived cfDNA in maternal plasma. Maternal cfDNA includes fragments wrapping the nucleosome core unit and linker DNA, which is cut at linker DNA region, and fetal cfDNA only includes fragments wrapping the nucleosome core unit.