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Non-invasive preimplantation genetic testing – possibilities and challenges

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Preimplantation Genetic Diagnosis - case studies

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PGD diagnosis for reciprocal translocations - development, possibilities, prospects importance in the treatment of carriers of reciprocal translocations?

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Non-invasive preimplantation genetic testing – possibilities and challenges

Abstract

The recent reports that spent embryo culture medium contains embryonic DNA have created hopes of non-invasive genetic analysis of preimplantation embryos. However, studies evaluating the feasibility of spent medium DNA (smDNA) for genetic testing of the embryo have met with conflicting results. A major concern is the risk of maternal contamination originating from cumulus cells and follicular fluid. Even if contamination risks were eliminated it is not yet clear how well the smDNA represents the embryonic genome. Systematic studies of test parameters such as methods for sample retrieval, pretreatment and DNA amplification are needed to evaluate the true potential of smDNA in preimplantation genetic testing. A better characterization of the embryonic smDNA as well as resolving the mechanism of secretion into the culture medium may prove crucial in implementing non-invasive genetic testing of embryos.

Introduction

The last decade has witnessed major advances in the field of primplantation genetic testing (PGT). The implementation of new analysis technologies including arrayCGH, Next Generation Sequencing (NGS) and karyomapping (SNP Array) has increased the accuracy of testing and simplified the work flow. The change from cleavage stage biopsy to trophectoderm biopsy was a significant step in increasing the post-biopsy viability of the embryo [Scott et al. 2013]. Nevertheless, performing biopsy involves labour and it is hard to claim that removal of a few cells would not have some harmful effect on embryo viability. Recently, blastocoel fluid was shown to contain nuclear embryonic DNA [Palini et al. 2013] opening the possibility to do genetic testing without removal of cells. While this blastocentesis approach has resulted in encouraging results [Gianaroli et al. 2014, Magli et al. 2016] more recent studies suggest that it may not be sufficiently effective for genetic testing [Babariya et al. 2017, Romanelli et al. 2017]. Furthermore, it still is an invasive technique requiring the use of micromanipulators for direct aspiration of blastocoel fluid. The finding that spent embryo culture medium contains embryonic DNA [Stigliani et al. 2013, Assou et al. 2014, Wu et al. 2015, Shamonki et al. 2016] opened an opportunity for developing non-invasive genetic testing of individually cultured embryos. While some reports have yielded promising noninvasive aneuploidy testing results [Xu et al. 2016, Babariya et al. 2017, Huang et al. 2017] others have produced discouraging outcomes [Romanelli et al. 2017, Feichtinger et al. 2017, Vera-Rodriguez et al. 2018]. The most important question is how well the spent medium DNA (smDNA) sample represents the embryonic genome. Some studies have evaluated the possibility of using spent medium in testing for single gene disorders achieving DNA detection rates up to about 90% [Wu et al 2015, Liu et al. 2016]. Compared to aneuploidy testing the requirement of efficiency and accuracy is clearly higher for genetic analysis of single gene disorders and it remains questionable whether satisfactory levels can be achieved. This overview will mainly focus on the use of spent culture medium for aneuploidy testing of embryos.

Sources of DNA in embryo culture medium

DNA in the culture medium can originate from several sources. Often DNA can be detected from the culture medium itself, most likely due to DNA bound to human serum albumin. Hammond et al. [2017] found mean numbers of 1 nuclear copy and 5-15 mitochondrial copies in commercial embryo culture media supplemented either with 5 mg/mL human serum albumin or serum substitute supplement.

Vera-Rodriguez et al. [2018] reported a median of 1.4 pg human DNA in 20 ul control culture medium droplets, which corresponds to about 0.2 genomic copies. In our smDNA studies the DNA levels in control media (6 ul droplets) have typically been well below 1 pg. Decreasing the culture drop size would increase the embryonic-DNA to culture-medium-DNA ratio, assuming that the release of embryonic DNA is largely unaffected by the culture droplet volume. In case the culture medium derived DNA turns out to be an obstacle for medium based PGT one possiblity is to use serum albumin of a recombinant non-human source. Cumulus cells present a more potential source of DNA contamination. Vera-Rodriguez et al. [2018] attributed discordant PGT results between biopsy and spent culture medium mainly to the high percentage of maternal DNA present in the medium. Analysis of embryonic DNA from the spent medium requires not only a careful removal of cumulus cells from oocytes prior to culture but also thorough washing to remove DNA traces of cumulus cell and follicular fluid origin. DNA of polar bodies is another potential source of maternal DNA contamination, although the small amount of DNA they would contribute may be negligible [Ottolini et al. 2015]. Spermatozoa represent a possible source of DNA contamination when embryos are produced by conventional IVF. However, with appropriate sample handling this contamination risk can be reduced to a minimum.

Confirming embryo as the source of DNA in the culture medium

The comprehensive PGD diagnosis A simple way to demonstrate that the DNA in spent medium is of embryonic origin is to perform sex determination of the medium and the corresponding embryo (or a biopsy of it). However, due to the possibility of contamination the presence of Y-chromosomal DNA does not necessarily prove that the DNA is of embryonic origin [Hammond 2017]. We developed a duplex qPCR assay amplifying highly repetetive autosomal and Y-chromosomal sequences and used it to validate a sexing assay. Sixteen blastocysts were excessively washed to remove traces of maternal DNA and cultured in individual drops from Day 5 to Day 6. The qPCR result of the spent media and the PCR result (amelogenin X/Y) of the whole embryos were in full concordance with respect to gender. In a qPCR approach Ct ratios of the autosomal and Y-chromosomal products might also be used to detect contamination. For intance, an autosomal to Y-chromosomal Ct ratio above the expected range could indicate maternal contamination.A more definitive approach to assess the maternal DNA contribution in spent medium is to use multiple polymorphic markers to compare the embryo, spent medium and maternal DNA. Vera-Rodriguez et al. [2018] used this approch using single nucleotide polymorphism markers (SNPs) to identify and quantify embryonic and matenal DNA and in spent medium. The proportion of embryonic DNA in the spent medium was found to vary considerably (0-100 %, median 8 %). A further possibility to confirm embryonic source of the spent medium DNA is to compare the result with a trophectoderm biopsy using chromosomal aneuploidy testing. In the case of mosaicism, the trophectoderm biopsy and the spent medium sample may represent different cell lineages of the embryo. Hence a discordant result between the samples does not necessarily indicate contamination or that the medium sample is not of embryonic origin.

Amount of DNA in spent culture medium

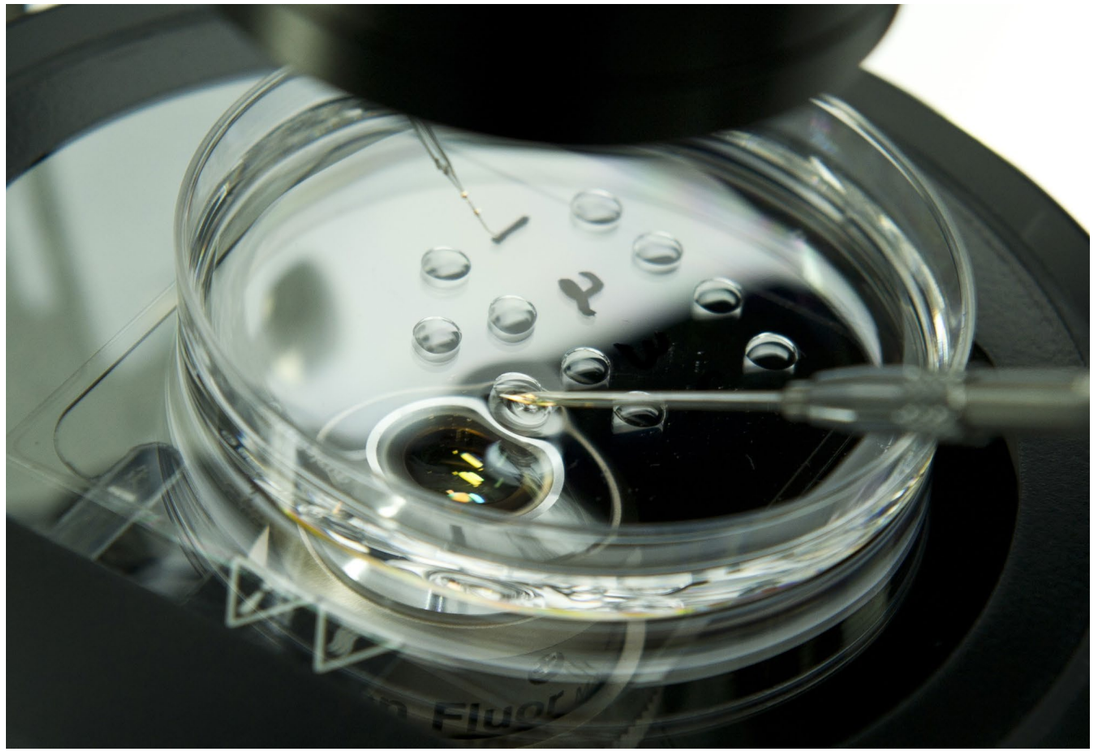
Embryonic DNA can be found in culture medium from Day 3 onwards and the accumulation increases, typically peaking at Day 5/6. Galluzzi et al. [2015] calculated a range of 3-257 pg and 2.1-633pg DNA in culture medium on Day 3 and 5/6 respectively. Using 5 µl culture drops, Wu et al. [2015] reported average concentrations of 14.24, 48.78 and 54.35 pg/ul on Day 4, 5 and 6, respectively. Vera-Rodriguez et al. [2018] found a relatively low median of 6.7 pg DNA after culturing embryos from Day 3 to Day 5. The differences in sampling, DNA extraction and qPCR protocols between these studies could explain the divergent results. Using a duplex qPCR amplifying highly repetetive autosomal and Y-chromosomal targets we found that the cumulative smDNA of embryos cultured from Day 3 to Day 6 ranged from 35 pg to 435 pg with a median of 136 pg. These amounts correspond to about 5, 66 and 21 diploid genomes, respectively. In over 80% of the time the daily DNA accumulation more than doubled from the previous day. The analysis of our multiplex qPCR results also indicated that a substantial proportion of the smDNA appear to consist of fragments smaller than ~200 bp. Since fragmented targets are not amplifiable by qPCR this would suggest that the results could in fact be understimates of the total smDNA quantity. The amounts of DNA in culture medium are at times remarkably high. Considering that the amount of nuclear DNA in a diploid human cell has been estimeted to be about 6.6 pg [Serth et al. 2000], the several hundred picograms of DNA often found in spent medium correspond the DNA content of roughly 50 blastomeres. Another notion, and perhaps a concern, is the high varitiation in smDNA between embryos.

Characteristics and possible origins of embryonic DNA

It has been suggested that the embryo-derived nuclear DNA in spent medium is fragmented [Gianaroli 2014,Hammond et al. 2016]. Apparently with this possibility in mind, Babariya et al. [2017] developed a novel multiple displacement amplification method and using a mixture of 70-100 bp fragments it was validated with encouraging results. In that study chromosomal copy number concordance between the spent medium and the trophectoderm samples was 95.65%. Our results with duplex-qPCR using different sized amplicons support the idea that smDNA is fragmented. Additionally, with conventional PCR we were able to amplify a 108 bp amelogenin-Y sequence in about 50% of spent culture media samples in which male embryos were cultured. However, no amplification was observed from the same samples using a ~900 bp amplicon of the same single copy gene, whereas amplification efficiency was 95% using male single cells as sample. If nuclear embryonic DNA in spent medium is fragmented the situation may be different in the case of embryonic mitochondrial DNA. Using long range PCR (15 kbp amplicon) Hammond et al. [2017] detected mtDNA in 40% of spent medium samples. A possibility is that nuclear and mitochondrial DNA are released into the culture medium by different mechanisms. The mechanism(s) underlying the release of embryonic DNA into the culture medium remain unclear. The most popular hypothesis is that nucleosome sized DNA fragments (~180-200bp) are being released from cells as a result of apoptosis [Hammond et al. 2016, Handyside 2016, Xu et al. 2016]. Other mechanisms include necrosis and blastomere fragmentation. Extrachromosomal microDNAs [Shibata et al. 2012] could possibly also be a source of embryonic nuclear DNA in the spent culture medium. The production of these microDNAs is a part of normal cellular physiology and has been linked to transcriptional activity and mismatch repair [Dillon et al. 2015]. Extrachromosomal microDNAs vary sin size from 60 to 2000 base pairs with the majority in the size range 100-400 bp. They are abundant in all tissue types of mammalian cells, including sperm [Dillon et al. 2015]. In contrast to accumulation of embryonic DNA in the culture medium by apoptosis or necrosis, this mechanism would not necessarily depend on cell death.

Challenges in smDNA analysis

In preimplantation genetic testing the standard procedure is to insert the biopsy into the reaction tube in a small amount of buffer, typically in a volume below 5 µl. Using spent culture medium this can be a problem. For instance, single culture wells of an EmbryoScope® slide contain about 25 µl of culture medium and using only a small fraction of the medium for analysis may not yield enough DNA for analysis. Using all or most of the culture medium would require at least some upscaling of the WGA protocol which in turn would increase the expenses. However, it may not be necessary to use all of the spent culture medium for analysis [Wu et al. 2015, Xu et al. 2016]. One way to avoid too diluted DNA is to culture embryos in small volumes. Babariya et al. [2017] cultured blastocysts overnight in ~3 µl droplets and, using this volume, achieved NGS-PGT results highly concordant with the trophectoderm biopsies of the embryos. This approach may not be lucrative as it would require IVF laboratories



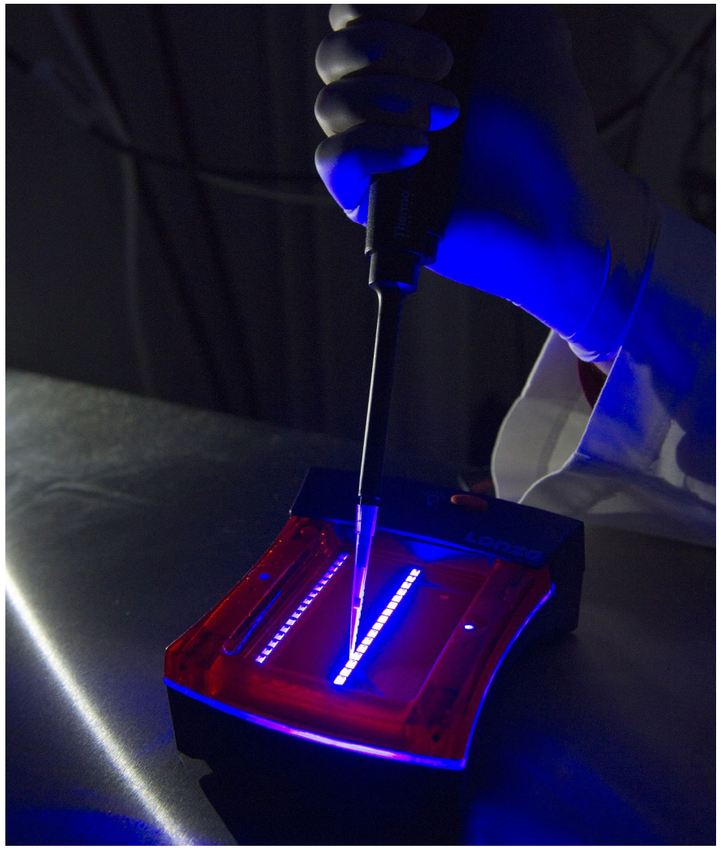
to test and validate such a culture system. Furthermore, the preparation of culture dishes becomes particularily critical to avoid increase of osmolarity due to evaporation. Another solution to concentrate the DNA by is by using commercial kits with spin columns designed for capturing cell free DNA [Stigliani et al. 2013, Hammond et al. 2017]. In case the embryonic DNA consists of relatively short DNA fragments the analysis of spent medium may require modifications of the WGA protocol. Babariya et al. [2017] developed a novel multiple displacement amplification method for whole genome amplification of smDNA with encouraging results using a mixture of 70-100 bp fragments in validation. NGS and array CGH have become the major embryo aneuploidy testing platforms. If the embryonic nuclear DNA in spent medium is highly fragmented NGS should theoretically have an advantage to approaches utilizing DNA probes in detecting the DNA of interest. Short fragments not fully covering the probe area would lead to ineffective hybridization efficiency. In contrast, the sequencing approach does not require specific areas of intact DNA. Very often smDNA results differ from biopsy results of the corresponding embryo. Using NGS to test for aneuploidy Xu et al. [2016] compared smDNA and biopsy results of 42 embryos. Of these 21 were diagnosed chromosomally normal with both samples, 4 tested abnormal with spent medium sample only and 2 were aneuploid with the biopsy sample only. The remaining 15 embryos were diagnosed aneuploid with both sample types with a a concordant karyotype result in only 3 of these biopsy/medium pairs. However, it is noteworthy that by both assays all these 15 embryos would be considered unsuitable for transfer. Result discrepancies between the sample types may be a result of unrepresentative or poor quality DNA, contamination or embryo mosaicism. Mosaic embryos can be a result of mitotic nondisjunction in a cleavage stage blastomere embryos resulting in some blastomeres with trisomy and others with monosomy of the same chromosome while other blastomeres can be euploid. Considering that the few cells of a trophectoderm biopsy may only contain one or two of the three cell lines it is not surprising if the result of the spent medium differ from the biopsy result. When comparing biopsy results with spent medium results Vera-Rodriguez et al. [2018] not only discovered a significant amount of maternal contamination in spent medium samples but also noticed several cases of complementary aneuploidies, i.e. monosomy in one type of sample and trisomy in the other. However, it is not self-evident that biopsy would always give a more reliable result than the spent medium sample.

Some practical advantages of noninvasive PGT

Noninvasive genetic testing offers other advantages in addition to preserved intactness of the embryo and less laborous sampling. In PGT a few percent of biopsy samples will not give an analysis result. Using spent medium samples as a backup these could be reanalysed without thawing, re-biopsy and refreezing the embryo. In cases where the result of the biopsy indicates mosaicism, spent medium analysis might give more information about the nature and extent of the mosaic condition. Furthermore, storing spent media for patients having their embryos frozen would open up the possibility of genetic testing at a later stage without the need to thaw, biopsy and refreeze the embryos.

Other potential benefits of spent medium nucleic acid analysis

If spent medium samples prove to be dissatisfactory for aneuploidy testing or single gene analysis, it may still have other uses. Stigliani et al. [2014] showed that the mtDNA/gDNA ratio in the spent medium can be used to assess blastocyst potential and impalntation outcome. Capalbo et al. [2016] examined microRNAs present in blastocyst culture medium to predict implantation potential. Using flow cytometry to quantify nucleic acid containing extracellular vesicles Pallinger et al. [2017] developed a test to identify embryos capable of implantation.



Conclusions

Whether spent medium analysis will be useful for genetic testing of embryos remains controversial. Many different methods have been used to perform the steps of the analysis flowchart and systematic studies comparing these are needed to find an optimal protocol. For instance, there is no standardized protocol for retrieval of spent culture medium [Romanelli et al. 2017] and in many papers the sample handling is not explained in enough detail to make meaningful comparisons. Solving the origins and characteristics of the embryonic DNA in culture medium will help in defining the true value of non-invasive preimplantation genetic testing.

References

Assou et al. 2014 Assou S, Aït-Ahmed O, El Messaoudi S, Thierry AR, Hamamah S. Non-invasive pre-implantation genetic diagnosis of X-linked disorders. Med Hypotheses 2014; 83: 506–508.
Babariya, D., Manoharan, A., Welch, C., Kung, A., Spaeth, K., Munne, S., Coates, A. & Wells, D. Development and application of a novel strategy to explore blastocoel fluid and spent culture media as a source of embryonic DNA. Abstracts of the 33rd annual meeting of the European society of human reproduction and embryology. Hum Reprod 2017: 32 Supp.1 Abstract book: i14.
Capalbo A, Ubaldi FM, Cimadomo D, Noli L, Khalaf Y, Farcomeni A, Ilic D,Rienzi L. MicroRNAs in spent blastocyst culture medium are derived fromtrophectoderm cells and can be explored for human embryo reproductive competence assessment. Fertil Steril 2016; 105: 225–235.
Dillon L, Kumar P, Shibata Y, Wang YH, Willcox S, Griffith JD, Pommier Y, Takeda S, Dutta A. Production of extrachromosomal microDNAs is linked to mismatch repair pathways and transcriptional activity. Cell Reports 2015; 11: 1749–1759.
Feichtinger M, Vaccari E, Carli L, Wallner E, Madel U, Figl K, Palini S, Feichtinger W. Non-invasive preimplantation genetic screening using array comparative genomic hybridization on spent culture media: a proof-of-concept pilot study. Reprod Biomed Online 2017; 34: 583–589
Galluzzi L, Palini S, Stefani SD, Andreoni F, Primiterra M, Diotallevi A, Bulletti C, Mangani M. Extracellular embryo genomic DNA and its potential for genotyping applications. Future Science OA 2015; 1: 4.
Gianaroli L, Magli C, Pomante A, Crivello AM, Cafueri G, Valerio M, Ferraretti AP. Blastocentesis: a source of DNA for preimplantation genetic testing. Results from a pilot study. Fertil Steril 2014: 102; 1692–1699.
Hammond E, Shelling A, Cree L. Nuclear and mitochondrial DNA in blastocoele fluid and embryo culture medium: evidence and potential clinical use. Hum Reprod 2016; 31: 1653–1661
Hammond ER, McGillivray BC, Wicker SM, Peek JC, Shelling AN, Stone P,Chamley LW, Cree LM. Characterizing nuclear and mitochondrial DNA in spent embryo culture media: genetic contamination identified. Fertil Steril 2017; 1: 220–228.
Handyside AH. Noninvasive preimplantation genetic testing: dream or reality? Fertil Steril 2016; 106: 1324-1325.
Huang L, Bogale B, Lu S, Xie XS, Racowsky C. High efficacy of non-invasive chromosome screening using spent culture medium for preimplantation genetic testing of human embryos. Fertil Steril 2017; 108; Supplement: e277–e278
Liu W, Liu J, Du H, ling JW, Sun XF, Chen DJ Non-invasive pre-implantation aneuploidy screening and diagnosis of beta thalassemia IVSII654 mutation using spent embryo culture medium. Annals of Medicine 2016; 49: 319–328.
Magli MC, Pomante A, Cafueri G, Valerio M, Crippa A, Ferraretti AP, Gianaroli L. Preimplantation genetic testing: polar bodies, blastomeres, trophectodermcells, or blastocoelic fluid? Fertil Steril 2016; 105: 676–683. e5.
Ottolini CS, Rogers S, Sage K, Summers MC, Capalbo A, Griffin DK, Sarasa J, Wells D, Handyside AH. Karyomapping identifies second polar bodyDNA persisting to the blastocyst stage: implications for embryo biopsy. Reprod Biomed Online 2015; 31: 776–782.
Palini S, Galluzzi L, De Stefani S, Bianchi M, Wells D, Magnani M, Bulletti C. Genomic DNA in human blastocoele fluid. Reprod Biomed Online 2013; 26: 603–610.
Pallinger E, Bognar Z, Bodis J, Csabai T, Farkas N, Godony K, Vargany A, Buzas E, Szekeres-Bartho J. A simple and rapid flow cytometry-based assay to identify a competent embryo prior to embryo transfer. Scientific Reports 2017; 7: 39927.
Romanelli V, Girardi L, Cimadomo D, Patassini C, Scarica C, Albricci M, Stoppa M, Cecchele A, Poli M, Ubaldi FM, Rienzi LF, Capalbo A. Spent Blastocyst Media and Blastocoel Fluid are not reliable DNA sources for preimplantation genetic diagnosis of aneuploidies and monogenic disorders. . Abstracts of the 33rd annual meeting of the European society of human reproduction and embryology. Hum Reprod 2017; 32 Supp.1 Abstract book: i13.
Scott RT Jr, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: a randomized and paired clinical trial. Fertil Steril 2013; 100: 624–630.
Serth J, Kuczyk MA, Paeslack U, Lichtinghagen R, Jonas U. Quantitation of DNA extracted after micropreparation of cells from frozen and formalin-fixed tissue sections. Am J Pathol 2000; 4: 1189–1196.
Shamonki MI, Jin H, Haimowitz Z, Liu L. Proof of concept: preimplantation genetic screening without embryo biopsy through analysis of cell-free DNA in spent embryo culture media. Fertil Steril 2016; 6: 1312–1318.
Shibata Y, Kumar P, Layer R, Wilcox S, Gagan JR, Griffith JD, Dutta A. Extrachromosomal microDNAs and chromosomal microdeletions in normal tissues. Science 2012; 336 (6077): 82–86.
Stigliani S, Anserini P, Venturini PL, Scaruffi P. Mitochondrial DNAcontent in embryo culture medium is significantly associated with human embryo fragmentation. Hum Reprod 2013; 28: 2652–2660.
Stigliani S, Persico L, Lagazio C, Anserini P, Venturini PL, Scaruffi P. Mitochondrial DNA in Day 3 embryo culture medium is a novel, non-invasive biomarker of blastocyst potential and implantation outcome. Mol Hum Reprod 2014; 20: 1238–1246.
Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, Mercader A, Meseguer M, Blesa D, Moreno I, Valbuena D, Rubio C, Simon C. Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. Hum Reprod 2018; 33: 745-756.
Wu H, Ding C, Shen X, Wang J, Li R, Cai B, Xu Y, Zhong Y, Zhou C. Medium-based noninvasive preimplantation genetic diagnosis for human α-thalassemias-SEA. Medicine 2015; 94: e669.
Xu J, Fang R, Chen L, Chen D, Xiao JP, Yang W, Wang H, Song X, Ma T, Bo S et al. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. Proc Natl Acad Sci USA 2016; 42: 11907–11912.