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Non-Invasive Preimplantation Genetic Testing: a Laboratory Review

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ABSTRACT

Since the presence of cell free DNA (cfDNA) was reported in blastocoel fluid and spent culture medium the possibility to introduce a non-invasive preimplantation genetic testing (niPGT) as a new tool in assisted reproductive technology (ART) has been investigated by different groups.

A variety of different approaches have been used until now to collect, extract, amplify and analyse embryonic DNA from BF, SCM and eventually both together. However, success rates in term of amplification, ploidy concordance, specificity and sensitivity vary widely among different papers.

This review aims at summarizing those different methodologies.

niPTG offers many advantages compared to the traditional biopsy method especially regarding safety of embryos and future children, nevertheless despite the growing amount of data, optimized and high reproducibility protocols are as for now, missing and clinical replacement is still not possible, and it should still be only classified as a screening method for optimizing noninvasive embryo prioritization.

KEYWORDS: non-invasive preimplantation genetic testing, cell-free DNA, spent culture medium, blastocoel fluid.

ABBREVIATIONS

cfDNA – cell free DNA.
BF – blastocoel fluid.
SCM – spent culture medium.
niPGT – non-invasive preimplantation genetic test.

MANUSCRIPT

Introduction

Since the first papers reporting the presence of cfDNA (cell-free DNA) in BF (blastocoel fluid) and SCM (spent culture medium) were published in 2013 [1], [2] an increasing interest has been set into the possibility to establish solid tools that allow the genetic screening of embryos without the necessity of biopsy.

As aneuploidy in human embryos is common and can result in implantation failure or miscarriage, preimplantation genetic testing (PGT) is used

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worldwide with the aim of improving pregnancy rates in poor IVF-prognosis patients, for example patients of advanced maternal age and recurrent pregnancy loss.

PGT nowadays is a standard procedure and is commonly performed through biopsy of trophectoderm cells of the expanded blastocyst from day 5 to day 7 of culture, or the biopsy of blastomere on the third day of embryo development. Additionally, polar body biopsy on day 1 after fertilization can reveal meiotic maternal aneuploidies. All those procedures are highly invasive, may compromise the embryonic development and consequently the embryo implantation potential and raise questions about long-term effects on the offspring´s health. From a more practical point of view, all the biopsy procedures require highly trained personal, special instrumentation, they are time consuming, they add risks into the lab workflow and significantly increase the treatment costs for patients.

The origin of cfDNA and the biological mechanisms responsible in both BF and SCM are still controversial. Nevertheless multiple studies in the last years have shown that cfDNA present in the BF and in the SCM is informative, and even if the clinical replacement of classic PGTA is not yet possible, it could provide a safer, easier and more economic source of genetic material for analysis in the future.

Cell Free DNA in Blastocoel Fluid

The first work introducing a minimally invasive preimplantation genetic testing (miPGT) in which BF was aspirated from blastocyst (blastocentesis) was published in 2013 followed from great interest of the potential of the new technique [1]. Blastocentesis is considered to be not detrimental for the embryo and used by many laboratories prior to blastocyst vitrification [3]. It is a well-established method that nevertheless still requires highly skilled personal and is both time and cost consuming. The sampling procedure has been described in detail: it consists of an ICSI pipette that enters the blastocoel cavity through the TE on the opposite side of the inner cell mass (ICM), ideally in the point of contact between two TE cells to minimize the amount of crossed cytoplasm and the possibility of cell damage. Through a slightly negative pressure, the fluid is collected until the embryo is fully collapsed, paying great attention to avoid the aspiration of any cellular material present inside the blastocoel. After the collection, the ICSI pipette is gently retracted and the specimens can be directly transferred to a PCR tube or released in a small drop of medium and then collected into a PCR tube before cryopreservation ([1], [4]–[9]). The reported BF´s aspired volume vary among different works between 0.005 µl and 1µl. Blastocentesis has been applied in different studies during years for both PGT-A and PGT-M with different degree of amplification success (35% to 88%) among different studies[1], [4]–[8], [10], [11] and independently from the genetic approach used, probably due to the restricted amount of DNA present in BF. The low amplification rate obtained and the resulting difficulty to reproduce data, with the consequent risk to have undiagnosed embryos strongly suggest the necessity of additional studies on the potential of BF in clinical use in order to optimize and standardize protocols for sampling, amplification and analysis of BF-cfDNA. Furthermore, blastocentesis needle aspiration protocols do not reduce cost and do not simplify the laboratory workflow compared to the traditional TE biopsy.

Cell Free DNA in Spent culture medium

The analysis of cfDNA in SCM is a potential alternative for the non-invasive preimplantation genetic testing of embryos in IVF and several publications in the last years explored this possibility. cfDNA has been reported to be detectable already on SCM of embryo on day 2 -3 of development [2], [10] and it has been shown that the amount of cfDNA increases during embryo culture, however not all cfDNA has embryonic origin [13]. In particular contamination with maternal DNA seems to be the main source of non-embryonic DNA in SCM. Xu and colleague published in 2016 [14] a comparison between 42 SCM of donated blastocyst and their subsequent whole embryo analysis. Embryos generated by ICSI and vitrified on day 3 were thawed and placed in 25µl single drop culture until day 5. On that day, after 2 days of culture, 5 to 20µl of SCM have been collected. A modified multiple annealing and looping based amplification cycles (MALBAC) followed by NGS was used to perform genome analysis. The corresponding D5 whole embryos were used as gold standard to evaluate the chromosome screening results from the culture media. They were able to amplify DNA in 100% of cases obtaining a ploidy concordance of 86%. The paper published by Shamonki and colleague in 2016 [15] first tried to assess whether PGT is possible by testing for cfDNA in SCM comparing the results with trophectoderm biopsy performed on the same embryos. Embryos were cultured in single 15µl media droplets from d3 until the biopsy (d5 or d6), assisted laser hatching was performed to facilitate the extrusion of TE and to allow the expulsion of cfDNA. Using the SCM in their proof-of-concept paper they were able to detect cfDNA in 55 out of 57 cases. Our group in 2017 ([16]) published a paper in which we demonstrated the presence of cfDNA in SCM after uninterrupted embryo culture after polar body biopsy from d1 to day d5/6. In this case 18 out of 22 samples could be amplified from 5µl of SCM and resulted in a 72% ploidy concordance rate. Ho and colleagues in 2018 ([17]) showed that cfDNA is detectable in SCM from thawed 2PNs stage obtained by ICSI. The zygotes have been cultured in a

continuous and uninterrupted way until d3 or d5 of embryo development. Both informativity and ploidy concordance were higher (39% vs 80.4%) and more accurate (56.3% vs 65%) in d5 samples. Vera-Rodriguez and colleagues ([13]) report a high percentage of maternal DNA in SCM, collected from embryos cultured after AH in single 25µl droplets until day 5, which can be the reason of low ploidy concordance between TE and SCM DNA analysis (33%). Conversely Huang and colleagues ([18]) reported extremely promising results in a cohort of 52 donated blastocysts previously biopsied and vitrified, then thawed and kept in culture for 24 hours. 10µl of SCM was collected from each sample. The results of the WGA (MALBAC) analysed through NGS have been compared with TE biopsy results and with the sequencing result of the corresponding whole embryo. The concordance rates for both embryo ploidy and chromosome copy numbers were higher between whole embryo and niPGT-A than the classical TE biopsy, suggesting that niPGT-A could be less prone to errors associated with mosaicism and that it could be more reliable than PGT-A in discovering aneuploidies. In 2019 Yeung published ([19]) one of the largest data sets on the subject: the authors did not modify any of their laboratory routine, performing assisted hatching and medium change on d3 of development and collecting SCM samples (3µl out of 30 µl) on the day of TE biopsy resulting in comparable results between niPGT-A and TE biopsy.

During the years different studies have been published: despite a big variety of embryo origin (ICSI/IVF), culture condition (single/double step, media type, drop size, volume of SCM collected) and embryo manipulations (assisted hatching, vitrification/thawing, zona pellucida removing) no significant differences have been found in term of amplification and concordance; conversely increasing the contact time between embryo and culture medium through the culture extension until d6 and d7 seems to improve both informativity and concordance of SCM with the TE biopsy. Rubio and colleagues ([20]) reported a significant increase of concordance rates between ploidy and sex from 78.7% to 84%, and an increase of sensitivity and specificity from 94.5% to 95.2% and from 71.7% to 82.1% respectively in embryos maintained in culture until d6/7 compared to d5.

Of paramount importance in niPGT is to avoid sample contamination with maternal and external DNA, and it is one of the big challenges of the technique. To minimize contamination several different strategies have been suggested: careful denudation before ICSI or after IVF, serial washes on the time of media changes with new capillaries for each embryo. In 2020 the largest study to date assessing ploidy concordance per embryo assessing traditional PGT and niPGT was published by Rubio and colleagues ([21]). In this prospective, observational, multicentre study TE and SCM from 1301 day 6-7 blastocyst were collected and analysed in 8 different centres. Data resulted in an overall ploidy concordance of 78.2% between cfDNA and TE analysis; when stratified by culture media used or incubators model concordance rates did not show significant differences. The authors point out the importance of the laboratory routine in obtaining good amplification rates and ploidy concordance. Especially they focused their attention in timing of SCM collection indicating in day 6 of development the ideal time for DNA sampling independently of embryo developmental stage on day 5 allowing the embryo to stay in contact with the medium for at least 40 hours. niPTG-A in this paper was applied to all d6 embryos that reach the blastocyst stage prolonging of 1 day the permanence in culture of d5 expanded blastocyst that normally would have been cryopreserved. The embryos were cultivated in small (10µl) medium droplets and the medium was changed on the 4th day of development. Special attention have been raised on careful denudation of oocytes and extensive washing in three sequential 20µl drops of fresh medium before media change on day 4 to minimise the possibility of samples contamination. Lledo and co-workers noticed in their study ([22]) that the results of niPGT-A are independent from the technique used for chromosomal analysis. They amplified 92 SCM samples with two different WGA protocols (MALBAC and Sureplex) obtaining genetic information in 92.4% of cases regardless of the method used; in 95.2% of the cases they reported consistency in the diagnosis. Furthermore they were able to investigate the causes of the discrepancy between niPGT-A and classic PGT-A using the TE of aneuploid donated embryos. In 22,2% of the cases the differences were due to embryo mosaicism and in 55,6% of cases due to DNA contamination (maternal origin) raising the attention on the protocols used to obtain contamination-free SCM. Hanson and colleague ([23]) collected 166 SCM samples from 30µl drops where embryos were cultivated, after AH, from day3 and day4 until blastocyst biopsy (day 5 to day 7). They compared the data obtained with NGS platform reporting 37.3% of amplification failure on niPGT-A samples and 40.4% of ploidy discordance with TE results. Due to the experimental design, they had in the cohort of SCM samples that were in contact for different durations with the embryos from 1 (dish change on day 4 and biopsy on day 5) to 4 days (dish change on day 3 and biopsy on day 7). They noticed statistically significant higher rates of DNA amplification with the increase of the time in culture of the embryo: after 1 day of exposure they amplified only 1 sample out of 26. After 2 days 36/63 samples where amplified, after 3 days 56/66 and finally all the samples collected after 4 days were amplified (11/11). The discrepancy in the

whole chromosome aneuploidies between niPGT-A and PGTA (40.4%) anyhow didn´t show any statistically relevant correlation with the day of contact. Using 75 previously vitrified donated blastocyst Yin and colleagues ([24]) were able to compare the analysis of SCM with TE and the whole embryo. After warming, blastocysts were placed in single 25µl drops and the SCM was collected after 24 hours of culture. The blastocyst were then biopsied and TE and the remaining embryos separately collected for the analysis. In a first phase of protocol optimization the thawed blastocysts were kept in contact 8 hours with the medium before the collection of SCM: the NGS profiles obtained after WGA were noisy providing the evidence of poor DNA samples and suggesting the necessity to increase to 24 hours the culture time. After this adjustment the group reported a good informative amplification rate (78.7%) nevertheless a significant lower percentage than their corresponding TE and WE groups. No statistically significant different ploidy concordance rate between WE and SCM (89,8%) and between WE and TE (94.8%) were reported, nevertheless they reported remarkable differences in full concordance rate (including mosaicism and segmental aneuploidies) between the two groups: WE to SCM 32.2% and WE to TE 69.3%. In order to assess the potential of zona pellucida (ZP) and its associates transzonal projection as source of cfDNA contamination, 6 ZP were placed in media drop and kept in culture for 24 hours. The media samples analyse showed amplification failure eliminating the possibility of ZP and associated projection as source of DNA in SCM. Interesting findings have been shown by Shitara and colleague ([25]) that compared niPGT-A results with classical PGT-A using outgrowth embryos as golden standard. 20 donated blastocysts have been thawed and placed in culture for 1 day (day 5 blastocyst) or 3 hours (day 6 blastocyst) before zona pellucida removing (embryo exposed to acidic Tyrode, Kitazato) and TE biopsy. After biopsy embryos were kept in long-term culture up to day 10 when the centre of the embryo was biopsied and sampled. Even though the study included only a small sample size the authors report a better autosomal chromosomal concordance between niPGT-A to outgrowth (56.3%) than between PGT-A to outgrowth (43.8%). They report as well for niPGT-A group a better sensitivity (100%), specificity (87,5%), positive predictive value (88,9%) and negative predictive value (100%) compared with the PGT-A group (sensitivity 87,5%, specificity 77.8%, PPV 87.5%, NPV 75%) stating that cfDNA in SCM reflects the chromosomal status of both ICM and TE, whereas the TE biopsy only reflects the chromosomal status of TE, thus the SCM could reflect the ploidy of the blastocyst better than TE biopsy samples. An further group in 2021 (Chen et al. 2021 [26]) published a study where whole embryo was used as a reference to calculate the ploidy concordance of SCM and TE. 265 embryos derived from ICSI and cultured from day 3 to blastocyst stage were donated and used for subsequent analysis. From 20 to 25µl of SCM were amplified with a MALBAC WGA strategy and then subjected to NGS. Using the NGS result from WE as the gold standard to evaluate PGT and niPGT performances they showed no differences in sensitivity (TE 89.6% vs SCM 86.5%), specificity (80.0% vs 73.1%), negative predictive value (92.8% vs 90.0%) and positive predictive value (72.9 % vs 65.9%).

The authors identified three main reason for low proportion of maternal contamination and higher concordance rates obtained in their study compared to Vera-Rodriguez [13]: (i) thorough precaution in removing as many as possible cumulus-corona radiata cells and extensive embryo rinsing on the time of medium change; (ii) MALBAC WGA technique; (iii) the utilization of whole embryos as gold standard for comparison, considering the fact that TE cells karyotype could be not fully representative of the WE. Like Rubio and colleague Chen group concluded that niPGT-A is a good rule-in assay and might be used for prioritizing embryos for transfer.

Collection of cfDNA from Spent culture medium and Blastocoel Fluid

The reported PCR amplification rates achieved from SCM are inferior to those using cellular biopsy and the potential presence of genetic contamination is a significant concern. In 2018 Kuznyetsov and colleague ([27]) published a study where they suggest a new approach to obtain sufficient embryonic DNA for aneuploidy screening with a non-invasive methodology. They combine the collection of SCM with BF. Expanded blastocyst were collapsed by single laser shot at the junction of TE cells allowing the content of blastocoel to flow into the culture medium. 100% of their samples, obtained after thawing previously cryopreserved embryos and maintained in culture for 24hours (28 blastocyst), were informative and the concordance with TE and WE was 87,5% and 96,4% respectively. Also the samples obtained from freshly cultured embryos from d4 to d5/6 were 100% informative and the ploidy concordance between niPGT and TE was 100%. In order to minimize the risk of contamination in fresh culture samples they modified the culture protocol introducing a more careful denudation and a medium drop change on day 4 of culture after extensive embryo wash. In 2020 the same group published a paper ([28]) where they report factors affecting accuracy of minimal invasive preimplantation genetic testing for aneuploidy (mi-PGT-a): according with their results blastocyst morphology has no effect neither on cfDNA quantity found in SCM + BF or in WGA-DNA fragment. According with this finding blastocyst morphology has

no effect on informativity and concordance of mi-PGT-A compared with classical TE biopsy and analysis: on a total of 102 samples 88.2% where informative and the overall ploidy concordance rate was 97,8%. In the same study they propose a new protocol for the whole genome amplification (WGA): in order to reduce the risk of maternal contamination by residual cumulus/corona cells, it avoids the cell lysis step and in this study on 43 blastocysts it showed no difference in terms of informative amplification and ploidy concordance analysis in case of WGA with or without lysis and compared with TE biopsy.

In combining BF and SCM, Li and colleague showed that DNA concentration in SCM together with BF were sufficient to obtain DNA amplification in 97,5% of cases with a ploidy concordance with TE and WE of 76,3% and 78.9% respectively. Several other studies describing collection of cfDNA from SCM together with BF have been published until now ([29]–[33]): anyway, a low number of samples were included in those papers and embryos were subjected to heterogeneous manipulation (vitrification, thawing, TE before or after cfDNA sampling) in addition to artificial collapsing. The genetic analysis results were compared with TE and WE and the results were heterogeneous in terms of informativity and ploidy concordance, nevertheless they never drop under 87,5% and 70%. Jiao and colleague collected SCM and BF from donated and thawed embryos kept in culture for 15 hours. They obtain 100% of amplification rate, 90.5% clinical concordance for aneuploidies and 100% clinical concordance for chromosomal structural rearrangements with embryos NGS analysis. Notably they developed a modified MALBAC approach to reduce the time of WGA and library preparation to 2.5 hours, introducing the possibility to undergo a fresh blastocyst transfer after PGT ([29]). In 2022 Tsai and colleague published a study where cfDNA where collected from SCM and BF using a routine IVF laboratory workflow. They used both IVF (64%) and ICSI (36%) as fertilization methods, the embryos were cultivated in a time-laps system in sequential media (change on d3). They reported an amplification rates consistent with those reported in previous studies but with a drop in the overall ploidy concordance to 67.7% ([34]). The ploidy concordance was higher in ICSI samples compared to IVF but the difference was not statistically significant and like shown by Rubio ([21]) sensitivity and specificity are not affected by the fertilisation methods used.

Discussion

Recently, after pioneering studies reported the presence of cfDNA in both BF and SCM, niPGT methods have been suggested and shown promising potentiality as an alternative to classical PGT. ([1], [2], [4], [15]). Although the biological mechanisms responsible for the presence of embryonic DNA in SCM and BF are elusive, a growing body of literature has demonstrated the possibility to collect, detect and amplify cfDNA and to use it for clinical application. Some authors suggested that embryonic cfDNA comes both from ICM and TE as results of apoptosis mechanisms happening during pre-implantation development, making it superior to traditional invasive PGT, since TE might be non-representative of the whole embryo due to mosaicism ([17], [25], [27]). Different studies report that the amount of cfDNA available in SCM is directly related with the time of culture of embryos in culture drops, obtaining better amplification rates and concordance after cfDNA sampling on day 6/7 of culture ([17], [18], [20]). Anyway the ideal time for sample collection has not been established yet and there are concerns about the risk connected with the prolongation of culture time regarding the potential negative effects that it could have on embryos viability and developmental potential ([23]). In the last years an interesting approach has been used from different groups to increase the amount of cfDNA present in SCM: a single laser pulse between two TE cells generated an artificial blastocyst shrinking, considered not armful for the embryo, that resulted in the flow of BF into the culture drop. Encouraging results have been achieved in terms of amplification rate in different studies, nevertheless most of them used prior vitrified and then donated embryos as samples. It is possible that the use of vitrified-thawed embryos introduced a bias, due to the possible damage of the freezing-thawing method, resulting in increased levels of DNA in the medium ([27]–[31]). Small media (10-15µl) droplets have been used in different works in order to obtain higher cfDNA concentration and improved DNA amplification rates but the necessity of this protocol modification is still debate [18], [20], [21], [29], [33]. Maternal DNA contamination is one of the main issues that must be solved to avoid misdiagnosis using niPGT. Gentle and careful denudation is of paramount importance to avoid contamination with cumulus and granulosa cells, together with serial extensive washing steps of embryos before transferring them to the culture drops and on the day of culture media change. Each embryo must be manipulated singularly using new capillaries to avoid cross-contamination between samples. Particular attention during all laboratory procedure and sterile condition must be used to prevent contamination from external DNA sources ([18], [21], [27]). From a laboratory point of view, it has been reported that concordance rates between PGT and niPGT is not influenced neither by the culture media used nor by incubator brand, potentially allowing the introduction of this technology in any IVF laboratory without the need to have any new instrumentations [21].Blastocysts laser collapse and assisted hatching have been used in order to increase the amount of cfDNA and its

release into the culture drop. The results obtained look promising, and some of the highest niPGT amplification rates have been reported, indicating that sampling SCM and BF together might be a valid approach to the DNA collection, nevertheless results must be evaluated and confirmed by further studies ([27]–[29], [35]).

CONCLUSIONS

cfDNA of embryonic origin is present in SCM and in BF and can be used to obtain genetic information. However, at present the application of niPGT in routine clinical setting has to be viewed critically due to the lack of clinical data. Further studies using larger sample sizes and laboratories condition that reflect the clinical practice should be encouraged in order to define new standardized laboratory protocols widely accepted. Since the biological mechanisms involved are at the moment not clear, more studies that try to solve the mystery of the origin of cfDNA are fundamental and necessary to improve genetic methodologies used for amplification and analysis of cfDNA and result interpretation. A lot of work needs to be done before niPGT can be used in clinical programs as a diagnostic tool. Nevertheless it is a very promising approach, which can be already considered a good tool in clinical practice to prioritise the order of transfer and possibly reduce the time to conceive for couples undergoing assisted medical reproduction treatments, without imposing biopsy related risks for the embryos and the deriving new-borns related with classical PGT approach.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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