

Divining the genetic status of embryos - Consult the medium?

Dhruti Babariya (1), Megan Leaver (2) and Dagan Wells (1,2).

(1) IVI-RMA Global, Magdalen Centre, Oxford Science Park, Oxford, OX4 4LP, UK; (2) University of Oxford, Nuffield Department of Women's and Reproductive Health, John Radcliffe Hospital, Oxford, OX3 9DU, UK.

Chromosomal abnormalities are extremely common in human preimplantation embryos, affecting the majority of blastocysts produced by women in their late thirties and forties. It has long been known that aneuploidy is the principal cause of miscarriage, while in recent years it has become clear that it is also responsible for many instances of implantation failure during *in vitro* fertilization (IVF) cycles. Preimplantation genetic testing for aneuploidy (PGT-A) has been proposed as a method for enhancing embryo selection, with the aim of distinguishing embryos affected by chromosomal abnormalities, which are predicted to have reduced developmental potential, from those that are euploid. Currently, PGT-A is an invasive methodology, typically involving the biopsy of several trophectoderm cells from embryos at the blastocyst stage. Most embryo biopsy techniques require specialist equipment, including a precise laser, as well as highly skilled embryologists. Together, the training of personnel and the purchase and maintenance of equipment add significantly to the costs of PGT-A. In addition to the financial implications of biopsy, there have also been concerns that the procedure could be damaging to some embryos, especially if the method is carried out suboptimally (it is worth noting that there is no standardized protocol for biopsy). In theory, avoiding biopsy could significantly reduce the costs of genetic testing, while eliminating any potential risk to the embryo. Given these perceived benefits, it is not surprising that there has been great interest in the development of non-invasive strategies for preimplantation genetic testing. Research activity in this area has grown substantially following recent reports indicating that embryonic DNA can be detected in spent culture medium (SCM) (Stigliani et al., 2016). However, the clinical applicability of non-invasive PGT-A (niPGT-A) remains the subject of debate, with some studies producing conflicting data and others unclear in their conclusions due to insufficient statistical power or because of confounding variables affecting the study design.

The most recent investigation of embryonic DNA in SCM, reported by Rubio and colleagues (2019), describes interesting results that add to the developing story of niPGT-A. Unlike most previous reports in this research area, the study had the advantage of being prospective and blinded. Furthermore, the protocol employed could be considered to be truly non-invasive, avoiding interventions that might artificially increase the release of embryonic DNA (e.g. embryos were not previously cryopreserved and did not undergo assisted hatching). Briefly, the authors assessed 115 samples of medium, each of which had been used for the culture a single embryo from Day-4 until Day-5, -6 or -7. While the number of samples can be considered relatively modest, this nevertheless represents the largest niPGT-A study to date. An attempt was made to amplify DNA from each SCM sample and detect aneuploidy using a next generation sequencing (NGS) approach. Results were compared to those obtained from the same embryos following routine TE biopsy and PGT-A (also using NGS). Amplification of DNA was successful in 94.8% of SCM samples, which

compares favorably with previous publications and is only slightly lower than rates typically seen for trophoctoderm biopsies.

When classifying embryos into simple categories of euploid or aneuploid, the concordance between SCM samples and corresponding TE biopsies was 78.7%, which is in a similar range to previous studies. If it is assumed that the TE biopsy result is an accurate representation of the cytogenetic status of the embryo, the sensitivity for SCM samples was 94.5%. This rate exceeds sensitivity values reported in two previous niPGT-A studies conducted on Day-3-Day-5 spent media (Xu et al. 2016; Li et al., 2018), in which rates of 88.2% and 89.5% were recorded with respect to the remaining whole blastocyst.

Given the possibility of embryonic mosaicism, which means that even sequential TE biopsies fail to yield 100% concordant results, the high sensitivity for aneuploidy detection in SCM samples is encouraging. However, the overall accuracy of the SCM diagnoses was compromised by an appreciable incidence of false positive (FP) aneuploidy detection, resulting in a specificity of only 71.7%. The problem was particularly pronounced for Day-4 to Day-5 SCM, where the specificity was just 42.1% - considerably lower than the 84% and 64.8% reported for Day-3 to Day-5 SCM by Xu et al. (2016), and Li et al. (2018), respectively. An important note, however, was that FPs were less prevalent when the culture period was extended by an additional 24-48 hours (to Day-6/7), resulting in an increase of specificity rates to 82.1%. Amplification success rates were also superior when culture was extended to Day-6/7 (100% vs 93.9% for Day-5) as were the overall concordance rates for ploidy and gender (84.0% vs 63.0% for Day-5). The incidence of samples with false negative (FN) results was similar regardless of the length of time that media samples were exposed to embryos (3.7% for Day-4 to Day-5 SCM and 2.5% for samples when culture was extended until Day-6/7).

Interestingly, half of the SCM samples with false positives displayed a complex profile with several aneuploid chromosomes detected. While results of this type correctly indicate the presence of multiple anomalies on some occasions, they can also be caused by inadequate DNA quantity and/or degradation. This led the authors to speculate that the higher rate of FPs observed at Day-5 might be a technical artefact, associated with restricted time in culture and concomitant reduction in the amount of genetic material released. This hypothesis might also explain the lower specificity at Day-5, compared to the aforementioned studies, each of which involved an earlier media change (on Day-3 rather than Day-4) and the use of vitrified-thawed embryos or assisted hatching - factors that could conceivably increase the amount of embryonic DNA in the SCM. However, while the exclusion of chaotic results might improve diagnostic concordance with respect to TE biopsy, it would also mean a significant increase in the proportion of samples failing to provide a result from SCM. If all samples with chaotic chromosome arrangements were omitted from the study of Rubio and colleagues the proportion of samples that could be considered to have yielded a result would fall to 80%. It is intriguing that this is not the first study to note an excess of FPs compared with FNs in SCM. This observation has led to speculation that mosaic embryos, composed of a mixture of normal and aneuploid cells, might preferentially eliminate the chromosomally abnormal cells.

It is also worth noting that Rubio and colleagues, like most previous investigators evaluating niPGT-A methods, considered the results of a single TE biopsy to be a true representation of the cytogenetic status of the remainder of the embryo. While this assumption is likely to be correct in the great majority of cases, the appreciable frequency of chromosomal mosaicism means that a single biopsy specimen may not always reflect the karyotype of the inner cell mass. It will, therefore, be important for future studies to examine embryos donated for research purposes, in order to determine levels of concordance between specimens obtained from SCM, TE and also ICM. Such information may be provided from an ongoing multicenter study, by the same group, in which an ICM biopsy is being performed in addition to the niPGT-A assay (ClinicalTrials.gov Identifier: NCT03520933).

A potentially important aspect of the study by Rubio and colleagues is the fact that pregnancy outcomes were available for a subset of blastocysts ($n=29$), which had been transferred to the uterus after routine TE biopsy and PGT-A had indicated them to be euploid. This provided an opportunity to retrospectively evaluate whether niPGT-A might provide additive information concerning embryo viability, above and beyond that obtained from TE biopsy alone. There was an indication that pregnancy and implantation rates for blastocysts with euploid findings in both TE and SCM samples might be superior in comparison to embryos that had a euploid TE result but an abnormal (discordant) SCM sample (64.7% positive pregnancy test for embryos with euploid TE + euploid SCM versus 33.3% for euploid TE + aneuploid SCM). While the number of samples is much too small to draw any conclusions, it is also interesting that no pregnancy losses were detected for embryos with euploid TE and euploid SCM results (0/9), whereas half (2/4) of pregnancies achieved following the transfer of blastocysts with euploid TE and aneuploid SCM ended in a miscarriage. The possibility that results from the SCM could help further divide embryos with a euploid TE sample into groups of higher or lower developmental potential warrants further investigation. A relevant consideration in this respect is the question of whether the embryonic DNA in the SCM is primarily derived from the ICM or TE, or whether it can equally come from either lineage. This remains to be determined but is likely to have implications for the accuracy and clinical value of SCM analysis.

Although the possibility of using SCM for genetic diagnostics is attractive, several technical challenges still remain to be fully resolved. Not only is it important to optimize DNA amplification in order to obtain results from as many samples as possible, but it is also vitally important that the culture medium used is free of DNA contaminants and that genetic material from other external sources is avoided. In a previous study, Vera-Rodriguez et al. (2018) reported high levels of contamination of a maternal origin (presumably from cumulus cells) and predicted that only ~8% of the genetic material in the SCM was embryonic. In order to mitigate the issue of maternal DNA contamination, several groups have advocated a two-step approach to embryo culture, wherein the embryo is transferred to a new drop of medium, usually on Day-3. Rubio et al. (2019) deployed this tactic, changing the medium on day-4, yet still observed ten instances (9.3% of SCM samples) where the sex of the DNA in the SCM did not match that of the corresponding TE biopsy. In most cases of sex mismatch,

the TE gave a male result while the SCM sample was apparently female. Such discrepancies involving the sex of embryos are extremely rare when comparing PGT-A results, based upon TE biopsy, with the sex of resulting pregnancies/births. The authors speculated that the high incidence of sex discordance was a consequence of maternal DNA contamination. However, in the absence of DNA fingerprinting it is not possible to confirm the exact source of contamination, which could also have been introduced during handling of the embryo or during testing in the genetic laboratory.

Thus far, published studies investigating the possibility of niPGT-A have yielded highly variable results. Although differences in the molecular methods used for analysis may explain part of this variation, it is likely that differences in embryological techniques also contribute significantly. There is no consensus on whether culture systems should be continuous, potentially allowing more time for embryonic DNA to accumulate, or sequential, which may reduce the risk of contamination from cumulus cells. Similarly, culture volumes, types of culture (fresh vs vitrified-warmed) and timing and length of media exposure to the embryo (from Day-3 to Day-5/6 or from Day-4 to Day-5/6/7) have all varied between studies and may each have contributed to the differences in the reported results.

For a PGT protocol to be successful, not only does the genetic test need to be accurate, but it is vitally important that the embryo is not adversely affected as a result of testing. It is questionable whether some of the embryological protocols that have been utilized in niPGT-A studies are practical for embryology laboratories or safe for embryos. Rubio et al. (2019) used a 'continuous' culture system (CSCM, Irvine Scientific), but transferred the embryo to a fresh 10µl droplet of the same medium on Day-4 of culture, following three 'thorough' washes in separate droplets. This represents a substantial deviation from the protocol recommended by the manufacturer (Irvine), which specifies uninterrupted group culture of 2-5 embryos in a much larger volume of medium (50-100µl). Additionally, the washing of embryos prior to transfer into the new drop of medium, could lead to inadvertent damage and inevitably means extending the time that the embryo is outside the optimal environment of the incubator. Rubio and coworkers found the accuracy of niPGT-A to be highest when culture was extended until Day-6/7. While many clinics will consider the culture of embryos to Day-6 (at least for those developing slower than the usual rate), a routine extension to Day-7 might have implications for costs, maintenance of embryo viability and the difficulty of subsequent cryopreservation. The effects of alterations in culture conditions are difficult to predict, but it is conceivable that the developmental potential of embryos could be altered and it would be advisable to avoid implementation in a clinical setting until it has been confirmed that embryo viability is not negatively impacted.

In conclusion, several crucial questions regarding the clinical potential of niPGT-A still remain to be answered. Is the embryonic DNA in the medium derived from the ICM, TE or both? In the case of mosaic embryos, is a disproportionate quantity of the DNA in the SCM derived from aneuploid cells? Do results from SCM samples have the potential to provide information on embryo viability that can be considered additional to that obtained from TE biopsy alone? Can the challenge of DNA contamination be overcome? Will clinics be prepared to adopt niPGT-A if accurate results can only be achieved by altering standard

embryological practice? Are such deviations from routine protocols entirely without risk to embryos? While the study by Rubio and colleagues does not provide a full answer to any of these questions, its findings are generally positive. The paper represents another useful step along the road, which encourages continued efforts to refine, optimize and validate this fascinating, and potentially revolutionary, approach to embryo genetic testing.

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References:

- Li, P., Song, Z., Yao, Y., Huang, T., Mao, R., Huang, J., Ma, Y., Dong, X., Huang, W., Huang, J. and Chen, T., 2018. Preimplantation genetic screening with spent culture medium/Blastocoel fluid for in vitro fertilization. *Scientific reports*, 8(1), p.9275. S
- Stigliani, S., Anserini, P., Venturini, P.L. and Scaruffi, P., 2013. Mitochondrial DNA content in embryo culture medium is significantly associated with human embryo fragmentation. *Human Reproduction*, 28(10), pp.2652-2660.
- Vera-Rodriguez, M., Diez-Juan, A., Jimenez-Almazan, J., Martinez, S., Navarro, R., Peinado, V., Mercader, A., Meseguer, M., Blesa, D., Moreno, I. and Valbuena, D., 2018. Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. *Human Reproduction*, 33(4), pp.745-756.
- Xu, J., Fang, R., Chen, L., Chen, D., Xiao, J. P., Yang, W., & Shi, C. (2016). Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. *Proceedings of the National Academy of Sciences*, 113(42), 11907-11912.