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REVIEW ARTICLE

Current data on the use of cytogenetic techniques in the study of spontaneous abortions

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ABSTRACT
Spontaneous abortion is the spontaneous loss of the product of conception before its viability, that is before the 22nd week of amenorrhea or a fetal weight of less than 500 gr. Several etiologies can cause loss, but the genetic cause alone is responsible for more than half of pregnancy failures. Diagnosis of a chromosomal abnormality as a cause of pregnancy loss provides important information for recurrence-risk, and give valuable information for genetic counselling, identify new genes involved in the pathology of embryonic development and reproductive planning. Traditionally, karyotyping has been used for genetic testing of perinatal losses, however, this technique is labour-intensive and requires actively dividing live cells and led to several challenges. Considerable efforts have been made to develop robust molecular biology techniques that do not require cultivation prior to analysis and are amenable to automation, thus enabling results to be achieved more quickly and at a lower cost. Each technique has its strengths but also limitations The different cytogenetic techniques of standard karyotyping and molecular biology must be combined and used in a complementary way to optimize the results and provide a reliable explanation for pregnancy failure.
Key words: Spontaneous, Abortion, Aneuploidies, Cytogenectis, Molecular, Techniques.

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INTRODUCTION

Spontaneous abortion (SA) is the spontaneous loss of the product of conception (POC) before its viability; this involves all pregnancy losses occurring between conception and the 22nd week of amenorrhoea [1]. Stillbirth is defined as fetal loss after 22 weeks of gestation and occurs in approximately three to six of every 1000 pregnancies in developed countries [2,3]. It is estimated that 10-15% of newborn pregnancies end in a miscarriage [1], but the exact frequency of these losses is unknown and underestimated because many POC are eliminated before implantation or before women realize their pregnancy. The overall prevalence of pregnancy losses is generally assumed to be 4–5 times higher [1]. In general, a miscarriage does not compromise the possibility of subsequent pregnancy, however, and in about 10% of cases, this is repeated, we are then talking about repeated spontaneous abortion, defined by the occurrence of at least three spontaneous abortions. The definition may vary but starts when at least two or more miscarriages have occurred [4]. The causes of spontaneous abortions are numerous and diverse, but the genetic cause alone explains 45–70% of sporadic miscarriages and in around 25–57% of recurrent cases [5-8]. The frequency of genetic abnormalities depends on the developmental stage of pregnancy, maternal age and the number of previous miscarriages [9]. Most chromosomal abnormalities in pregnancy loss are aneuploidies and have been detected traditionally through standard cytogenetic analysis [10,11]. Diagnosis of a chromosomal abnormality as a cause of pregnancy loss provides important information for recurrence-risk counseling and helps identify familial chromosomal rearrangements that may predispose couples to recurrent losses or to birth of children with congenital abnormalities and/or intellectual disability [12]. Thus, cytogenetic analysis of spontaneous miscarriages is essential to establish the etiology of fetal losses and to assess patients with risks of recurrence in future

pregnancies [13]. Traditionally, karyotyping has been used for genetic testing of perinatal losses [14], involved culture of chorionic villi or fetal tissue, followed by G-banded chromosome analysis. Standard G-band karyotyping has classically been used to detect chromosomal anomalies at a resolution of 5–10 Mb [15]. However, this technique is labour-intensive and requires actively dividing live cells and led to several challenges, such as culture failure from nonviable pregnancies, poor quality sample, maternal cell contamination (MCC), as well as microbial contamination resulting in culture failure [14]. Different strategies have been used in order to improve the finding of chromosomal abnormalities, trying to circumvent the limits of the cytogenetic analyses [16-18]. Techniques such as Chromosomal Comparative Genomic Hybridization (CGH), array–Comparative Genomic Hybridization (array-CGH), Fluorescence in situ hybridization (FISH), Multiplex Ligation-dependent Probe Amplification (MLPA) and Quantitative Fluorescent Polymerase Chain reaction (QF-PCR) and more recently Bacc On Beads ™ (BoBs ™) have overcome some disadvantages inherent to conventional cytogenetic techniques. Apart from various methodological limitations, the effectiveness of genetic analysis depends on the quality of the tested tissue, and the majority of inconclusive or discordant results are caused by MCC and DNA degradation resulting from improper pre-laboratory material collection or storing processes [19,20]. Until now little is known about the contribution of the newer techniques to resolving the clinical problem [21]. In this review, we will present what is currently known about the use of different conventional and molecular cytogenetics techniques in the analysis of spontaneous abortion products. We will discuss the clinical implications of these genetic tests, the advantages and disadvantages of each technique by paying attention to the capacity of the specific tests.

Karyotype

Since the introduction of standard caryotype in the cytogenetic study of misarriage more than 30 years ago, it has remained the selection technique of most cytogenetic laboratories [22]. Indeed, cytogenetic studies carried out since the 80s are mostly based on the analysis of metaphasic chromosomes in GTG bands obtained from chorial villus cultures or fetal mesenchymal cells [23]. Conventional karyotyping is defined as the morphological characterization of the chromosomal complement of an individual including number, form and size of the chromosomes. It can detect abnormalities throughout the entire genome and is therefore used as the standard for detecting chromosome abnormalities in miscarriages samples [21]. Despite the emergence of molecular cytogenetic techniques, standard caryotype remains the first-line examination in genetic testing of spontaneous abortion products. Recent studies have again demonstrated its reliability and usefulness in detecting aneuploidies most frequently associated with chromosomally abnormal POCs.Soler et *al*., in the largest Spanish series of cytogenetic analysis of first trimester spontaneous miscarriages [13], achieved a 90.3% pass rate on chromosome analysis, which is higher than signal rates in other major studies that performed cytogenetic analyses on miscarriages. The reason for this high success rate is that chorionic villi (CV) samples were obtained before evacuation and were processed within a few hours, thus minimizing microbiological contamination and allowing a high success in short-term culture karyotype achievement [13]. The main point is that conventional cytogenetics is not a cost-efficient and low throughput study [17]. The fact remains that despite the limitations of the standard karyotype, namely failure of culture, maternal contamination, the low resolution and the very high cost of the technique given the impossibility of its automation and the necessity to have a highly qualified staff. However, cytogenetic analysis (CA) is still the gold standard in the detection of chromosome aberrations in spontaneous miscarriages until further work is done before the absolute detection rate can be answered with newer techniques [25]. Indeed, Van den Berg and coworkers [21] reported that more chromosome aberrations were detected by CA compared to FISH or MLPA or QF-PCR [25].

Comparative Genomic Hybridization array (CGH a)

The comparative genomic DNA microarray hybridization (CGHa) technique has changed the diagnostic approach to chromosomal abnormalities. Historically and technically, CGHa derives from the comparative genomic hybridization known as metaphase (CGHm) described by Kallioniemi et *al.* [27]. The genomic DNA of an individual to be studied (test DNA) and that of a control individual (reference DNA) are marked with different fluorochromes emitting in two dissociable wavelengths, respectively. These two DNA are cohybridated in equivalent amounts on targets. Initially, according to the classic CGH technique, the targets were normal metaphasic chromosomes. The resolution of the technique has improved considerably with the Chromosomal microarray analysis (CMA) referring to molecular karyotyping using different array platforms such as bacterial artificial chromosome (BAC) arrays, oligonucleotide arrays, or singlenucleotide polymorphism (SNP) arrays [28,29] , whose targets are made up of genomic DNA fragments deposited in the form of microarrays on a support (glass blade or miniaturised support). Although described several years ago, it is only now that the use of CMA is being introduced effectively

into clinical practice, allowing far better resolution (10–100 kb) than the 10Mb that is achievable using conventional karyotyping. Another advantage is that CMA does not require cells to be in metaphase for analysis, resulting in a higher test success rate given that cell culture is unnecessary so culture failure is avoided and maternal cell and bacterial contamination minimized [30]. It can be performed on DNA extracted from direct fetal samples and does not require live cells. However, this technology has also revealed a new dimension of the complex nature of the human genome [31, 32]. Scattered regions within the genome show a variation in the number of copies (CNV) leading to microduplications and microdeletions. Several studies in control populations have considered some of these variations as relatively frequent polymorphisms. The correlations between genotype and phenotype for some of these variations are still very uncertain, leading to any diagnostic interpretation. Recent observations have also demonstrated the variability in clinical expression of some inherited micro-redesigns of a parent whose phenotype is mitigated, incomplete penetration with the parent with the same genomic imbalance having no associated clinical signs [33,34]. Large, clinically well-described cohort studies will be needed to discriminate a pathogenic CNV from a neutral variant. This current uncertainty of interpretation must therefore be taken into account in the diagnostic use of the CGH array. The interpretation and deferral of CNV detected by aCGH in miscarriage samples are complex; given the potential impact on family testing and future pregnancies; and best practice has yet to be determined [15]. Chromosomal analysis on microarrays of DNA offers many advantages over caryotype in the study of pregnancy losses at all gestational stages with a higher detection rate; which is a major factor in the Amerian College of Obstertiians and Gyneologists (ACOG) recommendation for using the test in stillbirths [2]. Study of Rosenfeld et al. (2015) showed higher overall detection rates in miscarriages than in stillbirths [12]; this led them to advocate the extension of ACOG recommendations to miscarriages. In the same study, the success rate of CGH was also higher than caryotype (comparison caryotype 2015) since this technique could be performed on at least 21 cases that failed caryotype. Reddy et al. [35] and Rosenfeld et al. [20] showed a superior yield of CGHa compared to caryotype of 2 and 5%, respectively. However, tests on chromosomal microarrays cannot detect all abnormal caryotypes. Indeed, Chromosomal microarrays cannot detect low-level mosaicism, although the threshold varies according to the type of array and size of the abnormality [36]. For marker chromosomes, microarrays will give normal results if the marker does not contain euchromatic material; which may be useful since heterochromatic markers are unlikely to cause phenotypic abnormalities [37]. Finally, the CGHa, carried out alone, cannot highlight the female triploidies [12]. Another major problem of cytogenetic analysis, the increased risk of fetal-maternal contamination even when procedures are scrupulously followed. Studies recommend that CCM tests be carried out in a standard manner with microarray analysis [31, 36, 38]. In conclusion, the usefulness demonstrated by numerous studies of chromosomal microarray tests for cytogenetic diagnosis makes it a method of choice for determining the genetic causes of pregnancy loss. It's an analysis capable of detecting any cytogenetic abnormalities that can be detected by caryotype, in addition to identifying other clinically significant abnormalities not visible by standard techniques with a higher rate of results. Thus, chromosomal microarray testing is a preferable, robust method of analyzing cases of pregnancy loss to better delineate possible genetic etiologies, regardless of gestational age [12].

Quantitative Fluorescent Polymerase Chain Reaction (QFPCR)

QF-PCR is a relatively new method that can be used to determine the number of copies of a DNA sequence [39,40]. QF-PCR is a multiplex PCR coupled with capillary electrophoresis. From DNA extracted from chorial villi or amniocytes that have not been cultured, QF-PCR amplifies the polymorphic DNA markers specific to the targeted chromosomes (13, 18, 21 and sometimes X and Y). QFPCR has many advantages. Undoubtedly, the main advantages of this technique are that the results are obtained quickly and require only a small amount of amniotic fluid and no cell culture. Therefore, QF PCR is more cost-effective than caryotyping [41]. In addition, QF PCR is able to detect >90% of clinically significant chromosomal abnormalities [42-44]. Numerous studies have shown that QF-PCR is significantly more robust than aCGH, MLPA or karyotype analyses; failure rates are 0.4%, 1.3%, 5% [17] and 30% [45] respectively. The discovery of chromosomal imbalance can be used to predict the risk of recurrence of miscarriage and fetal abnormality, as well as to provide the reasons for pregnancy loss, thereby reducing investigations [15]. QF-PCR genotyping also identifies and quantifies MCC; an important quality control. Other published cohorts found clinically significant sub-microscopic imbalance in 0.6% [46], 0.8% [47], 1.6% [48] of the samples, although the Levy study [48] included CNVs and CNVs of unknown importance in his study. Relatively little is known about the genes and pathways involved in miscarriages; many identified CNVs will therefore be classified as of unknown importance and even those containing genes involved in fetal development will not be of diagnostic or clinical use without further evidence; and studies [49]. The value

of QF PCR as a screening test is controversial, but can be used to confirm the diagnosis of common fetal aneuploidies [42,50,51].

Bacs-on-Beads™ (or Bobs)

A new technology has been developed to overcome this limitation of cytogenetic diagnosis: comparable to in situ fluorescence hybridization (FISH) in liquid suspension. The analysis uses immobilization of DNA probes generated from BAC (Bacterial Artificial Chromosome) artificial bacterial chromosomes, selected and amplified by PCR, on fluorescence-coded beads. It can simultaneously test multiple chromosomal abnormalities from minute amounts of DNA sample, and provide results in less than 24 hours [52,53]. This technique can analyse more than 40 samples simultaneously with high-throughput diagnostic capability The molecular caryotype targets DNA gains and losses in relevant genomic regions, namely aneuploidal chromosomes 13, 18, 21, X and Y, as well as changes in the number of copies of DNA in 9 regions of microredesign: Digeorge, Williams-Beuren, Prader-Willi, d'Angelman, Smith- Magenis, Wolf-Hirschhorn, Langer-Giedion, Miller-Dieker, and the cat's cry. By focusing on regions related to causes of serious constitutional diseases, this DNA analysis kit provides more decision-making information than is obtained from other commonly used methods, however, avoiding meaningful responses unknown [54]. Pioneering work with this technology [52-55] quickly demonstrated its robustness and effectiveness. The sensitivity of the Bobs™ on a sample of 2153 POC analyzed by Choy et *al*. was 96.7% (95% CI 92.6–98.7%), while the specificity was (95% CI 99.8–100%), Bobs' results were higher than those of the QFPCR in detecting major structural anomalies (53.3 versus 1 3.3%) and mosaicisms 28.6 versus 0%). The technique was also able to identify six microdeletion syndromes and two cases of microduplications not detected by caryotype and QFPCR [56].

Mellali et *al*. (2015) found a microdeletion of 17p13.3 (2.3 MB) that could not be detected by karyotyping due to its low resolution [57]. Hunag et *al.* (2018) demonstrated that the Bobs™ test success rate in identifying chromosomal aneuploidies was higher than caryotyping (100% vs. 94.88) as they were able to detect seven other cases of anomalies not detected by conventional caryotyping [58]. For this reason, many authors believe that Bob's test could offer additional diagnostic benefits in comparison with karyotyping because it provides greater diversity and sensitivity to detect microdeletions and microduplications [58-61].

Where Bobs™ analysis loses to QF-PCR is the detection of polyploid [62] and maternal contamination [56]. Indeed, a key limitation of the Bobs™ technique is that it does not detect triploids, tetraploids, or structural abnormalities, which together account for approximately 10% of the total etiology of POCs. A report in the literature has demonstrated the ability of this technique to detect male prenatal triploids [55]. Vialard et al. 12 identified six cases of false negatives, including five triploids and one case of 48,XXXY which were recognized only by karyotyping. In the study by Shaffer et al. [54] , a normal male contaminated with maternal cells was incorrectly identified as a sample of 69,XXY. False positives were also noted by Vialard et *al.* [55], who reported three false positives out of 1,653 cases (0.18%) [56]. The BoBs™ assay may not have a significant edge over CMA as a rapid diagnostic test. On the other hand, CMA is expensive and may not be affordable in many countries. The interpretation and counselling arising from the detection by CMA of copy-number variants of uncertain significance (VOUS) continue to be important clinical problems. Therefore, the BoBs™ assay would remain the preferred complementary test for countries that still rely on karyotyping [56]. The BoBs™ assay is superior to QF-PCR or other RATs as a triaging test for prenatal diagnosis because of its high specificity, yet it has a similar cost and turnaround time to current RATs. BaC-on-Beads™ can replace QF-PCR for triage in prenatal diagnosis, and gives better diagnostic performance than current rapid aneuploidy tests [56]. It is cheaper than the CMA method and without the worry of creating the dilemma of finding variants of unknown significance. Demonstrating this is a good and cost-efficient strategy that could be used in countries with low economical resources [63].

CONCLUSION

Compared to caryotype, the various molecular techniques were shown to detect between 3 and 13 additional pathogenic abnormalities and Nvs in SA products diagnosed as normal by conventional techniques [47, 64]. However, despite high potential to improve genetic analysis and develop new knowledge of genetic causes of failure of early human development, microarray studies of miscarriages are still not widely used due to high cost, difficulties of CNV interpretation, inability to detect balanced rearrangements and limited ability for ploidy change detection of some microarray platforms [65]. Several professional societies (American College of Medical Genetics [ACMG], American College of Obstetricians and Gynecologists [ACOG], Canadian College of Medical Genetics, and Italian Society of Human Genetics) do not encourage replacing prenatal karyotyping with CMA but recommend it as an

adjunct test in specific cases only [2,66]. The different cytogenetic techniques of standard karyotyping and molecular biology must be combined and used in a complementary way to optimize the results and provide a reliable explanation for pregnancy failure.

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