Preimplantation genetic diagnosis: new reproductive options for carriers of haemophilia

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Summary. Preimplantation genetic diagnosis for haemophilia offers couples at risk for transmitting the condition the opportunity to embark on a pregnancy knowing that the embryo is unaffected by the disease. The technique aims to increase the range of reproductive options available to these couples and remove the need for invasive prenatal diagnosis and the difficult decision on whether to terminate an affected pregnancy. This aims to reduce the anxiety associated with reproduction often seen in these couples. Patients undergo a cycle of *in vitro* fertilization followed by embryo biopsy. The single blastomeres are then analysed using fluorescent *in situ* hybridization to detect the sex of the embryo, and only female embryos are transferred to the uterus. Recently a PCR based approach has allowed specific mutation detection, and therefore the transfer of unaffected male and female embryos.

Keywords: haemophilia, preimplantation genetic diagnosis, reproduction, specific reproduction

Introduction

Preimplantation genetic diagnosis (PGD) is a form of very early prenatal diagnosis. The technique combines assisted reproductive technology with molecular genetics and cytogenetics to allow the identification of abnormalities in embryos prior to implantation. It was originally introduced with the aim of extending the range of choices available to couples at risk of having children with severe genetic disease such as haemophilia, thereby reducing the anxiety these couples associate with reproduction. Prior to the introduction of PGD carriers of genetic disorders had limited reproductive options: remain childless, adopt, gamete donation, or play 'reproductive roulette' with spontaneous conception [1]. Conventional prenatal diagnosis of haemophilia involves sampling cells of fetal origin by amniocentesis in the second trimester of pregnancy, or chorionic villus sampling in the first trimester. This is then followed by the detection of the genetic defect by DNA analysis. Couples at risk of having affected children face the risks associated with these diagnostic procedures and the difficult decision of whether to continue with an affected pregnancy. Some couples repeatedly terminate pregnancies in an attempt to have a normal child [2,3]. As an alternative to conventional prenatal diagnosis, PGD has the advantage that by selecting only those embryos identified as unaffected for replacement in the uterus, couples know from the beginning that any pregnancy should be unaffected by the genetic condition carried in that family, eliminating the need for prenatal diagnosis and termination of pregnancy.

The field is still very much in its infancy, the successful biopsy of human preimplantation embryos and their genetic analysis was first reported in 1989 [4]. The concept was first discussed in 1967 after experiments at sexing rabbit embryos at the blastocyst stage had proved successful [5,6]. Work in our own laboratory had shown that biopsy and removal of one or two cells at the 8-cell stage of the human preimplantation embryo does reduce cellular mass, but does not affect development of these embryos to the blastocyst stage [7]. This preliminary work paved the way for the first trials of preimplantation genetic diagnosis in the human and the first pregnancies [8].

PGD treatment cycle

Patients are referred for PGD of haemophilia from a variety of sources including general practitioners,

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assisted conception units, and regional genetics centres, an increasing number are self-referrals. At the initial consultation a detailed medical history is taken and a genetic pedigree constructed. Of particular note is the complicated and often tragic previous obstetric and family histories. The first 16 couples to achieve a pregnancy from our programme had between them 10 miscarriages, 25 terminations of pregnancy and eight affected children; three of the women had been sterilized before the age of 30. It is a requirement of the programme that all couples have received appropriate genetic evaluation and counselling before embarking on treatment. Couples are given information about the *in vitro* fertilization (IVF) cycle, embryo biopsy, diagnostic procedures and current success rates. The possible failure of the cycle such as non-fertilization or failure of the genetic analysis are discussed; complications such as the ovarian hyperstimulation syndrome and misdiagnosis are clearly described. The couple are then given some time to consider matters and are invited to make use of the counselling service. A detailed information booklet is given out. Couples who wish to proceed are then booked to have a transvaginal ultrasound examination, semen-analysis, cervical assessment, hormone analysis, and if appropriate evaluation of the uterine cavity by HSG (hysterosalpingography).

The female partner must undergo a treatment cycle of IVF similar to the approach used for assisted conception in couples with infertility. Initially her pituitary gland is down-regulated with a GnRH (gonadotrophin-releasing hormone) antagonist, this is usually commenced on the second day of her menstrual period and continued for approximately 14 days. During this time, the suppression of pituitary FSH (follicle stimulating hormone) and LH (luteinizing hormone) production produces a profoundly hypo-oestrogenic state and the patient may experience menopausal like side-effects such as hot flushes and night sweats. Following confirmation of pituitary suppression by hormone analysis and pelvic ultrasound, the patient commences controlled ovarian stimulation using daily injections of human recombinant FSH. A cohort of ovarian follicles are recruited and matured under the influence of recombinant FSH administration. This normally takes 12–14 days, with the patient having regular monitoring of ovarian follicular number and diameter with serial pelvic ultrasounds and serum oestradiol measurements. When a minimum of three follicles reach a diameter of 17 mm a trigger of hCG (human chorionic gonadotrophin) is injected. This mimics the physiological LH surge and results in final

maturation of the oocytes. Approximately 36 h later the patient has her oocytes surgically collected by a transvaginal procedure under ultrasound control and local anaesthetic with sedation. The male partner produces a semen sample by masturbation. Sperm are then injected into the oocytes using a procedure called ICSI (intracytoplasmic sperm injection) or inseminated around the oocyte in conventional IVF. On average between 50 and 70% of the oocytes will fertilize normally. These embryos are incubated in culture media.

Embryo biopsy

Embryo biopsy of cleavage stage embryos is performed on the morning of day 3 when embryos have between six and 10 cells [9]. The embryo is held by suction on a holding pipette with the blastomere to be biopsied in approximately the 9 o'clock position. A hole is made in the zona pellucida using a fine stream of acid Tyrodes solution directed onto the zona using a fine pipette. The embryo is then moved into another area of the drop to limit its exposure to the acid. A wider pipette is then used to remove one or two blastomeres from the embryo using gentle suction. Ideally blastomeres should be removed intact; lysed blastomeres are used if the nucleus is not lost but these cells are associated with a higher incidence of amplification failure [10]. Biopsied embryos are then washed and are returned to culture in the incubator. Blastomeres are then spread for fluorescent *in situ* hybridization (FISH) or tubed for PCR (polymerase chain reaction) analysis.

The main advantages of cleavage stage biopsy are that biopsy at this stage does not appear to adversely affect development to blastocyst stage [7], and a large series of live births have been reported [11]. Additionally this practice is easy to fit into the general running of an IVF programme where transfers are routinely performed on day 3. The disadvantages of cleavage stage biopsy include the small template of DNA (single cell) available for analysis. The impact of mosaicism on the accuracy of diagnosis at the cleavage stage has still to be fully evaluated. Ethical objections may be raised to the removal of a cell that may have contributed to the development of the fetus. Currently blastomere biopsy (usually of two cells) is the method of choice in the majority of centres around the world [12].

Single cell genetic analysis

Various scientific investigations have been employed to analyse the biopsied blastomeres. Originally for

PGD of haemophilia, the sex of the embryo was determined as it was not possible to diagnose a specific mutation for haemophilia. This was follwed by the transfer of only female embryos. Although this prevented the birth of affected children it resulted in all of the male embryos being discarded, including the 50% that were normal. The fact that 50% of the female embryos that were transferred were haemophilia carriers was also not considered ideal by many couples. Initially a PCR protocol was used to detect the sex of the embryos in a variety of X-linked disease including haemophilia [4]. A Y-linked repetitive sequence was amplified by the polymerase chain reaction: a female being diagnosed if the expected fragment was not detected. The coamplification of sequences from both the X and Y chromosomes was later used to improve accuracy [13], but both sequences could fail independently resulting in a possible misdiagnosis.

This lead to an entirely different approach to use *in situ* hybridization with X and Y chromosome specific probes to metaphase or interphase nuclei spread on microscope slides. Early attempts with biotin or radiolabelled probes were promising but detection of hybridization by autoradiography took several days, and neither method was sufficiently reliable at the single cell level with the X or Y probe alone [14]. FISH is a technique for enumerating chromosomes and is particularly useful where interpretable metaphase chromosomes are difficult to prepare such as in preimplantation human blastomeres [15]. FISH uses fluorescently labelled DNA probes that bind to complementary sequences on specific chromosomes and can then be detected under a fluorescent microscope. Using directly labelled probes is both rapid and sensitive and has the major advantage that combinations of probes can be detected by multicolour fluorescence [16]. A dual FISH method for simultaneous detection of X- and Y-chromosomes was developed and a number of pregnancies and births achieved [17]. Protocols using these probes that bind to repeat sequences have been designed to take advantage of their short hybridization time allowing the whole FISH procedure to be performed within 2 h [18]. The vast majority of PGD cycles performed worldwide for haemophilia now utilize this FISH approach to sex the embryos.

Recently work in our own hospital has lead to the development of protocols for the specific diagnosis of haemophilia in human preimplantation embryos (K. Michaelides and E. Tuddenham, pers. comm.) using a restriction enzyme approach for the diagnosis of point mutations. Diagnosis is then confirmed using a fluorescent sequencing strategy. This has several advantages: more embryos are available for transfer (75% of the total embryo pool as opposed to 50% when using sexing) and therefore the likelihood of better quality embryos being transferred leading to improvements in implantation and pregnancy rates; patients have the opportunity of having unaffected sons.

Data on referral practice, cycle details and pregnancy outcome is collected by two groups, the International Working Group on preimplantation genetics [19], and the ESHRE (European Society of Human Reproduction and Embryology) PGD Consortium [20]. Over 3000 cycles have been reported for PGD for a variety of indications with approximately 1000 children born. Pregnancy rates following PGD for haemophilia in our unit are 24%, which is consistent with the results reported by most centres.

Sperm sorting

An alternative approach to avoid a male pregnancy is to select X-carrying sperm for use in intrauterine insemination procedures. Sperm can be differentiated into X-carrying or Y-carrying by the addition of a fluorescent dye to the sperm nucleus and then passing the sample through a modified flow cytometer. Based on work in three species of animals through five generations, a clinical trial is now underway in humans. It is important to understand that this technology does not guarantee a female pregnancy but aims to 'enrich the proportion of X-carrying sperm and therefore increase the chance of a female child. The average enrichment in a sorted sample is to 88% X-carrying sperm. Although the accuracy is significantly compromised, an insemination of sorted sperm is far less invasive and avoids surgical egg collection, costly gonadotrophin drugs, intensive monitoring and may be an acceptable compromise for some couples. Pregnancy rates are approximately 16% per cycle of insemination [21]. It should be stressed that this approach is still experimental and only available at one or two centres in the USA.

The demand for PGD

Although the demand for PGD for haemophilia remains unknown, a few studies have considered the attitudes of potential users in women awaiting prenatal diagnosis for other genetic diseases such as thalassaemia [22]. Pergament [23] observed that 50% of women at high risk of having a child with genetic disease considered PGD to be a viable

alternative to prenatal diagnosis. These studies have shown that the acceptability of PGD is highest in women who have undergone termination of an affected pregnancy [24]. Patient acceptability is one crucial issue that may ultimately determine the value of PGD as an alternative to prenatal diagnosis in high-risk couples. We analysed the responses of 67 couples who had undergone PGD in our unit to evaluate their experiences and motivation [3]. One third had an affected child, over half had previous experience of conventional prenatal diagnosis (either amniocentesis or chorionic villus sampling) and over one third had had terminations of pregnancy because of a genetic risk. The responses suggested that the experience of prenatal diagnosis and termination of pregnancy is an unwelcome memory and this leads to a demand for an alternative approach. The main perceived advantage to couples of PGD was that only unaffected embryos are transferred to the uterus and thus therapeutic termination of pregnancy can be avoided. Couples at risk of transmitting genetic disease to their children frequently experience stress, depression and anxiety [25,26]. The psychological impact of termination of pregnancy is stressful and traumatic [27,28] and the grief experienced following termination for abnormality can be similar to that following neonatal death [29]. PGD is an alternative that may be more acceptable but, of course, undergoing IVF itself is associated with stress and anxiety [30,31]. These couples are therefore uniquely vulnerable. PGD is not an easy solution for high-risk genetic couples [32]. Responses from couples who had experienced both PGD and conventional prenatal diagnosis showed of those couples who contemplated a further pregnancy 76% felt they would choose PGD, whilst 16% would opt for prenatal diagnosis, and 8% no tests at all.

Safety concerns

The safety of the procedure relating to the outcome of pregnancies and children born is perhaps of most importance. Three safety issues need to be considered: the potential harm of biopsy to the viability and development of the human embryo; the risks of multiple pregnancy associated with assisted conception; and the chance of misdiagnosis. Hardy and colleagues had shown that removal of one or two cells at the 8-cell stage, while reducing the cellular mass, does not adversely affect the preimplantation development of biopsied embryos *in vitro* and suggested that this approach could be used for preimplantation diagnosis of genetic defects [7]. Initial data from the first 16 pregnancies following

PGD (12 singletons, four twins) showed a normal obstetric outcome [33]. Biochemical analysis and ultrasound assessment of the early stages of PGD pregnancies has shown that pregnancies resulting from biopsied embryos behave similarly to control IVF pregnancies, however, the reduction in cell mass following embryo biopsy occasionally results in reduced levels of circulating serum hCG in early pregnancy [33]. The safety of PGD has been the main motivator behind the European Society of Human Reproduction and Embryology (ESHRE) collecting data on treatment cycles. In the 2000 report 163 pregnancies were reported with 138 progressing to the second trimester [11]. Of the 123 deliveries 31% were multiples (37 sets of twins, 1 set of triplets). Complications before and after birth were similar to a comparable ICSI population as were parameters such as birthweight and length [34]. Data on 109 infants from a single-centre programme using polar body biopsy has also been presented [35]. Information on gestational age, mode of delivery, perinatal mortality, birth weight and length, the presence of birth defects and developmental milestones was collected. There was no significant decrease in birth length or weight, or the frequency of small for gestational age infants. Six infants did have birth defects, two were major and four minor: amniotic band syndrome, neonatal seizures, haemangioma, strawberry haemangioma, thickened tricuspid valve and webbed toes. No specific pattern of birth defects was observed. Although clearly the numbers are still small and no statistical conclusions can be made, the initial evidence that no observable detrimental effects of PGD on children born after the procedure is reassuring.

There is an intrinsic risk associated with PGD and assisted conception of multiple pregnancy. In the Chicago series there was a 9% incidence of twins, a 7% incidence of triplets and one case of five fetuses that spontaneously miscarried [35]. In our own programme we very rarely transfer three embryos as we consider the hazard of a triplet pregnancy in generally young fertile women who may already be caring for an affected child to be significant [36].

Since the first clinical applications of PGD there have been sporadic reports of misdiagnosis. These have been more common in the early years of development and are fortunately rare occurrences [37]. Problems leading to misdiagnosis may be intrinsic to the human embryo (mosaicism of blastomeres, anucleate blastomeres) or extrinsic (allele drop out, contamination or nonamplification). Misdiagnoses have occurred in most of the major international centres. The first case report was from

the original series of patients who underwent sexing for X-linked disease using PCR for a Y-linked sequence. Failure of amplification lead to the embryo being misdiagnosed as female and transferred to the uterus [8]. The error was detected by conventional prenatal diagnosis. Confirmatory prenatal diagnosis is still recommended in our own programme as we consider PGD to still be somewhat experimental.

Ethical challenges

PGD has become a frequent target for ethical commentary and speculation [38,39]. There have been several ethical concerns about PGD, these include: the discarding or destruction of affected embryos (the seriousness of the inherited disease may have an influence over this concern); the use of embryo sexing for X-linked disease where the specific mutation cannot be identified results in the exclusion and destruction of normal male embryos such as in haemophilia; and the anxiety over the potential abuse of the technology for non-medical reasons. There is a deontological argument that it is wrong to select the traits of offspring no matter how well intentioned. Dr Leon Kass Chairman of the Presidents Bioethics Council in the USA makes the case that human reproduction is a gift that any form of selection or manipulation turns the child into a manufacture or commodity. The first report of the ESHRE task force on ethics and law reported on the moral status of the human preimplantation embryo stating that the embryo is owed respect as a symbol of future human life [40].

Supporters of PGD need to ensure that the ethical domains of beneficence, non-maleficence, autonomy and justice are all satisfied. Beneficence from a medical ethical perspective includes curing disease, improving function, relieving pain and preventing disease. This has been the fundamental ethical justification for PGD: that the welfare of the child is improved by avoiding harm due to genetic disease [41]. There is however, a potential for malficience. Subjecting the early human preimplantation embryo to the biopsy procedure and the removal of up to 12.5% of its entire genetic mass may involve a degree of risk. So far the outcome data on pregnancy and congenital malformations is reassuring [20]. The number of live births is still small and many in the field consider PGD still experimental. PGD would still be considered ethically acceptable if the chance of benefit were felt to outweigh the risk for harm. It is a much more difficult argument to consider whether the embryos that have been discarded have been 'harmed'. This will ultimately be decided on whether one holds deontological beliefs or utilitarian ones. From a parental point of view PGD places a high value on autonomy and the respect for procreative liberty. It also aims to reduce the physical and psychological trauma associated with repeated terminations of pregnancy [42,43].

There has been a vigorous debate about the eugenic potential of PGD due to its ability or indeed purpose of embryo selection and a consequentialist 'slippery slope' argument has developed. It could be said that the intent of prenatal diagnosis and PGD is eugenic in that it aims to reduce number of people with genetic disorders. Indeed PGD has the ability to arouse greater disquiet and concern than its predecessors because of its perceived acceptability [44]. The attempt to seize the moral high ground by applying the term eugenics is somewhat emotive, others would merely call PGD the parental choice used to reduce disease in their offspring. Importantly, PGD involves no coercion and places a premium individual freedom of choice and parental autonomy. It therefore cannot be compared previous state eugenic programmes that removed individual freedom in a societal master plan to improve the human race [44]. Practicalities should also not be forgotten, the low delivery rates associated with PGD mean as eugenic tool it would be somewhat ineffectual.

Conclusion

There is now an increasing body of evidence to support the efficacy and safety of PGD as a valuable and acceptable alternative reproductive option for patients who carry haemophilia. There remains an understandable and genuine public concern about the use of PGD. Over the last 13 years the original indications for PGD such as screening for severe mendelian disorders like haemophilia have become legitimized through national regulatory guidelines and public policy. Welfare of the child legislation may help reinforce this feeling of public reassurance. Simultaneously the ethical arguments supporting this technology have become more broadly accepted. Opponents of PGD point to the possibility of undoing the struggle to tolerate disability and diversity leading to discrimination in areas of marriageability or insurability and an undermining of compassion for the sick or disabled who did not have PGD [45]. At the same time it is the parents who are most likely to educate, socialize and share religious and cultural heritage with children. Only a limited consensus concerning appropriate boundaries between parental/patient desires and community or social interests exists.

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