Six unaffected livebirths following preimplantation diagnosis for spinal muscular atrophy

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Spinal muscular atrophy (SMA) is a severe neurodegenerative autosomal recessive disorder, second only in frequency to cystic fibrosis. In its most severe form, SMA type I (Werdnig–Hoffman), death invariably ensues before age 2 years from respiratory failure or infection. Around 98% of clinical cases of SMA are caused by the homozygous absence of a region of exons 7 and 8 of the telomeric copy of the SMN gene (SMN1) on chromosome 5. We have developed a novel means of preimplantation diagnosis of SMA using a nested polymerase chain reaction (PCR) amplification of exon 7 of SMN, followed by a HinfI restriction digest of the PCR product enabling the important SMN1 gene to be distinguished from the centromeric SMN2 gene which has no clinical phenotype. This method was designed to reduce the likelihood of misdiagnosis. Five couples were treated using this method. Four proceeded to embryo transfer which resulted in six liveborns (one singleton, one twin and one triplet), all free of SMA. Embryo transfer was not performed in one cycle because of PCR contamination.

Key words: preimplantation genetic diagnosis/SMN1/spinal muscular atrophy

Introduction

Spinal muscular atrophy (SMA) is a neurodegenerative disorder, being the second most common lethal autosomal recessive disease in Caucasians, after cystic fibrosis. It has an incidence of 1 in 6000 livebirths and a carrier frequency of 1 in 40. The disorder is characterized by degeneration of the anterior horn cells of the spinal cord, leading to symmetrical muscle weakness and wasting of voluntary muscle. The clinical diagnosis is based upon a clinical picture of proximal and symmetrical weakness with wasting of voluntary muscle. The clinical diagnosis is based upon a clinical picture of proximal and symmetrical weakness with muscular atrophy. It is a heterogeneous group of diseases with a clinical classification dependent upon the age of onset, milestones in development, and the age of survival. There are three recognized types (I–III), of which type I [Werdnig–Hoffman Disease, online Mendelian Inheritance in Man (OMIM) OMIM #253300] is the most severe form. It can be recognized at, or even prior to, birth, and is usually diagnosed within the first 6 months of life. The children are hypotonic and are unable to sit unaided. Respiratory difficulty develops rapidly, and this is responsible for the child’s demise, usually before the age of 18 months.

All three types of SMA have been mapped by linkage analysis to 5q13.3 (Brzustowicz et al., 1990). The genomic region encompassing the disease gene is particularly unstable and is prone to large-scale deletions (Burlet et al., 1996). SMA appears to involve at least four genes, each of which is present in a telomeric (t) and a centromeric (c) copy: SMN, survival motor neurone gene—telomeric SMN1 (SMNt) and centromeric SMN2 (SMNc); neuronal apoptosis inhibitory protein gene NAIP and ψNAIP; basal transcription factor subunit p44 (BTFp44t and BTFp44c); and H4F5t and H4F5c (Wirth, 2000). Homozygous deletions of the SMN1 gene are found in >98% of patients with the disease (Lefebvre et al., 1995), while the number of SMN2 copies present seems to modulate the disease severity (Taylor et al., 1998). SMN1 and SMN2 are 99% homologous, differing at only five single nucleotide positions, including one in exon 7 and one in exon 8. Prenatal genetic testing is commonly based on the detection of exon 7, or exon 7 and 8 of SMN1. Exon 7 is deleted in >95% of SMA patients.

Preimplantation genetic diagnosis (PGD) is now possible for a number of single gene disorders and chromosome rearrangements (Scriven et al., 1998; Wells, 1999). We set up preimplantation diagnosis for SMA in response to the demand from patients who had usually had at least one child who had died from SMA, and often had had late terminations for an affected fetus. These couples sought a realistic option for having an unaffected child without having to resort to prenatal
diagnosis and further therapeutic abortions. We now report on five couples treated at our centre using this technically improved approach to preimplantation diagnosis of SMA.

Materials and methods

Lymphocyte preparation

Single lymphocytes were prepared as described previously (Ray and Handside, 1996). Briefly, a blood sample was taken (5 ml), and diluted 1:1 with sterile phosphate-buffered saline (PBS; Gibco BRL, Life Technologies Ltd, Paisley, UK). Three millilitres of Ficoll Paque (Pharmacia Biotech Products, St Albans, Herts, UK) was overlaid with 4 ml of a diluted blood sample, and was centrifuged according to the manufacturer’s instructions. The lymphocyte layer was aspirated and washed through three changes of sterile PBS before being resuspended in ~5 ml of sterile PBS containing 5 mg/ml polyvinylpyrrolidone (PVP; Sigma–Aldrich, Gillingham, Dorset, UK) to prevent cell clumping. The lymphocytes were diluted through small drops (3 µl) of PBS/PVP until single lymphocytes could be picked up. These single cells were picked up, washed through another two drops of PBS/PVP and put into PCR tubes containing 3 µl lysis buffer. At this point, wash-drop blanks were taken from the last drop through which the cell was washed. These consisted of a similar volume (<1 µl) of the PBS + PVP to that taken during the cell pick-up.

Lymphoblast/lymphocyte preparation

A cell line (T131; European Collection of Animal Cell Cultures, Salisbury, Wiltshire, UK) derived from a patient homozygous for a deletion of exon 7 of SMN1 was used as an affected control (−/−). The cells were centrifuged and washed three times in sterile PBS, and single cells obtained as described above.

Lymphocytes were isolated from blood samples obtained from couples wishing to undergo PGD for SMA, or from individuals within our unit. The couples’ lymphocytes were known to be SMN1 exon 7 heterozygotes (+/−), whereas the lymphocytes from the donors in our unit were of undetermined genotype but likely to carry two normal SMN1 alleles (+/+).

Ovarian stimulation

A standard long protocol (Khalaf et al., 2000) was used to induce superovulation and control timing of oocyte retrieval. Pituitary suppression was achieved using buserelin nasal spray (Suprefact; Shire, Andover, Hampshire, UK), followed by ovarian stimulation with between 225 and 450 IU daily of recombinant human FSH (Gonal-F; Serono, UK; or Puregon; Organon, UK) for 9–14 days. When adequate follicular development had been demonstrated by transvaginal ultrasonography, 10 000 IU of human chorionic gonadotrophin was administered to trigger ovulation. Transvaginal oocyte retrieval was performed 34–36 h later.

Intracytoplasmic sperm injection (ICSI) was used to maximize the chances of fertilization in vitro and to reduce the risk of contamination with spermatozoa. The ICSI procedure was carried out on the heated stage of an inverted microscope (Nikon Diaphot) using Hoffman optics at ×400 magnification. Embryos were biopsied on day 3 of culture with one or rarely two blastomeres being removed for analysis. Available unaffected embryos were usually replaced on day 4. Luteal support was with progesterone pessaries (Cyclogest; Shire) 400 mg daily for 14 days. A pregnancy test was performed on day 14 post embryo transfer.

Embryo culture and biopsy

For the first four cases, oocytes and subsequent embryos were cultured using Medicult universal IVF medium (Medicult, Redhill, Surrey, UK) under oil (Squibb, Princeton, NJ, USA) for the first 2 days. At midday on day 2, the embryos were transferred into individual 100 µl drops of Medicult M3 under oil for overnight culture. Medicult Embryo Biopsy Medium (calcium/magnesium ion free) and acidified Tyrode’s solution were used for the embryo biopsy procedure. The embryos were then rinsed in M3 medium and replaced into fresh M3 medium under oil and were cultured in this medium until embryo transfer on day 4. For the fifth cycle, oocytes and embryos were cultured in sequential media under oil (IVF Sciences, Scandinavian, Gothenberg, Sweden). IVF Sciences IVF medium was used for oocyte collection and overnight fertilization. Pronucleates were transferred to G1.2 micro-drops on day 1, and then into fresh G2.2 micro-drops late on day two for overnight culture. On day 3, the embryo biopsy procedure was performed using IVF Science calcium/magnesium-free Embryo Biopsy Medium (EB) and acidified Tyrode’s solution (ZD) used for zona drilling. The embryos were then rinsed to remove remnants of acid and biopsy medium before replacement into G2.2 micro-drops until embryo transfer the following day.

Biopsy was undertaken as previously described (Handside and Thornhill, 1998) using an Olympus IX70 Microscope fitted with Hoffman modulation contrast optics and the TDU500 micromanipulator with double tool holder (Research Instruments, Penryn, Cornwall, UK). Generally only embryos with more than six cells were deemed suitable for biopsy, although a 5-cell and two 4-cell embryos were biopsied as the cohort was insufficient. Blastomeres were assessed for the presence of nuclei prior to biopsy, and one blastomere with a distinct nucleus identified for removal from each embryo. The embryo was held by gentle suction with a flame-polished holding pipette (Research Instruments), and a hole drilled in to the zona pellucida by blowing a stream of acidified Tyrode’s solution (pH 2.5) towards the zona from a micropipette with a maximum outer diameter of 10 µm (Cook IVF, Letchworth, Herts, UK). The acid Tyrode’s pipette was moved to the edge of the biopsy drop and an in-house pulled, heat-polished, biopsy pipette (inner diameter of 35–40 µm) was moved adjacent to the embryo. A single nucleated blastomere was removed from each embryo by gentle suction aspiration using the SAS Air syringe (Research Instruments). Following extraction of the blastomere for diagnosis, the embryos were rinsed in culture medium and returned immediately to normal culture conditions as described above. The blastomere was washed in PBS/PVP before tubing for lysis and analysis.

Donated blastomere preparation

Blastomeres were obtained by disaggregation of cleavage stage embryos donated by patients who did not have their surplus embryos cryopreserved or where these embryos were of too poor quality to recommend cryopreservation. This research was approved by the St Thomas’ Hospital ethics committee as part of a project to improve methods for PGD, and licensed by the Human Fertilisation and Embryology Authority (R0072). These cells were processed as for biopsied blastomeres.

Lysis procedure

The lysis procedure was the same for both lymphocytes and blastomeres (El-Hashemite and Delhanty, 1997). The lysis buffer consisted of 850 µg/ml proteinase K and 5 mmol/l sodium dodecyl sulphate (SDS) in PCR grade water. Once the cells had been placed in the PCR tubes, the lysis buffer was overlaid with mineral oil and frozen at −20°C for at least 20 min. The frozen samples were then loaded in the thermal cycler and incubated at 37°C for 1 h. The Proteinase K was then denatured by heating at 99°C for 15 min and allowed to cool before the PCR mix was added.
digestion. Therefore following from SMN1 additional fragments (160 and 102 bp) (Figures 1 and 2). This is an improvement whereas the product from SMN2 (262 bp) is only cut into two bands: uncut secondary PCR product; 160 and 102 bp bands: results of restriction digestion of SMN1 homozygous non-deleted exon 7 or heterogeneous SMN1 exon 7 PCR products. Lane B: complete digest of SMN1 homozygous non-deleted exon 7 or heterogeneous SMN1 exon 7 PCR products. Lane C: complete digest of SMN1 homozygous exon 7 deleted PCR products, 262 bp band: results of restriction digestion of SMN1/SMN2 exon 7 deleted PCR products, 78 and 24 bands: digest products of the SMN1/SMN2 exon 7 allele which is normally determined during case work-up.

**PCR and mutation detection**

We designed a single cell PCR protocol for PGD of SMA based on the detection of a *Hinf*I restriction fragment originating from the amplified region containing SMN1 exon 7. The 5’ primer of the nested reaction incorporates a mismatch two base pairs along from the single base change in the SMN1 gene, so that, following the simultaneous amplification of SMN1 and SMN2, a restriction site for *Hinf*I is created in the PCR products from SMN1 and not in those from SMN2. Both PCR products from SMN1 and SMN2 contain an additional *Hinf*I site which provides an internal control for restriction digestion. Therefore following *Hinf*I digestion, the nested product from SMN1 (262 bp) is cut into three fragments (160, 78, and 24 bp), whereas the product from SMN2 (262 bp) is only cut into two fragments (160 and 102 bp) (Figures 1 and 2). This is an improvement over previous approaches (Dreesen et al., 1998; Fallon et al., 1999) because partial or complete failure of digestion does not result in affected cells appearing to be normal or carrier, thereby avoiding a misdiagnosis.

The PCR mix for the primary round of PCR [50 µl reaction volumes containing 20 pmol of each primer; forward TGC AGC CTA ATA ATT GTT TTC TTT G; reverse CCA ACC AGT TAA](CTA ATA ATT GTT TTC TTT G; reverse CCA ACC AGT TAA) and spare blastomeres

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Amplification n (%)</th>
<th>PCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT/WT (+/+)</td>
<td>32</td>
<td>32 (100)</td>
<td>26 normal</td>
</tr>
<tr>
<td>WT/del7 (+/−)</td>
<td>26</td>
<td>22 (85)</td>
<td>4 AF SMN1</td>
</tr>
<tr>
<td>Del7/del7 (−/−)</td>
<td>120</td>
<td>115 (96)</td>
<td>2 AF SMN1</td>
</tr>
<tr>
<td>Blanks unknown</td>
<td>187</td>
<td>2 (1)</td>
<td>1 AF SMN1</td>
</tr>
</tbody>
</table>

AF = (specific) amplification failure.

GTA TGA GAA TTC TAC; 2.5 mmol/l MgCl2, 100 µmol/l dNTP (Promega), 1×PCR Buffer II Mg-free (Perkin Elmer), 1.5 units AmpliTaq (Perkin Elmer) was added to the lysed cells, reagent or wash-drop blanks and heated for 5 min at 96°C before being cycled through 30 cycles (10 cycles at 96°C for 45 s, 56°C for 45 s, 72°C for 1 min, then 20 cycles at 94°C for 45 s, 56°C for 45 s, 72°C for 1 min and a final extension of 7 min at 72°C) on an MJ PTC200 DNA Engine thermal cycler (MJ Research Inc.). A sample (2 µl) of this primary reaction product was then used as a template in the 50 µl secondary PCR assay which contained 20 pmol each primer: forward CTT CCT TTT ATT TTC ATT ACA GGG ATT (mismatch underlined); reverse CTA GGG ATG TAG ATT AAC TTA TCT; 2.5 mmol/l MgCl2, 1×PCR buffer, 100 µmol/l dNTP, 1.5 units AmpliTaq (Perkin Elmer). These were then put through 35 cycles at 94°C for 45 s, 56°C for 45 s, 72°C for 1 min and a final extension of 7 min at 72°C on a Geneamp PCR system 9700 thermal cycler (Applied Biosystems). 10 units of *Hinf*I were added directly to the secondary PCR tube post cycling, and this was incubated at 37°C for 2 h. The products of restriction digestion were separated by electrophoresis on an 8% vertical polyacrylamide gel (20 V/cm for 20–40 min), stained with ethidium bromide and photographed under UV light for analysis.

**Results**

**Development of the single cell PCR assay for SMA**

The efficiency and accuracy of the assay was examined using single cells, and the results are presented in Table I. Fifty-eight lymphocytes (32 undetermined, 26+−) were analysed...
with an overall amplification efficiency of 93%. Due to the nature of the assay, in cases of known genotype, specific amplification failure (AF) at the SMN1 and SMN2 loci could be distinguished (see Figures 2 and 3). Amplification was successful in all 32 samples in the undetermined group, although specific AF occurred twice for SMN1 and four times for SMN2. In the SMN1 heterozygote deletion group (−/+), only 22 of 26 samples amplified (85%). Of these, 18 gave the expected pattern (82%), although the assay cannot specifically distinguish a heterozygous pattern. In this group, there were two AF SMN1 events and two AF SMN2 events detected (9% each, 18% total AF rate). Although these AF rates are higher than those quoted for other assays using the proteinase K method of cell lysis (El-Hashemite and Delhanty, 1997), none of these AF events would have resulted in the transfer of an affected embryo. However, AF at SMN1 would decrease the number of embryos available for transfer.

The efficiency and accuracy of detection of the SMN1 homozygous deleted (−−) state (disease causing) was determined using lymphoblasts isolated from an SMN1 deleted cell line, T131 (ECACC). For this group 96% (115/120) amplification was obtained with no observed specific AF events. A possible explanation for this lack of AF events in the SMN1 (−−) cells could be postulated if the SMN1 (−−) cell line has two copies of SMN2 while the SMN1 (++−) cells have only a single copy of SMN2. Thirty-seven single blastomeres were isolated from embryos surplus to therapeutic need obtained from the IVF programme. The blastomeres were designated to be of unknown genotype. Amplification was obtained from 30 (81%), and all showed a non-deleted pattern.

During the course of these experiments, 187 blank reactions were also run consisting of both reagent blanks and ‘wash-drop’ blanks (reactions containing a small amount of the PBS/PVP solution through which the cells had been washed). Of these, two unrelated contamination events were detected (1%).

**PGD of SMA in five couples**

Five couples were treated from December 1998 to April 1999. All five patients had at least one child die from SMA and one had had a termination for an affected pregnancy following chorionic villus sampling (CVS). The ages of the female partners ranged from 26 to 38 years with a mean of 31 (Table II). All patients responded reasonably to the stimulation protocol with a median of 12 oocytes being obtained. Fertilization following ICSI was surprisingly variable, ranging from 39 to 90%. The reason for the poor fertilization in three of the patients was not obvious. Almost all of the fertilized oocytes cleaved adequately to allow biopsy on day 3. The amplification following PCR was within the range expected from previous experiments (70–100%) apart from case 1 (45%). At least one embryo was found to be unaffected for each patient, although in case 2 the contamination of six of the 15 wash-drop blanks (Table II and Figure 3) made it unsafe to proceed to transfer (see below).

Between one and three embryos were transferred in the four remaining cases resulting in three pregnancies, one singleton, one twin and a triplet. All pregnancies proceeded to livebirth at 40, 32 and 35 weeks respectively. Although offered, none of the patients opted for prenatal testing by CVS or amniocentesis. Testing of the cord blood obtained at delivery confirmed that none of the babies had an exon 7 homozygous deletion and hence were expected to be free of the disease. All six infants were developing normally after 1 year of follow-up.

**Details of contaminated case 2**

An amplification of 100% was achieved from the five blastomeres obtained from the embryos suitable for biopsy. Of these, three showed an unaffected pattern, and two an affected pattern. However, PCR product was also detected in six of 15 reaction (wash-drop) blanks (Figure 3, lanes 2, 6 and 9) as well as in three of the reaction mix (reagent) controls (Figure 3, lanes 14, 15 and 19). The second PCR round was repeated in case the contamination had occurred in the second round but the same pattern emerged, making embryo transfer unsafe. All the embryos were frozen prior to reanalysis. New primers were obtained, and the embryos were either disaggregated for PCR, or, if they did not survive the thawing process, were used whole after the zona pellucida had been removed using acid Tyrode’s solution. Nine single blastomeres and five embryos were analysed. Of these, a single blastomere failed to amplify (Table III). The diagnoses made during the case were consistent for all five embryos with no contamination detected in the reaction blanks.

**Discussion**

Although this is the third report of successful PGD of spinal muscular atrophy (Dreesen et al., 1998; Fallon et al., 1999),
it is the only one to use \textit{Hinf}I restriction to avoid misdiagnosis due to incomplete digestion of \textit{SMN}, and the only one to report postnatal testing of the offspring. Dreessen \textit{et al}. (1998) reported two cycles for one couple which resulted in a twin pregnancy, but the outcome of this pregnancy and the follow-up of the diagnosis was not reported. Fallon \textit{et al}. (1999) described a twin pregnancy confirmed as normal on CVS, and, in a note added in proof, the birth of three healthy children from eleven attempts at PGD. In our series we have been able to confirm that the diagnosis on cord blood samples and examination of the children at 1 year was normal.

We have demonstrated that PCR to amplify a fragment containing \textit{SMN1} is a workable method for the PGD of SMA from single cells. An amplification efficiency of 95\% was achieved using lymphocytes and the T131 cell line. Amplification failures of both \textit{SMN1} and \textit{SMN2} were detected despite modifications of the cell lysis process and cycling conditions to optimize the assay. However, as the basis of this assay is the positive appearance of a restriction product of \textit{SMN1} before embryo transfer would be contemplated, amplification failure of one allele of \textit{SMN1} would only result in the transfer of a carrier where a normal embryo was anticipated.

Transfer of the PCR technology from lymphocytes to blastomeres yielded a reduced rate of amplification. The lower reliability of PCR assays from blastomeres is well known (Pickering \textit{et al}., 1992) although its origins are less clear. The absence of a visible nucleus at biopsy is one possible cause. Others include absence of relevant chromosomes due to postzygotic aneuploidy or mosaicism (Munne \textit{et al}., 1994; Kuo \textit{et al}., 1998). Nevertheless, confirmatory analysis of blastomeres from embryos not suitable for transfer yielded high amplification efficiencies in nucleated cells. The sensitivity of the assay might be improved by the use of fluorescent PCR (Blake \textit{et al}., 1999; Sermon \textit{et al}., 1999).

Despite stringent precautions, amplification of extraneous DNA is an omnipresent threat (Pickering \textit{et al}., 1994). In case 2, six of the wash-drop blanks produced amplified product. In order to try to identify whether this had occurred during the nested reaction, the second round of PCR was repeated using the primary product, but to no avail as the blanks were still shown to have amplified. The absence of amplification in some of the reaction mix blanks would tend to exclude the reagents as a source of the DNA contamination. The randomness of its appearance tended to favour carryover product as the source (despite stringent precautions to try to prevent it), although other less likely explanations could be mooted (e.g. miniscule random primer contamination). The source was never identified convincingly but could have been due to the use of a PCR tube rack that had previously been used in the product analysis area. This contamination event is sobering, not only for the disappointment that the couples experienced in not having had transferred embryos that were on follow-up shown to be unaffected, but that the source of contamination was never identified precisely. All patients were, and still are, counselled not only about the risk of misdiagnosis due to undetected contamination, but about the intense disappointment should no diagnosis, or no safe diagnosis, be realized after such intensive and stressful treatment. Furthermore, although the technical aspects of stimulation, culture and biopsy, continued in-vitro culture and day 4 replacement can be mastered relatively easily, and can lead to successful pregnancies, they are subject to the same vagaries of ICSI/IVF in infertility where stimulation, fertilization and cleavage can be dishearteningly unpredictable. The attrition that occurs between detection of suitable follicles and having suitable embryos for replacement is enormous (62 oocytes yielded 14 unaffected embryos), a factor that needs to be taken into account when counselling couples about PGD.

### Table II. Outcome of cycles

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Maternal age (years)</th>
<th>No. of oocytes</th>
<th>No. of 2PN/ Blastomeres</th>
<th>No. of Blastomeres for assay</th>
<th>No. amplified (%)</th>
<th>No. of embryos transferred</th>
<th>Pregnancy Implantation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38</td>
<td>12</td>
<td>9/8</td>
<td>8</td>
<td>4 (45)</td>
<td>0</td>
<td>6/15 (40)</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>10</td>
<td>9/5</td>
<td>5</td>
<td>5 (100)</td>
<td>2</td>
<td>0/12 (0)</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>10</td>
<td>4/3</td>
<td>4</td>
<td>3 (75)</td>
<td>1</td>
<td>0/17 (0)</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>12</td>
<td>5/5</td>
<td>5</td>
<td>4 (80)</td>
<td>3</td>
<td>0/24 (0)</td>
</tr>
<tr>
<td>5</td>
<td>29</td>
<td>18</td>
<td>7/7</td>
<td>7</td>
<td>5 (71)</td>
<td>2</td>
<td>0/96 (6%)</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>34/28</td>
<td>29</td>
<td>21</td>
<td>14</td>
<td>7</td>
<td>6/9 (67%)</td>
</tr>
</tbody>
</table>

PN = pronuclei.

### Table III. Follow up of affected and non-transferred embryos

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Embryos available for analysis</th>
<th>No. of blastomeres</th>
<th>No. of nucleated blastomeres</th>
<th>No. amplified</th>
<th>Amplification efficiency (%)</th>
<th>Efficiency per nucleated blastomere (%)</th>
<th>Specific amplification failure</th>
<th>Discordant results</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>31</td>
<td>24</td>
<td>9</td>
<td>29</td>
<td>38</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>9 (+5 embryos)</td>
<td>13</td>
<td>9</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>27</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>73</td>
<td>1X SMN2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>9</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>34</td>
<td>32*</td>
<td>31</td>
<td>91</td>
<td>93</td>
<td>1X SMN2</td>
<td>0</td>
<td>*One binucleate</td>
</tr>
</tbody>
</table>

Despite stringent precautions, amplification of extraneous DNA is an omnipresent threat (Pickering \textit{et al}., 1994). In case 2, six of the wash-drop blanks produced amplified product. In order to try to identify whether this had occurred during the nested reaction, the second round of PCR was repeated using the primary product, but to no avail as the blanks were still shown to have amplified. The absence of amplification in some of the reaction mix blanks would tend to exclude the reagents as a source of the DNA contamination. The randomness of its appearance tended to favour carryover product as the source (despite stringent precautions to try to prevent it), although other less likely explanations could be mooted (e.g. miniscule random primer contamination). The source was never identified convincingly but could have been due to the use of a PCR tube rack that had previously been used in the product analysis area. This contamination event is sobering, not only for the disappointment that the couples experienced in not having had transferred embryos that were on follow-up shown to be unaffected, but that the source of contamination was never identified precisely. All patients were, and still are, counselled not only about the risk of misdiagnosis due to undetected contamination, but about the intense disappointment should no diagnosis, or no safe diagnosis, be realized after such intensive and stressful treatment. Furthermore, although the technical aspects of stimulation, culture and biopsy, continued in-vitro culture and day 4 replacement can be mastered relatively easily, and can lead to successful pregnancies, they are subject to the same vagaries of ICSI/IVF in infertility where stimulation, fertilization and cleavage can be dishearteningly unpredictable. The attrition that occurs between detection of suitable follicles and having suitable embryos for replacement is enormous (62 oocytes yielded 14 unaffected embryos), a factor that needs to be taken into account when counselling couples about PGD.
Despite all these potential pitfalls, a successful outcome in three out of the four of the women who proceeded to transfer is remarkable, although, to judge from more general published data (Geraedts et al., 1999) the success is unlikely to continue at this rate. It was fortunate that both multiple pregnancies proceeded to livebirths at reasonable gestations. Beside the obstetric hazards, and the technical difficulty and risk associated with prenatal diagnosis performed to confirm the genetic status of the PGD pregnancy if requested, the personal challenges of raising twins or triplets is further increased where there is already a sick or special needs child in the family. We now strongly discourage transfer of more than two embryos in women, especially under 37 years of age (Templeton, 2000a,b).

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References

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