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Birth of a healthy infant following trophectoderm biopsy from blastocysts for PGD of β-thalassaemia major: Case report

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PGD is a well accepted reproductive choice for couples at genetic risk and involves the diagnosis and transfer of unaffected IVF embryos. PGD for monogenetic diseases is most commonly accomplished by the biopsy of one or two blastomeres from cleavage stage embryos, followed by PCR-based protocols. However, PCR-based DNA analysis of one or two cells is subject to several problems, including total PCR failure, or failure of one allele to amplify. Trophectoderm biopsy at the blastocyst stage enables the removal of more than two cells for diagnosis while being non-invasive to the inner cell mass which is destined for fetal development. The aim of this study was to develop a safe, reliable technique for the biopsy of trophectoderm cells from human blastocysts. This case report demonstrates that removal of trophectoderm cells prior to blastocyst transfer is compatible with implantation and development to term. Here we report successful PGD for β-thalassaemia following trophectoderm cell biopsy from blastocysts and the birth of a healthy infant.

Key words: biopsy/blastocyst/β-thalassaemia/laser/PGD

Introduction

β-Thalassaemia is a common severe monogenic disorder, prevalent in the Mediterranean region, with a carrier frequency of ~10% in the Greek population (Loukopoulos, 1996). β-Thalassaemia is caused by mutations in the β-globin gene and affected pregnancies can be diagnosed by DNA mutation analysis prenatally, following amniocentesis at 16 weeks or chorionic villous biopsy at 10 weeks (Kanavakis *et al.*, 1997; Vrettou *et al.*, 2003). Parents usually select to terminate an affected pregnancy. PGD and single cell genetic analysis has become increasingly more popular since its first application (Handyside *et al.*, 1989) as an alternative to prenatal diagnosis to avoid termination of pregnancy in couples at high risk of transmitting a genetic defect. PGD for β-thalassaemia has been performed by analysing genetic material obtained from polar body biopsy of both first and second polar bodies (Kuliev *et al.*, 1998) or more commonly by blastomere biopsy from day 3 cleavage stage embryos. The genetic diagnosis of both polar bodies and blastomeres is achieved with the application of PCR-based protocols (Ray *et al.*, 1996; El-Hashemite *et al.*, 1997; Vrettou *et al.*, 1999, 2004; Piyamongkol *et al.*, 2001; Hussey *et al.*, 2002). Pregnancies have been reported following day 3 blastomere biopsy and genetic screening for the β-globin gene defect (Kanavakis *et al.*, 1999; Chamayou *et al.*, 2002; Palmer *et al.*, 2002; Jiao *et al.*, 2004). The main disadvantage of PGD based on analysis of polar body or blastomere biopsy procedures, however, is the limited amount of material available for genetic analysis.

Once the genetic diagnosis has been completed, the embryo transfer may be performed the same day (Boada *et al.*, 1998) or delayed, and pregnancies have resulted from embryo transfer on day 4 or later (Grifo *et al.*, 1998; Palmer *et al.*, 2002). Since the development of sequential media (Gardner and Lane, 1998; Jones *et al.*, 1998a), reports indicate improved pregnancy rates following blastocyst stage transfers (Gardner *et al.*, 1998a,b; Jones *et al.*, 1998a,b; Rijnders and Jansen, 1998; Pantos *et al.*, 2001). Trophectoderm biopsy at the blastocyst stage enables the removal of more than two cells for diagnosis while being non-invasive to the inner cell mass which is destined for fetal development (Dokras *et al.*, 1990; Veiga *et al.*, 1997; de Boer *et al.*, 2004). This case report describes the first live birth following trophectoderm biopsy at the blastocyst stage of development

Table I. Details of IVF cycles

Cycle no.	Oocytes (MII)	2PN	Biopsy		PCR result				Embryo transfer		β-HCG (IU/ml)
			Day	No. of embryos	Normal	Abnormal	ADO	No result	Number	Day	
1 (fresh)	20	10	D3	10	5	1	2	2	5	D4	0
2 (fresh)	28	(28) 18[a]	D3	12	6	4	2	–	2	D6	0
3 (thawed)		(10) 8[b]	D5	3	2	1	–	–	2	D6	236

MII = metaphase II; PN = pronucleus.
[a]Eighteen zygotes were cultured for 3 days to undergo blastomere biopsy and 10 zygotes were cryopreserved.
[b]Ten zygotes were thawed and eight survived, of which three went to blastocyst stage.

for the PGD of β-thalassaemia. Another facet of this success is that the blastocysts were derived from thawed embryos previously cryopreserved at the pronucleate stage.

Case report

A 39 year-old β-thalassaemia carrier female patient with primary infertility after 4 years of marriage, and her β-thalassaemia carrier spouse, approached our IVF unit, the Centre for Human Reproduction in Athens, to discuss the possibility of PGD for β-thalassaemia. The patient underwent two IVF cycles involving controlled ovarian stimulation, initiated using a GnRH long downregulation protocol (Pantos *et al.*, 1994). The mature eggs collected (Table I) were fertilized following ICSI and, in the second cycle, 10 of 28 zygotes were frozen at the pronucleate stage using a slow cooling protocol (Testart *et al.*, 1986). Cleavage-stage biopsy was performed following zona ablation using a non-contact laser (ZILOS-tk; Hamilton Thorne Biosciences, Beverly, MA) on all the embryos with six or more cells (Table I). One blastomere was removed from each embryo, placed immediately in RNase–DNase-free 0.2 ml PCR tubes containing 15 μl of proteinase K (final concentration 500 μg/ml; Roche Molecular Biochemicals, Mannheim, Germany) and transferred (at 4–8°C) immediately to the Laboratory of Medical Genetics in Athens for molecular diagnosis as previously described (Traeger-Synodinos *et al.*, 2003; Vrettou *et al.*, 2004). The details of each cycle are summarized in Table I. In both cycles, day 16 post-oocyte collection serum β-HCG was negative.

The patient returned for a frozen embryo transfer cycle 5 months after the negative pregnancy test following the second cycle. The 10 cryopreserved zygotes were thawed and the eight that survived were cultured in 10 μl microdrops of G1:3 culture medium (Vitrolife, Goteborg, Sweden) and then transferred to 20 μl microdrops of G2:3 (Vitrolife, Goteborg, Sweden) for culture to the blastocyst stage (Jones *et al.*, 1998a) under standard incubation conditions.

On day 5, three early blastocysts had developed and a hole was made in the zona pellucida directly opposite the inner cell mass of each blastocyst, using the lowest setting of the ZILOS-tk non-contact laser (Hamilton Thorne Biosciences, Beverley, MA). Blastocysts were incubated for a further 4 h to allow further growth and herniation of the trophectoderm cells from the zona. After 4 h in culture, the blastocysts had grown and were expanding but no significant herniation was observed. A single cell had herniated from one blastocyst whereas no herniation was observed for the other two blastocysts. The blastocysts were placed individually in 20 μl of G-MOPS medium (Vitrolife, Goteborg, Sweden) under oil for biopsy. Applying gentle suction with the biopsy pipette (Cook Australia, Eight Mile Plains, Qld, Australia), trophectoderm cells were encouraged to herniate from the zona. Four to six trophectoderm cells were dissected from each of the blastocysts using four laser pulses of 3 msec duration. The biopsied cells were placed immediately in RNase–DNase-free 0.2 ml PCR tubes exactly as for biopsied blastomeres. β-Globin gene mutation analysis for the maternal IVS1-110 G>A and paternal cd39 C>T mutations was performed by the protocol for genotyping blastomeres as described by Vrettou *et al.* (2004), which applies real-time multiplex PCR. Briefly, first round multiplex PCR was carried out directly in the tube containing the biopsied samples in a final volume of 50 μl containing primers for the β-globin gene and the two hypervariable microsatellite markers, GABRB3 and D13S314 (Table II), the latter to monitor extraneous DNA contamination in the PCRs. All PCR and cycling conditions were as previously reported (Vrettou *et al.*, 2004). Nested PCR amplifications for the β-globin gene mutation analysis were carried out on the LightCycler™ (Roche Diagnostics GmbH, Manheim, Germany) using 0.5 μM of each nested β-globin gene PCR primer (Table II) and 0.15 μM of the relevant fluorescent mutation detection probes (TIB Molbiol, Berlin, Germany) as follows: Ac IVSI-110, 5′-tct gcc tat tgg tct att ttc cc-3′, LC Red 640; Ac Cd39, LC Red 705 5′-acc ctt gga ccc aga ggt tct t-3′ P; donor set C,

Table II. Primers used for real-time PCR analysis of biopsied cells

Primers	Sequence of primers (5′ → 3′)	Product size (bp)
First round multiplex PCR		
β gene (outer)		
Forward	GAA GTC CAA CTC CTA AGC CA	689
Reverse	CAT CAA GGG TCC CAT AGA CTC	
GABRB3		
Forward	CTC TTG TTC CTG TTG CTT TCA ATA CAC	191–201
Reverse	CAC TGT GCT AGT AGA TTC AGC TC	
D13S314		
Forward	GAG TGG AGG AGG AGA AAA GA	137–155
Reverse	GTG TGA CTG GAT GGA TGT GA	
Real-time nested PCR		
β gene (inner)		
Forward	GCT GTC ATC ACT TAG ACC TCA	587
Reverse	CAC AGT GCA GCT CAC TCA G	

FITC 5′-ccc tta ggc tgc tgg tgg tc-3′ FITC (Vrettou *et al.*, 2003, 2004). Immediately following the amplification stage, the β-globin gene alleles were assigned by melting curve analysis.

Embryo genotypes with respect to the parental β-globin mutations were available 6 h following delivery of the trophectoderm biopsied samples to the genetics laboratory. A diagnosis was achieved for all three biopsied blastocysts, with one blastocyst identified as a carrier heterozygote (IVS1-110/N), one identified as unaffected (N/N) for β-thalassaemia and the third blastocyst identified as affected (IVS1-110/cd39). On the morning of day 6, the morphology and growth of the three biopsied blastocysts was assessed. The affected blastocyst had re-expanded and had totally hatched from the zona. The carrier blastocyst had re-expanded and 50% of the blastocyst had herniated through the biopsy hole. The normal blastocyst had reformed the blastocoel cavity but had undergone no significant expansion from the preceding day. Both the carrier and the normal blastocyst were transferred to the patient's uterus using a Wallace catheter (SIMS Portex Ltd., Hythe, UK). On day 10 post-embryo transfer, serum β-HCG was 236 IU/ml. Pregnancy was confirmed at 6 weeks by ultrasound diagnosis of a single fetal heart. At 12 weeks of gestation, chorionic villous biopsy was performed, and cytogenetics analysis confirmed the unaffected status of the embryo (N/N) and a normal 46XX karyotype. The blastocyst of origin did not expand following trophectoderm biopsy yet remained viable. A healthy female baby girl was delivered at 38 weeks of gestation by Caesarean section.

Discussion

This is the first pregnancy reported following PGD of β-thalassaemia on biopsied trophectoderm cells rather than polar bodies or biopsied day 3 blastomeres. It is also the first pregnancy reported following trophectoderm biopsy of cryopreserved zygotes cultured to the blastocyst stage for PGD.

There are three potential sources of embryonic genetic material for preimplantation genetic analysis. In clinical practice, polar body biopsy has been used primarily for aneuploidy screening (Verlinsky *et al.*, 1996) and also for the detection of maternal single gene defects (Verlinsky *et al.*, 1997) such as β-thalassaemia (Kuliev *et al.*, 1998), but cleavage stage biopsy and aspiration of the blastomere(s) remains the most commonly used source of embryonic genetic material for PGD (ESHRE PGD Consortium Steering Committee, 2002). A number of modifications have been incorporated into the biopsy procedure for cleavage stage embryos. These include non-contact laser drilling of the zona pellucida instead of chemical digestion using acidified Tyrodes solution, which avoids exposure of embryos to low pH (Boada *et al.*, 1998; Joris *et al.*, 2003), and the use of Ca^{2+}/Mg^{2+}-free medium for pre-biopsy incubation to reverse intercellular adhesion which makes the removal of blastomeres less difficult (Dumoulin *et al.*, 1998). These modifications to the biopsy procedure have made cleavage stage biopsy highly efficient. According to the ESHRE PGD Consortium report, cleavage stage biopsy was applied with 99% efficiency in embryos biopsied for chromosomal and single gene defect analysis. A diagnosis was successful in 85% of the embryos biopsied in PGD cycles for single gene defects (ESHRE PGD Consortium Steering Committee, 2002). PCR-based genetic analysis in one or two cells is not 100% efficient as it is subject to several problems, including sample contamination with extraneous DNA, PCR amplification failure or failure of one allele to amplify (allele drop-out, ADO) (ESHRE PGD Consortium Steering Committee, 2002). Biopsy of the embryo at the blastocyst stage has the advantage that more cells may be removed and be available for genetic analysis, which potentially reduces the occurrence of amplification failure and ADO. In this case report, 4–6 cells were biopsied from each blastocyst and a diagnosis was available in each case, achieving a diagnosis for all three embryos with no ADO. The biopsy of several trophectoderm cells allows the possibility of making a diagnosis on duplicate samples. However, in our experience, the separation of biopsied trophectoderm cells appears to be technically difficult, due to strong cell–cell contacts between individual cells. In addition, further manipulation of the sample to achieve duplicates runs the risk of introducing extraneous DNA contamination. In our opinion, the risks in relation to creating duplicate samples far outweigh any potential benefit.

Extended culture of cleavage stage embryos to the blastocyst stage has the advantage of selecting developmentally competent embryos for diagnosis. Reports of extended culture using more complex media or new generation sequential media have reported high rates of development to the blastocyst stage, regardless of whether all embryos or only surplus embryos were cultured to the blastocyst stage (36–66%) (Muggleton-Harris *et al.*, 1995; Scholtes and Zeilmaker, 1996; Desai *et al.*, 1997; Gardner *et al.*, 1998a,b; Jones *et al.*, 1998a,b; Rijnders and Jansen, 1998). The ability of the zygote to develop to the blastocyst stage may not necessarily reflect the viability of the embryo (Bolton *et al.*, 1991; Winston *et al.*, 1991). Embryo biopsy on day 3 allows selection of embryos that have at least demonstrated the potential of continued development under embryonic genomic control. However, it is not rare that embryos of a patient following day 3 biopsy and diagnosis are normal with respect to the genetic defect but fail to develop to blastocysts. Trophectoderm biopsy from day 5 blastocysts and subsequent genetic diagnosis results in transferring genetically tested and developmentally competent embryos to the uterus.

The success of PGD at the blastocyst stage is dependent on the capability of the biopsied blastocysts to produce pregnancies and the suitability of the biopsied material for analysis. The ability of blastocysts to implant after trophectoderm biopsy has been reported in animal studies (Gardner and Edwards, 1968; Gardner, 1971; Betteridge *et al.*, 1981; Monk *et al.*, 1988; Summers *et al.*, 1988). Trophectoderm biopsy has been performed on human blastocysts, and sufficient extra-embryonic material can be obtained for preimplantation diagnosis of genetic disorders (Dokras *et al.*, 1990, 1991; Veiga *et al.*, 1997). Furthermore, the biopsy of up to 10 trophectoderm cells from human blastocysts has been demonstrated to have no impact on the amount of HCG

secreted by the surviving blastocyst (Dokras *et al.*, 1991), indicating that biopsied blastocysts may remain viable. Recently, de Boer *et al.* (2004) reported 25 fetal hearts resulting from the transfer of biopsied human blastocysts that had 2–5 trophectoderm cells removed for PGD for genetic indications such as balanced translocations, aneuploidy and familial genetic disease. However, the genetic indications for the single gene disorders were not described in any detail.

In the present report, a singleton pregnancy resulted from the transfer of two blastocysts biopsied for the PGD of β-thalassaemia. All three blastocysts were at the early blastocyst stage prior to zona drilling, and after 4 h of incubation they had grown to expanding blastocysts. No cells were herniating out of the zona at the time of biopsy; however, application of gentle suction on the trophoectoderm cells through the zona hole was effective to encourage trophectoderm cells to herniate outside the zona pellucida. With a few pulses of short duration, it was possible to dissect 4–6 trophectoderm cells for genetic analysis without any obvious signs of damage. This is further evidenced by the subsequent re-establishment of the blastocoel cavity and the continued development and expansion of two of the three biopsied blastocysts until the time of transfer. It is interesting to note that the blastocyst responsible for the pregnancy was the least advanced of the cohort at the time of embryo transfer and the lack of expansion following biopsy was not indicative of a loss of viability.

Patients undergoing assisted reproduction treatment and PGD procedures invest both financially and emotionally in the expected outcome of both a pregnancy and a healthy offspring. With the availability of new embryology and molecular techniques, it is now possible for PGD laboratories to offer patients at genetic risk the transfer of developmentally competent embryos unaffected by genetic disease. In the future, trophectoderm cells could potentially provide sufficient material for multiple genetic tests, allowing the simultaneous diagnosis of more than one genetic defect. The diagnosis of trophectoderm cells destined to the placenta could now be considered the earliest form of prenatal diagnosis.

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