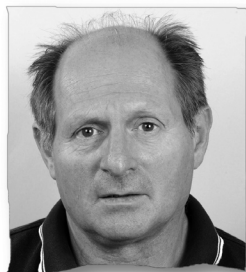# Article

Polar body biopsy for Curschmann–Steinert disease and successful pregnancy following embryo vitrification

Dr Ervin Macas studied biology at the University of Zagreb, Croatia, completing an MSc and a PhD, the latter on the study of chromosomal aberrations after IVF (1992). At present he is Head of the IVF laboratory at the University Hospital of Zurich. He has recently published a book, the first to be written in Croatian and German, on laboratory aspects of assisted reproduction techniques. His current scientific interests include preimplantation genetic diagnosis and cryopreservation.

Dr Ervin Macas

Ervin Macas[1,3,4], Gábor Mátyás[2,3], Philippe Reuge[2], Wolfgang Berger[2], Bruno Imthurn[1]
[1]Clinic for Reproductive Endocrinology, Department of Gynaecology and Obstetrics, University Hospital Zurich, Switzerland; [2]Division of Medical Molecular Genetics and Gene Diagnostics, Institute of Medical Genetics, University of Zurich, Switzerland
[3]These authors contributed equally to this work.
[4]Correspondence: e-mail: ervin.macas@usz.ch; matyas@medgen.uzh.ch

Abstract

This report describes the first successful case of preimplantation genetic diagnosis (PGD) for myotonic dystrophy type Curschmann–Steinert (DM1) using polar body biopsy with vitrification. A 39-year-old woman with expansion of a CTG trinucleotide repeat in the *DMPK* gene was included into the study centre's PGD programme. After intracytoplasmic sperm injection, a total of 13 fertilized oocytes were successfully biopsied for the first and second polar body. Nested multiplex polymerase chain reaction was used to amplify the CTG repeat region in *DMPK* along with two linked polymorphic markers. Six pronuclear stage (PN) oocytes were diagnosed as unaffected and four as affected by the CTG expansion, while analysis of the remaining PN oocytes was inconclusive. Three normal PN oocytes were left in culture to develop to cleavage-stage embryos and the remaining three were vitrified by applying the Cryotop method. On the following day, only one embryo was transferred into the patient's uterus and the remaining two were vitrified because of the progressive threat of ovarian hyperstimulation syndrome. Since the fresh cycle did not result in a pregnancy, 6 months later the two vitrified cleavage-stage embryos were warmed and transferred back to the patient. A clinical pregnancy was established and a healthy boy was born following Caesarean section in week 39 of gestation.

Keywords: myotonic dystrophy type Curschmann–Steinert, polar body biopsy, vitrification

Introduction

Myotonic dystrophy type Curschmann–Steinert (DM1) is a common multisystemic disorder that affects skeletal and smooth muscle as well as the eye, heart, endocrine system and central nervous system.

Among known single-gene defects, there is a group of genetic conditions which has been shown to result from the expansion of unstable trinucleotide repeats (Cummings and Zoghbi, 2000). One of these specific conditions is DM1. As an autosomal dominant trait, DM1 affects almost five in 100,000 individuals. Its phenotypic expression is highly variable and correlates with the length of the CTG repeat expansion within the 3' untranslated region of the *DMPK* gene (Brook *et al*., 1992; Mahadevan *et al*., 1992). Individuals with 5–150 CTG repeats are only mildly affected with symptoms of the disease, whereas persons with more than 1000 repeats suffer from the congenital form of DM1. In such cases, symptoms are usually expressed by severe hypotonia, respiratory distress, delayed motor development, and loss of mental function in cases of survival.

Currently, to avoid transmission of DM1 from parent to offspring, two principal diagnostic strategies are carried

out during preimplantation stages of embryo development. One involves testing of cells obtained from an embryo (blastomeres), and the other genetic analysis of the first and second polar bodies of inseminated oocytes (Harper and Thornhill, 2001; Hehr *et al.*, 2004; Verlinsky and Kuliev, 2005). The first strategy can be used in cases where either the male or female partner is a carrier of DM1, whereas the second is applicable only to female carriers. In clinical practice, however, diagnosis involving polar body biopsy has been used only rarely. Indeed, from an overall total of 314 preimplantation genetic diagnosis (PGD) cycles reported recently for DM1, only three cases were accomplished using polar body biopsy (Harper *et al.*, 2008). Obviously, one of the principal reasons for this disparity is that embryo biopsy is restricted by law only in some countries. The Swiss Reproductive Medicine Act (*Fortpflanzungsmedizingesetz*) prohibits embryo biopsy for PGD, allowing only polar body diagnosis, as is also the case in Austria, Germany and Italy. Consequently, clinical experience regarding diagnosis of DM1 with polar body biopsy is still lacking. As far as is known, no data are available on the outcome of pregnancies achieved using this method of biopsy for DM1 diagnosis. The purpose of this report was therefore to describe conditions that led to the establishment of a viable pregnancy and delivery of an unaffected child in a woman with DM1 following polar body diagnosis.

The second point to note in this report is that this pregnancy was obtained following vitrification of cleavage-stage embryos derived from oocytes biopsied at the pronuclear (PN) stage. Although vitrification has now been accepted as an efficient technique for cryopreservation of zona intact PN oocytes and embryos, this method has so far found little application in patients undergoing PGD. In one study, the authors were not convinced of the advantages of vitrification over slow freezing of PN oocytes following polar body biopsy (Van der Ven *et al.*, 2007). However, in the only other report available, promising results were observed regarding vitrification of PN oocytes after polar body diagnosis (Naether *et al.*, 2008).

Cryopreservation of embryos is currently forbidden in Switzerland. According to Swiss federal law on reproductive medicine, a maximum of only three PN oocytes can be cultured to the embryonic stage, and all surplus PN oocytes must be frozen before the onset of the first cleavage division. However, in exceptional circumstances, embryos may be frozen if their replacement is unavoidably and unintentionally impossible on the day of transfer.

Materials and methods

Patient

A 39-year-old woman affected with a DM1 mutation was included in the study centre's PGD programme. At the age of 22 years she had been diagnosed as having an expanded repeat (~150 CTG) on one allele of the *DMPK* gene. Genetic evidence for DM1 has also been verified in her family (**Figure 1**). At the age of 29 years she delivered a healthy child, but 5 years later her second pregnancy ended in the miscarriage of a DM1-affected child. After termination of a third pregnancy following positive prenatal diagnosis of DM1, she was referred to the clinic.

Ovarian stimulation, oocyte retrieval, and microinsemination

Ovarian stimulation was carried out by a desensitizing protocol of a daily injection of 0.1 mg s.c. D-triptoreline (Decapeptyl; Ferring, Switzerland) in association with human menopausal gonadotrophins (HMG/FSH; Puregon, Serono, Switzerland) (Imthurn *et al.*, 1996; Macas *et al.*, 1996). At 36 h after human chorionic gonadotrophin (HCG; Ovitrelle, Serono) injection, oocytes with cumulus cells were collected from antral follicles by transvaginal ultrasound-guided follicular aspiration. The cumulus cells were removed 1 h after oocyte retrieval using a combination of an enzymatic (Hyase; VitroLife, Sweden) and a mechanical procedure.

For the intracytoplasmic sperm injection (ICSI) procedure, oocytes with the first polar body (PB1) were transferred into drops of G-MOPS medium (VitroLife), covered with sterile paraffin oil (Ovoil, VitroLife), and placed on a warm plate (37°C) in an inverted Olympus IX microscope equipped with Hoffman optics (Olympus, Switzerland). The ICSI procedure was performed 3 h after oocyte retrieval using the method described by Palermo *et al.* (1992) and Van Steirteghem *et al.* (1993).

Polar body biopsy

Following ICSI, oocytes with PB1 were transferred into drops of G-MOPS medium, covered with paraffin oil and placed on a warm plate under an inverted microscope. Biopsy of PB1 was performed 5 h after oocyte retrieval. An opening in the zona pellucida was made using a 1.48 μm diode laser system (Octax; MTG Medical Technology, Germany) using a single pulse of 4.5 ms. After biopsy, PB1 was rinsed in a drop of G-MOPS medium and finally transferred into a polymerase chain reaction (PCR) tube with 5 μl of 1× HotStar PCR buffer (Qiagen, Hilden, Germany) containing 1.5 mmol/l $MgCl_2$. The second polar body (PB2) was isolated 12 h after oocyte retrieval using a freshly prepared biopsy capillary. When the opening in the zona pellucida was found, the capillary was inserted close to PB2, which was then aspirated into the lumen of capillary by applying a slightly higher pressure. PB2 was rinsed and transferred as described above for PB1.

Polar body analysis

The two polar bodies (PB1, PB2) were analysed separately and immediately after biopsy as outlined by Hehr *et al.* (2004). Briefly, after proteinase K treatment, multiplex PCR was performed in a final volume of 50 μl using primers for *DMPK*, D19S219, and rs2070737, with a low-stringency annealing temperature (50°C, 45°C) and long annealing time (90 s, 60 s) in a total of 38 cycles. Subsequently, second-round PCR amplifications were carried out separately for each marker by using 2 μl of the multiplex PCR product as template and (hemi-)nested locus-specific primers, with a high-stringency annealing temperature (58°C) and short annealing time (20 s) in a total of 35 cycles. Nested forward primers were fluorescently labelled with 6-FAM. For the single nucleotide polymorphism (SNP) rs2070737 (T/A), two separate second-round PCRs were performed using a nested 6-FAM-labelled forward primer specific for the T or the A allele. All second-round PCR

fragments were separated by capillary electrophoresis on an ABI Prism 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) under routine conditions. In order to avoid possible contamination by extraneous DNA, separate laboratory rooms and stringent precautions were used as recommended by the European Society for Human Reproduction and Embryology (ESHRE) PGD consortium (Thornhill *et al.*, 2005).

Vitrification and warming procedures

Vitrification and warming solutions were prepared by mixing cryoprotectants with G-MOPS medium (VitroLife, Sweden) containing 10% synthetic serum substitute (Irvine Scientific, USA) and 0.4% human serum albumin (Sigma, St. Louis, USA). Vitrification was performed following the method reported by Kuwayama *et al.* (2005) with slight modification. Briefly, all surplus PN oocytes or embryos were transferred to the first solution containing 7.5% ethylglycol (Sigma) and 7.5% dimethyl sulphoxide (DMSO; Sigma) for a total time of 15 min. After equilibration, PN oocytes or embryos were placed in vitrification solution containing 15% ethylglycol, 15% DMSO, and 0.5 mol/l sucrose (Sigma) for 30 s, and transferred for a further 30 s in a minimum amount of vitrification solution on the upper surface of a tip obtained by cutting a conventional 0.25 ml straw (CryoBio System, France). Upon loading, the straw was immediately submerged in liquid nitrogen, inserted into a 0.5 ml straw (CryoBioSystem, France) and finally stored in liquid nitrogen.

Warming was performed by immersing the tip of the straw in 1 ml of solution containing 1.0 mol/l sucrose (37°C) and keeping it there for approximately 60 s. The embryos were then transferred to a drop of solution with 0.5 mol/l sucrose for 2.5 min and finally placed in G-MOPS medium containing no sucrose for the same period of time. Embryos were washed four times in equilibrated G1-plus medium (VitroLife) and then cultured in an atmosphere of 6% CO_2 in air at a temperature of 37°C for approximately 21 h. On the morning of day 3, embryos were transferred into G2-plus medium (VitroLife) and 3 h later were replaced in the patient's uterus.

Results

The patient underwent a fresh ICSI cycle and produced 17 morphologically mature cumulus–oocyte complexes. Following denudation, each of these oocytes had a normally shaped PB1 within the perivitelline space and showed no cytoplasm dysmorphism.

Three hours after follicle aspiration, a total of 17 oocytes in metaphase II (MII) were inseminated using ICSI. A total of 14 MII oocytes were successfully biopsied for PB1. Only one oocyte was seen to be unfertilized (oocyte 14; **Table 1**), and approximately 8 h after biopsy of the first polar body, biopsy of PB2 was successfully accomplished on the remaining 13 fertilized oocytes (**Table 1**).

Preliminary molecular genetic testing revealed two polymorphic markers (D19S219, rs2070737) linked to the affected *DMPK* allele (**Figure 1**). Molecular analysis of both polar bodies using these two linked markers and primers flanking the CTG repeat region in *DMPK* detected six unaffected PN oocytes (oocytes 2, 4, 5, 6, 10, 12). Four of these oocytes extruded homozygous affected PB1 and normal PB2. In two unaffected oocytes (oocytes 2, 12; **Table 1**) crossover was shown by heterozygous PB1 and affected PB2 extrusion. Four PN oocytes were identified as affected by the CTG expansion (oocytes 7, 8, 9, 11), three of which underwent crossover (oocytes 8, 9, 11; **Table 1**), while the analysis in four cases was inconclusive due to putative allele dropout (ADO), unspecific results, or because of the lack of PB2 (oocyte 14). ADO of the markers D19S219 and rs2070737 was assumed in two and one case, respectively. No expanded *DMPK* allele could be amplified, most likely due to the size of the expansion (≥150 CTG).

Immediately after diagnosis, three of the six unaffected PN oocytes (oocytes 2, 4, 5) were left in culture to develop to cleavage-stage embryos, and the remaining three (oocytes 6, 10, 12) were pre-selected for cryopreservation and vitrified a few minutes later using the cryotop method.

A second course of cryopreservation was performed on day 2 of culture, applying the same method of vitrification. Due to the increasing risk of development of severe ovarian hyperstimulation syndrome (OHSS), contrary to the planned procedure, only one embryo at the 6-cell stage (day 2 of culture) could be replaced into the patient's uterus; the remaining two were vitrified at the 4-cell stage immediately after embryo transfer.

Since the fresh cycle did not result in a pregnancy, 6 months later the two vitrified embryos were warmed and cultured for 24 h. Both embryos showed regular morphology and were transferred back to the patient at the 8-cell stage. Pregnancy was confirmed 2 weeks after embryo transfer by rising concentrations of HCG. An intact pregnancy with positive fetal heart action was verified ultrasonographically 3 weeks later. Prenatal diagnosis by chorionic villus sampling was performed in week 12 of gestation and the presence of an unaffected fetus was confirmed using PCR and Southern blot analyses of the CTG repeat region in *DMPK*. The patient delivered a healthy son with normal body weight (3100 g) by Caesarean section in week 39 of gestation (38 weeks + 5 days).

Discussion

As far as is known, this study describes the first case of polar body-based PGD for DM1 leading to a successful pregnancy after vitrification of unaffected cleavage-stage embryos. The protocol involved the amplification of the non-expanded *DMPK* allele as well as two linked polymorphic markers in order to indicate not only the expanded allele but also crossover, allele dropout, nondisjunction, and contamination by extraneous DNA (not detected). Analysis of both polar bodies can identify oocytes with certain gene defects but cannot exclude other genome abnormalities affecting chromosomes or additional genes (Sermon *et al.*, 2004).

Shortly after the introduction of PGD into routine clinical practice, it became apparent that this method is one of the most technically difficult procedures in the field of IVF. Due to the technical complexity and labour intensity of PGD, the role of cryopreservation is particularly important in cases where genetically normal PN oocytes or embryos are surplus

Table 1. Results of haplotype analyses for the CTG expansion in *DMPK* using two linked markers (D19S219, rs2070737) in the mother's leukocytes and polar bodies (PB).

Cell type	Polar body	DMPK (CTG)	D19S219[a]	rs2070737[a]	Prediction[b]
Leukocytes	–	**5**/*~150*	**181**/*173*	**A**/*T*	Affected
Oocyte 1	1	**5**/–[c]	**181**/*173*	**A**/*T*	No prediction
	2	**5**/–[c]	Unspecific	**A**/*T*	
Oocyte 2	1	**5**/–[c]	**181**/*173*	**A**/*T*	Normal
	2	–[c]	*173*	*T*	
Oocyte 3	1	–[c]	*173*	*T*	No prediction
	2	**5**	Unspecific	**A**	
Oocyte 4	1	–[c]	*173*	*T*	Normal
	2	**5**	**181**	**A**	
Oocyte 5	1	–[c]	*173*	*T*	Normal
	2	**5**	**181**	**A**	
Oocyte 6	1	–[c]	*173*	*T*	Normal
	2	**5**	**181**	**A**	
Oocyte 7	1	**5**	**181**	**A**	Affected
	2	–[c]	–[c]	–[c]	
Oocyte 8	1	**5**/–[c]	**181**/*173*	**A**/*T*	Affected
	2	**5**	**181**	**A**	
Oocyte 9	1	**5**/–[c]	**181**/*173*	**A**/*T*	Affected
	2	**5**	Unspecific	**A**	
Oocyte 10	1	–[c]	*173*	*T*	Normal
	2	**5**	**181**	**A**	
Oocyte 11	1	**5**/–[c]	**181**/*173*	**A**/*T*	Affected
	2	**5**	**181**	**A**	
Oocyte 12	1	**5**/–[c]	**181**/*173*	**A**/*T*	Normal
	2	–[c]	*173*	*T*	
Oocyte 13	1	**5**/–[c]	–[c]/*173*	**A**/*T*	No prediction
	2	**5**	Unspecific	**A**	
Oocyte 14	1	**5**/–[c]	**181**/*173*	**A**/*T*	No prediction
	2	Not available	Not available	Not available	

Normal (bold), affected (italic), and unexpected (unspecific) alleles are indicated.
[a]D19S219 and rs2070737 are located proximal (~280 kb) and distal (~9.5 kb) to the CTG expansion, respectively.
[b]Prediction was made considering crossover, nondisjunction, allele dropout and PCR failure in both PB1 and PB2. Unexpected alleles (unspecific) resulted either in no prediction in case of oocytes with uncertain (oocytes 1, 13) and otherwise normal (oocyte 3) status, respectively, or were interpreted as affected in case of otherwise abnormal results (oocyte 9). Prediction was only confirmed using prenatal diagnosis by chorionic villus sampling.
[c]No PCR amplification product due to putative allele dropout, no PCR-amplifiable CTG expansion, or nondisjunction.

in the fresh cycle (Magli *et al.*, 2006). However, at present, the slow-freezing protocol does not offer optimal conditions for cryopreservation of PN oocytes and embryos after PGD. Studies conducted on biopsied PN oocytes and embryos following application of slow freezing are limited, and the few results obtained so far have demonstrated that the viability of these PN oocytes and embryos was negatively influenced by the biopsy method (Joris *et al.*, 1999; Magli *et al.*, 1999; Ciotti *et al.*, 2000; Jericho *et al.*, 2003; Montag *et al.*, 2004; Stachecki *et al.*, 2005).

The present study, therefore, did not use the conventional slow-freezing protocol but applied vitrification in order to store unaffected PN oocytes and embryos for future transfers. Vitrification is becoming increasingly recognized as offering significant improvements over all previously tested ultra-rapid cooling techniques. In addition, vitrification of embryos has recently led to higher clinical success with respect to survival

and pregnancy rates than any other known slow-freezing method (Kuwayama *et al.*, 2005; Zheng *et al.*, 2005). Furthermore, very acceptable pregnancy and implantation rates have recently been reported by Al-Hasani *et al.* (2007) following vitrification of human PN oocytes.

Several vitrification protocols, such as Cryoleaf, CryoTip, Cryoloop and Cryotop, are currently proposed for cryopreservation of human MII and PN oocytes, cleavage-stage embryos and blastocysts after IVF (Lane *et al.*, 1999; Chian *et al.*, 2005; Kuwayama *et al.*, 2005; Escribá *et al.*, 2008). Of these, Cryotop was selected as the most efficient method available for cryopreservation of human PN oocytes and cleavage-stage embryos following polar body biopsy. The main reason for this choice was that this method had recently been demonstrated to be a very effective procedure for cryopreservation of mouse and human PN oocytes after polar body biopsy (Macas *et al.*, 2007; Naether *et al.*, 2008).
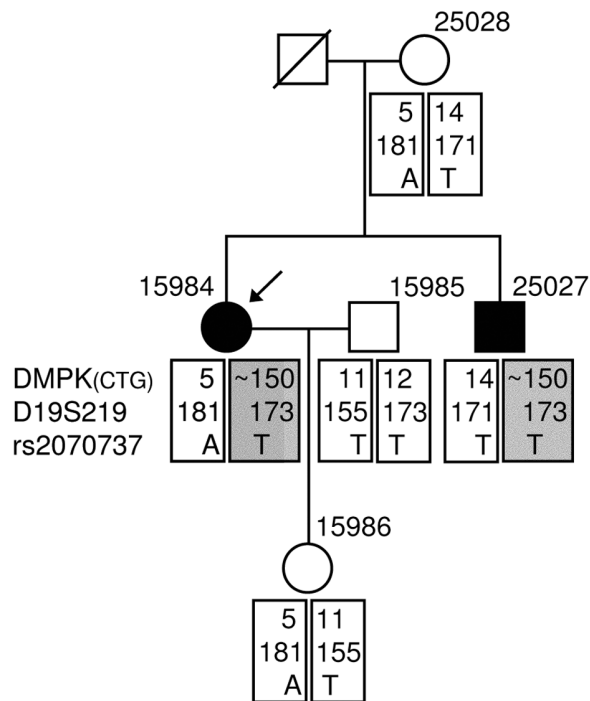
Figure 1. Pedigree with results of haplotype analysis in leukocytes of family members of the female patient with myotonic dystrophy type Curschmann–Steinert (DM1; *arrow*). Haplotypes of D19S219 and rs2070737 linked to the number of CTG repeats in *DMPK* are boxed. The grey-filled box represents the chromosome with the affected *DMPK* allele. Circles = females; squares = males; filled symbols = heterozygous mutation carriers.

In Switzerland, freezing and thawing procedures are limited by law to the cryopreservation of MII and PN oocytes only. Despite these legal regulations, it was necessary to make an exception and two embryos were vitrified following polar body biopsy. In human IVF, there are a few rare medical situations in which embryo transfer is unexpectedly and unintentionally impossible, and where the cleaved embryos must be frozen for a short period of time until the problem is resolved. Such situations may arise, for example, because of a severe stenosis of the cervical channel, which makes it impossible to replace an embryo into the patient's uterus. Cancellation of embryo transfer may also arise because of an acute illness on the day of transfer, or, as reported in this study, due to the progressive risk of the development of severe OHSS. However, in the latter instance, embryo transfer can be either abandoned completely (to prevent a pregnancy at all and to limit the duration of an OHSS until the onset of menstrual bleeding) or reduced to the replacement of only a single embryo to avoid a multiple pregnancy, which has a higher OHSS risk than a singleton pregnancy. Hence, being aware of the increasing threat of a severe OHSS in this case, the only ethically and medically acceptable and legally tolerated solution was chosen, i.e. to transfer only one embryo, and, exceptionally, to vitrify the two remaining embryos.

In the current case study, the prediction based on polar body analysis was only confirmed using prenatal diagnosis by chorionic villus sampling. Re-testing of embryos was not possible due to the restrictive law on PGD in Switzerland. In future studies, re-testing of each oocyte found to be affected either with aneuploidy (with fluorescence in-situ hybridization) or single gene defect (with PCR) following polar body biopsy is planned.

In conclusion, the case reported here indicates that a human embryo derived from a biopsied PN oocyte is able to induce a viable pregnancy following vitrification and warming. Further studies including a sufficient number of biopsied PN oocytes are required to confirm the full efficiency of the vitrification protocol used here.

Acknowledgements

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