Live birth following serial vitrification of embryos and PGD for fragile X syndrome in a patient with the premutation and decreased ovarian reserve

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Abstract

Purpose To present a live birth resulting from serial vitrification of embryos and pre-implantation genetic diagnosis (PGD).

Methods A 31-year-old with primary infertility, fragile-X premutation, and decreased ovarian reserve (DOR) (baseline FSH level 33 IU/L), presented after failing to stimulate to follicle diameters >10 mm with three cycles of invitro fertilization (IVF). After counseling, the couple opted for serial invitro maturation (IVM), embryo vitrification, and genetic testing using array comparative genomic hybridization (aCGH) and PGD. Embryos were vitrified 2 days after intracytoplasmic sperm injection (ICSI). Thawed embryos were biopsied on day-three and transferred on day-five.

Results The couple underwent 20 cycles of assisted reproductive technology. A total of 23 in-vivo mature and five immature oocytes were retrieved, of which one matured in-vitro. Of 24 embryos, 17/24 (71 %) developed to day two and 11/24 (46 %) survived to blastocyst stage with a biopsy result available. Four blastocysts had normal PGD and aCGH results. Both single embryo transfers resulted in a successful implantation, one a blighted ovum and the other in a live birth.

Capsule Serial vitrification of embryos may be a viable option in younger patients with diminished ovarian reserve that desire pre-implantation genetic diagnosis.

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Conclusions Young patients with DOR have potential for live birth as long as oocytes can be obtained and embryos created. Serial vitrification may be the mechanism of choice in these patients when PGD is needed.

Keywords In-vitro maturation (IVM) · Embryo vitrification · Diminished Ovarian Reserve (DOR) · Fragile X Syndrome (FXS) · Preimplantation Genetic Diagnosis (PGD) · Array Comparative Genomic Hybridization (aCGH)

Introduction

Decreased ovarian reserve (DOR) is a common condition among women with infertility and is highly correlated with advanced maternal age. However, in younger women with infertility there are numerous causes of DOR, including maternal Fragile X Syndrome (FXS) premutation. FXS is an X-linked neurodevelopment disorder and is the most common cause of inheritable mental retardation [6]. Males with FXS represent a more severe condition than females, although females may also have a spectrum of intellectual disabilities. FXS is a genetic disease involving an unstable trinucleotide repeat expansion of cytosine guanine guanine (CGG) in the FMR1 gene. Individuals with >200 repeats of CGG are classified as having the full mutation and those with 55–200 repeats are classified as having the premutation or being carriers [21]. There is growing evidence that FMR1 genotypes (premutations) are associated with increased rates of follicular recruitment and resultant decreased ovarian reserve [7–9]. Furthermore, maternal transmission of the premutation is associated with further expansion of the trinucleotide repeat in the offspring with the propensity to reach a full mutation [13]. Therefore, patients with FMR1 associated DOR have two significant reproductive concerns: genetic disease transmission and subfertility. Although both concerns can be addressed with oocyte donation, this is not a viable option for many

patients nor is it readily available worldwide. With respect to an FMR1 premutation, a couple may opt for no genetic screening, pre-implantation genetic diagnosis (PGD) or early antenatal diagnosis. Women with a FXS premutation that choose to undergo PGD require an adequate number of embryos to screen and therefore concomitant DOR represents a uniquely problematic scenario.

This is the first published case of a live birth after serial embryo vitrification obtained by following an IVM protocol for DOR requiring PGD for the FXS premutation. It is also among the few successful published cases of reproductive technology in a patient with a day 3 serum FSH >30 mIU/mL, who did not have a return of menses after resolving premature ovarian failure.

Methods

A 31-year-old woman with primary infertility, FXS premutation and DOR (maximum day 3 serum FSH=33 mIU/mL; antral follicle count=3) presented to the MUHC Reproductive Center in September 2009. The semen analysis revealed the following results: volume 3.5 mL; concentration 81.5 million/mL; motility 84 %; and WHO normal morphology 31 %. The patient had regular menstrual cycles lasting from 21 to 28 days. The patient's mother and maternal aunt had undergone premature ovarian failure (POF) at the age of 35 and 34 years respectively. The patient had a fragile X premutation of 91 repeats on a single X chromosome. A medical genetics consultation yielded an estimate of 40 % risk of having an offspring with FXS. Although males with FXS clearly represent a more severe condition, female offspring have an estimated 16 % risk of being mentally impaired, so genetic screening by sex selection alone was deemed as inadequate in this case. Furthermore, the couple did not wish to terminate an affected pregnancy, so relying on the antenatal diagnosis was not an option. Therefore, PGD was opted for, even with the functional limitations given the patient's DOR.

The couple underwent three previous IVF cycles at a different clinic resulting in failure to stimulate follicles >10 mm in mean diameter with 600 IU of gonadotropins daily, requiring cycle cancellation. The couple was counseled for oocyte donation but in their opinion this was not an acceptable option. Due to the patient's failure to stimulate with exogenous gonadotropins, an IVM protocol was attempted with both minimal and unstimulated cycles. Numerous IVM cycles were undertaken in order to generate a sufficient number of vitrified embryos for PGD and array comparative genomic hybridization (aCGH).

It is controversial whether IVM embryos have an increased risk of genetic abnormalities compared to embryos derived from IVF [14, 22, 23]. This may be an alternate explanation for the lower implantation and increased miscarriage rates

noted in IVM embryos in comparison to IVF and ICSI [4]. The couple planned to augment their genetic diagnostics with aneuploidy screening (aCGH) prior to embryo transfer. The use of aCGH prevents an aneuploid embryo from inappropriately being transferred. Furthermore, aCGH minimizes the risk of subjecting an unselected euploid embryo to the additional stress of freezing and thawing when an untested aneuploid embryo is transferred instead. The Applied Genomic Technology laboratories at Genesis Genetics have examined over 50,000 biopsied single cells from day 3 embryos with only one aneuploidic pregnancy resulting from a negative biopsy (unpublished data). Blastocysts from IVF cycles with normal aCGH result in high implantation rates approaching 70 % in some centers [15].

The McGill flexible IVM protocol begins with transvaginal ultrasonography monitoring after the onset of menstruation (cycle day 2–5) and repeated on cycle day 8, or as clinically indicated. When the leading follicle (LF) is between 10 mm and 12 mm, patients are administered 10,000 IU HCG subcutaneously. Patients undergo oocyte collection 38-h after HCG administration. It was previously demonstrated that an increase in in-vivo and in-vitro oocyte maturation rates, as well as an improved pregnancy outcome, are obtained when HCG is administered at 38-h as opposed to 35-h prior to collection [16]. The follicles are retrieved using a 19-gauge single-lumen needle (Cook Medical, Bloomington, IN) with aspiration pressure of 7.5 kPa and collected into 10 mL culture tubes with 2 mL warm 0.9 % normal saline with 2 IU heparin.

The nuclear maturity of the collected oocytes is assessed under the dissecting microscope with high magnification (x80) using the spreading method. Oocyte immaturity is assessed by the presence of germinal vesicle and the first polar body, while mature oocytes are identified when the first polar body is extruded. Oocytes that are mature on the oocyte retrieval day (Day 0: 0–6 h) are placed into routine IVF fertilization medium and inseminated on the same day. Immature oocytes are cultured in IVM medium (Cooper Surgical, Trumbull, CT) supplemented with 75 mIU/mL FSH and LH. Following culture day 1 (24–30 h), the oocytes are denuded of cumulus cells with hyaluronidase and mechanical pipetting. All matured oocytes are inseminated by intracytoplasmic sperm injection (ICSI). Fertilization is assessed 16–18 h after insemination for the appearance of two distinct pronuclei and two polar bodies. The zygotes are cultured in COOK cleavage medium (Cook company, Australia). Embryonic development is assessed and the embryos are vitrified 2 days after ICSI. A previous study showed that freezing embryos on day 2 or 3 post-fertilization doesn't have any significant effect on embryo survival and PGD test success rates [10, 24].

For vitrification, the embryos are suspended in equilibration medium containing 7.5 % (v/v) ethylene glycol (EG) and 7.5 % (v/v) dimethylsulfoxide (DMSO) for 5 min at room temperature, then transferred to vitrification medium

containing 15 % (v/v) EG, 15 % (v/v) DMSO and 0.5 M sucrose at room temperature for 45–60 s. The embryos are then loaded onto a CryoTop (Kitazato Biopharma Co., Ltd. Japan) and are immediately plunged into liquid nitrogen (LN2) for storage. At the time of warming the CryoTop is directly inserted into thawing medium (1.0 M sucrose) for 1 min at 37 °C. Warmed embryos are transferred to dilution I (0.5 M sucrose) and dilution II (0.25 M sucrose) respectively, for 3 min each. The embryos are then washed twice in washing medium (HEPES-buffered human tubal fluid containing 10 % human serum albumin). Embryos are considered to have survived if more than 50 % of the blastomeres are intact after warming.

During the designated PGD cycle, the frozen embryos were thawed on post-retrieval day 2 and cultured for another 24–28 h. Fresh or thawed day 3 embryos were biopsied for PGD and aCGH analysis and the decision for embryo transfer was made on day 5. Biopsy samples underwent whole genomic amplification (WGA) followed by independent analysis at nine polymorphic, genetically-informative alleles that flank the FXS gene. PGD data was compared to the haplotypes of the gamete providers to determine genetic risk status. A separate aliquot of the WGA of the same biopsy was used to

interrogate all 24 chromosomes using BAC microarrays (Cambridge BlueGnome, LTD).

Results

Of the 20 IVM cycles, 9 were minimally stimulated with clomiphene 200 mg daily for 5 days and 11 were unstimulated (natural). Once monofolliculogenesis had consistently occurred with clomiphene we transitioned to an unstimulated IVM cycle. On two occasions the patient requested we re-attempt clomiphene. Of the 20 IVM cycles, 17 cycles resulted in at least one oocyte retrieved while three cycles produced no oocytes at collection. There were a total of 28 oocytes retrieved; at the time of collection, 5 were germinal vesicles (GV) and 23 were mature oocytes (MII). Of the five GV, one was successfully matured and subsequently fertilized into a 2PN embryo, while among the 23 MII oocytes, 17 were fertilized into a 2PN embryo (Table 1).

HCG triggering occurred between cycle days 4–12 (median=cycle day 9) (Table 1). Among patients undergoing IVM cycles at our center, only the mean diameter (two largest perpendicular planes) of the LF is routinely documented at the

Table 1 Parameters and outcome per IVM cycle

Cycle #	Stimulation	HCG trigger cycle day	Largest Follicle (mm) on collection	GV oocytes	GV matured and fertilized (ICSI)	MII oocytes	MII fertilized (ICSI)
1	Clomid	7	10	0	–	2	1
2	Clomid	9	12	0	–	2	2
3	Clomid	7	12	0	–	2	2
4	Clomid	9	15	0	–	1	1
5	Clomid	10	12	0	–	1	1
6	None	7	10	2	0	2	1
7	Clomid	11	12	0	–	1	1
8	Clomid	12	10	0	–	1	0
9	None	8	12	0	–	1	1
10	None	12	10	1	1	1	1
11	None	8	Not recorded	0	–	0	0
12	None	12	8	1	0	1	1
13	None	11	10	0	–	1	1
14	Clomid	4	12	0	–	2	1
15	Clomid	7	Not recorded	0	–	0	0
16	None	6	10	1	0	2	1
17	None	6	Not recorded	0	–	0	0
18	None	9	6	0	–	1	0
19	None	10	12	0	–	1	0
20	None	11	10	0	–	1	1
Total				5	1	23	16

GV germinal vesicle; MII matured oocyte; ICSI intracytoplasmic sperm injection

No MI oocytes were collected in any cycle

time of oocyte retrieval, and in this case ranged from 6 mm to 15 mm (median=10 mm) (Table 1). The IVM cycle monitoring was performed by an ultrasonographer in the United States (US). Decisions regarding HCG triggering were based on these monitoring results and the patient came to Montreal, Canada for the oocyte retrievals accordingly. However, discrepancies were noted between the maximum follicular diameter in the US and on the day of oocyte retrieval in Montreal. The collection of lead follicles of <10 mm would not be anticipated based on the McGill IVM protocol and can be attributed to differences in monitoring at two different sonographic centers. All follicles were aspirated during the oocyte retrieval procedure, but specific data on how many MII oocytes were collected from follicles ≤ 8 mm is unavailable. However, based on lead follicle diameter data (Table 1) it is evident that several MII oocytes were collected from follicles ≤ 8 mm.

On the 13th cycle (natural IVM; Table 1) a single MII oocyte was retrieved, successfully fertilized and was not frozen. At this time six (out of 13) frozen embryos (day 2) were thawed and all six (100 %) survived and reached day 3 development. A total of 7 day 3 embryos were biopsied in our center and transported to Genesis Genetics in Detroit, Michigan for aCGH to screen for aneuploidy and PGD to rule out FXS mutation. In the interim, 6/7 (85.7 %) embryos reached the blastocyst stage. Only one blastocyst was diagnosed as both euploidic (3/6) and without the FMR1 gene mutation (1/6) and was transferred accordingly (Table 2). Daily luteal phase support included 100 mg progesterone intramuscularly and 6 mg estrace orally. At 5 weeks gestational age the patient acquired food poisoning accompanied by several days of fever ≥ 38.4 °C. Two ultrasounds, at 6 and 7 weeks gestational age, were consistent with a blighted ovum. Although there is no conclusive evidence that maternal fever caused the miscarriage, we are tempted to attribute the patient's first trimester illness to the arrested development of a chromosomally normal embryo.

A second attempt at pregnancy was undertaken with a similar protocol. On the 20th cycle (natural IVM; Table 1) a single mature oocyte was retrieved, successfully fertilized and was not frozen. At this time all remaining nine frozen embryos (day 2) were thawed, of which 8/9 (88.9 %) survived. Again, biopsies were performed on day 3 and embryos were cultured to blastocyst stage. Of the eight biopsies, only three had aCGH results available. Among the 5 day 3 embryos without a biopsy result, two developed into good quality blastocysts and a trophoectoderm re-biopsy was performed on day 5. In total there were five blastocysts with biopsy results available, of which three had both a normal PGD (4/5) and aCGH (3/5) diagnosis (Table 2). One blastocyst was transferred and the two others were frozen. The two embryos that were transferred (on cycle 13 and cycle 20) both originated from thawed embryos. The patient had a viable pregnancy and an amniocentesis

Table 2 Embryo biopsy and genetic screening results

	1st PGD and aCGH	2nd PGD and aCGH
Fresh Embryos (#) (Day 3)	1	1
Frozen/Thawed embryos (#) (Day 3)	6	9
Surviving Embryos (#) to Day 5	5	8
Total (Fresh+Thawed) embryos biopsied (#)	6	9
Embryos with results (#)	6	5^a
Embryos without FXS premutation (#)	1	4
Embryos without Aneuploidy (#)	3	3
Embryos without FXS premutation and Aneuploidy (#)	1	3
Embryos transferred (#)	1	1
Outcome	Blighted Ovum	Pregnancy

^a Two biopsy results originated from a trophoectoderm re-biopsy of a blastocyst

confirmed a chromosomally normal female without the FXS premutation. A live birth of a healthy term baby occurred.

Discussion

Given the history of three cancelled IVF cycles even with 600 IU daily of gonadotropins, this patient had two options for care with her own oocytes: either modified natural cycle IVF or IVM. Minimal stimulation IVF requires the use of 2 to 3 days of gonadotropins combined with gonadotropin releasing hormone (GnRH) antagonist. Minimal stimulation IVF is reported to have a 40 to 50 % cancellation rate due to premature ovulation, and failure to collect the oocyte [1]. This patient also would have had a risk of follicular arrest or regression when the GnRH antagonist was started, since 600 IU of gonadotropins daily failed to cause follicular development previously. Therefore, an IVM protocol was selected. It is a common error to believe that IVM results in the collection of only immature oocytes. Often mature oocytes are collected from both the LF, as well as follicles under 10 mm diameter, as demonstrated in this case. IVM may be a mechanism to collect several mature oocytes from young women with severe decreases in ovarian reserve, who would otherwise have had only a single oocyte collected with modified natural cycle IVF or stimulated IVF.

HCG-priming before egg retrieval in IVM cycles allows collection of both in-vivo mature oocytes as well as immature oocytes [20]. In HCG-primed IVM cycles in-vivo matured oocytes can be retrieved from follicles <10 mm with similar quality as larger follicles [17]. Although the chance to retrieve an in-vivo mature oocyte is greater when the LF is larger, we

have previously reported that with the McGill protocol >90 % of oocytes are MII when the LF is 12 mm at collection. Embryos produced from in-vivo matured oocytes had improved pregnancy outcomes in comparison to embryos from oocytes matured in-vitro [16]. Therefore, the McGill protocol specifies HCG administration when the LF reaches 10–12 mm. This timing is to ensure the presence of in-vivo matured oocytes on the day of collection and to avoid detrimental effects on the sibling immature oocytes [16, 18].

At our center, patients with a high AFC who undergo an IVM protocol and have more than one in-vivo mature oocyte at retrieval have a fertilization rate of 72.5 %, of which 90.4 % reach the cleavage stage [17]. Interestingly, in this case of DOR, 73.9 % (17/23) of the mature oocytes were fertilized, of which 100 % (17/17) reached at least day 2 development. Previous analysis at our center of 16 vitrified-thawed IVM cycles that included 83 cleavage stage embryos demonstrated an 85.5 % survival rate [19], which is similar to a thaw survival rate of 86.7 % (13/15) in this case.

The cost of the IVM cycles in this case was \$10,000 per three IVM treatments, for a total cost of \$70,000 (CAN), which is currently at par with US currency. The cost of two rounds of PGD and aCGH was \$7,700 (US) and \$4,150 (US) respectively. Therefore, the cost was \$20,462 per normal blastocyst and \$40,925 per pregnancy, noting that an additional two normal frozen blastocysts are available and may also result in a future pregnancy. Although cost-effectiveness analysis is noteworthy, it is important to take into context the clinical case of a patient with three previously cancelled IVF cycles and FMR1 associated DOR that may not have had an alternate option to conceive. Although there are case reports of utilizing IVM for PGD, patients have routinely been high responders allowing adequate embryos for biopsy [2, 3].

One of the most important factors affecting success in this case is the rather young age of the patient, likely permitting the collection of oocytes with a relatively high likelihood of creating a competent embryo. Although oocyte number was limited by the decreased ovarian reserve, young women with DOR have higher pregnancy rates than older women with equivalent clinical measures [12]. The rate of aneuploidy was 45.5 % (5/11) of cleavage biopsied embryos, which likely benefited from the patient's age. It is likely that the use of the IVM protocol in young women with severe DOR may potentiate the ability to collect more MII oocytes than IVF by exploiting the potential of small follicles to deliver mature eggs.

Our report is among only a handful of cases describing successful pregnancies and live births from using an IVM protocol in poor responders [5, 10, 11]. This subject represents the first case of a live birth obtained with an IVM protocol in FMR1 associated DOR. It should be noted that on several occasions mature oocytes were collected from follicles ≤ 8 mm in diameter, which may not have been possible if modified

natural cycle IVF was performed. This case represents a novel approach of vitrification of embryos to accumulate a sufficient sample for PGD and aCGH screening. A total of four chromosomally and genetically normal blastocysts were obtained and both transferred embryos resulted in a successful implantation. The role for IVM in young patients with DOR is still being investigated and this successful case adds support to its potential use.

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