Birth of a Healthy Male After Frozen Thawed Blastocyst Transfer Following Intracytoplasmic Injection of Frozen Thawed Testicular Spermatozoa From a Man with Nonmosaic Klinefelter's Syndrome

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We here report on a birth of a male, with normal karyotype, after frozen thawed blastocyst transfer following intracytoplasmic injection of frozen thawed testicular spermatozoa from an azoospermic 27-year-old man with nonmosaic Klinefelter's syndrome. Testicular sperm were retrieved by percutaneous needle biopsy.

KEY WORDS: Blastocyst; cryopreservation; intracytoplasmic sperm injection; Klinefelter's syndrome; testicular spermatozoa.

INTRODUCTION

Since Bourne *et al.* (1) reported in 1997 the first birth following intracytoplasmic sperm injection (ICSI) using ejaculated spermatozoa from a nonmosaic man with Klinefelter's syndrome, there have been other reports (2,3) on births after ICSI using testicular spermatozoa of nonmosaic 47,XXY men. We report a case where a combination of assisted reproductive technologies—testicular biopsy needle biopsy, cryopreservation of testicular sperm, intracytoplasmic sperm injection (ICSI), cryopreservation of blastocysts, and transfer of frozen thawed blastocysts—resulted in the birth of a healthy male infant to a man with nonmosaic Klinefelter's syndrome.

CASE REPORT

A 27-year-old man and his 26-year-old wife were referred to our fertility clinic after 4 years of primary infertility. Primary investigation had shown azoospermia in repeated semen samples.

Physical examination of the man revealed normal appearance and normal hair distribution. No gynecomastia was seen. Testicular volume as determined by orchidometry was estimated to be 6 mL bilaterally. Chromosome analysis of 25 blood cells showed a 47,XXY karyotype in all cells. Laboratory tests revealed FSH 36 IU/L, LH 16 IU/L, and testosterone 18 nmol/L. The spouse had been healthy, and her ovulatory cycles were regular.

Three months after a diagnostic bilateral percutaneous testicular needle biopsy (4) had revealed a few motile spermatozoa in specimens from both testes, the first ICSI treatment was carried out.

Ovarian hyperstimulation was achieved using down regulation with buserelin as nasal spray 0.9 mg daily (Suprecur[®], Hoechst, Frankfurt, Germany) and recombinant follicle stimulating hormone (r-FSH) (Gonal-F[®], Serono Nordic, Stockholm, Sweden) 150 IU for 12 days. Final oocyte maturation was

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induced with 10,000 IU hCG (Profasi[®], Serono Nordic, Stockholm, Sweden). Two oocytes were obtained at oocyte retrieval. Testicular biopsy was performed using a 14G Bard percutaneous cutting needle in local anaesthesia. A few spermatozoa, of which approximately 30% were motile, were identified after incubation of the biopsy specimen in Sperm Rinse medium (Vitrolife, Gothenburg, Sweden) and used for ICSI. However, neither of the two oocytes were fertilized. Cryopreservation of the biopsy material was performed by diluting the sample 1:1 with Freezing Medium in Test Yolk Buffer (Irvine Scientific, Santa Ana, California, USA) with glycerol as a cryoprotectant and cooling the samples in a programmable freezer (CryoLogic, Mount Waverley, Victoria, Australia) by $-8^{\circ}\text{C}/\text{min}$ to -6°C and $-5^{\circ}\text{C}/\text{min}$ to -30°C . The samples were held at -30°C for 15 min and cooled further to -43°C by $-4^{\circ}\text{C}/\text{min}$ before being plunged into liquid nitrogen.

A second similar treatment was carried out 4 months later using the same protocol but increasing the daily dose of r-FSH to 225 IU for the first 5 days, followed by 150 IU/day for six more days. This time 11 oocytes were retrieved. No spermatozoa were identified in a fresh testicular needle biopsy taken on the day of oocyte retrieval. The cryopreserved spermatozoa were thawed to room temperature, washed, and resuspended in Sperm Rinse medium and used for injection of all the oocytes. The oocytes were cultured in IVF medium (Vitrolife, Gothenburg, Sweden) and five of them showed fertilization with two pronuclei and two polar bodies. After checking for fertilization the oocytes were cultured in G.1.2 medium (Vitrolife, Gothenburg, Sweden). Two four cell embryos were transferred on Day 2 after ICSI. A pregnancy test carried out 14 days after embryo transfer was negative.

The other three embryos were cultured to Day 5 after fertilization, changing to G.2.2 medium (Vitrolife, Gothenburg, Sweden) on Day 3. Two blastocysts were cryopreserved on Day 5. One of them was expanded with a well-defined inner cell mass and many cells in the trophectoderm. The other blastocyst was not expanded and had an average number of cells in the inner cell mass and trophectoderm. For cryopreservation, a glycerol-based cryoprotectant medium (Freeze kit II, Vitrolife, Gothenburg, Sweden) was used. The two blastocysts were loaded into a straw after equilibration with cryoprotectant solution. They were placed in a programmable freezer (CryoLogic, Mount Waverley, Victoria, Australia), cooled from

room temperature to -6.5°C at a rate of $-2.0^{\circ}\text{C}/\text{min}$, and seeded during a 10-min holding period. They were then cooled to -35°C at a rate of $-0.3^{\circ}\text{C}/\text{min}$, held at -35°C for 5 min, and plunged into liquid nitrogen for storage.

The two blastocysts were thawed and transferred in a natural cycle 3 months later. They were rapidly thawed, first in air for 30 s, and then in a water bath at room temperature for 30 s. The cryoprotectant was removed stepwise using thaw kit II Vitrolife. The two blastocysts were transferred 2 h after recovery. This time the pregnancy test was positive 2 weeks after the frozen thawed blastocyst transfer. An early ultrasound scan showed that a singleton pregnancy had been established. Amniocentesis for karyotyping was carried out in pregnancy week 15, showing normal male, karyotype 46,XY. A healthy boy, weighing 4400 g, was delivered after 41 weeks of gestation in December 2000.

DISCUSSION

Two pregnancies among 11 transfers of frozen thawed human blastocysts were reported in 1985 (5). The blastocysts had been cultured in Earle's solution containing pyruvate and human serum, and glycerol had been used as a cryoprotectant. A large retrospective analysis of 516 transfers of frozen thawed blastocysts obtained by co-culture on Vero-cells gave a pregnancy rate of 21.7% per transfer (6). Slow freezing and two-step addition of glycerol had been used. Sequential medium has been increasingly used in blastocyst cultures and successful pregnancies after cryopreservation of such blastocysts have been reported (7). We describe a term pregnancy and birth of a healthy infant using frozen thawed blastocysts, after ICSI with frozen thawed testicular spermatozoa from a nonmosaic 47,XXY man. To our knowledge, no similar combination of procedures has been reported before.

Culturing embryos to the blastocyst stage is one strategy to improve pregnancy rates by better selection of viable embryos (8). There have been reports on high pregnancy rates after transfer of fresh blastocysts due to the use of new sequential culture media (9,10). Adopting culturing of embryos to the blastocyst stage will also increase the need for cryopreservation of blastocysts after IVF and ICSI.

In our unit, a programmed slow freezing method, using a medium containing glycerol, has been successful for survival of the blastocysts after thawing

and also for the establishment of pregnancies, among others the one reported here.

In men with nonmosaic Klinefelter's syndrome, the chromosomes of the spermatozoa have been studied using fluorescent in situ hybridization, showing aneuploidy in 3.48–21.76% (11,12). Of the testicular spermatozoa 6.3% had chromosomal abnormalities (13). In mosaic 47,XXY men the chromosomal abnormalities have been fewer (14).

Although the percentage of normal spermatozoa is much higher than that of the abnormal ones, there is an increased possibility of having offspring with an extra X chromosome. Individuals with karyotypes 47,XXY are not severely affected. However, these males are infertile. Therefore, if the couple wishes, prenatal genetic diagnosis can be carried out. In our case the cells cultured from amniotic fluid showed a normal male karyotype.

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