Seven pregnancies and deliveries from non-mosaic Klinefelter syndrome patients using fresh and frozen testicular sperm

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Received: 26 September 2006 / Accepted: 4 October 2006 / Published online: 20 December 2006
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Abstract *Purpose:* The aim of this study was to investigate the feasibility of using frozen-thawed testicular sperm as well as the timing of testicular sperm extraction (TESE) in patients with non-mosaic Klinefelter syndrome.

Methods: Intracytoplasmic sperm injection (ICSI) was performed in six of 17 (35%) patients whose sperm was recovered by TESE. Multiple biopsies of both testes were performed on the day of oocyte retrieval in all but one of the six patients.

Results: Seven pregnancies and deliveries were achieved in five couples, and one couple was unsuccessful. Five pregnancies were achieved using fresh motile sperm, and two were achieved using frozen-thawed sperm. Sperm cryopreservation was not possible in one of the five couples because of the small number of recovered sperm, and possible in four other couples for subsequent ICSI. One woman whose husband had TESE performed prior to ovarian stimulation did not become pregnant. This may be due to the attainment

of only a few immotile sperm following the frozen-thawed procedure.

Conclusion: The outcome of ICSI using fresh or frozen-thawed testicular sperm in patients with non-mosaic Klinefelter syndrome was identical; however, TESE should be performed on the day of oocyte retrieval until such time as a procedure with a higher sperm yield from TESE is available. Moreover, an improved recovery procedure after cryopreservation-thawing of a single spermatozoon must be developed.

Keywords Non-mosaic Klinefelter syndrome · ICSI · Cryopreserved-thawed testicular sperm · Testicular sperm extraction · TESE · TESE timing

Introduction

Approximately one of 1,000 male newborns has the 47, XXY Klinefelter syndrome, which is one of the most common sex chromosome anomalies in azoospermic Japanese men; its prevalence is 7.4% [1]. Several reports have described successful pregnancy outcomes in these patients using fresh testicular sperm obtained on the day of oocyte retrieval [2–7]. However, few reports in the literature exist in regard to using cryopreserved sperm for intracytoplasmic sperm injection (ICSI) [8–11].

We report five pregnancies and deliveries following ICSI using fresh sperm and two pregnancies and deliveries following ICSI using cryopreserved-thawed testicular sperm from five patients with non-mosaic Klinefelter syndrome. We also discuss the timing and the improvement of the sperm yield for testicular sperm extraction (TESE); furthermore, we describe the cryopreservation procedure for the scant testicular sperm retrieved from these patients.

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Case reports

Seventeen of 214 azoospermic patients referred to our institution between 1997 and 2005 were confirmed to have non-mosaic Klinefelter syndrome by previously described procedures [3]. The couples underwent genetic counseling in regard to the etiology of their infertility and the potential risks of using retrieved testicular sperm for ICSI. All 17 non-mosaic couples agreed to TESE.

Multiple biopsies of both testes were performed under local anesthesia with sedation on the day of oocyte retrieval except patient 6. And the testicular tissue was examined for hours. Sperm were found in the minced testicular tissue from six of these patients. For the initial treatment, fresh sperm were used in patients 1 through 5; however, in patient 6, a few immotile frozen-thawed sperm were used as the initial treatment because TESE was performed prior to ovarian stimulation. We obtained ample sperm for cryopreservation in patients 1, 2, 4, and 5; however, we obtained an inadequate amount of testicular sperm for cryopreservation in patient 3. For subsequent treatments, cryopreserved testicular sperm were used for ICSI in patients 1 and 2.

Testicular tissue was excised and minced using a sterile glass in a dish with 7 ml of modified-human tubal fluid (m-HTF) medium. An inverted microscope (400×) was used to check for motile sperm. When no sperm were observed, collagenase was added to the minced specimen in a test tube and incubated for 30 min at 37°C. The specimen was agitated, and then incubated for an additional 30 min at 37°C. The specimen was placed in the upper 3 ml of Isolate (Irvine Scientific; Irvine, CA, USA), and centrifuged at 400 g for 20 min; it was then washed with m-HTF and finally suspended in about 0.1 ml of the medium.

In the cryopreserved cases, the final sample was mixed in a 1:0.7 ratio with sperm freezing medium and aliquoted into one or more cryo-tubes, depending on the amount of sperm obtained. Those cryo-tubes were suspended in vapor approximately 1 cm above liquid nitrogen (LN₂) for 15 min, and then immersed in LN₂ for storage at −196°C.

Following retrieval from the LN₂ tank, the cryo-thawed tube was placed at room temperature for 10 s, and then incubated in water at 30°C for 10 min. The thawed specimen was placed in the upper 3 ml of Isolate, centrifuged at 400 g for 20 min, subsequently washed with m-HTF, and finally suspended in approximately 0.1 ml of the medium. This sample was divided into several aliquots for thorough examination under an inverted phase-contrast microscope. ICSI was performed using motile fresh or cryopreserved-thawed testicular sperm.

Ovarian stimulation was carried out by pituitary desensitization with a GnRH agonist (Suprecur; Aventis, Japan) or a GnRH antagonist (Cetrorelix; Serono, Switzerland) combined with recombinant FSH (either Gonal F; Serono,

Switzerland or Puregon; Organon, The Netherlands) or hMG (Humegon; Organon, The Netherlands) and hCG (Profasi; Serono, Switzerland). Oocyte retrieval was carried out using ultrasound-guided puncture 36 h following hCG administration.

After assessment of the cleaved embryo quality on day 2 or day 3 by the modified Veeck's criteria [12], and the blastocyst quality on day 5 or day 6 by Gardner's criteria [13], embryo transfer was performed. Clinical pregnancy was defined as a gestational sac with a fetal heart beat by ultrasonography. Characteristics of the six couples obtained testicular sperm by TESE and the outcome of ICSI are presented in Table 1.

Blood hormone levels (LH, FSH, and T) and testicular volume were not significantly different; however, the patient age in the positive group for sperm recovery was significantly lower than that of the negative group ($P < 0.05$) in Table 2. The Student's t-test was used for statistical analysis. $P < 0.05$ was considered to be statistically significant.

Testicular histology revealed hypospermatogenesis in Patient 1, and Sertoli cell only syndrome in Patients 2 through 6. Fortunately, all eight infants from the seven pregnancies had a normal karyotype at birth.

In Patient 1, four oocytes were collected following the GnRH agonist long protocol, and two of the four were fertilized with fresh motile sperm; these two embryos (8 cell, grade 2; and 5 cell, grade 4) were transferred. She delivered a single 3542 g healthy female. Two years later, six oocytes were retrieved following the GnRH agonist long protocol; following ICSI with motile frozen-thawed sperm, five of the six were fertilized. Five cleaved embryos on day 3 (8 cell, grade 2; 6 cell, grade 2; 6 cell, grade 2; 6 cell, grade 4; and 3 cell, grade 5) developed into two blastocysts on day 5. These two blastocysts (grade 1 and grade 5BC) were transferred on that day, and a successful pregnancy ensued. Two gestational sacs developed, and the patient subsequently delivered healthy twin infants: a 2664 g female and a 2602 g male.

In Patient 2, seven oocytes were collected following the GnRH antagonist with low dose hCG protocol [14]. ICSI was performed using motile fresh sperm. All of the seven mature oocytes were fertilized. Cleaved embryos on day 3 were: 8 cell, grade 1; 7 cell, grade 2; 7 cell, grade 3; 5 cell, grade 3; 4 cell, grade 1; 4 cell, grade 2; and 2 cell, grade 1. An 8 cell embryo (grade 1) on day 3 and a blastocyst (grade 3AA) on day 5 were transferred. The patient subsequently delivered a 3290 g healthy female. Three years later, four oocytes (one MI, immature oocyte and three MII mature oocytes) were collected following the GnRH antagonist with the low dose hCG protocol. ICSI was performed using motile frozen-thawed sperm. All three MII oocytes were fertilized, and three good-quality cleaved embryos (9 cell, 10 cell, and 11 cell; all grade 2 on day 3) developed. After extending the culture, two better-quality blastocysts (grade 3BC and grade 4BC on day 5) were transferred. One gestational sac

Table 1 Characteristics of patients with non-mosaic Klinefelter syndrome and ICSI outcome using fresh and cryopreserved sperm

	KS patient age (yr)	Spouse age (yr)	LH (mIU/ml)	FSH (mIU/ml)	PRL (ng/ml)	T (ng/ml)	TV (ml)	Sperm	Clinical Outcome
1	28	31	8.4	18.7	15.8	3.7	4/5*	Fresh	Delivered 3542 g F
								Frozen	Delivered 2664 g M; 2602 g F)
2	34	35	12.8	48.6	11.5	5.7	4/3	Fresh	Delivered 3290 g F
								Frozen	Delivered 3008 g F
3	32	32	7.2	20.1	12.4	1.1	1/1	Fresh	No pregnancy
								Fresh	Frozen-thawed blastocyst transferred; delivered 3188 g M
4	36	36	11.4	27.1	6.0	2.6	2/2	Fresh	Delivered 2478 g F
5	25	24	11.6	22.4	37.0	3.8	4/4	Fresh	Delivered 3100 g M)
6	26	26	25.1	54.1	8.1	1.9	4/4	Frozen	No pregnancy

Note. KS: Klinefelter syndrome, TV: testicular volume, F: female, M: male, *: left/right, WG: weeks' gestation. All infants were found to have a normal karyotype at birth.

Table 2 Sperm recovery from non-mosaic Klinefelter syndrome patients

	Testicular sperm recovery		
	Positive	Negative	
Number of patients	6	11	
Age	30.2 ± 3.9	37.6 ± 4.4	P < 0.05
Testicular	R 3.2 ± 1.4	R 2.1 ± 1.2	NS
Volume (cm ³)	L 3.2 ± 1.6	L 1.8 ± 1.3	NS
FSH (mIU/ml)	31.4 ± 16.8	38.1 ± 11.1	NS
LH (mIU/ml)	12.8 ± 7.1	11.6 ± 4.5	NS
T (ng/ml)	3.0 ± 1.7	2.2 ± 1.6	NS

Note. Mean ± SD. Student's *t*-test was used for statistical analysis.

developed, and the patient subsequently delivered a 3005 g healthy female.

In Patient 3, eight oocytes were collected following the GnRH antagonist with low dose hCG protocol and seven of seven mature oocytes were fertilized using motile fresh sperm. Scant sperm were retrieved; therefore, cryopreservation was not done. On day 3, three of the seven embryos (8 cell, grade 1; 7 cell, grade 2; 6 cell, grade 2; 6 cell, grade 3; 4 cell, grade 4; 3 cell, grade 5; and 3 cell, grade 5) developed into blastocysts. Two blastocysts (grade 4AB and grade 5AC) were transferred. A blastocyst (grade 3BB) was cryopreserved for the next attempt. The first blastocyst transfer using fresh sperm was unsuccessful. One year later, the frozen-thawed blastocyst (grade 3BB) was transferred; subsequently, a 3188 g healthy male was born.

In Patient 4, six oocytes were collected following the GnRH agonist long protocol. Using motile fresh sperm, three of the five mature oocytes were fertilized; two good-quality cleaved embryos (both 8 cell, grade 1) were transferred, and one embryo (7 cell; grade 4) was discarded. One gestational sac developed and a 2478 g healthy female was born.

As yet, this patient has not undergone another ICSI.

In Patient 5, 12 oocytes were collected following the GnRH antagonist with low dose hCG protocol. Nine of the twelve mature oocytes were fertilized by ICSI using motile fresh sperm. All nine fertilized zygotes cleaved (8 cell, grade 1; 8 cell, grade 2; 7 cell, grade 3; 7 cell, grade 4; 6 cell, grade 4; 5 cell, grade 5; 4 cell, grade 5; 4 cell, grade 5; and 4 cell, grade 5). A single high-quality embryo (8 cell, grade 1) was transferred on day 3 and subsequently a gestational sac formed. She delivered a healthy male infant weighing 3100 g. The eight surplus embryos were cultured until day 5; two developed to the blastocyst stage. Using vitrification, a blastocyst (grade 5BB) was cryopreserved on day 5 and a blastocyst (grade 5BC) was cryopreserved on day 6.

In Patient 6, 18 oocytes were collected following the GnRH agonist long protocol. TESE was performed two months prior to oocyte retrieval and cryopreserved. We obtained 17 mature oocytes; however, ICSI was performed on only 11 of them because of a low yield of sperm

after thawing (regrettably, six good-quality oocytes had to be discarded). We were concerned that the use of the hypo-osmotic swelling test could result in a loss of the 11 sperm; therefore, we performed ICSI with thawed immotile sperm without utilizing the test. Only two were fertilized using these frozen-thawed sperm, and one of the embryos cleaved. A six cell embryo (grade 4) was transferred; however, a pregnancy did not ensue.

Discussion

Few reports exist in the literature concerning the use of cryopreserved sperm for ICSI in patients with non-mosaic Klinefelter syndrome. We previously reported that testicular frozen sperm from patients with non-obstructive azoospermia were of poorer quality than frozen specimens from patients with obstructive azoospermia [15]. Two investigators [8, 10] compared fertilization rate, embryo cleavage rate, embryo transfer rate, and implantation rate between ICSI series using fresh testicular spermatozoa and using cryopreserved-thawed testicular spermatozoa in patients with non-mosaic Klinefelter syndrome. They concluded that the outcome of ICSI using either type of sperm was similar. Both those previous reports and our present results of two couples who delivered using both fresh and cryopreserved-thawed testicular sperm support the use of cryopreserved-thawed testicular spermatozoa from patients with non-mosaic Klinefelter syndrome.

Our results were less favorable in patients 3 and 6; only a few fresh sperm were obtained and cryopreservation was not done in patient 3; only a low yield of sperm was obtained after thawing in patient 6. The method using the zona pellucida from a hamster has been reported to be an ideal sterile vehicle for cryopreservation of a single spermatozoa [16]. This method is very reliable; however, an ethical problem exists. Although several methods using the cryoloop, the cryotop, the straw, and the cryo-tube have subsequently been employed, the recovery rate of motile sperm was low [17, 18]. A higher recovery procedure must be developed for the cryopreservation and thawing of a single human spermatozoon. Based on the two aforementioned studies, the percentage of successful sperm attainment by TESE in Klinefelter patients ranges between 21% and 45% [19, 20].

Microdissection TESE reported by Schlegel et al. improved the sperm retrieval rate to 60% or more [10, 21, 22]. In an attempt to improve the sperm retrieval rate, in January 2006 we began using microdissection TESE. Pretreatment with an aromatase inhibitor, clomiphene citrate, hCG, and rFSH before TESE may improve the sperm retrieval rate [21, 22, 23].

Another group of researchers [24] reported a sperm detection rate of up to 60% that was markedly age-dependent in patients with non-mosaic Klinefelter syndrome, and rec-

ommended that the procedure be done before the critical age of 35 years.

Although most investigators have noted that there are no clinical parameters predicting successful sperm retrieval in the patients with non-mosaic Klinefelter syndrome [25, 26], we found similar results to those of Okada et al. in our patient population. The patients with retrievable sperm (30.2 years) were significantly younger ($p < 0.05$) than those with no detectable sperm (37.6 years) (Table 2).

If no sperm are obtained from the testes on the day of oocyte retrieval, the couple must determine whether to undergo similar future attempts or to use donor sperm. Alternatively, oocytes can be cryopreserved [27] in the anticipation of medical advances (i.e., in vitro culture).

In conclusion, microdissection TESE in patients, especially under the age of 35 years with non-mosaic Klinefelter syndrome should be performed on the day of oocyte retrieval. The ultimate goal of the couple is to achieve a healthy infant, not to obtain testicular sperm. At present, microdissection TESE is the best method for achieving a pregnancy in these couples.

The frequency of sex chromosomal hyperploidy and autosomal aneuploidies is higher in sperm from patients with Klinefelter syndrome than that from normal men [28]. One investigative group [4] reported the conception of a 47, XXY fetus after ICSI of spermatozoa from a patient with non-mosaic Klinefelter syndrome. Another group [29] reviewed 21 reports and noted that a 47, XXY was one of 39 successful pregnancies fathered by non-mosaic Klinefelter patients. Another group [30] demonstrated that a significant drop in the percentage of normal embryos occurred in couples with Klinefelter syndrome (54.0%) compared to controls (77.2%). Moreover, a significantly increased risk of abnormalities was observed for both sex chromosomes and autosomes. The use of ICSI + PGD or prenatal diagnosis should be carefully considered. Genetic counseling is of great value for men with Klinefelter syndrome and their spouses; they can be apprised of the genetic disorder and the potential risk to their offspring. Following counseling, these couples can make the final decision in regard to treatment.

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