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Outcome of Intracytoplasmic Injection of Sperm Obtained by Testicular Sperm Extraction from 14 Azoospermic Men Suffering from 47,XXY Non-mosaic Klinefelter's Syndrome

Chun-Ming Chiang*, Chuan-Ju Lin, Liang-Ming Lee[1], Shih-Min Chen[2]

Jiahn-An OB/GYN Clinic and Infertility Center, Keelung, [1]Department of Urology, Taipei Municipal Wan-Fang Hospital, and [2]Taipei Municipal Women's and Children's Hospital, Taipei, Taiwan.

SUMMARY

Objective: The purposes of this study were to evaluate the potential for testicular sperm extraction (TESE) from azoospermic patients with non-mosaic Klinefelter's syndrome and to determine the outcome of intracytoplasmic sperm injection (ICSI) using the extracted testicular sperm sample.

Materials and Methods: Fourteen couples suffering from primary infertility in which the male partner had the azoospermic non-mosaic 47,XXY karyotype (Klinefelter's syndrome) participated in this study. All of the women underwent controlled ovarian hyperstimulation. Open testis biopsies were conducted 1 day prior to or on the day of oocyte retrieval. Motile sperm, extracted from the biopsied tissues in a wet preparation, were used for ICSI. The outcome of ICSI was evaluated from the fertilization rate, embryo-cleavage rate, clinical pregnancy rate, and chromosomal status of resultant fetuses or delivered babies.

Results: Sperm retrieval was successful in eight of 14 patients (sperm retrieval rate, 57%). In total, 118 mature oocytes were injected with extracted motile spermatozoa. This resulted in the production of 70 fertilized oocytes (fertilization rate, 59%) and 67 embryos (cleavage rate, 96%). Among the eight women who underwent embryo transfer, six achieved clinical pregnancies (clinical pregnancy rate, 75%). The outcome of these pregnancies included one blighted ovum and the birth of four male and five female healthy babies. The live delivery rate was 62.5%. All of these babies were chromosomally and physiologically normal.

Conclusion: This study demonstrates that azoospermic patients suffering from non-mosaic Klinefelter's syndrome can father their own genetic offspring when the TESE procedure is combined with ICSI and embryo transfer techniques. [*Taiwanese J Obstet Gynecol* 2004;43(2):88–96]

Key Words: azoospermia, intracytoplasmic sperm injection, 47,XXY karyotype, Klinefelter's syndrome, testicular sperm extraction

Introduction

Klinefelter's syndrome, initially described in 1942, is characterized by hypogonadism, small testes, azoospermia, and gynecomastia [1]. Patients with Klinefelter's syndrome have an additional X chromosome [2]. About 90% of affected patients have the non-mosaic form of this syndrome, with a chromosomal constitution of 47,XXY, and this form is believed to arise due to meiotic non-disjunction of the chromosomes during gametogenesis [3]. The remaining 10% of patients have the mosaic form, the usual chromosomal constitution of which is 46,XY/47,XXY; this form is thought to result from chromosomal mitotic non-disjunction arising after fertilization of the zygote [3].

Correspondence to: Dr. Chun-Ming Chiang, Jiahn-An OB/GYN Clinic and Infertility Center, 78 Zen-Er Road, Keelung, Taiwan.
E-mail: k0869091@ms23.hinet.net
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Klinefelter's syndrome is the most frequent sex chromosomal abnormality, occurring in 0.1% of the general phenotypic male population [4]. The incidence can be 3.1% in the infertile male population [5] and 11% in the azoospermic male population [6,7]. Klinefelter's syndrome in the mosaic form sometimes presents with severe oligospermia, although these patients are able to father their own genetic offspring by a combination of oocyte retrieval, intracytoplasmic sperm injection (ICSI), and embryo-transfer techniques [8]. By contrast, men suffering from non-mosaic Klinefelter's syndrome are typically azoospermic and are considered by many to be sterile. The observation that altered X chromosome dosage elicits germ-cell loss during testicular development has been made for virtually all male mammals, including humans [9], such that virtually no sperm cells are present in adult XXY testes [10–12]. However, rare break-through patches of spermatogenesis-active tissue have been reported in the testes of non-mosaic 47,XXY males [13–15], and occasional motile spermatozoa have been observed in the ejaculate of patients exhibiting the non-mosaic 47,XXY karyotype [10,11,15].

As pregnancy has been achieved using testicular sperm extraction (TESE) combined with ICSI in patients with abnormal seminiferous tubular function [16], several successful attempts at achieving pregnancy have been made in azoospermic patients suffering from non-mosaic Klinefelter's syndrome using this TESE-ICSI technique. This procedure offers such patients the opportunity to bear their own genetic offspring. According to our review of the literature, about 23 deliveries have been reported following ICSI using testicular spermatozoa extracted from the testes of azoospermic patients suffering from non-mosaic Klinefelter's syndrome [17–24].

The purpose of this study was to evaluate the feasibility and outcome of ICSI using testicular spermatozoa extracted from azoospermic individuals suffering from non-mosaic Klinefelter's syndrome.

Materials and Methods

Patient characteristics

During the period from May 1998 to December 2001, 14 consecutive azoospermic patients, aged 21 to 39 years, were diagnosed as suffering from non-mosaic Klinefelter's syndrome following cytogenetic evaluation of peripheral blood lymphocytes. These individuals all sought infertility treatment at our infertility clinic and were enrolled into this study.

All patients underwent a complete history and physical examination. Serum hormones were analyzed at the initial interview in 12 of these patients, and included luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, and prolactin. The serum inhibin B level was checked in four patients. Prior to undergoing testicular biopsy for TESE, all patients needed to undergo at least three separate medical examinations to evaluate ejaculate using extensive sperm preparation (ESP) to search for the occasional motile spermatozoa that would be suitable for use in subsequent ICSI [25]. Such activity would have enabled us to avoid a testicular biopsy in some patients.

Prior to undergoing TESE-ICSI, all patients were advised regarding the potential genetic risks to their future offspring resulting from the use of their own sperm. All patients gave informed consent for participation in the study.

Testis biopsy, testicular sperm retrieval, and sperm preparation

A testicular biopsy was conducted on either the day prior to or the day of oocyte retrieval. Open testicular biopsy for TESE was performed as described by Tournaye et al [15]. Briefly, all patients received spinal anesthesia and, subsequent to a hemiscrototomy, the scrotal contents were inspected and small multifocal biopsies, rather than a single large biopsy, were taken. If an intraoperative search of the fresh tissues in the wet preparation identified motile spermatozoa, testicular sampling was halted. If no motile spermatozoa were observed, more biopsies were taken until the entire testicular mass had been randomly sampled. Careful hemostasis was performed by means of unipolar cauterization, and both testicular and scrotal incisions were closed using interrupted resorbable sutures.

Biopsied testicular samples intended for sperm extraction were transferred to a Petri dish (Falcon 3002; Becton Dickinson Labware, Franklin Lakes, NJ, USA) with pre-warmed HEPES-human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA, USA) and 3% synthetic serum substitute (SSS; Irvine Scientific). The method of sperm extraction from testicular tissue was modified from that reported by Schlegel et al [26]. One small sample of tissue per testis was sent for histology. Initially, testicular samples were dispersed and stretched to isolate individual seminiferous tubules using sterile glass slides. Subsequently, mechanical dispersal of the tubules was accomplished by mincing of the extended tubules in pre-warmed HEPES-HTF/3% SSS using a sterile pair of scissors.

Intraoperatively, a wet preparation of the suspension was observed under an inverted microscope at 200× and 400× magnification to identify motile spermatozoa. The testicular tissue was then processed for sperm retrie-

val according to the following technique. The dispersed and minced testicular tissue was gently mixed with prewarmed HEPES-HTF/3% SSS and centrifuged at 500*g* for 5 minutes after incubation at 37°C under 5% CO_2 in air for 1 hour. The supernatant was further centrifuged at 1,800*g* for 5 minutes and the pellets were examined for the presence of motile spermatozoa. If motile spermatozoa were observed, the pellet was resuspended in 100 μL of HEPES-HTF/3% SSS and incubated at 37°C in 5% CO_2 in air for subsequent ICSI. If more than 1.0×10^6 erythrocytes/mL were observed in the sample, it was treated with erythrocyte-lysing buffer (Sigma Chemical Co, St Louis, MO, USA) to increase the chance of visualizing any motile spermatozoa that may have been present [26]. In some cases, extensive searching, sometimes for several hours, was needed to find motile spermatozoa. If we failed to detect motile spermatozoa following mechanical mincing and erythrocyte lysis, all residual tissue pieces from the shredded biopsies were collected and further dissociated by the addition of 800 IU/mL collagenase (Sigma C-5138) and 10 μg/mL DNase (Sigma DN-25) in an attempt to maximize the chance of sperm retrieval [27].

Protocol for ovarian stimulation, oocyte retrieval, and ICSI

Stimulating the growth of multiple oocytes in female partners was conducted using either an ultra-short or a long intranasal spray ovarian-stimulation protocol. For the ultra-short protocol, female partners received a daily subcutaneous injection of 0.5 mg buserelin acetate (Supremon; Hoechst AG, Frankfurt, Germany) from days 2 to 4 of their menstrual cycle. Both human menopausal gonadotropin (HMG; Serono, Auboune, Switzerland) and human urinary FSH (Serono) or recombinant FSH (Gonado-F, Serono, or Puregon, Organon, Oss, Netherlands) were administered from day 3. The initial dose of human gonadotropin depended on the basal serum LH and FSH levels and the total antral follicle count in both ovaries as revealed by a precycle examination on day 3. After day 9, the gonadotropin dose was adjusted according to both the serum absolute estradiol (E2) level and the patterns of follicular growth and rise in serum E2 level. When the three leading follicles reached 18 mm in diameter and the serum E2 level was about 100–200 pg/mL per follicle larger than 12 mm in diameter, a bolus of 10,000 IU of human chorionic gonadotropin (HCG; Serono) was injected intramuscularly to encourage final maturation of the oocytes.

For the long protocol, buserelin acetate intranasal spray (Supremon) was administered at 800 μg/day, divided into four separate doses, in the luteal phase of the participant's menstrual cycle preceding the IVF cycle. On day 2 of the participant's menstrual cycle, the serum E2 level was determined to ensure that pituitary desensitization had been successfully achieved. When the serum E2 level was less than 30 pg/mL, the dose of buserelin acetate was dropped to 400 μg/day and both HMG and urinary FSH or recombinant FSH were administered to stimulate multiple oocyte growth. The criteria for administering the initial dose of HMG and FSH, those for the adjustment of the dose subsequent to day 9, and those appropriate for the time of HCG injection were similar to those for the ultra-short protocol.

Ultrasound-guided transvaginal oocyte retrieval was performed under light general anesthesia at about 34 to 36 hours after administration of HCG. ICSI was performed according to the method of Van Steirteghem et al [28].

Embryo transfer, luteal support, embryo cryopreservation, and fetal karyotyping

At about 16 to 20 hours following the injection of oocytes with sperm, fertilization was assessed by looking for the presence of pronuclei within the injected oocytes. Fertilization was considered normal when two pronuclei were present. Appropriate grading criteria for embryo quality were applied, and the process was conducted according to the standard described by Scott et al [29].

Under normal circumstances, no more than three of the best-quality embryos were transferred into the uterus of the participating woman via the uterine cervix on day 3 following oocyte retrieval, under full-bladder status and abdominal ultrasound guidance using a Labotec (Labotec GmbH, Labor–Technik, Gottingen, Germany) or an Edwards-Wallace (SIMS Portex, Hythe, Kent, UK) embryo-transfer catheter. On a few occasions, we transferred more than three embryos, but never more than six, if we had specifically been requested to do so by a participating couple who felt that the quality of a frozen embryo was inferior to that of a "fresh" embryo, and that the transfer of an embryo via a frozen cycle might result in a decrease in the pregnancy rate. All couples who requested transfer of more than three embryos were informed of the possibility of the pregnancy resulting in a relatively high order of multiple births. All such prospective parents consented to fetal reduction if multiple pregnancies (≥ 3) were achieved. If present, surplus embryos were frozen using a slow-cooling method [30].

For luteal support, natural micronized progesterone (Utrogestan; Piette International Laboratories, Brussels, Belgium) 200 mg/day was administered in three doses into the vagina from the day of oocyte retrieval and for the following 16 days, at which time the serum β-HCG

level was determined to assess pregnancy status. All pregnant women received additional micronized progesterone (600 mg/day) until a gestational period of 10 weeks was reached. Clinical pregnancy was defined as the sonographic demonstration of a gestational sac with a visible fetal heart beat. Amniocentesis for fetal karyotyping was recommended for all women who achieved clinical pregnancy of 16 weeks' gestation. For couples who achieved pregnancy but were not willing to undergo amniocentesis, cytogenetic evaluation of their fetuses was not conducted until delivery had occurred.

Results

Physical features and serum hormone characteristics of azoospermic patients with non-mosaic Klinefelter's syndrome observed in our study are presented in the Table. The mean age of male patients was 33 ± 4 years (range, 28–39 years) and of female partners was 29 ± 5 years (range, 21–39 years). The mean serum baseline FSH level in male patients (39.33 ± 19.99 mIU/mL; range, 13.2–83.6 mIU/mL) was somewhat elevated compared to that in the normal population (2.0–10.0 mIU/mL), while mean serum baseline testosterone level (1.72 ± 0.85 ng/mL; range, 0.46–3.18 ng/mL) appeared to be below or near normal levels (2.7–10.7 ng/mL). The levels of patient serum inhibin B varied from less than 15 pg/mL to 164.25 pg/mL. Around half of our patients exhibited mild gynecomastia and all patients had small testicles (volume, 2–4 mL). Six individuals had previously undergone testis biopsy for simultaneous histologic examination and investigation for the presence of spermatozoa at other infertility centers prior to coming to our infertility clinic for treatment. At that time, two of these six individuals had undergone orchiopexy to treat an undescended testis in connection with the testicular biopsy. No spermatozoa had been recovered from any of these patients during the procedure. We found sperm in the ejaculate of two of the 14 patients by ESP. Sperm from one of these patients was completely non-viable and could not be used for ICSI. Sperm from the other patient could be used, avoiding the need for testicular biopsy, so this patient was not enrolled in the study.

Motile spermatozoa were found in the wet preparation of biopsied testicular tissue from eight patients, giving a retrieval rate of 57%. Two individuals who had spermatozoa retrieved in this attempt had previously undergone testicular biopsy at another medical center, one of whom had undergone orchiopexy in connection with the testicular biopsy at that time. Motile spermatozoa were retrieved by TESE from the patient whose ejaculate had revealed dead spermatozoa following the ESP. For all patients, histologic assessment of the biopsied testicular tissue revealed tubal hyalinization, no evidence of spermatogenesis, the absence of germ cells, and relative hyperplasia of observed Leydig cells.

Table. Physical and serum hormone characteristics of 14 azoospermic patients with non-mosaic Klinefelter's syndrome

Patient no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Mean ± SD
Female age (yr)	31	29	39	30	21	27	28	26	27	25	35	29	26	34	29 ± 4.58
Male age (yr)	37	30	38	36	29	34	35	31	29	28	39	32	30	39	33 ± 3.97
Body height (cm)	176	169	ND	173	170	180	176	169	ND	172	ND	175	175	170	173 ± 3.54
Body weight (kg)	86	64	ND	62	76	92	106	55	ND	91	ND	54	78	78	77 ± 16.59
LH (mIU/mL)	32.7	19.75	ND	18.6	18	12.32	28.6	28.5	18.9	21.6	16.1	34.1	46.84	ND	24.67 ± 9.77
FSH (mIU/mL)	15.6	46	ND	40.1	65.9	29.72	32.7	30.2	49.7	13.2	36.05	29.2	83.6	ND	39.33 ± 19.99
Prolactin (ng/mL)	10.4	13.54	ND	20.6	19.8	9.85	7.31	11.4	11.4	13.2	7.29	19.7	13.32	ND	13.15 ± 4.63
Testosterone (ng/mL)	2.24	3.18	ND	0.91	0.51	2.95	1.32	1.58	2.01	1.58	2.08	1.78	0.46	ND	1.72 ± 0.85
Inhibin B (pg/mL)				66.1	164		64.2			< 15.0					
Gynecomastia	+	–	–	–	+	+	–	–	+	+	–	+	–	+	
Previous testis biopsy*	+	+	–	–	–	–	–	–	–	+	+	+	–	+	
Orchiopexy	–	–	–	–	–	–	–	–	–	+	–	+	–	–	
Sperm found by ESP	+	–	–	–	–	–	–	–	–	–	–	–	–	–	
Sperm found by TESE	+	–	+	+	+	+	–	+	+	+	–	–	–	–	
Clinical pregnancy	quads	–	single	twins	–	–	–	quads	single	triplets	–	–	–	–	
Fetal reduction	+	–	–	–	–	–	–	+	–	+	–	–	–	–	
Outcome	2 girls	–	Blighted ovum	1 boy, 1 girl	–	–	–	1 boy, 1 girl	1 boy	1 boy, 1 girl	–	–	–	–	

**These patients had previously undergone testicular biopsy for TESE at another medical center. SD = standard deviation; ND = no data available; LH = luteinizing hormone; FSH = follicle-stimulating hormone; ESP = extensive sperm preparation; TESE = testicular sperm extraction.*

In total, 118 mature oocytes were injected with retrieved spermatozoa using ICSI, resulting in two distinct pronuclei in 70 oocytes, for a fertilization rate of 59%. Of the 67 fertilized oocytes that developed into cleaved embryos (cleavage rate, 96%), 62 were transferred, which resulted in the development of 15 gestational sacs featuring a visible heart beat (implantation rate, 24%). Of the eight women who underwent embryo transfer, six had clinical pregnancies (clinical pregnancy rate, 75%), including two singletons, one set of twins, one set of triplets, and two sets of quadruplets. Multiple pregnancies greater than twins were reduced to twins at the 10th gestational week after parents were informed of the relative risks of high-order multiple pregnancies. The six clinical pregnancies led to one blighted ovum and the birth of nine healthy babies, four boys and five girls, for a live delivery rate (no. of deliveries/no. of transfer cycles) of 62.5%. All these babies were karyotypically and physiologically normal.

Discussion

Almost all patients participating in our study exhibited an elevated serum FSH level, a low to normal level of serum testosterone, and atrophic testes, while half of the patients had mild gynecomastia. The clinical and hormonal pictures of our patients were similar to those characteristic of non-mosaic Klinefelter's syndrome as reported by a variety of other authors [15,17–24].

Prior to conducting TESE-ICSI, we carefully searched for the presence of viable sperm in a patient's ejaculate using an ESP method on at least three occasions, in order to use ejaculated sperm as first-line treatment and thus avoid testicular biopsy for TESE. As a result of the application of ESP, Ron-El et al [25] and Schlegel [31] detected viable spermatozoa in 35% and 20% of patients with non-obstructive azoospermia, respectively. These spermatozoa could be used for ICSI, avoiding the need for testicular biopsy in these patients. In Friedler et al's study involving 12 patients with non-mosaic Klinefelter's syndrome, two patients had totally dead spermatozoa in their ejaculate, although the authors did not mention whether spermatozoa were recovered by TESE in these two patients [23]. Mature spermatozoa were retrieved from four of nine patients with the azoospermic non-mosaic 47,XXY karyotype in Tournaye et al's study [15], and from four of seven patients in Reubinoff et al's study [19]. In both of these studies, three of the four individuals from whom spermatozoa were recovered had a history of spermatozoa in their ejaculates. In our study, one patient from whom spermatozoa were recovered revealed a history of the presence of spermatozoa in his ejaculate. Verifying the relative value of using the past demonstration of spermatozoa in the ejaculate as a predictor of the success of TESE, however, warrants the collection of further data from a larger study.

The TESE retrieval rate in our study was 57%. This is similar to the average TESE success rate of 56% among non-mosaic Klinefelter's syndrome patients [23]. It is also similar to the reported chance of finding spermatozoa following TESE in the general population of patients with non-obstructive azoospermia [32]. A relatively new sperm-retrieval technique, micro-TESE [30], may be associated with a higher sperm-recovery rate than other techniques, and may thus become popular in the future.

In this study, six patients had previously undergone testicular biopsy for histopathologic examination at other infertility centers, during which no spermatozoa had been recovered. Spermatozoa were recovered during this current attempt in two of these six individuals, one of whom had undergone orchiopexy for an undescended testis and simultaneous testicular biopsy during a previous attempt. This suggests that neither earlier negative histologic findings nor a lack of sperm recovery at a previous attempt necessarily precludes the success of subsequent TESE procedures for azoospermic patients with non-mosaic Klinefelter's syndrome. Similar results have been reported in patients suffering from non-obstructive azoospermia [33,34]. Moreover, azoospermic individuals suffering from non-mosaic Klinefelter's syndrome and an undescended testis are not necessarily precluded from successful future TESE for ICSI. In this study, six of eight patients who had not previously undergone testicular biopsy had successful recovery of sperm. The difference in success rates between patients who had or had not undergone previous testicular biopsy suggests that a previous testicular biopsy might have compromised the tiny germinal epithelium in the testis of patients afflicted with non-mosaic Klinefelter's syndrome. Determining whether a previous TESE attempt might elicit a decrease in the success rate of subsequent attempts requires further data from a larger study.

Our observation of the range of serum baseline FSH level, from 13.2 to 83.6 mIU/mL, in patients from whom spermatozoa were recovered in our study suggests that a high serum baseline FSH level is not necessarily a predictor of unsuccessful spermatozoa recovery for azoospermic patients with non-mosaic Klinefelter's syndrome. This point has been raised by other authors [15,17–24]. Serum inhibin B, a direct product of Sertoli cells, is a marker of spermatogenesis [35]. In our study, the serum inhibin B level, determined in four patients, ranged from less than 15 to 164 pg/mL. Spermatozoa

were recovered from three of these individuals, who had serum inhibin B levels of less than 15, 66.1, and 164 pg/mL, respectively. The normal range of serum inhibin B concentration for a proven father is 94–327 pg/mL [36]. Although there was a small number of cases in our study, the results appear to suggest that serum inhibin B level may not necessarily be an appropriate predictor of the success of sperm recovery from azoospermic patients suffering from non-mosaic Klinefelter's syndrome. Similar results have been reported for patients suffering from non-obstructive azoospermia [36,37].

Our fertilization and embryo-cleavage rates (59% and 96%, respectively) are similar to those of Friedler et al, who reported a 66% fertilization rate and a 98% embryo-cleavage rate [23]. The spermatozoa retrieved in our study were able to induce similar fertilization and embryo-cleavage rates as those achieved by spermatozoa retrieved from patients exhibiting non-obstructive azoospermia due to other etiology [38]. Our high implantation rate (24%), high clinical pregnancy rate (75%), and high live delivery rate (62.5%) may be attributable to the fairly young age of the women. Silber et al reported that pathology, source, quantity, or quality of spermatozoa did not elicit any discernible effect upon either fertilization or pregnancy rates, and that the principal factor that dramatically affected implantation, pregnancy, and delivery rates was maternal age [38]. Therefore, for cases of male infertility associated with severe oligospermia or azoospermia, we recommend that couples seek treatment for their infertility using ICSI or TESE-ICSI techniques when the female partners are still young, in order to achieve as high a pregnancy rate as possible.

Although the couples participating in our study were informed of the likelihood of achieving high-order multiple pregnancies following ICSI or TESE-ICSI, and were also informed of the risks associated with continuing multiple pregnancies subsequent to transferring more than two embryos to the female partner, several couples requested that more than three embryos of "the best quality" be transferred, if available, in order to increase the chances of achieving a successful pregnancy. Of the six women achieving clinical pregnancy in our study, five proved to have multiple pregnancies, including one pair of twins, two sets of triplets, and two set of quadruplets. Subsequent to informing the couples of the risk of high-order multiple pregnancies, all couples with a multiple pregnancy beyond twins consented to fetal reduction via a transvaginal approach during the 10^{th} to 12^{th} week of gestation, to reduce the fetal number to two. All women who underwent fetal reduction withstood the operation well and demonstrated a smooth prenatal course until birth.

A major concern associated with using testicular spermatozoa derived from patients with Klinefelter's syndrome for ICSI is the potential genetic risk to their offspring. Various studies of chromosomal abnormalities in ejaculated spermatozoa from patients with mosaic Klinefelter's syndrome have revealed an increased rate of sperm–nuclei abnormalities, the frequency of which appears to vary from 3.34% to 9.76% [39–43]. The rate of sperm–nuclei abnormalities in ejaculated spermatozoa from patients with non-mosaic Klinefelter's syndrome varies from 3.70% to 21.45% [41,44,45]. While sex-chromosomal hyperploidy has an incidence of 0.90% to 2.50% for mosaic Klinefelter's syndrome [39–43], its incidence in the non-mosaic form appears to vary from 2.62% to 17.58% [41,44,45]. The rate of abnormal sperm nuclei and sex-chromosomal hyperploidy in ejaculated sperm among the normal population appears to be 0.61–1.32% and 0.37–0.55%, respectively [40–42,46]. Both successful pregnancies and births have now been achieved using ICSI with testicular spermatozoa obtained from azoospermic patients with non-mosaic Klinefelter's syndrome. Two studies report that the aneuploidy rate of retrieved spermatozoa from the testicular tissues of azoospermic patients with non-mosaic Klinefelter's syndrome varies from 6.30% to 6.75%, and that the rate of corresponding sex-chromosomal hyperploidy varies from 2.70% to 3.57% [21,22]. Although the rates of both abnormal sperm nuclei and sex-chromosomal hyperploidy appear to be elevated for patients afflicted with Klinefelter's syndrome compared to the corresponding rates for the normal population, to the best of our knowledge, at the time of writing, all babies who were conceived using ICSI with testicular spermatozoa obtained from azoospermic patients with non-mosaic Klinefelter's syndrome have been both genetically and physiologically normal and healthy. This includes the 23 children reported in the literature [17–24] and the nine children in our current study, but not the one fetus reported by Ron-El et al [47]. In this study, karyotype analysis from chorionic-villous sampling during the 10^{th} gestational week in one woman who achieved a triplet pregnancy using a TESE-ICSI technique revealed a non-mosaic 47,XXY genotype for one of the three fetuses. This fetus was reduced during the 14^{th} gestational week. Due to the experimental nature of the TESE-ICSI technique and the relatively unknown risk of transmission of gonosomal aneuploidy to embryos obtained by testicular spermatozoa from patients suffering from Klinefelter's syndrome, pre-implantation genetic diagnosis (PGD) by embryo biopsy may serve as a potent tool to detect chromosomal aneuploidy in an embryo prior to transfer to the uterus.

However, not all infertility centers that offer assisted reproductive technology procedures have the facilities and skills necessary to undertake PGD. Furthermore, Munne et al suggest that PGD has several limitations for aneuploidy detection [48,49], with an error rate of 5.4% for PGD by fluorescence *in situ* hybridization (FISH) for common aneuploidies [49]. This suggests that PGD cannot yet be considered a reliable technique to detect chromosomal aneuploidy in an embryo.

Therefore, at present, PGD cannot replace invasive prenatal diagnostic techniques for fetal karyotyping, including chorionic-villous sampling or amniocentesis [19]. In our study, we recommended that couples who achieve pregnancy undergo amniocentesis for chromosomal karyotyping of their fetuses, although only one of the five couples with an ongoing pregnancy in our study agreed to this. The remaining four couples were willing to take the risk of potential genetic anomalies in their fetuses and took no action to determine whether any abnormal sex chromosomal constitution was apparent in the fetus. For these four couples, we requested frequent monitoring of the developing fetuses by ultrasound until the babies were born. Subsequent to birth, all babies from these pregnancies underwent cytogenetic evaluation using peripheral blood lymphocytes; all the babies had normal karyotypes. Our findings suggest that the risk of transmission of gonosomal aneuploidy using testicular spermatozoa derived from non-mosaic 47,XXY patients is probably not high, although we do acknowledge a rather small sample size. Our result is similar to those reported by others [17–24].

Whether spermatozoa produced by patients suffering from non-mosaic Klinefelter's syndrome are the consequence of meiotic segregation in a 47,XXY germ cell or a 46,XY germ cell is currently a hotly debated issue. Some authors have concluded that a 47,XXY spermatogonium may enter and complete meiosis, basing their conclusion on indirect evidence, such as an increase in the 24XY or 24XX spermatozoa rate in ejaculate potentially derived from 47,XXY spermatogonia [39,40,43,44] and a distorted sex ratio of the spermatozoa examined (excess of 23,X over 23,Y spermatozoa) [40,45]. On the other hand, other authors have concluded that only 46,XY spermatogonia are able to enter and complete meiosis, based on three observations: only 46,XY pachytene primary spermatocytes have been found in testicular cells obtained from patients with non-mosaic Klinefelter's syndrome using FISH [21,50]; only 46,XY cells enter and complete meiosis as revealed by both histology and microscopy [51,52]; and, most testicular sperm retrieved from patients with non-mosaic Klinefelter's syndrome demonstrate a normal pattern of sex chromosome segregation [21,22]. Using a murine Klinefelter model, Hunt et al discovered the demise of all XXY germ cells at a perinatal age [9], and Morz et al reported that two X-chromosome reactivations occurred in germ cells during the early stage of XXY mouse testis differentiation [53]. This altered X chromosome dosage in XXY germ cells acts to impair their continued survival [53]. Morz et al also found that exceptional germ cells which survive in the postnatal XXY testis are exclusively XY and result from rare mitotic non-disjunction events that give rise to clones of XY cells, and concluded that all spermatozoa found in an XXY mouse testis were exclusively yielded by XY germ cells [53]. Authors who previously advocated that only XY spermatogonia could enter and complete meiosis thought that a slight increase in chromosomal abnormalities and, in particular, the sex chromosome aneuploidy rate in spermatozoa found in patients with Klinefelter's syndrome were most probably related to meiotic errors of normal XY spermatogonia in a compromised testicular environment rather than to abnormal spermatogenic cell lines [21,22,50]. Morz et al substantiated this hypothesis using a murine Klinefelter model [54]. Consistent with this view, several studies have also reported a slight increase in chromosomal abnormalities, in particular, the rate of sex chromosome aneuploidies, for other types of infertile and subfertile males [55–57]. We determined the sex ratio among deliveries of all pregnancies achieved following TESE-ICSI for azoospermic patients suffering from non-mosaic Klinefelter's syndrome in reported studies [17–24] and our current study, and found no excess of girls over boys for either literature-based studies or our own study. Furthermore, all babies proved to be chromosomally normal, apart from a single XXY fetus [47]. This evidence may indirectly support the notion that spermatozoa retrieved from patients with non-mosaic Klinefelter's syndrome are produced by XY germ cells rather than XXY germ cells. Thus, the risk of using sperm from a non-mosaic 47,XXY patient would appear to be lower than expected; the offspring produced would not yield any resultant excess of females over males.

In conclusion, our results suggest that spermatozoa can be retrieved from azoospermic patients with non-mosaic Klinefelter's syndrome using a TESE technique. Furthermore, it may be possible to use the spermatozoa to induce normal fertilization by ICSI, normal embryo cleavage, and the birth of a karyotypically and physiologically normal baby. Our results should encourage and reassure physicians and their patients with non-mosaic Klinefelter's syndrome who are pursuing the use of patients' own sperm in treatment for infertility. Based on the literature and our results, it appears that

babies resulting from the application of an ICSI technique using testicular spermatozoa obtained from azoospermic patients with non-mosaic Klinefelter's syndrome are likely to be normal and healthy. We acknowledge that only limited data have yet been obtained pertaining to the potential risk of transmission of gonosomal aneuploidies to babies produced using testicular spermatozoa from azoospermic patients with non-mosaic Klinefelter's syndrome, and suggest that it is necessary to undertake detailed counseling of such patients regarding the potential genetic risks facing the offspring produced using the patients' own sperm.

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