Preliminary Results of a Prospective Study of Testicular Sperm Extraction in Young Versus Adult Patients With Nonmosaic 47,XXY Klinefelter Syndrome

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Context: Testicular sperm extraction (TESE) in adult patients with nonmosaic 47,XXY provides a sperm retrieval rate (SRR) of approximately 50%. Age is the only significant prognostic factor. Whether TESE should be performed in adolescent patients for sperm cryopreservation remains to be determined.

Objective: The objective of the study was to compare SRR between young (15–23 y) and adult (> 23 y) patients with 47,XXY, and to determine whether previous androgenic treatment had a deleterious effect.

Design: We designed a prospective comparative study between two groups enrolled in parallel from September 2010 onward.

Setting: University hospital.

Patients: Forty one patients with nonmosaic 47,XXY karyotype and azoospermia were included. Twenty five patients from 15–22 years of age were assigned to the "Young" group, and 16 patients age 23 years or more, to the "Adult" group.

Intervention: A bilateral testicular open biopsy was performed by a single surgeon. The reproductive biologist who performed TESE was blind to the patient's age.

Principal Outcome Measure: The main outcome measure was the SRR. The TESE procedure was considered positive if at least 20 sperm cells could be cryopreserved for intracytoplasmic sperm injection.

Results: SRR was 13/25 = 52% in the Young group and 10/16 = 62.5% in the Adult group, the difference being nonsignificant ($P = .73$). Ages were 24.3 ± 7.4 years in the 23 cases of positive TESE, and 23.7 ± 7.4 in the 18 cases of negative TESE, the difference being nonsignificant ($P = .42$). SRR was 9/17 = 52.9% for patients with and 14/24 = 59.1% for patients without previous testosterone (T) treatment, the difference being nonsignificant ($P = .98$).

Conclusions: According to the present results, performing TESE at a younger age (15–23 y) in patients with azoospermic nonmosaic 47,XXY Klinefelter did not increase SRR relative to adult patients (25–39 y). Previous replacement treatment with moderate doses of T did not seem to be deleterious for the recovery of sperm cells by TESE. **(*J Clin Endocrinol Metab* 100: 961–967, 2015)**

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Abbreviations: TESE–ICSI, Testicular Sperm Extraction–Intracytoplasmic Sperm Injection; IVF, in vitro fertilization; SRR, sperm retrieval rate; TESE, Testicular sperm extraction.

Most patients with a nonmosaic 47,XXY karyotype are azoospermic with small testes, high FSH, and low/undetectable inhibin B plasma levels, indicating severe spermatogenic failure (1). They were considered definitively sterile until the development of Testicular Sperm Extraction-Intracytoplasmic Sperm Injection (TESE–ICSI) in the 1990s (2, 3). As recently reviewed (4–6), published series of TESE in these patients showed a mean sperm retrieval rate (SRR) of 50%, similar to that in nonobstructive azoospermia with normal karyotype. This was confirmed in comparative studies (7, 8). The only significant prognostic factor found in several studies was the patient's age, with a better chance of positive TESE for patients younger than approximately 35 years (8–14). Several authors suggested that it might be favorable to perform TESE in younger patients and even adolescents, as in the first case reported (15). To address this issue, we designed a prospective controlled study comparing SRR between two groups of patients with nonmosaic 47,XXY Klinefelter enrolled in parallel from September 2010 onward, a "Young" (15–23 y) and an "Adult" group (> 23 y). Because findings could modify the current workup, advice based on recently published noncontrolled studies, we have decided to report our preliminary results with the first 41 patients included.

Materials and Methods

Patients and protocol

The inclusion criterion was nonmosaic 47,XXY Klinefelter syndrome, found to be azoospermic on two semen analyses at 3-month intervals. Any T treatment must have been withdrawn for at least 6 months before first semen analysis. Exclusion criteria were history of chemo- or radiotherapy, testicular torsion or violent testicular trauma, and untreated cryptorchidism. In case of temporary medical condition (fever, etc.) or any treatment liable to disturb spermatogenesis within the 3 months before first semen analysis (the Summaries of Product Characteristics of all medications were checked), inclusion was delayed for at least 3 months. In case of varicocele, the patient could be included provided embolization was successfully performed more than 3 months before first semen analysis.

Patients age 15–22 years at first semen analysis were allocated to the Young group and those older than 23 years to the Adult group.

Because the timing of onset of puberty in Klinefelter syndrome is similar to that of normal boys (1), the lower age limit for inclusion was set at 15, because no normal boys remained azoospermic after 15 years of age in an investigation of semen analysis according to age in normal boys (15, 16). Tanner stage was IV (9/25) or V (16/25) in the Young group.

The cutoff age between the two groups at 23 years was chosen as being the lowest age for Assisted Reproduction Technology in our institution.

Young patients were referred by pediatric endocrinologists for fertility preservation and adult patients were referred for infertility.

Except for psychological followup of young patients and their parents, both groups followed the same protocol: written informed consent was obtained from the patients (and parents for patients age under 18) at least 1 week after the protocol was explained by one of the investigators (I.P. or H.L.). History and clinical data were recorded following a systematic questionnaire administered on the day of the first sperm analysis and blood sampling for hormonal analysis. In case of azoospermia on first semen analysis, a second analysis was scheduled for 3 months later. In case of azoospermia on second semen analysis, the patient was included and TESE was scheduled within 1 month. Bilateral open testicular biopsy was performed under general anesthesia by a single urologist (B.C.). TESE was performed by a reproductive embryologist blind to the patient's age (J.L., M.B., or S.G.d.E.). TESE was considered positive if it was possible to cryopreserve at least 20 sperm cells.

T serum level, measured 1 month after biopsy, decreased from 10.4 ± 4.8 to 8.9 ± 4.6 nmol/l. Nineteen patients were in the normal adult range for T serum (> 10 nmol/l) before biopsy. For 10 of them, the postbiopsy level decreased to the hypogonadal range (≤ 10 nmol/l) and nine remained in the normal range. Given that the great majority of patients with Klinefelter syndrome develop clinical hypogonadism during adulthood, even without testicular biopsy, we systematically implemented T replacement therapy after biopsy, as currently recommended (17).

The protocol was approved by the institutional review board, in line with French legislation.

Methods

Karyotype

Cytogenetic study of peripheral blood samples used the standard procedure on 25 cells in culture and was confirmed on fluorescence in situ hybridization.

Genetic testing

All patients were tested for Y chromosome microdeletions by multiplex PCR.

Hormones

FSH blood concentration was measured on immunologic chemiluminescence assay (Abbott). Intra- and interassay coefficients of variation at 20 mIU/L were 1.9% and 7.6%, respectively.

LH blood concentration was measured using an immunologic chemiluminescence assay (Abbott). Intra- and interassay coefficients of variation at 20 mIU/L were 3.6% and 6.9%, respectively.

T blood concentration was measured using an in-house radioimmunologic assay after organic extraction and diatomaceous earth (celite) chromatography (18). Mean intra-assay coefficients of variation were 4.4, 3.3, 2.2, 3.7, 4.3, and 3.7%, respectively for concentrations of 0.17, 1.0, 2.6, 6.9, 15.6, and 26 nmol/L; interassay coefficients of variation were 7.1, 7.9, and 7.1% for T concentrations of 1.8, 3.5, and 21.3 nmol/L, respectively.

Semen analysis

Semen collection

Semen samples were collected by masturbation into a sterile container after 3–5 days' sexual abstinence. Immediately after 30 minutes' semen liquefaction at room temperature, sperm was analyzed according to the World Health Organization guidelines (2010).

TESE and cryopreservation

TESE

Under general anesthesia, a small incision was performed along the scrotal raphe. Then each testicle was extracted and stabilized for multiple biopsies. A second incision was performed in the tunica vaginalis and albuginea. A substantial part of the extruding testicular tissue was cut with scissors at the two extremities and washed with 1 ml in vitro fertilization (IVF) medium (Ferticult) to eliminate traces of blood. Surgical procedure was the same in the Young and Adult groups. The biopsies were placed in a sterile tube containing 2 ml IVF medium at room temperature. To preserve the totality of the tissue, no direct microscopic observation was performed in the surgical room; a specimen was sent for histological analysis.

After transfer to the laboratory at 33°C, the testicular tissue was placed in a small Petri dish with 3 ml preincubated IVF medium. Samples were delicately microdissected mechanically with small sterile needles to open the seminiferous tubes for sperm extraction. After 45 minutes' incubation (5% CO_2 in air, at 37°C), the supernatant was layered on a 1-step gradient of SupraSperm diluted with IVF medium (50%). After centrifugation (394 g, 20 min), the purified testicular sperm, covered by the 50% layer, was washed in IVF medium (Origio) (701 g, 10 min.). Supernatant was removed and the pellet was resuspended in a suitable volume of IVF medium. A small amount (10 μl) of the resuspended pellet was observed under an inverted microscope for an extended microscopic assessment of the presence, motility, and morphology of spermatozoa. If at least 20 spermatozoa were observed, they were cryopreserved.

Sperm cryopreservation

In positive sperm screening, the sample was cryopreserved for future use. After dilution in an equal volume of cryoprotectant (Spermfreeze, FertiPro) and 10 minutes' incubation at room temperature, the sperm suspensions were loaded into sterile, high-security straws (CBS, Cryo-Bio Systems) and frozen on a standard protocol in a Freezal freezer (Air Liquide Santé). The straws were then plunged into liquid nitrogen and stored in an RCB500 container (Air Liquide Santé).

Sperm thawing

To perform intracytoplasmic sperm injection (ICSI), one or more straws were thawed at 35°C. The thawed preparation was washed with Ferticult medium to eliminate the cryoprotectant. After centrifugation (701 g, 10 min), the pellet was washed resuspended to eliminate the cryoprotectant. Testicular spermatozoa were treated with 5mM pentoxifylline to induce mobility. Spermatozoa with slight tail movement were considered viable and were selected for injection after elimination of pentoxifylline in polyvinylpyrrolidone medium (Origio).

ICSI and embryo transfer

Monitored ovarian hyperstimulation was performed using a GnRH protocol with r-FSH (Gonal-F, Organon, Puregon, Merck Serono). Oocytes were retrieved 36 hours after administration of human chorionic gonadotropin (Ovitrelle, Merck Serono). ICSI was performed on metaphase II oocytes using thawed mobile testicular spermatozoa. Embryo transfer was performed at day 2 or 3 development.

Statistics

The χ^2 test was used to compare SRRs between groups (Young vs Adult; previous T treatment vs none; prenatal diagnosis vs detected during childhood vs not detected before adulthood).

Comparison of numeric variables used Student *t* test and ANOVA or Mann-Whitney *U* test according to distribution normality.

To establish the number of patients to be included, the percentage positive TESE results, which would change the current therapeutic attitude was considered to be 75% in young patients and 50% in adults. Sixty patients were required in each group. Thus the present report can only be presented as preliminary results.

However, given that the difference between the Young and Adult groups was far from significant with the present number of patients, the risk of having missed a clinically relevant difference between the two groups is low: statistical power was estimated to be 17% (ie, 17% chance of getting a statistically significant difference with two groups of 60 patients, conditional on the present results).

Results

Forty one azoospermic patients with nonmosaic 47,XXY karyotype were included. None had Yq microdeletion. Global SRR was 56.1% (23/41). As shown in Figure 1, giving the distribution of SRR according to age, and in Table 1, TESE procedure was positive for 13/25 patients (52%) in the Young group vs 10/16 patients (62.5%) in the Adult group, the difference being nonsignificant (P = .73). As indicated in Figure 1, the chances of retrieving sperm were similar in teenagers ($<$ 20 y) and in adults ($\geq$ 20 y): SRR, 57% (12/21) and 55% (11/20), respectively (P = .99).

As shown in Table 2, ages were 24.3 $\pm$ 7.4 years in the 23 cases of positive TESE, and 23.7 $\pm$ 7.4 in the 18 cases of negative TESE, the difference being nonsignificant (P = .42).

As shown in Table 3, 17 patients (10 in the Young group and seven in the Adult group) had history of previous T treatment at replacement doses: oral T undecanoate, 2 $\times$ 80 mg/d (n = 2); im T enanthate, 50–250 mg/3–4 wk (n = 13); or im T undecanoate, 1000 mg/3 mo (n = 1). Following the protocol, these treatments were stopped at least 6 months before first semen analysis: ie, at least 9 months before testicular biopsy. TESE was positive for

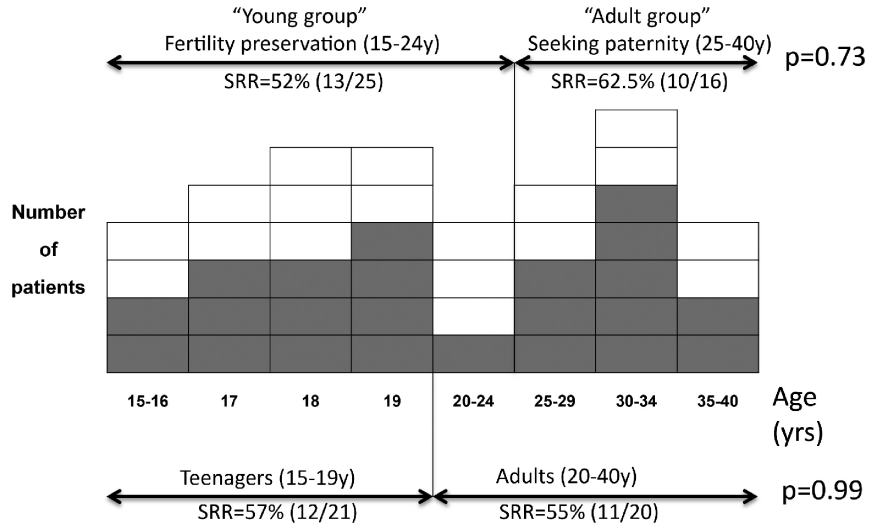

Figure 1. Distribution of azoospermia patients with nonmosaic 47,XXY with positive (gray squares) and negative (open squares) TESE, according to age at testicular biopsy.

9/17 (52.9%) of the patients who had previously received T and for 14/24 (59.1%) of those who had not, the difference being nonsignificant ($P = .98$). In the 17 patients who had received T, mean ± SD [median; min-max] T treatment duration was 4 ± 5.8 [1.5; 0.3–17] years for the nine patients with positive TESE and 3.2 ± 2.8 [2.3; 0.3–9] years for the eight with negative TESE, the difference being nonsignificant ($P = .53$).

FSH plasma level and T serum level and bitesticular volume are given according to the Young/Adult group and TESE results in Table 4. No significant differences were detected by two-way ANOVA.

Because phenotype severity might affect both the circumstances of initial karyotyping and TESE results, SRR was compared according to initial karyotyping circumstances: SRR was 6/9 (66.6%) in case of prenatal diagnosis (performed because of maternal age), 9/18 (50%) in cases recognized during childhood (during assessment for learning disability or micropenis in childhood, or gynecomastia and small testes at puberty) and 8/14 (57.1%) in cases not recognized before adulthood (referred for infertility and/or small testes), the differences being nonsignificant ($P = .71$).

To date, seven of the 10 couples having cryopreserved sperms began an ICSI program and three did not. Ten cycles were performed (one couple had three cycles, one couple two, and five couples had one cycle). In all these ICSI attempts, it was possible to microinject one cryopreserved/thawed sperm into all the retrieved mature oocytes. Four pregnancies resulted (four couples), three healthy babies (one girl, two boys) were born, and one pregnancy is ongoing.

Table 1. Number of Nonmosaic 47,XXY Azoospermic Patients According to TESE Results and Age Group

Subjects, No. (%)	TESE+	TESE−	Total
Young group	13 (52.0)	12 (48.0)	25 (100)
Adult group	10 (62.5)	6 (37.5)	16 (100)
Total	23 (56.1)	18 (43.9)	41 (100)

Discussion

Since 1996, TESE–ICSI has been applied in azoospermic men with nonmosaic 47,XXY Klinefelter syndrome (3). In published series, positive TESE rate average 50% (4, 5). The only prognostic factor regularly found was patient age, with a decrease in positive TESE with aging (8–14). A statistical threshold was found at approximately 35 years in most studies (12). In the light of this inverse relationship between age and SRR, several authors suggested testicular biopsy in adolescent patients with Klinefelter syndrome. The first publication was a case report (15) of the presence of sperm cells in the biopsy of a 15-year-old boy with nonmosaic 47,XXY. Other studies were recently published: testicular biopsies have been performed for seven nonmosaic 47,XXY adolescents age (13.3–16 y) (19, 20). No sperm cells could be isolated, but spermatogonia were identified in some subjects and testicular tissue was cryopreserved in the hope of maturing it in the future.

Another series of TESE was reported for five adolescents age 15–16.5 years, with isolation and cryopreservation of spermatozoa in one case and of elongated spermatids in another (21). The lower rate of sperm recovery in these young adolescents in comparison with published adult series could be related to the fact that focal spermatogenesis was not fully developed in these young boys. Conversely to these results, Schlegel and collaborators (22) reported a series of 10 young patients with nonmosaic 47,XXY age 14–22 years, with positive TESE in 70% of cases. In this study, patients received treatment with T gel and antiaromatase. The same authors reported SRRs of 68% and 69% in adults in previous series (13, 23). Although these studies were not conducted in parallel, the positive TESE rate seemed to be rather similar in young and adult patients, as in the present study. Nevertheless, because these reports (13, 22, 23) were not comparative, it could not be accurately determined whether the high positive TESE rates could be due to the procedure of mi-

Table 2. Age of Nonmosaic 47,XXY Azoospermic Patients According to TESE Results

Age, y	TESE+	TESE−	Total
Young group			
Mean ± SD	18.7 ± 2.0	19.0 ± 2.1	18.9 ± 2.0
[Median; min-max]	[18.3; 15.7–23.7]	[18.4; 16.5–22.5]	[18.3; 15.7–23.7]
Adult group			
Mean ± SD	31.6 ± 4.8	33.0 ± 4.8	32.1 ± 4.7
[Median; min-max]	[32.6; 25–38.8]	[34.3; 25.8–38.1]	[33.3; 25–38.8]
Total			
Mean ± SD	24.3 ± 7.4	23.7 ± 7.4	24.0 ± 7.3
[Median; min-max]	[19.9; 15.7–38.8]	[20.9; 16.5–38.1]	[20; 15.7–38.8]

cro-TESE developed by these authors, patient selection (several of the adults had diagnostic biopsy before TESE) (23), and/or the hormonal treatment systematically prescribed to young patients (22) conversely to the different treatments used for the adults patients (13, 23).

In the present prospective study, comparability between the Young and the Adult groups seems to us to be as good as possible. The only inevitable bias is that diagnosis of Klinefelter syndrome was earlier for the young patients than for the infertile patients, raising the question of possible global phenotypic variability, with more severe spermatogenic failure in case of a more severe phenotype that could be recognized during childhood. However, the positive TESE rate did not seem obviously different according to initial karyotype circumstances, whether systematic prenatal screening (for maternal aging), in case of pediatric symptoms, or late diagnosis in adulthood.

The present results did not confirm the hypothesis that, in case of nonmosaic 47,XXY karyotype, young azoospermic patients could have a better chance of positive TESE than adult azoospermic patients seeking paternity. According to the present results, the calculated statistical power suggests that the chance of having a clinically relevant result is low. Our results are not in opposition to previous series because none of the patients in our Adult group were older than 38.8 years.

The use of micro-TESE vs standard open biopsy has been discussed in the literature. According to the systematic review of comparative studies in nonobstructive azoospermia, by Deruyver et al (24), microTESE performed by trained surgeons could give rise to higher SRRs than standard open biopsy (54% vs 33%). For Klinefelter's syndrome, Aksglaede and Juul (4) presented an overview of reported SRRs in different studies, with higher values with micro-TESE (mean [min-max] = 57% [47–69%]) than with macro-TESE (42% [28–57%]). The present results using standard open biopsy (SRR = 56.1%) were close to the average value reported in the literature with micro-TESE. Whether these comparative results between younger and adult patients would be similar using micro-TESE remains to be determined.

A deleterious effect of previous T treatment was suggested in the series of Schlegel and coworkers (13, 23), in which only one of the five patients who had received previous T treatment had positive TESE (20%), compared with 69% in the population as a whole. No other report gave more powerful arguments in favor of a deleterious effect of previous T replacement treatment. The present results did not confirm this hypothesis, with similar SRR whether the patient had received androgen replacement treatment or not. However, in the present study, T was withdrawn 6 months before first semen analysis and at least 9 months before testicular biopsy.

Thus, in this prospective comparative clinical study between two groups of 47,XXY azoospermic patients, Young (15.3–23.7 y) vs Adult (25–38.8 y), included in parallel, age, duration of previous T treatment, age at ini-

Table 3. Positive TESE Rate According to Age Group and Previous T Treatment

	Previous T Treatment		
TESE + rate (%)	Yes	No	Total
Young group	6/10 (60.0)	7/15 (46.2)	13/25 (52.2)
Adult group	3/7 (42.9)	7/9 (77.8)	10/16 (62.5)
Total	9/17 (52.9)	14/24 (59.1)	23/41 (56.4)

Table 4. Plasma FSH, Serum T Levels, and Testicular Volume R + L According to Age Group and TESE Results

Level	TESE+, Mean ± SD	TESE−, Mean ± SD
FSH, IU/L[a]		
Young group	47.6 ± 24.0	46.8 ± 27.1
Adult group	45.7 ± 18.1	42.5 ± 27.8
T, nmol/L[b]		
Young group	12.2 ± 6.4	9.1 ± 2.8
Adult group	9.3 ± 4.2	8.9 ± 3.5
Testicular volume R + L, ml[c]		
Young group	6.8 ± 1.5	6.8 ± 2.3
Adult group	6.1 ± 1.4	7.0 ± 2.0

[a] Two-way ANOVA: TESE, $P = .82$; age, $P = .72$; interaction, $P = .89$.

[b] Two-way ANOVA: TESE, $P = .31$; age, $P = .35$; interaction, $P = .42$.

[c] Two-way ANOVA: TESE, $P = .42$; age, $P = .67$; interaction, $P = .44$.

tial karyotyping (prenatal, childhood or adulthood), plasma FSH level, serum T level, and bitesticular volume were not predictive of TESE results.

Our results can be discussed in the light of recent advances in the physiopathology of spermatogenesis in Klinefelter syndrome. The physiopathology of focal spermatogenesis became clearer with the demonstration by Sciurano et al (25) that spermatogonia and spermatocytes were 46,XY in spermatogenic foci, conversely to other testis areas where spermatogonia were 47,XXY and spermatogenesis did not progress. Thus spermatogenic foci seems to arise from spermatogonia that lost one X chromosome during mitosis, giving rise to a clone of 46,XY spermatogonia able to progress through the spermatogenic process. This is in good agreement with the normal karyotype found in most the sperm cells and children at birth (26). However, it is not known at what time the spermatogonial karyotype is corrected or the time needed for 46,XY spermatogonia to produce a focus of spermatogenesis with a sufficient number of sperm cells for positive TESE.

The decrease in the positive TESE rate observed in the older patients of the adult series is related to the concept of "compromised testicular environment" described by Mroz et al (27). It seems to be due to disruption of normal paracrine interactions within the Klinefelter testis, in which spermatogonia progressively decrease from birth to adulthood, Sertoli cells secrete a very small amount of inhibin B (1), antimullerian hormone (28, 29), and probably other paracrine factors, and Leydig cells produce small amounts of T despite their hyperplasia. From the adult series, it would seem that this phenomenon becomes significant only after approximately 35 years.

T at supraphysiological doses has been proved to decrease LH and FSH secretion and sperm production (30). However, the effect of replacement doses in patients with 47,XXY Klinefelter syndrome did not seem to be deleterious for the positive TESE rate, probably because at replacement doses, LH and FSH were not fully down-regulated. We were, however, unable to know whether the positive TESE rate would be conserved if treatment was being continued at the time of biopsy.

In conclusion, because the chances of sperm recovery are similar between teenagers and adults, the timing of sperm retrieval and preservation can be chosen by the patient. Nevertheless, it does not seem necessary to perform TESE at a very young age in patients with nonmosaic 47,XXY, as previously advocated. Previous replacement treatment with moderate doses of T does not seem to be deleterious for recovery of sperm cells by TESE. We still do not know if and when this treatment should be withdrawn before performing the TESE procedure. Further well-designed controlled studies could help give better advice for enhancing the chances of paternity in 47,XXY azoospermic patients.

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