

Testicular sperm recovery in nine 47,XXY Klinefelter patients

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Klinefelter's syndrome is generally characterized by hypergonadotrophic hypogonadism and azoospermia. The clinical features, however, are variable, and occasionally severe oligozoospermia may be present. Usually in these cases a 46,XY/47,XXY mosaic karyotype is involved. However, focal spermatogenesis and severe oligozoospermia have been reported in 47,XXY individuals too. In the present study we investigated whether testicular spermatozoa can be recovered in 47,XXY patients with a view to intracytoplasmic sperm injection (ICSI). In four out of nine apparently non-mosaic 47,XXY patients, spermatozoa were recovered from the wet preparations of testicular tissue and ICSI was performed in three couples. In one patient in whom spermatozoa were successfully recovered and used for ICSI, no spermatozoa were retrieved at a second trial. Although these results show that in some 47,XXY individuals testicular spermatozoa can be successfully recovered and even used for ICSI, at present this approach should be considered experimental. There may indeed be some concern about the chromosomal normality of the embryos generated through this infertility treatment. Patients with Klinefelter's syndrome should therefore be counselled about the complexity of this treatment, which involves multiple testicular biopsies from hypogonadal testes, ICSI and preimplantation diagnosis by fluorescence-in-situ hybridization.

Key words: 47,XXY/Klinefelter's syndrome/ICSI/spermatozoa/testicular biopsy

Introduction

Infertile patients with azoospermia and small scrotal gonads may have a primary testicular failure with dysfunctional seminiferous tubules or a pituitary–hypothalamic disorder with gonadotrophin deficiency leading to a testicular maturation failure. In these patients, serum follicle stimulating hormone (FSH) and testosterone should be assessed and, if gonadotro-

phins are found to be elevated, a karyotype should be performed to look for the presence of an extra X chromosome indicative of Klinefelter's syndrome. This syndrome, originally described by Klinefelter *et al.* (1942) was only found later to be the result of chromosomal aneuploidy (Jacobs and Strong, 1959). Mosaicisms and variants having more than two X chromosomes have also been described (Paulsen *et al.*, 1968). Males with a sex-determining region Y (SRY) gene-negative 46,XX karyotype may also develop a phenotype similar to that of patients with Klinefelter's syndrome (de la Chapelle *et al.*, 1964; Turner *et al.*, 1995). The incidence of 47,XXY Klinefelter syndrome is 0.1% in the general population (Nielsen and Wohler, 1991) and 3.1% in the infertile male population (Guichaoua *et al.*, 1993).

Klinefelter patients have intrinsically abnormal testicles. At the onset of puberty serum concentrations of luteinizing hormone (LH) will rise above normal limits since defective Leydig cells will secrete inadequate quantities of testosterone but high amounts of oestradiol. This condition will ultimately lead to a gynaecoid habitus. The clinical habitus, however, may vary and involves a whole spectrum from eunuchoid hypogonadism to normally virilized, albeit sterile males. Because of the constantly elevated gonadotrophins, the seminiferous tubules gradually become fibrotic and hyalinized. Their lumen will obliterate and their germ cells will gradually disappear. Hyperplasia of Leydig cells is generally found.

Since the introduction of testicular sperm recovery for patients with abnormal seminiferous tubular function (Devroey *et al.*, 1995; Tournaye *et al.*, 1995, 1996), we have been looking into the possibility of recovering spermatozoa from the testes of 47,XXY patients. The present paper reports our experience in nine patients.

Materials and methods

Nine individuals who were referred with 47,XXY karyotypes in their peripheral blood lymphocytes underwent testicular biopsy for sperm recovery. Six patients (patient nos. 1–5 and 9) were orthodox Jewish patients in whom at least one sperm cell had been observed during at least one previous fertility investigation, e.g. semen analysis or post-coital test. These patients were taking part in our intracytoplasmic sperm injection (ICSI) programme and, for religious reasons, their biopsies were scheduled at the time of ovum retrieval in their spouses. In another, non-orthodox Jewish patient, testicular biopsy was scheduled at the time of the ovum retrieval, but here a sperm donor was organized in case no spermatozoa were retrieved. The two remaining patients had a testicular biopsy as a diagnostic procedure aiming to explore the possibility of recovering testicular spermatozoa. Their ages ranged from 23 to 37 years. All had a physical examination, assessment of gonadotrophins and testosterone and a karyotype from

Table I. Clinical, genetic and endocrinological features in nine patients with 47,XXY Klinefelter's syndrome Values in parentheses are numbers of metaphases counted

Patient no	Age (years)	Karyotype			Physical examination	Testicular volume ^a (TU/l)	Serum FSH (IU/l)	Serum LH (IU/l)	Serum testosterone (µg/l)	Semen
		Lymphocyte	Skin fibroblasts	Testicular fibroblasts						
1	27	47,XXY (57)	47,XXY (24)	47,XXY (11)	Beard, gynaecomastia	L: 2 R: 5	24.8	13.1	2.0	Three amorphous spermatozoa in pellet
2	36	47,XXY (30)	47,XXY (24)	47,XXY (16)	Beard, gynaecomastia	L: <3 R: 6	54.8	39.0	2.9	One immotile spermatozoon at postcoital test
3	37	47,XXY (17)	NA	NA	Beard	L: 3 R: 3	60.1	35.7	3.2	One immotile spermatozoon
4	34	47,XXY (15)	47,XXY (10)	47,XXY (15)	Beard, gynaecomastia	L: 2 R: 2	27.0	NA	1.3	Two spermatozoa in micro-SEM
5	23	47,XXY (30)	47,XXY (24)	47,XXY (16)	Beard	L: 3 R: 3	38.1	23.1	3.2	Three spermatozoa in two micro-SEM
6	33	47,XXY (15)	NA	NA	Beard, gynaecomastia	L: <3 R: 3	74.0	29.1	3.8	None
7	28	47,XXY (15)	NA	NA	Beard	L: 3 R: 3	24.8	17.3	2.2	None
8	34	47,XXY (20)	NA	NA	Gynaecomastia	L: 3 R: 4	36.8	25.0	3.3	None
9	35	47,XXY (15)	47,XXY (15)	NA	Beard	L: <3 R: <3	20.0	NA	NA	Spermatozoa in pellet

^aL = left and R = right testis

NA = not available, micro-SEM = micro-scanning electron microscopy

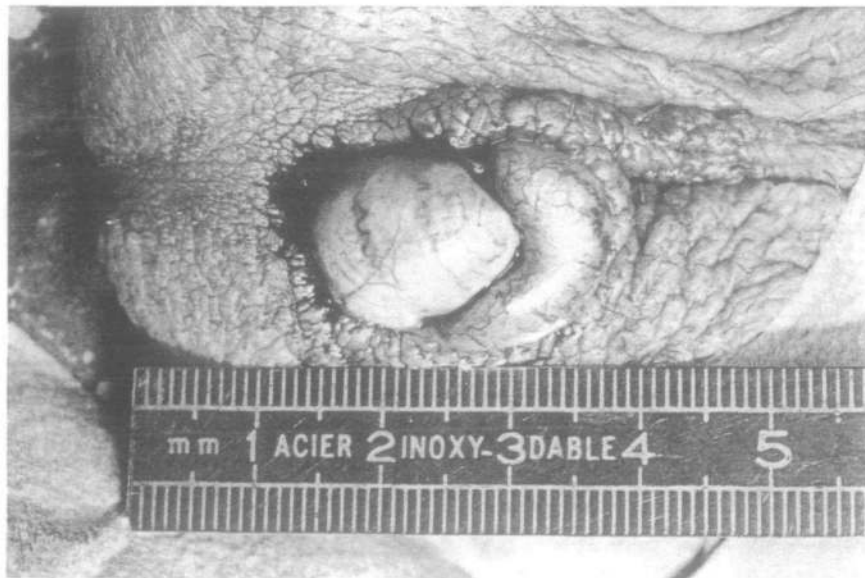


Figure 1. Hypogonadal testis from a 47,XXY Klinefelter patient after unilateral scrototomy for excisional testicular sperm recovery

peripheral blood lymphocytes. Whenever possible, karyotypes of skin fibroblasts and testicular fibroblasts were obtained. Patients willing to undergo testicular sperm recovery for ICSI were counselled about the experimental character of the procedure. All agreed to have a preimplantation diagnosis by fluorescence-in-situ hybridization (FISH) to determine the number of sex chromosomes if embryos should be available for transfer (Staessen *et al.*, 1996). They also agreed to take part in the follow-up programme for ICSI pregnancies which included a prenatal karyotyping by chorionic villus sampling or amniocentesis and paediatric follow-up of the children born (Bonduelle *et al.*, 1994, Wisanto *et al.*, 1995).

Testicular biopsies were performed under general anaesthesia by an open-excisional technique. After hemiscrototomy the scrotal contents were inspected and, according to our current practice, small

multifocal biopsies rather than one large biopsy were taken (Tournaye *et al.*, 1995, 1996). If spermatozoa were found in the wet preparation, testicular sampling was stopped. If no spermatozoa were found, more biopsies were taken until the whole testicular mass had been randomly sampled. A careful haemostasis was performed by means of bipolar cautery and both testicular and scrotal incisions were closed by interrupted resorbable sutures.

The testicular tissue was rinsed in HEPES-buffered Earle's medium and finally placed in a Petri dish with Earle's medium. In the adjacent laboratory the testicular samples were minced in a Petri dish by means of two microscope slides and jeweller's forceps. The minced tissue was examined under the inverted microscope at $\times 400$ magnification for the presence of spermatozoa. One specimen from each testicle was sent for histology. These specimens were taken according

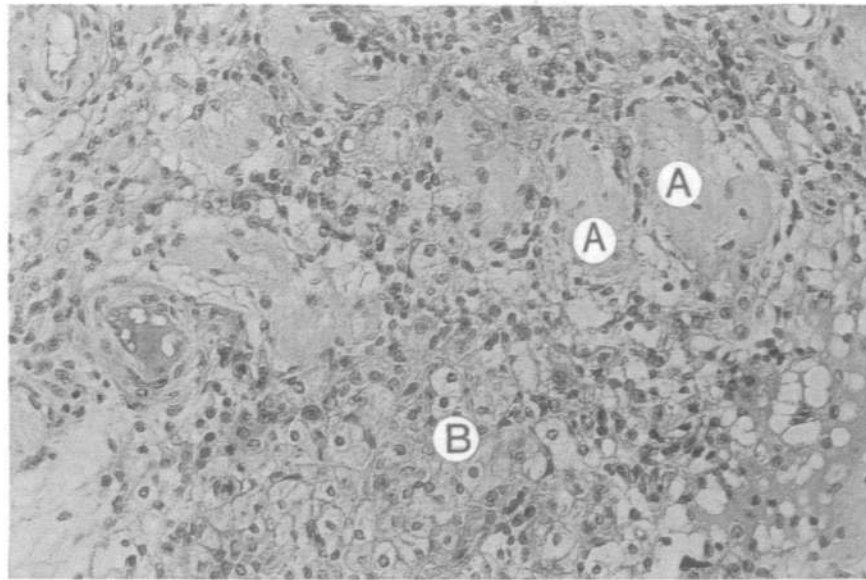


Figure 2. Testicular biopsy specimen from the same 47,XXY Klinefelter patient as in Figure 1. The seminiferous tubules (A) are completely hyalinized. In the interstitial space, hyperplasia of the Leydig cells (B) can be observed. Magnification: $\times 400$.

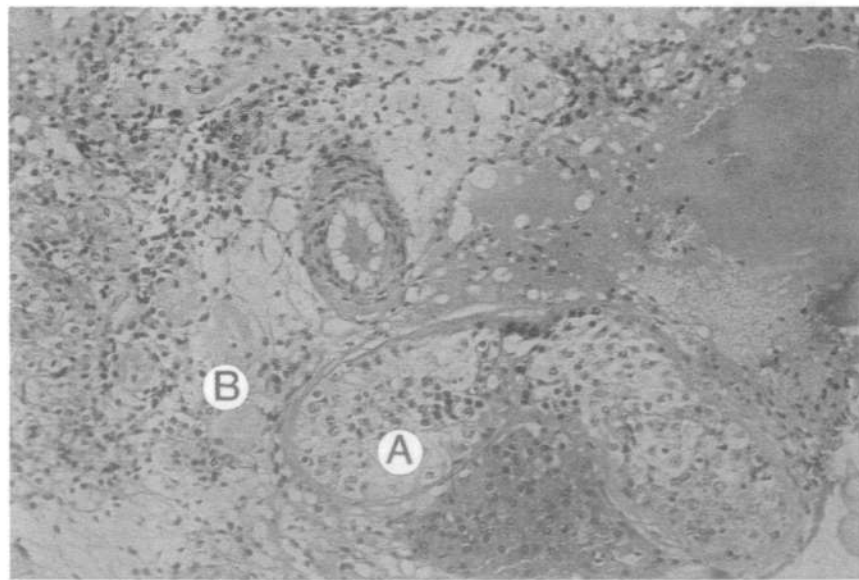


Figure 3. Testicular biopsy specimen from the same 47,XXY Klinefelter patient as in Figure 1, showing one tubule containing both Sertoli cells and germinal cells (A) adjacent to hyalinized seminiferous tubules (B). However, no mature spermatozoa are present. Magnification: $\times 200$.

to the non-touch technique and placed in Bouin's fixative. For examination they were stained using eosin/haematoxylin.

Details of the ICSI procedure and preimplantation diagnosis by FISH as performed in some patients are discussed by Staessen *et al.* (1996)

Results

As shown in Table I, physical examination of the nine patients included in this series revealed varying clinical features. While no patient was treated with androgens, most patients (8/9) had a normal facial hair pattern (beard) and only about half (5/9) had gynaecomastia. All had small scrotal testes with volumes ranging from 2 to 6 ml (see Figure 1) with varying degrees

of penile development. Patient no. 4 had a history of cryptorchidism hormonally treated by human chorionic gonadotrophin (HCG) injections. Table I also shows the results of the cytogenetic analyses together with the number of cells counted. None of the karyotypes from the peripheral blood lymphocytes, skin fibroblasts and testicular fibroblasts showed mosaicism. Serum FSH and LH concentrations were elevated in all patients and ranged from 20.0 to 74.0 IU/l (normal range 1.5–12 IU/l) and 13.1 to 39.0 IU/l (normal range 0.6–13.5 IU/l) respectively. Serum testosterone values (normal range 2.0–15.9 $\mu\text{g/l}$) were in the low normal range except for patient no. 4. Only patient no. 1 showed two mature, but immotile spermatozoa in the preliminary semen analysis performed in our centre. Another

Table II. Histology and wet preparation of testicular biopsies from nine 47,XXY Klinefelter patients

Patient no.	Trial no	No of samples taken	Spermatozoa at wet preparation	Histology	Treatment
1	1	L 1 R 3	Non-motile	L extensive sclerosis and fibrosis ICSI and FISH R tubular atrophy with Sertoli cell-only pattern	One embryo replaced, biochemical pregnancy No ICSI
2	2 1	R 22 L 3 Motile R 3	None L tubular atrophy	NA ICSI and FISH R: tubular atrophy with Sertoli cells only, with focal spermatogenesis arrested at spermatocyte level Only one tubulus showed presence of spermatozoa	Two embryos replaced, not pregnant
3	1	L 3 R 3	None	L: extensive tubular atrophy and sclerosis R: extensive tubular atrophy and sclerosis, <5% of tubuli show Sertoli cell-only pattern	— No ICSI
4	1	L 2 R 2	None	L: extensive tubular atrophy with Sertoli cells only R: extensive tubular atrophy with Sertoli cells only	— No ICSI
5	1	L 5 R 5	None	L extensive tubular atrophy with Sertoli cells only R extensive tubular atrophy with Sertoli cells only	— No ICSI
6	1	L 4 R 4	None	L extensive tubular atrophy and sclerosis R extensive tubular atrophy and sclerosis, only one tubulus showed Sertoli cell-only pattern	— ICSI using donor spermatozoa
7	1	L 1 R 1	Motile	L extensive tubular atrophy and sclerosis R extensive tubular atrophy and sclerosis	— Donor insemination
8	1	L 2 R 3	None	L extensive tubular atrophy and sclerosis R extensive tubular atrophy and sclerosis	— Donor insemination
9	1	L 3 R 3	Motile	L extensive tubular atrophy and sclerosis R extensive tubular atrophy and sclerosis	ICSI FISH. one embryo replaced, not pregnant

L = left, R = right, NA = not available

five patients had a history of observation of at least one sperm cell at a previous fertility investigation. Except again for patient no. 2, all these were unique observations.

Table II shows the result of the wet preparations and the histology of the testicular biopsies. The histological findings in all patients showed evidence of impaired spermatogenesis, i.e. tubular atrophy, fibrosis and hyalinization (see Figure 2) and Leydig cell hyperplasia. The few seminiferous tubules present showed germinal aplasia, i.e. Sertoli cells only. However, the four patients in whom spermatozoa were found in the wet preparation showed focal spermatogenesis with maturation arrest at the spermatocyte level (Figure 3). The number of biopsies taken varied from two to 22 according to the findings in the wet preparation and the testicular volume. In three patients (nos. 1, 2 and 9), as well as in one (no. 7) where no spermatozoa had ever been observed in his previous ejaculates, mature spermatozoa were observed. In patient nos. 2, 7 and 9 these spermatozoa were motile. The spermatozoa from patients 1, 2 and 9 were used for ICSI. For all three of these patients cleaving embryos were obtained. Preimplantation diagnosis by FISH was used in order to check for sex chromosome normality of these embryos and the results of this procedure are reported separately (Staessen *et al.*, 1996). The partner of patient no. 1 had a biochemical pregnancy (HCG 210 IU/l). Eleven months later this couple was again scheduled for testicular sperm recovery and ICSI. However, this time no sperm cells were retrieved after taking 22 biopsies.

Discussion

47,XXY Klinefelter's syndrome is invariably viewed as a definite cause of male infertility. In the present study we report on the results of testicular sperm recovery in nine consecutive 47,XXY patients. The typical clinical features of Klinefelter's syndrome are reported to be variable (Paulsen *et al.*, 1968). At their physical examination, 81% of Klinefelter's patients will not show facial hair growth. Gynaecomastia is reported in 50% of patients and small testes in 98%. In our small series, five out of the nine patients had gynaecomastia and all had small testes. However, eight of the nine had normal facial hair growth without having any androgen therapy. This difference may be the result of an ascertainment bias, since our patients were virile enough to get married. Selection bias too may explain this difference: our first five patients were selected only by a history of the presence of at least one spermatozoon in at least one of their ejaculates. Our patients may thus represent one end of the spectrum of the Klinefelter population and may not be representative of the whole population.

Azoospermia is not a constant feature (Futterweit, 1967; Paulsen *et al.*, 1968; Foss and Lewis, 1971). Most 47,XXY Klinefelter patients are reported to show germinal aplasia at histology, while in some 46,XY/47,XXY patients, focal spermatogenesis may be present (Ferguson-Smith *et al.*, 1957; Gordon *et al.*, 1972). Only a few cases of apparently non-mosaic 47,XXY individuals showing focal spermatogenesis have been described (Paulsen *et al.*, 1968; Luciani *et al.*,

1970). Although the karyotype from peripheral blood lymphocytes, skin fibroblasts and testicular fibroblasts did not show any mosaicism in our patients, mosaicism of the germ cell line cannot be excluded in our series.

It is generally assumed that whenever germ cells are present, they degenerate at the spermatocyte level in most Klinefelter individuals. Yet, Paulsen *et al.* (1968) reported oligozoospermia in 12 out of 206 patients (47,XXY and mosaicisms), while Foss and Lewis found 15 apparently non-mosaic 47,XXY karyotypes among 466 oligozoospermic individuals. In our series, six out of nine patients had had at least one spermatozoon observed once before in their fertility investigations, on at least one occasion. However, this high incidence is probably due to selection bias since we started to perform testicular sperm recovery in patients with such a history.

Ejaculated spermatozoa from mosaic Klinefelter patients have been reported to be used successfully for ICSI (Harari *et al.*, 1994; Bourne *et al.*, 1995). However, the use of both ejaculated and testicular spermatozoa for ICSI may raise concerns about the chromosomal normality of these cells. Some authors claim that whenever spermatozoa are present in a mosaic Klinefelter patient, spermatogenesis only results from a clonal germ cell line producing spermatozoa with a normal haploid set of chromosomes (Steinberger *et al.*, 1965; Luciani *et al.*, 1970). Yet others found testes of mosaic Klinefelter patients to contain two different germ cell lines: a cell line of spermatogonia with a normal chromosome set and another line with an aneuploidic chromosome set (Paulsen *et al.*, 1968). Furthermore, Cozzi *et al.* (1994) studied the ejaculated spermatozoa of a 46,XY/47,XXY mosaic individual and found 0.9% of the spermatozoa to carry a 24 XY chromosome set. In another 46,XY/47,XXY patient they found 2.09% hyperploid 24,XY spermatozoa by FISH (Chevret *et al.*, 1995). The figure reported in normal men ranges from 0.08 to 0.24% (Moosani *et al.*, 1995). Embryos generated from spermatozoa from 46,XY/47,XXY individuals should therefore be analysed for sex chromosome normality, e.g. by FISH. Even assuming 47,XXY patients with focal spermatogenesis to have a testicular-tissue mosaicism, FISH should be applied to check for euploidy before transferring embryos generated with spermatozoa from these patients.

Although the nature of the histological changes at the testicular level has still to be defined, patients with Klinefelter's syndrome are known to show a progressive deterioration of their testicular architecture. After the onset of puberty seminiferous tubules will grow and eventually develop the final stages of spermatogenesis. In time their testes will become smaller and their consistency will become firm. The final histological picture is that of an 'end-stage testis' with extensive fibrosis and hyalinization. Although we could recover spermatozoa in patients aged 35 and 36 years, it may still be that in those patients with negative findings in the wet preparation, spermatozoa might have been successfully recovered if these patients had been younger. Progressive atrophy and probably post-operative fibrosis may explain sperm recovery failure in patient no. 1, where spermatozoa were found during the first but not during the second treatment cycle 11 months later.

In 47,XXY Klinefelter patients a testicular biopsy should

not be performed for diagnostic reasons. However, for patients willing to father their own genetic children, e.g. because of religious reasons or refusal to use donor spermatozoa, a testicular biopsy may be useful for therapeutic reasons. In this respect a multiple biopsy sampling method is to be preferred over a single voluminous sampling method. Although in four out of the nine patients spermatozoa were successfully recovered, testicular sperm recovery in 47,XXY Klinefelter's patients should not be considered a routine procedure as yet. Although we have reported on a series of nine consecutive patients, our series included six patients with a history of the presence of at least one spermatozoon on at least one occasion. These patients may not be representative of the Klinefelter population. Patients should therefore be counselled about the biased success rate, the possible genetic implications and the complexity of this therapeutic approach which involves testicular biopsy, ICSI and preimplantation diagnosis.

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