

Focal spermatogenesis originates in euploid germ cells in classical Klinefelter patients

R.B. Sciarano¹, C.V. Luna Hisano¹, M.I. Rahn¹, S. Brugo Olmedo²,
G. Rey Valzacchi³, R. Coco⁴, and A.J. Solari^{1,5}

¹Biología Celular, Facultad de Medicina, UBA, Paraguay 2155, C1121ABG Buenos Aires, Argentina ²Seremas, C1120AAN Buenos Aires, Argentina ³Procreate, C1176ABV Buenos Aires, Argentina ⁴Fecunditas, C1030AAP Buenos Aires, Argentina

⁵Corresponding address. Tel/Fax: +54-11-4961-2763; E-mail: ajsolari@mail.retina.ar

BACKGROUND: Klinefelter syndrome is the most frequent chromosome abnormality in human males. This paper aims to investigate the ploidy of meiotic and pre-meiotic germ cells found in spermatogenic foci, and furthermore, the sex chromosome constitution of Sertoli cells which surround these germ cells in non-mosaic Klinefelter patients.

METHODS AND RESULTS: A survey of 11 adult patients diagnosed with classical, non-mosaic Klinefelter syndrome who underwent testicular biopsies, showed that six of them had spermatogenesis foci. The topographical study of the biopsies showed that tubuli with germ cells are a minor fraction (8–24%) of all tubuli, although the overwhelming majority is devoid of germ cells. Using fluorescence *in situ* hybridization (FISH) with probes for the X-centromere and immunolocalization of meiotic proteins, the present work shows that all the 92 meiotic spermatocytes analyzed with FISH were euploid, 46,XY, and thus can form normal, haploid gametes. On the other hand, Sertoli cells show two marks for the X chromosome, meaning that they are 47,XXY.

CONCLUSIONS: These results provide a rationale for the high rate of success in the testicular sperm extraction plus ICSI procedures when applied to Klinefelter patients. It is also in agreement with previous studies in the XXY-mouse model. These spermatogenic foci most probably originate from clones of spermatogonia that have randomly lost one of the X chromosomes, probably during periods of life when high spermatogonial mitotic activity occurs.

Key words: Klinefelter syndrome / germ cells / meiosis / XY body / sperm aneuploidy

Introduction

The chromosome condition in Klinefelter syndrome is the most frequent chromosome abnormality in human males. Its actual prevalence is 1.72 per 1000 male births (Morris *et al.*, 2007); or approximately 1 in 600 male births (Aksglæde *et al.*, 2006). Although traditionally this syndrome has been associated with infertility and the absence of germ cells in the testis, in recent years the results of testicular sperm extraction (TESE) and assisted fertilization technologies have been successful in fathering about 50 normal children from Klinefelter patients (Nodar *et al.*, 1999; Denschlag *et al.*, 2004; Kyono *et al.*, 2007). Furthermore, TESE has shown a significant rate of success (about 50%, Schiff *et al.*, 2005) in finding post-meiotic germ cells in testes from non-mosaic Klinefelter patients. Thus, the chromosomal condition and fate of the remaining germ cells in the testis in patients with Klinefelter syndrome is both of scientific and practical medical interest.

One of the main questions raised on this subject is: what is the chromosome constitution of the spermatocytes found in non-mosaic

Klinefelter patients? In a recent review, Hall *et al.* (2006) state that human data provide no clear-cut answers to that question. Although in the XXY mice there is substantial evidence that only diploid, XY, germ cells are 'competent' to engage in meiosis (Mroz *et al.*, 1999a), and that XXY germ cells are absent in the testes of adult XXY mice (Hunt *et al.*, 1998), this subject remains controversial in humans (Foresta *et al.*, 1999; Blanco *et al.*, 2001; Bergère *et al.*, 2002; Yamamoto *et al.*, 2002; Egozcue *et al.*, 2002; Gonsalves *et al.*, 2005; Tanaka *et al.*, 2006).

This paper aims to demonstrate the ploidy of meiotic and pre-meiotic germ cells found in the spermatogenic foci, and furthermore, the sex chromosome constitution of Sertoli cells which surround these germ cells in non-mosaic Klinefelter patients.

Materials and Methods

All the research procedures on the tissues from patients were submitted to and accepted by the Ethics Committee of the School of Medicine

[Facultad de Medicina, Comité Independiente de Ética de Investigación, (CIEI), UBA, Buenos Aires, Argentina].

Testicular biopsies

Bilateral testicular biopsies were performed on eleven azoospermic men between 26 and 36 years old, whose lymphocyte karyotype is exclusively 47,XXY. Testicular biopsy was indicated for histopathological diagnosis and for the recovery of germ cells for possible intracytoplasmic sperm injection (ICSI) treatment. Karyotypically normal men with obstructive azoospermia but having complete spermatogenesis were used as controls for meiotic protein immunolocalization and for fluorescence *in situ* hybridization (FISH).

The tissue obtained was divided for light microscopy, for immunohistochemistry and for immunolocalization of proteins combined with FISH.

Immunohistochemistry

One piece of testicular tissue was processed for histopathological diagnosis with routine methods and for immunohistochemistry in paraffin sections. Another piece of tissue was fixed in 2% glutaraldehyde, post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) and embedded in Araldite (Sciurano et al., 2007). Semi-thin sections (0.5 μ m thick) were stained with toluidine blue to analyze the seminiferous epithelium in detail with the light microscope.

Paraffin sections were deparaffined and hydrated, first in two rinses in heptane 100%, followed by 5 min rinses in decreasing concentrations of ethanol (100°, 95°, 90°, 80°, 70°). Avidin–biotin–peroxidase immunostaining was carried out using a Vectastain ABC kit (Vector Laboratories, Inc., USA). Non-specific antibody binding was blocked with 5% non-immune goat serum for 30 min, and endogenous avidin and biotin were blocked using an avidin–biotin blocking kit according to the manufacturer's instructions. Then, tissues were incubated overnight at 4°C using mouse anti-MAGE-A4 at 1:5 dilution (G. Spagnoli, University of Basel, Basel, Switzerland). An 1 h incubation with biotinylated goat anti-mouse antibody at 1:100 was performed, followed by endogenous-peroxidase blocking with a 30 min incubation in 10% H₂O₂ in methanol. Then, sections were incubated in peroxidase-substrate solution until the desired stain intensity developed.

The sections were counterstained for 10 min with Harris' hematoxylin and then examined using a LEICA DM microscope (Leica Microsystems, Wetzlar, Germany) and photographed with a Leica DFC 300 FX digital camera (Cambridge, UK).

Spermatocyte microspreads

Spermatocyte microspreads for synaptonemal complexes (SCs) (Sciurano et al., 2007) were performed as previously described by Anderson et al. (1999), with modifications. Seminiferous tubules were squeezed in 200 μ l of 0.1 M sucrose that contained a cocktail of protease inhibitors (final concentration 0.1 μ g/ml each for pepstatin A, chymostatin, and leupeptin, and 1 μ g/ml for aprotinin) to give a final total volume of 1200 μ l. Eighty microliters of an aqueous solution of 2% paraformaldehyde (pH 8.5) and 0.15% Triton X-100 with the same protease inhibitor cocktail was dropped onto each slide. A 20 μ l aliquot of cell suspension was placed on each slide. Slides were maintained in a humid chamber for 2 h. Then, the slides were gently rinsed in 0.4% Photo-Flo 200 and air dried. These slides were kept at –70°C until used for fluorescence microscopy.

Immunolocalization and FISH

For immunolocalization of meiotic proteins, slides were blocked with PBT [3% bovine serum albumin (BSA) and 0.1% Tween 20 in phosphate-buffered saline (PBS)] for 30 min at room temperature before incubation.

The following primary antibodies were incubated at 4°C: a mouse anti-SC protein SYCP1 at 1:100 (P.J. Moens and B. Spyropoulos, York University, Toronto, Ontario, Canada) to label synapsed chromosomes; a rabbit anti-SYCP3 at 1:100 (P.J. Moens and B. Spyropoulos) to label chromosomal axes; a rabbit anti-BRCA1 (Santa Cruz Biotech, CA, USA) at 1:10, to label the axes in the XY body; a mouse anti-MLH1 at 1:10 (BD Pharmingen, USA) to label mature recombination nodules; human CREST serum at 1:10 (Laboratorios IFI, Buenos Aires, Argentina) to label kinetochores; and a mouse anti-MAGE-A4 at 1:5 (G.C. Spagnoli, University Hospital, Basel, Switzerland) to label germ cells. A rabbit anti- γ -H2AX antibody (Abcam Ltd, Cambridge, UK) was used at 1:500 dilution in PBS and incubated at 37°C to label the XY body. For MAGE-A4, microwave and pressure cooker epitope retrieval was performed in 0.01 M sodium citrate buffer (pH6) before the blocking step. The slides were heated in 250 ml of retrieval solution at 85–87°C for 4 min. After microwave heating, the slides were cooled in the buffer solution for 5 min and washed in PBS for 10 min. All incubations were performed overnight in a humid chamber. After washing, the following secondary antibodies were used at the specified dilutions in PBS for 2 h: a fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit at 1:50; a tetramethyl-rhodamine isothiocyanate (TRITC)-labelled goat anti-mouse at 1:25; a TRITC-labelled goat anti-rabbit at 1:25; a FITC-labelled goat anti-mouse at 1:25; and a FITC-labelled anti-human gammaglobulin IgG at 1:200. Slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (0.2 μ g/ml) and mounted in glycerol with 1,4-diazobicyclo-(2,2,2)-octane (DABCO) antifade. Slides were examined and photographed as described above.

After washing, FISH was done on slides that had been previously subjected to immunolocalization of proteins. A DNA probe was used, that binds specifically to the X centromere (DXZI locus) (Oncor Inc., Gaithersburg, MD, USA). Slides were treated with proteinase K in buffer Tris–CaCl₂ (final concentration 20 μ g/ml) for 20 min at 37°C (with the exception of slides preheated in epitope retrieval solution). Hybridization was according to the manufacturer's protocol. For detection, slides were blocked with 2.5% BSA in PBS for 15 min before each of the following antibodies: FITC-labelled sheep anti-digoxigenin (Detection kit, Oncor Inc., Gaithersburg, MD, USA), rabbit anti-sheep (Detection kit, Oncor Inc., Gaithersburg, MD, USA) and FITC-labelled goat anti-rabbit (Sigma, USA). All incubations were performed for 30 min at 37°C in a humid chamber. Slides were counterstained, mounted and photographed as described above. The separate images were superimposed using the program Adobe Photoshop CS (Adobe Systems Inc., USA).

The counts of seminiferous tubules with and without germ cells and the average total number of Sertoli cells, spermatogonia, spermatocytes and spermatids, were performed on one side of the bilateral testicular biopsies from each patient.

Results

Histology in paraffin and semi-thin sections

Paraffin sections showed that six of the eleven non-mosaic Klinefelter patients had spermatogenic foci (Fig. 1A and B). However, the vast majority of the seminiferous tubules showed only Sertoli cells (Table I). In a few tubules, early and late spermatids were identified near the lumen (Table I). Thus, the histological picture of the biopsied testes was that of some focal maintenance of spermatogenesis, in a global set of seminiferous tubules deprived of germ cells.

The interstitium showed hyperplasia of Leydig cells which formed large clumps of polygonal cells with round nuclei, and with fine lipid droplets in their cytoplasm (Fig. 1A and B).

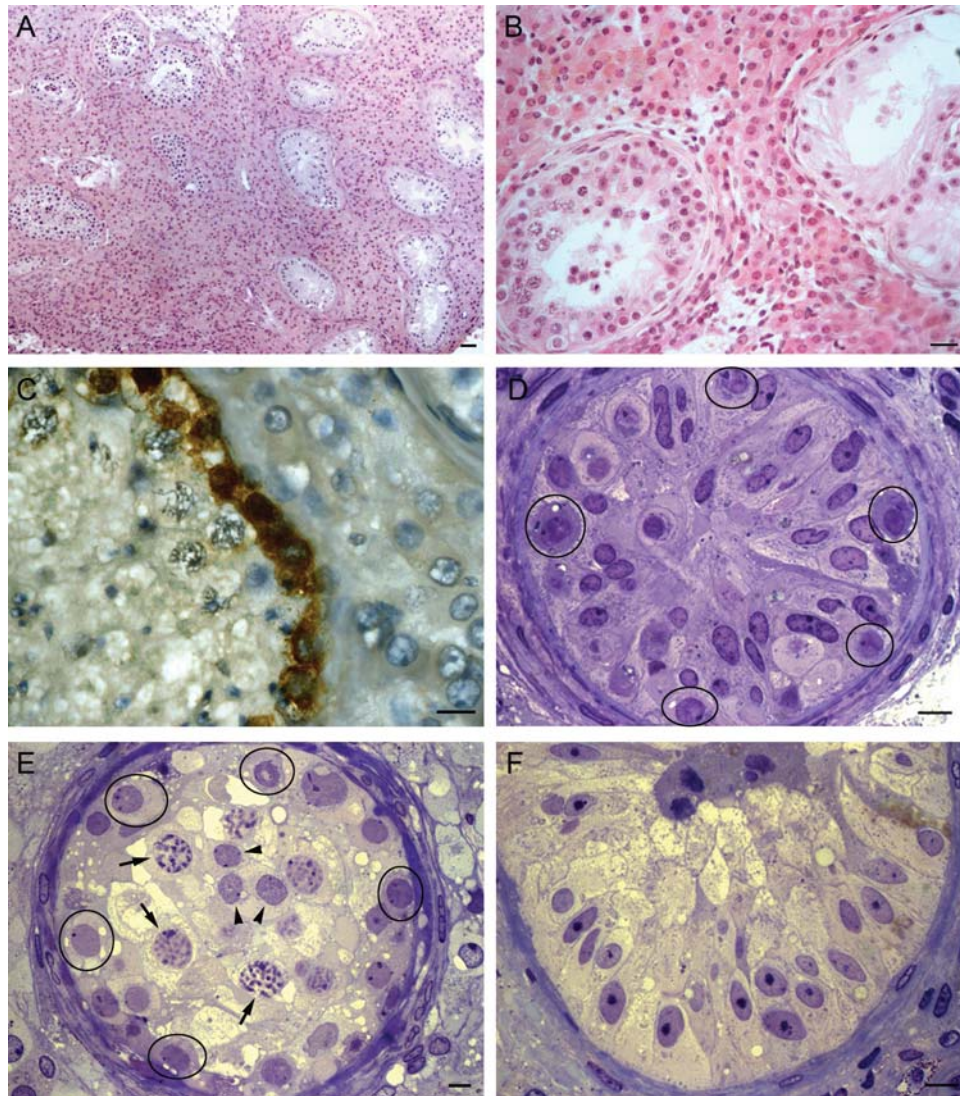


Figure 1 Histology in patients with Klinefelter syndrome.

(A) Testicular biopsies of six non-mosaic Klinefelter patients of our sample revealed isolated tubules with spermatogenic development surrounded by tubules with Sertoli cells only in paraffin sections. A massive Leydig cell hyperplasia and hypertrophy is seen in the testicular interstitium. (B) A detail of a testicular biopsy shows one seminiferous tubule having Sertoli cells only (right side) and another tubule with preserved spermatogenesis (left side). (C) Resting on the basal membrane of some seminiferous tubules, spermatogonia exhibit a strongly nuclear and cytoplasmic staining with MAGE-A4 antibody. Early primary spermatocytes are weakly stained. (D, E) In tubules with germ cells, semi-thin sections showed the different stages of spermatogenesis: spermatogonia (black circles), pachytene spermatocytes (arrows) and round spermatids (arrowheads). (F) Most of the tubules showed immature-like Sertoli cells arranged in rows. Bars scale: A, 40 μ m; B, 20 μ m; C-D, 6 μ m; E, 5 μ m and F, 6 μ m.

In the tubules having germ cells, both type A dark and A pale spermatogonia (as identified in semi-thin sections) (Fig. 1E) were labelled by MAGE-A4 immunohistochemical staining (Fig. 1C and Table 1). Early primary spermatocytes exhibited a weaker cytoplasmic staining with MAGE-A4 in a subset of these tubules (Fig. 1C).

Fine cytological details were seen in semi-thin sections (Fig. 1D–F). When followed in serial sections, tubules showing spermatogonia frequently also display spermatocytes. Spermatocytes were mainly in the pachytene stage (Fig. 1E), with an XY body of normal size and structure and a large, round main nucleolus.

There were pronounced morphological differences between Sertoli cells from tubules having some degree of spermatogenesis and those

tubules devoid of germ cells. Sertoli cells in tubules having germ cells have a mature morphology, with visible infoldings of the nuclear envelope, a distinct nucleolar complex, Charcott-Bottchner crystalloids and lipid droplets in the cytoplasm (Fig. 1D and E). In contrast, in germ-cell deprived tubuli, Sertoli cells were tall, palisade-forming rows, had oval nuclei without obvious folds of the nuclear envelope and a less-than-prominent nucleolar complex (Fig. 1F).

An abnormal feature was the presence of nuclear alterations suggestive of cell death among germ cells (16%, measured as [number of degenerating germ cells/total number of germ cells] \times 100) and Sertoli cells (10%, measured as [number of degenerating Sertoli cells/total number of Sertoli cells] \times 100). The frequencies of these

Table 1 Numbers of pre-meiotic and meiotic germ cells and Sertoli cells in patients with Klinefelter's syndrome

Patients ^a	Age (years)	Tubules without germ cells	Tubules with germ cells	n	Sertoli cells	Spermatogonia	Spermato-cytes I	Elongated spermatids	MAGE-A4 stained spermatogonia/Sertoli cells ratio	Euploid spermatocytes (FISH)
P1	31	25	8	33	17	20	20	5	54/20	NA
P2	36	46	4	50	10	22	19	9	34/15	NA
P3	35	78	7	85	10	21	35	4	30/11	NA
P4	33	11	2	13	56	26	2	0	NA	NA
P5	35	44	7	51	12	30	21	0	NA	45 (100%)
P6	30	NA	NA	NA	33	12	30	10 ^b	NA	47 (100%)

NA, not available.

^aFive of eleven non-mosaic patients with Klinefelter's syndrome showed only Sertoli cells in their seminiferous epithelium from the biopsy (Age of patients: P7, 26; P8, 31; P9, 29; P10, 28 and P11, 33).

^bSpermatozoa + spermatids.

nuclear alterations were increased in Klinefelter patients compared with control testes (3 and 2%, respectively).

Chromosomal constitution of meiotic and premeiotic germ cells

Spread cells attached to glass slides allowed the search and analysis of proteins specific to spermatocytes. Even though most were Sertoli cells, significant numbers of spermatogonia and pachytene spermatocytes were located and analysed (Fig. 2).

In all the analysed spermatocytes (absolute number = 92) the sex chromosomes were exclusively XY. The protein BRCA1 decorates the differential axes of the X and Y chromosomes in the XY body during the pachytene stage, as in normal cells (Sciurano et al., 2007). Using a combination of antibodies against BRCA1 and SYCP1, the spermatocytes show labelling of only one differential X axis, and one differential Y axis with BRCA1; and the marker protein for synapsed chromosomes (SYCP1) decorates all the autosomes, plus the small pseudoautosomal region (PAR) of the XY pair (Fig. 2A). This perfectly euploid picture was confirmed by the use of a combined labelling of kinetochores (CREST) and the axial protein SYCP3 (Fig. 2B). The XY body in Fig. 2B corresponds to a late pachytene substage, as the sex axes are convoluted, according to the general substaging of human spermatocytes (Solari, 1980). In this XY body there are only two kinetochores, those of one X and one Y chromosome (Fig. 2B). The immunolocalization of the variant histone γ -H2AX, revealed the presence of this protein in the XY chromatin domain (Fig. 2C) as in normal spermatocytes.

When the probe specific for the human X centromere was used after the protein immunolocalization, the cloud corresponding to the centromeric region of the X chromosome overlapped the CREST signal of the single X chromosome, and the remaining Y kinetochore was also labelled with CREST (Fig. 2D). Thus, there was a complete agreement of the results with both techniques: the sex chromosomes of all the spermatocytes are exclusively XY.

To elucidate the origin of the diploid spermatocytes, a MAGE-A4 staining of spermatogonia followed by FISH was done in the same cells. All MAGE-A4-positively-stained spermatogonia showed a single signal with the X-centromere probe corresponding to only one X chromosome (Fig. 2E and F). Thus, all the germ cells observed in spreads are euploid.

Sex chromosome constitution of Sertoli cells

The use of combined protein analysis first and FISH afterwards in the same cell, allowed a full confirmation of the sex complement in Sertoli cells.

Almost all nuclei of Sertoli cells (70–94%) gave two signals with the X-centromere probe (Fig. 3A). Generally, the two signals were far from each other and were not associated with the nucleolar complex. In the control slides from healthy men, a single signal, corresponding to the single X chromosome, was obtained (Fig. 3B).

Discussion

The success rate of TESE in finding sperm cells in testes of Klinefelter patients is reported to be 40–50% (Lanfranco et al., 2004; reviewed in Paduch et al., 2008) and this fact has reopened the question of the

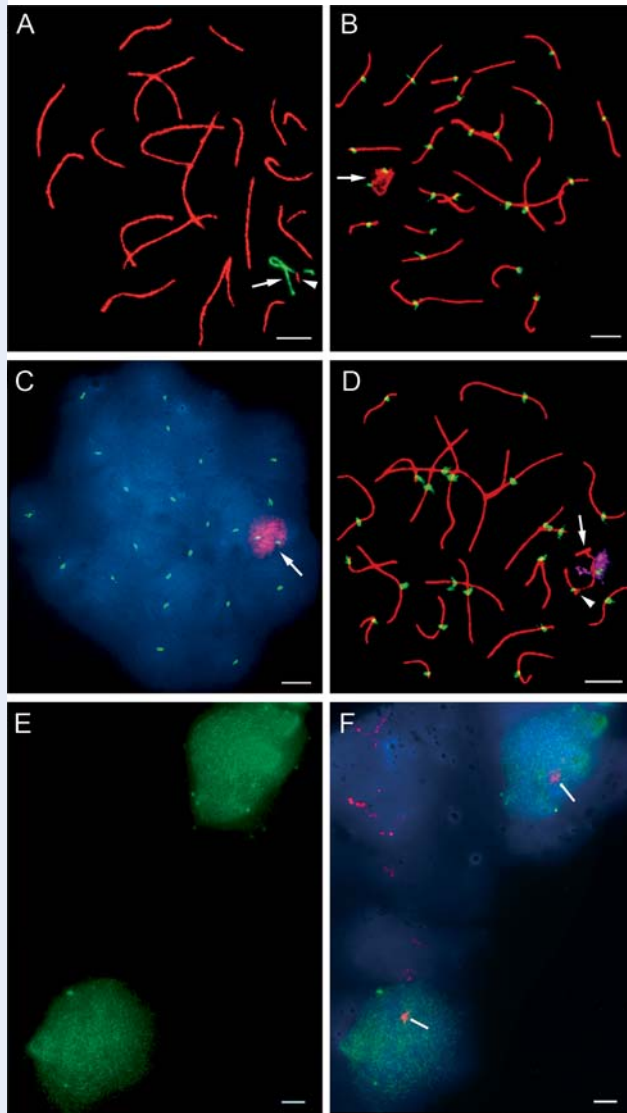


Figure 2 Immunolocalization of meiotic proteins in patients with Klinefelter's syndrome.

(A) Double immunolocalization of BRCA1 (green) and SYCP1 (red). The protein BRCA1 decorates the differential X and Y axes. SYCP1 is located along synapsed regions of autosomes and the pseudoautosomal region (PAR, arrowhead) from the XY pair (arrow). (B) Double immunolocalization of SYCP3 (red) and kinetochores by CREST (green) on a spermatocyte at a late stage of pachytene with the XY pair curled up (arrow). (C) The variant histone γ -H2AX (red) is present in the chromatin of the XY body (arrow). (D) Immunolocalization combined with FISH showed the XY constitution of spermatocytes. X centromere signal, in purple. The PAR is indicated by an arrowhead. All images show a euploid constitution (22 bivalents plus XY pair) in pachytene spermatocytes from these patients. (E) Immunostaining with MAGE-A4 antibody (green) enabled identification of spermatogonia in spreads. (F) FISH for X centromeres (red) in the same MAGE-A4-positive spermatogonia (green) revealed that all spermatogonia have only one chromosome X (lines). Bars scale: A–D, 6 μ m; E–F, 2 μ m.

chromosomal constitution and the origin of these remaining germ cells.

On one hand, some authors have suggested that the presence of a small but significant frequency of sex chromosome aneuploidy in

sperm from non-mosaic Klinefelter patients (Chevret *et al.*, 1996) is indirect evidence of the existence of meiotic XXY cells. From the observations on testicular tissue obtained by fine needle aspiration, Foresta *et al.* (1999) concluded that two out of 10 patients had 11 and 9 pachytene spermatocytes, respectively, all with an assumed XXY sex chromosome constitution, according to three-colour FISH. However, this paper did not include any pictures of such pachytene spermatocytes, and the small quantity of germ cells and the disruption of the cellular associations did not allow following the maturation of XXY cells. Yamamoto *et al.* (2002) studied testicular biopsies of Klinefelter patients with FISH, centring on the round spermatids, and suggested that XXY spermatogonia underwent meiosis, a conclusion that was strongly objected to by Egozcue *et al.* (2002). Recently, Gonsalves *et al.* (2005) described one patient among four Klinefelter patients that was assumed to contain 47% XXY meiotic germ cells and 53% XY meiotic germ cells. Despite this conclusion, the single picture of a spermatocyte from the Klinefelter group shows 22 paired kinetochores and two separate kinetochores in the presumable sex chromosomes (Gonsalves *et al.*, 2005). Thus, there is scarce, if not nil graphic evidence of the presence of two X and one Y chromosomes in spermatocytes retrieved from non-mosaic Klinefelter patients. This fact, added to the clear absence of meiotic competence of XXY cells in mice (Mroz *et al.*, 1999a) strongly suggests that only 46,XY germ cells enter meiosis in the human testis. This has already been suggested by Blanco *et al.* (2001) and by Bergère *et al.* (2002), but without showing definitive graphical evidence by FISH and immunolocalization on the XY constitution of meiotic spermatocytes from non-mosaic Klinefelter patients. However, in a general review on this subject Siffroi *et al.* (2003) clearly stated that Klinefelter patients who produce sperm are probably mosaics of XY germ cells and XXY somatic cells, the XY germ cells being the only ones able to engage in meiosis.

The present work shows definitive evidence for the XY constitution of all the meiotic and pre-meiotic germ cells, as analyzed by immunolocalization and FISH with the X-centromere probe, in Klinefelter patients having spermatogenesis foci.

Furthermore, the Sertoli cells obtained from these patients have an XXY constitution (two X-centromere signals), showing that there is no somatic mosaicism in any of the analysed Klinefelter patients. The possibility that the Sertoli cells surrounding the germ cells in spermatogenic patches could have also an XY constitution has not been experimentally excluded. However, as Sertoli cell populations have no stem cells (Sharpe *et al.*, 2003), the probability that each of the Sertoli cells in such patches underwent an accidental X-chromosomal loss, besides one such accident in one germ stem cell, is exceedingly low, making this hypothesis unlikely.

The immunological marker, MAGE-A4 (Yakirevich *et al.*, 2003) used to identify spermatogonia in spreads, enables us to elucidate the origin of the euploid spermatocytes: 'these euploid spermatocytes come from diploid, 46,XY, spermatogonia'. These observations suggest that spermatogonia that undergo mitosis may lose one of the original two X chromosomes, perhaps the inactive one, possibly because of the sluggishness of this chromosome in the capture of spindle microtubules, or perhaps simply as a random event. The results of losing one X chromosome may be to recover the ability to progress in cell differentiation and engage in meiotic prophase. This kind of event should produce a clone of diploid germ cells able to continue spermatogenesis, as in fact occurs in the XXY mouse (Mroz *et al.*, 1999a). In man, it is known that the number of spermatogonia is already decreased at

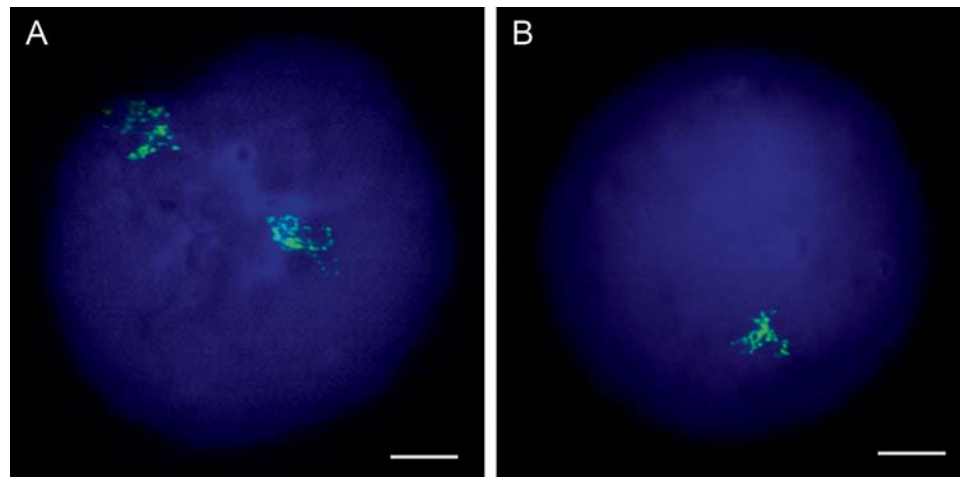


Figure 3 The human X-centromere mark (green) viewed in Sertoli cells by FISH.

(A) Two diffuse signals are evident in Sertoli cells from the Klinefelter patient. (B) In a karyotypically normal patient, the Sertoli cells showed only one signal. Bars scale: A–B, 6 μ m.

birth, and that this number is reduced dramatically at puberty (reviewed in Wikström and Dunkel, 2008), at a time when Sertoli cells acquire their maturity and form an adequate microenvironment for the differentiation of germ cells. It is then possible that 47,XXY germ cells become subject to stimuli that lead them to cell death, but the occasional loss of one of the X chromosomes may revert this detrimental condition and allow the formation of clones of diploid spermatogonia able to progress into meiotic prophase.

The observed fact that the diploid spermatocytes undergo an increased rate of cell death compared with germ cells in normal testes suggests that the testicular environment in Klinefelter patients is not normal. This abnormal medium may be the result of several causes: the nursing functions of XXY Sertoli cells may not be as effective as those of normal XY Sertoli cells or, alternatively, the established fact of a relative decrease of plasmatic androgen concentration in Klinefelter patients (Lahlou et al., 2004; Wikström et al., 2007) may affect the viability of the euploid spermatocytes. In any case, a comparison with XXY mice may help in the understanding of the human patients.

XXY mice, reproduced by special breeding techniques, have a testicular histological picture similar to that of Klinefelter patients (Lue et al., 2001) and a gonadal and behavioural phenotype that make these mice a suitable experimental model for the human XXY condition (Lue et al., 2001, 2005). The stepwise demise of germ cells in XXY mice occurs in the early post-natal period (Hunt et al., 1998). However, the same authors showed that the intrinsic ability of XXY germ cells to proliferate *in vitro* is as good as that of XY germ cells (Hunt et al., 1998). Thus, if germ cell proliferation is impaired *in vivo* in the XXY mice but not *in vitro*, the suggestion was made that the somatic-germ cell communication is at fault in XXY mice (Hunt et al., 1998). In a further search for the origin of the germ cell demise in XXY mice, Mroz et al. (1999a) showed that X chromosome reactivation in the gonads of XXY mice occurs when germ cells reach the genital ridge in the fetus, and that the surviving germ cells in the adult XXY mice are

exclusively XY cells, that may result from rare mitotic non-disjunctional events during development. Furthermore, 'sex reversed' mice, having an XX^{Sxr} chromosomal constitution, show a testicular phenotype very similar to that of XXY mice, as the loss of germ cells at the spermatogonial stage of differentiation is the rule, and the occasional germ cells found in the adult testes have lost one X chromosome (reviewed in Solari, 1994).

The loss of one of the X chromosomes in somatic cells of human females has been repeatedly described. However, in the case of female cells the loss seems to be age-dependent (Catalán et al., 1995, 2000; Surrallés et al., 1996).

As is the cases for all infertile males undergoing ICSI, the frequency of genetically imbalanced germ cells is the most important matter of concern in patients with Klinefelter's syndrome. FISH studies performed on sperm from Klinefelter men showed percentages of abnormal forms below a few per cent (reviewed in Siffrói et al., 2003). There are two hypotheses to explain the slightly increased frequency of aneuploidy in these patients. One hypothesis proposed that 47,XXY spermatogonia undergo meiosis to produce hyperploid spermatozoa (Yamamoto et al., 2002); the other one, suggested that normal diploid germ cells, found in spermatogenic foci, are more susceptible to meiotic abnormalities than controls, because of an adverse testicular environment which interferes with the mechanisms of proper chromosome disjunction in XY germ cells (Mroz et al., 1999b). The latter hypothesis seems to be the most suitable because if 47,XXY spermatogonia could undergo meiosis, they would produce a high aneuploidy rate (~40%) in sperm and preimplantation embryos, as was described in 47,XXY men (González-Merino et al., 2007). By contrast, in studies of chromosomal abnormalities in sperm from patients with Klinefelter's syndrome, the incidence of sex-chromosomal hyperploidy varied only from 2 and 15% (Guttenbach et al., 1997; Foresta et al., 1998; Rives et al., 2000; reviewed in Ferlin et al., 2005). Thus, as the frequency of aneuploid sperm in Klinefelter patients is different from both the theoretically expected from XXY cells (50% if the behaviour had been normal; Burnham, 1962) and from the actual

aneuploid frequency found in XYY men (see above) the mechanism involved in aneuploidy for the former seems to stem from a particular phenomenon proper of this syndrome and not from sex chromosome trisomy.

The present observations on non-mosaic Klinefelter patients point to the same basic phenomena as in XXY mice: the meiotic spermatocytes are exclusively XY cells, although Sertoli cells are XXY, and the focal pattern of remaining spermatogenic patches may be the result of relatively rare non-disjunctional events that form clones of XY germ cells.

Our study sample (11 patients), although larger than most of the published studies, is not sufficiently large to evaluate the frequency of spermatogenesis remnants in Klinefelter patients. It must also be cautioned that it could be biased, as the patients were selected for andrologists as those that may have a successful ICSI.

Our results contradict the assumed hypothesis that patients over 30 years of age would be deprived of germ cells (Emre Bakircioglu *et al.*, 2006). In fact, no correlation between germ cell contents and age is possible in our sample (Table I). Furthermore, other data, such as hormonal levels was not correlated with germ cell content (Emre Bakircioglu *et al.*, 2006; Koga *et al.*, 2007).

The testicular biopsies (15–30 mm³) sampled <1% of the testicular volume (3 ± 2.7 cm³, Koga *et al.*, 2007). Thus, we cannot disregard the possible presence of germ cells in other cases, including the five patients with tubuli having Sertoli cells only.

Even though the investigation of a larger sample of randomly chosen Klinefelter biopsies is needed to have a balanced estimation of the general presence of germ cells in these patients, the present work provides new perspectives to the reproductive life of men with a diagnosis that for decades has been associated with sterility.

Author's Role

R.B.S. performed the main experimental design and procedures and draft; C.V.L.H. participated in the acquisition of histochemical data and contributed to draft; M.I.R. participated in experimental procedures, FISH, and contributed to draft; S.B.O., G.R.V. and R.C. performed the present clinical study, testicular biopsies, TESE and ICSI and contributed to the draft discussion; A.J.S. participated in conception of study, collaborated with design and draft.

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