**Presence of spermatogonia in 47,XXY men with no spermatozoa recovered after testicular sperm extraction**

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**Objective:** To evaluate the presence of spermatogonia in men diagnosed with Klinefelter syndrome (KS), in whom no testicular spermatozoa were recovered by testicular sperm extraction.

**Design:** Retrospective case series.

**Setting:** University hospital.

**Patient(s):** Testicular samples from 22 nonmosaic 47,XXY men, aged 24–43 years, with no spermatozoa at multiple biopsies.

**Intervention(s):** Paraffin-embedded testicular tissue was sectioned and stained with hematoxylin-eosin, and immunostainings were performed for both MAGE-A4 and vimentin.

**Main Outcome Measure(s):** The presence of spermatogonia.

**Result(s):** Massive fibrosis and hyalinization were observed in all men with KS. Spermatogonia were observed in 4 of 22 men with KS, with differentiation up to the spermatocyte level in 2 of them.

**Conclusion(s):** A few men with KS, having no spermatozoa after testicular sperm extraction, still had few spermatogonia. These patients may eventually benefit from in vitro maturation using spermatogonial stem cells. The adult KS population can thus be divided into three subgroups: one subgroup showing focal spermatogenesis, a second having spermatogonia, and a third group in which no germ cells can be recovered. Further research is necessary to unravel the mechanism leading to these different patterns in patients with KS. (Fertil Steril 2012;97:319–23. ©2012 by American Society for Reproductive Medicine.)

**Key Words:** Klinefelter syndrome, spermatogenesis, spermatogonial stem cells, testicular sperm extraction

Klinefelter syndrome (KS) is characterized by the presence of one or more extra X chromosomes. Eighty percent of the cases are due to 47,XXY aneuploidy, whereas the remaining 20% can show 46,XY/47,XXY mosaicism or higher-grade chromosome aneuploidies like 48, XXXY or 49,XXXXY (1). Although it is a rather common syndrome (1 in 600 newborn males) (2) and one of the most frequent genetic causes of human infertility (3), it is also a commonly overlooked syndrome. It is estimated that <10% of the cases are diagnosed during childhood, whereas the remainder are diagnosed during infertility workup or remains undiagnosed (4). Infertility occurs due to the degeneration of germ cells starting in early infancy and accelerating at the onset of puberty. The histologic picture of the adult KS testis is characterized by extensive fibrosis and hyalinization of the seminiferous tubules and hyperplasia of the interstitial Leydig cells (5). In a small number of patients with KS, spermatozoa can be observed in the ejaculate, although the majority of patients are azoospermic (6). However, the presence of intratesticular residual foci of spermatogenesis was reported in azoospermic patients with KS (7). The introduction of intracytoplasmic sperm injection (ICSI) provided hope for fertility in Klinefelter patients. Successful recovery of spermatozoa by testicular sperm extraction (TESE) for ICSI was reported in approximately half of the azoospermic patients with KS referred to centers specialized in assisted reproductive techniques (8–12). To date, the remaining patients have been considered infertile. However, it is not clear whether stem cells or foci with maturation arrest are still present in these patients. If not all spermatogonial stem cells (SSCs) are lost, these cells could be cryopreserved and eventually matured in vitro. Spermatogonial stem cell cryopreservation has been introduced.
in the clinic mainly to prevent fertility loss in children undergoing gonadotoxic treatments (13). Postmeiotic differentiation of SSCs in vitro was reported in mice (14, 15). In vitro maturation of human SSCs is not yet possible, but recently long-term proliferation was achieved using adult as well as prepubertal SSCs (16, 17). This study aimed to evaluate whether spermatogonia could still be observed in adult testis from men in whom no spermatozoa were recovered by TESE.

MATERIALS AND METHODS

Patients

A total of 69 Klinefelter patients had a TESE procedure. Testicular sperm extraction was performed under general anesthesia by an open excision technique, as previously described (9). After hemiscrototomy the scrotal contents were inspected, and after opening of the tunica albuginea, small multifocal biopsies were taken. The approach involved micro-TESE with random TESE when no dilated tubules or when no spermatozoa were observed perioperatively. Testicular sampling was continued until spermatozoa were found or until the whole testicular mass was sampled without finding spermatozoa (9). The TESE procedure was successful in 33 patients (i.e., a recovery rate of 47.8%). For 24 of the 36 patients from whom no sperm were recovered after TESE, a sample was sent for histopathology. However, in two of them, after processing for histopathology, no testicular material was left over. Embedded testicular biopsies were thus obtained from 22 patients, aged 24–43 years, all showing a nonmosaic 47,XXY karyotype in their peripheral blood lymphocytes. All patients gave written consent to be involved in this study protocol, which was approved by the internal review board of the hospital.

Histology

Two testicular biopsy samples were available for evaluation in 15 patients (four depths per patient), whereas for the remaining 7 patients only one biopsy sample was evaluated (two depths per patient). The testicular biopsies were fixed and embedded in paraffin at the pathology department of the Universitair Ziekenhuis Brussel. Five-micrometer-thick sections were stained with hematoxylin and eosin to evaluate structural integrity, and immunohistochemical stainings were performed for melanoma-associated antigen 4 (MAGE-A4; provided by Dr. Giulio Spagnoli, University of Basel, Switzerland) and vimentin (mouse monoclonal antibody clone V9, M0725, Dako) as previously described (18). The seminiferous tubules were classified as normal (intact basement membrane, good attachment of cells to the basement membrane, and intercellular adhesion), degenerated (degenerative Sertoli cells with pyknotic nuclei, detachment of cells from the basement membrane, the loss of intercellular contacts, and moderate thickened basal lamina),

FIGURE 1

Hematoxylin-eosin staining showing structural integrity of the testicular tissue. (A) Biopsy sample from a 28-year-old patient with KS, showing mainly hyalinized tubules and fibrosis. (B) Biopsy sample from a 43-year-old patient, showing hyalinized tubules and fibrosis. (C) Biopsy sample from a 30-year-old patient with KS, showing degenerated and hyalinized tubules. Degenerated tubules can be recognized by detachment of cells from the basement membrane and a moderately thickened basal lamina. (D) Biopsy sample from a 34-year-old patient; normal tubules are visible (no detachment of cells from the basement membrane and good intercellular contacts). HT = hyalinized tubules; DT = degenerated tubules; NT = normal tubules. *Fibrosis.

and hyalinized (massive thickening of the basement membrane and complete loss of seminiferous epithelial cells).

All histologic examinations were performed on an inverted light microscope (Olympus IX81). Digital images were made using a digital camera (CC12 Soft Imaging System, Olympus).

RESULTS

Structural integrity was evaluated by hematoxylin and eosin staining. Most of the testicular biopsies showed severe fibrosis and hyalinization of tubules, although degenerated or even normal seminiferous tubules were observed in some biopsy samples (Fig. 1A–1D).

The presence of spermatogonia was evaluated by the immunohistochemical marker MAGE-A4, which is often used as a marker for spermatogonia but is also expressed in primary spermatocytes (19). MAGE-A4 positive staining was observed in 4 of 22 patients (aged 28, 29, 30, and 34 years). In the 28-, 29-, and 30-year-old patients not only spermatogonia were observed, but differentiation up to spermatocytes was also present (Fig. 2A). In the testicular biopsy from the 34-year-old patient only spermatogonia were observed (Fig. 2B). Positive staining for MAGE-A4 was only observed in one of two evaluated biopsies in these four patients. In all other patients no MAGE-A4 staining was detected (Fig. 2C) When MAGE-A4+ cells were observed, this was confirmed by vimentin staining, in which germ cells are visible as negative cells between the positive Sertoli cells. Corresponding vimentin staining was respectively seen in A–C'. Scale bars represent 100 μm.

confirmed by vimentin staining, in which germ cells are visible as negative cells between stained Sertoli cells (Fig. 2A’–2C’).

**DISCUSSION**

Although patients with KS with a nonmosaic 47,XXY karyotype were considered sterile for many years, the recovery of spermatozoa for ICSI has changed the fertility outcome for some of them. Approximately half of men with KS show spermatozoa after TESE (8–12). However, in the other half of patients no testicular spermatozoa can be found. Although the mechanisms behind stem cell loss in KS remain to be elucidated, it may be possible that spermatozoa for ICSI may one day be generated in vitro from spermatogonia obtained from men with KS in whom TESE was unsuccessful. Therefore, this study aimed to evaluate whether the presence of spermatogenesis and/or spermatogonia are still present in these patients. Spermatogonia were observed in 4 of 22 (18%) evaluated adult men with KS, and spermatogenesis up to the spermatocyte level was found in 3 of them (14%). Currently, fertility preservation strategies in men with KS aim to preserve spermatozoa or spermatogonia before germ cell depletion is occurring. Spermatogonia were observed in approximately half of the adolescent patients with KS (aged 10–16 years), whereas no meiotic germ cells were observed (20, 21). Because longitudinal studies are lacking, at present it remains unclear whether the subpopulation of peripubertal boys still having spermatogonia is the same as that still showing spermatogonia or spermatogenesis in adulthood.

From our data, we assume that the adult Klinefelter population may be divided into three subgroups: one subgroup in which mature spermatozoa can be retrieved by TESE; a second subgroup having no testicular spermatozoa but in which germ cells can still be detected in testicular biopsies; and a third group without any germ cells left. Yamamoto et al. (22) observed that in nonmosaic KS with spermatozoa, most of the spermatogonia had the 46,XY karyotype, although 47,XXY spermatogonia were also present in a much smaller amount. However, most of the spermatids and spermatozoa evaluated showed a normal haploid karyotype. On the other hand, only 47,XXY spermatogonia were observed in men with KS without spermatozoa in their testicular tissue (22). The reason why some patients with KS have spermatogenesis, whereas others only have spermatogonia or a complete lack of germ cells is, however, not clear.

It is well known that the KS phenotype is highly variable. Multiple genetic mechanisms may explain this phenotypic variability, all related to the presence of a supernumerary X chromosome. The excessive dosage of X-linked genes that escape inactivation, skewed X-inactivation, and the parental origin of the supernumerary X chromosome may all influence the phenotypical characteristics of men with KS (23–26). Because 10% of protein-encoding genes on the X chromosome are testis-specific, it is not surprising that testicular function is affected in men with KS (5). Inactivation of the excessive X chromosome(s) occurs in men with KS, but increased expression of genes located on the X chromosome that escape inactivation might play an important role. It is assumed that 15% of X-linked genes escape inactivation (26). It is, however, not clear whether the abnormal karyotype affects the germ cells or rather the somatic niche cells. In a mouse study it was shown that XXY germ cells are able to proliferate, as well as germ cells from XY controls (27). Yamamoto et al. (22) observed a greater degree of Sertoli cell dysfunction in men without testicular spermatozoa, compared with men with testicular spermatozoa after TESE (22). Further research is necessary to unravel the molecular causes of testicular failure in patients with KS. However, if the somatic cell compartment is deficient in patients with KS, showing spermatogonia but no further spermatogenesis, then in vitro culture of fresh or frozen–thawed spermatogonia may be a potential future fertility preservation strategy.

In conclusion, we observed spermatogonia in 4 of 22 patients with KS not showing testicular spermatozoa after TESE. These patients may eventually benefit from in vitro maturation using spermatogonial stem cells.

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**REFERENCES**


